

INFECTION WITH TAURA SYNDROME VIRUS

1. Scope

Infection with Taura syndrome virus means infection with the pathogenic agent Taura syndrome virus (TSV), Genus Aparavirus, Family *Dicistroviridae*, Order Picornavirales.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

TSV was described as the cause of the disease commonly known as Taura syndrome by Bonami *et al.* (Bonami *et al.*, 1997) and Mari *et al.* (Mari *et al.*, 1998; Mari *et al.*, 2002). At least four genotypes (strains) of TSV have been documented based on the gene sequence encoding VP1, the largest and presumably dominant of the three major structural proteins of the virus. Based on VP1 sequence variations, these genotypic groups are: 1) the Americas group; 2) the South-East Asian group; 3) the Belize group; and 4) the Venezuelan group (Chang *et al.*, 2004; Erickson *et al.*, 2002; Erickson *et al.*, 2005; Nielsen *et al.*, 2005; Tang & Lightner, 2005; Wertheim *et al.*, 2009).

At least two distinct antigenic variants of TSV have been identified by their differential reactivity to monoclonal antibody MAb 1A1, produced to a reference isolate from the Americas (TSV USA-HI94 – GenBank AF277675) (Mari *et al.*, 2002; Poulos *et al.*, 1999): Type A represents those that react with MAb 1A1 (in the enzyme-linked immunosorbent assay [ELISA], Western blots and immunohistochemistry [IHC] 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 (Erickson *et al.*, 2002; Erickson *et al.*, 2005), nor does a TSV isolate from the 2005 epizootic in Venezuelan shrimp farms.

TSV particles are 32 nm in diameter, non-enveloped icosahedrons and have a buoyant density of 1.338 g ml⁻¹ in CsCl. The genome of TSV consists of a linear, positive-sense single-stranded RNA 10,205 nucleotides in length, 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 (Bonami *et al.*, 1997; Mari *et al.*, 1998; Mari *et al.*, 2002; Robles-Sikisaka *et al.*, 2001).

Other reported causes of TS: Taura syndrome in Ecuador was initially linked to fungicide contamination of shrimp farms, a contention that was supported by litigation for ~ 16 years after the disease was scientifically shown to have a viral aetiology (Bonami *et al.*, 1997; Hasson *et al.*, 1995; Lightner, 2005). Hence, several papers in the literature propose a toxic aetiology for TS (Intriago *et al.*, 1997; Jimenez, 1992; Jimenez *et al.*, 2000).

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

Species that fulfil the criteria for listing as susceptible to infection with TSV according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include: greasyback shrimp (*Metapenaeus ensis*), northern brown shrimp (*Penaeus aztecus*), giant tiger prawn (*P. monodon*), northern white shrimp (*P. setiferus*), blue shrimp (*P. stylirostris*), and whiteleg shrimp (*P. vannamei*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with TSV according to Chapter 1.5. of the *Aquatic Code* include: fleshy prawn (*Penaeus chinensis*), giant river prawn (*Macrobrachium rosenbergii*), the copepod (*Ergasilus manicatus*), and the barnacles (*Chelonibia patula* and *Octolasmis muelleri*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: northern pink shrimp (*Penaeus duorarum*), kuruma prawn (*Penaeus japonicus*), southern white shrimp (*Penaeus schmitti*), gulf killifish (*Fundulus grandis*), blue crab (*Callinectes sapidus*), the crabs (*Uca vocans* and *Sesarma mederi*), and Indo-Pacific swamp crab (*Scylla serrata*).

2.2.3. Susceptible stages of the host

Infection with TSV has been documented in all life stages (i.e. PL, juveniles and adults) of *P. vannamei* except in eggs, zygotes and larvae (Lightner, 1996a).

2.2.4. Species or subpopulation predilection (probability of detection)

All postlarval stages of *P. vannamei*, and populations of other known susceptible species.

