DEVELOPMENT OF DOUBLE-STRANDED RNA-DELIVERY SYSTEM FOR CONTROL OF LAEM-SINGH VIRUS (LSNV) IN THAI *PENAEUS MONODON*

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ABSTRACT

RNA interference (RNAi) technology through the trigger of double-stranded RNA (dsRNA) has been applied to inhibit viral replication in penaeid shrimp. In this study, we have developed a new methodology to deliver dsRNA for controlling Laem-Singh virus (LSNV), a causative agent of Monodon Slow Growth Syndrome in *Penaeus monodon* (*P. monodon*). First, the transformed *Escherichia coli (E. coli*) expressing red fluorescent protein (RFP) was tested in the *Artemia* enrichment process. RFP signals detectable in the gut of *Artemia* under confocal microscope were evident for the successful encapsulation. Second, the *Artemia* enrichment process was performed using *E. coli* producing LSNV-specific dsRNA. By reverse transcription-PCR (RT-PCR), dsRNA-LSNV was detected in *Artemia* after enriching with 4.3 x 10^{11} CFU of *E. coli* expressing dsRNA-LSNV for 2 hours. *Artemia* containing dsRNA-LSNV were subjected to the feeding test with *P. monodon* postlarvae 1-15. According to RT-PCR analysis, 38% and 74% LSNV-infected shrimp were observed in the dsRNA-treated and control groups, respectively. Quantitative RT-PCR indicated that a number of LSNV copies in most of the treated shrimp were, at least, 1000-fold lower than the untreated controls. During 11-17 weeks after feeding, the average body weight of the treated group was markedly increased relative to the control group. These results suggest that feeding shrimp with the dsRNA-enriched *Artemia* can eliminate LSNV infection, which is the cause of retarded growth in *P. monodon*. An additional investigation on feed formulated with dsRNA-LSNV suggests the potential of this method for delivering dsRNA to juvenile shrimp. In conclusion, therapeutic dsRNA can be delivered to post larval and juvenile shrimp via *Artemia* enrichment and feed formulation, respectively.

KEY WORDS: PENAEUS MONODON, LAEM-SINGH VIRUS (LSNV), DOUBLE-STRANDED RNA (DSRNA), ORAL DELIVERY, *ARTEMIA*

73 pages

การพัฒนาระบบการส่งผ่านอาร์เอ็นเอสายคู่เพื่อใช้ในการป้องกันไวรัสแหลมสิงห์ในกุ้งกุลาดำ

DEVELOPMENT OF DOUBLE-STRANDED RNA-DELIVERY SYSTEM FOR CONTROL OF LAEM-SINGH VIRUS (LSNV) IN THAI *PENAEUS MONODON*

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บทคัดย่อ

กระบวนการ RNA interference (RNAi) ซึ่งอาศัยการทำงานของอาร์เอ็นเอสายคู่ ได้มีการประยุกต์ใช้เพื่อป้องกันการเพิ่มจำนวนของไวรัสในกุ้งอย่างกว้างขวาง โดยงานวิจัยนี้ได้ทำการพัฒนาระบบการส่งผ่านอาร์เอ็นเอสายคู่ขึ้น เพื่อป้องกันไวรัสแหลมสิงห์ซึ่งเป็นสาเหตุของโรคโตช้าในกุ้งกุลาดำ เริ่มต้นจากการทดสอบการส่งผ่านแบคทีเรียที่สามารถผลิตสาร red fluorescent protein (RFP) เข้าสู่อาร์ทีเมีย โดยพบสัญญาณสีแดงของสาร RFP ภายในลำไส้ของอาร์ทีเมียเมื่อส่องใต้กล้อง confocal ซึ่งแสดงให้เห็นอย่างชัดเจนถึงความสำเร็จในการนำส่งสารโดยใช้อาร์ทีเมียเป็นตัวกลาง ในส่วนที่สองคือการใช้อาร์ทีเมียเป็นตัวกลางในการส่งผ่านอาร์เอ็นเอเข้าสู่กุ้ง จากวิธี reverse-transcription PCR (RT-PCR) พบสารอาร์เอ็นเอสายคู่ ภายในอาร์ทีเมียที่ได้รับอาร์เอ็นเอสายคู่จากแบคทีเรียปริมาณ 4.3 x 10^{11} CFU เป็นเวลา 2 ชั่วโมง เพื่อทดสอบประสิทธิภาพในการต้านทานไวรัสแหลมสิงห์ในกุ้ง ได้ทำการให้อาร์ทีเมียที่มีอาร์เอ็นเอสายคู่แก่กุ้งในระยะ postlarvae (PL) 1 ถึง PL15 ผลการทำ RT-PCR พบไวรัสในกุ้งร้อยละ 38% และ 74% ในกุ้งกลุ่มที่ได้รับและไม่ได้รับอาร์เอ็นเอสายคู่ตามลำดับ และเมื่อวิเคราะห์หาปริมาณไวรัสด้วยวิธี real-time RT-PCR พบว่ากุ้งที่ได้รับอาร์เอ็นเอสายคู่มีปริมาณไวรัสที่น้อยกว่ากลุ่มที่ไม่ได้รับถึง 1000 เท่า สำหรับน้ำหนักกุ้งพบว่าสัปดาห์ที่ 11-17 หลังจากได้รับอาร์ทีเมีย กุ้งที่ได้รับอาร์เอ็นเอสายคู่มีน้ำหนักเฉลี่ยมากกว่ากลุ่มที่ไม่ได้รับ จากการทดลองนี้แสดงให้เห็นว่าการส่งผ่านอาร์เอ็นเอสายคู่โดยผ่านอาร์ทีเมียให้ผลในการกำจัดไวรัสแหลมสิงห์ได้ อย่างมีประสิทธิภาพ นอกจากนี้การใช้อาหารเม็ดซึ่งมีส่วนผสมของแบคทีเรียที่มีอาร์เอ็นเอสายคู่แสดงผลในการลดลงของไวรัสแหลมสิงห์ ซึ่งนำไปสู่การเพิ่มขึ้นของน้ำหนักกุ้งเช่นกัน จากการพัฒนาระบบการส่งผ่านสารอาร์เอ็นเอสายคู่นี้ สามารถส่งผ่านอาร์เอ็นเอสายคู่เข้าสู่กุ้งในระยะ PL และ juvenile โดยการใช้อาร์ทีเมียและอาหารเม็ดเป็นตัวกลางในการขนส่งอาร์เอ็นเอสายคู่

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LIST OF ABBREVIATIONS

Abbreviation or symbol	
%	percent
α	alpha
bp	base pair
^{o}C	degree Celsius
CV	coefficient of variation
DNA	deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
dsRNA	double-stranded ribonucleic acid
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
e.g.	exampli grati (Latin) for examples
et al.	*et. alli* (Latin) and others people
g	gram/gravity
h	hour(s)
LB	Lauria-Bertani
LSNV	Laem singh virus
MBW	mean body weight
mg	milligram
min	minute(s)
mL	milliliter
mM	millimolar
µm	micrometer
µg	microgram
µL	microliter
MSGS	Monodon Slow Growth Syndrome
ng	nanogram

LIST OF ABBREVIATIONS (cont.)

Abbreviation or symbol	
nm	nanometer
OD	optical density
RdRp	RNA dependent RNA polymerase
RISC	RNA-inducing silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction
sec	second(s)
SD	standard deviation
Tris-HCl	Tris-(hydroxymethyl)-aminoethane hydrochloric acid
UV	ultraviolet
w	weight
WSSV	White Spot Syndrome Virus
YHV	Yellow Head Virus

CHAPTER I
INTRODUCTION

Monodon slow growth syndrome (MSGS) is one of the most serious problems in the black tiger shrimp *Penaeus monodon* (*P. monodon*) causing massive economic loss in shrimp cultivation. Since the retarded growth problem has been presented, the exportation of *P. monodon* of Thailand rapidly declined from 83,040.26 metric tons (t) valued at 28,300.28 million baht in 2002 to 6,080.20 t valued at 1,986.78 million baht in 2012 (http://www.oae.go.th; Office of Agricultural Economics). During the investigation of causes for MSGS, Laem-singh virus (LSNV) was found (1) and had a potential link to slow growth problems (2).

Many approaches have been used to inhibit viral replication in shrimp including RNA interference (RNAi) technique. RNAi is a process which triggered by double-stranded RNA (dsRNA) targeting its homologous mRNA, and leading to regulation of gene expression. The RNAi application has been demonstrated that it can inhibit replication of several shrimp virus species such as yellow head virus (YHV), white spot syndrome virus (WSSV), and LSNV (3-5). Despite, RNAi has shown potential for protection against viral disease in shrimp, the development of dsRNA delivery system on large scale is important for the successful application of the technology in shrimp farms.

The virus infection in *P.monodon* occurs not only in juvenile or adult, but also in postlarvae (PL) stage (6). So, the protection from viral infection more than one stage of shrimp is necessary for a complete elimination of viral infection. To develop the dsRNA-delivery system for shrimp farm application, the oral administration by using live organism and shrimp feed is the interesting choices. During development, *P. monodon* has a different feeding in each specific stage. In shrimp farm, post larvae (PL) shrimp was normally fed with zooplankton such as *Artemia*, while juvenile was fed with pellet feed. Recently, the delivery of antibody targeting shrimp virus to shrimp by using *Artemia* was reported (7), so *Artemia* is one of the carriers which has

a possibility to transfer dsRNA in to shrimp. For juvenile shrimp, using feed pellets coated with dsRNA is the common methods that have been reported to prevent viral infection in *P. monodon* (4).

All of the reasons, the aims of this study are to develop dsRNA delivery system by two different carriers including *Artemia* nauplii and feed pellets to deliver dsRNA for anti-LSNV infection in *P. monodon*. The results can be applied with the other viruses as model to apply dsRNA to prevent the outbreak of viral infections in shrimp farming.

CHAPTER II
OBJECTIVES

This study was to explore the possibility of protection of *Penaeus monodon* (*P. monodon*) against laem-singh virus (LSNV) infection via RNA interference technology through oral administration of double-stranded RNA (dsRNA) targeting RNA dependent RNA polymerase (RdRp) gene of LSNV (dsRNA-LSNV).

The objective proposed the development of dsRNA-delivery system for antiviral application in shrimp farm. Oral administration was used as a model of dsRNA delivery and divided into two methods. In the first method, shrimp were given dsRNA via *Artemia* containing bacteria expressed dsRNA-LSNV, and in the second, pellet feed mixed with bacteria expressed dsRNA-LSNV. The main objectives were:

1. The oral administration of dsRNA-LSNV through *Artemia* :
    - 1.1. To enrich the bacteria expressing dsRNA-LSNV and red fluorescence protein to *Artemia* nauplii.
    - 1.2. To optimize the condition of dsRNA-LSNV enrichment.
    - 1.3. To test RNAi-mediated inhibitory effect in *P. monodon* after feeding with *Artemia* containing dsRNA-LSNV-expressing bacteria.
2. The oral administration of dsRNA-LSNV through pellet feed :
    - 2.1. To evaluate dsRNA-LSNV in feed after mixed with bacteria expressing dsRNA-LSNV
    - 2.2. To test RNAi-mediated inhibitory effect in *P. monodon* after feeding with pellet feed mixed with dsRNA-LSNV-expressing bacteria.

CHAPTER III
LITERATURE REVIEW

3.1. The black tiger shrimp, *Penaeus monodon*

The black tiger shrimp, *Penaeus monodon* (*P. monodon*), is one of the largest shrimp among *penaeid* in the world, it can grow-up to a large size 200-320 g and 25-30 cm in body length (Fig. 3.1). *P. monodon* is the most popular marine crustacean in aquaculture which is widely reared for food. It has natural inhabited in the coasts of Australia, South East Asia, South Asia and East Africa.

Figure 3.1: The black tiger shrimp, *P. monodon*

3.1.1. Life cycle of *P. monodon*

The life cycle of *P. monodon* starts with three larvae stages including nauplii, protozoea, and mysis (Fig.3.2). Nauplii are the first stage which hatches from the fertilized eggs about 12-15 hour after spawning. They are free swimming and look like tiny aquatic spider. At this stage the larvae do not need a food because it lives on its yolk reserve. After that they have rapid six molts to next stage, called protozoea. The protozoea have feathery appendages and increased their body size and length. After molting three times, protozoea develop into the mysis stage. Mysis have characteristics of adult shrimp including segmented bodies, eyestalk and tails. At this stage, they have three times of molting to become post-larvae. For protozoea and mysis, they feed phytoplankton while the post-larvae (PL) changes habit to feed on zooplankton such as *Artemia*. At PL stage, they molt for 15 times (PL15) and become to juvenile phase. The juvenile takes 4-6 months into sub-adult with body weight of 25-40 g, normally they are found mainly in coast and mangroves. In shrimp farming, juvenile stages are fed with shrimp pellet feed. Finally, they move out to the sea to adult stage with 80-100 g.

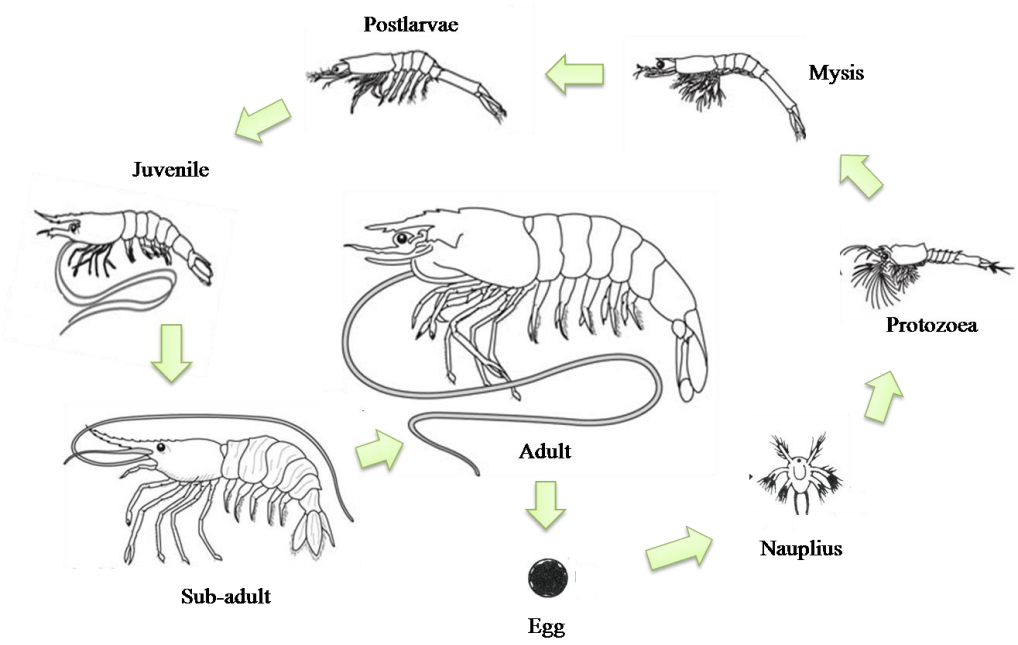


Figure 3.2: life cycle of *P. monodon*

3.1.2. Shrimp *P. monodon* production

The global aquaculture production of *P. monodon* progressively increased from 21,000 tons in 1989 to nearly 800,000 tons in 2010 (FAO - Fisheries and Aquaculture Information and Statistics Service). The main producing countries of *P. monodon* include Viet Nam, Indonesia, Thailand and Malaysia.

In Thailand, shrimp farming started in early 1980s and had become a leader in export of cultivated shrimp in mid 1990. The most popular species for farming in that time was the *P. monodon*. But since 2002, the *P. monodon* farming has been attacked with outbreak syndrome especially monodon slow growth syndrome (MSGS), leading to heavy production losses in Thailand. Resulting of MSGS, the exportation of *P. monodon* of Thailand rapidly declined from 83,040 tons with a value at 28.3 billion baht to 33,054 tons with valued at 8.5 billion baht in 2005 and continued to present. Until 2012, Thailand has exported *P. monodon* 6,080.20 tons with valued at 2.0 billion baht (Office of Agricultural Economics) (Fig. 3.3).

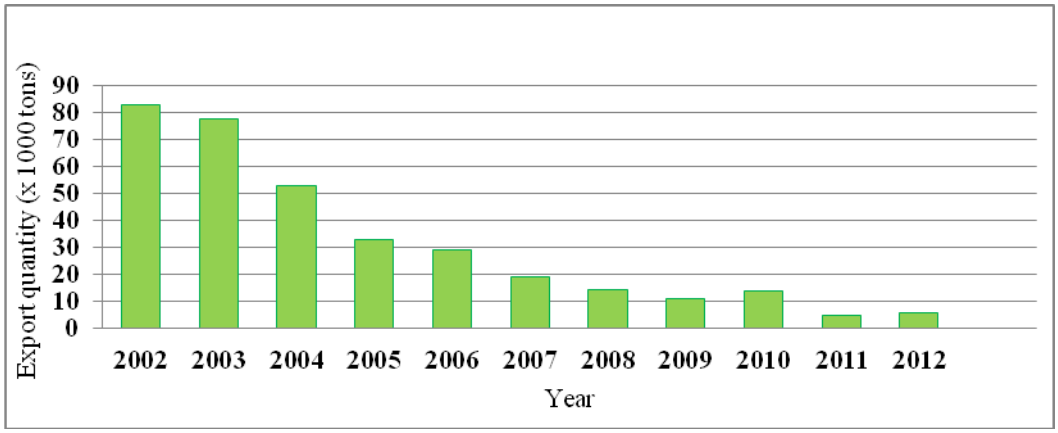


Figure 3.3: Thailand exportation of *P. monodon*

(http://www.oae.go.th/oae_report/export_import/export_result.php)

3.2. Monodon slow growth syndrome (MSGS)

Monodon Slow Growth Syndrome (MSGS) has caused severe production losses in culture of black tiger shrimp *Penaeus monodon* (*P. monodon*). MSGS was first reported in Thailand in the early 2002 by Thai shrimp farmers and has continued to affect cultivation of *P. monodon*. As a result, many farmers have switched to cultivate Pacific shrimp, *Penaeus vannamei*.

