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ORIGINAL ARTICLE

Inhibition of *Penaeus monodon* densovirus replication in shrimp by double-stranded RNA

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Abstract Stunted shrimp caused by *Penaeus monodon* densovirus (*Pm*DNV) infection is one of the main problems leading to a significant economic loss in Thailand. To control this pandemic disease, a double-stranded-RNA-mediated virus-specific gene silencing approach was applied to inhibit viral replication. In this study, two dsRNAs corresponding to the non-structural protein (ns1) and the structural protein (vp) genes of *Pm*DNV were synthesized and introduced into shrimp haemolymph prior to viral challenge. After allowing viral replication for two weeks, the suppression effect by each dsRNA was evaluated by semi-quantitative PCR and compared with the control. A reduction of *Pm*DNV in shrimp treated with each dsRNA was observed. In contrast, a high level of viral infection was detected in the control group (NaCl). Based on a limited sample number, we reached the tentative conclusion that virus-specific dsRNA can inhibit *Pm*DNV replication, in which the dsRNA-ns1was more effective than the dsRNA-vp.

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 [11]. Although it does not cause an abrupt mortality crisis of cultured shrimp as do 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 [12]. 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 have demonstrated that the replication of both RNA [21, 22, 25] and DNA viruses [3, 13, 19] 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 case all of the shrimp were dead [3, 18, 21, 24].

*Pm*DNV is a non-enveloped virus with an icosahedral-shaped particle [14]. It contains a linear single-stranded DNA genome with typical palindromic termini [6]. According to a report of the complete sequence of a Thai isolate [20], it contains three large open reading frames (ORFs): two non-structural protein genes (ns1 and ns2) and one structural protein gene (vp). The ns1 protein of parvoviruses is known to have multiple functions, including ATP-dependent site-specific DNA binding and nicking and also helicase activity, which is essential for viral replication [10, 16, 24]. Meanwhile, the phospholipase A2 activity of the vp protein has been reported to be critical for viral

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infectivity [7]. However, the biological function of ns2 during the parvovirus life cycle is still obscure [9, 15].

In this study, we determined the effectiveness of dsRNA for the inhibition of *Pm*DNV in *P. monodon*. Two dsRNAs specifically targeted to genes that are essential for viral infection, ns1 and vp, were synthesized in bacterial cells and subsequently injected into shrimp followed by viral challenge. The suppression of *Pm*DNV by virus-specific dsRNA was then evaluated by semi-quantitative PCR analysis.

Materials and methods

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 shrimp pellet diet.

Virus source

Hepatopancreas isolated from naturally *Pm*DNV-infected shrimp was utilized for viral infection via the oral route. To quantify the amount of virus, the level of *Pm*DNV in each hepatopancreas was determined by PCR. Only hepatopancreas samples with equal amounts of virus were then selected for further experimental feeding. The tissue was kept at −20°C until required.

Stem-loop RNA expression plasmid construction

For virus-specific dsRNA: Two regions of the *Pm*DNV genome corresponding to the ns1 and vp genes shown in Fig. 1a were selected for dsRNA targeting. In order to minimize misfolding of RNA during the dsRNA production, the Clone Manager program was used for scanning any repeats throughout the regions of ns1 and vp before selection. Moreover, a survey of the specificity of the selected region using the BLAST program was also performed to minimize the off-target effect. 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 (primers C and D of each gene for the 400-bp product and A and B of each gene for the 600-bp product). These two fragments were then cloned into the pET17b vector in an inverted orientation, directed by restriction enzymes flanking the fragments (*Nde*I and *Eco*RV for 600 bp, *Eco*RV and *Xho*I for 400 bp) (Fig. 1b). 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. By this cloning strategy, the transcribed RNA can form a stem-loop structure with a 400-bp stem and a 200-bp loop due to complementary binding of the RNA ends.

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 for specific dsRNA except that pET3a was used as a cloning vector instead of pET17b, *Xba*I was used for joining the two fragments, and *Nde*I was used for inserting them into the plasmid vector [25].

