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Trial Vaccine and immune function analysis of *Penaeus monodon* against *Vibrio harveyi* and *Vibrio parahaemolyticus*

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ABSTRACT

This paper describes the details of vaccination trials in *Penaeus monodon* carried out by administering formalin-killed, virulent strains of *Vibrio harveyi* (VHV) and *Vibrio parahaemolyticus* (VPV), isolated from *P. monodon* with symptoms of vibriosis, as vaccines through immersion and oral (feeding) methods. The efficacy of the vaccination was evaluated in terms of relative percent survival (RPS) upon challenge with the respective live virulent strains of vibrio, total haemocyte count (THC), haemolymph phenoloxidase (PO) activity and haemolymph antibacterial activity on 7, 14 and 21 days post-vaccination (PV). It was observed that vaccination with both VHV and VPV by both immersion and oral methods induced maximum immunity in *P. monodon* in terms of all the parameters investigated within a short period of about 7 days PV. The increased immune response gradually declined to a lower level during the next 14 to 21 days PV. From the study, it can be concluded that *P. monodon* can be protected from vibriosis by oral and immersion method of vaccination in a short time of 7 days.

Keywords: *Penaeus monodon*; *Vibrio harveyi*; *Vibro parahaemolyticus*; Vaccine trial.

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INTRODUCTION

In Asia, shrimp production has been predominantly focused on the Black Tiger shrimp, *Penaeus monodon*. However, bacterial infection of penaeid shrimp with *Vibrio* species (Vibriosis) are known to cause most serious diseases and often resulting in tremendous economic losses [1]. *Vibrio harveyi* and *Vibrio parahaemolyticus* are considered to be important to cause disease outbreaks of vibriosis in giant tiger prawn [2]. Several methods are practiced such as chemotherapeutants, immunostimulant, etc. to protect the shrimp from disease outbreak and to reduce the losses due to mortality in culture farms. Further, the use of drugs for the treatment of vibriosis can naturally shift the bacteria towards the resistant species. The increased occurrence of vibriosis in shrimp culture coupled with the problems associated with the use of antibiotics has led researchers to move towards the field of vaccination studies, i.e., vaccination of shrimp against one or more pathogens. Developing a commercial vaccine against diseases in shrimp needs investigations on model shrimps taking into consideration the different parameters of immune components and functions which have already been well established. In crustaceans especially in shrimps, investigations have been made to characterize the immune system [3,4]. Their immunological defense represents only the natural mechanism involving various morphological barriers, blood cells and several humoral factors. Though shrimps, like many other invertebrates, are assumed to lack an adaptive immune response and solely depend on innate immune response, several studies have shown that shrimps do have adaptive immune response and immunological memory. Immunostimulation and vaccination of shrimp with inactivated *Vibrio* species have been reported to provide some protection [5,6]. Plasma from the surviving infected shrimp could neutralize white spot syndrome virus (WSSV) from 20 days up to 2 months after infection.

However the relative importance of the above mentioned immunological factors and the interactions of cellular components and humoral components are not yet clearly understood in decapods. Hence, monitoring the immune system constitutes an important index for accessing the health status of the shrimp. There is a general agreement that shrimps with low immunity are more vulnerable to diseases and result in loss of productivity. In this context enhancement of the above mentioned immunological components and functions could be taken as a criterion to assess the potency and efficacy of a vaccine for protection in the short and long terms.

The objective of present study is to conduct vaccination trials in *P. monodon* using formalin-killed highly virulent strains of *V. harveyi* and *V. parahaemolyticus*, isolated from *P. monodon* with symptoms of vibriosis. Vaccines were administrated through immersion and oral (feeding) methods. The parameter for the evaluation of the efficacy of vaccination is the relative percent survival of vaccinated *P. monodon* on days 7, 14 and 21 post-vaccination upon challenge with the respective live virulent strains. In addition, total haemocyte count, haemolymph phenoloxidase activity and haemolymph antibacterial activity are to be estimated in vaccinated shrimps on different days of post vaccination.

