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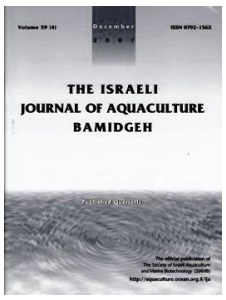

Protection of *Litopenaeus vannamei* against White Spot Syndrome Virus (WSSV) Using Bacterially Expressed Recombinant Envelope Proteins VP39 and VP28

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Key words: WSSV, *Litopenaeus vannamei*, VP39, envelope protein, recombinant vaccine

Abstract

White spot syndrome virus (WSSV) is a highly virulent shrimp pathogen, causing huge economic loss to the aquaculture industry. We investigated the efficacy of recombinant VP39 protein against WSSV infection in *Litopenaeus vannamei* by intramuscular and oral administration, and also compared the efficacy with recombinant VP28 (rVP28). The VP39 is a 283 amino acid protein encoded by the structural gene vp39 which acts as an important mediator for virus entry to the host. Shrimp orally vaccinated with rVP39 and rVP28 showed a cumulative mortality of 50% and 60% respectively following challenge, and this indicates that rVP39 had a better protective effect against WSSV infection compared with rVP28. Vaccination by intramuscular injection with rVP39 and rVP28 resulted in survival rate of 60% and 50%, respectively. The transcriptional profiling of viral genes of vaccinated shrimp with recombinant viral proteins of VP28 showed higher transcriptional levels than VP39. The transcriptional level of rVP39 vaccinated animals was delayed by 6 days after WSSV infection, while the delay was only 4 days in the case of rVP28. These results indicate that vaccination delays the transcription of envelope genes of WSSV in shrimp. The present study could demonstrate the importance of VP39 as a prominent candidate for vaccination against WSSV.

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Introduction

White spot syndrome virus (WSSV) is a rapidly replicating and highly virulent shrimp pathogen, which can cause up to 100% mortality within 7 to 10 days in shrimp farms, resulting in huge economic losses to the aquaculture industry (Witteveldt et al., 2004a). The virus has a broad host range which comprises shrimps, prawns, lobsters, copepods, crabs, and other arthropods (Lo et al., 1996; Maeda et al., 1998; Shi et al., 2000; Chakraborty et al*.,* 2002). The infection is characterized by clinical signs such as lethargy, anorexia, loose cuticle from underlying epidermis, and the appearance of white spots on the inner side of the carapace, appendages, and the abdominal segments (Lightner, 1996; Hameed et al*.,* 1998; Wang et al*.,* 1999; Escobedo-Bonilla et al., 2007). WSSV belongs to the unique genus called *Whispovirus* in the family *Nimaviridae*, reported in the 8th Report of the International Committee on Taxonomy of Viruses (ICTV) (Vlak et al., 2005). It is a large, oval, enveloped DNA virus, with tail-like appendages at one end (Wongteerasupaya et al., 1995; Chang et al., 1996; Durand et al., 1997; Nadala et al., 1998). The size of the virion particles ranges between approximately 210-420 nm in length and 70-167 nm in diameter (Guoxing et al., 1997; Lotz and Soto, 2002). The viral nucleocapsid contains circular double stranded DNA with an approximate size of 300 kbp, with 181 non-overlapping open reading frames consisting of 39 structural proteins (van Hulten et al., 2001 and Yang et al., 2001).

An adaptive immune system based on clonal expansion of activated lymphocytes, as found in vertebrates, is not present in shrimp and hence it is thought that they rely mostly on innate immune response for internal defense against parasites and pathogens (Söderhäll and Thornqvist, 1997). However *in vivo* experiments with *Penaeus japonicus* infected with WSSV revealed the presence of a quasi-immune response when the shrimp were re-challenged with WSSV (Venegas et al., 2000). This suggests that there is some sort of adaptive immune response in shrimp (Kurtz and Franz, 2003; Kurtz, 2005). A short memory of antigen in shrimp might involve an induced secretion of neutralizing substances or other defense proteins against WSSV invasion (Granof and Webster, 1999) and this could be used to protect these animals against the disease.

