JOURNAL OF THE
WORLD AQUACULTURE SOCIETY

Vol. 46, No. 6
December, 2015
doi: 10.1111/jwas.12236

Long-lasting Effect Against White Spot Syndrome Virus in Shrimp Broodstock, *Litopenaeus vannamei*, by LvRab7 Silencing

Píndaro Alvarez-Ruiz[1], Antonio Luna-González, and Ruth Escamilla-Montes

Centro de Interdisciplinario de Investigación para el Desarrollo Integral Regional, Instituto Politécnico Nacional, Guasave, México

Claudio H. Mejía-Ruiz and Francisco J. Magallón-Barajas

Centro de Investigaciones Biológicas del Noroeste, S.C. Instituto Politécnico Nacional 195, La Paz, México

Raúl Llera-Herrera

Centro de Investigación en Alimentación y Desarrollo, A.C. Avenida Sábalo Cerritos s/n, Mazatlán, México

Diego A. Galván-Alvarez

Centro de Investigaciones Biológicas del Noroeste, S.C. Hermosa 101, Hermosillo, México

Abstract

The white spot syndrome virus (WSSV) remains the most devastating viral pathogen of shrimp culture worldwide. Gene silencing by RNA interference (RNAi) using double stranded RNA (dsRNA) has been considered a powerful tool for conferring protection against WSSV when viral genes are silenced, as documented in several shrimp species. However, this effect is not long lasting. Our results provide the first evidence that long-term silencing of the LvRab7 endogen produced antiviral effect against WSSV, which endured at least 21 d after dsRNA treatment (dat). Until now, the most efficient way to implement RNAi with dsRNA into the shrimp is by injection. Consequently, its application to broodstock in hatcheries is possible, minimizing the risk of vertical transmission of the virus. We show that the expression of Rab7 in hemocytes is lowest at 2 dat and finally recovers to basal status. In contrast, in gills and pleopods, gene expression silencing continued for at least 21 d. We challenged *Litopenaeus vannamei* broodstock with WSSV at 7, 14, or 21 dat reaching mortality rates of 0, 40, and 27%, respectively. In conclusion, the LvRab7 gene silencing is progressive and effective against WSSV. However, further studies are necessary to elucidate the functions of Rab7 in shrimp cells before applying this methodology at a commercial level.

The white spot syndrome virus (WSSV) remains the most devastating viral pathogen of shrimp culture worldwide (Lightner 2011). Since its first appearance, the white spot disease has caused mass mortalities in a few days after the onset of signs, being a serious threat to shrimp aquaculture (Lightner 1996). To counteract its impact, several biosecurity strategies have been implemented, such as the control of broodstock (Lightner 2005), water filtration and disinfection (Schuur 2003), use of certified specific pathogen-free (SPF) postlarvae (Lightner and Redman 1998), and eradication of vectors and/or carriers (Lotz 1997). However, these actions have not been effective in preventing development of the disease. Hence, some biotechnological tools have been tested as recombinant subunit vaccines (Witteveldt et al. 2004), probiotic microorganisms and antiviral plants (Peraza-Gómez et al. 2009), synthetic antiviral products, macroalgae and

[1] Correspondence to: pindaroalvarez@hotmail.com

microalgae (Rahman et al. 2006; Huynh et al. 2011), and recently RNA interference (RNAi) triggered by short interfering RNA (siRNA) or double-stranded RNA (dsRNA) (Robalino et al. 2005; Kim et al. 2007). RNAi is a cellular pathway initiated by long dsRNA that is processed to small RNAs (21–26 bp) by an RNase III (Dicer). These small RNAs act within silencing effector complexes that are targeted to complementary sequences in the mRNA, starting their specific degradation (Fire et al. 1998). This pathway has been described in numerous eukaryotic organisms including shrimp (Tirasophon et al. 2007; Xu et al. 2007; Rijiravanich et al. 2008). RNAi has been applied to shrimp in three ways: (a) interfering transcription and, therefore, with the synthesis of viral proteins; (b) interfering proteins involved in shrimp viral infection; and (c) to identify biological, physiological or immunological consequences of the interference of endogenous genes. RNAi has been denoted as a powerful tool against WSSV when viral genes are silenced. This effect has been documented in several shrimp species such as *Marsupenaeus japonicus* (Wu et al. 2007; Xu et al. 2007; Sudhakaran et al. 2011), *Penaeus chinensis* (Kim et al. 2007), *Penaeus monodon* (Westenberg et al. 2005; Attasart et al. 2009), and *Litopenaeus vannamei* (Robalino et al. 2005; Wu et al. 2007). The efficiency of RNAi to inhibit the replication of WSSV in shrimp depends on the protein target, dsRNA size, dsRNA dosage applied, and the number of inoculated virions (Robalino et al. 2005; Wu et al. 2007; Alvarez-Ruiz et al. 2013). Some results suggest that RNAi pathway in shrimp is more effective when target mRNAs are present within the cell (Mejía-Ruiz et al. 2011). Moreover, by silencing endogenous shrimp genes associated with the infection of WSSV, as the integrin β-cell receptor, it has been possible to successfully reduce viral replication and, therefore, increase survival in *M. japonicus* (Li et al. 2007). Silencing of caspase-3 and Toll gene in *L. vannamei* activated an antiviral response against WSSV (Rijiravanich et al. 2008; Lebreuche et al. 2009). Silencing of tetraspanin-3 in *Fenneropenaeus chinensis* significantly reduced mortality in shrimp challenged with WSSV (Gui et al. 2012). In addition, Rab7 gene silencing has shown an impact against the replication of WSSV in *P. monodon* juveniles (Ongvarrasopone et al. 2008).

