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# The immune response of white shrimp *Penaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under sulfide stress

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**Abstract**

White shrimp *Penaeus vannamei* held in 34‰ seawater were injected with tryptic soy broth (TSB)-grown *Vibrio alginolyticus* ($6.2\times10^5$ cfu shrimp$^{-1}$), and then placed in water containing different concentrations of sulfide at 0 (control), 48, 111, 492 and 1026 μg l$^{-1}$, respectively. After 48–144 h, mortality of *V. alginolyticus*-injected shrimp exposed to = or > ~ 490 μg l$^{-1}$ sulfide was significantly higher than that in the control solution. In another experiment, *P. vannamei* which had been exposed to 0, 49, 105, 488 and 967 μg l$^{-1}$ sulfide for 6, 12, 24 and 48 h were examined for immune parameters, phagocytic activity and clearance efficiency of *V. alginolyticus*. Sulfide concentrations = or > ~ 490 μg l$^{-1}$ for 24 h resulted in decreased hyaline cell count, total haemocyte count, phenoloxidase activity, phagocytic activity and bacterial clearance efficiency, whereas a sulfide concentration at = > ~ 490 μg l$^{-1}$ for 24 h caused a significant increase in respiratory burst and superoxide dismutase activity of *P. vannamei*. It is concluded that concentrations of sulfide = or > ~ 490 μg l$^{-1}$ increased the susceptibility of *P. vannamei* against *V. alginolyticus* infection by a depression in immune ability.


*Keywords:* *Penaeus vannamei*; *Vibrio alginolyticus*; Sulfide; Challenge; Haemocyte count; Phenoloxidase activity; Respiratory burst; Phagocytic activity

## 1. Introduction

White shrimp *Penaeus* (*Litopenaeus*) *vannamei* are the primary penaeid shrimps currently being cultured not only in Central and South Americas, but also in China, Indonesia, Thailand and Taiwan. However, since 1998, shrimp farmers have experienced disease problems linked to production declines due to virus infections like Taura Syndrome Virus (TSV) (Yu and Song, 2000), and vibriosis like *Vibrio alginolyticus* and *Vibrio harveyi* (Liu et al., 2004a). Disease outbreak is often a result of deteriorated environment and stress associated with intensification of shrimp farming, and is associated with increases in the proportion of potentially pathogenic species in the *Vibrio* population of cultured pond waters (Lavilla-Pitogo et al., 1998).

In marine habitats, sulfide which is produced under the activity of sulfate-reducing bacteria and through the decomposition of remaining organic matter in anaerobic condition is commonly found in the bottom layer and sediment of aquatic environment. Sulfide concentrations as high as 600 μM in the overlying water have been reported (Fenchel, 1969). Crustaceans have low tolerance to sulfide as compared to other benthic invertebrates (Vismann, 1991; Bagarinao, 1992). The 1 h LC50 of sulfide is 640 μg l$^{-1}$ in shrimp *Crangon crangon* (Vismann, 1996). The 96 h LC50 of sulfide is 340 and

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378 μg $l^{-1}$ in Kadal shrimp *Metapenaeus dobsoni* for the size of 25–40 mm and 20–25 mm, respectively, and 144, 281 and 342 μg $l^{-1}$ in the Indian white shrimp *Penaeus* (*Fenneropenaeus*) *indicus* for the size of 85–90, 35–40 and 20–25 mm, respectively (Gopakumar and Kuttyamma, 1996). Sulfide concentrations in the range of 0.1–2.0 mg $l^{-1}$ and 4.0 mg $l^{-1}$ have been reported to loss equilibrium, and instantly succumb to death in kuruma shrimp *Penaeus* (*Marsupenaeus*) *japonicus* (Shigueno, 1972). However, nothing is known regarding the effect of sulfide on the immune response of white shrimp *P. vannamei* and its resistance against *Vibrio* species.

Total haemocyte count (THC), differential haemocyte count (DHC), phenoloxidase activity, respiratory burst (release of superoxide anion), SOD activity, phagocytic activity and clearance efficiency are commonly used as immune parameters to evaluate the effects of physico-chemical changes like temperature, salinity, dissolved oxygen and pH, and the effects of toxicants like ammonia, nitrite, and heavy metals on the immune responses of penaeid shrimps (Le Moullac and Haffner, 2000; Rodriguez and Le Moullac, 2000).

