รายงานวิจัยฉบับสมบูรณ์

โครงการ การใช้เทคนิค RNAi ในการยับยั้งการติดเชื้อ HPV ในกุ้งกุลาดำ

RNA interference for inhibition of Hepatopancreatic parvovirus (HPV) Infection in *Penaeus monodon*

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บทคัดย่อ

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ชื่อโครงการ: การใช้เทคนิค RNAi ในการยับยั้งการติดเชื้อ HPV ในกุ้งกุลาดำ

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ด้วยระบบการจัดการในฟาร์มเลี้ยงกุ้งที่ไม่เหมาะสมมีผลส่งให้เกิดปัญหาสำคัญต่าง ๆตามมาเช่น การเกิดโรคระบาดจากไวรัส ซึ่งไวรัส HPV เป็นหนึ่งในนั้นที่ทำให้กุ้งมีลักษณะแคระแกรน มีราคาขายที่ต่ำกว่ากุ้งปกติอย่างมาก ดังนั้นในการศึกษาครั้งนี้จึงได้นำเอาเทคนิค RNAi ซึ่งมีประสิทธิภาพสูง มาใช้ในการยับยั้งไวรัสชนิดนี้ โดยสร้างอาร์เอ็นเอสายคู่ 3 ชนิดที่มีความจำเพาะกับยีนที่ไม่ได้เป็นส่วนประกอบโครงสร้างไวรัส (ns1 และ ns2) และยีนที่เป็นส่วนประกอบโครงสร้างของไวรัสเช่น vp แล้วจึงทำการฉีดอาร์เอ็นเอสายคู่ดังกล่าวเข้าสู่กุ้งก่อนทำให้กุ้งติดไวรัส ในการศึกษานี้ยังได้แสดงเป็นครั้งแรกที่สามารถทำให้กุ้งในระยะ juvenile ติดไวรัส HPV โดยการกินได้สำเร็จ หลังจากทำให้กุ้งติดไวรัสแล้วเป็นเวลา 2 อาทิตย์ ผลการยับยั้งไวรัสจากการฉีดอาร์เอ็นเอสายคู่จะถูกแสดงโดยวิธี semi-quantitative PCR พบว่ามีการลดลงของไวรัสในกุ้งที่ได้รับอาร์เอ็นเอสายคู่ ในขณะที่กลุ่มกุ้งควบคุมที่ไม่ได้รับอาร์เอ็นเอสายคู่สามารถตรวจสอบไวรัสได้ปริมาณมาก นอกจากนี้ยังเห็นผลยับยั้งบ้างในกุ้งที่ได้รับอาร์เอ็นเอสายคู่ที่จำเพาะกับ gfp จากผลการทดลองด้วยปริมาณกุ้งที่มีจำกัดทำให้สามารถสรุปได้ว่าอาร์เอ็นเอสายคู่ที่จำเพาะกับไวรัสสามารถยับยั้งการเพิ่มจำนวนไวรัส HPV ได้ โดยอาร์เอ็นเอสายคู่ที่จำเพาะกับยีน ns1 ให้ผลยับยั้งสูงกว่ายีน vp ในการศึกษาครั้งนี้ยังได้ทดสอบประสิทธิภาพของอาร์เอ็นเอสายคู่ที่จำเพาะกับยีน ns1 ในการกำจัดไวรัสในกุ้งที่ติดไวรัสมาแล้วจากธรรมชาติ ซึ่งหลังจากทำการฉีดอาร์เอ็นเอสายคู่ต่อ ns1 เข้าสู่กุ้งเป็นจำนวนหลายครั้งพบว่าสามารถกำจัดไวรัสออกจากกุ้งได้อย่างชัดเจน ในขณะที่กุ้งในกลุ่มที่ไม่ได้รับอาร์เอ็นเอสายคู่และได้รับอาร์เอ็นเอสายคู่ต่อ gfp มีปริมาณไวรัสอยู่จำนวนมาก แสดงให้เห็นว่าอาร์เอ็นเอสายคู่ต่อ ns1 สามารถนำมาใช้ในการป้องกันและรักษากุ้งจากการติดไวรัส HPV ได้ ทั้งนี้ยังต้องมีการปรับปรุงในเรื่องการนำอาร์เอ็นเอสายคู่เข้าสู่กุ้งโดยการกินต่อไป เพื่อนำไปประยุกต์ใช้ในฟาร์มเลี้ยงกุ้งในอนาคต

Abstract

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Project Title: RNA interference for inhibition of Hepatopancreatic parvovirus (HPV) infection in *Penaeus monodon*

