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# *Vibrio parahaemolyticus* infection in cultured *Fenneropenaeus indicus*: Impact on immune status and oxidative stress response with special reference to *in-vitro* antibiotic resistance pattern

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**Abstract**
In the present study, 150 pieces of marine shrimp "*Fenneropenaeus indicus*" were collected during heavy mortalities attack at El-Deba triangle area, Damietta governorates, Egypt. Samples were collected for clinical, postmortem examination and different microbiological assessments for determination of the causes of mortality. Bacterial identification results revealed infection with *Vibrio parahaemolyticus.* In addition, studying the effect of bacterial infection on the oxidative stress biomarkers in the infected shrimp revealed a significant increase in SOD activity in comparison with the control group in gills, hepatopancreas, and musculature. The activity of GP x significantly increased in the infected group, Where GP x activity in gills, hepatopancreas and musculature revealed a 3.25, 1.64- and 2.18-fold increases when compared with the control group, respectively. Similarly, levels of lipid peroxidation (MDA) showed a significant increase in Vibriosis infected groups in comparison with the control group by 2.1, 1.6 and 2.1 folds for gills, hepatopancreas, and musculature, respectively. The total protein, hemolytic count and lysozyme activity of shrimps exposed to Vibriosis showed a significant decrease compared to control while phenol oxidase activity increased. There was a non-significant difference between infected and the apparently healthy shrimps regarding respiratory burst. There was a significant increase in glucose concentration in shrimp group exposed to Vibriosis in comparison with control healthy shrimps. ($P \leq 0.05$). Regarding antibiotic sensitivity test, *Vibrio parahaemolyticus* isolate was sensitive to Ciprofloxacin and Novobiocin but it was resistant to Amoxicillin and Colistin sulphate.

**Keywords:** *Vibrio parahaemolyticus, Fenneropenaeus indicus*, immune response, oxidative stress

## 1. Introduction
Shrimp farming was used extensively in the last four decades to produce a product of high protein quality that satisfies consumer taste. The sustainability of shrimp production requires providing of adequate sanitary conditions and improving pond management criteria through a selection of appropriate species that can tolerate the adverse environmental condition especially with the worldwide spread of bacterial and viral infection in the shrimp causing high mortalities and economic losses. These have led to the use of closed-life cycle rearing system as a priority to guarantee biosecurity, and avoid the vertical disease transmissions (Martínez-Córdova *et al.*, 2015) [19]. In Egypt, in the last years, several projects and Mari cultures for shrimp production were established around the Suez Canal from 2016 until now. Moreover, the establishment of a culturing system in Lake Galion in Kafr El sheikh and other projects on east Port Said are promising for fish and shrimps production.
Aquatic animals are become integrated to their environment where potential pathogens have the ability to survive patently in their outer environment (Water), such pathogens enter to their bodies through osmoregulation and feeding constantly and can freely propagate and cause serious effects on their hosts.
*Vibrio* species are part of the normal microflora of shrimps causing high mortalities when the natural immune mechanisms of the cultured species are immunosuppressed especially in a bad environment or under severe stress conditions. Vibriosis is a bacterial disease caused by gram-negative bacteria; it is abundant in marine aquaculture farms and ecosystems in general and become pathogenic especially under stressful conditions. It is considered the most prevalent disease and widely responsible for mortality for cultured

Aquaculture organisms worldwide. The major recorded *Vibrio* sp. include *V. parahaemolyticus, V. harveyi, V. alginolyticus, V. vulnificus*, and *V. anguillarum* are usually associated with shrimp diseases. *V. harveyi* is associated with luminescent Vibriosis in shrimps (Lavilla-Pitogo *et al.*, 1998, CR & LD, 1998, Aguirre-Guzmán *et al.*, 2010) [2]. Pathogenic strains of *V. parahaemolyticus* also recorded to be the main cause of massive epidemics in shrimp culture. The main route of infection mainly involves the penetration of bacterium to the host tissue through the chemotactic activity, followed by placement of iron appropriating system and ultimately damages the aquatic organism through excretion of extracellular products including toxins. This study was aimed to identify the main causes of high mortalities during culture of non-native species of shrimps in Egypt *(P. indicus).* In addition, studying the effect of bacterial infection on oxidative stress biomarkers of the infected shrimp.

