CHAPTER 2.2.4.

TAURA SYNDROME

1. Scope

Taura syndrome (TS) is a virus disease of penaeid shrimp caused by infection with Taura syndrome virus (TSV) (3, 15, 27, 42). The principal host species in which TSV can cause significant disease outbreaks and mortalities are *Penaeus vannamei* and *P. stylirostris*. TS has three distinct phases: acute, transition and chronic. In acute disease, pathognomonic lesions are typically present in histological sections of the cuticular epithelium, while in the transition and chronic phases of the disease there are no pathognomonic lesions and molecular and antibody-based methods for TSV detection are necessary for diagnosis (27). Chronic TSV infections in individual shrimp may persist for life. Vertical transmission is likely, but not experimentally confirmed (27).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent is TSV, as described by Bonami *et al.* (3) and Mari *et al.* (42, 43). At least four genotypes (strains) have been documented based on the sequence of the VP1 (= CP2), the largest and presumably dominant structural protein of the virus. Based on the sequence of VP1 (= CP2) these genotypic groups are: 1) the Americas group; 2) the South-East Asian group; 3) the Belize group; and 4) the Venezuelan group (8, 13, 14, 45, 56).

At least two distinct antigenic variants of TSV have been demonstrated using the monoclonal antibody MAb 1A1, produced to a reference isolate from the Americas (TSV USA-HI94 – GenBank AF277675) (43, 50): Type A represents those that react to MAb 1A1 (in the enzyme-linked immunosorbent assay [ELISA], Western blots and *in-situ* hybridisation [ISH] with infected tissues) and those that do not. The MAB 1A1 non-reactors were subdivided into Types B (TSV 98 Sinaloa, Mexico) and Type C (TSV 02 Belize), based on host species and virulence. All TSV isolates of the Americas and most, if not all, South-East Asian genotypes react with MAb 1A1. In marked contrast, none of the Belize genotype group reacts with MAb 1A1 (13, 14), nor does a TSV isolate from the 2005 epizootic in Venezuelan shrimp farms.

TSV particles are 32 nm, non-enveloped icosahedrons with a buoyant density of 1.338 g ml^{-1}. The genome of TSV consists of a linear, positive-sense single-stranded RNA of 10,205 nucleotides, excluding the 3' poly-A tail, and it contains two large open reading frames (ORFs). ORF 1 contains the sequence motifs for nonstructural proteins, such as helicase, protease and RNA-dependent RNA polymerase. ORF 2 contains the sequences for TSV structural proteins, including the three major capsid proteins VP1, VP2 and VP3 (55, 40, and 24 kDa, respectively). The virus replicates in the cytoplasm of host cells (3, 42, 43, 53).

TSV was listed as an unassigned species in the Family Dicistroviridae in the most recent report of the International Committee on Taxonomy of Viruses (ICTV; 15).

Other reported causes of TS: Taura syndrome in Ecuador was initially linked to pesticide contamination of shrimp farms, a contention that was supported by litigation for ~8 years after the disease was scientifically shown to have a viral aetiology (3, 21, 30). Hence, several papers in the literature propose a toxic aetiology for TS (22–24).

2.1.2. Survival outside the host

No information available.

2.1.3. Stability of the agent (effective inactivation methods)

No information available.

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species

The principal host species for TSV are the Pacific white shrimp, *Penaeus vannamei*, and the Pacific blue shrimp, *P. stylirostris.* While the principal host species for TSV all belong to the penaeid subgenus *Litopenaeus*, other penaeid species can be infected with TSV by direct challenge, although disease signs do not develop. Documented natural and experimental hosts for TSV include: *P. setiferus, P. schmitti, P. monodon, P. chinensis, P. japonicus, P. aztecus, P. duorarum* and *Metapenaeus ensis* (4, 5, 7, 8, 27, 28, 48, 55).

2.2.2. Susceptible stages of the host

TSV has been documented in all life stages (i.e. PL, juveniles and adults) of *P. vannamei* (the most economically significant of the two principal host species) except in eggs, zygotes and larvae (27).

2.2.3. Species or subpopulation predilection (probability of detection)

No data.

2.2.4. Target organs and infected tissue

TSV infects and has been shown to replicate (using ISH with specific DNA probes) principally in the cuticular epithelium (or hypodermis) of the general exoskeleton, foregut, hindgut, gills and appendages, and often in the connective tissues, the haematopoietic tissues, the lymphoid organ (LO), and antennal gland. The enteric organs (endoderm-derived hepatopancreas, midgut and midgut caeca mucosal epithelia) and smooth, cardiac, striated muscle, and the ventral nerve cord, its branches and its ganglia typically show no histological signs of infection by TSV and are usually negative for TSV by ISH (4, 18–20, 24, 27, 31, 32, 34, 55).

