Vol. 52: 1–10, 2002 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published November 7

Detection of Taura syndrome virus (TSV) strain differences using selected diagnostic methods: diagnostic implications in penaeid shrimp

Heidi S. Erickson[1,*], Martha Zarain-Herzberg[2], Donald V. Lightner[1]

[1]Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, The University of Arizona, Tucson, Arizona 85721, USA

[2]Centro de Ciencias de Sinaloa, APDO Postal 1889, Culiacan, Sinaloa, Mexico

ABSTRACT: Anecdotal industry reports of Taura syndrome (TS) epizootics in a Taura syndrome virus (TSV) tolerant strain of *Penaeus stylirostris* and collected evidence of field TS epizootics in *P. stylirostris* suggested that a distinct new TSV strain might have emerged since 1994. The Ecuadorian 1992 TSV genome published in GenBank is virtually identical to the Hawaiian 1994 TSV isolate (HI94TSV) used as reference throughout this investigation. Three other geographic and year isolates of TSV from naturally occurring TS epizootics of cultured penaeid shrimp were obtained from Mexico (SIN98TSV and MX99TSV from *P. vannamei* and SON2KTSV from *P. stylirostris*). Selected TSV diagnostic methods set forth by the Office International des Epizooties were utilized as the basis for isolate analysis. By Southern blot, TSV probes P15 and Q1 reacted specifically with all the diagnostic reverse transcription polymerase chain reaction (RT-PCR) fragments. Additionally, labeled RT-PCR amplicons from the TSV isolates amplified by routine diagnostic RT-PCR primers gave positive *in situ* hybridizations with TSV, indicating that all 4 isolates shared homology. By Western blot, immuno-dot blot, and immunohistochemistry, all TSV-purified isolates reacted with TSV polyclonal antibody (PAb). However, with TSV monoclonal antibody (MAb) 1A1 all isolates, except SIN98TSV, reacted, indicating that the difference in isolate SIN98TSV is within VP1, the target for MAb 1A1. The amino acid (AA) sequence of SIN98TSV VP1, MX99TSV VP1 and SON2KTSV VP1 has a 98% homology with the reference HI94TSV VP1. A span of 12 AAs are identified in SIN98TSV VP1 containing significant AA substitutions which may account for a conformational change of the antigenic epitope sufficient to prevent MAb 1A1 from binding. The implications of these results with respect to the antibody-based diagnosis of TSV are discussed.

KEY WORDS: TSV · Taura syndrome virus · Diagnostic methods · Immunodetection

INTRODUCTION

Taura syndrome virus (TSV), the etiologic agent of Taura syndrome (TS), was tentatively classified in the family *Picornaviridae* (Bonami et al. 1997), and recent work has shown that it should be classified within the proposed new genus 'Cricket paralysis-like viruses' because it more closely resembles certain presently unclassified insect ssRNA viruses, such as Drosophila C Virus (DCV; Mari et al. 2002). TSV is a 32 nm, icosahedreal, non-enveloped viral particle with a buoyant density of 1.338 g ml^{-1}. Its protein capsid is comprised of 3 major polypeptides (55, 40, 24 kDa designated VP1, VP2 and VP3, respectively) and 1 minor polypeptide (58 kDa designated V0) (Bonami et al. 1997, Mari et al. 2002). The TSV genome consists of a linear, positive-sense, single-stranded ribonucleic acid (ssRNA) of 10 205 bases (Mari et al. 2002).

TS was first recognized as a distinct disease in shrimp farms located near the mouth of the Taura River

*E-mail: heidiserickson@hotmail.com

in the Gulf of Guayaquil, Ecuador, in mid-1992 (Jimenez 1992, Lightner et al. 1995), where the disease caused catastrophic losses with cumulative mortality rates from 60 to >90% of affected pond-cultured juvenile *Penaeus vannamei*. Reports of occurrences of the disease now include Ecuador, Peru, Colombia, Honduras, Jamaica, Guatemala, El Salvador, Brazil, Nicaragua, Belize, the USA, the Mexican states of Sonora, Sinaloa, Chiapas Guerrero, Yucatan, and most recently Taiwan (Lightner 1996a,b, Chien et al. 1999, Yu & Song 2000). To date, it is estimated that the worldwide economic impact of TS to shrimp farming may have exceeded $2 billion dollars (Rosenberry 1997, 2000).

The worldwide geographic distribution of TSV indicates that the same virus or closely related viruses are responsible for the TS epizootics that have occurred throughout the Americas since 1992 (Lotz 1997b, Hasson et al. 1999). Ecuadorian 1992 and Hawaiian 1994 TSV isolates were found to be identical in their biophysical, biochemical and biological characteristics (Bonami et al. 1997). However, anecdotal evidence of the occurrences of TS epizootics in *Penaeus stylirostris* since 1999 in Mexico suggested that a closely related virus might have evolved from the original Ecuadorian/Hawaiian isolate characterized by Bonami et al. (1997) and Mari et al. (2002). If TSV has changed since 1994, it is possible that a change in the virus, potentially in its structural proteins, is responsible for the emergence of TS in a previously TS refractive species, *P. stylirostris*.

The Office International de Epizooties (OIE) list of crustacean diseases considered for notification and certification presently contains 3 'Diseases Notifiable to the OIE' and 5 'Other Significant Diseases'. Seven of the 8 crustacean diseases listed by the OIE occur in penaeid shrimp and of the 3 notifiable diseases of crustaceans TSV is one. Current OIE recommended diagnostic and detection methods for TSV include histopathology, bioassay, *in situ* hybridization (ISH) with TSV-specific gene probes, reverse-transcription polymerase chain reaction (RT-PCR) with TSV-specific primers, and immunoassays with TSV-specific antibodies (OIE 2000). Except for histopathology, the TSV recognized detection methods are either specific to a portion of the TSV genome (ISH and RT-PCR) (Mari et al. 1998, Nunan et al. 1998) or specific to an antigenic epitope on TSV VP1 (Poulos et al. 1999). The potential change in TSV and its ability to cause TS in *Penaeus stylirostris* raise serious questions about the potential limitations in the OIE (2000) recognized detection methods for TSV. The current report details the analysis of 4 geographic and year isolates of TSV from Hawaii and Mexico, utilizing selected OIE recommended TSV diagnostic and detection methods, and discusses the implications newly demonstrated TSV strain differences have for the detection of TSV in penaeid shrimp.

