

Taura syndrome of marine penaeid shrimp: characterization of the viral agent

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The causative agent of Taura syndrome (TS) was recognized in 1994 to be viral in nature and tentatively classified as belonging to either the family *Picornaviridae* or *Nodaviridae*. The work reported here has led to a more definitive classification of this new penaeid virus. Located within the cytoplasm of infected cuticular epithelial cells of penaeid shrimp, the virus is a 31 to 32 nm icosahedral particle with a buoyant density of 1.338 ± 0.001 g/ml. Three major (55, 40 and

24 kDa) and one minor (58 kDa) polypeptides constitute its proteinic capsid. Its genome contains a single molecule of ssRNA, which is polyadenylated at the 3' end and approximately 9 kb in length. Based on these characteristics, we believe that TS virus should be included in the family *Picornaviridae*. Ecuadorian and Hawaiian TS virus isolates were found to be identical in their biophysical, biochemical and biological characteristics, and should be considered as the same virus.

Introduction

Taura syndrome (TS) of penaeid shrimp was first described in 1992 by Jimenez in samples of *Penaeus vannamei* collected from shrimp farms located near the mouth of the Taura river (Guayas province, Ecuador; Jimenez, 1992). Originally attributed to fungicides used for the treatment of banana plantations suffering from black leaf wilt disease or sigatoka negra, TS was subsequently shown to have a viral aetiology (Hasson *et al.*, 1995). The rapid spread of TS throughout the Americas, together with the resulting economic loss suffered by *P. vannamei* farmers, makes this one of the most important and detrimental pathogens affecting the shrimp industry in the Western hemisphere. The geographic distribution of the disease now includes Ecuador, the adjacent region of Peru, the Pacific and Caribbean coasts of Colombia, Honduras, Guatemala, El Salvador, north-east Brazil, Nicaragua, Belize, the States of Hawaii, Florida and Texas in the USA, and the Mexican States of Sonora, Sinaloa, Chiapas and Guerrero (Lightner, 1996). Naturally occurring Taura syndrome virus (TSV) infections have been reported in farm-raised *P. vannamei*, *P. setiferus* and *P. stylirostris* (Brock *et al.*, 1995; R. M. Overstreet, D. V. Lightner, K. W. Hasson, S. McIlwain & J.

Lotz, unpublished results). The penaeid species most adversely affected by the disease is *P. vannamei*, with cumulative percentage mortality ranging from 50 to 90% among populations with either experimentally induced or naturally acquired infections. In comparison, *P. stylirostris* and *P. setiferus* are moderately susceptible. Experimental infections have been successfully produced in *P. setiferus*, *P. chinensis*, *P. schmitti*, *P. vannamei* and *P. stylirostris*, but not in *P. duorarum* or *P. aztecus*, which appear to be resistant to TSV (R. M. Overstreet, D. V. Lightner, K. W. Hasson, S. McIlwain & J. Lotz, unpublished results).

TSV was initially classified as being either a member of the family *Picornaviridae* or *Nodaviridae* based strictly on limited morphological characteristics (Hasson *et al.*, 1995). Prior to the discovery of TSV, no member of either of these two virus families had been reported in penaeid shrimp. The only exception to this was infectious hypodermal and haematopoietic necrosis virus, which was initially believed to be closely related to the family *Picornaviridae* before it was characterized as a parvovirus (Bonami *et al.*, 1990; Bonami & Lightner, 1991). In marine crustacea, only three picornavirus representatives have been identified, all in crabs; however, the characterization of these isolates was based solely on virion size, morphology and cytoplasmic location, pending analysis of the genome (Bonami & Lightner, 1991).

We report here our results on the purification, physico-

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chemical characterization and pathogenicity of two geographically distinct TSV isolates. For this purpose, two separate lots of TSV isolates were purified from naturally diseased *P. vannamei* originating from Ecuador (Ec-TSV) and Hawaii (Hi-TSV). All experiments that were conducted on these two isolates were done in parallel with the intent of comparing the two viruses.

Methods

■ **Virus isolates.** Two groups of TSV-infected *P. vannamei* carcasses, one originating from Hawaii (Hi-TSV, provided by J. Brock in 1994, Aquaculture Development Program, Department of Land and Natural Resources, Hawaii, USA) and the second from Ecuador (Ec-TSV, provided by S. Stern in 1993, Desarrollo Industrial Bioacuatico, Guayaquil, Ecuador), were utilized. Both groups of shrimp were collected during naturally occurring TSV epizootics and stored frozen at -70°C . Confirmation that the frozen shrimp were TSV-infected was done by histological analysis (Lightner *et al.*, 1995) of parallel representative samples preserved at the time of collection and detection of pathognomonic TSV lesions.