## 3. Materials and Methods

### 3.1 Naturally infected shrimp (*Fenneropenaeus indicus*) samples

A total of 150 pieces of shrimp samples were collected during grow out stages with an average body weight range (22 ± 5 gm) in 2016 during high mortality attack from different private farms in at Deba triangle area, Damietta governorates, Egypt. Samples were collected for clinical, postmortem examination and different microbiological assessments for determination of the causes of mortality. Clinical examination of the collected naturally infected shrimp was performed to examine any clinical abnormalities. Necropsy of freshly dead and moribund shrimp samples was done to detect any postmortem lesions according to (Raidal *et al*., 2004).

### 3.2 Bacteriological examination

Sampling from hepatopancreas from all the collected samples was carried out under complete aseptic conditions then inoculated into tryptase soya broth (Oxoid) with 2% NaCl and incubated at 25ºC for 24hr. A loop full of incubated broth streaked on thiosulfate citrate bile salt sucrose agar (TCBS) (Oxoid). The plates were incubated at 25 ºC for 24hr. After the recommended incubation period (25 ºC for 24hr), a single colony from each morphologically diverse colony was picked up and re-streaked on a new plate culture media and re-incubated at the same condition. When pure colonies have been obtained, colonial characters were recorded (Shape, size, color and margin of the colony). Pure colonies were preserved on to semi-solid agar at 4 C until used in identification. API® 20E (Bio Merieux, France) used for identification of bacterial isolates according to the instruction of Bio Merieux company.

### 3.3 Antibiogram test

The disc diffusion method described by (Zhou *et al.*, 2005). Purified bacterial colonies were suspended in 0.85 % sterile saline. Then the turbidity of the broth culture was adjusted to McFarland Opacity Standard No 0.5 equal density (Approximately $1.5x10^8$/ml). Then the bacterial suspension was inoculated onto Mueller Hinton salt agar supplemented with 2% NaCl. Commercially available antibacterial disks (Oxoid) was dispensed on the surface of the medium with sterile forceps and incubated for 24 h at 25°C. Antibiotic Sensitivity Discs (Oxoid) as Oxytetracycline (OT 30 ug), Ciprofloxacin (CIP 5 ug), Colistin sulphate (CT 10 ug), Amoxycillin (Aml 10 ug). After incubation of the plates, the degree of sensitivity was determined by measuring the zone of inhibition around each disk which produced by diffusion of antimicrobial agents from the discs into the surrounding medium.

### 3.4 Determination of antioxidant defense system:

Shrimps from infected and apparently healthy (control) groups were dissected and slices from gills, hepatopancreas and musculature were collected for preparation of tissue homogenate by adding phosphate buffer saline (pH 7.4).

#### 3.4.1 Superoxide dismutase activity

The activity of superoxide dismutase was estimated according to the method of (Nishikimi *et al.*, 1972)

#### 3.4.2 Glutathione peroxidase activity

The activity of glutathione peroxidase activity was determined according to the method (Paglia & Valentine, 1967).

#### 3.4.3 Catalase activity

Catalase activity was determined spectrophotometrically by estimating the action of catalase enzyme on hydrogen peroxide that decreases the intensity of horseradish peroxidase activity according to the protocol of (Aebi, 1974).

#### 3.4.4 Malondialdehyde concentration

The concentration of lipid peroxidation product was determined according to the method of (Draper & Hadley, 1990) where thiobarbituric acid reacted with Malondialdehyde to produce red colored complex that directly proportional to the increase in concentration.

### 3.5 Statistical analysis

The data were statistically analyzed using Chi-square and ANOVA tests according to (*SAS, 1987*).

## 4. Results

### 4.1 Clinical signs and postmortem findings

Clinical examination of diseased shrimp revealed that presence of hemorrhages at swimming legs, appendages, and tail (a) compared to apparently healthy (b) control group (Figure 1).

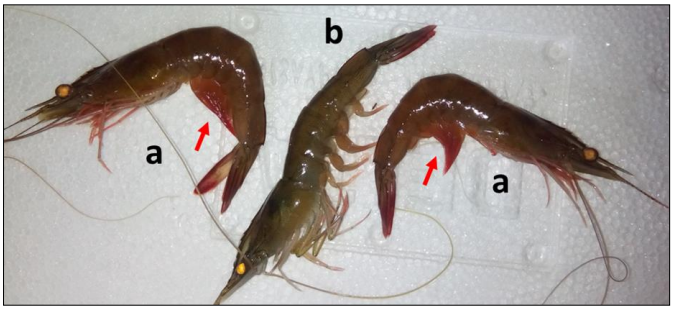


**Fig 1:** Adult shrimp "*Fenneropenaeus indicus*" showing reddening of hemorrhages at swimming legs, appendages, and tail infected with *Vibrio parahaemolyticus*, (a) compared to apparently healthy (b) control group.

