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Simultaneous detection of white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) by multiplex reverse transcription-polymerase chain reaction (RT-PCR) in Pacific white shrimp *Penaeus vannamei*

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ABSTRACT: An assay using a single-tube, 1-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) was established for the simultaneous detection of white spot syndrome virus (WSSV) and Taura syndrome virus (TSV). Three primer sets, 9195 F/9992 R, 94 F2/R2, and ITS F/28S R, were mixed at a ratio of 3:1:1 to amplify specific fragments of the TSV, WSSV, and *Penaeus vannamei* genome, respectively, in the RT-PCR reaction. Shrimp samples were experimentally infected with WSSV and TSV. PCR-amplified products detected in the nucleic acid extraction of shrimp pleopods produced 4 kinds of results. With no virus infection, 1 fragment of 892 base pairs (bp) was amplified from a ribosomal RNA gene by primer set ITS F/28S R as an internal control. In samples only infected by WSSV or TSV, 2 fragments could be seen: either from WSSV (530 bp) plus the internal control or TSV (231 bp) plus the internal control, respectively. In cases of co-infection with both viruses, all 3 amplified products were detected simultaneously. This study is the first report of *Penaeus vannamei* specimens co-infected with WSSV and TSV being detected using a PCR method via experimental infection.

KEY WORDS: RT-PCR · WSSV · TSV · Penaeid shrimp

INTRODUCTION

Over the past several years, white spot syndrome virus (WSSV) has devastated the tiger shrimp *Penaeus monodon* culture industry in Taiwan and other Asian countries (Lo & Kou 1998). *Penaeus vannamei* (Pacific white shrimp, also called *Litopenaeus vannamei*) was introduced into Taiwan from the Americas; many farmers switched to raising this newly introduced species. By early 1998, *P. vannamei* had become the most popular and highest yield cultured shrimp in southern Taiwan. However, from late 1998 to early 1999, due to importation of Taura syndrome virus (TSV)-contaminated post larvae (PL) or spawners, there were outbreaks of Taura syndrome in cultured Pacific white shrimp (Tu et al. 1999, Yu & Song 2000). Shrimp production dramatically dropped to as low as 10% of the production level in early 1998. After the introduction of *P. vannamei*, both WSSV and TSV have become important shrimp viral pathogens in Taiwan. WSSV has a

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wide host range among crustaceans (Flegel 1997) and distinctive clinical signs (white spots) in penaeid shrimps. The entire sequence of the double-stranded, circular DNA genome has been determined (van Hulten et al. 2001). TSV is a single-stranded RNA virus that causes serious disease in the PL, juvenile and adult stages of *P. vannamei* exclusively (Lightner & Redman 1998).

Reverse transcription (RT) and polymerase chain reaction (PCR) technologies have proven to be powerful diagnostic tools for shrimp viral infections and for detection of viral reservoirs in asymptotic carriers. Methods for PCR diagnosis have been published for WSSV (Kimura et al. 1996, Lo et al. 1996, Takahashi et al. 1996, Kim et al. 1998, Tapay et al. 1999) and TSV (Nunan et al. 1998). This study was carried out to develop a modified method using a template comprised of total nucleic acid for simultaneous detection of WSSV and TSV in a single tube, 1-step multiplex RT-PCR.

MATERIALS AND METHODS

Experimental infections. *Penaeus vannamei*, weighing approximately 3.5 g and originating from a commercial shrimp hatchery in Tungkang, southern Taiwan, were reared from post larvae to the juvenile stage in an indoor recirculation system. Randomly selected specimens were checked using TSV RT-PCR (Nunan et al. 1998) and then WSSV PCR (Lo et al. 1996), and all were found to be PCR negative. Juveniles were stocked at a density of about 20 shrimp per 90 l aquarium tank. Inocula were prepared from patently infected (carapace with white spots) *P. monodon* for WSSV and from *P. vannamei* for TSV. Shrimp collected from cultivation ponds experiencing disease outbreaks in I-lan, north Taiwan, were proven to be virally infected by WSSV PCR and TSV RT-PCR. The carapace was removed from whole moribund specimens, chopped, and fed to juveniles in the tank. The shrimps were initially given 15% (body weight) infected tissue, and this was repeated 1 d later. The TSV inoculum was introduced first, and WSSV was given 10 d later.

