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Comparative Efficacy of dsRNA VP24, VP26, RR1 and WSV477 Gene against WSSV Infection in *Penaeus monodon*

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A B S T R A C T

White spot syndrome virus (WSSV) is considered as dangerous shrimp pathogen which causes severe economic loss to the shrimp production. Unavailability of protective and therapeutic measures is a serious constraint in aquaculture and till date, there is no effective preventive measure to treat WSSV. In this context, dsRNA was synthesised and used to prevent the replication of WSSV. RNA interference (RNAi) is a promising tool to fight against viral infections. Thus the application of RNAi technology was evaluated by examining the efficacy of virus-specific dsRNA (VP24, VP26, RR1 and *wsv*477) to inhibit WSSV replication in shrimp, *Penaeus monodon*. The efficacy of the synthesised virus-specific dsRNA for inhibition of WSSV replication was done by injection and oral delivery method. Shrimps injected with *wsv*477, RR1, VP26 and VP24 dsRNA provided survival rates of 90%, 80%, 60% and 50% respectively. Shrimps fed with WSV477, RR1, VP26 and VP24 dsRNA showed mortality rates of 80%, 70%, 50%, and 50% respectively. Among all the tested genes WSV477 showed higher potency against WSSV infection. Overall results of these studies clearly indicate the success of RNAi strategy which mainly depends on the functionality of the targeted gene.

Introduction

Shrimp aquaculture is a multi-billion dollar income generating sector, contributing to the major economic growth of several countries across the world. The expansion in shrimp culture practices has also led to significant economic losses worldwide due to the viral disease outbreak. White spot disease (WSD) is caused by dsDNA virus known as the white spot syndrome virus (WSSV), considered as one of the deadly shrimp viral pathogens reported to bring about 100% mortality immediately after infection. This virus infects a wide range of aquatic crustaceans (Sanchez-Paz 2010). WSSV is a rod-shaped, non-occluded, enveloped virus having tail-like appendage at one end of the virion.

As of now genome of the four WSSV isolates like Thailand 293 kbp [WSSV-TH; Acc. No. AF369029] (van Hulten *et al.*, 2000a; 2001a), Taiwan 307 kbp [WSSV-TW; Acc. No. AF440570] (Tsai *et al.*, 2000a, b), China 305 kbp (WSSV-CN; Acc. No. AF332093) (Yang *et al.*, 2001) and Korean 295 kbp (WSSV-

KW; Acc. No. JX515788) has been reported to be fully sequenced.

As shrimps do not possess adaptive immune system they rely solely on cellular and humoral innate immune mechanisms to fight against the invading pathogen. Hence understanding the immune system of shrimps in much detailed manner is the need of the hour to establish greater and efficient methods to control the infectious diseases and also to ensure the long-term viability of the shrimp aquaculture practices. To overcome the impacts of WSSV various therapeutic approaches are followed, that includes manipulating environmental conditions, administrating various immune modulators (Chang *et al.,* 2003; Balasubramanian *et al.,* 2007), vaccination (Huang *et al.,* 2005; Jha *et al.,* 2006), and RNA interference (RNAi) strategies (Robalino *et al.,* 2004; Xu *et al.,* 2007). RNAi, an evolutionary conserved natural mechanism that was initially studied in the nematode, *Caenorhabditis elegans* (Fire *et al*.1998). RNAi is a cellular process where a sequence-specificdouble-stranded RNA (dsRNA) molecule interferes with the target gene regulates the gene expression through sequence-specific silencing of target mRNA, chromatin modification, and translation repression (Robalino *et al.,* 2005). RNAi is initiated by a dsRNA that is cleaved by RNase III enzyme (Dicer) into small interfering RNA (siRNA) of 21-23 nucleotide length that targets complementary mRNAs for degradation by an endonuclease containing RNA-induced silencing complex (RISC) (Meister and Tuschi, 2004).