### 4.2 Phenotypical, biochemical identification and antimicrobial susceptibility testing of the collected samples

The phenotypical and biochemical identification of the isolated bacteria revealed motile Gram-negative slightly curved rods with yellow pale small colonies on TSA agar with

2% salt and round green opaque colored colonies 2-3 mm diameter on Thiosulphate Citrate Bile Salt Sucrose agar (TCBS) agar. Oxidase and catalase positive reactions and identified as *Vibrio parahaemolyticus.* It was sensitive to Novobiocin (30 µg) and Vibrio static disc (O/129) (150 µg). *Vibrio parahaemolyticus* was sensitive to Ciprofloxacin (Cip5µg), Novobiocin (NV30µg), while it was resistance to Colistin sulphate (CT 10 µg) and Amoxicillin (Aml 10 µg) (Table 1).

**Table 1:** Antimicrobial resistance patterns of the identified *V. parahaemolyticus* from cultured *F. indicus*.

| Antimicrobial agents | Inhibition zone (mm) | Interpretation |
|---|---|---|
| Novobiocin (NV 30 µg) | 17 | Sensitive |
| Oxytetracycline (OT 30 µg) | 12 | Intermediate |
| Colistin sulphate (CT 10µg) | - | Resistant |
| Amoxicillin (Aml 10µg) | - | Resistant |
| Ciprofloxacin (Cip 5 µg) | 19 | Sensitive |

### 4.3 Antioxidant defense system and oxidative stress markers of shrimp infected with *Vibrio parahaemolyticus*

#### 4.3.1 Superoxide dismutase activity

Infected species with Vibriosis showed a significant increase in SOD activity in Comparison with control non-infected group in gills, hepatopancreas, and musculature of shrimps ($P \leq 0.05$). Figure 2.

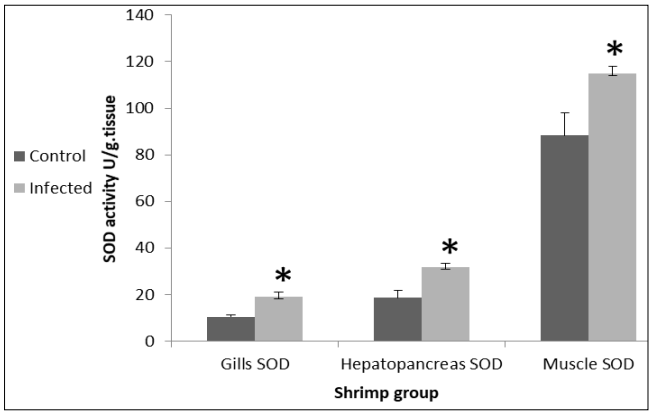


**Fig 2:** SOD activity in gills, hepatopancreas, and musculature of shrimps infected with Vibriosis. Columns contained asterisk indicated a significant difference (P≤0.05)

#### 4.3.2 Glutathione peroxidase activity

The activity of GP x showed a significant increase in Vibriosis infected group in comparison with the control group, where GP x activity significantly ($P \leq 0.05$) increased in all studied tissues when compared with the control group. Figure 3.

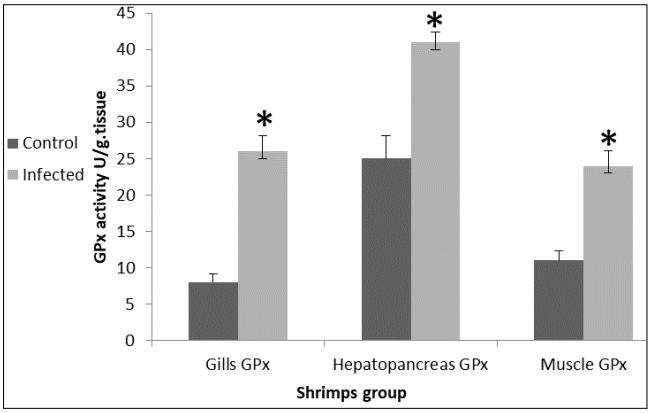


**Fig 3:** GP x activity in gills, hepatopancreas, and musculature of shrimps infected with Vibriosis. Columns contained asterisk indicated a significant difference (P≤0.05).