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MATERIALS AND METHODS

Rearing of Shrimp

Healthy *P. monodon* weighing 14-18 g were obtained from shrimp farms along the east coast of Tamilnadu, India and acclimatized for 5 days to filtered seawater with a salinity range of 31-33 ppt, pH 7.8 - 8.2 and temperature of 28 ± 2ºC. The tank had continuous aeration with a daily 30% exchange of water. The shrimps were fed with sterile pelletized feed at a rate of 5% of bodyweight per day.

Bacterial culture and dose preparation

Virulent strains of *V. harveyi* and *V. parahaemolyticus* that were isolated from diseased *P. monodon* in east coast of Tamilnadu, India were used for vaccine preparation, antibacterial activity assay and challenge experiment. Pure isolates of *V. harveyi* and *V. parahaemolyticus* were suspended in sterile phosphate buffer saline (PBS) and diluted to standard concentrations equal to a particulate suspension with an optical density of 0.9 at 600 nm. This suspension contained approximately 8×10^7 colony forming units per ml of culture (CFU/ml) as determined by dilution and plating methods. The standard concentrations of the isolates were serially diluted 10 times to obtain bacterial suspensions of different concentrations (10^7, 10^6, 10^5, 10^4, 10^3 CFU/ml).

Vaccine preparation

Vaccines of *Vibrio harveyi* (VHV) and *Vibrio parahaemolyticus* (VPV) consisted of formalin killed *V. harveyi* and *V. parahaemolyticus* strains, respectively. Vaccines were prepared as per the method of Alabi et al., (1999). The bacterial culture at a concentration of about 8×10^7 CFU/ml was inactivated in 0.5% formalin and incubated at 20ºC for 12 h. The culture-formalin mixture was centrifuged at 11,000 rpm for 2 min at 4ºC to remove the formalin. The pellet of inactivated bacteria was then resuspended in sterile phosphate buffer saline (PBS), such that the suspension had an OD of 0.9, indicating an inactivated bacterial concentration of about 8×10^7 cells/ml. After checking the inactivation level, the vaccines were stored at 4ºC for further use.

Vaccination

Vaccination was carried out by both immersion and oral methods. Vaccination trials were conducted in triplicate for each of the methods.

Immersion method

Immersion vaccination with VHV was carried out by the method of Teunissen et al. [7]. Thirty healthy shrimps were selected randomly and maintained in a tank (1.2 m × 0.8 m × 0.45 m) with 400 l of filtered seawater. Vaccination was performed by replacing 2% of the tank water with equal volume of VHV preparation. The shrimps were left immersed in vaccine containing water for 5 h. At the end of the vaccination period the vaccinated animals were randomly divided into 3 groups of 10 shrimps each. Each group was

maintained in 250 l of filtered seawater. Unvaccinated (control) shrimps (n = 10) were maintained for each of the three vaccinated groups. Immersion vaccination with VPV was also carried out using above procedure.

Oral method

The feed pellets were coated with VHV and VPV separately, following the method of Witteveldt et al. [8]. Thirty randomly selected healthy shrimps, maintained in a tank with 400 l of filtered seawater, were fed with VHV coated feed pellets. The shrimps were fed twice a day (9 AM and 6 PM), at an initial daily rate of 3-5 % of bodyweight, for about 2 days. This rate was adjusted thereafter according to the feeding response of the shrimp, for the next 5 days. At the end of vaccination period, the vaccinated shrimps were randomly divided into 3 groups of 10 shrimps each. Each group was maintained in 250 l of filtered seawater. Unvaccinated control shrimps (n = 10), i.e., those fed with control feed, were maintained for each group. Control feed consisted of feed pellets mixed with sterile phosphate buffer saline, and coated with cod liver oil. Oral vaccination with VPV was carried out using the above procedure.