2.2.5. Persistent infection with lifelong carriers

Some members of populations of *P. vannamei* or *P. stylirostris* that survive TSV infections and/or epizootics may carry the virus for life (19, 20) and, although not documented, pass the virus to their progeny by vertical transmission. Survivors of TSV infections may carry the virus for life (7, 20, 27–29, 33, 35, 39).

2.2.6. Vectors

Sea birds: TSV has been demonstrated to remain infectious for up to 48 hours (after ingestion of TSV-infected shrimp carcasses) in the faeces passed by wild or captive sea gulls (*Larus atricilla*) and chickens (*Gallus domesticus,* used as a laboratory surrogate for all shrimp-eating birds). These findings implicate birds as being an important mechanical vector for the transmission of the virus within affected farms or farming regions (17, 59).

Aquatic insects: the water boatman (*Trichocorixa reticulata* [*Corixidae*], an aquatic insect that feeds on shrimp carcasses in shrimp farm ponds), has also been shown to serve as a mechanical vector of TSV (5, 26–28).

Frozen TSV-infected commodity products: TSV has been found in frozen commodity shrimp (*P. vannamei*) products in samples from markets in the USA that originated in Latin America and South-East Asia. Improper disposal of wastes (liquid and solid, i.e. peeled shells, heads, intestinal tracts, etc.) from value-added reprocessing of TSV-infected shrimp at coastal locations may provide a source of TSV that may contaminate wild or farmed stocks near the point of the waste stream discharge (28, 47).

2.2.7. Known or suspected wild aquatic animal carriers

No data.

2.2.8. Zoonotic potential

TSV was reported to infect human and monkey cell lines, suggesting a zoonotic potential for this virus and that penaeid shrimp could serve as reservoirs for TSV and other members of the 'picornavirus superfamily' that infect humans (2). As a result of the experimental design and the improbable results reported by Audelo del Valle *et al.* (2), two other laboratories repeated the study and both found that TSV does not infect or replicate in primate or human cell lines with known susceptibility to human picornaviruses (41, 49), effectively refuting the contention that TSV has zoonotic potential.

2.3. Disease pattern

TS is best known as a disease of nursery- or grow-out-phase *P. vannamei* that occurs within ~14–40 days of stocking PLs into grow-out ponds or tanks, hence, shrimp with TS are typically small juveniles of from ~0.05 g to <5 g. Larger shrimp may also be affected, especially if they are not exposed to the virus until they are larger juveniles or adults (5, 6, 27, 28, 36).

2.3.1. Transmission mechanisms

Transmission of TSV can be by horizontal or vertical routes. Horizontal transmission by cannibalism or by contaminated water has been demonstrated (5, 21, 27, 28, 60). Vertical transmission from infected adult broodstock to their offspring is strongly suspected but has not been experimentally confirmed.

2.3.2. Prevalence

In regions where the virus is enzootic in farmed stocks, the prevalence of TSV has been found in various surveys to range from 0 to 100% (5, 24, 25).

2.3.3. Geographical distribution

TS is widely distributed in the shrimp-farming regions of the Americas and South-East Asia (4, 5, 8, 19, 27, 28, 37, 45, 56, 58, 63).

The Americas: following its recognition in 1992 as a distinct disease of cultured *P. vannamei* in Ecuador (6, 23), TS spread rapidly throughout many of the shrimp-farming regions of the Americas through shipments of infected PL and broodstock (5, 7, 19, 27, 28). Within the Western Hemisphere, TS and TSV have been reported from virtually every penaeid shrimp-growing region in the Americas and Hawaii (1, 5, 53). TSV is enzootic in cultured penaeid shrimp stocks on the Pacific coast of the Americas from Peru to Mexico, and it has been occasionally found in some wild stocks of *P. vannamei* from the same region (31, 34). TSV has also been reported in farmed penaeid stocks from the Atlantic, Caribbean, and Gulf of Mexico coasts of the Americas, but it has not been reported in wild stocks from the these regions (19, 27, 28, 30).

Asia: TSV was introduced into Chinese Taipei in 1999 with infected imported Pacific white shrimp, *P. vannamei*, from Central and South American sources (58, 63). Since that original introduction, the virus has spread with movements of broodstock and PL to China (People's Rep. of), Thailand, Malaysia, and Indonesia where it has been the cause of major epizootics with high mortality rates in introduced unselected stocks of *P. vannamei* (8, 45, 56).

2.3.4. Mortality and morbidity

In on-farm epizootics of TS involving unselected stocks of *P. vannamei*, the principal host species for TSV, typical cumulative mortalities range from 40 to >90% in cultured populations of PL, juvenile, and subadult life stages. TSV-resistant lines of *P. vannamei* are available which show survival rates of up to 100% in laboratory challenge with all four TSV genotypes (33, 44).

2.3.5. Environmental factors

Outbreaks of TS are more frequent when salinities are below 30 ppt (24).

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccines for TSV are available.

2.4.2. Chemotherapy

No scientifically confirmed reports.

