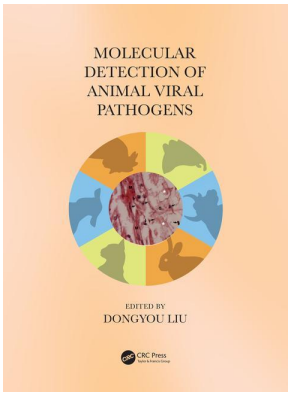

Molecular Detection of Animal Viral Pathogens

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Taura Syndrome Virus

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3 Taura Syndrome Virus

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3.1 INTRODUCTION

Taura syndrome (TS) of peneid shrimp was described in 1992 in *Penaeus vannamei* harvested from shrimp farms near the Taura River in Ecuador [1]. TS later spread to many countries, including the United States, Taiwan, China, Thailand, and Indonesia [2–4]. The viral etiologic agent, Taura syndrome virus (TSV), was identified in 1994 through infectivity studies using specific pathogen-free *P. vannamei* as the host [5]. The principal hosts of TSV are white shrimp *P. vannamei* and blue shrimp *P. stylirostris*. However, other shrimp species such as *P. setiferus*, *P. monodon*, *P. chinensis*, *P. aztecus*, *P. duorarum*, *P. indicus*, and *Metapenaeus ensis* can be infected by the virus [6–12]. The disease often results in cumulative mortalities ranging from 40% to >90% of cultured populations of postlarval, juvenile, and subadult shrimp. Shrimp with TS are typically small juveniles weighing between 0.05 g and less than 5 g [6,8,13]. The horizontal transmission of TSV by cannibalism or contaminated water has been documented [5,6,8,14], but vertical transmission has not been experimentally verified. Currently, there are no vaccines or chemotherapy treatments available. Therefore, to reduce the risk of TSV infection during shrimp farming, screening of brood stock and spawned eggs/nauplii using polymerase chain reaction (PCR) has been applied. The development of specific pathogen-free shrimp stocks of *P. vannamei* and *P. stylirostris* has proven to be the most successful husbandry practice for the prevention and control of TS [15].

3.1.1 Classification

Characteristics of TSV virions include a buoyant density of approximately 1.338 g mL^{-1} in CsCl, icosahedral morphology, a size of 31–32 nm in diameter, and a positive sense single-stranded RNA (ssRNA) genome that is polyadenylated at the 3′ end [16]. Therefore, the virus was initially classified as a member of the family *Picornaviridae* [5,16]. However, genomic characterization and sequence comparison revealed that TSV is closely related to the cricket paralysis-like virus [17]. Presently, TSV has been classified by the International Committee on Taxonomy of Viruses into the novel genus *Aparavirus* in a new family *Dicistroviridae* in the order *Picornavirales* [18].

3.1.2 Genome Organization, Biology, and Epidemiology

TSV is small, with a nonenveloped icosahedral shape. The ssRNA genome is 10,205 nucleotides in length, excluding the 3′-poly(A) tail, and contains two large open reading frames (ORFs) separated by an intergenic region (IGR) of 207 nucleotides [17]. The amino acid sequence of ORF1 consists of helicase, a protease, and an RNA-dependent RNA polymerase (RdRp). ORF2 contains the sequences of structural proteins, including three major capsid proteins, VP1 (CP2, 55 kDa), VP2 (40 kDa), and VP3 (24 kDa). ORF1 and ORF2 represent 92% of the TSV genome, and the remaining 8%

consists of an untranslated region (UTR). In the 3′-UTR, no putative polyadenylation signal (AAUAAA) was identified [17]. Sequence homology of the IGR between TSV and other members of the cricket paralysis-like viruses suggested that TSV ORF2 was translated by an internal ribosome entry site (IRES) [17]. Unlike other IRES-mediated translation initiations, the IGR-IRES-mediated initiation of translation in discistroviruses was shown to occur in the absence of base-pair interactions between the initiation codon and the anticodon triplet in initiator methionine tRNA (Met-tRNAi). Therefore, the IRES did not require an AUG initiation [19]. It has been shown that TSV translation from IGR-IRES started from a non-AUG start codon and did not involve initiator methionyl-$tRNA^{Met}$. Translation from the TSV IGR-IRES initiated with alanyl-$tRNA^{Ala}$ even though there was an in-frame AUG codon positioned two codons upstream of the GCU-alanine codon of the TSV structural polyprotein [20].

