6.2.2 Taura Syndrome of Penaeid Shrimp

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A. Name of Disease and Etiological Agent

Taura Syndrome (TS) (a.k.a. "colita roja" or "little red tail") is a viral disease of penaeid shrimp whose name was derived from a region of Ecuador located southwest of Guayaquil (Taura River) where the disease was first recognized in farmed Pacific White shrimp, *Litopenaeus vannamei*, during 1992 (Jimenez 1992, Wigglesworth 1994, Lightner 1996a, Lightner et al. 1994, 1995, Brock et al. 1995, 1997). The early descriptive name given to the disease,"little red tail", was based on one of the gross clinical signs observed in acutely infected *L. vannamei*. TS disease was initially attributed to waterborne toxic effects of two agricultural fungicides (Tilt™ (Propicanizole), Ciba-Geigy and Calixin™ (Tridemorph), BASF) being utilized in Ecuador to control Black Leaf Wilt disease in banana plantations within the vicinity of the stricken shrimp farms (Brock et al. 1995, 1997; Stern 1995, Wigglesworth 1994, Lightner et al. 1994, 1995, Intriago et al. 1997). During 1993 to early 1994, the spread of TS into other *L. vannamei* shrimp farming regions (Peru, Colombia) where neither of the two reported fungicides were being employed, together with the failure of repeated attempts to experimentally induce the disease through fungicide exposure bioassays, cast doubt on the toxic etiology theory (Brock et al. 1995, 1997; Hasson et al. 1995, 1999a; Lightner et al. 1994, 1995, 1996). During 1994 a series of infectivity studies were conducted culminating with fulfillment of River's postulates for a previously unknown virus, which definitively demonstrated that TS was virus-caused and the agent named Taura Syndrome virus (TSV) (Brock et al. 1995, Hasson et al. 1995, Lightner et al. 1995). Investigators in Ecuador have suggested changing the name of the virus to Infectious Cuticular Epithelial Necrosis virus (ICENV) (Jimenez et al. 2000), but TSV will be used herein. Since 1992, TSV has caused global losses among cultured *L. vannamei* stocks conservatively estimated at ~1-2 billion dollars (Lightner 2003) and is categorized as a notifiable viral pathogen by the OIE (OIE, 2003). TSV was originally classified as a member of the Picornaviridae based on limited physicochemical characteristics including intracytoplasmic replication, 32 nm diameter, nonenveloped, icosahedral morphology (Figure 1), linear positive sense ssRNA genome, a buoyant density of 1.338 g/ml and containing three major (VP1, 55kDa; VP2, 40 kDa; VP3, 24 kDa) and one minor (58 kDa) capsid proteins (Hasson et al. 1995, Bonami et al. 1997). These characteristics, together with subsequent sequence analysis of the 10,205 kb genome and finding of two distinct open reading frames (ORFs) resulted in reclassification of TSV as a member of a newly designated genus, Cripavirus within the family Dicistroviridae (Mari et al. 2002, Dhar et al. 2004, Robles-Sikisaka et al. 2001, Mayo 2002a,b). Like many other RNA viruses, TSV has been found to rapidly mutate and at least three distinct variants (1994 Hawaii reference isolate, 2000 Mexican isolate and 2002 Belize isolate) have been reported in the Americas to date based on genomic comparisons and immuohistochemical reactions of different geographic isolates (Erickson et al. 2002, Robles-Sikisaka et al. 2002, Dhar et al. 2004). Changes in virulence and host specificity among these TSV isolates have been documented (Erickson et al. 2002, 2005; Robles-Sikisaka et al. 2002, Tang et al. 2005). A recent comprehensive phylogenetic

analysis of 40 TSV geographic isolates originating from both hemispheres by Tang et al. (2005) determined that three distinct groups of TSV genetic variants exist; Americas, SE Asia and Belize.