2.4.3. Immunostimulation

No scientifically confirmed reports

2.4.4. Resistance breeding

Penaeus stylirostris were found to be resistant to TSV (genotype 1, MAb 1A1 type A) after TS emerged in Ecuador in 1992–1994. Owing to this characteristic, by 1995, selected lines of *P. stylirostris* became the dominant stocks farmed in western Mexico after TSV had reached Mexico in 1994 and caused crop failures in farms with *P. vannamei*. However, in 1998–1999, a new 'strain' of TSV (Type B; 14, 16, 29, 30, 64) emerged and caused massive epizootics in *P. stylirostris*. The emergence of this new 'strain' of TSV was soon followed in late 1999 by the introduction of white spot syndrome virus (WSSV) into shrimp farms in western Mexico, to which *P. stylirostris* had no resistance, effectively ending any interest in the culture of *P. stylirostris*.

TSV-resistant domesticated stocks of *P. vannamei* and *P. stylirostris* have been developed. Some domesticated lines of TSV-resistant *P. vannamei* (that are also TSV-free) are in widespread use by the shrimp-farming industries of the Americas and South-East Asia (10, 44, 60). After the appearance of TS in Central America, improved TS resistance was reported in wild caught *P. vannamei* PLs used to stock shrimp farms in the region (25).

2.4.5. Restocking with resistant species

Selected lines of TS resistant *P. vannamei* have been developed and are commercially available (10, 25, 44, 60).

2.4.6. Blocking agents

Resistance to TSV infection was reported by expression of the TSV coat protein antisense RNA in *P. vannamei* zygotes. Transgenic juveniles reared from zygotes protected in this manner showed improved resistance to TSV challenge by *per os* or intramuscular (IM) injection routes (40). Similar results have been produced by introducing random double-stranded RNA sequences into juvenile *P. vannamei* (52).

2.4.7. Disinfection of eggs and larvae

While TSV is believed to be transmitted vertically (transovarian transmission), there have been no published report documenting this route of transmission. Disinfection of eggs and larvae (9) is good management practice and it is recommended for its potential to reduce TSV contamination of spawned eggs and larvae produced from them.

2.4.8. General husbandry practices

Some husbandry practices have been successfully applied to the prevention of TSV infections and TS disease. These include the application of polymerase chain reaction (PCR) prescreening of wild or pond-reared broodstock and/or their spawned eggs/nauplii and discarding those that test positive for the virus (16), fallowing and restocking of entire culture regions with TSV-free stocks (12), and the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (28, 30, 38, 44, 51, 61, 62). The adoption of the latter technology (SPF stocks) has proven to be among the most successful husbandry practice for the prevention and control of TS. Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status. The development of SPF *P. vannamei* that were free not only of TSV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its challenging *P. monodon* by 2004–2005 as the dominant farmed shrimp species in Asia, as well as the Americas where the SPF stocks were developed (30, 54).

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection by TSV include PL, juveniles and adults. While TSV may infect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in the larval stages, so these life stages may not be suitable samples for TSV detection or certification of TS disease freedom.

3.2. Preservation of samples for submission

For routine histology or molecular assays, and guidance on preservation of samples for the intended test method see Chapter 2.2.0.

3.3. Pooling of samples

Samples taken for molecular tests may be combined as pooled samples of no more than five specimens per pooled sample of faecal strands, juveniles, subadults and adults. However, for eggs, larvae and PL pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs or tissues

TSV infects tissues of ectodermal and mesodermal origin. The principal target tissue in the acute phase of TS is the cuticular epithelium. In chronic infections the LO is the principal target tissue.

Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary.

3.5. Samples/tissues that are not suitable

TSV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of infection by TSV.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Only acute-phase TS disease can be presumptively diagnosed from clinical signs. See Section 4.2 for a description of gross clinical signs presented by shrimp with acute-phase TS disease.

4.1.2. Behavioural changes

Only shrimp with acute-phase TS disease present behavioural changes. Typically, severely affected shrimp apparently become hypoxic and move to the pond edges or pond surface where dissolved oxygen levels are higher. Such shrimp may attract seabirds in large numbers. In many TS disease outbreaks, it is the large numbers of seabirds attracted to the moribund shrimp that first indicate the presence of a serious disease outbreak (which is often either TS or WSD when sea birds are observed) to the farm manager.

4.2. Clinical methods

4.2.1. Gross pathology

TS disease has three distinct phases, acute, transition, and chronic, which are grossly distinguishable (19, 20, 27, 28, 34). Gross signs presented by juvenile, subadult and adult shrimp in the transition phase of TS are unique and provide a provisional diagnosis of the disease.

