

NOTE

Detection of infectious myonecrosis virus (IMNV) of penaeid shrimp by reverse-transcriptase polymerase chain reaction (RT-PCR)

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ABSTRACT: Infectious myonecrosis virus (IMNV) infecting cultured *Litopenaeus vannamei* in Brazil is a double-stranded RNA virus that causes a slowly progressive disease with cumulative mortalities of up to 70%. The disease is currently diagnosed using a combination of gross signs (primarily skeletal tail muscle necrosis with white opaque discoloration), histopathology, and *in situ* hybridization with a digoxigenin-labeled gene probe. A rapid and sensitive method for definitive diagnosis of the disease was developed using reverse-transcriptase polymerase chain reaction (RT-PCR). Two primer sets were used to detect 328 and 139 bp amplicons in a nested RT-PCR assay. Using RNA extracted from purified virions, the first step reaction detected 100 copies of the IMNV viral genome whereas the nested step detected 10 copies. The primers were shown to be specific for IMNV and no amplicons were detected using RNA extracted from shrimp infected with other penaeid shrimp viruses (Taura syndrome virus [TSV], yellowhead virus [YHV], infectious hypodermal hematopoietic necrosis virus [IHHNV] and white spot syndrome virus [WSSV]).

KEY WORDS: Infectious myonecrosis · IMNV · RT-PCR · Penaeid shrimp

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INTRODUCTION

Infectious myonecrosis (IMN) affecting cultured *Litopenaeus vannamei* in Brazil is caused by a double-stranded RNA virus that is named infectious myonecrosis virus (IMNV) (Poulos et al. 2006). Shrimp infected with IMNV develop gross lesions that are characterized by focal to extensive areas of necrosis in skeletal muscle tissues primarily in the distal abdominal segments that are visible as opaque, whitish, discolorations (Lightner et al. 2004a,b). Histologically, lesions are characterized by coagulative muscle necrosis, usually accompanied by fluid accumulation between muscle fibers, hemocytic infiltration and fibrosis. Dark basophilic inclusion bodies may also be seen within the cytoplasm of muscle cells, in hemocytes and connective tissue cells, and in lymphoid organ spheroids, which are often present in the lymphoid organ

and ectopically in the hemocoel and loose connective tissues. Tang et al. (2005) developed an *in situ* hybridization method for definitive diagnosis of IMNV in shrimp displaying these lesions. Subsequently, the viral genome was characterized and sequenced (Poulos et al. 2006; GenBank accession number AY570982). As described herein, a nested reverse-transcriptase polymerase chain reaction (RT-PCR) method was developed and tested to provide a rapid, sensitive and specific test to detect IMNV in penaeid shrimp.

MATERIALS AND METHODS

Primer sets from 3 different regions of the IMNV genome were designed using Primer Designer 4 software (Scientific & Educational Software) and conditions for optimal amplification were determined. One

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primer set was chosen for further development as a nested RT-PCR test for detection of the virus in infected shrimp tissues. The primers for the first step reaction, IMNV-F (5'-CGACGCTGCTAACCATACA A-3') and IMNV-R (5'-ACTCGGCTGTTTCGATCAAG T-3'), amplified a 328 bp fragment and the primers for the second, nested step, IMNV-NF (5'-GGCACAT-GCTCAGAGACA-3') and IMNV-NR (5'-AGCGCTGA GTCCAGTCTTG-3'), amplified a 139 bp fragment. The first step reaction was performed using the rTth DNA polymerase and 5X EZ buffer system (Applied Biosystems). The optimized reaction mixture for the first step reaction was 1X EZ buffer, 300 μ M each dNTP, 0.465 μ M each primer, 2.5 mM manganese acetate and 2.5 U rTth DNA polymerase; 1 to 5 μ l of RNA template (untreated or boiled for 3 min) were used for the 25 μ l reaction. The cycling parameters for the first step RT-PCR were 1 cycle of 60°C for 30 min and 95°C for 2 min followed by 39 cycles of 95°C for 45 s and 60°C for 45 s and ending with a 7 min extension at 60°C. The nested second step reaction was performed using puReTaq Ready-To-Go PCR Beads according to manufacturer's directions (Amersham Biosciences, GE Healthcare). When the beads were resuspended in distilled water the final concentration of the reagents contained in the beads was 200 μ M each dNTP, 1.5 mM magnesium chloride and 2.5 U of puRe Taq DNA polymerase in 25 μ l total volume. The nested primers were added at a final concentration of 0.465 μ M each. A volume of 0.5 μ l from the first step reaction was used as template for the second step reaction. The cycling parameters for the second step reaction were 1 cycle at 95°C for 2 min followed by 39 cycles of 95°C, 65°C and 72°C for 30 s each and a final extension at 72°C for 2 min. The products of the first and second step reactions were visualized after electrophoresis in 2% agarose gels in the presence of ethidium bromide.