MATERIALS AND METHODS

Shrimp. Shrimp taxonomy as used in this paper is according to Holthius (1980). For bioassays and virus generation, specific pathogen free (SPF) (Lotz 1997a) Kona stock *Penaeus vannamei* obtained from the Oceanic Institute's SPF breeding program (Hawaii) (Wyban et al. 1992, Carr et al. 1996) were reared and maintained according to the protocol of Williams et al. (1992). The SPF Kona stock *P. vannamei* is the reference shrimp line (indicator species) used in all University of Arizona Aquaculture Pathology Laboratory (UAZAPL) penaeid shrimp virus research including that for TSV (Hasson et al. 1995, 1999), white spot syndrome virus (WSSV) and yellow head virus (YHV) (Durand et al. 2000, Lightner et al. 2001).

Virus isolates and bioassay. The origin of the viral materials used in this study is shown in (Table 1). The TS disease status of the shrimp was confirmed by routine histopathology with hematoxylin and eosin-phloxine (H&E) staining (Lightner et al. 1995, 1997b, Lightner 1996a). The isolates were processed for bioassay analysis and experimental quantities of the viruses were obtained by feeding or intramuscular injection of SPF *Penaeus vannamei* (Wyban et al. 1992, Lotz 1997b) with TSV-infected shrimp homogenates according to the protocol of Hasson et al. (1995). All moribund shrimp were collected for histopathology and/or virus purification and mortality data was recorded daily. All analyses utilized the 1994 isolate from Hawaii (HI94TSV) as the reference isolate. For the purposes of this paper the term 'TSV isolate(s)' refers to 'TSV geographic and year isolate(s)'. All viral isolates from different geographic regions and years were kept strictly separate during bioassay, purification and testing (Table 1).

Table 1. Description of the TSV isolates collected from cultured juvenile penaeid shrimp during reported TSV natural infections and mean ($\bar{x}$) percent mortality from bioassays

TSV isolate	Collection location	Source species	Collection year	Bioassay $\bar{x}$ mortality (%)
HI94TSV	Hawaii, USA	*Penaeus vannamei*	1994	95
SIN98TSV	Sinaloa, Mexico	*P. vannamei*	1998	43
MX99TSV	Sonora, Mexico	*P. stylirostris*	1999	95
SON2KTSV	Sonora, Mexico	*P. stylirostris*	2000	80

Virus purification. TSV isolates used for testing were purified from experimentally infected *Penaeus vannamei* tissue according to the procedure of Bonami et al. (1997). Purity of viral extracts was assessed by transmission electron microscopy (TEM) using 2% phosphotungstic acid (PTA) as the negative stain and by routine diagnostic RT-PCR (Nunan et al. 1998, OIE 2000).

Antibodies. Mouse anti-TSV polyclonal antibody (PAb) used in this study was produced as described by Poulos et al. (1999) and stored at –20°C until used. The mouse anti-TSV monoclonal antibody (MAb) used was MAb 1A1 supernatant fluid maintained and produced by UAZAPL as described by Poulos et al. (1999). This antibody is also available from DiagXotics, in their commercial TSV immuno-dot blot and immunohistochemistry kits.

Immuno-dot blot assays. An MAb titration assay was conducted to determine the concentration of MAb 1A1 in the supernatant fluid used for immunoassays. Specificity assays for PAb and MAb to individual TSV isolates, followed the protocol of Poulos et al. (1999). Reactions were graded using the PAb at a dilution of 1:500 against purified HI94TSV isolate, with G4 being the strongest and G1 being the weakest. A negative reaction was one in which no color spot was visible in the well. Normal mouse serum was used as a negative control for the primary antibody. A 'no template control' (i.e. no dotting of purified virus) was also employed in each assay.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The structural proteins of individual purified TSV isolates were denatured in Laemmli buffer (Laemmli 1970) containing 10 M urea and separated using 10% SDS-PAGE according to the protocol of Bonami et al. (1997). A premixed protein molecular weight marker (Boehringer Mannheim), with proteins ranging from 14.4 to 97.4 kDa, was co-electrophoresed in the SDS-PAGE for reference. A prestained SDS-PAGE standard, low range marker (BIO-RAD), was also run to provide a visual reference for Western blot analysis. The marker proteins had apparent molecular weights of 106, 81, 47.5, 36.3, 28.2, and 20.8 kDa. Two simultaneous gels containing the 4 purified TSV isolates were electrophoresed. One of the gels was silver stained according to the protocol of Nielsen & Brown (1984) and the other gel was used for transfer to nitrocellulose. A third gel containing the four purified TSV isolates was electrophoresed and used for transfer to a polyvinyliden diflouride-type membrane (PVDF).

Western blot analysis. The TSV structural proteins of the 4 isolates separated by 10% SDS-PAGE were transferred to a nitrocellulose membrane (Towbin et al. 1979, Poulos et al. 1999) and the presence of proteins were visualized with 0.5% Ponceau Red (0.5 ml Ponceau Red, 1ml glacial acetic acid, 98.5 ml DDH_2O) (Sigma) according to the protocol of Nakamura et al. (1985). The Western blot assay was conducted according to the protocol of Poulos et al. (1999, 2001). Each TSV isolate on the membrane was reacted with anti-TSV PAb (diluted 1:500) or anti-TSV MAb 1A1 (undiluted) and the reactions to the proteins were visualized and graded as in the immuno-dot blot assay. Leibovitz's Medium L-15 (Irvine Scientific) was used as a negative control for the primary antibody.

Immunohistochemistry on fixed sections. Experimentally infected moribund shrimp were fixed according to standard methods (Bell & Lightner 1998) with Davidson's alcohol-formalin-acetic acid (AFA) for 24 h. Tissues were processed and immunohistochemistry on fixed sections was performed according to the protocol of Poulos et al. (2001), using anti-TSV PAb or anti-TSV MAb as the primary antibodies. The slides were examined by light microscopy for the presence of a blue-black precipitate in the cytoplasm of TSV-infected cells.