Rab7 belongs to the family of Rab proteins (Ras-associated binding). These are small GTPases that regulate docking and fusion of transport vesicles through the endocytosis and exocytosis pathways (Rodman and Wandinger-Ness 2000). Some viruses use endocytosis pathway to enter the cell and replicate (Sieczkarski and Whittaker 2002). Sritunyalucksana et al. (2006) first reported Rab7 in *P. monodon* (named PmRab7) as a protein related to WSSV infection. Later, it was discovered that silencing Rab7 produces an effect against WSSV in *P. monodon* postlarvae and juveniles (Ongvarrasopone et al. 2008; Attasart et al. 2009) as well as in broodstock of *L. vannamei* (Alvarez-Ruiz et al. 2013). In addition, shrimp treated with dsRNA-PmRab7 inhibited infection against other shrimp viruses, such as the yellow head virus (YHV) (Ongvarrasopone et al. 2008), Laem-Singh virus (LSNV) (Ongvarrasopone et al. 2010), and the Taura syndrome virus (TSV) (Ongvarrasopone et al. 2011). Recently, a Rab7 cDNA sequence was isolated from *L. vannamei* (LvRab7) and silencing of this transcript showed a modest antiviral response against WSSV challenge after 48 h post-dsRNA inoculation in broodstock; however, the highest gene silencing was registered up to 120 h post-treatment (Alvarez-Ruiz et al. 2013). Therefore, the aims of this research were to assess the antiviral effect against WSSV at several times after dsRNA treatment in broodstock and to determine long-term silencing in different shrimp tissues.

Materials and Methods

Virus Inoculum and Infectious Doses

The WSSV inoculum was titrated *in vivo* in a previous study and inoculation was done intramuscularly (Alvarez-Ruiz et al. 2013). The infectious titer of the inoculum was $10^{6.2}$ infectious doses with 50% endpoint (shrimp infectious dose, SID_{50}/mL) (Reed and Muench 1938). A deadly dose (20 SID_{50}) was chosen for challenge experiments.

TABLE 1. *Primers used for expression, synthesis of dsRNA, and WSSV detection.*

Gene	Primer	Sequence	Product size (bp)	Efficiency (%)	Analyses
LvRab7	LvRab7-F	5′ ATGGCATCTCGCAA GAAG 3′	618		dsRNA production
	LvRab7-R	5′ TTAGCAAGAGCATGC ATCC 3′			
LvRab7	qLvRab7-F	5′ GCAACCATTGGAGCA GATTT 3′	195	94.1	LvRab7 expression
	qLvRab7-R	5′ ACGCCATGAATCGAG AGACT 3′			
WSSV VP28	VP28-F	5′ AACTGCAGATGGATCT TTCTTTC 3′	615		WSSV detection
	VP28-R	5′ AACTGCAGTTACTCGGT CTCAG 3′[a]			
β-actin	β-actinF	5′ GAAGTAGCCGCCCTG GTTG 3′	338		Internal control
	β-actinR	5′ CGGTTAGCCTTGGGG TTGAG 3′			
Ribosomal protein 40S-S24	qLv40S-S24-F	5′ CAGGCCGATCAACTGT CC 3′	204	93.1	
	qLv40S-S24-R	5′ CAATGAGAGCTTGCCT TTCC 3′			
Elongation factor 2	qLvEf-F	5′ CTGTGGTCTGGTTGGT GTTG 3′	141	99.2	
	qLvEf-R	5′ TCAGATGGGTTCTTGG GTTC 3′			
Ubiquitin	qLvUBIQ-F	5′ GGGAAGACCATCACC CTTG 3′	146	90.2	qPCR reference genes
	qLvUBIQ-R	5′ TCAGACAGAGTGCGA CCATC 3′			
18S rRNA	qLv18S-F	5’ GCAGGCTGGTTTTTG CTTAC 3’	185	86.9	
	qLv18S-R	5′ ATGCTTTCGCAGTAGG TCGT 3′			
Tubulin	qLvTUB-F	5′ GTCCGCAAGGAAGCAG AA 3′	210	89.9	
	uLvTUB-R	5′ GATTGAGAGGGTTGCG TTGT 3′			

WSSV = White Spot Syndrome Virus.
[a]Restriction site PstI is underlined.

WSSV Diagnosis by PCR

Total DNA from hemocytes was extracted using GENECLEAN SPIN Kit (MP Biomedicals, Santa Ana, CA, USA). DNA from gills was extracted using DNAzol (Gibco®, Invitrogen, Waltham, MA, USA), following manufacturer's instructions.

In order to confirm that the organisms used in this work were WSSV free, the experimental shrimp collected were screened for WSSV by PCR using primers (VP28 F/R) (Table 1) and DNA (80–100 µg) from hemocytes as template. Viral infection in dead shrimp was confirmed by PCR, extracting DNA from gills. β-actin sequence was used as an internal control (Table 1). PCR conditions for amplification were denaturation at 95 C for 3 min, followed by 35 cycles of denaturation (95 C for 30 s), annealing (55 C for 30 s), and extension (72 C for 1 min), with a final extension (72 C for 5 min). The PCR products were resolved on a 1% agarose gel.

RNA Extraction, cDNA Synthesis, and Reverse Transcription PCR

Total RNA was extracted using Trizol (Gibco, Invitrogen, Waltham, MA, USA), following

manufacturer's instructions. Total RNA was quantified on a NanoPhotometer® (Nano-Drop 2000c, Thermo Scientific, Vantaa, Finland), considering the absorbance ratio 260/280 nm between 1.8 and 2.0 as acceptable purity range.

cDNAs were synthesized from 1.0 µg of total RNA from each sample using Improm-II Reverse Transcriptase (Promega, Madison, WI, USA) and oligo dT20. cDNA was diluted 10 times with water; 5 µL of this cDNA dilution was used as template in RT-PCR reactions. Conditions for amplification were the same of the PCR (above).

qPCR Analyses

The qPCR mix contained 2.5 mM $MgCl_2$, 50 µM of each dNTP, 0.45 U of GoTaq DNA polymerase (Promega, Madison, WI, USA), 0.20–0.46 µM of each primer, and 1× EvaGreen fluorescent dye (Biotium Inc., Hayward, CA, USA), in 15 µL of final volume. The efficiency of the amplification was determined by calculating a slope with at least five serial dilutions (dilution factor of 5) of a representative pool of cDNAs. The amount of primers was optimized for each gene when the best efficiency was recorded, analyzing different dilution curves with different amounts of primers. The amplification conditions were as follows: 95 C for 3 min followed by 40 cycles of 95 C for 10 s, 60 C for 15 s, 72 C for 30 s, and 79 C for 5 s (to acquire fluorescence), using a CFX96 Real Time PCR thermal cycler (Bio-Rad). After each reaction, a dissociation curve from 60 to 90 C was recorded at increments of 0.5 C and examined for unique and specific products. All reactions were conducted in duplicate (expression analyses) or triplicate (efficiency curves), and spurious amplification of C_q 38 and/or with unexpected dissociation curves were considered as not amplified.

