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Quantitative assay for measuring the Taura syndrome virus and yellow head virus load in shrimp by real-time RT-PCR using SYBR Green chemistry

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Abstract

Taura syndrome virus (TSV) and yellow head virus (YHV) are the two RNA viruses infecting penaeid shrimp (*Penaeus* sp.) that have caused major economic losses to shrimp aquaculture. A rapid and highly sensitive detection and quantification method for TSV and YHV was developed using the GeneAmp® 5700 Sequence Detection System and SYBR Green chemistry. The reverse transcriptase polymerase chain reaction (RT-PCR) mixture contained a fluorescent dye, SYBR Green, which exhibits fluorescence enhancement upon binding to double strand cDNA. The enhancement of fluorescence was found to be proportional to the initial concentration of the template cDNA. A linear relationship was observed between input plasmid DNA and cycle threshold (C_T) values for 10^6 down to a single copy of both viruses. To control for the variation in sample processing and in reverse transcription reaction among samples, shrimp β-actin and elongation factor-1α (EF-1α) genes were amplified in parallel with the viral cDNA. The sensitivity and the efficiency of amplification of EF-1α was greater than β-actin when compared to TSV and YHV amplification efficiency suggesting that EF-1α is a better internal control for the RT-PCR detection of TSV and YHV. In addition, sample to sample variation in EF-1α C_T value was lower than the variation in β-actin C_T value of the corresponding samples. The specificity of TSV, YHV, EF-1α and β-actin amplifications was confirmed by analyzing the dissociation curves of the target amplicon. The C_T values of TSV and YHV samples were normalized against EF-1α C_T values for determining the absolute copy number from the standard curve of the corresponding virus. The method described here is highly robust and is amenable to high throughput assays making it a useful tool for diagnostic, epidemiological and genetic studies in shrimp aquaculture.

Keywords: Shrimp; Taura syndrome virus; Yellow head virus; SYBR Green RT-PCR; Real-time PCR

1. Introduction

Taura syndrome virus (TSV) and yellow head virus (YHV) are the two most important RNA viruses of penaeid shrimp (*Penaeus* sp.) (Lightner et al., 1996). In the Western Hemisphere, TSV has

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caused serious economic losses, whereas, YHV is viewed as one of the most significant viral pathogens in the Eastern Hemisphere (Brock, 1997; Flegel, 1997). The cumulative losses due to TSV in the Americas from 1992 to 1996 were estimated to be US$1.2–2.0 billion (Lightner et al., 1996). YHV has affected significantly shrimp farming in South East Asian countries including Thailand, China, Malaysia, Indonesia and India (Lightner et al., 1996).

Taura syndrome disease, caused by the TSV, was first described in samples collected from shrimp farms located near the mouth of the Taura river in Ecuador in 1992 (Jimenez, 1992; Brock et al., 1995; Hasson et al., 1995). TSV virions are non-enveloped, icosahedral, 31–32 nm in diameter and contain a single stranded positive sense RNA genome of $\sim$10 kb capable of encoding three major (55, 40 and 24 kDa) and one minor (58 kDa) capsid proteins (Hasson et al., 1995; Bonami et al., 1997). We have cloned and sequenced the 3′-end of TSV genome (Robles-Sikisaka et al., 2001). Sequence analysis showed that, unlike mammalian picornaviruses, TSV capsid protein genes are located at the 3′-end of the genome and the TSV genome organization is similar to insect picornaviruses (Robles-Sikisaka et al., 2001).

YHV was first reported in 1990 with the occurrence of mass mortalities in farm reared black tiger shrimp (*P. monodon*) in Thailand (Chantanachookin et al., 1993). YHV virions have an enveloped bacilliform shape of 150–170 $\times$ 40–50 nm in size (Wongteerasupaya et al., 1995). The viral genome contains a single stranded, positive sense RNA and encodes four major structural proteins of 170, 135, 67 and 22 kDa. The partial nucleotide sequence (open reading frame 1b) revealed that the genome organization of YHV is very similar to the gill-associated virus (GAV), reported from Australia. It has been proposed that YHV and GAV should belong to a new taxon (proposed name *Okavirus*) in the order Nidovirales that also included Coronaviruses, toroviruses and arteriviruses (Walker et al., 2001).

The current diagnostic methods for TSV and YHV include bioassay using indicator hosts, monitoring clinical signs, histopathology, dot blot, in situ hybridization using virus specific gene probe, immunohistochemistry and by the polymerase chain reaction (PCR) (Lightner and Redman, 1998). Although conventional PCR is most sensitive among these methods, it is unable to detect a single copy of the viral genome in the infected tissue. This is critical for the development of a specific pathogen free shrimp-breeding program and for monitoring movement of live and frozen shrimp between countries. To address these issues, we have developed a rapid and highly sensitive real-time quantitative PCR method using the GeneAmp® 5700 Sequence Detection System coupled with SYBR Green chemistry. SYBR Green dye has a high affinity for double-stranded DNA (dsDNA) and exhibits enhancement of fluorescence upon binding to the dsDNA. In the GeneAmp® 5700 Sequence Detection System, the fluorescence of the SYBR Green dye is monitored at the end of the each cycle and the increase in fluorescence above background is dependent on the initial template concentration (PE Biosystem GeneAmp® 5700 User Manual, 1998). The method does not need any post PCR analyses and the specificity of the product is monitored by analyzing the melting curve (Ririe et al., 1997).

The objectives of the present study were (1) to determine the sensitivity and specificity of SYBR Green RT-PCR using the GeneAmp 5700 Sequence Detection system in detecting TSV and YHV; and (2) to determine the TSV and YHV load in laboratory challenged shrimp.