RT-PCR primers. Two TSV-specific oligonucleotide primers amplified a 231 bp region of the TSV genome were employed to confirm the presence of TSV (Nunan et al. 1998) in each purified TSV preparation. Two additional TSV-specific oligonucleotide primers were designed to amplify the entire VP1 structural protein region of the TSV genome (Mari et al. 2002). Primers designated as TSV55P1 and TSV55P2 (Table 2) amplify a 1303 bp region of the TSV genome. Digoxigenin (DIG)-labeling of TSV gene probes P15 and Q1 was achieved by PCR employing cloned DNA and utilizing Q1-specific primers (9193 and 9194) and P15-specific primers (9195 and 9196) (Table 2, Fig. 1). All primers were chosen using Primer Designer 4 (Scientific and Educational Software) from the TSV nucleotide and amino-acid sequence information for the Ecuadorian 1992 TSV (EC92TSV)/HI94TSV isolate, GenBank accession #AF277674 (Mari et al. 2002).

Table 2. Oligonucleotide primers employed in RT-PCR amplification of the TSV diagnostic fragment (DF) and VP1 and for PCR production of TSV-specific gene probes Q1 and P15, using the nucleotide sequence of HI94TSV, GenBank accession #AF277674

Amplicon designation	Primer	Nucleotide position	Length of primer	Amplicon length (bp)
Q1	9194	3218	18-mer	905
	9193	4122	18-mer	
P15	9196	5915	18-mer	1209
	9195	7123	18-mer	
VP1	TSV55P1	7901	20-mer	1303
	TSV55P2	9203	20-mer	
DF	9992	6910	18-mer	231
	9195	7140	18-mer	

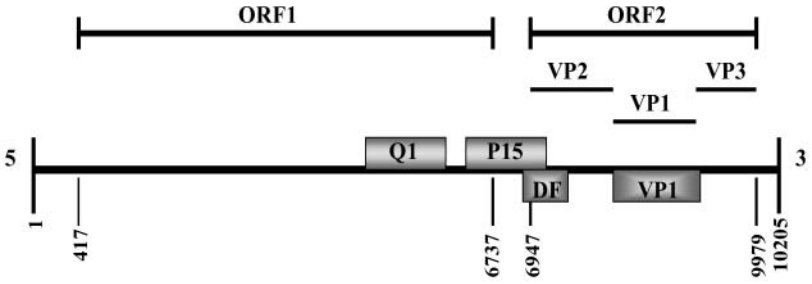

Fig. 1. Diagram of TSV genome regions amplified by RT-PCR using the oligonucleotide primers employed in RT-PCR in Table 2. This is based upon the TSV genome organization proposed by Mari et al. (2002), GenBank accession #AF277674. TSV gene probes Q1 and P15 are indicated in gray boxes above the line. VP1 and diagnostic fragment (DF) amplicons are shown by gray boxes below the line. Note the overlap of genome regions recognized by gene probe P15 and amplified by the diagnostic RT-PCR primers 9195/9992 (DF)

RT-PCR of purified TSV. The RT-PCR assays were done using 1.0 µl of purified virus as the RNA template. The 231 bp region of the TSV genome was amplified according to the protocol of Nunan et al. (1998). The Sigma® Enhanced Avian RT-PCR Kit (Sigma-Aldrich) was used for all TSV VP1 (1303 bp fragment) amplification reactions. The conditions (final concentration in 50 µl total volume) for 1-step RT-PCR amplification of TSV VP1 from purified TSV isolates were those set forth in the manufacturer's protocol. The RNA template and all the reagents were combined and reverse transcription was allowed to proceed at 50°C for 45 min, followed by 95°C for 5 min. After reverse transcription, the samples were amplified for 40 cycles using the following conditions: denaturation at 95°C for 1 min; annealing at 60°C for 45 s; and extension at 72°C for 1.5 min. A final extension at 72°C for 5 min followed the last cycle. RT-PCR products were visualized by standard agarose gel electrophoresis (Nunan et al. 1998).

Nucleotide and derived amino acid sequence and analysis. TSV nucleotide sequence data for the VP1 portion of the coat protein of SIN98TSV, MX99TSV, SON2KTSV and HI94TSV (GenBank accession #AF-510515, #AF510516, #AF510517, #AF510518, respectively) was obtained by sequencing the 1303 bp RT-PCR products at the University of Arizona Genomic Analysis and Technology Core on an ABI PRISM® 377 DNA Sequencer with 'XL' upgrades (Applied Biosystems). The HI94TSV VP1 nucleotide and derived amino acid (AA) sequence was compared to the EC93TSV sequence data from GenBank accession #AF277674 (Mari et al. 2002) and confirmed to be virtually identical. The regions and nucleotide sequences of the TSV genome, represented by the TSV specific clones P15 and Q1 (Mari et al. 1998) and the diagnostic TSV RT-PCR amplicon (Nunan et al. 1998, OIE 2000) were identified within the EC93TSV/HI94TSV sequence and the VP1 nucleotide and derived AA sequences from each of the TSV isolates were analyzed using the University of Wisconsin Genetics Computer Group (GCG) program (Devereux et al. 1984).

Protein sequencing. The TSV structural proteins of the 4 isolates separated by 10% SDS-PAGE were transferred to Immobilon-P^{SQ} PVDF (0.2 µm pore size) (Millipore) and the presence of proteins were visualized with 0.1% Coomassie blue (Wilson 1983, Matsudaria 1987). The VP1 amino termini (NH_3^+) from each isolate were sequenced out 12 cycles at the University of Arizona Laboratory for Protein Sequencing and Analyses using an ABI 477A pulsed-liquid protein sequencer (Applied Biosystems).

Southern blot hybridization and *in situ* hybridization. Southern blot analysis (Maniatis et al. 1982) of the RT-PCR amplified 231 and 1303 bp products of each TSV isolate was performed using TSV DIG-labeled TSV-specific gene probes P15 and Q1 (Mari et al. 1998, Nunan et al. 1998). Shrimp used for immunohistochemistry were also evaluated by *in situ* hybridization (Bruce et al. 1993, Lightner 1996a) utilizing the same TSV-specific DIG-labeled probe mixture that was used for the Southern blot analysis (Maniatis et al. 1982) and according to the procedures of Lightner (1996a), Mari et al. (1998) and Hasson et al. (1999). The TSV-specific gene probes were DIG-labeled as described in the section on RT-PCR primers.

RESULTS AND DISCUSSION

The purpose of this study was to determine whether or not 4 Hawaiian and Mexican year isolates of TSV that showed possible differences in virulence and host range could be detected and distinguished by selected OIE TSV diagnostic and detection methods. These isolates from natural infections in cultured *Penaeus vannamei* and *P. stylirostris* were obtained during a period of apparent transition to a broader host range than was previously recognized and they represented clinical samples that aquaculture diagnostic laboratories would receive for TSV diagnostic testing. Bioassays were performed in *P. vannamei*, but not in SPF *P. stylirostris* since such shrimp were not available during the course of the study.

