Leukocyte Response and Phagocytic Activity in Common Carp, *Cyprinus carpio* Experimentally Infected With Virulent *Aeromonas allosaccharophila*

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Abstract: Total and differential leukocyte count, and the phagocytic activity in blood of common carp, *Cyprinus carpio* (Linnaeus, 1758), experimentally infected with virulent *Aeromonas allosaccharophila* was evaluated in this study. For experimental study, common carp fingerlings (10.0 ± 1.5 g Weight, 8.5 ± 2.3 cm Length) were divided into five groups in duplicates of un-injected fish, fish injected with sterile phosphate buffered saline (PBS), and fish injected with 4.2 x 10^4, 1.9 x 10^6 and 7.3 x 10^8 colony forming units per milliliter (CFU ml^-1) of suspension of *A*. allosaccharophila. Forty eight hours after infection, the total and differential leukocyte count and phagocytic activity of blood leukocytes of common carp was determined. The leukocyte, lymphocyte and monocyte count were significantly (P< 0.05) high in experimentally infected fishes, while no significant change in neutrophils number was observed. In fishes injected with 4.2 x 10^4, 1.9 x 10^6 and 7.3 x 10^8 CFU ml^-1 of bacterial suspension, the percentage of phagocytic activity in the blood were 55.3, 54.7 and 58.2, respectively. The result suggests that, *A*. allosaccharophila could influence the leukocyte number and phagocytic activity of blood of common carp, which is an integral component of innate immune system of fish.

Keywords: Bacteria, Blood, Fish, Innate immunity, Phagocytosis.

Introduction

Innate immunological parameters are important indicator of fish health and its physiological status. It is one of the most important tool for fish disease diagnosis, and it’s alter depend on the health condition of fish (Hrubec et al., 2000). Increase or decrease in neutrophil, monocyte and lymphocytes number may occur during microbial infections (Haney et al., 1992). Phagocytic activity is a cellular mediated nonspecific defense mechanism in fish, and may also vary according to its health status. Phagocytic activity of fish blood can be used as a significant nonspecific immunological indicator of immune-suppression in fish (Anderson, 1990). Increase in phagocytic activity in the spleen and blood of bacterial infected fish was reported earlier (Cai et al., 2004).

Outbreaks of bacterial diseases have been causing severe economic losses to fish farmers in India (Shahi and Mallik, 2013). Among bacterial diseases, motile aeromonad septicemia (MAS) caused by *Aeromonas* spp. is commonly observed in freshwater cultured fish. Several *Aeromonas* such as *A. hydrophila*, *A. sobria*, *A. bestiarum*, and *A. salmonicida* are responsible for bacterial septicemia and ulcers in cultured fishes, worldwide. The outbreak of disease by *Aeromonas* spp. is a common problem in the intensive carp culture system, where stress is generated by higher stocking density and other adverse biotic and abiotic conditions. As carp farming system they also cause tail rot, fin rot, blood hemorrhage and epizootic ulcerative syndrome in rainbow trout culture practice of India (Shahi et al., 2013) The mortality caused by Aeromonads to cultured fishes can be over 95 % under unfavorable condition (Zhan, 2004). *Aeromonas allosaccharophila* is a mesophilic bacterium, first isolated from diseased elvers, *Anguilla anguilla* in Spain (Martinez-Murcia et al.,...
Shahi et al.

Preparation of bacterial inoculum & its administration

Bacterium was cultured overnight in tryptic soy broth (TSB, Difco) at 27 °C. Culture was centrifuged at 10,000 rpm for 5 min, to pellet the cell. After removal of supernatant, the pellet was washed thrice in sterile phosphate buffered saline (PBS, pH 7.2). The concentration of the bacterial cell was determined and adjusted by taking OD at 540 nm. Fishes were injected with 100 µl of bacterial suspension in three different cell concentrations $4.2 \times 10^4$, $1.9 \times 10^6$ and $7.3 \times 10^8$ colony forming unit (CFU) ml$^{-1}$. The two other groups were un-injected fish and fish injected with 100 µl of sterile PBS. All the injections were given in swim bladder, according to the method described earlier (Martins et al., 2004).