3.2.1. MSGS infected shrimp

MSGS is characterized as the unusual growth of shrimp, and a ratio of retarded to normal shrimp is observed in the same pond (Fig. 3.4). Significant difference in the growth rates results in a high coefficient of size variation (CV) of 30 - 80%. Sritunyalucksana *et al.* define the characteristics of MSGS as follows. Differential growth rate CV is more than 35 %. Shrimp must not be infected by hepatopancreatic parvovirus (HPV) or any other severe hepatooancreas infection, plus any 3 out of the 5 following characteristics: (1) unusually dark color, (2) average daily weight gain of less than 0.1 g/day at 4 months, (3) unusually bright yellow markings, (4) “bamboo-shaped” abdominal segments, and (5) brittle antennae (1).

Figure 3.4: Gross sign of the MSGS, unusual retarded growth in MSGS pond of *P. monodon*

3.2.2. Possible causative agent of MSGS

After the outbreak of MSGS in Thailand, several shrimp pathogens such as hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), and infectious hypodermal hematopoietic necrosis virus (IHHNV) were found in both normal and retarded shrimp, consequently these pathogens were not affected to the MSGS. So, this led to the hypothesizes that MSGS caused by a new type of pathogen (8). A new type of yellow head virus (YHV) were observed in some slow growth shrimp of MSGS ponds but the subsequently results revealed that it was hardly to be caused of MSGS (9). It has been reported about a new virus named Laem-Singh Virus (LSNV) (1). This virus was found at optic nerve in MSGS shrimp and not in normal shrimp (10). LSNV could cause growth retardation by damaging optic nerve and inhibiting the release of crustacean hyperglycemic hormone (CHH) which related to growth of the shrimp (2). For these reasons, LSNV infection may be linked to stunting of shrimp.

Laem-singh virus (LSNV) was identified by shot-gun cloning technique. The results revealed the cDNA clone called 20A (GenBank DQ127905) which related to the RNA dependent RNA polymerases (RdRp) of the viruses in the family *Luteoviridae*. However, the phylogenetic anlysis showed that this sequence did not match with the Luteoviridae or others known RNA virus. Thus, this new RNA virus was named Laem-singh virus (LSNV) according to the area where the first infected samples were collected at Laem-Singh district, Chantaburi, Thailand (1).

The characteristic of LSNV was studied and the results showed LSNV is an RNA virus. By Transmission electron micrograph (TEM) showed non-envelope, icosahedral viral-like particles with approximate size 25-30 nm in diameter as shown in Fig 3.5.

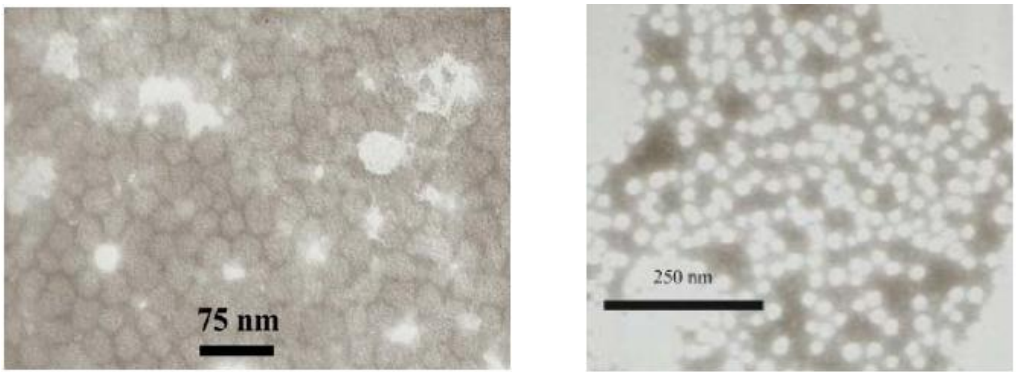


Figure 3.5: Transmission electron micrograph (TEM) of LSNV virions (29).

LSNV can be detected using reverse transcription-PCR (RT-PCR), nested RT-PCR and reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick (RT-LAMP-LFD) (11, 12).

3.3. RNA interference (RNAi)

The RNA interference (RNAi) process was first discovered in the nematode *Caenorhabditis elegans* (13). RNAi is a natural process that cells use to restrain the function of specific genes. This pathway occurs at post-transcriptional level by using double-stranded RNA (dsRNA) to target and eliminates their homologous mRNA, which results in sequence-specific gene silencing (14).

3.3.1. RNAi mechanism

The RNAi pathway starts with long dsRNA was cut by ribonuclease III (RNase III) called Dicer into short dsRNA (21-23 nucleotides) as small interfering RNA (siRNA) as showed in Fig. 3.6. These siRNA were incorporated into multimeric protein complex, as RNA-induced silencing complex (RISC). And the main component of RISC protein complex is an Argonaute (Ago) protein. The siRNA was bound with RISC and unwound into single-stranded RNA. Then, the sense strand will be degraded and the antisense strand remains bind to RISC. The RISC complex will be guided by the antisense strand siRNA to cleaved messenger RNA (mRNA) that has a complementary base-pairing. Finally, target mRNA will be destroyed, silencing the function of specific genes target (15, 16).

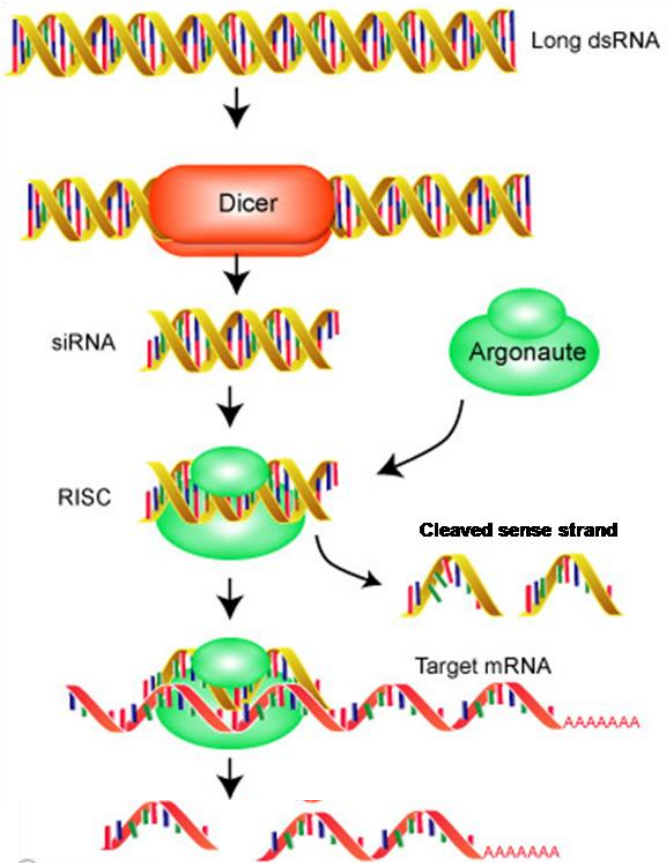


Figure 3.6: The mechanism of RNA interference (http://rnaiweb.com/RNAi/What_is_RNAi/).

3.3.2. RNAi application in shrimp

RNAi is a powerful technique which is widely used in gene functional characterization, and also developed as antiviral agent to prevent infection. In shrimp, the RNAi technology through dsRNA was first reported by Robalino and collaborators in 2004. They shown that dsRNA can be protected shrimp (*Litopenaeus vannamei*) from viral infection (17). Then, there are many publications reported about dsRNA which both specific to viral gene and host gene having ability to inhibit the replication of virus such as white spot syndrome virus (WSSV), taura syndrome virus (TSV), yellow head virus (YHV), gill-associated virus (GAV), densovirus (DNV) and laem-sigh virus (LSNV) (16). For these reasons, RNAi technique is a powerful tool which

high potential for use as a therapeutic treatment against viral disease in shrimp. However, the delivery system of dsRNA on a large scale should be considered and improved for the RNAi application successfully in shrimp farming.

3.3.3. Oral delivery of dsRNA into shrimp

To apply RNAi technology in shrimp cultivation, oral administration was a method of choice to deliver dsRNA into shrimp. The practical way was done by feeding shrimp with feed pellets or live organism that contained dsRNA.

3.3.3.1 Oral delivery of dsRNA by using pelleted feed: There are studies showing possibility of oral administration using dsRNA-contained feed can protect shrimp from viral infections. For example, the inhibition of white spot syndrome virus (WSSV) replication in *Penaeus monodon* was observed by feed pellets that coated with inactivated bacteria expressing VP28-targeted dsRNA (4). Moreover, this method can be applied with the viral component protein as protein vaccination. Feed coated with bacteria expressing recombinant VP28 protein prevented WSSV infection (18, 19). For these reasons, oral administration of dsRNA using feed pellets is a potential method for antiviral infection in farm application.

3.3.3.2 Oral delivery of dsRNA by using *Artemia* as carriers: *Artemia* is widely used as a carrier for various dietary components such as essential nutrients, hormones, and therapeutic vaccine (20, 21). Bioencapsulation of *Artemia* nuaplii for against disease has been studied in many groups. For example, Lin *et al*., 2007 reported the use of *Artemia* as a carrier for combating infectious virus in early stage of marine culturing such as larvae of grouper (22). Recently, *Artemia* nauplii have been exploited to deliver antibody into shrimp for controlling diseases, such as white spot syndrome virus and vibriosis, in aquaculture (7). According to these researches, oral administration of dsRNA by using *Artemia* could be possible applied for protection of shrimp against viruses.

3.4. The brine shrimp, *Artemia*

The brine shrimp known as *Artemia* is zooplankton which is used as a live food for newly hatched fish and shrimp larvae (Fig 3.7). *Artemia* has been known an excellent food for aquatic animals, because of convenience in production and their suitable biochemical composition and size for many stages of different aqua species.

Figure 3.7: *Artemia spp.*

The first stage of *Artemia* is called instar I has a brownish-orange color with size about 400 to 500 µm**.** The instar I do not feed but thrive on their yolk reserves. It is important to feed instar I to larvae animal rather than second stage (instar II), because instar II are about 50% larger in length and contain lower amounts of free amino acids.

To manipulate the nutritional value of *Artemia*, the method of bioencapsulation, also called *Artemia* enrichment or boosting is widely applied to improve larvae output. This method not only enhances growth and quality of many fish and crustaceans, but same method also being developed for oral delivery of chemotherapeutic and vaccines (23-25).

CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemical reagents

Chemicals used in this study were analytical and biotechnological grade and purchased from BD (Oxford, UK), Bio-basic (Markham, ON, Canada), Merck (Damstadt, Germany), Qiagen (Limburg, Netherlands), Roche (Indianapolis, IN, USA), Sigma (St. Louis, MO, USA), and Thermo Scientifics (Manchester, UK).

These included 70% ,75% ethanol, absolute ethanol, agarose, tetracycline, ampicillin, chloroform, isopropanol, diethyl pyrocarbonate (DEPC), dideoxynucleotide triphosphates (dNTPs), DNase/RNase free distilled water, ethidium bromide, glycerol, phenol, sodium acetate, sodium chloride, Tripure reagent, tryptone, isopropyl-β-D-thaiogalactopyranoside (IPTG), and yeast extract.

4.1.2 Restriction enzymes and miscellaneous materials

Restriction and DNA modifying enzymes were acquired from New England Biolabs (Massachusetts, USA) and Promega (Madison, USA), and QuantiTect® SYBR® Green RT-PCR kit was purchased from Qiagen (Limburg, Netherlands). Standard DNA markers were supplied by New England Biolabs (Massachusetts, USA). and Transcripter III One-Step RT-PCR was purchased from Roche (Indianapolis, IN, USA).

4.1.3 Equipments

Equipment used in this study including a -80^{o}C deep freeze (Sanyo, Model 790), gel electrophoresis sets (Mupids, Japan and BioRad, Hercules, CA, USA), a heat block (Accublock^{TM} Digital Dry Bath, LabNet International Inc., USA), a high speed centrifuge (Hettich, Germany), a high speed micro, refrigerated centrifuge (Hettich, universal 32 R, Germany), 37^{o}C incubators (Heraeus instrument, B6120, Germany), an incubator shaker (Thermoelectron corporation, USA), a PCR machine (ABI 7500 SDS Applied biosystem, Foster, CA, USA and Biometra Tgradient (Germany), a spectrophotometer (UV121 UV-VIS SHIMADZU, Japan), a vortex mixer (Vortex-Genie2, Scientific Industries Inc., USA), a water bath (Memmert, Germany) , ABI 7500 Real-time PCR System (Applied Biosystems, USA) and confocal laser microscopy (FV1000, Olympus, Japan).

4.1.4. Bacterial strains and media

Escherichia coli (*E. coli*) strain HT115 was used as hosts for RNA expression. *E. coli* was grown in Lauria-Bertani (LB) medium and selected on LB media containing antibiotics containing 100 μg/mL ampicillin and 12 μg/mL tetracycline.

4.1.5. Primers

All primers were manually designed or using Primer3 program and synthesized by Bio-basic Laboratories (Markham, ON, Canada). The nucleotide sequences of the primers were shown in Table 4.1.

Table 4.1 Nucleotide primer sequences used for RT-PCR amplification of dsRNA-LSNV, LSNV and host gene

Primer	**Sequence (5'→3')**	**%GC**	**Tm (˚C)**
LV_sense 484	GGGTGAGCCCGTGACTCCTA	65	64
LV_As 484	GCCCCAGAAACGTATTGGCAC	57	62
Luteo-F600	CGTTGCCTTCTCCCGAGTGGT	62	67
Luteo-R600	TTGCCCCAGAAACGTATTGGCA	50	63

Table 4.1 Nucleotide primer sequences used for RT-PCR amplification of dsRNA-LSNV, LSNV and host gene (cont.)

Primer	**Sequence (5'→3')**	**%GC**	**Tm (˚C)**
20AR	CCGGCTGAGGTAGCTGCTTG	65	64
Actin_arte_F	GGTCGTGACTTGACGGACTATCT	52	62
Actin_arte_R	AGCGGTTGCCATTTCTTGTT	45	56
Pm_EF1	GAACTGCTGACCAAGATCGACAGG	54	64
Pm_ER1	GAGCATACTACTGTTGGAAGGTCTCCA	50	62

4.1.6. Experimental shrimp

Black tiger shrimp *Penaeus monodon* (*P. monodon*) were carried out at Shrimp Genetic Improvement Center (SGIC), Surat Thani, Thailand. Shrimp was divided into two experiments.

- In dsRNA delivery using *Artemia* nauplii, *P. monodon* post larvae 1 (PL1) were reared in 200-L tank with a density of 30 PL per L, 28 ±2 °C, and 25-ppt salinity. They were fed with commercial diet (TNT 3, Charoen Pokphand food PCL., Thailand) before and during experiment.
- In dsRNA delivery using feed pellet, *P. monodon* with the average size of 10-15 g were reared in 113 m^2 pond area with a density of 11 shrimp per m^2 at 28 ±2 °C, and 25-ppt salinity. They were fed with feed diet (done by SGIC) before experiment.

4.2. Methods

4.2.1. *Artemia* nauplii preparation

Artemia cysts (Aqua brand, USA) were incubated in artificial seawater at 35 ppt salinity at temperature of 28±2℃ with aeration. After 24-h incubation, *Artemia* nauplii (instar I stage) hatched, and were separated from cyst shells and stocked in the tank containing seawater for enrichment study.

4.2.2. Shrimp pellet feed

Formulation of feed containing *E. coli* expressing dsRNA was performed according the method described by Saksmerprome *et al.*, 2013 (3) (feed ingredient and method was described in Appendix A). There were two formulas of feed based on dsRNA-LSNV concentration produced approximately 6 and 12 mg dsRNA-LSNV, formulas-1 and 2 respectively.

In this part, feed pellet was produced and the experiments were done by Shrimp Genetic Improvement Center (SGIC), Surat Thani, Thailand. The responsibility in this part were 1) preparation of *E. coli* expressing dsRNA, 2) detection of dsRNA in feed pellet after formulation, and 3) detection of LSNV in shrimp after fed with developed-feed.

