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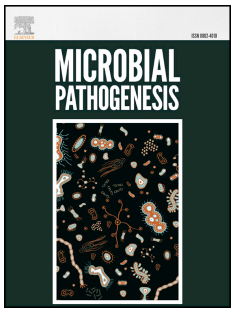


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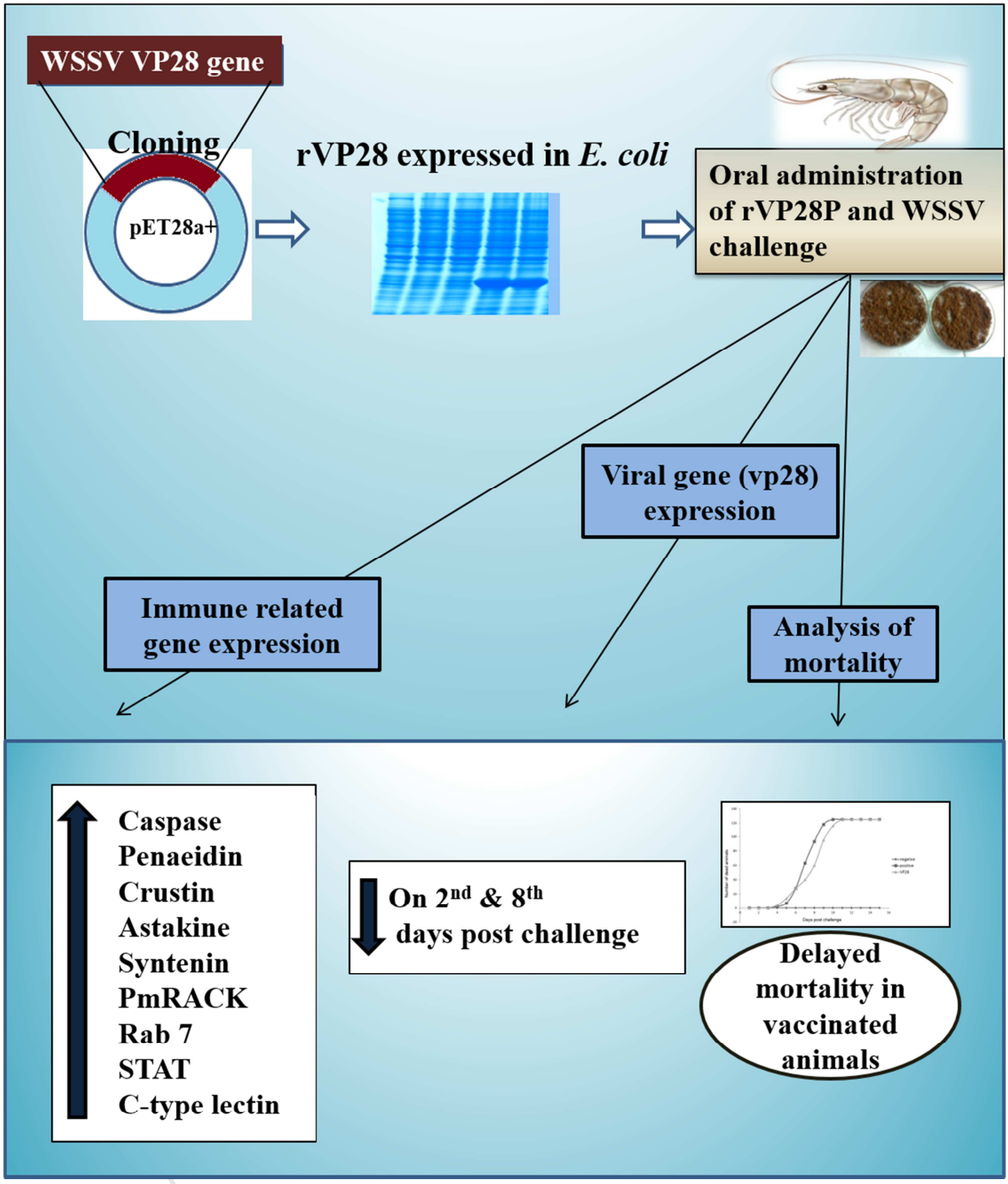
WSSV VP28 gene
Cloning
pET28a+
rVP28 expressed in E. coli
Oral administration of rVP28P and WSSV challenge
Viral gene (vp28) expression
Immune related gene expression
Analysis of mortality
Caspase
Penaeidin
Crustin
Astakine
Syntenin
PmRACK
Rab 7
STAT
C-type lectin
On 2nd & 8th days post challenge
Delayed mortality in vaccinated animals

# Expression profile of key immune-related genes in *Penaeus monodon* juveniles after oral administration of recombinant envelope protein VP28 of White Spot Syndrome Virus

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## Abstract

White spot syndrome virus (WSSV) is the most catastrophic pathogen the shrimp industry has ever encountered. VP28, the abundant envelope protein of WSSV was expressed in bacteria, the purified protein administered orally to *Penaeus monodon* juveniles and its immune modulatory effects examined. The results indicated significant up-regulation of caspase, penaeidin, crustin, astakine, syntenin, PmRACK, Rab7, STAT and C-type lectin in animals orally administered with this antigen. This revealed the immune modulations in shrimps followed by oral administration of rVP28P which resulted in the reduced transcription of viral gene *vp28* and delay in mortality after WSSV challenge. The study suggests the potential of rVP28P to elicit a non-specific immune stimulation in shrimps.

## 1. Introduction

Penaeid shrimp, one of the most economically important crustaceans, faces significant outbreaks of many diseases that have damaged aquaculture. White spot syndrome virus (WSSV) is the most catastrophic pathogen the shrimp industry has ever encountered [1,2]. Since the first report of WSSV in 1993, the major concern of world aquaculture industry has been to stave off this disease emerging in aquaculture settings [3]. There is increasing evidence based on laboratory trials that the administration of antigens or immunostimulants is

a promising approach to stimulate shrimp immune system against viral or bacterial infections. Administration of inactivated whole virus, oral recombinant protein antigens and DNA and dsRNA molecules provides remarkable protection to shrimp from WSSV as reviewed by Rowley and Pope [4] and Haq et al. [5].