Immune function analysis

All the vaccinated shrimps and the control shrimps were subjected to immune function analysis which included total haemocyte count (THC), haemolymph phenoloxidase activity (PO) and haemolymph antibacterial activity. The first group of vaccinated shrimps and the corresponding control shrimps were analyzed for 7 days post-vaccination (PV), the second group, 14 days PV and the third group, 21 days PV. 0.5 ml of haemolymph from each vaccinated and control shrimp was withdrawn from the ventral sinus using a 2 ml syringe containing 1.5 ml of anticoagulant (K-199 medium + 5% L-cysteine). This anticoagulant-haemolymph mixture was used for total heamocyte count and assessment of phenoloxidase activity. For antibacterial activity assay, haemolymph was collected as described above without anticoagulant in the syringe. Total heamocyte count (THC) was made in anticoagulant-haemolymph mixture using a haemocytometer, and total heamocytes/mm ^{3} of haemolymph was calculated. The method of Supamattaya et al. [4] as modified by Purivirojkul et al. [9] was adopted for assessing the phenoloxidase activity in the haemolymph. Measurement of protein content in HLS was made by the method of Lowry et al. [10]. The phenoloxidase activity was calculated as the increase of optical density (OD) per minute per mg protein.

$$\text{One unit of phenoloxidase} = \Delta\ OD_{490}/\text{min/mg protein}$$

The antibacterial activity of the haemolymph was assayed as described by Sritunyalucksana et al. [11], however with necessary modifications. The principle of this assay is to expose known number of bacteria to the haemolymph and then estimate how many of these exposed bacteria were killed. The estimation is done by subjecting the exposed bacteria to plating method and obtaining the number of live bacteria in terms of CFU/ml of bacterial culture. The calculated CFU/ml of culture was inversely proportional to the antibacterial activity, i.e., lower the CFU, higher was the antibacterial activity.

The LD_{90} was selected as challenge dose and it was about 8×10^5 CFU/shrimp for *V. harveyi* and 8×10^7 CFU/shrimp for *V. parahaemolyticus*. The doses were determined from the pathogenicity studies using Probit method [12]. The required dose of bacterial suspension for challenge was prepared as described earlier in vaccine preparation. All the three vaccinated groups (7, 14 and 21 days PV) from each experiments (VHV and VPV) and control shrimps were challenged immediately after the collection of haemolymph sample for immune function analysis. The challenge was made between the 3rd and 4th abdominal segments by intramuscular injection of 50 µl of *Vibrio harveyi* suspension at a dose of 8×10^5 CFU/shrimp and *Vibrio parahaemolyticus* suspension at a dose of 8×10^7 CFU/shrimp. Both the control and experimental shrimps were observed for 7 days and relative percent survival (RPS) was calculated.

Statistical Analysis

Arithmetic mean and standard deviation (SD) were calculated for all quantitative measurements. One-way classification Analysis of Variance (ANOVA) followed by Tukey's test for multiple comparisons, was performed to test the significance of the differences among more than two means at 0.05 level of significance. In the immune function analysis experiment where triplicate samples were used, the data were pooled since the differences among the triplicate means were not significant. Student's t-test was employed to test the significance of difference between two means. "MINITAB" statistical software, 13.1 and "MS-Excel" software were used for all the statistical analysis.

RESULTS

Relative percent survival

The relative percent survival (RPS), i.e., survival rates of *Penaeus monodon* vaccinated with VHV by immersion and oral methods and subsequently challenged with the pathogenic strain of *Vibrio harveyi* on different days PV are shown in Table 1. The RPS values of *Penaeus monodon* vaccinated with VPV by immersion and oral methods and subsequently challenged with the pathogenic strain of *Vibrio parahaemolyticus* on different days PV are shown in Table 2. Comparison of RPS (Fig. 1) between the two methods of vaccination (immersion and oral) by both the vaccines (VHV and VPV) showed that maximum immunity was on day 7 PV. The RPS on day 7 PV by the immersion method was significantly ($P>0.05$) higher than that by the oral method for both the vaccines. This level of immunity decreased gradually to a significantly ($P<0.05$) lower level on day 21 PV. There was no significant difference ($P>0.05$) in the RPS values of *P. monodon* vaccinated with VHV and with VPV vaccine by both immersion and oral methods.

