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Therapeutic effect of *Artemia* enriched with *Escherichia coli* expressing double-stranded RNA in the black tiger shrimp *Penaeus monodon*

Thitiporn Thammasorn [a,b], Parinyachat Somchai [a,b], Chaowanee Laosutthipong [a], Sarocha Jitrakorn [a,c], Somjai Wongtripop [c,d], Siripong Thitamadee [a,b], Boonsirm Withyachumnarnkul [a,d,e,f], Vanvimon Saksmerprome [a,c,*]

[a] Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Bangkok, Thailand
[b] Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
[c] National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani 12120, Thailand
[d] Shrimp Genetics Improvement Center (SGIC), Surat Thani, Thailand
[e] Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
[f] Aquatic Animal Biotechnology Research Center, Faculty of Science and Industrial Technology, Prince of Songkla University, Surat Thani Campus, Surat Thani, Thailand

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ABSTRACT

We exploited *Artemia* as a double-stranded (ds)RNA-delivery system to combat viral diseases in shrimp. 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 Laem-Singh virus (LSNV)-specific dsRNA, which has been previously shown to inhibit the viral infection in the black tiger shrimp *Penaeus monodon* by intramuscular injection and oral administration. The enriched *Artemia* nauplii were confirmed to contain dsRNA-LSNV by RT-PCR, and were subjected to the feeding test with *P. monodon* postlarvae. 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, average body weight of the treated group was markedly increased relative to the control group. A smaller differential growth rate of the treated group as compared to the control was also noticed. These results suggested that feeding shrimp with the dsRNA-enriched *Artemia* can eliminate LSNV infection, which is the cause of retarded growth in *P. monodon*. The present study reveals for the first time the therapeutic effect of dsRNA-enriched *Artemia* for shrimp disease control.

1. Introduction

Delivery of therapeutics/vaccine is a key for developing the practical methods for disease control in aquaculture. Various molecules that can act against viruses, including antibodies, shrimp viral binding proteins, and double-stranded (ds)RNA, are widely used to prevent shrimp diseases (Saksmerprome et al., 2009; Sritunyalucksana et al., 2006b; Tirasophon et al., 2007; Westenberg et al., 2005; Wu et al., 2005). However, most of the successful reports are the results of laboratory efforts. A limited number of work on improving "drug" delivery methods for shrimp farming include applications of chitosan nanoparticles and the recombinant bacteria strains (Sarathi et al., 2008; Theerawanitchpan et al., 2012; Attasart et al., 2013; Saksmerprome et al., 2013). Therefore, it is much needed to seek the efficient and inexpensive methods to deliver therapeutics/protective agents for farm applications.

Gene silencing triggered by RNA duplex or double-stranded (ds)RNA is a promising technique for antiviral and therapeutic applications. In this study, we are interested in using *Artemia* (brine shrimp), the most common live diets used in shrimp aquaculture, for delivering dsRNA to the black tiger shrimp (*Penaeus monodon*) at post-larval stage. Through the filtration process, *Artemia* encapsulate various dietary components, such as pigment and essential nutrients, necessary for post-larvae (Fernández, 2001). A study by Subhadra and his group supported the use of *Artemia* nauplii to deliver antibody into shrimp for controlling viral diseases (Subhadra et al., 2010).

Target virus of interest is Laem-Singh virus (LSNV), a possible causative agent of Monodon slow growth syndrome (MSGS) in cul-

* Corresponding author at: Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Bangkok, Thailand. Tel.: +66 2 201 5870; fax: +66 2 354 7344.
E-mail address: vsaksmer@gmail.com (V. Saksmerprome).

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tured black tiger shrimp in Thailand (Sritunyalucksana et al., 2006a). Retarded growth and wide variation of size (>35% of coefficient of variation) are among the main characteristics of MSGS shrimp that leads to economical loss in Thai shrimp farms since 2002. Here, *Escherichia coli* (*E. coli*)-expressed dsRNA specific to LSNV previously shown to effectively inhibit the virus by injection and oral administration (Saksmerprome et al., 2013) was encapsulated in *Artemia* via the enrichment process. *P. monodon* postlarvae (PL1–15) were fed with the enriched *Artemia* nauplii, and were examined for the antiviral effect against LSNV in comparison to the control group. This study proposed a novel dsRNA-delivery system by using *Artemia* as a live carrier for shrimp farm application.