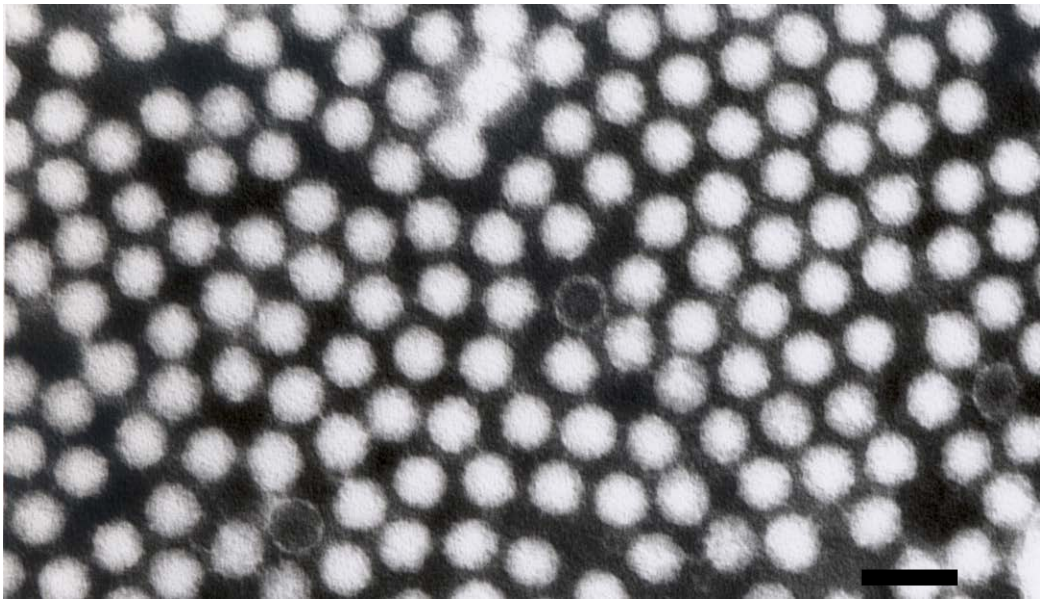

Figure 1. Transmission electron micrograph of negatively stained (2% PTA) CsCl gradient-purified TSV virions. Scale Bar= 64 nm

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Following the recognition of TS disease in Ecuador in 1992, the virus spread to 13 shrimp farming countries (Colombia, Peru, USA, Honduras, Costa Rica, Guatemala, Panama, Mexico, Nicaragua, El Salvador, Brazil, Belize, Dominican Republic) in the Western hemisphere within a four year period and is currently considered endemic along the Pacific coast from northern Peru up into southern Mexico (Lightner 1996a,b, Hasson et al. 1999a, Brock et al. 1995, 1997, Zarain-Herzberg and Ascencio-Valle, 2001). The most recent major shrimp farming country to be affected in the West was Venezuela during 2005. During 1998, TSV was detected in *L. vannamei* stocks in Taiwan (Tu et al.1999, Yu and Song 2000), marking the first incursion of the virus into the Eastern Hemisphere. International sales and movement of *L. vannamei* stocks of unknown health history within Asia is believed to be responsible for the subsequent spread of the disease into China, Thailand, Indonesia and South Korea (Nielsen et al.2005, Hanggono et al. 2005, Do et al. 2006).

2. Host Species

The Pacific White shrimp, *Litopenaeus vannamei*, is considered the principal shrimp host of TSV. All stages of this species are TSV susceptible from postlarvae (PL11) to adults (Wigglesworth 1994, Lightner et al. 1994, Lightner 1996a, Brock et al. 1995, Brock 1997, Lotz 1997). However, mortalities are highest among early juvenile stocks and outbreaks typically occur between 15-40 days post-stocking of nursery or growout ponds (Wigglesworth 1994, Lightner et al. 1994, Lightner 1996a, Brock et al. 1995). TSV has been reported in wild and cultured *L. vannamei* in the Western hemisphere (Hasson et al. 1999a, Brock 1997, Brock et al. 1997, Lightner 1995, 1996a,b) and cultured *L. vannamei* in the Eastern hemisphere (Tu

et al. 1999, Yu and Song 2000, Nielsen et al.2005, Hanggono et al. 2005, Do et al. 2006). Juvenile *L. setiferus* and *Penaeus schmitti* are susceptible to TSV and can develop severe infections (Brock et a. 1997, Overstreet et al. 1997, Lightner 1996a). Laboratory exposure studies have demonstrated that *P. japonicus*, *P. chinensis*, *F. aztecus*, and *F. duorarum* are only mildly susceptible to TSV, resulting in low grade acute and chronic infections with little or no mortality (Brock et al. 1997, Overstreet et al. 1997, Hasson 1998). Recent injection-mediated exposure studies of *P. monodon* juveniles have shown this species is susceptible to TSV (Srisuvan et al. 2005). *Litopenaeus stylirostris* stocks were initially found to be refractive to TSV infection and, as a result, were widely farmed throughout Mexico from the mid to late 1990's (Brock et al 1995, 1997; Clifford 2000). However, during 1999-2000, a TSV genotypic variant emerged in Mexico that caused high mortality in farmed *L. stylirostris* stocks and resulted in a rapid industry-wide return to *L. vannamei* culture (Robles-Sikisaka et al 2002, Erickson et al 2002). A third distinct strain of TSV was found in farmed *L. vannamei* stocks in Belize where it caused significant losses (Erickson et al. 2005). In response to the threat posed by TSV to U.S. shrimp mariculture, the US Marine Shrimp Farming Program has developed various strains of TSV-resistant (SPR) specific-pathogen-free (SPF) *L. vannamei* stocks for distribution to the U.S. industry. These SPF-SPR lines are currently the principal shrimp strain cultured in the U.S. and selection programs for additional TSV-resistant lineages are ongoing (Carr et al. 1997, Argue et al. 2002, Lightner 1995).