2. Materials and methods

2.1. Virus challenge

Juvenile shrimp (*Penaeus vannamei*, $\sim$2–3 g) of a TSV susceptible line (Kona stock) developed by the Oceanic Institute, Hawaii and a TSV resistant line of shrimp (*P. stylirostris*) developed by Super Shrimp, Inc. were used for this study. For YHV detection work, Super Shrimp, Inc. *P. stylirostris* that are susceptible to YHV were used. Virus inoculum (TSV Mexican 1999 isolate, courtesy of Dr K.W. Hasson, Super Shrimp Inc. and YHV, courtesy of Dr D.V. Lightner, University

of Arizona, Arizona) was prepared by homogenizing PCR confirmed TSV and YHV infected tail tissue in 2% saline (1:10 w/v) and centrifuging the homogenate in a tabletop centrifuge (Beckman Microfuge Lite Model) at 12 000 rpm for 5 min. The supernatants were diluted to 1:10 before injecting the animals. Healthy juvenile shrimp were injected with a virus inoculum (30 μl $\approx 10^6$ copies) using a 26-gauge needle and 1 ml tuberculin syringe between the last 2–3 tail segments on the ventral surface. Control group animals were injected with a tail muscle homogenate from PCR-confirmed virus negative healthy animals. Healthy tissue homogenate was prepared same as described above. Animals were kept indoors within environmentally controlled tanks, reared on a commercially available feed formulation (MADMAC-MS Dry pellet, Bio-Marine, Inc. Hawthorne, CA) and routinely monitored during the course of the study.

2.2. Isolation of total RNA

Virus challenged moribund animals were killed at 3–4 days post-injection (p.i.) for TSV susceptible *P. vannamei* and YHV susceptible *P. stylirostris*. For TSV resistant Super Shrimp *P. stylirostris*, animals were sacrificed 3–5 days p.i. The sampling time was based on the observation that in Super Shrimp *P. stylirostris* TSV titer attains a high level after 3–5 days p.i., as determined by real-time reverse transcriptase polymerase chain reaction (RT-PCR) (K.R. Klimpel, unpublished). Tail muscle tissue (~ 50 mg) from virus challenged as well as control animals were taken for the extraction of RNA using TRI Reagent™ (Molecular Research Center, Inc. Ohio). The RNA pellets were dissolved in DNase, RNase free distilled water and the yield of total RNA was measured by using a spectrophotometer (Shimadzu UV-1201). The RNA quality was assessed by running the samples in a 1% formaldehyde agarose gel following standard protocol (Sambrook et al., 1989). Total RNA was treated with DNase I using the MessageClean® kit of GenHunter Corp. (Nashville, TN) before synthesizing cDNA for SYBR Green RT-PCR.

2.3. Cloning and sequencing of TSV, YHV, β-actin and elongation factor-1α genes

A list of primers used for the RT-PCR amplification of TSV, YHV, β-actin, and elongation factor-1α (EF-1α) is given in Table 1. For TSV, YHV and β-actin RT-PCR, cDNA was synthesized using Omniscript™ cDNA synthesis protocol (Qiagen, CA) and 1 μg total RNA in a 20 μl reaction volume. The RT-PCR mixture contained, 4 μl cDNA reaction mixture, 1 × PCR buffer (Sigma, St. Louis, MO), 1 μM dNTP, 1.25 μM of

Table 1
Details of the primers used for the RT-PCR amplification of TSV, YHV, EF-1α and β-actin genes

Virus/Control gene	Primer name	Primer sequence (5′-3′)	Product size (bp)	Reference
TSV	TSVF1 TSVR1	TCAATGAGAGCTTGGTCC AGTAGACAGCCGCGCTTG	220	Nunan et al. (1998)
YHV	141F 962R	CGTCCCGGCAATTGTGAT GAATGGTATCACCGTTCAGTGTC TT	821	Tang and Lightner (1999)
EF-1α	H-AP1 H-T_{11}A	AAGCTTGATTGCC AAGCTTTTTTTTTTTA	382	Dhar et al. (2001b)
β-actin	AD-65F AD-566R	CCCTTGTGGTTGACAATGGCT GCATGAGGAAGAGCGAAACCT	510	GenBank Accession No. AF100986

Table 2
List of primers used for the detection of TSV, YHV, EF-1α and β-actin gene by SYBR Green RT-PCR

Virus/Control gene	Primer	SYBR Green Primer sequence (5′–3′)	%GC	T_m[a]	Amplicon size (bp)
TSV	112F	For: CTGTTTGTAACACTACCTCCTGGAATT	40	52	50
	162R	Rev: TGATACAACAACCAGTGGAGGACTAA	42	51	
YHV	141F	For: CGTCCCGGCAATTGTGAT	55	45	65
	206R	Rev: CCAGTGACGTTCGATGCAATA	47	47	
EF-1α	123F	For: TCGCCGAACTGCTGACCAAGA	57	51	55
	123R	Rev: CCGGCTTCCAGTTCCTTACC	60	51	
β-actin	178F	For: GGTCGGTATGGGTCAGAAGGA	57	51	50
	228R	Rev: TTGCTTTGGGCCTCATCAC	52	46	

[a] At 50 mM Na^+.

each forward and reverse primer and 0.6 U of RED *Taq* DNA polymerase (Sigma) in a 25 μl reaction volume. The temperature profile for the PCR amplification was 94 °C 2 min followed by 35 cycles of 94 °C 1 min, 55 °C 2 min, 72 °C 1 min with extension at 72 °C 7 min. The PCR amplified products were run in a 1% agarose gel at 80 V for 1 h and stained with ethidium bromide to visualize the products on a UV transilluminator. The EF-1α gene was previously isolated from a white spot syndrome virus (WSSV) challenged *P. stylirostris* shrimp by using the mRNA differential display technique (Dhar et al., 2001b).

TSV, YHV and β-actin cDNAs were cloned into a TOPO cloning vector (Invitrogen, CA) and the EF-1α cDNA was cloned into a PCR-TRAP vector (GenHunter Corp., Inc.). The recombinant plasmid DNA was sequenced in an automated DNA sequencer (model ABI 373A, PE Applied Biosystems). The Sequence analyses were carried out using the NCBI BLAST search program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to confirm identity between the cloned and the published sequences based on which the primers were designed (Table 1).

2.4. SYBR Green RT-PCR

The primers used for SYBR Green RT-PCR are listed in Table 2. The primers were designed based on the sequence of the cloned segment of TSV, YHV, β-actin and EF-1α genes and using the Primer Express Software version 1.0 (PE Applied Biosystem). The primers were checked by running a virtual PCR and the amplifications were analyzed for expected product, mispairing and primer dimer formation using a computer program (Amplify v1.2B, Dr William Engles, University of Wisconsin, Department of Genetics). The best primer set was taken for amplification.

