รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาเพื่อเพิ่มความต้านทานต่อโรค Taura syndrome ในกุ้งด้วยเทคนิค RNAi

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สัญญาเลขที่ RSA4780026

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ผู้วิจัยและคณะขอขอบคุณหลายๆฝ่ายที่มีส่วนช่วยให้การทำงานวิจัยชิ้นนี้สำเร็จลงได้แม้ไม่ครบถ้วนตามทีหวังไว้แต่ก็อยู่ในระดับที่น่าพอใจ ขอขอบคุณศาสตราจารย์เกียรติคุณสกล พันธุ์ยิ้ม ผู้ทำหน้าที่คอยให้คำแนะนำ ติชม ต่างๆที่มีประโยชน์ต่องานวิจัยนี้อย่างเต็มที่ นอกจากนี้ความช่วยเหลืออย่างดียิ่งที่ผู้วิจัยและคณะได้รับจากเจ้าหน้าที่ทุกฝ่ายของสถาบันอณูชีววิทยาและพันธุศาสตร์ มหาวิทยาลัยมหิดล ที่มีส่วนช่วยทำให้การทำงานวิจัยมีความสะดวกขึ้นมาก ท้ายที่สุดผู้วิจัยขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ให้ทุนสนับสนุนงานวิจัยชิ้นนี้ ตลอดจนเจ้าหน้าที่ของสกว.ที่ให้ความช่วยเหลือเป็นอย่างดีตลอดมา

บทคัดย่อ

กุ้งขาว (*Penaeus vannamei*) เป็นกุ้งทะเลที่มีความสำคัญในอุตสาหกรรมเพาะเลี้ยงสัตว์น้ำของประเทศไทย โรคติดเชื้อที่สำคัญของกุ้งขาวแปซิฟิกคือโรคจากไวรัสทอร่าสร้างความเสียหายอย่างมากต่ออุตสาหกรรมการเลี้ยงกุ้งชนิดนี้ ในปัจจุบันนี้ยังไม่พบวิธีการรักษาหรือวัคซีนสำหรับโรคติดเชื้อไวรัสทอร่าในกุ้งทะเล

ในการศึกษาครั้งนี้ได้แยกเชื้อไวรัสทอร่าจากตัวอย่างกุ้งจากแหล่งเพาะเลี้ยงกุ้งขาว 3 แห่งในประเทศไทย การวิเคราะห์ลำดับเบสโนในบริเวณที่มีการสร้างโปรตีน (open reading frame) ของยีนที่ 1 (ORF1) ของไวรัสทอร่าแสดงให้เห็นว่าในบริเวณนี้ของจีโนมไวรัสมีการอนุรักษ์สูง (conserved region) มาก ลำดับเบสใน ORF1 บางส่วนถูกเลือกเป็นบริเวณเป้าหมายสำหรับศึกษาการยับยั้งการเพิ่มจำนวนของเชื้อไวรัสโดยเทคนิคกระบวนการยับยั้งการแสดงออกของยีนที่เรียกว่า RNA interference (RNAi) เพื่อศึกษาถึงความเป็นไปได้ในการประยุกต์ใช้กลไกนี้ป้องกันโรคติดเชื้อจากไวรัสทอร่าในกุ้งขาวแปซิฟิกโดยการฉีดอาร์เอ็นเอสายคู่ที่มีลำดับเบสจำเพาะกับยีนของไวรัสทอร่า (TSV-specific dsRNA) เข้าสู่ตัวกุ้งจากนั้นติดตามว่าสามารถยับยั้งการเพิ่มจำนวนของเชื้อไวรัส ซึ่งพบว่ากุ้งที่ได้รับอาร์เอ็นเอสายคู่ที่มีลำดับจำเพาะกับยีน helicase, protease และ polymerase ก่อนได้รับเชื้อไวรัส การติดเชื้อไวรัสทอร่าในกุ้งกลุ่มนี้ลดลงเมื่อเทียบกับกุ้งที่ไม่ได้รับอาร์เอ็นเอสายคู่ โดยอาร์เอ็นเอสายคู่ที่จำเพาะกับยีน helicase ของไวรัสพบมีสามารถในการยับยั้งการเพิ่มจำนวนของเชื้อไวรัสมากที่สุด ขณะที่อาร์เอ็นเอสายคู่ที่มีลำดับเบสจำเพาะกับยีน polymerase นั้นแสดงความสามารถในการยับยั้งการเพิ่มจำนวนของเชื้อไวรัสได้น้อยที่สุด จากผลการทดลองแสดงให้เห็นว่า อาร์เอ็นเอสายคู่ที่มีลำดับเบสจำเพาะกับยีนของไวรัสทอร่าสามารถยับยั้งการเพิ่มจำนวนของเชื้อไวรัสได้ในกุ้งที่ติดเชื้อไวรัสทอร่าได้

Abstract

Pacific white shrimp (*Penaeus vannamei*) is an economically important farmed penaeid shrimp species in Thailand. One of the most important diseases of the white shrimp is caused by Taura syndrome virus (TSV), which has recently affected shrimp cultivation throughout the world. At present, there is no known treatment or vaccine available for this viral disease.

TSV were isolated from three different cultivation areas of Thailand. Analysis of nucleotide and deduced amino acid sequences in open reading frame 1 (ORF1) of the TSV revealed that this region of the viral genome is highly conserved with overall identity. Subregions of the ORF1 (helicase, protease and polymerase) were chosen as targets for RNAi mediated viral suppression study. The feasibility of RNAi-based technique to prevent TSV infection was assessed in the pacific white shrimp. Shrimps were injected with TSV specific dsRNAs, followed by virus infection. Shrimp treated with specific dsRNA targeting the nonstructural gene of TSV (ORF1) showed a reduction in the viral level as compared with untreated shrimp. The dsRNA targeting helicase domain exhibited the strongest inhibitory effect while the domain targeting polymerase exhibited the least effect. These results suggested that virus specific dsRNAs could suppress TSV replication in white shrimp.

Introduction

Shrimp is an aquatic animal with high economic value that earns many countries with billions of foreign currency from exportation. Although there are hundreds of marine shrimp species, most important farmed shrimps are about dozen species in Penaeidae family. Of these species, *Penaeus monodon*, *Penaeus vannamei* and *Penaeus chinensis* are the most widely cultured shrimp (1-3).

P. vannamei (also known as *Litopenaeus vannamei*) is a scientific name of pacific white shrimp which is native *spp.* to the western Pacific coast of Latin America. Following the incidence of sustained outbreak of viral disease in black tiger shrimp (*P. monodon*) in East and Southeast Asia, most farmed shrimp shifted towards *P. vannamei* (4). To date, *P. vannamei* has become the most important farmed shrimp in many countries including Thailand. Compared to *P. monodon*, *P. vannamei* is easier to culture in a higher populated pond and able tolerate to a wide range of salinities. More importantly certified Specific Pathogen Free (SPF) of domesticated broodstock of this species has been developed to assure the production of disease free postlavae. Despite this safeguard, a huge demand for postlarvae from shrimp farming industry lead to illegally imported of non SPF broodstock into Thailand.

Taura syndrome disease in *Penaeus vannamei*

Infectious diseases have long negative impact on *P. vannamei* shrimp farming industry affecting shrimp survival and growth thus lead to significant economic losses. Number of infectious agents was reported to cause infection and mortality in the shrimps including protozoa, fungi, bacteria, however viral diseases are among the most important.

Taura syndrome or TS was first recognized in *P. vannamei* shrimp in 1992 and became the biggest problem for *P. vannamei* cultivation as they can cause mass shrimps mortality (varies from 5 to more than 95%) (5, 6). The causative agent is named Taura syndrome virus (TSV) is principally targets the

tissue just underneath the shell, the cuticle epidermis of the infected shrimp. TS could be divided into acute and chronic phases. The acute phase shrimps appear weak, have a soft shell, an empty digestive tract and may have diffuse expansion of the red chromatophores (pigment spot) in the appendages especially uropod. The shrimp typically die during the molting process (7). Individuals survive from acute phase progress into the chronic phase and show sign of recovery. Shrimp in the chronic phase has scattered, pitted, melanized (black spot) lesions along their outer skin of shell. The shrimps in this stage are often asymptomatic carriers of TSV (8).

TSV has been classified in the family *Picornaviridae* based on its morphological characteristics. The study of the genome analysis suggested that it is a member of the genus *Cricket paralysis-like viruses*. TSV has a non-enveloped icosahedral particle with diameter of 31-32 nm. Its capsid comprised of three major (55, 40 and 24 kDa) and one minor (58 kDa) polypeptides. Its genome is a linear, positive-sense single stranded RNA of approximately 10.2 kb in length containing two large open reading frames (ORFs) separated by an intergenic region of 207 nucleotides (figure 1). The predicted amino acid sequence of ORF1 revealed sequence motifs characteristic of a helicase, a protease and an RNA-dependent RNA polymerase. Amino acid sequences on the N-terminus of all three TSV capsid proteins were mapped in the ORF2. Although many strategies have been attempted to control the disease, however, there is no specific treatments or vaccines has been proven for success.

RNA interference

In recent years, a large number of research works have described the RNA interference (RNAi) phenomenon in various organisms. Identifications of various components involved in silencing pathway by genetic mutant screens in plants and nematodes led to a model to explain how RNAi works (9) (Figure. 2). A simplified model for RNAi pathway is based on two steps. The initiation step, long dsRNAs enter the cell where they are cleaved by the RNase III-like

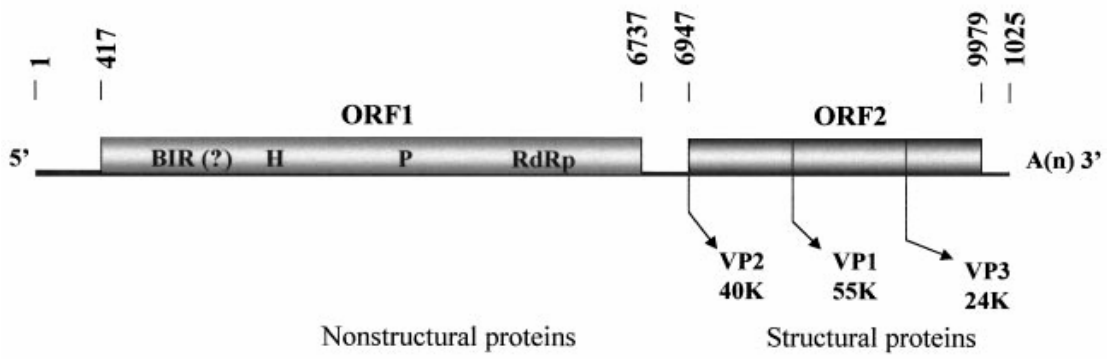

Figure 1: Schematic diagram of the genome of TSV (20)

The figure showed the gene organization of TSV RNA genome. Number indicates nucleotide positions. Open reading frames (ORFs) 1 and 2 are shown as open boxes and untranslated regions (UTRs) as a single line. The approximate positions of the BIR-like sequence (BIR), helicase (H), protease (P) and RNA-dependent RNA polymerase (RdRp) are indicated. Arrows represent the N-terminal of the capsid proteins.

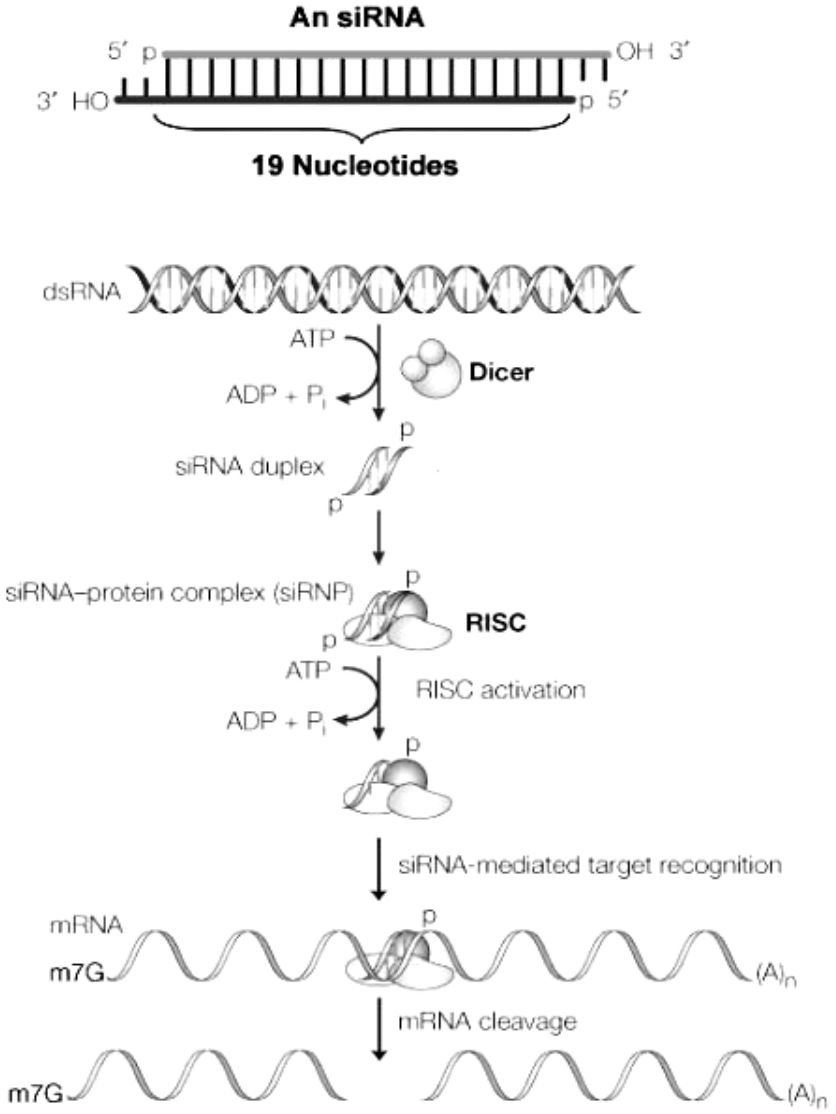

Figure 2: A model for RNAi mechanism (modified from 43)

First, the long dsRNAs get processed into 21-25 nucleotide (nt) small interfering RNA (siRNA) by RNase III-like enzyme called Dicer (initiation step). Then, the siRNAs assemble into multicomponent nuclease protein complex known as RNA induced silencing complex (RISC), unwinding the siRNA duplex. The antisense siRNA subsequently guide the RISC to target complementary mRNA molecule, where RISC cleave and destroy the full-length cognate mRNA (effector step).

enzyme Dicer to generate discrete 21 to 25 nucleotide small RNA fragments containing 2 nucleotides 3’ overhangs and 5’ phosphate group (10). These small RNA fragments are called small interfering RNA (siRNAs) (11,12). In the effector step, siRNAs are loaded into the multicomponent nuclease protein complex to form a RNA induced silencing complex (RISC) (13). The entire components of RISC have not yet been identified, but they might include an endonuclease, and exonuclease, a helicase and a homology searching activity. One of the protein components of this complex was identified as a member of the Argonaute gene family (14,15).