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Shrimps with improper farm management leads to many serious problems such as pandemic diseases particularly from virus infection. Hepatopancreatic parvovirus (HPV) causing stunted shrimps is one of such pathogens. Even though the HPV infected *P. monodon* has survived, the price is much lower than that of the normal one. Therefore, the most promising strategy so far, RNA interference (RNAi), was applied to combat with this virus in this study. Three dsRNAs corresponding to the non-structural protein (ns1 and ns2) and the structural protein (vp) genes of HPV were synthesized and introduced into shrimp haemolymph prior to viral challenge. Successful experimental infection of HPV via the oral route in juvenile shrimp was first demonstrated. After allowing viral replication for two weeks, the suppression effect by each dsRNA was evaluated by semi-quantitative PCR and compared with the control (NaCl or non-related dsRNA-gfp). A reduction of HPV in shrimp treated with each dsRNA was observed. In contrast, a high level of viral infection was detected in the control group (NaCl). Moreover, partial viral inhibition was detected while using the dsRNA-gfp. Based on a limited sample number, we reached the tentative conclusion that virus-specific dsRNA can inhibit HPV replication in which the dsRNA-ns1was more effective than the dsRNA-vp. Moreover, the curative effect of dsRNA-ns1 in the natural HPV infected-shrimps was investigated. After multiple injection of dsRNA-ns1, the clearance of HPV was observed whereas the strong viral infection was detected in the control group (NaCl and non-specific dsRNA-gfp). It demonstrates that dsRNA-ns1 gives both protection and therapeutic effects on HPV infection in shrimp. The delivery approach by oral feeding will be further established before the benefits to shrimp farming.

Keywords: Hepatopancreatic parvovirus, *Penaeus monodon* densovirus, shrimp, RNAi, double-stranded RNA

1. Introduction

During the past few years the shrimp culturing industry has encountered a tremendous economic loss which is mainly from viral infectious diseases. Among the causative pathogens, *Penaeus monodon* densovirus (*Pm*DNV) (formerly hepatopancreatic parvovirus or HPV) has been reported to be associated with a slow growth rate and stunted appearance of shrimp (Flegel et al., 1999). Although it does not cause an abrupt mortality crisis of cultured shrimp as WSSV and YHV-associated outbreaks, the infected shrimp have the same value as the dead ones. This problem leads to the significant reduction of shrimp production and a consequent loss of profit. At present, there is no available treatment for this disease.

The recently described phenomenon of RNA interference (RNAi) provides a powerful means for silencing gene expression in a sequence-specific manner (Fire, 1999; Hannon, 2002). Moreover, it has been shown to be applicable to viral protection in a number of organisms, including shrimp, through the introduction of exogenous, specific double-stranded RNA (dsRNA). Previously published data has demonstrated that the replication of both RNA (Yodmuang et al., 2006; Tirasophon et al., 2005, 2007) and DNA viruses (Robalino et al., 2005; Kim et al., 2006; Attasart et al., 2009) was effectively inhibited by dsRNAs corresponding to their viral gene targets. Consequently, the shrimp mortality was significantly diminished when compared to shrimp without treatment with dsRNA in which all shrimp were dead.

*Pm*DNV is a non-enveloped virus with icosahedral shaped particle (Lightner and Redman, 1985). It contains a linear single-stranded DNA genome with typical palindromic termini (Bonami et al., 1995). According to the report of the complete sequence of Thai isolate (Sukhumsirichart et al., 2006), it composes three large open reading frames (ORFs); two of non-structural protein genes (ns1 and ns2) and one structural protein gene (vp). The ns1 protein of parvovirus is the protein known to have multiple functions including ATP-dependent site-specific DNA binding and nicking and also helicase activities which are essential for viral replication (Cotmore et al. 1994; Nuesch et al. 1995; Wilson et al., 1991). Meanwhile, a phospholipase A2 activity of vp protein has been reported to be critical for viral infectivity (Canaan et al., 2004). However, the biological function of ns2 during the parvovirus life cycle is still obscure (Cotmore et al. 1997; Naeger et al., 1990).

In this study, we determined the effectiveness of dsRNA for the inhibition of *Pm*DNV in *P. monodon* in both protective and curative modes.

2. Materials and Methods

2.1 ***Shrimp specimens***

The 300 mg juvenile *P. monodon* was used for all experiments in this study. The post-larva stage shrimps (P15-20) obtained from a hatchery farm were reared in a 500 L tank containing artificial sea water at 10 parts per thousand (ppt) salinity with aeration until they reached juvenile stage with the appropriate size. The shrimps were fed daily with a pellet shrimp diet.

The natural *Pm*DNV infected- *P. monodon* (300 mg) were obtained from a hatchery farm since they were at post-larva stage. They were reared in 10 ppt artificial sea water with aeration and fed daily with a pellet shrimp diet until they reached the juvenile stage.

2.2 Virus source

Hepatopancreas isolated from naturally *Pm*DNV-infected shrimp was utilized for viral infection through an oral route. To quantify the amount of virus present before feeding, the level of *Pm*DNV in each hepatopancreas was determined by PCR. The tissue was kept at -20°C until required.

2.3 Stem loop RNA expression plasmid construction

2.3.1. For viral specific dsRNA: Two regions of *Pm*DNV genome corresponding to ns1 and vp gene shown in Fig. 1 were selected for dsRNA targeting. PCR products (400 and 600 bp) of each targeted gene were amplified using Vent DNA polymerase and the specific primer pair shown in table 1 (nos. 1 and 2 of each gene for 400 bp while nos. 3 and 4 of each gene for 600 bp). These two fragments were then cloned into pET17b vector in an inverted manner directed by restriction enzymes flanking the fragments (*Nde*I and *Eco*RV for 600 bp, *Eco*RV and *Xho*I for 400 bp). PCR and restriction enzyme analysis were then used to screen the recombinant clones. Furthermore, the nucleotide DNA sequence of the insert was confirmed by automated DNA sequencing.