To determine the most stable reference genes to be used in relative expression analyses, we analyzed the expression of five genes as potential reference genes (Table 1). The relative stability of reference genes was analyzed in 86 samples obtained from the experiment. The expression stability of the potential reference genes was analyzed using two algorithms: GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004), using the RefFinder web application (http://www.leonxie.com/referencegene.php). The expression of target genes was normalized to the geometric mean of the three most stable genes.

C_q values were exported to Excel (Microsoft) and transformed to relative quantities (RQ) using the equation $RQ_{ij} = E^{(C_q(\min) - C_q(ij))}$. Where E is the gene-specific efficiency, and $(C_q(\min) - C_q(ij))$ is the absolute difference for each C_q sample against the lowest C_q in the data set for each gene. Relative expression was calculated with the equation RQ^{target}/Geometric mean of the three most stable reference genes $RQ^{reference\ genes}$ (Vandesompele et al. 2002).

Construction of LvRab7-Specific Double-Stranded RNA (dsRNA)

A 618 bp Rab7 dsRNA fragment was synthesized using an *in vitro* transcription kit (Block-iT RNAi TOPO Transcription Kit; Invitrogen) as follows. Briefly, PCR products of the LvRab7 gene were amplified and linked to the T7 promoter. Two secondary PCR reactions were performed separately combining the T7 primer and LvRab7-specific forward or reverse primers, respectively, to amplify linked forward or reverse PCR products. Each of these amplifications produced sense or antisense DNA strands linked to T7 promoter sequence in the 5′ end, to be used next as templates for ssRNA transcription. Transcription of sense or antisense ssRNA templates was achieved by incubating separate templates for 6 h at 37 C with BLOCK-iT T7 enzyme mix. After transcription, DNAse I was added to digest DNA templates. Sense and antisense ssRNAs were purified and quantified using a NanoPhotometer (Nano-Drop 2000c; Thermo Scientific, Vantaa, Finland). Equimolar concentrations of sense and antisense ssRNA transcripts were mixed to form dsRNA and stored in aliquots of 100 µg each at −70 C until used. To test whether RNA was double stranded, an aliquot of dsRNA was incubated with RNaseA and DNAase I, which specifically cleave ssRNA and DNA, respectively, and the intact dsRNA was assessed by agarose electrophoresis.

LvRab7 Silencing in Hemocytes, Gills, and Pleopods

To evaluate LvRab7 gene silencing in different shrimp tissues, 200 *L. vannamei* (15 ± 2.5 g mean body weight [MBW]) were obtained from experimental ponds of CIBNOR-La Paz and placed in plastic containers of 1500 L with filtered sea water (35 g/L salinity) and continuous aeration.

After 24 h acclimation, 60 shrimp were intramuscularly injected with dsRNA-LvRab7 (5 µg/shrimp) and placed in six plastic tanks with 80 L of filtered seawater and continuous aeration. Water temperature was 27 ± 1.0 C and water exchange (50%) was done daily. Shrimp were fed twice daily with commercial diet during the experiment. A total of 60 shrimp, under the same conditions and injected with phosphate buffer saline (PBS) buffer (1×), were used as a negative control.

Seven treated and seven untreated shrimp were collected at 2, 7, 14, and 21 d after dsRNA treatment (dat). Hemolymph (300 µL), pleopods, and gills were taken from each animal. Gills and pleopods were fixed with Trizol (1.0 mL) and stored at −70 C until used. The hemolymph was processed, and subsequently hemocytes were fixed with Trizol (500 µL).

At the end of the experiment, all samples were processed to obtain total RNA. cDNAs were synthesized, and expression of LvRab7 was assessed through qRT-PCR.

WSSV Challenge at 7, 14, and 21 d After dsRNA Treatment

Shrimp *L. vannamei* broodstock ($n = 300$, MBW 27 ± 4.0 g) were obtained from a hatchery in Sonora, Mexico. Shrimp were maintained in a glass fiber tank (2000 L) contained 1800 L of filtered sea water (25 g L^{-1} salinity) provided with continuous aeration. Animals were kept in these conditions 10 d before the experiments. DNA was extracted from hemocytes and analyzed by PCR to discard WSSV presence. After that, 225 shrimp broodstock were distributed among three batches in separate tanks: Batch (1), 75 shrimp injected with 5 µg of LvRab7-dsRNA (treatment); Batch (2), 75 shrimp injected with PBS-1× alone (used as positive control); and Batch (3), 75 intact shrimp (used as negative control).

The challenge experiments were performed in controlled temperature chambers at 27 ± 0.1 C using sterile seawater at 25 g/L salinity. The experimental units consisted of plastic tanks of 80 L with lids. To eliminate water exchange, each tank was equipped with a sand filter and continuous water recirculation impelled by aeration. Five shrimp were placed in each tank and acclimated 24 h before challenge. Each experiment was performed in triplicate. Shrimp were fed ad libitum in two portions daily.

At 7 dat, five shrimps from treatment and positive control (Batches 1 and 2) were challenged intramuscularly with a lethal dose of WSSV (20 SID_{50}); five shrimp from negative control (Batch 3) were mock-treated with 100 µL of PBS 1×. Clinical signs of infection and shrimp mortality were monitored twice daily. Moribund shrimp were collected during the experiment and processed for DNA-PCR and qRT-PCR analyses. Dead shrimp were collected and fixed only for WSSV detection by PCR. At 10 d after challenge (dac), survivors were euthanized and processed for analyses. Identical experiments were done with shrimp from the initial batches (1, 2, and 3) at 14 and 21 dat.

Statistical Analyses

The significance of the mortality rates and LvRab7 expression of the different experimental groups was determined by one-way ANOVA followed by Tukey's multiple comparison test. Mortality percentage values were converted to arcsine values for statistical analysis. A $P \leq 0.05$ value was considered statistically significant.

Results

Stability Analyses of Reference Genes

Amplification efficiencies (E) of each reference gene primers (Table 1) were calculated from the slope curve with a pool of shrimp cDNAs. The efficiencies ranged between 86.9 and 99.2% (Table 1). These values were used as a correction parameter for the relative quantification of samples.

