Accepted Manuscript

Expression profile of key immune-related genes in *Penaeus monodon* juveniles after oral administration of recombinant envelope protein VP28 of white spot syndrome virus

Ancy Thomas, Naduvilamuriparampu Saidumuhammed Sudheer, Viswanath Kiron, Issac S. Bright Singh, Rangarajan Badri Narayanan

Please cite this article as: Thomas A, Sudheer NS, Kiron V, Bright Singh IS, Narayanan RB, Expression profile of key immune-related genes in *Penaeus monodon* juveniles after oral administration of recombinant envelope protein VP28 of white spot syndrome virus, *Microbial Pathogenesis* (2016), doi: 10.1016/j.micpath.2016.05.002.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
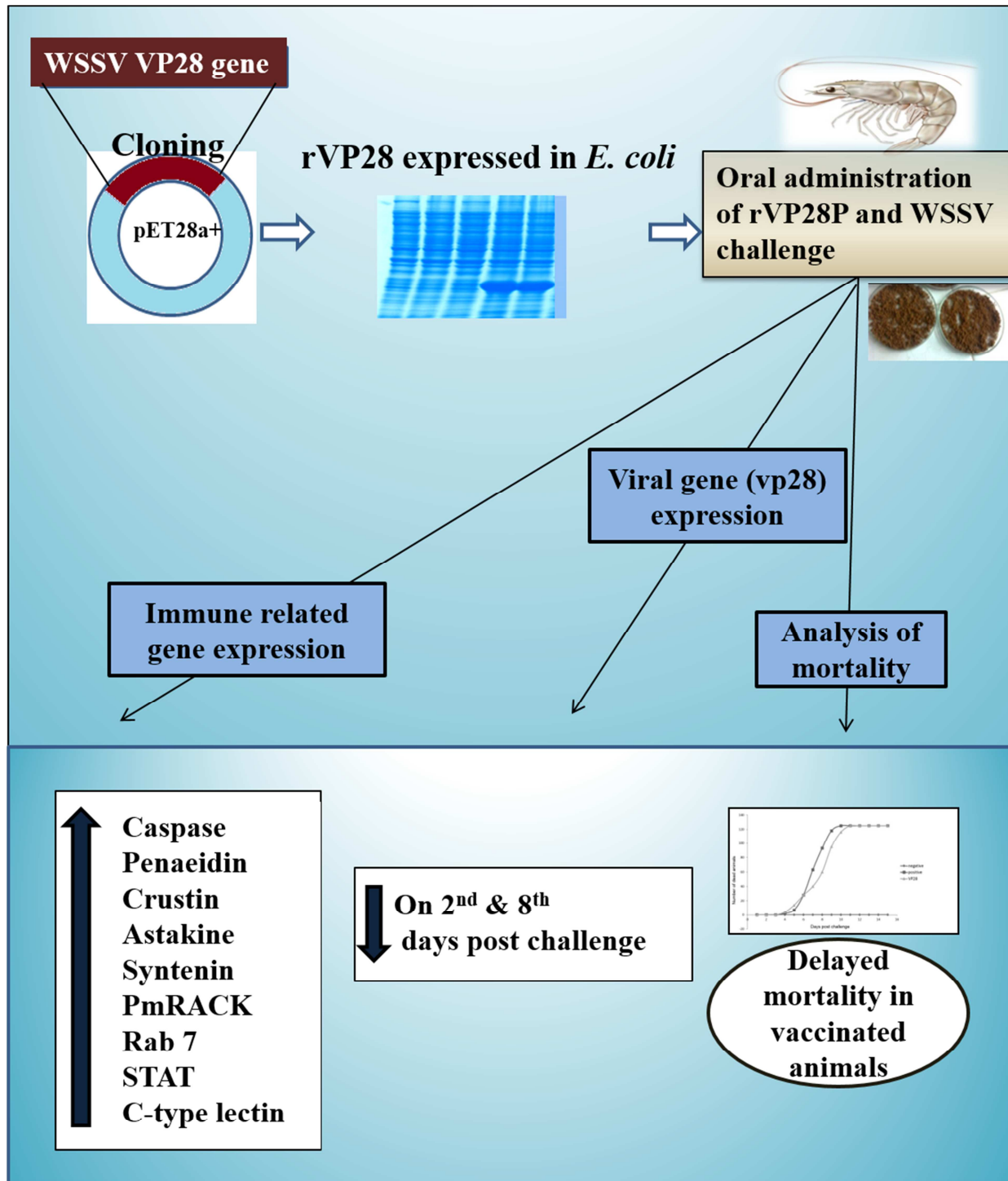
1 **Expression profile of key immune-related genes in *Penaeus monodon* juveniles after oral**

2 **administration of recombinant envelope protein VP28 of White Spot Syndrome Virus**

3 Ancy Thomas[1], Naduvilamuriparampu Saidumuhammed Sudheer[2], Viswanath Kiron [3], Issac

4 S Bright Singh[2], Rangarajan Badri Narayanan[1*]

5 [1] Centre for Biotechnology, Anna University, Chennai, India

6 E-Mails: anci.thomas@gmail.com, rbn@annauniv.edu

7 [2] National Centre for Aquatic Animal Health, Cochin University of Science and Technology,

8 Cochin, India

9 E-Mails: cusatmarine@gmail.com, isbsingh@gmail.com

10 [3] Faculty of Biosciences and Aquaculture, University of Nordland, 8049 - Bodø, Norway

11 E-Mail: kiron.viswanath@hibo.no

12 **Abstract**

13 White spot syndrome virus (WSSV) is the most catastrophic pathogen the shrimp

14 industry has ever encountered. VP28, the abundant envelope protein of WSSV was expressed

15 in bacteria, the purified protein administered orally to *Penaeus monodon* juveniles and its

16 immune modulatory effects examined. The results indicated significant up-regulation of

17 caspase, penaeidin, crustin, astakine, syntenin, PmRACK, Rab7, STAT and C-type lectin in

18 animals orally administered with this antigen. This revealed the immune modulations in

19 shrimps followed by oral administration of rVP28P which resulted in the reduced

20 transcription of viral gene *vp28* and delay in mortality after WSSV challenge. The study

21 suggests the potential of rVP28P to elicit a non-specific immune stimulation in shrimps.

22 **1. Introduction**

23 Penaeid shrimp, one of the most economically important crustaceans, faces significant

24 outbreaks of many diseases that have damaged aquaculture. White spot syndrome virus

25 (WSSV) is the most catastrophic pathogen the shrimp industry has ever encountered [1,2].

