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Determining the Dosage and Time of Injection for WSSV VP28 Double Stranded RNA to *Penaeus indicus* in Providing Effective Protection Against WSSV

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ABSTRACT

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White spot syndrome virus of shrimp is best known for its wide distribution and high virulence in bringing mass mortality and huge economic loss to farmers. Though the virus has been causing wide spread mortality to all the cultured penaeid shrimps for quite a long time, no effective treatment strategies have been identified so far. RNAi has been recognized as a powerful tool and found to be functional in shrimp. An attempt was made to verify the possibility of using this technology in laboratory-based experiment through double stranded RNA (dsRNA) construction and injection to Indian white leg shrimp, *Penaeus indicus*. Amongst the different dosages tried, 1 µg/gm of shrimp administered 24-48 hours before WSSV infection was found to be effective in providing full protection. While trying to further refine the time interval by injecting the shrimp with 1 µg of VP28 dsRNA/gm of shrimp at 3, 6, 12 and 18 hours before WSSV infection, no protection was observed in any of these time intervals indicating that administration of dsRNA 24 hours before infection to be the most suitable. Real time PCR analysis further indicated that only the shrimps injected with dsRNA 24 hours prior to infection were virus free, and the others were with high copy numbers of WSSV. This experiment indicates that dsRNA can be used as a prophylaxis to further prevent the infection by WSSV.

ADDITIONAL INDEX WORDS: *dsRNA, protection, real time PCR, shrimp, WSSV.*

INTRODUCTION

Shrimp aquaculture has evolved as a major food industry and serves as a good household income for the major coastal population of the world. About 17% of seafood with respect to value comes from the shrimp industry. However, its production has been severely affected by varying aquatic pathogens (Jiravanichpaisal *et al*., 1994; Lightner *et al*., 1997). White Spot Syndrome Virus (WSSV) remains one of the most devastating pathogens of all time because of its virulence, and it can cause mass mortality in 3 to 10 days post-infection (Lightner, 1996).

WSSV is a 300 kb double stranded DNA virus of family Nimaviridae, and its host range is very broader in crustacean species like crayfish, crab and shrimp (Wang *et al*., 1998). Infection in shrimp first starts from the stomach, gill and rapidly spreading to mesodermal and ectodermal tissues (Chang *et al*., 1996). The virus consists of structural (VP28, VP26, VP19, VP24) and non-structural (RR1, ie1, tk) proteins, of these the structural proteins are responsible for establishing contact with host cells. VP28 and VP19 are present on the viral envelope, whereas VP26, VP24 and VP15 are present on nucleocapsid (van Hulten *et al*., 2000).

There's no potential vaccine candidate available yet against WSSV as the success of vaccination involves the presence of specific effector and memory cells in shrimp. Unfortunately, being an invertebrate, shrimps lack humoral immune response system and thus believed that it cannot be vaccinated (Thomas *et al.*, 2014). RNAi has been recognized as a powerful tool ever since its discovery in Caenorhabditis elegans (Fire *et al*., 1998) and shrimps do have a functional RNAi pathway (Hirono *et al*., 2011).

Numerous studies have used RNAi to control WSSV infection in shrimp, and most of them were targeted towards virion genes encoding for structural proteins (Kim *et al*., 2007; Robalino *et al*., 2004, 2005; Tirasophon, Roshorm, and Panyim, 2005; Yodmuang *et al*., 2006). VP28 and VP26 are responsible for virus trafficking into the host cell (Tang *et al*., 2007; van Hulten *et al*., 2001a; Youtong *et al*., 2011; Zhang *et al*., 2002). Reports also show RNAi towards these structural proteins like VP28 are effective in controlling the WSSV infection or multiplication (Kim *et al*., 2007; Nilsen *et al*., 2017; Robalino *et al*., 2005; Sanjuktha *et al*., 2012; Sarathi *et al*., 2008; Sudhakaran *et al*., 2011; Westenberg *et al*., 2005; Xu, Han, and Zhang, 2007).

To date, in all RNAi oriented protection studies against WSSV, dsRNA or siRNA specific to VP28 was administered either 24 hours or 48 hours prior to WSSV challenge. Time course between the activation of dsRNA or initiation of WSSV

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replication also plays an important role in the effective functioning of RNAi. This study was attempted to understand the dosage of VP28 specific dsRNA required to protect shrimps from WSSV under different time points of dsRNA injection prior to WSSV challenge.