In the absence of initiation factors, TSV IGR-IRES elements can recruit ribosomes and direct protein synthesis in a reconstituted system containing only purified ribosomal subunits, eukaryotic elongation factors 1A and 2, and aminoacylated tRNAs [20]. A computational-based structure search suggested that TSV had an internal stem-loop structure in the predicted IRES, and *in vitro* translation analysis showed that TSV had a structurally distinct IGR-IRES [21]. The IGR-IRESs contain three pseudoknots, one of which, called pseudoknot I (PKI), is essential for the function of IGR-IRES [22]. A cryomicroscopy structure analysis revealed that PKI of TSV IRES occupied the ribosomal decoding center at the aminoacyl (A) site in a manner similar to that of the tRNA anticodon/mRNA codon. Moreover, the structure indicated that TSV IRES initiated translation by an unprecedented mechanism. Specifically, the ORF of the IRES-driven mRNA was established by the placement of the tRNA–mRNA-like structure in the A site, whereas the 40S P site remained unoccupied during the initial step [23].

VP1 (equivalent to capsid protein 2; CP2) demonstrates greater variation in its amino acid sequence (3.5%) than VP2 (CP1) and VP3 (CP3) (both 0.8%) [24]. Therefore, the CP2 region has been used to establish the genetic relationship among TSV isolates. Currently, at least four genotypic variants have been identified according to the sequence of the VP1 structural protein. These variants are the Americas group, the Southeast Asian group, the Belize group, and the Venezuelan group [12,25,26]. In 2010, a newly emerged strain of TSV isolated from Colombian shrimp farms called CO 10 was analyzed. The phylogenetic analysis based on the CP2 amino acid sequence indicated that the CO 10 strain forms a new cluster and differs from the previous Colombia isolates. The results revealed the diversity of TSV CP2 in the Americas group leading to two new subclusters in South America (Venezuela and Colombia) and a new subcluster in Mexico in the Central America variant [27].

Infection of TSV occurs in tissues derived from the ectoderm and mesoderm. Infection in the cuticular epithelium was most prevalent in shrimp with acute infections, and the infection in lymphoid organs was observed in shrimp with chronic infections. Histological analysis revealed no signs of infection of TSV in the enteric organs [10,28–30].

In TSV vector studies, it has been demonstrated that the feces of seagulls (*Larus atricilla*) can contain infectious TSV particles for up to 6 h after consumption of viral-infected shrimp tissues. Therefore, this wild seabird may serve as a vector for transmission of TSV to aquatic environments [31]. In addition, the water boatman *Trichocorixa reticulata* (*Corixidae*), an aquatic insect collected from ponds with active TS, was shown to be TSV positive using *in situ* hybridization (ISH) assay in the gut [6,32].

A study on potential carriers of TSV was conducted in Thailand with five common native crustaceans found near shrimp ponds including two palaemonid shrimp species, *Palaemon styliferus* and *Macrobrachium lanchesteri*, and three crab species, *Sesarma mederi*, *Scylla serrata*, and *Uca vocans.* The results showed that *U. vocans*, *S. serrata*, and *S. mederi* gave strong reverse transcription polymerase chain reaction (RT-PCR) at 10, 15, and 50 days (d), respectively, after feeding with TSV-infecting *P. vannamei* carcasses. Also after feeding, a strong RT-PCR reaction was observed in *P. stylirostris* at 5 d postchallenge but no reaction occurred at 15 d. In the case of *M. lanchesteri*, a light RT-PCR reaction was observed at 2 d after feeding but no reaction occurred at 15 d. These results suggest that these crabs and palaemonid shrimp species can pose a risk as potential carriers for TSV transmission [33].