26 Since the first report of WSSV in 1993, the major concern of world aquaculture industry has

27 been to stave off this disease emerging in aquaculture settings [3]. There is increasing

28 evidence based on laboratory trials that the administration of antigens or immunostimulants is

29 a promising approach to stimulate shrimp immune system against viral or bacterial infections.

30 Administration of inactivated whole virus, oral recombinant protein antigens and DNA and

31 dsRNA molecules provides remarkable protection to shrimp from WSSV as reviewed by

32 Rowley and Pope [4] and Haq et al. [5].

33   Several investigations suggest that the administration of recombinant WSSV proteins,

34 particularly VP28, imparts a form of immune stimulation, described as immune priming,

35 resulting in increased survival of shrimps in subsequent virus infections [6]. VP28 is the

36 major envelope protein of WSSV that expedites entry of the virion into shrimp cells [7]. The

37 mRNA transcription of WSSV genes was delayed for 4~10 days in various organs of shrimp

38 vaccinated with recombinant VP28 (rVP28) [8]. However, the underlying mechanism by

39 which rVP28 activates the shrimp defense system remains unknown.

40   The crustacean immune system eliminates pathogens very efficiently through humoral

41 and cellular immune processes. The biological defense molecules produced in shrimps in

42 response to the invading pathogens include proteins and peptides related to immunity,

43 homeostasis, host pathogen interaction and other cellular processes. Identification and

44 functional analysis of immune related genes such as AMPs, proteinases and their inhibitors,

45 anti-apoptotic proteins, pattern recognition receptors (PRRs) revealed its specific roles in

46 shrimp defense system and in disease process [9]. Shrimps evolved to use diverse

47 antimicrobial peptides (AMPs) and proteins with diversity in structure and function as the

48 first-line of innate immune response [10]. The identification of thousands of high quality

49 Expressed Sequence Tags (EST) in shrimps suggested that WSSV infection modulates the

50 genes involved in several cellular and metabolic immune processes in post larvae [11].

51 Administration of immune stimulants alters the expression of wide range of cellular and

52 immune related genes and triggers the innate immune reactions in shrimps by upregulation of

53 penaeidin, lysozyme, crustin and JAK-STAT pathway genes and downregulation of AMPs

54 and clotting related proteins in WSSV resistant animals [12,13].

55   The high frequency of occurrence and differential expression of immune-related

56 genes in shrimps infected with WSSV, *Vibrio harveyi* or administered with probiotics further

57 emphasize the molecular changes during an elevated immune response [14,15]. Earlier

58 reports also demonstrated enhanced expression of few immune-related genes upon VP28

59 based DNA vaccination [16,17]. However, immune modulations at molecular level upon

60 VP28 administration in shrimps [6] is scanty, which point to the relevance of the present

61 study. Genes associated with immune functions such as pathogen recognition, clotting,

2

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,

92 assessed for the presence of bacterial endo-toxin using Limulus Amebocyte Lysate (LAL)

93 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

95 2.2.1 Experimental animals

96 Specific Pathogen Free (SPF) *P. monodon* juveniles (PL 40) maintained in a

97 recirculating aquaculture system developed by National Centre for Aquatic Animal Health,

98 Cochin University of Science and Technology, Cochin, Kerala, India was used for the study.

99 The WSSV-free status of animals was tested by nested PCR as described by Lo et al. [18]

100 using viral DNA extracted from whole animal. The shrimps were transferred to rectangular

101 fiber reinforced plastic (FRP) tanks of 30 litre capacity prior to the experiment and

102 acclimatized to 20 g L^{-1} salinity filtered sea water at 28 ± 1 $^{\circ}$C, having total ammonia < 0.1

103 ppm, alkalinity 70-80 ppm at pH 8.4, for one week. The animals were maintained at this

104 optimum conditions through partial exchange of water whenever required. During this period,

105 shrimps were fed with a commercial feed (Amalgam Nutrients and Feeds Limited, Cochin,

106 India) at the rate of 10% body weight per day.

107 2.2.2 Preparation of rVP28P protein coated feed

108 rVP28P was produced in large scale and a quantity of 100 µg purified rVP28P

109 blended with 1ml phosphate buffered saline (PBS-pH 7.4). This was mixed with 4 g (at the

110 rate of 25 µg protein g $^{-1}$ feed) commercial shrimp feed pellets (Amalgam Nutrients and

111 Feeds Limited, Cochin, India) and incubated overnight at 4 $^{\circ}$C to allow the absorption of

112 protein. The pellets were further coated with a commercial binder (Trubind, Wockhardt,

113 Mumbai, India) to prevent dispersion of the protein in water and maintained at 4 $^{\circ}$C.

114 Similarly, control feed was prepared by soaking in PBS and coating with the same

115 commercial binder.

116 2.2.3 Oral administration of rVP28P and WSSV challenge

117 Four groups of animals (groups of 125) each animal weighing 2-4 g were used for the

118 experiment and the design was as shown in Table 1. Feed mixed with PBS alone was

119 administered to two batches of animals (PBS 1 and PBS 2) and maintained as controls and

120 two batches administered with rVP28P in PBS (rVP28P 1 and rVP28P 2) served as the tests.

121 All the animals were starved for 6 hr and offered PBS and rVP28P dissolved in PBS as

4

122   control and test feeds respectively. The feeding rate was of 10% of the body weight twice a

123   day for a period of ten days and thereafter, resorted to normal commercial feed. On 1st day

124   post administration of the coated feed, a batch of rVP28P (rVP28P 2) and PBS administered

125   (PBS 2) animals were challenged with WSSV by feeding freshly infected tissue (soft tissue

126   from cephalothorax confirmed WSSV positive through nested PCR) at a rate 10% of the body

127   weight [19]. The uneaten WSSV infected tissue pieces were removed from the tanks after 4

128   hours of feeding along with complete replacement of water with fresh sea water. The

129   percentage survival of the rVP28P administered animals at the event of challenge with WSSV

130   compared with that in the control group was recorded.

131   **2.3 Tissue sampling, RNA extraction and cDNA synthesis**

132   Animals survived were collected from unchallenged and WSSV challenged groups on

133   2nd, 5th and 8th days post administration (dpa) and post challenge (dpc) respectively. Three

134   batches of animals comprising three animals each from each group were collected from tests

135   and controls, euthanized by immersing in ice flakes, washed with diethyl pyrocarbonate

136   (DEPC) treated water, preserved in Tri- Reagent (Sigma, USA) and maintained at -80 °C.