2.2.5. Target organs and infected tissue

TSV infects and has been shown to replicate 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 by ISH (Bondad-Reantaso et al., 2001; Hasson et al., 1997; Hasson et al., 1999a; Hasson et al., 1999b; Jimenez et al., 2000; Lightner, 1996a; Lightner & Redman, 1998a; Lightner & Redman, 1998b; Lightner et al., 1995; Srisuvan et al., 2005).

2.2.6. Persistent infection

Some members of populations of *P. vannamei* or *P. stylirostris* that survive infection with TSV may carry the virus for life (Hasson et al., 1999a; Hasson et al., 1999b) and, although not documented, are assumed to pass the virus to their progeny by vertical transmission.

2.2.7. 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 gallus*, used as a laboratory surrogate for all shrimp-eating birds) thus suggesting that the virus can retain infectivity when passed through the gastro-intestinal system of any bird species. These findings implicate birds as being an important mechanical vector for the transmission of the virus within affected farms or farming regions (Garza et al., 1997; Vanpatten et al., 2004).

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 (Brock, 1997; Lightner, 1995, Lightner, 1996a, Lightner, 1996b).

2.3. Disease pattern

Infection with TSV 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 infection with TSV 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 (Brock, 1997; Brock et al., 1995; Lightner, 1996a, Lightner, 1996b; Lotz, 1997b).

2.3.1. Transmission mechanisms

Horizontal or vertical routes. Horizontal transmission through cannibalism or by contaminated water has been demonstrated (Brock, 1997; Hasson et al., 1995; Lightner, 1996a, Lightner, 1996b; White et al., 2002). Vertical transmission 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 infection with TSV has been found in various surveys to range from 0 to 100% (Brock, 1997; Jimenez et al., 2000; Laramore, 1997).

2.3.3. Geographical distribution

TSV is now widely distributed in the shrimp-farming regions of the Americas, South-East Asia and the Middle East (Bondad-Reantaso et al., 2001; Brock, 1997; Chang et al., 2004; Hasson et al., 1999a; Lightner, 1996a, Lightner, 1996b; Lightner et al., 2012; Lotz et al., 2005; Nielsen et al., 2005; Tang & Lightner, 2005; Tu et al., 1999; Yu & Song, 2000; Wertheim et al., 2009).

The Americas: following its recognition in 1992 as a distinct disease of cultured *P. vannamei* in Ecuador (Brock et al., 1995; Jimenez, 1992; Lightner et al., 1995), TSV spread rapidly throughout many of the shrimp-farming regions of the Americas through shipments of infected PL and broodstock (Brock, 1997; Brock et al., 1997; Hasson et al., 1999a; Lightner, 1996a; Lightner, 1996b; Lightner et al., 2012). Infection with TSV have been reported from virtually every penaeid shrimp-growing country in the Americas and Hawaii (Aguirre Guzman & Ascencio Valle, 2000; Brock, 1997; Lightner, 2011; Lightner et al., 2012; Robles-Sikisaka et al., 2001). 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 (Lightner & Redman, 1998a; Lightner et al., 1995). 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 these regions (Hasson et al., 1999a; Lightner, 1996a; Lightner, 2005; Lightner, 2011; Lightner et al., 2012).

Asia and the Middle East: Infection with TSV was introduced into Chinese Taipei in 1999 with infected imported Pacific white shrimp, *P. vannamei*, from Central and South American sources (Tu et al., 1999; Yu & Song, 2000). 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 stocks of *P. vannamei* not selected for resistance (Chang et al., 2004; Nielsen et al., 2005; Tang & Lightner, 2005; Lightner, 2011). During 2010 and 2011, infection with TSV was associated with significant mortalities in farmed *P. indicus* in Saudi Arabia (Wertheim et al., 2009). By a phylogenetic analysis based on the viral capsid protein 2 (also named as VP1) sequence, the Saudi Arabian TSV clustered into a new, distinct group (Tang et al., 2012; Wertheim et al., 2009).