Several investigations suggest that the administration of recombinant WSSV proteins, particularly VP28, imparts a form of immune stimulation, described as immune priming, resulting in increased survival of shrimps in subsequent virus infections [6]. VP28 is the major envelope protein of WSSV that expedites entry of the virion into shrimp cells [7]. The mRNA transcription of WSSV genes was delayed for 4~10 days in various organs of shrimp vaccinated with recombinant VP28 (rVP28) [8]. However, the underlying mechanism by which rVP28 activates the shrimp defense system remains unknown.

The crustacean immune system eliminates pathogens very efficiently through humoral and cellular immune processes. The biological defense molecules produced in shrimps in response to the invading pathogens include proteins and peptides related to immunity, homeostasis, host pathogen interaction and other cellular processes. Identification and functional analysis of immune related genes such as AMPs, proteinases and their inhibitors, anti-apoptotic proteins, pattern recognition receptors (PRRs) revealed its specific roles in shrimp defense system and in disease process [9]. Shrimps evolved to use diverse antimicrobial peptides (AMPs) and proteins with diversity in structure and function as the first-line of innate immune response [10]. The identification of thousands of high quality Expressed Sequence Tags (EST) in shrimps suggested that WSSV infection modulates the genes involved in several cellular and metabolic immune processes in post larvae [11]. Administration of immune stimulants alters the expression of wide range of cellular and immune related genes and triggers the innate immune reactions in shrimps by upregulation of penaeidin, lysozyme, crustin and JAK-STAT pathway genes and downregulation of AMPs and clotting related proteins in WSSV resistant animals [12,13].

The high frequency of occurrence and differential expression of immune-related genes in shrimps infected with WSSV, *Vibrio harveyi* or administered with probiotics further emphasize the molecular changes during an elevated immune response [14,15]. Earlier reports also demonstrated enhanced expression of few immune-related genes upon VP28 based DNA vaccination [16,17]. However, immune modulations at molecular level upon VP28 administration in shrimps [6] is scanty, which point to the relevance of the present study. Genes associated with immune functions such as pathogen recognition, clotting,

melanisation, agglutination, apoptosis, proPO pathway and signal transduction are characterized in shrimps. In the present study, expression of genes associated with these immune processes such as caspase, penaeidin, crustin, astakine, syntenin, *P. monodon* activated protein kinase C (PmRACK), Rab7 receptor, signal transducer and activator of transcription (STAT), superoxide dismutase (SOD), toll like receptor (TLR) and C-type lectin are assessed. We investigated the immune modulation in *P. monodon* juveniles (PL 40) after oral administration of rVP28P and assessed the survival rate of animals challenged with WSSV.

## 2. Materials and Methods

### 2.1 Expression and purification of WSSV VP28 from Indian WSSV isolate

The VP28 gene (*vp28*) identified from an Indian WSSV isolate was retrieved from Genbank (AY422228.1), and putative trans-membrane regions of VP28 were predicted by using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) (Data not shown)**.** Expression construct of VP28 was generated in fusion with His6X tag. The full length ORF of 612 bp (VP28) and a truncated form of 546 bp (VP28P) without N-terminal transmembrane region (amino acids 1-22) were PCR amplified. Gene specific primers carrying BamHI and NotI restriction sites were used for the amplification of the insert sequences from TOPO vector and cloned into pET28a$^{+}$ expression vector (Novagen, Madison, Wisconsin, USA). Transformation and expression of rVP28 and rVP28P were achieved in *E. coli* BL21 (Invitrogen, Carlsbud, MA, USA). Their over expression in *E. coli* was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot using 6X Histidine specific monoclonal antibody (1:20000; Genei, Banglore, India).

The rVP28 and rVP28P expressed *E. coli* BL21 cultures were re-suspended in binding buffer (50 mM $NaH_2PO_4$, 20 mM Tris, 100 mM NaCl) and sonicated. As the full length rVP28 accumulated as inclusion bodies, rVP28P available in the soluble form was selected for further purification by immobilized metal affinity chromatography (IMAC). The total cell lysate was loaded onto Ni-Sepharose column and gradient elution was carried out by adding imidazole at concentrations of 50–500 mM after incubation for 3 hr. The purified rVP28P was concentrated by ultrafiltration (Amicon Ultra 15, Millipore, MA, USA), dialyzed and,

assessed for the presence of bacterial endo-toxin using Limulus Amebocyte Lysate (LAL) assay as per manufacturer's (E-Toxate Kit, Sigma, St. Louis, MO, USA) protocol.

## 2.2 Oral administration of rVP28P to *P. monodon* juveniles and WSSV challenge

### 2.2.1 Experimental animals

Specific Pathogen Free (SPF) *P. monodon* juveniles (PL 40) maintained in a recirculating aquaculture system developed by National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin, Kerala, India was used for the study. The WSSV-free status of animals was tested by nested PCR as described by Lo et al. [18] using viral DNA extracted from whole animal. The shrimps were transferred to rectangular fiber reinforced plastic (FRP) tanks of 30 litre capacity prior to the experiment and acclimatized to 20 g $L^{-1}$ salinity filtered sea water at 28 ± 1 $^{\circ}$C, having total ammonia < 0.1 ppm, alkalinity 70-80 ppm at pH 8.4, for one week. The animals were maintained at this optimum conditions through partial exchange of water whenever required. During this period, shrimps were fed with a commercial feed (Amalgam Nutrients and Feeds Limited, Cochin, India) at the rate of 10% body weight per day.

### 2.2.2 Preparation of rVP28P protein coated feed

rVP28P was produced in large scale and a quantity of 100 µg purified rVP28P blended with 1ml phosphate buffered saline (PBS-pH 7.4). This was mixed with 4 g (at the rate of 25 µg protein $g^{-1}$ feed) commercial shrimp feed pellets (Amalgam Nutrients and Feeds Limited, Cochin, India) and incubated overnight at 4 $^{\circ}$C to allow the absorption of protein. The pellets were further coated with a commercial binder (Trubind, Wockhardt, Mumbai, India) to prevent dispersion of the protein in water and maintained at 4 $^{\circ}$C. Similarly, control feed was prepared by soaking in PBS and coating with the same commercial binder.

