

Shrimps survive white spot syndrome virus challenge following treatment with vibrio bacterin

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Taking an innovative approach, a vaccination study using five bacterial strains viz. *Vibrio campbelli* (B60), *V. alginolyticus* (B73), *V. parahaemolyticus*-like (B79), *V. parahaemolyticus* (R8) and *V. harveyi* (RG203) was conducted in *Penaeus monodon* against white spot syndrome virus (WSSV) infection, considered as one of the serious pathogens of shrimps. Oral challenge with shrimps infected with WSSV showed a relative percentage survival of 5 and 47 % in the *P. monodon* juveniles vaccinated with *V. parahaemolyticus* and *V. harveyi*, respectively. Results showed that there is a possibility of specifically immunising the shrimps against WSSV using bacterin prepared out of *Vibrio harveyi* isolates taken from shrimps infected with WSSV. Also, there was a level of protection attained by the shrimps due to immunisation with *Vibrio* strains.

Keywords: Bacterin, *Penaeus monodon*, Shrimp, Vaccination, *Vibrio harveyi*, White spot syndrome virus

The white spot syndrome virus (WSSV) has been a serious pathogen of cultured penaeid shrimps since 1993. WSSV infection is characterised by very high (70–100%) mortalities occurring within 2-7 days after the onset of clinical signs in grow-out populations¹⁻³. Apart from shrimps, WSSV has a wide host range which includes crabs, crayfish, lobsters and copepods⁴⁻⁷. In India, the annual loss in fisheries due to WSSV has been estimated to be over 600 crores⁸. Considered as an obligate pathogen in the early days of the disease, field observations showed that WSSV has become an opportunistic pathogen in the present scenario. The cultured shrimps, may therefore, become susceptible to WSSV infection when subjected to physiological stress. An enhancement of the immune status of shrimps can prevent the onset of WSSV infection in the farm-reared shrimps.

Immunisation, a prophylactic measure and a protective management tool⁹ is designed to aid in the prevention of disease. Chemicals or antibiotics used in preventing the disease in shrimp aquaculture could lead to development of resistance in the microorganisms and render them ineffective. Considering the seriousness of the disease, wide host range and nature of farming practices, immunisation,

due to its inherent advantages need to be developed as an effective tool for the prevention of WSSV infection in shrimps. Although invertebrates generally lack an adaptive immune system, they use the innate immune responses for effectively recognising and destroying the foreign materials including pathogens^{10,11}.

Development of resistance to infection by probable immune response like mechanism following vaccination has been documented against bacterial pathogens. Song and Hsieh¹² demonstrated the generation of microbiocidal substances by haemocytes following immunostimulation of shrimp with products of microbial origin. Itami *et al.*¹³ vaccinated shrimps with *Vibrio* spp. and Alabi *et al.*¹⁴ used formalin killed cells of bacteria for shrimp vaccination against vibriosis with success. George¹⁵ reported a relative percentage survival (RPS) of 34% for *Penaeus monodon* immunised with *V. alginolyticus* bacterin.

Apart from the reports of immunisation against vibriosis, vaccination against viral infections was also reported in shrimps. Presence of a quasi-immune response to WSSV has been demonstrated in *Penaeus japonicus* following challenging the survivors of natural and experimental WSSV infections¹⁶. Artificial infection leading to the presence of WSSV neutralising activity of plasma was observed from 20

days up to well over two months in infected *Penaeus japonicus*¹⁷. In view of the above information and to find out whether the bacterial antigen preparations have any effect in controlling the WSSV, an innovative approach of immunising *Penaeus monodon* with formalin killed bacterin has been undertaken in order to increase its immune response against WSSV challenge. Cue for this was taken from the observations of increased virulence of vibrio isolates obtained from white spot infected shrimps¹⁵ and reports of the lysogenic conversion of *Vibrio cholerae* and *V. harveyi* by phage DNA integration into the bacterial genome causing increased virulence¹⁸⁻²⁰. Therefore, while selecting the bacteria, it was taken into consideration that they were obtained from white spot infected *Penaeus monodon*.

Materials and Methods

Shrimp specimens—Post-larvae of *Penaeus monodon* (Fabricius) (tiger prawn) were obtained locally. The seeds were tested for the presence of white spot virus with two sets of diagnostic nested primers^{21, 22} and were found to be negative. The post-larvae were maintained in a 5000 l capacity cement tank containing 2000 l of seawater provided with continuous aeration. The seeds were fed *ad libitum* with artificial commercial feed, twice daily. The animals were used for the experiment following a rearing period of one month. Average size of shrimp seeds at the time of start of the experiment was 3.728 ± 0.121 cm length and 0.386 ± 0.036 g in weight.

Bacterial strains—Five different cultures, B60, B73, B79, R8 and RG203, belonging to genus *Vibrio* were used for the bacterin treatment studies. These strains were isolated from white spot infected *P. monodon* collected from farms/hatcheries of the southern districts of Tamil Nadu (Table 1). Bacteriological media for isolation and growth of the bacteria were obtained from Himedia, Mumbai.

