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NOTE

A multiplex RT-PCR for simultaneous differentiation of three viral pathogens of penaeid shrimp

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ABSTRACT: A multiplex reverse transcription polymerase chain reaction (mRT-PCR) was developed and optimized to simultaneously detect 3 viral pathogens of shrimp. Three sets of specific oligonucleotide primers for Taura syndrome virus (TSV), white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) were used in the assay. The mRT-PCR DNA products were visualized by gel electrophoresis and consisted of fragments of 231 bp for TSV, 593 bp for WSSV and 356 bp for IHHNV. No specific bands of the same size were amplified from other penaeid shrimp pathogenic viruses or bacteria. As little as 10 pg of TSV RNA and 100 pg of WSSV DNA and IHHNV DNA could be detected using gel electrophoresis. Studies are in progress to further test the specificity and sensitivity of this mRT-PCR method on viral isolates, as well as on clinical samples.

KEY WORDS: Infectious hypodermal and hematopoietic necrosis virus · Multiplex · Polymerase chain reaction · Taura syndrome virus · White spot syndrome virus

INTRODUCTION

Infectious hypodermal and hematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV) and white spot syndrome virus (WSSV) are 3 major viral pathogens that infect penaeid shrimp (Bonami et al. 1990, 1997, Brock 1997, Lightner & Redman 1998, Erickson et al. 2002). Mixed infections involving these viruses have been described in penaeid shrimps (Manivannan et al. 2002) and can cause high mortality, leading to economic losses that are detrimental to the shrimp farming industry (Lightner 1996, Plumb 1997, Wang et al. 1998).

Multiple diagnostic methods, such as histological examination, electron microscopy and histological studies using *in situ* hybridization, are required to detect and differentiate these viral pathogens (Takahashi et al. 1994, Lightner 1996, Plumb 1997, Lightner & Redman 1998). However, these methods are time consuming and labor intensive. Molecular assays, such as DNA probes (Nunan & Lightner 1997, Mari et al. 1998) and PCR methods, have been used for rapid and sensitive detection of these viruses (Lo et al. 1996, Wang et al. 1996, Nunan et al.1998, Kiatpathomchai et al. 2001, Tang et al. 2004). Traditionally, specific probes and PCR have been developed to detect a specific nucleic acid of one pathogen. Recently, real-time PCR assays have been developed to detect WSSV, TSV and IHHNV (Dhar et al. 2001, Yue et al. 2006) and to differentiate 2 viruses at a time (Tsai et al. 2002, Yang

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et al. 2006). Conventional singleplex PCR is potentially expensive and resource intensive, whereas the cost of real-time PCR equipment and the specific technical training required limit the usefulness of such assays as routine laboratory tests. In our study, we have developed a specific and sensitive multiplex PCR that can simultaneously detect and differentiate these 3 important viral agents infecting shrimp.

MATERIALS AND METHODS

Viral isolates and clinical samples. TSV, WSSV and IHHNV isolates are listed in Table 1.

Isolation of nucleic acids from viruses. RNA and DNA extractions from TSV, IHHNV and WSV isolates were carried out using Trizol according to the manufacturer's protocol (Invitrogen).

Extraction of nucleic acids from tissue samples. Total nucleic acid extracts from tissue samples from disease-free *Penaeus vannamei* were extracted by homogenizing 10 mg frozen infected tissues (stomach tissue homogenate) in 700 µl lysis buffer containing 2% hexadecyl-trimethy-ammonium-bromide, 1.4 mM NaCl, 20 mM EDTA and 20 mM Tris-HCl (pH 7.5), adding isoamyl alcohol to the final concentration of 2.5%. Samples were incubated at room temperature for 1 h. After incubation, 500 µl phenol/chloroform (1:1) was added to each sample and mixed vigorously by vortexing and incubated for 15 min at room temperature. The mixtures were centrifuged at 12 000 rpm (14 000 × *g*) for 15 min at 4°C, and supernatants were transferred to 1.5 ml tubes. Equal volumes of isopropanol were added to precipitate the nucleic acid, and the mixtures were inverted several times during the incubation at room temperature for 15 min. The mixtures were centrifuged at 12 000 rpm (14 000 × *g*) for 15 min at 4°C. Pellets were rinsed with 500 µl 75% ethanol, then air dried for about 15 min.

Pellets containing nucleic acid were resuspended in 20 µl of RNase-free distilled water. The purity and concentration of DNA and RNA were determined spectrophotometrically by 260:280 nm ratios and 260 nm readings, respectively, using a spectrophotometer (Shimadzu UV-1200), and the nucleic acids were stored at –20°C until use. Extracted DNA of *Vibro* spp. and *Streptococcus* spp. and RNA of yellow head virus were kindly provided by the China Institute of Veterinary Drug Control, Beijing.