137   Total RNA was extracted individually from each whole animal of all experimental groups and

138   converted to cDNA within 12 hr of collection of samples under RNase free condition. The

139   total RNA was isolated using TRI reagent (Sigma, St. Louis, MO, USA) following

140   manufacturer's protocol with slight modifications. Briefly, the samples were homogenized

141   and 200 μL chloroform was added per ml of TRI reagent. The RNA was precipitated with

142   70% isopropyl alcohol, dissolved in 20 μL RNase free water followed by DNase I treatment

143   at 37 °C. Further, the quality and quantity of RNA was determined using UV–visible

144   spectrophotometer and confirmed using Qubit RNA assay kit (Life technologies, USA). RNA

145   was subjected to electrophoresis on denaturing gel (5% polyacrylamide gel in Tris -borate

146   EDTA (TBE buffer) containing 7 M urea) to assess the integrity of RNA. Immediately after

147   RNA extraction, 5 μg RNA was reverse transcribed to cDNA using Moloney Murine

148   Leukemia Virus (M-MuLV) Reverse Transcriptase and oligo dT$_{(12-18)}$ (New England Biolabs,

149   MA, USA) as per the standard protocol and the quality and quantity of cDNA were also

150   assessed spectrophotometrically.

151   **2.4 Expression of immune-related genes upon rVP28P administration - semi**

152   **quantitative reverse transcriptase - polymerase chain reaction (RT-PCR)**

153   All extracted RNA samples had Abs_{260}/Abs_{280} ratio of 1.8-2.0 in water and were used

154   for cDNA preparation from 5 μg of total RNA. The 25 μL PCR mixture with 200 μM dNTPs,

155   10 pmol each forward and reverse primers, 1 X PCR buffer (Thermopol), 0.5 U Taq DNA

156   Polymerase and 1 μL cDNA each were used. The cycle parameters were initial denaturation

157   at 94 ℃ for 5 min, 25-30 cycles of 94 ℃ for 30 s, annealing for 30 s, extension for 1 min at

158   72 ℃ and a final extension at 72 ℃ for 10 min. The PCR cycles and the cDNA dilution had

159   been optimized so that the target gene and reference gene amplification could be obtained at

160   logarithmic phase. In order to check the consistency, the reactions were repeated three times

161   for each gene per sample. The target gene, predicted amplicon size, cycling conditions and

162   primer sequences for each target gene and its source are presented in Table 2.

163   Shrimp elongation factor-5A (EF-5A) was used as the reference gene based on its

164   consistency in the previous experiments. The PCR products were analyzed by horizontal gel

165   electrophoresis and the average integrated density values (IDV) of the amplicons were semi-

166   quantitatively measured using Quantity One® software (Bio-Rad, Philadelphia, USA) and

167   normalized with the expression of EF-5A.

168   **2.5 Statistics**

169   Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software

170   Inc., San Diego, USA). The amplification plots were constructed and represented as mean ±

171   standard deviation of the IDV. Differences between the gene expression of the unvaccinated

172   and rVP28P vaccinated animals at three time points were calculated using non-parametric t-

173   test having $p \leq 0.05$ considered significant. Mean values of target gene expression in three

174   samples, normalized with the expression of the reference gene EF-5A are presented.

175   **3. Results**

176   **3.1 Expression and purification of rVP28P**

177   The WSSV *vp28* gene was cloned and expressed in *E. coli* BL21. The VP28 protein

178   sequence contained N-terminus hydrophobic membrane anchoring domain at amino acids 1-

179   22, as elucidated by TMpred (Data not shown). The full length ORF rVP28 (612bp) and a

180   partial reading frame VP28P (546 bp) were expressed in bacteria using pET28a expression

181   system. Bands corresponding to 27 kDa and 25 kDa of full length and truncated protein

182   respectively were visualized in an SDS-PAGE stained with Coomassie Blue G-250 (Fig.1a).

183   The truncated rVP28P expressed in large quantity as soluble protein was further purified

184 using Ni-NTA affinity chromatography, and eluted in 250 mM imidazole (Fig.1b). The

185 purified and dialyzed rVP28P exhibited reactivity with antibodies to His6X (Fig.2a). The

186 endotoxin level in the purified rVP28P was negligible (<1pg/ml of lipopolysaccharide) as

187 determined by LAL assay.

3.2 Oral administration of rVP28P and WSSV challenge

189 The animals used in the study had been confirmed WSSV free through two step

190 nested PCR for virus detection prior to the experiment. The rVP28P administered and

191 unchallenged animals did not show any sign of infection. Expression of *vp28* was

192 significantly low in rVP28P administered animals on 2nd and 8th dpc compared to the control

193 group (Fig.2b). The reduced expression of *vp28* indirectly evidenced the low proliferation of

194 WSSV in the rVP28P administered shrimps.

3.3 Expression of immune-genes in shrimps administered with rVP28P

196 Shrimps were orally administered with rVP28P for 10 days and expression of selected

197 immune genes assayed, which indicated the effect of this antigen in the immune system. The

198 statistically significant ($p < 0.05$) up and down regulations when compared with the PBS

199 control group at each time point alone have been brought under this section. Accordingly,

200 caspase transcripts were found up-regulated in the rVP28P administered animals on 2nd dpa.

201 However, it was low in both administered and non-administered groups on 5th and 8th dpa.

202 (Fig.3a). Crustin was up- regulated in the rVP28P administered group on 8th dpa (Fig. 3b).

203 Expression of penaeidin was high on 2nd dpa without any variations on other two time points,

204 as shown in Fig. 3c. Peneidin transcripts were up regulated in rVP28P administered group on

205 2nd dpa compared with that of the control group (Fig. 3c). Astakine was up regulated on 8th

206 day in the rVP28P administered group (Fig. 3d). Up regulation of syntenin on 5th and 8th dpa

207 in the rVP28P administered groups is shown in Figs 3e. A significant upregulation of

208 PmRACK was noticed on 2nd dpa. PmRab7 transcripts were up-regulated on 5th and 8th dpa (p

209 <0.05) (Fig. 4a). STAT expression was high on 8th dpa (Fig. 4b). Low level expression of

210 TLR was observed on 8th dpa compared with that of the control (Fig. 4c). C-type lectin

211 exhibited up-regulation on 8th dpa in the rVP28P administered animals (Fig. 4d) with no

212 variation between administered and control groups at any time points.

213 Among the seven immune-related genes analyzed, caspase, penaeidin, crustin,

214 astakine, syntenin, PmRACK, Rab7, STAT and C-type lectin transcripts exhibited up-

7

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

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

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

4. Discussion

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

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

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

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

8

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

245   WSSV replication [21].

246   The expression of caspase in rVP28P administered shrimps was upregulated on the

247   2nd dpa compared with PBS control. This may indicate the immediate activation of caspase

248   by the oral administration of rVP28P. Suppression of caspase gene in *M. japonicas* prior to

249   WSSV invasion led to inhibition of apoptosis and increase in WSSV copy number [22]. The

250   role of caspase in anti-WSSV immunity is also evident from the high expression of caspase in

251   shrimps infected with WSSV [23, 24]. In the present study, the high level expression of

252   caspase only on 2nd dpa indicates that the antigen administration needs further optimization to

253   maintain the enhanced expression in the rVP28P administered animals to provide prolonged

254   protection. The enhanced and prolonged caspase expression through rVP28P vaccination may

255   help the animals to resist subsequent WSSV infections.

