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Differential diagnosis of active hypodermal and hematopoietic necrosis virus based on gene choice and reverse transcription coupled with PCR

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ABSTRACT. The Pacific whiteleg shrimp *Litopenaeus vannamei* (Penaeidae) is one of the most important cultivated species in world aquaculture. In Brazil, the northeastern states are home to the main shrimp producers. As shrimp aquaculture has expanded and intensified, diseases have progressively become one of the most serious threats to this industry. Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is an enzootic viral agent in Brazilian shrimp farms. Its is usually diagnosed by histological methods. However, to detect sub-clinical or acute IHHNV infection, more refined methods based on molecular techniques have been utilized. We found that by using "universal" primers and a single-step PCR diagnostic test, it was difficult to distinguish between non-infective forms of the virus and

active IHHNV. Detection of IHHNV was more accurate when we used two alternative molecular strategies, namely 1) single-step PCR amplification based on gene choice and 2) reverse transcription coupled with PCR.

Key words: Molecular diagnosis; Shrimp aquaculture; RT-PCR; *Litopenaeus vannamei*; Shrimp virus; IHHNV

INTRODUCTION

Shrimp aquaculture is an industry that has experienced a vigorous and worldwide economic growth. The gradual increase of such activity is most prominent in tropical and subtropical countries (Joventino and Mayorga, 2008). Today, the Pacific whiteleg shrimp *Litopenaeus vannemei* is the commercial species predominantly farmed in the Americas (FAO, 2007). In Brazil, the northeastern region concentrates practically all the shrimp culture, where excellent climate conditions contribute to the high productivity and performance of this activity. The States of Ceará, Rio Grande do Norte and Piauí have all emerged as the main national shrimp producers (IBAMA, 2007).

Owing to the intensive rearing techniques required for efficient and cost-effective shrimp farming, outbreaks of epizootic diseases eventually occur, resulting in high mortality and significant economic losses (Bachère, 2000). Thus, good practice in aquaculture depends on, among other factors, disease control measures, which include routine diagnostic programs.

Infectious hypodermal and hematopoietic necrosis (IHHN) is one of the most prevalent diseases in cultured shrimp, which is caused by a very stable parvovirus (acronym, IHHNV). IHHNV is an icosahedral, non-enveloped virus with a diameter of 22 nm. The IHHNV genome consists of single-stranded DNA that encodes three open-reading frames (ORFs), which correspond to a non-structural protein, ORF2 and a 37-kDa coat protein (Bonami et al., 1990; Mari et al., 1993). IHHNV is a ubiquitous virus that infects penaeid shrimp species in Asia, Central America and South America. However, the Pacific blue shrimp (*Litopenaeus stylirostris*) is the most susceptible and severely affected species. In the case of *L. vannamei*, despite the relatively low rate of morbidity and mortality caused by IHHNV, the typical manifestation of IHHNV-induced disease includes growth retardation, which is characterized by high levels of energy consumption, and runt deformity syndrome, manifested by cuticular deformities and bent rostrum (Kalagayan et al., 1991). These symptoms are responsible for decreasing the market value of commercial shrimp.

For the diagnosis of IHHNV-infected shrimp, histological methods are currently used for the confirmation of the clinical signs that result from IHHN syndrome. However, these methods are laborious, time-consuming and not suitable for detecting low levels of pathogens that occur in acute viral infection. More robust and sensitive techniques, based on DNA technology, such as polymerase chain reaction (PCR) and *in situ* hybridization, have emerged as routine methods for molecular diagnosis of IHHNV, principally when the aim is to detect chronic infection or to obtain pathogen-free shrimp broodstock (OIE, 2009).

In the present study, as part of routine monitoring program for viral acute and chronic IHHN disease, we found that gene choice is essential for accurate single-step PCR-based diagnosis of IHHNV infection in *L. vannamei*. In addition, the use of reverse-transcription

coupled to PCR (RT-PCR) proved to be useful to discriminate between active (infective) and non-infective genome-integrated IHHNV. These results are useful as a reference for management plans in shrimp culture and hatchery.

MATERIAL AND METHODS

Samples

Approximately two hundred samples including postlarvae, juveniles and subadults of *L. vannamei* were collected from different shrimp farms located in the States of Ceará, Piauí and Rio Grande do Norte. Whole postlarvae or excised shrimp tissues (gill or pleopode) were either immediately immersed in 95% ethanol in clean collection tubes, when the purpose was the purification of genomic DNA, or transferred to microtubes containing NAsafe solution (5 M $(NH_4)_2SO_4$, 10 mM EDTA, 0.1 M MOPS, pH 4.6) for the preparation of total RNA. Samples were maintained at 4°C until processing, and the samples were processed within one week following collection.