### 3.3 Catalase activity

There was a non-significant change between the infected group with Vibriosis and control group in all studied tissues ($P \geq 0.05$) Figure 4.

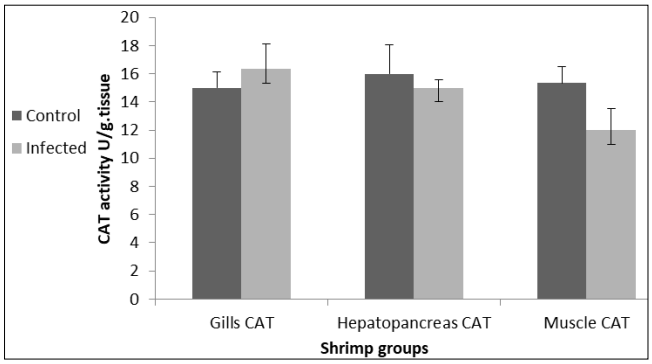


**Fig 4:** CAT activity in gills, hepatopancreas, and muscle of shrimps infected with Vibriosis. Columns contained asterisk indicated a significance difference

#### 4.3.4 Malondialdehyde concentration

The levels of lipid peroxidation (MDA) showed a significant increase in Vibriosis infected groups in comparison with the control group by 2.1, 1.6 and 2.1 for gills, hepatopancreas, and musculature, respectively ($P \leq 0.05$) Figure 5.

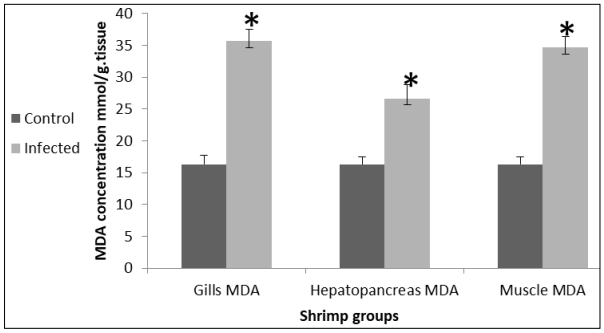


**Fig 5:** MDA concentration in gills, hepatopancreas, and musculature of shrimps infected with Vibriosis. Columns contained asterisk indicated a significant difference (P≤0.05).

### 3.4 Glucose and total protein concentrations

There was a significant increase in glucose concentration in shrimp group exposed to Vibriosis in comparison with control healthy shrimps. ($P \leq 0.05$). While a significant decrease in the concentration of total protein in shrimps infected with Vibriosis when compared with another apparently healthy control group ($P \leq 0.05$) Figure 6.

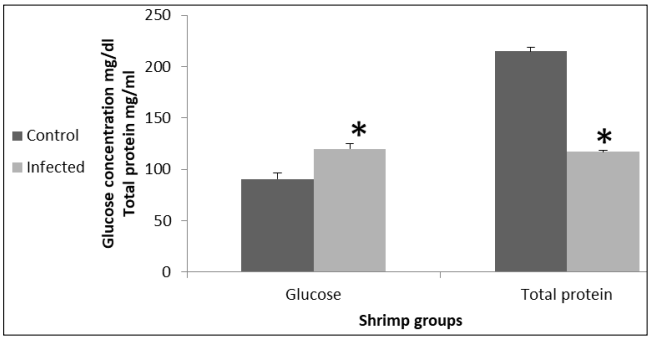


**Fig 6:** Glucose and total protein concentration in the Haemolymph of shrimps infected with Vibriosis. Columns contained asterisk indicated a significance difference

### 4.4 Immunological parameters of hemolymph in shrimps

The hemocytes count of shrimps exposed to Vibriosis showed a significant decrease in comparison with normal healthy individuals ($P \leq 0.05$) (Figure 7). Similarly, Vibriosis infection produced a significant decrease in lysozyme activity in comparison with the control group ($P \leq 0.05$). While there was a significant increase in phenol oxidase activity in the infected group compared with the control group by sevenfold ($P \leq 0.05$) (Figure 8). In contrast, there was a non-significant difference between infected and apparently, health shrimps regarding respiratory burst activity ($P \geq 0.05$) (Figure 7).