### 2.2.3 Oral administration of rVP28P and WSSV challenge

Four groups of animals (groups of 125) each animal weighing 2-4 g were used for the experiment and the design was as shown in Table 1. Feed mixed with PBS alone was administered to two batches of animals (PBS 1 and PBS 2) and maintained as controls and two batches administered with rVP28P in PBS (rVP28P 1 and rVP28P 2) served as the tests. All the animals were starved for 6 hr and offered PBS and rVP28P dissolved in PBS as

control and test feeds respectively. The feeding rate was of 10% of the body weight twice a day for a period of ten days and thereafter, resorted to normal commercial feed. On 1st day post administration of the coated feed, a batch of rVP28P (rVP28P 2) and PBS administered (PBS 2) animals were challenged with WSSV by feeding freshly infected tissue (soft tissue from cephalothorax confirmed WSSV positive through nested PCR) at a rate 10% of the body weight [19]. The uneaten WSSV infected tissue pieces were removed from the tanks after 4 hours of feeding along with complete replacement of water with fresh sea water. The percentage survival of the rVP28P administered animals at the event of challenge with WSSV compared with that in the control group was recorded.

### 2.3 Tissue sampling, RNA extraction and cDNA synthesis

Animals survived were collected from unchallenged and WSSV challenged groups on 2nd, 5th and 8th days post administration (dpa) and post challenge (dpc) respectively. Three batches of animals comprising three animals each from each group were collected from tests and controls, euthanized by immersing in ice flakes, washed with diethyl pyrocarbonate (DEPC) treated water, preserved in Tri- Reagent (Sigma, USA) and maintained at -80 °C. Total RNA was extracted individually from each whole animal of all experimental groups and converted to cDNA within 12 hr of collection of samples under RNase free condition. The total RNA was isolated using TRI reagent (Sigma, St. Louis, MO, USA) following manufacturer's protocol with slight modifications. Briefly, the samples were homogenized and 200 µL chloroform was added per ml of TRI reagent. The RNA was precipitated with 70% isopropyl alcohol, dissolved in 20 µL RNase free water followed by DNase I treatment at 37 °C. Further, the quality and quantity of RNA was determined using UV–visible spectrophotometer and confirmed using Qubit RNA assay kit (Life technologies, USA). RNA was subjected to electrophoresis on denaturing gel (5% polyacrylamide gel in Tris -borate EDTA (TBE buffer) containing 7 M urea) to assess the integrity of RNA. Immediately after RNA extraction, 5 µg RNA was reverse transcribed to cDNA using Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase and oligo $dT_{(12\text{-}18)}$ (New England Biolabs, MA, USA) as per the standard protocol and the quality and quantity of cDNA were also assessed spectrophotometrically.

### 2.4 Expression of immune-related genes upon rVP28P administration - semi quantitative reverse transcriptase - polymerase chain reaction (RT-PCR)

All extracted RNA samples had $Abs_{260}/Abs_{280}$ ratio of 1.8-2.0 in water and were used for cDNA preparation from 5 μg of total RNA. The 25 μL PCR mixture with 200 μM dNTPs, 10 pmol each forward and reverse primers, 1 X PCR buffer (Thermopol), 0.5 U Taq DNA Polymerase and 1 μL cDNA each were used. The cycle parameters were initial denaturation at 94 ºC for 5 min, 25-30 cycles of 94 ºC for 30 s, annealing for 30 s, extension for 1 min at 72 ºC and a final extension at 72 ºC for 10 min. The PCR cycles and the cDNA dilution had been optimized so that the target gene and reference gene amplification could be obtained at logarithmic phase. In order to check the consistency, the reactions were repeated three times for each gene per sample. The target gene, predicted amplicon size, cycling conditions and primer sequences for each target gene and its source are presented in Table 2.

Shrimp elongation factor-5A (EF-5A) was used as the reference gene based on its consistency in the previous experiments. The PCR products were analyzed by horizontal gel electrophoresis and the average integrated density values (IDV) of the amplicons were semi-quantitatively measured using Quantity One® software (Bio-Rad, Philadelphia, USA) and normalized with the expression of EF-5A.

### 2.5 Statistics

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA). The amplification plots were constructed and represented as mean ± standard deviation of the IDV. Differences between the gene expression of the unvaccinated and rVP28P vaccinated animals at three time points were calculated using non-parametric t-test having $p \leq 0.05$ considered significant. Mean values of target gene expression in three samples, normalized with the expression of the reference gene EF-5A are presented.

## 3. Results

### 3.1 Expression and purification of rVP28P

The WSSV *vp28* gene was cloned and expressed in *E. coli* BL21. The VP28 protein sequence contained N-terminus hydrophobic membrane anchoring domain at amino acids 1-22, as elucidated by TMpred (Data not shown). The full length ORF rVP28 (612bp) and a partial reading frame VP28P (546 bp) were expressed in bacteria using pET28a expression system. Bands corresponding to 27 kDa and 25 kDa of full length and truncated protein respectively were visualized in an SDS-PAGE stained with Coomassie Blue G-250 (Fig.1a). The truncated rVP28P expressed in large quantity as soluble protein was further purified

using Ni-NTA affinity chromatography, and eluted in 250 mM imidazole (Fig.1b). The purified and dialyzed rVP28P exhibited reactivity with antibodies to His6X (Fig.2a). The endotoxin level in the purified rVP28P was negligible (<1pg/ml of lipopolysaccharide) as determined by LAL assay.

### 3.2 Oral administration of rVP28P and WSSV challenge

The animals used in the study had been confirmed WSSV free through two step nested PCR for virus detection prior to the experiment. The rVP28P administered and unchallenged animals did not show any sign of infection. Expression of *vp28* was significantly low in rVP28P administered animals on $2^{nd}$ and $8^{th}$ dpc compared to the control group (Fig.2b). The reduced expression of *vp28* indirectly evidenced the low proliferation of WSSV in the rVP28P administered shrimps.

### 3.3 Expression of immune-genes in shrimps administered with rVP28P

Shrimps were orally administered with rVP28P for 10 days and expression of selected immune genes assayed, which indicated the effect of this antigen in the immune system. The statistically significant ($p$ <0.05) up and down regulations when compared with the PBS control group at each time point alone have been brought under this section. Accordingly, caspase transcripts were found up-regulated in the rVP28P administered animals on $2^{nd}$ dpa. However, it was low in both administered and non-administered groups on $5^{th}$ and $8^{th}$ dpa. (Fig.3a). Crustin was up- regulated in the rVP28P administered group on $8^{th}$ dpa (Fig. 3b). Expression of penaeidin was high on $2^{nd}$ dpa without any variations on other two time points, as shown in Fig. 3c. Peneidin transcripts were up regulated in rVP28P administered group on $2^{nd}$ dpa compared with that of the control group (Fig. 3c). Astakine was up regulated on $8^{th}$ day in the rVP28P administered group (Fig. 3d). Up regulation of syntenin on $5^{th}$ and $8^{th}$ dpa in the rVP28P administered groups is shown in Figs 3e. A significant upregulation of PmRACK was noticed on $2^{nd}$ dpa. PmRab7 transcripts were up-regulated on $5^{th}$ and $8^{th}$ dpa ($p$ <0.05) (Fig. 4a). STAT expression was high on $8^{th}$ dpa (Fig. 4b). Low level expression of TLR was observed on $8^{th}$ dpa compared with that of the control (Fig. 4c). C-type lectin exhibited up-regulation on $8^{th}$ dpa in the rVP28P administered animals (Fig. 4d) with no variation between administered and control groups at any time points.