Acute phase: gross signs displayed by moribund *P. vannamei* with acute-phase TS include expansion of the red chromatophores giving the affected shrimp a general, overall pale reddish coloration and making the tail fan and pleopods distinctly red; hence ‘red tail’ disease was one of the names given by farmers when the disease first appeared in Ecuador (34). In such shrimp, close inspection of the cuticular epithelium in thin appendages (such as the edges of the uropods or pleopods) with a ×10 hand lens reveals signs of focal epithelial necrosis. Shrimp showing these gross signs of acute TS typically have soft shells, an empty gut and are often in the late D stages of the moult cycle. Acutely affected shrimp usually die during ecdysis. If the affected shrimp are larger than ~1 g, moribund shrimp may be visible to sea birds at the pond edges and surface. Thus, during the peak of severe epizootics, hundreds of sea birds (gulls, terns, herons, cormorants, etc.) may be observed feeding on affected moribund shrimp that accumulate at the surface of the affected pond surface and edges (5–7, 17, 27, 28, 34, 59).

Transition (*recovery*) *phase:* although only present for a few days during TS epizootics, the gross signs presented by shrimp in the transition phase can provide a tentative diagnosis of TSV infection. During the transition phase (which may be occurring while many shrimp in the affected populations are still in the acute phase and daily mortalities are high), fair to moderate numbers of shrimp in affected ponds show random, multifocal, irregularly shaped melanised cuticular lesions. These melanised spots are haemocyte accumulations indicating the sites resolving TS lesions in the cuticular epithelium. Such shrimp may or

may not have soft cuticles and red-chromatophore expansion, and may be behaving and feeding normally (5, 20, 27).

Chronic phase: after successfully moulting, shrimp in the transition phase move into the chronic phase of TS in which persistently infected shrimp show no obvious signs of disease (5, 20, 27, 28, 34). However, *P. vannamei* that are chronically infected with TSV may be less resistant to normal environmental stressors (i.e. sudden salinity reductions) than uninfected shrimp (38).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

TS disease in the acute and chronic phases can be diagnosed most reliably using histological methods (20, 27). Pathognomonic TSV-induced pathology is unique in acute-phase infections (6, 27). In chronic TSV infections, the only lesion typically presented by infected shrimp is the presence of an enlarged LO with multiple LO spheroids (LOS) (20), which cannot be distinguished from LOS induced by chronic infections of other RNA viruses (27). When LOS are observed by routine histology and chronic TSV infection is suspected, a molecular test (ISH with TSV-specific probes, or reverse-transcription [RT] PCR [see Section 4.3.1.2.3]) is recommended for confirmation of TSV infection.

4.2.3.1. Acute phase of Taura syndrome

Diagnosis of TS in the acute phase of the disease is dependent on the histological demonstration (in haematoxylin and eosin [H&E] stained preparations) of multifocal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hindgut, and foregut (the oesophagus, anterior and posterior chambers of the stomach). Cells of the subcuticular connective tissues and adjacent striated muscle fibres basal to affected cuticular epithelium are occasionally affected. In some severe cases of acute-phase TS, the antennal gland tubule epithelium is also destroyed. Prominent in the multifocal cuticular lesions are conspicuous foci of affected cells that display an increased eosinophilia of the cytoplasm and pyknotic or karyorrhectic nuclei. Cytoplasmic remnants of necrotic cells are often extremely abundant in these TS acute-phase lesions and these are generally presented as spherical bodies (1–20 µm in diameter) that range in staining from eosinophilic to pale basophilic. These structures, along with pyknotic and karyorrhectic nuclei, give acute-phase TS lesions a characteristic 'peppered' or 'buckshot-riddled' appearance, which is considered to be pathognomonic for TS disease when there is no concurrent necrosis of the parenchymal cells of the LO tubules. The absence of necrosis of the LO in acute-phase TSV infections distinguishes TS disease from acute-phase yellowhead disease in which similar patterns of necrosis to those induced by TSV may occur in the cuticular epithelium and gills (27).

In TSV-infected tissues, pyknotic or karyorrhectic nuclei give a positive (for DNA) Feulgen reaction, which distinguishes them from the less basophilic to eosinophilic cytoplasmic inclusions that do not contain DNA. The absence of haemocytic infiltration or other signs of a significant host-inflammatory response distinguishes the acute phase of TS from the transitional phase of the disease (4–7, 13, 14, 19–21, 27, 34).

4.2.3.2. Transition (recovery) phase of Taura syndrome

In the transitional phase of TS, typical acute-phase cuticular lesions decline in abundance and severity and are replaced by conspicuous infiltration and accumulation of haemocytes at the sites of necrosis. The masses of haemocytes may become melanised giving rise to the irregular black spots that characterise the transition phase of the disease. In H&E sections, such lesions may show erosion of the cuticle, surface colonisation and invasion of the affected cuticle and exposed surface haemocytes by *Vibrio* spp. (20, 27). Sections of the LO during the transition phase of TS may appear normal with H&E staining. However, when sections of the LO are assayed for TSV by ISH with a specific cDNA probe (or by ISH with MAb 1A1 for TSV type A, genotype 1), large quantities of TSV are shown accumulating in the more peripheral parenchymal cells of the LO tubules (20, 55).