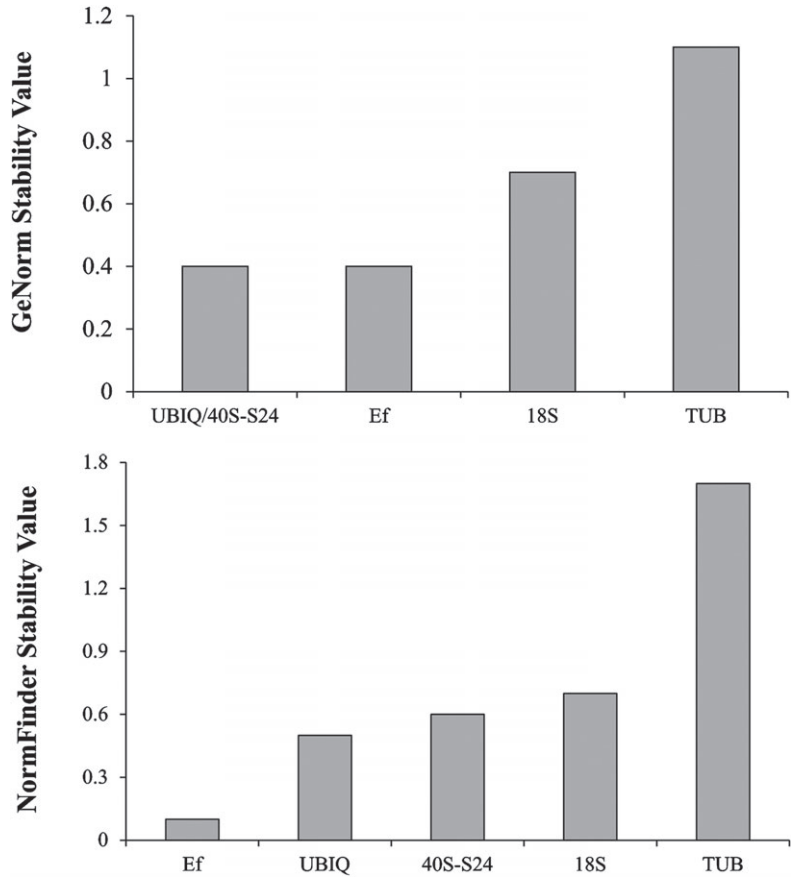

FIGURE 1. *Stability values of reference genes, by geNorm and NormFinder.*

When the Cq of reference genes was analyzed using geNorm. UBIQ, 40S-S24, and Ef remained as the most stable genes, giving stability-values (SV) of 0.4 (Fig. 1). Analyses using NormFinder determined Ef as the most stable gene reaching SV = 0.1, the second and the third were UBIQ and 40S-S24 giving SV values of 0.5 and 0.6, respectively (Fig. 1). Hence, they were selected to obtain LvRab7 expression.

Silencing of LvRab7 in Different Tissues

The silencing effect of LvRab7 dsRNA during 21 days was quantified in three shrimp tissues. The monitoring was carried out in hemocytes, gills, and pleopods (Fig. 2). All treated shrimp had the lowest expression levels at 2 dat, whereas expression was clearly shown in the PBS-injected shrimp. However, expression in hemocytes at 7, 14, and 21 dat was not significantly different from that of untreated shrimp. In contrast, expression in gills and pleopods remained significantly different at least until 21 dat. These results demonstrate that silencing of LvRab7 was active at 2 dat and persisted in some shrimp tissues for at least 21 dat.

Long-term Protection Against WSSV

The antiviral effect of LvRab7 silencing was estimated in shrimp broodstock by three experiments. A lethal dose of WSSV (20 SID_{50}) was injected into experimental shrimp at 7, 14, or 21 dat, and development of disease and LvRab7 gene expression were analyzed.

No mortality was recorded in the negative control (no WSSV and no dsRNA). On the other hand, mortality reached 100% in the positive