The SYBR Green RT-PCR amplifications were undertaken in a GeneAmp 9600 Thermocycler coupled with a GeneAmp® 5700 Sequence Detection System (PE Applied Biosystems). The cDNA synthesis was carried out in a 20 μl reaction volume containing 1 μg DNase I treated total RNA, 1 × RT-PCR buffer, 1 mM dNTPs (PE Applied Biosystems), 0.75 μM oligo dT, 4 U of RNase inhibitor (PE Applied Biosystems) and 5 U of MutiScribe™ reverse transcriptase (PE Applied Biosystems). The cDNA reaction mixture was diluted 1:10 using DNase, RNase free molecular biology grade water and 1 μl was taken for each amplification reaction. The amplifications were carried out in a 96 well plate in a 25 μl reaction volume containing 7.1 μl of 2 × SYBR® Green Master Mix (PE Biosystems), 0.24 μM each of forward and reverse primers and 1 μl of the 1:10 diluted cDNA. The thermal profile for SYBR RT-PCR was 50 °C 2 min, 95 °C 10 min followed by 40 cycles of 95 °C 10 s and 60 °C 1 min. In each 96 well plate, a dilution series of the plasmid standard for the respective virus was run along with the unknown samples for the corresponding virus and the EF-1α control. Each sam-

ple had 2–3 replicates and all reactions were repeated at least 3 times independently to ensure the reproducibility of the results.

For comparing the efficiency of amplification of EF-1α and β-actin genes with TSV and YHV, cDNA was synthesized in a 20 μl reaction volume as described above. A serial dilution was then made using sheared salmon sperm DNA (5 ng/ml) as a diluent. SYBR Green RT-PCR was performed in a 96 well plate using 1 μl of each of the cDNA dilutions for TSV and YHV detection along with EF-1α and β-actin controls following the reaction parameters as described above.

2.5. TSV and YHV Plasmid standard for quantification by SYBR Green PCR

The plasmid DNAs containing 220 bp TSV insert and 821 bp YHV insert were separately linearized by *Hin*dIII (Promega, WI) digestions. An aliquot of the digested plasmids were run in a 1% agarose gel to confirm the digestion before purifying the remaining digestion reactions by Qiaquick Gel Purification kit (Qiagen, CA). DNA was quantified using a spectrophotometer (Shimadzu UV-1201) and dilutions were made using sheared salmon sperm DNA (5 ng/ml) as a diluent.

2.6. Data analyses

After a SYBR Green PCR run, data acquisition and subsequent data analyses were done using the 5700 Sequence Detection System (SDS Version 1.3). In the 5700 Sequence Detection System, the fluorescence of SYBR Green against the internal passive reference dye, ROX (ΔR_n) is measured at the end of each cycle. A sample is considered positive when ΔR_n exceeds the threshold value. The threshold value is set at the midpoint of ΔR_n vs. cycle number plot. For all the amplifications described in this paper, the threshold value of ΔR_n was taken as 0.25. The threshold cycle (C_T) is defined as the cycle at which a statistically significant increase in R_n is first detected. Target cDNA copy number and C_T values are related inversely. A sample containing higher copies of the target cDNA will cross the threshold at an earlier cycle compared to a sample with lower copies of the same target. The copy number of TSV and YHV samples were determined by normalizing the C_T values of the samples with respect to EF-1α and then extrapolating the normalized C_T values to the standard curve of the corresponding virus.

For further statistical analyses, the C_T values were exported into a Microsoft Excel Worksheet. Regression analyses of the C_T values of the cDNA dilution series were used to determine the amplification efficiency for TSV and YHV compared to the corresponding EF-1α and β-actin controls.

3. Results

3.1. Analytical sensitivity of SYBR Green PCR using plasmid DNA template

The analytical sensitivity of SYBR Green PCR was determined by using a serial dilution of TSV and YHV plasmid DNA as template for amplification. Dilution series of plasmid standard contained $1.51–2.42 \times 10^6$ copies for TSV and $1.33–2.12 \times 10^6$ copies for YHV. A linear relationship between the input plasmid DNA and the C_T values with regression coefficient (r^2) greater than 0.99 were obtained for both the viruses. The mean C_T values of replicate assays ranged from 19.383 ± 0.267 (for 2.42×10^6 copies) to 40.0 ± 0.0 (for 1.51 copies) for TSV and 18.601 ± 0.169 (for 2.12×10^6 copies) to 38.944 ± 0.337 (for 1.33 copies) for YHV, respectively (Fig. 1, Table 3). The coefficient of variation was less than 4.0% for both TSV and YHV samples (Table 3).

3.2. Comparison of amplification efficiency of TSV and YHV with EF-1α and β-actin controls

To compare the amplification efficiency of TSV and YHV with the internal control genes, EF-1α and β-actin, a serial dilution of the cDNA derived from TSV and YHV infected samples were made. If the amplification efficiency of TSV and YHV with the corresponding internal controls, EF-1α and β-actin, is very similar then the difference in slope (Δs) of curves for the virus and the corresponding internal controls will approach to 0. The

Δs value of TSV and EF-1α was −0.139 and TSV and β-actin was −0.833 (Fig. 2A). The Δs value of YHV and EF-1α (+0.033) was closer than the Δs value of YHV and β-actin (+0.378) (Fig. 2B).