This study is aimed at examining 1) the susceptibility of *P. vannamei* to *V. alginolyticus*, and 2) the immune parameters of *P. vannamei* under stress from sulfide. For the immune parameters assays, THC, DHC, phenoloxidase activity, respiratory burst, SOD activity, phagocytic activity and bacterial clearance efficiency were used as indicators.

## 2. Materials and methods

### 2.1. Experimental shrimp

White shrimp *Penaeus vannamei* were obtained from a commercial farm in Iilan, Taiwan, and acclimated in the laboratory for 2 weeks before experimentation. Only shrimp in the intermoult stage were used for the study. The molt stage was identified by the examination of uropoda in which partial retraction of the epidermis could be distinguished (Robertson et al., 1987). For the susceptibility experiment, there were six treatments (five challenged test groups and one unchallenged control group). Test and control groups were comprised of 10 shrimp and in triplicate tanks. For the experiment of immune parameter assays, there were 25 treatments (five concentrations of sulfide at 0, 50, 100, 500 and 1000 μg $l^{-1}$ combined with five exposure times at 0, 6, 12, 24 and 48 h). Tests were carried out in two replicate test groups consisting of four shrimp each in 20 l PVC tanks. In all tests, the shrimp were fed twice daily with a formulated shrimp diet (Tairoun Feed Company, Taipei, Taiwan). The shrimp ranged from 9.68 to 12.31 g, averaging 10.28 ± 1.27 g (mean ± SD) with no significant size difference among the treatments. During experiments, water conditions were 24 ± 1 °C, pH 7.95 to 8.08 and salinity at 34‰.

### 2.2. Culture of *V. alginolyticus*

A pathogenic strain of *V. alginolyticus* isolated from diseased *P. vannamei* with 96 h LD50 (median lethal dose) of $6.6 \times 10^4$ colony forming units (CFU) × $g^{-1}$ was used for the study (Liu et al., 2004). Stocks were cultured on tryptic soy agar (TSA supplemented with 3% NaCl, Difco) for 24 h at 25 °C and transferred to 10 ml tryptic soy broth (TSB supplemented with 3% NaCl, Difco) for 24 h at 25 °C as stock bacterial broth. The broth cultures were centrifuged at 7155 ×*g* for 15 min at 4 °C. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution at $3.1 \times 10^7$ and $2.3 \times 10^7$ colony forming units (cfu) $ml^{-1}$ as bacterial suspensions for the susceptibility test, and for the test of phagocytic activity and clearance efficiency of *P. vannamei* to *V. alginolyticus*, respectively.

### 2.3. Effect of sulfide on the susceptibility of *P. vannamei* to *V. alginolyticus*

The stock solution of sulfide was prepared by dissolving 7.49 g of $Na_2S{\cdot}9H_2O$ (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) in 1 l distilled water to achieve a concentration of 1000 mg $l^{-1}$ sulfide. Challenge tests were conducted in triplicate with 10 shrimp per replicate following the method described before (Liu and Chen, 2004). Into the ventral sinus of the cephalothorax of each shrimp, 20 μl of bacterial suspension ($3.1 \times 10^7$ cfu $ml^{-1}$) was injected resulting in $6.2 \times 10^5$ cfu $shrimp^{-1}$. After injection, shrimp were then kept in separate 60 l glass aquaria (10 shrimp each) containing 40 l of water with different added concentrations of sulfide (zero control and 50, 100, 500 and 1000 μg $l^{-1}$) that was renewed daily. The actual mean measured concentrations of sulfide were 0, 48, 111, 492 and 1026 μg $l^{-1}$ based on the methylene blue method (Clesceri et al., 1998). Shrimp injected with an equal volume of sterile saline solution and kept in water containing 1026 μg $l^{-1}$ sulfide served as the unchallenged controls (Table 1). There were five treatments in the challenged test and one treatment in the unchallenged test. A total of 180 shrimp (30 × 5 + 30) were used for the study. Mortality of shrimp was observed, and the experiment lasted 6 days (144 h).