The IMNV-infected tissues used for this study were from *Litopenaeus vannamei* collected during outbreaks of the disease in Brazil in 2003 and *L. vannamei* that were experimentally infected with IMNV as described by Poulos et al. (2006). Sensitivity of the nested RT-PCR for IMNV was determined using 10-fold serial dilutions of RNA extracted from purified virions in which the copy number of IMNV genome was calculated. IMNV was purified from the shrimp from Brazil according to Poulos et al. (2006). RNA was isolated from the purified virions and from shrimp tissues using the High Pure RNA Tissue kit (Roche) according to the manufacturer's directions. RNA was eluted from the columns using RNase-free water. The copy number of IMNV contained in the RNA preparation from purified virions was calculated based on a genome size of 7560 bp, which is equivalent to 4.99×10^6 Da. Multi-

plying this value by the conversion factor of 1.66×10^{-24} yields 8.28×10^{-18} g. Thus, 1 ng (10^{-9} g) of extracted IMNV double-stranded RNA contained 1.21×10^8 copies of the viral genome. The amount of RNA isolated from the purified virions was quantified from the spectrophotometric reading at a wavelength of 260 nm and this value was used to determine the copy number in the preparation and diluted samples. The specificity of the nested RT-PCR was determined using total RNA extracted from IMNV-infected *L. vannamei* obtained from Brazil and from an infectivity assay using purified IMNV as inoculum (Poulos et al. 2006), from specific pathogen free (SPF) *L. vannamei* (Wyban et al. 1992), from Taura syndrome virus (TSV)-infected *L. vannamei*, from yellowhead virus (YHV)-infected *L. stylirostris*, from infectious hypodermal and hematopoietic necrosis virus (IHHNV)-infected *L. vannamei*, and from white spot syndrome virus (WSSV)-infected *L. vannamei*. The disease status of all shrimp was determined by a combination of PCR tests, histology and *in situ* hybridization (OIE 2003).

RESULTS

A nested RT-PCR method was developed to detect the IMN virus in IMNV-infected penaeid shrimp. Boiling the template for 3 min just prior to the addition to the first RT-PCR step increased the sensitivity of detection (results not shown); therefore, all templates were boiled for 3 min just prior to use. For determination of the sensitivity of the assay, RNA extracted from purified IMNV was adjusted to 10^8 copies μ l⁻¹ and serial 10-fold dilutions were made in RNase-free water to obtain copy numbers from 10^8 to 1 copy μ l⁻¹. The first step of the nested RT-PCR method, using the diluted and boiled RNA templates, was capable of detecting 100 copies of the IMNV genome, whereas in the second step, the detection limit was increased to 10 copies (Fig. 1). The RT-PCR method was able to detect the presence of the virus in the *Litopenaeus vannamei* specimens originally obtained from Brazil and in the shrimp injected with purified virions (Fig. 2). The RT-PCR assay detected IMNV in pleopods as well as muscle tissue from the tail. The RT-PCR method did not detect any amplicons in RNA extracted from SPF shrimp nor from shrimp infected with TSV, YHV, IHHNV or WSSV (Fig. 2).

DISCUSSION

The availability of sensitive and specific tests for detection of pathogens in shrimp is essential for accurate diagnosis of diseases affecting cultured popula-

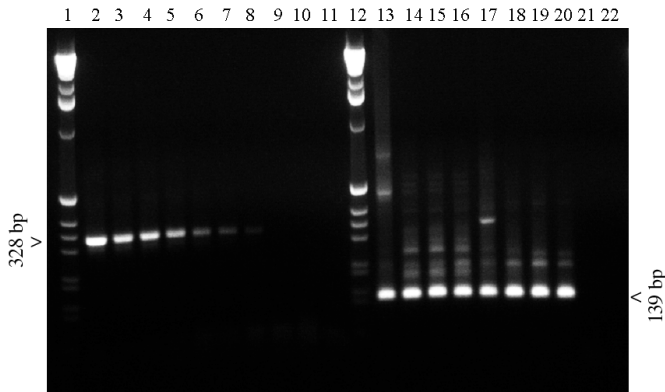


Fig. 1. Determination of the sensitivity of the RT-PCR assay using RNA extracted from purified IMNV, starting with 10^8 copies of the genome in the reaction mixture and serially diluted 10-fold down to 1 copy of the genome. Lanes 1 and 12 contain the 1 kbp molecular weight marker. Lanes 2 to 10 contain products from the first step reaction starting with 10^8 copies and ending with 1 copy per reaction. Lane 8, with 100 copies, is the last detectable band from the first step reaction. Lane 11 contains the no template control product from the first step reaction. Lanes 13 to 21 contain products from the second, nested step starting with 10^8 copies and ending with 1 copy per reaction. Lane 20, with 10 copies, is the last detectable band from the second, nested step. Lane 22 contains the no template control product from the second step reaction. Arrowheads indicate position of the amplicon from the first step (328 bp) and second step (139 bp) reaction