TS first appeared in Ecuador in 1992 [1] and may have caused up to US$100 million worth of damage [6]. TS has spread progressively to the United States, including the states of Hawaii, Texas, and Florida [34]. It was estimated that the outbreaks of TS in Southern and Central Texas led to US$10 million losses [6]. TS also caused an impact on shrimp farms in other countries in South and Central America, including Peru, Honduras, Guatemala, El Salvador, Brazil, Nicaragua, Belize, and Mexico [34]. TSV may have spread from South America to Asia due to importation of infected live *P. vannamei*, which became a primary shrimp species in Asia during the 1990s. The first TS outbreak in Asia occurred in Taiwan in late 1998 to early 1999, and shrimp production dropped abruptly to as low as 10% of the volume produced in early 1998. It was assumed that TSV was transferred to Taiwan by contaminated postlarvae and spawners from Ecuador and elsewhere [2]. In Thailand, the first TS outbreak was confirmed in 2003 after legal importation of *P. vannamei* in mid-2002. During that time, over 150,000 brood stock shrimp were officially imported from China and Taiwan [35]. In 2004, mass mortality of *P. vannamei* shrimp cultured in Korea occurred, and it was suggested that TSV was introduced to Korea via imported stocks of *P. vannamei*. Based on the partial nucleotide sequences of VP1, two Korean TSV isolates were 96%–99% similar to those of TSV isolates from the Americas, Taiwan, and Thailand [36].

During the mid-1990s, TSV caused devastation to the shrimp industries of Ecuador and Colombia. At that time, 20%–30% of shrimp survived, and various staff including technicians and researchers speculated that the survivors could be genetically resistant animals. Therefore, one of

the major shrimp producers in Colombia initiated a program to select the survivors from infected ponds and used them as parents for the next generation in a simple mass selection process. Within two to three generations, the success of this simple scheme increased survival rates to previous levels [37].

In response to TSV outbreaks in the United States, the U.S. Marine Shrimp Farming Program initiated a selective breeding program to improve TSV resistance in *P. vannamei*, operated by the Oceanic Institute (OI). From 1995 to 1998, the OI (Waimanalo, Hawaii, United States) operated a breeding program based on a selection index weighted equally for growth and resistance to TSV. One line demonstrated an 18.4% increase in survival to TSV after one generation of selection compared with unselected control families [38]. The OI had been selecting *P. vannamei* for TSV resistance for over 15 generations, and several families per generation exhibited 100% survival after exposure [39]. Over the past decade, several shrimp breeding programs have distributed TSV-resistant brood stock to commercial shrimp hatcheries worldwide. Therefore, the availability of TSV-resistant shrimp is of great benefit to the shrimp farming industry, and TSV is no longer considered to be a major threat in many shrimp farming regions [40].

The first transcriptomic study in hemolymph and hemocytes of TSV-resistant and susceptible *P. vannamei* was recently conducted using high-throughput RNA-seq. The comparison of gene expression between resistant and susceptible shrimp revealed several differentially expressed genes involved in the immune response activity. These included (1) pathogen recognition through cues such as immune regulators, adhesive proteins, and signal transducers, (2) coagulation, (3) proPO pathway, (4) antioxidation, and (5) protease [41].

3.1.3 Clinical Features and Pathogenesis

TSV infection in *P. vannamei* exhibits gross pathology in three distinct phases including acute, transition, and chronic phases.

During the acute phase of disease outbreak in *P. vannamei*, onset of mortality is often sudden and massive with moribund shrimp approaching the pond edge and surface water. Moribund shrimp in the acute phase demonstrate symptoms such as a soft shell, pink to red coloration due to the expansion of red cuticular chromatophores especially in the tail fan, and visible necrosis of the epithelial tissue. Infected shrimps are lethargic, stop feeding despite having an empty gut, and typically survive less than 24 h. However, the acute phase in a shrimp farm pond may last for several days in an affected population. Death often occurs in the acute phase during molting [6,42,43].

In the transition phase, some affected shrimp may recover from the infection. The shrimp typically demonstrate randomly distributed variably sized melanized lesions in or under the cuticle where the acute phase necrosis occurs. Shrimp with these black lesions are at some risk of mortality during their succeeding molt. Shrimp that survive the next molting process typically look normal and the lesions disappear from the cuticle. In the transition phase, death may also occur due either to osmotic failure as a consequence of widespread destruction of cuticular epithelium or to systematic infection from opportunistic bacteria [6,42,43].

