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Reverse transcription polymerase chain reaction (RT-PCR) used for the detection of Taura Syndrome Virus (TSV) in experimentally infected shrimp

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ABSTRACT: Taura Syndrome Virus (TSV) has adversely affected the shrimp culture industries of the Americas. First recognized in 1992, this viral agent has spread throughout the shrimp growing regions of South and Central America to become established in North America in the short span of 5 yr. Diagnostic methods for TSV include histopathology, bioassay using susceptible *Penaeus vannamei* as the indicator species and *in situ* hybridization with TSV specific complimentary DNA (cDNA) gene probes. An additional method for detecting TSV is through the use of reverse transcription polymerase chain reaction (RT-PCR). Two oligonucleotide primers were selected using the sequence information from a cloned cDNA segment of the TSV genome. The primers, designated 9195 and 9992, used in the RT-PCR procedure amplify a 231 base pair (bp) fragment of the cDNA. Using the RT-PCR technique, TSV has been detected in the hemolymph of *P. stylirostris* and *P. vannamei* with experimentally induced TSV infections.

KEY WORDS: RT-PCR · Taura Syndrome Virus (TSV) · Penaeid shrimp

INTRODUCTION

The shrimp farming industries of the Americas have been adversely affected by epizootics due to viral pathogens. Taura Syndrome Virus (TSV) has had an enormous negative impact on shrimp culture in the Western Hemisphere (Lightner 1995). First recognized in commercial penaeid shrimp farms located near the mouth of the Taura River in the Gulf of Guayaquil, Ecuador in mid-1992 (Jimenez 1992), this viral pathogen has spread throughout South and Central America into North America in the short span of 5 yr (Lightner 1996). TSV has been the causative agent of economically disastrous epizootics in *Penaeus vannamei*, causing mass mortalities of 40 to 95% in affected postlarval and juvenile populations (Lightner et al. 1995, 1997, Brock et al. 1995, 1997). Other American species, including *P. setiferus*, *P. schmitti* and *P. stylirostris*, are less seriously affected by TSV (Hasson et al. 1995, Overstreet et al. 1997).

TSV causes 3 distinguishable disease phases in infected shrimp. The peracute/acute phase of the disease is characterized by moribund shrimp displaying an overall pale reddish coloration caused by the expansion of the red chromatophores. Shrimp in this phase usually die in the process of molting. If the shrimp survive through the peracute/acute phase of TSV infection, the recovery phase begins. Multifocal, melanized cuticular lesions are the major distinguishing characteristic of the recovery phase (Lightner 1996). In the chronic phase of TSV infection, infected shrimp appear and behave normally, but remain persistently infected, perhaps for life (Hasson et al. 1997b).

The current diagnostic and detection methods for TSV include histopathology, *in situ* hybridization and bioassay (Lightner 1996). Most recently, reverse transcription polymerase chain reaction (RT-PCR) has been developed to detect TSV in the hemolymph of infected shrimp. Diagnostic histopathology in shrimp with acute, natural or experimental TSV infections display multifocal areas of necrosis of the cuticular epithelium and subcutis. Lesions are characterized by

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the presence of variably sized eosinophilic to basophilic cytoplasmic inclusion bodies creating a 'peppered' or 'buckshot' appearance which is considered to be pathognomonic for this disease (Lightner et al. 1997). *In situ* hybridization assays, employing a non-radioactively digoxigenin (DIG)-labeled cDNA probe, provide excellent sensitivity when used on properly fixed tissues (Hasson et al. 1997a). A bioassay diagnostic method, using juvenile specific pathogen-free (SPF) *Penaeus vannamei* (Wyban et al. 1992) as the indicator species for TSV, can enhance the virus from asymptomatic carrier shrimp (Brock et al. 1995, Lightner 1996).

TSV is a positive sense single stranded RNA virus, tentatively classified in the Picornaviridae family (Hasson et al. 1995, Lightner 1996, Bonami et al. 1997). RT-PCR must first convert the ssRNA to cDNA through reverse transcription. Using sequence information from cloned cDNA segments of the TSV genome (Mari et al. 1998) 2 primers were selected from a cDNA TSV clone. These 18 mer primers amplify a 231 base pair (bp) fragment of the TSV genome.

MATERIALS AND METHODS

Indicator shrimp. *Penaeus stylirostris* weighing approximately 4.5 g, originating from a commercial shrimp hatchery in Venezuela, and SPF *P. vannamei* (Wyban et al. 1992, Carr et al. 1996), from the Oceanic Institute, Hawaii, average weight of 4.0 g, were reared from post larvae to the juvenile stage, using the methods described in Williams et al. (1992).

