

# Comparison of four Taura syndrome virus (TSV) isolates in oral challenge studies with *Litopenaeus vannamei* unselected or selected for resistance to TSV

Thinnarat Srisuvan<sup>1,2,\*</sup>, Brenda L. Noble<sup>2</sup>, Paul J. Schofield<sup>2</sup>, Donald V. Lightner<sup>2</sup>

<sup>1</sup>Department of Livestock Development, 69/1 Phayathai Road, Bangkok 10400, Thailand

<sup>2</sup>Department of Veterinary Science and Microbiology, University of Arizona, 1117 E. Lowell, Tucson, Arizona 85721, USA

**ABSTRACT:** Taura syndrome virus (TSV) infection in TSV-resistant (TSR) and TSV-susceptible (Kona) *Litopenaeus vannamei* (also called *Penaeus vannamei*) was investigated using histology, *in situ* hybridization (ISH), conventional reverse transcription polymerase chain reaction (RT-PCR) assays, and SYBR-Green real-time RT-PCR analysis. The shrimp were challenged by feeding with minced tissues of *L. vannamei* infected with 4 genotypic variants of TSV (Bz01, Th04, UsHi94, and Ve05). Survival probabilities of TSR shrimp were higher than those for Kona shrimp with all 4 variants. Th04, UsHi94, and Ve05 gave no Taura syndrome lesions with TSR shrimp. In contrast, TSR shrimp challenged with Bz01 and Kona shrimp with all 4 TSV variants exhibited severe necrosis of cuticular epithelial cells and lymphoid organ spheroids, indicative of acute and chronic phases of TSV infection, respectively. TSV was not detected by RT-PCR in TSR shrimp infected with Th04, UsHi94, and Ve05, or in Kona shrimp infected with Ve05 but was detected in TSR shrimp infected with Bz01 and in Kona shrimp infected with Bz01, Th04, and UsHi94. Real-time RT-PCR revealed that mean TSV copy numbers in TSR shrimp infected with Bz01, Th04, and UsHi94 were significantly ( $p < 0.0005$ ) lower than those in Kona shrimp. In contrast, mean TSV copy numbers in TSR and Kona shrimp infected with Ve05 were not significantly different ( $p > 0.4$ ). The results show that TSR *L. vannamei* are susceptible to infection but give high survival rates following challenge by all 4 variants of TSV.

**KEY WORDS:** Taura syndrome virus · TSV · TSV variants · *Litopenaeus vannamei*

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## INTRODUCTION

Taura syndrome (TS) is an important disease of cultured penaeid shrimp. It has been reported in many countries and has caused considerable economic loss in the last decade (Hasson et al. 1995, 1999, Lightner et al. 1995, Tu et al. 1999, Yu & Song 2002). It is caused by Taura syndrome virus (TSV), a member of the family *Dicistroviridae* (Mayo 2005). TSV is a non-enveloped icosahedral virus with a diameter of 32 nm (Bonami et al. 1997) and a single-stranded, positive-sense RNA genome of 10 205 nucleotides comprising 2 open reading frames (ORF1 and ORF2) (Mari et al. 2002). ORF1 may code for non-structural proteins, including helicase, protease, and RNA-dependent RNA polymerase,

whereas ORF2 codes for structural proteins such as the 3 major capsid proteins CP1, CP2, and CP3.

TSV isolated at different times and/or from different locations has been reported to display phenotypic variations. For instance, Erickson et al. (2005) demonstrated that Pacific white shrimp *Litopenaeus vannamei* (also called *Penaeus vannamei*) (Farfante & Kensley 1997) experimentally infected with a Belize isolate (Bz02) showed higher mortality than they did when infected with a Hawaiian isolate (UsHi94) (referred to as BLZ02TSV and Hi94TSV, respectively, in the cited paper). Phylogenetic analysis revealed that Bz02 was distinct among 29 isolates of TSV, despite the fact that TSV displays low genetic variation from 0 to 5.6% in nucleotide sequence and from 0 to 7% in deduced amino

\*Email: thinnarat@hotmail.com

acid sequence (Tang & Lightner 2005). There were 3 distinct phylogenetic lineages in the Americas, Asia, and Belize. Nielsen et al. (2005) also reported that TSV isolates from Asia and the Americas were distinct.

Because TS causes high mortalities in *Litopenaeus vannamei* (Lightner 1996), scientists have attempted to develop TSV-resistant shrimp populations (Wyban 2000, White et al. 2002, Xu et al. 2003). This is important because *L. vannamei* is the predominant cultivated species worldwide (FAO 2004). We used histology, *in situ* hybridization (ISH), conventional reverse transcription polymerase chain reaction (RT-PCR), and SYBR-Green real-time RT-PCR to study the course of TSV infection in a population of *L. vannamei* selected for TSV resistance and in an unselected population.

## MATERIALS AND METHODS

**Experimental challenges.** We performed experimental challenges with 2 populations of *Litopenaeus vannamei* to determine possible differences in susceptibility to TSV. These 2 populations were a specific-pathogen-free (SPF) Taura syndrome resistant (TSR) stock obtained from High Health Aquaculture, Kona, Hawaii, USA, and an SPF Kona stock (Moss et al. 2005) obtained from the Oceanic Institute, Oahu, Hawaii, USA. These shrimp were derived from stocks certified SPF for a list of shrimp pathogens including TSV for the preceding 2 yr. There were 4 viral challenge groups (Groups 1 to 4) and 1 negative control group (Group 5) for each shrimp stock. Groups 1 to 4 of each stock were challenged with TSV isolates, Bz01, Th04, UsHi94, and Ve05, respectively (Table 1). Each challenge group was held in a separate aquarium. Each TSR group contained ~150 shrimp (avg. wt = 2 g), and each Kona group contained 20 shrimp (avg. wt = 2 g) (see Fig. 1). The challenged groups were fed for 3 d (Days 0, 1, and 2) at 10% body wt d<sup>-1</sup> with minced shrimp tissues prepared from frozen *L. vannamei* infected with an appropriate TSV isolate. Starting at Day 3 post-infection (p.i.), all shrimp groups were fed once a day with a commercial pelleted feed (Rangen 35%, Buhl) for 12 d. Group 5 of each stock (negative control) that consisted of 20 shrimp per aquarium (avg.

wt = 2 g) were fed only commercial feed throughout the test period of 15 d. All of the aquaria were checked daily for moribund and dead shrimp.