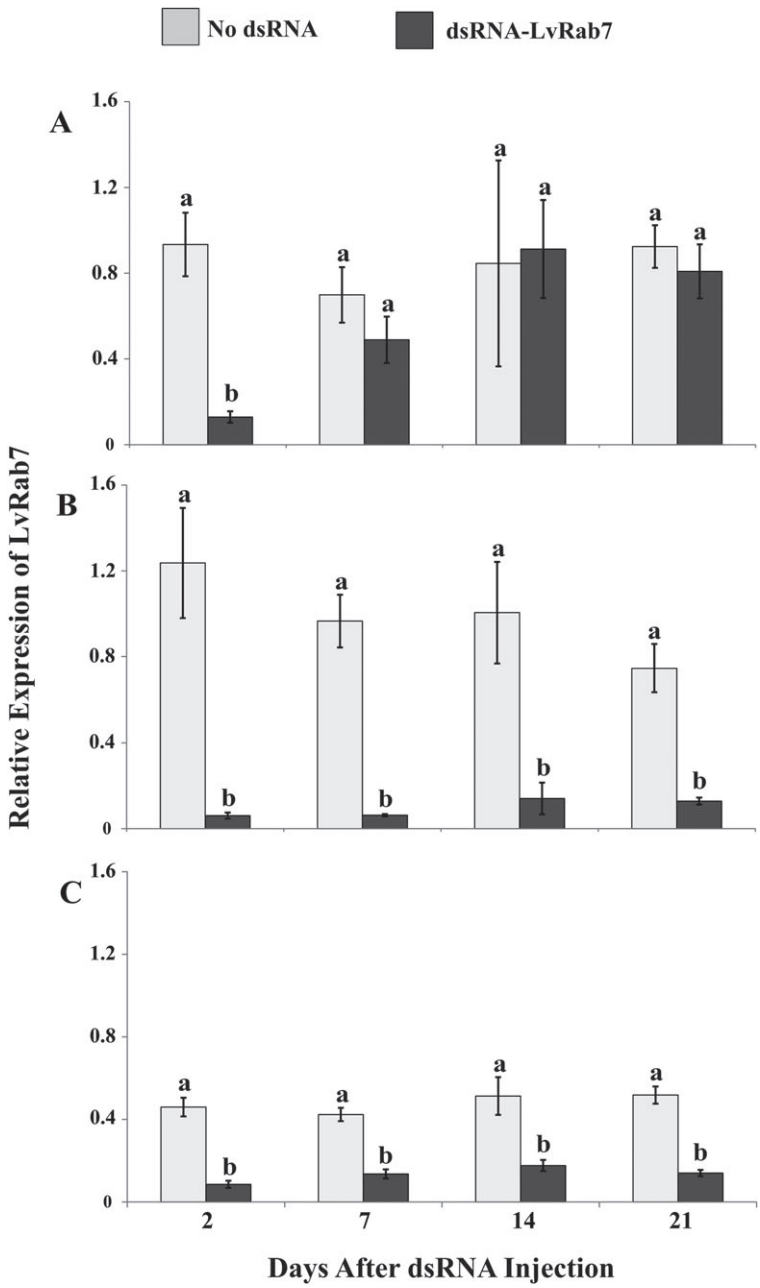

FIGURE 2. *Relative expression of LvRab7 mRNA in (A) hemocytes, (B) gills, and (C) pleopods. Samples were taken at different times after dsRNA-LvRab7 injection (2, 7, 14, and 21 d). Relative expression was normalized with geometric means of 40S-S24, Ef, and UBIQ. Different letters indicate significant differences* ($P \leq 0.05$).

control (no dsRNA + WSSV) (Fig. 3). WSSV presence was verified by PCR.

Experiment 1: At the first WSSV challenge (7 dat), shrimp treated with dsRNA as well as the negative control group showed no signs of disease during the experiment. At 10 dac, no mortality was recorded in either treated animals or negative control (Fig. 3A).

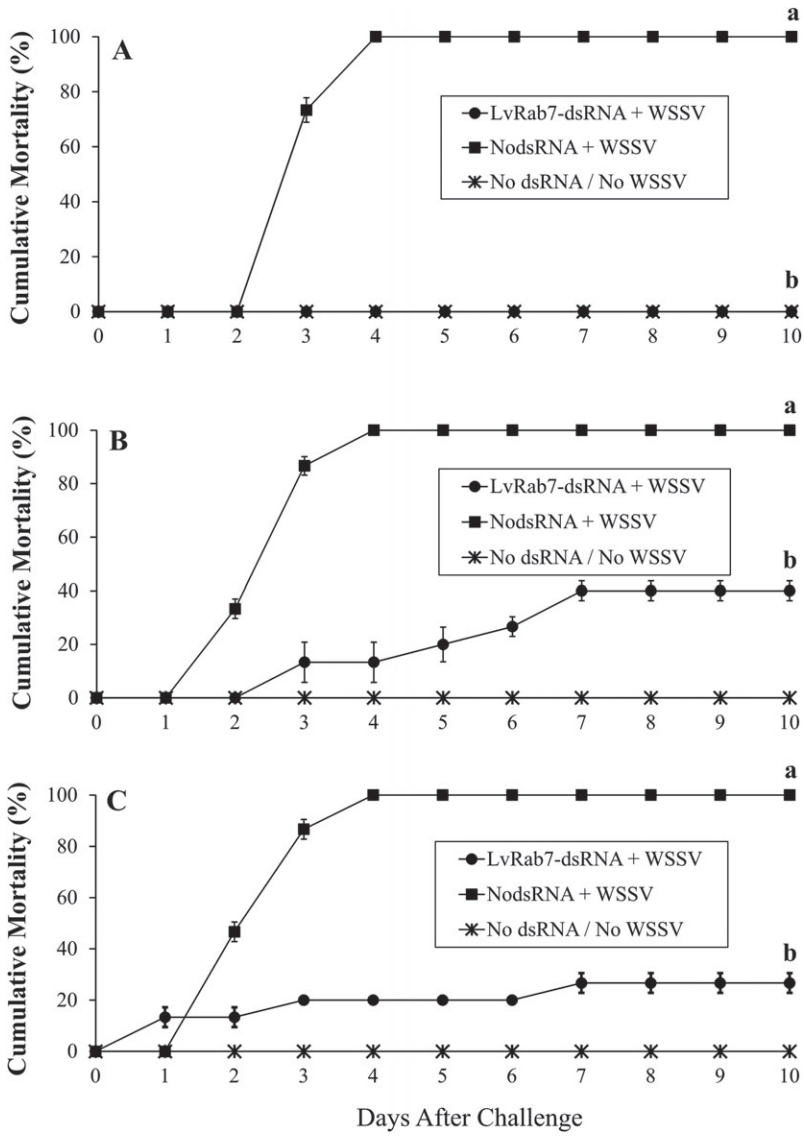

FIGURE 3. *Cumulative mortality of* Litopenaeus vannamei *broodstock challenged with WSSV (20 SID_{50}). No mortality was recorded in negative control groups (no dsRNA, no WSSV) while positive control groups (no dsRNA + WSSV) had a cumulative mortality of 100% at 4 days after challenge. (A) No mortality was recorded in shrimp challenged at 7 days after treatment (dat). (B) Challenged at 14 dat (40% mortality). (C) Challenged at 21 dat (27% mortality). Different letters indicate significant differences (*$P \leq 0.05$*).*

Experiment 2: Shrimp dsRNA-treated and challenged with WSSV at 14 dat showed cumulative mortality of 40% (Fig. 3B).

Experiment 3: At 21 dat, shrimp were inoculated with WSSV. At the end of the experiment mortality reached 27% in the LvRab7-dsRNA treatment (Fig. 3C).