Table 1: Relative percent survival (RPS) of *Penaeus monodon*

S.No.	Replicates	Day PV	RPS*	
			Immersion	Oral
1	3 × 10	7	76.26 ± 8.25	55.53 ± 9.58
2	3 × 10	14	66.66 ± 7.21	42.90 ±14.30
3	3 × 10	21	33.33 ± 7.21	16.66 ± 7.21

* Mean ± SD of 3 replicates of 10 shrimps each

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Table 2: Relative percent survival (RPS) of *Penaeus monodon*

S.No.	Replicates	Day PV	RPS*	
			Immersion	Oral
1	3 × 10	7	70.83 ± 7.21	52.43 ± 8.25
2	3 × 10	14	61.96 ± 8.25	45.83 ± 7.21
3	3 × 10	21	28.70 ± 14.15	14.30 ± 0.00

* Mean ± SD of 3 replicates of 10 shrimps each

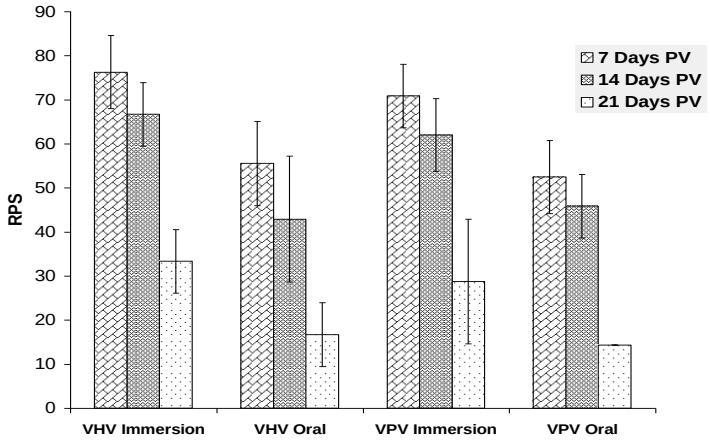

Fig. 1: Comparison of Relative Percent Survival (Mean ± SD) of *Penaeus monodon* vaccinated with VHV and VPV

(PV - Post-vaccination; **RPS** - Relative Percent Survival; **VHV** - *Vibrio harveyi* vaccine; **VPV** - *Vibrio parahaemolyticus* vaccine)

Immune function analysis

The THC, PO activity and antibacterial activities in control and vaccinated *P. monodon*, on different days PV by both immersion and oral methods are given figure 2 for both VHV and VPV vaccines.