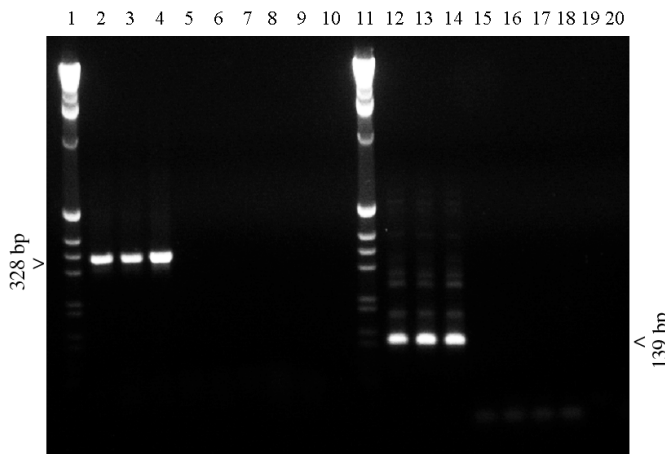


Fig. 2. Determination of the specificity of the RT-PCR assay using RNA extracted from penaeid shrimp tissue samples. Lanes 1 and 11 contain the 1 kbp molecular weight marker. Lanes 2 to 10 contain the products from the first step reaction. Lanes 12 to 20 contain the products from the second, nested step. RNA templates used for the RT-PCR were extracted from the following samples: IMNV-infected pleopod tissue from Brazil (Lanes 2 and 12); pleopod tissue (Lanes 3 and 13) and muscle tissue (Lanes 4 and 14) from infectivity study using purified virus as inoculum; TSV-infected tissue (Lanes 5 and 15); YHV-infected tissue (Lanes 6 and 16); IHHNV-infected tissue (Lanes 7 and 17); WSSV-infected tissue (Lanes 8 and 18); SPF tissue (Lanes 9 and 19). Lanes 10 and 20 contain first and second step products from the no template control. Arrowheads indicate position of the amplicon from the first step (328 bp) and second step (139 bp) reaction

tions. Histological methods coupled with *in situ* hybridization using specific gene probes provides accurate and sensitive diagnosis; however, the time involved for tissue fixation, embedding and sectioning and subsequent microscopic analysis is extensive and gene probes for penaeid shrimp pathogens are not readily available commercially. The PCR and RT-PCR tests developed for the shrimp aquaculture industry provide sensitive and specific methods that are also rapid and do not require extensive preparation of the tissue specimens. In this study, a nested RT-PCR method was developed for the detection of IMNV that was found to be both sensitive and specific for IMNV. Boiling of the RNA prior to the first RT step enhanced the detection, presumably by denaturing the double-stranded RNA viral genome so that the primers were able to bind to a single-stranded template prior to reverse transcription (Gouvea et al. 1990). The detection limit of 10 copies of IMNV genome was demonstrated using RNA extracted from purified virions, although in clinical specimens the limit of detection will likely be 10- or 100-fold less due to the presence of shrimp RNA and other factors from shrimp tissue that may co-elute with the specific RNA template.

Specificity of the test method was demonstrated using RNA extracted from shrimp infected with other penaeid shrimp viruses. The other viruses tested are the most common viral pathogens found in cultured populations of *Litopenaeus vannamei* and they were shown not to produce any amplicons with the IMNV primers chosen for development of the assay method. The nested RT-PCR method described here can be performed in less than 1 d, including the time necessary for RNA extraction, thus providing a rapid diagnostic test for IMNV.

In recent months, cultured shrimp from other geographic regions have been received at the University of Arizona Aquaculture Pathology Laboratory that demonstrate gross and histological lesions similar to those seen with IMNV infections (D. V. Lightner pers. comm.). However, specimens obtained from regions other than Brazil have failed to react with the IMNV-specific gene probe or with the RT-PCR method described here, indicating the presence of other agents that cause similar disease signs and the need for definitive tests such as *in situ* hybridization and RT-PCR for accurate diagnosis of IMNV.

Acknowledgements. Financial support for this research was provided by the Gulf Coast Research Laboratory Consortium Marine Shrimp Farming Program, CSREES, under grant no. 2002-38808-01345. The authors thank Dr. A. J. P. Nunes, Mr. T. Andrade, and the Brazilian Shrimp Farmers Association (Associação Brasileira de Criadores de Camarões-ABCC) for their valuable assistance in obtaining the IMNV specimens that were used for this research.

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Editorial responsibility: Timothy Flegel, Bangkok, Thailand

*Submitted: April 21, 2006; Accepted: August 1, 2006
Proofs received from author(s): October 27, 2006*