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Immune response of shrimp (*Penaeus monodon*) against *Vibrios furnissii* pathogen

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PEER REVIEW

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Comments

This is a valuable research work in which authors have focused the study on the innate immune response of shrimps against *Vibrios*. In addition, the results can be used by the scientific community for the better disease resistant shrimp culture.
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ABSTRACT

Objective: To analyse experimental infection and immune system of shrimp (*Penaeus monodon*) against *Vibrios furnissii* (*V. furnissii*).
Methods: Experimental animals were collected and acclimatized by maintaining specific temperature, pH and salinity to avoid mortality. Shrimps were experimentally infected with *V. furnissii* and their immune responses were monitored. After the infection all the shrimps were monitored for any symptoms, death rate in 0, 12, 24, 36, 48 h. Then haemolymph were collected and tetrahydrocannabinol, phenol oxidase, nitroblue tetrazolium and lysozyme were monitored in every 12 h at the interval of 48 h.
Results: Shrimps infected by live *V. furnissii* had showed gradual increase in tetrahydrocannabinol, phenol oxidase activity, nitro−blue−tetrazolium and lysozyme activity comparing with the killed and control.
Conclusions: The live *V. furnissii* had showed infection in the shrimp immune system. The live *V. furnissii* shows infection in experimental shrimps comparing with killed *V. furnissii*. So the *V. furnissii* in nature cause the infection in shrimp *Penaeus monodon* immune system. This report could be applied to control of the infection in shrimp hatchery.

KEYWORDS
Shrimp, *Vibrios furnissii*, Immune response, Pathogenesis

1. Introduction

Marine shrimp constitutes the most prominent seafood product in international trade, and aquaculture has been the major source for increased shrimp trading during the past decade. Marine shrimp was regarded as the traded seafood product internationally, and half of total shrimp productions was derived from aquaculture[1]. During fiscal year 2009−2010, according to India's Marine Exports Product Development Authority, total aquaculture production reached 106 000 metric tons, an increase of more than 30% over the previous fiscal year.

In many countries, diseases are major constraint to aquaculture productions. Especially in the shrimp production sector, infectious diseases are considered as the most limiting factor for further development. Since shrimp farmers still rely mainly on wild shrimps for the production of seed stock, genetic selection of resistant

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domesticated shrimp stock is still not feasible. In addition, epidemiological surveys and knowledge to determine the health status of shrimp are scarce so adequate measures to control diseases rather than management practices are not available yet. However, such measures to prevent and control diseases are essential for further development of a sustainable shrimp culture. Moreover shrimp aquaculture is threatened by changes in temperature, precipitation, drought and storms/floods that affect infrastructure and people's livelihoods which can impact aquaculture both negatively and positively[2].

Vibrios are highly abundant in aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide. Ecologically, *Vibrios* plays an important role in the degradation of organic matter and act as a linkage to transfer dissolved organic carbon to higher trophic levels of the marine food web[3]. Several cultivation-dependent and independent studies have showed that *Vibrios* appears at particularly high densities in and on marine organisms, *e.g.* corals, fish, molluscs, sea grass, sponges, shrimp and zooplankton[4]. Plankton colonized by pathogenic *Vibrio* species can potentially act as a vehicle of disease transmission, such as in the case of cholera[5]. However, the details of the interactions between other members of the *Vibrio* genus and the plankton community remain largely unknown.

The *Vibrio* genus includes more than 30 species, at least 12 of which are pathogenic to humans and/or have been associated with food-borne diseases[6]. Among these species, *Vibrio cholerae*, serogroups O1 and O139, are the most important cases, since they are associated with epidemic and pandemic diarrhea outbreaking in many parts of the world[7]. However, other species of *Vibrios* capable of causing diarrheal disease in humans have received greater attention finally. These bactiera include *Vibrios parahaemolyticus*, a leading cause of food-borne disease outbreak in Japan and Korea[8], such as *Vibrios vulnificus*, *Vibrios alginolyticus*, *Vibrios damsela*, *Vibrios fluvialis*, *Vibrios furnissii* (*V. furnissii*), *Vibrios hollisae*, *Vibrios metschnikovii* and *Vibrios mimicus*[9]. Recently, a number of reports have highlighted the pathogenic potential of *Vibrios* toward humans and marine animals (*e.g.* corals, gorgonians and shrimp), which may be coupled with rising of sea water temperature due to global warming[10].