Among the seven immune-related genes analyzed, caspase, penaeidin, crustin, astakine, syntenin, PmRACK, Rab7, STAT and C-type lectin transcripts exhibited up-

regulation after rVP28P administration at least at one-time point, whereas TLR was down-regulated.

### 3.4 Time - mortality relationship of animals after rVP28P administration and WSSV challenge

In the survival analysis, rVP28P administered animals exhibited delayed mortality compared to the PBS administered group (positive) which exhibited 100% mortality on $10^{th}$ dpc. The unchallenged animals (negative) exhibited no mortality (Fig. 5).

## 4. Discussion

In the present study, we conducted an investigation of the gene expression pattern in the rVP28P immunized shrimps using the whole animal (*P. monodon* juveniles) instead of specific shrimp tissue or organ. WSSV is systemic and infects ectodermal and mesodermal originated tissues and organs of shrimps and the immune response vary among different tissues and organs [20]. In different cell types, the virus is likely to modulate the gene expression differently in order to promote its multiplication. Therefore, the assessment of immune molecules in whole animal reveals the overall molecular changes which might be underlying rVP28P mediated protective immunity in shrimps.

*E. coli* based protein expression system yields the highest quantity of rVP28P from a given biomass. The expression analysis of full-length and truncated VP28 in *E. coli* indicates higher level of soluble expression of the latter while the former accumulated as inclusion bodies. The high yield and solubility of rVP28P aids large scale production for commercial purposes, and may enhance the bioavailability and absorption of the protein in the gut of the animal after oral administration. The high solubility of rVP28P also facilitates its purification in non-denaturant condition.

Despite several reports demonstrating the efficacy of crude rVP28 against WSSV [6], we used purified rVP28P, which was tested endotoxin-free. This facilitated the assessment of immune responses highly specific to the WSSV antigen VP28.

The expression of WSSV gene *vp28* transcripts correlated with the WSSV proliferation and immune-related gene expression indirectly. Transcriptional analysis of WSSV genes, particularly *vp28,* is a well-known approach to assess the viral replication after

rVP28 administration [8]. Silencing studies of *vp28* gene further demonstrated its vital role in WSSV replication [21].

The expression of caspase in rVP28P administered shrimps was upregulated on the 2nd dpa compared with PBS control. This may indicate the immediate activation of caspase by the oral administration of rVP28P. Suppression of caspase gene in *M. japonicas* prior to WSSV invasion led to inhibition of apoptosis and increase in WSSV copy number [22]. The role of caspase in anti-WSSV immunity is also evident from the high expression of caspase in shrimps infected with WSSV [23, 24]. In the present study, the high level expression of caspase only on 2nd dpa indicates that the antigen administration needs further optimization to maintain the enhanced expression in the rVP28P administered animals to provide prolonged protection. The enhanced and prolonged caspase expression through rVP28P vaccination may help the animals to resist subsequent WSSV infections.

The significant upregulation of crustin on 5th and 8th dpa in vaccinated animals when compared with the PBS control group indicates the efficacy of rVP28P as a candidate antigen to stimulate immune system. This can be further correlated with the reduced viral load in the vaccinated animals as evidenced from the low gene expression of *vp28*. Crustin-like AMPs were found to be constitutively expressed in shrimps and upregulated after the administration of immune stimulants and WSSV challenge [25]. The significant increase of crustin in the vaccinated animals indicates the effect of rVP28P vaccination in the shrimp immune system by increasing the expression of AMPs.

The recombinant vaccine administration significantly enhanced the expression of penaeidin on 2nd dpa. This peptide is constitutively synthesized and released into plasma after microbial challenge [26] and, upregulated during *Vibrio* infection in post larvae [27]. These studies demonstrated its role in shrimp host defense and rVP28P mediated protective immunity.

In crayfish, *Pacifastacus leniusculus*, hematopoiesis is regulated by an invertebrate cytokine astakine, critical for hemocyte count [28]. The enhanced expression of astakine after rVP28P vaccination in the test animals can positively elevate the hemocyte count and improve the immune status. The high levels of astakine may also contribute to the low expression of *vp28* in the vaccinated animals after WSSV challenge.

Syntenin is an adaptor protein that links various molecules in signal transduction pathway. Previous studies reported enhanced expression of syntenin in WSSV infected shrimps which declined rapidly as the infection progressed. This implied the role of syntenin as an important molecule in shrimp immune response subsequent to viral infection [29]. In the present study syntenin levels were significantly higher in rVP28P administered animals on $5^{th}$ and $8^{th}$ days after rVP28P administration. The stable maintenance of high levels of syntenin in the animals could attribute to the reduced severity of infection and may offer protection against subsequent viral attacks.

PmRACK-1 was elevated in the rVP28P administered animals at all the three time points compared with its PBS control with a significant upregulation on $2^{nd}$ dpa. This kinase receptor interacts with the VP9 viral protein of WSSV and involves in the response against viral infection in *P. monodon* [30]. It can also participate in the shrimp antioxidant response induced by the formation of ROS [31]. This postulates the rVP28P binding mediated signal transduction activation of the kinase receptor and its role in vaccination mediated protective immunity in shrimps.

Rab7 is a VP28 binding protein that aids the recognition and entry of WSSV into shrimp cells. The real-time PCR analysis demonstrated that PmRab7 transcripts were constitutively expressed during the course of WSSV infection and PmRab7-VP28 binding reduces the severity of infection [32]. The enhanced expression of Rab7 on $5^{th}$ and $8^{th}$ dpa in the rVP28P vaccinated shrimps implies that rVP28P immunization may aid the neutralization of WSSV in the future viral exposures through Rab7-VP28 interaction and successive immune activation.