Total nucleic acid purification. Pleopods from virus-treated *Penaeus vannamei* were collected from moribund shrimp and other remaining survivors randomly during the period of the bioassay experiment. Pleopods sampled from juveniles on Day 0 were used as the negative control in each experiment. Approximately 15 to 20 mg of pleopod was excised from each specimen. The protocol for total nucleic purification was developed, with modifications, from the procedure of Miller et al. (1988). The pleopods were homogenized in a 1.5 ml microcentrifuge tube with a microcentrifuge pestle. Next, 600 µl lysis buffer (100 mM NaCl, 10 mM EDTA, 50 mM Tris-base, pH 7.5, 0.5% SDS) and 15 µl Proteinase K stock solution (20 mg ml^{-1}) were added to the tube. Each sample was heated to 60°C for 30 min and mixed every 10 min. After incubation, the tube was placed on ice for 3 to 5 min. To remove protein in the sample, 200 µl of 10 M NH_4OAC was added and mixed vigorously before centrifugation at 12 000 × *g* for 10 min at 4°C with a swing bucket rotor. To minimize the risk of contaminating the sample with possible inhibitory compounds in the pellet, only 650 µl of the supernatant was transferred to a new 1.5 ml tube. To precipitate the nucleic acid, 700 µl isopropanol was added to the tube, which was inverted several times, and centrifuged for 10 min at 12 000 × *g* and 4°C. The pellet was rinsed twice with 500 µl 75% ethanol and then air dried for 10 to 15 min. The nucleic acid pellet was resuspended in 150 µl of DEPC-treated water at 60°C for 10 min and stored at –20°C until use.

Oligonucleotide primers. Several specific primer sets were used in the 1-step multiplex RT-PCR, including: 9195 F/9992 R (Nunan et al. 1998) for TSV; 94 F2/R2 (Tsai et al. 1999) for WSSV; and ITS F/28S R for the internal control (Table 1). The internal control was included so that the quality of extracted nucleic acid and PCR inhibitor conditions could be checked. The primer set ITS F and 28S R was designed from the Internal Transcribed Spacer 2 (ITS2) and 28S rRNA sequences of *Penaeus vannamei*, based on unpublished sequences available in GenBank under Accession No. GI 5825422 (www.ncbi.nlm.nih.gov/Genbank/index.html). Shrimp DNA was expected to yield a PCR product of 892 bp pairs corresponding to the nucleotide sequence from Positions 1063 to 1954 of the ribosomal RNA gene in the GenBank data file. These primers were synthesized by Genset Singapore Biotech.

One-step multiplex RT-PCR. Ready-To-Go™ RT-PCR Beads (Amersham Pharmacia Biotech) were used in each amplification reaction. This kit is designed so that all components for RT and PCR can be run in a single tube by a simple non-interrupted thermal cycle. Each bead in the tube contains M-MuLV reverse transcrip-

Table 1. Nucleotide sequence and melting temperature (T_m) of the 6 primers used in this study

Primer	Nucleotide sequence	T_m (°C)
9195 F	5′-TCAATGAGAGCTTGGTCC	54
9992 R	5′-AAGTAGACAGCCGCGCTT	56
94 F2	5′-CGATACTGCCATTGAAAGC	56
94 R2	5′-GCCCTGGAGAACACTTCC	58
ITS F	5′-AAAGGCCGCTACGAGAATCTCT	55
28S R	5′-AGGTCGACGTGAGCAAGGAAT	55

tase, buffer, nucleotide, and Taq DNA polymerase. The only additional reagents required are water, primers, and template. Each oligonucleotide primer was diluted to 10 µM of the working solution. Three primer sets for TSV, WSSV, and the internal control were mixed in 1 tube in the ratio of 3:1:1 (6 µM:2 µM: 2 µM) as the primer cocktail solution. Each reagent was added to the reaction tube in the following order: 43 µl water was added to dissolve the beads, and then the mixture was cooled in an ice bath for 5 min. Next, 5 µl primer cocktail solution was added and mixed thoroughly. To reduce the cost, the original final volume (50 µl) of each reaction was halved (to 25 µl). Twenty-four µl of reaction solution was transferred to a new PCR tube for the other reaction, and finally, 1 µl shrimp nucleic acid was added to both tubes as a template. Each tube was incubated in a thermal cycler (PE 9700; Perkin Elmer) set to the following program: 15 min at 42°C (for RT reaction), then 4 min at 95°C (for inactivation of reverse transcriptase and denaturation of DNA); 40 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, followed by a final incubation for 7 min at 72°C. The amplified product was analyzed following electrophoresis (100 V, 30 min) in 2% agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

During the experimental infections, there were no obvious acute-phase mortalities among samples after TSV experimental infection. The inoculum was prepared from crude samples of virus-infected shrimp which were fed directly to juveniles, not by injection as done, for example, by Nunan et al. (1998). Thus, it is possible that some *Penaeus vannamei* survived into the chronic phase of TSV infection, and then were co-infected with the WSSV inoculum.