■ **Virus purification.** All purification steps, including preparation of the gradient solutions, were done in TN buffer (0.4 M NaCl, 0.01 M Tris-HCl pH 7.4). Whole frozen animals were homogenized in TN buffer (1:8) using an 'Ultra-turrax' tissue blender and clarified twice with an SS34 rotor in a RC5B Sorvall centrifuge (15 min at 4000 g and 20 min at 27000 g). The final supernatant was pelleted at 205 000 g for 2 h using a T647.5 rotor in a OTD80B Sorvall ultracentrifuge. The pellet was then resuspended in TN buffer, the suspension was freon (1,1,2 trichloro-1,2,2 trifluoroethane)-extracted three times and re-pelleted at 205 000 g for 2.25 h. After being resuspended using a Potter tissue grinder, the pellet suspension was layered onto a 15–30% (w/w) linear sucrose gradient and centrifuged for 2 h at 131 000 g in a Sorval AH629 rotor. Fractions of the gradient were removed using an Autodensiflow IIC (Buchler Instruments), collected with an ISCO Retriever II and absorbance readings of the fractions simultaneously plotted at a wavelength of 254 nm using an ISCO UA5 UV monitor. Fractions containing virus were diluted in TN buffer and pelleted at 233 000 g during 1.75 h in a TH641 rotor. Further purification was achieved by layering the resuspended pellet on a 15–45% (w/w) CsCl gradient and centrifuging for at least 12 h. The fractions were then recovered exactly as described above for the sucrose gradient. The final purified virus fraction was diluted with TN buffer, pelleted at 220 000 g for 2 h in a TH641 rotor, and resuspended in either TN buffer or in DEPC-treated water, depending on the type of analysis to be conducted on the virus suspension.

The density of the virus was determined by measuring the refractive index of every other CsCl gradient fraction (prepared with $0.1 \times \text{PBS}$) using an Abbe refractometer and plotting the corresponding density values on the absorbance chart.

■ **Electron microscopy.** To monitor the different purification steps and to check the quality of the final suspensions, each virus preparation was negatively stained with 2% phosphotungstic acid (PTA) pH 7 on collodion-carbon-coated grids. Observations were made using either a Hitachi HU11C or Hitachi HU12 transmission electron microscope (TEM).

■ **Spectrophotometry.** A Beckman DU7 spectrophotometer was used for absorbance measurements using either visible or UV wavelengths. Virus nucleic acid concentrations were determined by colorimetric methods with Orcinol (Mejbaum, 1939) and diphenylamine (Giles & Myers, 1965). The amount of virus proteins in each purified suspension

was estimated using the mini-Bradford method (Bradford, 1976) and read at a wavelength of 595 nm.

The quality of the purifications and quantification of extracted RNA were estimated by recording absorbance between the wavelengths of 220 and 320 nm and determination of the $A_{260}:A_{280}$ ratio (Sambrook *et al.*, 1989).

■ **SDS-PAGE.** 12% polyacrylamide vertical slab gels were run for 3 h at a constant 200 V using the Laemmli buffer system (25 mM Tris-HCl, 192 mM glycine pH 8.3) containing 0.1% SDS (Laemmli, 1970). Samples were mixed with an equal volume of gel sample buffer (2% SDS, 5 M urea, 1% 2-mercaptoethanol, 15% glycerol and 0.001% bromophenol blue) and heated to 100°C for 3 min prior to loading onto the gel. Molecular mass markers (Sigma) were BSA, egg albumin, carbonic anhydrase, trypsin inhibitor and lactalbumin (66, 45, 29, 20.1 and 14.2 kDa, respectively). The gels were stained for 1 h with 0.1% Coomassie blue (Wilson, 1983). The molecular mass of the polypeptides was estimated by measurement of the electrophoretic mobilities according to the method of Weber & Osborn (1969).