4.2.3.3. Chronic phase of Taura syndrome

Shrimp in the chronic phase of TS display no gross signs of infection, and histologically the only sign of infection is the presence of numerous prominent LOS, which may remain associated with the main body of the paired LO, or which may detach and become ectopic LOS bodies that lodge in constricted areas of the haemocoel (i.e. the heart, gills, in the subcuticular connective tissues, etc.). Such LOS are spherical accumulations of LO cells and haemocytes and may be distinguished from normal LO tissues by their spherical nature and the lack of the central vessel that is typical of normal LO tubules. When

assayed by ISH with a cDNA probe for TSV (or with MAb 1A1 using ISH) some cells in the LOS give positive reactions to the virus, while no other target tissues react (20, 27, 28)

4.2.4. Wet mounts

Direct microscopy of simple unstained wet mounts from excised pieces of the gills, appendage tips, etc., examined by phase- or reduced-light microscopy may be used to demonstrate (and make a tentative diagnosis of acute-phase TS) focal lesions of acute-phase TS in cuticular epithelial cells. Preparations presenting TS acute-phase lesions will contain numerous spherical structures (see the histopathological methods in Section 4.2.3 above), which are pyknotic and karyorrhectic nuclei and cytoplasmic remnants of necrotic cells.

4.2.5. Smears

Not applicable.

4.2.6. Electron microscopy/cytopathology

Not currently applicable for diagnostic purposes.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

Not applicable.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.1.4. Bioassay method

Confirmation of TSV infection may be accomplished by bioassay of TSV-suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the virus (7, 17, 20, 21, 27, 36, 48). Oral or injection protocols may be used. The oral method is relatively simple to perform and is accomplished by feeding chopped carcasses of suspect shrimp to SPF juvenile *P. vannamei* in small tanks (60). The use of a negative control tank of indicator shrimp, which receive only a normal feed, is required. When the carcass feeding (*per os*) protocol is used to bioassay for TSV, TS-positive indicator shrimp (by gross signs and histopathology) are typically apparent within 3–4 days of initial exposure, and significant mortalities occur by 3–8 days after initial exposure. The negative control shrimp must remain negative (for at least 10–15 days) for gross or histological signs of TS disease and unusual mortalities (20, 27, 60).

With the injection bioassay protocol, a variety of sample types may be tested for TSV. Whole shrimp are used if they were collected during a TSV epizootic. Heads only should be used if shrimp display gross transition-phase lesions (multifocal melanised spots on the cuticle) or no clinical signs of infection (chronic phase) as the virus, if present, will be concentrated in the LO (20, 27). For non-lethal testing of broodstock, haemolymph samples may be taken and used to expose the indicator shrimp by IM injection (27).

To perform the IM (injection) bioassay for TSV:

i) Prepare a 1:2 or 1:3 ratio of TSV-suspect shrimp heads or whole shrimp with TN buffer (see Chapter 2.2.2, infectious hypodermal and haematopoietic necrosis [IHHN], for the composition of this buffer) or sterile 2% saline prepared with distilled water.

ii) Homogenise the mixture using a tissue grinder or blender. Do not permit the mixture to heat up by excessive homogenisation or grinding. Tissues and the resulting homogenate should be kept cool during the entire protocol by maintaining on ice.

iii) Clarify the homogenate by centrifugation at 3000 ***g*** for 10 minutes. Decant and save the supernatant fluid. Discard the pellet.

iv) Centrifuge the supernatant fluid at 27,000 ***g*** for 20–30 minutes at 4°C. Decant and save the supernatant fluid. Discard the pellet.

v) Dilute the supernatant fluid from step iv to 1/10 to 1/100 with sterile 2% saline. This solution may now be used as the inoculum to inject indicator shrimp (or filter sterilised as described in step vi).

vi) Filter the diluted supernatant fluid from step v using a sterile syringe (size depends on the final volume of diluted supernatant) and a sterile 0.45 µm syringe filter. Multiple filters may have to be used as they clog easily. Filtrate should be collected in a sterile test tube or beaker. The solution can now be stored frozen (recommend –20°C for short-term [weeks] storage and –80°C for long-term [months to years] storage) or used immediately to inject indicator shrimp.

vii) Indicator shrimp should be from TSV-susceptible stocks of SPF *P. vannamei* (such as the ‘Kona stock’) (44), which are commercially available from a number of sources in the Americas, and not from selected lines of known TSV-resistant stocks.

viii) Inject 0.01 ml per gram of body weight using a 1 ml tuberculin syringe. Indicator shrimp should be injected intramuscularly into the third tail segment. If the test shrimp begin to die within minutes post-injection, the inoculum contains excessive amounts of proteinaceous material and should be further diluted prior to injecting additional indicator shrimp. Sudden death occurring post-injection is referred to as ‘protein shock’, and is the result of systemic clotting of the shrimp’s haemolymph in response to the inoculum (27, 60).