2.3.2. For non-related dsRNA (green fluorescent protein, gfp): The recombinant plasmid was kindly provided by Dr. Witoon Tirasophon, Mahidol University. The cloning strategy was similar to that of specific dsRNA except pET3a was used as a cloning vector instead of pET17b and *Xba*I was used for the joining of the two fragments and *Nde*I for inserting into the plasmid vector (Yodmuang et al., 2006).

2.4 ***Double-stranded RNA production***

dsRNA was expressed in *Escherichia coli* HT115 according to the protocol of Ongvarrasopone et al. (2007). The overnight bacterial culture in LB medium with antibiotic was diluted with new medium and grown until an OD_{600} of 0.4 at 37 °C was achieved. The

expression of dsRNA was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) and the culture was further incubated for 4 h. The bacterial cells were then harvested by centrifugation at 6000 x g for 5 min at 4 °C. The cell pellet was resuspended in 0.1% sodium dodecyl sulphate (SDS) with a ratio of 50 µl per 1 OD. To lyse the cells, the cell suspension was boiled for 2 min. Next, the bacterial single-stranded RNAs (ssRNAs) and the loop region of the expressed RNA were eliminated by incubation with RNaseA (1 µg RNaseA per 1 OD cells) in the reaction buffer (300 mM sodium acetate, 10 mM Tris-Cl pH 7.5) at 37°C for 30 min. The remaining dsRNA (400 bp) was subjected to purification by TRI reagent (Molecular Research Center) following the manufacturer's protocol. Finally, the concentration of each dsRNA was estimated by gel electrophoresis using a standard DNA marker.

2.5 Verification of dsRNA

To ensure the integrity of the synthesized dsRNA before introducing into shrimp, treatment with RNaseA (specific to ssRNA) and RNaseIII (specific to dsRNA) was carried out. An equal amount of dsRNA was separately digested with RNaseA (0.01 µg RNaseA per 2 µg dsRNA) and RNaseIII (0.5 units RNaseIII per 2 µg dsRNA) in the reaction buffer of RNaseA (300 mM sodium acetate, 10 mM Tris-Cl) and of RNaseIII (10 mM Tris-Cl, 0.1 mM $CaCl_2$, and 2.5 mM $MgCl_2$) respectively at 37 °C for 5 min. The patterns of the digested RNAs were then determined by gel electrophoresis with a standard size marker.

2.6 ***Experimental condition***

2.6.1. For infectivity of PmDNV

In order to determine the infectivity profile of *Pm*DNV in shrimp, shrimps (300 mg) were kept in individual petridish (90 mm x 15 mm) in 10 ppt artificial sea water. Thirty six hours pre-fasting shrimps were fed with *Pm*DNV-infected hepatopancreas (approximately 10% of its body weight per one meal) 3 times with roughly 12 h intervals. Subsequently, the shrimps were fed daily with pellet shrimp diet up to two weeks. Sampling of 2-3 shrimps at 4, 8, and 14 days after first feeding were performed to investigate the viral production in hepatopancreas by PCR analysis.

2.6.2. For viral protection

According to the infectivity test, shrimp of the same size were injected with approximately 750 ng of each viral specific dsRNA (ns1 and vp) 24 h before viral infection by oral feeding as previously described. To maintain the effect of dsRNA during the experiment, another dsRNA administration was carried out on day 5 post the first injection. Thereafter, the shrimp were fed daily with pellet shrimp diet. Two weeks after oral feeding with *Pm*DNV-infected tissue, the hepatopancreas of each shrimp was isolated for further DNA extraction. The inhibitory effect of delivered dsRNA was then evaluated by semi-quantitative PCR.

2.6.3. For viral clearance

In order to determine the effect of dsRNA-ns1 in the therapeutic mode, the 300-400 mg naturally *Pm*DNV infected-shrimps were treated with the dsRNA-ns1 2-4 times (as indicated in each experiment). An approximate 800 ng of dsRNA-ns1 was used for each injection. During the experiment, shrimps were kept in an individual cage maintaining in the same tank in 10 ppt artificial sea water with aeration. Five days after the last injection, hepatopancreas of each shrimp was collected for DNA extraction. The amount of viral DNA in each shrimp was subsequently determined by semi-quantitative PCR.

2.7 ***DNA extraction***

Total DNA was extracted from hepatopancreas tissues (or as indicated) using TRI reagent (Molecular Research Center). The 50-100 mg of tissue was ground in 1 ml TRI reagent. The DNA in the interphase and organic phase was precipitated by absolute ethanol. The DNA pellet was washed with 0.1 M Tri-sodium citrate and 75% ethanol and then resuspended in sterile distilled water. To increase solubilization, the DNA pellet was heated at 65 °C for 10 min. The absorbance at 260 nm of each DNA sample was measured in order to calculate its concentration.

2.8 Sample analysis

Semi-quantitative PCR

Total amount of extracted DNA (approximately 600 ng for almost PCR amplification except 200 ng for the curative experiment) was used for amplification of the viral specific gene (vp) using primers (nos. 5 and 6 for infectivity, protection experiment and curative experiment, nos. 7 and 8 for tissue distribution test) listed in table 1. To determine the relative amount of virus between samples, PCR of an internal control gene (shrimp beta actin) was included (primers nos.1 and 2 for infectivity and protection experiment, nos. 3 and 4 for tissue distribution test). The temperature profile for PCR amplification was as follows; 94 °C for 2 min, denaturation at 94ºC for 10 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 1 min. After 20 cycles (35 cycles for tissue distribution experiment), the reaction was held at 72ºC for another 5 min. The PCR product was analyzed by agarose gel electrophoresis.