LvRab7 Expression in Shrimp Challenged Against WSSV

LvRab7 expression was quantified using qRT-PCR in gill tissue of surviving shrimp in all three assays (7, 14, and 21 dat), as well as in control groups. The expression was significantly higher in the control groups shrimp (no dsRNA)

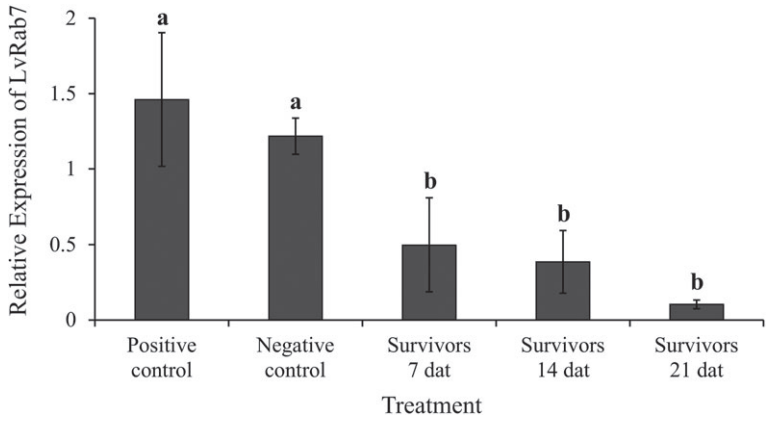

FIGURE 4. *mRNA expression levels of LvRab7 estimated in gill tissue of experimental shrimp broodstock. Positive control [no dsRNA + white spot syndrome virus (WSSV)]. Negative control (no dsRNA + no WSSV) (survivors). Shrimp treated with LvRab7 dsRNA that survived to WSSV challenge. Relative expression was normalized with geometric means of 40S-S24, Ef, and UBIQ. Different letters indicate significant differences* ($P \leq 0.05$).

than in those treated with dsRNALvRab7, besides decreasing gradually (Fig. 4). These results reveal that silencing of LvRab7 persisted during all the experiments.

Discussion

The Rab proteins are small GTPases with a significant function in endocytosis and exocytosis (Stenmark and Olkkonen 2001; Sieczkarski and Whittaker 2002). Several enveloped viruses enter into cells by endocytosis-mediated Rab5 and/or Rab7. Venezuelan equine encephalitis virus (VEEV) and dengue virus (DENV) require Rab5 and Rab7 to enter the mosquito cells (Colpitts et al. 2007; van der Schaar et al. 2008). Semliki Forest virus and vesicular stomatitis virus need exclusively Rab5 to infect cells (Sieczkarski and Whittaker 2002). Moreover, it has been found that silencing of Rab5 and Rab7 reduces viral replication of hepatitis C virus (HCV) (Manna et al. 2010). In shrimp, blocking of Rab7 protein with anti-Rab7 or silencing gene with dsRNA increased survival in broodstock and juvenile shrimp challenged against WSSV (Sritunyalucksana et al. 2006; Ongvarrasopone et al. 2008; Attasart et al. 2009; Alvarez-Ruiz et al. 2013). Furthermore, it has been documented that silencing of Rab7 in *P. monodon* and *L. vannamei* inhibits the replication of other viruses such as YHV (Ongvarrasopone et al. 2008), LSNV (Ongvarrasopone et al. 2010), and TSV (Ongvarrasopone et al. 2011). Hence, it is clear the potential of silencing Rab7 as a preventive strategy against WSSV. The first study concerned Rab7 silencing in juveniles (7–10 g) of *P. monodon*, experimentally infected at 2 d dat, with duration of 9 d (Ongvarrasopone et al. 2008). Then, *P. monodon* (300 mg) were infected at 3 dat, and observed for 11 d (Attasart et al. 2009) and *L. vannamei* broodstock (27.5 ± 2.5 g) infected at 2 dat and maintained for 12 days (Alvarez-Ruiz et al. 2013). Regarding the Rab7 silencing against other viruses (LSNV or TSV), monitoring has been less than a week (Ongvarrasopone et al. 2010, 2011). To our knowledge, there are no studies on the long-term protection against WSSV by silencing endogenous genes or the effect of silencing LvRab7 in shrimp after 12 d. In this study, for the first time *L. vannamei* treated with dsRNA-LvRab7 were observed during 31 dat.

Rab7 silencing in juvenile shrimp reaches its highest level at 2 dat, evaluated by qRT-PCR (Ongvarrasopone et al. 2008). However, in a previous study, LvRab7 dsRNA-treated shrimp broodstock showed lower expression in hemocytes up to 5 dat, evaluated by RT-PCR (Alvarez-Ruiz et al. 2013). Besides, there are no reports on the development of Rab7 silencing in other shrimp tissues. In this study, the expression

of Rab7 in hemocytes, gill, and pleopods at 2, 7, 14, and 21 dat were evaluated. The results showed that silencing in hemocytes is lowest at 2 dat, but recovers to basal status at 7 dat. This result agrees with research published by Ongvarrasopone et al. (2008). On the other hand, silencing of Rab7 in gills and pleopods was significantly different from the control (no dsRNA) at all sampling times. This suggests that silencing of Rab7 depends on the lifetime of the cell. In this case, it could be that hemocytes were renewed after 2 d, and the new cells were not silenced.

To assess the durability of the antiviral effect, dsRNA Rab7-treated broodstock were challenged against WSSV at 7, 14, and 21 dat. Previous studies using Rab7-dsRNA to inhibit virus infection in *P. monodon* (1–2 g) showed a modest inhibition and 45% cumulative mortality (Attasart et al. 2009). *L. vannamei* broodstock showed 33 and 40% mortality at 10 d after WSSV challenge (Alvarez-Ruiz et al. 2013). However, both challenge experiments were carried out by inoculating the virus at 2 dat. In this study, the results showed a potent antiviral effect at 7 dat. In addition, there was no mortality in this experiment, indicating that the antiviral effect is optimal after 7 d. This is the first experiment where dsRNA-Rab7-treated shrimp were kept uninfected for long intervals of time before being challenged with WSSV. Moreover, it is the first report of zero mortality in dsRNA-Rab7-treated shrimp and challenged with WSSV. It is possible that the antiviral treatment described in this study improved survival because the elapsed interval between the dsRNA injection and the WSSV infection was longer than 2 d.

The lifetime of human Rab7 is ~28 h (Ganley et al. 2004). However, in this study, the antiviral effect of dsRNA-Rab7 administered at 7 dat was stronger than at 2 dat, probably due to the half-life of the LvRab7 synthesized before RNAi triggered. Some studies revealed that the last amino acid of the protein chain (N-terminal) indicates its lifetime (Bachmair et al. 1986). LvRab7 has cysteine in its N-terminal region, which classifies it as a half-life protein. However, no information is available on the lifetime of Rab7 in shrimp.

On the other hand, in this study, we have shown that the shrimp inoculated with dsRNA-LvRab7 resulted in eliminating the virus from the experimental shrimp after 7, 14, or 21 dat. These results contrast with a previous research where shrimp treated with dsRNA of the WSSV gene (VP28 and VP26) were challenged against WSSV at 10 or 20 d after dsRNA injection. They reported a gradual loss of the antiviral effect and suggest that if there is no target mRNAs to degrade (viral mRNAs), the RNAi pathway gradually loses its effect in shrimp (Mejía-Ruiz et al. 2011).