C. Epizootiology

Cumulative mortality among TSV-susceptible *L. vannamei* stocks typically range from 60-95% (Wigglesworth 1994, Brock et al. 1995, 1997, Lightner et al. 1994, 1995, Hasson et al. 1995). Vertical transmission of TSV has not been documented and remains an open question, although rare findings of acute phase TSV lesions within the ovaries and epithelium of the vas deferens of severely infected *L. vannamei* have been reported (Lightner 1995, Dhar et al. 2004, Hasson 1998). Transmission within a pond population occurs as a result of cannibalism of moribund or dead infected shrimp by healthy, naive members of the same population (Brock et al 1995, Lightner et al. 1995, Hasson et al. 1995). Prior et al. (2000) demonstrated that the virus remains viable within dead shrimp carcasses for up to two weeks postmortem and waterborne transmission is possible if viral concentrations are high. *L. vannamei* survivors of a TSV epizootic develop a long term chronic TSV infection localized within the lymphoid organ that may last for life (Hasson et al. 1995, 1999a,b). These TSV carriers can initiate renewed epizootics if they are not removed from farms between culture cycles and are consumed by newly stocked naïve shrimp during subsequent production cycles. Fecal transmission of viable TSV and its movement between ponds, farms or neighboring countries has been linked to marine birds (Laughing Gulls, *Larus atricilla*) and a flying aquatic insect (Water Boatmen, *Trichocorixa reticulata*) following their ingestion of TSV-infected shrimp tissue (Garza et al. 1997, Lightner 1995, 1996b, Hasson et al. 1995). International movement of TSV is principally attributed to the sale of infected *L. vannamei* nauplii, postlarvae and broodstock (Brock et al. 1997, Lightner 1996a,b, Hasson et al. 1999a). The spread of the virus into the Eastern hemisphere has been linked to the importation and culture of TSV-infected *L. vannamei* (Tu et al. 1999, Yu and Song 2000). As with many penaeid viruses, TSV can withstand long term freezing in shrimp tissues and still remain viable (Lightner 1995, Hasson et al. 1995). As a result, imported frozen shrimp pose a risk of virus introduction if used as fishing bait or if solid waste from shrimp processing plants or roadside markets are introduced into salt water systems (Lightner et al. 1997, Prior et al. 2001, Lightner 2002).

D. Disease Signs

TSV infection in susceptible *L. vannamei* populations has three clinically and histologically distinct overlapping phases; a ~7 day peracute to acute phase, a short term transition phase (~5 d) and a long term chronic phase (10 mo- life) (Hasson et al. 1999b,c). Acutely infected *L. vannamei* typically display lethargy, anorexia, atactic swimming behavior, flaccid bodies, soft cuticles, muscle opacity and chromatophore expansion resulting in reddening or darkening of the uropods, antennae and general body (Figure 2) (Brock et al. 1995, Lightner 1995, 1996a, Lightner et al. 1994, 1995, Hasson et al. 1995). A high percentage of TSV-infected *L. vannamei* typically die during or immediately post-molt and this accounts for the high percentage of moribund and dead shrimp with flaccid bodies and soft cuticles (Lightner 1996a, Hasson et al. 1999b). Transition phase infections are grossly identifiable by the presence of numerous, irregularly shaped, black melanized superficial cuticular lesions on the cephalothorax, abdomen and appendages (Figure 2). Shrimp with transition phase infections are typically lethargic and anorexic. Mortality continues among transition phase *L. vannamei*, but rates are greatly reduced when compared to losses among acutely infected shrimp (Hasson et al. 1999b). Chronic phase infections begin following shrimp molt and loss of the melanized exocuticle that formed during the transition phase infection. Chronically infected *L. vannamei* can easily go undetected as they feed and behave normally, displaying no outward clinical signs of disease (Hasson et al. 1999b,c). This phase of a TSV infection can persist for 10-12 months, possibly for the remainder of the shrimp's life.