3. Results and Discussion

3.1 ***Double-stranded RNA verification***

To proof whether RNA was double-stranded RNA, the RNA extracted from bacterial cells was incubated with RNaseA and RNaseIII which specifically cleaved ssRNA and dsRNA, respectively. The result showed that all synthesized RNA was digested by RNaseIII but not RNaseA indicating that the RNA was double-stranded (Fig. 2). In contrast, the control single-stranded RNA (ss) was completely digested with RNaseA. It clearly demonstrated that the synthesized dsRNAs (ns1, vp and gfp) were suitable for injection into shrimp to trigger the anti-virus pathway.

3.2 ***Localization of PmDNV in various tissues of shrimp***

To date, the distribution of this virus in the tissues of shrimp has not been investigated. In this study, the localization of *Pm*DNV in various organs of shrimp (hepatopancreas, gill, pleopod, periopod and muscle) was determined. Semi-quantitative PCR of the specific viral vp gene was performed by using an equal amount of DNA template extracted from each tissue. By normalizing to the β-actin control, it could be seen that the predominance of *Pm*DNV was in the hepatopancreas (Fig. 3). Much less was noted in periopod, gill, pleopod and muscle. For this reason, tissue from the hepatopancreas was used for all subsequent experiments.

3.3 Experimental infection of PmDNV in shrimp

In order to determine an infectivity profile, shrimps were fed with *Pm*DNV-infected hepatopancreas and randomly selected for testing at three different time points (4, 8, and 14 days post first feeding). Viral replication was then assayed by PCR. A reduction in the amount of virus present was observed at day 8 compared with total virus uptake at day 4. The amount of virus was then increased significantly at day 14 (Fig. 4). This phenomenon may be due to the cellular composition of the hepatopancreas. It is widely known that parvoviral replication depends on cellular factors associated with the S phase of the cell cycle to convert its ssDNA genome into a double-stranded RF for further genome amplification (Bashir et al., 2000). There are four main epithelial cell types present in the hepatopancreas, E (embryonic), F (fibrilar), B (blister-like), and R (absorptive) cells (Al-Mohanna et al., 1989). Out of these, only the E cells show mitotic activity. Hence, it is possible that the large number of virus particles initially entered into all of the types of epithelial cells during the feeding period but only the dividing E cells, which are a small proportion (less than 10%) of the hepatopancreatic epithelial cells (Zilli et al., 2003), were able to support viral replication. On the other hand, the other differentiated epithelial cells (F, B, and R cells) would have eventually been disintegrated and extruded from the tubule epithelium (Vogt, 1994) releasing a digestive enzyme together with the viral particles into the lumen of hepatopancreatic tubule. The virus would then later be excreted out via feces (Pantoja and Lightner, 2001). This explains the significant reduction of *Pm*DNV at day 8 compared to the initial uptake. Subsequently, propagation of the virus was promoted due to division of the infected E

cells.This indicates that the infection of shrimp with *Pm*DNV can be experimentally investigated over a time period of up to 14 days.

3.4 Suppression of PmDNV replication by dsRNA

To test the efficacy of dsRNA for inhibition of viral replication, long dsRNA (approximately 400 bp) targeted to viral genes, including non-structural (ns1) and structural protein genes (vp), were introduced into the shrimp's haemolymph followed by *Pm*DNV challenge. The amount of virus in each shrimp that received viral specific dsRNA was analyzed and compared with the control (without dsRNA) and non-specific dsRNA (gfp). Fourteen days post-infection, viral DNA in the hepatopancreas of each shrimp was monitored by PCR co-amplified with shrimp actin gene for normalization. The reduction of *Pm*DNV in shrimp treated with dsRNA-ns1 was more pronounced than dsRNA-vp. A high level of viral infection was observed in the control group (Fig. 5). In addition to this, a partial viral inhibition was detected from dsRNA-gfp. Our findings are in agreement with a number of previous studies demonstrating dsRNA mediated viral suppression in shrimp (Robalino et al., 2005; Tirasophon et al., 2005; Kim et al., 2006; Attasart et al., 2009). It has been documented that silencing of the expression an essential viral protein gene can significantly prohibit the infection of that particular virus. According to the strength of the inhibitory effect, the different gene targets of dsRNA provided different degrees of effect for viral protection. The non-structural protein gene was more potent than the structural protein gene. In the case of *Pm*DNV, the most effective target was probably the multi-functional ns1 protein gene. The ns1 protein has been reported to be involved in the early step of parvovirus infection via the binding and nicking of the double-stranded replicative form (RF) of DNA generating the single-stranded DNA. This then further facilitates the replication process (Astell et al., 1985; Baldauf et al., 1997; Bashir et al., 2000). In addition to this, the ns1 of parvovirus minute virus of mice (MVM) has been shown to regulate the expression of the late protein gene (vp) through promoter activation (Christensen et al., 1995). Because of these essential functions, the ns1 of *Pm*DNV may represent a potential target for further development of anti-*Pm*DNV approach.