In this sense, it is known that RNA-dependent RNA polymerase (RdRP) is a required component of the RNA silencing pathway (Wassenegger and Krczal 2006). A role for RdRP in eukaryotic RNA silencing has been demonstrated in a wide range of eukaryotic species, including fungi (Cogoni and Macino 1999), plants (Hamilton and Baulcombe 1999), nematodes (Sijen et al. 2007), and insects (Lipardi and Paterson 2010). Besides, the RNAi pathway potentiates its effect using the mRNAs target as a substrate to generate secondary short interfering RNA (siRNAs) synthesized by RdRPs in *Caenorhabditis elegans* (Sijen et al. 2007). In this study, we registered a potent antiviral effect, when an endogenous gene of shrimp (LvRab7) was silenced, so that the mRNAs were present before virus infection. Therefore, our results suggest that *L. vannamei* has a homologue of RdRP to enhance the RNAi potency. However, until now no RdRP has been identified in shrimp.

To determine the status of LvRab7 mRNA in experimental shrimp, the expression was assessed using qRT-PCR. Statistical analysis of results showed that samples were separated in two groups (treated and untreated). However, LvRab7 expression diminished gradually according to the elapsed time after LvRab7 dsRNA application.

Despite the efforts made so far, WSSV continues to cause mortalities to the shrimp farming industry. RNAi pathway has proven to be a powerful tool to prevent WSSV replication. Currently, applying large-scale RNAi is not profitable. However, because the broodstock are

the source of postlarvae, implementing RNAi as a prophylactic measure in hatcheries could be a strategy to obtain WSSV-free postlarvae. The reproductive period of female shrimp in hatcheries is about 60 d. Therefore, it is imperative to find strategies to protect broodstock against pathogens for prolonged periods of time. This is the first study in which LvRab7 silencing provides a lasting effect against WSSV. But, although our work adds to the molecular knowledge related to WSSV infection, the particular complexity of Rab proteins, interacting in significant cellular pathways, implies that much more work is required to define the specific function of LvRab7 in viral pathogenesis.

Acknowledgments

This project was supported by Consejo Nacional de Ciencia y Tecnologia (CONACyT) AERII consortium (87684), CIBNOR La Paz, CIBNOR Hermosillo, CIBNOR Guaymas, CIIDIR-Sinaloa. Special thanks to Ana Maria Ibarra, Alejandra Garcia, and Cesar Escobedo, for their help during this study.

Literature Cited

Alvarez-Ruiz, P., C. H. Mejía-Ruiz, F. J. Magallón-Barajas, and C. M. Escobedo-Bonilla. 2013. Silencing Pacific White shrimp, *Litopenaeus vannamei* LvRab7 reduces mortality in brooders challenged with white spot syndrome virus. Aquaculture Research 44:772–782.

Andersen, C. L., J. L. Jensen, and T. F. Ørntoft. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64:5245–5250.

Attasart, P., R. Kaewkhaw, C. Chimwai, U. Kongphom, O. Namramoon, and S. Panyim. 2009. Inhibition of white spot syndrome virus replication in *Penaeus monodon* by combined silencing of viral rr2 and shrimp PmRab7. Virus Research 145:127–133.

Bachmair, A., D. Finley, and A. Varshavsky. 1986. In vivo half-life of a protein is a function of its amino-terminal residue. Science 234:179–186.

Cogoni, C. and G. Macino. 1999. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature 399:19–21.

Colpitts, T. M., A. C. Moore, A. A. Kolokoltsov, and R. A. Davey. 2007. Venezuelan equine encephalitis virus infection of mosquito cells requires acidification as well as mosquito homologs of the endocytic proteins Rab5 and Rab7. Virology 369:78–91.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811.

Ganley, I. G., K. Carroll, L. Bittova, and S. Pfeffer. 2004. Rab9 GTPase regulates late endosome size and requires effector interaction for its stability. Molecular Biology of the Cell 15:5420–5430.

Gui, L., B. Wang, F. H. Li, Y. M. Sun, Z. Luo, and J. H. Xiang. 2012. Blocking the large extracellular loop (LEL) domain of FcTetraspanin-3 could inhibit the infection of white spot syndrome virus (WSSV) in Chinese shrimp. *Fenneropenaeus chinensis*. Fish & Shellfish Immunology 32:1008–1015.

Hamilton, A. J. and D. C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950–952.

Huynh, T. G., S. T. Yeh, Y. C. Lin, J. F. Shyu, J. C. Chen, and L. L. Chen. 2011. White shrimp *Litopenaeus vannamei* immersed in seawater containing *Sargassum hemiphyllun* var. Chinese powder and its extract showed increased immunity and resistance against *Vibrio alginolyticus* and white spot syndrome virus. Fish & Shellfish Immunology 31:286–293.

Kim, C. S., Z. Kosuke, Y. K. Nam, S. K. Kim, and K. H. Kim. 2007. Protection of shrimp (*Penaeus chinensis*) against white spot syndrome virus (WSSV) challenge by double-stranded RNA. Fish & Shellfish Immunology 23:242–246.

Lebreuche, Y., N. A. O'Leary, E. de la Vega, A. Veloso, P. S. Gross, R. W. Chapman, C. L. Browdy, and G. W. Warr. 2009. Lack of evidence for *Litopenaeus vannamei* Toll receptor (lToll) involvement in activation of sequence-independent antiviral immunity in shrimp. Developmental and Comparative Immunology 33:806–810.

Li, D. F., M. C. Zhang, H. J. Yang, Y. Zhu, and X. Xun. 2007. Beta-integrin mediates WSSV infection. Virology 368:122–132.

Lightner, D. V. 1996. A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Lightner, D. V. 2005. Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. Journal of the World Aquaculture Society 36:229–248.

Lightner, D. V. 2011. Virus diseases of farmed shrimp in the western Hemisphere (the Americas): a review. Journal of Invertebrate Pathology 106:110–130.

Lightner, D. V. and R. M. Redman. 1998. Strategies for the control of viral diseases of shrimp in the Americas. Fish Pathology 33:165–180.