4.2.3. Preparation of double-stranded RNA (dsRNA)

4.2.3.1. Induction of bacterially (*E. coli* HT115) expressed dsRNA targeting LSNV gene (dsRNA-LSNV): The recombinant plasmid containing LSNV-RNA dependent RNA polymerase (RdRp) hairpin gene was provided by Dr.Vanvimon Saksmerprome and transformed into RNase III deficient, *E. coli* HT115 (DE3) strain. dsRNA targeted LSNV RdRp gene (dsRNA-LSNV) was produced from bacterial culture according the method described by Saksmerprome *et al*., 2009 (3). Briefly, single transformant colony was inoculated into 3 mL of Luria-Bertani (LB) medium containing ampicillin and tetracycline. The bacterial culture was shaken 250 rounds per minute (rpm) at 37°C for 16 hour and subsequently inoculated bacterial culture at the ratio 1:100 into fresh LB medium containing ampicillin and tetracycline.

The culture was shaken until $OD_{600\ nm}$ of 0.4. Over-expression of LSNV-RdRp hairpin gene was induced by isopropyl-β-D-thaiogalactopyranoside (IPTG) at a final concentration of 0.5 mM and incubated again for 4 hour. The *E. coli* HT115 without transformed was used as the negative control.

4.2.3.2. Evaluation of dsRNA-LSNV in *E. coli* HT115: The quality of dsRNA-LSNV produced from bacterial culture was checked by RNA extraction. Bacterial cells were collected from 12.5 mL bacteria culture by centrifugation at 4,500 x g for 5 min at 4°C. The cell pellet was dissolved in 700 µl Tris – EDTA buffer (pH8). To extract total nucleic acid, 700 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the cell solution and vortex for 15 second then incubated at 65 °C for 10 minutes. The sample was separated into three phases was collected by centrifugation at 12,000 x g for 10 min at 4°C. The upper phase (aqueous phase) was transferred to fresh tube. Total nucleic acid was precipitated from aqueous phase with 0.2 volumes of 3M NaOAC and 1.1 volumes of absolute ethanol per 1 mL of aqueous phase. After 16 hour of incubation at – 20°C, Total nucleic acid pellet was collected by centrifugation at 12,000 x g for 10 min at 4°C and washed with cold 75% ethanol, followed by centrifugation at 7,500 x g for 5 min at 4°C. Total nucleic acid pellet was dried and dissolved with diethylpyrocarbonate (DEPC) water. To eliminate the DNA and single-stranded (ss) RNA loop region of hairpin dsRNA, 0.33 M NaCl, RNase A and DNase was added to the solution and incubated at 37°C for 1 hour. To remove RNase A and DNase from the sample, 1:1 (v/v) of phenol: chloroform: isoamyl alcohol was added and followed by step as explain above. Finally, dsRNA-LSNV was determined by measuring UV absorbance (UV121 UV-VIS SHIMADZU, Japan) at 260 and 280 nm. The size and quality of dsRNA-LSNV were analyzed by 1.5% agarose gel electrophoresis.

4.2.4. Preparation of marker protein

The pCS2 containing red fluorescence protein gene (pCS2-RFP) was provided by Dr. Siripong Thitamadee. The pCS2-RFP was used as the template for PCR amplification of RFP gene with the primers, RFP_for (5'-GAT CCA CCA TGG CCT CCT-3') and RFR_rev (5'- ATC CTT AGG CGC CGG TG-3'). The RFP product was cloned into pDrive cloning vector (Qiagen, Valencia, CA, USA). The

recombinant plasmid, pDrive-RFP, was confirmed by DNA sequencing. The resulting pDrive-RFP was transformed into *E. coli* HT115 using heat shock method and induced with IPTG to express RFP marker protein. After over-expression of RFP gene, RNA was extracted by phenol: chloroform: isoamyl alcohol (25:24:1) and treated with DNase to remove DNA, according to the method described in 4.2.3.2. The size and quality of RNA of RFP gene were analyzed by 1.5% agarose gels electrophoresis.

4.2.5. dsRNA delivery using *Artemia* nauplii

4.2.5.1. Enrichment of *Artemia* nauplii with *E. coli* expressing marker protein, red fluorescence protein (RFP): The harvested *Artemia* nauplii at a density of 100 nauplii per 1 mL seawater were stocked for enrichment. Total of 10^5 nauplii were enriched with 1.6 x 10^{10} CFU *E. coli* expressing RFP. At enrichment durations of 4, 6, 8, 22, 24, 26, 28, and 30 h, 10-20 nauplii were collected, fixed by immersion into 70% ethanol and stored at 4°C. To examine RFP signals, the enriched nauplii were suspended in 70% ethanol solution on a microscopic slide for confocal microscopy (FV1000, Olympus, Japan).

4.2.5.2. Enrichment of *Artemia* nauplii with *E. coli* expressing dsRNA-LSNV:

4.2.5.2.1. Laboratory scale (small scale): Approximately 2 x 10^5*Artemia* nauplii in 2,000 mL of sea water were enriched with 8 x 10^9 CFU of *E. coli* expressing dsRNA-LSNV. At 0, 2, 6, 22 h of enrichment, the 2 x 10^4 *Artemia* were collected, washed with fresh artificial seawater for 10 times and stored in 500 µl TriPure isolation reagent (Roche, USA) at -20 °C for further analysis. The *E. coli* HT115 without expressing dsRNA-LSNV was used as a control group.

4.2.5.2.2. Pilot scale (large scale): Approximately 2 x 10^6*Artemia* nauplii in 100 mL of seawater were enriched with 1.1 x 10^{11}, 2.2 x 10^{11}, 4.3 x 10^{11} and 6.5 x 10^{11} CFU of *E. coli* expressing dsRNA-LSNV. At 0, 0.5, 1, 2, and 3 h of enrichment, the 2.0 x 10^4 *Artemia* were collected, washed with fresh artificial seawater for 10 times and stored in 500 µl Tripure isolation reagent at -20°C for further analysis. The *E. coli* HT115 without expressing dsRNA-LSNV was used as a control group.

4.2.5.3. Evaluation of dsRNA-LSNV in *Artemia* nauplii

4.2.5.3.1. Extraction of dsRNA-LSNV from *Artemia* nauplii: Total RNA from *Artemia* sample was extracted using Tripure isolation reagent, according to the manufacturer's instructions. Briefly, the *Artemia* sample was homogenized in Tripure isolation reagents. Then, chloroform was added into the sample tube and vortexed for 15 second. The sample was separated into three phases by centrifugation. The upper phase (aqueous phase) was transferred to fresh tube and precipitated RNA from aqueous phase with 3M NaOAC and absolute ethanol. After 16 hours of incubation at – 20°C, RNA pellet was collected by centrifugation and washed with cold 75% ethanol. RNA pellet was dried and dissolved with DEPC water. To eliminate DNA contamination, 1 µl of DNase was added to the 100 µl RNA solutions and incubated at 37°C for 1 h. After that, DNase was removed from the solution by phenol: chloroform: isoamyl alcohol, according to the method described in 4.2.3.2. Finally, the concentration of total RNA was determined by spectrophotometer at 260 and 280 nm.

4.2.5.3.2. Detection of dsRNA-LSNV in *Artemia* nauplii: dsRNA- LSNV in total RNA of *Artemia* sample was detected using transcriptor one-step RT-PCR kit (Roche, Germany).The 1-step RT-PCR was performed with two LSNV-specific primers (LV_sense 484 and LV_As 484) and two *Artemia* actin primers (Actin_arte_F and Actin_arte_R) was used as internal control. Approximately 150 ng of total RNA were used for 1-step RT-PCR analysis. Each 12.5 µl RT-PCR master mix contained 3 µl diluted template, 400 nM of each primer, 2.5µl of 5x reaction buffer, 0.25 µl of transcriptor enzyme mix and 5.75µl of RNase free water. Thermal cycling was performed on a TPersonal Thermocycler (Biometra). The 1-step RT-PCR reaction was carried out with the following sequential profile:

•	50°C	5 min
• 35 cycles:	94°C	10 sec
	60°C	30 sec
	68°C	30 sec
•	68°C	5 min

Then, PCR product was analyzed with 1.5 % agarose gel in TAE buffer. The PCR bands in the gel were used to determine dsRNA-LSNV positive or negative results by comparison to the pattern of the PCR product of pGEM containing LSNV gene used as a positive control.

4.2.5.4. Evaluation of RNAi-mediated inhibitory effect in *P. monodon*: The study was carried out at Shrimp Genetic Improvement Center (SGIC), Surat Thani, Thailand. Shrimp *P. monodon* post larvae 1 (PL1) were reared in 200 – L tank with a density of 30 PL/L, 28 ± 2 °C, and 25-ppt salinity. Shrimp were divided into 2 groups, one received *Artemia* containing dsRNA-LSNV and the other fed with *Artemia* were incubated without enrichment (non enriched *Artemia*). The feeding rate for PL1 was 0.5 g *Artemia* per 10^3 PL at every 6 h, and was then increased to meet feed requirement of other PL as showed in Fig 4.1. The shrimp were fed with the enriched *Artemia* nauplii for 15 consecutive days until PL15. Mean body weights (MBW) of shrimp (n=50) were recorded during 4-17 weeks after feeding with *Artemia*. The MBW values were used to calculate percentage of coefficient of variations, CV (%). A statistical analysis was performed using paired-samples T Test, and $P < 0.05$ was considered statistically significant. At 15 weeks after feeding with *Artemia*, 50 shrimp from each group were randomly selected, and their pleopods were diagnosed for LSNV.

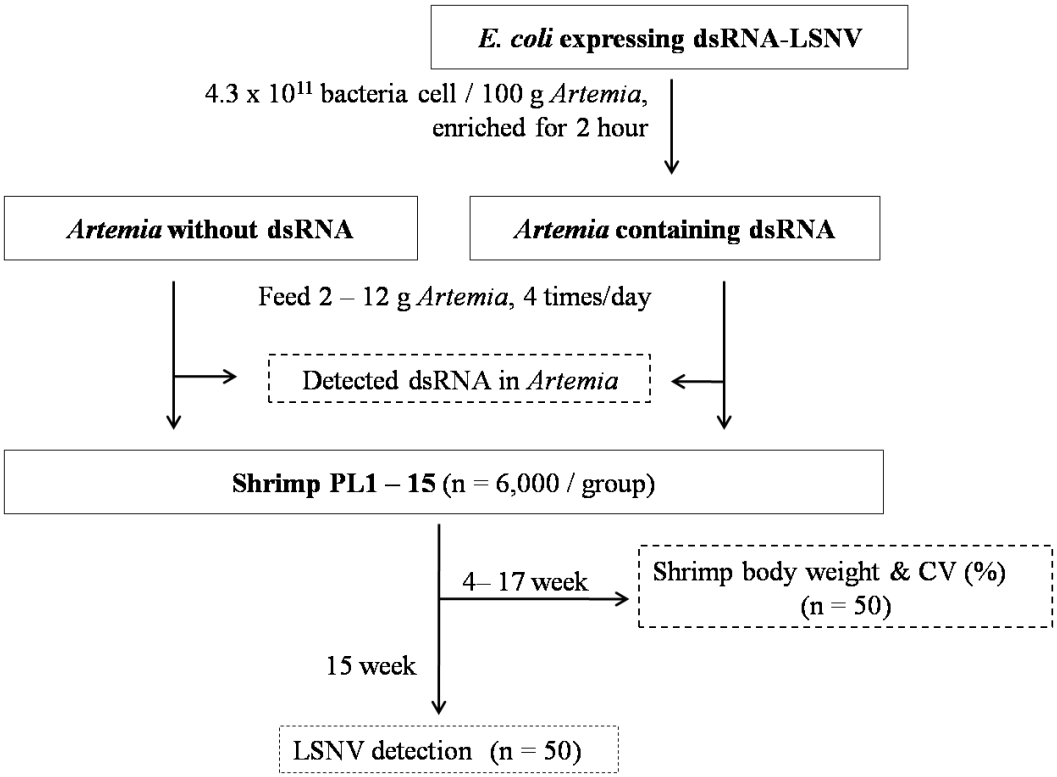


Figure 4.1: Evaluation of RNAi-mediated inhibitory effect in *P. monodon* through *Artemia* nauplii

4.2.5.4.1. Evaluation of *Artemia* containing dsRNA-LSNV: The enriched-*Artemia* was randomly collected daily (15 days) and the non-enriched samples were collected on days 1, 5, 10 and 15and subjected to dsRNA-LSNV detection. The total RNA of *Artemia* was extracted and detected dsRNA-LSNV by 1-step RT-PCR, according to the method described in 4.2.5.3.

4.2.5.4.2. Extraction of shrimp RNA: Using Tripure isolation reagent, total RNA was extracted from shrimp pleopods. The same method of RNA extraction as described in 4.2.5.3.1. was used. The concentration of total RNA was determined by spectrophotometer at 260 and 280 nm and subjected to LSNV detection.

4.2.5.4.3. Detection of LSNV in shrimp

Reverse-transcription PCR (RT-PCR) analysis: To detect LSNV in total RNA of shrimp sample using transcriptor one-step RT-PCR kit. The 1-step RT-PCR was performed with two LSNV-specific primers including Luteo-F600 and Luteo-R600. The primers derived from shrimp elongation factor including Pm_EF1 and Pm_ER1 was used as internal control. Approximately 150 ng of total RNA were used in same RT-PCR master mix as described in 4.2.5.3.1. The 1-step RT-PCR reaction was carried out with the following sequential profile:

•	50°C	5 min
• 35 cycles:	94°C	10 sec
	55°C	30 sec
	68°C	30 sec
•	68°C	5 min

Then, PCR product was analyzed with 1.5 % agarose gel in TAE buffer. The PCR bands in the gel were used to determine LSNV positive or negative results by comparison to the pattern of the PCR product of pGEM containing LSNV gene used as a positive control.

Real-time RT-PCR analysis: Approximately 250 ng of total RNA were used for real-time RT-PCR analysis. To detect the level of RdRp mRNA of LSNV in shrimp, the primer pairs were Luteo-F600 and 20AR. Each 25 µl PCR master mix contained 5 µl diluted template, 400 nM of each primer, 12.5µl of 2× SYBR® Green PCR Mix (Applied Biosystems) and 5.25 µl of RNase free water. Thermal cycling was performed on a 7500^{TM} Real-Time PCR System (Applied Biosystems) using the conditions as follows:

•	50°C	30 min
•	95°C	15 min
• 35 cycles:	94°C	15 sec
	65°C	30 sec
	72°C	30 sec

All reactions were run in triplicates and the same PCR master mix and thermocycler conditions as described above were used. To generate a standard curve, a pGEM containing LSNV gene was used to make 10-fold serial dilutions from 10^{10} copies of plasmid DNA down to one starting molecule. The same PCR master mix and thermocycler conditions as described above were used.

4.2.6. dsRNA delivery using feed pellet containing dsRNA-LSNV

4.2.6.1. Evaluation of dsRNA-LSNV in feed pellet: To confirm the presence of dsRNA in feed, total RNA were extracted from 3-5 pellets (approximately 0.05 g) using Tripure isolation reagent, according to the method in 4.2.5.3.1. The concentration of total RNA was determined by spectrophotometer at 260 and 280 nm and subjected to dsRNA-LSNV detection.

To detect dsRNA-LSNV from each feed types, the 1- step RT-PCR analysis was performed. The same method of dsRNA- LSNV detection described in 4.2.5.3.2. was used. Then, PCR product was analyzed with 1.5 % agarose gel in TAE buffer to indicate the presence of dsRNA-LSNV product.

4.2.6.2. Study RNAi-mediated knockdown efficiency: Shrimp with the average size of 10–15 g were divided into 3 groups. Shrimp in group #1 were treated with control feed (without addition of dsRNA-LSNV), whereas groups 2 and 3 were treated with the developed formulas 1 and 2, respectively. Feeding dose rate was at 3% of their body weight, and shrimp were fed 4 times a day. Initial and final body weights of random samples of 100 shrimp were recorded and used for calculating percentage of coefficient of variations (%CV) of each group. A statistical analysis was performed using one-way ANOVA, and $P < 0.0001$ was considered statistically significant. At the end of experiments, nine to ten shrimp from each group were randomly selected, and their pleopods were collected LSNV detection as described in Fig. 4.2.

For LSNV detection, total RNA was extracted using Tripure reaction according to the method in 4.2.5.3.1. and detected LSNV by 1-step RT-PCR following 4.2.5.3.2.