Double-stranded RNA production

dsRNA was expressed in *Escherichia coli* HT115 according to the protocol of Ongvarrasopone et al. [17]. An overnight bacterial culture in LB medium with antibiotic was diluted with fresh medium and grown at 37°C until an OD_{600} of 0.4 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 6,000×g for 5 min at 4°C. The cell pellet was resuspended in 0.1% sodium dodecyl sulphate (SDS) at a ratio of 50 µl per OD unit. To lyse the cells, the cell suspension was boiled for 2 min. Next, the bacterial single-stranded RNAs (ssRNAs) and the loop region (200 bp) 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.

Verification of dsRNA

To ensure the integrity of the synthesized dsRNA before introducing it into shrimp, treatment with RNaseA (specific for ssRNA) and RNaseIII (specific for dsRNA) was carried out. An equal amount of dsRNA was digested separately with RNaseA (0.01 µg RNaseA per 2 µg dsRNA) and RNaseIII (0.5 units RNaseIII per 2 µg dsRNA) in the reaction buffer for RNaseA (300 mM sodium acetate, 10 mM Tris–Cl) and for 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.

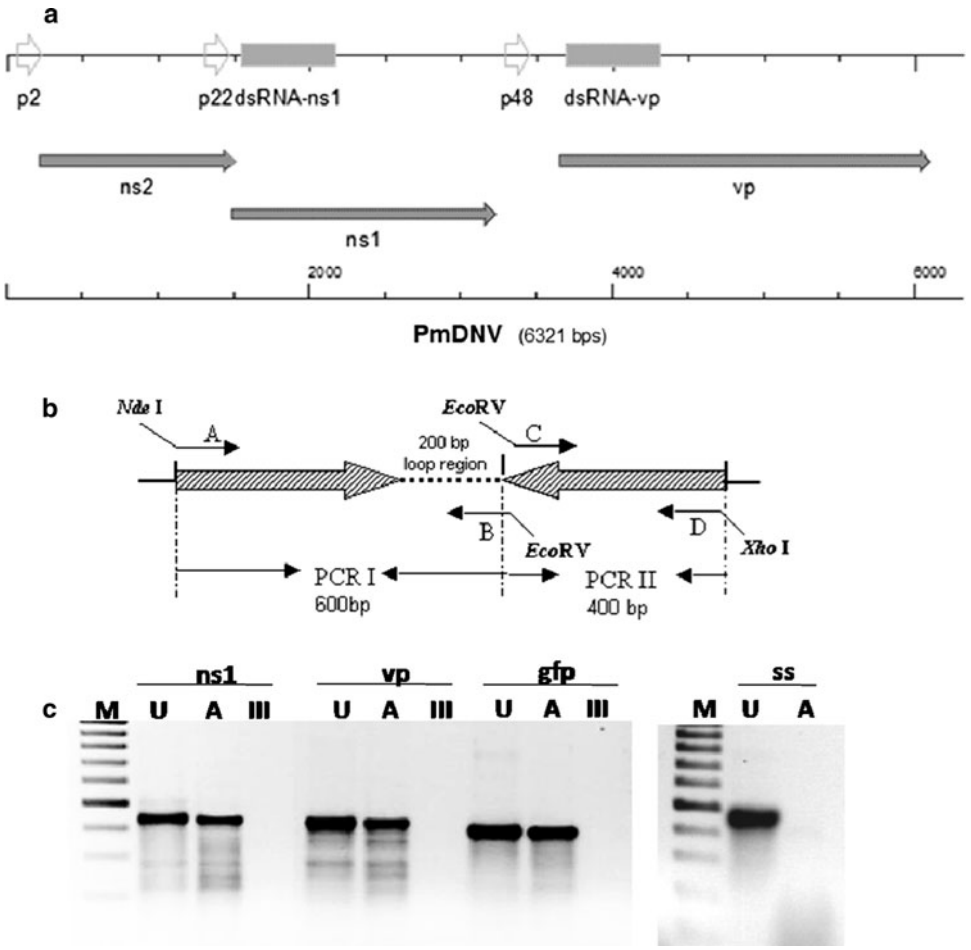

Fig. 1 Double-stranded RNA production. **a** Schematic diagram of dsRNA-targeted DNA regions of the *Pm*DNV genome. Each gray arrow represents a particular gene [ns2 (nt 216–1,502), ns1 (nt 1,487–3,226), and vp (nt 3,642–6,098)], lying below a line representing the *Pm*DNV genome. The regions targeted by dsRNA-ns1 (nt 1,547–2,170) and dsRNA-vp (nt 3,689–4,303) are shown in *gray boxes*, while the *open arrows* show tentative promoters of each gene. The *scale bar* relative to the full length of the PmDNV genome (6,321 bp) is shown below the diagram. **b** Schematic diagram of stem-loop RNA expression plasmid construction. Primers A + B and C + D were used for amplification of a 600-bp PCR fragment (*PCR I*) and a 400-bp fragment (*PCR II*) of each virus-specific gene. The restriction enzyme sites allowing directional cloning are indicated at the 5′ end of each primer. The *shaded arrow* and *dotted line* represent an inverted repeat of the target and the spacer between the inverted repeats, respectively. **c** dsRNAs verification by RNase treatment. The integrity of each dsRNA targeted to the ns1 gene (*ns1*), vp gene (*vp*) or gfp gene (*gfp*) was confirmed by incubation with RNase A (*A*) and RNase III (*III*), which specifically digest ssRNA and dsRNA, respectively. The expected sizes of dsRNA-ns1 and dsRNA-vp are 425 bp and 409 bp, respectively. The untreated dsRNAs (*U*) and ssRNA (*ss*) were used as a control. Approximately 50 ng of dsRNA was loaded in each lane of a normal 1× TBE 2% agarose gel. *M* 100-bp DNA ladder