METHODS

In this section, dsRNA design, *in vitro* synthesis followed by WSSV challenge experiments is explained in detail.

Experimental Animals

Healthy *P. indicus* weighing ~8 g in size were procured and acclimatized in 500 litre FRP tank containing sea water of salinity 30 ppt. Shrimps were screened for WSSV infection by PCR.

Preparation of WSSV Virion

WSSV infected shrimp collected from culture ponds were homogenized in TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4) to prepare a 10% (W/V) suspension. The homogenate was clarified by centrifuging at 3000 x g for 5 min, and the supernatant was filtered using 0.45 µm syringe filter. The filtrate containing the WSSV was stored at -80°C until further use.

dsRNA Design and Synthesis

A siRNA specific region from VP28 gene (Gene specific) and GFP (Control) was determined *in silico* using RNAz server from University of Vienna RNA Webservices (http://rna.tbi.univie.ac.at/cgi-bin/RNAxs/RNAxs.cgi). Primers were designed in such a way that after PCR, amplified fragments contain this siRNA sequence within the recognition site for ECoRI and Bam HI (Table 1). Amplified VP28/GFP fragment and LITMUS38i vector (New England Biolabs) was restriction digested with ECoRI/BamHI and ligated to form VP28 – LIT38i and GFP – LIT38i. The ligated plasmid was transformed to *E. coli* DH5 alpha competent cells and positive recombinant clones were selected by sequencing.

This VP28-LIT38i and GFP-LIT38i plasmid was used as a template for *in vitro* dsRNA synthesis using HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) as per manufacture's instruction. The dsRNA synthesized were quantified and stored in -80°C.

Experimental Design and WSSV Challenge

Initially, an experiment was conducted to know the dosage of dsRNA required to neutralize WSSV virions. 0.25, 0.5, 1 and 2 µg of dsRNA per gram body weight of shrimp was injected into the third abdominal segment, and after 24 hours, the shrimp were challenged with WSSV. In the second experiment, 6 groups of shrimps with 10 animals in duplicates were taken (Totally 12 groups) and named as 3 h, 6 h, 12 h, 18h, 24 h and control GFP group.

To each group, 1 µg VP28-dsRNA per gram body weight of shrimp was injected and to control group GFP-dsRNA was injected. After the specified time for each group, 10^5 copies of WSSV virion was injected intramuscularly and monitored daily for 10 days post challenge. Dead and moribund animals were collected, DNA extracted as per Otta, Karunasagar, and Karunasagar (2003).

Quantitative Real Time PCR

Quantified DNA was used to amplify WSSV using specific TaqMan Primer and Probe. The amplification conditions used is: a preliminary holding step of 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s and isothermal annealing and extension at 60°C for 60s. Conventional PCR was also performed prior to qPCR following Kimura *et al*. (1996) to know the presence of WSSV.

RESULTS

The primary results of the study are explained below.

dsRNA Synthesis

Both vp28 – dsRNA and GFP – dsRNA construct was cloned onto LITMUS 38i vector, which has siRNA sequence specific regulatory region. This plasmid construct was used as template for *in vitro* dsRNA synthesis. The synthesized dsRNA was subjected to RNase treatment as to check its quality; both VP28-dsRNA (Figure 1) and GFP-dsRNA were not cleaved by RNase H and RNase III denoting their true double stranded nature when compared to RNase A and RNase I treatment.

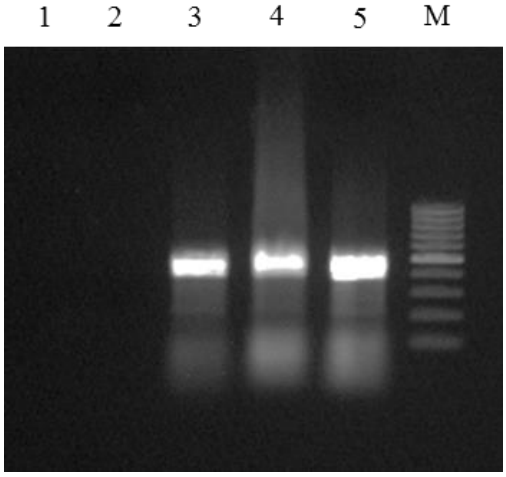

Figure 1. RNase treatment of synthesized VP28-dsRNA: Lane 1–RNase A treated, Lane 2– RNase I treated, Lane 3– RNase H treated, Lane 4–RNase III treated, Lane 5– Untreated, Lane M– 100 bp ladder.

dsRNA Dosage Determination

The amount of VP28 – dsRNA required for suppression of VP28 gene expression was determined by administering varying amounts of VP28 – dsRNA 24 h prior to WSSV challenge. Of the different doses of VP28 – dsRNA tried, 1 and 2 µg per gram of body weight of shrimp almost displayed the same level of gene silencing, whereas 0.25 and 0.5 µg failed to silence the VP28 mRNA (data not shown).