The significantly increased expression of STAT gene on $8^{th}$ dpa may help the immune system to respond to a later infection through some unknown STAT-mediated immune mechanism. WSSV uses shrimp STAT as transcription factor to enhance viral gene expression [33]. Here, an increase in STAT gene expression correlates with reduced copies of *vp28* transcripts after WSSV challenge. The STAT-mediated immune activation may reduce the expression of *vp28* and other viral genes thus, negatively affect the WSSV establishment.

The innate immune system of shrimps recognizes and binds to specific patterns on the surfaces of pathogens through PRRs [34] and this recognition mechanism and clearance of the pathogen is the supreme part of the shrimp innate immune system. TLR is an important

PRR in shrimps that recognize molecules derived from microbes [35]. Down-regulation of TLR in the rVP28P administered animals on 8$^{th}$ dpa evidences to the low significance of TLR mediated immune recognition in this context. C-type lectin is also a major PRR in shrimps and its reduction increases mortality of shrimps during WSSV infection [36]. The significant up-regulation on 8$^{th}$ dpa with rVP28P vaccination may help the animals to resist virus establishment in the future exposures.

Time - mortality relationship of shrimps after rVP28P administration and WSSV challenge demonstrated delayed mortality of animals administered with rVP28P. Earlier reports demonstrated the efficacy of VP28 as an oral vaccine which offered more than 60% survival in administered animals [37,38]. In the present study, the oral administration of rVP28P did not show similar trend in survival, except a delay in mortality when compared to that of the controls. More investigations are required to delineate the situation.

The present study suggested significant up-regulation of caspase, penaeidin, crustin, astakine, syntenin, PmRACK, Rab7, STAT and C-type lectin in the rVP28P vaccinated animals. This points out the immunomodulation followed by oral administration of rVP28P in *P. monodon* juveniles at molecular level. This resulted in the low expression of viral gene *vp28* and enhanced resistance of shrimps to WSSV and thereby delayed mortality of animals in the subsequent viral challenge. These results are supported by our earlier studies on the modulation of immune-related genes in shrimp juveniles administered with rVP24 [39]. Even though, shrimps lack adaptive immunity and antibodies, investigations on immune modulations and the underlying mechanisms involved, can pave way for a better understanding of how shrimp immune system interacts with pathogen components, particularly rVP28P.

The study suggests that rVP28P alone may not protect shrimp from WSSV, and a cocktail of recombinant viral proteins along with immunostimulants might accord protection by enhanced elicitation of non -specific immune mechanism coupled with management of the environment favorable to the animal. The study thus opens up challenging new opportunities for investigation.

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## Conflicts of Interest

The authors declare no conflict of interest.

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**Table-1** Experimental design for oral administration of rVP28P in *P. monodon* juveniles with timeline of sample collection

| Group | Coating material (administered for 10days) | No. of shrimps | | Tissue collection |
|---|---|---|---|---|
| | | Unchallenged | WSSV challenged (1dpa) | |
| PBS 1 (control) | PBS | 3x125 | - | 2nd, 5th and 8th dpa* |
| PBS 2 (control) | PBS | | 3x125 | 2nd, 5th and 8th dpc# |
| rVP28P 1 (test) | PBS+rVP28P | 3x125 | - | 2nd, 5th and 8th dpa* |
| rVP28P 2 (test) | PBS+rVP28P | | 3x125 | 2nd, 5th and 8th dpc# |

dpa - days post administration ; dpc – days post challenge

* Immune gene expression analysis; # WSSV gene *vp28* expression analysis

**Table-2** Amplicon size and primers used to amplify WSSV genes and immune-related genes

| Target | Amplicon size (bp) | Annealing tem (°C) | Primer sequence | Reference/GenBank Accession Number |
|---|---|---|---|---|
| EF-5A | 737 | 55 | F-5’GCTCTCTCGCTCCTCCTTTCA3’<br>R-5’CCCATGGACAAAGCAAAGG3’ | Loongyai et al.,2007 |
| *vp28* | 555 | 55 | F-5’CTGCTGTGATTGCTGTATTT3’<br>R-5’CAGTGCCAGAGTAGGTGAC3’ | Liu et al.,2005 |
| Caspase | 827 | 57 | F-5’GGAGGAACCTGCGAAGAA3’<br>R-5’AGCGTCGAGTGGATGTAAG3’ | Wongprasert et al.,2007 |

| | | | | |
|---|---|---|---|---|
| Crustin | 430 | 55 | F-5’CGCACAGCCGAGAGAAACACT3’ | GQ334395.1 |
| | | | R-5’GGCCTATCCCTCAGAACCCA3, | |
| Penaeidin-3 | 240 | 55 | F-5’AGGATATCATCCAGTTCCTG3’ | JX961662.1 |
| | | | R-5’ACCTACATCCTTTCCACAAG3’ | |
| Astakine | 455 | 56 | F-5’GTCGCGCATTTAACAAGGAG3’ | EU980446.1 |
| | | | R-5’CCCTGTGGATTGAGCTCACT3’ | |
| Syntenin | 750 | 55 | F-5’GATTTGGCCGTCTCACA3’ | AF335106.1 |
| | | | R-5’GCCTTAAGTTACAGGTCGG3’ | |
| PmRACK-1 | 937 | 60 | F-5’CTGCGCGGGACCCTGGTG3’ | KF041001.1 |
| | | | R-5’CACGGGAAGTAACGCTGACCT3’ | |
| Rab7 | 887 | 55 | F-5’TTCCCTCCCAAAGTACAT3’ | DQ231062.1 |
| | | | R-5’AGGCCAATCCCCATGTGAA3’ | |
| STAT | 620 | 55 | F-5’AGCCCCTGTCTGAGCGAA3’ | EU367985.1 |
| | | | R-5’CTGAGGCTTCATGAAGTTGG3’ | |
| TLR | 670 | 56 | F-5’CTGAGAAACAACAGTTTG3’ | GU014556.1 |
| | | | R-5’GCATTTTGAAATCGAGCG 3’ | |
| C-type lectin | 546 | 57 | F-5’CGTGGATCCCAACCTCTTCAGG3’ | DQ078266.1 |
| | | | R-5’CGTGAATTCAGCATTGTACTG3’ | |