Protection studies targeting novel genes that code for viral envelope and coat proteins are ideal due to their basic interactions inside host during the pathogen entry and proliferation (Chazal and Gerlier, 2003). Protective efficacies of various dsRNA targeting structural and nonstructural protein coding genes against WSSV infection is evidenced by several studies (Kim *et al.,* 2007; Robalino *et al.,* 2004, 2005;Tirasophon *et al.,* 2005; Yodmuang *et al.,* 2006). Structural gene VP26 is one among the major component of the virus envelope (Zhang *et al.,* 2002; Tang *et al.,* 2007) involved in thecytoplasmic transport of the nucleocapsid by interacting with actin or cellular actin-binding proteins (Xie and Yang 2005). VP24 is a nucleocapsid protein of WSSV that interacts with VP28 during viral infection. RR1 non-structural protein is a large subunit of Ribonucleotide reductase enzyme that aids in nucleotide metabolism and converts ribonucleotides into deoxyribonucleotides which serve as immediate precursors of DNA (Jordan and Reichard 1998). Non-structural gene, WSV477 encodes for 208 amino acid and these peptides have GTP-binding activity (Han *et al.,* 2007). Antisense construct on WSV477 offered potent and significant antiviral immunity against WSSV infection in comparison to other targeted envelope protein in *Litopenaeus vannaemi* (Akhila *et al.,* 2015). The present study aimed at comparing the potentiality of dsRNA coding structural (VP26, VP24) and non-structural (RR1, WSV477) genes of WSSV and their possible effects against WSSV infection in Indian black tiger shrimp (*Penaeus monodon*).

Materials and Methods

Healthy Shrimps *P. monodon*, weighing 7-10 g were harvested from shrimp culture farm near Kumta region, Karnataka. The shrimp were acclimatised in 1000 litres capacity tanks containing filtered seawater having salinity between 20-25 parts per thousand (ppt) with constant aeration at a temperature of 28-30^{0}C. The commercial pelleted feed was fed to the shrimps. Salinity, temperature and dissolved oxygen (DO) were monitored regularly. The animals were screened for DNA and RNA viruses of shrimp by

polymerase chain reaction (PCR) (Otta *et al.*, 2003) using virus-specific primers.

Preparation of viral inoculum

Shrimps infected with WSSV having characteristic white spots were collected from the shrimp farm. The virus was isolated from gill of the WSSV infected animals using reaction protocol (Otta *et al.*, 2003) with minor modification. The isolated virus was passed through a 0.45µm membrane filter and stored at -80^{0}C until future use. Virus copy number in the stock was calculated by performing the standard curve experiments using quantitative real-time PCR (data not shown). The viral inoculum is expressed as copies per microliter.

Synthesis of dsRNA molecule

In this study, bacterial expression system was used for the production of dsRNA *in vivo*. The structural and non-structural genes of WSSV (VP26, VP24, RR1 and WSV477) were amplified using the specific primers (Table 1). PCR amplifications was done in a thermocycler (Bio-Rad, Germany) with an initial denaturation of 95^{0}C for 5min followed by 30 cycles of 30 sec denaturation at 95^{0}C, annealing at 55^{0}C and extension at 72^{0}C for 30 sec, with a final extension step of 10 min at 72^{0}C. The obtained PCR products were purified using the purification kit (Roche life science, USA) and then cloned into the pLITMUS38i vector (NEB) which is flanked by two T7 opposing promoters. The recombinant plasmids were transformed into an RNase III-deficient chemically competent *Escherichia coli* HT115 (DE3) for the production of dsRNA. The transformed colonies were screened by PCR using gene specific primers (Fig.1-4). For a starter culture, a single transformed clone was inoculated into 5 ml of LB broth (HiMedia, Mumbai) containing 100µg/mL ampicillin and 12.5µg/mL tetracycline. For the bulk production of dsRNA, 1000µl of pre-culture were transferred to 100mL of LB broth containing 100µg/mL ampicillin and 12.5µg/mL tetracycline. The culture was incubated at 37^{0}C at 250 rpm until the OD_{600} nm of 0.4–0.5 was obtained and cultures were induced with 0.4mM isopropyl-β-D-thiogalactopyranoside (IPTG). Following induction for 4hr, bacterial culture was harvested and extracted for dsRNA using TRIzol method according to the manufacturer's instructions. Purity and concentration of the extracted dsRNA were determined by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). The integrity of dsRNA was confirmed by digesting with RNase A (HiMedia, Mumbai) and RNase III (New England Biolabs, USA) enzymes (Fig. 5).

In vivo RNAi assay in shrimp

Protective efficacy of bacterially expressed dsRNA was determined by oral feeding and intramuscular injection to shrimp.

Preparation of dsRNA feed

Recombinant *E. coli* HT115 (DE3) containing different genes of WSSV were over expressed by using IPTG and inactivated in 0.5% formaldehyde for 15 min by incubating at 20^{0}C, checked for inactivation levels and stored at 4^{0}C until further use. Following inactivation, the bacterial cells were centrifuged, washed and suspended in phosphate buffer saline (PBS) and approximately 10^8 cells of the inactivated bacteria were mixed with 0.02g of commercially available shrimp feeds (Charoen Pokphand, India Pvt Ltd), incubated on ice for 15 min and coated with cod liver oil to prevent the dispersion of bacteria in the water. The prepared feed was kept at 4^{0}C until use.