The farm information on differences in TSV virulence in *Penaeus vannamei* populations was supported by results from primary, standard, individual bioassays

and from subsequent bioassays performed using infected tissue generated from the primary bioassays. Hawaiian isolate HI94TSV (i.e. the reference isolate) and the Mexican isolate MX99TSV (Table 1) affected *P. vannamei* similarly, with both causing 95% mortality (Table1) during the acute phase of TS. By contrast, isolates SIN98TSV and SON2KTSV gave 43 and 80% mortality, respectively (Table 1), during the acute and transition phases of TS. Moribund shrimp samples collected during the bioassays of all 4 isolates presented pathognomonic acute phase TSV lesions characterized by the classic 'peppered or buckshot appearance' in the cuticular epithelium (Lightner 1996a). The presence of TSV was confirmed in the serial samples by *in situ* hybridization with TSV-specific gene probes giving positive results similar to those seen in Fig. 4a (see Fig. 4).

The biological characteristics and the genome of TSV isolate EC92TSV is identical to that of the reference isolate HI94TSV and the 2 isolates are considered to be the same virus (Bonami et al. 1997, Mari et al. 2002). The major structural polypeptides found in the second open reading frame (ORF) have been designated VP1 for the 55 kDa protein, VP2 for the 40 kDa protein, and VP3 for the 24 kDa protein (Fig 1; Bonami et al. 1997, Robles-Sikisaka et al. 2001, Mari et al. 2002). The HI94TSV VP1 nucleotide and derived AA sequence (GenBank accession #AF510518) was compared to the EC93TSV sequence data from GenBank accession #AF277674 (Mari et al. 2002) and confirmed to be virtually identical. The purity of the TSV isolate preparations used in this study was confirmed by TEM (data not shown).

Routine diagnostic TSV RT-PCR (OIE 2000) produces a 231 bp fragment, amplified by primers 9195 and 9992 (Nunan et al. 1998). The nucleotide sequence comprises a portion of the VP2 structural protein region of the TSV genome. In addition to these primers, 2 TSV-specific oligonucleotide primers (TSV55P1 and TSV55P2 in Table 2) were also designed to amplify the 1303 bp fragment that encompasses the entire VP1 structural protein region of the TSV genome (Mari et al. 2002) that does not overlap the region amplified by primers 9195 and 9992 (Fig. 1). The TSV RT-PCR system described by Nunan et al. (1998) could not be utilized due to the inability of the GeneAmp® EZ rTth RNA PCR kit (Perkin Elmer Cetus) to efficiently transcribe a large target fragment. By switching to the Sigma® Enhanced Avian RT-PCR Kit (Sigma-Aldrich) and using a novel RT-PCR protocol, the 1303 bp fragment that encompasses the entireVP1 region of the genome was obtained from all 4 TSV isolates (Fig. 2a). No differences were observed in the ability of the diagnostic primers 9195 and 9992 or those for VP1 (TSV55P1 and TSV55P2) to amplify their respective fragments by routine TSV RT-PCR (Fig. 2a) from all 4 TSV isolates.

Two TSV gene probes, P15 and Q1 (Mari et al. 1998) are used together as a cocktail in TSV gene probe analysis (OIE 2000), with Q1 spanning a region at the 3 prime end of the first ORF and P15 spanning a region beginning in the first ORF at and ending in the second ORF in VP2 (Table 2). By Southern blot analysis with TSV gene probes P15 and Q1 hybridized with the 231 bp RT-PCR products of VP2 (Lanes 3, 5, 7, and 9 in Fig. 2b) while they did not hybridize with the 1303 bp RT-PCR products from VP1 (Lanes 2, 4, 6, and 8 in Fig. 2b). Therefore, the commercially available gene probes used in this study did not hybridize to fragments from other regions of the TSV genome such as VP1 (Fig. 1).

The Southern blot (Fig. 2b) confirmed the specificity of the gene probes to react with the diagnostic RT-PCR fragment of the TSV genome (Nunan et al. 1998, OIE 2000) and further demonstrated that the gene probes do not hybridize with the VP1 portion of the amplified

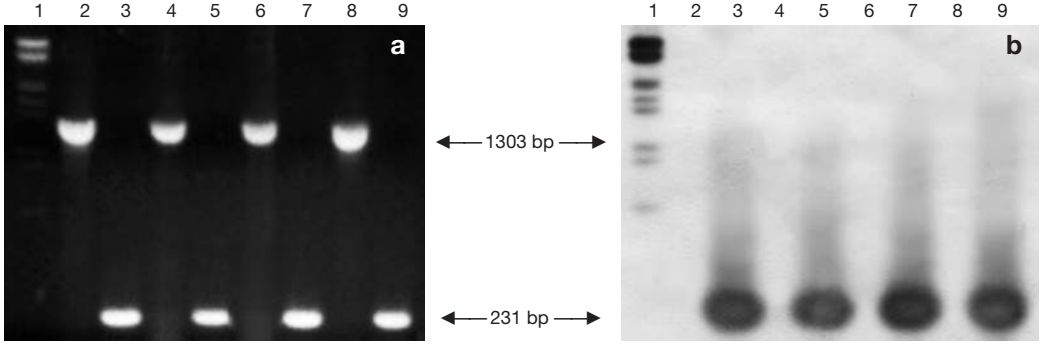

Fig. 2. RT-PCR products of TSV demonstrating no detectable strain differences between the 4 TSV isolates by gel electrophoresis or Southern blot hybridization using diagnostic TSV gene probes P15 and Q1. (a) Electrophoretic gel showing amplicons (231 bp) from primers 9195 and 9992 and amplicons (1303 bp) from primers TSV55P1 and TSV55P2 that amplify VP1. (b) Southern blot showing P15 and Q1 hybridization with the 231 bp amplicons (Lanes 3, 5, 7, and 9) but not with the 1303 bp amplicons of VP1. Lane 1, DIG-labeled DNA ladder. Lanes 2 to 9, primers TSV55P1/P2 and 9195/9992, respectively, used with HI94TSV (Lanes 2 and 3), SIN98TSV (Lanes 4 and 5), MX99TSV (Lanes 6 and 7) and SON2KTSV (Lanes 8 and 9)

sequence. Together with the *in situ* hybridization results obtained after proper sample collection, fixation, and histological processing protocols (Lightner 1996a, Hasson et al. 1997), the data indicate that all of the TSV isolates analyzed shared homology in the regions of the genome recognized by the TSV gene probes.

