In vitro and in vivo biocontrol of Vibrio harveyi using indigenous bacterium, Bacillus spp.

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The present study was undertaken to find out the potential of indigenously isolated Bacillus spp. in controlling virulent luminous Vibrio harveyi infection in shrimp in vitro and in vivo. Luminescent bacteria were isolated from shrimp farms of east coast of India. It is biochemically and genetically identified as Vibrio harveyi. Two isolates of Vibrio harveyi obtained from infected shrimps were used for pathogenicity studies in juvenile shrimps and the LD50 values for the above isolates ranged between $3.7 \times 10^6$ to $8.9 \times 10^7$ CFU/ml. Indigenously isolated and identified Bacillus spp. exhibiting antagonism were used for controlling the Vibrio infection both in vivo and in vitro. In vivo antagonistic activity was studied with three selected Bacillus isolates. The same had reduced the mortality of Penaeus monodon juveniles when challenged with V. harveyi with an RPS of 87.5 - 50%. This is without affecting the multiplication of the pathogen in water. Present study found that indigenously isolated antagonistic Bacillus sp. could be used as effective biocontrol agents against pathogenic luminous V. harveyi.

[Keywords: Shrimp, Biocontrol, luminescent bacterial infection, Vibrio harveyi, Bacillus]

Introduction

Aquaculture is considered as one of the fastest growing food production industries in the world with the cultured shrimp production increasing at an annual rate of 16.8%1. Global losses resulting from shrimp disease are estimated around 3 billion US dollars. Vibrio harveyi has caused 100% losses in the larval production in many parts of the world2-4. The use of probiotic bacteria such as Bacillus sp. has been recommended for successful control of luminescent vibrios in shrimp farms5,6. Antagonism of Bacillus sp. against luminescent vibrios in vitro has been reported by various authors7-12. Verschueren et al.13 emphasised the need for isolating indigenous microflora to be identified for the better advantage in shrimp farming systems since they establish in minimum time within the systems.

Present study was undertaken to isolate antagonistic bacteria from the shrimp farms and coastal region of Southeast Indian Coast and to study their efficiency to control shrimp pathogenic bacteria in vivo.

Materials and Methods

Shrimp (Penaeus monodon) specimens, water and sediments were collected from three shrimp farms and one hatchery situated along the Tamilnadu coast in the regions of Marakkanam and Kattumavadi. Nine samples of water and sediments obtained from different shrimp farms of the same region were used for screening of antagonistic bacteria against V. harveyi. Tiger shrimp (Penaeus monodon) postlarvae (PL-13) were purchased from a shrimp hatchery at Marakkanam (Tamilnadu) and brought to the laboratory in oxygenated polybags, acclimated and reared in cement tanks fed with commercial shrimps feed (CP Aquaculture (India) Pvt. Ltd.). The juvenile shrimps with an average size of 0.8 – 2 g were used for the biocontrol experiment. In order to find out the pathogenicity of the isolates, juvenile Indian white shrimp Fenneropenaeus indicus (av. wt.0.7 - 4.7 g) obtained from wild collection was used. Water, soil and shrimp tissue (haemolymph, gut, gill and hepatopancreas) samples were inoculated on thiosulphate citrate bile salt sucrose agar (TCBS) and trypticase soya agar (TSA) (Himedia Laboratories Pvt. Ltd. Mumbai) supplemented with 1% sodium chloride. Presumptive Vibrio colonies collected at random from the TCBS

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agar plates were further purified and identified based on standard biochemical tests\textsuperscript{14} and following the identification schedule of Alsina and Blanch for \textit{Vibrio} \textit{sp.}\textsuperscript{15-16}. Bacterial colonies from sediment and water samples obtained from TSA were selected for bacterial antagonistic study and identified using standard biochemical tests\textsuperscript{17}.

\textit{Vibrio harveyi} strains were confirmed by PCR in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using \textit{gyrB} gene specific primers\textsuperscript{18}. Selected antagonistic \textit{Bacillus} spp. were further confirmed by PCR protocol of Ochoa-Salano and Olmos-Soto\textsuperscript{19}.

