CHAPTER IV
RESULTS

4.1. Determination of IHHNV titer

The IHHNV lysate was prepared from gills of IHHNV-infected shrimp, *Penaeus monodon*, size about 20 g by lysis method. The viral nucleic acid was purified by High pure viral nucleic acid kit. The purified nucleic acid was serially diluted from 10^0 (undiluted) to 10^{-8} and determined the viral titer by PCR. The PCR products were run on agarose gel electrophoresis. The first band observed on the gel was estimated about 100 particles. The factor from all purification steps were used to calculate the viral titer.

The first band was shown at 10^{-6} dilution and was estimated about 100 viral particles. Calculation of the viral titer indicated that the IHHNV virus stock was about 6×10^7 particles/μl (Figure 4-1). The titer of IHHNV stock about 6×10^6 and 6×10^7 particles was used to test the IHHNV infectivity.

4.2. IHHNV infectivity in *L. vannamei*

To test the IHHNV infectivity, viral-free shrimps were challenged with 6×10^6 or 6×10^7 IHHNV lysate by injection into hemolymph of about 2 grams shrimps. After 3 and 5 days viral injection, pleopods of each shrimp were collected and extracted DNA in order to analyse IHHNV infectivity. The control group is shrimps injected with 150 mM NaCl. The plan of shrimp injection followed to this diagram.

Viral replication was assayed by PCR. The results in Figure 4-2 showed that shrimp injected with 6×10^6 and 6×10^7 particles of IHHNV showed IHHNV genome expression in 3 of 10 and 8 of 9 shrimps at day 3, respectively. In addition, expression of IHHNV genome can be detected in all shrimp at day 5 after viral challenge. Therefore, investigation of IHHNV replication in the following experiments was performed at 5 days after IHHNV challenge.

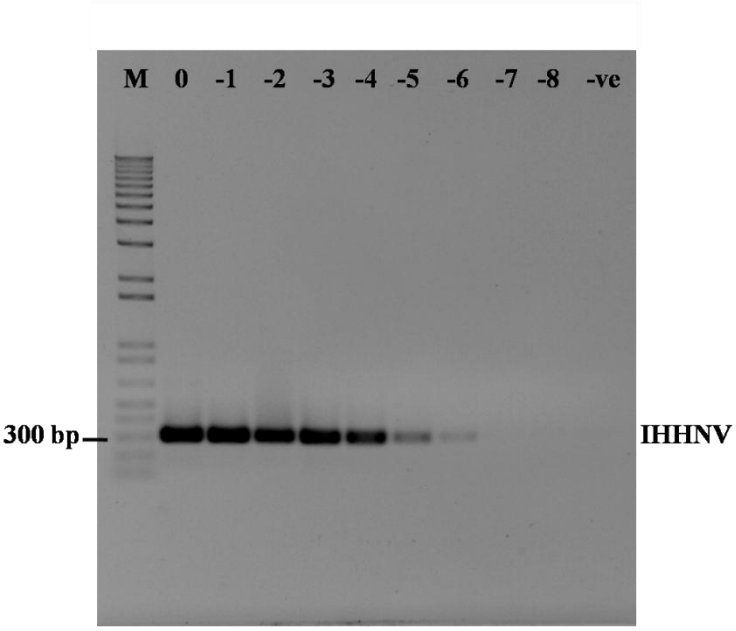


Figure 4-1. Determination of IHHNV titer. The IHHNV viral nucleic acid was extracted from gills and determined the viral titer by PCR in several dilutions, from 1 fold (10^0) to 10^8 fold (10^{-8}) (Lane 0 to -8). The viral band is about 309 bp. Virus stock is about 6×10^7 particles/μl. M is 1 kb plus DNA marker. –ve is negative control using sterile water instead of DNA.

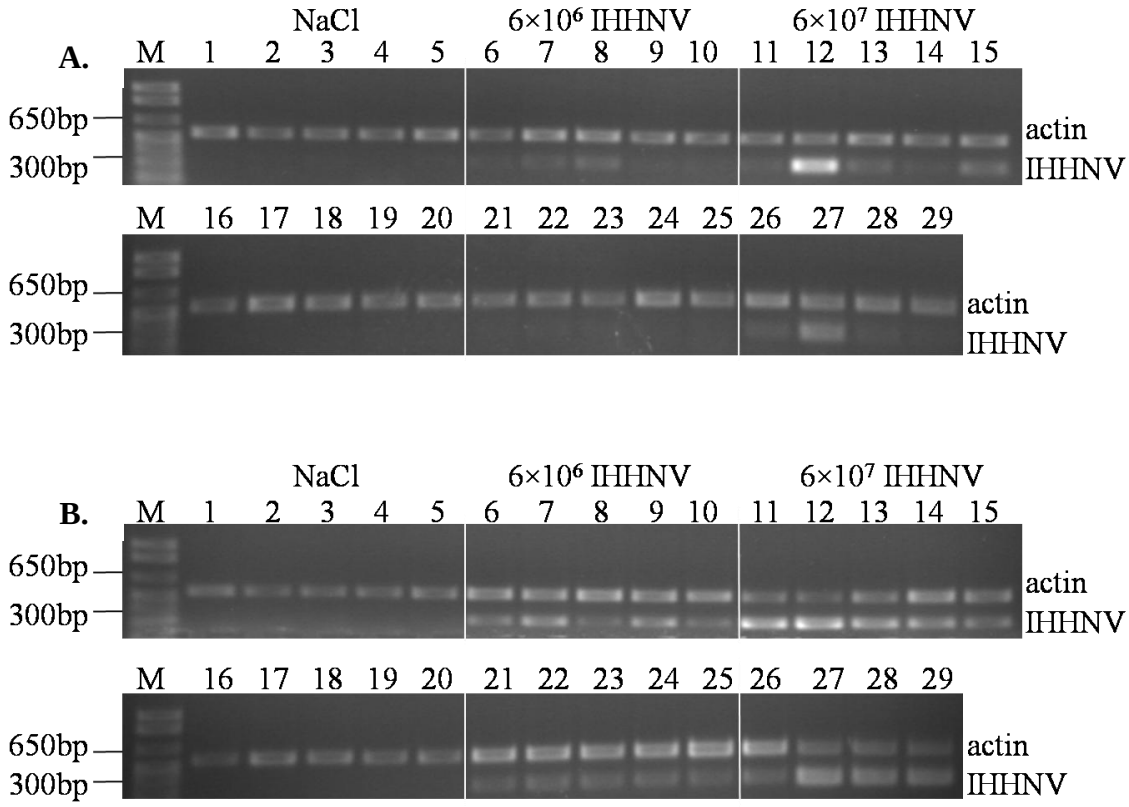


Figure 4-2. The IHHNV infectivity in *L. vannamei.* Shrimp were injected with 150 mM NaCl (n=10) (lane 1-5, 16-20), 6×10^6 IHHNV (n=10) (lane 6-10, 21-25) and 6×10^7 IHHNV (n=9) (lane 11-15, 26-29). The expression level of IHHNV (309 bp) and actin (550 bp), an internal control, were determined by PCR after 3 (A) and 5 (B) days IHHNV challenge. M is 1 kb plus DNA marker.