**Figure 1**

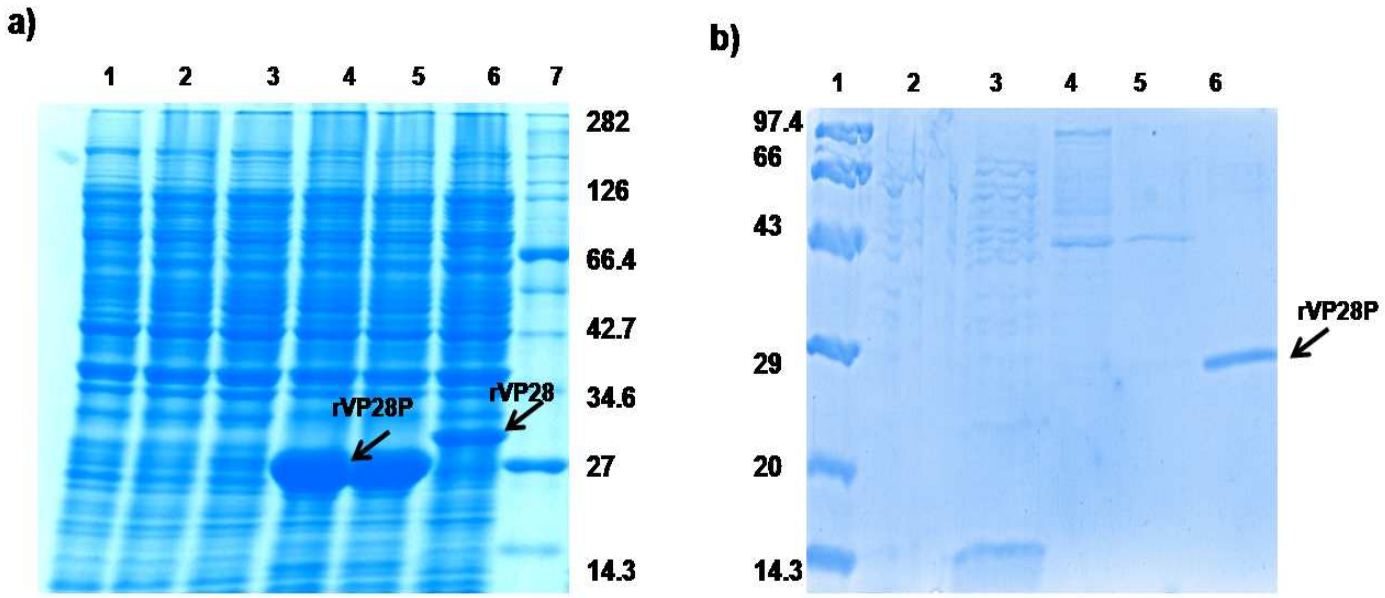
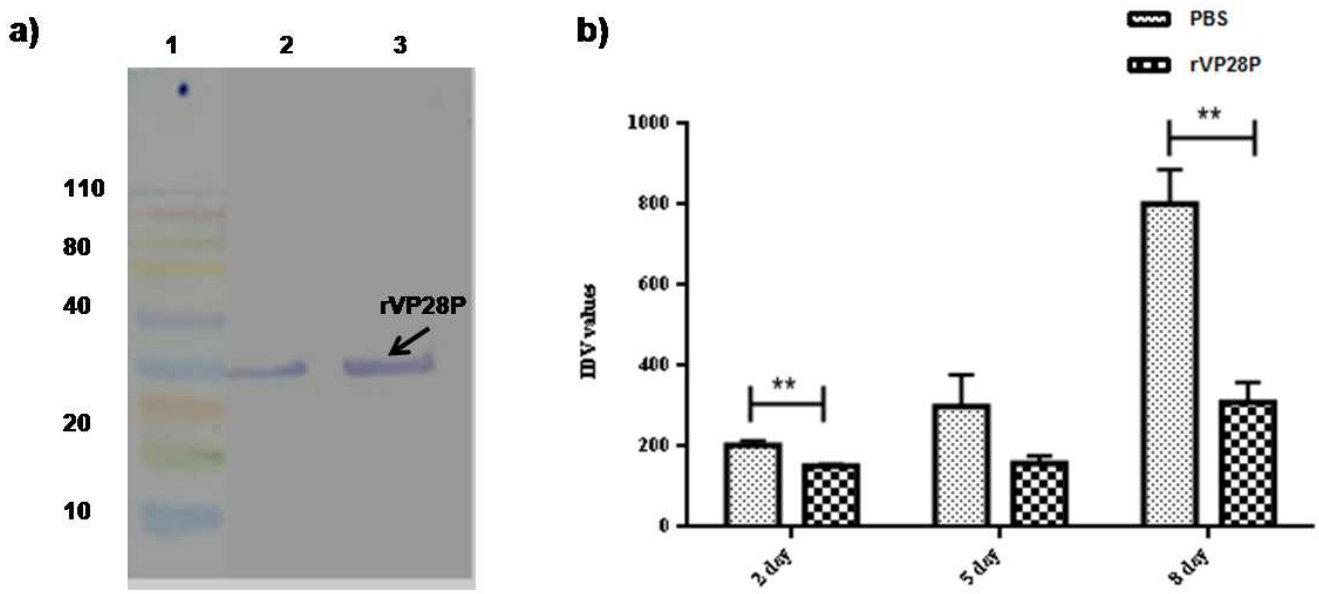



**Fig. 1(a)** SDS-PAGE of rVP28 and rVP28P expression in *E. coli* BL21. Lane 1-*E. coli* BL21 host induced, Lane 2-pET28a *E. coli* BL21 induced, Lane 3-VP28P pET28a+ uninduced, Lane 4 to 5-rVP28P expressed in *E. coli* BL21, Lane 6- rVP28 expressed in *E. coli* BL21, Lane 7-Protein molecular weight marker (kilodaltons). **1(b)** SDS-PAGE of the purified rVP28P by immobilised metal affinity chromatography. Lane 1-Protein molecular weight marker (kilodaltons), Lane 2-Flow through fraction containing the unbound proteins, Lane 3-Wash fraction that removes all unbound proteins, Lane 4 & 5- Eluted fraction in 100 and 200 mM imidazole fractions, Lane 6-Purified rVP28P eluted in 250 mM imidazole.