3.5 Clearance of PmDNV in infected-shrimp by dsRNA-ns1

To investigate the inhibitory effect of dsRNA-ns1 for curing the *Pm*DNV from the naturally infected-shrimp, dsRNA-ns1 was multiple injected into shrimp haemolymph. The remaining of virus in shrimp treated with dsRNA-ns1 was evaluated and compared with the control (no dsRNA) and non-related dsRNA (gfp). The clearance of *Pm*DNV was obviously detected in the shrimp injected with dsRNA-ns1 (Fig. 6, 7). In contrast, high amount of *Pm*DNV was observed in both control groups (no dsRNA and dsRNA-gfp). The result strongly demonstrates that the viral specific dsRNA-ns1 confer the *Pm*DNV inhibition in the therapeutic modes in shrimp whereas the non-related dsRNA-gfp could not.

In conclusion, an effective tool for both prevention and curative of *Pm*DNV in shrimp using virus-specific dsRNA has been demonstrated in this study. This finding would provide the beneficial tool for further application to control this disease in the shrimp farm.

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5. Tables and Figures

gene	Primer sequences
ns1	ns1-1: 5′ CCCCAGATATCTTGCGTATTTCCTGTCCCTG 3′ ns1-2: 5′ CCGCTCGAGACATTTTACTCTGGTCTCATTG 3′ ns1-3: 5′ CGGAATTCATATGACATTTTACTCTGGTCTCATTG 3′ ns1-4: 5′ CCCCAGATATCGCCCTAGAACTGCTTAGTC 3′
vp	vp-1: 5′ CCCCCCGATATCTCTGTGCTGTCTGAAAATCCT 3′ vp-2: 5′ CCGCTCGAGCCAAAGTAAGCGAAAGAATAAAC 3′ vp-3: 5′ CGGAATTCATATGCCAAAGTAAGCGAAAGAATAAAC 3′ vp-4: 5′ CCCCCCGATATCATTTCTTAGCGTTTTCTATGCG 3′ vp-5: 5′ AATCTGCAGGGTACGGAAAAAAC 3′ vp-6: 5′ TGTGGAACCATCTCAAATGCC 3′ vp-7: 5′ ATCTGGATAGTATACATGTC 3′ vp-8: 5′ GGAGATATTAAGCACAGTTTC 3′
actin	actin-1: 5′ GACTCGTACGTCGGGCGACGAGG 3′ actin-2: 5′ AGCAGCGGTGGTCATCACCTGCTC 3′ actin-3: 5′ CAAGTGCTTCTAAGGATACTG 3′ actin-4: 5′ CATGATTATTTTGTATATATTATCG 3′

Table 1 Primer sequences for PCR amplification in this study.

Viral gene specific primers were designed according to the sequence of *Pm*DNV genome Thai-isolate (GenBank accession no. **DQ002873**).

Fig. 1 Schematic diagram of dsRNA targeted DNA regions on *Pm*DNV genome. Each arrow represents a particular gene (ns2, ns1, and vp) lying on a line of *Pm*DNV genome. The regions targeted by dsRNAs are shown in gray boxes.

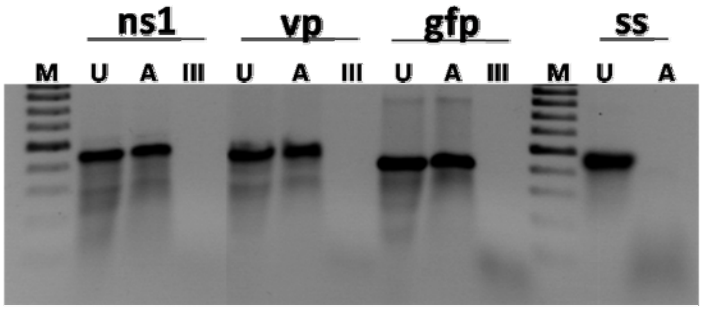

Fig. 2 Double-stranded RNAs verification by RNase treatment. The integrity of each dsRNA targeted to ns1 gene (ns1) or vp gene (vp) or gfp gene (gfp) was confirmed by incubation with RNase A (A) and RNase III (III) which specifically digested ssRNA and dsRNA, respectively. The untreated dsRNAs (U) and ssRNA (ss) were used as the control. M is marked for the 100 bp DNA ladder.