Oligonucleotide primers. Three sets of primers that specifically amplify WSSV, IHHNV and TSV were used. WSSV primers GATGAGACAGCCAAGTTGTTAAAC and GCATCAACTTCCACAGCTTTATC amplify 593 bp DNA product, IHHNV primers ATCGGTGCACTACTCGGA and TCGTACTGGCTGTTCATC amplify 356 bp DNA product and TSV primers TCAATGAGAGCTTGGTCC and AAGTAGACAGCCGCGCTT amplify 231 bp DNA product. The primers for WSSV were designed using DNASTAR software against a conserved region of the WSSV genomic sequence (GenBank No. AF 369029) that encodes for a non-structural protein. IHHNV primers were from a region in between the non-structural and the structural protein-coding regions of the genome (GenBank No. AF 218266). TSV-specific primers had been reported previously in the literature (Nunan et al. 1998). All 3 sets of primers were synthesized at Takara Shuzo Stet. Primers were diluted to a final concentration of 100 pmol μl^{-1} using RNase-free distilled water and stored at –20°C.

Multiplex reverse transcription polymerase reaction (mRT-PCR). The mRT-PCR consisted of a 2-step procedure as described (Pang et al. 2002), which includes reverse transcription (RT) and PCR amplification. The RT-PCR kit (Takara Shuzo Co.) was used for all RT and PCR amplifications. RT is performed in 20 µl volumes, each RT mixture containing 5 mM $MgCl_2$; 500 mM KCL; 100 mM Tris HCL, pH 8.3; 1 mM of each deoxyadenosine triphosphate (dATP), de-oxythymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP) and deoxyguanosine (dGTP); 2 U RNase inhibitor; 0.25 U avian myeloblastosis virus (AMV) reverse transcriptase; and 1.25 pmol of TSV forward primer. Different concentrations of DNA or RNA

Table 1. Shrimp pathogens and field samples used in this experiment. TSV: Taura syndrome virus; WSSV: white spot syndrome virus; IHHNV: infectious hypodermal and hematopoietic necrosis virus; GXPRC: Guangxi, People's Republic of China; CIVDC: China Institute of Veterinary Drug Control

Shrimp pathogens	Source
TSV (GXPRC/1/02)	Beihai, Guangxi, PR China
TSV (GXPRC/1/03)	Hepu, Guangxi, PR China
TSV (GXPRC/2/03)	Qinzhou, Guangxi, PR China
WSSV (GXPRC/1/02)	Beihai, Guangxi, PR China
WSSV (GXPRC/2/02)	Hepu, Guangxi, PR China
WSSV (GXPRC/1/03)	Qinzhou, Guangxi, PR China
IHHNV (GXPRC/1/03)	Beihai, Guangxi, PR China
IHHNV (GXPRC/2/03)	Hepu, Guangxi, PR China
IHHNV (GXPRC/1/04)	Qinzhou, Guangxi, PR China
Vibrio spp. (extracted DNA)	CIVDC, Beijing, PR China
Streptococcus spp. (extracted DNA)	CIVDC, Beijing, PR China
Yellow head virus (extracted RNA)	CIVDC, Beijing, PR China
Tissue sample	Disease-free *Penaeus vannamei*

of WSSV, IHHNV and TSV in 4 µl volumes were then added to the mixture. Diethylpyrocarbonate (DEPC)-treated distilled H_2O was added to bring the final volume to 20 µl. RT was performed in a thermal cycler (Model 9600, Perkin Elmer Cetus) for 1 cycle at 42°C for 25 min, 99°C for 3 min and 4°C for 5 min.

For the multiplex PCR reaction, 5 mM $MgCl_2$; 500 mM KCL; 100 mM Tris HCL, pH 8.3; 10 mM each dATP, dTTP, dCTP and dGTP; 0.5 pmol of each WSSV and IHHNV forward and reverse primers with TSV reverse primer; and 1.25 U TaKaRa LA *Taq* (Takara Shuzo Co.) polymerase were added in the above RT reaction tubes, and 50 µl of the total volume was obtained by adding DEPC-treated distilled water. The mPCR was carried out in the same thermal cycler used for RT. After extensive preliminary trials with different annealing temperatures and times and with various concentrations of DNA and RNA, the thermal cycler was programmed for optimum conditions. The optimized cycling protocol consisted of an initial denaturing at 94°C for 5 min, then 35 cycles that each consisted of denaturing at 94°C for 45 s and annealing and extension at 68°C for 2 min. The sample was then heated to 68°C for 10 min for a final extension. The negative control did not contain template cDNA/DNA; it consisted of PCR master mix, all 3 sets of primers and de-ionized water.

Detection of amplified mRT-PCR products. Agarose gel electrophoresis was used to detect mRT-PCR nucleic acid products. A volume of 10 µl of amplified product was subjected to 1% agarose horizontal gel electrophoresis with 0.5 µg ethidium bromide ml^{-1} at 5 v/m V using Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA) (Sambrook et al. 1989). Gels were visualized and photographed using the Bio-Vision Post-Electrophoresis Instrument (Vilber Lourmat).