Shrimp were sampled for histological analysis. For TSR Group 1, 1 and 2 shrimp were sampled at Days 7 and 14 p.i., respectively. For TSR Group 2, 1 and 2 shrimp were sampled at Days 8 and 14 p.i., respectively. For TSR Groups 3 and 4, 3 shrimp were sampled from each group at Day 14 p.i. For Kona Group 1, 1 shrimp was sampled at Day 3 p.i. For Kona Group 2, 1 shrimp was sampled on Days 3 and 14 p.i. For Kona Group 3, 1 and 2 shrimp were sampled on Days 8 and 14 p.i., respectively. For Kona Group 4, 1 shrimp was sampled at Days 8 and 14 p.i. These shrimp were not tested for TSV by RT-PCR or real-time RT-PCR, but the cephalothoraxes were fixed overnight in Davidson's fixative and transferred to 70% alcohol for histological analysis by hematoxylin and eosin (H&E) staining and ISH using standard methods (Lightner 1996, Mari et al. 1998).

In addition to histological analysis, shrimp were also collected for RT-PCR and real-time RT-PCR analyses. These shrimp were not used for histological analysis. These specimens were either dead shrimp collected during the challenge study, Days 0 to 13 p.i., or surviving shrimp at Day 14 p.i. Numbers of shrimp collected at specific days are provided later in Table 3 and Fig. 4. Total RNA was extracted from the pleopods using a High Pure RNA tissue extraction kit (Roche Biochemical) and stored at -70°C.

**ISH.** A mixture of probes, TS624 and TS622, was used for ISH. Probes TS624 and TS622 hybridize with the UsHi94 genome at nucleotides 3218 to 3841 and 5899 to 6520, respectively. The probes were prepared from 2 cDNA clones TSV837–5575 and TSV5049–10205, respectively, by polymerase chain reaction (PCR) labeling with digoxigenin (DIG)-11-dUTP as described by Mari et al. (1998). Primers 3218F (5'-CAC TAC GTT AGC AGG CAA TG-3') and 3841R (5'-CAC TTC ACT GCA CTC GAC AC-3') were used to label probe TS624 (624 bp), while primers 5899F (5'-TTA AGC GCG TTG GTG ACA AG-3') and 6520R (5'-GCA TCC TGC GCA TCG ATA TT-3') were used to label probe TS622 (622 bp). Following PCR, the DIG-labeled probes TS624 and TS622 were precipitated

Table 1. Taura syndrome virus (TSV) isolates used for the oral challenge studies

TSV isolates	Collection locations	Source species	Collection years	GenBank no.
Bz01	Belize	<i>Litopenaeus vannamei</i>	2001	AY590471
Th04	Chumporn, Thailand	<i>L. vannamei</i>	2004	AY997025
UsHi94	Hawaii, USA	<i>L. vannamei</i>	1994	AF277675
Ve05	Venezuela	<i>L. vannamei</i>	2005	DQ212790

with ethanol, re-suspended in distilled water, and stored at  $-20^{\circ}\text{C}$ .

**SYBR-Green real-time RT-PCR.** Primer Express software (Applied Biosystems) was used to design forward and reverse primers, 401F (5'-GAC TGG CTC ATA TAC TAT GGC CTC TTA T-3') and 545R (5'-CCG TCG CAA AGT TCC AAT TAA-3'), respectively, from ORF1 of the Th04 genome to amplify a product of 145 bp from nucleotide positions 401 to 545. Real-time assays were performed using an ABI GeneAmp 5700 sequence detection system with SYBR-Green RT-PCR reagents (Applied Biosystems). The reaction mixture contained 1  $\mu\text{l}$  of RNA sample, 12.5  $\mu\text{l}$  of 2 $\times$  SYBR-Green Master Mix, 200 nM each of forward and reverse primers, and 0.0025 unit of Multiscribe reverse transcriptase in a final volume of 25  $\mu\text{l}$ . The RT-PCR profile was 30 min at  $48^{\circ}\text{C}$  and 10 min at  $95^{\circ}\text{C}$ , followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 1 min. Data analysis was performed with GeneAmp 5700 sequence detection software.

To prepare an RNA standard for the real-time assay, a TSV fragment (nucleotides 20 to 1600) was amplified from original shrimp specimens of Th04. The amplification product was then cleaned with a QIAquick PCR purification kit (Qiagen) and ligated to pGEM-T-Easy vector (Promega). Recombinant plasmids were cloned into competent *Escherichia coli* JM109 cells (Promega), and a recombinant plasmid, pTSV-1, from 1 clone was verified by sequencing with an ABI Prism automated DNA sequencer (Applied Biosystems) at the University of Arizona. Then, pTSV-1 isolated with a PerfectoPrep plasmid isolation kit (Eppendorf Scientific) was linearized by *Sa*I digestion and used as the template for an *in vitro* transcription with T7 RNA polymerase (Fermentas). A volume of 1  $\mu\text{g}$  of plasmid was used in a 50  $\mu\text{l}$  reaction at  $37^{\circ}\text{C}$  for 2 h, followed by DNase I digestion at  $37^{\circ}\text{C}$  for 30 min. The synthesis of RNA (~800 nucleotides) was confirmed by electrophoresis in a 1.2% agarose gel containing ethidium bromide. The RNA standard thus prepared was cleaned using a QIAquick PCR purification kit, quantified by a spectrophotometer, and stored at  $-70^{\circ}\text{C}$ .