With the method described here, 3 kinds of viral infections were detected using nucleic acids from shrimp pleopods (Fig. 1). One internal control fragment of 892 bp was the only fragment amplified by the internal control primer set from the negative control shrimp. Two fragments displayed single infection with either WSSV or TSV, from WSSV a 530 bp fragment plus the internal control, and from TSV a 231 bp fragment plus the internal control. In cases of co-infection by both viruses, all 3 amplified products were observed in the agarose gel. Wongteerasupaya et al. (1995) reported a *Penaeus monodon* specimen experimentally co-infected with WSSV (called systemic ectodermal and mesodermal baculovirus or SEMBV in their paper) and yellow-head virus (YHV) as determined by transmission electron microscopy. Our study is the first report of an experimental co-infection of WSSV and TSV in *P. vannamei* being detected using a PCR method.

The multiplex RT-PCR conditions using 3 primer sets were optimized using different primers and mixing ratios. According to the size distribution of the amplified products, the 3 primer sets (9195 F/9992 R, 94 F2/R2, and ITS F/28S R) were used to amplify fragments of 231, 530, and 892 bp respectively. Empirically, the mixing ratio was optimized to 3:1:1 in the primer cocktail solution. Generally, there is a high concentration of ribosomal RNA and DNA in total nucleic acid extracts. Thus, primers specific to ribosomal RNA gene sequences used as the internal control (e.g. as in Lo et al. 1996), would compete with the TSV primers in the reverse transcription step. In order to avoid this unbalanced competition, the reverse primer of the internal control, 28S R, was designed to anneal at the splice junction of the ITS2 and 28S genes (Bagshaw & Quiel 1998). Thus one-half of the primer was hybridized to the 3' end of the 28S gene and the other half to the 5' end of the adjacent ITS2 gene. This primer would anneal to ribosomal DNA but not to spliced ribosomal RNA, and this eliminated reverse transcription of rRNA to cDNA.

The extraction procedure used in the sample preparation was easy to carry out, and it yielded nucleic acids including both DNA and RNA from shrimp

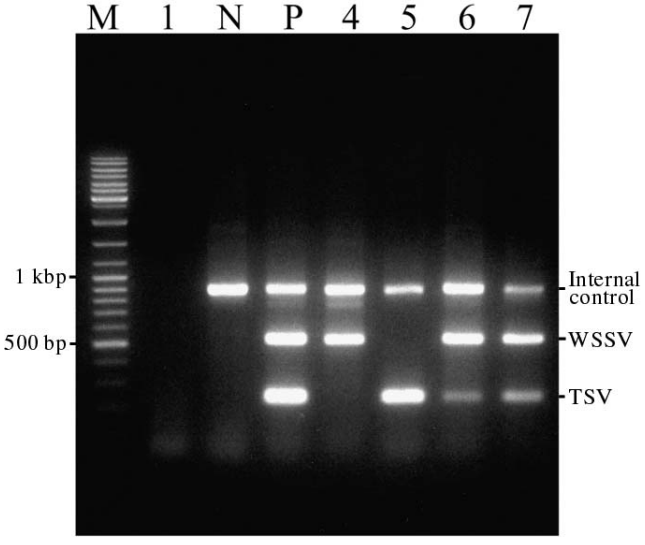

Fig. 1. Agarose electrophoresis gel of RT-PCR products from a multiplex RT-PCR assay using the 3 primer sets, 9195 F/9992 R, 94 F2/R2, and ITS F/28S R. The nucleic acid from pleopods of experimentally infected *Penaeus vannamei* was used as the template. Lane M: DNA molecular weight markers; Lane 1: water as the template for RT-PCR; Lane N: no virus infection, Day 0 pleopod as the negative control; Lane P: mixed templates of Lanes 4 and 5 as the positive control; Lane 4: infected by WSSV only; Lane 5: infected by TSV only; Lanes 6 and 7: co-infection with both viruses, WSSV and TSV

pleopods. Some problems have been reported with co-purification of PCR inhibitors in nucleic acid extraction tests with alternative template sources such as hepatopancreatic tissue and post larvae (Wang et al. 1996, Belcher & Young 1998). The salting-out procedure does not eliminate inhibitors. A different method, such as CTAB (Lo et al. 1996), should be developed to deal with these kinds of samples.

The Ready-To-Go dry beads are designed to be stable at room temperature, enabling temperature-sensitive enzymes to be carried on trips for viral disease screening at farms. In the case of screening post larvae or broodstock, where greater sensitivity is required, the Ready-To-Go system may prove less sensitive than other reagents. This could potentially be overcome by the use of nested PCR.

In routine testing of large numbers of *Penaeus vannamei* specimens in the culture industry, this the 1-step multiplex RT-PCR method described above may be useful for simultaneous detections of WSSV and TSV.

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