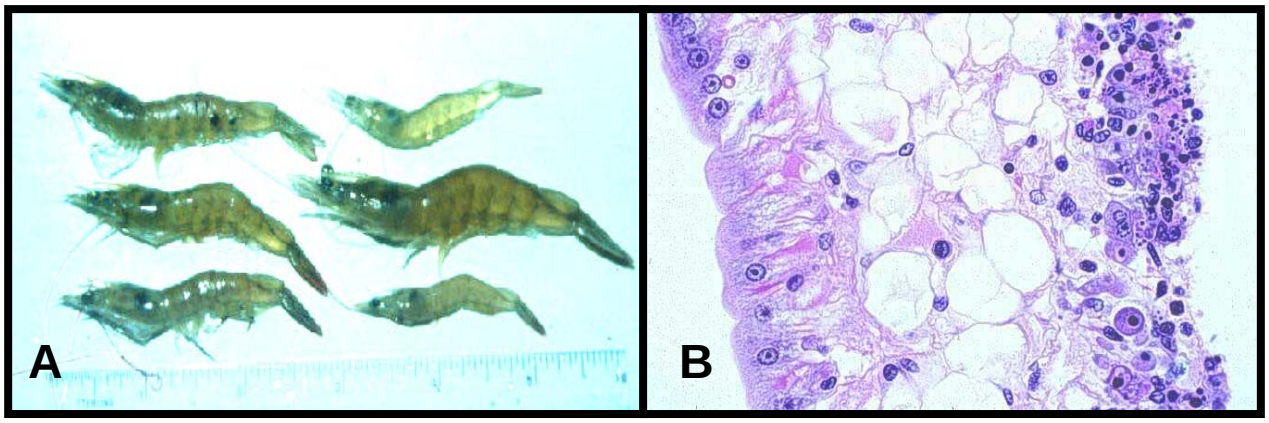

Figure 2. A) Gross clinical signs of acute phase TSV infection in *Litopenaeus vannamei*. Note chromatophore expansion producing a dark to reddish coloration of the general body, uropods and antennae. Shrimp in upper right corner is a normal, healthy specimen and appears transparent in contrast to the other five TSV-infected specimens. B) Histological section of an *L. vannamei* juvenile appendage illustrating an acute phase TSV lesion. The right side of the tissue illustrates the characteristic nuclear pyknosis, karyorrhexis and desquamation of rounded cuticular epithelial cells, which typically display cytoplasmic eosinophilia. The left side illustrates normal, uninfected cuticular epithelial cells. The exocuticle normally covering the epithelium was lost during processing. Hematoxylin and eosin stain. 600X magnification.

E. Disease Diagnostic Procedures

Numerous diagnostic methods have been developed for the detection of TSV including histological, immunological and molecular techniques (Lightner 1996a, 1999, Mari et al. 1998, Poulos et al. 1999, OIE 2003). The three principal diagnostic methods currently used for this disease and advocated by the OIE are histopathology, in situ hybridization and conventional RT-PCR (OIE 2003).

1. Histology

Shrimp are preserved with Davidson's AFA fixative (Humason 1972) through injection of the hepatopancreas, anterior cephalothorax and first, third and sixth tail segments following the methods of Bell and Lightner (1988). Tissues or whole shrimp are maintained in Davidson's for up to 24 hr prior to transfer into 70-95% ethanol for temporary storage or shipment. Dissected gill tissue, mid-sagittal sections of the cephalothorax, transverse sections of the first and third tail segments and a mid-saggital section of the sixth tail segment are dehydrated through a series of alcohol and xylene (or xylene substitutes) baths and paraffin embedded according to the methods of Bell and Lightner (1988). Larval through early juvenile stages can be processed whole and multiple shrimp embedded together. Stain 4-5 μm sections using Mayer-Bennett hematoxylin-eosin phloxine (H&E), coverslip and observe by light microscopy (400 to 600 X).