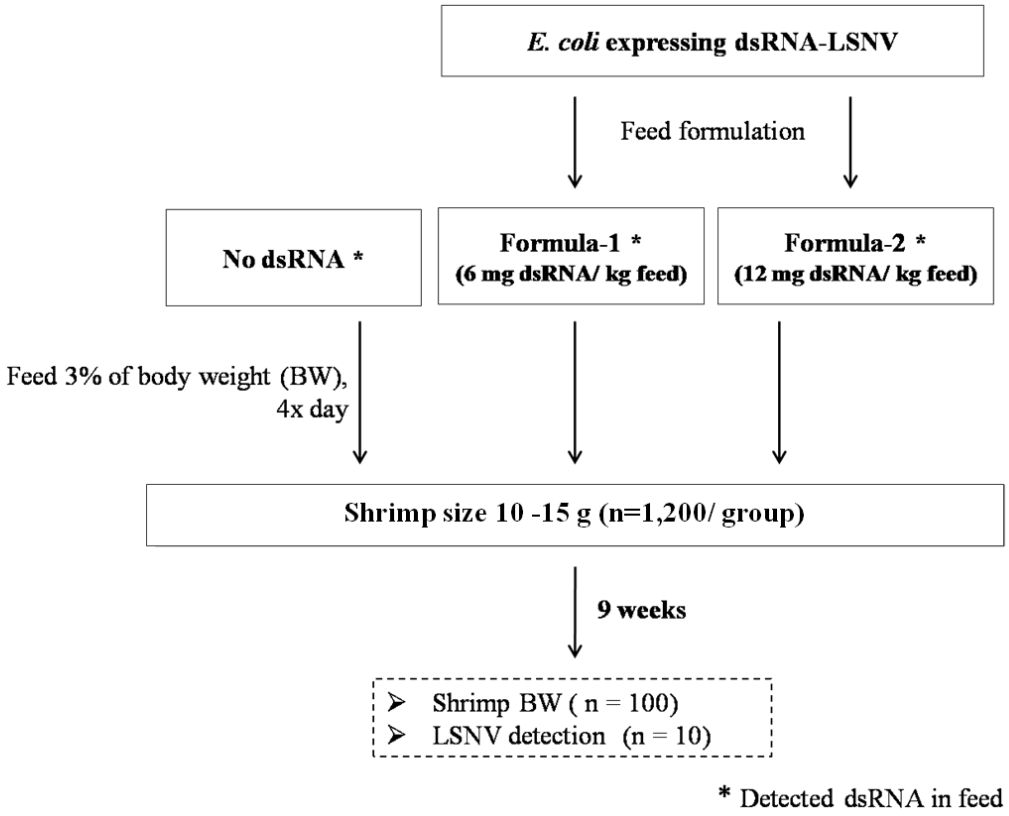


Figure 4.2 : Evaluation of RNAi-mediated inhibitory effect in *P.monodon* through feed pellets

CHAPTER V
RESULTS

5.1 Preparation of bacterially (*E. coli* HT115) expressed double-stranded RNA (dsRNA)

The bacterial expression system was used to produce hairpin RNA targeting LSNV-RNA dependent RNA polymerase (RdRp) gene. After removal of hairpin loop by RNase A, the size of dsRNA targeting LSNV (dsRNA-LSNV) was 484 bp (red arrow) and the quality of the transcribed RNAs was examined by 1.5% agarose gel electrophoresis (Fig. 5.1). Lanes 2 and 3 indicated undiluted and diluted (1:5) dsRNA-LSNV, respectively. Lanes 4 and 5 indicated undiluted and diluted (1:5) of induced HT115 (DE3) without transformation of the LSNV- specific hairpin plasmid as a control group.

5.2 Preparation of bacterially (*E. coli* HT115) expressed marker protein, red fluorescence protein (RFP)

The red fluorescence protein gene (RFP) was provided by Dr. Siripong Thitamadee. The pDrive-RFP was constructed by Miss Parinyachat Somchai and Miss Sarocha Jitrakorn. The recombinant plasmid was transformed into *Escherichia coli (E. coli)* HT115 and used for RFP expression. After eliminating DNA with DNase enzyme, the size of mRNA of RFP gene was 800 bp (red arrow) and examined the quality of the transcribed RNAs by 1.5% agarose gel electrophoresis (Fig. 5.2)

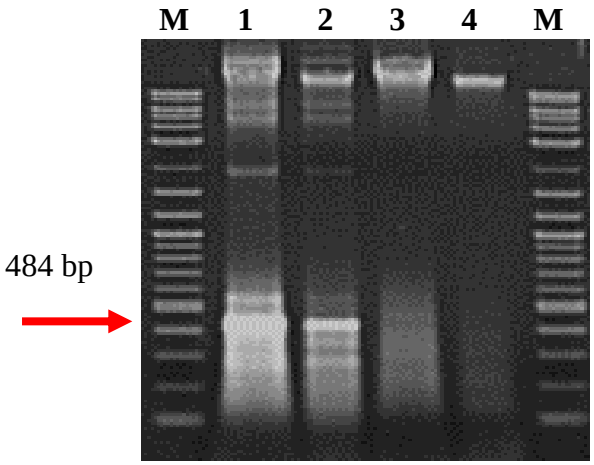


Figure 5.1: Analysis of RNA produced by the hairpin-expressed plasmid. Lane M, 2 log DNA marker. Lane 1 and 2, undiluted and dilution 1 : 5 of dsRNA-LSNV from HT115 (DE3) transformed with the LSNV- specific hairpin plasmid, respectively. Lane 3 and 4, undiluted and dilution 1: 5 of induced HT115 (DE3) without transformation of the LSNV- specific hairpin plasmid.

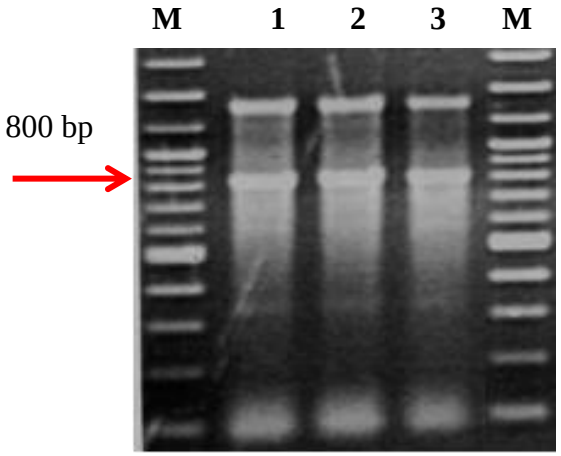
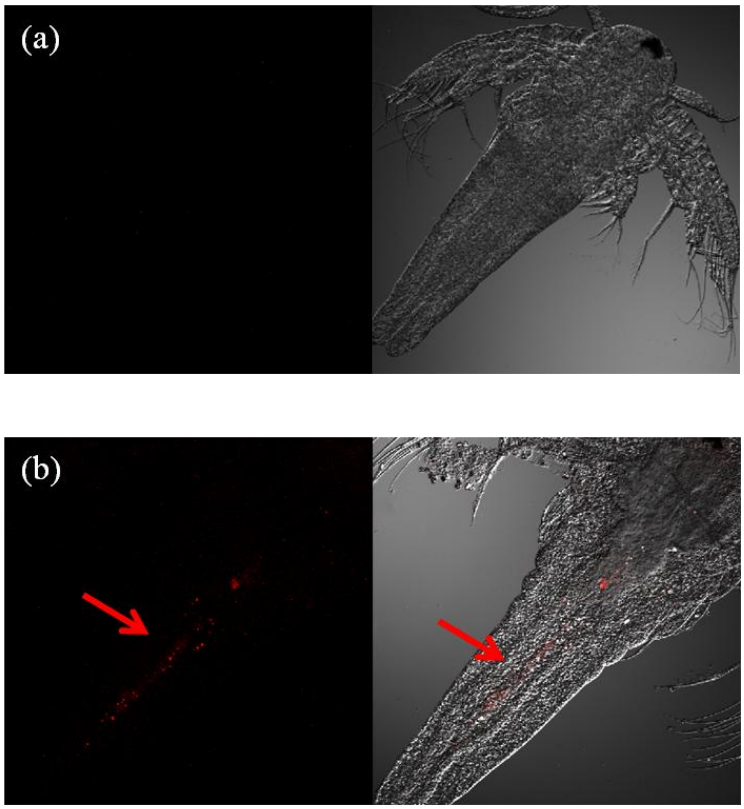


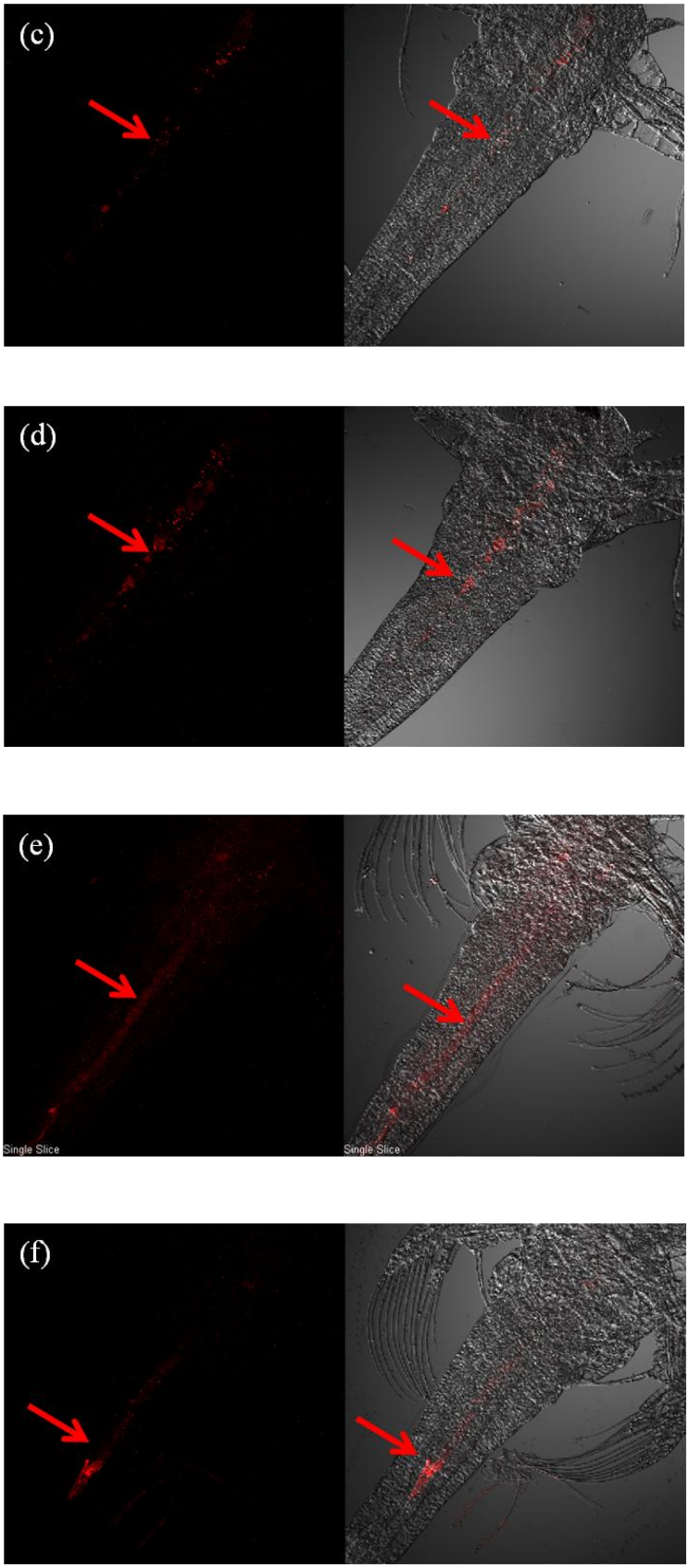
Figure 5.2: Analysis of RNA produced by the pDrive-RFP plasmid. Lane M: 2 log DNA marker. Lane 1-3, mRNA of RFP gene from HT115 (DE3) transformed

5.3. dsRNA delivery using *Artemia* nauplii

5.3.1. Detection of red fluorescence protein (RFP) in the gut of *Artemia* using confocal microscopy

At duration of enrichments 0, 4, 6, 8, 22, 24, 26, 28, and 30 h, *Artemia* nauplii were collected, and fixed in 70% ethanol. RFP was analyzed by confocal microscopy. The results shown that red fluorescent signals were detected in the *Artemia* gut (red arrow) at 4 to 30 h, whereas no red signal was observed from *Artemia* at 0 h (before enrichment) (Fig. 5.3- A). Moreover, red signal in *Artemia* gut showed a progressive increase in red color density from 4 to 28 h, and a small decrease in 30 h (Fig 5.3- B-I). Thus, this experiment suggested that RFP was delivered into *Artemia* by enrichment with *E. coli* expressing RFP. Moreover, RFP can accumulate in *Artemia* gut for a long time (at least 28h).




(c)
(d)
(e)
Single Slice
Single Slice
(f)

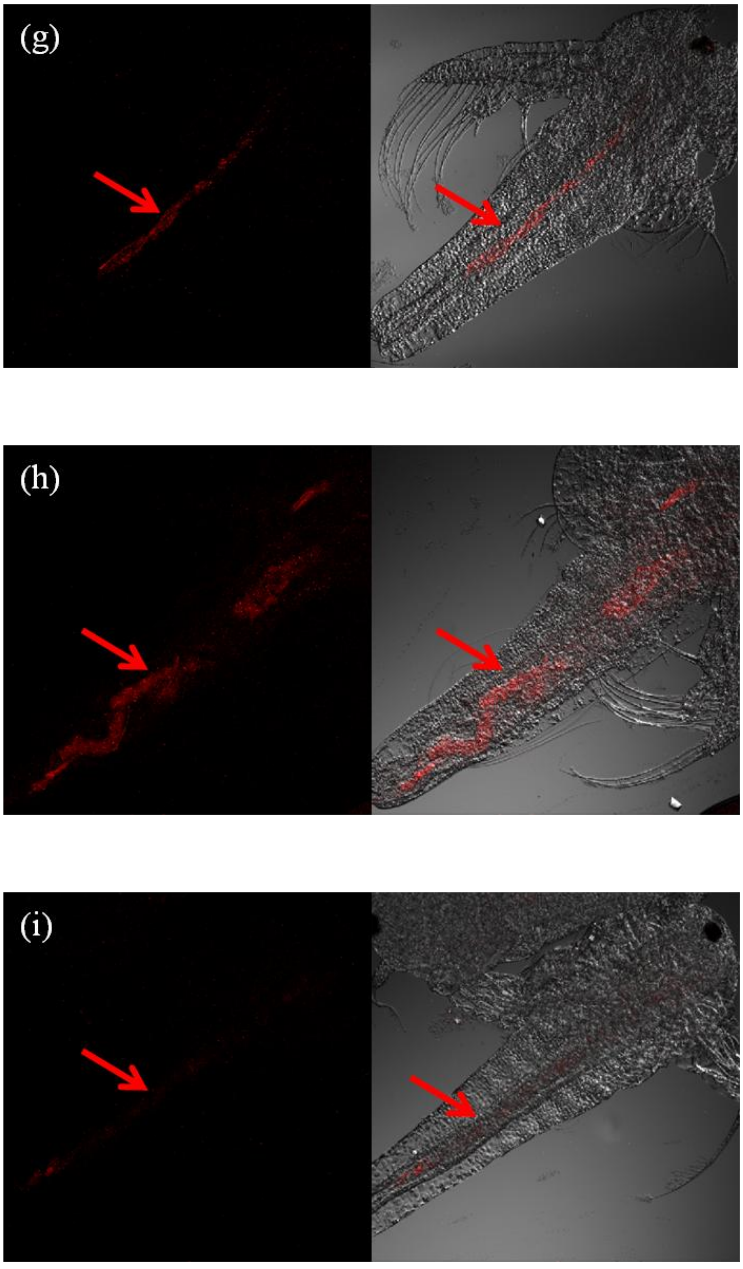


Figure 5.3 : Confocal microscopy of the *Artemia* gut with the *E. coli* RFP-enrichment periods of 0 (a); 4 (b); 6 (c); 8 (d); 22 (e); 24 (f); 26 (g); 28 (h) and 30 h (i)

5.3.2. Evaluation of dsRNA-LSNV in *Artemia* nauplii

5.3.2.1. Laboratory scale: *Artemia* nauplii were enriched with 8 x 10^9 CFU of *E. coli* expressing dsRNA- LSNV. The samples were collected at 2, 6, 22 h of enrichment and *Artemia* nauplii which enriched with *E. coli* HT115 without expressing dsRNA-LSNV were used as a control group. The total RNA was extracted from *Artemia* samples by using Tripure isolation reagent. The level of dsRNA- LSNV in each RNA sample was amplified by 1- step RT-PCR with specific primer to LSNV. Gel electrophoresis results indicated the expected size of dsRNA-LSNV observed only in *Artemia* enriched with *E. coli* expressing dsRNA-LSNV group at 2 and 6 h. In contrast, dsRNA-LSNV was not observed in *Artemia* before enriched and in control group (Fig. 5.4). Therefore, *Artemia* could be enriched with *E. coli* expressing dsRNA-LSNV and could be used to deliver dsRNA into shrimp.

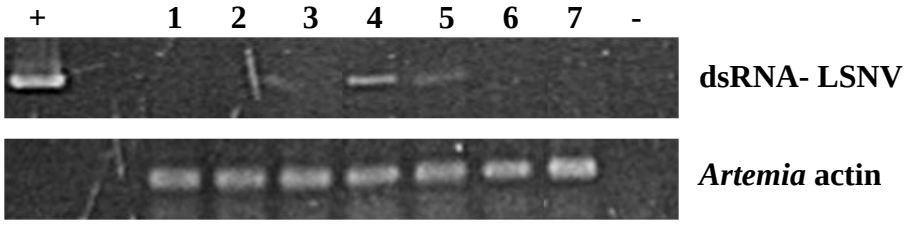


Figure 5.4 : RT- PCR amplification of dsRNA targeting LSNV in total RNA of *Artemia* samples. Lane +, LSNV-cloned plasmid as positive control; lanes 1-3, *Artemia* enriched with *E. coli* HT115 without expressing dsRNA-LSNV at 2, 6 and 22 h; lanes 4-6, *Artemia* enriched with *E. coli* HT115 expressing dsRNA-LSNV at 2, 6 and 22 h; lane 7, *Artemia* before enrichment and lane -, DEPC instead of RNA template as a negative control.