256   The significant upregulation of crustin on 5th and 8th dpa in vaccinated animals when

257   compared with the PBS control group indicates the efficacy of rVP28P as a candidate

258   antigen to stimulate immune system. This can be further correlated with the reduced viral

259   load in the vaccinated animals as evidenced from the low gene expression of *vp28*. Crustin-

260   like AMPs were found to be constitutively expressed in shrimps and upregulated after the

261   administration of immune stimulants and WSSV challenge [25]. The significant increase of

262   crustin in the vaccinated animals indicates the effect of rVP28P vaccination in the shrimp

263   immune system by increasing the expression of AMPs.

264   The recombinant vaccine administration significantly enhanced the expression of

265   penaeidin on 2nd dpa. This peptide is constitutively synthesized and released into plasma after

266   microbial challenge [26] and, upregulated during *Vibrio* infection in post larvae [27]. These

267   studies demonstrated its role in shrimp host defense and rVP28P mediated protective

268   immunity.

269   In crayfish, *Pacifastacus leniusculus*, hematopoiesis is regulated by an invertebrate

270   cytokine astakine, critical for hemocyte count [28]. The enhanced expression of astakine after

271   rVP28P vaccination in the test animals can positively elevate the hemocyte count and

272   improve the immune status. The high levels of astakine may also contribute to the low

273   expression of *vp28* in the vaccinated animals after WSSV challenge.

274   Syntenin is an adaptor protein that links various molecules in signal transduction

275   pathway.   Previous studies reported enhanced expression of syntenin in WSSV infected

276   shrimps which declined rapidly as the infection progressed. This implied the role of syntenin

277   as an important molecule in shrimp immune response subsequent to viral infection [29]. In

278   the present study syntenin levels were significantly higher in rVP28P administered animals

279   on 5th and 8th days after rVP28P administration. The stable maintenance of high levels of

280   syntenin in the animals could attribute to the reduced severity of infection and may offer

281   protection against subsequent viral attacks.

282   PmRACK-1 was elevated in the rVP28P administered animals at all the three time

283   points compared with its PBS control with a significant upregulation on 2nd dpa. This kinase

284   receptor interacts with the VP9 viral protein of WSSV and involves in the response against

285   viral infection in *P. monodon* [30]. It can also participate in the shrimp antioxidant response

286   induced by the formation of ROS [31]. This postulates the rVP28P binding mediated signal

287   transduction activation of the kinase receptor and its role in vaccination mediated protective

288   immunity in shrimps.

289   Rab7 is a VP28 binding protein that aids the recognition and entry of WSSV into

290   shrimp cells. The real-time PCR analysis demonstrated that PmRab7 transcripts were

291   constitutively expressed during the course of WSSV infection and PmRab7-VP28 binding

292   reduces the severity of infection [32]. The enhanced expression of Rab7 on 5th and 8th dpa in

293   the rVP28P vaccinated shrimps implies that rVP28P immunization may aid the neutralization

294   of WSSV in the future viral exposures through Rab7-VP28 interaction and successive

295   immune activation.

296   The significantly increased expression of STAT gene on 8th dpa may help the immune

297   system to respond to a later infection through some unknown STAT-mediated immune

298   mechanism. WSSV uses shrimp STAT as transcription factor to enhance viral gene expression

299   [33]. Here, an increase in STAT gene expression correlates with reduced copies of *vp28*

300   transcripts after WSSV challenge. The STAT-mediated immune activation may reduce the

301   expression of *vp28* and other viral genes thus, negatively affect the WSSV establishment.

302   The innate immune system of shrimps recognizes and binds to specific patterns on the

303   surfaces of pathogens through PRRs [34] and this recognition mechanism and clearance of

304   the pathogen is the supreme part of the shrimp innate immune system. TLR is an important

305　PRR in shrimps that recognize molecules derived from microbes [35]. Down-regulation of

306　TLR in the rVP28P administered animals on 8th dpa evidences to the low significance of TLR

307　mediated immune recognition in this context. C-type lectin is also a major PRR in shrimps

308　and its reduction increases mortality of shrimps during WSSV infection [36]. The significant

309　up-regulation on 8th dpa with rVP28P vaccination may help the animals to resist virus

310　establishment in the future exposures.

311　　　Time - mortality relationship of shrimps after rVP28P administration and WSSV

312　challenge demonstrated delayed mortality of animals administered with rVP28P. Earlier

313　reports demonstrated the efficacy of VP28 as an oral vaccine which offered more than 60%

314　survival in administered animals [37,38]. In the present study, the oral administration of

315　rVP28P did not show similar trend in survival, except a delay in mortality when compared to

316　that of the controls. More investigations are required to delineate the situation.

317　　　The present study suggested significant up-regulation of caspase, penaeidin, crustin,

318　astakine, syntenin, PmRACK, Rab7, STAT and C-type lectin in the rVP28P vaccinated

319　animals. This points out the immunomodulation followed by oral administration of rVP28P in

320　*P. monodon* juveniles at molecular level. This resulted in the low expression of viral gene

321　*vp28* and enhanced resistance of shrimps to WSSV and thereby delayed mortality of animals

322　in the subsequent viral challenge. These results are supported by our earlier studies on the

323　modulation of immune-related genes in shrimp juveniles administered with rVP24 [39]. Even

324　though, shrimps lack adaptive immunity and antibodies, investigations on immune

325　modulations and the underlying mechanisms involved, can pave way for a better

326　understanding of how shrimp immune system interacts with pathogen components,

327　particularly rVP28P.

328　　　The study suggests that rVP28P alone may not protect shrimp from WSSV, and a

329　cocktail of recombinant viral proteins along with immunostimulants might accord protection

330　by enhanced elicitation of non -specific immune mechanism coupled with management of the

331　environment favorable to the animal. The study thus opens up challenging new opportunities

332　for investigation.

336

337   **Conflicts of Interest**

338   The authors declare no conflict of interest.

339   **References**

340   1) Flegel, T.W.; Major viral diseases of the black tiger prawn *Penaeus monodon* in

341   Thailand. World. *J. Microb. Biot.* 1997, 13(4), 433-442.

342   2) Lotz, J.M.; Viruses, biosecurity and specific pathogen-free stocks in shrimp

343   aquaculture. World. *J. Microb. Biot.* 1997, 13(4), 405-413.