Acute TSV infections induce a distinctive histological lesion within the cuticular epithelium and subcutis of the foregut, gills, appendages, body cuticle and hindgut that is considered pathodiagnostic for this disease (Jimenez 1992, Lightner et al. 1995, Lightner 1996a, Brock et al. 1995, 1997, Hasson et al. 1995, 1999b). In susceptible moribund shrimp species, cuticular epithelial lesions are typically severe, multifocal to diffuse, and characterized by cytoplasmic eosinophilia, nuclear pyknosis and nuclear karyorhexsis in the absence of any inflammatory response. Rounding and desquamation of infected epithelial cells is commonly seen within lesions during this phase of the disease (Figure 2), which terminates with cell lysis. Numerous pyknotic and karyorrhectic nuclei impart a "peppered" or "buckshot laden" appearance to the lesion in H&E histological sections (Jimenez 1992, Lightner et al. 1995, Lightner 1996a, Brock et al. 1995, 1997, Hasson et al. 1995, 1999b). Lesions within the subhypodermal skeletal muscle, connective tissues, antennal gland, hematopoietic tissue and gonads have been observed in severely infected *L. vannamei*, but are rare to infrequent.

Marked hemocytic infiltrates and melanization within foci that previously contained cuticular epithelial cells are the primary characteristics of transition phase TSV lesions (Figure 3). Rare to infrequent scattered mild acute phase lesions of the cuticular epithelium may still persist and low numbers of spheroids may be observed within the lymphoid organ during this phase.

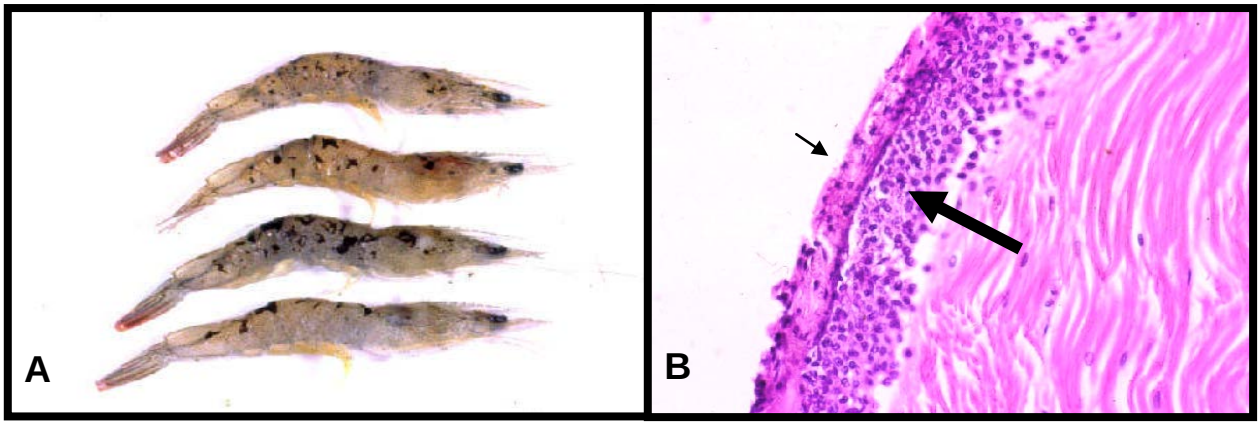

Figure 3. A) *Litopenaeus vannamei* juveniles with grossly visible TSV transition phase melanized lesions (black spots) on the abdomen and cephalothorax. B) H&E histological section of a transition phase melanized lesion illustrating numerous layered infiltrating hemocytes (large arrow), lack of epithelial cells and a melanized layer covering the surface of the lesion (small arrow).

A chronic TSV infection is characterized by numerous irregularly shaped and sized aggregates of hemocytes called spheroids (Figure 4) that are predominantly observed within the interstitium of the

lymphoid organ and along the external surface of the subgastric artery (Hasson et al. 1999b,c, Anggraeni et al. 2000). No acute or transition phase lesions are detectable histologically in chronic phase TSV infections. In severely infected shrimp, spheroids are sometimes observed in association with tegmental glands and within connective tissues of the cephalothorax and appendages (ectopic spheroids) (Hasson et al. 1999c). Lymphoid organ spheroid production is found in conjunction with at least 7 different penaeid shrimp viral diseases (TSV, LPV, IMNV, YHV, LOVV, RPV, MoV) and were recently shown to be induced by bacterial antigens (Hasson et al. 1999c, Van de Braak et al. 2002). The development of spheroids is believed to represent a cell-mediated immune response mounted by the shrimp host to sequester and eliminate foreign biotic and abiotic substances. As a result, only a presumptive diagnosis of chronic TSV infection can be made based on histologic observation of spheroids. Additional diagnostic tests (e.g. ISH or PCR) are necessary to arrive at a definitive diagnosis of chronic TSV infection (Hasson et al. 1997, 1999b, c).