Lipardi, C. and B. M. Paterson. 2010. Identification of an RNA-dependent RNA polymerase in *Drosophila* establishes a common theme in RNA silencing. Fly 4:30–35.

Lotz, J. M. 1997. Viruses, biosecurity and specific pathogen-free stock in shrimp aquaculture. World Journal of Microbiology & Biotechnology 13:405–413.

Manna, D., J. Aligo, C. Xu, W. S. Park, H. Koc, W. D. Heo, and K. V. Konan. 2010. Endocytic Rab proteins are required for hepatitis C virus replication complex formation. Virology 398:21–37.

Mejía-Ruiz, C. H., S. Vega-Peña, P. Alvarez-Ruiz, and C. M. Escobedo-Bonilla. 2011. Double-stranded RNA against white spot syndrome virus (WSSV) cp28 or vp26 reduced susceptibility of *Litopenaeus vannamei* to WSSV, and survivors exhibited decreased susceptibility in subsequent re-infections. Journal of Invertebrate Pathology 107:65–68.

Ongvarrasopone, C., M. Chanasakulniyom, K. Sritunyalucksana, and S. Panyim. 2008. Suppression of PmRab7 by dsRNA inhibits WSSV or YHV infection in shrimp. Marine Biotechnology 10:374–381.

Ongvarrasopone, C., E. Chomchay, and S. Panyim. 2010. Antiviral effect of PmRab7 knock-down in inhibition of Leam-Singh virus replication in black tiger shrimp. Antiviral Research 88:116–118.

Ongvarrasopone, C., P. Saejia, M. Chanasakulniyom, and S. Panyim. 2011. Inhibition of Taura syndrome virus replication in *Litopenaeus vannamei* through silencing the LvRab7 gene using double-stranded RNA. Archives of Virology 156:1117–1123.

Peraza-Gómez, V., A. Luna-González, A. I. Campa-Córdova, M. López-Meyer, J. A. Fierro-Coronado, and P. Alvarez-Ruiz. 2009. Probiotic microorganisms and antiviral plants reduce mortality and prevalence of WSSV in shrimp (*Litopenaeus vannamei*) cultured under laboratory conditions. Aquaculture Research 40:1481–1489.

Rahman, M. M., C. M. Escobedo-Bonilla, M. Wille, V. Alday Sanz, L. Audoorn, J. Neyts, M. B. Pensaert, P. Sorgeloos, and H. J. Nauwynck. 2006. Clinical effect of cidofovir and a diet supplemented with *Spirulina platensis* in white spot syndrome virus (WSSV) infected specific pathogen-free *Litopenaeus vannamei* juveniles. Aquaculture 255:600–605.

Reed, L. J. and H. Muench. 1938. A simple method of estimating 50 per cent end-points. The American Journal of Hygiene 27:493–497.

Rijiravanich, A., C. L. Browdy, and B. Withyachumnarnkul. 2008. Knocking down caspase-3 by RNAi reduces mortality in Pacific white shrimp *Penaeus* (*Litopenaeus*) *vannamei* challenged with a low dose of white-spot syndrome virus. Fish & Shellfish Immunology 24:308–313.

Robalino, J., T. Bartlett, E. F. Shepard, S. Prior, G. Jaramillo, E. Scura, R. W. Chapman, P. S. Gross, C. L. Browdy, and G. W. Warr. 2005. Double-stranded RNA induces sequence-specific antiviral silencing in addition to non-specific immunity in marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response? Journal of Virology 79:13561–13571.

Rodman, J. S. and A. Wandinger-Ness. 2000. Rab GTPases coordinate endocytosis. Journal of Cell Science 133:183–192.

van der Schaar, H. M., M. J. Rust, C. Chen, H. van der Ende-Metselaar, J. Wilschut, X. Zhuang, and J. M. Smit. 2008. Dissecting the cell entry pathway of dengue virus by single-particle tracking in living cells. PLoS Pathogens 4:e1000244.

Schuur, A. M. 2003. Evaluation of biosecurity applications for intensive shrimp farming. Aquacultural Engineering 28:3–20.

Sieczkarski, S. B. and G. R. Whittaker. 2002. Dissecting virus entry via endocytosis. Journal of Virology 83:1535–1545.

Sijen, T., F. A. Steiner, K. L. Thijssen, and R. H. A. Plasterk. 2007. Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. Science 315:244–247.

Sritunyalucksana, K., W. Wannapapho, C. F. Lo, and T. W. Flegel. 2006. PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp. Journal of Virology 80:10734–10742.

Stenmark, H. and V. M. Olkkonen. 2001. The Rab GTPase family. Genome Biology 2(5):REVIEWS3007.

Sudhakaran, R., T. Mekata, T. Kono, M. Inada, S. Okugawa, M. Yoshimine, T. Yoshida, M. Sakai, and T. Itami. 2011. Double-stranded RNA-mediated silencing of the white spot syndrome virus VP28 gene in kuruma shrimp, *Marsupenaeus japonicus*. Aquaculture Research 42:1153–1162.

Tirasophon, W., S. Yodmuang, W. Chinnifunvong, N. Plongthongkum, and S. Panyim. 2007. Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA. Antiviral Research 74:150–155.

Vandesompele, J., K. D. Preter, F. Pattyn, B. Poppe, N. V. Roy, A. D. Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3:research0034.

Wassenegger, M. and G. Krczal. 2006. Nomenclature and functions of RNA-directed RNA polymerases. Trends in Plant Science 11:142–151.

Westenberg, M., B. Heinhuis, D. Zuidema, and J. M. Vlak. 2005. siRNA injection induces sequence-independent protection in *Penaeus monodon* against white spot syndrome virus. Virus Research 114:133–139.

Witteveldt, J., J. M. Vlak, and M. C. van Hulten. 2004. Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. Fish & Shellfish Immunology 16:571–579.

Wu, Y., L. Lü, L. S. Yang, S. P. Weng, S. M. Chan, and J. G. He. 2007. Inhibition of white spot syndrome virus in *Litopenaeus vannamei* shrimp by sequence-specific siRNA. Aquaculture 271:21–30.

Xu, J., F. Han, and X. Zhang. 2007. Silencing shrimp white spot syndrome virus (WSSV) genes by siRNA. Antiviral Research 73:126–131.