5.3.2.2. Large scale: *Artemia* nauplii were enriched with 1.1 x 10^{11}, 2.2 x 10^{11}, 4.3 x 10^{11} and 6.5 x 10^{11} CFU of *E. coli* expressing dsRNA-LSNV. At 0, 0.5, 1, 2, and 3 h of enrichment, the 2.0 x 10^{4} *Artemia* were collected and subjected to RNA extraction. The *E. coli* HT115 without expressing dsRNA- LSNV was used in the control group. The total RNA of *Artemia* was extracted and determined the level of dsRNA-LSNV by 1-step RT-PCR. The results revealed that dsRNA-LSNV could be detected within 1 h of enrichment with 2.2 x 10^{11} CFU of *E. coli* expressing dsRNA-LSNV (Fig. 5.5- A), 0.5 h of enrichment with 4.3 x 10^{11} and 6.5 x 10^{11} CFU (Fig. 5.5 - B) but not in control groups (Fig. 5.6 - A, B).

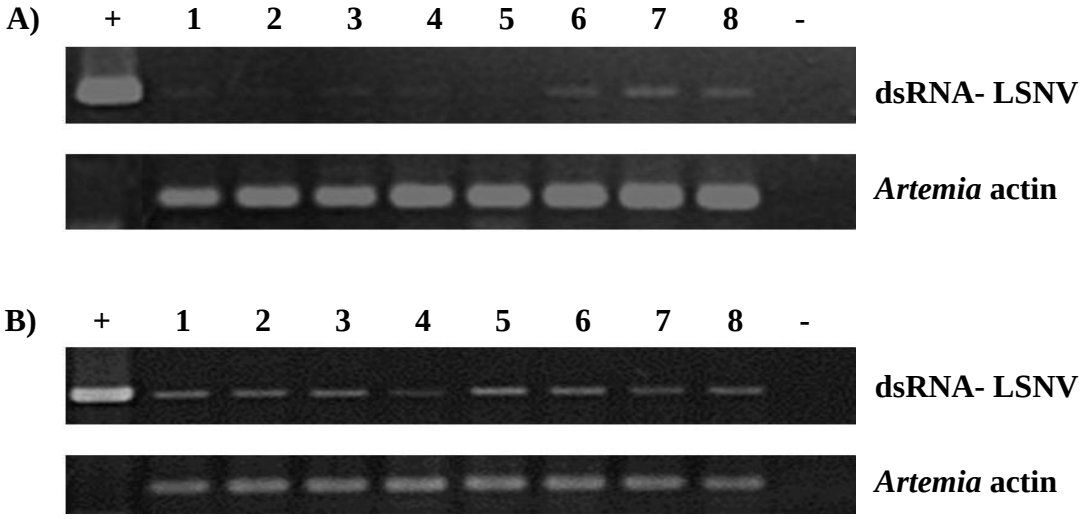


Figure 5.5 : RT- PCR amplification of dsRNA-LSNV in total RNA of *Artemia* samples which enriched with *E. coli* expressing dsRNA-LSNV. A), B) lane +, pGEM containing LSNV gene as a positive control and lane -, DEPC instead of RNA template as a negative control. *Artemia* enriched with *E. coli* expressing dsRNA-LSNV at A) lane 1-4, 1.1 x 10^{11} CFU; A) lane 5-8, 2.2 x 10^{11} CFU; B) lanes 1-4, 4.3 x 10^{11} CFU; B) lanes 5-8, 6.5 x 10^{11} CFU collected at 0.5, 1, 2, and 3 h respectively.

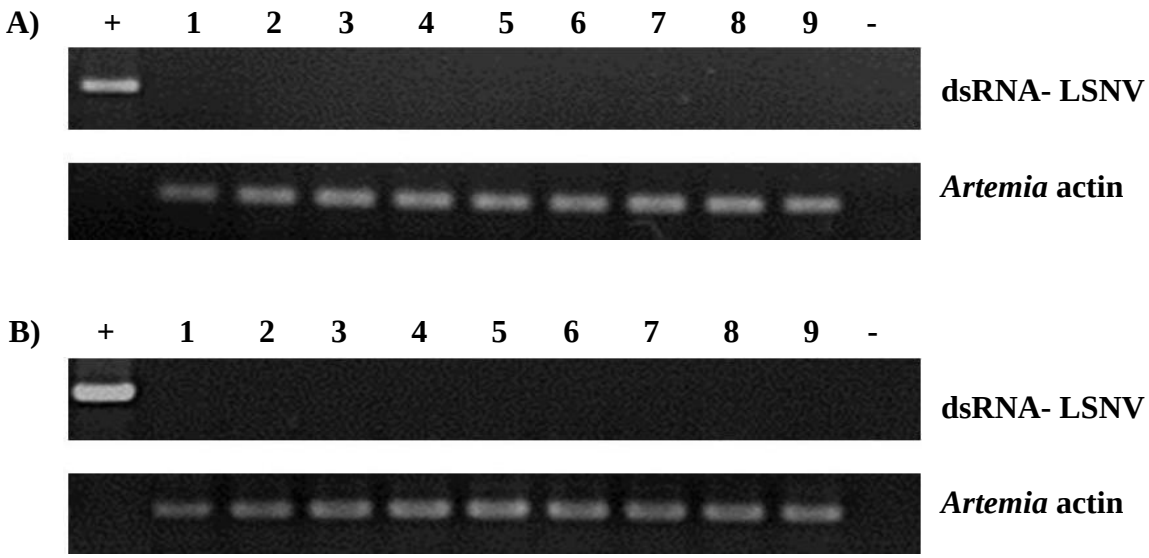


Figure 5.6 : RT- PCR amplification of dsRNA-LSNV in total RNA of *Artemia* samples which enriched with *E. coli* without expressing dsRNA-LSNV. A), B) lane +, pGEM containing LSNV gene as a positive control; lane 1, *Artemia* before enrichment and lane -, DEPC instead of RNA template as a negative control. *Artemia* enriched with *E. coli* without expressing dsRNA- LSNV at A) lanes 2-5, 1.1 x 10^{11} CFU; A) lanes 6-9, 2.2 x 10^{11} CFU; B) lanes 2-5, 4.3 x 10^{11} CFU; B) lanes 6-9, 6.5 x 10^{11} CFU collected at 0.5, 1, 2, and 3 h respectively.

The large scale results suggested that *Artemia* can be used as a carrier to preserve and deliver dsRNA into shrimp. Under the optimal condition, *Artemia* were produced with a suitable size for shrimp larvae, and their nutritional values are well maintained. *Artemia* which enriched with 4.3 x 10^{11} CFU of *E. coli* expressing dsRNA-LSNV for 2 h were selected for feed to post larval shrimp to test RNAi-mediated inhibitory effects in *Penaeus monodon* (*P. monodon*).

5.3.3. Evaluation of RNAi-mediated inhibitory in *Penaeus monodon*

The enriched *Artemia* were randomly collected everyday (15 days) and the non-enriched samples were collected on days 1, 5, 10 and 15and subjected to dsRNA-LSNV detection. The total RNA of *Artemia* was extracted and detected dsRNA-LSNV by 1-step RT-PCR.

5.3.3.1. Detection of dsRNA-LSNV in *Artemia* nauplii: RT-PCR was performed using total RNA extracted from *Artemia* after 2 h of the enrichment. The amplified products shown in Fig.5.7-A indicated the presence of dsRNA-LSNV in the enriched *Artemia* for PL1-15 feeding, whereas no RT-PCR product was observed in the non-enriched *Artemia* (Fig. 5.7- B).

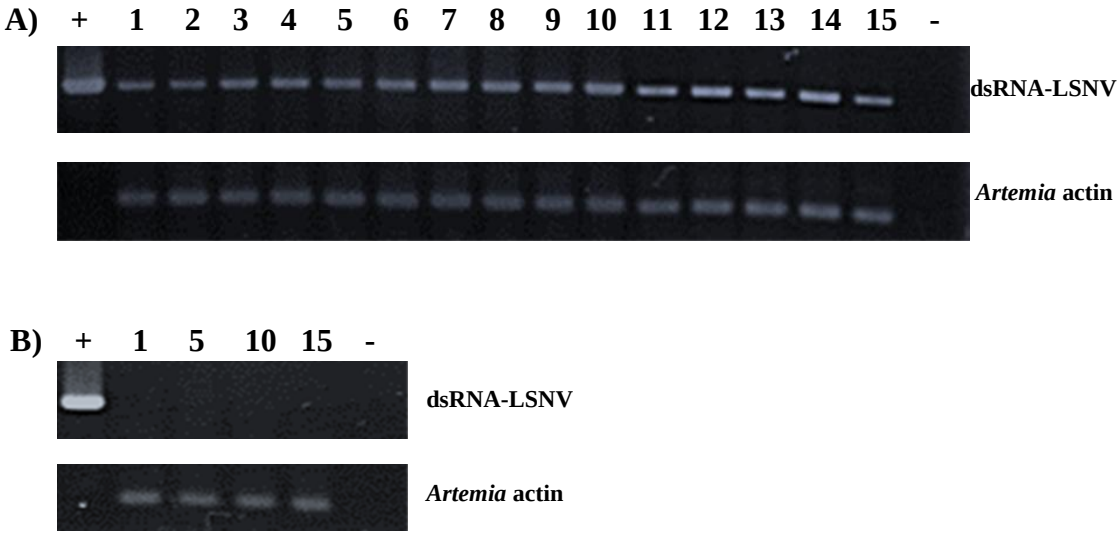


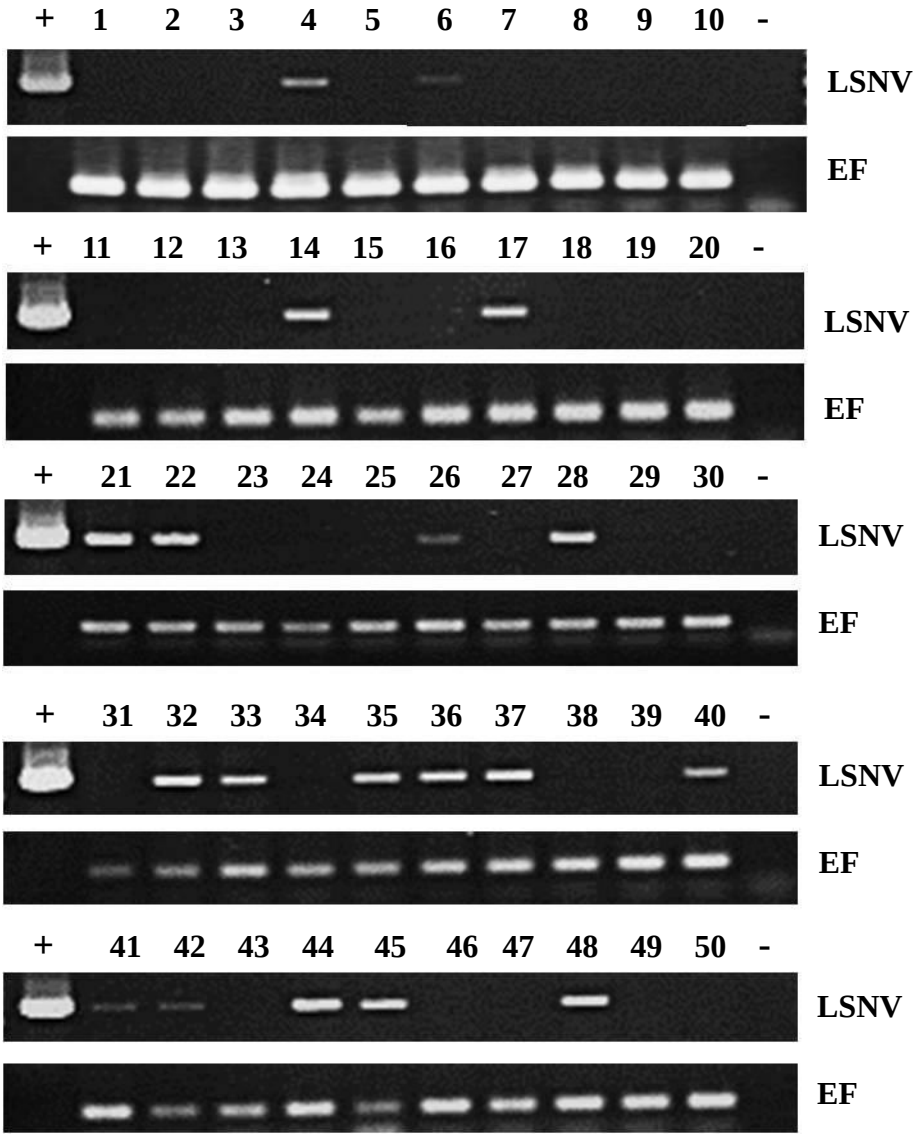
Figure 5.7 : RT-PCR analysis for dsLSNV detection in the enriched (A) and non-enriched (B) *Artemia*. Lane +, LSNV-cloned plasmid as positive control; lanes 1-15, *Artemia* samples in day 1-15 of enrichment; lane -, DEPC instead of RNA template as a negative control

5.3.3.2. Detection of LSNV in shrimp after fed with *Artemia* containing dsRNA-LSNV:

The effectiveness of protection of shrimp from LSNV via feed with *Artemia* containing dsRNA-LSNV were done by extracted total RNA extracted from pleopod at 15 weeks after feeding. RT-PCR analysis was performed to verify the presence of LSNV infection. The RT-PCR product showed that only 38% (19 from 50 shrimp) of shrimp fed with enriched *Artemia* were positive by showing LSNV infection; whereas 74% (37 from 50 shrimp) from shrimp received the non-enriched *Artemia* presented LSNV-positive (Fig. 5.8- A and B).

Relative real-time RT-PCR analysis was conducted to compare the level of LSNV expression in shrimp by using 20A as a reference gene. The level of LSNV expression in 20 shrimp (individual shrimp number 1- 20) of each group was analyzed (Fig. 5.9- A and B). From the results of real-time RT-PCR, the lower levels of LSNV were observed from shrimp received the enriched *Artemia*. The 10% of shrimp in this group contained more than 100 LSNV copies/total RNA (ng). In contrast, 65% of shrimp received the non-enriched *Artemia* contained more than 100 LSNV copies/total RNA (ng).

A)


+ 1 2 3 4 5 6 7 8 9 10 -
LSNV
EF
+ 11 12 13 14 15 16 17 18 19 20 -
LSNV
EF
+ 21 22 23 24 25 26 27 28 29 30 -
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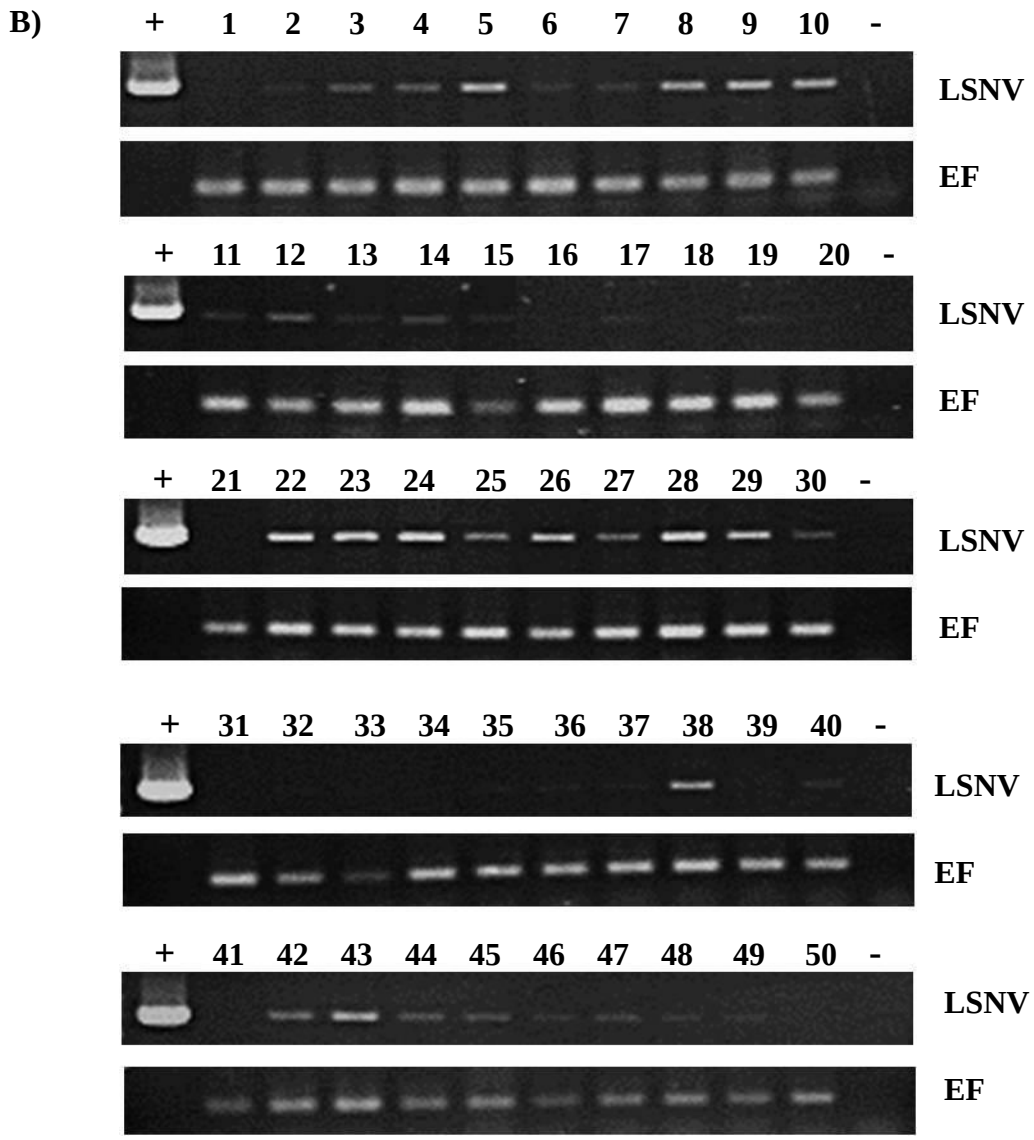