Preparation of inoculum. Whole animal TSV-infected *Penaeus vannamei* with carapace removed, originating from a shrimp farm in Mexico, were minced and fed *per os* to SPF, juvenile *P. vannamei* (Wyban et al. 1992), weighing approximately 1 g. The infected shrimp were collected, the carapace removed, minced and homogenized in TN buffer (0.02 M Tris-HCl, 0.4 M NaCl, pH 7.4). Following centrifugation at 1000 × *g* for 10 min, the supernatant fluid was diluted 1:10 with 2% NaCl. Ten *P. stylirostris* (4.5 g) and 20 *P. vannamei* (4.0 g) were inoculated with 0.1 ml of inoculum, by injecting dorso-laterally between the tergal plates of the third and fourth abdominal segments into the underlying muscle.

RNA template. The hemolymph from TSV-infected *Penaeus vannamei* was collected from moribund shrimp during the acute phase of the bioassay experiment. The hemolymph from the surviving shrimp, which entered into the chronic stage of infection, was also collected. A 1 ml syringe fitted with a 25 g needle was inserted into the sinus located beneath the coxa segment of the third pereiopod. Approximately 0.2 to 0.5 ml of the hemolymph was obtained from each specimen. The hemolymph from the TSV-infected *P. stylirostris* was collected by the same procedure as was used for the *P. vannamei*. In this bioassay, there were no acute phase mortalities. Samples were collected over a period of 56 d.

Day 0 hemolymph sampled from the *Penaeus vannamei* and *P. stylirostris* was used as the negative control in the appropriate experiments. The hemolymph was put on ice and stored in a –70°C freezer until needed for the RT-PCR reactions. All PCR assays used hemolymph directly as the RNA template, extraction of the RNA was not necessary.

RT-PCR primers. Two oligonucleotide primers were used in the RT-PCR amplification procedure. Primers designated as 9195 and 9992 (Fig. 1) amplify a 231 bp sequence of the TSV genome. The primers were chosen using the Primer 2, Primer Designer Program Version 2.2 (Scientific and Educational Software, Stateline, PA) from the cDNA sequence information from a TSV P15 clone (Mari et al. 1998). These primers were synthesized at the University of Arizona, Arizona Research Laboratory, Division of Biotechnology Macromolecular Structures Facility, using a 4 column ABI 394A DNA synthesizer. After purification, the oligonucleotide primers were dispensed into aliquots and stored at –70°C.

Reverse transcription of TSV RNA. The RT-PCR assays were done in solution, using 1.0 µl of shrimp hemolymph as the RNA template. The GeneAmp®, EZ rTth RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) was used for all amplification reactions. This enzyme both reverse transcribes the RNA to cDNA and subsequently amplifies the cDNA. The optimized RT-PCR conditions (final concentrations in 50 µl total volume) for detection of TSV in *Penaeus vannamei* were: primers (0.46 µM each), dNTPs (300 µM each), rTth DNA polymerase (2.5 U/50 µl), manganese acetate (2.5 mM), in 5× EZ buffer (25 mM Bicine, 57.5 mM potassium acetate, 40% [w/v] glycerol, pH 8.2). The optimized RT-PCR conditions for detec-

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1   TCAATGAGAG CTTGGTCCTG GACTTCAAAC AGGTTATCTG TTACATTCTT
    AGTTACTCTC GAACCAGGAC CTGAAGTTTG TCCAATAGAC AATGTAAGAA

51  AGACAGCACT ACATTCTGAT TAGTGATTGG GATATCATTC ATCAAGATTG
    TCTGTCGTGA TGTAAGACTA ATCACTAACC CTATAGTAAG TAGTTCTAAC

101 TAGAACCTTC ACTGTTTGTA ACACTACCTC CTGGAATTAG TCCTCCACTG
    ATCTTGGAAG TGACAAACAT TGTGATGGAG GACCTTAATC AGGAGGTGAC

151 GTTGTTGTAT CAAAATTATC AATTTCAACT GGGTTAGCAG GCATTAATTA
    CAACAACATA GTTTTAATAG TTAAAGTTGA CCCAATCGTC CGTAATTAAT

201 AGTCCCACCA CGCAAGCGCG GCTGTCTACT T
    TCAGGGTGGT GCGTTCGCGC CGACAGATGA A
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Fig. 1. Sequence of the 231 bp TSV fragment. Underlined portion represents the position of the primers 9195F and 9992R

tion of TSV in the hemolymph from infected *Penaeus stylirostris* were the same as for *P. vannamei*, except that the amount of the rTth DNA polymerase was increased to 5.0 U/50 µl. Light mineral oil (50 µl) was overlaid on the top of the 50 µl reaction mixtures to prevent evaporation during thermal cycling.