Experimental conditions

For infectivity of PmDNV

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

For viral inhibition

According to the infectivity test, shrimp of the same size, kept in individual Petri dishes, were injected with approximately 750 ng of each virus-specific dsRNA (ns1 and vp) 24 h before viral infection by oral feeding as described above. To maintain the effect of dsRNA during the experiment, another dsRNA administration was carried out on day 5 after the first injection. Thereafter, the shrimp were fed daily with a shrimp pellet 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 the delivered dsRNA was then evaluated by semi-quantitative PCR.

DNA extraction

Total DNA was extracted from hepatopancreas tissues (or as indicated) using TRI Reagent (Molecular Research Center). Samples of 50–100 mg of tissue were ground in 1 ml TRI Reagent. The DNA in the interphase and organic phase was precipitated using absolute ethanol. The DNA pellet was washed with 0.1 M trisodium 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.

Sample analysis

Semi-quantitative PCR

The total amount of extracted DNA (approximately 600 ng) was used for amplification of the virus-specific gene (vp) using the primers listed in Table 1 (primers 1 and 2 for the infectivity and inhibition experiment and primers 3 and 4 for the tissue distribution test). To determine the relative amount of virus in the samples, PCR of an internal control gene (shrimp beta actin) was included (primers 1 and 2 for the infectivity and inhibition experiment and primers 3 and 4 for the tissue distribution test). The temperature profile for PCR amplification was as follows; 94°C for 2 min, denaturation at 94°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. After 20 cycles (35 cycles for the tissue distribution experiment), the reaction was held at 72°C for another 5 min. The PCR product was analyzed by agarose gel electrophoresis.

Statistical analysis

The data were analyzed statistically using GraphPad software. One-way ANOVA followed by Tukey–Kramer multiple comparison test was used for analysis. The data are presented as mean ± standard error (standard error of the mean, SEM).