Figure 5.8 : RT-PCR analysis for LSNV detection in shrimp fed with the enriched (A) and non-enriched (B) *Artemia*. Lane +, LSNV-cloned plasmid as positive control; lanes 1-50, individual shrimp samples at 15 after feeding with *Artemia*; lane -, DEPC instead of RNA template as a negative control

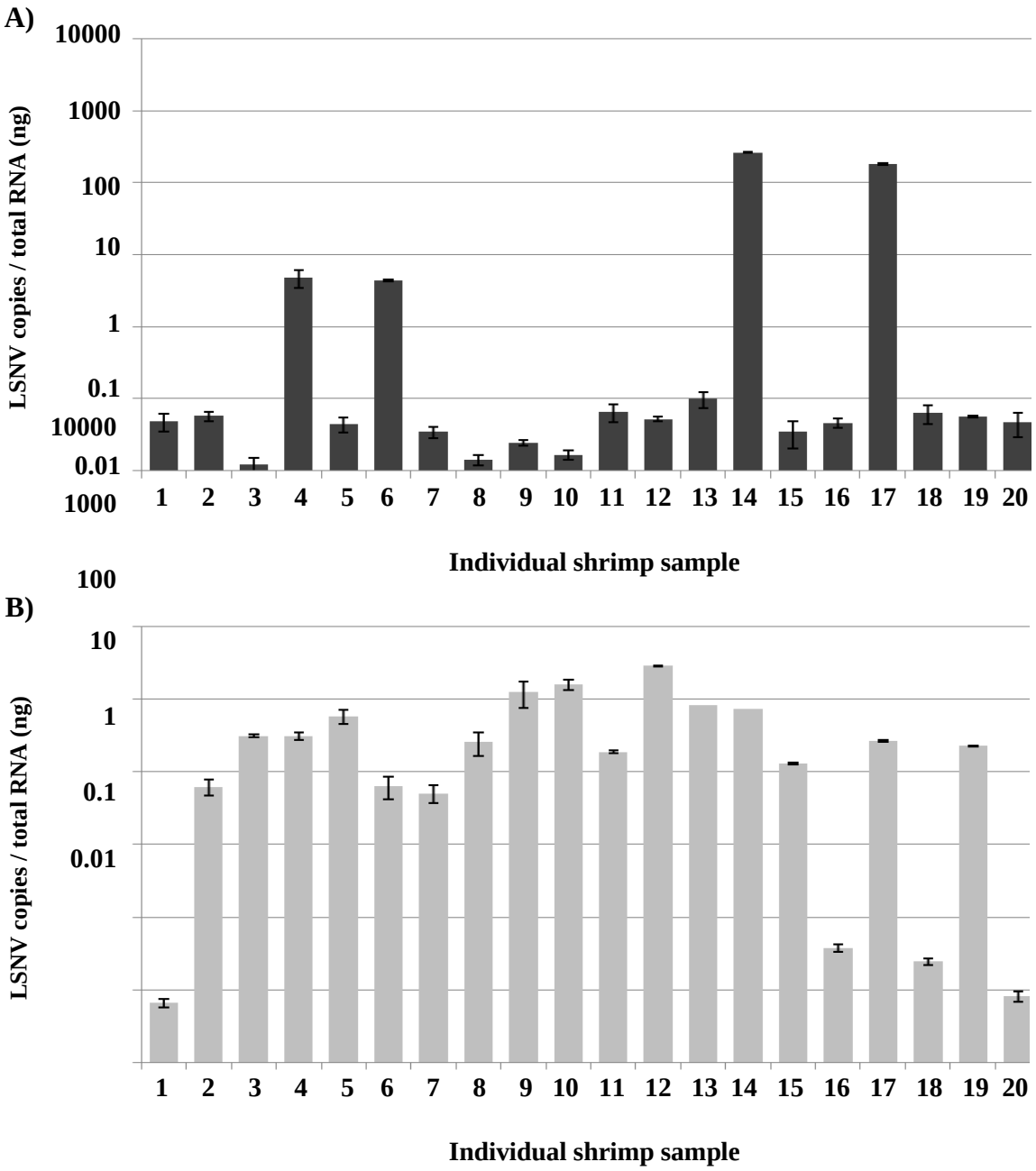

Figure 5.9 : Real-time RT-PCR analysis of the number of LSNV copies per total RNA (ng) in individual shrimp at 15 weeks after feeding the enriched *Artemia* (A) and non-enriched *Artemia* (B). Error bars indicate the standard deviation

5.3.3.3. Observation of shrimp body weight:

The culture performances were indicated by mean body weight (MBW) and percentage of coefficient of variations, CV (%). During 11-17 weeks after *Artemia* feeding, MBW of the shrimp fed with the enriched *Artemia* group (dsRNA) were significantly ($P<0.05$) higher than the shrimp fed with the non- enriched *Artemia* group (No dsRNA) (Fig. 5.10). To compare size variation of each group CV (%) values were calculated from standard deviation divided by mean. A CV (%) value in dsRNA group (37.29, 38.99, and 32.26) was lower than the no dsRNA group (51.40, 44.38, and 35.41) (Table 5.1).

All data suggested that shrimp received the dsRNA through enriched *Artemia* showed low level of LSNV infection which detected with both RT-PCR and real-time RT-PCR analysis. The protection from LSNV infection led to no decrease in shrimp body weight ($P < 0.05$).

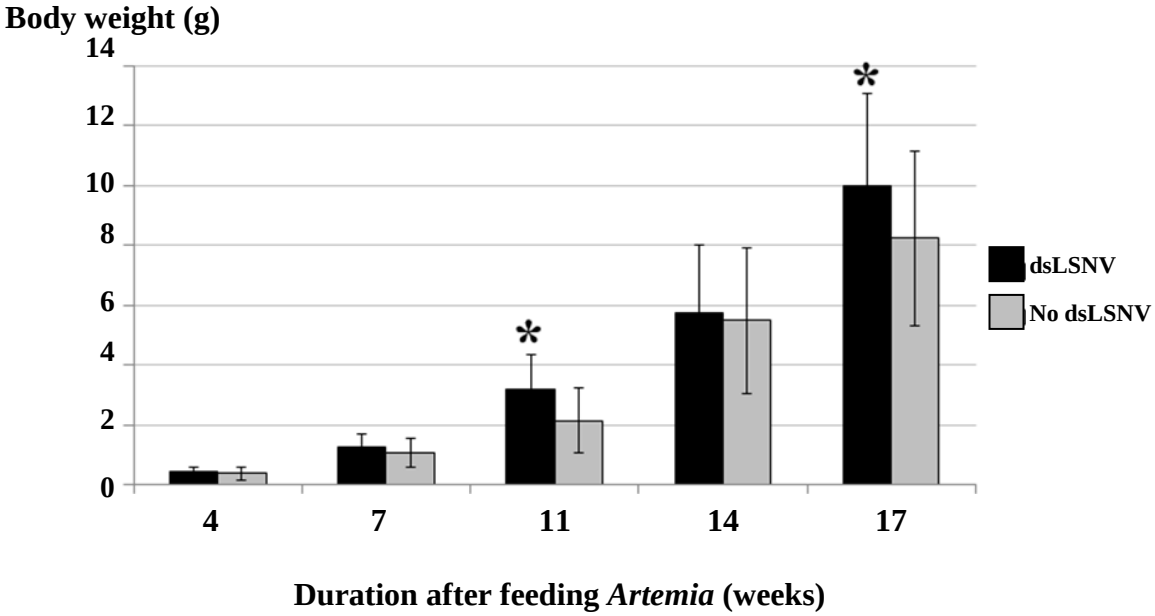

Figure 5.10: Mean body weights of shrimp received the dsRNA-enriched *Artemia* as compared to those of the non-enriched *Artemia*. Data were collected at 4, 7, 11, 14 and 17 weeks after shrimp were fed with *Artemia*. Error bars indicate the standard deviations, and asterisk indicates significant difference from the pair-wise comparison with the control group ($P < 0.05$).

Table 5.1: Shrimp production performance at 4, 7, 11, 14 and 17 weeks after feeding with *Artemia* (N=50). MBW= mean body weight, SD = standard deviation, and CV=coefficient of variation.

Enriched *Artemia*					
Duration (weeks)	4	7	11	14	17
MBW (g)	0.42	1.24	3.17	5.76	9.98
SD	0.15	0.47	1.18	2.25	3.12
% CV	36.36	37.37	37.29	38.99	31.26
Non- enriched *Artemia*					
Duration (weeks)	4	7	11	14	17
MBW (g)	0.37	1.06	2.14	5.48	8.24
SD	0.22	0.50	1.10	2.44	2.92
% CV	59.35	46.73	51.40	44.58	35.41

5.4. dsRNA delivery using feed pellets containing dsRNA-LSNV

Formulation of feed containing *E. coli* expressing dsRNA was performed according the method described by Saksmerprome et al., 2013 (26). There were two formulas of feed based on dsRNA-LSNV concentration produced approximately 6 and 12 mg dsRNA-LSNV, formulas-1 and 2 respectively.

In this part, feed pellet was produced and the feeding experiments were performed by Shrimp Genetic Improvement Center (SGIC), Surat Thani, Thailand. The responsibility in this part were 1) evaluation of dsRNA in feed pellet after formulation, and 2) Evaluation of RNAi-mediated inhibitory of feed containing dsRNA in *P. monodon***.**

5.4.1. Evaluation of dsRNA-LSNV in feed pellets

RT-PCR analysis was done using total RNA extracted from the developed formula feed pellets 1 and 2. The presence of dsRNA-LSNV PCR amplification were observed in the developed formula-1 (lanes 4-6) and formula-2 (lanes 7-9) whereas no PCR products were amplified in control feed lane 1-3 (Fig. 5.11)**.**

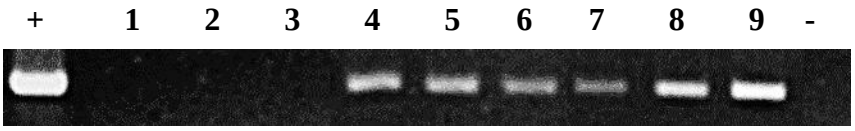

Figure 5.11: RT-PCR amplification of dsRNA-LSNV from the developed formula feed pellets. Lane +, LSNV-cloned plasmid as positive control; lanes 1-3 , control feed; lanes 4-6, the developed formula-1; lanes 7-9, the developed formula-2; -, DEPC instead of RNA template as a negative control

5.4.2. Evaluation of RNAi-mediated inhibiting of feed containing dsRNA in *P. monodon*

The developed feed pellet containing dsRNA-LSNV including formular-1 and -2 and normal feed were fed to shrimp. After 9 weeks of feeding, the shrimp were randomly collected (number of shrimp = 10) and subjected to LSNV detection. The total RNA of shrimp was extracted from shrimp pleopod and detected LSNV by 1-step RT-PCR analysis. Moreover, the effect of LSNV infected in shrimp was demonstrated via body weight observation.

5.4.2.1. Detection of LSNV in shrimp after feed with pellet containing dsRNA-LSNV:

The effectiveness of protection shrimp from LSNV with pellet feed containing dsRNA-LSNV was detected from total RNA extracted from shrimp pleopod by using RT-PCR analysis. The results showed that all control shrimp which fed with normal feed were LSNV-positive (Fig. 5.12- A), whereas the shrimp that received dsRNA-LSNV feed presented 20-60% LSNV reductions (Fig. 5.12- B and C). The LSNV infection in shrimp that received formula-1 (6mg dsRNA/kg feed) was higher than shrimp received formula-2 (12 mg dsRNA/kg feed).

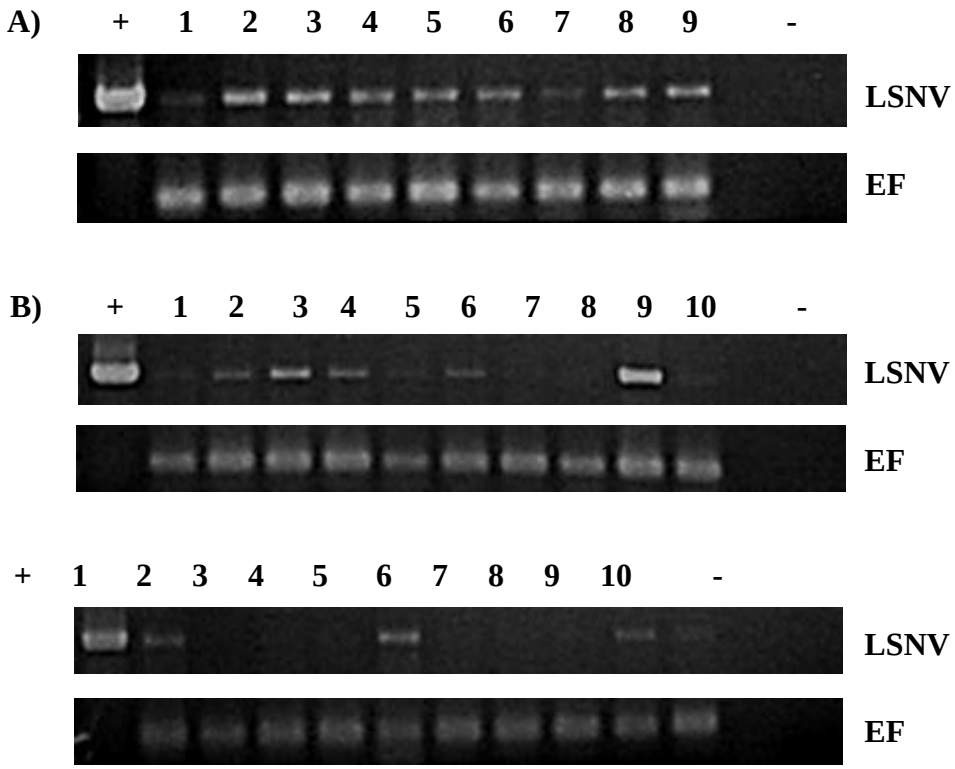

Figure 5.12: RT-PCR amplification of LSNV in shrimp fed with control feed (A), the developed feed pellets formulas 1 (B) and formulas 2 (C). Lane +, LSNV-cloned plasmid as positive control; lanes 1-10, individual shrimp samples at the end of experiment; lane -, DEPC instead of RNA template as a negative control

5.4.2.2. Observation of shrimp body weight:

The mean body weight (MBW) of shrimp (number of shrimp = 100) and percentage of coefficient of variations, CV (%) was exhibited for growth condition of shrimp. The initial MBW and CV (%) were not different in all groups (Fig. 5.13). After 9 weeks, the final MBW of control shrimp was significantly (P<0.0001) lower than shrimp received dsRNA feed, but not significant difference between the two developed dsRNA feed. In addition, CV (%) values of shrimp fed with dsRNA-feed (21.43% for formula-1 and 19.07% for formula-2) were lower than control shrimp (30.84%) as shown in Table 5.2.