2.3.4. Mortality and morbidity

At a farm level outbreaks of infection with TSV involving stocks of *P. vannamei* not selected for resistance, the principal host species for infection with 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 (Lightner et al., 2009; Moss et al., 2001).

2.3.5. Environmental factors

Outbreaks of infection with TSV are more frequent when salinities are below 30 ppt (Jimenez et al., 2000).

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccines for TSV are available.

2.4.2. Chemotherapy

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3. Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4. Breeding for resistance

After TS emerged in Ecuador in 1992–1994, *P. stylirostris* were found that possessed resistance to infection with TSV (genotype 1, MAb 1A1 Type A). Following from this discovery and due to disease occurrence in Mexico in 1994 where it caused crop failures of *P. vannamei*, selected lines of TSV-resistant *P. stylirostris* became the dominant shrimp farmed in western Mexico from 1995. However, in 1998–1999, a new 'strain' of TSV (Type B; Erickson et al., 2002; Fegan & Clifford, 2001; Lightner, 1999; Lightner, 2005; Zarain-Herzberg & Ascencio-Valle, 2001) 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* in Mexico.

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 (Clifford, 1998; Moss et al., 2001; White et al., 2002). After the appearance of infection with TSV in Central America, improved TSV resistance was reported in wild caught *P. vannamei* PLs used to stock shrimp farms in the region (Laramore, 1997).

2.4.5. Restocking with resistant species

Selected lines of TSV resistant *P. vannamei* have been developed and are commercially available (Clifford, 1998, Laramore, 1997, Moss et al., 2001, White et al., 2002).

2.4.6. Blocking agents

Resistance to infection with TSV 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 (Lu & Sun, 2005). Similar results have been produced by injection of short random double-stranded RNAi sequences into juvenile *P. vannamei* (Robalino et al., 2004).

2.4.7. Disinfection of eggs and larvae

It is possible TSV might be transmitted vertically (transovarian transmission), despite the lack of published report documenting this route of transmission. Disinfection of eggs and larvae (Chen et al., 1992) 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 and disease control and management practices have been used successfully to reduce the risks of infection with TSV occurring during farm grow-out. These include the application of PCR assays for prescreening of wild or pond-reared broodstock or their spawned eggs/nauplii and discarding those that test positive for the virus (Fegan & Clifford, 2001), fallowing and restocking of entire culture regions with TSV-free stocks (Dixon & Dorado, 1997), and the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; Lightner, 2005; Lotz et al., 1995; Moss et al., 2001; Pruder et al., 1995; Wyban, 1992; Wyban et al., 2004). The adoption of the latter technology (SPF stocks) has proven to be among the most successful husbandry practice for the prevention and control of infection with TSV.

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection with 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 freedom from infection with TSV.

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

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, small life stages, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing.

3.4. Best organs or tissues

TSV infects tissues of ectodermal and mesodermal origin. The principal target tissue in the acute phase of infection with TSV 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 clinical infection with TSV can be presumptively diagnosed from clinical signs. See Section 4.2 for a description of gross clinical signs presented by shrimp with acute-phase clinical infection with TSV

4.1.2. Behavioural changes

Only shrimp with acute-phase clinical infection with TSV 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 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 infection with TSV or white spot syndrome virus) to the farm manager.

4.2. Clinical methods

4.2.1. Gross pathology

Infection with TSV has three distinct phases, acute, transition, and chronic, which are grossly distinguishable (Hasson et al., 1999a; Hasson et al., 1999b; Lightner, 1996a; Lightner, 1996b; Lightner, 2011; Lightner et al., 1995). Gross signs presented by juvenile, subadult and adult shrimp in the transition phase of infection with TSV are unique and provide a presumptive diagnosis of the disease.

Acute phase: gross signs displayed by moribund *P. vannamei* with acute-phase infection with TSV 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 (Lightner et al., 1995). In such shrimp, close inspection of the cuticular epithelium in thin appendages (such as the edges of the uropods or pleopods) with a $\times 10$ hand lens reveals signs of focal epithelial necrosis. Shrimp showing these gross signs of acute infection with TSV 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.

