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Selection of *Vibrio harveyi*-resistant *Litopenaeus vannamei* via a three-round challenge selection with a pathogenic strain of *V. harveyi*

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ABSTRACT

To obtain *Vibrio harveyi*-resistant *Litopenaeus vannamei* shrimp used for study on immune response of shrimp avoid vibriosis, a three-round challenge selection procedure was applied. In this procedure, resistant shrimp were selected gradually via three rounds challenge experiment with a pathogenic strain of *V. harveyi* at a median and controllable lethal dose of 96-h LD50 (the median lethal dose). After this procedure, the cumulative mortality of selected shrimp during 96 h after injection of *V. harveyi* at 2.0×10^6 cfu shrimp^{-1} significantly decreased from 93.3% to 26.7%, the hours of beginning of death and the hours of attaining of the maximum cumulative mortality of shrimp prolonged from 4 h and 10 h to 8 h and 24 h, respectively. The LD50 of 6 h, 12 h, 24 h, 48 h and 96 h of selected shrimp significantly increased to $1.4 \pm 0.1 \times 10^7$ ($p < 0.01$), $5.5 \pm 0.4 \times 10^6$ ($p < 0.01$), $3.1 \pm 0.2 \times 10^6$ ($p < 0.01$), $2.7 \pm 0.1 \times 10^6$ ($p < 0.01$) and $2.7 \pm 0.1 \times 10^6$ cfu shrimp^{-1} ($p < 0.01$), about 15.9, 15.3, 9.4, 10.0 and 10.4 times of that of normal shrimp, respectively. In conclusion, the resistance of shrimp to *Vibrio* significantly increased after the three-round challenge selection procedure.

1. Introduction

Shrimp culture has become a very important department for food production of the world [1]. However, with the spectacular developing, shrimp cultivation has also suffered enormous losses due to viral and bacterial diseases, especially white spot syndrome virus (WSSV) disease and vibriosis in past decades. This loss to shrimp industry was estimated to be approximately one billion dollars per year since 1990s in the world [2].

To resist diseases bringing about economic losses, disease-resistant shrimp should be selectively bred and then the immune responses of those resistant shrimp to virus or bacterial infection also should be researched [3]. There were several papers about selection and breeding of disease-resistant shrimp. To resist Taura syndrome virus (TSV), Argue et al. [4] built a line for Pacific white shrimp, *Litopenaeus vannamei*, which 18.4% higher in survival rate to TSV than control shrimp. Huang et al. [5] selectively bred a WSSV-resistant *L. vannamei* family, which the survival rate increased from 0.78% to 22.7%, after WSSV infection. Meanwhile, several disease-resistant shrimp have been selective bred for research on immune response of shrimp to pathogens [6–9]. A WSSV-resistant *L. vannamei* family was bred to explore immunological variables of parent shrimp and their selective filial families after infection of WSSV [6]. An experimental *Litopenaeus stylirostris* line was developed by mating animals surviving from "Syndrome 93" episodes to investigate the relationship between antimicrobial peptide gene expression and capacity of a selected shrimp line to survive a *Vibrio* infection [7]. A selective breeding *L. vannamei* family was developed to study on immune characteristics of WSSV-resistant shrimp [8]. Unlike selective breeding, García et al. [9] collected the final 1.8% *L. vannamei* shrimp surviving from a pre-challenge of WSSV as to be WSSV-resistant shrimp to research on differential gene expression in those resistant shrimp. Unfortunately, a long period even lasting for several years was needed for collecting a disease-resistant shrimp line or family via a 3–4 generation selective breeding [6–8]. Otherwise, adequate disease-resistant shrimp would not be obtained via the challenge selection approach due to the low survival rate [9], or a large quality of normal shrimp from which resistant shrimp was selected should be

Abbreviations: LD50, the median lethal dose; WSSV, white spot syndrome virus; TSV, Taura syndrome virus; TCBS, thiosulfate citrate bile salts sucrose plate; TSB, tryptic soy broth; SSS, shrimp salt solution.

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collected and consumed. Although those meaningful works has been done for selection of disease-resistant shrimp, little knowledge accumulation could be obtained to assist to collect disease-resistant shrimp rapidly and conveniently.

In the present study, a three-round selection procedure was applied to obtain *Vibrio harveyi*-resistant *L. vannamei* shrimp used for the study on immune response of shrimp against vibriosis. After this procedure, adequate *V. harveyi*-resistant shrimp were collected in a short period of about three month via three rounds challenge selection with a pathogenic *V. harveyi* strain at a median and controllable lethal dose of 96 h LD50.

2. Methods and materials

2.1. Shrimp acclimation

All of *L. vannamei* shrimp used in the experiment were collected from Nanjiang Marine Biotechnology Co, Ltd (Hainan, China). Shrimp were acclimated for 2 weeks with salinity, temperature, pH and density conditions similar to those of the culture ponds before experimentation. During the acclimation period, shrimp were fed three times daily with a commercial formulated shrimp diet (Yuehai Corporation, Zhanjiang, China). No sexual distinction was made and only shrimp in the intermoult stage were used in this study. There was no significant size difference among shrimp. Feeding was stopped 24 h prior to treatment.