Development of a vaccine based on the envelope proteins of WSSV could be more effective since they are the first molecules to interact with the host. Among the structural proteins, VP28 has been reported to be the most abundant and exposed protein of WSSV which facilitates the entry of the virion into the host cell at the early stage of infection (van Hulten et al., 2001). Several studies have shown that a recombinant vaccine based on VP28 provides notable protective effect against WSSV infection and has proven to be the best and most promising vaccine (Namikoshi et al., 2004; Witteveldt et al., 2004a, b; Jha et al., 2006; Du et al., 2006). Another structural gene, 852 bp long VP39, encodes a 283 amino acid protein with a calculated molecular mass of 32 kDa which seems to be an important mediator for virus entry into the host (Zhu et al., 2006), and has been identified as an integument protein (Tsai et al., 2006). Temporal analysis of VP39 gene expression has shown that it is expressed 12 h after viral infection, and immunoelectron microscopy suggests that this is an envelope protein, but western blot analysis indicated its presence in both virions and viral envelopes (Zhu et al., 2006). VP39 has been identified as an integument protein (Tsai et al. 2006). However, the role of this protein in the infectivity of WSSV, or its interaction with shrimp receptors facilitating virus attachment and invasion, remains to be elucidated. There have been no reports in the literature regarding the efficiency of VP39 in inducing protection. Therefore, we investigated the efficacy of recombinant VP39 expressed in *E. coli* and delivered through intramuscular and oral administration, against WSSV infection in Pacific white shrimp, *Litopenaeus vannamei*.

Materials and Methods

Collection and maintenance of shrimp. Shrimp (*L. vannamei*) approximately 7-10 g body weight, were collected from a shrimp farm located in Kundapura, Karnataka and kept in 1000 liter fiberglass tanks containing sand filtered and ozone treated natural seawater [salinity between 15-20 parts per thousand (ppt)], with vigorous aeration at 28-30°C. The shrimp were fed with artificial feed pellets and were acclimatized in the tanks for a week prior to the experiment. The shrimp were randomly screened for WSSV, IHHNV, MBV and HPV by polymerase chain reaction (PCR). Only healthy shrimp were used in the experiments.

Preparation of WSSV inoculum. The viral inoculum was prepared from the gills of WSSV infected shrimp according to the method described by Xie et al. (2005) with minor modifications. The infected tissues were homogenized in TNE buffer (50mM Tris-Cl, 400mM NaCl, 5mM EDTA; pH 8.5) with protease inhibitors and centrifuged, filtered through nylon net and again centrifuged for 30 min at 4°C. The pellets were suspended in 10 ml TN buffer (50mM Tris-HCl, 10mM $MgCl_2$, pH 7.5), and centrifuged for 20 min at 4°C followed by re-suspension in 10 ml TN buffer containing 0.1% NaN_3 and stored at -80°C. 50 µl of viral filtrate was used for the challenge study. The WSSV copy number was determined by quantitative real-time PCR (data not shown).

Bacterial expression of VP39 and VP28 proteins. The complete VP28 and VP39 open reading frame (ORF) was amplified from the genome of WSSV using primer VP28 F (5′-CCGGGATCCATGGATCTTTCTTTCACTCTTTC-3′), VP28 R (5′-CGCAAGCTTCTCGGTCTCAGTGCCAG-3′) and VP39 F (5′-CGCAAGCTTATGTCGTCTAACGGAGATGA-3′) and VP39 R (5′-CGGGATCCAAAAACAAACAGATTG-3′) with *Bam*HI and *Eco*RI restriction enzyme sites (underlined). PCR was carried out under the following conditions: 95°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C (VP28) and 50°C (VP39) for 1 min, extension step at 72°C for 1 min and final extension of 72°C for 10 min. The products of vp28 and vp39 genes were cloned into pET-32a (+) expression vector and transformed into *E. coli* BL21 (DE3) (pSBET A). To determine the expression of recombinant proteins, *E. coli* BL21 (DE3) (pSBET A) harboring recombinant plasmid pET-32a-VP28 and pET-32a-VP39 were cultured in Luria-Bertani (LB) broth (HiMedia) containing 100 mg/ml ampicillin, 50 mg/ml kanamycin, and incubated at 37°C to exponential phase with constant agitation at 150 rpm. Expressions of recombinant proteins were then induced with 1mM IPTG (isopropyl-1-1-thio-b-d-galactoside) for 4 h. Expressed VP28 and VP39 proteins were purified by Ni-NTA affinity chromatography and purity of these proteins were analyzed by SDS-PAGE and specificity of the proteins were confirmed by western blotting (Towbin et al., 1979). The purified recombinant proteins were used for vaccination experiments.