■ **Nucleic acid extraction.** Purified virus was resuspended in DEPC-treated water (Sambrook *et al.*, 1989). Virus proteins were digested with proteinase K (50 mg/ml final concentration) for 1 h at 37°C , followed by 1 h of Sarkosyl treatment (0.5% final concentration) at 65°C . The nucleic acid was extracted twice with water-saturated phenol, once with phenol-chloroform-isoamyl alcohol (25:24:1) and then twice with chloroform-isoamyl alcohol (24:1). The nucleic acid was then precipitated with cold absolute ethanol in the presence of LiCl (0.4 M final concentration) and the pellet washed with 70% ethanol. The final pellet was resuspended in DEPC-treated water. All pipette tips and plastic tubes used were RNase-free.

■ **Nucleic acid electrophoresis.** Fully denaturing formamide 1% agarose gels (Sambrook *et al.*, 1989) were used. A 0.24–9.5 kb RNA ladder (Gibco BRL) was used as a marker for genomic size determination. The enzymes mung bean nuclease (MBN), RNase A and DNase (Boehringer Mannheim) were used according to the manufacturer's instructions.

■ **Bioassays.** Two virus suspensions, one containing CsCl gradient-purified Ec-TSV and the other Hi-TSV, were prepared. The virus RNA concentrations of both inocula were adjusted to 0.15 mg/ml using 2% NaCl as a diluent. Each suspension was used to intramuscularly inject (0.02 ml per shrimp) 25 specific pathogen-free (SPF) *P. vannamei* (Mexican strain, 1.8 g average weight) (Wyban *et al.*, 1992; Pruder *et al.*, 1995) in the third tail segment. Extracted Hi-TSV RNA was similarly diluted with 2% NaCl to a final concentration of 1.65 mg/ml just minutes prior to injecting an additional 25 shrimp. In like manner, 25 shrimp were injected with DEPC-treated TN buffer to serve as negative controls. Each group of 25 shrimp was maintained in a 90 l salt water aquarium (23 to 25°C , 25 p.p.t. salinity) and fed daily according to Williams *et al.* (1992).

The shrimp were observed four times daily for signs of the disease over a period of 7 days. All moribund shrimp were processed for routine histological analysis and TSV lesion severity was determined by light microscopy according to a modified grading method of Bell & Lightner (1987) and Hasson *et al.* (1995). Briefly, TS-negative shrimp received a severity grade of 0; mild focal TS lesions were assigned a grade of 1; moderate, locally extensive to multifocal lesions received a grade of 2 to 3; and severe, multifocal to diffuse TS lesions were assigned a grade of 4. Moreover, all shrimp removed from the treatment tanks (dead or moribund) were counted as mortalities.

In addition, TEM was utilized to examine negatively stained haemolymph samples collected from moribund *P. vannamei* with

experimentally induced, acute phase TSV infections. Haemolymph samples were collected from the base of the fifth periopod using a 1 ml tuberculin syringe containing 10 to 30 µg of 10% sodium citrate and then stored frozen (−70 °C).

Results

Virus purification

Illumination of the Hi-TSV and Ec-TSV sucrose density gradients revealed a visible, opaque band in approximately the middle of each tube. A single, sharply delineated, opaque band was similarly observed within the CsCl gradients of each of the two isolates after isopycnic centrifugation (Fig. 1). Observation by TEM of negatively stained preparations of both sucrose and CsCl gradient fractions corresponding to these bands demonstrated that they consisted of numerous TSV virions (Fig. 2). Very few empty particles were observed in the purified preparations or within the lower density fractions of the gradient. Particles were icosahedral in shape with a size of 31 to 32 nm as determined by comparison with tobacco mosaic virus, used as an internal size marker. The density of the virus, determined after plotting the corresponding density values derived from the refractive index measurements of the first drops of the two fractions, was 1.338 ± 0.001 g/ml.

Absorbance values of purified TSV suspensions, recorded over a wavelength range of 220 to 320 nm, demonstrated typical nucleoproteic spectra with a maximum at 258–259 nm and a minimum at approximately 240 nm. The $A_{260}:A_{280}$ ratio varied slightly from one purification to another and ranged between 1.78 and 1.95. Values were typically higher than 1.8, which suggests the presence of RNA (Sambrook *et al.*, 1989). Corroborating these findings, the Orcinol reaction demonstrated that the TSV genome consisted of RNA, while the diphenylamine reaction for DNA remained negative. Analysis of purified TSV suspension aliquots, using both the Orcinol reaction and the mini-Bradford, indicated that the percentage of nucleic acid within the virions ranged from 14% to 16%. Based on this information and the A_{260} readings, the virus concentration in the aliquoted samples was determined as follows: one A_{260} (1 unit) indicates 61 to 65 mg/ml of virions, i.e. about 8.5 to 9 mg/ml of virus RNA or 53 to 55 mg/ml of virus protein. Thus, application of the purification protocol to 60 g of frozen, TSV-infected *P. vannamei* yielded 120 to 170 mg of highly purified TSV (Fig. 2). Identical purification results were obtained using the Hawaiian and Ecuadorian TSV isolates.