Transition (recovery) phase: although only present for a few days during outbreaks of infection with TSV, the gross signs presented by shrimp in the transition phase can provide a tentative diagnosis of infection with TSV. 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 TSV 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 (Brock, 1997; Hasson et al., 1999b; Lightner, 1996a; Lightner, 2011).

Chronic phase: after successfully moulting, shrimp in the transition phase move into the chronic phase of infection with TSV in which persistently infected shrimp show no obvious signs of disease (Brock, 1997; Hasson et al., 1999b; Lightner, 1996a; Lightner, 1996b; Lightner, 2011; Lightner et al., 1995). 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 (Lotz et al., 1995).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology (for penaeid hosts)

TS disease in the acute and chronic phases can be diagnosed using histological methods (Hasson et al., 1999b; Lightner, 1996a). Pathognomonic TSV-induced pathology is unique in acute-phase infections (Brock et al., 1995; Lightner, 1996a; Lightner, 2011). In chronic infections with TSV, the only lesion typically presented by infected shrimp is the presence of an enlarged LO with multiple LO spheroids (LOS) (Hasson et al., 1999b; Lightner, 2011), which cannot be distinguished from LOS induced by chronic infections of other RNA viruses (Lightner, 1996a). When LOS are observed by routine histology and chronic infection with TSV is suspected, a molecular test (ISH with TSV-specific probes, or reverse-transcription [RT] PCR [see Section 4.3.1.2.7]) is recommended for confirmation of infection with TSV.

4.2.3.1. Acute phase of infection with *Taura syndrome virus*

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 infection with TSV, 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 infections with TSV 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 infections with TSV distinguishes TS disease from acute-phase yellowhead virus genotype 1 in which similar patterns of necrosis to those induced by infection with TSV may occur in the cuticular epithelium and gills (Lightner, 1996a).

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 infection with TSV from the transitional phase of the disease (Bondad-Reantaso et al., 2001; Brock, 1997; Brock et al., 1995; Brock et al., 1997; Erickson et al., 2002; Erickson et al., 2005; Hasson et al., 1995; Hasson et al., 1999a; Hasson et al., 1999b; Lightner, 1996a; Lightner et al., 1995).

4.2.3.2. Transition (recovery) phase of infection with *Taura syndrome virus*

In the transitional phase of infection with TSV, 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. (Hasson et al., 1999b; Lightner, 1996a; 2011). Sections of the LO during the transition phase of infection with TSV 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 (Hasson et al., 1999b; Srisuvan et al., 2005).

4.2.3.3. Chronic phase of infection with *Taura syndrome virus*

Shrimp in the chronic phase of infection with TSV 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 (Hasson et al., 1999b; Lightner, 1996a; Lightner, 1996b; 2011).

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 infection with TSV) focal lesions of acute-phase infection with TSV in cuticular epithelial cells. Preparations presenting acute-phase infection with TSV 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. Fixed sections

See Section 4.2.3

4.2.7. 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*

See Section 4.2.5

4.3.1.1.3. *Fixed sections*

See Section 4.2.3

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 (Lightner, 1996a; Pantoja et al., 2004). Although one publication incorrectly reported that TSV infected human and monkey cell lines (Audelo del Valle et al., 2003), two other laboratories that repeated the study both found that TSV does not infect or replicate in primate or human cell lines that are known to have susceptibility to human picornaviruses (Luo et al., 2004; Pantoja et al., 2004).

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 (Erickson et al., 2002; Erickson et al., 2005; Poulos et al., 1999). TSV MAb 1A1 may be used to distinguish some variants or 'strains' of TSV from other strains (Erickson et al., 2002; Erickson et al., 2005).