ix) Haemolymph samples may be diluted (1/10 or 1/20 in TN buffer), filter sterilised (if necessary), and injected into the indicator shrimp without further preparation.

x) If TSV was present in the inoculum, the indicator shrimp should begin to die within 24–48 hours post-injection. Lower doses of virus may take longer to establish a lethal infection and shrimp should be monitored for at least 10–15 days post-injection.

xi) The presence (or absence) of TSV in the indicator shrimp should be confirmed by histological analysis (and/or ISH by gene probe, if available) of Davidson’s fixed moribund shrimp.

4.3.1.1.5. Sentinel shrimp bioassay method

As a variation to the bioassay technique, a ‘sentinel shrimp’ system may be used. For example, TSV-sensitive stocks of small juvenile SPF *P. vannamei* may be held in net-pens in tanks, or in the same water system, with other shrimp of unknown TSV status to bioassay for the presence of infectious agents such as TSV.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

TSV has not been grown *in vitro*, as no crustacean cell lines exist (27, 49). Despite a recent publication that incorrectly reported that TSV infected human and monkey cell lines (2), two other laboratories repeated the study and both found that TSV does not infect or replicate in primate or human cell lines with known susceptibility to human picornaviruses (41, 49).

4.3.1.2.2. Antibody-based antigen detection methods

An MAb for detection of TSV may be used to assay samples of haemolymph, tissue homogenates, or Davidson’s AFA-fixed tissue sections from shrimp (13, 14, 50). TSV MAb 1A1 may be used to distinguish some variants or ‘strains’ of TSV from other strains (13, 14).

4.3.1.2.2.1. Dot-blot immunoassay method

i) For the dot-blot immunoassay method, 1 µl of test antigen (purified virus, infected shrimp haemolymph or SPF shrimp haemolymph) is dotted on to the surface of MA-HA-N45 assay plates (Millipore, South San Francisco, California [CA], USA)[1].

ii) After air drying, the wells are blocked for 1 hour at room temperature with 200 µl of a buffer containing phosphate-buffered saline and 0.05% Tween 20 (PBST) mixed with 10% normal goat serum (Life Technologies, Gibco BRL) and 2% Hammersten casein (Amersham Life Sciences, Arlington Heights, Illinois, USA).

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1 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

iii) The wells are washed three times with PBST and then reacted with 100 µl primary antibody (MAb or mouse polyclonal antibodies) for 30 minutes at room temperature.

iv) Alkaline-phosphatase-labelled goat anti-mouse IgG, γ chain specific, secondary antibody (Zymed, South San Francisco, CA) diluted 1/1000 in PBST plus 10% normal goat serum is used for detection (30 minutes at room temperature).

v) After washing three times with PBST, once with PBS and once with distilled water, the reactions are visualised by development for 15 minutes at room temperature with nitroblue tetrazolium and bromo-chloro-indoyl phosphate (Roche Diagnostics, Corp.) in Tris-NaCl (100 mM each) buffer containing 50 mM $MgCl_2$, pH 9.5.

vi) Reactions are stopped with distilled water.

vii) The reactions are graded using a scale from 0 to +4, with the highest intensity reaction being equivalent to the reaction generated using the MAb against the reference control consisting of semi-purified TSV. A negative reaction is one in which no coloured spot is visible in the well.

4.3.1.2.2.2. Other antibody-based methods

The TSV MAb 1A1 may be applicable to other antibody-based test formats (i.e. indirect fluorescent antibody [IFAT] or immunohistochemistry [IHC] tests with tissue smears, frozen sections, or deparaffinised fixed tissues). MAb 1A1 is applicable for use in an IHC format using Davidson's AFA-fixed tissue sections (13, 14).

It is recommended that unexpected results from MAb-based tests for TSV should be interpreted in the context of clinical signs, case history, and in conjunction with other test results (e.g. RT-PCR test results, or findings from histology or ISH with a TSV-specific DNA probe – see appropriate sections in this chapter).

4.3.1.2.3. Molecular techniques

ISH and RT-PCR tests for TSV have been developed, and kits of RT-PCR methods for TSV are commercially available. The dot-blot method for TSV detection is not available.

4.3.1.2.3.1. DNA probes for ISH applications with non-radioactive cDNA probes

Non-radioactive, DIG-labelled cDNA probes for TSV may be produced in the laboratory. The ISH method provides greater diagnostic sensitivity than do more traditional methods for TSV detection and diagnosis that employ classic histological methods (19, 27, 29, 32, 42). The ISH assay of routine histological sections of acute- and transition-phase lesions in the cuticular epithelium, other tissues, and of LOS in transition and chronic phase with a specific DIG-labelled cDNA probe to TSV, provides a definitive diagnosis of TSV infection (19, 20, 27, 28). Pathognomonic TSV-positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the cDNA probes. Not reacting to the probe are the prominent karyorrhectic nuclear fragments and pyknotic nuclei that contribute to the pathognomonic 'buckshot riddled' appearance of TS lesions (27, 28, 42). (See Chapter 2.2.2 IHHN for details of the ISH method, and Chapter 2.2.0 Section B.5.3.ii for detailed information on the use of Davidson's AFA fixative.)