Attempts were made to utilize a 25 to 50% CsCl gradient to purify further sucrose gradient-purified Ec-TSV samples that had been frozen and thawed approximately three times. Monitoring of the CsCl gradient by spectrophotometric analysis generated a curve which did not display a virus peak, suggesting that the virions had degraded and that few to no whole particles remained in the preparation.

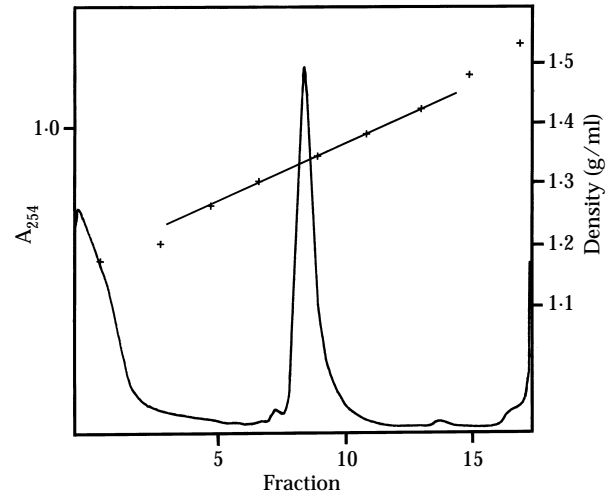


Fig. 1. TSV purification. Record of the UV absorbance (A_{254}) and density (g/ml) of the extracted CsCl gradient.

Protein composition

Utilizing SDS-PAGE (12% acrylamide gels), three major polypeptides (24, 40 and 55 kDa), designated VP1 to VP3, present in approximately equal amounts (similar intensity of the stained bands) were found to be present in purified TSV particles (Fig. 3). A fourth minor polypeptide VPO (58 kDa) was also observed in all experiments.

Virus RNA

Spectrophotometric analysis of extracted TSV RNA typically produced an $A_{260}:A_{280}$ ratio higher than 1.9 and often closer to 2. These values are in agreement with those for purified RNA (Sambrook *et al.*, 1989). Quantification of extracted RNA was done by measuring the A_{260} value of a diluted aliquot.

In fully denaturing 1% gel electrophoresis, the extracted TSV RNA migrated as a single band and was determined to have an approximate length of 9 kb when compared to ssRNA markers (Fig. 4). The TSV RNA was sensitive to digestion with both MBN and RNase A, but resistant to treatment with RNase-free DNase (data not shown). No differences were observed between the Ec-TSV RNA and the Hi-TSV RNA when co-migrated in agarose gels.

Infectivity assays

Peak mortalities and the highest prevalence of moribund shrimp occurred approximately 48 h post-injection among the treatment groups injected with the Hi-TSV and Ec-TSV whole virus inocula. These inocula had identical virus RNA concentrations (0.15 mg/ml) and induced cumulative mortalities of 60% among the Hi-TSV treatment group and 64% among the