In the chronic phase, shrimp may carry TSV throughout their life as a persistent infection. They may appear and behave normally but will show slightly less tolerance to low salinity stress than uninfected shrimp. The lymphoid organ of *P. vannamei* in this phase usually has spheroids (proliferative nodules of pale staining), vacuolated cells, and a lack of the central vessel that is typical for normal lymphoid organ tubules [6,42,43].

3.1.4 Diagnosis

3.1.4.1 Conventional Techniques

Histopathology changes in acute TSV-infected shrimp present as multifocal areas of necrosis in the cuticular epithelium and subcuticular connective tissue. Infection in the underlining muscle, heart, and lymphoid organ is occasionally observed. Pyknotic and karyorrhectic nuclei are scattered throughout the infected areas. Cytoplasmic remnants of the necrotic cells are abundant and present as spherical inclusion bodies that range from 1 to 20 μm in diameter and are often described as "peppered" or "buckshot" in appearance. In hematoxylin and eosin (H&E) staining, the inclusions show eosinophilic to pale basophilic remnants [6,42,43].

Shrimp that survive the acute infection may undergo the transition phase in which the typical acute-phase cuticular lesions decline in both abundance and severity. In the affected areas, infiltration and accumulation of hemocytes at the site of necrosis are conspicuous and become melanized, giving rise to the irregular black spots. Invasion of the *Vibrio* spp. may be observed on the corrosive cuticle. The lymphoid organ may appear normal with H&E staining [43,44].

In shrimp with chronic infection, the irregularly shaped and sized melanized lesions on the cuticle mostly disappear and display no gross signs of infection. The lymphoid organ in the chronic phase usually has spheroids, which are proliferative nodules of pale staining, vacuolated cells, and lack of a central vessel. When assayed via ISH with TSV probe or immunohistochemistry with monoclonal antibodies (MAbs), some cells gave positive signals for the virus [10,43,44].

3.1.4.2 Molecular Techniques

The early molecular assays consist of ISH and RT-PCR following the successful identification of TSV-specific gene targets [29,45,46]. The ISH method has been used for the routine histopathology diagnosis of TSV infection. However, false-negative results may be obtained if the shrimp tissues were preserved in Davidson's acetic acid–formaldehyde–alcohol solution for more than 24–48 h. The highly acidic pH (~3.5–4.0) of Davidson's fixative causes acid hydrolysis of TSV genomic RNA leading to false-negative probe results [28]. This problem can be avoided by using a neutral pH (~6.0–7.0)

fixative called RNA-friendly fixative or through proper fixation (24–48 h) in Davidson's solution [28].

In the case of RT-PCR, Nunan et al. [46] designed a pair of primers called 9992F and 9195R based on a clone from TSV cDNA library. Using the one-step RT-PCR approach, the primers amplify a 231 base pair (bp) sequence of the TSV genome. Primer 9992F is located near the 3′ end of the IGR, and primer 9195R is located in the VP2 (CP1) gene of the TSV genome [17,46]. This RT-PCR assay allows detection of all known TSV genetic variants [43] and is recommended by the Office International des Epizooties (OIE) [15] for surveillance and diagnostic purposes.

An improved RT-PCR assay was developed by Navarro et al. [47] using newly designed primers, designated 7171F and 7511R, which amplify a 341 bp fragment. The detection limit of the improved RT-PCR assay was 20 copies of the TSV genome, 100 times more sensitive than the RT-PCR using the 9992F/9195R primers recommended by OIE. The primers 7171F/7511R were demonstrated to successfully detect TSV isolates from four phylogenetic lineages including Belize, the Americas, Southeast Asia, and Venezuela and displayed no cross-reactivity to infectious hypodermal and hematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV), yellow-head virus (YHV), or infectious myonecrosis virus (IMNV) [47]. In addition to single RT-PCR methods, multiplex RT-PCR for detection of TSV and WSSV was developed and could be used to detect coinfection with WSSV and TSV in *P. vannamei* [48].