4.3. Double-stranded RNAs production

The DsRNAs, dsRNA-PmRab7 and dsRNA-GFP, were produced by *in vivo* bacterial expression method. The recombinant plasmid containing an inverted sequence of dsRNAs were transformed into *E.coli* HT115 which lacked ribonuclease III (RNaseIII) activity. The addition of IPTG induced dsRNAs expression into the culture. The bacterial cells were colleted by centrifugation after IPTG induction. DsRNAs were extracted by TRI reagent according to the manufacturer's protocol. The dsRNA integrity was confirmed by RNaseA and RNaseIII digestion assay. DsRNAs concentration was determined by agarose gel electrophoresis.

The results showed a single band of dsRNA-GFP of size about 400 bp, and 3 bands of dsRNA-PmRab7 of size about 400 bp, 300 bp and 100 bp. DsRNA-GFP and dsRNA-PmRab7 integrity were confirmed by RNaseA and RNaseIII digestion assay (Figure 4-3). The result indicated that both dsRNAs can be digested by RNaseIII but cannot be cleaved by RNaseA. The 3 major bands of dsRNA-PmRab7 were due to the mismatches in some bases of the plasmid (pET17b-stRab7) that used to express dsRNA-PmRab7. Therefore, the mismatched bases of the expressed dsRNA-PmRab7 was cleaved by RNase A in the purification step and resulted in 3 major bands of dsRNA-PmRab7. These bands can be cleaved by RNaseIII but not by RNaseA suggesting that the synthesized dsRNA-PmRab7 is really in the form of dsRNA that can be used for RNAi study in the next step.

The determination of the dsRNAs concentration was performed in 1% agarose gel electrophoresis. DsRNAs were serially diluted from 1 to 200 fold. The result (Figure 4-4) demonstrated that dsRNA-GFP concentration is 0.7 µg/µl and the yield of dsRNA-GFP is 14 µg/OD. DsRNA-PmRab7 concentration is 1.5 µg/µl and the yield of dsRNA-PmRab7 is 30 µg/OD.

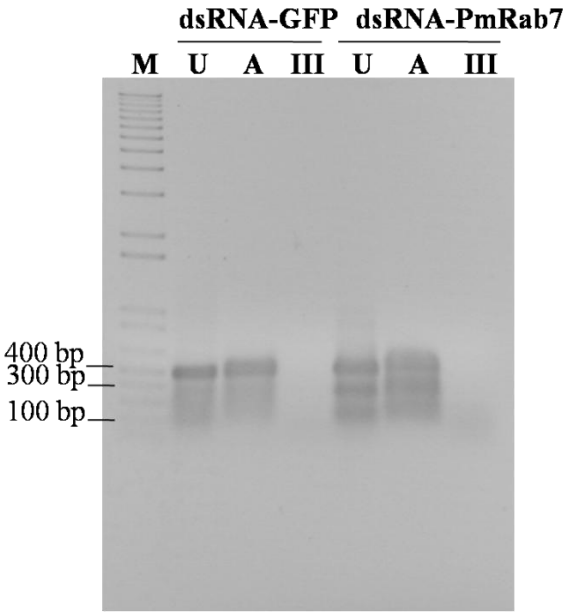


Figure 4-3. Analysis of the integrity of dsRNA-GFP (left) and dsRNA-PmRab7 (right) by ribonuclease digestion assay. The integrity of dsRNAs were analysed by RNaseA and RNaseIII digestion assay: U is undigested dsRNA, A is RNaseA digested dsRNA, III is RNaseIII digested dsRNA and M is 1 kb plus DNA marker.

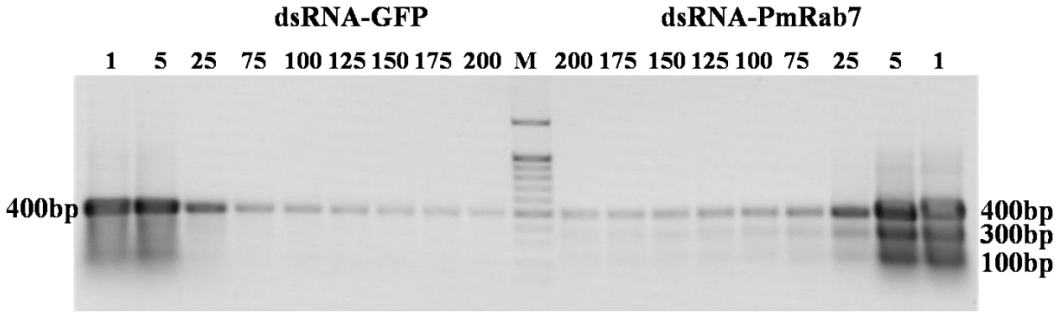


Figure 4-4. Quantitation of dsRNAs. DsRNA-GFP and dsRNA-PmRab7 were serially diluted from 1 to 200 fold. The samples (5μl) were loaded in each lane. Their concentrations were determined by running on 1% agarose gel electrophoresis. The band intensity of the 500 bp band of the standard is equivalent to 33 ng. M is 100 bp DNA marker.

4.4. Suppression of LvRab7 by dsRNA-PmRab7

To investigate how long LvRab7 gene was suppressed by dsRNA-PmRab7 in *L. vannamei*, shrimps of size about 2 g were injected with 0.63 μg/g shrimp of dsRNA-PmRab7. The injection with dsRNA-GFP (0.63 μg/g shrimp, size 2 g) was used to investigate the specific effect of dsRNA-PmRab7. The negative control group is shrimps injected with 150 mM NaCl. Two and four days after injection, pleopods were collected and extracted total RNA for RT-PCR analysis to determine LvRab7 expression levels. Actin gene was used as an internal control.

The result in Figure 4-5 showed RT-PCR analysis of the knockdown effect of LvRab7 from pleopods of shrimps injected with 0.63 μg/g shrimp of dsRNA-PmRab7, 0.63 μg/g shrimp of dsRNA-GFP and 150 mM NaCl after 2 and 4 days dsRNA injection. Quantitation of the relative expression of LvRab7 normalized with actin (Figure 4-6) showed 96% and 100% inhibition of LvRab7 expression after 2 and 4 days dsRNA-PmRab7 injection, respectively. LvRab7 expression levels were not suppressed in groups of shrimp injected with dsRNA-GFP and 150 mM NaCl. It showed specific knockdown of dsRNA-PmRab7 for LvRab7 in *L. vannamei*.