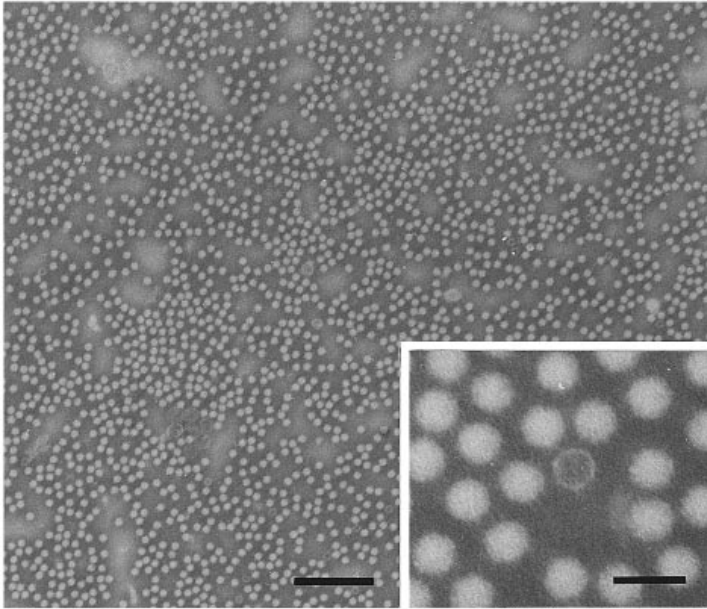


Fig. 2. Purified TSV suspension. Negative staining, 2% PTA. Bar represents 300 nm. At higher magnification (inset), virions appear icosahedral in shape. Bar represents 50 nm.

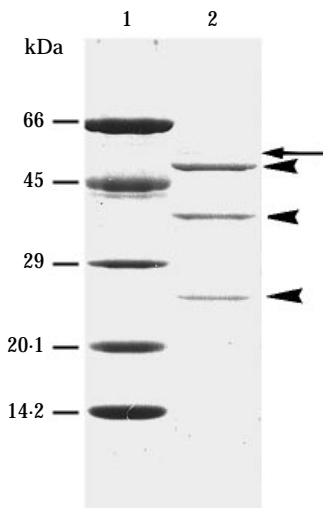


Fig. 3. SDS-PAGE (12% acrylamide gel) of purified TSV. Lane 1, marker proteins; lane 2, TSV polypeptides, three major (arrowheads) and one minor (arrow).

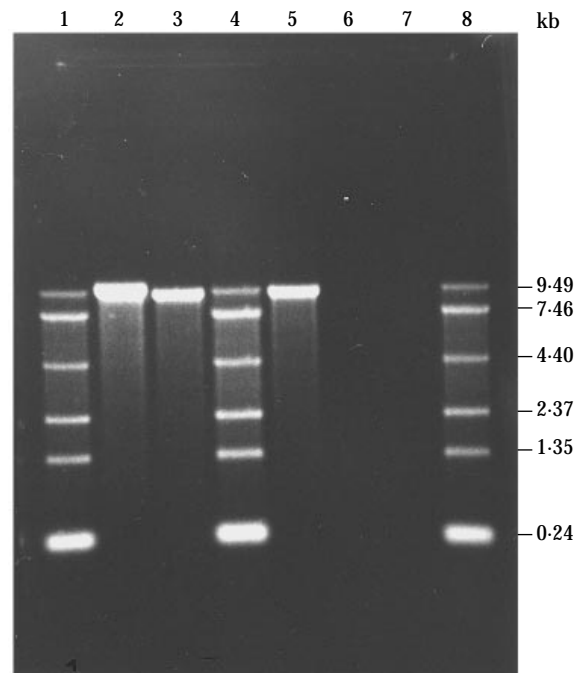


Fig. 4. TSV RNA gel electrophoresis. Fully denaturing (formamide) 1% agarose gel. Lane 1, 4 and 8, ssRNA markers; lane 2, Hi-TSV RNA; lane 3, Ec-TSV RNA; lane 5, Hi-TSV RNA; lane 6, MBN-digested Hi-TSV RNA; lane 7, RNase A-treated Hi-TSV RNA.

Ec-TSV treatment group (Table 1; Fig. 5). Peak mortalities among the treatment shrimp injected with extracted Hi-TSV RNA (1.65 mg/ml extracted virus RNA) occurred 72 h p.i. and resulted in a cumulative mortality of 40% (Table 1; Fig. 5). This experiment was a first attempt to study virus RNA infectivity and the results must therefore be taken as indicative rather than definitive. No mortalities were noted among the control shrimp during the 7 day bioassay.

All moribund shrimp in each of the three treatment groups displayed the same clinical signs associated with acute phase TSV infection as described by Hasson *et al.* (1995). Furthermore, beginning on day 4 p.i., characteristic TSV chronic

or recovery phase lesions were visible on one to three shrimp per treatment group, as was previously described by Hasson *et al.* (1995).

Histological analysis of all moribund treatment shrimp, sampled up through day 4, revealed moderate to severe (G1–4) pathodiagnostic TSV lesions (Table 1). Treatment

Table 1. Daily mortality of shrimps and severity of induced TS lesions

P. vannamei juveniles (1.82 g average weight) were injected with purified Hi-TSV, Ec-TSV or TSV RNA (from the Hi isolate). Each shrimp received a single intramuscular injection containing 0.02 ml of inoculum on day 0. Severity of lesions was graded from G0 to G4.