Results

Double-stranded RNA verification

To test whether RNA was double-stranded, 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 it was double-stranded RNA (Fig. 1c). In contrast, the control single-stranded RNA (ss) was completely digested with RNaseA. This clearly demonstrated

Table 1 Primer sequences for PCR amplification in this study

Gene	Primer sequences
ns1	
ns1-A	5′ CGGAATTCATATGACATTTTACTCTGGTCTCATTG 3′
ns1-B	5′ CCCCAGATATCGCCCTAGAACTGCTTAGTC 3′
ns1-C	5′ CCCCAGATATCTTGCGTATTTCCTGTCCCTG 3′
ns1-D	5′ CCGCTCGAGACATTTTACTCTGGTCTCATTG 3′
vp	
vp-A	5′ CGGAATTCATATGCCAAAGTAAGCGAAAGAATAAAC 3′
vp-B	5′ CCCCCCGATATCATTTCTTAGCGTTTTCTATGCG 3′
vp-C	5′ CCCCCCGATATCTCTGTGCTGTCTGAAAATCCT 3′
vp-D	5′ CCGCTCGAGCCAAAGTAAGCGAAAGAATAAAC 3′
vp-1	5′ AATCTGCAGGGTACGGAAAAAAC 3′
vp-2	5′ TGTGGAACCATCTCAAATGCC 3′
vp-3	5′ ATCTGGATAGTATACATGTC 3′
vp-4	5′ GGAGATATTAAGCACAGTTTC 3′
Actin	
Actin-1	5′ GACTCGTACGTCGGGCGACGAGG 3′
Actin-2	5′ AGCAGCGGTGGTCATCACCTGCTC 3′
Actin-3	5′ CAAGTGCTTCTAAGGATACTG 3′
Actin-4	5′ CATGATTATTTTGTATATATTATCG 3′

Viral gene-specific primers were designed according to the sequence of the genome of a Thai *Pm*DNV isolate (GenBank accession no. *DQ002873*). The sequence of a shrimp beta actin gene (accession no. *AW600735*) was used for actin primers

that the synthesized dsRNAs (ns1, vp and gfp) were suitable for injection into shrimp to trigger the anti-virus pathway.

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 normalization with the β-actin control, it could be seen that *Pm*DNV was predominantly in the hepatopancreas (Fig. 2). Much less was noted in the periopod, gill, pleopod and muscle. Based on this information, tissue from the hepatopancreas was used for all subsequent experiments.

Experimental infection of PmDNV in shrimp

In order to establish an infectivity profile, shrimp were fed with *Pm*DNV-infected hepatopancreas and randomly selected for testing at three different time points (4, 8, and 14 days after the first feeding). Viral replication was then assayed by PCR. A reduction in virus levels was observed at day 8 compared with total virus uptake at day 4. The amount of virus then increased significantly at day 14 (Fig. 3). This indicates that the infection of shrimp with *Pm*DNV can be performed experimentally, and viral replication can also be investigated over a time period of up to 14 days.

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

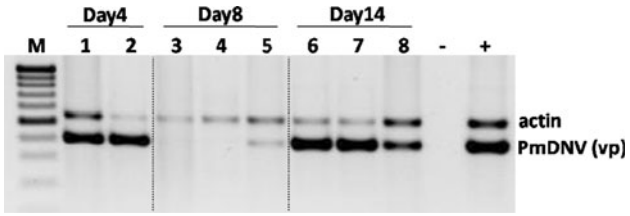

Fig. 3 Infectivity profile of *Pm*DNV in shrimp after feeding with hepatopancreas of *Pm*DNV-infected shrimp. The 300-mg shrimp were fed three times (approximately 30 mg each meal, at 12-h intervals) with *Pm*DNV-infected hepatopanceas. Shrimp were picked randomly and sacrificed at 4 (*Day4*), 8 (*Day8*), 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 (*PmDNV*) and the internal control actin primers (*actin*). Each shrimp is indicated by a number at the top of the gel, while *M* is the 100-bp marker. Negative (−) and positive (+) controls are included

followed by *Pm*DNV challenge by feeding. The amount of virus in each shrimp that received virus-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 a shrimp actin gene for normalization (Fig. 4b). A high level of viral infection was observed in the control group. The reduction of *Pm*DNV in shrimp treated with dsRNA-ns1 was more pronounced than with dsRNA-vp and significantly different from the control (no dsRNA) ($P = 0.004$) and non-related dsRNA (dsRNA-gfp) ($P = 0.02$) (Fig. 4c). These results demonstrate that the virus-specific dsRNA can suppress the replication of *Pm*DNV in shrimp, with dsRNA-ns1 being more potent than the dsRNA-vp.