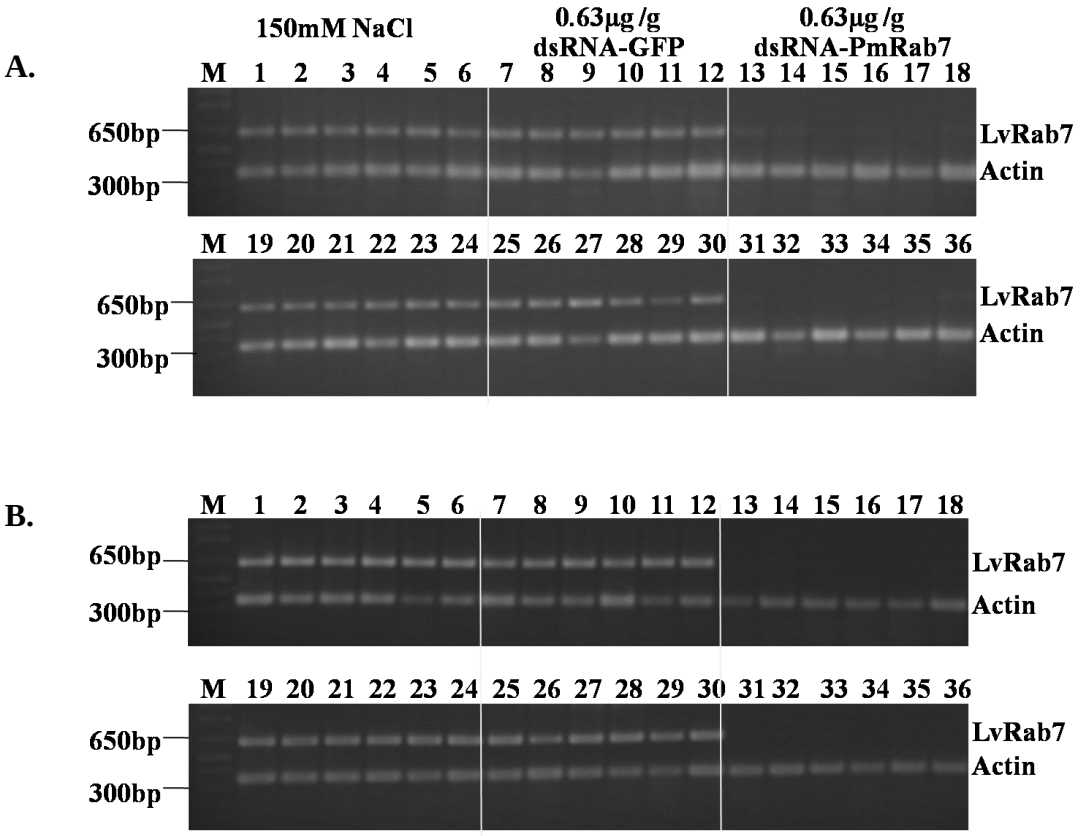
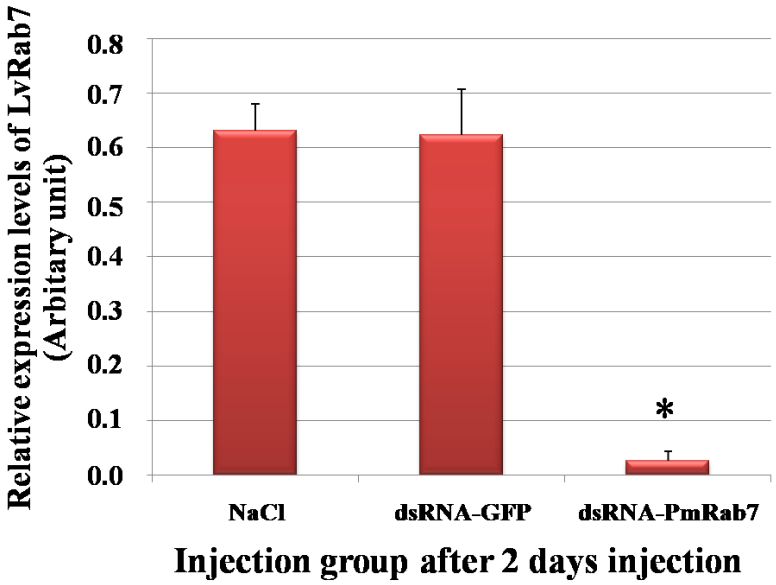


Figure 4-5. Suppression of LvRab7 after 2 (A) and 4 (B) days dsRNA-PmRab7 injection. Expression of LvRab7 (617 bp) and actin (350 bp) were determined by RT-PCR in shrimp injected with 150 mM NaCl (lane 1-6, 19-24), dsRNA-GFP (0.63 µg/g shrimp) (lane 7-12, 25-30) and dsRNA-PmRab7 (0.63 µg/g shrimp) (lane 13-18, 31-36) (n=12 each). M is 1 kb plus DNA marker.

A.



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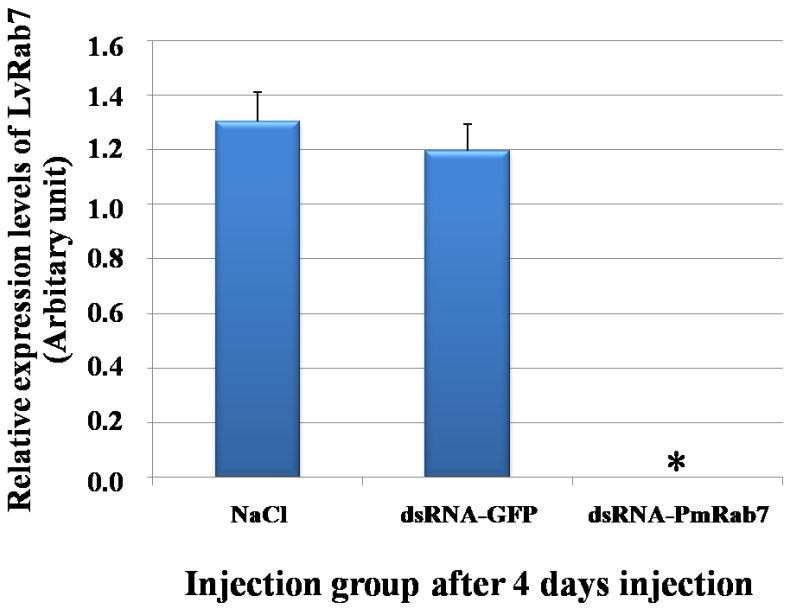


Figure 4-6. The relative mRNA expression levels of LvRab7 after 2 (A) and 4 (B) days suppression by dsRNA-PmRab7. LvRab7 was normalized with actin. Data represents mean±SEM, n=12 each. * showed statistically significant difference in shrimp injected with 150 mM NaCl and dsRNA-PmRab7 ($p<0.05$).

4.5. Inhibition of IHHNV replication in *L. vannamei* through LvRab7 knockdown

4.5.1 Preventive effect of dsRNA on IHHNV replication in *L. vannamei*

To study the preventive effect of the dsRNA-PmRab7 on IHHNV replication, shrimps size about 2 g were injected into hemolymph with dsRNA-PmRab7 (2.5 and 0.63 μg/g shrimp) 2 days before challenging with IHHNV (6×10^7 particles). Injection of 150 mM NaCl was used as a control group in this study. In addition, injection of dsRNA-GFP (2.5 μg/g shrimp) 2 days before challenging with IHHNV (6×10^7 particles) was used as a non-specific control group. The pleopod of an individual shrimp was collected before (day 0) and 7 days after injection to extract DNA and RNA. PCR and RT-PCR analysis were performed to detect expression levels of LvRab7, IHHNV and LvActin.