During RISC assembly, this complex might undergo activation in the presence of ATP so that the antisense strand of the unwound siRNA becomes exposed. This antisense siRNAs in the activated RISC subsequently guide the RISC to complementary mRNA targets by Watson-Crick base pairing. This interaction induced an endonuclease specific siRNA subsequently guide the RISC to target complementary mRNA to initiate its cleavage in the middle of mRNA sequence in duplex region by the action of RNaseH activity in Argonaute (16). Finally, RISC is liberated from the cleaved mRNA and is recycled for the next rounds of catalysis. This property of RISC is responsible for the potent nature of the silencing effect.

RNAi has been recognized as machinery involved in the control of developmental stages by posttranscriptional and transcriptional gene silencing mechanisms (17-19). RNAi also limits transposon mobilization, presumably by degrading transposon encoded mRNA and by ensuring and shaping chromosomal functions (20, 21). RNAi was mostly considered to be a cellular defense mechanism against incoming foreign and parasitic genetic information and against aberrant endogenous mRNA. It is now generally accepted that RNAi silencing is a major antiviral mechanism in plants (22). Furthermore, RNAi also contributes to antiviral defense in invertebrates. In this respect, RNAi can be observed as an innate antiviral response.

Given the sequence specific nature of RNAi, it provides a new idea that this technique will play an important role in medical applications such as the use of RNAi-based therapeutic strategy to target virus and viral gene expression. To date, RNAi has been used effectively to inhibit the replication of several viruses in cultured cell lines, including human immunodeficiency virus (HIV), respiratory syncytial virus (RSV), hepatitis C virus (HCV) and poliovirus (23-27). The inhibition of virus by RNAi required very high level of sequence homology since escape of resistant strain of poliovirus was appeared as a result of single mismatch between siRNA and targeted mRNA (24). Although single mismatches have been shown to render a siRNA ineffective, it may be tolerate by the use of multiple siRNAs targeting of multiple regions or regions that are very important for viral replication (26). Recently, the therapeutic potential of siRNA treatment has been demonstrated *in vivo* in mouse models infected with HCV (28).

Although much researches on RNAi remains to be intensively investigated, application of RNAi seems to play an important role in determining cellular gene function and shows a great deal of promise as a potential medical therapeutic agent.

Materials and methods

TSV specimens

TSV infected *Penaeus vannamei* was collected from shrimp farms in Chanthaburi, Rayong and Samut Sakorn provinces, Thailand. Samples were stored at -80 °C until used. To prepare viral stock used in the study, pacific white shrimp, *Penaeus vannamei*, infected with TSV was minced then grinded with mortar and pestle in 5 ml of TN buffer. The mixture prepared from the infected tissue was divided into several aliquots of equal volume. All aliqouts were centrifuged at 5000 x g for 10 min at 4 ºC and the supernatant containing virus from each aliquot was then carefully recovered so as not to disturb the pellet to a new tube. The supernatant was kept at -80 ºC as a viral stock.

Oligonucleotide primers

All primers used in this study listed in Table 2 were synthesized by PROLIGO Primers and Probes, Singapore.

Miscellaneous

TRI REAGENT®	Molecular Research Center
TRI REAGENT®-LS	Molecular Research Center
QIAquick Gel Extraction Kit	QIAGEN
QIAGEN Plasmid Maxi Kit	QIAGEN
RiboMAX™ Large Scale RNA Production Systems	Promega
Lambda DNA/*Hind* III markers	Promega
100 bp DNA Ladder	Invitrogen
1 Kb Plus DNA Ladder	Invitrogen

Table 1. Description of the primers used in this study

Name	Sequence 5'to 3'	T_m (ºC)
F1	GAA CCC CTG TTG CCG ACC GAG C	65
R1	CTC GAC TAC CAT CAC CAC ATG TCA G	57
F2	CAC GAC ACG GTA GAT GCT AAT GTG C	60
R2	GGC ATC AAC AAC ATT CGT CCA TAC GCG	64
F3	CCA GAC TAT CAG GTG GAG TGG ACC G	62
R3	CGA TGC AGA TGG GTA ACC AAT ACA TGG	63
F1.2	CAT ATC ACA CTA TGG GAG TTT CAG G	54
R1.2	CAG TGA TTC CAA GAC ACA TGA CTG C	57
F2.2	CAA AAG CCA AAT CCT GAG CTA TTC G	59
R2.2	GTA ACA CCT TCC GAG CGA TTT CGG	62
F3.2	GAC TTT GTA GCC AAG TAC ATT GCT GC	57
R3.2	GAG GAA GGT AAC CTC ATT TAA GGA GC	56
Fhel	GGA GGC ACG CAA TTC AGT TAA GG	59
Rhel	GAT GAC CCT TAG ATC CAG CTA CG	54
Fpro	GTA GAG TGG ACC GAT TTG AGA ACT G	55
Rpro	GTC ACG CGA CCT GCA TGA GTT GTC G	66
Fpoly	GTT TCT TGG ACC ATG TGA TGA CG	56
Rpoly	CTC CAC ATG CAC ATA TCT TCA ATC G	57

TSV-stem-Hel-PstI	GAA TCT GCA GGG CAC GCA ATT CAG TTA AGG	68
TSV-stem-Hel-SpeI	ATA TAC TAG TGG ATT TAC GTT TGA GGT TGC	57
TSV-stem-Poly-PstI	AAT TCT GCA GGA CCA TGT GAT GAC GAA CAG	66
TSV-stem-Poly-SpeI	GGG TAC TAG TAT GCT GGT TGA ACC ATT CAC	61
Pv actinF	GAC TGC TAC GTC GGC GAC GAG G	63
Pv actinR	GCA CGG TGG TCA TCT CCT GCT CG	68
YHV-Hel-Sense-1	CAA GGA CCA CCT GGT ACC GGT AAG AC	62
YHV-Hel-Anti-1	GCG GAA ACG ACT GAC GGC TAC ATT CAC	66
RR1 sense	AAC TCG GTA CCC GGT CCA CCC TCG GAA CTT G	73
RR1 antisense	AAT CCA AGC TTT ATT TTC CTA TAC GTC TTC TCGG	64

Plasmid vectors

pGEM®-Teasy vector (Promega, USA) is used for cloning of PCR products at A overhang. pLITMUS™ 28i plasmid vector (NEB Inc., USA) designed for efficient transcription of double stranded RNA using T7 RNA polymerase flanked on both ends of the multiple cloning sites.

Total RNA isolation from shrimp tissue

Total RNA was isolated from gill of TSV infected shrimps. The sample was ground in TN buffer (0.4 M NaCl, 0.01 M Tris-HCl pH 7.4) in liquid nitrogen as described by Bonami *et al*. (6). Tissue debris was eliminated by centrifugation at 5000 x g for 10 min at 4 ºC, 250 µl of supernatant was mixed in 750 µl of TRI REAGENT®-LS (Molecular Research Center, Inc., USA). The homogenate was added with 200 µl of chloroform then vigorously shaking and centrifuged at 12,000 x g for 15 min to separate RNA into the aqueous phase. Subsequently, RNA was precipitated with 500 µl of isopropanol and washed with 1 ml of ice-cold 75% ethanol. The RNA pellet was dissolved in 10 µl DEPC (Diethylpyrocarbonate) treated water and stored at -80 ºC.

Reverse Transcription-Polymerase Chain Reaction

1 µg of total RNA and 1 µl of 10 µM specific antisense primer in 5 µl volume (Table 1) was heated at 80 ºC for 3 min then directly cooled down on ice. The mixture of 4.1 µl of DEPC treated water, 4 µl of ImProm-II™ 5X Reaction buffer (Promega, USA), 2.4 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs mix, 0.5 µl of Recombinant RNasin® Ribonuclease Inhibitor and 1 µl of ImProm-II™ Reverse Transcriptase (Promega, USA) was added. The reverse transcription (RT) reaction was carried out at 25 ºC for 5 min, followed by 42 ºC for 60 min. The reaction was terminated by incubation at 70 ºC for 15 min.

Two microliters of the first stranded cDNAs were added to PCR reaction composing of 15.5 µl of DEPC treated water, 2.5 µl of 10X thermophilic polymerase reaction buffer (Promega, USA), 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs mixture, 0.5 µl of 10 µM F2 primer, 0.5 µl of 10 µM R1 primer, 0.5 µl of 10 µM YHV-Hel-Sense-1 primer, 0.5 µl of 10 µM YHV-Hel-Anti-1 primer (Table 1), and 0.5 µl of *Taq* polymerase (Promega, USA) for TSV and YHV detection. PCR amplification for 35 cycles was done at 94 ºC denaturation for 30 s, annealing at 55 ºC for 15 s and extension at 72 ºC for 45 s.

10 µl of the amplicons were resolved in 1.2% TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0) agarose gel. The gel was stained with ethidium bromide and visualized by UV transilluminator.

Production of anti-TSV structural protein polyclonal antibodies

To produce specific antibodies to TSV particle, two regions of TSV structural genes corresponding to VP1 and VP2 were PCR amplified and subcloned into pGEX-5X-1 expression plasmid in the same translation frame of Glutathion S Transferase. After transforming into *E. coli*, GST-VP1 and GST-VP2 fusion proteins were produced in the soluble fraction with the expected molecular weight upon IPTG induction. Individual fusion protein was partial purified by affinity binding to Glutathione agarose bead and subjected to immunizing into BALB/C mice.

The fusion protein was expressed in the cells when its optical density at 600 nm reach 0.3 by addition of IPTG to final concentration of 0.1 µM. Total protein was harvested after 6 hr IPTG induction and purified as described by Frangioni and Neel (1993) except that the induction was performed at 30^{o}C. 40 µg of purified GST-VP1 or GST-VP2 fusion proteins was mixed with freund's adjuvant and repeatedly injected into femaled BALB/C mice as described by Harlow and Lane (1988). Sera collected from tail bleed were used for determining the titer by Western blot analysis. When optimal titer was obtained, the mice were injected with the sarcoma cell line S180 (10^6 cells) to induce ascites fluid (Harlow and Lane 1988). Antibodies in the ascites were collected by centrifugation to eliminate the cells. The antibodies were aliqouted and stored at -80^{o}C until use.

Construction of recombinant plasmid for dsRNA synthesis

The template for dsRNA was designed based on the TSV sequence of Thai isolates to target three regions of the helicase, protease and polymerase within the ORF1 of TSV. To generate recombinant plasmids for conventional dsRNA

synthesis, three primer pairs (Fhel/Rhel, Fpro/Rpro, and Fpoly/Rpoly; Table 1) were used to PCR amplify DNA fragments using the $Vent_R$®DNA polymerase. After amplification, DNA fragments were gel purified and cloned into pGEM®T-easy vector. To facilitates *In vitro* transcription, DNA fragments from pGEM®T-easy vector were cut with *Eco*RI restriction enzyme and subcloned into LITMUS 28i vector at the same site (Figure. 3).

For stem loop dsRNAs, two primer pairs (TSV-stem-Hel-PstI/TSV-stem-Hel-SpeI and TSV-stem-Poly-PstI/TSV-stem-Poly-SpeI; (Table 1) were synthesized to amplify additional DNA fragments of helicase and polymerase, repectively. The DNA fragments were then cloned into *Spe*I and *Sal*I restriction site of previous pGEM®T-easy vector containing corresponding DNA fragment in antisense direction (Figure. 4). The recombinant plasmids with designated insert were purified by using QIAGEN Plasmid Maxi Kit. The DNA pellets were dissolved in 300 µl of sterile milli-Q water prior determining thier concentration by spectrophotometer.