2. Materials and methods

2.1. Preparation of transformed E. coli variants

pCS2 Containing red fluorescence protein gene (pCS2-RFP) was used as the template for PCR amplification of RFP gene with the primers, RFP_for (5′-GATCCACCATGGCCTCCT-3′) and RFR_rev (5′-ATCCTTAGGCGCCGGTG -3′). The RFP product was cloned into pDrive cloning vector (Qiagen, Valencia, CA, USA). The resulting pDrive-RFP was transformed into *E. coli* HT115 using heat shock method and induced with IPTG to express RFP marker protein. Transformed *E. coli* expressing dsRNA-LSNV was prepared according to the method described by Saksmerprome et al. (2013).

2.2. Artemia hatching

Artemia cysts (Aqua brand, USA) were incubated in artificial seawater at 35 ppt salinity at temperature of 28 ± 2 °C 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.

2.3. Artemia enrichment procedure

2.3.1. Enrichment of Artemia with E. coli expressing Red Fluorescent Protein (RFP)

The harvested *Artemia* nauplii were stocked for enrichment at a density of 100 nauplii per 1 mL of seawater at 35 ppt salinity with aeration, temperature of 28 ± 2 °C, pH 7–8. Total of 10^5 nauplii were enriched with 1.6×10^{10} CFU *E. coli* expressing RFP. At enrichment durations of 4, 6, 8, 22, 24, 26, 28, and 30 h, 10–20 *Artemia* were collected and washed with 200 ml of seawater. After that, *Artemia* sample was 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 micro-scopic slide for confocal microscopy. Fluorescent microscopic images (20× objective magnification) were taken using a Fluoview FV1000 confocal laser scanning biological microscope (Olympus, Japan). All images were analyzed in FV10-ASW3.0 viewer software (Olympus, Japan) and exported as JPEG file format.

2.3.2. Enrichment of Artemia with E. coli expressing dsRNA-LSNV

Approximately 2×10^6 *Artemia* nauplii were enriched with 4.3×10^{11} CFU *E. coli* expressing dsRNA-LSNV for 2 h in 100 mL of 35 ppt seawater with aeration, temperature of 28 ± 2 °C, pH 7–8. In parallel, the separated nauplii were incubated without enrichment as a negative control group. The 2.0×10^4 naupill of enriched *Artemia* were randomly collected daily and the non-enriched samples were collected on days 1, 5, 10 and 15. The *Artemia* sample was washed with 500 ml of seawater and subjected to dsRNA-LSNV detection. The enriched and non-enriched *Artemia* were harvested and rinsed with seawater prior to feeding shrimp PL. Each enrichment experiment was performed in triplicate.

2.4. Detection of dsRNA- LSNV in Artemia

To extract total RNA, the 2.0×10^4 *Artemia* were homogenized in 500 μl Tripure isolation reagent (Roche, Germany). Total RNA yield was determined by measuring UV absorbance at 260 nm. To indicate the presence of dsRNA-LSNV product, 150 ng total RNA were subjected to one-tube RT-PCR analysis (Titan, Roche, Germany). The LSNV-specific primers used were forward primer (5′ GGGTGAGCCC GTGACTCCTA 3′) and reverse primer (5′ GCCCCAGAAACGTATTGG-CAC 3′). The primers derived from *Artemia* actin including forward primer (5′ GGTCGTGACTTGACGGACTATCT 3′) and reverse primer (5′ AGCGGTTGCCATTTCTTGTT 3′) were used as the internal control. The conditions for RT-PCR amplification were as follows: 5 min at 50 °C, 5 min at 94 °C, 35 cycles of 10 s at 94 °C, 30 s at 60 °C and 30 s at 68 °C and extension at 68 °C for 5 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

2.5. Detection of LSNV in shrimp

Using Tripure isolation reagent (Roche, Germany), total RNA was extracted from shrimp pleopods. Total RNA yield was determined by measuring UV absorbance at 260 nm. 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-F (5′ CGTTGCCTTCTCCCGAGTGGT 3′) and 20AR (5′-CCG GCT GAG GTA GCT GCT TG-3′). Each 25 μl PCR master mix contained 5 μl diluted template, 4008 nM of each primer, 12.5 μl of 2 × SYBR® Green PCR Mix (Applied Biosystems) and 5.25 μl of RNase free water. Thermal cycling was performed on a 7500™ Real-Time PCR System (Applied Biosystems) using the conditions as follows: one cycle of 50 °C for 30 min and initial PCR activate step at 95 °C for 15 min followed by 35 cycles of 94 °C for 15 s, 65 °C for 30 s, and 72 °C for 30 s. 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 plasmid (3015-bp backbone) containing a 600-bp insert of 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.