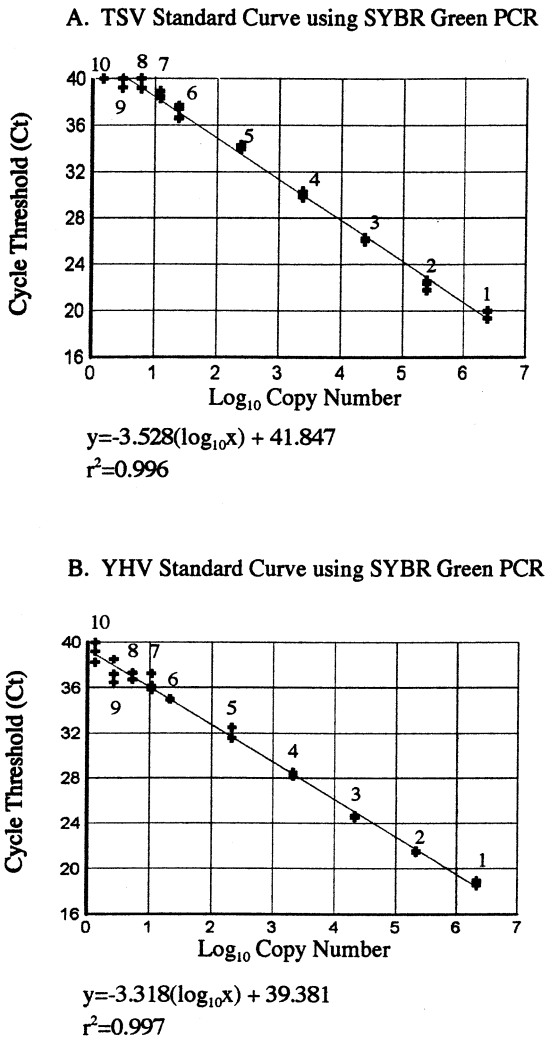

Fig. 1. The standard curve for TSV (A) and YHV (B) obtained by SYBR Green PCR using plasmid DNA as template. The number of copies of TSV plasmid DNA added to each reaction mixture (corresponding to the numbers on the linear curve in panel A) were as follows: (1) 2.42×10^6, (2) 2.42×10^5, (3) 2.42×10^4, (4) 2.42×10^3, (5) 2.42×10^2, (6) 2.42×10^1, (7) 12.1, (8) 6.05, (9) 3.03 and (10) 1.51. For YHV sample, the plasmid copy numbers (corresponding to the numbers on the linear curve in panel B) were as follows: (1) 2.12×10^6, (2) 2.12×10^5, (3) 2.12×10^4, (4) 2.12×10^3, (5) 2.12×10^2, (6) 2.12×10^1, (7) 10.6, (8) 5.3, (9) 2.6 and (10) 1.33.

To determine the sample to sample variation in the EF-1α and β-actin C_T values, SYBR Green RT-PCR was run for TSV, EF-1α and β-actin or YHV, EF-1α and β-actin in parallel in the same 96 well plate. For the TSV samples, the C_T values for EF-1α ranged from 21.502 to 25.023 and the C_T values of β-actin ranged from 19.437 to 25.955 (Table 4). For the YHV samples, the C_T values for both EF-1α and β-actin genes were quite variable although the variability for β-actin C_T values (20.418–29.405) were slightly higher than EF-1α C_T values (21.395–29.807) for the corresponding samples (Table 4).

3.3. Amplification specificity for TSV, YHV, EF-1α and β-actin genes

Since the SYBR Green RT-PCR does not involve any post-PCR analysis, amplification of specific vs. non-specific products was confirmed by analyzing the dissociation curve of the target amplicons. A dissociation curve with a single peak at temperature expected for that amplicon indicated specific amplification. The amplification profiles and the dissociation curves for TSV and YHV along with their corresponding internal controls (EF-1α and β-actin) are shown in Fig. 3 and Fig. 4. When amplification was undertaken with cDNA from TSV infected shrimp, a significant increase in SYBR Green fluorescence was recorded with a C_T value of 31.25 (Fig. 3A). Amplification using cDNA from healthy shrimp, did not provide any significant increase in fluorescence indicating absence of TSV specific target (Fig. 3A). The dissociation curves showed a single peak at melting temperature ($T_m = 72.0$ °C) expected for the TSV amplicon only in the TSV infected, but not in the healthy sample (Fig. 3B). However, both healthy and the TSV infected sample provided successful amplification of EF-1α and β-actin genes (Fig. 3C and E) with a single peak at expected melting temperature ($T_m =$ 79.2 °C for EF-1α and $T_m = 78.8$ °C for β-actin) (Fig. 3D and F).

For YHV sample, only the cDNA from infected but not healthy animals provided the am-

Table 3
The cycle threshold (C_T) values of replicate assays for TSV and YHV plasmid DNA dilutions

Plasmid standards	C_T values[a]			Mean	SD[b]	CV[c]
	Expt. 1	Expt. 2	Expt. 3			
TSV plasmid copy no.						
2.42×10^6	19.383	19.117	19.650	19.383	0.267	1.376
2.42×10^5	23.403	22.703	22.237	22.781	0.587	2.578
2.42×10^4	27.130	26.840	26.103	26.691	0.529	1.983
2.42×10^3	30.737	30.557	30.040	30.444	0.362	1.188
2.42×10^2	34.443	33.777	34.153	34.124	0.334	0.980
2.42×10^1	35.747	38.220	37.237	37.068	1.245	3.359
12.1	36.703	38.543	38.580	37.942	1.073	2.828
6.05	37.017	39.447	39.727	38.730	1.490	3.848
3.03	39.580	39.713	39.747	39.680	0.088	0.222
1.51	40.000	40.000	40.000	40.000	0.000	0.000
YHV plasmid copy no.						
2.12×10^6	18.503	18.503	18.797	18.601	0.169	0.910
2.12×10^5	21.520	21.520	21.707	21.582	0.108	0.499
2.12×10^4	24.640	24.757	24.873	24.757	0.117	0.471
212×10^3	28.323	28.263	28.263	28.283	0.035	0.122
2.12×10^2	32.083	31.750	32.197	32.010	0.232	0.725
2.12×10^1	34.727	35.557	35.443	35.242	0.450	1.277
10.6	36.443	36.470	36.467	36.460	0.015	0.040
5.3	38.033	38.100	38.813	38.316	0.432	1.128
2.6	37.383	38.417	39.510	38.437	1.063	2.767
1.33	39.113	38.557	39.163	38.944	0.337	0.865

[a] The C_T value is the average of 3 replicates of SYBR Green PCR run of that experiment.
[b] Standard deviation.
[c] Coefficient of variation.

plification of virus-specific product (Fig. 4A). The dissociation curves indicated that the amplicon had melting temperature ($T_m = 79.2$ °C) as expected for the YHV specific product (Fig. 4B). However, both healthy and infected samples provided successful amplification of EF-1α and β-actin genes (Fig. 4C and E) with each dissociation curve showing a single peak at the expected melting temperature for EF-1α and β-actin (Fig. 4D and F).