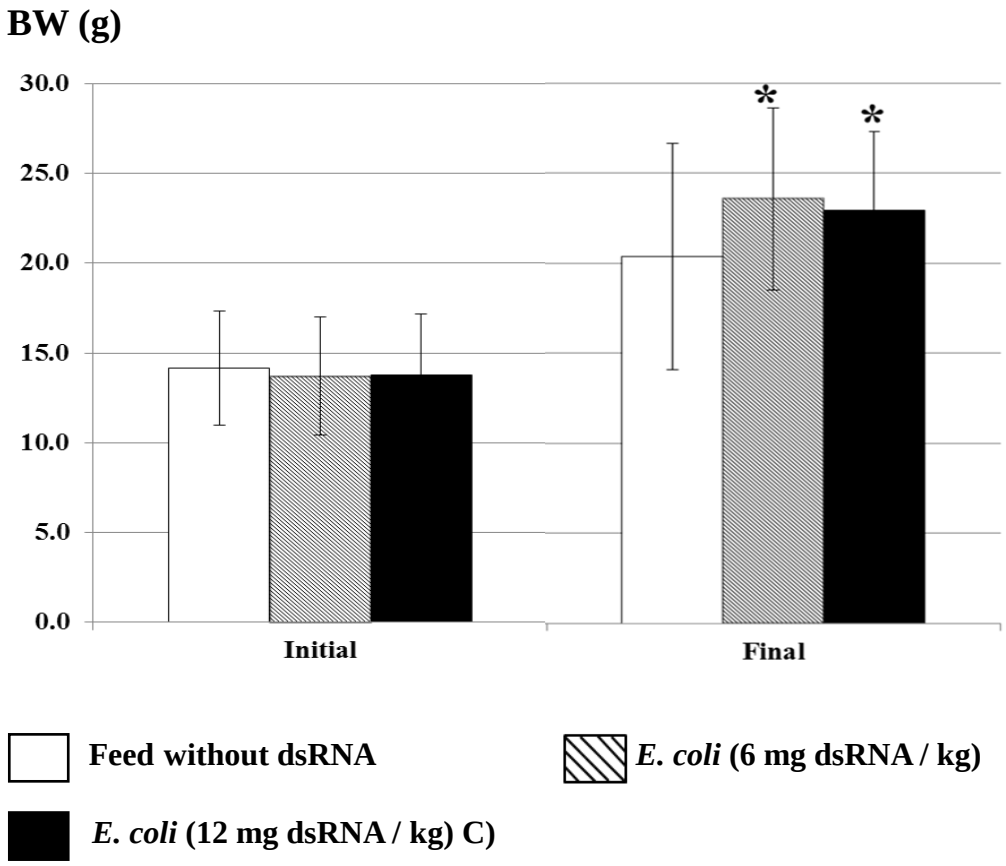

Figure 5.13: Mean body weight of shrimp received control feed compared with dsRNA-LSNV feed. Data collected at the beginning ("initial") and the end ("final") of feed trials. Error bars indicate the standard deviation, and asterisk indicates significant difference from the pair-wise comparison with group received control feed ($P < 0.0001$).

Table 5.2 : Shrimp production performance before and after received control and dsRNA-LSNV feed for 9 weeks (N=100). MBW= mean body weight, SD = standard deviation, and CV=coefficient of variation.

	Initial		
	No dsRNA	6 mg dsRNA/ kg feed	12 mg dsRNA/ kg feed
Sampling (pcs.)	100	100	100
MBW (g)	14.140	13.730	13.790
SD	3.18	3.28	3.38
CV (%)	22.52	23.87	24.51
	Final		
	No dsRNA	6 mg dsRNA/ kg feed	12 mg dsRNA/ kg feed
Sampling (pcs.)	100	102	100
MBW (g)	20.370	23.588	22.960
SD	6.28	5.05	4.38
CV (%)	30.84	21.43	19.07

CHAPTER VI
DISCUSSION

Laem - singh virus (LSNV) has been proposed as a causative agent of monodon slow growth syndrome (MSGS), resulting in massive production loss in the black tiger shrimp *Penaeus monodon* (*P. monodon*) cultivation (2). In this study, double-stranded RNA (dsRNA) specific to RNA dependent RNA polymerase (RdRp) gene of LSNV (dsRNA-LSNV) has been applied to control the LSNV replication in shrimp. The previous studies (5) reported that dsRNA targeted Pmrab7 gene could be successfully was to inhibit LSNV infection. Even though an introduction of dsRNA targeting host essential gene showed very promising result in viral inhibition, virus specific dsRNA was widely used and successful to combat viral infection in shrimp. Despite, RNAi has shown potential for protection shrimp from viral diseases, the development of large-scale dsRNA delivery system is important for successful application of RNAi technology in shrimp farms.

In this study, oral application of dsRNA by using live organism and shrimp feed was developed as carriers to deliver dsRNA and evaluated to LSNV inhibitory effect in *P. monodon*. Previous studies indicated that *Artemia*, standard living feed, can be encapsulated with a variety of compounds such as essential nutrients, hormones, therapeutic vaccine and fed to cultured fish and shrimp larvae for enhance growth and survival (20, 21). Lin *et al*., 2007 reported the use of *Artemia* as a carrier for combating infectious virus in early stage of marine culturing such as larvae of grouper. The developed system also could be applied in early infection due to vertical transmission (22). Furthermore, *Artemia* nauplii have been exploited to deliver antibody into shrimp for controlling diseases, such as white spot syndrome virus and vibriosis, in aquaculture (7). Recently, Treerattrakool *et al*., 2013 reported that the successfully to enrichment of adult *Artemia* with dsRNA targeted gonad-inhibiting hormone (GIH) through *E. coli* expression system and it could be used to knockdown the GIH gene in *P. monodon* after feeding with enriched-*Artemia* (27).

In the present study, we are interested in using *Artemia* to deliver dsRNA into shrimp at postlarvae (PL) stage. The dsRNA-LSNV and red fluorescent protein (RFP) were produced in *Escherichia coli* (*E. coli*) expression system (Fig. 4.1-2), for *Artemia* enrichment processes. In this experiment, dsRNA-LSNV could be produced in high amount of dsRNA, approximately 0.2 mg dsRNA from 1.8 x 10^{10} CFU of bacterial culture (3).

First, the transformed *E. coli* expressing RFP was tested in the *Artemia* enrichment process. *Artemia* were enriched with *E. coli* expressing RFP and monitored by confocal microscope. During 4-30 hour of enrichment, the red fluorescent signals were observed in *Artemia* gut (Fig 4.3b-i). Red fluorescent in *Artemia* gut also showed a progressive increase in red color density from 4 to 28 h after enrichment, suggested that RFP can be delivered to *Artemia* and retained their biological activity. These findings were in agreement with the previous studies (7) reported that *Artemia* enriched with *E. coli* expressed fluorescent markers, green fluorescent protein could be accumulated for up to 10 h. Second, *Artemia* were enriched with *E. coli* expressing dsRNA-LSNV. The laboratory scale of dsRNA delivery was performed to test feasibility of *Artemia* as a carrier of dsRNA. By reverse transcription PCR (RT-PCR) assay, dsRNA-LSNV was detected in *Artemia* at 2 h and decreased in 6 h after enrichment. The results showed that *Artemia* could be enriched with *E. coli* expressing dsRNA-LSNV and used as a carrier to deliver dsRNA. Taken together, both RFP and dsRNA-LSNV enrichment assays suggest that biomaterials can be effectively transported to *Artemia* through the developed system and retained their biological activity.

The large scales of enrichment process were performed by increasing concentration of *Artemia* from 100 *Artemia* per ml sea water in lab scale to 20,000 *Artemia* per ml sea water. Moreover, the dsRNA-LSNV enrichment period was carried out soon after *Artemia* newly hatched (Instar I nauplii) to retain the most nutritious diet of *Artemia* for PL shrimp. In this study, dsRNA-LSNV could be detected in *Artemia* within 0.5-3 h after enrichment (Fig 5.5A-B). Not only achievable enriched *Artemia* in large scale but also had many benefits such as have a suitable size and more nutrition for shrimp PL. From our results, the enrichment with 4.3 x 10^{11} CFU of *E. coli* for 2 h condition was used to produce *Artemia* containing dsRNA-LSNV.

RNAi-mediated efficiency to inhibit LSNV in *P. monodon* was evaluated after fed with *Artemia* containing dsRNA to PL1 continues to PL15. After 15 weeks of *Artemia* feeding processes, the results suggested that shrimp received the dsRNA through enriched-*Artemia* showed low level of LSNV infection when detected with both RT-PCR and real-time RT-PCR analysis (Fig 5.8-9). The prohibition of LSNV infection led to more body weight in shrimp and low percentage of coefficient of variations, CV (%) (Fig 5.10, Table 5.1). Taken together, our results revealed that *Artemia* can be used as carriers of dsRNA delivery into shrimp in farm application and inhibited LSNV replication.

Feed pellets were used to deliver dsRNA into shrimp at juvenile stage. Feed pellets containing dsRNA-LSNV were prepared with two different amounts of *E. coli* expressing dsRNA-LSNV, 6 (formula-1) and 12 mg (formula-2) dsRNA-LSNV per kg feed. The presence of dsRNA-LSNV was detected in both formulas (Fig. 5.11) and these pellets were further used to feed the juvenile shrimp for 9 weeks. The RT-PCR results showed that the two types of feed had a capability of reducing LSNV in a dose-dependent manner (Fig. 5.12). In addition, MBW of the groups receiving formula-1 and 2 treatments were significantly higher than shrimp fed with normal feed, but there was no significant difference in MBW of those receiving the two types of formulated feed (Fig. 5.13). The initial and final calculated CV (%) values of dsRNA group are lower relative to normal group (Table 5.2). From MBW results, suggested formula-2 (dsRNA-LSNV dose: 12 mg/kg feed) was not affected the growth of shrimp compared to formula-1. Formula-1 feed which had lower amount of dsRNA-LSNV (6 mg/kg feed) could be efficiently enough to significantly inhibit LSNV infection and, more likely, to minimize cost of feed production. Thus, formulated-1 should be selected for future farm-scale application. There are many reports show that a feed pellets have a capacity in protection marine animal as well. For example, the inhibition of white spot syndrome virus (WSSV) replication in *Penaeus monodon* by feed pellets that coated with inactivated bacteria expressing VP28-targeted dsRNA (4). In addition, Kumar *et al*., 2013 showed successful to inhibited *Macrobrachium rosenbergii* nodavirus (*Mr*NV) replication in *Macrobrachium rosenbergii* (*M. rosenbergii*) by feed pellets that coated with bacterial expressing dsRNA targeted B2 gene of *Mr*NV (28). Nevertheless, this is the first

report on feed formulated with LSNV-specific dsRNA that can inhibit LSNV replication and promoted the overall growth of *P. monodon*.

Our study reports the successful development of dsRNA delivery into shrimp for antiviral purpose in farm application. The delivery systems were proved to be more practical method than injection in farm. The large-scale dsRNA production was performed using the bacteria system that is simple and cost-effective (3). For both dsRNA delivery systems, the additional cost came from the bacteria culture expressing dsRNA-LSNV. In *Artemia* experiment, the cost of bacteria culture were calculated from cultivated 5,000 PL shrimp which fed for 4 times per day, continues to 15 day. The cost was approximately 225 baht. For feed pellets, total cost of bacteria culture for 9 weeks of feeding 1,200 shrimp were 1,680 and 3,360 bath for formula-1 and 2, respectively. In our results, we showed that shrimp body weight were increased in each experiment about 1 - 3 g which estimated increase of 240 - 720 kg of total shrimp mass in one-hectare pond (240,000 shrimps). In *Artemia* experiment, the extra cost on bacteria culture for feeding one -hectare pond is 10,800 baht. The increase of body weight could be an extra income (subtracted by the cost of bacteria culture) at least 37,200 - 133,200 baht per one-hectare pond. Therefore, dsRNA-delivery systems by *Artemia* not only could be protected shrimp from viral infection but also had a highly potential and achievable in cost effective to be used in shrimp farm. In contrast, feed pellets have higher extra cost more than extra income (336,000 and 672,000 for formula-1 and 2, respectively per one-hectare pond). Nonetheless, this delivery system could be protected shrimp from viral infection.

Overall results suggested the potential of the two types of dsRNA-delivery methods for controlling viral disease in shrimp farming.

CHAPTER VII
CONCLUSIONS

The main purpose of this work is to develop the system of double stranded RNA (dsRNA) delivery by oral administration using *Artemia* and feed pellets containing dsRNA as carriers and evaluate RNAi-mediated inhibitory effected of these carriers in *Penaeus monodon (P. monodon)*. For the conclusions of this study are summarized as follows:

1. Double-stranded RNA targeting LSNV (dsRNA-LSNV) and red fluorescence protein (RFP) are successfully produced from bacteria *Escherichia coli* (*E. coli*) stain HT115 culture.

2. The dsRNA delivery system by using *Artemia* nauplii

   2.1. Both RFP and dsRNA-LSNV enrichment assays suggest that biomaterials in their normal functions can be delivered effectively into *Artemia* through the strategy developed here in.

   2.2. The dsRNA-enriched *Artemia* can inhibit LSNV infection and relieve MSGS conditions in *P. monodon*.

   2.3 This is the first report on the antiviral effect of dsRNA-encapsulated *Artemia* in the larvae culture of shrimp. The work offers a practical and inexpensive dsRNA delivery-system for controlling viral disease in shrimp farming.

3. The dsRNA delivery system by using feed pellets

   3.1 Feed containing with dsRNA-LSNV promotes the overall growth of *P. monodon* and reduces MSGS condition in LSNV-infected shrimp. This work confirms the potential of dsRNA application for controlling viral disease in shrimp farming

In summary, this research demonstrates that both delivery methods are successfully used to control the infection of LSNV in shrimp and have a highly potential to apply in real shrimp farming.

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APPENDICES

APPENDIX A

Reagents for double-stranded RNA induction

LB (Luria-Bertani) Broth (1% Tryptone, 0.5% Yeast Extract, 1% NaCl)

To make 100 ml of LB dissolves

1 g	tryptone
0.5 g	yeast extract
1 g	NaCl

Add distilled water to 100 ml. Autoclave at 121°C for 15 min to sterile the medium. Let cool to ~55°C and add desired antibiotics at this point.

IPTG (Isopropyl-beta-D-thiogalactopyranoside)

0.595 g	IPTG

Add distilled water up to 5 ml and filtrate through 0.45 µm membrane filter.

3M Sodium Acetate, pH 5.2 (NaOAc)

40.8 g	NaOAc

Adjust pH to 5.2 with glacial acetic acid then add distilled water up to 100 ml. Autoclave at 121°C for 15 min to sterile solution.

10X TBE (0.89 M Tris-HCl, 0.02 M EDTA, 0.89 M Boric acid, pH 8.3)

108 g Tris-Base
55 g Boric acid
9.3 g Na_4EDTA

Add distilled water to 950 ml. Adjust pH to 8.3 with NaOH then make final volume of 1 liter. Autoclave to sterilize.

0.5 M EDTA pH 8.0

93.06 g EDTA

Add distilled water to 400 ml, adjust pH to 8.0 then add distilled water to final volume 500 ml. Autoclave to sterilize. Store at room temperature.

STOCK ANTIBIOTIC

100 mg/ml Ampicillin

1 g ampicillin

Add 10 ml of distilled water. Filter through 0.2 µm membrane. Store at -20°C.

12 mg/ml Tetracycline

1.2 g tetracycline

Add 100 ml of distilled water. Filter through 0.2 µm membrane. Store at -20°C.

APPENDIX B

Formulation of feed containing double-stranded RNA (dsRNA)

Composition of ingredient of feed for *Penaeus monodon* (*P. monodon*)

Table B. 1: Composition of ingredients (% by weight) of baseline feed for *P. monodon*

Ingredient	% by weight
Fish meal	10
Squid meal	3
Poultry meal	25
Soy bean meal	17
Polychaete *Perinereisnuntia* meal	3
Seaweed *Gracilariafisheri* powder	1
Wheat gluten	4.4
Fish oil	2
Palm oil	1
Bran	7
Wheat flour	23.7
Vitamin/mineral premix	2.5
Vitamin C	0.1
Choline chloride	0.3
Total	100

Formulation of shrimp feed containing dsRNA

Formulation of feed containing dsRNA-LSNV was performed according the method described by Saksmerprome *et al*., 2013 (3). Briefly, the ground ingredients listed in Table B. 1 were weighed according to the calculated formula. Vitamins and minerals were individually weighed and combined to wheat flour before being thoroughly mixed with other ingredients for 10 min. Vegetable oil and attractants, including fish oil and lectithin, were added and mixed for 10 min. For preparation of feed with dsRNA-LSNV, 1 kg feed was mixed with 30 mL of concentrated *E.coli* culture, prior to being pelleted. There were two formulas of feed base on concentrated culture produced approximately 6 and 12 mg dsRNA-LSNV, formulas-1 and 2. Once the ingredient mixture was thoroughly homogenized, and the mixture of mashed feed was passed through a pellet machine with a 3 mm die. Feed pellets were dried at 60 °C and stored until use.

Fig B. 1: Normal shrimp feed

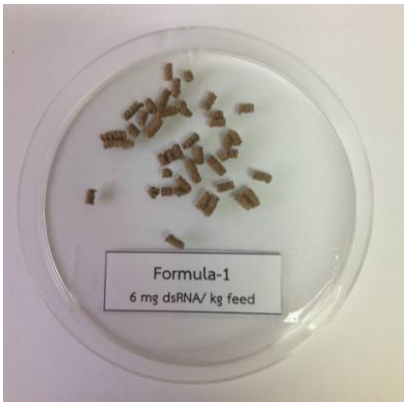


Fig B. 2: Feed formula-1 (6 mg dsRNA/kg)

Fig B. 3: Feed formula-2 (12 mg dsRNA/kg)

APPENDIX C

Observation of shrimp body weight for RNAi-mediated inhibitory in *P. monodon*

I. dsRNA delivery by using *Artemia* nauplii

Mean body weights (MBW) of shrimp (n=50) were recorded during 4-17 weeks after feeding with *Artemia*. The MBW values were used to calculate percentage of coefficient of variations, CV (%) (Table C.1). A statistical analysis was performed using paired-samples T Test, and $P < 0.05$ was considered statistically significant.