Inoculum	Daily mortality								Survival on day 7 (%)	No. examined (day sampled)	No. with TSV (severity)
	0	1	2	3	4	5	6	7			
Saline (2%)	0	0	0	0	0	0	0	0	25/25 (100)	8 (0) 5 (7)	0/8 0/5
Ec-TSV	0	1	9	2	2	0	0	2	9/25 (36)	6 (2) 1 (4) 6 (7)	6/6 (G3-4) 1/1 (G2-3) 0/6
Hi-TSV	0	2	8	2	2	1	0	0	10/25 (40)	2 (1) 3 (2) 3 (3) 2 (4) 6 (7)	2/2 (G1) 3/3 (G3-4) 3/3 (G3-4) 2/2 (G2-3) 2/6 (G1-3)
Hi-RNA	0	0	0	4	2	1	2	1	15/25 (60)	4 (3) 1 (4) 2 (6) 10 (7)	4/4 (G2-4) 1/1 (G3-4) 2/2 (G2-4) 2/10 (G1-2)

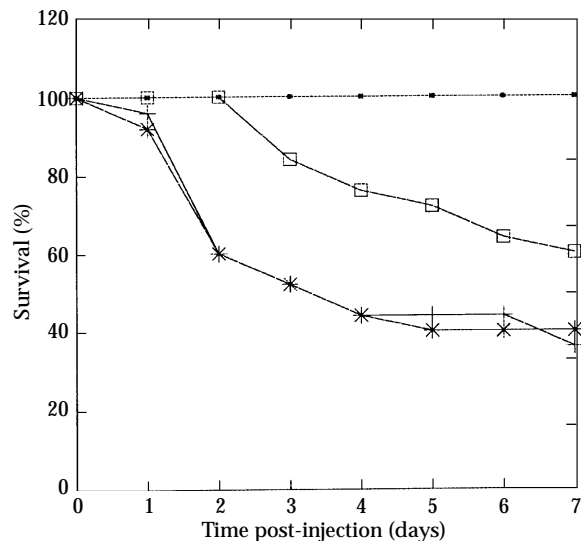


Fig. 5. Daily percentage survival of SPF *P. vannamei* injected with purified Hi-TSV (*), purified Ec-TSV (I), Hi-TSV RNA (□) and controls (shrimp injected with $1 \times$ TN buffer, DEPC-treated, diluted 1:100 with sterile 2% saline; ■).

shrimp sampled at termination on day 7 demonstrated a sharp decline in the number of animals with acute phase lesions. When observed, lesions were focal and mild in severity (G1-2, Table 1). Histological analysis of eight control shrimp sampled prior to initiating the experiment (day 0) and five shrimp sampled at the end (day 7) demonstrated that the shrimp were not infected before and remained TSV-free for the duration of the experiment.

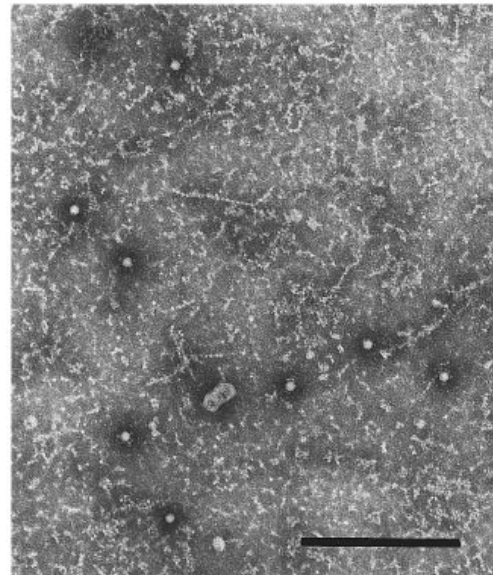


Fig. 6. Negative staining of haemolymph of a TSV-infected shrimp bled during the acute phase of the disease. Note the background constituted of haemocyanin molecules. TEM; bar represents 500 nm.

TEM examination of negatively stained haemolymph samples collected from moribund *P. vannamei* with acute phase TSV revealed the presence of virions with the same morphological characteristics as those previously described (Fig. 6). These findings further indicate the systemic nature of the acute phase infection and that whole TS virus particles are transported by the haemolymph.