Production dsRNA using *in vitro* transcription

The recombinant plasmids used as DNA templates were linearized with restriction enzymes to generate 5'-overhang. Sense and antisense strands were transcribed *in vitro* by using RiboMAX™ Large Scale RNA Production Systems (Promega, USA) in 20 µl reaction containing 1-2 µg of linear DNA template, 1X T7 transcription buffer (80 mM HEPES-KOH pH 7.5, 24 mM $MgCl_2$, 40 mM DTT and 2 mM spermidine) 25 mM rNTPs and 2 µl of T7 enzyme mix (Promega,USA). The reaction was mixed gently and incubated at 37 ºC for 4 h. DNA templates were then removed by digestion with 1-2 U of DNase at 37 ºC for 15 min. The reaction was adjusted to 100 µl with DEPC treated water. The RNA was extracted by phenol:chloroform. The mixture was precipitated with 3M sodium acetate (pH 5.2) and isopropanol at -30 ºC for 2 h. The RNA pellet was

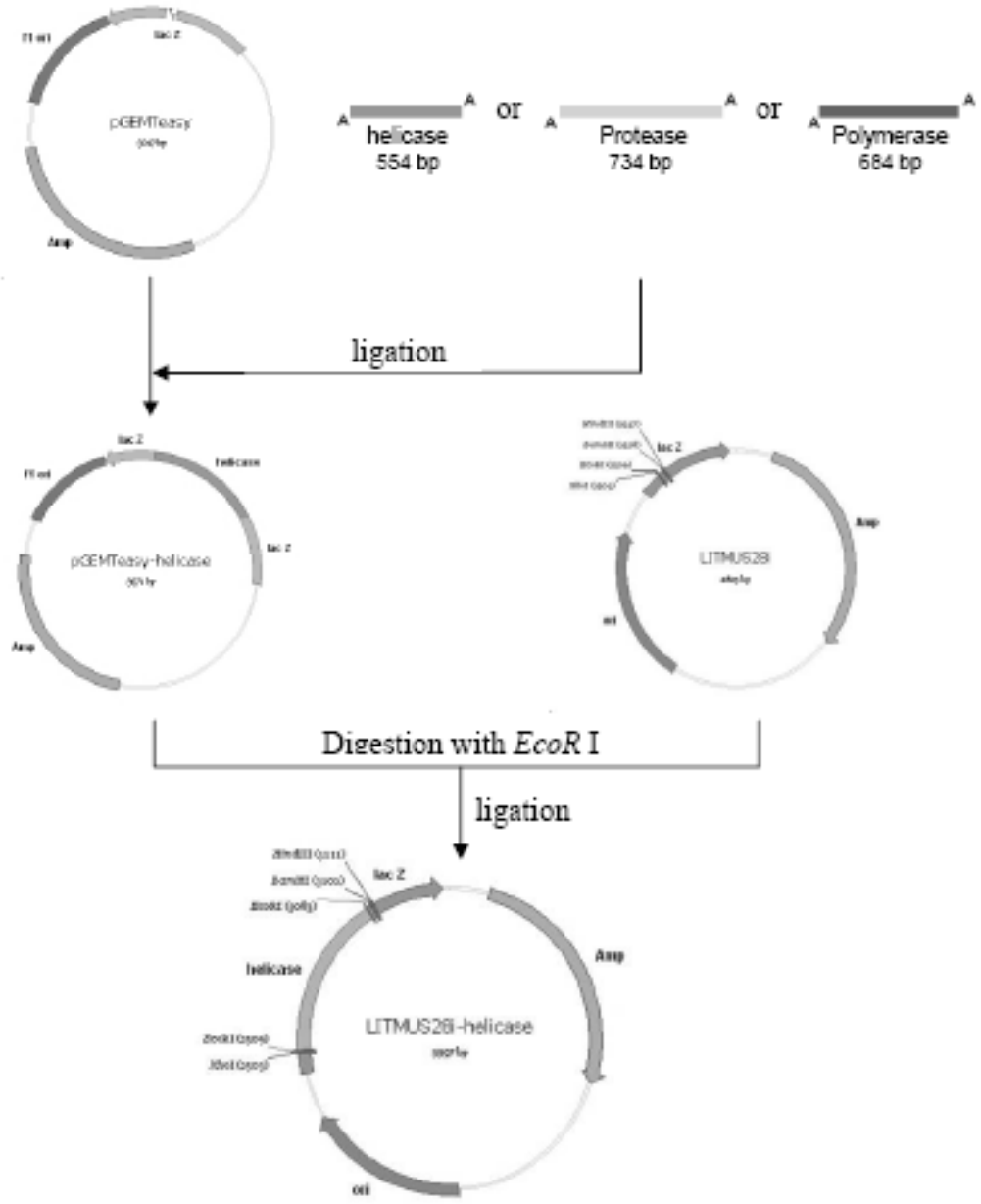

Figure 3: A schematic diagram for construction of LITMUS28i-Helicase

The purified PCR products of the helicase, protease and polymerase were ligated into pGEM®T-easy vector before subcloned into the *EcoR* I site of LITMUS 28i vector.

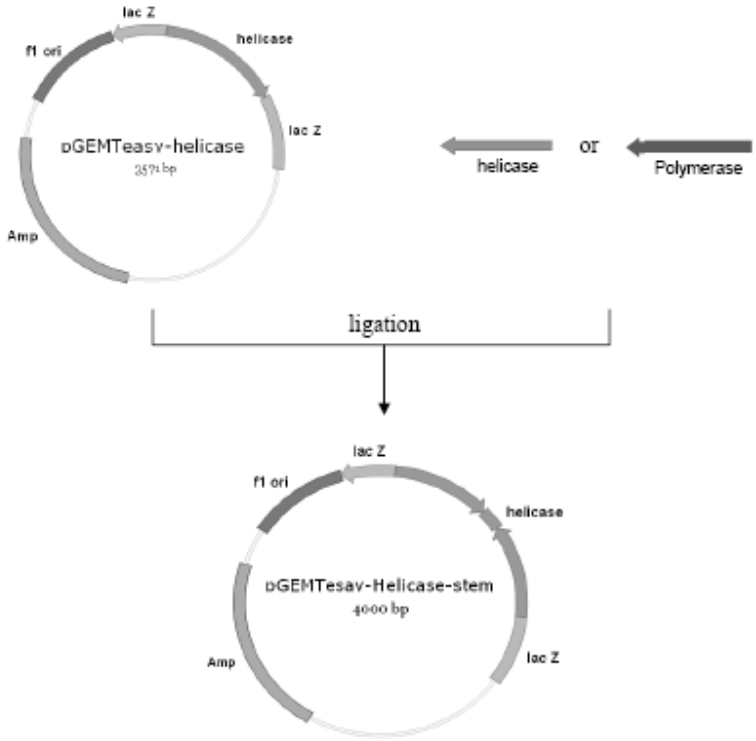

Figure 4: A schematic diagram for construction of pGEMTeasy-Helicase-stem

The PCR products of the helicase and polymerase were cloned into the *Spe* I/*Pst* I site of pGEM®T-easy vector containing the corresponding DNA fragment in antisense direction.

pelleted by centrifugation at 7500 x g at 4 ºC for 5 min. The RNA pellet was resuspended in DEPC treated water. To generate conventional dsRNA, equal amounts of *in vitro* transcribed sense and antisense stranded RNAs (100 µg) were added to annealing buffer at 1X concentration (20 mM potassium acetate, 6 mM HEPES-KOH pH 7.4 and 0.4 mM magnesium acetate). The sample was heated at 80°C for 3 minutes then cooled down to room temperature and left for additional 1 h at room temperature to allow efficient annealing of the complementary strands..

To produce stem loop dsRNA, the T7 transcription template was prepared by digesting with a restriction enzyme that linearized the DNA downstream from the insert. *In vitro* transcription reaction was performed as above. The single stranded RNA that was produced in the 4 hours reaction annealed spontaneously to form dsRNA. Following transcription, The DNA template was removed as described above.

Characterization of dsRNA

To verify the formation of dsRNAs, both conventional dsRNAs and stem loop dsRNAs were digested with RNase A and III ribonuclease enzymes and compared to undigested dsRNAs on agarose gel electrophoresis. For RNase A digestion reaction, 2 µg of dsRNA were incubated with 0.01 µg of RNase A in 1X RNase A buffer (300 mM sodium acetate, 10 mM Tris, 5 mM EDTA) in a total volume of 10 µl. For RNase III digestion reaction, 2 µg of dsRNA were incubated with 0.5 U of RNase III in a final 1X RNase III buffer (10 mM Tris, 0.1 mM $CaCl_2$ and 2.5 mM $MgCl_2$) concentration. The digestion reactions were incubated at 37 ºC for 5 min and immediately loaded onto gel.

Preparation of primary cell culture from lymphoid organ

The primary cell culture from lymphoid (Oka) organ of *P. monodon* was prepared as described by (30, 31). Briefly, shrimps were killed and lymphoid organs were excised and placed into washing solution [2X Leibovitz's L-15

medium, 1% D-glucose, 0.5% NaCl, 200 IU/ml penicillin and 200 µg/ml streptomycin]. Lymphoid organs were washed for ten times with the washing solution and minced into small pieces. The ground tissue were transferred to a 50 ml centrifuged tube containing approximately 15 ml of working medium [2X Leibovitz's L-15 medium, 1% D-glucose, 0.5% NaCl, 200 IU/ml penicillin and 200 µg/ml streptomycin supplement with 15% (v/v) fetal bovine serum, 15% (v/v) shrimp meat extract and 5% (v/v) lactabumin] and left at room temperature for 5-10 min. The upper cell suspension was transferred to a new 50 ml centrifuged tube and seeded into 24-well tissue culture plate for monolayer culture and incubated at 26-28 ºC for 3 days. Cultured cells were observed daily with an inverted microscope (Nikon) for the propagation of cells.

Primary lymphoid cells were inoculated with 1:1 filtered TSV in LHM [10.1 g Minimum Essential Medium with L-glutamine, 3 mM $Na_2HPO_4 \cdot 7H_2O$, 28 mM $MgSO_4 \cdot 7H2O$, 15 mM $CaCl_2 \cdot 2H_2O$, 10 mM KCl, 500 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$, 36 mM $NaHCO_3$ and 3 mM D-glucose] on Day 3 after plating. Cells were incubated at 28 ºC for 14 h and washed 2 times with washing solution after inoculation. Cells were periodically observed under a microscope for any sign of cytopathic effect as well as 50 µl of culture medium were collected at 0, 24, 48, 72 and 96 h. Cells were lysed with 250 µl of TRI REAGENT®-LS for RNA extraction at the same time points.

RNA interference experiment

Shrimps were kept in tanks (5-7 shrimps/tank) containing artificial seawater with 10 ppt salinity at room temperature and fed with commercial feed. Shrimps were administered with 25 or 35 µg dsRNA in 100 µl through haemolymph. Twenty-four hours after injection, shrimps were infected by intramuscular injection at 1:50 dilution except negative control group. Injection volumes were 100 µl. Finally, haemolymph was drawn from individual shrimp and shrimps were collected at 2 days post infection for RT-PCR analysis.

Result

Characterization nucleotide sequence identification of TSV nonstructural genes of field isolates

3 different isolates of TSV were collected from TSV infected shrimps obtained from different farming areas of Thailand. Total RNA of the infected shrimp each isolate was prepared and conversed into complementary DNA. A 6 kb region of open reading frame 1(ORF 1) of TSV genome was PCR amplified as three overlapping fragments (Figure 5, Figure. 6) and cloned into pGEM-T Easy vector. Nucleotide of each fragment was determined by automate DNA sequencing an assembled into entire 6 kb (Figure 7). Alignment the nucleotide sequence among the 3 isolates as well as to the reference sequence reported in public data base was shown in figure 1. The ORF 1 of our three TSV isolates showed high degree of overall sequence identity (97.8 - 99.2%) which is slightly higher than compared sequence in public database (96-97% identity) (Table 2). Variation of the nucleotide sequence distribute throughout the entire 6 kb of ORF1 without any sign of hot spot for sequence variation.