**Conventional RT-PCR.** Conventional RT-PCR assays were performed with a GeneAmp EZ rTth RNA PCR kit (Applied Biosystems) using 5  $\mu\text{l}$  of extracted RNA as the template and primers 9195 (5'-TCA ATG AGA GCT TGG TCC-3') and 9992 (5'-AAG TAG ACA GCC GCG CTT-3') to produce an amplicon of 231 bp (Nunan et al. 1998). The RT-PCR protocol comprised 30 min at  $60^{\circ}\text{C}$  and 2 min at  $94^{\circ}\text{C}$  followed by 40 cycles of 45 s at  $94^{\circ}\text{C}$ , 45 s at  $60^{\circ}\text{C}$  and a final extension step for 7 min at  $60^{\circ}\text{C}$ . An aliquot of amplified products was analyzed in 1.8% agarose gel containing ethidium bromide.

**Statistical analyses.** Statistical analyses were performed on resultant data according to Milton (1999). First, the Kaplan-Meier survival curves and cumulative survival probabilities were computed by SPSS 14.0 software for Windows (Bland & Altman 1998). Second, an analysis-of-variance (ANOVA) technique was used to determine the reproducibility of the SYBR-Green real-time RT-PCR and to test whether there was no linear regression between the coefficient of variations (CVs) and threshold cycle ( $C_T$ ). Third, a 1-way classification ANOVA, completely random design with fixed effect, was used to compare mean TSV copies among 8 different shrimp subgroups and to test whether the population means were equal. Fourth, once a 1-way ANOVA had been run to compare population means and the hypothesis of equality had been rejected, the investigation was continued to pinpoint the differences within 4 pairs of mean TSV copy number using the Bonferroni *t*-test. Finally, assuming that variances were unequal, the Smith-Satterthwaite *t*-test was performed to make inferences on difference between 2 mean TSV copy numbers, (1 found to be negative and 1 found to be positive by conventional RT-PCR). The statistical analyses from steps 2 to 5 were performed using Microsoft Excel 2002 software.

## RESULTS AND DISCUSSION

### Survival and lesions in TSV-resistant *Litopenaeus vannamei* after TSV infection

The selected TSR *Litopenaeus vannamei* showed greater survival than did the unselected TSV-susceptible (Kona) stock after challenge with 4 different TSV variants. Because of the experimental design (i.e. intermittent sampling and differences in test group sizes), simple estimation of percent survivals over time could not be precisely calculated and compared. However, a mean estimated survival, also known as a cumulative survival probability, for each group could be obtained using the Kaplan-Meier analysis (Bland & Altman 1998). Survival of the selected TSR and unselected TSV-susceptible (Kona) populations was determined at termination of the bioassays, Day 14 p.i.; for TSR shrimp, survival probabilities were from 0.775 to 1, while for Kona shrimp survival probabilities were from 0 to 0.215 (Fig. 1). Both TSR and Kona shrimp showed the lowest survival probabilities of 0.775 and 0, respectively, when infected with Bz01 (Group 1) (Fig. 1A). In contrast, both TSR and Kona shrimp showed the highest survival probabilities of 1 and 0.215, respectively, when infected with UsHi94 (Group 3) (Fig. 1C). Our results were also consistent with Bz01 being more virulent than UsHi94 (Erickson et al. 2005, Tang & Lightner 2005). The data in Fig. 1 also