False-negative ISH results may occur with Davidson's fixed tissues if tissues are left in fixative for more than 24–48 hours. The low pH of Davidson's fixative causes acid hydrolysis of the TSV single-stranded RNA genome, resulting in false-negative probe results. This artefact can be avoided through the use of neutral fixatives, including an 'RNA-friendly' fixative developed for shrimp, or by the proper use (avoiding fixation times over 24 hours) of Davidson's fixative (18, 27, 32).

4.3.1.2.3.2. Reverse-transcription (RT)-PCR method

Tissue samples (haemolymph, pleopods, whole small shrimp, etc.) may be assayed for TSV using RT-PCR. Primers designated as 9195 and 9992, amplify a 231 base pair (bp) sequence of the TSV genome (46). The fragment amplified is from a conserved sequence located in the intergenic region and ORF 2 of TSV. Primer 9992F is located near the 3' end of intergenic region and 9195R is located on ORF 2 within VP2 (= CP1) (43, 46).

Primer	Product	Sequence	G+C%	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 RT-PCR method outlined below for TSV generally follows the method used in Nunan *et al.* (46).

i) *Preparation of RNA template:* RNA can be extracted from fresh, frozen and ethanol-preserved tissues. Extraction of RNA should be performed using commercially available RNA tissue extraction kits, such as the High Pure RNA Tissue Kit (Roche, Penzberg, Germany) and following the manufacturer's procedures for production of quality RNA templates.

ii) The RT-PCR assay is carried out in solution, using 10 µl of total RNA extracted from haemolymph, frozen shrimp tissues, ethanol fixed tissue as the template (concentration of RNA = 1–100 ng ml^{-1}).

iii) The following controls should be included in every RT-PCR assay for TSV: a) known TSV-negative tissue sample; b) a known TSV-positive sample (tissue or purified virus); and c) a 'no-template' control.

iv) The GeneAmp® EZ rTth RNA PCR kit (Applied Bioscience, Forster City, CA) is used for all amplification reactions described here.

v) The optimised RT-PCR conditions (final concentrations in 50 µl total volume) for detection of TSV in shrimp tissue samples are: primers (0.46 µM each), dNTPs (300 µM each), rTth DNA polymerase (2.5 U 50 μl^{-1}), manganese acetate (2.5 mM), in 5 × EZ buffer (25 mM Bicine, 57.5 mM potassium acetate, 40% [w/v] glycerol, pH 8.2).

vi) If the thermal cycler does not have a heated lid, then light mineral oil (50 µl) is overlaid on the top of the 50 µl reaction mixtures to prevent condensation or evaporation during thermal cycling.

vii) The RNA template and all the reagents are combined and reverse transcription is allowed to proceed at 60°C for 30 minutes, followed by 94°C for 2 minutes.

Note: The reaction conditions described here were optimised using an automatic Thermal Cycler GeneAmp 980 (Applied Biosystems). The conditions should be optimised for each thermal cycler using known positive controls.

viii) At the completion of reverse transcription, the samples are amplified for 40 cycles under the following conditions: denaturation at 94°C for 45 seconds, and then annealing/extension at 60°C for 45 seconds. A final extension step for 7 minutes at 60°C follows the last cycle and the process is terminated in a 4°C soak file.

ix) Following the termination of RT-PCR, the amplified cDNA solutions are drawn off from beneath the mineral oil and placed into clean 0.5 ml microfuge tubes.

x) A 10 µl sample of the amplified product can then be added to the well of a 2.0% agarose gel, stained with ethidium bromide (0.5 g ml^{-1}), and electrophoresed in 0.5 × TBE (Tris, boric acid, ethylene diamine tetra-acetic acid [EDTA]).

xi) A 1 kb DNA ladder (Invitrogen, Carlsbad, CA) is used as a marker.

xiii) Details of the composition of the reagents and buffers used here may be found in Chapter 2.2.2 IHHN.

4.3.1.2.3.3. Real-time PCR method for TSV

Real-time RT-PCR methods have been developed for the detection of TSV. These methods have the advantages of speed, specificity and sensitivity. The sensitivity of real-time RT-PCR is ~100 copies of the target sequence from the TSV genome (11, 57).