Amplification and purification of TSV

To amplify TSV for future purification, crude lysate from TSV infected *P. vannamei* was injected into haemolymph of 300 healthy white shrimps. TSV level in these shrimps was increase at 4-5 days post-infection. Hemolymph from all infected shrimps were collected and subjected to sucrose gradient centrifugation in order to purify TSV according to the method described by Lightner and coworker (). Unfortunately, after 2 attempts of purification TSV level in the purified fraction was not be enriched compared to the viral level in the crude sample. Moreover testing for the infectivity of TSV, TSV after purification appeared inactive and cannot propagate in white shrimps (data not shown). TSV in the crude lysate, on

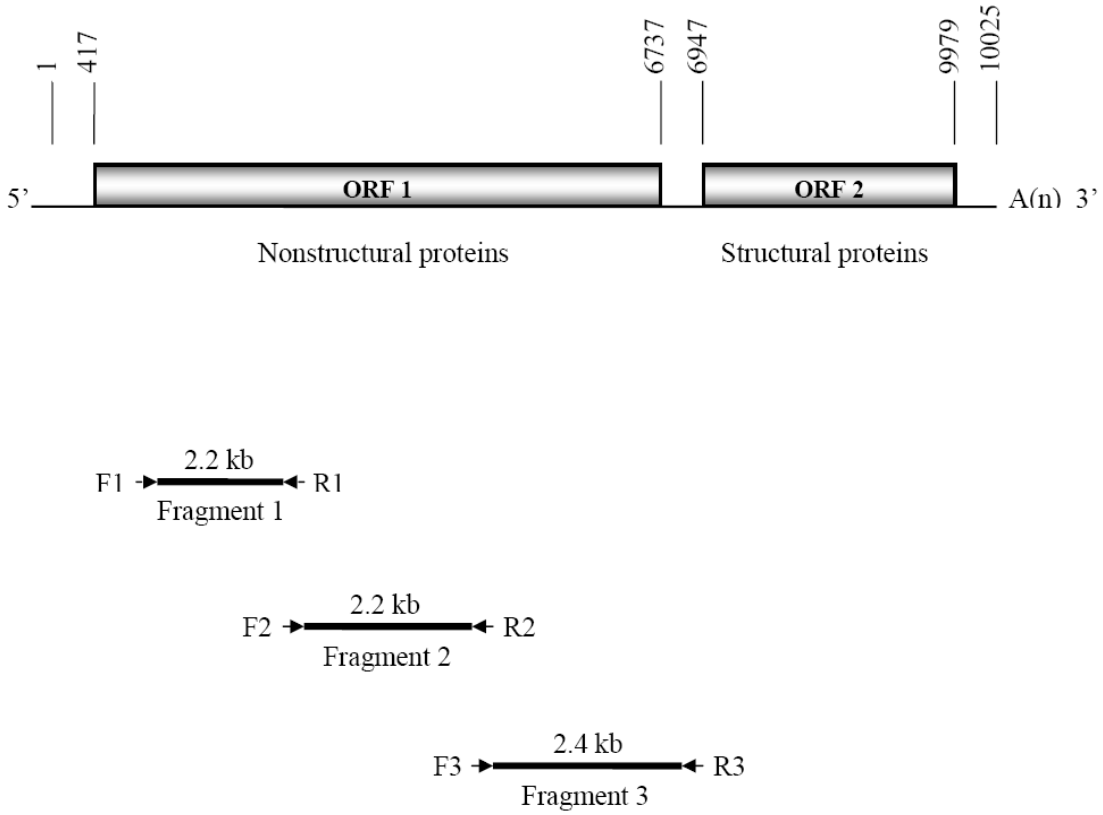

Figure 5: A schematic representation of Taura syndrome virus genomic RNA along with the location of primers used in the RT-PCR

The three pairs of primer were used to amplify the overlapping fragments of the TSV ORF1 gene by reverse transcriptase-polymerase chain reaction (RT-PCR).

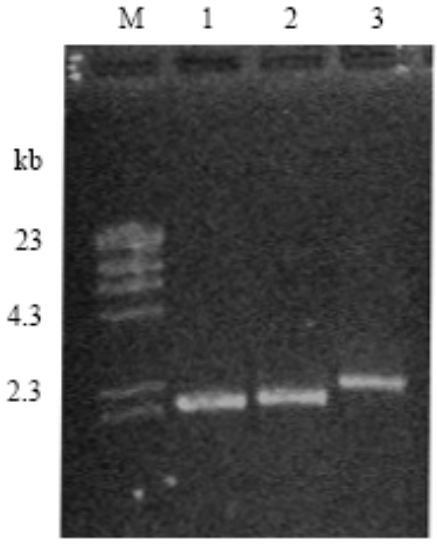

Figure 6: RT-PCR amplification of fragments representing the TSV ORF1 gene of Samut Sakorn sample

The ORF1 gene of TSV was amplified as overlapping fragments with TSV-specific primer pairs.

Lane M: λ *Hin*d III marker

Lane The 2.2 kb amplification products of fragment 1

Lane The 2.2 kb amplification products of fragment 2

Lane The 2.4 kb amplification products of fragment 3

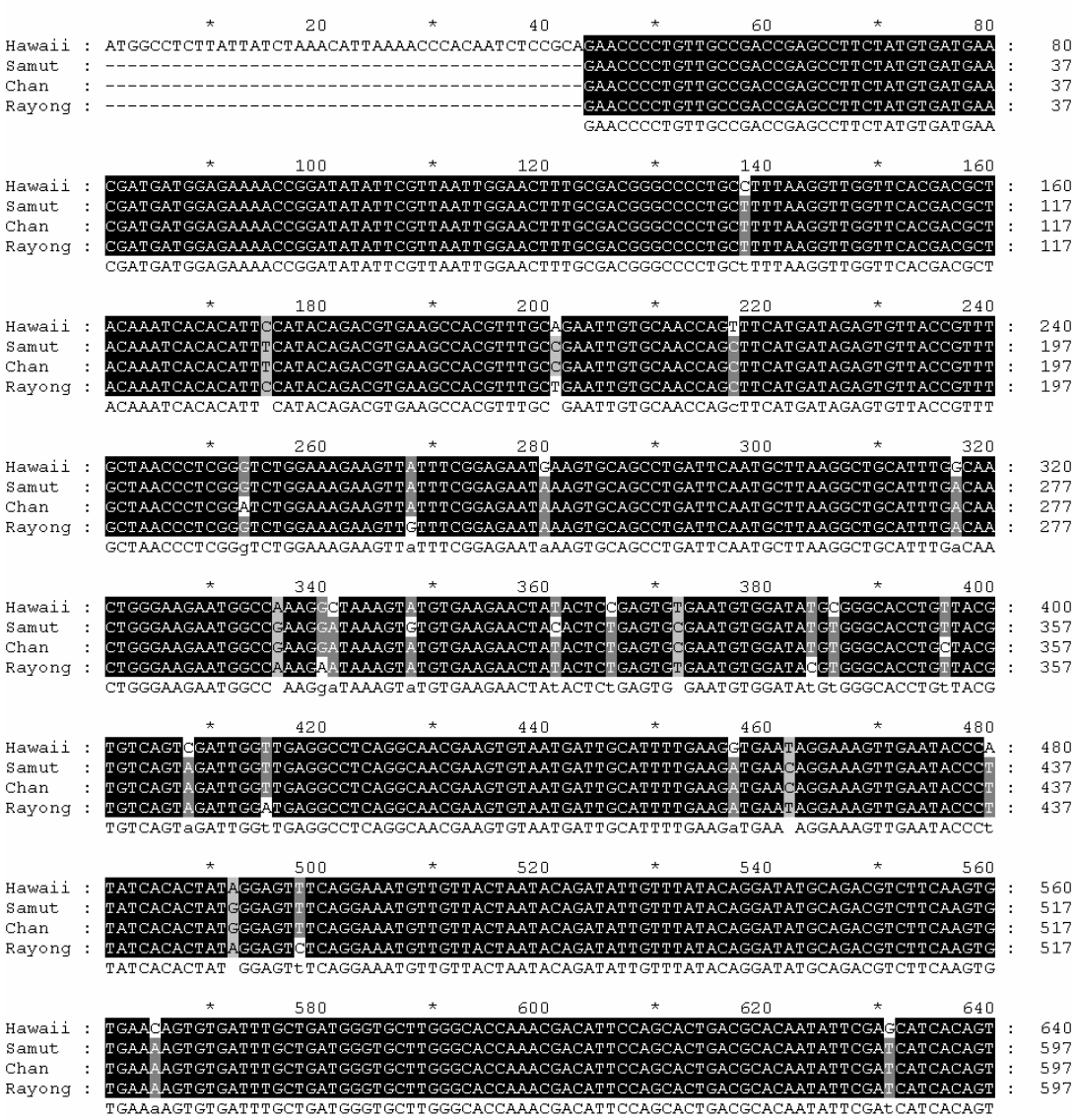

Figure 7: Multiple sequences alignment of Thai TSV isolates

The nucleotide sequences of the ORF 1 gene of TSV were compared between three Thai isolates and the Hawaii isolate. The first 43 nucleotides at the 5'end were not determined. The Hawaii sequence is shown in the top line. The followings are sequences from Samut Sakorn (Samut), Chanthaburi (Chan) and Rayong (Rayong) isolates. Black shadings represent matching nucleotides. Gray shadings represent matching nucleotides between two or three sequences. Numbers on the right are position of nucleotide relative to the start codon of ORF1.

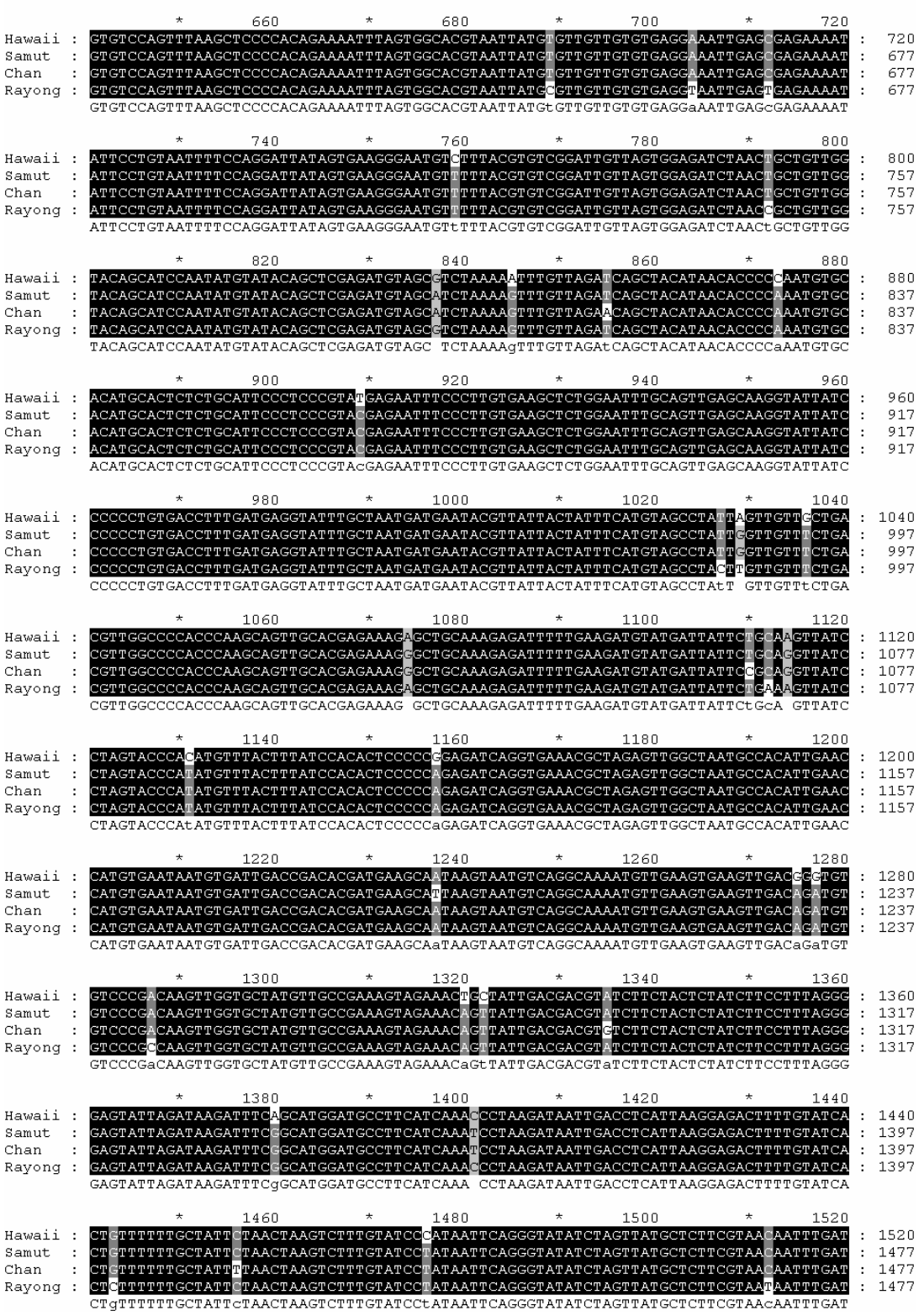

Figure 7: Multiple sequences alignment of Thai TSV isolates (Continued)