The bacterial isolates purified were grown in Luria - Bertani (LB) broth (1.0% tryptone, 0.5% yeast, 1.0% NaCl, pH 7.5) at 32°C for 48 h. The fully grown tubes were harvested at 48 h by centrifuging at 5000 g for 10 min in sterile polypropylene tubes. The cell pellet was then washed in 2 mL of sterile phosphate buffer solution (PBS) and were transferred into eppendorf microfuge tube and added with 100 µl each of sodium dodecyl sulphate (SDS) (25 mg/100 ml) and sodium hydroxide (0.05 N) solution. The solution was mixed well and immersed in boiling water for 3 min and cooled immediately on ice and centrifuged at 10000 g in a microfuge (Eppendorf, Germany) for 10 min and the supernatant was used as template for PCR analysis after diluting 10 times with sterile deionised water (Millipore Biocel, France) immediately or stored at -20°C until used.

Three luminescent strains of \textit{V. harveyi} (DS 144, LS 21 and LS 22) were used for the pathogenicity study. The bacteria were grown in TSB (5 mL) for 24 h and centrifuged at 5000 g for 10 min. The pelleted cells were washed with sterile saline, serially diluted and injected to twelve juvenile \textit{Fenneropenaeus indicus} at 50 µl per shrimp intramuscularly in the 5\textsuperscript{th} segment while control group received same amount of saline. The animals were maintained in troughs of 50 litre capacity with 6 animals per trough with full aeration and \textit{ad libitum} feeding. The salinity of the water was maintained at 25 +/- 1.0 ppt and temperature varied between 28-30°C. The mortality pattern was observed for 5 days. The LD50 value was calculated by the technique of Reed and Muench\textsuperscript{20}.

\textbf{In vitro antagonism of bacterial isolates towards \textit{V. harveyi}}

Antagonistic effect of bacteria was studied based on cross streak method described by Maeda\textsuperscript{21} with modification. Bacterial isolates were streaked (5 ± 1 mm wide) across the diameter of each petri plate containing sterile TSA supplemented with 1% NaCl. Following to the incubation at room temperature for 24h, two \textit{V. harveyi} strains (LS 21 and LS 22) were streaked in the same way but perpendicular and apart by ≤ 1 mm to the central strip of the first strain. The plates were re-incubated for 24 h at room temperature. The growth inhibition of the \textit{V. harveyi} at the confluence was recorded.

The bactericidal effect of selected bacterial strain was tested against \textit{V. harveyi} by double layer method described by Riquelme \textit{et al.}\textsuperscript{22}. Briefly, TSA plates supplemented with 1% NaCl were spot inoculated with 5 µl of overnight cultures of each bacterial strain to be tested. Following incubation for 24h at room temperature, the developed colonies were killed with chloroform vapour over a period of 45 min. These plates were overlaid using 6 ml of TSB supplemented with 1% NaCl and 0.9% agar, containing 100 µl of a 1/10 dilution of 12 h culture of \textit{V. harveyi} in trpticase soya broth. After 24 h incubation, antagonistic effects of bacterial strains tested were observed by measuring the zone of inhibition, which appeared as clearance zone around these bacterial colonies.

\textbf{In vivo action of antagonistic bacteria against \textit{V. harveyi}}:

\textbf{In vivo} antagonistic activity of bacterial strains against one (LS 22) of the three \textit{V. harveyi} strains selected at random among three similar isolates was studied by the co-inoculation of both \textit{V. harveyi} and antagonistic bacterial isolates as per the method of Roque \textit{et al.}\textsuperscript{23} with modifications. Five tiger shrimp juveniles each (average length: 6.26±0.427 cm and average weight 1.40±0.256 g) were maintained in 51 capacity plastic jars were filled with 4 l of filtered seawater (30 ppt). Four treatments along with control in duplicate were set up.

