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Effect of Aeromonas Hydrophila Infection on Immunological Parameters in Nile Tilapia

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Abstract Background and Aim: The lack of disease control has the potential of being the limiting factor of fish production. Improving disease resistance of cultured fish are major challenges facing fish culturists, especially Aeromonas Hydrophila (A. Hydrophila) bacterial infections that cause mass mortalities. There was a positive link between bacterial infection and immunity in fish. The aim of the current study is to assess the effect of A. Hydrophilaon immunological parameters (serum total protein, serum globulin, serum IgM, phagocytic activity, phagocytic index and lysosomal activity) in Nile tilapia. Materials and methods: Nile tilapia (n = 110) with average size between 70 and 90gwere obtained from local farms in Kafrelsheikh. After 2 weeks of acclimation, fish were intraperitoneally injected by A. hydrophila to get two divergent lines of fish in response to disease: one susceptible/diseased and the other resistant to disease. Results: All immunity parameters were significantly increased in the resistant fish than the control and susceptible fish, except albumin which showed nonsignificant increase. On the other hand, susceptible fish exhibited nonsignificant changes in these parameters as compared to control fish, except for lysozyme activity which was significantly elevated in susceptible than control fish. Conclusion: This suggests a positive link between bacterial infection and immunological parameters in susceptible fish as However; this link was negative in resistant fish.

Keywords Nile Tilapia, Aeromonas Hydrophila, immunological parameters

Introduction

The tilapia is a group of cichlid fishes, which includes three economically important genera namely, Tilapia, *Oreochromis*, and *Sarotherodon*. It is an important commodity, ranking ninth in global aquaculture production [1-3]. China, Egypt, Indonesia, Philippines, and Thailand are the principal producing countries of tilapia. The world tilapia production had been growing increasingly in recent years with 1.7 million metric tons in 2016 [4]. The

ubiquitous bacterium *A. hydrophila* is a freshwater, facultatively anaerobic, chemoorganoheterotroph [5]. and the etiologic agent of disease in amphibians, birds, fishes, mammals, and reptiles, with the most common forms of disease being gastroenteritis, septicemia, and necrotizing fasciitis [6]. Virulence in A. *hydrophila* is multifactorial, with disease resulting from the production and/or secretion of virulence factors, such as adhesins, cytotoxins, hemolysins, lipases, and proteases as well as the capacity to form biofilms, use specific metabolic pathways, and mediate virulence factor expression through quorum sensing [7]. Fish, like other vertebrates, respond to infectious agents in both nonspecific and specific ways, although they depend to a much greater extent on the nonspecific mechanisms [8]. In the immune system of fish, non-specific immunity occupies a larger extent of fish immunity rather than specific immunity, compared to higher vertebrates [9]. As in all vertebrates, fish have cellular and humoral immune responses. Most generative and secondary lymphoid organs in mammals are also found in fish, except for lymphatic nodules and bone marrow [10]. The aim of the current study is to assess the effect of Aeromonas Hydrophila on the immunological parameters in nile tilapia.

Material and Methods

Fish and Rearing Conditions

This work was approved by the Animal Care and Welfare Committee of Kafrelsheikh University which follow the general guidelines of the Canadian Council on Animal Care. Genetically unrelated Nile tilapia (n = 110, weight 60± 2.90 g) with average size between 70 and 90 g were obtained from local farms in Kafr El- Sheikh. After 2 weeks of acclimation, fish reared in fiberglass tanks (20 fish per tank). These tanks filled with appropriate amount of de-chlorinated water at pH ranged from 6.5 -7, temperature at a range of 26-28 °C, and light cycle of 13:00 hours light and 11:00 hour dark. Fish were fed a commercial ration with 30.0% crude protein, 4.0% crude lipid, 4.4% pure crude fiber have total energy of ≥3754 kcal/kg at a rate of 3% body weight daily.