2.6. Feeding trials

Feeding trials were carried out at shrimp genetic improvement center (SGIC), Surat Thani, Thailand. *P. monodon* post larvae 1 (PL1) were reared in 200-L tank with a density of 30 PL/L, 28 °C, and 25-ppt salinity. Shrimp were divided into two groups, one received *Artemia* containing dsRNA-LSNV and the other fed with 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. 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 at 4–23 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.

3. Results and discussion

3.1. Successful Artemia enrichment with E. coli expressing RFP and dsRNA-LSNV

Prior to the enrichment process, *Artemia* nauplii were randomly picked and subjected to RT-PCR analysis for LSNV detection (not shown). This was performed to ensure that they were disease-free. Two variants of transformed *E. coli* were tested in the encapsulation process. First, *Artemia* enrichment assay using *E. coli* expressing RFP was performed. Under confocal microscope, red fluorescent signals were observed in the *Artemia* gastrointestinal system at the enrichment durations of 0–30 h (Fig. 1a–d), indicating RFP uptake by *Artemia* through the encapsulation. The highest intensity of RFP signal was achieved at the enrichment of 28 h, and started to decline afterwards. Similar to previous reports (Allender et al., 2011; Immanuel et al., 2007; Subhadra et al., 2010), our results suggest that the bioenrichment process is time dependent.

In addition, the *Artemia* enrichment process was carried out using *E. coli* expressing dsRNA-LSNV that is previously shown to inhibit LSNV by injection and oral delivery (Saksmerprome et al., 2013). It is important that the dsLSNV-enrichment period was carried out soon after *Artemia* hatched or became Instar I nauplii, so *Artemia* remained the most nutritious diet for shrimp postlarvae. RT-PCR analysis was performed using total RNA extract from *Artemia* after 2 h of the enrichment. The amplified products shown in lanes 1–15 (Fig. 2a) indicated the presence of dsRNA-LSNV in the enriched *Artemia* for PL1–15 feeding, whereas no RT-PCR product was obtained from the non-enriched *Artemia* (Fig. 2b). Taken together, both RFP and dsRNA-LSNV enrichment assays suggest that biomaterials in their normal functions can be transported effectively to *Artemia* through the strategy developed herein.

3.2. Using Artemia for dsRNA delivery in shrimp postlarvae provides the therapeutic effect in P. monodon

To evaluate if the *Artemia* enriched with dsRNA-LSNV can control LSNV in shrimp, PL1 were divided into two groups that were daily fed with the enriched and non-enriched (control) *Artemia* for 15 days. Shrimp from each group were randomly selected at 15 weeks after *Artemia* feeding and subjected to quantitaive RT-PCR analysis. As shown in Fig. 3a, thirteen of twenty shrimp that received the non-enriched *Artemia* (control) contained more than 100 LSNV copies/total RNA (ng). In contrast, most of the shrimp in the enriched group (Fig. 3b) contained 0.1 LSNV copies/total RNA (ng), and only two specimen were shown to have more than 100 viral copies/total RNA(ng). qRT-PCR indicated that a number of LSNV copies in most of the treated shrimp were, at least, 1000-fold lower than the control animals, suggesting the efficient LSNV inhibition in shrimp by the dsRNA-enriched *Artemia*.

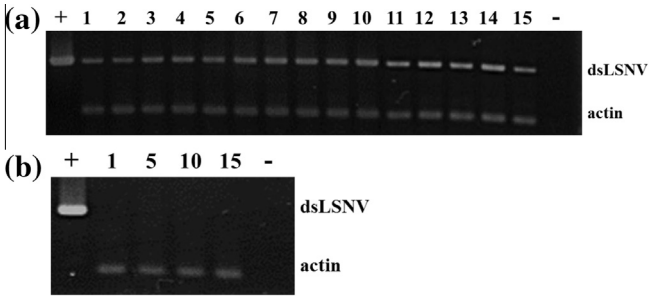

Fig. 2. RT-PCR analysis for dsLSNV detection in the enriched (A) and non-enriched (B) Artemia. Lane +, LSNV-cloned plasmid as positive control; lanes 1–15, Artemia samples in day 1–15 of enrichment; lane –, negative control (using water instead of the extracted RNA).