Subunit vaccination with rVP28 and rVP39 proteins. L. vannamei weighing approximately 7-10g were divided into 5 groups in triplicate (n=10 in each) and all shrimp in these groups were intramuscularly (IM) injected with 100 µl purified recombinant proteins rVP28, rVP39, mixture of both (rVP28+rVP39), and two groups were injected using a 29 gauge needle, with phosphate buffered saline (PBS) in the 4^{th} or 5^{th} tail segment as positive and negative control. The dose of recombinant proteins injected was 10 µg/g per shrimp. Ten days after the first dose, shrimp were immunized with a booster dose of the same concentration of the recombinant proteins. On the 20^{th} day post initial vaccination (dpv), shrimp were challenged intramuscularly with a known concentration of WSSV (2.4×10^4 copies) and were then monitored daily until 10 days post challenge. The dead shrimp were analyzed for the presence of WSSV by PCR. Hemolymph was collected from the cephalothorax of moribund shrimp using a 3-ml syringe with 23G needle. The hemolymph and organs were stored at -80°C until RNA extraction.

Oral vaccination experiment. The recombinant proteins over-expressed in *E. coli* BL21 (DE3) (pSBET A) cells were inactivated with 0.5% formaldehyde and incubated for 15 min at 20°C followed by mixing with the feed pellets (approximately 10^7 cells). They were then incubated on ice for 15 min to facilitate absorption of the bacterial suspension and coated with cod liver oil to prevent dispersion of the inactivated bacteria in the water (Witteveldt et al., 2004b). The prepared feed pellets were used for oral vaccination by feeding three times daily at the rate of 3.5% of shrimp body weight. The shrimp were divided into six groups (10 per group) and vaccinated as shown in Table 1 for 7 consecutive days. Seven days after final vaccination, the shrimp in 5 groups were challenged by injecting WSSV (2.4×10^4 copies). The 6^{th} group received only PBS injection (negative control). Shrimp were monitored daily and the dead shrimp were analyzed for WSSV infection by PCR.

Table 1. Experimental set up for oral vaccination.

SI No.	*Groups*	*No.of shrimps/group*
1	VP 28	10 × 3
2	VP 39	10 × 3
3	Recombinant *E. coli* BL21 (DE3) (pSBET A) cells with empty pET-32a (+) vector	10 × 3
4	Non-recombinant *E. coli* BL21 (DE3) (pSBET A) cells	10 × 3
5	Positive Control	10 × 3
6	Negative Control	10 × 3

Total RNA extraction and cDNA synthesis. Total RNA was extracted from the hemolymph of WSSV challenged shrimp by using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. Complementary DNA (cDNA) was prepared from 1 µg of each RNA sample in a 20 µl reaction volume by using reverse transcriptase (Fermentas, UAB, Lithuania) and gene specific reverse primer; reaction was carried out at 42°C for 60 min. The expression levels of VP39 and VP28 genes were determined by PCR by using cDNA as template. Shrimp β-actin gene was used as internal control by using primers Actin F (5- GTGACTCACTTTGTCCCCGT- 3'), Actin R (5- TTACGCTCAGGAGGAGCAAT- 3'). The PCR products obtained were analyzed on agarose gel electrophoresis.

Statistical analysis. The protection conferred by the recombinant coat proteins against WSSV infection in shrimp was statistically represented as relative percentage survival (RPS). It was calculated as RPS = [1 – (mortality in vaccinated group / mortality in control group)] × 100 (Amend, 1981).