The analyses of the shrimp immune response to infections or to immune stimulations will lead to a better understanding of their involvement in defence reactions against some specific harmful bacteria or viral pathogens. The non-self-recognition factors activate several immediate defence systems mediated by the haemocytes. The defence reactions also include the haemocytic process of encapsulation, phagocytosis and the microbicidal mechanism based on the production of cytotoxic reactive oxygen intermediates demonstrated in shrimps[11]. Haemocyte activation in shrimps results in rapid clotting cellular degranulation, and activation of the proPO system and subsequently the production of sticky molecules[12]. On the other hand, phenoloxidase (PO), part of the immune system of penaeid shrimp, is regulated by digestive enzymes from the hepatopancreas. Circulating haemocytes also play a central role in Crustacean immune defence. The hyaline cells readily attach to foreign particles, and are capable of phagocytosis, encapsulation and nodulation[13].

Shrimp culturing is the primary important aquaculture in coastal area of India especially in southeast coast of India. Monsoon season in that coastal area usually occurrs in October to the beginning of December. We noticed that the shrimp ponds water qualities were completely changed with reference to pH reduction and low salinity. This condition may induce the invasion of pathogenic forms into shrimp culturing ponds through rainfall and inflow of fresh water with sewage contamination. In shrimp ponds near Vellar estuary, Parangipettai, India, some shrimps culturing systems were infected at 70 days of culture and the mortality were approximately 40% occurred in most of the ponds. Therefore the objectives of this study are in the pathogenicity of *Vibrios* and immune response of shrimp against *Vibrios*.

However immune responses need to be analysed under stress conditions and pathological injuries in cultured shrimp for the validation of health monitoring at population level. Immune parameters like total haemocyte count, phenol oxidase activity, respiratory burst activity, lysozyme assay studies will be carried out.

2. Materials and methods

2.1. Experimental shrimp

Eighty five black tiger shrimps *Penaeus monodon* (*P. monodon*) were obtained from a commercial farm and acclimated in the laboratory for 5 d before the experimentation. During the acclimation period, shrimps were fed twice daily with a commercial pellet feed. Only shrimps in the intermoult stage were used for the study. The molt stage was identified by examination of the uropoda in which partial retraction of the epidermis could be distinguished[14]. Shrimps ranged in weight from 16.5 to 19.0 g were used in these experiments. During the experiments, water (temperature, pH, salinity) conditions were 20-28 °C, pH 7.8-8.0, 22-24%.

2.2. Bacterial preparation

To determine lethal dose 50 concentration and pathogenicity of the pathogens (*V. furnissi*) the bacterium culture obtained in Annamalai University Centre of Advanced Study in Marine Biology was cultured in lysogeny broth at 37 °C for 24 h and then the cells were

separated by centrifugation at 8000 r/min for 10 min at 10 °C. Supernatant was discarded and pellet containing cells was suspended in phosphate buffer (pH 7.4) and preserved at 4 °C.

2.3. Experimental infection

Ten shrimps for 2 treatments control shrimp, shrimps injected with *V. furnissii* were used in this study. Therefore, 60 shrimps in total were used for the study. Bacterial cells in phosphate buffer were serially diluted as 3×10^5 CFU/mL. In the ventral sinus of the first abdominal segment of each shrimp, 250 μL of bacterial suspension was injected with heat killed and live *V. furnissii* (each 125 μL). After the infection all the shrimps were monitored for any symptoms and death rate from 1, 2, 4, 6, 12, 24, 36, 48 h. Haemolymph were extracted after every 12 h of infection.

2.4. Haemolymph extraction

Haemolymph was extracted with an insulin syringe with 28 gauge needle from the ventral region of the cephalothorax. The isotonic solution was used as an anticoagulant solution (30 mmol/L trisodium citrate, 0.34 mol/L sodium chloride and 10 mmol/L ethylene diamine tetraacetic acid, at pH 7.55) [15]. The ratio of anticoagulant and haemolymph was 1:1. From this mixture, 25 μL were used for tetrahydrocannabinol analysis and 100 μL were centrifuged at 1400 r/min for 10 min at 4 °C. The supernatant was used to measure PO activity and lysozyme activity. Whole haemolymph was used to measure nitro-blue-tetrazolium (NBT) assay.