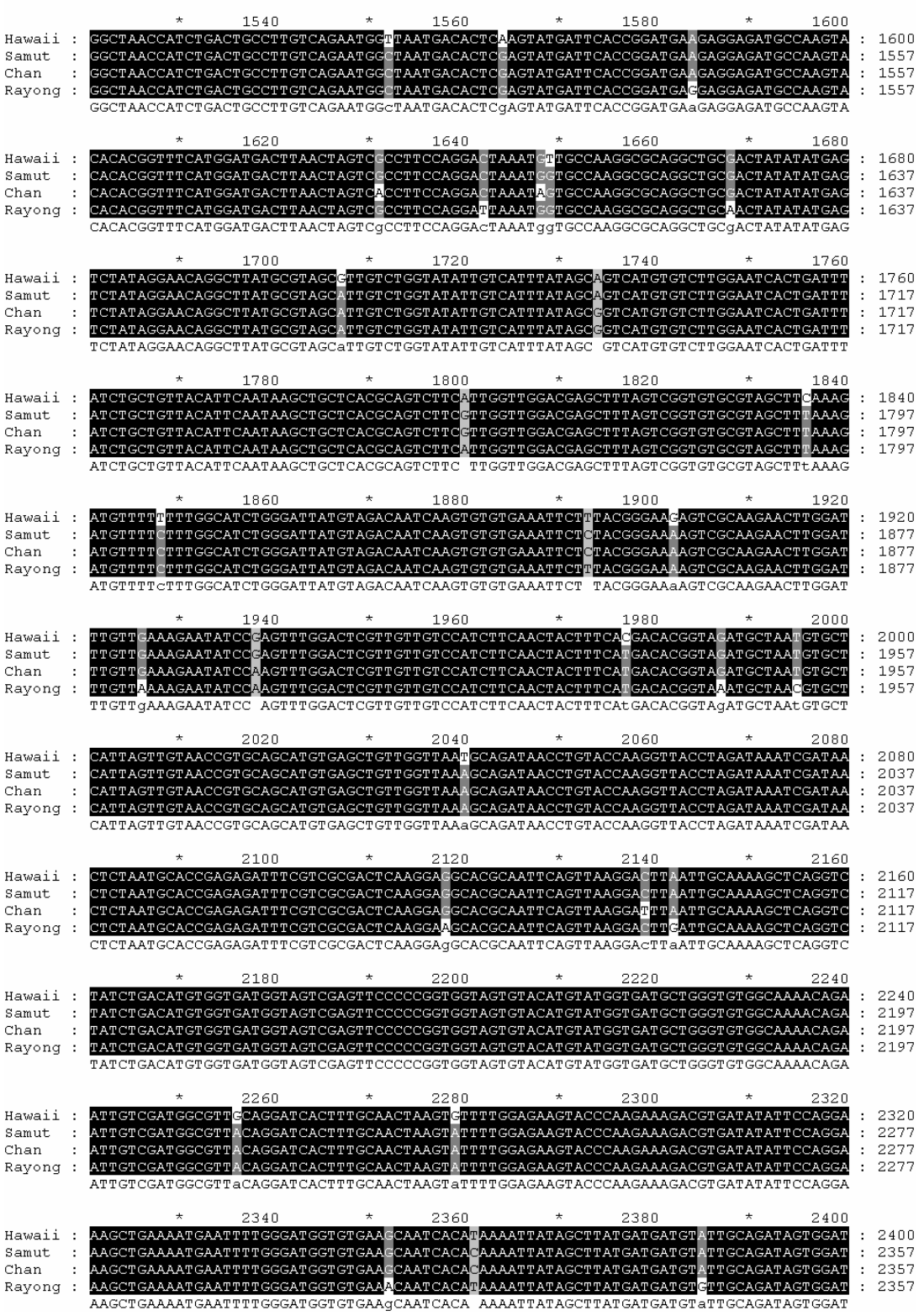

Figure 7: Multiple sequences alignment of Thai TSV isolates (Continued)

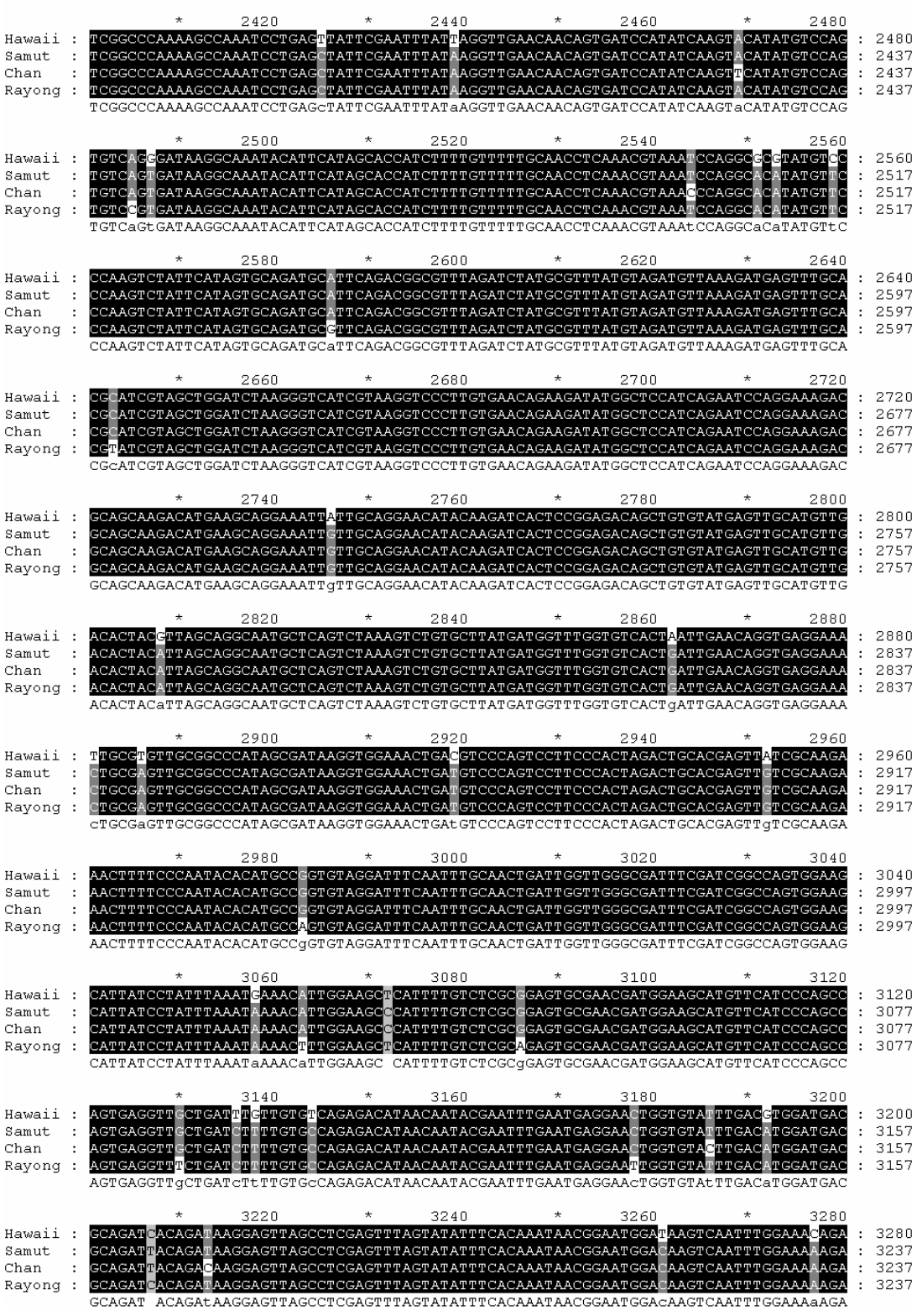

Figure 7: Multiple sequences alignment of Thai TSV isolates (Continued)

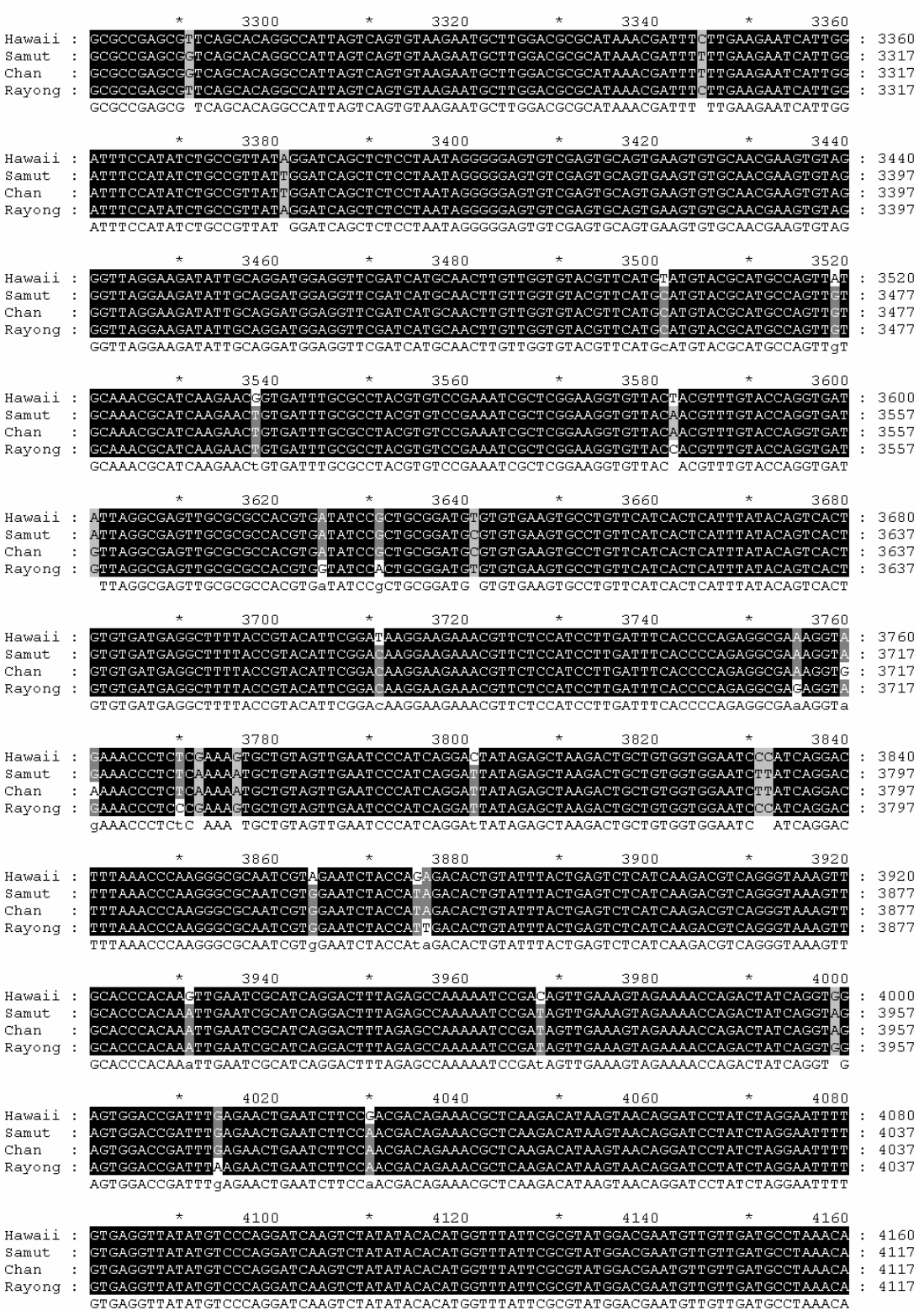

Figure 7: Multiple sequences alignment of Thai TSV isolates (Continued)

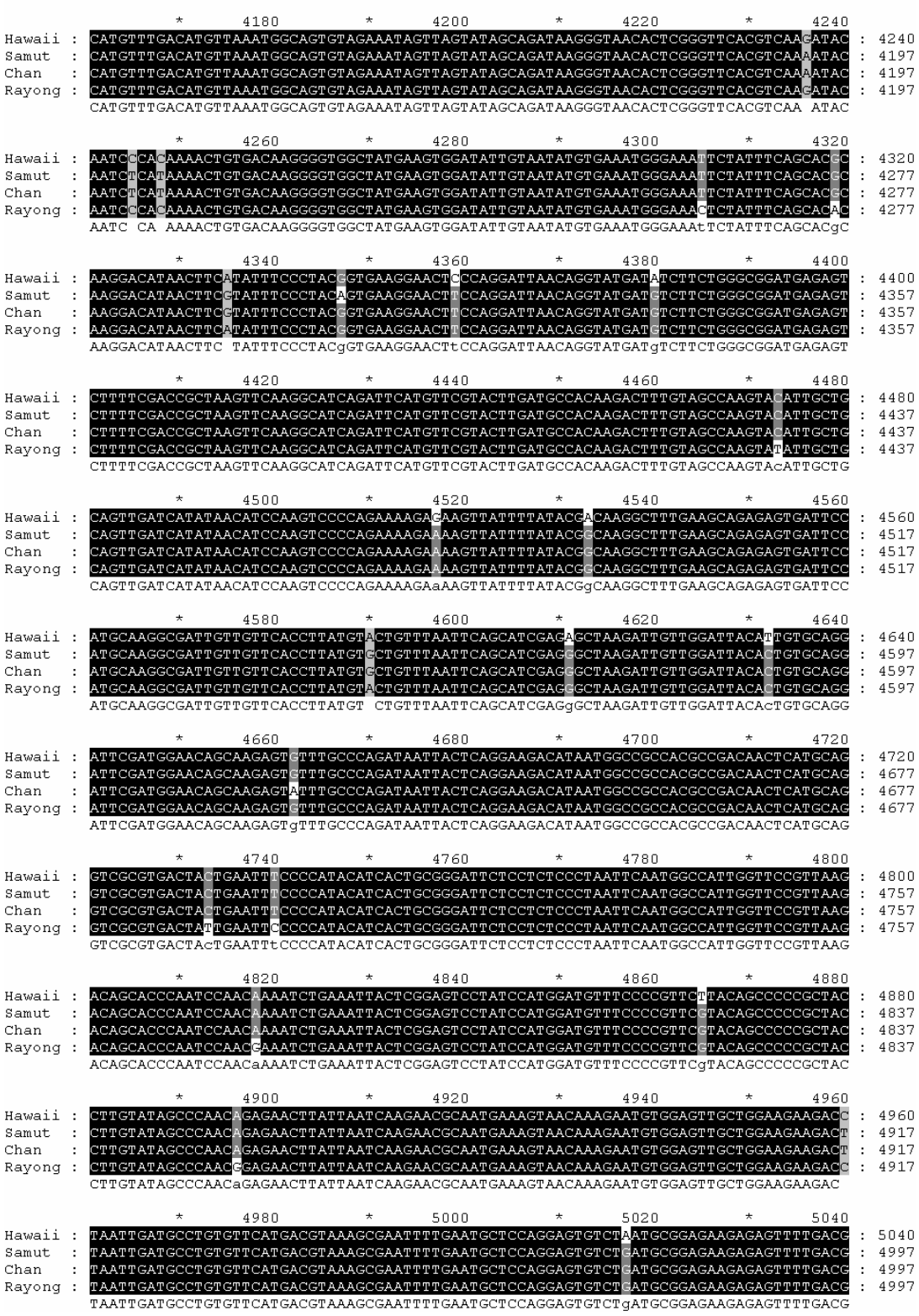

Figure 7: Multiple sequences alignment of Thai TSV isolates (Continued)