2.2. Isolation of V. harveyi strain pathogenic to shrimp

Several abnormal *L. vannamei* shrimp were collected from a shrimp culture pond of Nanjiang Marine Biotechnology Co, Ltd (Hainan, China) and transferred to 100 L sea water to acclimate. After an acclimation of overnight, those shrimp all died with fluorescence in legs, gills, abdominal segments and uropoda. Then their hepatopancreas were dissected, mixed and crushed with a sterile shrimp salt solution (SSS, 450 mM NaCl, 10 mM KCl, 10 mM HEPES, at a pH of 7.0 and with the osmolality adjusted with glucose to 780 mOsm kg^{-1}) buffer in aseptic condition. One hundred microliters of this mixture were coated on Thiosulphate Citrate Bile Salt (TCBS) agar and cultured for 24 h at 30 °C. Green colonies with fluorescence were randomly selected and tested using API 20E strips (bioMérieux, Shanghai, China) according to the manufacture's instruction. Then, colonies with different morphological or biochemical characteristics were identified via 16S rRNA gene sequence analysis using the universal primer set of 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) according to the method described by Marchesi et al. [10]. Strains identified as *V. harveyi* were collected for pathogenicity detection.

2.3. Preparation of bacterial suspension

Bacterium was inoculated on TCBS agar and incubated for 24 h at 30 °C to form colonies 2–3 mm in diameter. Then one of those colonies was transferred to 10 ml tryptic soy broth (TSB supplemented with 2.5% NaCl) and incubated for 6 h at 30 °C. The broth culture was centrifuged at 7500 × g for 10 min at 4 °C. The precipitate was rinsed with sterile SSS buffer, and re-suspended in sterile SSS buffer as bacterial suspensions for studies.

2.4. Detection of pathogenicity of V. harveyi strain to normal shrimp

For detection of pathogenicity of *V. harveyi* isolated from diseased shrimp, rechallenged experiment was carried out in the present study. Briefly, 1 mL of the broth culture prepared according to the method discussed above was centrifuged at 7500 × g for 10 min at 4 °C, the precipitate was rinsed with sterile SSS buffer, and re-suspended with 1 ml SSS buffer to prepare bacterial solution for rechallenged experiment. Then, ten healthy shrimp was used for rechallenged experiment, and 20 μL of this bacterial solution were intramuscularly injected into the second abdominal segment of per shrimp. After injection, those ten shrimp were transferred to a tank containing 100 L sterile sea water with conditions similar to the culture pond. Ten shrimp injected with sterile SSS buffer at a dose of 20 μl $shrimp^{-1}$ were as control. Three replicates were set up for rechallenged experiment group as well as control group.

2.5. Determination of virulence of V. harveyi strain to shrimp

Bacterial suspension prepared above was diluted with SSS buffer to make a series of injection solutions with different concentrations. Ten shrimp were challenged for each concentration. Briefly, 20 μl of injection solution were intramuscularly injected into the second abdominal segment of each shrimp, then transferred to a tank containing 100 L sterile sea water with conditions similar to the culture pond. Ten individuals injected with 20 μl of sterile SSS buffer were as control. Four replicates were set up for challenge experiment of each concentration as well as control. During experimental period, no feeding and no water exchanging were executed, and dead shrimp were recorded and removed every 2 h until 96 h post injection. Mortality was expressed as cumulative mortality and measured by following equation [11]:

$$\begin{aligned}\text{Cumulative mortality}(\%) = [&(\text{Mortality of test} \\ &- \text{Mortality of control}) \\ &/(100 - \text{Mortality of control})]^{*}100\end{aligned}$$

Then, the median lethal dose (LD50) of *V. harveyi* to shrimp was determined. Five LD50 doses were determined, LD50 dose of 6 h, 12 h, 24 h, 48 h and 96 h, which was measured according to the cumulative mortality at 6 h, 12 h, 24 h, 48 h and 96 h after injection, respectively. The LD50 dose was defined as colony-forming units (cfu) $shrimp^{-1}$.

2.6. Selection of V. harveyi-resistant shrimp

To select *V. harveyi*-resistant *L. vannamei* shrimp, a three-round challenge selection procedure was applied. In this procedure, resistant shrimp were selected gradually by three rounds challenge experiment in which shrimp were injected with a pathogenic *V. harveyi* strain at the 96-h LD50 dose. Before this procedure, the LD50 6 h, 12 h, 24 h, 48 h and 96 h of *V. harveyi* to normal shrimp were determined according to the method discussed above.

In the first round selection, normal shrimp was challenged with pathogenic *V. harveyi*. Briefly, a bacterial suspension at a proper concentration was prepared with SSS buffer, and then 20 μl of this suspension was intramuscularly injected into the second abdominal segment of each shrimp to make an injection at a 96 h LD50 dose. After injection, shrimp were transferred to sterile sea water. No feeding and no water exchanging were executed, and dead shrimp was removed instantaneously during 96 h after injection. Survivors were collected as the resistant shrimp from the first round selection, and were acclimated for 2 weeks with normal feeding, water exchanging and other administration for the next round selection. The LD50s of *V. harveyi* strain to the survival shrimp were measured according to the method discussed above, and was used as the resistance of the resistant shrimp from the first round selection to the *V. harveyi* strain.

In the second round selection, the shrimp surviving from the first round selection was challenged with pathogenic *V. harveyi*. Briefly, a bacterial suspension at proper a concentration was