3.4. Reproducibility of the SYBR Green assay

To assess the reproducibility of SYBR Green assays, amplifications were carried out independently on different days. In a 96 well plate, each sample had 3–4 replicates. The coefficient of variation for the C_T values of TSV, YHV, EF-1α and β-actin genes were less than 5.0% indicating that the assay was highly reproducible (Table 4).

3.5. Determining the load of TSV and YHV in laboratory challenged shrimp by SYBR Green RT-PCR

The TSV and YHV viral load in the laboratory-challenged shrimp was determined by normalizing the C_T values of the virus with EF-1α C_T values and then extrapolating the normalized C_T values of the samples to the standard curves of the corresponding virus. The TSV load in *P. vannamei* (Kona stock) varied from 54 to 6745 copies/μg of total RNA and the TSV load in Super Shrimp *P. stylirostris* was 5–135 copies/μg of total RNA. The YHV load in the Super Shrimp *P. stylirostris* varied from 4.5×10^3 to 2.89×10^5 copies/μg of

total RNA. This indicated that Super Shrimp *P. stylirostris* has greater resistance to TSV compared to *P. vannamei* (Kona stock) but it is highly susceptible to YHV.

4. Discussion

The shrimp aquaculture industry has expanded rapidly over the last three decades. This has coincided with the emergence of new viral pathogens that were unknown previously to shrimp farming. In addition, there have been considerable movements of live and frozen shrimp from one country to another increasing the risk of spread of diseases into naive populations (Lightner et al., 1996). For example, until 1998 the geographic distribution of TSV was restricted to the Americas. From late 1998 to early 1999, TSV epizootics were recorded in Taiwan that was attributed to the introduction of TSV contaminated postlarvae and spawners from Ecuador and elsewhere in the Latin America to Taiwan (Tu et al., 1999). To prevent the spread of viral epizootics and to monitor the movement of live and frozen shrimp among countries and continents, there is a growing and urgent need to develop rapid and highly sensitive detection methods.

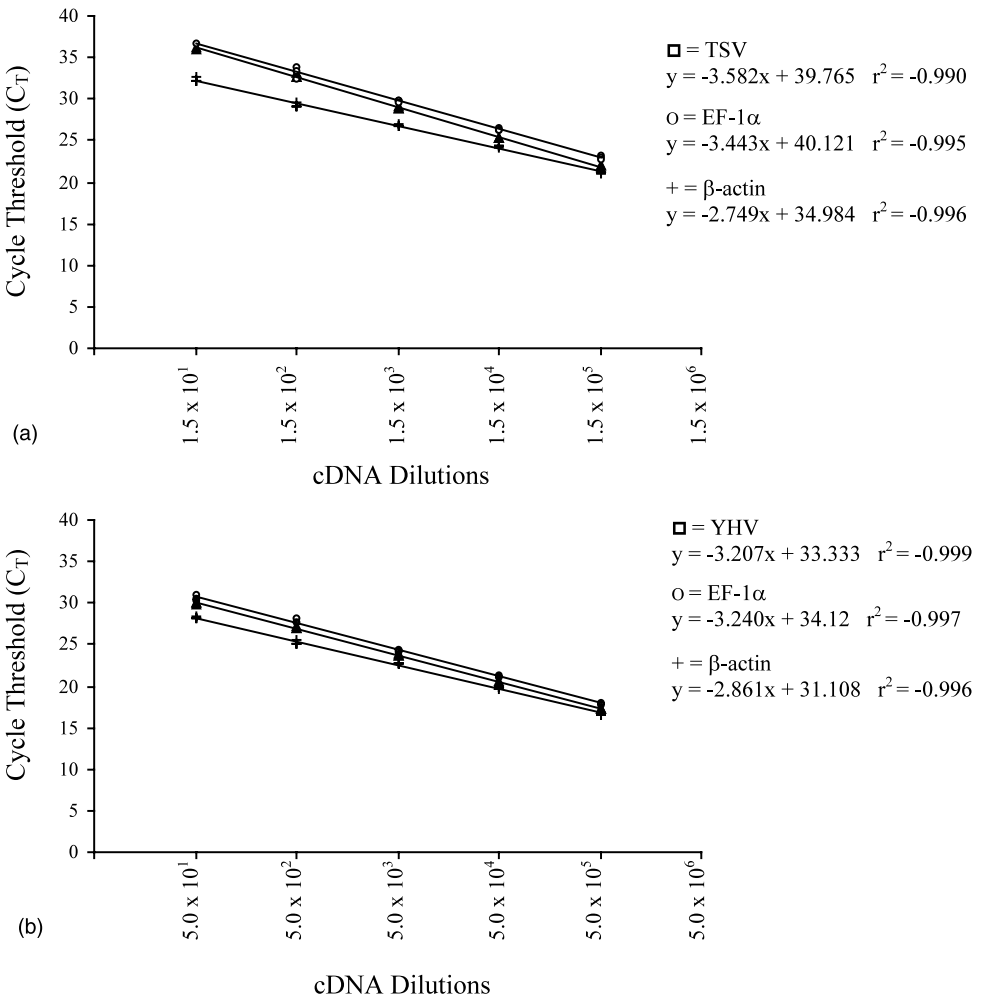

Fig. 2. Relative amplification efficiency curves for (A) TSV and (B) YHV compared to EF-1α and β-actin using cDNA as template.