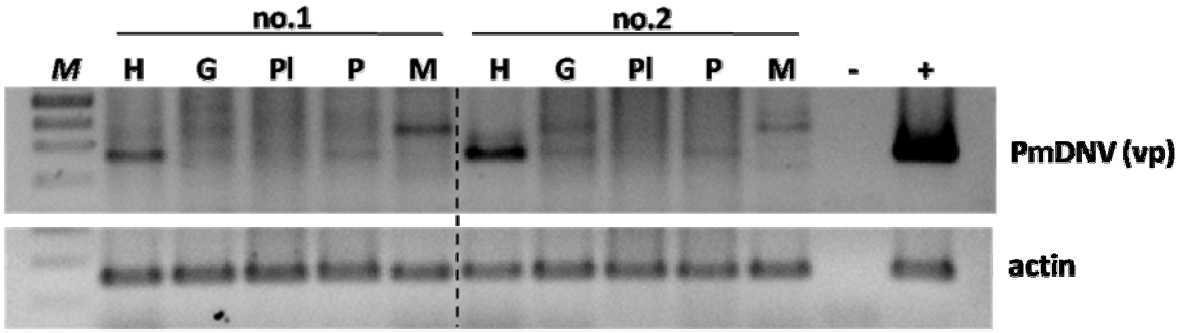

Fig. 3 Tissue distribution of *Pm*DNV in a naturally infected shrimp. Equal amount of total DNA extracted from various tissues (hepatopancreas (H), gill (G), pleopods (Pl), periopods, (P), and muscle (M)) of each naturally *Pm*DNV-infected shrimp were subjected to PCR analysis to determine the viral localization. The product was separately amplified using viral specific primers (*Pm*DNV, vp) and host beta-actin primers (actin). To control the experiment, negative (-) and positive (+) control were included. Number on the top indicates an individual shrimp. The size of PCR product was compared with the 100 bp DNA marker (*M*).

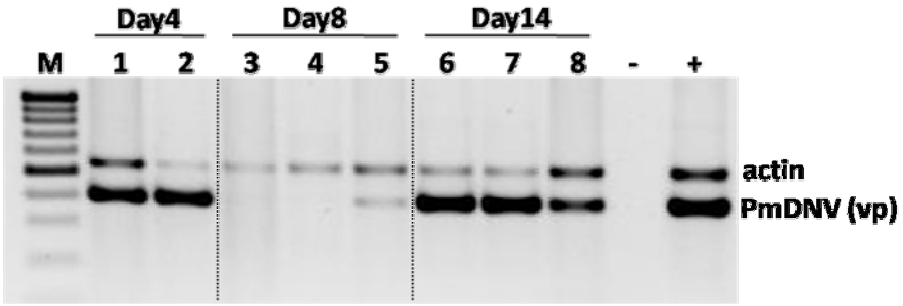

Fig. 4 Infectivity profile of *Pm*DNV in shrimp after feeding with hepatopancreas of *Pm*DNV-infected shrimp. The 300 mg shrimps were fed 3 times (approximately 30 mg each meal and 12 h intervals) with *Pm*DNV-infected hepatopanceas. Shrimps were randomly picked up and sacrificed at 4 (Day4), 8 (Day8), and 14 (Day14) days post-feeding. Total DNA extracted from hepatopancreas of each shrimp was used as a template for semi-quantitative PCR using the viral specific primers (*Pm*DNV) and the internal control actin primers (actin). Each shrimp is numbered on the top of gel while M is the 100 bp marker. Negative (-) and positive (+) control of the reaction are added.

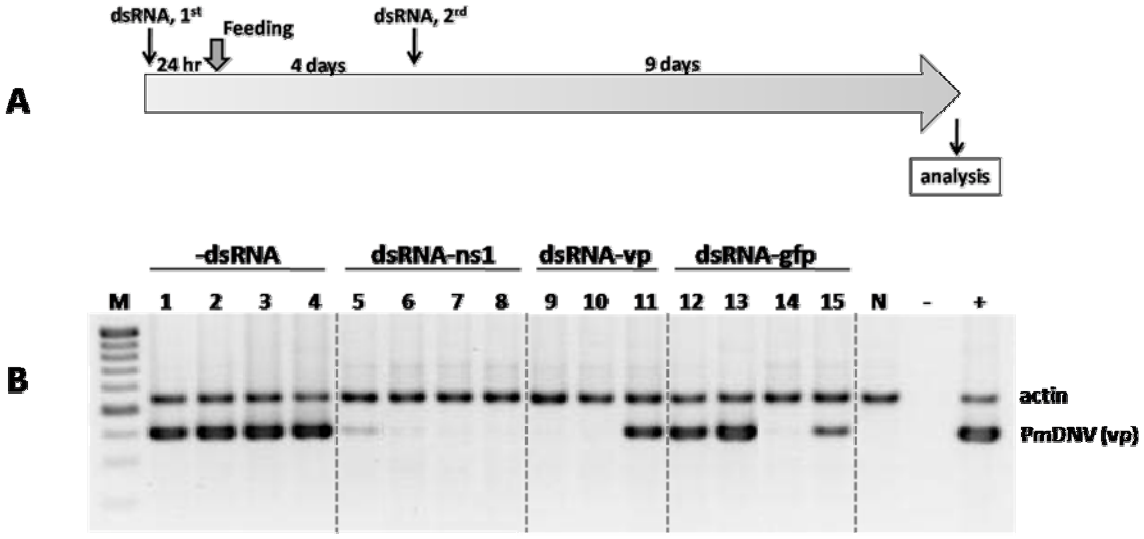

Fig. 5 Treatment of dsRNA for inhibition of *Pm*DNV in shrimp. The 300 mg shrimps were injected with 750 ng dsRNA specific either to ns1 gene or vp gene or non-related gfp gene two times (as indicated in the diagram) before challenging with *Pm*DNV by oral feeding with infected tissue. Shrimps were treated with NaCl instead of dsRNA in the control group. Fourteen days after first dsRNA administration, hepatopancreas of each shrimp was collected for DNA extraction and further PCR analysis was performed. (A) Diagram represents the experimental condition. (B) Semi-quantitative PCR of *Pm*DNV DNA. The amount of *Pm*DNV in an individual shrimp was determined by multiplex PCR using the viral specific primers (vp) together with the host control gene (actin) primers for normalization. Lanes 1-4 represent individual shrimp receiving no dsRNA; 5-8 dsRNA-ns1; 9-11 dsRNA-vp; 12-15 dsRNA-gfp. N is the normal shrimp. Negative (-) and positive (+) control of the reaction are included with the 100 bp DNA marker in lane M.