The results in Figure 4-7A showed the RT-PCR (LvRab7 and Actin) products and PCR (IHHNV and LvActin) products (Figure 4-7B) from pleopods injected with 150 mM NaCl, 2.5 μg/g shrimp of dsRNA-GFP, 2.5 and 0.63 μg/g shrimp of dsRNA-PmRab7. The relative mRNA expression of LvRab7 normalized with LvActin was shown in Figure 4-8A. The result showed that LvRab7 expression levels were reduced approximately 90% and 83% after dsRNA-PmRab7 injection at 2.5 and 0.63 μg/g shrimp, respectively. Suppression of LvRab7 resulted in an inhibition of approximately 95% and 97% IHHNV replication (Figure 4-8B).

The results demonstrated that LvRab7 was knocked down in shrimps injected with dsRNA-PmRab7 when compared with shrimps injected with NaCl or dsRNA-GFP. Silencing of LvRab7 by dsRNA-PmRab7 can prevent IHHNV replication in *L. vannamei*. These results suggested the involvement of LvRab7 during IHHNV replication.

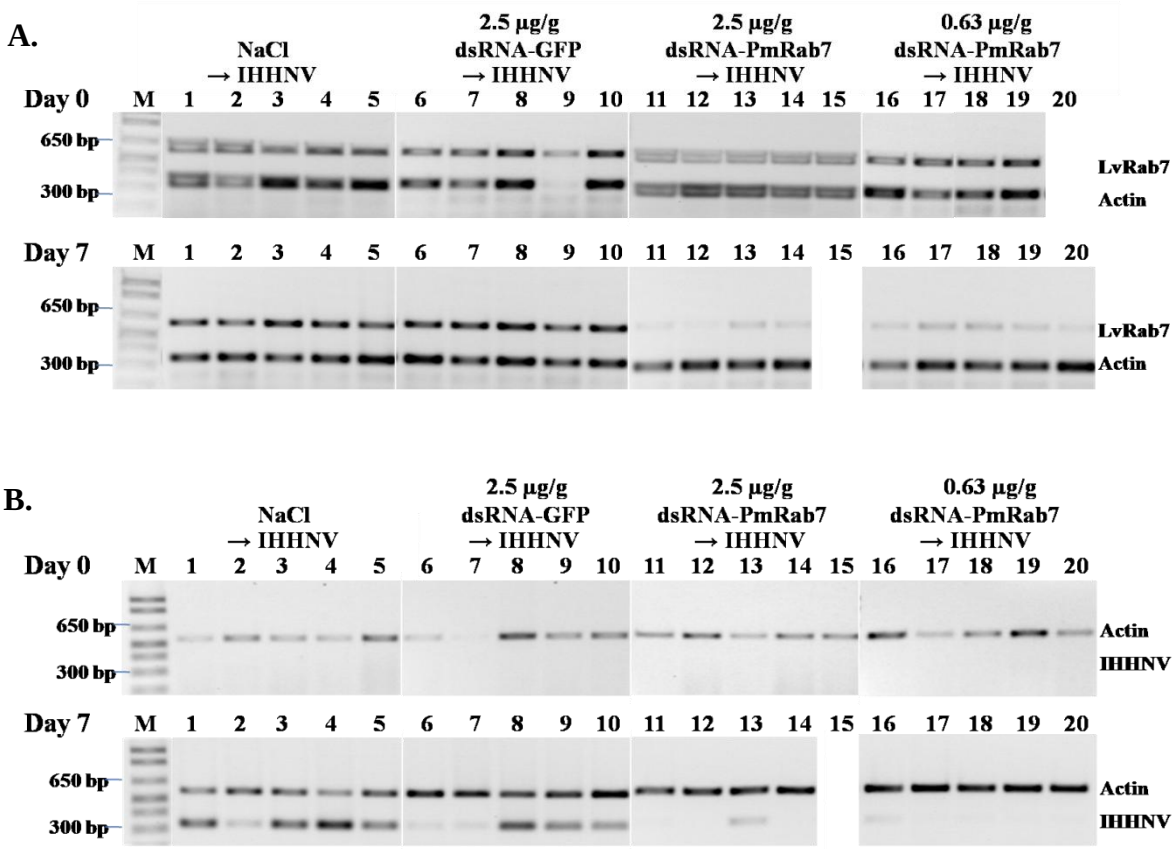


Figure 4-7. Prevention of IHHNV genome expression in LvRab7-knockdown shrimp before (day 0) and after 7 days dsRNA-PmRab7 injection. (A) Expression of LvRab7 (617 bp) and actin (350 bp) were determined by RT-PCR. (B**)** Expression of IHHNV (309 bp) and actin (550 bp) were determined by PCR. Shrimp injected with 150 mM NaCl (lane 1-5), dsRNA-GFP (2.5 µg/g shrimp) (lane 6-10) and dsRNA-PmRab7 (2.5 (lane 11-15) and 0.63 (lane 16-20) µg/g shrimp). M is 1 kb plus DNA marker. The same shrimp number on day 0 and day 7 means the samples were collected from the same shrimp. Missing data was due to the samples were degraded (#20) or shrimp died before day 7 (#15).