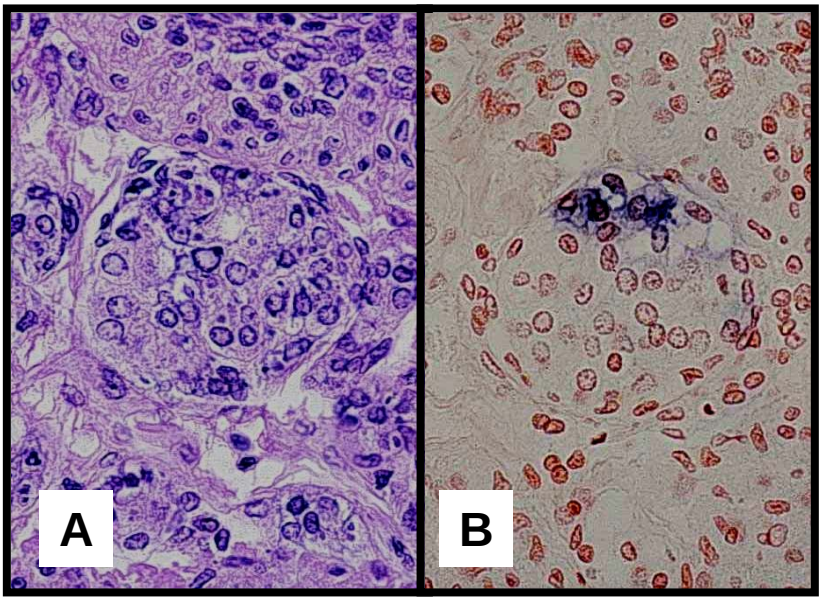

Figure 4. Consecutive histological sections illustrating a chronic phase TSV spheroid within the lymphoid organ of a *Litopenaeus vannamei* juvenile by routine H&E histology (A) and following in situ hybridization analysis with a TSV-specific DNA probe (B). Note presence of pyknotic and karyorrhectic cells within the H&E stained spheroid and a TSV probe positive signal (blue stain) within the ISH section. 400X.

2. In situ hybridization

In situ hybridization can be conducted to confirm suspect histologic findings (Figures 4 & 5). Since TSV-specific *in situ* hybridization kits (Mari et al., 1998) are no longer commercially available, randomly-labeled, digoxigenin TSV-specific probes can be generated by PCR based on the TSV PCR protocol outlined by Nunan et al. (1998).

a. Slide processing

1. De-paraffinize embedded tissue sections mounted on positively-charged slides in a xylene-based solvent.
2. Rehydrate tissue sections through a series of graded ethanols from 100% to 50% ethanol including one 10 min acid/ethanol soak.

b. Permeabilization

1. Incubate sections in Proteinase K for 8 min. at 37°C to permeabilize the tissues to allow probe penetration.
2. Inactivate the Proteinase K by heating slides up to 95-100°C for 10 min.
3. Then incubate the slide in 0.4% paraformaldehyde/PBS for 10 min at 4°C to enhance tissue adherence to the slide.

c. ISH hybridization & stringency washes

1. Incubate tissue section(s) in the hybridization solution at 100°C for 10 min followed by a 2 hr (or overnight) incubation at 42°C.

2. Wash slides in decreasing concentrations of SSC (Standard Saline Citrate Buffer, 2X to 0.2X) with the last soak at 37°C.

d. ISH detection step

1. Incubate slides in an Anti-digoxigenin-alkaline phosphatase conjugate for 30 min at 37°C.
2. Then incubate in development solution (NBT/BCIP substrate) for 30-60 min. at 37°C.
3. Counterstain with Bismarck Brown stain and dehydrate slides through graded ethanols (95%-100%) and a xylene-based solvent prior to cover-slipping for histologic evaluation (Figures 4 & 5).