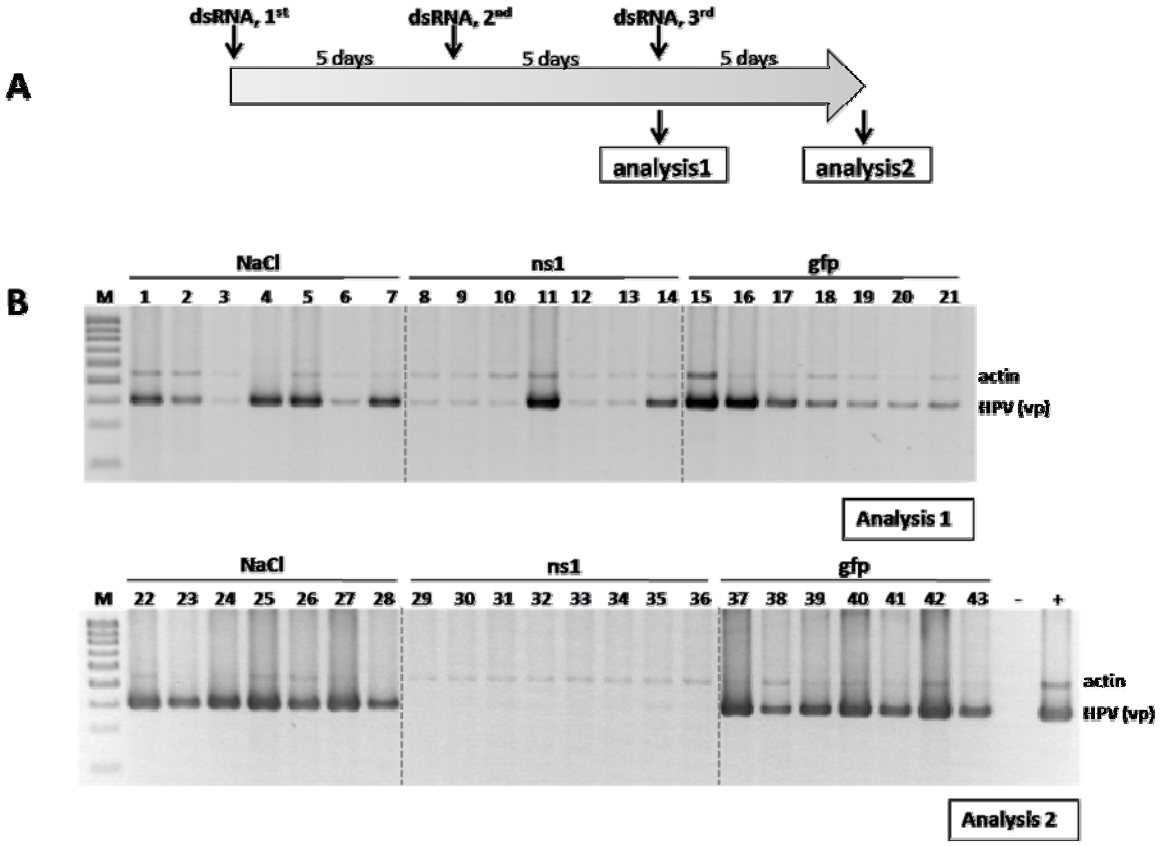

Fig. 6 Clearance of *Pm*DNV in the naturally infected-shrimp (experiment 1). The 300-400 mg shrimps which already infected with *Pm*DNV were injected with dsRNA-ns1 three times (5 days intervals) as shown in the diagram (A). The 2.5 µg of dsRNA-ns1 per one gram shrimp was used for each injection. The NaCl and dsRNA-gfp were used to treat the shrimp instead of dsRNA-ns1 for experimental control. Five days post the second (analysis 1) and the last injection (analysis 2), the hepatopancreas of each shrimp was isolated and the viral DNA was detected by PCR. (A) Diagram represents the experimental condition. (B) Semi-quantitative PCR of *Pm*DNV DNA. The amount of *Pm*DNV in an individual shrimp was determined by multiplex PCR using the viral specific primers (vp) together with the host control gene (actin) primers for normalization. Lane 1-21 demonstrates shrimp receiving NaCl (1-7); dsRNA-ns1 (8-14), and dsRNA-gfp (15-21) two times. Lane 22-43 represents shrimp receiving NaCl (22-28); dsRNA-ns1 (29-36); dsRNA-gfp (37-43) three times. M is the 100 bp DNA marker. Negative (-) and positive (+) control of the reaction are included.