344   3) Bachère E.; Shrimp immunity and disease control. *Aquaculture.* 2000, 191(1), 3-

345   11.

346   4) Rowley, A.F.; Pope, E.C.; Vaccines and crustacean aquaculture—A mechanistic

347   exploration. *Aquaculture.* 2012, 334, 1-11.

348   5) Haq, M.A.; Vignesh, R.; & Srinivasan, M.; Deep insight into white spot syndrome

349   virus vaccines: A review. *Asian Pac. J. Trop. Dis.* 2012, 2(1), 73-77.

350   6) Mavichaak, R.; Kondo, H.I.D.E.H.I.R.O.; Hirono, I.K.U.O.; & Aoki, T.A.K.A.

351   S.H.I.; The utilization of VP28 gene to protect penaeid shrimps from white spot

352   syndrome virus disease: a review. *Dis. Aqua.* 2011, 7, 157-169.

353   7) Tsai, J.M..; Wang H.C.; Leu, J.H.; Wang, A.H.J.Y.; Zhuang, P.J.; Walker, G.H.

354   Kou.; & Lo, C.F.; Identification of the nucleocapsid, tegument, and envelope

355   proteins of the shrimp white spot syndrome virus virion. *J. Virol.* 2006, 80, 3021-

356   3029.

357   8) Choi, M.R.; & Jang, J.S.; Transcriptional analysis for oral vaccination of

358   recombinant viral proteins against white spot syndrome virus (WSSV) in

359   *Litopenaeus vannamei. J. Microbiol. Biotechnol.* 2011, 21(2), 170-175.

360   9) Tassanakajon, A.; Somboonwiwat, K.; Supungul, P.; & Tang, S.; Discovery of

361   immune molecules and their crucial functions in shrimp immunity. *Fish. Shellfish.*

362   *Immun.* 2013, 34(4), 954-967.

363   10) Tassanakajon, A.; Amparyup, P.; Somboonwiwat, K.; & Supungul, P.; Cationic

364   antimicrobial peptides in penaeid shrimp. *Mar. Biotechnol.* 2011, 13(4), 639-657.

365   11) Leu, J.H., Chang, C.C., Wu, J.L., Hsu, C.W., Hirono, I., Aoki, T., Juan, H.F., Lo,

366   C.F., Kou, G.H. & Huang, H.C.; Comparative analysis of differentially expressed

genes in normal and white spot syndrome virus infected *Penaeus monodon. BMC genomics.* 2007, 8(1), 120.

12) Fagutao, F.F., Yasuike, M., Caipang, C.M., Kondo, H., Hirono, I., Takahashi, Y. & Aoki, T., Gene expression profile of hemocytes of kuruma shrimp, *Marsupenaeus japonicus* following peptidoglycan stimulation. *Mar. Biotechnol.* 2008, 10(6), 731-740.

13) Wongpanya, R., Aoki, T., Hirono, I., Yasuike, M. & Tassanakajon, A., Analysis of gene expression in haemocytes of shrimp *Penaeus monodon* challenged with white spot syndrome virus by cDNA microarray. *Sci. Asia.* 2007,33, 165-174.

14) Pope, E.C.; Powell, A.; Roberts, E.C.; Shields, R.J.; Wardle, R.; & Rowley, A.F.; Enhanced cellular immunity in shrimp *Litopenaeus vannamei* after 'vaccination'. *Plos one.* 2011, 6(6), e20960.

15) Tseng, D.Y.; Ho, P.L.; Huang, S.Y.; Cheng, S.C.; Shiu, Y.L.; Chiu, C.S.; & Liu, C.H.; Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei* by the probiotic *Bacillus subtilis* E20. *Fish..Shellfish.. Immun..*2009, 26(2), 339-344.

16) Kono, T.; Fall, J.; Korenaga, H.; Takayama, H.; Iizasa, T.; Mekata, T.; ... & Sakai, M.; Immunomodulation by DNA vaccination against white spot syndrome virus (WSSV). *Asian. Fish. Sci.* 2010, 23, 435-446.

17) Rajesh Kumar, S.; Ishaq Ahamed, V.P.; Sarathi, M.; Nazeer Basha, A.; & Sahul Hameed, A.S.; Immunological responses of *Penaeus monodon* to DNA vaccine and its efficacy to protect shrimp against white spot syndrome virus (WSSV). *Fish. Shellfish. Immun.* 2008, 24(4), 467-478.

18) Lo, C.F.; Leu, J.H.; Ho, C.H.; Chen, C.N.; Peng, S.E.; Chen, Y.T. .... & Kou, G.H., Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis. Aquat. Org.* 1996b, 25, 133-141.

19) Sudheer, N.S.; Philip, R.; & Bright Singh, I.S.; Anti-white spot syndrome virus activity of *Ceriops tagal* aqueous extract in giant tiger shrimp *Penaeus monodon. Arch. Virol.* 2012, 157, 1667-1675.

20) Robalino, J.; Almeida, J. S.; McKillen, D.; Colglazier, J.; Trent III, H. F.; Chen, Y. A.; .. & Gross, P. S.; Insights into the immune transcriptome of the shrimp

Litopenaeus vannamei: tissue-specific expression profiles and transcriptomic responses to immune challenge. *Physiol. genomics.* 2007, 29(1), 44-56.

21) Sarathi, M.; Simon, M.C.; Venkatesan, C.; & Hameed, A.S.; Oral administration of bacterially expressed VP28dsRNA to protect *Penaeus monodon* from white spot syndrome virus. *Mar. Biotechnol.* 2008, 10(3), 242-249.

22) Wang, L.; Zhi, B.; Wu, W.; & Zhang, X.; Requirement for shrimp caspase in apoptosis against virus infection. *Dev. Comp. Immunol.* 2008, 32(6), 706-715.

23) Phongdara, A.; Wanna, W.; & Chotigeat, W.; Molecular cloning and expression of caspase from white shrimp *Penaeus merguiensis. Aquaculture.* 2006, 252(2), 114-120.

24) Wongprasert, K.; Sangsuriya, P.; Phongdara, A.; & Senapin, S.; Cloning and characterization of a caspase gene from black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus (WSSV). *J. Biotechnol.* 2007, 131(1), 9-19.

25) Antony, S. P.; Singh, I. S.; Sudheer, N. S.; Vrinda, S.; Priyaja, P.; & Philip, R.; Molecular characterization of a crustin-like antimicrobial peptide in the giant tiger shrimp, *Penaeus monodon*, and its expression profile in response to various immunostimulants and challenge with WSSV. *Immunobiology.* 2011, 216(1), 184-194.