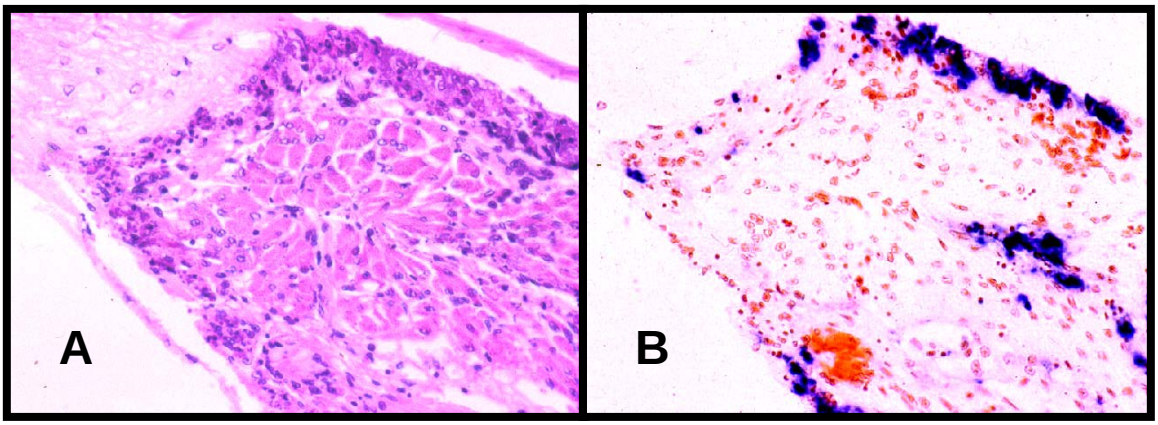

Figure 5. Consecutive histological sections of an *L. vannamei* pleopod containing acute phase TSV lesions by routine H&E histology (A) and following in situ hybridization (ISH) with a digoxigenin-labeled, TSV-specific cDNA probe (B). The probe positive signals (blue stain) observed in the ISH section identify foci containing TSV RNA within the cuticular epithelium along the outer perimeter of the appendage and skeletal muscle/connective tissue within the center of the appendage. 200X.

3. Polymerase Chain Reaction

The reverse transcription PCR protocol of Nunan et al (1998) is utilized for routine screening and diagnostic purposes and is recommended by the OIE Manual of Diagnostic Tests for Aquatic Animals (2003). Other conventional RT-PCR assays (Erickson et al. 2002, Tu et al. 1999, Do et al. 2006) and Real-time PCR assays (Dhar et al. 2002, Tang et al. 2003, Mouillesseaux et al. 2003) for TSV detection have also been developed. This technique is used to screen shrimp populations for both acute or chronic phase TSV infections, as well as to confirm suspect TSV-positive histological findings. Reverse transcription is followed by cDNA synthesis in this one step protocol, which amplifies a portion of the VP1 gene producing a 231 bp product (Nunan et al. 1998, OIE 2003). All currently known TSV serotypes

(A, B and C) are detectable by this method. Tissues used for RNA extraction can consist of pleopods, hemolymph, cephalothoracies or whole animals in the case of larval (Nauplii, Zooplankton, Mysis stages) or postlarval samples (PL5-10). Fresh, frozen (-80^{o}C) or alcohol (70-95% ethanol) preserved tissues can be utilized. Removal of eyestalks during tissue preparation of whole body postlarval samples is recommended as they may contain PCR inhibitors.

a. RNA extraction

Excise a 1-2 mm^3 piece of pleopod or cephalothorax from up to 30 fresh, frozen or ethanol preserved shrimp to obtain a pooled sample not exceeding 150 mg or pooled samples of 10% sodium citrate (anticoagulant) treated hemolymph not exceeding 100 µl can be utilized. According to Nunan et al. (1998), frozen hemolymph samples lacking anticoagulant can be assayed without prior RNA extraction. Ethanol preserved tissues are soaked in ddH_2O until saturated (15-30min) prior to extraction to remove the alcohol. Tissues are then homogenized in 1000 µl Trizol and the extracted RNA precipitated and resuspended in 100 µl of DEPC treated water for immediate use or frozen at -20 to -80^{o}C for <24 hr prior to PCR analysis. Both undiluted and diluted (1:100) samples are run for each test sample due to possible inhibition by excessive extracted RNA.

b. Amplification

1. The primers utilized are as follows:
   Forward primer TSV9195: 5' TCA ATG AGA GCT TGG TCC 3'
   Reverse primer TSV 9992: 5' AAG TAG ACA GCC GCG CTT 3'
2. Prepare a 25 µl reaction using the GeneAmp EZ rTth RNA PCR kit (Applied Biosystems):