4.3.1.2.3. 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 (Brock et al., 1997; Garza et al., 1997; Hasson et al., 1999b; Hasson et al., 1995; Lightner, 1996a; Lotz, 1997b; Overstreet et al., 1997). 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 (White et al., 2002). The use of a negative control tank of indicator shrimp, which receive only SPF (TSV-free) tissue and normal shrimp feed is required. When the carcass feeding (*per os*) protocol is used to bioassay for TSV, TSV-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 (Hasson et al., 1999b; Lightner, 1996a; White et al., 2002).

With the injection bioassay protocol, a variety of sample types may be tested for TSV. Whole shrimp are used if they were collected during an outbreak of infection with TSV. 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 (Hasson et al., 1999b; Lightner, 1996a). For non-lethal testing of broodstock, haemolymph samples may be taken and used to expose the indicator shrimp by IM injection (Lightner, 1996a).

To perform the IM (injection) bioassay for TSV:

Note that tissues and the resulting homogenate should be kept cool during the entire protocol by maintaining on ice.

- i) Prepare a 1:2 or 1:3 ratio of TSV-suspect shrimp heads or whole shrimp with TN buffer (20 mM Tris-HCl, pH 7.4, 0.4 M NaCl) 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.
- iii) Clarify the homogenate by centrifugation at 3000 **g** for 10 minutes. Decant and save the supernatant fluid.
- iv) Centrifuge the supernatant fluid at 27,000 **g** for 20–30 minutes at 4°C. Decant and save the supernatant fluid.
- 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. Filtrate should be collected in a sterile test tube or beaker. The solution can now be stored frozen at –20°C (or –80°C for a long-term storage) or used immediately to inject indicator shrimp.
- vii) Indicator shrimp should be from infection with TSV-susceptible stocks of SPF *P. vannamei* (such as the 'Kona stock') (Moss et al., 2001), which are commercially available from a number of sources in the Americas, and not from selected lines of known infection with 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 (Lightner, 1996a, White et al., 2002).
- 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. If additional confirmation is needed beyond demonstration of pathognomonic TSV lesions, RT-PCR with sequencing of the resulting amplicon can be carried out.

4.3.1.2.4. 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 infection with TSV status to bioassay for the presence of infectious agents such as TSV.

4.3.1.2.5. 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)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) and 2% Hammersten casein (Amersham Life Sciences).
- 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) 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) in 100 mM Tris-HCl, 100 mM NaCl buffer containing 50 mM MgCl₂, 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.

4.3.1.2.6. 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 (Erickson et al., 2002; Erickson et al., 2005).

It is recommended that unexpected results from MAb-based tests for detection of 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).

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*.

4.3.1.2.7. Molecular techniques

ISH and RT-PCR tests for detection of 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.

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

Non-radioactive, DIG-labelled cDNA probes for detection of 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 (Hasson et al., 1999a; Lightner, 1996a; Lightner, 1999; Lightner & Redman, 1998b; Mari et al., 1998). 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 infection with TSV (Hasson et al., 1999a; Hasson et al., 1999b; Lightner, 1996a; Lightner, 1996b). 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 (Lightner, 1996a; Mari et al., 1998). (See Chapter 2.2.4 Infection with infectious hypodermal and haematopoietic necrosis virus for details of the ISH method, and Chapter 2.2.0 Section 7.1.6.3. 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 hydrolysis 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 (Hasson et al., 1997; Lightner, 1996a; Lightner & Redman, 1998b).

4.3.1.2.7.2. Reverse-transcription (RT)-PCR method

Tissue samples (haemolymph, pleopods, whole small shrimp, etc.) may be assayed for TSV using RT-PCR. Primers designated as 9992F and 9195R, amplify a 231 base pair (bp) sequence of the TSV genome (Nunan et al., 1998). 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) (Mari et al., 2002; Nunan et al., 1998). A new pair of TSV primers (7171F and 7511R) has been developed and shown to have an improved sensitivity for TSV detection (Navarro et al., 2009). These replacement primers are 9992F/9195R and they are located within ORF 2.