Genomic DNA preparation

Genomic DNA of each *L. vannamei* sample was prepared by the proteinase K/SDS method, from 30 to 50 mg minced tissue, according to current protocols in molecular biology (Sambrook and Russel, 2001). The purified shrimp DNA was resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and stored at 4°C. DNA quality and integrity were confirmed by agarose gel electrophoresis.

Total RNA purification

Total RNA was prepared from whole postlarvae or minced tissues (gill and pleopode) of adult and subadult *L. vannamei* using the SV total RNA isolation system (Promega, Madison, WI, USA), according to manufacturer recommendations. The quality and yield of the total RNA was verified by the following methods: 1) assessments of the integrity of 28S and 18S rRNA, using denaturing agarose gel electrophoresis; and 2) spectrophotometric assessments using the 260/280 nm ratio.

Single-step PCR-based IHHNV diagnostic test

For diagnostic purposes, genomic DNA from *L. vannamei* postlarvae and tissues were investigated by means of a single-step touchdown PCR (TD-PCR) for the presence of IHHNV. The selected primer pair was in accordance with the recommendations of the World Organisation for Animal Health (OIE): sense primer 77012F: 5'-AGC GGA ACA CAA CCC GAC TTT A-3', and anti-sense 77353R: 5'-TGG CCA AGA CCA AAA TAC GAA-3'. PCR was carried out in a final volume of 15 µL, consisting of 1X PCR buffer (60 mM Tris-SO_4, pH 8.4, 18 mM ammonium sulfate, 2.5 mM $MgSO_4$), 1.5 µL template (50-100 ng genomic DNA), 1 U GoTaq DNA polymerase (Promega), 2.0 mM $MgCl_2$, 0.2 µM dNTPs, and 0.2 µM of each specific primer. The PCR conditions involved an initial denaturation step at 95°C for

5 min, followed by 10 cycles of denaturation at 95°C for 50 s, annealing at 60°C for 50 s, minus 1°C per cycle, and an extension at 72°C for 50 s. The touchdown steps were sequentially followed for additional 25 cycles of 95°C for 50 s, 50°C for 50 s, and 72°C for 50 s. A final step at 72°C for 8 min was added for termination of DNA synthesis. The PCR products were separated by 1.5% agarose gel electrophoresis and analyzed after ethidium bromide staining and image digitization.

RT-PCR for detection of infectious IHHNV type

Complementary DNA (cDNA) of *L. vannamei* was used as template for detection of active IHHNV through RT-PCR.

For cDNA synthesis, 1 μg of each DNase I treated-total RNA purified from whole postlarvae and shrimp tissues was mixed with 500 ng random primers (Promega), in a final volume of 10 μL, heated to 70°C for 10 min, and quickly chilled on ice. A total of 100 U ImProm-II reverse transcriptase (Promega) was added to the reaction mixture with 1 mM of each deoxynucleoside triphosphate (dNTP), 2 mM $MgSO_4$, 1 mM DTT, and 20 U recombinant RNase inhibitor. Nuclease-free water was finally added to make a final volume of 20 μL. Reverse transcription was allowed to proceed at 42°C for 60 min, and terminated by heating the reaction mixture at 70°C for 15 min. The samples were diluted 10 times with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and 1.5-μL aliquots (approximately 10 ng) were combined with 18.5 μL TD-PCR mixture. The amplification parameters were the same as for single-step TD-PCR, described above.

Gene choice for differential diagnosis of IHHNV

For the purpose of distinguishing infective and non-infective viruses, specific primers for the complete IHHNV ORF2 (GenBank accession No. AAF59416.1) and IHHNV non-structural protein gene sequence (GenBank accession No. AAF59415.1) were synthesized and used in single-step touchdown PCR, as described above.

RESULTS AND DISCUSSION

In a recent routine diagnostic screening for IHHNV, we analyzed almost 200 samples of *L. vannamei*, which were believed to be naturally infected. These samples were collected from different shrimp farms located along the coast of three northeastern Brazilian states, where most of these farms have management control programs to improve the quality and productivity of shrimp farming.