2.5. Total haemocytes count

Total haemocyte counts (THCs) were performed by previously reported method[16]. Briefly, 20 μL haemolymph was withdrawn from the ventral sinus of each shrimp using a 100 μL syringe with 80 μL cold anticoagulant (8.2 g/L sodium chloride, 5.5 g/L citric acid, 19.8 g/L glucose, 8.8 g/L sodium citrate, pH 7.45). So the haemolymph was diluted by 5 times with the anticoagulant. Then, the THCs in the diluted haemolymph were immediately determined by using a Neubauer hemocytometer. The THCs on the hemocytometer were observed under contrast microscope (Olympus DP70) and counted in all 25 squares by adaptation of routine methods of medicine and clinical assay.

2.6. PO activity

PO activity was determined spectrophotometrically (490 nm) by the formation of the red pigment DOPA-chrome, after oxidation of the enzyme substrate L-dihydroxyphenylalanine (L-DOPA). Briefly, serum samples (four pools of three animals per treatment) were diluted (1:8) in tris-buffered saline solution and 50 μL of this solution was pre-incubated with an equal volume of the enzyme inducer trypsin (Hi Media, 1 mg/mL) for 15 min at 20 °C in 96-microwell plates (flat bottomed). In controls, trypsin and serum were replaced by tris-buffered saline. After incubation, 50 μL of L-DOPA (3 mg/mL) was added to the wells and the formation of DOPA-chrome was monitored after 0, 5, 15 and 25 min. PO activity was expressed as the variation in absorbance (490 nm) per minute and per milligram of total protein in the samples. Reading was recorded by using microplate reader ($VERSA_{max}$ tunable microplate reader). One unit of enzymatic activity is equivalent to a change of 0.001 in the absorbance/min/milligram of protein at 20 °C[17].

2.7. Reduction of NBT by haematocytes

To determine the amount of superoxide anion the reduction of NBT by haematocytes (respiratory burst activity) was measured[18]. Hemolymph (100 μL) was placed in a microplate and incubated for 30 min at room temperature. The supernatant was discarded and 50 μL of 0.3% NBT were added and incubated for 2 h at room temperature. The supernatant was again discarded, and the haematocytes were fixed with 200 μL of absolute ethanol. Haematocytes were washed twice with 200 μL 70% methanol and let dry. The formazan deposits generated were dissolved in 120 mL 2 mol/L potassium hydroxide and 140 mL dimethyl sulfoxide, and the absorbance (620 nm) was recorded using microtiter plate reader.

2.8. Lysozyme assay

Lysozyme activity was measured by the standard method of minor modifications[19]. In this turbidimetric assay, 0.03% lyophilized micrococcus lysodeikticus in 0.05 mmol/L sodium phosphate buffer (pH 6.2) was used as substrate. Ten microlitres of fish serum was added to 250 mL of bacterial suspension in duplicate wells of microtitre plate and the reduction in absorbance at 490 nm was determined after 0.5 and 4.5 min of incubation at 22 °C by using a microtiter plate reader ($VERSA_{max}$ tunable microplate reader). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min.

3. Results

3.1. Total haemocytes count

All the experimental shrimps were alive after 10 h of injection and control. There was no significant difference in THC of shrimp at the beginning. After 12 h, there were slight increases in both live and heat killed *V. furnissii*. After

24 h, THC of all the experimental shrimp were significantly decreased and during 36 h and 48 h, both injected shrimps shows drastically decreased in THC (Figure 1).

3.2. Total PO activity

There was no significant difference in the PO activity of shrimps at the beginning. After 12 h of infection, all the experimental groups had showed a drastic increase in PO activity compare with control. During the 24, 36 and 48 h, shrimps injected with *V. furnissii* live and *V. furnissii* heat killed had showed gradual increase in PO activity. Finally high PO activity was observed more in shrimps injected with live than heat killed *V. furnissii* and control respectively (Figure 2).

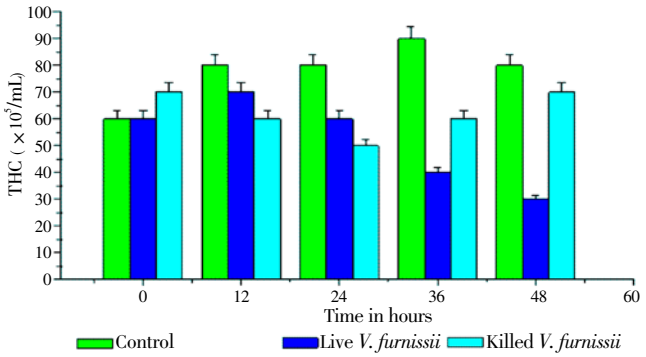

Figure 1. Total haemocyte of *P. monodon* injected with live and killed *V. furnissii* and control at the beginning and after 0, 12, 24, 36 and 48 h.
Each bar represents the mean value from three determinations with the standard deviation, mean±SD.