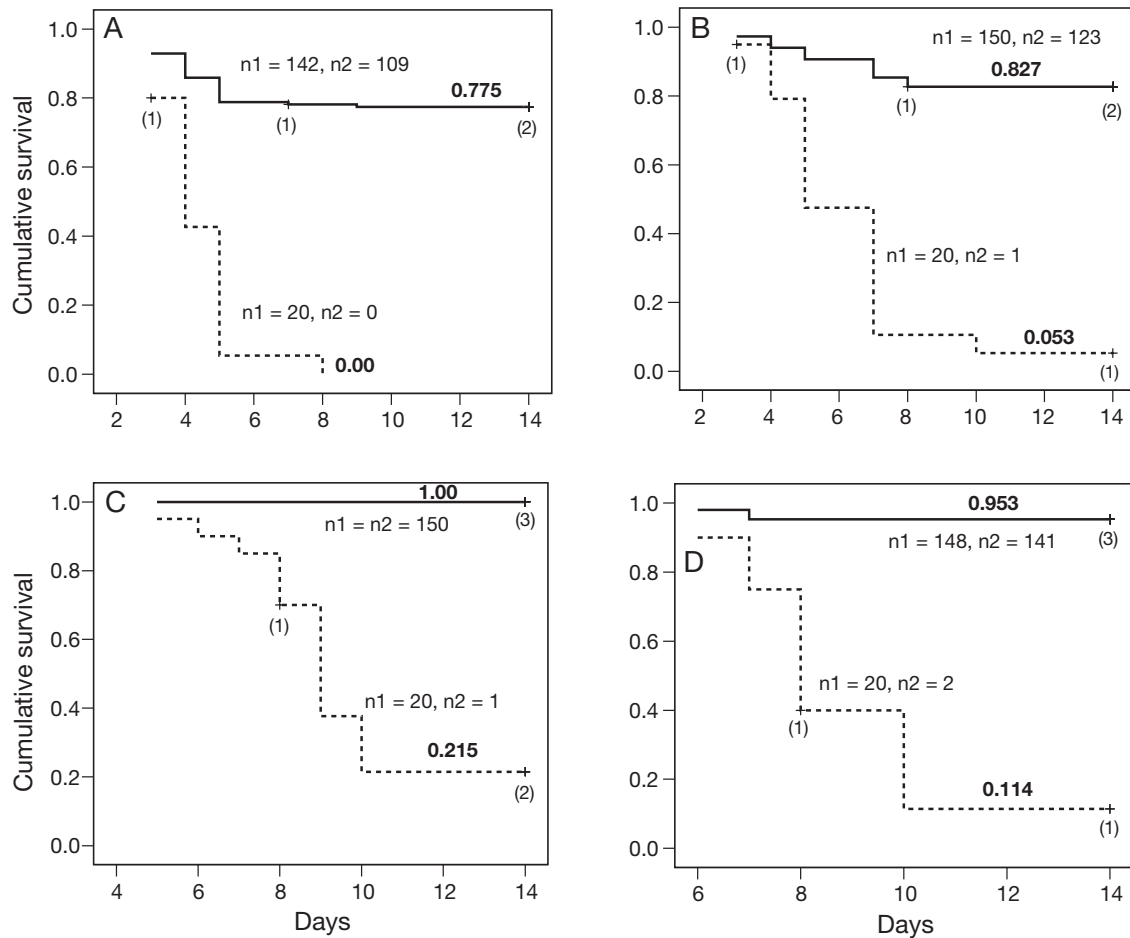
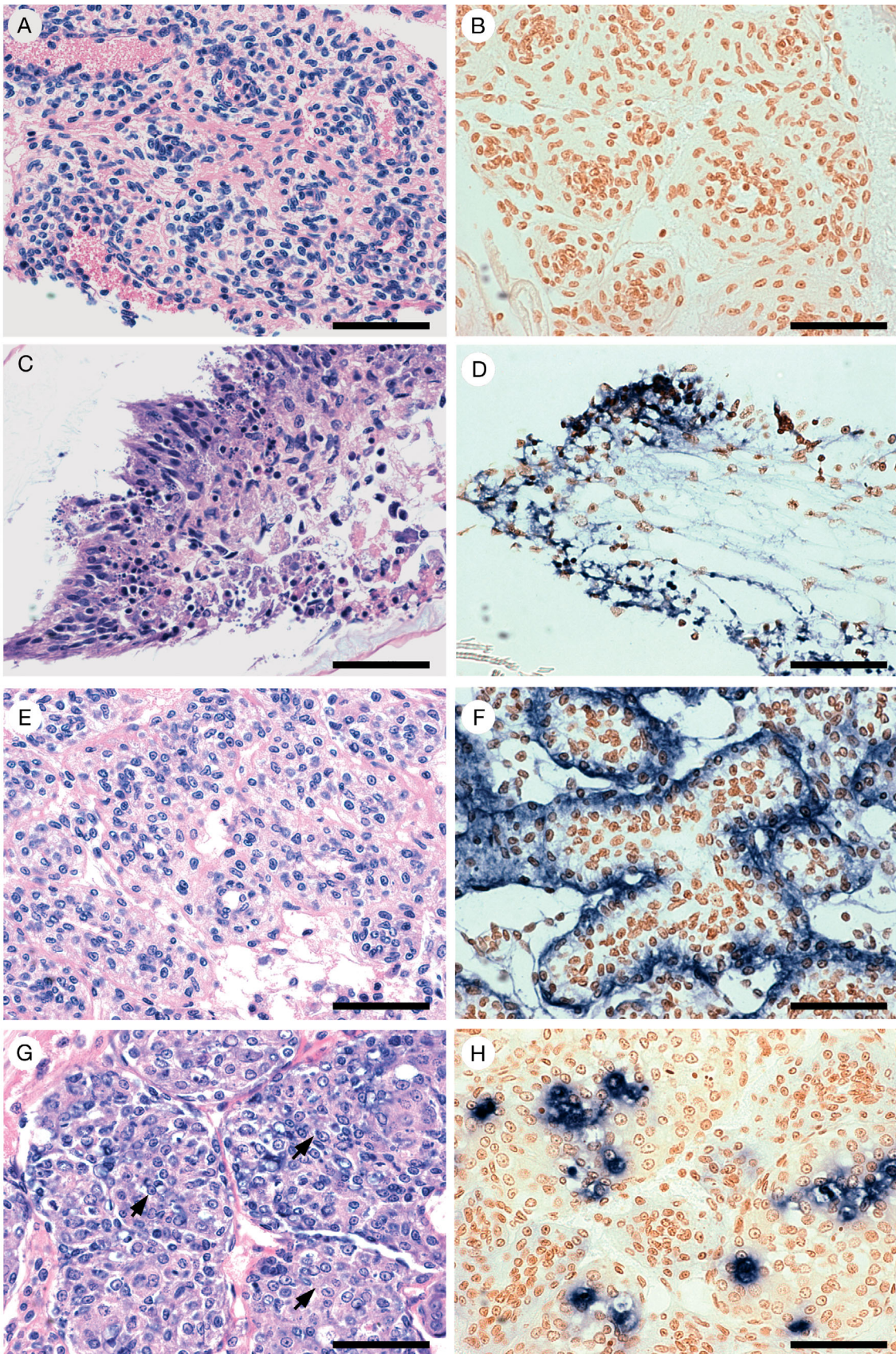


Fig. 1. *Litopenaeus vannamei*. Survival curves of Taura syndrome virus (TSV)-resistant (TSR, solid lines) and TSV-susceptible (Kona, dashed lines) shrimp after challenge by feeding with 4 TSV isolates: (A) Bz01, (B) Th04, (C) UsHi94, and (D) Ve05. Numbers in **bold**: Kaplan-Meier survival probability values. Numbers in parentheses: number of shrimp sampled for histological analysis at specific days (+). n1: group total numbers of shrimp stocked at Day 0 post-infection (p.i.). n2: numbers of survivors at Day 14 p.i.

allowed comparison of survival between TSR and Kona *L. vannamei* that were infected with the same TSV isolate. Specifically, survival probabilities of TSR shrimp were higher than those of Kona shrimp challenged with all 4 variants. No mortality was observed among *L. vannamei* in the TSR and Kona *L. vannamei* negative control group (Group 5).