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
7171F	341 bp	5'-CGA-CAG-TTG-GAC-ATC-TAG-TG-3'	50%	
7511R		5'-GAG-CTT-CAG-ACT-GCA-ACT-TC-3'	50%	

The RT-PCR method outlined below for TSV generally follows the method used in Nunan et al., 1998.

- 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) was used for all amplification reactions described here. Alternative kits can be used and adjusted for use for this assay.

- v) The optimised RT-PCR conditions (final concentrations in 50 µl total volume) for detection of TSV in shrimp tissue samples are: primers (0.62 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.
- xii) Details of the composition of the reagents and buffers used here may be found in Chapter 2.2.4. Infection with infectious haematopoietic necrosis virus.

4.3.1.2.7.3. 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 (Dhar et al., 2002; Tang et al., 2004).

The real-time RT-PCR method using TaqMan chemistry described below for TSV generally follows the method used in Tang et al. (Tang et al., 2004).

- i) The PCR primers and TaqMan probe were selected from the ORF1 region of the TSV genomic sequence (GenBank AF277675) that encodes for nonstructural proteins. The primers and TaqMan probe were designed by the Primer Express software (Life Technologies). 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-carboxyfluorescein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end.
- 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 conventional RT-PCR.
- iii) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of contaminants in the reaction mixture. 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.
- iv) The real-time RT-PCR reaction mixture contains: TaqMan Fast virus 1-Step Master Mix (Life Technologies), 0.3 µM of each primer, 0.1 µM of TaqMan probe, 5–50 ng of RNA, and water in a reaction volume of 10 µl.
- v) Amplification can be performed with the StepOnePlus PCRSystem (Life Technologies or equivalent real-time PCR systems). The cycling consists of reverse transcription at 50°C for 30 minutes and initial denaturation at 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds and annealing/extension at 60°C for 30 seconds.

- vi) At the end of the reaction, real-time fluorescence measurements are analysed. A threshold will be set to be above the baseline. Samples will be defined as negative if there is no Ct (threshold cycle) value after 40 cycles. Samples with a Ct value lower than 40 cycles are considered to be positive.

4.3.1.2.7.4. Sequencing

RT-PCR products may be sequenced when necessary to confirm infection by TSV or to identify false positives or nonspecific amplification (Mari et al., 2002; Nielsen et al., 2005; Srisuvan et al., 2005; Tang & Lightner, 2005; Wertheim et al., 2009).

4.3.1.2.8. Agent purification

Methods for TSV isolation and purification are available (Bonami et al., 1997, Hasson et al., 1995, Mari et al., 2002, Poulos et al., 1999), but these are not recommended for routine diagnosis of TS.

4.3.2. Serological methods

Not applicable because shrimps 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 surveillance, detection, and diagnosis of infection with 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 and/or not available 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 surveillance, detection and diagnosis

Method	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 (wet mount)	d	d	c	c	c	d
Histopathology	d	b	b	c	a	a
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	c	c	b	b
<i>In situ</i> DNA probes	d	c	b	b	a	a
RT-PCR, Real-time 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 infection with Taura syndrome virus

As indicated in Table 5.1., RT-PCR (Section 4.3.1.2.7.2) or real-time RT-PCR (Section 4.3.1.2.7.3) are the recommended methods 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

Infection with TSV is suspected if at least one of the following criteria is met:

- i) histopathology consistent with infection with TSV
- or
- ii) a positive result by RT-PCR or real-time RT-PCR.

7.2. Definition of confirmed case

Infection with TSV is considered to be confirmed if two or more of the following criteria are met:

- i) histopathology consistent with infection with TSV
- ii) ISH positive result in target tissues
- iii) RT-PCR (followed by sequencing), or real-time RT-PCR with positive results for TSV.

8. References

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NB: There is an OIE Reference Laboratory for Infection with Taura syndrome virus
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:

<http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).

Please contact the OIE Reference Laboratories for any further information on Infection with Taura syndrome virus

NB: FIRST ADOPTED IN 2000; MOST RECENT UPDATES ADOPTED IN 2017.