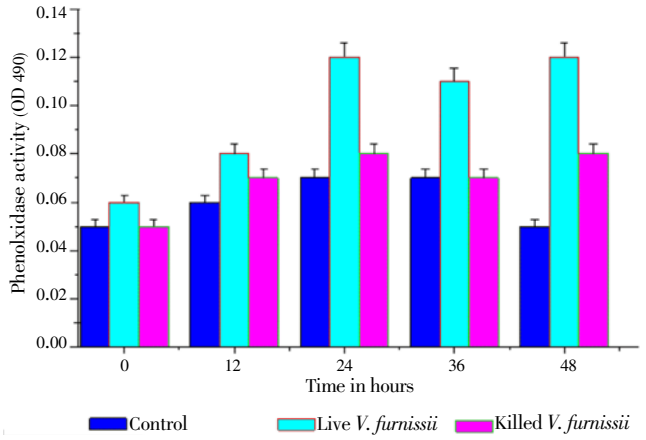

Figure 2. Total PO activity of *P. monodon* injected with live and killed of *V. furnissii* and control at the beginning and after 0, 12, 24, 36 and 48 h.
Each bar represents the mean value from three determinations with the standard deviation, mean±SD.

3.3.Reduction of NBT by haematocytes

There was slight difference in the respiratory burst activity of shrimp at the beginning and after 12 h among the two treatments during the 24, 36 and 48 h, NBT activity of shrimps injected with *V. furnissii* live and *V. furnissii* heat killed was increased significantly. Finally high NBT activity was observed more in shrimps injected with heat killed *V. furnissii* cells than live and control respectively (Figure 3).

3.4. Lysozyme activity

There was a consistent increase of lysozyme activity observed in shrimps after 12 h infection compared with control. During the 24, 36, and 48 h, shrimps injected with *V. furnissii* heat killed showed more activity in lysozyme activity than that in live *V. furnissii* and control. Finally high lysozyme activity was observed in shrimp injected with heat killed cells than lives *V. furnissii* and control respectively (Figure 4).

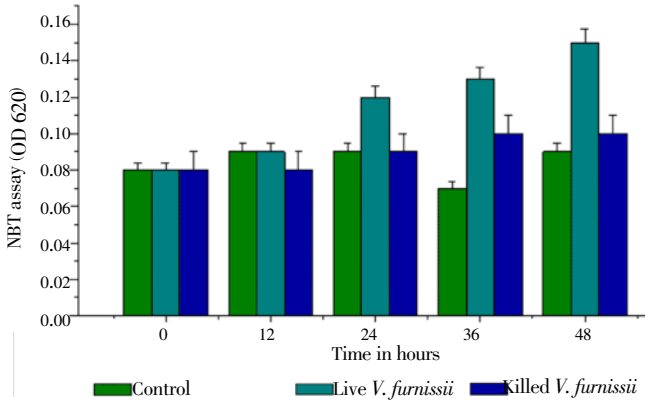

Figure 3. NBT assay of *P. monodon* injected with live and killed of *V. furnissii* and control at the beginning and after 0, 12, 24, 36 and 48 h.
Each bar represents the mean value from three determinations with the standard deviation, mean±SD.

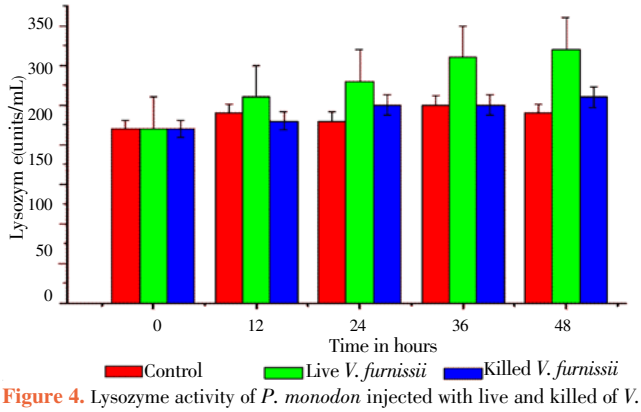

Figure 4. Lysozyme activity of *P. monodon* injected with live and killed of *V. furnissii* and control at the beginning and after 0, 12, 24, 36 and 48 h.
Each bar represents the mean value from three determinations with the standard deviation, mean±SD.