We did not detect characteristic acute or chronic lesions of TSV infection, as described by Lightner et al. (1995) and Hasson et al. (1995, 1999), in the TSR *Litopenaeus vannamei* that were infected with Th04, UsHi94, or Ve05 (Fig. 2A,B). The TSR *L. vannamei*, however, developed TSV lesions when infected with Bz01. One specimen (sampled at Day 7 p.i.) displayed

Fig. 2. *Litopenaeus vannamei*. Photomicrographs of consecutive shrimp tissue sections tested for Taura syndrome virus (TSV) lesions by hematoxylin and eosin (H&E) staining (left column) and *in situ* hybridization (ISH) (right column). (A,B) Example of a normal appearing lymphoid organ (LO) from TSV-resistant (TSR) shrimp challenged by feeding with Th04, UsHi94, Ve05, and from both TSR and TSV-susceptible (Kona) shrimp in the negative control group collected at Day 14 post-infection (p.i.). (C–H): Examples of the characteristic acute (C,D) and chronic phase (E–H) lesions seen in TSR shrimp challenged with Bz01 and in Kona shrimp challenged with all 4 TSV isolates used in this study. (C,D) Cuticular epithelium of the stomach illustrating an acute phase lesion in a TSR shrimp challenged with Bz01 and collected at Day 7 p.i. Note the severe necrosis of infected cuticular epithelial cells as shown by H&E and blue-black precipitates by ISH with TSV-specific probes. (E,F) LO from the same TSR specimen as shown in (C,D) illustrating the early to transition phases of TSV infection. Note the normal tissue appearance by H&E but blue-black precipitates at the outermost parenchymal cells of the LO tubules by ISH. (G,H) LO from a TSR shrimp challenged with Bz01 and collected at Day 14 p.i. Note the presence of lymphoid organ spheroids (arrows in G) exemplifying the chronic phase lesion by H&E staining and a positive ISH reaction. Scale bars = 50  $\mu$ m



the characteristic acute phase lesions of TSV infection, indicated by severe necrosis in various epithelial tissues, including gills, cuticular, and stomach epithelia (Fig. 2C). When the consecutive tissue section of this shrimp was analyzed by ISH, a positive reaction to TSV-specific gene probes was detected as blue-black precipitates (Fig. 2D). This TSR specimen exhibited a normal lymphoid organ by histology (Fig. 2E). However, by ISH, its lymphoid organ section showed a strong positive reaction, and the reaction was only seen at the peripheral cells in the lymphoid organ tubules (Fig. 2F). A similar distribution of TSV-positive cells in the lymphoid organ was seen by ISH in *L. vannamei* and *Penaeus monodon* (Srisuvan et al. 2005) in the transition phase of TSV infection and by immunohistochemistry in *P. monodon* infected with yellow head virus (YHV) (Soowannayan et al. 2002). Two specimens infected with Bz01 and sampled at Day 14 p.i. exhibited lymphoid organ spheroids (LOS) characteristic of chronic phase lesions of TSV infection (Fig. 2G) and their consecutive sections reacted to the TSV-specific gene probes by ISH (Fig. 2H), confirming that the LOS formation was associated with TSV infection. Since only Bz01 produced the characteristic TSV lesions in the TSR *L. vannamei*, this population was clearly shown to be partially resistant to TSV infection using the 4 TSV variants tested in the present study.

As expected, the Kona *Litopenaeus vannamei*, infected with all 4 TSV isolates, displayed acute and chronic phase lesions of TSV infection at Days 3, 8, and 14 p.i., as illustrated in the photomicrographs for Bz01 infections in the TSR stock (Fig. 2C to H). In contrast, no lesions indicative of TSV infection were detected in any shrimp from the negative control group (Group 5) of either TSR or Kona stock at termination (Fig. 2A,B).

#### Development and validation of a SYBR-Green realtime RT-PCR

The SYBR-Green real-time RT-PCR analysis was capable of detecting Bz01, Th05, UsHi94, and Ve05, despite the fact that there is 1 nucleotide mismatch at the primer 401F binding region for UsHi94. The dissociation curve showed a single peak for the 145 bp amplification product at a melting temperature of 79°C. The assay gave negative results for RNA extracted from YHV-infected shrimp, infectious myonecrosis virus (IMNV)-infected shrimp and specific-pathogen-free shrimp ( $C_T = 40$ ).