Oral delivery

Shrimps (*P. monodon*) were divided into 8 groups of 10 animals each in triplicates and maintained in fibreglass troughs filled with filtered sea water at ambient temperature. Animals were fed with pelleted feed at 3.5 % of body weight for 15 days as indicated in table 2. The feed coated with *E. coli*, HT115 (DE3) cells without vector served as host control. After 15 days, the shrimps were challenged by injecting $6x10^4$copies/µl of WSSV, except for the negative control that was injected with PBS and fed by normal feed until completion of the experiment. The quality of water was monitored by changing the water daily. The experiment was performed up to 30 days post-challenge with WSSV.

Delivery of dsRNA and WSSV challenge

Experimental shrimps were divided into 7 groups in triplicates (experimental layout is given in table 3). Shrimps of these groups received 50 µl bacterially expressed dsRNA (2.5 µg/g of shrimp) intramuscularly. Following 24h of dsRNA injection, the shrimps were challenged with 10^{-2} dilution of WSSV. At 96h of the first dose, shrimps were re-injected with abooster dose ofthesame concentration of dsRNA at the 3rd abdominal segment of the shrimp. The positive control shrimps received PBS injection. The dsRNA from a pLITMUS38i vector without any insert served as control dsRNA experiment.

Results and Discussion

Effect of dsRNA

Oral feeding

To evaluate the ability of dsRNAs produced by *E. coli* HT115 in conferring protection against WSSV, shrimps were treated with bacterially expressed dsRNA (VP24, VP26, RR1 and WSV477) independently. The cumulative mortality of orally fed animals is shown in figure 6 where, shrimps fed with VP26 dsRNA, VP24 dsRNA, RR1 dsRNA and WSV477 dsRNA showed cumulative mortality of 50%, 50%, 30% and 20%, respectively. Whereas, positive control pLITMUS38i with *E. coli* HT115 and *E. coli* HT115 group of shrimps showed 100% mortality on the 10th day of post viral infection. Negative control groups showed 100% survival till the end of the experiment. Shrimp fed with WSV477 dsRNA showed survival till 45th-day post viral infection.

Injection

Intramuscular injection of dsRNA at 2.5 µg/g body weight to the shrimp was found to afford the highest protection against WSSV in *P. monodon*, and the protective efficacy of the dsRNA showed varied results with the gene that was used for silencing. Shrimps treated with VP24, VP26, RR1 and *wsv*477 dsRNA showed a survival of 50%, 60%, 80% and 90% respectively at 15 days post viral challenge (Fig. 7). Positive control group shrimps showed 100% mortality at 10th-day post viral infection. Throughout the experimental course, no mortality was found in negative control group.

The present study aimed at documenting the protective efficacy of dsRNA targeting structural and non-structural genes of WSSV by oral and injection mode of delivery. Injection of dsRNA/ siRNA or hnRNA targetted on the specific genes was able to reduce the severity of WSD. Previous works on shrimps treated with sequence specific dsRNA that was able to provide longer days of survival 15 days post infection (dpi) in contrast to the shrimp injected with non-specific dsRNA (Robalino *et al.,* 2007; Sarathi *et al.,* 2010) . Structural protein plays a vital role in the viral attachment, infection and also plays a critical role in activating host

defence of shrimp. *wsv*477 is an early gene of WSSV containing ATP/GTP-binding with GTP-binding activity which helps in regulating the expression of late genes (Sanchez-Paz 2010). Ponprateep *et al.,* (2013) validated the interaction of *wsv*477 protein with SPIPm2, where SPIPm2 hindered the functional property of *wsv*477 which was also proved by RNAi studies. Shrimp treated with *wsv*477dsRNA offered higher protection compared to other targeted gene and this endorses with earlier work where antisense targeted on this gene provided 90% survival against WSSV infection (Akhila *et al.,* 2015).