4. Disscussion

Shrimps were collected from Vellar estuary in Parangipettai, Southeast Coast of Tamil Nadu, India. *V. furrnissii* was obtained from CAS in Marine Biology Centre. After injection with live and heat killed *V. furnissii* in the first abdominal segments of the shrimps they were monitored for sign of infection. After 24 h the shrimps showed red colour over the body. If the shrimp cephalothorax were infected, all the infected shrimp would be unable to feed pellet and become lethargy. This result was already reported by Wang

and Gu[20], as *Vibrio* sp. was isolated from the hemolymph of *Penaeus orientalis* with red leg disease. Shrimp ponds with red leg disease have often encountered mortalities of more than 90%. The disease is characterized by an expansion of chromatophores on the periopods and pleopods, giving the appendages a reddish colouration. Yellow pigmentation on the branchial region of cephalothorax is also observed in prawns with red leg disease. Jayasree, *et al*.[21] also reported that 30 moribund shrimps affected by red disease were subjected to bacteriological study and they identified 4 species of *Vibrio*, *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrios alginolyticus* and *Vibrio anguillarum*, from culture ponds at Kakinada and Amalapuram.

After every twelve hours haemolymph were collected from all the experimental animals. In the initial experiments samples and control samples haemolymph were clotted suddenly. But the haemolymph infected by live and heat killed *V. furnissii* take some time to clot. The similar results were reported by Jose, *et al*.,[22] that disease states in crustaceans are associated with decreased clotting of haemolymph. Little attention has been given to the practical use of these observations. However the clotting factor is important to decide whether the animal is infected or not. Sahul, *et al*.,[23] also observed in experimentally infected larvae which were expansion of chromatophores. Opaqueness of the body, loss of setae and bending, twisting and gradual degeneration of appendages as observed in natural epizootic. After twelve hours of post infection gradual increase and sudden increase in PO were observed in shrimps infected by live and heat killed *V. furnissi*. The best study about haemolymph enzymatic system of crustaceans is the PO cascade. PO catalyses the oxidation of phenol to quinines, which subsequently polymerize into melanin, a black pigmented polymer[24]. Similar results were observed when PO activity was assayed. Several studies have reported that the total PO (tot PO) activity increased when white shrimp and crab were exposed (via feed) to a variety of compounds (β-glucans and peptideglucans) commonly found in biological contaminants such as fungi and bacteria.

The respiratory burst is an aerobic process which generates free radicals having potent microbicidal activity. Shrimp's haemocytes produced super oxidase ions. After 12 h infection super oxidase anion's activity were measured in experimental to control animals. The respiratory burst activity was show increase intent to the invaded pathogen except animals infected by live *V. furnissii* shown reduced activity after 48 h. The low level of superoxide anion detected is in accordance with the decreased level of haematocytes observed in white shrimps' hemolymph, suggesting a depression of the cellular immune activity due to the amount of immune cells present[25].

Lysozyme is widely distributed its catalyses, the hydrolysis of bacterial cell walls between eukaryotes and prokaryotes and act as a non-specific innate immune molecules against the invasion of bacterial pathogens[26]. A lysozyme activity gradually increased in all the infected animals comparing with control. Highest lysozyme activity was observed in infected strains for heat killed *V. furnissii*. Hikama, *et al*.[27], found that in Kuruma shrimps' lysozymes was active against *Vibrio* species that are infectious to the penaeid shrimp, suggesting that lysozymes are active against infectious pathogens.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Marine shrimp constitute the prominent seafood product and aquaculture has been major source. But diseases are a major constraint to aquaculture production. So it is necessary to study on the immunological aspects of shrimps, which will overcome this problem.

Research frontiers

The present research work focused on the innate immune response of shrimps to control the disease and productivity loss.

Related reports

Sahul, *et al.*, (1996), Flegel *et al.*, (2010) and Amar and Faisan, (2012) have been conducted various researches on the innate immune responses of infected and vaccinated shrimps.

Innovations and breakthroughs

The authors reported that a few shrimp ponds were

infected recently and the mortality was occurred in and around Vellar estuary, South East Coast of India. So the study is a fine documentation for the pathogenicity of *Vibrios* and immune response of shrimps.

Applications

The analyses of the shrimp immune response to infections or to immune stimulations will lead to a better understanding of their involvement in defence reactions against some specific harmful bacteria or viral pathogens.

Peer review

This is a valuable research work in which authors have focused the study on the innate immune response of shrimps against *Vibrios*. In addition, the results can be used by the scientific community for the better disease resistant shrimp culture.

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