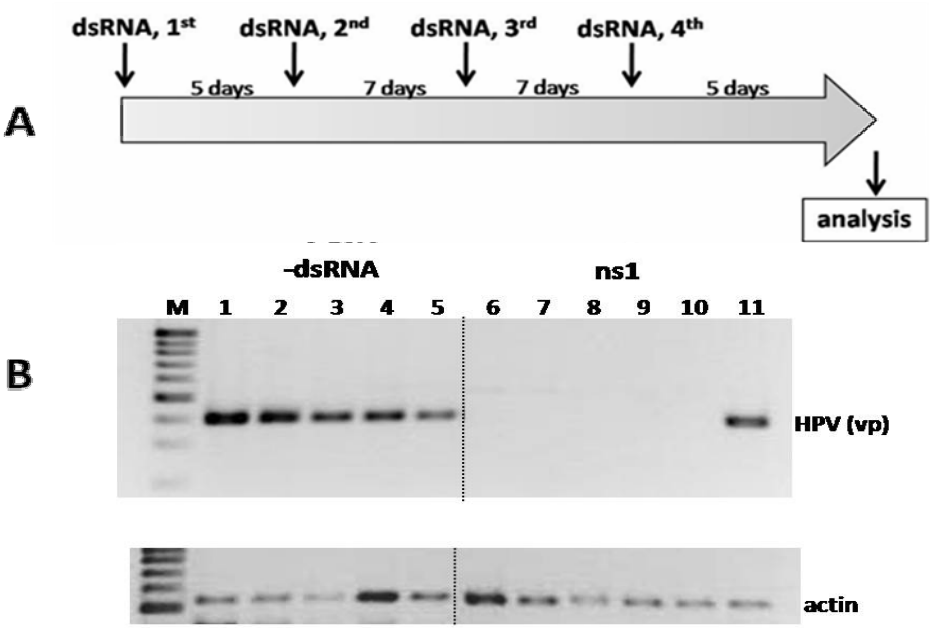

Fig. 7 Clearance of *Pm*DNV in the naturally infected-shrimp (experiment 2). The 300-400 mg shrimps which already infected with *Pm*DNV were injected with dsRNA-ns1 four times (5-7 days intervals) as shown in the diagram (A). The 2.5 µg of dsRNA-ns1 per one gram shrimp was used for each injection. The NaCl was used to treat the shrimp instead of dsRNA-ns1 for experimental control. Five days after the last injection, the hepatopancreas of each shrimp was isolated and the viral DNA was detected by PCR. (A) Diagram represents the experimental condition. (B) Determination of *Pm*DNV DNA by PCR. The amount of *Pm*DNV in an individual shrimp was detected by PCR using the viral specific primers (vp). The same amount of DNA for vp DNA detection was used to amplify the host actin gene for normalization. Lane 1-5 demonstrates shrimp receiving NaCl (-dsRNA); dsRNA-ns1 (6-11). M is the 100 bp DNA marker.

6. Outputs

6.1 International Publication

6.1.1 Attasart, P., Kaewkhaw, R., Chimwai, C., Kongphom, U., Namramoon, O., Panyim, S. Prevention of *Penaeus monodon* densovirus (*Pm*DNV) infection in shrimp by double-stranded RNA. Virus Res. 2009 (submitted, impact factor 2.429).

6.1.2 Attasart, P., Kaewkhaw, R., Chimwai, C., Kongphom, U., Panyim, S. Therapeutic activity of dsRNA-ns1 for *Pm*DNV inhibition in black tiger shrimp. (manuscript in preparation for submission to Antiviral Res., impact factor 3.613)

6.2 Presentations and Posters

6.2.1 Kaewkhaw, R., **Attasart, P**. and Panyim, S. 2006. Inhibition of hepatopancreaic parvovirus (HPV) infection in shrimp by dsRNA. The Eleventh Biological Sciences Graduate Congress, Chulalongkorn University, Bangkok, Thailand (December 15-17).

6.2.2 Kaewkhaw, R., **Attasart, P**. and Panyim, S. 2007. Inhibition of hepatopancreaic parvovirus (HPV) infection in shrimp by dsRNA. The 6thNational Symposium on Marine Shrimp, BIOTEC, Science Park, Patumthani (March 29-30).

*6.2.*3 **Attasart, P**., Kaewkhaw, R., Chimwai, C., Tanwanchai, O. and Panyim, S. 2007. RNA interference for inhibition of Hepatopancreatic parvovirus (HPV) infection in *Penaeus monodon*. The 7th TRF Junior and Senior Researchers Meeting, The Ambassador City Jomtien Hotel, Chonburi, Thailand (October 11-13) (ได้รางวัลเสนอผลงานยอดเยี่ยมแบบโปสเตอร์).

6.2.4 **Attasart, P**., Kaewkhaw, R., Chimwai, C., Kongphom, U., Namramoon, O., Panyim, S., 2009. Application of RNA interference to DNA virus inhibition in *Penaeus monodon*. Agricultural Biotechnology International Conference, Queen Sirikit National Convention Center, Bangkok, Thailand (September 23-25).

6.2.5 Chimwai, C., Kongphom, U., Panyim, S., **Attasart, P**., 2009. Survey of potential *Pm*DNV (HPV) natural carrier from aquatic animals. The 35 th Congress on Science and Technology of Thailand, The Tide resort (Bangsaen beach), Chonburi, Thailand (October 15-17).

6.3 Student

6.3.1 Miss Rossukon Kaewkhaw (M.Sc., 2007)