In addition to gel-based conventional RT-PCR, quantitative real-time RT-PCR assays were developed [49–51]. The real-time RT-PCR, using primers and a TaqMan probe designed from the ORF1 region of the TSV genomic sequence, demonstrated amplification with TSV isolates from Hawaii, Texas, Colombia, Mexico, Belize, Indonesia, and Thailand. No amplification was observed with RNA from healthy shrimp or an isolate of YHV. This real-time RT-PCR method has a detection limit of 100 copies and could be used to detect both acutely and chronically infected shrimp [49]. This TaqMan probe RT-PCR assay has been recommended by OIE [15]. The real-time multiplex PCR method was also developed for the detection of WSSV, IHHNV, and TSV using three sets of oligonucleotide primers and TaqMan probes specific for each virus. However, the assay has a sensitivity of 2000 copies of TSV, which is 20 times lower than that afforded by the single RT-PCR assay described earlier [50].

The SYBR Green RT-PCR was evaluated for TSV detection, and the results indicated that increasing the amplicon size from ~50 to ~70–100 bp enhanced its specificity and sensitivity [51].

The alternative nucleic acid amplification techniques, including nucleic acid–based amplification (NASBA) and loop-mediated isothermal amplification (LAMP), were also employed for specific detection of TSV [52–55]. NASBA coupled with dot-blotting procedure was approximately five times less sensitive than that of commercial nested RT-PCR kit IQ2000 TSV Detection and Prevention System (Farming IntelliGene Technology Corporation, Taiwan). No false-positive reaction was obtained with nontarget shrimp RNA viruses including gill-associated virus (GAV), YHV, and IMNV [52].

The agarose gel–based RT loop-mediated isothermal amplification (RT-LAMP) demonstrated 10 times more sensitivity than the RT-PCR recommended by OIE but less sensitivity than the nested RT-PCR IQ2000 kit. No amplification was observed with nucleic acids from other shrimp pathogens including YHV and WSSV [53]. The colorimetric dot-blot hybridization (DBH) was used to detect RT-LAMP amplicons and exhibited the same sensitivity as the nested RT-PCR IQ2000 method. There was no cross-reaction with other shrimp viruses, including WSSV, IHHNV, IMNV, GAV, monodon baculovirus, and hepatopancreatic parvovirus. However, in some cases, tRNA samples or very low concentrations of the target templates could lead to the production of the aberrant products. Due to the utilization of hybridization probe specific to LAMP products, the DBH has the capacity to eliminate false-positive reactions [55].

Further development of RT-LAMP combined with lateral flow dipstick (LFD) for detection of LAMP amplicons was demonstrated [54]. This strip detected biotin-labeled LAMP products that had been hybridized with a fluorescein isothiocyanate (FITC)-labeled probe. Without relying on special equipment, the LFD is immersed into the appropriate buffer containing LAMP amplicons and the results are available within 10 min. This assay had a detection limit comparable to that of nested RT-PCR and exhibited high specificity to TSV with no cross-reactivity with WSSV, IHHNV, or YHV [54].

As the early RT-LAMP procedures included a step in which the reaction tube must be opened for further detection, the risk of contamination between samples could occur. Given the LAMP amplification produced magnesium pyrophosphate ($Mg_2P_2O_7$) by-products correlated with the amount of amplified LAMP amplicons, resulting in turbidity, a portable turbidimeter was designed and used to measure the turbidity without opening the reaction tubes. The results showed that this RT-LAMP coupled with turbidimeter is equally sensitive to agarose gel RT-LAMP and IQ2000-nested RT-PCR kit, with reduced contamination risk [56].

Recently, a hyperbranched rolling circle amplification (HRCA) assay combined with a strip test was developed for TSV detection. The sensitivity was approximately 10 copies, 100 times higher than that of RT-PCR. The HRCA test was TSV-specific and showed no cross-reaction to shrimp viruses including WSSV, YHV, and IHHNV [57].

3.1.4.3 Immunological Techniques

Chicken and mouse polyclonal TSV antisera and MAbs were produced using purified TSV as the antigen [58]. One of the MAbs designated 1A1, specific to VP1, was used to detect TSV via western blot dot and immunohistochemistry. However, while this MAb can react with TSV isolates from Taiwan, the United States, and Hawaii, it does not recognize TSV isolates from Mexico, Nicaragua, Belize, or Venezuela [25,26,59]. MAbs against recombinant structural capsid proteins, including VP1, VP2 and VP3, were also developed [60–62].