The real-time RT-PCR method using TaqMan chemistry described below for TSV generally follows the method used in Tang *et al.* (57).

i) The PCR primers and TaqMan probe were selected from the ORF1 region of the TSV genomic sequence (GenBank AFAF277675) that encodes for nonstructural proteins. The primers and TaqMan probe were designed by the Primer Express software (Applied Biosystems). The upstream (TSV1004F) and downstream (TSV1075R) primer sequences are: 5'-TTG-GGC-ACC-AAA-CGA-CAT-T-3' and 5'-GGG-AGC-TTA-AAC-TGG-ACA-CAC-TGT-3'), respectively. The TaqMan probe, TSV-P1 (5'-CAG-CAC-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-3'), which corresponds to the region from nucleotide 1024 to 1051, is synthesised and labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end (Applied Biosystems, catalog no. 450025).

ii) *Preparation of RNA template:* the extraction and purification of RNA template from haemolymph, or shrimp tissue, is the same as that described in the section for traditional RT-PCR.

iii) The RT-PCR reaction mixture contains: TaqMan One-step RT-PCR Master Mix (Applied Biosystems, part no. 4309169), 0.3 µM of each primer, 0.1 µM of TaqMan probe, 5–50 ng of RNA, and water in a reaction volume of 25 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iv) Amplification is performed with the GeneAmp 5700 Sequence Detection System (Applied Biosystems; ABI PRISM 7000, 7300, or 7500 can also be used). The cycling consists of reverse transcription at 48°C for 30 minutes and initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.

v) At the end of the reaction, real-time fluorescence measurements will be taken with a built in charge-coupled device (CCD) camera. A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase in PCR product. Samples will be defined as negative if the Ct (threshold cycle) value is 40 cycles. Samples with a Ct value lower than 40 cycles are considered to be positive. To confirm the real-time RT-PCR results, an aliquot of RT-PCR product can be subjected to electrophoresis on a 4% ethidium bromide-agarose gel and photographed. A 72-bp DNA fragment can be visualised in the samples that are positive for TSV.

vi) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture or in the heat block of the thermal cycler. A positive control should also be included, and this can be an *in-vitro* transcribed RNA containing the target sequence, purified virions, or RNA extracted from TSV-infected tissue.

4.3.1.2.3.4. Sequencing

RT-PCR products may be cloned and sequenced when necessary to confirm infection by TSV or to identify false positives or nonspecific amplification (43, 45, 55, 56).

4.3.1.2.4. Agent purification

Methods for TSV isolation and purification are available (3, 21, 43, 50), but these are not recommended for routine diagnosis of TS.

4.3.2. Serological methods

Not applicable because shrimp are invertebrate animals which do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to TSV.

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of TSV are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	b	c
Bioassay	d	d	d	d	c	b
Direct LM	d	d	c	d	c	d
Histopathology	d	b	b	c	a	a

***Table 5.1.* (*cont.*)** *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	c	c	b	b
DNA probes – *in situ*	d	c	b	b	a	a
RT-PCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; RT-PCR = reverse-transcription polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from Taura syndrome

As indicated in Table 5.1, RT-PCR (Section 4.3.1.2.3.2) is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic TSV-induced lesions in the cuticular epithelium by histology (with or without confirmation by ISH with TSV-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case is represented by:

- sudden high mortalities in late PL, juvenile or subadult *P. vannamei* or *P. stylirostris* in regions where TSV is enzootic;
- the sudden presence of numerous sea birds (gulls, cormorants, herons, terns, etc.) ‘fishing’ in one or more shrimp culture ponds;
- samples of cultured *P. vannamei* or *P. stylirostris* from ponds with feeding sea birds that present gross signs indicative of acute- or transition-phase TS, such as a general reddish colouration, lethargy, soft shells, empty guts, and the presence of numerous irregular black spots on the cuticle; or
- demonstration of foci of necrosis in the cuticular epithelium using low magnification (i.e. a ×10 hand lens or by direct microscopic examination of wet mounts) to examine the edges of appendages such as uropods or pleopods, or the gills.

7.2. Definition of confirmed case

Any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results):

- Histological demonstration of diagnostic acute-phase TSV lesions in (especially) the cuticular epithelia of the foregut (oesophagus, anterior, or posterior chambers of the stomach) and/or in the gills, appendages, or general cuticle. Such TSV lesions are pathognomonic for TSV only when they occur without accompanying severe acute necrosis (with nuclear pyknosis and karyorrhexis) of the parenchymal cells of the lymphoid organ tubules (which may occur in acute-phase yellowhead virus infections).
- ISH-positive (with a TSV-specific cDNA probe) signal to TSV-type lesions in histological sections (i.e. cuticular acute-phase TS lesions) or to distinctive lymphoid organ spheroids (LOS) in the lymphoid organs of shrimp with chronic phase TS lesions.
- RT-PCR positive results for TSV.
- Sequencing of PCR product encompassing CP2 may be accomplished, as needed, to determine the TSV genotype (56).

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NB: There is an OIE Reference Laboratory for Taura syndrome (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).