Table 4
Inter-experimental variabilities in the cycle threshold (C_T) values of TSV and YHV samples and their corresponding internal controls (EF-1α and β-actin) using cDNA as template

Virus	cDNA samples	C_T values[a]			Mean	SD[b]	CV[c]
		Expt. 1	Expt. 2	Expt. 3			
TSV	*P. vannamei* (Kona) stock						
	K1	28.440	30.490	29.280	29.403	1.031	3.505
	K2	28.475	29.210	29.120	28.935	0.401	1.386
	K3	33.355	33.940	33.210	33.502	0.386	1.154
	K4	24.135	26.005	24.965	25.035	0.937	3.743
	K5	33.775	33.930	33.585	33.763	0.173	0.512
	K6	26.900	28.395	28.160	27.818	0.804	2.890
	K7	35.365	36.465	35.460	35.763	0.610	1.704
	K8	27.795	28.535	28.485	28.272	0.414	1.463
	K9	26.535	26.600	26.350	26.495	0.130	0.490
	K10	28.985	31.415	30.725	30.375	1.252	4.123
	EF-1α						
	K1	23.655	25.495	24.565	24.572	0.920	3.744
	K2	21.860	23.105	23.045	22.670	0.702	3.097
	K3	23.275	24.290	23.850	23.805	0.509	2.138
	K4	21.535	23.765	22.615	22.638	1.115	4.926
	K5	20.950	21.690	21.865	21.502	0.486	2.259
	K6	21.220	22.695	22.285	22.067	0.761	3.450
	K7	22.135	23.640	23.265	23.013	0.783	3.404
	K8	21.270	22.280	22.345	21.965	0.603	2.744
	K9	21.960	22.940	22.870	22.590	0.547	2.420
	K10	24.125	25.505	25.440	25.023	0.779	3.112
	β-Actin						
	K1	24.130	23.715	23.875	23.907	0.209	0.876
	K2	22.440	22.255	22.355	22.350	0.093	0.414
	K3	26.090	25.930	25.845	25.955	0.124	0.479
	K4	27.590	21.065	20.260	22.972	4.020	3.350
	K5	23.870	24.035	23.880	23.928	0.093	0.387
	K6	19.760	19.380	19.170	19.437	0.299	1.539
	K7	21.400	21.090	21.290	21.260	0.157	0.739
	K8	20.115	19.440	20.075	19.877	0.379	1.905
	K9	20.765	20.270	20.495	20.510	0.248	1.208
	K10	21.385	NT	20.975	21.180	0.290	1.369
YHV	Super Shrimp (SS) *P. stylirostris*						
	SS1	20.635	21.330	20.865	20.943	0.354	1.691
	SS2	28.780	27.110	27.305	27.732	0.913	3.293
	SS3	24.725	24.015	23.270	24.003	0.728	3.031
	SS4	20.535	20.780	20.810	20.708	0.151	0.728
	SS5	26.575	25.500	26.770	26.282	0.684	2.602
	SS6	26.300	27.455	25.530	26.428	0.969	3.666
	SS7	22.430	22.905	22.075	22.470	0.416	1.853
	SS8	24.805	25.270	25.020	25.032	0.233	0.930
	SS9	26.135	26.170	25.805	26.037	0.201	0.773
	SS10	25.505	25.095	25.040	25.213	0.254	1.008
	EF-1α						
	SS1	22.395	22.330	22.580	22.435	0.130	0.578
	SS2	26.415	25.065	26.405	25.962	0.777	2.991
	SS3	21.650	21.650	21.360	21.553	0.167	0.777

Table 4 (*Continued*)

Virus	cDNA samples	C_T values[a]			Mean	SD[b]	CV[c]
		Expt. 1	Expt. 2	Expt. 3			
	SS4	21.165	21.510	21.510	21.395	0.199	0.931
	SS5	29.275	30.040	30.105	29.807	0.462	1.549
	SS6	28.195	26.240	27.270	27.235	0.978	3.591
	SS7	25.020	24.220	25.150	24.797	0.504	2.031
	SS8	24.800	25.125	25.215	25.047	0.218	0.872
	SS9	24.865	24.030	24.920	24.605	0.499	2.027
	SS10	24.150	25.865	25.040	25.018	0.858	3.428
	β-Actin						
	SS1	22.055	23.180	NT	22.618	0.795	3.517
	SS2	30.430	28.460	28.865	29.252	1.040	3.557
	SS3	20.325	20.935	20.655	20.638	0.305	1.479
	SS4	19.855	20.700	20.700	20.418	0.488	2.389
	SS5	29.020	29.255	29.940	29.405	0.360	1.225
	SS6	27.390	28.185	26.210	27.262	0.994	3.645
	SS7	25.345	26.055	25.085	25.495	0.502	1.969
	SS8	26.750	27.290	27.020	27.020	0.270	0.999
	SS9	25.250	25.730	24.710	25.230	0.510	2.023
	SS10	26.325	27.930	27.790	27.348	0.889	3.251

NT, not tested.

[a] For each sample, the C_T value is the average of 2–3 replicates of SYBR Green PCR run of that day.

[b] Standard deviation.

[c] Coefficient of variation.

The real-time RT-PCR described here is highly sensitive. It is capable of detecting up to a single copy equivalent of the TSV or the YHV genome (Fig. 1). In SYBR Green RT-PCR, it takes 40 cycles ($C_T = 40$) to detect a single copy of a viral genome (Perkin Elmer User Manual, GeneAmp® 5700 Sequence Detection System, User Manual, 1998). A linear relationship between the input plasmid DNA and the C_T values was observed from 10^6 down to a single copy of both TSV and YHV. Detection of viruses over such a large dynamic range is useful for measuring the viral load in animals with different levels of infection. Thus SYBR Green RT-PCR provides a continuous scale for measuring the viral load. In addition, since SYBR Green RT-PCR is capable of detecting a single copy of viral genome, it will be useful to detect sub-clinical infections. Due to exquisite sensitivity of SYBR Green PCR, it is highly susceptible to PCR carry over or other contamination. Therefore, laboratory hygiene practices should be followed very strictly to prevent any potential contamination that may give false positive result. In addition, any negative result as well as samples with C_T values close to 40 should be tested at least twice for confirmation.