Specificity and sensitivity of the mRT-PCR. To determine the specificity of the mRT-PCR, the amplified DNA fragments from TSV, WSSV and IHHNV were cloned into the pMD18-T cloning vector (Takara Shuzo Co.). The recombinant plasmid DNA was sequenced in an automated DNA sequencer (Takara Shuzo Co.), and the sequence data were analyzed by DNASTAR software and compared with the corresponding sequences in GenBank. For further confirmation of specificity, 200 ng of RNA from yellow head disease virus and 200 ng of DNA from both *Vibrio* spp. and *Streptococcus* spp., as well as same amount of nucleic acid isolated from specific-pathogen-free (SPF) *Penaeus vannamei* were also tested by mRT-PCR. Sensitivity of this mPCR was determined by making 10-fold dilutions of a mixture containing 100 ng of template of each TSV RNA, WSSV DNA and IHHNV DNA.

RESULTS AND DISCUSSION

A method of mRT-PCR was developed and optimized to detect and simultaneously differentiate 3 viral pathogens in a single tube reaction through 35 cycles of PCR. The optimal mRT-PCR yielded 3 amplified fragments, i.e. 231 bp for TSV, 593 bp for WSSV and 365 bp for IHHNV (Figs. 1 & 2). The identity of each mRT-PCR product was further confirmed by DNA sequencing. The sequences of the mRT-PCR products were matched to those of TSV, WSSV and IHHNV based on sequence data in GenBank using DNASTAR software. The mRT-PCR method was found to be specific and to be able to detect and differentiate TSV, WSSV and IHHNV. No amplifications were observed when nucleic acid from *Vibrio* spp. and *Streptococcus* spp., as well as SPF *Penaeus vannamei* were used as mRT-PCR templates (Fig. 1). The detectable limit of the mRT-PCR was 10 pg for TSV and100 pg for both WSSV and IHHNV (Fig. 2).

Throughout development of the mRT-PCR method, various modifications were made to the annealing temperature, extension time, cycle quantity and primer concentrations in order to obtain optimal conditions.

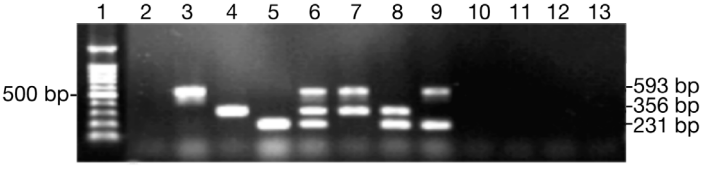

Fig. 1. Specificity of multiplex RT-PCR. Lane 1: 100 bp DNA size marker; Lane 2: specific pathogen-free (SPF) *Penaeus vannamei*; Lane 3: WSSV (GXPRC/1/02); Lane 4: IHHNV (GXPRC/1/03); Lane 5: TSV (GXPRC/1/02); Lane 6: WSSV (GXPRC/1/02) + IHHNV (GXPRC/1/03) + TSV (GXPRC/1/02); Lane 7: WSSV (GXP- RC/1/02) + IHHNV (GXPRC/1/03); Lane 8: IHHNV (GXPRC/1/03) + TSV (GXPRC/1/02); Lane 9: WSSV (GXPRC/1/02) + TSV (GXPRC/1/02); Lane 10: yellow head disease virus; Lane 11: *Vibrio* spp.; Lane 12: *Streptococcus* spp.; Lane 13: buffer control. For pathogen abbreviations, see Table 1

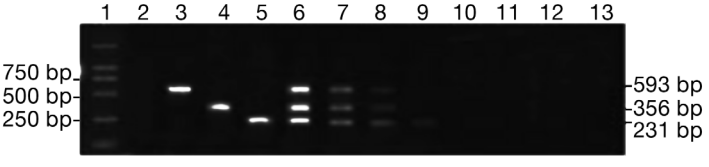

Fig. 2. Sensitivity of multiplex RT-PCR. Lane 1: 200 bp DNA size marker; Lane 2: SPF *Penaeus vannamei*; Lane 3: WSSV (GXPRC/2/02); Lane 4: IHHNV (GXPRC/1/04); Lane 5: TSV (GXPRC/2/03). Lanes 6 to 13: different amounts of DNA or RNA from each of WSSV, IHHNV and TSV; Lane 6: 100 ng; Lane 7: 10 ng; Lane 8: 100 pg; Lane 9: 10 pg; Lane 10: 1 pg; Lane 11: 100 fg; Lane 12: 10 fg; Lane 13: 1 fg. For pathogen abbreviations, see Table 1

No spurious PCR amplification reactions among any shrimp or other pathogens were noted with various amounts of RNA and DNA mixtures. All negative controls included RNA/DNA samples from disease-free *Penaeus vannamei.* DNASTAR software analysis indicated that the mRT-PCR-amplified DNA products were similar to the TSV-, WSSV- and IHHNV-specific gene sequences.

An mRT-PCR that can rapidly identify and differentiate these 3 viral infections, and possibly detect multiple infections, will be useful for the control of viral diseases in shrimp. Further studies are in progress to test the specificity and sensitivity of this mRT-PCR method on viral isolates of TSV, WSSV and IHHNV from various diagnostic and research laboratories, as well as on clinical samples originating from shrimp farms in South China.

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