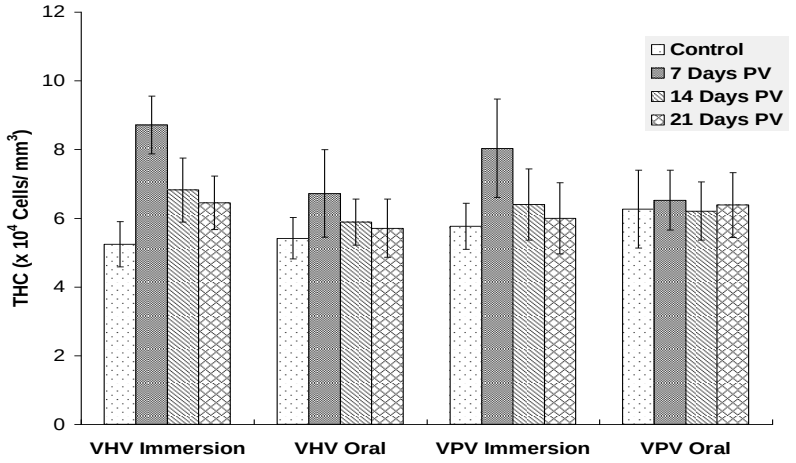

Fig. 2(a): Phenoloxidase activity

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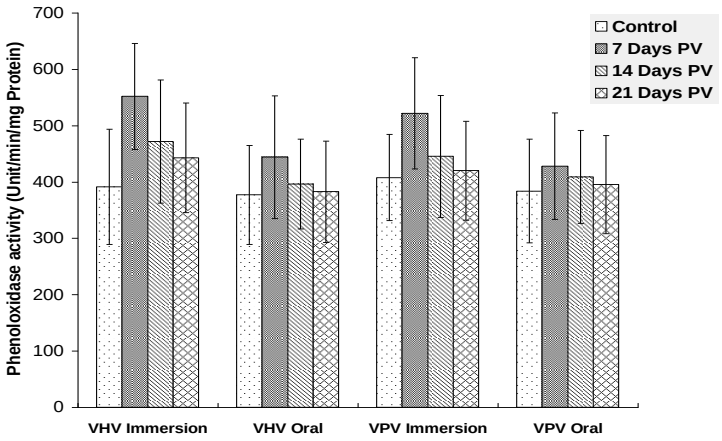

Fig. 2(b): antibacterial activity

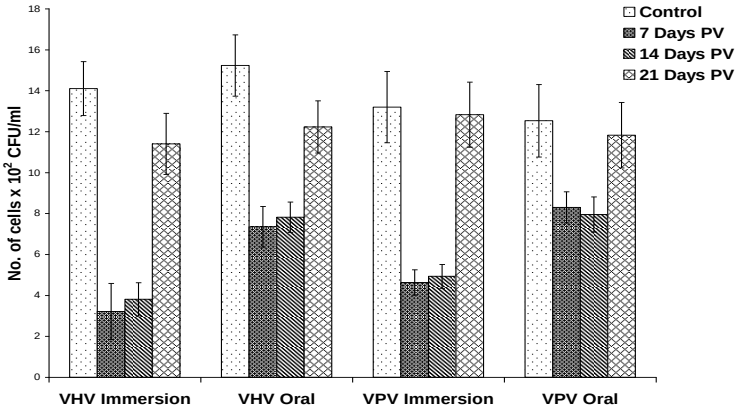

Fig. 2 (c): Haemolymph of *Penaeus monodon*

Fig. 2: (Mean ± SD) total haemocyte count (a), phenoloxidase activity (b), and antibacterial activity(c) of haemolymph of *Penaeus monodon*

(PV - Post-vaccination; **THC -** Total haemocyte count; **VHV** - *Vibrio harveyi* vaccine; **VPV** - *Vibrio parahaemolyticus* vaccine)

Total haemocyte count

The THC in vaccinated *P. monodon* was generally significantly (P<0.05) higher than in the control shrimps. The THC on day 7 PV by immersion method was significantly (P<0.05) higher than that by the oral method for both the vaccines. However, there was marked decline in the THC of the vaccinated shrimps on days 14 and 21 PV. The differences in the THC on day 7 PV between VHV immersion vaccinated and VPV immersion vaccinated *P. monodon* was very slight, though statistically significant (P<0.05). On the other hand the difference in the THC between VHV oral vaccinated and VPV oral vaccinated shrimps was not significant (P>0.05). In the case of *P. monodon* vaccinated with VPV by oral method, the day 7 PV count, though higher, was not significantly different from those of the other groups.

Phenoloxidase activity

The phenoloxidase activity in shrimps vaccinated with VHV by both immersion and oral methods was significantly (P<0.05) higher on day 7 PV than in the respective control shrimps and in the respective day 14 and day 21 PV shrimps. In the case of shrimps vaccinated with VPV, significantly (P<0.05) higher phenoloxidase activity on day 7 PV was found only in the immersion group. The activity of this enzyme in shrimps vaccinated with VPV by oral method did not differ significantly (P>0.05) on different days PV, though the activity on day 7 PV was greater than those on day 14 PV, day 21 PV and control. The general trend of the phenoloxidase activity on *P. monodon* vaccinated with VHV and VPV by immersion and oral methods was maximum on day 7 PV that declined to minimum on day 21 PV. Immersion vaccination with either of the vaccines produced significantly (P<0.05) greater phenoloxidase activity on day 7 PV than by the oral vaccination. There was no significant difference (P>0.05) in phenoloxidase activity on day 7 PV in shrimps vaccinated with VHV and VPV by both immersion and oral methods.