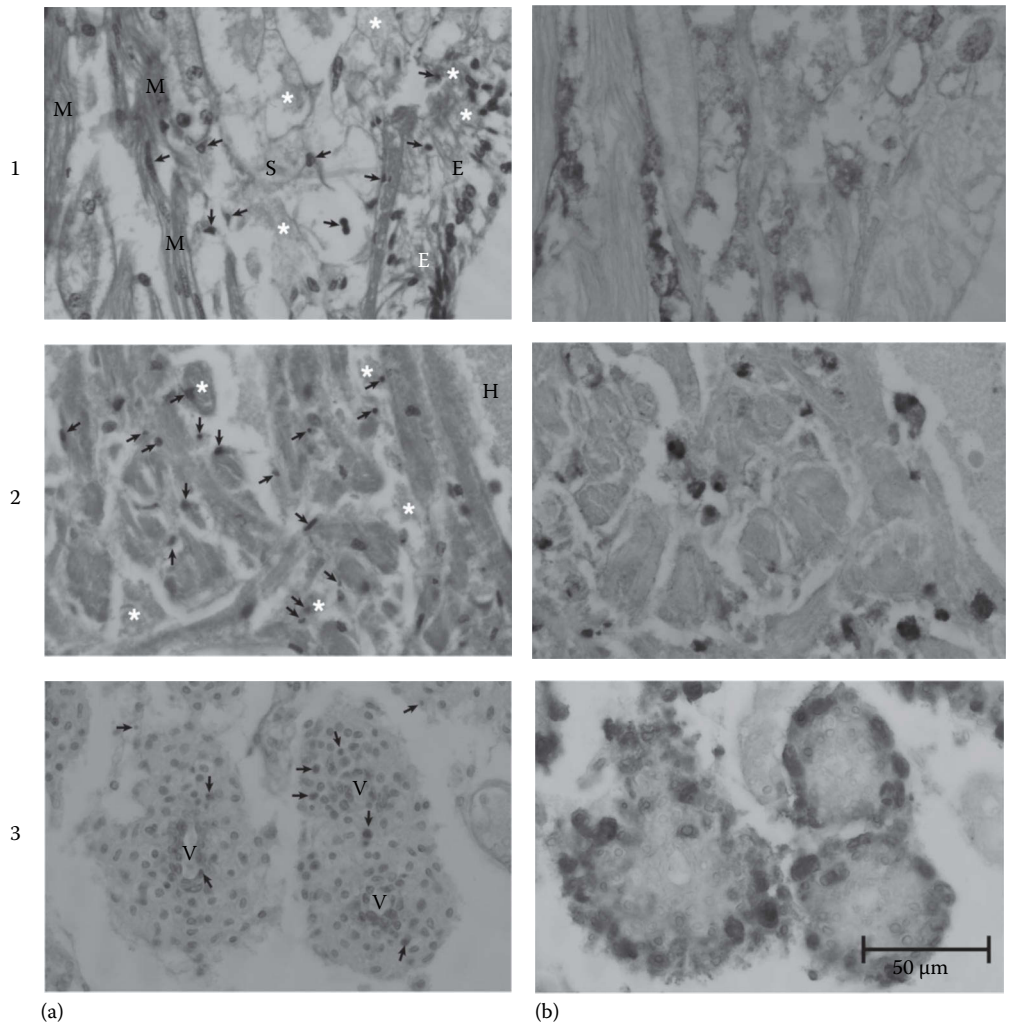

FIGURE 3.1 Histopathology and immunohistochemistry of tissues from naturally TSV-infected *P. vannamei* with asymptomatic infection in (1) cuticular epithelium, (2) heart, and (3) lymphoid organ. Two consecutive sections: (a) tissues were stained with hematoxylin and eosin (H&E) and (b) tissues were immunohistochemically stained with monoclonal antibody specific to VP2 of TSV and counterstained with eosin. Positive immunoreactivity appeared as brown staining. Intensely basophilic staining of pyknotic and karyorrhectic nuclei (arrow) and eosinophilic cytoplasmic remnants referred as "buckshot" appearance (*) are scattered in the lesion areas. *Note*: Lymphoid organ (3) demonstrated histologically normal in H&E staining; however, it demonstrated strong immunoreactivity of TSV infection. E, epithelium; S, subcuticular connective tissue; M, skeletal muscle; H, hemolymph; V, central vessel.