Table C. 1: Shrimp production performance at 4, 7, 11, 14 and 17 weeks after feeding with *Artemia* (N=50). SD = standard deviation, and CV=coefficient of variation.

	Treated shrimp					Untreated shrimp				
No.	4	7	11	14	17	4	7	11	14	17
1	0.56	1.54	4.92	7.00	14.00	0.23	0.97	2.74	10.00	6.00
2	0.75	2.22	2.59	3.00	12.00	0.40	0.60	1.55	5.00	10.00
3	0.38	1.48	2.66	5.00	9.00	0.17	0.78	3.35	10.00	14.00
4	0.38	1.16	2.49	4.00	3.00	0.71	0.20	3.98	3.00	6.00
5	0.32	1.63	6.62	6.00	10.00	0.54	1.09	1.58	5.00	6.00
6	0.25	1.58	1.13	6.00	8.00	0.48	1.74	5.35	10.00	5.00
7	0.41	1.08	1.88	8.00	10.00	0.19	1.42	3.39	4.00	10.00
8	0.79	1.60	2.60	7.00	14.00	0.43	1.14	5.14	7.00	10.00
9	0.76	1.78	2.54	9.00	5.00	0.14	1.92	1.28	6.00	6.00
10	0.37	1.33	3.57	6.00	15.00	0.45	1.57	1.64	5.00	11.00
11	0.14	1.09	3.25	7.00	14.00	0.43	0.62	1.95	14.00	8.00
12	0.38	1.25	2.46	4.00	7.00	0.59	0.97	3.22	6.00	13.00
13	0.60	1.84	2.25	8.00	7.00	0.23	0.90	3.65	2.00	8.00
14	0.45	1.87	2.19	3.00	12.00	0.21	0.48	1.89	5.00	13.00

No.	Treated shrimp					Untreated shrimp				
	4	7	11	14	17	4	7	11	14	17
15	0.37	1.99	2.19	7.00	9.00	0.29	0.84	4.12	5.00	8.00
16	0.47	0.64	3.32	4.00	14.00	0.22	2.33	1.29	2.00	10.00
17	0.40	0.61	4.49	5.00	9.00	0.16	2.19	2.55	5.00	7.00
18	0.34	1.58	1.74	5.00	11.00	0.93	2.49	1.73	8.00	4.00
19	0.40	1.25	3.21	7.00	4.00	0.20	1.18	1.24	6.00	13.00
20	0.37	1.15	3.37	4.00	13.00	0.38	1.70	1.93	6.00	11.00
21	0.34	0.91	4.59	8.00	8.00	0.41	1.07	1.06	9.00	7.00
22	0.49	0.95	2.21	12.00	15.00	0.22	1.38	2.42	7.00	4.00
23	0.55	0.79	5.31	5.00	11.00	0.23	1.02	2.51	3.00	13.00
24	0.59	0.83	3.66	7.00	7.00	0.11	1.27	1.80	5.00	9.00
25	0.40	0.97	3.00	5.00	10.00	0.48	1.12	0.98	5.00	3.00
26	0.66	0.89	2.86	4.00	8.00	0.65	0.70	1.60	5.00	10.00
27	0.57	0.47	2.18	7.00	9.00	0.34	0.77	2.41	6.00	7.00
28	0.37	0.90	3.10	5.00	9.00	0.59	0.74	1.47	5.00	6.00
29	0.46	0.92	1.75	3.00	11.00	0.15	0.70	2.81	4.00	6.00
30	0.14	0.87	2.03	8.00	9.00	0.32	0.99	1.71	5.00	5.00
31	0.73	0.99	6.21	6.00	12.00	0.31	0.98	2.49	8.00	8.00
32	0.46	0.58	4.36	11.00	8.00	0.73	0.75	2.28	8.00	11.00
33	0.19	0.56	4.70	7.00	7.00	0.28	0.66	0.97	5.00	12.00
34	0.44	0.94	3.58	2.00	17.00	0.14	1.20	2.24	4.00	7.00
35	0.31	0.55	1.15	4.00	13.00	1.19	0.56	1.32	2.00	4.00
36	0.47	1.01	4.09	7.00	7.00	0.11	0.77	4.17	6.00	8.00
37	0.49	1.41	2.83	6.00	5.00	0.34	1.05	2.57	1.00	9.00
38	0.56	1.64	3.06	5.00	8.00	0.43	0.88	2.38	5.00	12.00
39	0.42	0.75	3.59	10.00	8.00	0.25	1.01	1.37	5.00	3.00
40	0.24	1.27	1.41	4.00	9.00	0.22	1.37	2.40	2.00	8.00
41	0.27	1.39	2.95	1.00	10.00	0.14	0.97	1.32	7.00	7.00
42	0.45	1.31	3.76	4.00	10.00	0.53	0.35	0.97	7.00	7.00
43	0.31	2.85	3.53	7.00	13.00	0.24	0.93	1.50	3.00	12.00
44	0.27	1.60	3.90	9.00	15.00	0.20	1.23	1.56	4.00	5.00
45	0.32	1.51	2.54	6.00	13.00	0.17	0.54	0.72	6.00	7.00
46	0.36	1.15	2.20	4.00	10.00	0.38	1.85	2.04	7.00	11.00

	Treated shrimp					Untreated shrimp				
No.	4	7	11	14	17	4	7	11	14	17
47	0.34	1.68	2.76	5.00	6.00	0.36	1.29	0.99	6.00	6.00
48	0.28	1.29	3.90	3.00	9.00	0.47	0.60	0.86	3.00	12.00
49	0.59	1.25	3.48	4.00	13.00	0.36	0.80	1.73	4.00	7.00
50	0.22	1.31	4.55	4.00	9.00	0.53	0.47	0.56	3.00	7.00
Total	21.18	62.21	158.71	288.00	499.00	18.26	53.15	106.78	274.00	412.00
Avg.	0.424	1.244	3.174	5.760	9.980	0.365	1.063	2.136	5.480	8.240
Max.	0.8	2.9	6.6	12.0	17.0	1.2	2.5	5.4	14.0	14.0
Min.	0.1	0.5	1.1	1.0	3.0	0.1	0.2	0.6	1.0	3.0
SD	0.15	0.47	1.18	2.25	3.12	0.22	0.50	1.10	2.44	2.92
CV%	36.36	37.74	37.29	38.99	31.26	59.35	46.73	51.40	44.58	35.41

II. dsRNA delivery by using feed pellets

Initial and final body weights of random samples of 100 shrimp were recorded and used for calculating percentage of coefficient of variations (%CV) of each group (Table C.2). A statistical analysis was performed using one-way ANOVA, and $P < 0.0001$ was considered statistically significant.

Table C. 2: Shrimp production performance before and after received control and tested feed for 9 weeks (N=100). SD = standard deviation, and CV=coefficient of variation.

	Initial			Final		
No.	No dsRNA	Formula-1 (6mg dsRNA/kg)	Formula-2 (12mg dsRNA/kg)	No dsRNA	Formula-1 (6mg dsRNA/kg)	Formula-2 (12mg dsRNA/kg)
1	21.0	14.0	11.0	27.0	31.0	21.0
2	13.0	17.0	21.0	28.0	12.0	23.0
3	18.0	20.0	14.0	15.0	24.0	24.0
4	16.0	11.0	17.0	21.0	35.0	20.0
5	13.0	11.0	11.0	18.0	24.0	26.0
6	19.0	15.0	12.0	28.0	21.0	31.0
7	12.0	13.0	16.0	18.0	12.0	21.0
8	11.0	18.0	14.0	22.0	22.0	32.0
9	12.0	19.0	15.0	19.0	27.0	24.0
10	14.0	17.0	18.0	25.0	17.0	22.0
11	18.0	19.0	15.0	20.0	23.0	19.0
12	13.0	15.0	15.0	22.0	21.0	20.0
13	8.0	16.0	15.0	18.0	16.0	31.0
14	16.0	14.0	18.0	23.0	32.0	34.0
15	12.0	17.0	17.0	28.0	17.0	22.0
16	15.0	18.0	16.0	28.0	21.0	26.0
17	13.0	15.0	14.0	18.0	25.0	22.0
18	12.0	13.0	17.0	27.0	18.0	24.0
19	11.0	16.0	18.0	19.0	22.0	16.0

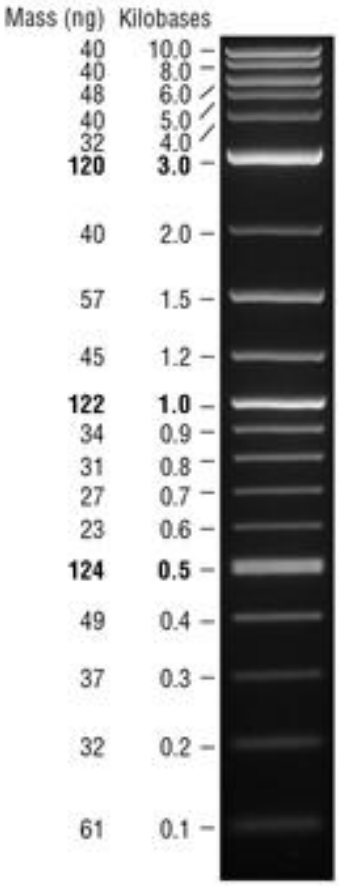
	Initial			Final		
No.	No dsRNA	Formula-1 (6mg dsRNA/kg)	Formula-2 (12mg dsRNA/kg)	No dsRNA	Formula-1 (6mg dsRNA/kg)	Formula-2 (12mg dsRNA/kg)
20	17.0	9.0	10.0	27.0	12.0	25.0
21	16.0	13.0	10.0	25.0	17.0	13.0
22	19.0	14.0	16.0	23.0	18.0	20.0
23	20.0	12.0	12.0	27.0	14.0	22.0
24	11.0	15.0	14.0	15.0	23.0	22.0
25	13.0	14.0	16.0	17.0	23.0	20.0
26	17.0	16.0	12.0	21.0	41.0	20.0
27	16.0	14.0	18.0	18.0	19.0	24.0
28	10.0	14.0	14.0	24.0	31.0	22.0
29	12.0	16.0	11.0	25.0	21.0	24.0
30	14.0	11.0	17.0	27.0	17.0	24.0
31	9.0	14.0	15.0	34.0	27.0	14.0
32	12.0	14.0	10.0	23.0	31.0	23.0
33	15.0	12.0	11.0	23.0	11.0	31.0
34	11.0	17.0	16.0	17.0	39.0	16.0
35	10.0	17.0	19.0	38.0	31.0	14.0
36	24.0	13.0	14.0	24.0	17.0	23.0
37	15.0	16.0	12.0	23.0	15.0	16.0
38	13.0	15.0	16.0	23.0	29.0	21.0
39	10.0	13.0	25.0	27.0	21.0	19.0
40	12.0	12.0	12.0	29.0	11.0	23.0
41	13.0	15.0	14.0	21.0	21.0	26.0
42	16.0	16.0	18.0	21.0	19.0	17.0
43	17.0	15.0	21.0	22.0	13.0	24.0
44	12.0	12.0	19.0	24.0	16.0	31.0
45	13.0	9.0	18.0	26.0	14.0	20.0
46	12.0	16.0	10.0	30.0	17.0	34.0
47	20.0	9.0	18.0	22.0	21.0	39.0
48	11.0	8.0	15.0	21.0	19.0	21.0
49	8.0	11.0	16.0	26.0	21.0	21.0

	Initial			Final		
No.	No dsRNA	Formula-1 (6mg dsRNA/kg)	Formula-2 (12mg dsRNA/kg)	No dsRNA	Formula-1 (6mg dsRNA/kg)	Formula-2 (12mg dsRNA/kg)
50	14.0	21.0	14.0	27.0	21.0	29.0
51	13.0	12.0	20.0	21.0	34.0	31.0
52	16.0	14.0	17.0	23.0	26.0	26.0
53	9.0	17.0	9.0	29.0	20.0	27.0
54	14.0	17.0	16.0	19.0	18.0	33.0
55	16.0	11.0	20.0	24.0	13.0	21.0
56	16.0	16.0	13.0	18.0	16.0	40.0
57	11.0	11.0	17.0	26.0	16.0	26.0
58	12.0	13.0	15.0	24.0	18.0	23.0
59	11.0	16.0	10.0	29.0	23.0	26.0
60	12.0	14.0	14.0	18.0	19.0	23.0
61	21.0	14.0	20.0	24.0	17.0	19.0
62	20.0	16.0	10.0	20.0	21.0	18.0
63	12.0	17.0	9.0	26.0	15.0	28.0
64	16.0	11.0	17.0	22.0	23.0	18.0
65	16.0	12.0	13.0	22.0	31.0	15.0
66	11.0	14.0	14.0	33.0	18.0	22.0
67	8.0	13.0	16.0	21.0	25.0	13.0
68	12.0	12.0	18.0	20.0	26.0	26.0
69	18.0	11.0	17.0	24.0	17.0	26.0
70	8.0	19.0	16.0	26.0	12.0	16.0
71	13.0	15.0	13.0	24.0	18.0	26.0
72	12.0	16.0	10.0	22.0	16.0	27.0
73	16.0	9.0	14.0	18.0	15.0	30.0
74	8.0	16.0	13.0	23.0	18.0	22.0
75	16.0	17.0	15.0	23.0	23.0	24.0
76	18.0	16.0	22.0	24.0	16.0	22.0
77	10.0	14.0	8.0	19.0	29.0	26.0
78	19.0	18.0	8.0	32.0	18.0	26.0

	Initial			Final		
No.	No dsRNA	Formula-1 (6mg dsRNA/kg)	Formula-2 (12mg dsRNA/kg)	No dsRNA	Formula-1 (6mg dsRNA/kg)	Formula-2 (12mg dsRNA/kg)
79	10.0	17.0	14.0	24.0	15.0	22.0
80	11.0	13.0	7.0	24.0	18.0	25.0
81	10.0	9.0	16.0	24.0	14.0	31.0
82	14.0	9.0	18.0	23.0	19.0	20.0
83	18.0	15.0	12.0	21.0	26.0	14.0
84	15.0	11.0	13.0	23.0	34.0	27.0
85	12.0	9.0	15.0	21.0	15.0	32.0
86	8.0	9.0	15.0	15.0	18.0	22.0
87	14.0	16.0	12.0	22.0	17.0	17.0
88	19.0	13.0	16.0	27.0	23.0	25.0
89	16.0	12.0	14.0	18.0	18.0	21.0
90	13.0	16.0	12.0	15.0	16.0	16.0
91	15.0	13.0	17.0	16.0	16.0	19.0
92	15.0	13.0	11.0	23.0	14.0	22.0
93	11.0	12.0	15.0	18.0	17.0	21.0
94	15.0	9.0	18.0	32.0	20.0	13.0
95	14.0	16.0	10.0	21.0	15.0	18.0
96	14.0	12.0	14.0	22.0	16.0	27.0
97	18.0	10.0	14.0	19.0	22.0	28.0
98	10.0	14.0	16.0	17.0	15.0	21.0
99	12.0	15.0	15.0	24.0	12.0	18.0
100	12.0	29.0	13.0	19.0	26.0	17.0
Total	1,414	1,373	1,379	2,037	2,359	2,296
Avg.	14.14	13.73	13.79	20.37	23.59	22.96
SD	3.18	3.28	3.38	6.28	5.05	4.38
CV%	22.52	23.87	24.51	30.84	21.43	19.07

MARKER

2 log DNA ladder



BIOGRAPHY

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PRESENTATIONS

1. **Thammasorn T**., Jitrakorn S., Barvornpinyo, S., Saksmerprome V., Development of dsRNA-delivery system for antiviral application in shrimp, MUSC Graduate Research Exposition 2012, Faculty of science, Mahidol Unniversity, Phayathai, Thailand. 2008. (Award : popular vote)
2. Saksmerprome V., **Thammasorn T**., Jitrakorn S., Wongtripop S., Borwornpinyo S., Withyachumnarnkul B., Using double-stranded RNA for the control of Laem-Singh Virus (LSNV) in Thai *P.monodon*, National Genetic Conference 2013 (NGC2013), Ambassador Hotel, Bangkok, Thailand. 2013.

PUBLICATIONS

1. Saksmerprome V, **Thammasorn T**, Jitrakorn S, Wongtripop S, Borwornpinyo, S. and Withyachumnarnkul B., Using double-stranded RNA for the control of Laem-Singh Virus (LSNV) in Thai *P. monodon.* J Biotechnol. 2013 Feb 13;164(4):449-53
2. **Thammasorn T**, Somchai P, Laosutthipong C, Jitrakorn S, Wongtripop S, Thitamadee S, et al. Therapeutic effect of *Artemia* enriched with *Escherichia coli* expressing double-stranded RNA in the black tiger shrimp Penaeus monodon. Antiviral Res. 2013;100(1):202-6.

PATENT

1. Saksmerprome V, **Thammasorn T**, Jitrakorn S, Wongtripop S, Withyachumnarnkul B. Feed formulation for inhibition of Laem-Singh virus in the black tiger shrimp *Penaeus monodon*. Thai Patent Application no. 1301000289