Leukocytes count & Phagocytic activity

Forty eight hours after injection, the fish were anesthetized with tricaine methanesulphonate (MS-222) solution (4 mg ml$^{-1}$). The blood was withdrawn from the caudal vein with a syringe rinsed with 2.5 % EDTA solution and kept in 2.0 ml centrifuge tube coated with 20 µl of 2.7 % EDTA. The collected blood was used for total leukocyte count and differential leukocytes count (Martins et al., 2004). The blood was also used for evaluation of phagocytic activity of leukocytes. Total leukocytes were calculated by indirect methods as follows:

\[
\text{Leukocytes} \; \mu l^{-1} = \frac{(\text{Leucocyte number in the smear} \times \text{erythrocyte number per microlitre})}{(2000 \text{ erythrocytes counted in the blood smear})}
\]

Leukocyte phagocytic function was carried out by the method described previously (Cai et al., 2004) with slight modifications. Five hundred microlitre of blood was taken in 2 ml centrifuge tubes, to which 0.25 ml of $5 \times 10^8$ CFU ml$^{-1}$ A. allosaccharophila culture was added. After mixing the tubes were incubated at 28 °C in a dry bath for 30 min, with occasional shaking. Incubated tubes were centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the upper layer of the precipitate was used to make blood slides (three slides for each fish).
Leukocyte response and phagoctic activity

The number of leukocytes that engulfed bacteria was calculated as follows:

Phagocytic percentage (%) = \[ \frac{100 \times (\text{Phagocytotic leukocyte number})}{(\text{Total leukocyte number})} \]

Statistical Analysis

The studied parameters were subjected to one way analysis of variance (ANOVA) and F-test \((P < 0.05)\), and the averages to Tukey tests (SPSS version 19.0). Differences between experimental groups were expressed at a significant level of \(P < 0.05\).

Results and Discussion

The blood smear of injected common carp with different cell types is shown in Figure 1 and Figure 2.

The leukocyte count was higher \((P < 0.05)\) in injected fishes compared to un-injected and fishes injected with PBS (Table 1). Significantly higher \((P < 0.05)\) lymphocytes and monocytes numbers were observed in injected group than the control group. There was no significant change in the neutrophil number in experimental and control group. Higher \((P < 0.05)\) percentage of phagocytosis by leukocytes was observed in challenged groups (Table 1). The highest phagocytosis was found in fish injected with \(7.3 \times 10^8\) CFU ml\(^{-1}\) of \(A.\) allosaccharophila.

Leukocyte count is an indicator of fish health status, due to its role in non-specific or innate immune response. An increase in the leukocyte count and its functions is most likely results in an enhancement of the non-specific defense, because macrophages and other phagocytic cells are the key cells of immune system. The present study describes the influence of \(A.\) allo\- saccharophila on leukocyte count and phagocytic function of common carp experimentally infected with the bacterium. Leukocyte number was significantly higher in experimentally infected fish at \(1.9 \times 10^6\) and \(7.3 \times 10^9\) CFU ml\(^{-1}\), which indicates that \(A.\) allosaccharophila could induce the non specific innate defense mechanism of fish.

An increase in the number of circulating lymphocyte is observed in bacterium infected fish (Martins et al., 2009). Increased number of lymphocytes is an integral part of defense mechanism of any living organism. However, there are reports of lymphopenia under certain conditions, especially when fishes are intraperitoneally injected with bacterium (Lamas et al., 1994; Balfry et al., 1997). The lymphopenia in
injected fishes might be caused by localized migration of lymphocytes to tissues.

Neutrophils are the first line of innate immune against infectious diseases. Apart from releasing various antimicrobial molecules, neutrophils also release neutrophil extracellular traps (NETs) for the containment of infection. Activated neutrophils provide signals for the activation and maturation of macrophages as well as dendritic cells. Neutrophils are also involved in the regulation of T-cell immune response against various pathogens. The number of neutrophils in fish injected with bacterium are not significantly different from the un-injected and PBS injected fish, which is in concurrence with the earlier finding (Ranzani-Paiva et al., 2004), while few other researchers found significant increase in the number of neutrophils and monocytes after bacterial infections (Garcia et al., 2007). In this study, monocytes were higher in injected fish in comparison to control fish.

Phagocytosis is one of the important processes in poikilothermic animals because it is the process that is least influenced by temperature (Blazer, 1991; Lange and Magnadottir, 2003). Phagocytic cells are the important cellular components of the innate immune system of fish (MacArthur and Fletcher, 1985), and their phagocytic activity is a defence mechanism, which is an important characteristic of the non specific immune system. The main cells involved in phagocytosis in fish are neutrophils and macrophages (Secombes and Fletcher, 1994). In this study the higher percentage of phagocytosis by leukocytes was observed in challenged groups (Table 1), as well as the highest phagocytosis was found in fish injected with $7.3 \times 10^8$ CFU ml$^{-1}$ of bacterium.

A. allosaccharophila is a rare bacterium, known to cause disease in fish. However, so far there are no reports of the effect of this bacterium on innate immune cells of carps. The results showed that, there is significant difference in some of the studied immune related blood parameters of common carp, experimentally challenged by A. allosaccharophila, which indicates that this virulent bacterium could induce the migration of immune cells. Future studies are needed to know the effect of this bacterium on hematological parameters of carp as well as antimicrobial activity of the serum.

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References