Since the TSV isolates purified from tissue collected during bioassays and used in immuno-dot blot and Western blot analysis were purified prior to the optimization of TSV real time RT-PCR, the quantitation of the number of virions present in each preparation was not determined. However, based upon the Ponceau Red visualization of the purified TSV isolates present on the electroblotted membrane, prior to Western blot analysis, the preparations of purified isolates HI94TSV, MX99TSV, and SON2KTSV were present in approximately equal quantities, while the preparation of purified isolate SIN98TSV appeared to be present at 2 times the concentration of the other preparations (see Fig. 5a).

By immuno-dot blot, PAb successfully detected all 4 TSV isolates. By contrast, MAb 1A1 detected all except isolate SIN98TSV (Fig. 3). When the standard grading system (G4 = strongest and G1 = weakest) was applied to the immuno-dot blot with TSV PAb, isolate HI94TSV produced a G4 reaction as expected, isolate SIN98TSV produced a G4 reaction, and isolates MX99TSV and SON2KTSV produced a G3 reaction (Fig. 3.). When MAb 1A1 was used to develop the immuno-dot blot, isolate SIN98TSV did not react, while isolates MX99-TSV and SON2KTSV produced a markedly reduced reaction signal (G1), compared to the reaction produced in the wells inoculated with isolate HI94TSV (G4) (Fig. 3). Poulos et al. (1999) stated that immuno-dot blot with MAb 1A1 is highly specific for TSV (HI94TSV). Considering the Ponceau Red staining results indicating comparable amounts of protein present in the preparations, the demonstration of a weaker immuno-dot reaction of MAb 1A1 with isolates MX99TSV and SON2KTSV when compared to the reference isolate HI94TSV supports the hypothesis that

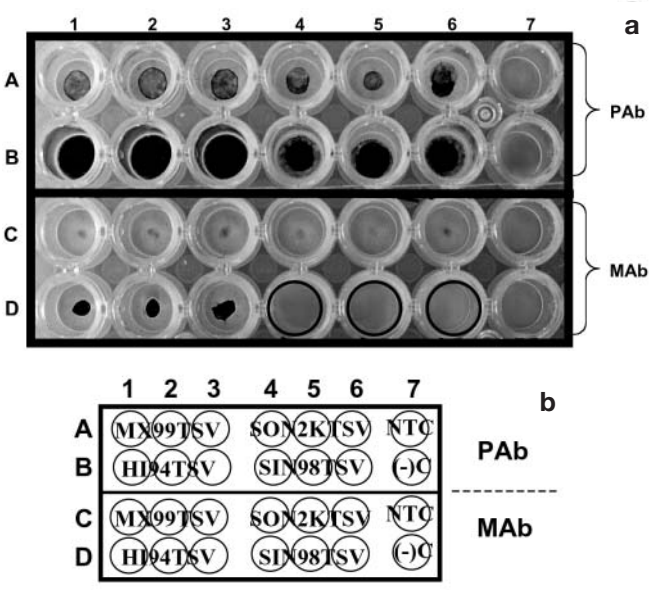

Fig. 3. (a) Immuno-dot blot of TSV PAb (Rows A and B) and TSV MAb 1A1 (Rows C and D) reacted with MX99TSV (A1-3 and C1-4), SON2KTSV (A4-6 and C4-6), HI94TSV (B1-3 and D1-3), and SIN98TSV (B4-6 and D4-6). A7 and C7 are no-template controls while B7 and D7 are negative control mouse serum. Circled wells D4-6 for SIN98TSV indicate no reaction to MAb 1A1. (b) Map diagram showing locations of TSV isolates in the wells

isolates MX99TSV and SON2KTSV have a structural difference to HI94TSV in the epitope recognized by MAb 1A1 (Poulos et al. 1999).

The immuno-dot blot results were corroborated by the immunohistochemistry (IHC) results, with SIN98-TSV being the only tissue not detected by MAb 1A1. A comparison of the serial tissue sections of SIN98TSV processed for ISH and IHC confirmed that isolate SIN98TSV can be detected by routine TSV ISH with TSV-specific gene probes P15 and Q1, but cannot be detected by TSV IHC with MAb 1A1 (Fig. 4). MAb 1A1 used in these studies recognizes an antigenic epitope somewhere in the VP1 region (Poulos et al. 1999) of the TSV genome (Mari et al. 2002), which is a completely

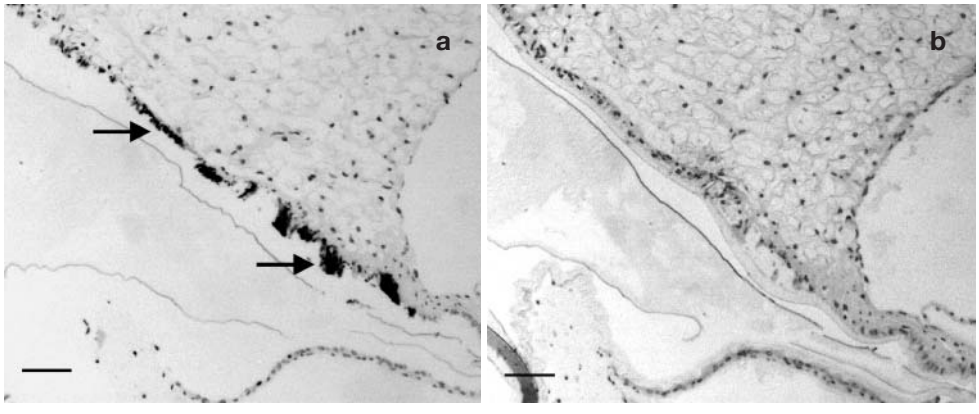

Fig. 4. SIN98TSV serial sections analyzed using gene probes P15 and Q1 and TSV MAb 1A1. In (a) arrows indicate a positive reaction to the TSV-specific gene probes P15 and Q1, while the parallel section in (b) shows no reaction to MAb 1A1, confirming that a positive ISH with a negative IHC, reacted with MAb 1A1, may be used to distinguish the strain, SIN98TSV. Scale bars = 50 µm

different region of the genome from that recognized by the TSV gene probes (Table 2, Fig. 1).