Indicators for culture performance including mean body weight (MBW) and percentage of CV were monitored for the therapeutic effect of dsRNA-enriched *Artemia*. During 11–17 weeks after *Artemia* feeding, MBW of the treated group was elevated as compared to the control group (Fig. 4). As shown in Table 1, CV values of the treated groups (37.29, 38.99, and 32.26) were lower than the control (51.40, 44.38, and 35.41) at all time points, indicating a smaller differential growth rate of the treated group relative to the control. Interestingly, MBW were not markedly improved, although dsRNA-mediated LSNV inhibition was clearly pronounced. With early infection of the shrimp, i.e. from vertical transmission, LSNV might have damaged the zona fasciculata already and by getting rid of them at postlarval stage may not be "early enough" since the damage could occur earlier in the embryogenesis stage. This study raises a possibility that if LSNV-

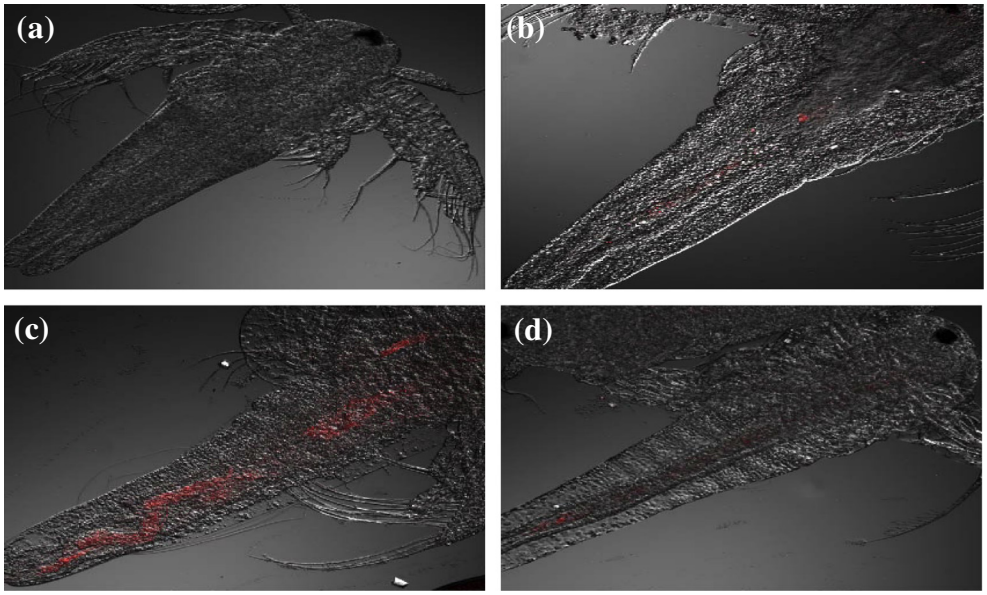

Fig. 1. Confocal microscopy of the *Artemia* gut at the *E. coli* RFP-enrichment periods of 0 (a) 4 (b) 28 (c) and 30 h (d).