Haemolymph antibacterial activity

Maximum antibacterial activity of the haemolymph of *Penaeus monodon* vaccinated with either VHV or VPV by either immersion or oral methods was seen in about 7 to 14 days PV. The difference between 7 and 14 days PV was not significant (P>0.05) or marginal (P = 0.04). In all the case, the haemolymph antibacterial activity on day 7 PV was significantly greater than the respective day 21 PV activity and the respective control activity. The haemolymph antibacterial activity of the immersion group was higher than that of the oral group, as the means of the 7 day PV immersion group were significantly (P<0.05) lower than the means of the respective oral groups. The haemolymph antibacterial activity of the *P. monodon* vaccinated with VHV was significantly (P<0.05) higher than that vaccinated with VPV by immersion and oral methods.

DISCUSSION

In this study, formalin was used to inactivate the virulent strains of *V. harveyi* and *V. parahaemolyticus*, which is commonly used in the preparation of viral (e.g. WSSV) and bacterial vaccines as well because of its ability to cross link proteins and stabilize antigenic epitopes. The vaccines prepared in this study were effective in inducing immune response in *P. monodon* against the respective bacterial species. Administration of vaccine by injection is the most effective method, because it ensures the antigenic specificity, potency and the dosage of the vaccine each animal receives. However, the injection method is labour-intensive and costly. The other two methods of vaccinations viz., immersion and oral methods are more commonly practiced in shrimp aquaculture for the reasons that they are practical and cheaper. Further, these methods mimic infection of shrimps in natural situations. The results obtained in the present study showed that both immersion and oral methods of vaccination were capable of generating immune responses in *P. monodon* against the respective bacterial pathogens. Further, the immune response following immersion vaccination was significantly greater than that after oral vaccination.

In the immersion and the oral methods of vaccination, the dose of vaccine, i.e., the number of formalin killed VHV and VPV, each prawn receives is rather difficult to estimate and by which route they entered and exactly how many were involved in immunostimulation cannot be estimated. The assumption is that a fairly large dose of vaccine was used to immunize each shrimp. The results obtained support this assumption that the doses of the vaccines used in the immersion and oral vaccination are large enough to produce an enhanced immune response in *Penaeus monodon* which are challenged with the respective pathogens.

In the present work, the shrimps *Penaeus monodon*, weighing 14-18 g were immunized by immersion in the vaccine containing medium for 5 h. The result showed that this duration was sufficient to increase the immune response. Various researchers have used different time schedules ranging from a minimum of 1 h to maximum of 6 h have been followed to vaccinate shrimps by immersion method [13,14]. The schedule for vaccination by oral method followed in the present study was feeding the shrimps with vaccine coated pellets, twice a day for 7 days.

In the present investigation, the challenge of the vaccinated *Penaeus monodon* was performed on 7, 14 and 21 days PV in order to determine when the peak immune response occurred and how long it lasted. A minimum of 7 day PV was chosen assuming that shrimps of the size used in this study (14-18 g) would require at least these many days to develop a peak immune response. The results showed that the immune response had peaked during the first 7 day PV. Silakes and Supamattaya [15] allowed a minimum of 10 day PV for analyzing the immunostimulant as vaccination effects in *Penaeus monodon*. The relative percent survival (RPS) is an index has been widely used in evaluating the efficacy of vaccination in shrimps and in other animals. The RPS values obtained in this study clearly show that vaccination with either VHV or VPV offers protection against the respective pathogens. In all the groups, maximum RPS was observed on day 7 PV. This level of immunity gradually decreased to significantly lower level on day 21 PV. Thus, vaccination of *Penaeus monodon* with VHV or VPV induces immune response in a short period, i.e., within about 7 day PV, the increased response lasting about 14 day PV. The vaccination effect persists even on day 21 PV but at significantly lower level.