Silver stained SDS-PAGE of the TSV isolates demonstrated the expected presence of TSV major and minor structural proteins (data not shown). Unstained SDS-PAGE of the TSV isolates was transferred to a nitrocellulose membrane and the presence of TSV structural proteins was visually confirmed by pre-staining with 0.5% Ponceau Red (Fig. 5a). By Western blot (Fig. 5b) all of the isolates reacted with the TSV PAb. However, VP1 from all isolates except SIN98TSV reacted with MAb 1A1 (Fig. 5b), again confirming the previous immuno-dot blot and IHC results.

Our data from electrophoresis indicates that VP1 of SIN98TSV migrates to a different position than does VP1 of the reference strain HI94TSV. Furthermore, when the VP1 bands are reacted with MAb 1A1 by Western blotting, SIN98TSV VP1 does not react whereas the VP1 band from HI94TSV does react. By protein sequencing 12 cycles of the amino termini of VP1 from isolates HI94TSV and SIN98TSV, we were able to confirm that SIN98TSV's putative VP1 demonstrated by SDS-PAGE was indeed VP1. The VP1 amino termini of all 4 isolates contained the AA sequence SKDRDMTKVNAY (Fig. 6), which corresponds to the first reading frame of the VP1 nucleotide sequences (GenBank accession #AF510515, #AF510516, #AF510517 and #AF510518 corresponding to SIN98TSV VP1, MX99TSV VP1, SON2KTSV VP1 and HI94TSV VP1, respectively) obtained from the 1303 bp amplicons.

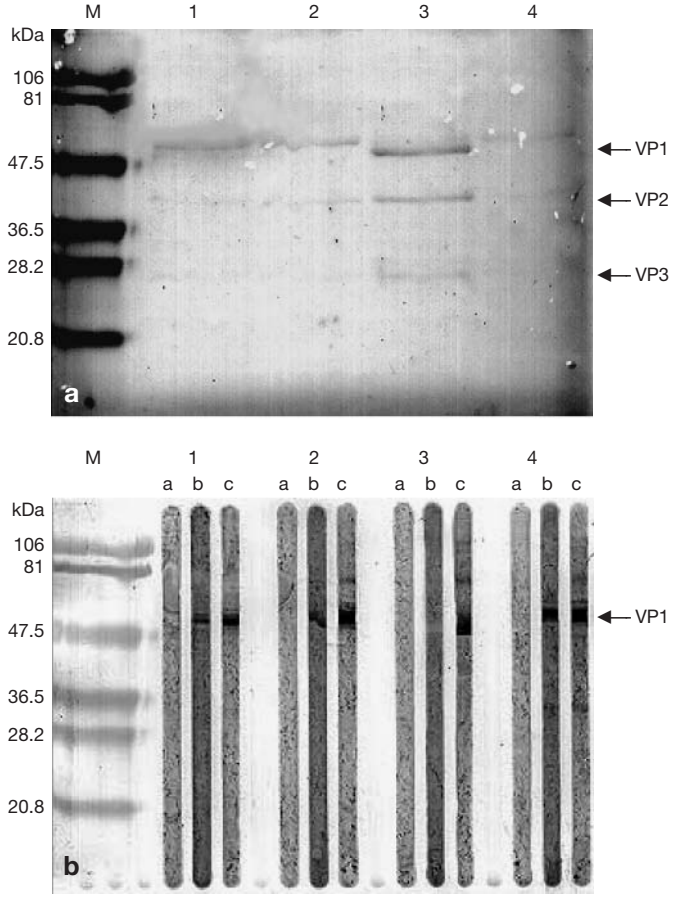

Fig. 5. Western blot membrane of proteins from purified TSV isolates (a) prestained with Ponceau Red and (b) reacted with TSV PAb and MAb 1A1. Lane M, prestained molecular weight marker. Lane 1, SON2KTSV. Lane 2, MX99TSV. Lane 3, SIN98TSV. Lane 4, HI94TSV. In (b), TSV proteins were reacted with TSV PAb (Sublanes c), MAb 1A1 (Sublanes b) and Leibovitzís Medium L-15 as the negative control (Sublanes a). PAb reacted with VP1 of all 4 isolates (Sublanes c). MAb 1A1 reacted with all isolates except SIN98TSV VP1 (Lane 3b). The position of TSV viral proteins is indicated on the right side of the photograph

The derived AA sequence of VP1 of isolates SIN98-TSV, MX99TSV and SON2KTSV have a 98% homology with the VP1 AA sequence of reference isolate HI94TSV. Isolate SIN98TSV contains 7 AA substitutions, while isolates MX99TSV and SON2KTSV contain only 4 AA substitutions (Fig. 6). Branden & Tooze (1999) state that an antibody generally recognizes an antigenic epitope of approximately 5 to 11 AAs and that MAb binding may be very susceptible to conformational changes in the antigen. The hypothesis that isolates SIN98TSV, MX99TSV and SON2KTSV have a structural difference to HI94TSV in the epitope recognized by MAb 1A1 is supported by the AA substitutions at positions 292, 300, 301 and 304 (Fig. 6). This is the only region in which all 3 isolates differ from HI94TSV and constitutes a span of 12 AA residues. SIN98TSV VP1 has 2 significant AA substitutions, one at AA 300 changes from a glycine (G; which only has a hydrogen for a side chain) to a serine (S; which is a polar AA) and one at AA 301 changes from a G to a lysine (K; which is a charged AA), which may account for a conformational change of the epitope significant enough to prevent MAb 1A1 from binding. MX99TSV VP1 and SON2KTSV VP1 contain identical substitutions at AA 301 (glycine to arginine: G to R) and AA 304 (arginine to lysine: R to K; insignificant substitution within the charged AA group), which may account for a subtle conformational change of the epitope responsible for the reduction in affinity of MAb 1A1 to the putative antigenic epitope.