Testing of 10-fold serial dilutions of the TSV RNA standard from  $10^9$  copies down to 1 copy revealed that 1 and 10 copies could be detected in 1 and 2 out of 5 assays, respectively, while 100 copies were detected in all 5 assays. Thus, the lower detection limit was consid-

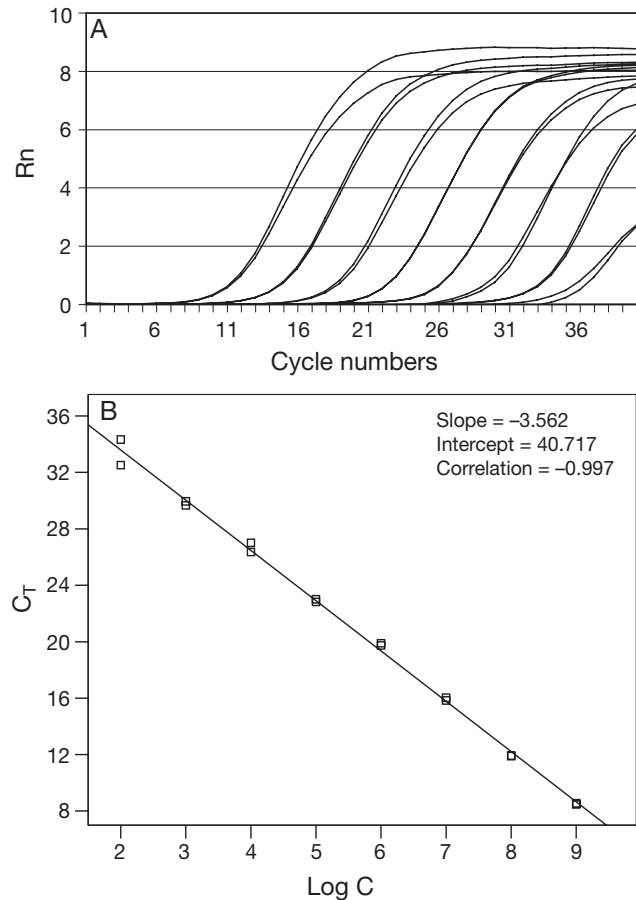


Fig. 3. SYBR-Green real-time reverse transcription polymerase chain reaction (RT-PCR). (A) Amplification plot and (B) standard curve from 10-fold serial dilutions of a Taura syndrome virus (TSV) RNA standard in duplicate measurements (squares). R<sub>n</sub>: normalized fluorescent intensity. C<sub>T</sub>: threshold cycle. Log C: logarithm values of TSV copy numbers

ered to be 100 copies. A strong linear correlation ( $r < -0.99$ ) was obtained between C<sub>T</sub> and RNA quantities over an 8-log range from  $10^2$  to  $10^9$  copies per reaction, indicating that this real-time assay has a large dynamic range (Fig. 3).

To determine the reproducibility of the SYBR-Green real-time RT-PCR assay, we compared 5 standard curves from  $10^2$  to  $10^9$  copies of the TSV RNA standard. The CVs within each run were between 0.11 and 0.87% for  $10^9$  copies and increased to 0.5 to 4.12% for  $10^2$  copies (Table 2). For these 5 independent runs, there was a significant linear relation between CVs and C<sub>T</sub> ( $p < 0.01$ ,  $F_{1,38} = 8.62$  [ANOVA, where  $df = 1$  and 38],  $r^2 = 0.1848$ ,  $n = 40$ ). This was in accordance with previous studies (Tang et al. 2004) showing that lower copies of RNA templates translated to higher C<sub>T</sub> and higher CVs. The CVs were 1.66% for  $10^9$  copies and increased to 6.28% for  $10^2$  copies, indicating that this real-time assay has little variation between runs.

Table 2. Reproducibility of the SYBR-Green reverse transcription polymerase chain reaction (RT-PCR) assay. TSV: Taura syndrome virus; CV: coefficient of variation;  $C_T$ : threshold cycle

TSV copies $\mu\text{l}^{-1}$	CV (%) within assay in 5 runs (mean $C_T$ from duplicate measurements)					CV (%) between assays (mean $C_T$ )
	1	2	3	4	5	
$10^9$	0.42 (8.79)	0.71 (8.51)	0.11 (8.44)	0.12 (8.50)	0.87 (8.66)	1.66 (8.58)
$10^8$	0.16 (12.13)	0.25 (11.91)	1.01 (11.84)	0.81 (11.86)	0.08 (12.18)	1.34 (12.11)
$10^7$	0.03 (15.25)	0.82 (15.94)	0.46 (15.26)	0.93 (15.56)	0.76 (15.84)	2.05 (15.57)
$10^6$	0.35 (20.09)	0.55 (19.82)	1.11 (19.74)	0.70 (20.13)	0.35 (20.20)	1.02 (20.00)
$10^5$	0.45 (22.81)	0.61 (22.92)	1.25 (23.13)	1.36 (23.54)	1.21 (23.99)	2.09 (23.27)
$10^4$	0.95 (26.26)	1.72 (26.69)	0.84 (26.23)	0.04 (26.16)	0.18 (26.75)	1.06 (26.42)
$10^3$	1.84 (27.43)	0.67 (29.81)	1.98 (27.42)	0.44 (27.20)	0.18 (27.80)	3.84 (27.93)
$10^2$	0.66 (29.02)	2.02 (33.43)	4.12 (30.63)	0.70 (28.59)	0.50 (30.03)	6.28 (30.34)

### Mean viral loads after TSV challenge

By real-time RT-PCR, the TSR and Kona groups showed significant differences in mean TSV copy number after challenge with Bz01, Th04, or UsHi94 ( $p < 0.0005$ ,  $t_{76} = 7.66$ , 4.82, and 10.71, respectively [Bonferroni  $t$ -test, where  $df = 76$ ]) (Fig. 4). In contrast, TSV copy number within the TSR and Kona *Litope-*

*naeus vannamei* infected with Ve05 showed no difference in mean effect ( $p > 0.4$ ,  $t_{76} = 0.0017$ ). We reasoned that high mortalities in the Kona *L. vannamei* infected with Bz01 may be associated with its high virus copy numbers. The real-time RT-PCR analysis showed that the TSR *L. vannamei* are susceptible to infection although they give high survival rates following challenge by all 4 variants of TSV. Fig. 4 also revealed that,