Apart from the analysis of RPS following challenge of vaccinated shrimps, the response of the animal's immune system to the administrated vaccine can be evaluated by analyzing certain haemolymph parameters like THC, PO activity and antibacterial activity. In crustaceans, haemocytes play a crucial role in non-specific cellular immunity against pathogens and parasites; they are involved in primary immune responses such as phagocytosis, encapsulation, nodule formation, cytotoxicity and cell agglutination. They also play a major role in regulating the physiological functions including hardening of exoskeleton, wound repair, carbohydrate metabolism, transport and storage of proteins and aminoacid, haemolymph coagulation and the confinement of invasive organisms by clot formation, phagocytosis and encapsulation.

Vaccination either with VHV or VPV, either by immersion or oral methods resulted in significant increase in the THC, over the control values. Maximum THC always occurred on day 7 PV and the values gradually decreased on day 14 and 21 PV. This trend in immune

response corresponds to that of RPS following challenge. There is no comparable data in the reported works that vaccination induces multiplication of haemocytes in shrimps. The observation of significant increase in the THC of the vaccinated *Penaeus monodon* in this study, suggest that the vaccines used (VHV and VPV), could have acted more like probionts than like pathogens. The increase observed in the THC of *Penaeus monodon* on day 7 PV and the subsequent decrease on days 14 and 21 PV correspond with the maximum RPS (above 60%) on day 7 PV and significantly lesser RPS on days 14 and 21 PV. This suggests that the increased THC has contributed to the immune protection in *Penaeus monodon* following vaccination with VHV or VPV. It is likely that the vaccinated shrimps having larger number of haemocytes are able to effectively counter the challenge virulent *V. harveyi* or *V. parahaemolyticus*.

Beside phagocytosis, haemocytes are also associated with secretion of proteins like prophenoloxidase (proPO) and phenoloxidase, which are involved in encapsulation and melanization. Phenoloxidase is the terminal enzyme in the proPO activating system and is activated by LPS or peptidoglycan from bacteria and β-1,3-glucan from fungi through recognition molecules. The results of the present study showed that the phenoloxidase activity in vaccinated shrimps by both immersion and oral methods was higher on day 7 PV than in the respective control shrimps. This high level of activity declined gradually on days 14 and 21 PV. However, the activity in vaccinated shrimps is always greater than that in unvaccinated control shrimps. Feeding *P. monodon* maintained in experimental tanks with vibrio bacterin with or without carboxymethyl β-1,3-glucan for 10 days, significantly increased the prophenoloxidase activity of haemolymph lysate supernatant fluid [16].

The increase in the phenoloxidase activity in the vaccinated *Penaeus monodon* corresponds with the similar trend in the THC of these shrimps. Thus it can be inferred that the haemocytes are likely to be one of the sources of this enzyme and that both VHV and VPV are capable of inducing the secretion and/or release of this enzyme from the haemocytes.

In the present study, the haemocyte free haemolymph of *Penaeus monodon*, vaccinated with either VHV or VPV by either immersion or oral methods, exhibited significantly higher antimicrobial activity lasting for about 14 days. This suggests that both VHV and VPV are capable of enhancing the antibacterial factor in the haemolymph of *Penaeus monodon*. Further, the humoral immune protection persists for a longer duration than the cellular immunity. While the levels of THC and phenoloxidase activity increased during day 7 PV, but declined significantly on day 14 PV. On the other hand, the haemolymph antibacterial activity remained significantly higher even on day 14 PV.

The results of the present study could only demonstrate the presence of an antibacterial factor in the haemolymph of *Penaeus monodon*, the activity of which can be enhanced by vaccination either with VHV or VPV. Whether the factor has lysozyme like activity or otherwise, can be elucidated by further studies. In this study, two vaccines viz., VHV and VPV were evaluated against the respective pathogens. Both vaccines were found to be equally effective in terms of RPS following challenge, and increase in THC, phenoloxidase activity and bactericidal activity. No attempt was made in this study to cross challenge, i.e., to find whether vaccination with VHV or VPV was equally effective against

both the pathogens. The results of these vaccination trials of the virulent strains *V. harveyi* and *V. parahaemolyticus* in *P. monodon* would contribute to the development of a comprehensive and economical multivalent vaccine, and effective method and schedule of vaccination.

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