Results

Cloning and expression of VP28 and VP39 proteins. The complete ORF of both VP28 and VP39 genes were amplified from the viral DNA, and products were of expected length of 615 and 852 bp, respectively (Fig.1).

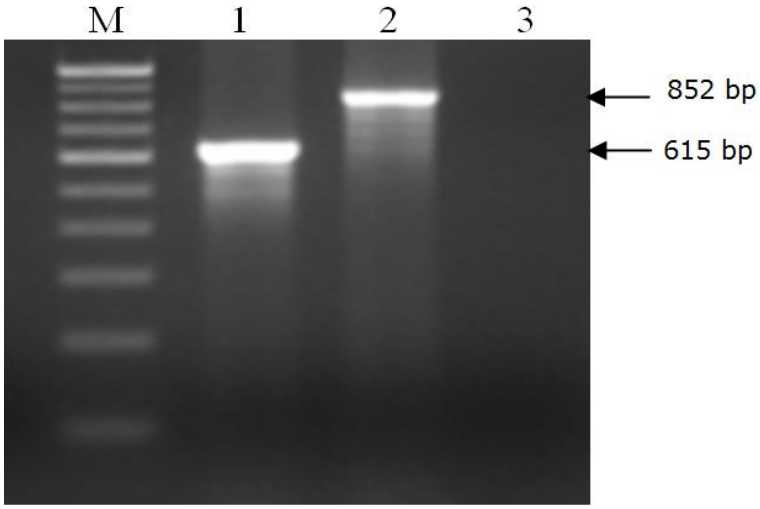

Fig. 1. Agarose gel electrophoresis showing PCR amplification of WSSV envelope genes. Lane M: 100 bp molecular weight marker; Lane 1: VP28 gene (615 bp); Lane 2: VP39 gene (852 bp); Lane 3: Negative control.

The amplified products were purified and cloned into pET-32a (+) expression vector followed by expression in *E. coli* BL21 (DE3) (pSBET A). The size of recombinant protein was about 40.12 kDa and 49.72 kDa including His-tag corresponding to VP28 and VP39 proteins respectively, by SDS-PAGE analysis. This was further confirmed by western blot analysis using polyclonal antibodies (Fig.2 A and B).

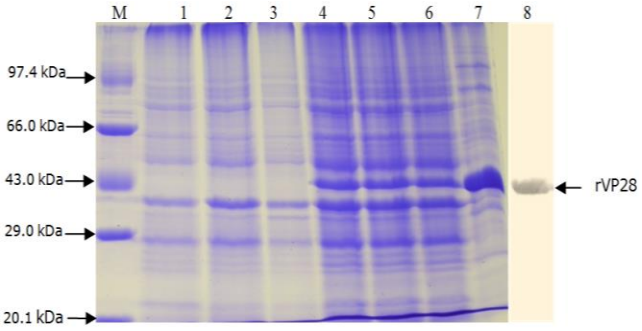

Fig. 2a. SDS–PAGE showing the expression of recombinant protein VP28 of WSSV.
Lane M: Molecular weight marker (PMW-M); Lane 1: Non-recombinant BL21 (DE3) (pSBET A) *E. coli*, cell without IPTG induction; Lane 2: Non-recombinant BL21 (DE3) (pSBET A) *E. coli*, cell with IPTG induction; Lane 3: Recombinant VP28 clone without IPTG; Lanes 4-6: Recombinant VP28 clones with IPTG; Lane 7: Purified VP28 protein; Lane 8: Western blot analysis of purified VP28 protein by using polyclonal antibody.