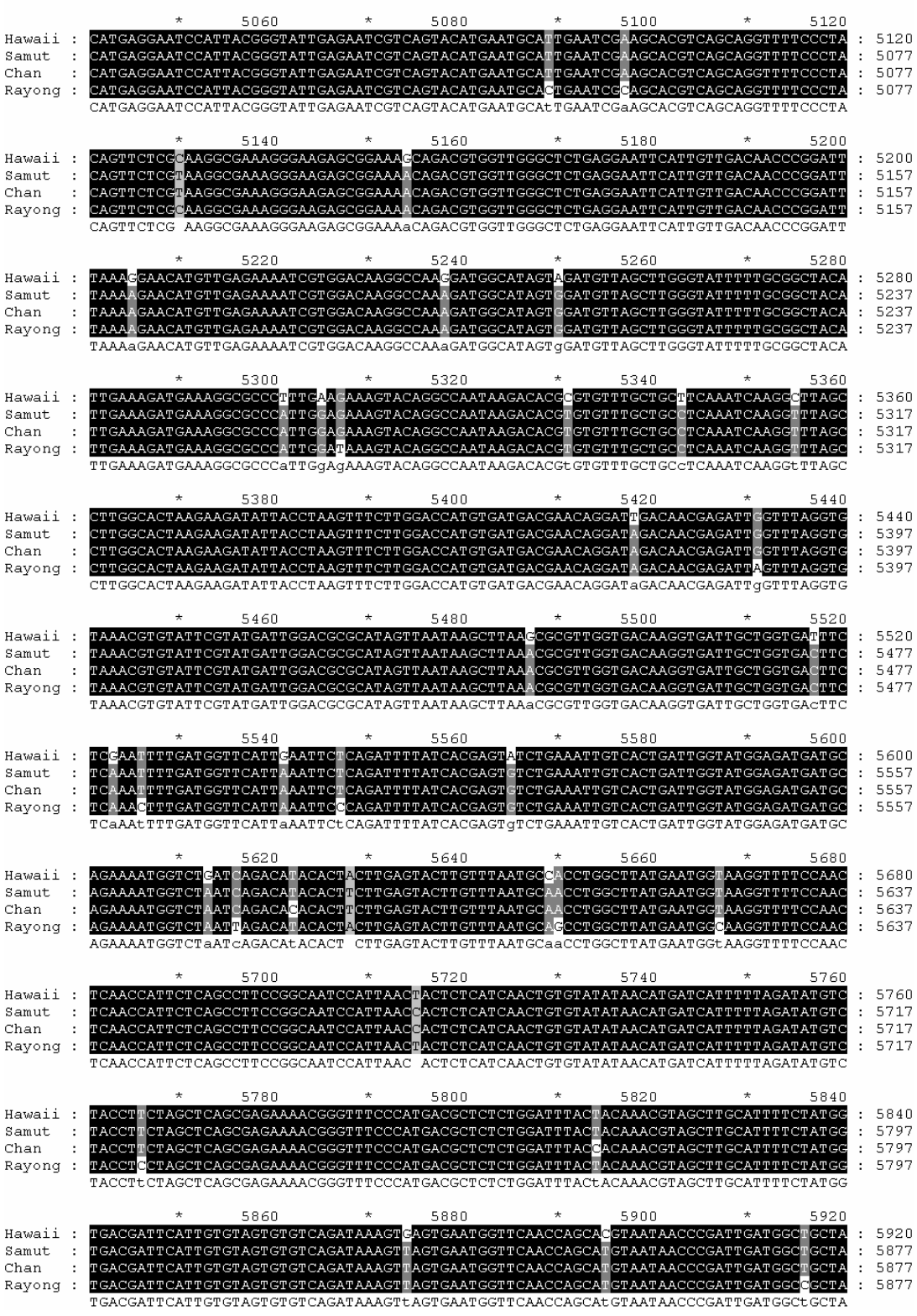

Figure 7: Multiple sequences alignment of Thai TSV isolates (Continued)

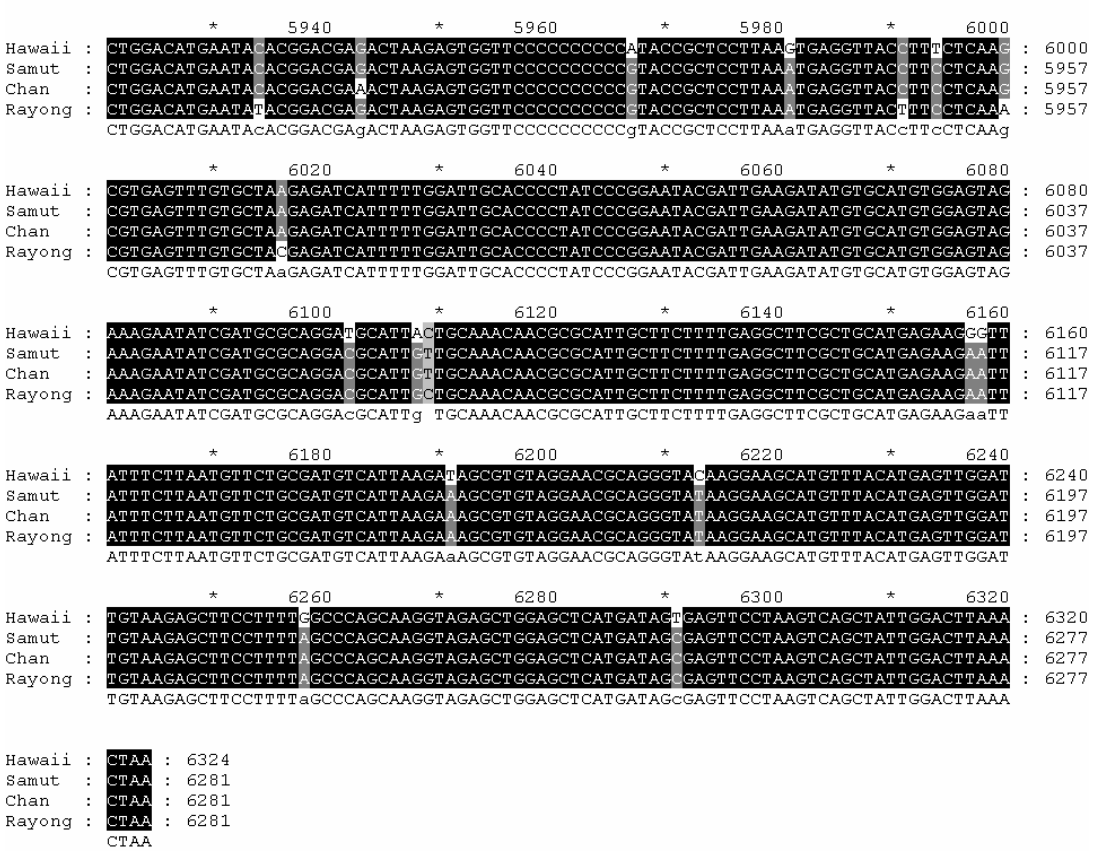

Figure 7: Multiple sequences alignment of Thai TSV isolates (Continued)

Table 2. Percent nucleotide (above diagonal) identities between the three Thai isolates and Hawaii isolate in the ORF1 gene of TSV.

	Isolate	Percent identity of nucleotide sequence			
		1	2	3	4
1	Hawaii	*	97.0	96.4	96.6
2	Samut Sakorn		*	99.2	98.4
3	Chanthaburi			*	97.8
4	Rayong				*

the other hand, was therefore tested for their infectivity. Upon injection, the presence of virus in the shrimps was monitored by RT-PCR. The result showed that the RNA prepared from haemolymph and epidermis of infected shrimp gave positive results (Figure 8). Thus we concluded that our TSV stock prepared from TSV infected shrimp was active and infectious.

Besides testing for the infectivity in *P. vannamei*, the same TSV stock was injected into *P. monodon* to determine whether the shrimp is experimentally susceptible to TSV infection. RT-PCR analysis was used for monitoring TSV replication. The result showed that TSV was detected in haemolymph sample at lower level compared that observed in *P. vannamei*, however it was not detected in the epidermis sample (Figure 9). The result is therefore suggested *P. monodon* may be used as model TSV infection.

Experimental infection in Oka cells with TSV

Because the culturing system of primary lymphoid cells (Oka cells) of *P. monodon* is available in our laboratory and successfully used for shrimp viral infection, therefore, the next experiment was to determine if TSV can replicate in Oka cell culture of *P. monodon*. The Oka cell culture was prepared and inoculated with with TSV in triplicate well. As compared with the YHV infected cells, no sign of Cytopatic effect was not observed in all samples inoculated with TSV in the course of 96 hr (Figure 10). Moreover no TSV was detected in these samples by RT-PCR as well. Taken together we concluded that the OKA cell from P. monodon is not suitable for TSV experiment.

Antisera for TSV structural protein

Antisera from the immunized mice were tested with proteins from TSV infected shrimp by Western blot analysis. The result indicated that antisera reacted to protein from TSV infected sample with the expected protein whose size was corresponded to structural protein VP1 and VP2 (Figure 11). This signal was not

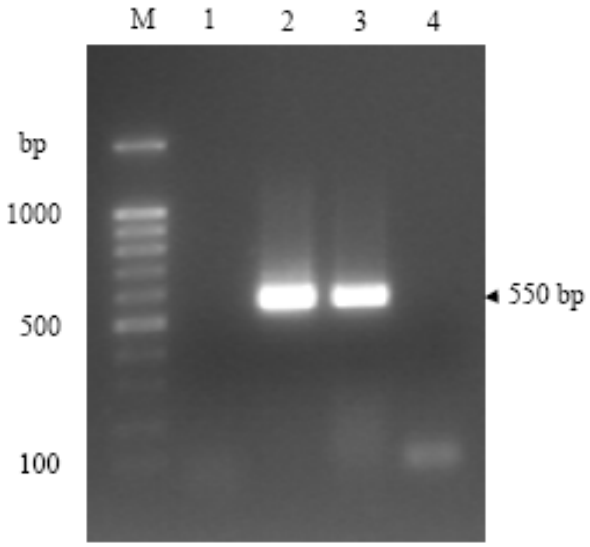

Figure 8: The experimental infection of TSV in *P. vannamei*

P. vannamei were injected with lysate of TSV infected shrimp. Five days later, total RNA was extracted from haemolymph or epidermis for TSV detection. The TSV specific fragment of 550 bp resulted from RT-PCR assay using the Fhel/Rhel primers, indicated the presence of TSV genomic RNA in the sample

Lane M: 100 bp DNA ladder

Lane 1: The haemolymph sample of shrimp injected with PBS

Lane 2: The epidermis sample of shrimp infected with TSV

Lane 3: The haemolymph sample of shrimp infected with TSV

Lane 4: Negative RT-PCR control

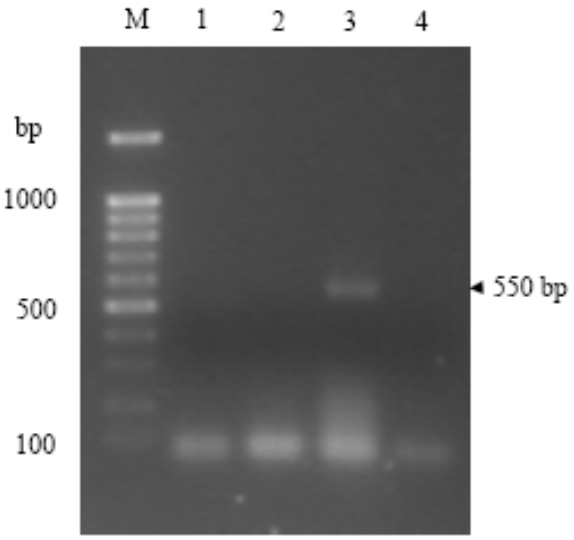

Figure 9: The experimental infection of TSV in *P. monodon*

P. monodon were injected with lysate of TSV infected shrimp. Five days later, total RNA was extracted from haemolymph or epidermis for TSV detection. The TSV specific fragment of 550 bp resulted from RT-PCR assay using the Fhel/Rhel primers, indicated the presence of TSV genomic RNA in the sample.

