

# The effect of *Aeromonas hydrophila* infection on the non-specific immunity of blunt snout bream (*Megalobrama amblycephala*)

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## Abstract

*Aeromonas hydrophila* is the main reason of epidemic septicaemia for freshwater fish. In the present study, the effect of *Aeromonas hydrophila* infection on the non-specific immunity of blunt snout bream (*Megalobrama amblycephala*) was studied. After *Aeromonas hydrophila* challenge, lysozyme activity was significantly increased at 4 h, 1 d, 3 d, 5 d, 14 d and 21 d. An increased level of lysozyme activity indicated a natural protective mechanism in fish. The significant increases of superoxide dismutase activity and catalase activity in treatment group were detected at 4 h, 1 d, 3 d, 5 d, 14 d and 21 d after *Aeromonas hydrophila* challenge. Increase in the superoxide anion and H<sub>2</sub>O<sub>2</sub> is considered to be beneficial for self-protection from disease. Acid phosphatase activity increased significantly at 1 d, 3 d and 5 d after *Aeromonas hydrophila* challenge. Alkaline phosphatase activity in treatment group showed significant increase at 4 h, 1 d, 3 d, 5 d, 14 d and 21 d compared to control group. Increased phosphatase activity indicates higher breakdown of the energy reserve, which is utilized for the growth and survival of fish. These results revealed that the non-specific immunity of fish played an important role in self-protection after pathogens infection.

**Key words:** Blunt snout bream (*Megalobrama amblycephala*), *Aeromonas hydrophila*, SOD, Catalase activity, lysozyme activity, non-specific immunity.

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## Introduction

*Aeromonas hydrophila* is a pathogenic organism that causes a broad spectrum of diseases in humans and animals [1]. While it was thought to be an opportunistic pathogen in humans, a growing number of intestinal and extra-intestinal cases of disease indicate that *Aeromonas hydrophila* is an emergent human pathogen, irrespective of the immunological aspects of the host [2]. *Aeromonas hydrophila* is a Gram-negative motile bacillus that causes motile aeromonad septicaemia [3].

Infectious diseases are a major cause of economic loss in commercial aquaculture [4]. Like higher vertebrates, fish rely on both specific and nonspecific mechanisms to protect themselves against invading pathogens. In fish, the primary lines of non-specific defences are the skin and mucus [5]. As a first line of defence, various peptides/proteins such as lysozymes, antibodies, complement factors, and other lytic factors are present in serum, where they pre-

vent colonisation of microorganisms, leading to prevention of infection and disease [6-8]. When pathogens enter the body, cellular and humoral non-specific defences are mobilised [5]. It is well known that the innate immune system in fish can be triggered by many immunostimulants, both synthetic and natural ingredients [5].

Blunt snout bream (*Megalobrama amblycephala*) is an economically important freshwater fish species in the aquaculture industry in China. However, diseases of the cultured fish have occurred frequently, and losses due to infectious diseases severely restrict the development of aquaculture. *Aeromonas hydrophila* infection was a major problem in the blunt snout bream culture industry in China. Until now, the effect of *Aeromonas hydrophila* infection on the immunity of blunt snout bream has been rarely studied, although it is very important for aquaculture [9-11]. Knowledge of the effect of *Aeromonas hydrophila* infection on non-specific immunity is of potential importance for the immunological control of

disease in the *M. amblycephala* farms. Therefore, the aim of present study is to determine the effect of *Aeromonas hydrophila* infection on the non-specific immunity of blunt snout bream. The blood serum lysozyme activity, superoxide dismutase (SOD) activity, catalase activity, alkaline phosphatase activity, and acid phosphatase activity were examined.

## Material and methods

### Experimental fish

Blunt snout bream (*M. amblycephala*) (body weight: 45–55 g) were collected from the breeding base of the Hunan University of Arts and Science. Before the experiments, fish were acclimatised in quarantine plastic tanks in aerated freshwater at  $24 \pm 2^\circ\text{C}$  for two weeks. The experiment was approved by the guidelines of Institutional Animal Care and Use Committees (IACUC) of Hunan University of Arts and Science, Changde, China.

### Challenge experiment

The bacteria *Aeromonas hydrophila* was isolated from diseased *M. amblycephala* in Dongxi Lake (Wuhan, China) by our laboratory and used for challenge experiment with intraperitoneal injection. A single colony was cultured in LB medium at  $28^\circ\text{C}$  to mid-logarithmic growth. In a pre-challenge experiment prior to the challenge trial, the concentration  $1 \times 10^7$  colony forming units/mL (CFU/ml) was determined as  $\text{LD}_{50}$ . A total of 240 individuals were injected with 0.1 ml ( $1 \times 10^7$  colony forming units [CFU]/ml) bacterial suspension per individual in the treatment group (infected group), while 240 individuals were injected with the same volume of phosphate-buffered saline (PBS, pH 7.2) as the control group (non-infected group). After the treatment, the fish were returned to tanks with a water temperature of  $27 \pm 0.5^\circ\text{C}$ . Fish were euthanised by exposure to 300 mg/l of MS-222 (Sigma, USA) before sampling. Thirty injected individuals from three pools of the infected group and 30 injected individuals from three pools of the non-infected group were randomly sampled at 4 hours and 1, 3, 5, 14, 21, and 28 days post injection. Each pool sampled 10 individuals, and the samples of 10 individuals were pooled respectively.