Table 1. *List of primers used in this study*.

Primer	Sequence	Amplicon size (bp)
VP28-dsR F	AAGGCGGATCCCAGACAATATCGAGACAAA	429
VP28-dsR R	AATTGGAATTCTAGCTGCAATTGGTACGC	
GFP-dsR F	AAGGCGGATCCAGGACGACGGCAACTACAAG	401
GFP-dsR R	AATTGGAATTCGTCCATGCCGAGAGTGATCC	
qWSV F	CCGACGCCAAGGGAACT	72
qWSV R	TTCAGATTCGTTACCGTTTCCA	
qWSV Probe	6-FAM – 5'-CGCTTCAGCCATGCCAGCCG-3' – 6-TAMRA	

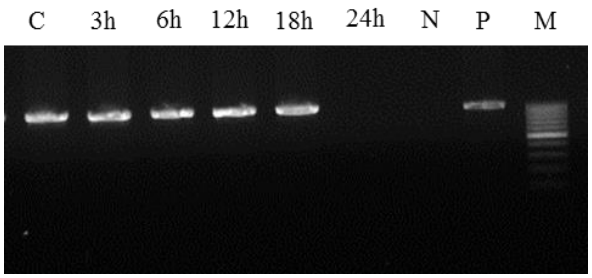

Figure 2. WSSV 1st Step PCR for VP28-dsRNA injected shrimp challenged with WSSV: Lane C – GFP Control group, Lane 3h, 6h, 12h, 18h, 24h – corresponds to respective time hours; N – Negative control, P – Positive control; Lane M – 100bp marker.

Time Point of dsRNA Administration

To know the minimum hours required for dsRNA to be functional before the WSSV takes over the host into its control, 5 different time points of VP28 - dsRNA administration were tried at 3, 6, 12, 18 and 24 h prior to WSSV challenge with non-specific GFP-dsRNA as control. DNA extracted from moribund and dead shrimps were first analyzed through conventional PCR to know the presence of WSSV infection. Animals in 3, 6, 12, 18 h and control group were WSSV positive (~980 bp) in 1st step (Figure 2), whereas 24 h group was negative for both direct and nested PCR (~560 bp) (Figure 3). Copy number detection using qPCR showed 6.84 x 10^9 copies for control GFP group, 6.93 x 10^9 copies for 3 hour group, 6.379 x 10^9 copies for 6 hour group, 6.820 x 10^9 copies for 12 hour group, 5.976 x 10^9 copies for 18 hour group and Zero copies (Undetermined) for 24 hour group respectively (Figure 4).

DISCUSSION

RNA interference mechanism is one of the promising tools to exploit the genes involved in cellular pathways by effectively knocking them down. So many molecular mechanisms have been uncovered using this tool in carcinogenesis pathway like multiple DNA repair pathway (Cortez *et al*., 2001), angiogenesis inhibitory pathway (Liu *et al*., 2003) and antiapoptotic factors (Crnkovic-Mertens, Hoppe-Seyler and Butz, 2003; Schmitt, 2003).

Even novel crustaceans genes such as metabolism related in *Litopenaeus vannamei* (Sonanez-Organis, Racotta, and Yepiz-Plascencia, 2010) and growth and reproduction-related in *P. monodon* were analysed by RNAi (De Santis *et al*., 2011; Treerattrakool, Panyim, and Udomkit, 2011). In this experiment minimum dosage of dsRNA required for effective silencing of VP28 gene was found to be 1 µg per gram body weight of shrimp which is less when compared to previous studies as 4 µg per gram body weight of shrimp (Escobedo-Bonilla, Vega-Pen, and Mejiala-Ruiz, 2015; Mejia-Ruiz *et al*., 2011) and 2.5 µg per gram body weight of shrimp (Sudhakaran *et al*., 2011).