Lane M: 100 bp DNA ladder

Lane 1; The haemolymph sample of shrimp injected with PBS

Lane 2: The epidermis sample of shrimp infected with TSV

Lane 3: The haemolymph sample of shrimp infected with TSV

Lane 4: Negative RT-PCR control

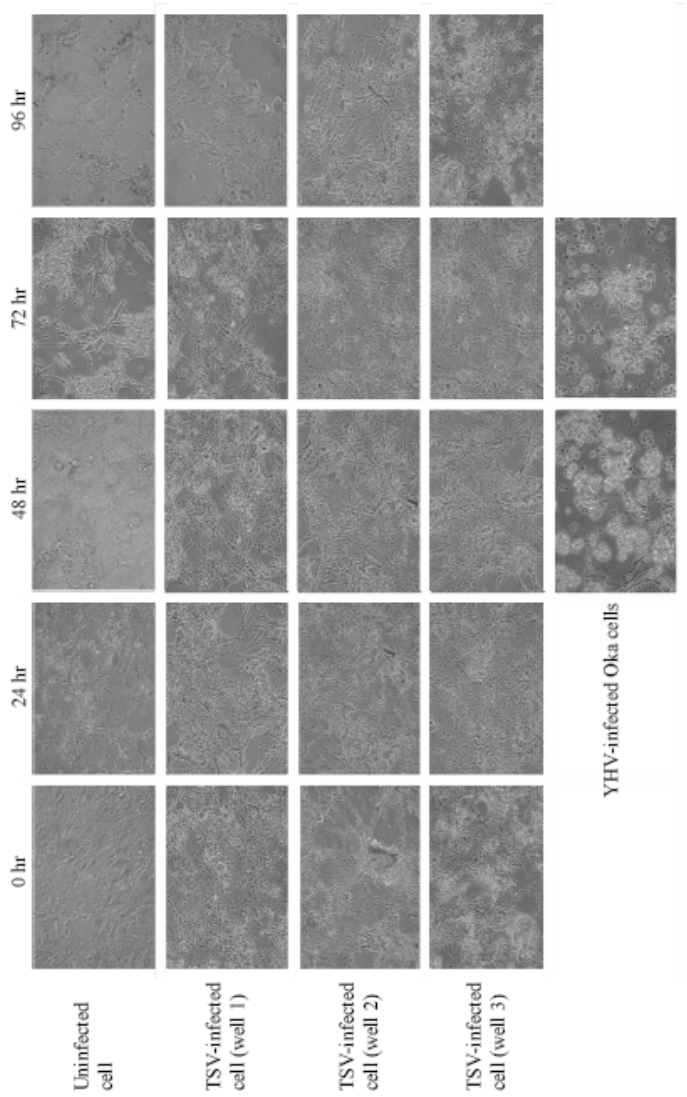

Figure 10: Morphology of primary Oka cells infected with haemolymph from shrimp infected with TSV

Primary Oka cells were infected with haemolymph from shrimp infected with TSV. Change of cell morphology was observed at 0, 24, 48, 72 and 96 hours post infection. Uninfected cell was shown at the top. Triplicate of TSV-infected cell were shown in the following. The characteristic of CPE was first observed at 48 hr post infection in Oka cells infected with YHV. The images were taken at a magnification of 40X with a microscope.

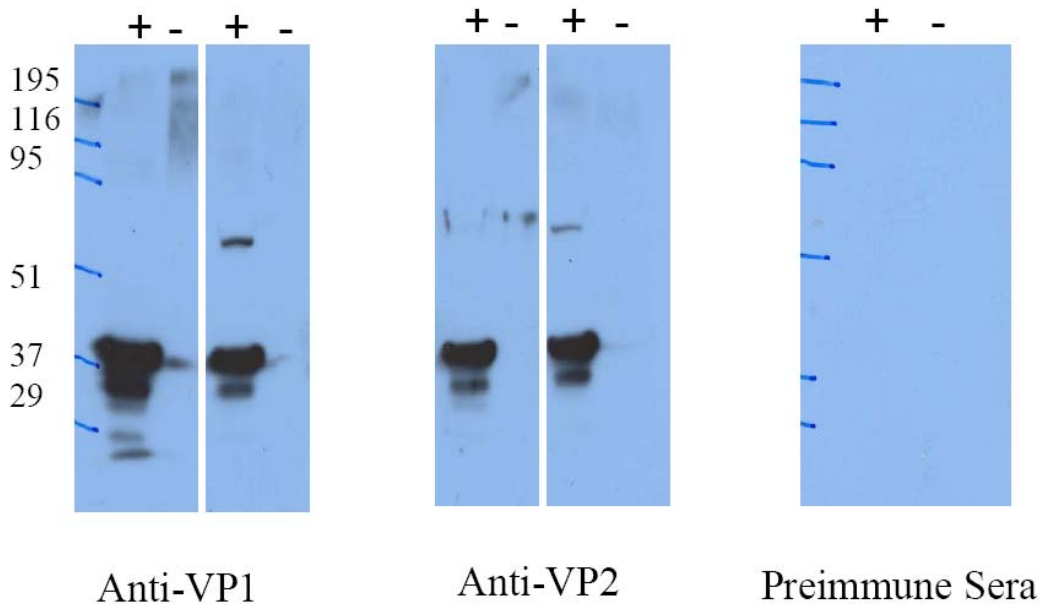

Figure 11. Characterization of antisera from mice immunized with GST-VP1 or GST-VP2 by western blot analysis

Equal amount of total protein from TSV infected shrimp (+) or non-infected shrimp were separated in 10% SDS-PAGE and blotted onto PVDF membrane. Antisera from two independent immunized mice with GST-VP1, GST-VP2, or preimmune sera were diluted at 1:4000 and used as primary antibody to probe the protein blot. The specific signals of TSV protein structural proteins were visualized by ECL detection system.

observed in sample from uninfected shrimp. In contrast preimmune sera collected from the same mice react to protein in neither non-infected nor TSV infected shrimps. Taken together, this result indicated that the antisera react specifically to the expected structural protein on the TSV particles.

dsRNA production by *In vitro* transcription

We designed specific primers to amplify cDNA corresponding to polymerase, helicase and protease gene from TSV genome. These PCR products were subcloned into plasmid vector p-Litmus and pGEM T Eazy and used as template for dsRNA/ hpRNA *in vitro* transcription. To verify the quality of RNA product generated by these methods, these dsRNA products were characterized by enzymatic digestion either by single stranded specific RNase (RNase A) or double stranded specific RNase (RNase III) (Figure 12). Majority of RNA sample generated from these experiments are resistant to RNase A, however, highly sensitive to RNase III implicated that these RNA exhibit the properties of double stranded RNA. Thus they are suitable to investigate their potency in inhibiting TSV replication in the future experiment.

Inhibition of TSV replication in *P. vannamei* shrimp by dsRNA

Since primary culture of Oka cells from *P. monodon* was not susceptible TSV *in vitro*, we therefore evaluated the inhibitory effect of dsRNA on TSV replication directly in *P. vannamei*. The dsRNAs targeted three different regions in helicase, protease and polymerase on the TSV genome were generated *in vitro* as described earlier. To investigate the inhibitory effects of dsRNAs on TSV replication, 25 µg of dsRNAs specific to those regions were injected intramuscularly into TSV free *P. vannamei*. The shrimps were then infected (24 hr later) by TSV (at 1:10 dilution of crude extract fro TSV infected shrimp). RNA from gill tissues was subjected to RT-PCR analysis at 3 days post-infection. We tested for efficiency of TSV infectivity upon experimentally challenge. From two

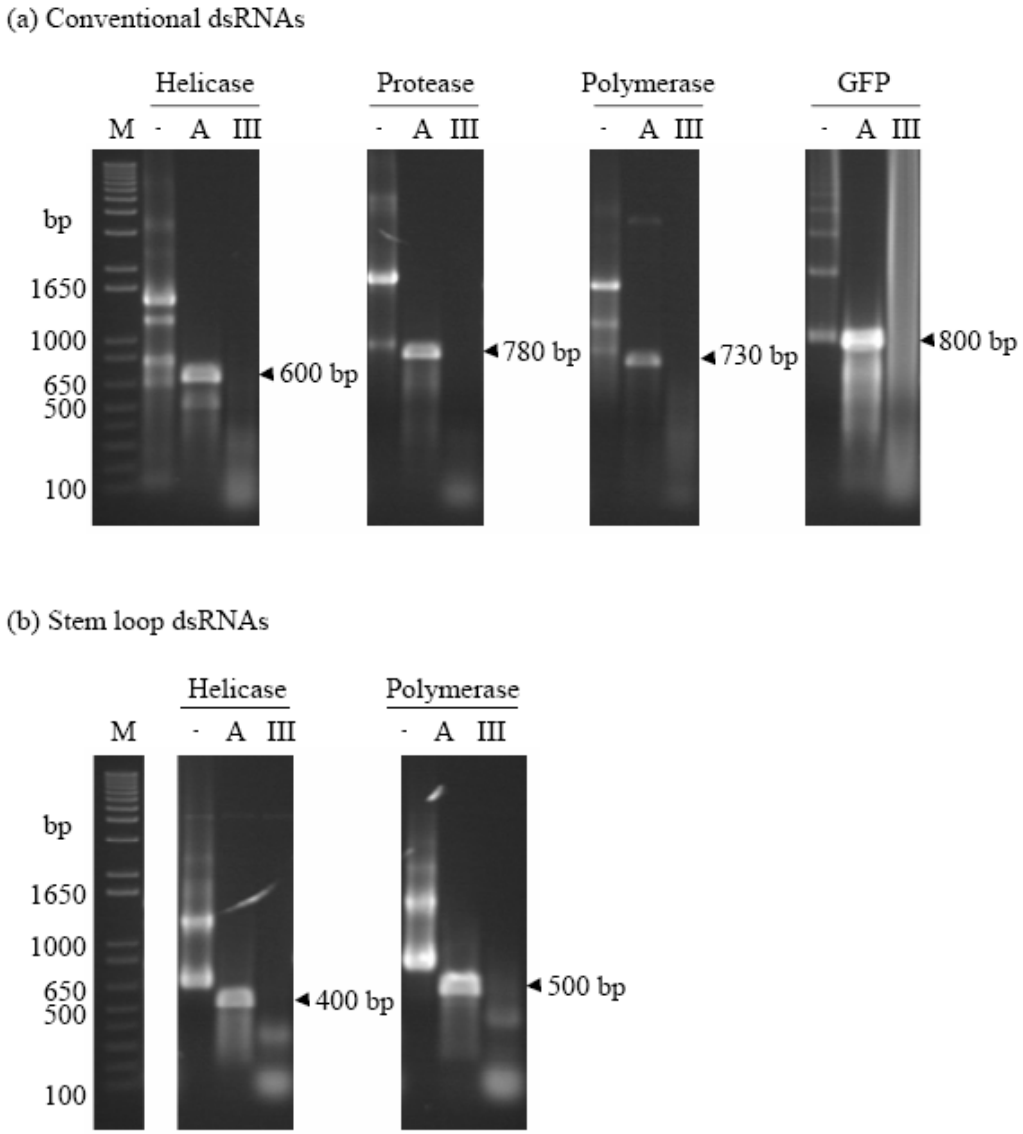

Figure 12: Characterization of conventional and stem loop dsRNAs by RNase digestion.

The conventional and stem loop dsRNAs were generated and tested for their integrity by RNase A and III digestion. Each dsRNAs were separated on 1.2% agarose gel and visualized by staining with ethidium bromide. Lane: M, 1 kb plus DNA ladder. Note that dsRNA migrates more slowly than double stranded DNA marker.

separated experiments, we found that approximately 80% of these shrimp were susceptible to TSV infection (data not shown). Identical result of RT-PCR detection of TSV was found in both gill and hemolymph of the same shrimps. Subsequently, these shrimps were divided into several groups then subject to test for the inhibitory effect of dsRNA on TSV replication (Figure 13). In the control groups, no TSV were detected in all unchallenged shrimps confirming that these shrimp were free from TSV whereas ~70% of shrimp challenged with TSV were clearly identified as positive for TSV at 2 day after infection. Injecting the shrimps with TSV specific dsRNA prior to TSV challenge resulted in strong inhibitory effect to TSV propagation. No shrimps received dsRNA-helicase was identified positive for TSV, whereas only 1 shrimp received dsRNA-protease (of 4 shrimps) or 1 shrimp received dsRNA-polymerase (of 5) was found TSV positive. In contrast, shrimps received unrelated dsRNA-GFP showed approximately 70% TSV infection.

To confirm the result from RT-PCR, western blot analysis was used to test for the presence of TSV in the experimental shrimp. Gill from the previously identified as TSV-infected shrimp were used for total protein extraction. Anti-VP2 polyclonal antibody was used to monitor the presence of structural protein of TSV-VP2 in the gill extract. The result in figure 14 showed example of the western blot result that high level of VP2 were detected in shrimps strongly positive by RT-PCR. Only faint bands of VP2 were presence in shrimps with low level of the TSV, however, some positive gill sample did not react with this antibody. This is most likely due to the lower sensitivity of the Western detection.