26) Destoumieux, D.; Muñoz, M.; Cosseau, C.; Rodriguez, J.; Bulet, P.; Comps, M.' & Bachère, E.; Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. *J. Cell. Sci.* 2000, 113(3), 461-469.

27) Nayak, S., Singh, S.K., Ramaiah, N. & Sreepada, R.A.; Identification of upregulated immune-related genes in *Vibrio harveyi* challenged *Penaeus monodon* postlarvae. *Fish. Shellfish. Immun*. 2010, 29(3), 544-549.

28) Lin, X.; Söderhäll, K.; & Söderhäll, I.; Invertebrate hematopoiesis: an astakine-dependent novel hematopoietic factor. *J. Immunol.* 2011, 186(4), 2073-2079.

29) Bangrak, P.; Graidist, P.; Chotigeat, W.; Supamattaya, K.; & Phongdara, A.; A syntenin-like protein with postsynaptic density protein (PDZ) domains produced by black tiger shrimp *Penaeus monodon* in response to white spot syndrome virus infection. *Dis. Aquat. Org*. 2002, 49(1), 19-25.

30) Tonganunt, M.; Saelee, N.; Chotigeat, W.; & Phongdara, A.; Identification of a receptor for activated protein kinase C1 (Pm-RACK1), a cellular gene product from black tiger shrimp (*Penaeus monodon*) interacts with a protein, VP9 from the white spot syndrome virus. *Fish. Shellfish. Immun*. 2009, 26(3), 509-514.

31) Saelee, N.; Tonganunt-Srithaworn, M.; Wanna, W.; & Phongdara, A.; Receptor for Activated C Kinase-1 protein from *Penaeus monodon* (Pm-RACK1) participates in the shrimp antioxidant response. *Int. J. Biol. Macromol.* 2011, 49(1), 32-36.

32) Sritunyalucksana, K.; Wannapapho, W.; Lo, C.F.; & Flegel, T.W.; PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp. *J. Virol.* 2006, 80, 10734–10742.

33) Liu, W.J.; Chang, Y.S.; Wang, A.H.J.; Kou, G.H.; & Lo, C.F.; White spot syndrome virus annexes a shrimp STAT to enhance expression of the immediate-early gene ie1. *J. Virol.* 2007, 81(3), 1461-1471.

34) Vazquez, L.; Alpuche, J.; Maldonado, G.; Agundis, C.; Pereyra-Morales, A.; & Zenteno, E.; Review: immunity mechanisms in crustaceans. *Innate. Immun.* 2009, 15(3), 179-188.

35) Li, F.; & Xiang, J.; Signaling pathways regulating innate immune responses in shrimp. *Fish. Shellfish. Immun*. 2013, 34(4), 973-980.

36) Ma, T.H.T.; Tiu, S.H.K.; He, J.G.; & Chan, S.M.; Molecular cloning of a C-type lectin (LvLT) from the shrimp *Litopenaeus vannamei*: Early gene down-regulation after WSSV infection. *Fish. Shellfish. Immun*. 2007, 23(2), 430-437.

37) Witteveldt, J.; Vlak., J.M. & van Hulten, M.C.; Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. *Fish Shellfish Immun*. 2004a, 16(5), 571-579.

38) Witteveldt, J.; Cifuentes, C.C.; Vlak, J.M; & van Hulten, M.C. Protection *of Penaeus monodon* against white spot syndrome virus by oral vaccination. *J. Virol*. 2004b, 78(4), 2057-2061.