Using shrimp genomic DNA as template, one of the OIE-recommended primer pairs, namely 77012F/77353R, which anneals to the 3' segment of the IHHNV non-structural protein gene and a single-step PCR protocol, 100% of samples scored positive for the presence of this virus (Table 1 and Figure 1, Panel A). For us, such percentage of affected shrimp was unexpectedly high. Still, it is known that in regions where the virus is enzootic, it may be typical for its prevalence to approach 100% (OIE, 2009). In addition, these suspected samples may be infected by other enzootic causative agents, such as infectious myonecrosis virus and *Vibrio* bacteria.

Table 1. Diagnostic test based on gene choice and single-step PCR, for the presence of IHHNV in shrimp suspected of IHHN disease.

Site of sampling	No. of shrimp samples with gross signs of IHHNV disease	Universal IHHNV primers	IHHNV ORF2	IHHNV non-structural protein
Ceará	93	93 (100.0%)*	44 (47.3%)	34 (36.5%)
Rio Grande do Norte	70	66 (94.3%)*	20 (28.5%)	21 (30.0%)
Piauí	34	34 (100.0%)*	12 (35.3%)	10 (29.4%)
Total	197	193 (98.0%)*	76 (38.6%)	65 (34.0%)

Data are reported as number with percent in parentheses. Universal primers refer to the OIE-recommended pair 77012F/77353R. *Number of positive samples. IHHNV = infectious hypodermal and hematopoietic necrosis virus; ORF2 = open-reading frame 2.

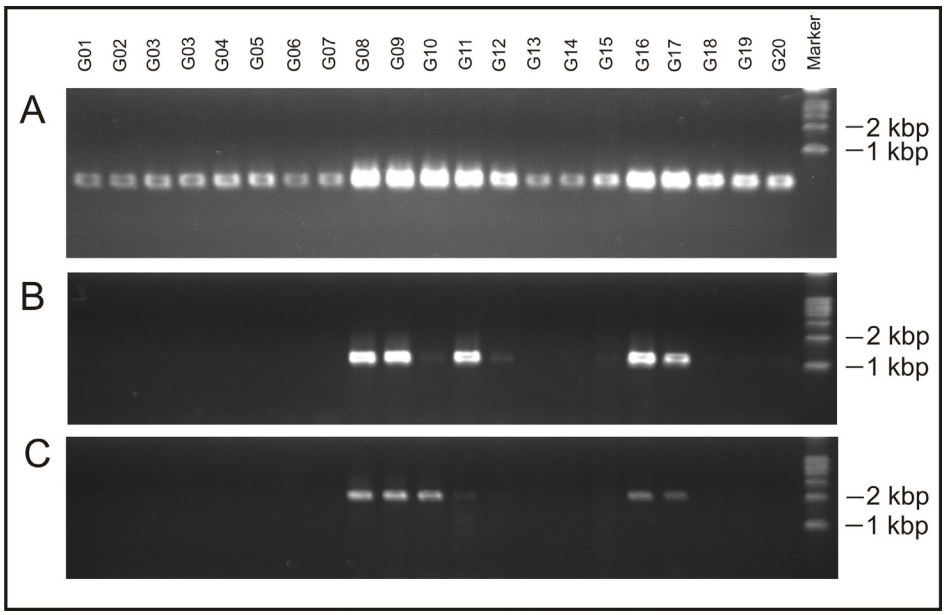

Figure 1. Diagnosis of IHHNV infection in *Litopenaeus vannamei*, by single-step arbitrary PCR. Shrimp genomic DNA was used as template. Representative samples (*lanes G01* to *G20*) are shown for visualization of samples, which resulted in 100% positive and diagnostically unsolved with the primer pair 77012F and 77353R (Panel A), and truly positive samples (*lanes G08* to *G12*; *lanes G16* and *G17*) distinguished with specific primers for ORF2 (Panel B) and non-structural protein gene (Panel C).

However, it was previously reported that IHHNV-related sequences were detected in the genome of *Penaeus monodon* from Africa (Madagascar) and Australia (Tanzania). These IHHNV sequences were found to be associated with genomic-integrated non-infective type A and B forms of IHHNV (Tang and Lightner, 2006). Thus, by using the OIE recommended primers (IHHNV 77012F/77353R), which anneal to the 3'-end of the ORF encoding a non-structural protein, the use of the single-step PCR diagnostic test was impracticable to distinguish between the so-called integrated non-infective forms of the virus and truly active IHHNV.