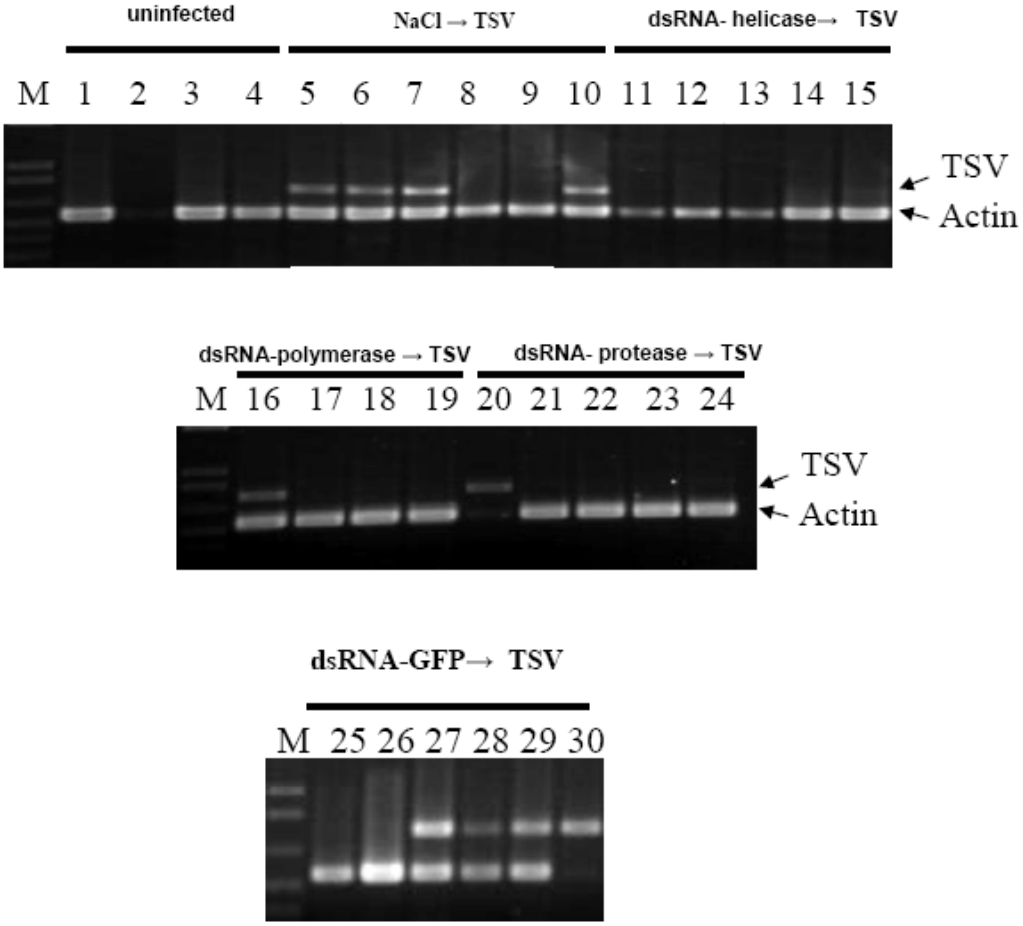

Figure 13: The inhibition effects of dsRNAs on TSV replication in live *P. vannamei*.

TSV-free white shrimps were infected with TSV alone or infected with TSV at 24 hours after dsRNAs administration (25 μg) as indicated. After 2 days post infection, the TSV in hemolymph was monitored by multiplex RT-PCR assay. The levels of TSV in each shrimp were shown in the upper band. Actin shown in the lower band served as internal control. Number represents RNA from individual shrimp.

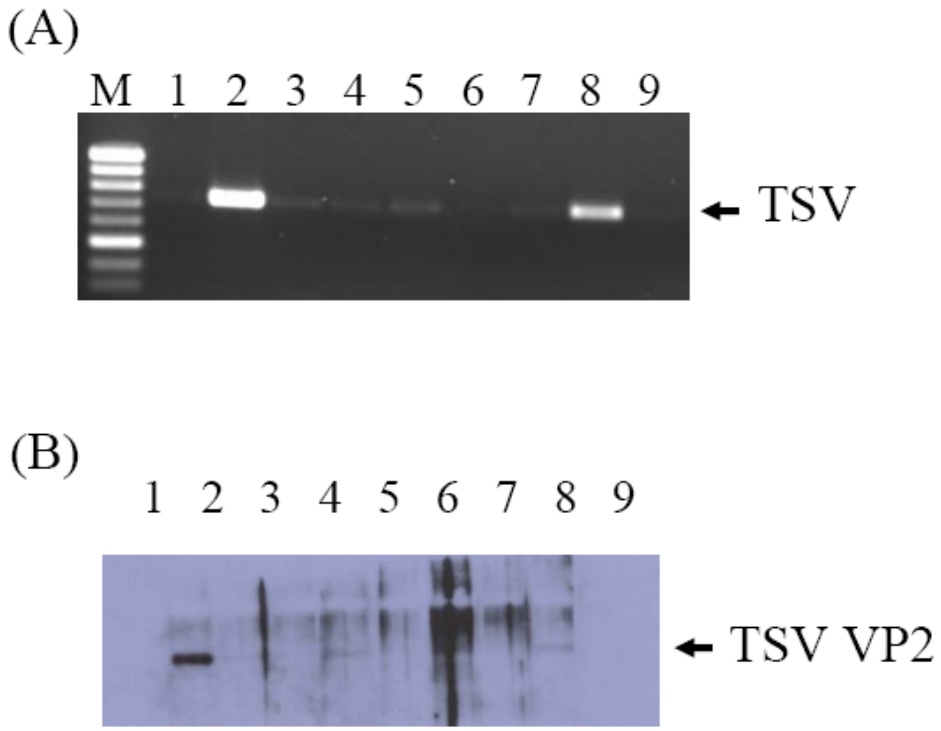

Figure 14: The inhibition effects of dsRNAs on TSV replication in live *P. vannamei*.

White shrimps identified positive for TSV by RT-PCR (A) were confirmed by western blot analysis (B). Equal amount of protein extracted from gill of the infected shrimps were resolved in 10% SDS-PAGE. The proteins profiles were blot onto nylon membrane and probed with anti-TSV-VP-2 antibodies followed by detection ECL-plus system. Number represents RNA from individual shrimp.

DISCUSSION

The poor performance, slow growth rate and disease susceptibility of the major cultured shrimp species, *P. monodon*, in Thailand have led many shrimp farmers shifted to cultivation of *P. vannamei*. Since then *P. vannamei* has risen in popularity to become the second species of shrimp farmed in Thailand. However, *P. vannamei* is known to be carriers of viral diseases such as TSV and WSSV. Unfortunately, the illegal importations of cheaper, non-disease free shrimp broodstocks have led to introduction of TSV to Thailand. As a consequent, TSV outbreak occurred in early 2003. Due to the lack of its effective means for controlling and prevention, the incident of TSV seems to continue with higher impact. Hence, development of effective method to control disease caused by this virus as well as other viruses would help to prevent economic lost of shrimp culture industry.

In this study, three isolates of TSV were collected from infected *P. vannamei* from farms in Samut Sakorn, Chanthaburi and Rayong, respectively. Nucleotide sequences ORF1 enncoding for nonstructural polyproteins (helicase, protease and RdRp) of these TSV isolates were determined and analyzed. The resulting nucleotide sequence identity of these isolates showed that they varied slightly from each other (range from 97.8 to 99.2%). In addition, these sequences were approximately 97% identical the sequence from the Hawaii isolate reported by Mari J. *et al.*, 2002 (32). These results clearly demonstrated the close relationship of our three TSV isolates to the Hawaii reference isolate. Based the high sequence conservation it is suggested that nucleotide sequence within ORF1 of TSV should be a good target for RNAi mediated viral suppression experiment.

Two approaches for dsRNA synthesis, conventional and stem loop structure, were chosen for comparison, both of which yields similar quality of dsRNA essential to initiate RNAi pathway. Generation of dsRNA as hairpin structure with long perfect complementary stem, up to 0.5 kb, was shown possible. The advantages of this alternative approach are: 1) it requires only a single *in vitro*

transcription reaction and 2) the inverted repeat on both ends of the transcript could automatically form hairpin structure without additional step. Hence, the stem loop approach seems more convenient and cost effective. Indeed, in our hand the quality of the hairpin RNA appears better quality since more prominent form of intact hairpin RNA, the precursor of dsRNA, was obtained. Thus synthesizing large quantity of this stem loop RNA for *in vivo* testing its inhibitory effect on TSV replication seems more appropriate.

In our preliminary experiments on TSV infectivity in *P. vannamei*, we demonstrated that viral inoculum prepared from crude extracts of TSV infected specimen was infectious. Although TSV multiplication and mortality was observed subsequent to infection, we were, however, unable to observe the clinical signs of TSV infection in our experimental infected shrimps. Investigation for the susceptibility of *P. monodon* to TSV infection was performed. The result implicates that *P. monodon* could be experimentally infected with TSV, although the level of virus that detected in haemolymph was lower than that detected in *P. vannamei.* This result is similar to the earlier observation by other group who demonstrated that cultured *P. monodon* originating from Indonesia was found RT-PCR positive for TSV (3). *P. monodon* is therefore served as potential carriers of TSV. Alternatively, the absent of clinical signs of TSV infected black tiger shrimps may resulted from probably other factors involving the host, virus and environmental conditions.

Since the system for culturing primary lymphoid cell (Oka cells) of *P. monodon* has been well established in our laboratory. This primary cell is highly susceptible to YHV and shown as excellent model to investigate RNAi mediated Yellow head virus suppression mechanism (33). The clear phenotypic changes are readily observed upon YHV infection and RNAi mediated YHV inhibition. We therefore attempted to investigate whether this system can be applied for testing TSV suppression by RNAi. First, we investigated the susceptibility of this primary lymphoid cell to TSV infection. Oka cells were infected with TSV then we

monitored the level of virus at various times of after inoculation as an index of viral replication in either cell or culture media by RT-PCR analysis. Only in one third of cell lysates was detected positive by TSV, unexpectedly, we were unable to show the presence of TSV in culture mediums of any of these triplicate. Base on this contradicted result, the primary lymphoid cells of *P. monodon* are not a good model for studying TSV inhibition.

To investigate the inhibitory effects of dsRNA molecules on TSV replication, *P. vannamei* were injected with designated dsRNA 24 hr prior to TSV challenge. The inhibitory effects of dsRNAs on TSV replication and viral mRNA transcripts were investigated by the analyzing the levels of viral RNA by RT-PCR analysis. Results from the first experiment showed the trend that virus specific dsRNAs particularly dsRNA targeting helicase can suppress TSV replication as the level of TSV was significantly reduced or to undetectable level in some shrimps. It is of interested to note that the suppressive effects of each TSV specific dsRNA are not equal. dsRNA targeting polymerase region did not seem to exhibit good inhibitory effect. Whereas unrelated dsRNA of GFP exhibit no inhibitory effect on TSV. Our subsequence results also confirmed that conventional dsRNAs of helicase, protease and polymerase and stem loop dsRNAs of helicase and polymerase had suppressive effects on viral mRNA transcripts and viral replication even though the differences in the ability of dsRNAs interfere with TSV replication was observed.

The differences in the ability of each dsRNA targeted to different regions of the same mRNA transcript suggested that target accessibility of the viral genome plays an important for RNAi effect. As not all virus specific dsRNAs were equally effective; among the dsRNAs tested, dsRNA directed against a helicase region was the most efficient while dsRNA directed against a polymerase region hardly had inhibitory effect on viral replication. These results may be due to a highly secondary structure of the polymerase, which may leave few single stranded gaps that siRNAs can access as described for other viruses such as poliovirus (34). In

contrast, some regions of the helicase are accessible to the RNAi machinery. These data also suggested that the most effective site for the inhibition of TSV replication may locate within the helicase region. In the second experiment, higher amount of dsRNAs were introduced into shrimps and viral level was assessed 2 day postinfection. Although the dose dependence of the dsRNA effect was not investigated in this RNAi study, there seems increasing suppression of viral RNA level as injection of the increasing amount of dsRNA. Thus, this experiment confirmed the trend of inhibitory effects on TSV replication by using virus specific dsRNAs. The sequence independent antiviral immune response in shrimp as reported early by Rabalino *et al* (35) was not clearly observed in our study. To our knowledge, the introduction of TSV specific dsRNAs tended to reduce the viral replication in shrimps and this is likely due to the induction of RNAi-related mechanism that exists in shrimp (36). Our unexpected finding that dsRNA mediated inhibitory effect on TSV observed only in some shrimps may be due to insufficient level of dsRNA introduced into the shrimps since these dsRNA mediated suppression is known as concentration dependent phenomenon (37).

Although our results on TSV suppression by RNAi present so far are quite convincing, more experiments using better control condition is necessary to further investigate for example the use of SPF shrimp. Unlike the potential use of RNAi technology in therapeutic application in human or big animal where direct administration is possible, the direct application of protective dsRNA into shrimp is impossible. Although it is not an over expectation that RNAi would exhibit inhibitory effect on TSV or other virus in shrimp, the ultimate goal of applying this technique to control the viruses in farm is a real challenge.

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Output

- 2 M.Sc. graduates

  Miss Varunya Vudijun graduated in 2006

  Mr. Pharanai Sukumunggoon graduated in 2007
- Two TSV specific antibodies (anti-VP1 and anti-VP2)
- 2 published articles related to RNAi mediated antiviral infection in shrimp
- A manuscript on inhibition of TSV infection in *Penaeus vannamei* is in preparation.