The data presented here demonstrate that MAb 1A1 based methods may not detect all TSV isolates and offer important implications for TSV surveillance and diagnostic detection in cultured penaeid shrimp. Presently, only 1 MAb is available for use in OIE (2000) recognized TSV antibody based detection methods and it is recommended that results from its use should not be relied on as the only confirmatory test following histopathology. OIE recommends that

```
HI94TSV VP1       1 skdrdmtkvnayenlpgkgfthgvgfdygvplslfpnnaidptiavpegl
SIN98TSV VP1      1 ....................................l.............
MX99TSV VP1       1 ....................................l.............
SON2KTSV VP1      1 ....................................l.............

HI94TSV VP1      51 demsieylaqrpymlnrytirggdtpdahgtiiadipvspvnfslygkvi
SIN98TSV VP1     51 ....................................l.............
MX99TSV VP1      51 ...........................e........l.............
SON2KTSV VP1     51 ...........................e........l.............

HI94TSV VP1     101 akyrtlfaapvslavamanwwrgninlnlrfaktqyhqcrllvqylpygs
SIN98TSV VP1    101 ...........g....l....l.....l.....l.....l.....l.....
MX99TSV VP1     101 ....................l.....l.....l.....l.....l.....
SON2KTSV VP1    101 ....................l.....l.....l.....l.....l.....

HI94TSV VP1     151 gvqpiesilsqiidisqvddkgidiafpsvypnkwmrvydpakvgytadc
SIN98TSV VP1    151 ....................l.....l.....l.....l.....l.....
MX99TSV VP1     151 ....................l.....l.....l.....l.....l.....
SON2KTSV VP1    151 ....................l.....l.....l.....l.....l.....

HI94TSV VP1     201 apgrivisvlnplisastvspnivmypwvnwsnlevaepgtlakaaigfn
SIN98TSV VP1    201 ......v.....l.....l.....l.....l.....l.....l.....
MX99TSV VP1     201 ....................l.....l.....l.....l.....l.....
SON2KTSV VP1    201 ....................l.....l.....l.....l.....l.....

HI94TSV VP1     251 ypadvpeeptfsvtrapvsgtlftllqdtkvslgeadgvfslyftnttg
SIN98TSV VP1    251 ....................l.....l.....v.....l.....l.....s
MX99TSV VP1     251 ....................l.....l.....v.....l.....l.....
SON2KTSV VP1    251 ....................l.....l.....g.....l.....l.....

HI94TSV VP1     301 grhrlayaglpgelgsceivklpqgqysieyaatsaptlvldrpifsepi
SIN98TSV VP1    301 k.....l.....l.....l.....l.....l.....l.....l.....
MX99TSV VP1     301 r..k..l.....l.....l.....l.....l.....l.....l.....
SON2KTSV VP1    301 r..k..l.....l.....l.....l.....l.....l.....l.....

HI94TSV VP1     351 gpkyvvtkvkngdvvgiseetlvtcgsmaaigeatval
SIN98TSV VP1    351 .....l.....l.....l.....l.....l.....l.....
MX99TSV VP1     351 .....l.....l.....l.....l.....l.....l.....
SON2KTSV VP1    351 .....l.....l.....l.....l.....l.....l.....
```

Fig. 6. VP1-deduced amino acid sequences from SIN98TSV, MX99TSV and SON2KTSV (GenBank accession #AF510515, #AF510516 and #AF510517, respectively) compared to the HI94TSV amino acid (AA) sequence (GenBank accession #AF510518). All 3 isolates (SIN98TSV, MX99TSV and SIN98TSV) demonstrate 98% homology with HI94TSV VP1 AA sequence. The only region in which all 3 isolates differ from HI94TSV constitutes a span of 12 AA residues (AA position 292 through 304). SIN98TSV VP1 has 2 significant AA substitutions, one at AA 300 and one at AA 301, which may account for a conformational change of the epitope significant enough to prevent MAb 1A1 from binding. MX99TSV VP1 and SON2KTSV VP1 contain identical substitutions at AA 301 and AA 304, which may account for a subtle conformational change of the epitope responsible for the reduction in affinity of MAb 1A1 to the putative antigenic epitope

more than one diagnostic detection method for viral pathogens of penaeid shrimp should be conducted whenever analyzing the disease status of samples for importation, exportation, routine health monitoring, population screening or research. Indeed, we have shown that screening of penaeid shrimp broodstock and postlarvae by MAb 1A1 testing will not detect all TSV isolates, possibly leading to false negative results, further spread of TSV and re-emergence of TS in regions where it has been eradicated. An additional battery of TSV MAbs is needed to recognize conserved and individual antigenic epitopes of all TSV strains.

Acknowledgements. Support for this research was provided by the Gulf Coast Research Laboratory Consortium Marine Shrimp Farming Program, CSREES, under Grant No. 99-38808-7431. Special thanks to Kenneth Hasson (Super Shrimp), and Max Lopez (Aquanova) for providing the isolates used in these studies. Thanks to Debbie Bradley-Dunlop, Kerri Kislin, Leone Mohney, Solangel Navarro, Carlos Pantoja, Bonnie Poulos, Rita Redman, Paul Schofield and Brenda White (UAZAPL) for their input and/or assistance.

LITERATURE CITED

Bell TA, Lightner DV (1988) A handbook of normal penaeid shrimp histology. World Aquaculture Society, Baton Rouge, LA

Bonami JR, Hasson KW, Mari J, Poulos BT, Lightner DV (1997) Taura Syndrome of marine penaeid shrimp: characterization of the viral agent. J Gen Virol 78:313–319

Bruce LD, Redman RM, Lightner DV, Bonami JR (1993) Application of gene probes to detect a penaeid shrimp baculovirus in fixed tissue using *in situ* hybridization. Dis Aquat Org 17:215–221

Carr WH, Sweeney JN, Nunan L, Lightner DV, Hirsh HH, Reddington JJ (1996) The use of an infectious hypodermal and hematopoietic necrosis virus gene probe serodiagnostic field kit for the screening of candidate specific pathogen-free *Penaeus vannamei* broodstock. Aquaculture 147:1–8

Chien T, Hsu-Tien H, Sheng-Hsiung C, Jung-Ping Hand 5 others (1999) Taura syndrome in Pacific white shrimp *Penaeus vannamei* cultured in Taiwan. Dis Aquat Org 38: 159–161

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the Vax. Nucleic Acid Res 12:387–395

Durand SV, Tang KFJ, Lightner DV (2000) Frozen commodity shrimp: potential avenue for introduction of white spot syndrome virus and yellow head virus. J Aquat Anim Health 12:128–135

Hasson KW, Lightner DV, Poulos BT, Redman RM, White BL, Brock JA, Bonami JR (1995) Taura syndrome in *Penaeus vannamei*: demonstration of a viral etiology. Dis Aquat Org 23:115–126