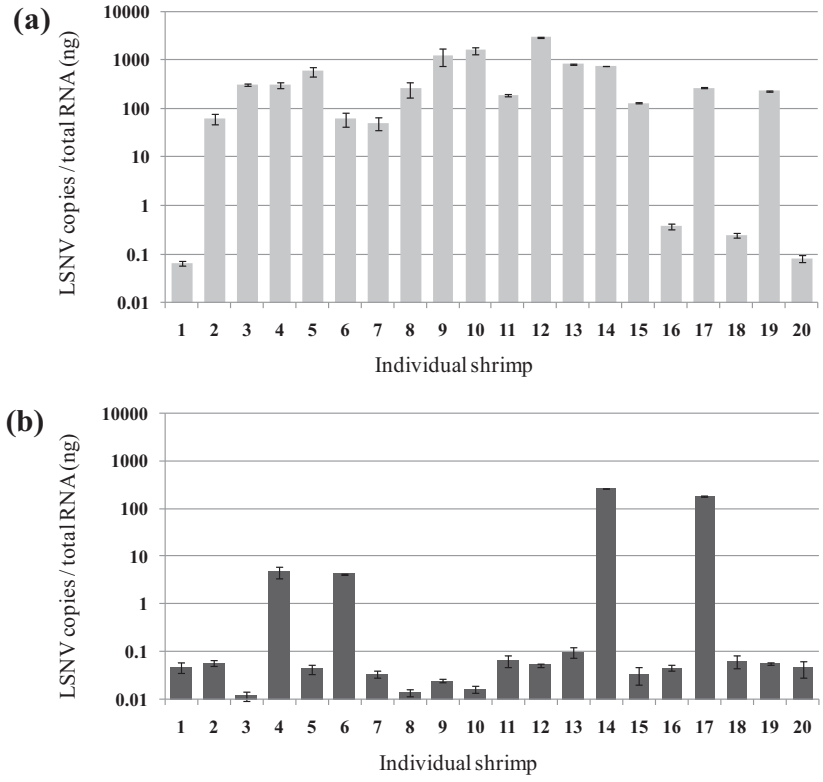

Fig. 3. Real-time RT-PCR analysis indicates the number of LSNV copies per total RNA (ng) in individual shrimp at 15 weeks after feeding the non-enriched *Artemia* (a) and the enriched *Artemia* (b). Error bars indicate the standard deviation.

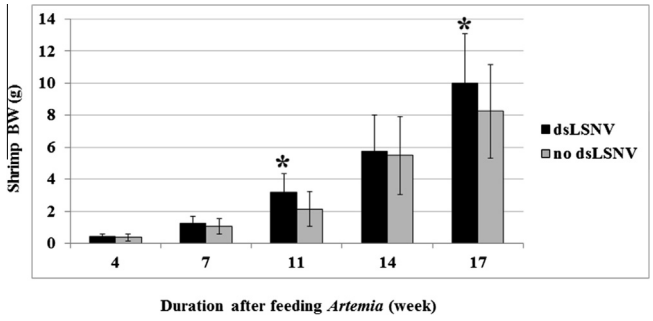

Fig. 4. Average body weights of shrimp *P. monodon* that received the dsRNA-enriched *Artemia* as compared to those of the non-enriched *Artemia*. Data were collected at 4, 7, 11, 14 and 17 weeks after shrimp were fed with *Artemia*. Error bars indicate the standard deviations, and asterisk indicates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

Table 1
Shrimp production performance at 4, 7, 11, 14 and 17 weeks after feeding with *Artemia* (number of shrimp = 50). MBW = mean body weight, SD = standard deviation, and CV = coefficient of variation.

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

dsRNA is also given to broodstock to make them LSNV-free, the growth rate of their offspring might be much higher.

In summary, the findings confirm that feeding shrimp with the dsRNA-enriched *Artemia* can inhibit LSNV infection and relieve MSGS conditions in *P. monodon*. Using *Artemia* enriched with dsRNA for shrimp disease control is practical and inexpensive for farm application. For shrimp production of one-hectare pond, the additional cost for the *Artemia* enrichment was estimated to be only 0.6-fold higher than the non-enrichment *Artemia*. On the other hand, an increase of 1 g in MBW, due to the application of therapeutic dsRNA-LSNV, is equivalent to 240 kg of total shrimp mass in 1-ha pond. The increase in mass production could result in an elevated income for at least 1600 US$ per 1-ha pond. As a result, benefits from the enhanced culture performance by using the dsRNA-enriched *Artemia* would be more likely to offset the enrichment cost.

4. Conclusions

In the present work, *Artemia* nauplii are shown to deliver dsRNA targeting LSNV to *P. monodon* postlarvae for inhibiting LSNV, a causative agent of MSGS conditions. The results suggest a dual function of *Artemia* nauplii as the nutritious diets and therapeutic delivery system. This is the first report on the antiviral effect of dsRNA-encapsulated *Artemia* in the larviculture of shrimp. The work offers a practical and inexpensive dsRNA delivery-system for controlling viral disease in shrimp farming. Ultimately, application of dsRNA for shrimp aquaculture via combinatorial approaches (i.e. bioencapsulated *Artemia* and oral administration by feed) should further improve viral protective performance of shrimp stock throughout domestication.

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