Bacterial Challenge Test and Fish Groups

Bacterial challenge using Aeromonas hydrophila, a virulent strain obtained kindly from the Fish Disease Lab, National Institute of Oceanography and Fisheries (NIOF), Alexandria branch, Egypt, was done to get two divergent lines of fish in response to disease: one susceptible/diseased and the other resistant to disease. A. hydrophila was cultured in Bacto tryptic soy broth and identical solid media containing 1.5% Bacto agar at 25°C with rotation at 250 rpm. Fish (n = 90) were challenged intraperitoneally with A. hydrophila ($0.5x10^6$ CFU/fish) and kept under observation for 14 days to record symptoms and survival rate daily. During observation period, 20 challenged-fish were dead and 40 out of the remaining 70 showed the symptoms (and was considered as diseased/susceptible group), while the remaining 30 showed no symptoms (and was considered as resistant group). Control (non-challenged) fish was intraperitoneally injected by PBS (n = 20). Re-isolation of injected bacteria from freshly dead fish during the period of observation was confirmed.

Blood Sampling

Nile tilapia was immobilized on absorbent paper and kept motionless. The body surface was then cleaned and blotted dry. The blood sample was collected from heart puncture with a disposable syringe and transferred into Eppendorf tubes with or without heparin. To get serum, some tubes without anticoagulants were left in stand position for 30 minutes at room temperature and then were centrifuged at 3000 r.p.m. for 20 minutes. Serum samples were carefully separated then transferred into clean dry tubes and kept frozen at -20 oC until used for biochemical analysis.

Total Protein Assay

Total protein was estimated according to the method (Vassault [11] and Yatzidis [12] following the manufacturer protocol Kit (Biomed diagnostic medical company). Proteins reacts with copper ions (II) to produce a blue violet

color compound in alkaline medium. The color intensity is proportional to the concentration of total proteins present in the sample.

Serum Albumin Assay

Serum albumin concentration was assayed by colorimetric method using commercial Kit (Diamond–Diagnostic, Egypt) according to the method described by Young and Friedman [13]. Albumin is bound by the BCG dye to produce an increase in the blue green color in a pH 3.8 acidic medium. The color increase is proportional to the concentration of albumin present in the sample.

Determination of Serum Globulins Level

Serum total globulins concentration was calculated mathematically by subtracting the albumin values from the total proteins values for the same samples [14].

Serum Immunoglobulin M (IgM) assay

This assay employs the competitive inhibition enzyme immuno assay technique. The microtiter plate provided in this kit has been pre-coated with goat-anti-rabbit antibody. Standards or serum samples are added to the appropriate microtiter plate wells with an antibody specific for IgM and Horseradish Peroxidase (HRP) conjugated IgM. The competitive inhibition reaction is launched between with HRP labeled IgM and unlabeled IgM with the antibody. A substrate solution is added to the wells and the color develops in opposite to the amount of IgM in the sample. The color development is stopped and the intensity of the color is measured.

Serum Lysozyme Activity

Lysozyme catalyzes hydrolysis of β-glucosidic bonds of polysaccharides in the cell membrane. The turbidity decrease of a suspension of Micrococcus lysodeikticus cell membranes is proportional to the enzymatic activity. Serum lysozyme activity was determined through the turbidimetry described by Abo-Al-Ela et al [15].

Phagocytosis Assay

Phagocytic functional assays were performed in vitro, using Candida albicans according to the method of Kawahara et al [16] & Soliman et al [17]. Phagocytosis has been traditionally assayed by measuring the engulfment of a cell "substrate". The most common substrates used in phagocytosis assays are erythrocytes (red blood cells) and zymosan (yeast) particles. When using red blood cells (RBCs) in the assay, the RBCs are first opsonized with serum or IgG; then they are incubated with phagocytes. The RBCs that are not engulfed by the phagocytes are removed, and the phagocytes are then lysed to release the engulfed RBCs. The detection of RBCs can then be quantified using a standard micro plate reader. The same assay principle is followed when using the Zymosan substrate; however, since Zymosan is prepared from yeast cell walls consisting of protein carbohydrate complexes, the opsonization step is not needed.

Results

Serum total protein

Among the three fish groups, the serum level of the total protein was significantly increased in the resistant fish than the control and susceptible fish. On the other hand, susceptible fish exhibited no significant changes in total protein as compared to control fish.

Serum albumin

No significant change in serum albumin level was noticed either among the three fish groups

Serum globulin

The serum level of the globulin was significantly increased in the resistant fish than the control and susceptible fish. However, susceptible fish showed no significant changes in globulin as compared to control fish.