Table.1 PCR primers designed in this study

Primers	Sequence (5'-3')	Restriction enzyme
VP26F	CGC*GGATCC*ATGGAATTTGGCAACCTAACAAACCT	BamHI
VP26R	CGC*AAGCTT*TTACTTCTTCTTGATTTCGTCC	HindIII
VP24F	CGC*GGATCC*ATGCACATGTGGGGGGTTTACG	BamHI
VP24R	CGC*AAGCTT*TTATTTTTCCCCAACCTTAAAC	HindIII
RR1F	CGC*GGATCC*CGGACTGAGGACGCTAGAAT	BamHI
RR1R	CGC*AAGCTT*CCCTCGTCCTCAAATCTTCA	HindIII
WSV477F	CGC*GGATCC*ATGTATATCTTCGTCGAAGG	BamHI
WSV477R	CGC*AAGCTT*TTATAAGAAATGTACAATCCTATGCC	HindIII

Table.2 Representing the grouping of shrimps for oral delivery

Group	**Feed composition**	**No. of shrimps / group**
VP26	Feed + VP26dsRNA + PBS + cod liver oil	10 X 3
VP24	Feed + VP24dsRNA + PBS + cod liver oil	10 X 3
RR1	Feed + RR1dsRNA + PBS + cod liver oil	10 X 3
*wsv*477	Feed + *wsv*477dsrna + PBS + cod liver oil	10 X 3
(*E. coli* HT115)	Feed + *E. coli* HT115 + PBS + cod liver oil	10 X 3
(pLITMUS38i)	Feed + pLITMUS38i + PBS + cod liver oil	10 X 3
Positive control	Feed + PBS + cod liver oil	10 X 3
Negative control	Feed + PBS + cod liver oil	10 X 3

Table.3 Grouping of shrimps based on various dsRNA for injection delivery

Group	**No. of shrimps/ Group**
VP26dsRNA	10×3
VP24dsRNA	10×3
RR1dsRNA	10×3
*wsv*477dsRNA	10×3
Vector control	10×3
Positive control	10×3
Negative control	10×3

Fig.1 PCR amplification of VP26 gene clones by gene specific primers. Lane M: 100 bp molecular weight marker; Lane 1: Positive control; Lane 2-4: VP26 gene positive clones

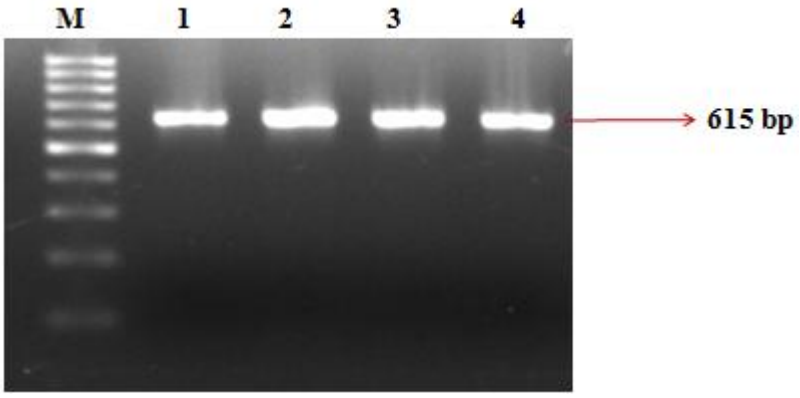


Fig.2 PCR amplification of VP24 gene clones by gene specific primers. Lane M: 100 bp molecular weight marker; Lane 1: Positive control; Lane 2: Negative control; Lanes 3- 5: VP24 gene positive clones

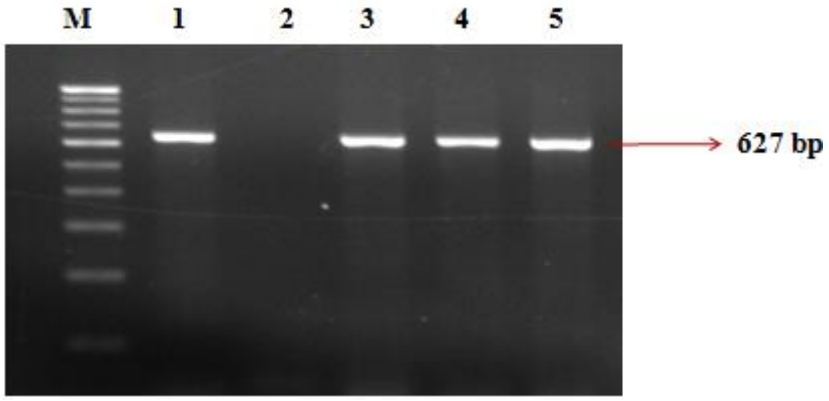


Fig.3 PCR amplification of RR1 gene clones by gene specific primers. Lane M: 100 bp molecular weight marker; Lane 1: Positive control; Lane 2: Negative control; Lanes 3- 5: RR1 gene positive clones