1. Control (Wounded shrimp) – C
2. Wounded shrimp (WS) with \textit{V. harveyi} immersion – VC
3. Wounded shrimp with co-inoculation of \textit{V. harveyi} (LS 22) and antagonistic bacteria (SAB-8) ~ VB1
4. WS with *V. harveyi* (LS 22) and antagonistic bacteria (SAB-15) – VB 2
5. WS with *V. harveyi* and antagonistic bacteria (SAB – 16) – VB 3

The shrimps were individually netted out and a small wound was (Approximately 2 mm long) made through the cuticle and into the muscle of the third abdominal segment by pushing a scalpel until it penetrated. The bacterial cells required for the study was grown in roux bottles containing 150 ml TSA (1% NaCl) for *V. harveyi* and 200 ml of TSB (1% NaCl) for antagonistic bacteria. The bacterial cells were centrifuged at 6000 rpm and added to the 4 l water. The bacterial load in each experimental set up was estimated as total plate count (TPC) and presumptive vibrio count (PVC) (Table 1a, 1b & 1c). Twenty five percent of the water in all the bottles was replaced with filtered seawater containing equal concentration of bacterial cells as in the initial stage. The animals were fed with *ad libitum* commercial feed and aerated continuously. The mortality percentage 24 and the bacterial count were examined for 4 days. The experimental setup included treatments in duplicate.

\[
\% \text{ Mortality} = \frac{\text{No. of dead/infected shrimp} - A}{\text{Total no. of inoculated shrimp} - A} \times 100
\]

where, A is the number of dead shrimp in 1 day after infection. Based on the mortality rate, the relative percentage survival (RPS) of challenged shrimps was evaluated.

\[
\text{RSP} = 1 - \left\{ \frac{\% \text{ of mortality (treated)}}{\% \text{ of mortality (control)}} \right\} \times 100
\]

Table 1a—Relative percentage survival (RPS) observed in the *in vivo* antagonistic study using selected *Bacillus* sp. during the 4-day experimental duration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>100 100 100 100</td>
</tr>
<tr>
<td>PC</td>
<td>100 37.5 12.5 0</td>
</tr>
<tr>
<td>VB1 (SAB8)</td>
<td>87.5 75 75 75</td>
</tr>
<tr>
<td>VB2 (SAB15)</td>
<td>100 100 87.5 62.5</td>
</tr>
<tr>
<td>VB3 (SAB16)</td>
<td>100 87.5 75 50</td>
</tr>
</tbody>
</table>

(NC negative control, PC positive control)

Table 1b. Average values of presumptive vibrio count (PVC) obtained in the *in vivo* antagonistic study using selected *Bacillus* sp. during the 4-day experimental duration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Presumptive vibrio count</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1.0 × 10^4 1.15 × 10^4 6.0 × 10^3 1.1 × 10^4</td>
</tr>
<tr>
<td>PC</td>
<td>2.29 × 10^7 5.6 × 10^6 2.35 × 10^6 1.2 × 10^6</td>
</tr>
<tr>
<td>VB1 (SAB8)</td>
<td>6.5 × 10^6 4.6 × 10^6 3.7 × 10^6 3.5 × 10^6</td>
</tr>
<tr>
<td>VB2 (SAB15)</td>
<td>1.0 × 10^7 3.0 × 10^6 7.0 × 10^5 9.0 × 10^5</td>
</tr>
<tr>
<td>VB3 (SAB16)</td>
<td>5.3 × 10^6 5.5 × 10^6 3.7 × 10^6 3.5 × 10^6</td>
</tr>
</tbody>
</table>

(NC negative control, PC positive control)

Table 1c—Average values of total plate count (TPC), obtained in the *in vivo* antagonistic study using selected *Bacillus* sp. during the 4-day experimental duration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total plate count</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1.37 × 10^4 1.29 × 10^4 4.8 × 10^4 8.6 × 10^4</td>
</tr>
<tr>
<td>PC</td>
<td>5.2 × 10^7 3.0 × 10^7 1.27 × 10^7 2.15 × 10^7</td>
</tr>
<tr>
<td>VB1 (SAB8)</td>
<td>1.15 × 10^7 7.5 × 10^7 1.3 × 10^7 2.0 × 10^7</td>
</tr>
<tr>
<td>VB2 (SAB15)</td>
<td>1.28 × 10^8 1.47 × 10^7 1.52 × 10^7 7.5 × 10^7</td>
</tr>
<tr>
<td>VB3 (SAB16)</td>
<td>1.27 × 10^7 1.35 × 10^7 1.33 × 10^7 2.08 × 10^7</td>
</tr>
</tbody>
</table>