Discussion

Two years after the first report of TS in Ecuador by Jimenez (1992), the disease was shown to be infectious (Brock *et al.*, 1995) and to have a viral aetiology (Hasson *et al.*, 1995). Information on the ultrastructure of infected cells remains scarce (Brock *et al.*, 1995), essentially due to the small size of the cytoplasmic particles and the apparent absence of paracrystalline arrays of virions. Owing to these characteristics, observation and interpretation of infected tissues by TEM (Lightner *et al.*, 1995) is difficult and one of the primary reasons for the late discovery and recognition of the viral nature of TS.

By its general characteristics (i.e. cytoplasmic development of non-enveloped, icosahedral, 31 to 32 nm particles containing ssRNA), TSV is closely related to the family *Picornaviridae* (Murphy *et al.*, 1995). Its density of 1.338 g/ml in CsCl is similar to that reported for the genera *Hepatovirus* and *Cardiovirus*. TSV also shares many traits with cricket paralysis virus (CrPV), *Drosophila* C virus (DCV) and *Gonometa* virus. These are, for the moment, unassigned to a specific virus family but considered as possible members of the *Picornaviridae* (Moore & Eley, 1991).

The TSV genome was determined to consist of RNA by the Orcinol reaction and further confirmed by both an $A_{260}:A_{280}$ ratio higher than 1.8 and by nuclease digestions. Its single-strandedness was demonstrated by MBN digestion and its high sensitivity to low concentrations of RNase. In fully denaturing agarose gels, its size was estimated to be about 9 kb. Based on the observation that extracted genomic RNA seemed to be infectious when inoculated into healthy shrimp, we speculated that the genome is polyadenylated at its 3' end, and that it acts as a polycistronic mRNA. This was recently confirmed by purification of the genome using oligo(dT) columns (J. Mari, unpublished results). Based on these genomic characteristics, TSV could be related to either the *Cardiovirus* genus or both CrPV and DCV (Moore & Eley, 1991; Murphy *et al.*, 1995).

The TSV capsid was found to contain three major (24, 40 and 55 kDa) and one minor (58 kDa) polypeptides. Based on these results, TSV does not greatly differ from the *Cardiovirus* genus (Murphy *et al.*, 1995) or from CrPV, DCV and *Gonometa* virus (Moore & Eley, 1991), particularly when the size classes of the three major polypeptides are considered (i.e. poliovirus has three polypeptides, VP1 to VP3, which range from 24 to 41 kDa). In contrast, and despite several attempts, our results did not reveal any TSV polypeptides within the size class of 5 to 13 kDa as seen in the virus groups mentioned above.

If we consider that TSV (as with the other picornaviruses) possesses 60 copies of each of the three proteins (24, 40 and 55 kDa) and a 9 kb ssRNA genome, it can be estimated that the virus is 85% protein and 15% ssRNA, values in agreement with our experimental determination (14–16% ssRNA).

Our results demonstrate that the physicochemical characteristics of the Hawaiian and Ecuadorian TSV isolates are

identical and indicate that the same virus was responsible for the epizootics that occurred in these two different shrimp-growing regions. This conclusion is further corroborated by the similar pathogenic effects (i.e. identical mortality patterns, identical clinical signs and induction of pathodiagnostic TSV lesions) produced by both isolates when injected into *P. vannamei*.

Failure to purify further and recover freeze-thawed sucrose gradient-purified TSV from CsCl gradients indicates that this virus is easily degraded by multiple freeze-thaw cycles when in purified or pre-purified form. In contrast, when present within the carcass of a shrimp, the virus can withstand multiple freeze-thaw cycles without degradation or becoming inactivated (Hasson *et al.*, 1995).

The presence of virions in the haemolymph during the peracute to acute phase of a TSV infection underlines the systemic nature of the disease, particularly during the acute phase.

Application of a newly developed, TSV-specific genomic probe (J. Mari, D. V. Lightner & J. R. Bonami, unpublished results) to archived samples from other affected shrimp-growing regions should help to clarify further the origin and distribution of TS throughout the Americas. This work is presently in progress.

At present, approximately 25 small RNA insect viruses within the family *Picornaviridae* are under review (Murphy *et al.*, 1995) and, based on all the characteristics discussed above, TSV constitutes *de facto* one more candidate.

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