Bacterin preparation—The five different isolates of vibrios were inoculated into trypticase soya broth and overnight grown cultures were further bulked up in Roux bottles with sterile trypticase soya agar. After 24 hr, these cultures were harvested and washed twice in sterile saline and resuspended in 5 ml sterile saline. The cell density in each of the preparation was calculated by standard plating techniques (Table 2). The cells were then killed by adding 0.5% formalin (v/v) to the bacterial suspension for 2 hr and were washed twice in sterile distilled water to remove formalin¹³.

Immunisation—The bacterins prepared by inactivating with formalin were diluted to 1 litre in seawater and twenty shrimp seeds each were immunised by immersion treatment by 5 bacterins for 1 hr¹³. Following treatment, ten seeds each were transferred to 50 l capacity plastic troughs containing 20 l of filtered seawater. The control set was immersed in sterile saline in the same manner. Duplicates were maintained for all the five bacterin treatments and control. Continuous aeration was provided in all the troughs. The seeds were fed *ad libitum* with artificial commercial pelleted feed. Excess feed was removed daily and the water was exchanged 20% per day. Shrimps were maintained for 35 days.

Challenge experiment—After 35 days of rearing, the immunised and non-immunised shrimps were subjected to challenge (*per os*) by feeding with WSSV infected shrimp meat²³. The infected shrimp meat was fed to the animals for 7 days followed by commercial pelleted feed. The infected shrimp were obtained from a farm in Kerala state and were tested positive for WSSV in single step PCR²¹. The mortality of shrimps was recorded daily for a period of 10 days and the dead ones were immediately removed from the experimental tanks.

Statistical analysis—The protection against WSSV after vaccination was calculated as the relative percentage survival (RPS):

$$RPS = 1 - \frac{\text{mortality of immunised shrimp (\%)}}{\text{mortality of control (\%)}} \times 100$$

Statistical tests such as mean, standard deviation and two-way analysis of variance (ANOVA) were carried out as per Snedecor and Cochran²⁴.

Table 1—Details of bacterial isolates used in the present study

Isolate No	Date of collection	Source tissue*	Species
B60	25-05-02	Muscle	<i>Vibrio campbellii</i>
B73	25-05-02	Gills	<i>V. alginolyticus</i>
B79	25-05-02	Hepatopancreas	<i>V. parahaemolyticus</i> -like
R8	19-07-02	Muscle	<i>V. parahaemolyticus</i>
RG203	14-07-03	Post larvae	<i>V. harveyi</i>

*Tissues obtained from WSSV infected *Penaeus monodon*

Table 2—Bacterial cell concentration used for vaccination study

Culture number	Cell concentration (CFU/ml)
B60	1.67×10^{11}
B73	1.18×10^{11}
B79	1.96×10^{11}
R8	1.65×10^{10}
RG203	2.07×10^{12}

Results

The growth performance of the shrimps during the post-treatment period is given in the Table 3. After 30 days, control shrimps recorded an average weight of 1.305 g whereas the immunised specimens showed average growth of 1.270 to 1.482 g with B60-immunised set showing the minimum and B73-immunised set showing maximum growth.

After the *per os* challenge with WSSV infected shrimp, all the animals in the control died on the 5th day post challenge (dpc). Of the five groups of *P. monodon* treated with bacterial strains, three immunised with B79, B60 and B73 showed 100% mortality in 5, 6 and 7 dpc. The shrimps treated with R8 (*V. parahaemolyticus*) and RG203 (*V. harveyi*) strains had only 95 and 59% mortality at the end of the 10 dpc, significantly lower ($P<0.05$) than that of the control and other treatments (Fig.1). RPS of the above two strains *viz.* R8 and RG 203 was 5 and 47% respectively.

Discussion

An innovative method of protecting shrimps against white spot syndrome virus using specific bacterial strains was attempted in the present study. Five different strains of vibrios belonging to *Vibrio campbellii*, *V. alginolyticus*, *V. parahaemolyticus* – like, *V. parahaemolyticus* and *V. harveyi* isolated from white spot virus infected *Penaeus monodon* were used for treating juvenile tiger prawns. Formalin inactivated bacterins were administered by a single, short duration immersion treatment of 1 hr. The result indicates that the shrimps were able to evoke a successful immune response against WSSV infection in at least one treatment with *V. harveyi* even 35 days after the singular immersion treatment carried out. Although the RPS values of 5 and 47% obtained at the end of 10 days post challenge for the *V. parahaemolyticus* (R8) and *V. harveyi* (RG203) were far from optimum, the results were highly encouraging considering the fact that the control test

animals were completely dead within halfway of the challenge duration. Teunissen *et al.*²⁵ who vaccinated *P. monodon* using formalin killed *V. alginolyticus* cells found that the vaccine was effective against vibriosis 50 days post vaccination. Similarly, Karunasagar *et al.*²⁶ reported the delayed onset of disease and significant reduction in mortality rate in white spot disease occurrence in farms where regular booster doses of vibrio cells were added. Specific immune response in shrimps has been documented by George *et al.*²⁷, who obtained an RPS of 66.6% when *P. indicus* was vaccinated and challenged with *V. alginolyticus*, at the end of 8 weeks of immunisation. Comparative analysis of the vaccination potential of *Vibrio alginolyticus* strains obtained from white spot syndrome virus infected shrimps and environmental samples has shown that the protection offered by the former was higher than that of the latter when challenged with *V. alginolyticus*²⁸.