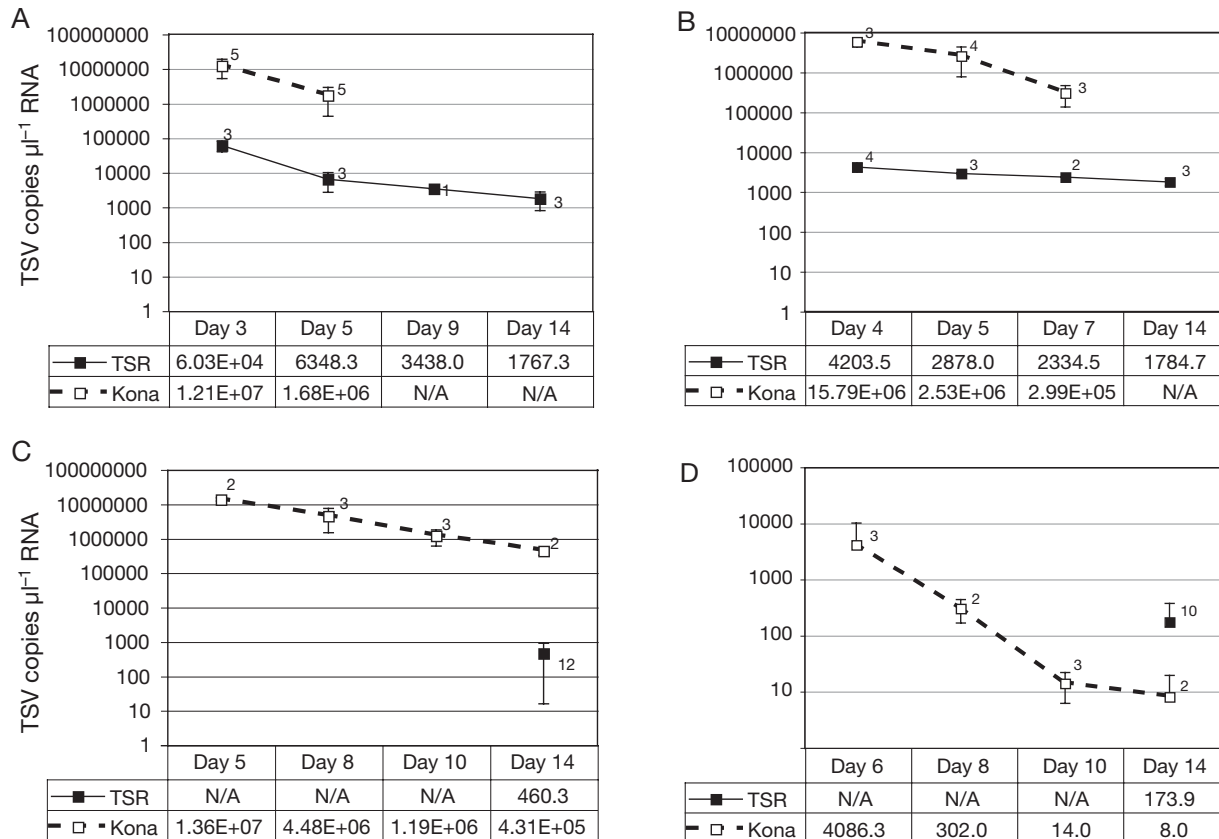


Fig. 4. *Litopenaeus vannamei*. Means  $\pm$  standard errors of Taura syndrome virus (TSV) copy number  $\mu\text{l}^{-1}$  RNA within pleopods of TSV-resistant (TSR) and TSV-susceptible (Kona) shrimp after challenge by feeding with 4 virus isolates: (A) Bz01, (B) Th04, (C) UsHi94, and (D) Ve05. Numbers near squares: number of shrimp collected on specific days. N/A: not applicable

for both TSR and Kona shrimp, the quantities of tissue-loaded viruses were relatively high at Days 3 to 6 p.i. and decreased at termination, Day 14 p.i. In addition, the results from the real-time RT-PCR analysis and those by histological analysis (Fig. 2) further support that the acute, transition, and chronic phases of TSV infection occurred following challenge as previously

described by Lightner et al. (1995) and Hasson et al. (1995, 1999). The quantities of tissue-loaded TSV may be responsible for the pathogenesis and the survivability of the affected shrimp.

In contrast to real-time RT-PCR, conventional RT-PCR did not detect TSV in all TSR *Litopenaeus vannamei* challenged with Th04, UsHi94, and Ve05, in 6

Table 3. *Litopenaeus vannamei*. Comparison of a conventional reverse transcription polymerase chain reaction (RT-PCR) with the SYBR-Green real-time RT-PCR for Taura syndrome virus (TSV) within pleopods of TSV-resistant (TSR) and TSV-susceptible (Kona) populations of *L. vannamei* after challenge by feeding with 4 virus isolates. (+) presence or (-) absence of the specific 231 bp amplicon