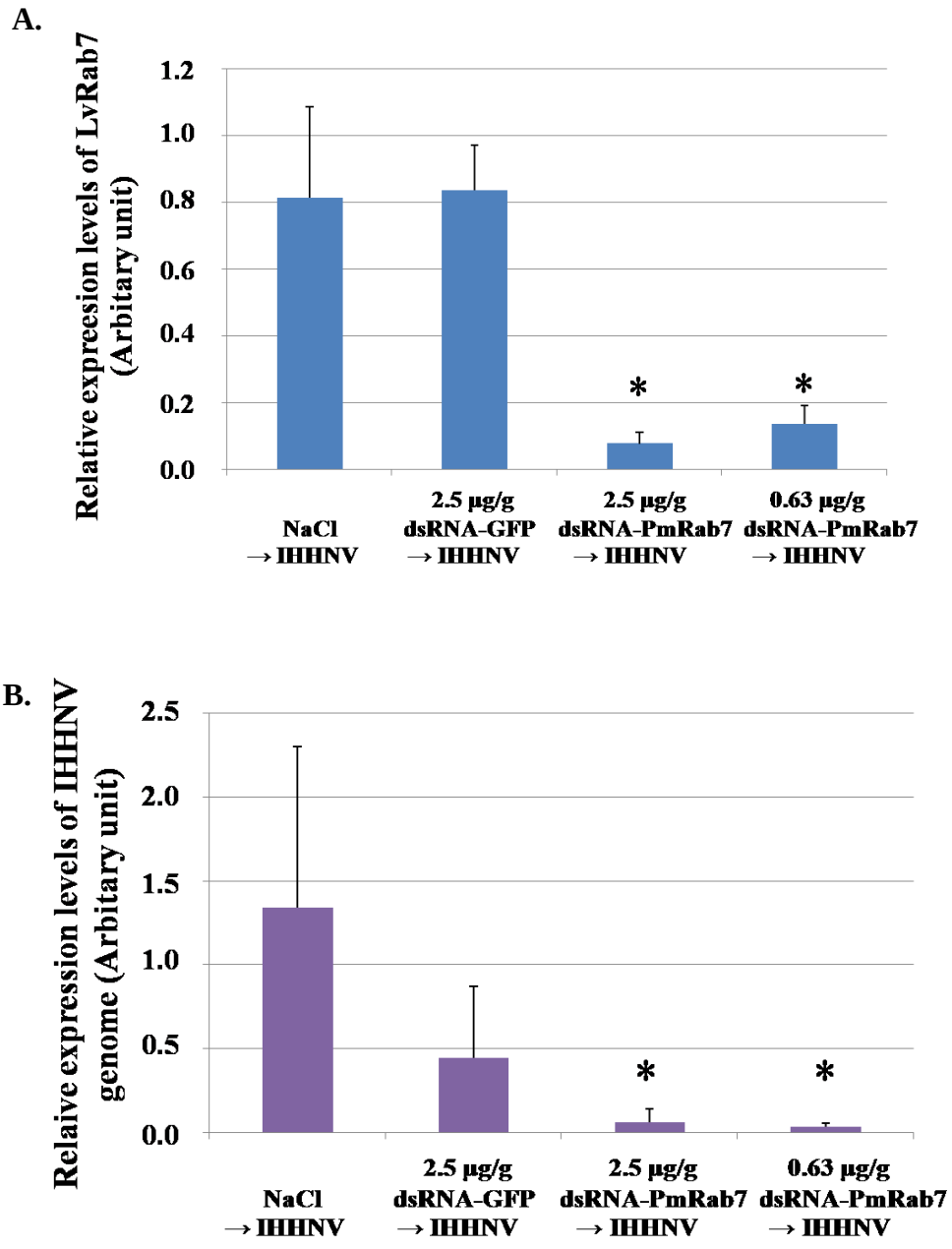


Figure 4-8. Knockdown effect of dsRNA-PmRab7 on LvRab7 mRNA expression levels (A) and IHHNV genome expression levels (B) after 7 days dsRNA-PmRab7 injection. Relative expression of LvRab7 and IHHNV were normalized with actin. Data represents mean±SEM, n=5 each. * showed statistically significant difference in shrimp injected with 150 mM NaCl and dsRNA-PmRab7 ($p<0.05$).

4.5.2 Co-injection effect of IHHNV and dsRNA-PmRab7

To study co-injection between IHHNV and dsRNA, shrimp of size about 3 g was co-injected into hemolymph with dsRNA-PmRab7 (0.63 μg/g shrimp) and IHHNV (6×10^7 particles). Injection of 150 mM NaCl or IHHNV alone was used as negative and positive control groups. In addition, dsRNA-GFP (0.63 μg/g shrimp) and IHHNV was co-injected into shrimp in order to use as a non-specific control group. Pleopods from individual shrimp were collected at 5 days post injection to extract genomic DNA. PCR analysis was performed to detect IHHNV expression level.

The results in Figure 4-9 showed PCR products of IHHNV and actin from pleopods of shrimp injected with 150 mM NaCl alone, IHHNV alone, 0.63 μg/g shrimp of dsRNA-GFP or 0.63 μg/g shrimp of dsRNA-PmRab7 and IHHNV. Relative expression levels of IHHNV genome normalized with actin was shown in Figure 4-10. The relative expression levels of IHHNV genome was 59% and 8% in the group that co-injected with dsRNA-GFP or dsRNA-PmRab7 and IHHNV, respectively, when compared to the group that injected with IHHNV alone. However, the relative expression levels of IHHNV genome was 37% in shrimps injected with 150 mM NaCl alone. It is indicated that shrimps in this experiment is not IHHNV-free shrimp. However, statistically significant difference was observed in the group injected with IHHNV alone and the group co-injected with IHHNV and dsRNA-PmRab7 ($P<0.05$). It is suggested that co-injection of IHHNV and dsRNA-PmRab7 can inhibit IHHNV replication in *L. vannamei*.

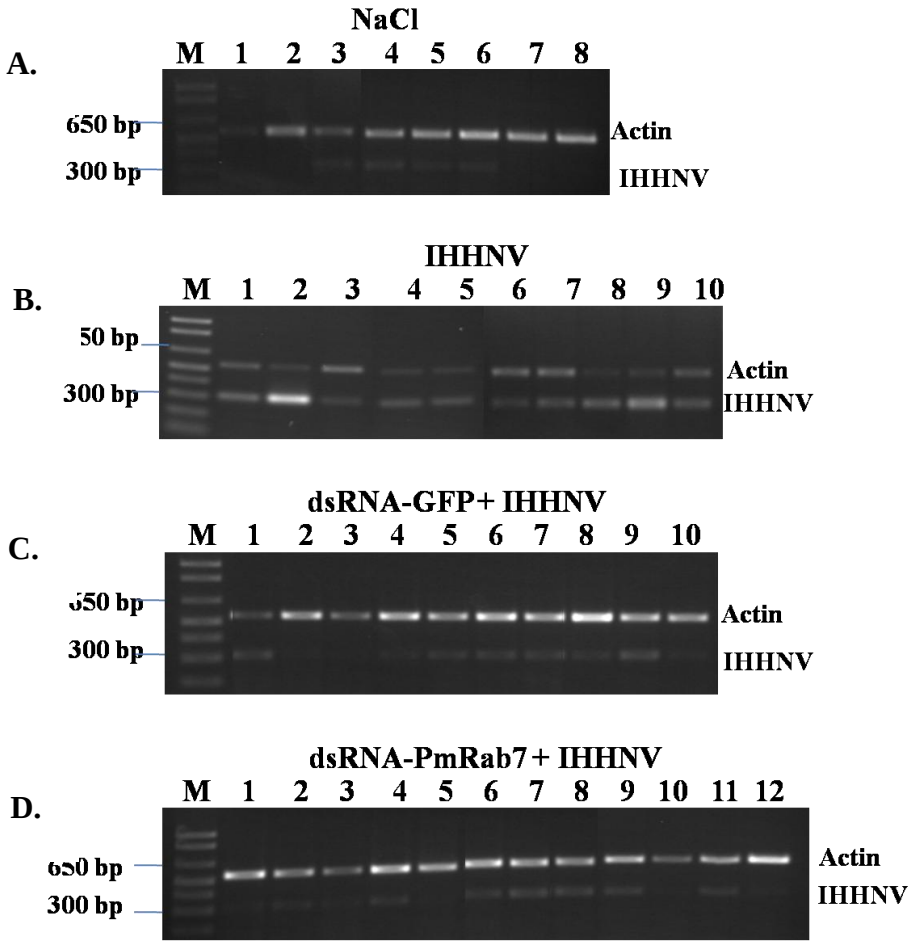


Figure 4-9. IHHNV genome expression in LvRab7-knockdown shrimp after 5 days co-injection of dsRNA and IHHNV. Expression of IHHNV (309 bp) and actin (550 bp) were determined by PCR in shrimp injected with (A) 150 mM NaCl alone (n=8), (B) IHHNV alone (n=10), (C) dsRNA-GFP (n=10) or (D) dsRNA-PmRab7 (n=12) and IHHNV. M is 1 kb plus DNA marker.