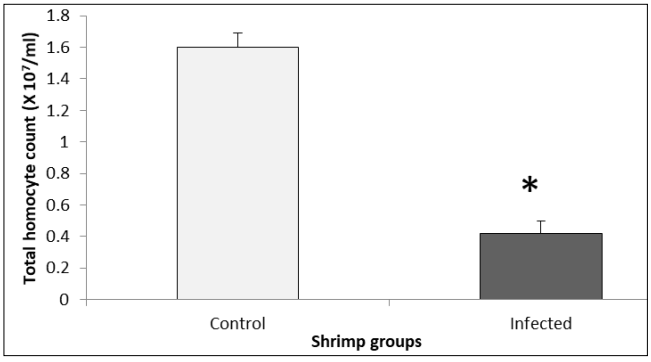


**Fig 7:** Total hemocyte count in the hemolymph of shrimps infected with Vibriosis. Columns contained Asterix indicated a significant difference ($P \leq 0.05$).

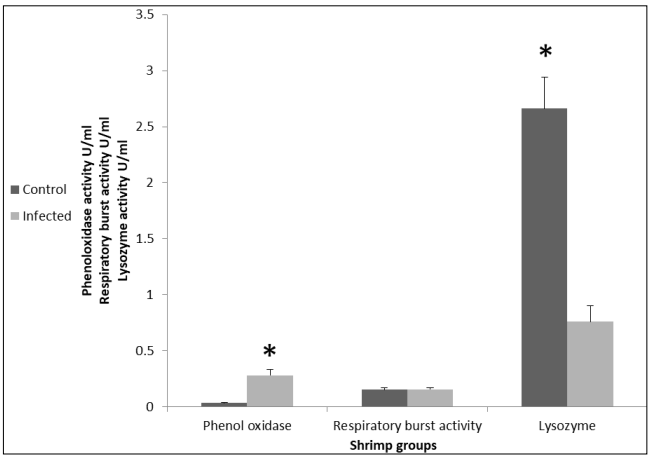


**Fig 8:** Phenol oxidase, respiratory burst and lysozyme activity in the hemolymph of shrimps infected with Vibriosis. Columns contained asterisk indicated a significant difference ($P \leq 0.05$).

## 5. Discussion

Shrimp culture is being threatened by many factors either environmental, culture system or infectious including virus, bacteria or parasites. Among bacterial infections, Vibriosis recorded on the top list of pathogens affecting shrimp culture causing high mortalities all over the world according to (Austin *et al*., 2012), (Wang *et al*., 2015).

Vibrio species are dominantly present in the aquatic environment. However, the presence of *Vibrio parahaemolyticus* in the collected shrimp samples considered a risk factor for a public health concern especially to those who eat shrimp either raw or undercooked. Clinical examination of diseased shrimp in the present study revealed the presence of hemorrhages at swimming legs, appendages, and tail of the collected shrimp samples. Similar mortalities have been recorded periodically all over the world caused by *Vibrio parahaemolyticus* (Lightner, 1996, Thakur *et al*., 2003, Vaseeharan & Ramasamy, 2003) [39]. In addition, (El Far *et al*., 2015) [10] reported the same clinical signs in the isolated shrimp samples infected with *Vibrio parahaemolyticus* supporting the recurrent infection recorded in the present study.

The trials to control Vibriosis infection in aquaculture either fish or shrimp culture were done through oral administration of antibiotic medicated diet. However, the frequent use of antimicrobial compounds caused many problems due to the development of antibiotic-resistant species of pathogens. Recorded that, in different states in Australia 74% of the Vibrio species isolates from aquaculture settings were resistant to at least one antibiotic. Furthermore, the presence of the antibiotic residues in commercialized aquaculture products negatively affecting human health and causing allergy and even toxicity (Cabello, 2006, Pridgeon & Klesius, 2012) [25].