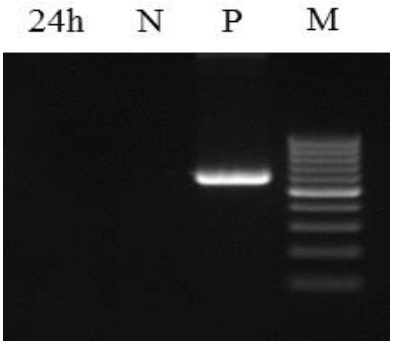

Figure 3. WSSV Nested PCR for VP28-dsRNA injected shrimp challenged with WSSV: 24h-24 hour group, N – Negative control, P – Positive control; Lane M – 100bp marker.

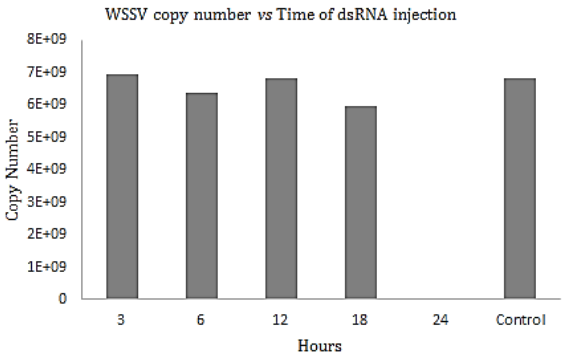

Figure 4. Quantitative Real Time PCR: Graph showing WSSV copy numbers obtained in shrimps challenged with WSSV at different time points post dsRNA injection.

The time course of dsRNA administration prior to WSSV challenge was also studied in the present work, starting with the minimum time point of 3, 6, 12, 18 and 24 hours prior to WSSV challenge. Out of these time points dsRNA administered 24 h prior to WSSV challenge was found to be the most effective in

protecting shrimps against WSSV (No virion detected) throughout the experiment (10 days). On the other side 3, 6, 12 and 18 h time point failed to give protection/silencing against WSSV (data not shown), and virion copy number were almost similar to control GFP-dsRNA treated group; which also denotes RNAi is sequence specific. Similar results were obtained when dsRNA was injected 24 h or 48 h prior to challenge (Kim *et al*., 2007; Nilsen *et al*., 2017; Robalino *et al*. 2005; Sanjuktha, *et al.*, 2012; Sarathi *et al*., 2008; Sudhakaran *et al*., 2011; Westenberg *et al*., 2005; Xu, Han, and Zhang, 2007). Shrimps do have enzymes required for RNAi machinery, especially Argonaute and Dicer protein components of the RNAi pathway are available in shrimp (Phetrungnapha *et al*., 2013 and Su *et al*., 2008). It is desirable to know whether much earlier treatment of dsRNA prior to WSSV infection can enhance the survivability of shrimps during WSSV infection. Our results suggest that a minimum time of 24 h is required for dsRNA, which is perhaps to process and convert the dsRNA into functional siRNA population to neutralize WSSV.

CONCLUSION

As evident from qPCR, no WSSV virion was detected when VP28-dsRNA was injected 24hours prior to WSSV challenge. Additionally, 24hour group animals continued to survive for 10 days even after WSSV challenge. On the other hand, 18, 12, 6 and 3 hour group animal attained mortality in 72 to 96 hours post WSSV challenge. Control non-specific GFP-dsRNA injected shrimps died in 72 hours of post-challenge, which denotes VP28 dsRNA specifically inhibits Viral VP28 mRNA and thus prohibit new viral infection and reduce the further infection. However, dsRNA injected either early hours (less than 24 hours) prior to challenge or after any time point post WSSV challenge did not show any effective protection. Thus this study indicates that dsRNA mediated WSSV control can be used only as a prophylactic in controlling the WSD. This also concludes that a minimum of 1 µg of dsRNA is required which need to be administered 24hrs before WSSV challenge or infection.

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LITERATURE CITED

Chang, P.; Lo, C.; Wang, Y., and Kou, G., 1996. Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by in situ hybridization. *Diseases of Aquatic Organisms*, 27, 131-139.

Cortez, D.; Guntuku, S.; Qin, J., and Elledge, S.J., 2001. ATR and ATRIP: partners in checkpoint signalling. *Science*, 294, 1713-1716.