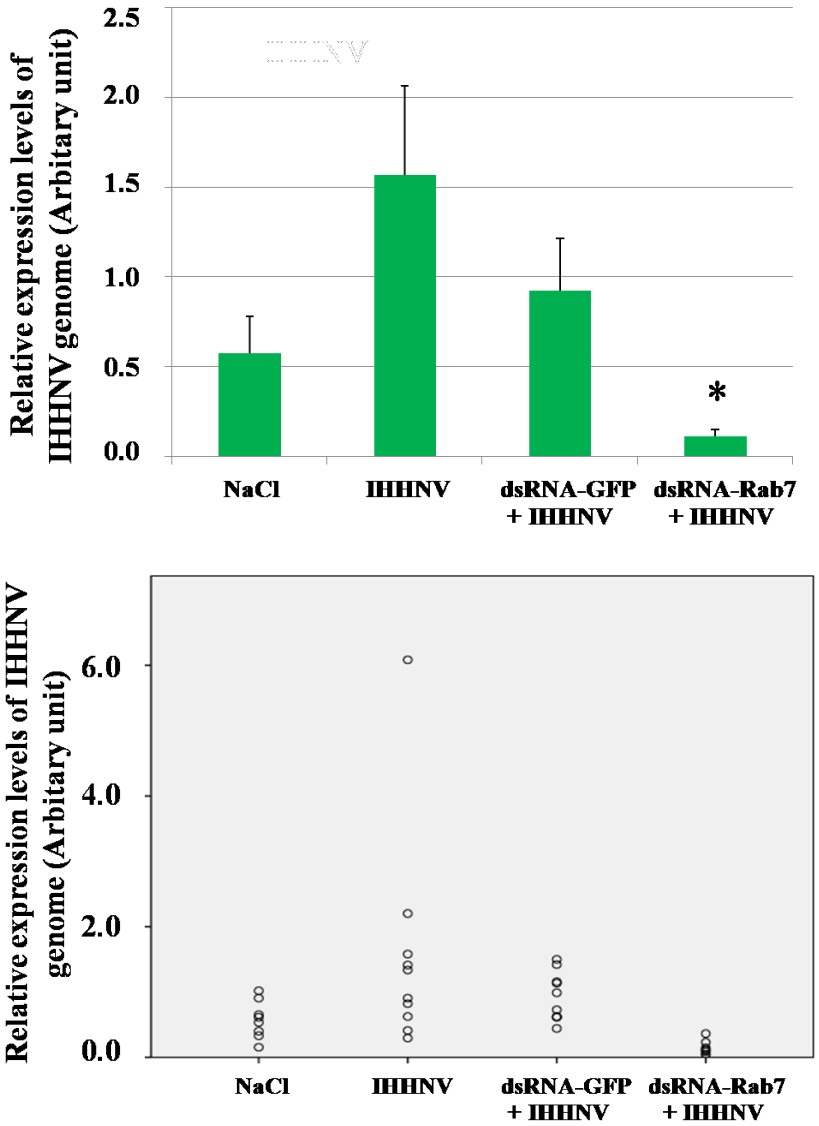


Figure 4-10. Relative expression levels of IHHNV genome in LvRab7-knockdown shrimp after 5 days co-injection of dsRNA and IHHNV. Relative expression of IHHNV was normalized with actin. Data represents mean±SEM, n=8-12. * represents statistically significant difference ($p<0.05$) between the group co-injected with dsRNA-PmRab7 and IHHNV and the group injected with IHHNV alone. The relative expression levels of IHHNV genome of an individual shrimp was plotted as a dot plot analysis in the lower panel.

4.5.3 Therapeutic effect of dsRNA on IHHNV replication in *L. vannamei* by single injection of dsRNA

To study the therapeutic effect of dsRNA-PmRab7 on IHHNV replication in *L. vannamei*, shrimps of size about 3 g were challenged with 6×10^7 viral particles. One day after IHHNV injection, shrimps were injected with 0.63 μg/g shrimp of dsRNA-PmRab7, 150 mM NaCl or IHHNV alone. In addition, shrimp injected with dsRNA-GFP was used to investigate the specific effect of dsRNA-PmRab7. Pleopods from individual shrimp were collected at 5 days post IHHNV challenge. Genomic DNA and total RNA were extracted and analyzed IHHNV and LvRab7 expression levels by PCR and RT-PCR, respectively.

Figure 4-11 presented the RT-PCR (LvRab7 and actin) products and PCR (IHHNV and actin) products of mRNA from pleopods injected with 150 mM NaCl, 0.63 μg/g shrimp of dsRNA-GFP and 0.63 μg/g shrimp of dsRNA-PmRab7. Relative expression levels of LvRab7 mRNA and IHHNV genome normalized with actin were shown in Figure 4-12. The result showed that LvRab7 expression levels were statistically significant reduced ($p<0.05$) approximately 65% after dsRNA-PmRab7 injection when compared to NaCl injected group. The expression levels of IHHNV were 35% and 35% in shrimps injected with dsRNA-GFP and dsRNA-PmRab7, respectively, when compared to shrimps injected with IHHNV alone. However, 32% of IHHNV expression can be detected in shrimp injected with 150 mM NaCl alone indicating that shrimps in this experiment were not IHHNV-free shrimp. Interestingly, 65% of IHHNV genome expression was inhibited in the groups injected with dsRNA-PmRab7 and with dsRNA-GFP. The results suggested that a single injection of dsRNA-PmRab7 or dsRNA-GFP after 1 day IHHNV challenge can reduce IHHNV replication in IHHNV-infected *L. vannamei*.