Through immunohistochemistry, these MAbs could be used to detect chronic and light TSV infection (Figure 3.1). It is likely that the combination of MAbs-specific to VP1, VP2, and VP3 may be useful for the detection of most TSV isolates from various geographic regions.

3.2 METHODS

3.2.1 Sample Preparation

RT-PCR methods are recommended by the OIE for TSV monitoring and diagnostic purposes. Tissue samples such as hemolymph, pleopods, or whole small shrimp can be used for RT-PCR assays. Various commercial RNA extraction kits are available for TSV RNA isolation from fresh, frozen, and ethanol-preserved tissues.

In case of the High Pure RNA Tissue Kit (Roche, Penzberg, Germany), 400 μL of Lysis/Binding Buffer is added to the appropriate amount of tissue (max. 20–25 mg) in a nuclease-free 1.5 mL microcentrifuge tube. The tissues are disrupted and homogenized using a rotor–stator homogenizer, and the lysate is centrifuged for 2 min at maximum speed in a microcentrifuge. The supernatant is mixed with 200 μL of absolute ethanol. Next, the mixture is applied to the spin column combined with the collection tube (maximal volume 700 μL) and centrifuged at maximal speed (13,000 × *g*) for 30 s, and then the flow-through is discarded. For each isolation, 10 μL of DNase I working solution is mixed with 90 μL of DNase incubation buffer, and the prepared solution is added to the spin column combined with the collection tube and incubated at 15°C–25°C for 15 min. The column is washed via the addition of 500 μL Wash Buffer I, centrifuged at 8000 × *g* for 15 s,

the flow-through is discarded, and the washing step is repeated with Wash Buffer II. Then, the column is washed by the addition of 300 µL Wash Buffer II and centrifuged at 13,000 × *g* for 2 min, and then the flow-through is discarded. Finally, the spin column is placed onto a new 1.5 mL microcentrifuge tube and 100 µL of elution buffer is added directly to the spin column and centrifuged at 8000 × *g* for 1 min. The eluted RNA can then be used for RT-PCRs or stored at −80°C for later analysis.

3.2.2 Detection Procedures

3.2.2.1 Gel-Based RT-PCR

Two oligonucleotide primers, designated 9992F and 9195R, are used in the RT-PCR, for specific amplification of a 231 bp product. The method outlined in the succeeding text for TSV detection is based on that described by Nunan et al. [46].

Primers	Product	Sequence	GC	Temperature
9992F	231 bp	5′-AAG TAG ACA GCC GCG CTT-3′	55%	69°C
9195R		5′-TCA ATG AGA GCT TGG TCC-3′	50%	63°C

The GeneAmp® EZ rTth RNA PCR kit (Applied Biosystems, Forster City, CA, USA) is used for the amplification reactions described in the succeeding text. The TSV positive and negative controls should be performed along with the no-template control.

3.2.2.1.1 Procedure

1. Prepare the RT-PCR reaction mix as follows (volumes listed are per specimen). (*Note*: Vortex and spin down all reagents before opening the tubes.)

Component	Volume (µL)
Diethyl pyrocarbonate (DEPC)-treated water	Variable
dNTPs (300 µM each)	Variable
9992F (0.46 µM)	Variable
9195R (0.46 µM)	Variable
Manganese acetate (2.5 mM)	Variable
5× EZ buffer	10
rTth DNA polymerase 2.5 U	1
RNA sample	10
Total volume	50

2. Add the RT-PCR reaction mix to a 0.2 mL PCR tube.
3. Spin the sample tubes in a microcentrifuge at 14,000 × *g* for 30 s at room temperature.
4. Place the sample tubes in a thermal cycler. RT may then proceed at 60°C for 30 min, followed by 94°C for 2 min. After the completion of RT, the samples are amplified for 40 cycles using the following conditions: denaturation at 94°C for 45 s, annealing/extension at 60°C for 45 s, and final extension at 60°C for 7 min.
5. Following the termination of the RT-PCR reaction, pulse spin the reaction tubes to pull down the condensation droplets at the inner wall of the tubes.
6. Analyze a 10 µL of the PCR products using 2.0% agarose gel, stained with ethidium bromide (0.5 g mL^{-1}), and electrophoresis in 0.5× TBE (Tris, boric acid, ethylenediaminetetraacetic acid). Then, visualize the sample under an ultraviolet light source.