Ability of shrimp immune system to specifically recognise the antigen was noticed by Witteveldt *et*

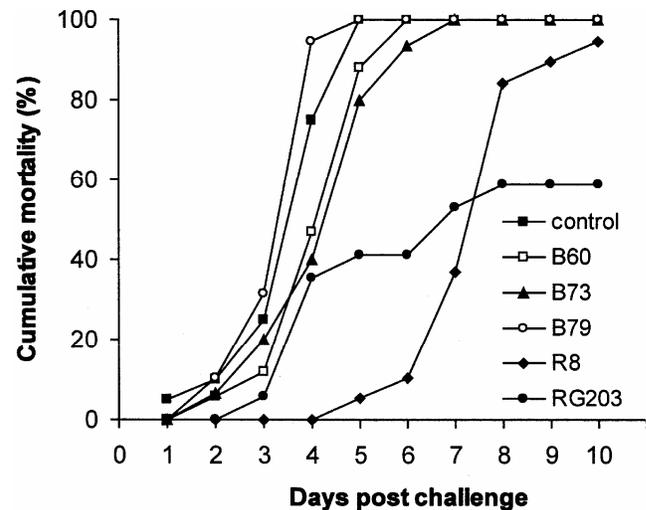


Fig.1—Cumulative mortalities of *Penaeus monodon* treated with formalin-inactivated bacterial strains and challenged with WSSV on 36th day post-immunisation.

Table 3—Average growth performance of shrimps in the immunisation study

Culture Number	0 day		15 th day		30 th day	
	Total length (cm)	Weight (g)	Total length (cm)	Weight (g)	Total length (cm)	Weight (g)
Control	3.933± 0.314	0.442± 0.059	5.217± 0.204	0.825± 0.081	5.800±0.443	1.305±0.231
B60	3.767± 0.378	0.413± 0.068	4.650± 0.315	0.602± 0.123	5.617± 0.453	1.270± 0.275
B73	3.667± 0.388	0.357± 0.052	5.033± 0.472	0.805± 0.179	6.012± 0.354	1.482± 0.171
B79	3.767± 0.326	0.393± 0.029	4.967± 0.383	0.727± 0.141	5.833± 0.821	1.353± 0.309
R8	3.617± 0.496	0.360± 0.062	5.033± 0.356	0.785± 0.185	5.800± 0.704	1.425± 0.332
RG203	3.617± 0.567	0.353± 0.078	4.800± 0.322	0.723± 0.129	5.717± 0.232	1.375± 0.249

*al.*²⁹ who immunised the animals with a subunit vaccine of VP19 of WSSV and observed an RPS of 33 and 57% at 2 and 25 days after vaccination of *P. monodon*. Compared to the above study, the RPS of 47% obtained in the present experiment is highly promising due to the fact that the immunisation was done by a single immersion treatment with a bacterin while the former was a subunit vaccine and had a booster dose applied after 5 days and both administrations were carried out by injection inoculation. A similar observation was also observed in a WSSV vaccination study in the *Penaeus japonicus* where the efficiency of formalin inactivated WSSV vaccine was increased by the additional injections of *Vibrio penaeicida*³⁰. Further, in the present study, the challenge was designed to increase the viral load drastically high in a manner similar to the field conditions and its effect was productively proved as all the control shrimps died halfway through the challenge test while the RG203 treated shrimps could successfully establish an RPS of 47%. This again contrasts with the study conducted by Witteveldt *et al.*²⁹ where the challenge test brought about only 90% mortality in the unvaccinated controls.

Despite the fact that all the five species of vibrios used in the present study were from white spot infected *P. monodon*, 3 species (*Vibrio campbelli*, *V. alginolyticus* and *V. parahaemolyticus*-like) did not give any RPS values as they failed to survive the challenge period. Of the remaining two, R8 (*V. parahaemolyticus*) had given only 5% RPS and only *V. harveyi* (RG203) could give an RPS of 47%. Compared to other strains used in the study, RG203 was recently isolated and underwent less than 4 passages before preparation of the bacterin while the other strains underwent over 7 passages during the last one year before the bacterins were prepared. Therefore it can be concluded that *Vibrio harveyi* strains that are recently isolated from WSSV infected *P. monodon* and underwent minimal passages outside the host body could function as potential vaccine/immunisation candidate against WSSV considering the severity of the disease and simplicity of the technique. While it could be argued that the protection offered by the *Vibrio* bacterin in the present study is a non-specific immune enhancement, the absence of protection in all the immunised groups and partial protection offered by the *V. parahaemolyticus* strain (R8) points to the specificity

of *V. harveyi* (RG203) in protecting *Penaeus monodon* against WSSV. Further work is essentially required to identify the molecular characteristics and dosage of bacterial antigens and the stage at which the shrimp could be better immunised using the bacterin to effectively protect them from WSSV infection.

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