(NC negative control, PC positive control)

Results

The *Vibrio harveyi* isolate used for the experiment was identified from a total of 151 presumptive *Vibrio* isolates obtained randomly for identification from shrimp farm samples. Of the different samples. Presumptive *Vibrio* count was highest in the sediment sample from shrimp farm of Marakkanam region (3.4 × 10^4 CFU/g). Among the 151 *Vibrio* isolates, 90 belonged to *V. harveyi* contributing to 59.6% followed by *V. vulnificus* (13.25%), *V. mimicus* (8.6%), *V. alginolyticus* (6.6%), *V. mediterranei* (1.99%), *V. metschnikovii* (0.66%) and unidentified *Vibrio* sp. (9.27%).

Pathogenic organism for the biocontrol experiment was selected randomly from the *Vibrio harveyi* isolates. These isolates were identified by gyr B PCR, which generated a confirmatory amplicon of 363 bp (Fig. 1). Three luminescent *V. harveyi* strains (LS 21, LS 22 & DS144), which comprised Gram-negative motile fermentative rods which produced enzymes like catalase, oxidase, gelatinase, lysine and ornithine decarboxylase, produced indole from tryptophan, with no growth in 0% NaCl while exhibited growth at 8%, unable to produce acetoin from glucose, thus corresponding with the description of *V. harveyi* 15,16 were used for the experiment after molecular confirmation with gyrB gene specific primers 18 were tested for their pathogenicity against *F. indicus*. Among the tested strains, LS 21 had shown highest pathogenicity with the lowest LD50 value of 3.7 × 10^6 CFU/ml (shrimp mean weight 0.864±0.114 g and mean
length 5.35±0.157 cm) whereas DS 144 and LS 22 had LD₅₀ values of 8.9 × 10⁷ CFU/ml (shrimp mean weight 0.892±0.0646g and mean length 5.37±0.21cm) and 4.2 × 10⁷ CFU/ml (4.31±0.2166 g and 9.38±0.1887 cm) respectively.

For obtaining antagonistic bacteria, 173 isolates were screened against luminescent V. harveyi strains LS21 and LS22. Among the tested isolates, 36 showed antagonism to V. harveyi. The antagonistic bacteria, which were gram positive, motile spore forming rods were identified as Bacillus spp. based on biochemical characteristics such as production of enzyme catalase, no production of acetoin from glucose, non-fermentative, further confirmed by Bacillus 16S rDNA specific PCR, which amplified a characteristic band of 640 bp (Fig. 2). These strains showed effective inhibition clearance zones of over 6mm (Fig. 3).

**Evaluation of the efficiency of Bacillus spp. in vivo challenge of P. monodon juveniles against V. harveyi**

The in vivo co-culture experiment was designed to find out whether the presence of Bacillus sp. exhibiting antagonism to V. harveyi were actually able to control them in water and to reduce the effect of pathogenic V. harveyi on wounded shrimps. The result of the challenge test is given in Table 1. The RPS values of the challenged shrimp were 75, 62 and 50 % for SAB 8, SAB15 and SAB 16 respectively (Fig. 4). The heterotrophic bacterial (Fig. 5) and presumptive vibrio load (Fig. 6) did not show any
Discussion

Present study revealed large numbers of luminescent V. harveyi (LVH) forming up to 59.6% of the vibrio population of the samples. Since the luminescence in V. harveyi is co-regulated with the production of the toxin and hence considered as a virulence factor\textsuperscript{25}, the dominance of luminescent V. harveyi in shrimp farms implies that they can cause serious damage by acting as potential pathogens in adverse environmental conditions and hence there is a compelling need to find out effective method for controlling them.