We confirmed that the integration of IHHNV-related sequences may be responsible for the high number of samples that scored positive for IHHNV, through the amplification of a 900-bp segment, which corresponds to part of the IHHNV genome and part of the shrimp genome (data not shown). Such product of PCR was amplified with the primers MG831F (specific for the virus) and PM2R (specific for shrimp genome) (Tang and Lightner, 2006).

Thus, to distinguish between the infective and non-infective forms of the virus, we utilized two strategies based on: 1) gene choice, with specific primers for the complete IHHNV ORF2 and the IHHNV non-structural protein gene sequence, and 2) RT-PCR amplification using the universal primers IHHNV 77012F/77353R.

In the first case, both of these sequences were suitable for distinguishing between active/infectious and inactive/non-infectious viruses (Figure 1, Panels C and D). Consequently, the suspected samples of moribund shrimp that scored positive for IHHNV, using the gene choice strategy, were between 34 and 38% (Table 1).

The second strategy of IHHNV diagnosis has additional steps to be carried out, where it detects exclusively actively replicating virus, which produces transcripts and which will be differentially and effectively diagnosed by the RT-PCR amplification (Figure 2).

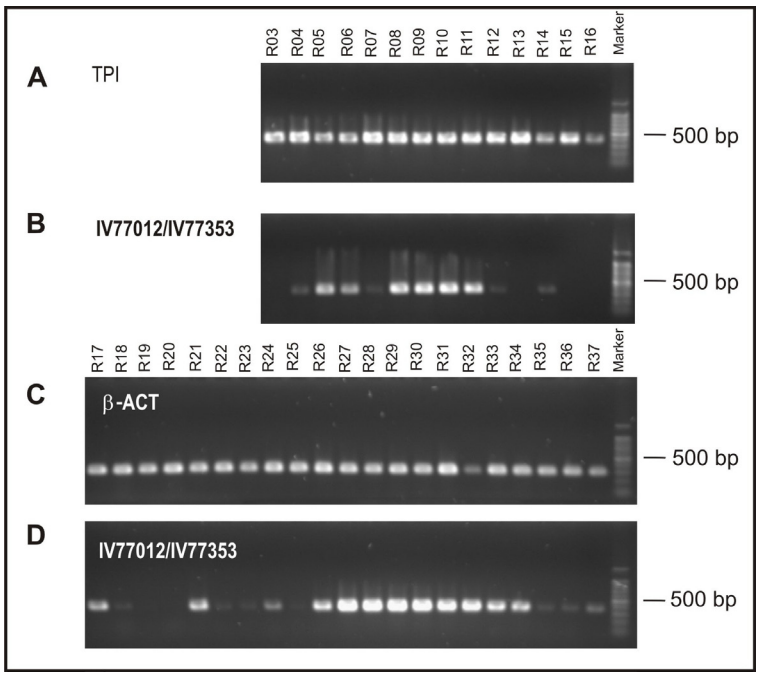

Figure 2. RT-PCR-based diagnostic test for detection of active infection with IHHNV in *Litopenaeus vannamei*. In this test, shrimp complementary DNA (cDNA) was used as template (samples R03 to R37). **A.** Amplification of *L. vannamei* triose phosphate isomerase (TPI) transcript, which was used as control of expressed resident gene (*lanes R03* to *R16*). **B.** Same samples as in Panel A, of which some are positive for IHHNV (amplification product with primer pair 77012F/77353R). **C.** Amplification of *L. vannamei* β-actin (β-ACT) transcripts, which were used as control of expressed housekeeping gene (*lanes R17* to *R37*). **D.** Same samples as in Panel C, which were positive for IHHNV (with the same primer pair 77012F/77353R).

In this situation, suspected samples that scored positive for IHHNV were between 23% (Rio Grande do Norte) and 36% (Ceará). Samples from Piauí were not analyzed by this strategy. These numbers of samples that score positive for IHHNV are more realistic, since they correlate better with shrimp health status and IHHN disease prevalence.

In summary, strategies for molecular diagnosis based on gene choice and RT-PCR amplification are essential to distinguish between infectious/active IHHNV and genome-integrated/inactive IHHNV-related sequences, as seen in this study. Moreover, such strategies are easily adaptable for routine diagnostic programs in shrimp culture for detecting the presence of IHHN virus.

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