Discussion

This study showed for the first time that the replication of *Pm*DNV in shrimp can be effectively inhibited by the

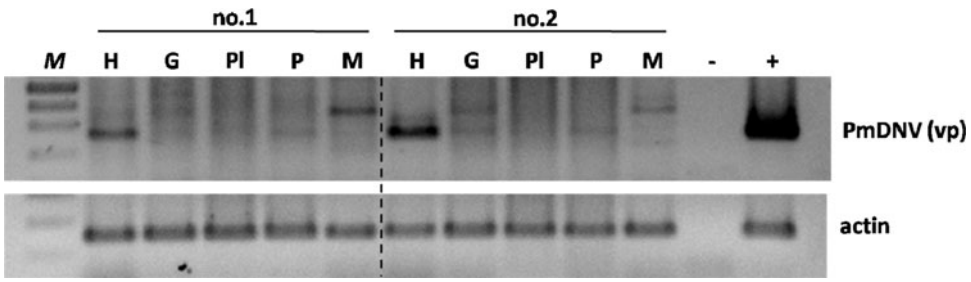

Fig. 2 Tissue distribution of *Pm*DNV in a naturally infected shrimp. Equal amounts of total DNA extracted from various tissues (*H* hepatopancreas, *G* gill, *Pl* pleopods, *P* periopods and *M* muscle) of each naturally *Pm*DNV-infected shrimp were subjected to PCR analysis to determine the viral localization. The product was amplified separately using virus-specific primers (*Pm*DNV, vp) and host beta-actin primers (*actin*). Negative (−) and positive (+) controls were included. Numbers at the top indicate individual shrimp. The size of the PCR product was compared with the 100-bp DNA marker (*M*)