**Figure 2**




**2(a)** Western blot for the confirmation of expression and purification of rVP28P. Protein resolved in 12% SDS-PAGE gel transferred onto nitrocellulose membrane. Anti-HIS monoclonal antibody (1:20000) was used as primary antibody. Secondary anti mouse antibody-ALP (1:10000) was detected using BCIP-NBT substrates. Lane 1-Protein molecular weight marker (kilodaltons), Lane 2-rVP28P expressed in *E. coli* BL21, Lane 3-rVP28P purified by immobilised metal affinity chromatography. **2(b**) Expression of WSSV gene *vp28* in the rVP28P administered *P. monodon* juveniles compared with the PBS administered group as control. Animals were collected at different time points (2, 5 and 8 day) post challenge and *vp28* transcripts were assessed by semi-quantitative RT-PCR. The expression level in the PBS and rVP28P administered animals were calculated as the mean of the average integrated density values (IDV) of the amplicons relative to the expression reference gene (EF-5A). (* indicates the level of significance; *-$p \leq 0.05$, **- $p \leq 0.01$ & ***- $p \leq 0.001$).

**Figure 3**

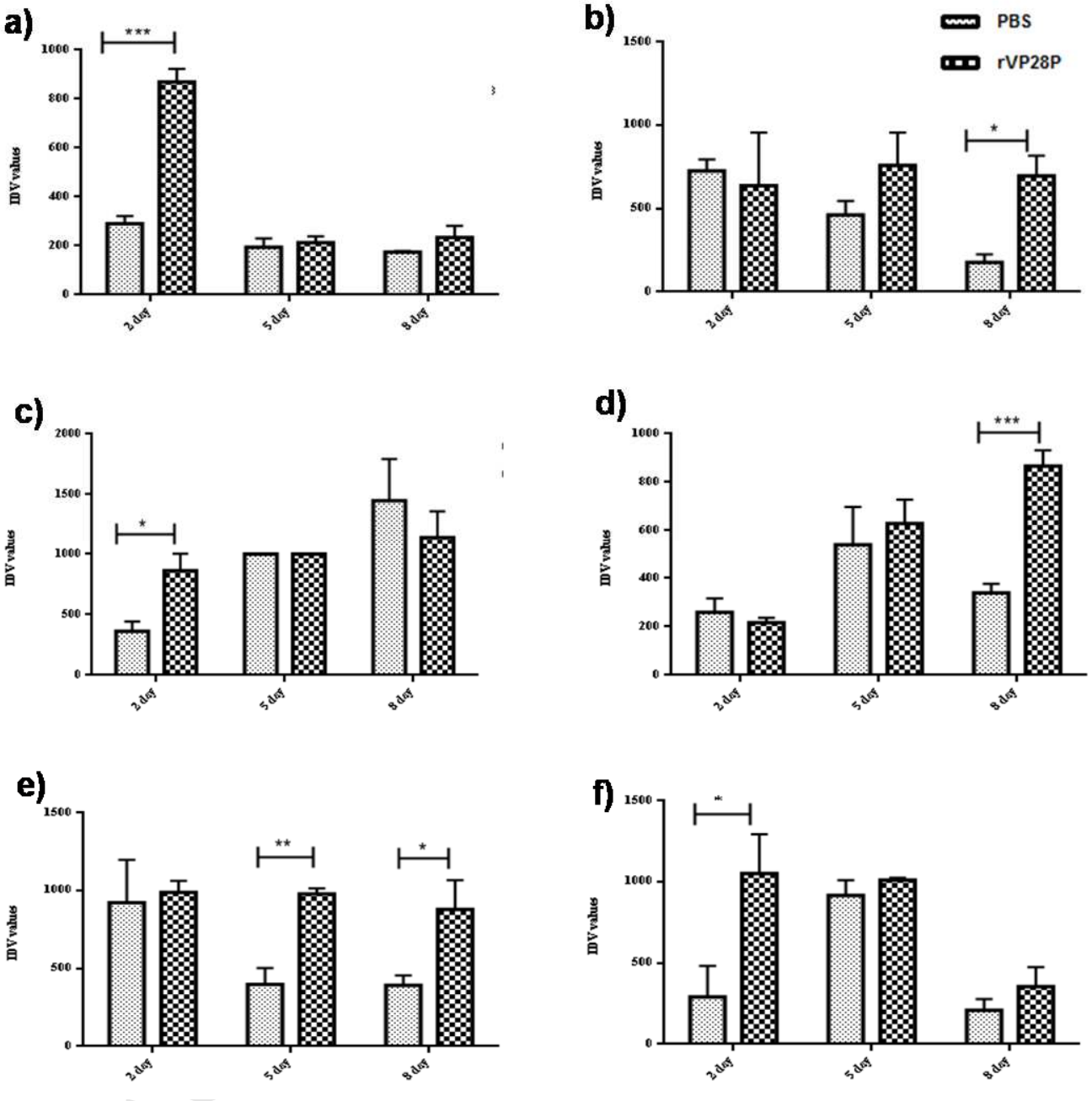



**Fig. 3)** Expression of immune related genes a) Caspase, b) Crustin, c) Penaeidin, d) Astakine, e) Syntenin and f) PmRACK in rVP28P administered animals compared with the PBS administered group as control on 2nd, 5th and 8th days post administration (dpa) as assessed by semi-quantitative RT-PCR. The expression level was calculated as mean of the average integrated density values (IDV) of the amplicons relative to the expression of reference gene (EF-5A) in the Y-axis and the data are expressed as mean ± SD of three individual animals. (* indicates the level of significance; *-$p \leq 0.05$, **- $p \leq 0.01$ & ***- $p \leq 0.001$).