In the present study, the isolated strain show sensitivity to both ciprofloxacin (Cip5µg) and Novobiocin (NV30µg), while it was resistance to Colistin sulphate (CT 10 µg) and amoxicillin (Aml 10 µg). The findings in the present study are different from other previous studies in which, V. parahaemolyticus was highly susceptible to some other antibiotics including chloramphenicol, trimethoprim-sulfame thoxazole, gentamicin, levofloxacin, and tetracycline. In addition, during the sampling campaign program, several Vibrio isolates were resistance to Erythromycin, Gentamycin, Tetracycline, Ampicillin, Amoxicillin, Cephalexin, Cephalothin, and Oxytetracycline. Based on these previous findings, besides the widely use of tetracyclines and Imipenem prescribed by doctors for bacterial infections. This may explain the production of recent resistance strains that differ from place to another or even the system used in shrimp culture or even handling till reaching the consumer. Moreover, to such problems including public health concern, there is another economic problem come from the frequent exports rejections from countries due to the presence of prohibited antibiotic residues. Recently, Japanese authorities have refused market entry to 464 Vietnamese shipments during (2003 – 2010) (Aya & Nam, 2017) [4]. Finally, from all these data the use of antibiotics not only caused a severe decline in the production but also lead to a loss of competitiveness in the export market from the food hygiene point of view.

Reactive oxygen species are produced as a result of the increase of pro-oxidant reactive oxygen species in relation to antioxidant defense system which is named as “oxidative stress” as a result of different pathological condition or stressors.

In the current study, the significant elevation in SOD and GP

x activities and Malondialdehyde concentration reflects the oxidative damage produced by Vibrio spp. On shrimps. Superoxide dismutase is an antioxidant enzyme that has an important role in scavenging superoxide radical, the initiator of reactive oxygen species, chain reaction. It is also has a critical role in the pathogenicity of a certain pathogenic agent such as *Vibrio cholera*, where expression of both copper and zinc superoxide dismutase increase significantly during infection. *Vibrio penaeicida* increased the activity of superoxide dismutase in muscle after 48 hours of infection in muscles of shrimps. *V. parahaemolyticus* injection increases the expression of Cu, Mn-SOD and catalase activity in shrimps. Catalase activity increased significantly by 7 fold when *V. parahaemolyticus* is injected in hepatopancreas in shrimps.

Similarly, in black tiger shrimp, *V. parahaemolyticus* managed to significantly increase the expression of GPx and other peroxidases activities with 12 hours of exposure in hepatopancreas and gills. Moreover, the expression of catalase with significantly lowered in shrimps' infected group when compared with the control group at 12 hours and 24 hours of exposure. Finally, the levels of lipid peroxidation are elevated markedly in *V. parahaemolyticus* exposed group with 6 to 24 hours of exposure in comparison with the control group.

Hemocytes are secreted from hemolymph in shrimps and characterized by the immune-regulatory role which an essential cellular compartment of hemolymph in protection against the infectious agent in invertebrates (van de Braak, 2002) [28]. There was a significant decrease in total hemocyte count in response to Vibrio infection in shrimps, which was discussed by the work of (Ford *et al.*, 1993). They found that decreasing of the total hemocyte count is occurred consequently in alteration of the normal physiological condition in shrimps because of bacterial infection. In another literature concluded by (Pipe & Coles, 1995), they suggested that the significant reduction in total hemocyte count could be as a result of cell lysis or the migration of cells from hemolymph to tissues.

Phenol oxidase system has a critical role in innate immunity of invertebrates in recognizing infective agent and defense against (Rodrıguez & Le Moullac, 2000) [28]. The activity of phenol oxidase in shrimp is positively correlated with the concentration of bacterial cells. Therefore, in the current study, the activation of phenol oxidase has occurred after challenge with Vibrio species which was also elevated by the use of lipopolysaccharide derived from *Escherichia coli*.

Lysozyme has a bactericidal effect on vertebrates and invertebrates, which showed a significant change in different stages of animals' life. Serum lysozyme activity showed a significant decrease in activity in comparison with the control group which is attributed to the stressors produced by the infectious agent in the current study (Möck & Peters, 1990) [20]. Artificially induced infection in common carp with *Pseudomonas alcaligenes* and *Aeromonas punctate* produced a significant decrease in lysozyme activity in comparison with the control group (Siwicki & Studnicka, 1987) [32].

Serum total protein significantly decreased, and glucose showed a significant increase in their concentration, respectively. In a similar study in Pacific white shrimp, a significant reduction in serum total protein was observed in shrimps injected with Taura syndrome virus. The increase in serum glucose concentration was attributed to the increase of stressors in the exposed fish due to the elevation of cortisol, gluconeogenic hormone.

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