Hasson, KW, Hasson J, Aubert H, Redman RM, Lightner DV (1997) A new RNA-friendly fixative for the preservation of penaeid shrimp samples for virological detection using cDNA genomic probes. J Virol Methods 66:227–236

Hasson KW, Lightner DV, Mohney DV, Redman RM, Poulos BT, Mari J, Bonami JR, Brock JA (1999) The geographic distribution of Taura syndrome virus (TSV) in the Americas: determination by histopathology and *in situ* hybridization using TSV-specific cDNA probes. Aquaculture 17: 13–26

Holthuis LB (1980) Shrimps and prawns of the world: an annotated catalogue of species of interest to fisheries. FAO species catalogue. FAO Fisheries Synopsis 125(1). Food and Agricultural Organization of the United Nations, Rome

Jimenez R (1992) Síndrome de Taura (Resumen) Acuacultura del Ecuador. Rev Esp Cámara Nacional Acuacultura, Guayaquil, Ecuador December 1:1–16

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lightner DV (1995) Taura syndrome: an economically important viral disease impacting the shrimp farming industries of the Americas including the United States. In: Campbell P Associates (ed) Proceedings of the Ninety-Ninth Annual Meeting USDAHA, Reno, NV. Campbell and Associates, Richmond, VA, p 36–52

Lightner DV (1996a) A handbook of shrimp pathology and diagnostic procedures for diseases of culture penaeid shrimp. World Aquaculture Society, Baton Rouge, LA

Lightner DV (1996b) Epizootiology, distribution and the impact on international trade of two penaeid shrimp viruses in the Americas. In: Hill BJ, Hastein T (eds) Preventing the spread of aquatic animal disease. Rev Sci Tech Off Int Epizoot 15:579–601

Lightner DV, Redman RM, Hasson KW, Pantoja CR (1995) Taura syndrome in *Penaeus vannamei* (Crustacea: Decapoda): gross signs, histopathology and ultrastructure. Dis Aquat Org 21:53–59

Lightner DV, Redman RM, Poulos BT, Nunan LM, Mari JL, Hasson KW (1997a) Risk of spread of penaeid shrimp viruses in the Americas by the international movement of live and frozen shrimp. Rev Sci Off Int Epizoot 16(1):146–160

Lightner DV, Redman RM, Poulos BT, Nunan LM, Mari JL, Hasson KW, Bonami JR (1997b) Taura syndrome: etiology, pathology, hosts, and geographic distribution, and detection methods. In: Inui Y (ed) Proceedings of the NRIA International Workshop. New approaches to viral discases of aquatic animals. January 21–24, 1997, Kyoto, Japan. National Research Institute of Aquaculture. Nansei, Watarai, Mie 516–01

Lightner DV, Durand SV, Redman RM, Mohney LL, Tang-Nelson K (2001) Qualitative and quantitative studies on the relative virus load of tails and heads of shrimp acutely infected with WSSV: implications for risk assessment. In: Browdy CL, DE Jory (eds) The new wave. Proceedings of the special session on sustainable shrimp culture. World Aquaculture Society, Baton Rouge, LA, p 285–291

Lotz JM (1997a) Special topic review: viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. World J Microbiol Biotechnol 13(4):405–413

Lotz JM (1997b) The effect of host size on virulence of Taura syndrome virus (TSV) to the marine shrimp *Penaeus vannamei* (Crustacea: Penaeidae). Dis Aquat Org 30(1):45–51

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Mari J, Bonami JR, Lightner DV (1998) Taura syndrome of penaeid shrimp: cloning of viral genome fragments and development of specific gene probes. Dis Aquat Org 33: 11–17

Mari J, Poulos BT, Lightner DV, Bonami JR (2002) Full nucleotide sequence and genome organization of the Taura syndrome virus (TSV) of penaeid shrimp. J Gen Virol 83(4): 917–928

Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem 262:10035–10038

Nakamura K, Tanaka T, Kuwahara A, Takeo K (1985) Microassay for proteins on nitrocellulose filter using protein dye-staining procedure. Anal Bioch 148(2):311–319

Nielsen BL, Brown LR (1984) The basis for colored silver-protein complex formation in stained polyacrylamide gels. Anal Bioch 141(2):311–315

Nunan L, Poulos BT, Lightner DV (1998) Reverse transcription polymerase chain reaction (RT-PCR) used for the detection of Taura syndrome virus (TSV) in experimentally infected shrimp. Dis Aquat Org 34:87–91

Office International de Epizooties (OIE) (2000) Diagnostic manual for aquatic animal diseases, 3rd edn. Chapt 4.1.1. Taura syndrome virus. Office International de Epizootics, Paris

Poulos BT, Kibler R, Bradley-Dunlop D, Mohney LL, Lightner DV (1999) Production and use of antibodies for the detection of Taura syndrome virus in penaeid shrimp. Dis Aquat Org 37:99–106

Poulos BT, Pantoja CR, Bradley-Dunlop D, Aguilar J, Lightner DV (2001) Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. Dis Aquat Org 47:13–23

Robles-Sikisaka R, Garcia DK, Klimpel KR, Dhar A (2001) Nucleotide sequence of 3′-end of Taura syndrome virus of shrimp suggests that it is related to insect picornaviruses. Arch Virol 146:941–952

Rosenberry B (1997) World shrimp farming, No. 10. Shrimp News International, San Diego

Rosenberry B (2000) World shrimp farming, No. 13. Shrimp News International, San Diego

Towbin HT, Staehelin T, Gordon J (1979) Electrophoretic

transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Williams RR, Dehdashti B, Lightner DV (1992) Performance of an aquatic multispecies system in evaluating the effects of a model microbial pest control agent on nontarget organisms. J Toxicol Environ Health 37:247–264

Wilson CM (1983) Staining of proteins on gels: comparison of dyes and procedures. Methods Enzymol 91:236–274

Wyban JA, Swingle JS, Sweeney JN, Pruder GD (1992) Development and commercial performance of high health shrimp using specific pathogen free (SPF) broodstock *Penaeus vannamei*. In: Wyban J (ed) Proceedings of the Special Session on Shrimp Farming. World Aquaculture Society, Baton Rouge, LA, p 254–259

Yu CI, Song YL (2000) Outbreaks of Taura syndrome in Pacific white shrimp *Penaeus vannamei* cultured in Taiwan. Fish Pathol 35(1):21–24

Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: March 22, 2002; Accepted: June 4, 2002
Proofs received from author(s): September 17, 2002