The RNA template and all the reagents were combined and reverse transcription was allowed to proceed at 60°C for 30 min, followed by 94°C for 2 min, in an automatic DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, CT). At the completion of reverse transcription, the samples were amplified for 40 cycles under the following conditions: denaturation at 94°C for 45 s; annealing/extension at 60°C for 45 s. A final extension step for 7 min at 60°C followed the last cycle and the process was terminated in a 4°C soak file.

Agarose gel electrophoresis. Following the termination of RT-PCR, the amplified cDNA solutions were drawn off from beneath the mineral oil and placed into clean 0.5 ml microfuge tubes. A 10 µl sample of the amplified product was analyzed on a 2.0% agarose gel (Perkin Elmer Cetus, Norwalk, CT), stained with ethidium bromide (0.5 mg ml^{-1}), and electrophoresed in 0.5% TBE (Tris, boric acid, EDTA) (Maniatis et al. 1982). A 100 bp DNA ladder (Gibco/BRL, Gaithersburg, MD) was used as a marker. To confirm the specificity of the amplified RT-PCR product, Southern blot analysis (Maniatis et al. 1982) was performed using DIG-labeled cDNA derived from the same cDNA sequence data as the RT-PCR primers. The cDNA was labeled by the random primed labeling procedure according to the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN).

Histological procedures. Two of the 20 *Penaeus vannamei* were sampled in a moribund state and their cephalothoracies were preserved in Davidson's AFA (alcohol, formaldehyde and acetic acid) fixative for histopathological examination according to Bell & Lightner (1988). Two of the 10 *P. stylirostris* were randomly sampled for histological evaluation. Histopathology was carried out by routine procedures (Lightner 1996). Histopathological examination of the processed samples was performed using standard light microscopy.

***In situ* hybridization.** The sample animals that were fixed in Davidson's AFA and embedded in paraffin for histological analysis were also evaluated by *in situ* hybridization (Bruce et al. 1993, Lightner 1996). A series of consecutive sections, 4 µm thick, were mounted onto Superfrost®/Plus positively charged microscope slides (Fisher Scientific, Pittsburg, PA). These tissue sections were evaluated using the same DIG-labeled probe mixture as was used for the Southern blot confirmation (Maniatis et al. 1982). The *in situ* procedure for TSV is outlined in detail by Lightner (1996) and Hasson et al. (1997a).

RESULTS

A 90% mortality rate was reached 4 d post-infection in the bioassay experiment conducted with *Penaeus vannamei* (Table 1). One shrimp survived into the chronic phase of TSV infection and was sampled on Day 9. All of the hemolymph samples tested by RT-PCR revealed the 231 bp fragment indicative of TSV when analyzed on a 2% agarose gel (Fig. 2). These fragments were confirmed to be TSV specific by Southern blot analysis (data not shown). Histology confirmed that the experimental shrimp had acute phase TSV infections.

No mortalities were observed following the injection of the TSV inoculum in *Penaeus stylirostris* (Table 2). Hemolymph was sampled randomly over a period of 56 d. TSV was detected consistently in the hemolymph

Table 1. Detection of TSV in moribund, acute phase or in chronic phase *Penaeus vannamei* using RT-PCR and histological methods

Species	Date of hemolymph draw	Date (post-injection)	No. of samples	RT-PCR results	Histology results
P. vannamei	18 May 1997	0	1	Negative	Negative
P. vannamei	19 May 1997	1	3	Positive	Positive
P. vannamei	20 May 1997	2	2	Positive	Positive
P. vannamei	21 May 1997	3	1	Positive	Not examined
P. vannamei	22 May 1997	4	1	Positive	Positive
P. vannamei	27 May 1997	9	1	Positive	Positive

Table 2. Detection of TSV in randomly sampled *Penaeus stylirostris* using RT-PCR, routine histology and *in situ* hybridization

Species	Date of hemolymph draw	Date (post-injection)	No. of samples	RT-PCR results	Histology & *in situ* results
P. stylirostris	29 Sep 1997	0	1	Negative	Negative
P. stylirostris	2 Oct 1997	3	1	Positive	Not examined
P. stylirostris	6 Oct 1997	7	2	Positive	Not examined
P. stylirostris	9 Oct 1997	10	2	Positive	Not examined
P. stylirostris	16 Oct 1997	17	1	Positive	Negative
P. stylirostris	30 Oct 1997	31	1	Negative	Not examined
P. stylirostris	5 Nov 1997	37	2	Positive	Negative
P. stylirostris	17 Nov 1997	49	2	Negative	Not examined
P. stylirostris	24 Nov 1997	56	2	Negative	Not examined