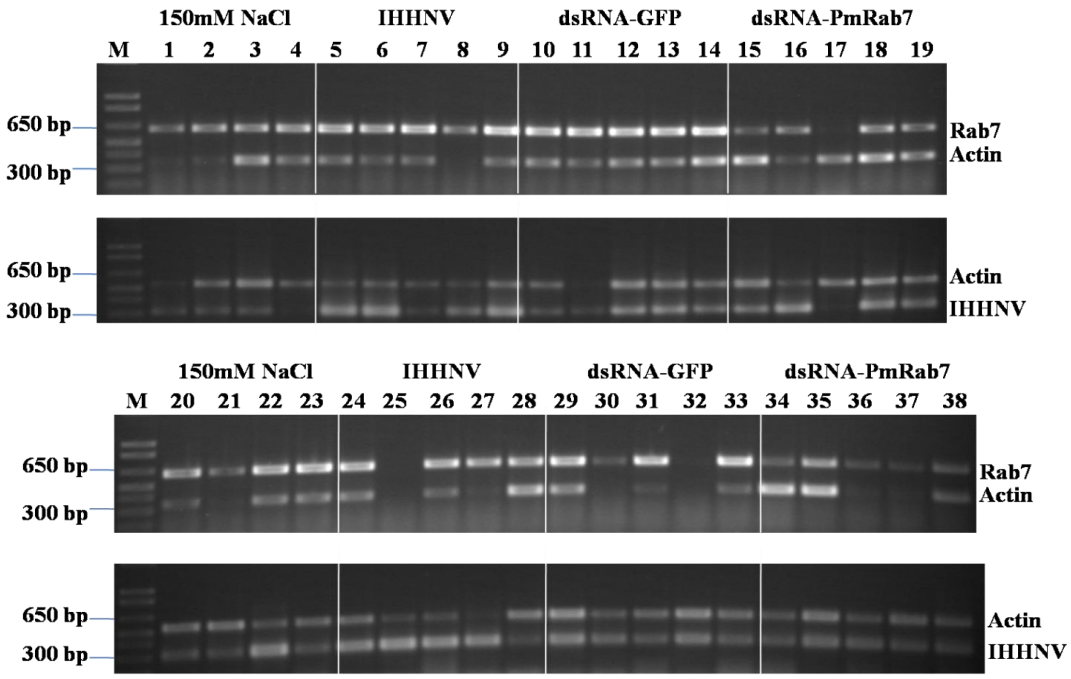
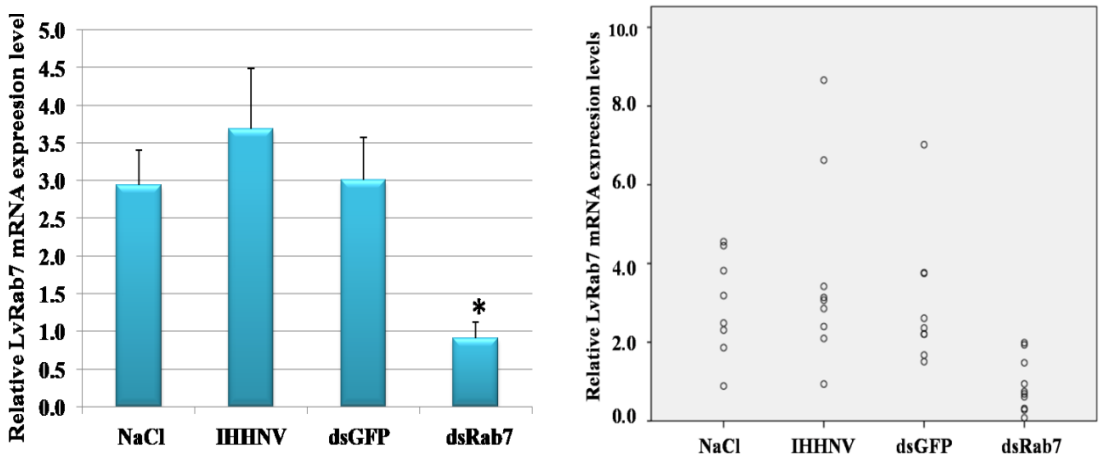


Figure 4-11. Therapeutic effect of dsRNA-PmRab7 in IHHNV-infected shrimp on day 5 after IHHNV challenge. LvRab7 and actin (617 bp and 350 bp) expression were determined by RT-PCR whereas IHHNV and actin (309 bp and 550 bp) expression were determined by PCR. Shrimps were injected with 150 mM NaCl only (lane 1-4, 20-23), IHHNV only (lane 5-9, 24-28), dsRNA-GFP (0.63 µg/g shrimp) (lane 10-14, 29-33) and dsRNA-PmRab7 (0.63 µg/g shrimp) (lane 15-19, 34-38). M is 1 kb plus DNA marker.

A.



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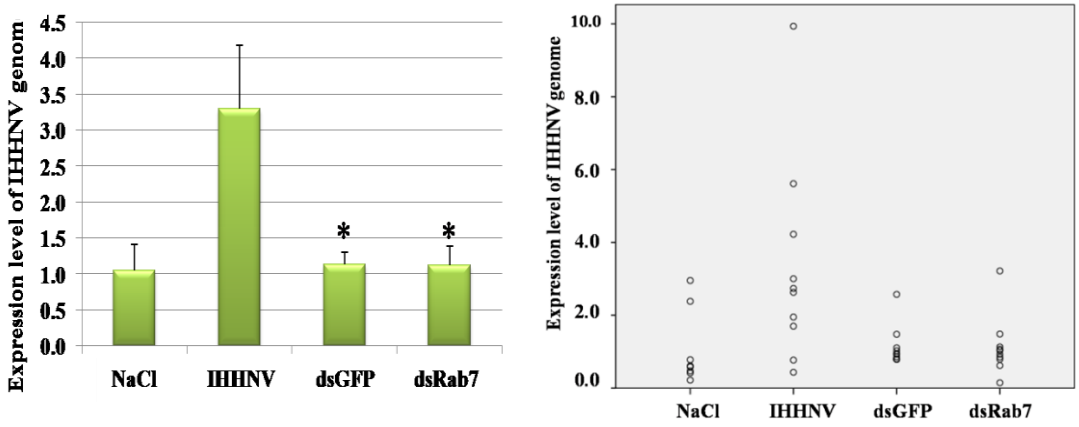

Figure 4-12. Relative expression levels of IHHNV genome in LvRab7-knockdown shrimp injected with 0.63 μg/g shrimp of dsRNA-PmRab7 1 day after IHHNV challenge. Relative expression of IHHNV was normalized with actin. Data represents mean±SEM, n=8-12. * represents statistically significant difference ($p<0.05$) between the group injected with dsRNA and IHHNV and the group injected with IHHNV alone. The dot plot distributions of an individual sample were shown on the right panel. DsGFP and dsRab7 represented the groups that injected with dsRNA-GFP or dsRNA-PmRab7 1 day after IHHNV challenge, respectively.

4.5.4 Therapeutic effect on IHHNV replication by double injection of dsRNA-PmRab7

To study the therapeutic effect of double injection of dsRNA-PmRab7 on IHHNV replication**,** shrimps, size about 1 g were challenged with 6×10^7 viral particles. One and 5 day (s) after viral challenge, *L.vannamei* was injected with 2.5 μg/g shrimp of dsRNA-PmRab7. Shrimp injected with 150 mM NaCl was used as a control group. Pleopods were collected at 0 (before) and 10 day (s) post IHHNV challenge. Genomic DNA was extracted and analyzed IHHNV expression levels by PCR.