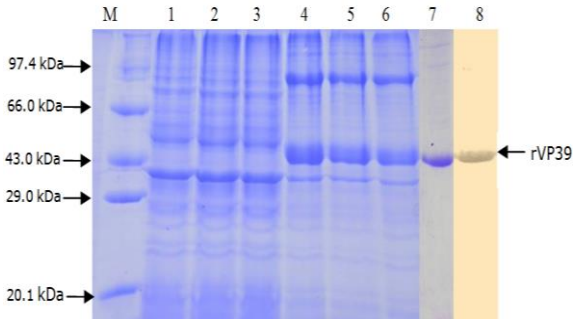

Fig. 2b. SDS–PAGE showing the expression of recombinant protein VP39 of WSSV.
Lane M: Molecular weight marker (PMW-M); Lane 1: Non-recombinant BL21 (DE3) (pSBET A) *E. coli*, cell without IPTG induction; Lane 2: Non-recombinant BL21 (DE3) (pSBET A) *E. coli*, cell with IPTG induction; Lane 3: Recombinant VP39 clone without IPTG; Lanes 4-6: Recombinant VP39 clones with IPTG; Lane 7: Purified VP39 protein; Lane 8: Western blot analysis of purified VP39 protein by using polyclonal antibodies.

Subunit vaccination with envelope proteins (rVP28 and rVP39). The cumulative mortality of the vaccinated shrimp following challenge with WSSV was 50%, 40% and 40% with rVP28, rVP39 and rVP39+rVP28, respectively (Fig.3).

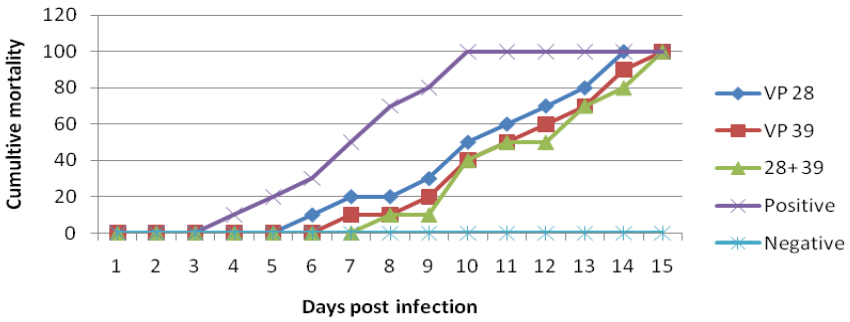

Fig. 3. Cumulative mortality of *L. vannamei* vaccinated with recombinant envelope protein VP28 and VP39 by injection method.

The cumulative mortality for these vaccinated groups was significantly lower than that of the control group. RPS values of rVP39 and rVP28 groups were found to be 60 and 50, respectively. Highest survival rate was in the group vaccinated with rVP39. All the dead shrimp tested positive for WSSV infection by PCR.

In vivo expression of vp28 and vp39 gene in intramuscularly injected shrimps. The RT-PCR of the transcripts from hemolymh of vaccinated shrimp challenged with WSSV showed a variation in the quantity of mRNA transcriptional level (Fig.4 and 5).

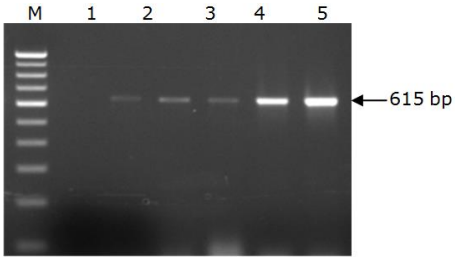

Fig. 4. RT-PCR analysis of viral gene transcription in vaccinated shrimp with recombinant envelope protein VP28. Lane M: 100 bp Molecular weight DNA marker; Lane 1: 1 day after post initial vaccination; Lanes 2-6: 2nd, 4th, 6th, 8th and 10th day of post initial vaccination.

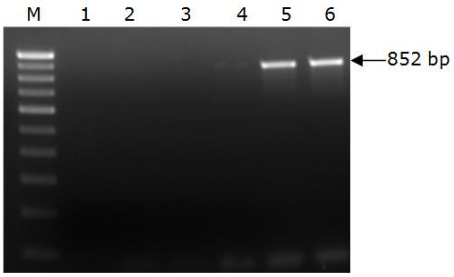

Fig. 5. RT-PCR analysis of viral gene transcription in vaccinated shrimp with recombinant envelope protein VP39. Lane M: 100 bp Molecular weight DNA marker; Lane 1: 1 day after post initial vaccination; Lanes 2-6: 2nd, 4th, 6th, 8th and 10th day of post initial vaccination.