3.2.2.2 Quantitative RT-PCR

The detection of TSV using quantitative RT-PCR is rapid, sensitive, and specific. The sensitivity of qRT-PCR is approximately 100 copies of the target sequence in the TSV genome [49]. The qRT-PCR method outlined in the succeeding text follows the procedure described by Tang et al. [49]. The designed primers and TaqMan probe are specific to the ORF1 region of the TSV genome (GenBank AF277675). The primers TSV1004F (5′-TTG GGC ACC AAA CGA CAT T-3′) and TSV1075R (5′-GGG AGC TTA AAC TGG ACA CAC TGT-3′) generate a 72 bp DNA fragment. The TaqMan probe TSV-P1 (5′-CAG CAC TGA CGC ACA ATA TTC GAG CAT C-3′) is labeled with fluorescent dyes 5-carboxylfluorescein (FAM) on the 5′ end and *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end (Applied Biosystems, Cat. no. 450025). The TSV-positive control and the no-template control should be included in each run.

3.2.2.2.1 Procedure

1. Extract RNA from shrimp tissue using the method described earlier.
2. Prepare the qRT-PCR reaction mix as follows using TaqMan One-Step RT-PCR master mixture (Applied Biosystems, part no. 4309169). (*Note*: Vortexing and spinning down the reaction mix before use is recommended.)

Component	Volume (µL)
2× Master mix without uracil-N-glycosylase (UNG)	12.50
40× MultiScribe and RNase Inhibitor Mix	0.63
TaqMan probe TSV-P1(0.1 µM)	Variable
TSV1004F (0.3 µM)	Variable
TSV1075R (0.3 µM)	Variable
RNA sample (5–50 ng)	Variable
DEPC-treated water	Variable
Total volume	25

3. Place the reaction tubes in the GeneAmp 5700 Sequence Detection System (Applied Biosystems); ABI PRISM 7000, 7300, and 7500 (newer models and brands are also suitable).
4. Perform the qRT-PCR cycling consisting of RT at 48°C for 30 min, initial denaturation at 95°C for

10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min.
5. Interpret the results from the threshold cycle obtained from real-time fluorescence measurements using a charge-coupled device camera. Samples will be interpreted as negative if the Ct (threshold cycle) value is 40 cycles or greater, whereas samples with Ct value lower than 40 cycles are interpreted as positive.
6. To confirm the results, the qRT-PCR product can be electrophoresed on a 4% agarose gel with ethidium bromide staining and exposed to UV light. The DNA fragment at 72 bp can be observed in the positive samples. (Optional)

3.3 CONCLUSIONS

TSV is a major viral pathogen in cultured white shrimp *P. vannamei*. First recognized in Ecuador in 1992 and later spread to many countries, TS disease usually results in mortalities between 40% and over 90% of infected shrimp. Based on genomic characterization and sequence comparison, TSV is classified to the novel genus *Aparavirus* in a new family *Dicistroviridae* (in the order *Picornavirales*). The ssRNA genome of TSV contains two ORFs separated by an IGR. It has been suggested that TSV ORF2 is translated by an IRES located within IGR. Currently, at least four genotypic lineages have been identified, including ones in Mexico, Southeast Asia, Belize/Nicaragua, and Venezuela/Aruba, according to the sequence of the VP1.

For molecular detection, OIE recommends the use of RT-PCR with a pair of primers called 9992F and 9195R, which can detect all known TSV genetic variants, and qRT-PCR using primers and TaqMan probes designed from the ORF1 region of the TSV genomic sequence for TSV surveillance and diagnostic purposes. However, several assays such as LAMP or monoclonal antibody-based techniques were developed for TSV detection with different sensitivities. The commercial nested RT-PCR kit is also available.

As no vaccine or chemotherapy treatment are presently available, breeding programs to improve TSV resistance in *P. vannamei* plays an important role in the control of TS. During the past several years, the shrimp breeding programs have distributed TSV-resistant brood stock to shrimp hatcheries worldwide, giving great benefit to the shrimp farming industry. As a consequence, in many shrimp farming regions, TSV is no longer considered a major threat.

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