Crnkovic-Mertens, I.; Hoppe-Seyler, F., and Butz, K., 2003. Induction of apoptosis in tumor cells by siRNA-mediated silencing of the livin/ML-IAP/KIAP gene. *Oncogene*, 22, 8330-8336.

De Santis, C.; Wade, N.M.; Jerry, D.R.; Preston, N.P.; Glencross, B.D.; Sellars, M.J., 2011. Growing backwards: An inverted role for the shrimp ortholog of vertebrate myostatin and GDF11. *Journal of Experimental Biology*, 214, 2671-2677.

Escobedo-Bonilla, C.M.; Vega-Pen, S., and Mejiala-Ruiz, C.H., 2015. Efficacy of double-stranded RNA against white spot syndrome virus (WSSV) non-structural (orf89, wsv191) and structural (vp28, vp26) genes in the Pacific white shrimp *Litopenaeus vannamei*, *Journal of King Saud University - Science*, 27, 182-188.

Fire, A.; Xu, S.Q.; Montgomery, M.K.; Kostas, S.A.; Driver, S.E., and Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391, 806-810.

Hirono, I.; Fagutao, F.; Kondo, H., and Aoki, T., 2011. Uncovering the mechanisms of shrimp innate immune response by RNA interference. *Marine Biotechnology*, 13, 622-628.

Jiravanichpaisal, P.; Huang, X.D,; Zhao, L.; Zhang, H.Q,; Xu, X.P.; Jia, X.T.; Chen, Y.H.; Wang, P.H.; Miyazaki, T., and Limsuawan, C., 1994. Histopathology, biochemistry and pathogenicity of *Vibrio harveyi* infecting black tiger *Penaeus monodon*. *Journal of Aquatic Animal Health*, 61:27-35.

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

Kimura, T.; Yamano, K.; Nakano, H.; Momoyama, K.; Hiraoka, M., and Inouye, K., 1996. Detection of penaeid rod-shaped DNA virus (PRDV) by PCR. *Fish Pathology*, 31, 93-98.

Lightner, D.V., 1996. *A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp*. Baton Rouge, LA: World Aquaculture Society, 256 p.

Lightner, D.V.; Redman, R.M.; Poulos, B.T.; Nunan, L.M.; Mari, J.L., and Hasson, K.W., 1997. Risk of spread of penaeid shrimp viruses in the Americas by international movement of live shrimp for aquaculture and frozen shrimp for commodity markets. *Scientific and Technical Review of the Office International des Epizooties*, 16,146-160.

Liu, L.T.; Chang, H.C.; Chiang, L.C., and Hung, W.C., 2003. Histone deacetylase inhibitor up-regulates RECK to inhibit MMP-2 activation and cancer cell invasion. *Cancer Research*, 63, 3069-3072.

Mejia-Ruiz, C.H.; Vega-Pena, S.; Alvarez-Ruiz, P., and Escobedo-Bonilla, C.M., 2011. Double-stranded RNA against white spot syndrome virus (WSSV) vp28 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.

Nilsen, P.; Karlsen, M.; Sritunyalucksana, K., and Thitamadee, S., 2017. White spot syndrome virus VP28 specific double-stranded RNA provides protection through a highly focused siRNA population. *Scientific Reports*, 7: 1028.

Otta, S.K.; Karunasagar, I., and Karunasagar, I., 2003. Detection of monodon baculovirus and white spot syndrome virus in

apparently healthy *Penaeus monodon* postlarvae from India by polymerase chain reaction. *Aquaculture*, 220, 59-67.

Phetrungnapha, A.; Ho, T.; Udomkit, A.; Panyim, S., and Ongvarrasopone, C., 2013. Molecular cloning and functional characterization of Argonaute-3 gene from *Penaeus monodon. Fish and Shellfish Immunology*, 35, 874 – 882.

Robalino, J.; Bartlett, T.; Shepard, E.F.; Prior, S.; Jaramillo, G.; Scura, E.; Chapman, R.W.; Gross, P.S.; Browdy, C.L., and Warr, G.W., 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.

Robalino, J.; Browdy, C.L.; Prior, S.; Metz, A.; Parnell, P.; Gross, P., and Warr, G., 2004. Induction of antiviral immunity of double stranded RNA in a marine invertebrate. *Journal of Virology*, 78, 10442-10448.