Serum IgM

Among the three fish groups, the serum level of the IgM was significantly increased in the resistant fish than the control and susceptible fish. In contrast, susceptible fish showed no significant changes in IgM as compared to control fish.

Phagocytic activity

Among the three fish groups, the percentage of the phagocytic activity was significantly increased in the resistant fish than the control and susceptible fish. In contrast, susceptible fish showed no significant changes in phagocytic activity as compared to the control fish.

Phagocytic index

The phagocytic index was significantly increased in the resistant fish than the control and susceptible fish. Moreover, susceptible fish showed significantly higher phagocytic index than control fish.

Lysosomal activity

Among the three fish groups, the percentage of the phagocytic indexwas significantly increased in the resistant fish than the control and susceptible fish. Moreover, susceptible fish showed significantly higher Lysosomal activity than control fish.

Table 1: Levels of serum total protein, globulin, IgM, lysozyme activity, phagocytic activity and phagocytic index in control, resistant and susceptible tilapia

	Control	**Resistant**	**Susceptible**
Total protein (g/dL)	3.77±0.19	5.82±0.35 **	4.10±0.21
Globulin (g/dL)	1.88±0.11	3.62±0.19 ****	2.10±0.10
IgM (µg/ml)	0.54 ± 0.04	0.99 ± 0.06 ****	0.45±0.04
Phagocytic activity (%)	28.34±1.52	47.51±1.72 ****	33.14±1.37
Phagocytic index	1.40±0.07 ^{C}	2.16±0.10 ^{A**}	1.60±0.08 ^{B*}
Lysosomal activity	8.49±0.32	12.78±0.50 ****	10.09±0.43 *

Means within the same column of groups are significantly different at $P < 0.05$. $^{****}P < 0.0001$ (resistant and susceptible vs control).

Discussion

The lack of disease control has the potential of being the limiting factor of fish production. Improving disease resistance of cultured fish are major challenges facing fish culturists, especially *A. hydrophila* bacterial infections that cause mass mortalities. Detection of IgM is a good indicator for the immune response of the fish following infection. Among the three fish groups, the serum level of the IgM was significantly increased in the resistant fish (especially those carrying GG genotype) than the control and susceptible fish. In contrast, susceptible fish showed no significant changes in IgM as compared to the control fish. These findings were consistent with that reported by Reyes- Becerril et al. [18].

In fish, the primary lines of non-specific defense are the skin and mucus [19]. As a first line of defense, various peptides/proteins such as lysozymes, antibodies, complement factors, and other lytic factors are present in serum, where they prevent colonization of microorganisms, leading to prevention of infection and disease [20]. When pathogens enter the body, cellular and humoral non-specific defenses are mobilized [19]. It is well known that the innate immune system in fish can be triggered by many immune-stimulants, both synthetic and natural ingredients. Lysozyme activity functions as a primary defense factor of non-specific humoral immunity in preference to cellular defense mechanisms [21]. Fish serum lysozyme is believed to be of leukocyte origin. Lysozyme plays an important role in innate immunity by lysis of bacterial cell wall, and thus stimulates the phagocytosis of bacteria. 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 [22]. Xia et al [23] reported that the serum lysozyme activity was significantly increased in Blunt snout bream fish after *A. hydrophila* challenge. Serum lysozyme is used as an indicator of innate immune response in fish [24]. An increased level has been considered to be a natural protective mechanism in fish [25].

The aforementioned previous studies agreed with our present study which showed that there was a significant reduction in the serum levels of total protein, globulin, IgM, phagocytic activity, phagocytic index, and lysosome activity in susceptible/diseased fish. On the other hand, enhanced the phagocytic activity, phagocytic index, and lysozyme activity in resistant fish after challenge by *A. hydrophila* as compared to the control (non-challenged) fish, suggesting that this bacterial pathogen can stimulate phagocytosis and lysozyme activity and subsequently the immune response in the challenged fish.

Conclusion

The level of the antioxidant parameters (CAT, GSH and SOD) in the spleen were significantly increased in the control and resistant fish than the susceptible fish. However, the lipid peroxidation marker MDA level in spleen was significantly increased in susceptible fish than the control and resistant fish.

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