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

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 (^{o}C)	Primer sequence	Reference/GenBank Accession Number
EF-5A	737	55	F-5'GCTCTCTCGCTCCTCCTTTCA3' R-5'CCCATGGACAAAGCAAAGG3'	Loongyai et al.,2007
vp28	555	55	F-5'CTGCTGTGATTGCTGTATTT3' R-5'CAGTGCCAGAGTAGGTGAC3'	Liu et al.,2005
Caspase	827	57	F-5'GGAGGAACCTGCGAAGAA3' 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
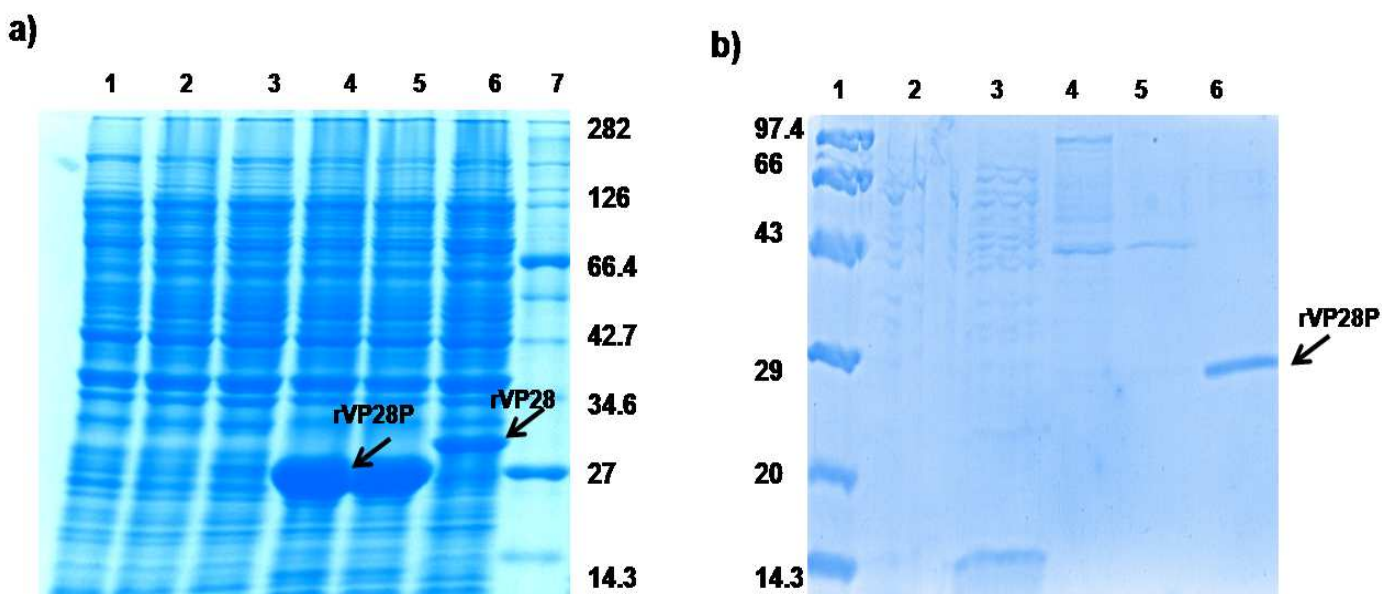
Fig. 1(a) SDS-PAGE of rVP28 and rVP28P expression in *E. coli* BL21. Lane 1-*E. coli* BL21 host induced, Lane 2-pET28a *E. coli* BL21 induced, Lane 3-VP28P pET28a+ uninduced, Lane 4 to 5-rVP28P expressed in *E. coli* BL21, Lane 6- rVP28 expressed in *E. coli* BL21, Lane 7- Protein molecular weight marker (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
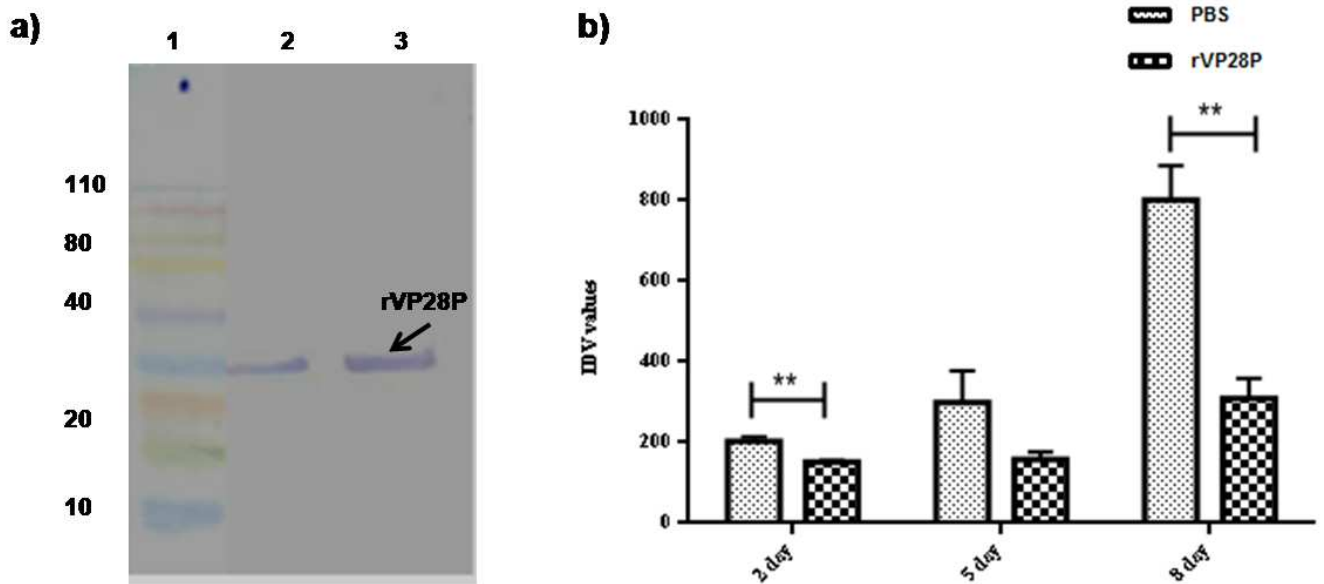
2(a) Western blot for the confirmation of expression and purification of rVP28P. Protein resolved in 12% SDS-PAGE gel transferred onto nitrocellulose membrane. Anti-HIS monoclonal antibody (1:20000) was used as primary antibody. Secondary anti mouse antibody-ALP (1:10000) was detected using BCIP-NBT 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; *-$p \leq 0.05$, **- $p \leq 0.01$ & ***- $p \leq 0.001$).
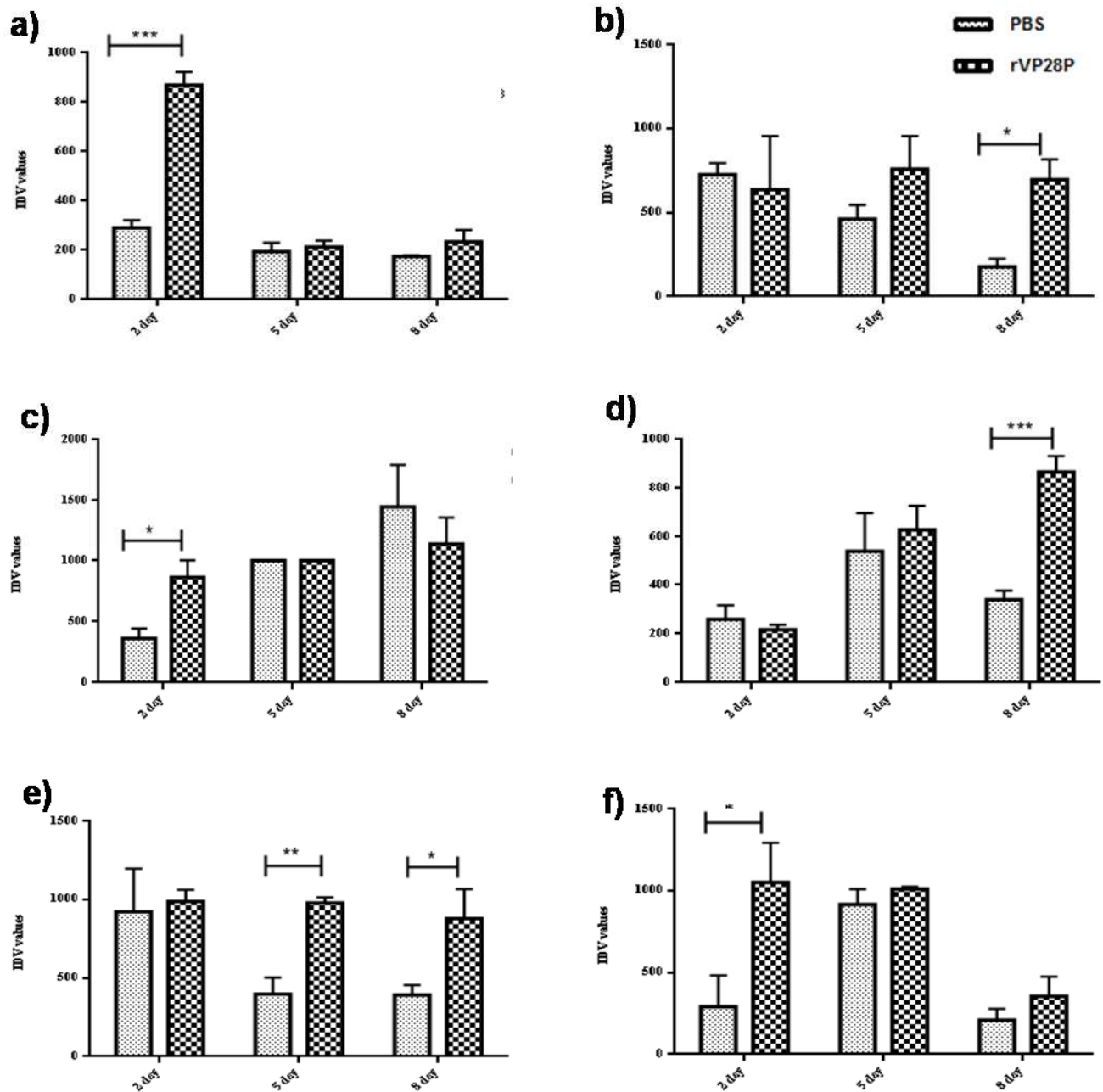
Figure 3