Reagent	Vol/RXn	Initial Conc.	Final Conc.
DEPC Water	10.25 µl	-	-
5X EZ Buffer	5.0 µl	5X	1X
10 X DNTP's	3.75 µl	2.0 mM each	0.3 mM each (dATP, dGTP, dCTP, dTTP)
rTH polymerase	0.5 µl	2.5U/µl	1.25U
25mM Mn^{+2}	2.5µl	25mM	2.5 mM
TSV-9195	0.5 µl	50µM	1.0 µM
TSV-9992	0.5 µl	50µM	1.0 µM
RNA	2.0 µl	-	-

3. Cycling parameters
   Reverse Transcription: 30 min at 60 °C, 2 min at 94°C
   40 cycles of:
   45 s at 94 °C (denaturation)
   45 s at 60 °C (annealing and extension)
   Final extension of 7 min at 60 °C
   4°C hold.

PCR products (10 µl) are electrophoresed in a 2% agarose gel submerged in 1x TBE, stained with ethidium bromide for 20 min, de-stained under running tap water for 3 min, and the resulting 210 bp band visualized by UV transillumination (Figure 6).

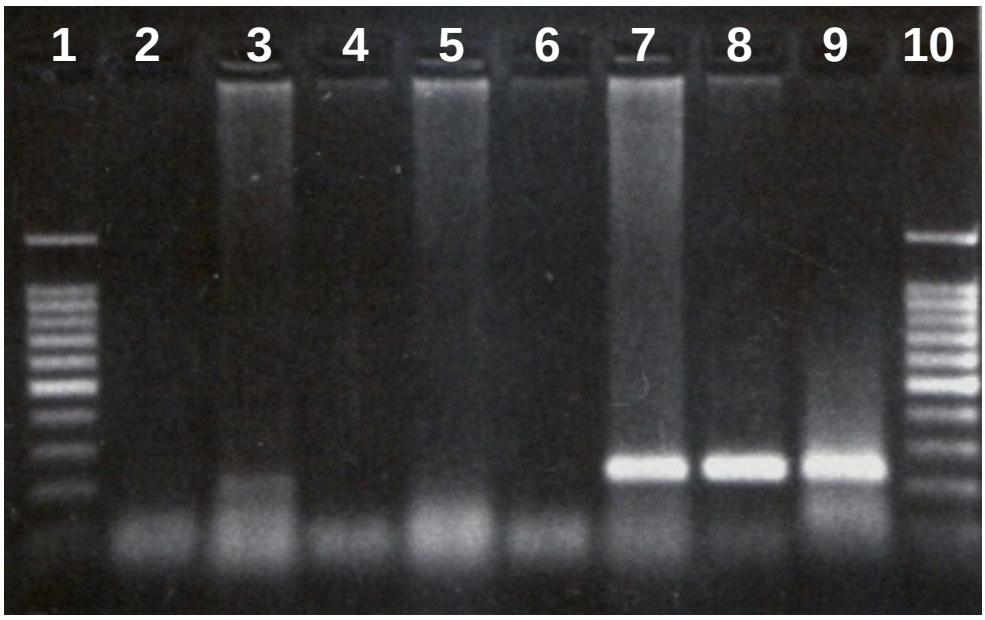

Figure 6. Electrophoretic band patterns of RT-PCR amplified TSV in an ethidium bromide- stained 2% agarose gel. A 231bp product is produced and observable in Lanes 7-9. Lanes 1 and 10: 1 kb ladder. Lane 2: Negative control (water template). Lanes 3 and 4: Undiluted and 1:100 dilution of a sample of shrimp RNA. Lanes 5 and 6: Undiluted and 1:100 dilution of a second sample of shrimp RNA. Lanes 7 and 8: Undiluted and 1:100 dilution of RNA extracted from TSV infected shrimp tissue (Positive control). Lane 9: TSV amplicons (positive control).

F. Procedures for Transportation and Storage of Samples

Davidson's preserved samples for routine histology should be transferred to either 70 or 95% ethanol within 24 hr post-fixation and prior to shipping or transport as described by Bell and Lightner (1988). Ideally, tissues for histological analysis should be processed and embedded within 24-48 hr following preservation to avoid potential hydrolysis of the viral RNA and consequent false-negative in situ hybridization results (Hasson et al. 1997). Alternatively, samples collected specifically for TSV in situ hybridization analysis can be preserved with R-F fixative (Hasson et al. 1997). Ethanol preserved pleopods, tissues or whole animals should be shipped in 70-95% ethanol. Frozen hemolymph, tissue samples or whole animals should be shipped on dry ice and maintained at -80^{o}C for long term storage.

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