Expression level of mRNA of β-actin from the host was similar in every sample and even it is included as an internal control for checking the quality of RNA (Fig.6).

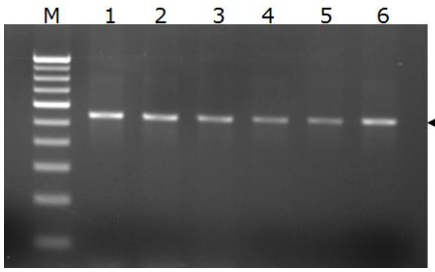

Fig.6. RT-PCR analysis of internal control β-actin gene. Lanes 1-6: 1st, 2nd, 4th, 6th, 8th and 10th day of post initial vaccination.

The transcriptional pattern of viral genes in the hemolymph of shrimp vaccinated with recombinant viral proteins VP28 showed higher transcriptional levels compared with the batch vaccinated with VP39 protein. In the case of the latter, the transcription level was delayed by 6 days, while the delay was 2 days in the case of shrimp vaccinated with VP28. These transcriptome results have clearly indicated that the vaccination of shrimp, delays the transcription of envelope and tegument genes of WSSV following challenge.

Oral vaccination. A comparison was made between the efficacy of orally administered bacterially expressed proteins rVP39 protein and rVP28. The cumulative mortality results from the experiment are shown in Fig.7.

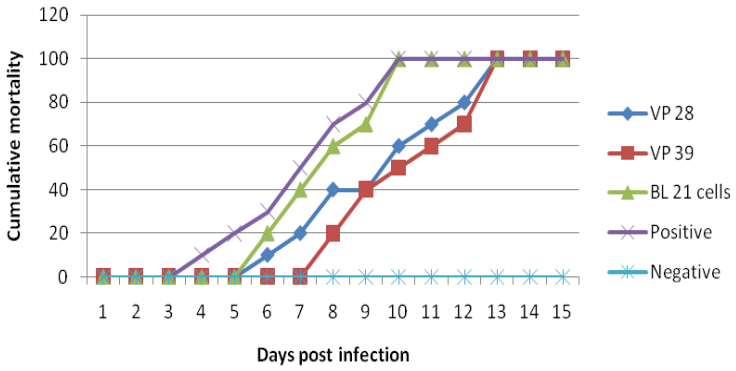

Fig. 7. Cumulative mortality of *L. vannamei* vaccinated orally with recombinant envelope protein VP28 and VP39.

Groups of shrimp vaccinated with rVP39 or rVP28 resulted in cumulative mortality of 50% and 60%, respectively, 10 days after viral challenge whereas positive control and group fed with *E. coli* BL21 cells containing empty vector resulted in 100% mortality at 10 dpi. Protective efficacy of rVP39 was higher compared to rVP28. The PCR analysis indicated that all the dead shrimp were infected with WSSV. There was no mortality in the negative control until the end of experimental period.

Discussion

In this study, we determined the efficacy of bacterially expressed recombinant envelope proteins VP28 and VP39, against WSSV infection by oral and intramuscular administration in Pacific white shrimp, *L. vannamei*. The envelope proteins are targeted mainly for vaccination studies because these proteins are the first to interact with the host defense system and stimulate protective immune response. These envelope proteins may play major roles in entry, assembly, and budding of viruses in the host (Chiu and Chang, 2002; Chazal and Gerlier, 2003). There have been number of reports regarding the role of VP28 as an effective vaccine candidate for providing long term protection against WSSV in shrimp (Witteveldt et al., 2004a; Jha et al., 2006; Satoh et al., 2008). Literature did not yield any information about the immunization trials using VP39. We have attempted to develop a VP39 based vaccine and compared its protective efficacy with VP28.

This study demonstrates that in *L. vannamei* vaccination by recombinant WSSV proteins VP28 and VP39 increased survival rate compared to the control groups. Oral vaccination of shrimp with rVP39 or rVP28 resulted in significantly lower mortality than that among the control shrimp. Vaccination with VP39 produced a lower cumulative mortality rate of 50% compared to VP28 (60%) and control groups (100%) 10 days post infection.