### Blood sampling

Whole blood (1 ml per individual) was collected from the caudal vein using syringes and needles that were rinsed with heparin. The blood sample was centrifuged at 1000 g for 5 min in order to separate the blood serum. Then the sample of blood serum was stored at  $-20^\circ\text{C}$  for CAT, AKP, ACP, T-SOD, and lysozyme activity test.

### Lysozyme activity

The lysozyme activity was measured using the turbidity assay. Lysozyme standard product powder (80,000

U mg<sup>-1</sup>) was used as a standard, and 1 mg lyophilised micrococcus lysodeikticus in sodium phosphate buffer (pH 5.75) was used as substrate. 20  $\mu\text{l}$  plasma sample was added to 2 ml of substrate, and the reduction in the transmittance at 530 nm was determined after 20 s and 8 min of incubation. One unit of lysozyme activity was defined as an increase in transmittance of 0.001 per min.

### Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical-dependent reactions using the Ransod Kit (Randox, Crumlin, UK). Briefly, the reaction mixture (1.7 ml) contained xanthine (0.05 mM) and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025 mM) dissolved in CAPS 50 mM (pH 10.2) and EDTA (0.94 mM). In the presence of xanthine oxidase (80 U/l, 250  $\mu\text{l}$ ), superoxide and uric acid were produced from xanthine. Then, the superoxide radical reacted with INT to produce a red formazan dye. The optical density was measured at 505 nm, and the rate of reaction was estimated from the absorbance readings 30 s and 3 min after adding xanthine oxidase. A reference standard SOD was supplied with the Ransod Kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units  $\text{ml}^{-1}$ .

### Catalase, alkaline phosphatase, and acid phosphatase activity

In the experiments, we measured catalase activity (CAT), alkaline phosphatase activity (AKP), and acid phosphatase activity (ACP) of blood serum. Catalase activity, alkaline phosphatase activity, and acid phosphatase activity were determined using Diagnostic Reagent Kits purchased from Nanjing Jian Cheng Bioengineering Institute (China).

### Statistical analysis

Data are presented as mean value  $\pm$  standard error (SE); mean values of treatments were compared using the one-way analysis of variance by Duncan's test using the STATISTICA software package (Version 6.0, Statsoft Inc.). Differences between the control and the treatment groups were considered to be statistically significant at  $p < 0.05$ .

## Results

### Lysozyme activity

Lysozyme activity in the treatment group showed significant increase ( $p < 0.01$ ) at 4 h, 1 d, 3 d, 5 d, 14 d, and 21 d compared to the control group (Fig. 1). In the treatment group, lysozyme activity was increased gradual-

ly after *Aeromonas hydrophila* challenge and reached the peak at 1 d, and finally there was a tendency to recover to control group level after 1 d (Fig. 1).

### Superoxide dismutase (SOD) activity

Superoxide dismutase activity in the treatment group showed a significant decrease at 1 d, 3 d, 5 d, 14 d, 21 d, and 28 d compared to the control group (Fig. 2). In the treatment group, superoxide dismutase activity was decreased gradually after *Aeromonas hydrophila* challenge (Fig. 2).

### Catalase activity

A similar tendency to that observed for SOD was found for CAT. The activity of CAT was found to be significantly reduced ( $p < 0.05$ ) at 1 d, 3 d, 5 d, 14 d, 21 d, and 28 d after *Aeromonas hydrophila* challenge (Fig. 3). In the treatment group, catalase activity was decreased gradually after *Aeromonas hydrophila* challenge (Fig. 3).

### Acid phosphatase activity

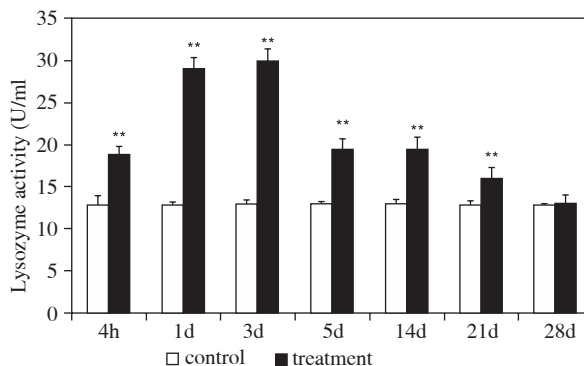
Acid phosphatase activity of the treatment group increased significantly ( $p < 0.01$ ) at 1 d, 3 d, and 5 d compared to the control group (Fig. 4). There was no significant difference between the treatment group and the control group at 4 h, 14 d, 21 d, and 28 d (Fig. 4). In the treatment group, catalase activity was increased gradually after *Aeromonas hydrophila* challenge and reached the peak at 3 d, and there was a tendency to recover to control group level (Fig. 4).