**Figure 4**

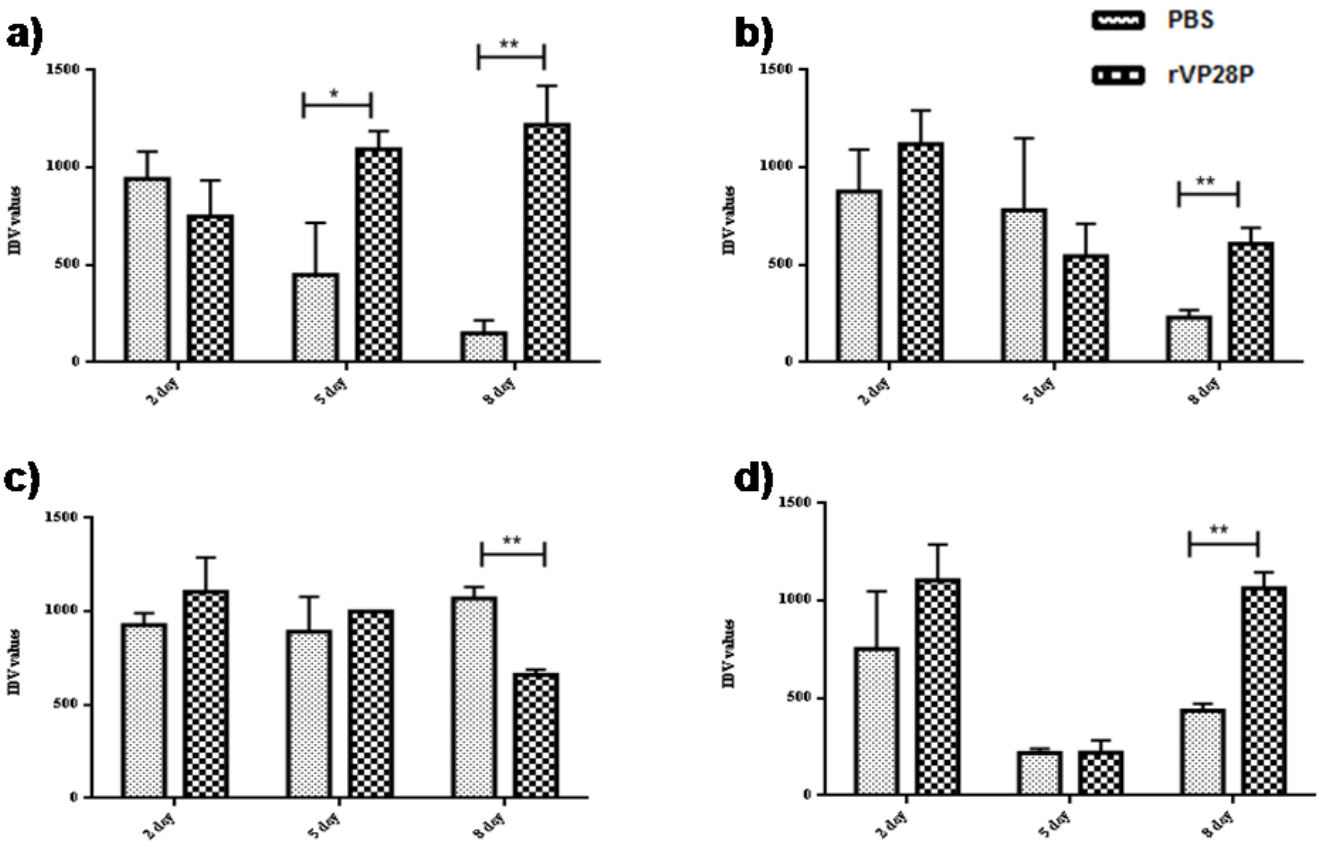



**Fig. 4)** Expression levels of immune related genes a) Rab7, b) STAT, c) TLR and d) C-type lectin in rVP28P administered animals compared with the PBS administered group as control on $2^{nd}$, $5^{th}$ and $8^{th}$ days post administration (dpa) as assessed by semi-quantitative RT- PCR. The expression level was calculated as mean of the average integrated density values (IDV) of the amplicons relative to the expression of reference gene (EF-5A) in the Y-axis and the data are expressed as mean ± SD of three individual animals. (* indicates the level of significance; *- $p \leq 0.05$, **- $p \leq 0.01$ & ***- $p \leq 0.001$).

**Figure 5**

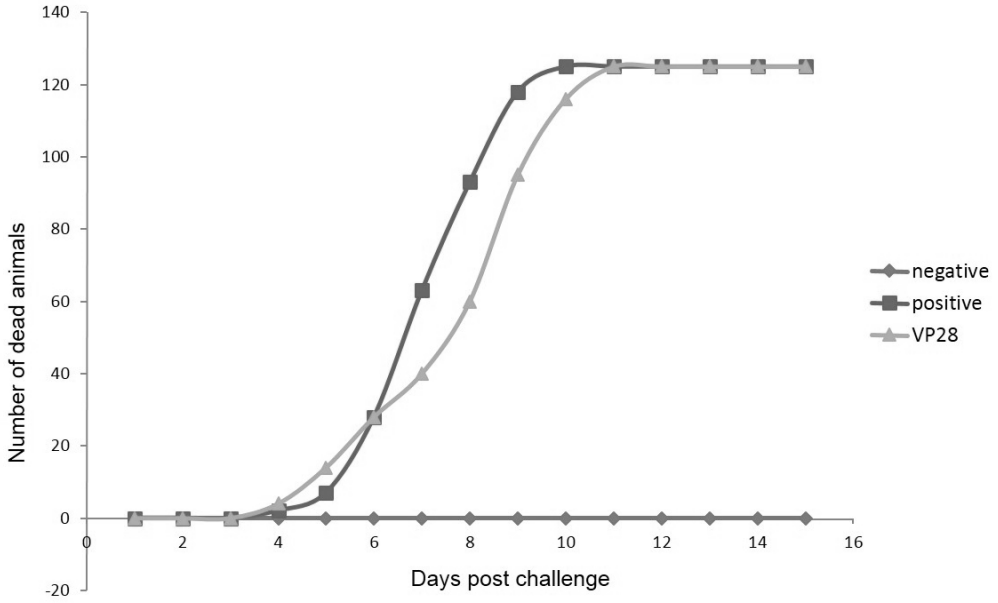



**Fig.5)** Time-mortality relationship of animals administered with rVP28P after WSSV challenge. Cumulative mortality rates of the *P. monodon* juveniles from the experimental groups, rVP28P administered and challenged as test (VP28), PBS administered and challenged as positive control (positive) and unvaccinated unchallenged as negative control (negative) are plotted against day post challenge. Each experimental group has 3 tanks with 125 animals in each as indicated in the Table 1.

**Highlights**

- WSSV recombinant envelope protein VP28 was produced as truncated protein and its immune modulatory effects were assessed at molecular level.

- Purified rVP28P was formulated as oral vaccine and administered to shrimp juveniles.

- Up-regulation of syntenin caspase, penaeidin, crustin, astakine, syntenin, PmRACK, Rab7, STAT and C-type lectin after the administration of rVP28P points to the ability of rVP28P to modulate the shrimp immune system.

- Lesser expression of WSSV gene *vp28* in the protein administered animals and delayed mortality of animals in the subsequent viral challenge reveals the efficacy of rVP28P as a vaccine.