Figure 4-13 presented PCR (IHHNV and actin) products of DNA from pleopods injected with 150 mM NaCl and double injection of 2.5 μg/g shrimp of dsRNA-PmRab7. Relative expression levels of IHHNV genome normalized with actin was shown in Figure 4-14. The results showed the replication level of IHHNV genome in 3 of 18 shrimps in both NaCl group and dsRNA-PmRab7 group at day 0 (before experiment). At day 10 after IHHNV challenge, the replication level of IHHNV genome can be detected in 8 of 18 shrimps in NaCl group but it cannot be detected in dsRNA-PmRab7 group. The replication level of IHHNV presented significantly difference between NaCl group and dsRNA-PmRab7 group at day 10. The results suggested that double injection of 2.5 μg/g shrimp of dsRNA-PmRab7 after 1 and 5 day (s) IHHNV challenge can reduce IHHNV replication in IHHNV-infected *L. vannamei*.

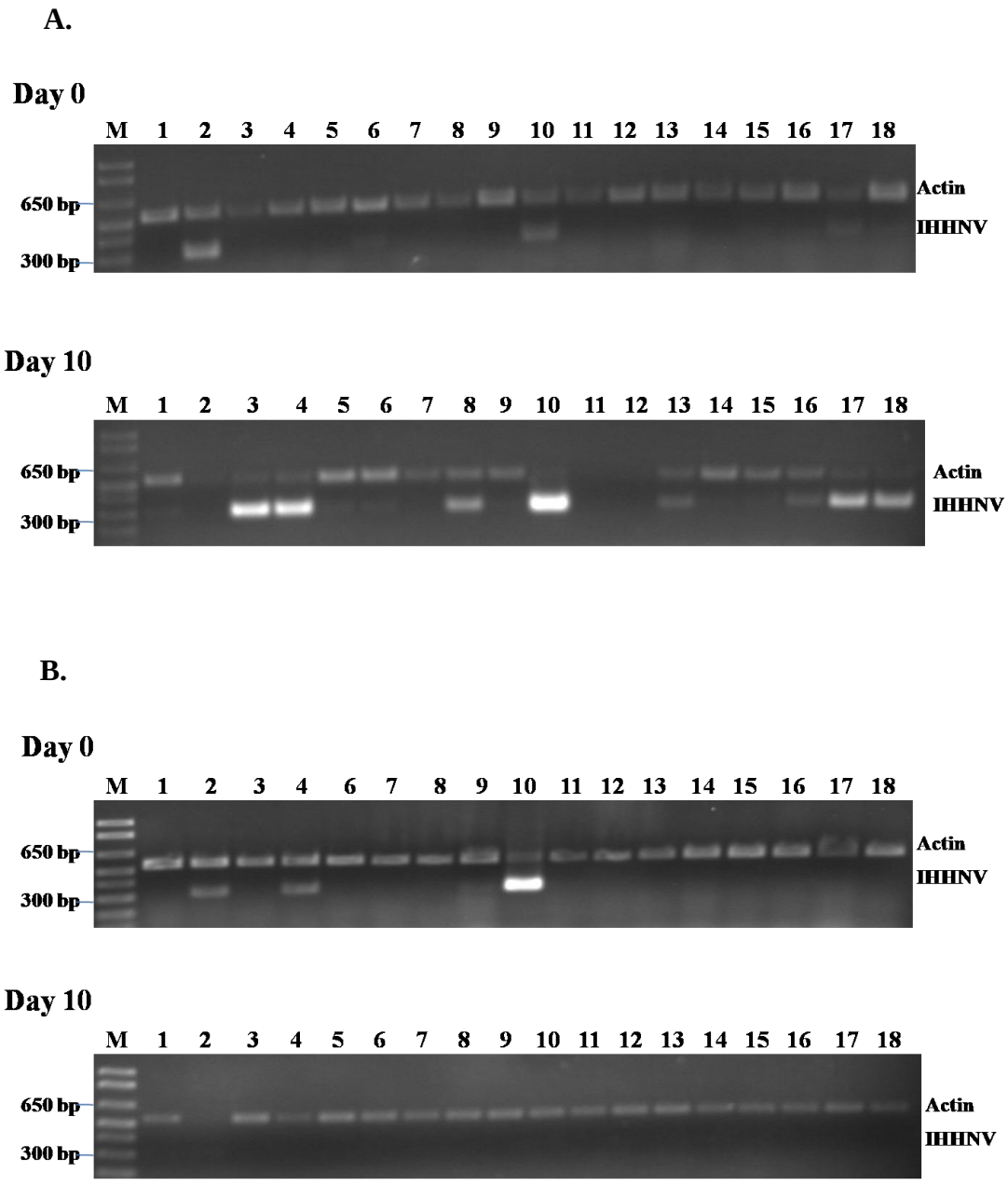

Figure 4-13. Therapeutic effect of dsRNA-PmRab7 in IHHNV-infected shrimp. IHHNV and actin (309 bp and 550 bp) expression were determined by PCR. Shrimps were injected with 150 mM NaCl only (A) and double injection of dsRNA-PmRab7 (2.5 µg/g shrimp) (B) at day 0 and 10 after IHHNV challenge. M is 1 kb plus DNA marker.

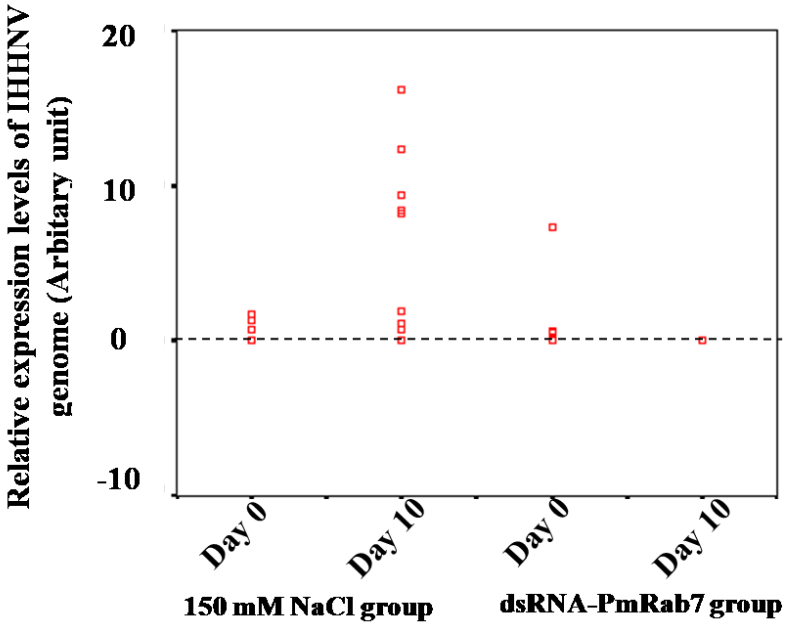

Figure 4-14. Dot plot distributions showing the therapeutic effect of double injection of 2.5 µg/g shrimp of dsRNA-PmRab7 in IHHNV-infected shrimp. The dot plot distributions of an individual sample were shown in shrimp injected with 150 mM NaCl and double injection of 2.5 µg/g shrimp of dsRNA-PmRab7 at day 0 and 10 after IHHNV challenge. Relative expression of IHHNV was normalized with actin. Data represents mean±SEM, n=18.