The duration and extent of protection provided by VP28 vaccine varies in different studies with different shrimp species. The term 'immunization' though used by several researchers in shrimp, is not a true vaccination as in vertebrates. It is a quasi immune response that suggests the existence of adaptive immunity. WSSV is a serious pathogen of shrimp and is known to cause 100% mortality in 3-10 days. Delaying mortality even by a few days is considered very significant. The difference in the number of days between the control and the treated groups of shrimp when 100% mortality was recorded suggests the usefulness of the molecules in delaying mortality by a quasi-immune response. It was reported that the oral administration of feed pellets coated with inactivated bacteria expressing envelope protein VP28 could confer 61% protection to *P. monodon* against WSSV, but at the same time rVP19 failed to give any protection to the same (Wittaveldt et al., 2004b). A double dose of rVP28 could confer protection to *P. japonicus* with a survival rate of 95% 30 days post vaccination (Namikoshi et al., 2004) whereas a cumulative mortality of 41.7% was shown by rVP28 vaccinated *L. vannamei* 21 days after viral challenging (Choi et al., 2011). The oral administration of *P. monodon* with baculovirus expressed VP28 showed survival rates of 81.7% and 76.7% on 3 and 15 days post immunization (Syed Musthaq and Kwang, 2011). In *Mar supenaeus japonicus,* the oral vaccination of rVP28 could give 100% protection 30 days after viral challenging whereas the immunization by immersion and injection could provide only 70% and 30% protection, respectively (Satoh et al., 2008). According to Satoh et al, the high survival rate after oral vaccination might be due to the prevention of horizontal transmission of WSSV through cannibalism in shrimp farms. The over expression of VP28 in silkworm pupae with recombinant baculovirus in *Procambarus clarkii* could effectively inhibit WSSV by supplementing as oral feed (Wei and Xu, 2009). Recombinant VP28 expressed in *Pichia pastoris* showed 78% survival of *P. clarkii* 21 days after virus challenge (Jha et al., 2006). *P. japonicus* treated with oral supplementation of feed pellets containing rVP28 expressed in gram positive bacteria exhibited high survival rates from WSSV, and the protection was dose dependent since the RPS increased from 60 to 80 with the use of 1-50 µg/shrimp (Caipang et al., 2008).

The recombinant vaccines are meant to be simple, non-infectious, and inexpensive to produce in large quantities (Jha et al., 2006; Witteveldt et al., 2004). The rVP28 was found to be a common antigen to control WSSV in shrimp (Choi et al., 2011; Mavichak et al., 2010; van Hulten et al., 2000). In this study, vaccination with the rVP39 temporarily delayed mortality compared to rVP28 in both oral and subunit vaccination, suggesting that VP39 is potentially suitable as a vaccine against WSSV. Vaccination of *P. monodon* was administered both orally and with intramuscular injection. Even though there are various strategies or modes of vaccination such as oral, immersion, intramuscular etc., for farmed shrimp and similar cultured aquatic animals, oral administration seems to be the most practical approach although there may be some destruction of the proteins in the digestive tract which leads to reduced efficiency (Wei and Xu, 2009). Oral administration is also a more convenient and inexpensive way of administering potential vaccines to cultured aquatic animals (Syed Musthaq and Kwang, 2011). The immersion vaccination method is more expensive, and the generated pathogen protection rate is lower than that observed with oral vaccination. In this study, vaccination by injection trials showed higher survival rate compared to oral vaccination and this result supports the work of Ha et al. (2008). Although, vaccination via injection is not feasible in aquaculture farms, it was carried out to ensure the application of a consistent amount of protein per shrimp and also to determine the potential of protein vaccine.

In conclusion, oral vaccination of shrimp with rVP39 showed more temporary protective efficacy than the more studied rVP28 in both intramuscular and oral vaccination studies. Hence, VP39 could potentially be a more potent vaccine than VP28.

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