The SYBR Green RT-PCR was not only highly sensitive but also very specific for detecting TSV, YHV and the internal control genes, EF-1α and β-actin. The specificity of SYBR Green RT-PCR was determined by monitoring the amplification profile and the dissociation curve of the target amplicons. In SYBR Green RT-PCR, a sample is considered positive when the amplification plot crosses the threshold value. For example, in Fig. 3A the amplification plot of TSV infected sample exceeds the threshold value at cycle number 31.45 whereas the amplification plot of the healthy sample did not exceed the threshold line. To ensure that the amplification plot obtained for TSV infected sample was indeed due to the amplification of TSV specific product, the dissociation curve of the product was analyzed (Fig. 3B). Since the dissociation curve of a product depends on its GC

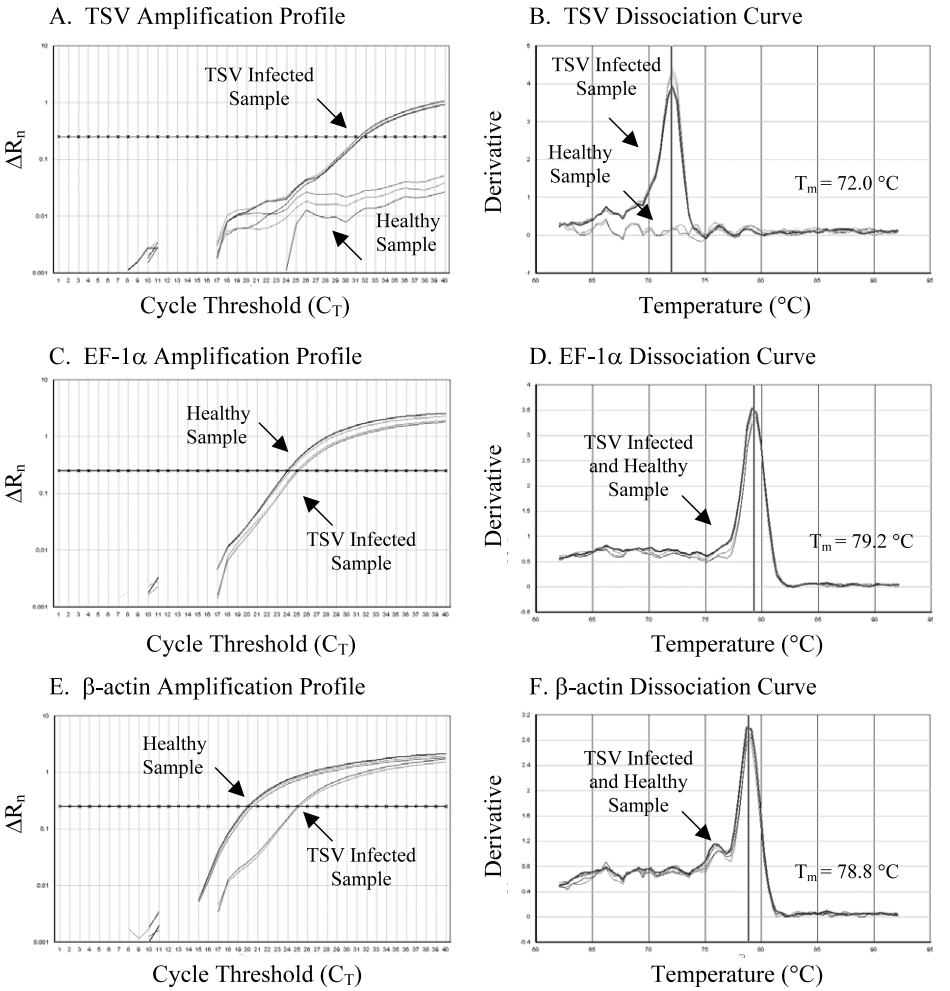

Fig. 3. The amplification plots and the corresponding dissociation curves of TSV, EF-1α and β-actin genes. The melting temperature (T_m) of each amplicon is shown alongside its dissociation curve.

content, length and sequence composition; amplification of a specific versus non-specific product could be differentiated by examining the dissociation curve. The TSV amplicon provided a dissociation curve with a single peak at 72.0 °C which is expected for the TSV specific amplicon. To determine the quality and any variation in the amount of input RNA as well as the efficiency of the reverse transcriptase reaction in both healthy and TSV infected samples, EF-1α and β-actin genes were amplified in parallel to the target virus. Both healthy and TSV infected samples provided successful amplification of EF-1α and β-actin genes with the dissociation curve showing a single peak at the expected temperature (T_m for EF-1α was 79.2 °C and T_m for β-actin was 78.8 °C, Fig. 3C–F). Similar observations were recorded for YHV amplicon and the EF-1α and β-actin controls for the corresponding samples (Fig. 4A–F).

In addition to sensitivity and specificity, SYBR Green RT-PCR is very rapid and robust in nature. It takes about 2 h to run a 96 well plate from the time the plate is put into the instrument. After amplification, the data analysis takes a few minutes. In a 96 well plate, 22 samples can be run at a time with two replicates for each virus sample,

internal control, positive control and negative control. Thus, SYBR Green RT-PCR can be used for high throughput assays for YHV and TSV detection. In recent years, real-time RT-PCR based on TaqMan chemistry, has been used for the detection of RNA viruses infecting plants (Roberts et al., 2000), animals (Moody et al., 2000; Komurian-Pradel et al., 2001; Oleksiewicz et al., 2001) and to quantitate cellular transcripts in yeast and mammals (Kang et al., 2000; Leutenegger et al., 1999; Schmittgen et al., 2000). Schmittgen et al. (2000) compared the endpoint RT-PCR to TaqMan and SYBR Green real-time RT-PCR to evaluate the time course of mRNA