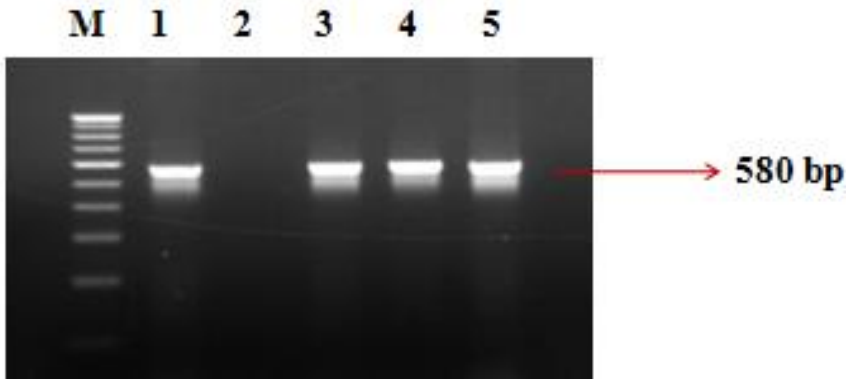


Fig.4 PCR amplification of wsv477 gene clones by gene specific primers. Lane M: 100 bp molecular weight marker; Lane 1: Positive control; Lanes 2- 5: wsv477 gene positive clones; Lane 6: Negative control

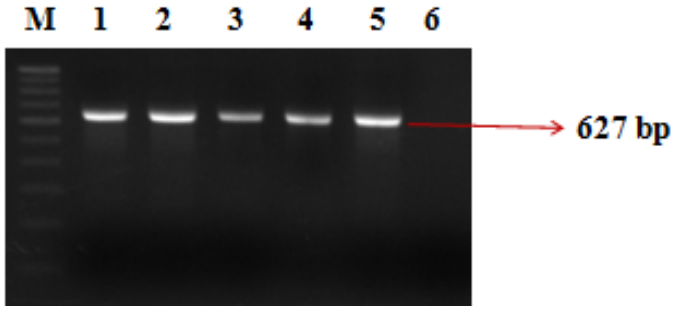


Fig.5 Verification of dsRNA integrity by RNase treatment. The integrity of dsRNA targeted to the VP26, VP24, RR1 and *wsv*477gene was confirmed by incubation with RNase A (Lane A) and RNase III (Lane III), which specifically digests sRNA and dsRNA, respectively. Lane M is standard DNA marker and lane U is untreated dsRNA

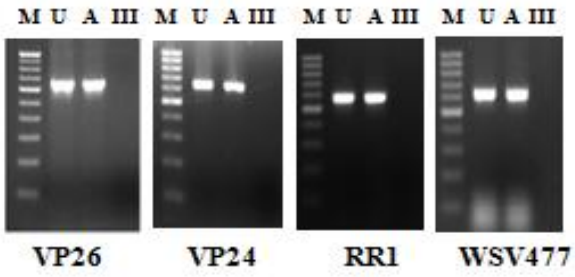


Fig.6 Cumulative mortality of *P. monodon* orally feed with VP26, VP24, RR1 and *wsv*477 dsRNA