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 $\pm$ SD of three individual animals. (* indicates the level of significance; *-$p \leq 0.05$, **- $p \leq 0.01$ & ***- $p \leq 0.001$).
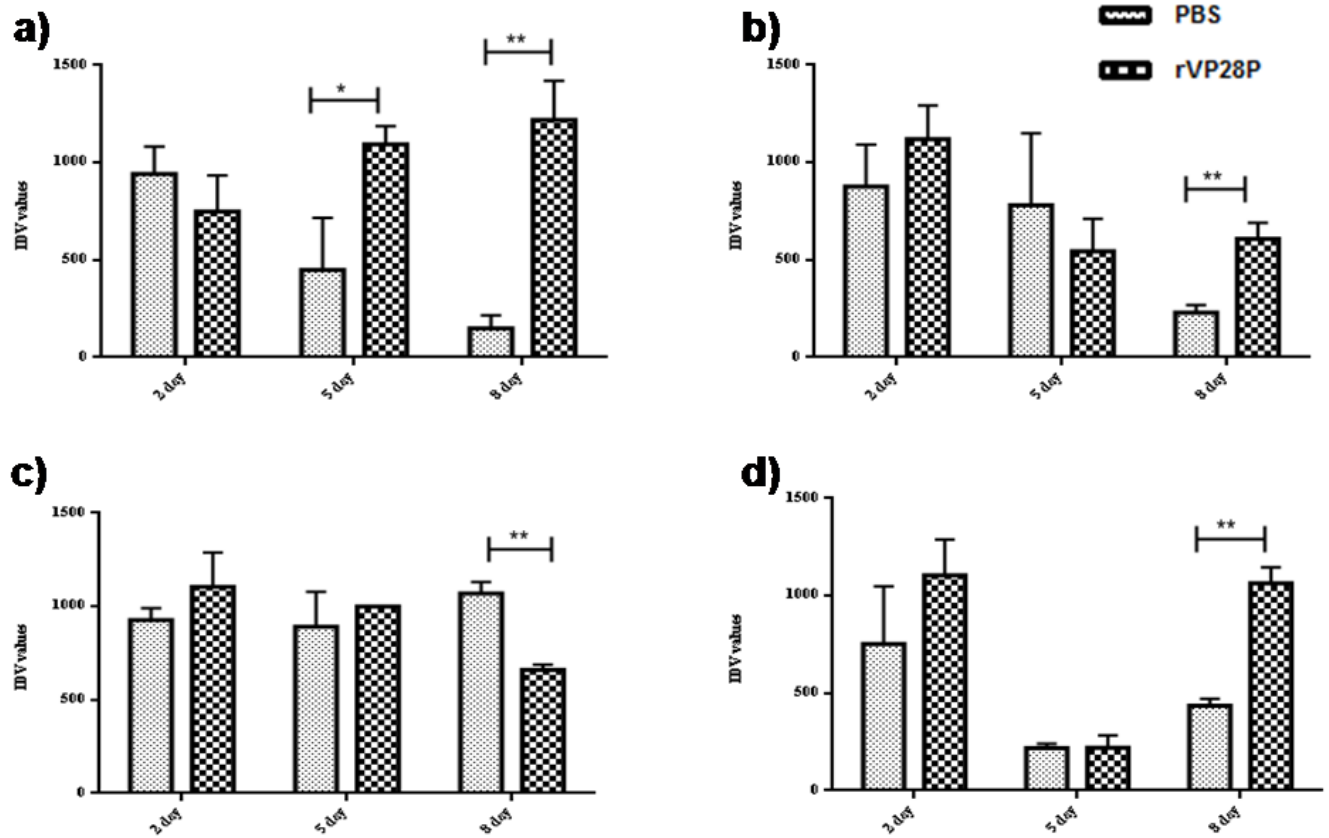
Figure 4

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 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 $\pm$ SD of three individual animals. (* indicates the level of significance; *- $p \leq$ 0.05, **- $p \leq 0.01$ & ***- $p \leq 0.001$).
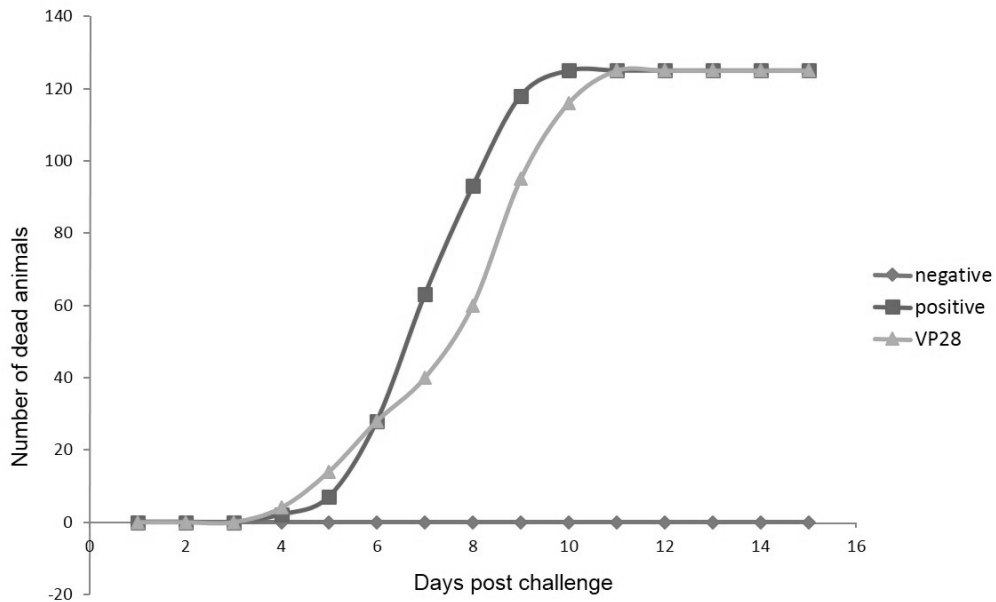
Figure 5

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.