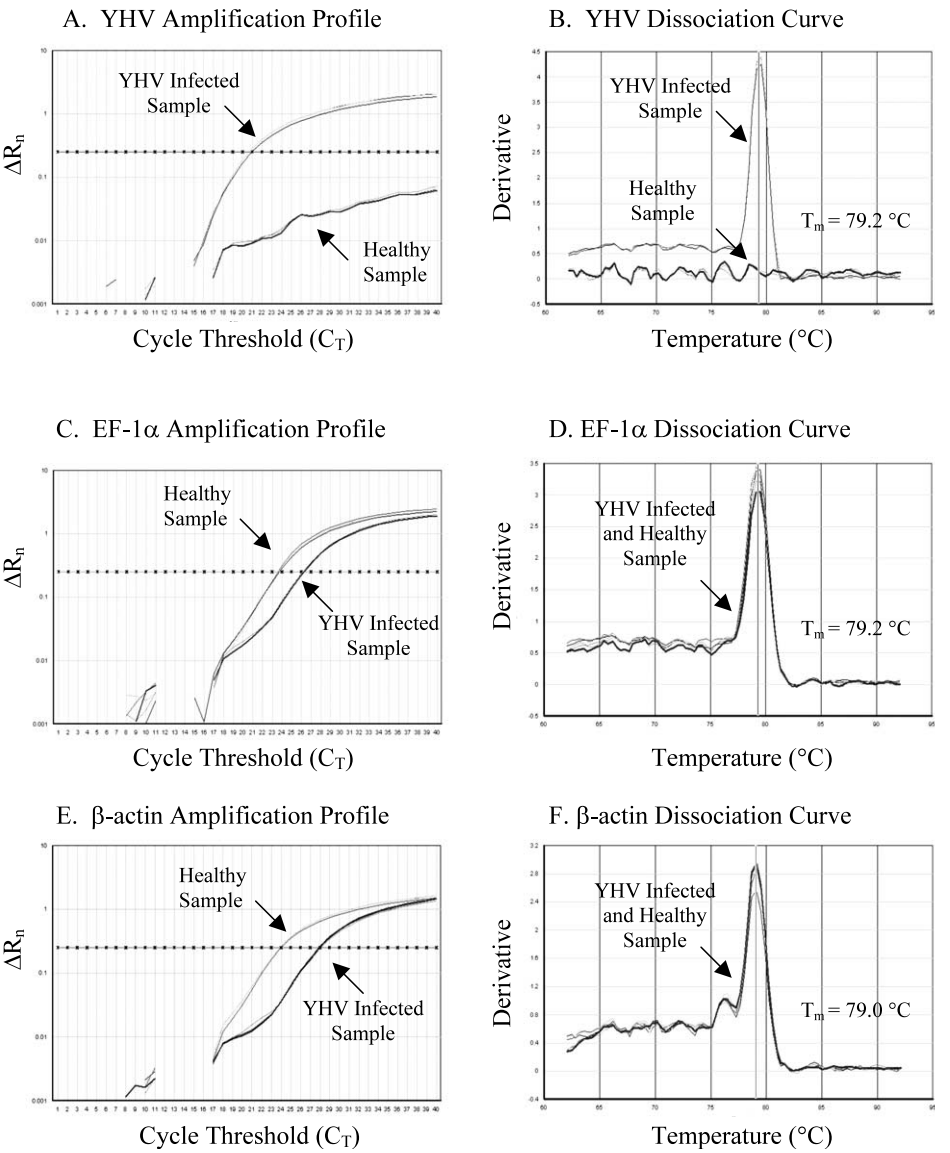

Fig. 4. The amplification plots and the dissociation curves of YHV, EF-1α and β-actin genes. The melting temperature (T_m) of each amplicon is shown alongside its dissociation curve.

formation and decay of human chimeric β globin gene. Both real-time RT-PCR methods produced a 4- to 5-log dynamic range of amplification compared to 1-log dynamic range for endpoint RT-PCR. They reported that although both real-time RT-PCR methods provided comparable dynamic range and sensitivity, SYBR Green detection was more precise and produced a more linear decay plot than the TaqMan detection system. In contrast to SYBR Green detection, multiple fluorogenic probes can be used in a TaqMan assay to detect more than one target in a reaction. However, TaqMan assay is more costly than SYBR Green assay.

Recently, we isolated EF-1α by mRNA differential technique while comparing the RNA fingerprints of healthy and WSSV, a double stranded DNA containing virus, infected shrimp (Dhar et al., 2001b). The EF-1α was expressed constitutively in both healthy and WSSV infected shrimp. We compared the amplification efficiency and sensitivity of EF-1α and β-actin to that of TSV and YHV to determine which of these two genes could serve as a better internal reference. Ideally, the amplification efficiency as well as the sensitivity of an internal control should be comparable to the RNA under study and the internal control should be expressed at an equivalent level irrespective of tissue used, stages of development and the experimental treatments (Bustin, 2000). The sensitivity (Y intercepts) and amplification efficiency (slope) of EF-1α was greater than β-actin when compared to both TSV and YHV amplification. For example, the sensitivity (Y intercept 39.765) and amplification efficiency (slope -3.582) of TSV was more similar to EF-1α (Y intercepts 40.121, slope -3.443) than β-actin (Y intercepts 34.984 and slope -2.749) (Fig. 2A). Similarly, the slope and the intercepts of YHV curve were more similar to the EF-1α than β-actin (Fig. 2B). There is considerable evidence that the β-actin transcription varies widely in response to experimental treatment in human breast epithelial cells, porcine tissues and canine myocardium (reviewed in Bustin, 2000). In the current study, Both EF-1α and β-actin showed variation in their level of expression in TSV and YHV infected samples. However, based on the sensitivity and the amplification efficiency, as well as the level of variation, EF-1α appeared to be a better internal reference for SYBR Green RT-PCR detection of TSV and YHV.

One of the obstacles in the development of virus resistant lines in shrimp is the lack of method(s) for quantification of viruses. Lack of established crustacean cell lines further emphasizes the need to develop methods for virus quantitation. Recently, we have developed a real-time PCR assay based on SYBR Green chemistry for the detection and quantification of two penaeid DNA viruses, infectious hypodermal and haematopoietic necrosis virus (IHHNV) and WSSV (Dhar et al., 2001a). We used the SYBR Green PCR method, along with random amplified polymorphic DNA (RAPD) technique, to identify genetic markers in *P. stylirostris* shrimp populations that differ in their IHHNV load (Hizer et al., 2002). Thus, SYBR Green RT-PCR, along with other molecular techniques, will be useful for developing TSV and YHV resistant lines in shrimp.

In summary, the SYBR Green RT-PCR method described above is a major development in the detection and quantification of TSV and YHV in shrimp. The method is very rapid, highly sensitive and is applicable to routine high throughput assay making it a suitable tool for diagnostic, epidemiological and genetic studies in shrimp aquaculture.

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