### Alkaline phosphatase

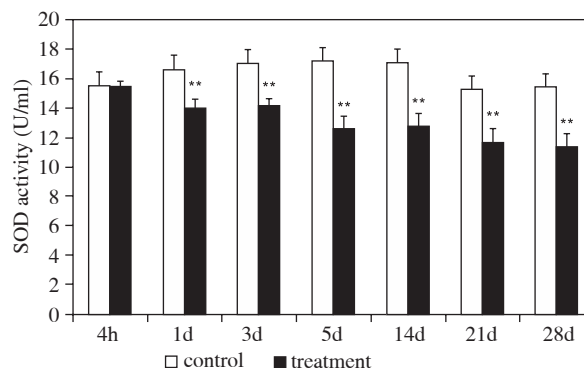
Alkaline phosphatase in the treatment group showed significant increase at 4 h, 1 d, 3 d, 5 d, 14 d, and 21 d compared to the control group (Fig. 5). In the treatment group, the level of alkaline phosphatase was increased gradually after *Aeromonas hydrophila* challenge and reached a peak at 3 d, and there was a tendency to recover to control group level (Fig. 5). Finally, there was no significant difference between the treatment group and the control group at 28 d (Fig. 5).

## Discussion

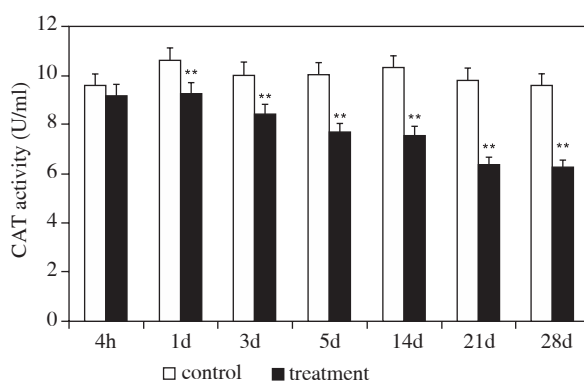
The present study preliminarily explored, for the first time, the effect of *Aeromonas hydrophila* infection on the non-specific immunity of blunt snout bream (*Megalobrama amblycephala*). Neutrophils are considered to be the source of lysozyme, and the enzyme appears to be much more bactericidal than lysozyme of higher vertebrates [12]. Lysozyme activity functions as a primary defence factor of non-specific humoral immunity in preference to cellular defence mechanisms [13]. Fish serum lysozyme is believed



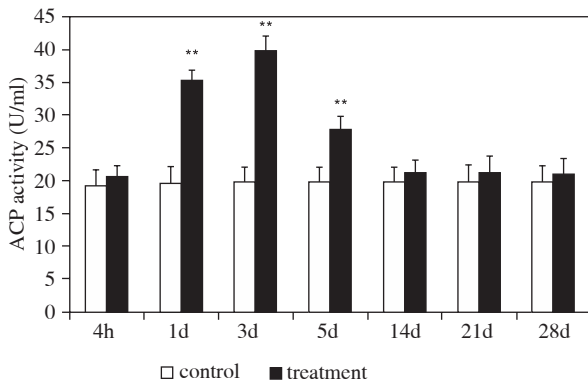
**Fig. 1.** Lysozyme activity of *M. amblycephala* after *A. hydrophila* infection. Data are presented as mean  $\pm$  SE ( $n = 30$ ). Differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences from the control group (\* $p < 0.05$ ; \*\* $p < 0.01$ )



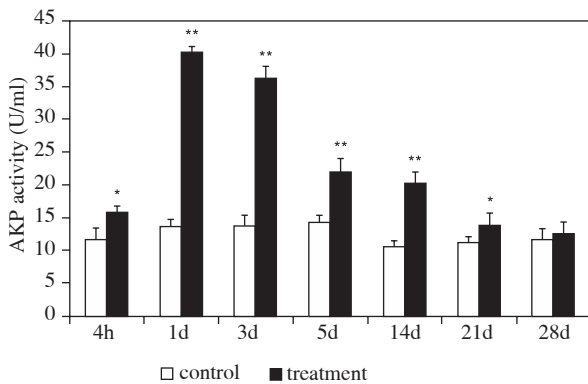
**Fig. 2.** Superoxide dismutase (SOD) activity of *M. amblycephala* after *A. hydrophila* infection. Data are presented as mean  $\pm$  SE ( $n = 30$ ). Differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences from the control group (\* $p < 0.05$ ; \*\* $p < 0.01$ )