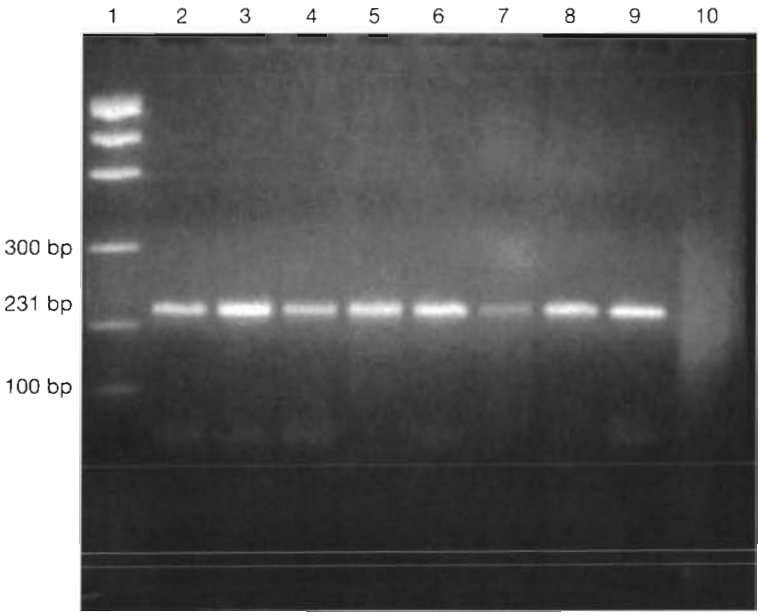

Fig. 2. Agarose electrophoresis gel of RT-PCR products from an RT-PCR assay using the primers 9195 and 9992. The 231 bp band indicates the presence of TSV. The hemolymph from TSV-infected *Penaeus vannamei* was used as the RNA template. Lane 1: 100 bp ladder; lanes 2–8: hemolymph from *P. vannamei* bioassay, corresponding to samples listed in Table 1 ; lane 9: positive TSV RT-PCR control; lane 10: Day 0 hemolymph negative RT-PCR control

samples to Day 17. A 31 d sample was negative by RT-PCR, but 6 d later (Day 37) 2 hemolymph samples were tested and TSV was once again detected. The next 2 hemolymph samples, taken on Days 49 and 56, were negative.

RT-PCR analysis of the *Penaeus stylirostris* hemolymph sampled on Day 37 indicated that TSV was present. These shrimp were also evaluated by histology and by *in situ* hybridization. The results from the 2 latter assays indicated that TSV was not detectable (Table 2). To determine if the RT-PCR reactions were valid, a bioassay using susceptible *P. vannamei* was performed using as the inoculum homogenized tails from the 37 d samples. The *P. vannamei* in this bioassay experienced a 90% mortality rate over a 4 d period and were determined TSV positive by histology. The bioassay results demonstrated that TSV was indeed present in the 37 d sample.

DISCUSSION

The primary methods for detection of TSV are histology, *in situ* hybridization and bioassay to enhance the virus. All of these methods are both labor and time consuming. RT-PCR provides an alternative non-lethal and rapid procedure for detection of the virus in hemolymph samples.

All of the experimental assays (RT-PCR, histology, *in situ* hybridization and bioassay) used to determine TSV infection demonstrated the presence of the virus in the acute phase of the disease. RT-PCR consistently detected TSV in hemolymph, during the acute phase of the bioassay using *Penaeus vannamei* as the indicator species. In the bioassay which used the less susceptible species *P. stylirostris*, RT-PCR consistently detected the virus until 17 d post-injection. Although negative on Day 31, a hemolymph sample from Day 37 indicated the presence of TSV. The corresponding Davidson's AFA-fixed samples, taken on the same days (Table 2), were determined to be negative for TSV when analyzed by histology and *in situ* hybridization. With the conflicting results from the RT-PCR assay in which TSV was detected, and the histology and *in situ* hybridization assays which indicated that TSV was not present, a bioassay using TSV susceptible *P. vannamei* became necessary to determine if, in fact, the shrimp were carriers of the virus. The confirmatory bioassay demonstrated the presence of TSV in the shrimp that were sampled on Day 37. In this study, RT-PCR was found to be a more sensitive assay when compared to histology or *in situ* hybridization for shrimp which have entered the chronic phase of this disease.

Presently, the limitations of detection of TSV by RT-PCR is approximately 14 d post-injection. Further work will concentrate on determining the length of time in which shrimp can carry TSV and remain infectious. Additional RT-PCR work will attempt to develop methods for using alternative RNA templates such as tissue homogenates in an assay with greater sensitivity in order to detect TSV in shrimp which may be asymptomatic carriers of the disease.

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