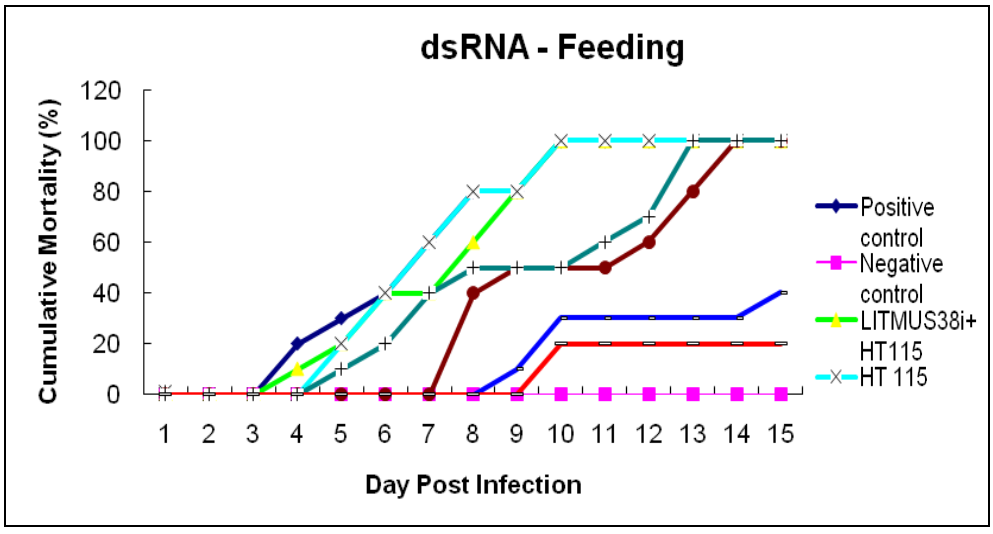
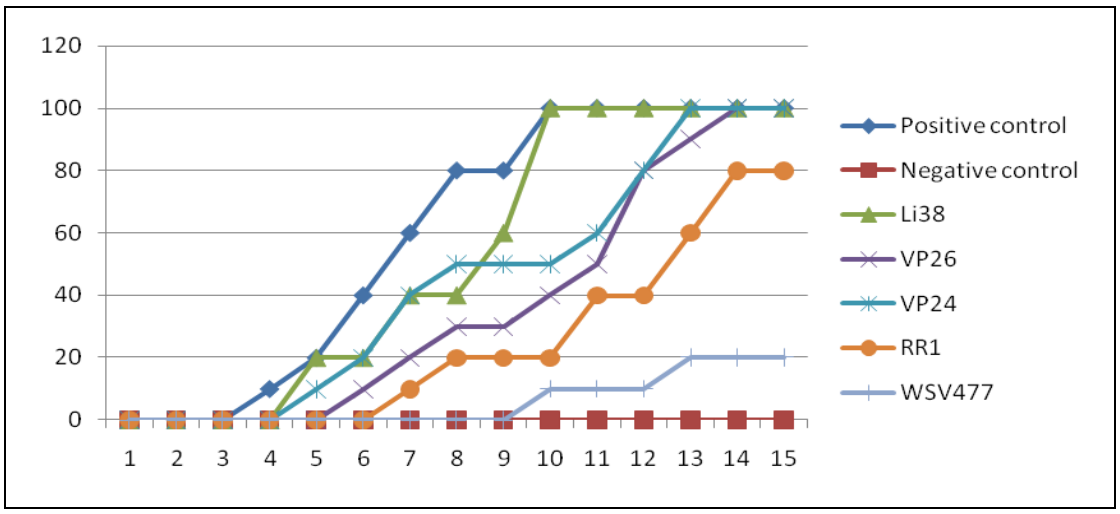


Fig.7 Cumulative mortality of *P. monodon* injected with VP26, VP24, RR1 and *wsv*477 dsRNA



In this study, shrimp injected with *wsv*477dsRNA offered higher protection (90%) compare to other targeted genes proving it to be one of the best dsRNA targets against WSSV infection.

Envelope proteins have a major role in viral infection since they are the first molecule to interact with the host (van Hulten *et al.*, 2000, 2001a; Xie and Yang 2005). VP26 protein anchors with the nucleocapsid protein called VP51 which helps for the close attachment of envelope to nucleocapsid (Xie *et al.*, 2005; Wan *et al.*, 2008). *P. monodon* challenged with VP26 dsRNA conferred 50% survival till the10th day of post viral infection. VP24, the chitin binding protein (Li *et al.*, 2015) play a vital role as an anchor protein with other structural proteins *viz*. VP28, VP26 and WSV010. Shrimps (*P. monodon*) challenged with VP24 dsRNA showed 40% protection till 10^{th}-day post viral infection whereas, in *L. vannamei* siRNA conferred 20% survival against WSSV infection (Wu *et al.*, 2007). In the current study shrimp treated with VP26 and VP24 dsRNA provided relatively lower survival rate in contrast to the *wsv*477 and RR1 protein. The potency and efficacy of dsRNA depend on the target gene and its role in the replication and pathogenesis of virus. Thus knowledge on the interaction of various structural and nonstructural proteins would help in understanding the viral morphogenesis and its infection (Chen *et al.*, 2007) to select a better vaccine candidate against infectious WSSV disease.

In conclusion, the present study demonstrates that the intramuscular injection delivery of dsRNA targeting structural and non-structural genes can provide better protection than oral delivery. Even though oral delivery has proven to be the best preventive strategy against pathogens in shrimp aquaculture, dsRNA was injected to shrimp to evaluate the potential of virus-specific dsRNA to inhibit viral replication. Similarly, non-structural proteins confer resistance to thepathogen in comparison with structural protein in terms of survival. These results provide valuable information on developing a novel strategy of RNAi-based therapeutics in shrimp farming against White spot disease.

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