**Fig. 3.** Catalase activity (CAT) of *M. amblycephala* after *A. hydrophila* infection. Data are presented as mean  $\pm$  SE ( $n = 30$ ). Differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences from the control group (\* $p < 0.05$ ; \*\* $p < 0.01$ )



**Fig. 4.** Acid phosphatase activity (ACP) of *M. amblycephala* after *A. hydrophila* infection. Data are presented as mean  $\pm$  SE ( $n = 30$ ). Differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences from the control group (\* $p < 0.05$ ; \*\* $p < 0.01$ )



**Fig. 5.** Alkaline phosphatase activity (AKP) of *M. amblycephala* after *A. hydrophila* infection. Data are presented as mean  $\pm$  SE ( $n = 30$ ). Differences were determined by one-way analysis of variance (ANOVA). The asterisks indicated statistically significant differences from the control group (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

to be of leukocyte origin [5]. Lysozyme plays an important role in innate immunity by lysis of bacterial cell wall, and thus stimulates the phagocytosis of bacteria [14]. Its ability to disrupt the cell walls of certain pathogens makes lysozyme a natural antagonist to harmful invaders like parasites, bacteria, and viruses. Lysozyme occurs prominently in fish serum and mucus [14]. Lysozyme activity may be enhanced at relatively low concentrations of pollutants, and it has been proven that lysozyme activity is induced by relatively low dosages of mercury exposure [15, 16]. The serum lysozyme activity was significantly increased in a low-dose MC group [17]. In the present study, the significant increases of serum lysozyme activity in treatment group were detected at 4 h, 1 d, 3 d, 5 d, 14 d, and 21 d

after *Aeromonas hydrophila* challenge. Serum lysozyme is used as an indicator of innate immune response in fish [18]. An increased level has been considered to be a natural protective mechanism in fish [19].

SOD is one of the main anti-oxidant defence enzymes generated in response to oxidative stress, which converts the highly toxic superoxide anions into hydrogen peroxide [20]. Chang *et al.* found that SOD decreases in *P. monodon* against WSSV-infected [21]. The activity of SOD was significantly lowered in WSSV-infected *F. indicus* [22, 23]. In the present study, the activity of SOD decreased significantly ( $p < 0.05$ ) at 1 d, 3 d, 5 d, 14 d, 21 d, and 28 d after *Aeromonas hydrophila* challenge. The activity of superoxide dismutase responsible for the scavenging of reactive oxygen species (ROS) decreased, leading to increases of superoxide anion [24]. An increase in the superoxide anion production against pathogens is considered to be beneficial after exposing shrimp to immunostimulants [25].

CAT is one of the primary antioxidant enzymes involved in ROS removal [26]. A similar tendency as that observed for SOD was found for CAT; the activity of CAT was found to be significantly reduced at 1 d, 3 d, 5 d, 14 d, 21 d, and 28 d after *Aeromonas hydrophila* challenge. These results were in line with previous studies showing that the reduction in CAT activity may be caused by poor efficiency of CAT in removing low ROS levels, or the SOD activity decline made the activity of CAT decrease [27]. Superoxide anion ( $O_2^{\cdot-}$ ) was reduced to  $H_2O_2$  by SOD, and  $H_2O_2$  was converted to water and oxygen by CAT [28]. The production of  $O_2^{\cdot-}$  has been reported as an accurate method to measure the effectiveness of potential immunostimulants [29, 30]. An increase in  $H_2O_2$  and superoxide anion is considered to be beneficially protective against disease with respect to increased immunity [29].

Alkaline phosphatase played an important role in metabolic regulation, which directly involved in the transfer of phosphate group and calcium phosphorus metabolism. Alkaline phosphatase also could change the surface structure of the pathogen to strengthen the recognition and phagocytosis of pathogens [31]. Activity of alkaline phosphatase was increased in the group of fish fed with turmeric over different days [32]. The level of alkaline phosphatase in the treatment group showed a significant increase at 4 h, 1 d, 3 d, 5 d, 14 d, and 21 d compared to the control group. An increase in the alkaline phosphatase activity is considered to be beneficial for fish disease resistance [31]. Das *et al.* found that the acid phosphatase activity was increased post-challenge with *Aeromonas hydrophila* in all groups [8]. In the present study, the acid phosphatase activity of the treatment group increased significantly ( $p < 0.01$ ) at 1 d, 3 d, and 5 d, which concurred with the findings of Das *et al.* [8]. Increased phosphatase activity indicates higher breakdown of the energy reserve, which is utilised for the growth and survival of fish [32].

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*The authors declare no conflict of interests.*

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