Pathogenicity of V. harveyi isolates was recorded as LD\textsubscript{50} value, which is the lethal dose for 50% of the experimental animals in the challenge study. In the present work, The Vibrio isolates tested had LD\textsubscript{50} values $3.6 \times 10^6$ and $8.9 \times 10^7$/ml towards juvenile shrimps upon immersion challenge. Naturally occurring bacterial flora have been used as disease control agents in farming systems as a new method\textsuperscript{21}. In the present study, 173 isolates of bacteria were selected at random and checked for antagonistic activity and 20% isolates showed antagonistic activity against LVH strains and the strains were found to belong to Bacillus spp. by biochemical and molecular tests. Bacillus sp. has been generally reported to exhibit antagonism towards Vibrio harveyi\textsuperscript{26,27}. 

significant variation during the experimental period. A ten-fold reduction of vibrio count was observed in case of the Bacillus strain SAB 15 inoculated treatment. In case of other treatments, heterotrophic counts were maintained at $10^7$ cells of and presumptive vibrios at $10^6$ cells/ml.
The average zone of inhibition obtained for the different isolates was 10 mm against the LVH strains used in this study by double layer as well as cross streak method. Earlier reports indicate the antagonistic effect of *Bacillus* spp. against *V. vulnificus* with a zone of inhibition as 41 mm and *B. subtilis* against *V. harveyi* zone of 6 mm.

Antagonistic effect of three *Bacillus* isolates in vivo (SAB 8, SAB15 and SAB 16) towards LVH was evaluated in this study by co-inoculation of *Bacillus* sp. and *V. harveyi*. It was observed that these strains could protect the shrimps challenged with virulent luminescent *V. harveyi* with an RPS of 75, 62 and 50% for SAB 8, SAB15 and SAB 16 respectively. The experiment had *V. harveyi* maintained at 10^3-10^7 CFU/ml of the water of all the tanks producing 100% mortality of shrimps in the control group. Certain strains of *V. alginolyticus* were reported to reduce the severity of luminescent vibriosis when used as probiotic in larval rearing tanks. Similarly, *Bacillus* spp. was found to eliminate luminescent bacterial disease in culture conditions.

Results of the present study showed that the number of the *V. harveyi* were unaffected in the experimental tanks while the strain SAB8 produced an RPS of 75%. This indicates that antagonistic *Bacillus* strains were able to quench the quorum sensing properties of *V. harveyi* and reduce its virulence but not its multiplication. In other words, in the presence of the specific *Bacillus* strains, *V. harveyi* were still possible to multiply in its non-virulent form. This assumes significance in shrimp farming sector because *Bacillus* is able to control the virulence alone of *V. harveyi* and not the total number thereby not disturbing ecological balance between the microorganisms and permitting these flora to carry out its natural role of decomposer of nitrogenous waste from aquatic systems. This could probably happen only in cases where the antagonistic bacteria is from indigenous strains as the de novo establishment of an allochthonous bacteria in the system is rather difficult and time consuming and underlie the importance of identification of native strains for controlling infections in seawater culture systems having increased nitrogenous waste products.

In the current study, the animals could survive despite the wounds in the abdominal segment which normally make the shrimps vulnerable to attack by opportunistic pathogens. The survival of the shrimps in the experiment could be due to prevention of colonization ability of *V. harveyi* in both gut and wound surfaces. Pathogenicity of *V. harveyi* was found to be due to the ingested bacteria colonizing the digestive tract of the larvae. The survival of shrimps with wound in *V. harveyi* and *Bacillus* sp. inoculated tanks could therefore be either due to the extracellular products of *Bacillus* sp. preventing the entry of pathogen into the shrimp or the reduced virulence characteristics of *V. harveyi*, which could prevent their multiplication in vivo in shrimps. This could also be due to the competition for colonization of shrimp by these two microbes, which effectively reduces the concentration of the pathogenic *V. harveyi* invading the shrimp. Pathogenicity studies using adult penaeid shrimps have revealed that the multiplication of pathogenic bacteria begin in the hepatopancreas, although in the first few minutes to hours of bacterial infection, many bacteria can be cleaned by the shrimp. Results of the current study indicate that the native antagonistic *Bacillus* strains, which do not prevent multiplication of *V. harveyi* could not only reduce the pathogenic effect of luminescent vibrios but leave the system undisturbed without reduction of the bacterial flora thereby protecting the water quality and health of the animals.

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References