Sanjuktha, M.; Stalin Raj, V.; Aravindan, K.; Alavandi, S.V.; Poornima, M., and Santiago, T.C., 2012. Comparative efficacy of double-stranded RNAs targeting WSSV structural and nonstructural genes in controlling viral multiplication in *Penaeus monodon*. *Archives of Virology*, 157, 993-998.

Sarathi, M.; Simon, M.C.; Venkatesan, C., and Sahul Hameed, A.S., 2008. Oral administration of bacterially expressed VP28dsRNA to protect *Penaeus monodon* from White Spot Syndrome Virus. *Marine Biotechnol*ogy, 10, 242-249.

Schmitt, C.A., 2003. Senescence, apoptosis and therapy cutting the lifelines of cancer. *Nature Reviews Cancer*, 3, 286-295.

Sonanez-Organis, J.G.; Racotta, I.S., and Yepiz-Plascencia, G., 2010. Silencing of the hypoxia inducible factor 1 -HIF-1- obliterates the effects of hypoxia on glucose and lactate concentrations in a tissue-specific manner in the shrimp *Litopenaeus vannamei*. *Journal of Experimental Marine Biology and Ecology*, 393, 51-58.

Sudhakaran, R.; Mekata, T.; Kono1, T.; Inada, M.; Okugawa, S.; Yoshimine, M.; Yoshida, T.; Sakai, M., and Itami. T., 2011, Double-stranded RNA-mediated silencing of the white spot syndrome virus VP28 gene in kuruma shrimp, *Marsupenaeus japonicus*. *Aquaculture Research*, 42, 1153-1162.

Su, J.; Dang, T.H.; Oanh, a.; Russell ,E.; Lyons, A.; Leeton, L.; Marielle, C.W. ; van Hulten, A.; Siok-Hwee Tan,; Linsheng Song.; Rajendran, K.V., and Walker, P.T., 2008. A key gene of the RNA interference pathway in the black tiger shrimp, *Penaeus monodon*: Identification and functional characterisation of Dicer-1 *Fish and Shellfish Immunology*, 24, 223-233.

Tang, X.; Wu, J.; Sivaraman, J., and Hew, C.L., 2007. Crystal structures of major envelope proteins vp26 and vp28 from white spot syndrome virus shed light on their evolutionary relationship. *Journal of Virology*, 81, 6709-6717.

Thomas, A.; Sudheer, N.S.; Viswanathan, K.; Kiron, V.; Bright Singh, I.S., and Narayanan, R.B., 2014. Immunogenicity and protective efficacy of a major White Spot Syndrome Virus (WSSV) envelope protein VP24 expressed in *Escherichia coli* against WSSV. *Journal of Invertebrate Pathology*, 123, 17-24.

Tirasophon, W.; Roshorm, Y., and Panyim, S., 2005. Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. *Biochemical and Biophysical Research Communication*, 334, 102-107.

Treerattrakool, S.; Panyim, S., and Udomkit, A., 2011. Induction of ovarian maturation and spawning in *Penaeus monodon* broodstock by double-stranded RNA. *Marine Biotechnology*, 13, 163-169.

van Hulten, M.C.W.; Westenberg, M.; Goodall, S.D., and Vlak, J.M. 2000. Identification of two major virion protein genes of white spot syndrome virus of shrimp. *Virology*, 266, 227-236.

van Hulten, M.C.W.; Witteveldt, J.; Peters, S.; Kloosterboer, N.; Tarchini, R.; Fiers, M.; Sandbrink, H.; Klein-Langhorst, R., and Vlak, J.M., 2001. The white spot syndrome virus DNA genome sequence. *Virology*, 286, 7-22.

Wang, Y.C.; Lo, C.F.; Chang, P.S., and Kou, G.H., 1998. Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. *Aquaculture*, 164, 221-231.

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

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

Yodmuang, S.; Tirasophon, W.; Roshorm, Y.; Chinnirunvong, W., and Panyim, S., 2006. YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. *Biochemical and Biophysical Research Communication*, 341, 351-356.

Youtong, W.; Deachamag, P.; Phongdara, A., and Chotigeat, W., 2011. WSSV: vp26 binding protein and its biological activity. *Fish and Shellfish Immunology*, 30, 77-83.

Zhang, X.; Huang, C.; Xu, X., and Hew, C.L., 2002. Transcription and identification of an envelope protein gene (p22) from shrimp white spot syndrome virus. *Journal of General Virology*, 83, 471-477.