Population TSV isolate	Shrimp no.	Collection days	Conventional RT-PCR	Real-time RT-PCR <sup>a</sup> (TSV copy numbers $\mu\text{l}^{-1}$ RNA)	Population TSV isolate	Shrimp no.	Collection days	Conventional RT-PCR	Real-time RT-PCR <sup>a</sup> (TSV copy numbers $\mu\text{l}^{-1}$ RNA)	
TSR					Kona					
Ve05	1	14	-	15	Ve05	45	6	-	11001	
	2	14	-	73		46	6	-	1258	
	3	14	-	572		47	6	-	0	
	4	14	-	85		48	8	-	207	
	5	14	-	83		49	8	-	397	
	6	14	-	328		50	10	-	19	
	7	14	-	450		51	10	-	18	
	8	14	-	32		52	10	-	5	
	9	14	-	46		53	14	-	16	
	10	14	-	55		54	14	-	0	
UsHi94	11	14	-	263	UsHi94	55	5	+	$1.46 \times 10^7$	
	12	14	-	300		56	5	+	$1.26 \times 10^7$	
	13	14	-	1692		57	8	+	2934997	
	14	14	-	913		58	8	+	2541995	
	15	14	-	145		59	8	+	7949984	
	16	14	-	249		60	10	+	1629794	
	17	14	-	232		61	10	+	1425576	
	18	14	-	261		62	10	+	516528	
	19	14	-	135		63	14	+	507199	
	20	14	-	545		64	14	+	355568	
	21	14	-	273		Th04	65	4	+	7509711
	22	14	-	516			66	4	+	4952226
Th04	23	4	-	5590	67		4	+	4916875	
	24	4	-	4233	68		5	+	1966963	
	25	4	-	3039	69		5	+	3503556	
	26	4	-	3952	70		5	+	4301637	
	27	5	-	2942	71		5	+	335719	
	28	5	-	2991	72		7	+	157968	
	29	5	-	2701	73	7	+	479278		
	30	7	-	2338	74	7	+	258309		
	31	7	-	2331	Bz01	75	3	+	$2.26 \times 10^7$	
	32	14	-	1831		76	3	+	$1.37 \times 10^7$	
	33	14	-	2124		77	3	+	$1.25 \times 10^7$	
	34	14	-	1399		78	3	+	5281579	
Bz01	35	3	+	78517		79	3	+	6401372	
	36	3	+	37799		80	5	+	3781850	
	37	3	+	64731		81	5	+	615517	
	38	5	+	10476		82	5	+	895659	
	39	5	-	3429	83	5	+	1355328		
	40	5	-	5140	84	5	+	1749467		
	41	9	-	3438						
	42	14	-	2843						
	43	14	-	954						
	44	14	-	1505						

<sup>a</sup>Means of duplicate copy numbers

out of 10 TSR shrimp challenged with Bz01, or in all Kona shrimp challenged with Ve05 (Table 3). However, it did detect TSV in 4 out of 10 TSR *L. vannamei* infected with Bz01 and in all Kona shrimp infected with Bz01, Th04, and UsHi94. Our results in Table 3 were not consistent with those of D. V. Lightner et al. (unpubl. data), who found that Ve05 could be detected by the conventional RT-PCR. To investigate this apparent inconsistency, we hypothesized that only samples containing relatively high copy numbers of TSV were positive by the conventional RT-PCR. Using the SYBR-Green real-time RT-PCR, it was determined that TSV was detected in the same shrimp specimens that had given negative TSV results by the conventional RT-PCR (Table 3). We found that mean TSV copy number among the TSR shrimp infected with Th04, UsHi94, Ve05 (Shrimp 1 to 34), and 6 TSR shrimp infected with Bz01 (Shrimp 39 to 44), which were tested as negative by the conventional RT-PCR, was  $1.50 \times 10^3$  (standard deviation [SD] =  $1.57 \times 10^3$ ), whereas mean TSV copy in 4 TSR shrimp infected with Bz01 (Shrimp 35 to 38), which were tested as positive by the conventional RT-PCR, was  $4.79 \times 10^4$  (SD =  $3.01 \times 10^4$ ). Interestingly, we found that there was a significant difference in TSV copy number between the TSR shrimp that TSV was not detected and those that were positive by conventional RT-PCR ( $p < 0.0005$ ,  $t_{\infty} = 4.87$  [Smith-Satterthwaite  $t$ -test where  $df = \infty$ ]). It was also determined that mean TSV copy number in the Kona shrimp infected with Ve05 (Shrimp 45 to 54), which were detected as negative by the conventional RT-PCR, was  $1.29 \times 10^3$  (SD =  $3.43 \times 10^3$ ), whereas mean TSV copy in the Kona shrimp infected with Bz01, Th04, and UsHi94 (Shrimp 55 to 84), which were detected as positive by the conventional RT-PCR, was  $2.65 \times 10^6$  (SD =  $2.39 \times 10^6$ ). Again, we found that there was a significant difference in TSV copy number between the Kona shrimp that TSV was not detected and those that were positive by conventional RT-PCR ( $p < 0.0005$ ,  $t_{\infty} = 6.08$ ). Thus, it is statistically necessary to conclude that the SYBR-Green real-time RT-PCR has a greater sensitivity compared to the conventional RT-PCR and that the conventional RT-PCR was only capable of detecting TSV at a relatively high virus copy number.

In conclusion, the selected line of TSR *Litopenaeus vannamei*, while susceptible to TSV infection, is resistant to development of severe TS. Using experimental challenge studies, we demonstrated the resistance to severe TSV infection (as indicated by lower TSV copy number in infected individuals) in the TSR *L. vannamei* by histological, ISH, RT-PCR, and real-time RT-PCR analyses. Resistance to TSV, as observed in the selected TSR line, may not be prevalent among wild

and cultured populations of *L. vannamei*. Finally, our results indicate that additional studies are needed to elucidate the genetic trait and markers associated to TSV resistance in *L. vannamei*.

**Acknowledgements.** This work was supported by Gulf Coast Research Laboratory Consortium Marine Shrimp Farming Program, USDACSREES, Grant no. 2002-38808-01345. We thank Dr. James Wyban (High Health Aquaculture) for supplying Taura syndrome resistant (TSR) *Litopenaeus vannamei* for the experimental challenges and Dr. Kathy F. J. Tang for technical assistance.

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*Editorial responsibility: Timothy W. Flegel, Bangkok, Thailand*

*Submitted: October 31, 2005; Accepted: March 3, 2006  
Proofs received from author(s): June 26, 2006*