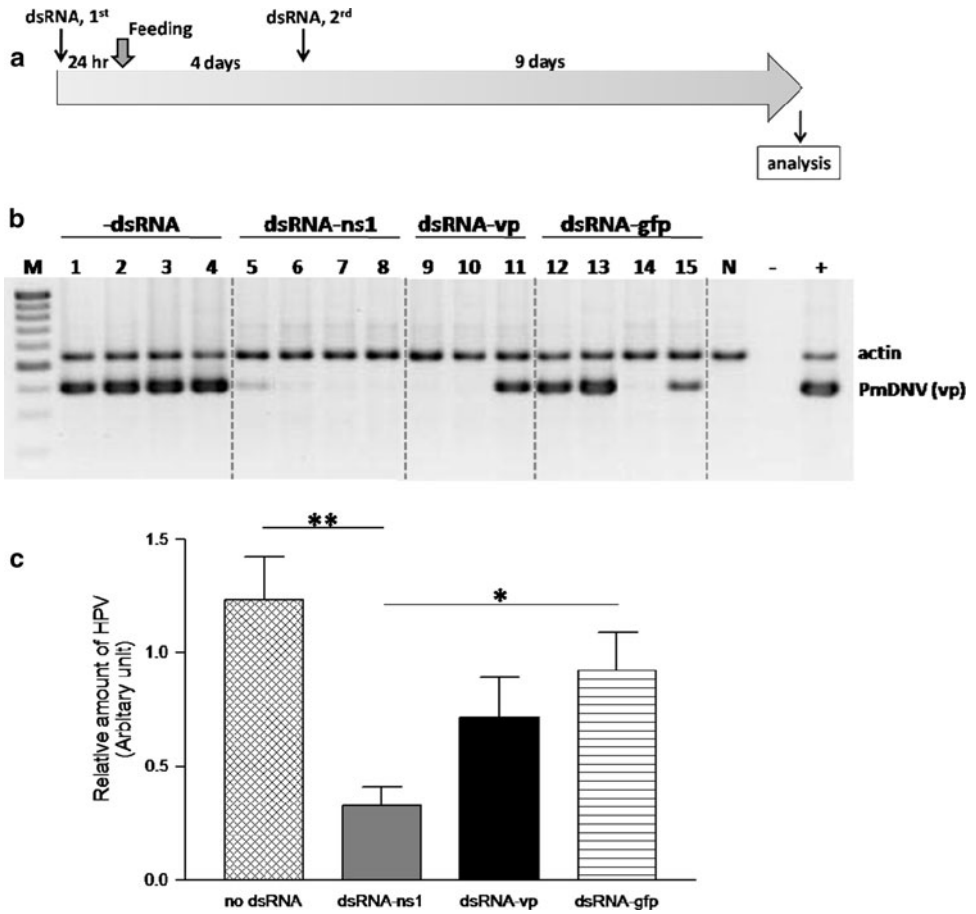

Fig. 4 Treatment with dsRNA for inhibition of *Pm*DNV in shrimp. The 300-mg shrimp were injected two times with 750 ng dsRNA specific either for the ns1 gene or vp gene or the unrelated gfp gene (as indicated in the diagram) before challenging with *Pm*DNV by oral feeding with infected tissue. In the control group, shrimp were treated with NaCl instead of dsRNA. Fourteen days after the first administration of dsRNA, the hepatopancreas of each shrimp was collected for DNA extraction, and further PCR analysis was performed. **a** The 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 virus-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; and *12–15*, dsRNA-gfp. N is the normal shrimp. Negative (−) and positive (+) controls are included, with the 100-bp DNA marker in *lane M*. **c** Relative amount of *Pm*DNV normalized with actin. Data are shown as means of duplicate experiments with *error bar*. Statistical significance was analyzed by ANOVA test. *P* values, * less than 0.05 and ** less than 0.01

administration of virus-specific long dsRNA. Our findings are in agreement with a number of previous studies demonstrating dsRNA-mediated viral suppression in shrimp [3, 13, 19, 21]. It has been documented that silencing the expression of an essential viral replication protein gene can significantly inhibit infection with 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 has been shown to be more potent than the structural protein gene [19, 21]. 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 for generating the single-stranded DNA. This then further facilitates the replication process [2, 4, 5]. 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 [8]. Because of these essential functions, the ns1 of *Pm*DNV may represent a potential target for further development of an anti-*Pm*DNV approach.

As described recently by several research groups [3, 13, 15, 25], the antiviral response in shrimp triggered by long dsRNA occurs through two pathways: sequence-dependent RNAi and sequence-independent innate immunity. In the case of nonspecific inhibition from dsRNA-gfp or other irrelevant dsRNAs, the protection was not high and could be observed at a low level of viral infection, whereas it was

overwhelmed by heavy infection. Our results show that a moderate inhibition was also found in the dsRNA-gfp-treated shrimp group (Fig. 4b, lane 14–15). This suggests that shrimp could be partially protected from *Pm*DNV infection by nonspecific dsRNA. This might be due to the slow rate of its infection (approximately 2 weeks after receiving viruses as shown in Fig. 3); therefore, nonspecific inhibition could be effective enough to block *Pm*DNV infection.

This report is the first to show successful infection of *Pm*DNV in juvenile *Penaeus monodon* (300 mg body weight). Interestingly, shrimp infected by feeding with virus-infected hepatopancreas had a specific infectivity profile. The amount of virus declined after initial infection but then rose significantly at a later time point. 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 [5]. There are four main epithelial cell types present in the hepatopancreas, E (embryonic), F (fibrilar), B (blister-like), and R (absorptive) cells [1]. Of these, only the E cells show mitotic activity. Hence, it is possible that a 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 total hepatopancreatic epithelial cells [26], 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, releasing a digestive enzyme together with the viral particles into the lumen of hepatopancreatic tubule [23]. The virus would then later be excreted out via feces [18]. 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. On the basis of this finding, approximately 14 days should be the appropriate experimental time course to assess *Pm*DNV replication in 300-mg juvenile shrimp.

In conclusion, an effective tool for inhibition of *Pm*DNV replication in shrimp using virus-specific dsRNA has been demonstrated in this study. However, several aspects, such as therapeutic effect as well as methodology for continuous delivery of dsRNA into shrimp, have to be further resolved in order to apply this RNAi technology for controlling this disease in the shrimp farm.

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