

Original Article

The response of New-season Nile tilapia to *Aeromonas hydrophila* vaccine

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Received January 12, 2015; Accepted February 25, 2015; Epub March 15, 2015; Published March 30, 2015

Abstract: The present study was conducted to recognize the response of new-season Nile tilapia to *Aeromonas hydrophila* vaccine. Four hundred new-season Nile tilapia were used in this study and divided into two equal groups, the first group served as control and the 2nd group was vaccinated with *Aeromonas hydrophila* vaccine via intraperitoneal injection. The antibody titer, Hematocrit level (HCV), Nitroblue tetrazolium activity (NBT) and lysozyme activity of new-season Nile tilapia was measured at the end of the 1st, 2nd, 3rd, 4th, 6th, 8th and 10th week post vaccination (PV). Challenge with *A. hydrophila* was carried out at the end of the 6th, 8th and 10th week PV. The antibody titer of vaccinated new-season tilapia showed significant higher values than unvaccinated group at all periods. The hematocrit and lysozymes activity values showed, a non significant increased in comparison with unvaccinated group at all periods PV. The NBT was significantly increased in vaccinated tilapia in comparison with unvaccinated group at all periods except one week PV. The relative level of protection of vaccinated tilapia after challenge infection was highest at 6th week PV in the new-season tilapia. We conclude that, vaccination against *A. hydrophila* increase the resistance of tilapia to such infection and consequently improve the survival and economic outcome. Other more applicable routes of vaccination should be investigated to be used on a large scale.

Keywords: Tilapia, vaccines, *Aeromonas hydrophila*, NBT, lysozyme

Introduction

Capture fisheries and aquaculture supplied the world with about 148 million tons of fish in 2010. World per capita food fish supply increased from an average of 9.9 kg (live weight equivalent) in 1960 to 18.4 kg in 2009, and preliminary estimates for 2010 point to a further increase in fish consumption to 18.6 kg [1]. Globally, fish provides more than 1.5 billion people with almost 20 percent of their average per capita intake of animal protein, and 3.0 billion people with at least 15 percent of such protein. Total aquaculture production in the Arab world was 587 tonnes, where Egypt represented 91.9% (540,000 tonnes) of commercial Arab aquaculture production [1]. Egypt ranks as second to China in terms of tilapia production worldwide.

Nile tilapia require a minimum temperature of 20°C to spawn and do not grow at tempera-

tures below 16°C. Poor survival is observed when water temperatures fall below 10°C for more than few days [2] as a consequence of bacterial infection as a second invader. Others have reported that reproduction is adversely affected at temperatures less than 20°C [3].

Disease outbreaks were recently identified as a major constraint to aquaculture production and trade, with a consequent effect on the industry's economic development [4]. The most common bacterial pathogens in the Egyptian aquaculture are short, Gram-negative rods belonging to the families Vibrionaceae where *Aeromonas hydrophila* are the most prevalent during the new season culture where they infect not only fish but human are also susceptible to infection. It is the causative agent of motile aeromonad septicaemia (MAS) with mortalities among tilapia and other fish species reared under the hatchery and farm environment in

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Table 1. Composition of the basal diet used throughout the experiment

Ingredients	Diet (%)	Protein (%)		Metabolic energy (Joules)	
		ingredients	Feed	ingredients	Feed
Fish meal	8.00	0.72	5.76	4000	32000
Soybean meal	52.9	0.48	25.392	2870	151823
Ground corn	29.1	0.109	3.1719	1240	36084
Wheat flour	5.00	0.134	0.67	2700	13500
Vegetable oil	2.00	0.00	0.00	9100	18200
Cod liver oil	2.00	0.00	0.00	9100	18200
Di calcium phosphate	1.00	0.00	0.00	0.00	0000
Mineral mix.	0.07	0.00	0.00	0.00	0000
Vitamin mix.	0.05	0.00	0.00	0.00	0000
Vitamin C	0.03	0.00	0.00	0.00	0000
Total	100.15	0.00	34.9939	0.00	269807

Ingredients were obtained from local markets.

virulence could be variable with localities, are recommended especially, commercial vaccines are expensive for fish producers, and may not be available against the encountered and emerging diseases. This study was designed to evaluate the response of new season Nile tilapia to *Aeromonas hydrophila* vaccine through evaluating some immunological parameters and challenge infection as well.

Egypt [5-7]. The symptoms of MAS include swelling of tissues, dropsy, red sores, necrosis, ulceration and haemorrhagic septicaemia [8]. Fish species affected by MAS include tilapia [9, 10], catfish [11], goldfish [12] and common carp [13]. Although *Aeromonas hydrophila* are usually considered as a secondary pathogen associated with disease outbreaks, it could also emerge as a primary pathogen [14], causing outbreaks in fish farms with high mortality rates and severe economic losses to the aquaculture industry worldwide. The chemotaxis, motility and attachment are all important factors for *Aeromonas hydrophila* to locate the host and eventually attach to the host, but the ability to invade the host might be directly linked to virulence [15]. The use of disinfectants and antimicrobials has shown limited success in preventing or curing aquatic diseases [16]. Furthermore, there is a growing concern about the use and abuse of the antimicrobials in aquaculture, as they increase the selective pressure exerted on the microbes and encourage the emergence of resistant bacteria by transferring resistant-genes to bacteria not exposed to antibiotics. Moreover, the antimicrobials lead to drug residues in the treated fish, besides having a negative impact on the environment [17]. Antimicrobials can generate cross-resistance against human antimicrobials, which could pose a hazard. Currently, the concern about bacterial resistance to antibiotics in livestock industry has led to legislation minimizing/eliminating the use of such compounds. Therefore, trials to develop vaccination program to control bacterial infection, at the national level where bacterial strains and their

Materials and methods

Fish

Four hundred new-season Nile tilapia fry, *O. niloticus* (initial weight 1.5 g) were collected from the WorldFish Hatchery, Abbassa, Sharkia, Egypt. They were divided into two equal groups and each group was equally reared in 4 glass aquaria (50 × 60 × 70 cm). The aquaria were filled with freshwater that was exchanged 20% daily through partial input and output of controlled tap water. Fish were acclimatized for 2 weeks prior to the experiment and fed on balanced ration (**Table 1**) throughout the experiment. The water quality was within the normal range throughout the experimental period [NO₃ (0.20 mg/L), NH₄ (0.2 mg/L), Chl at (42.27 mg/L), available P (0.02 mg/L). Water temperatures during the experiment was optimal (28 ± 2°C) for the culture of tilapia.

Preparation of diets

Diets containing 35% protein were prepared. Dietary ingredients (**Table 1**) were obtained from local suppliers and prepared in the WorldFish Center in the form of pellets. Ingredients were prepared by grinding the corn to granules (0.5 mm mesh size) (Thomes-Willey Laboratory Mill Model 4, Swedesboro, NJ 08085 U.S.A). Ingredients were mixed mechanically by horizontal mixer (Hobart model D300T, U.S.A.) at a low speed for 30 min. Oil (vegetable & cod liver) was added gradually to assure the homogeneity of the ingredients.

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Bacterial pathogen

A pathogenic *Aeromonas hydrophila* was obtained as a reference strain from Fish Health Management Division of the World Fish Center. The isolate was used in the vaccination trial and to test response of the overwintered vaccinated-fry.

Vaccine preparation

Formalin-killed *Aeromonas hydrophila* bacterin was prepared by the addition of formalin (0.3%) to the bacterial culture, which had been previously incubated at 35°C for 48 h [18]. The formalized bacterial culture was held at room temperature overnight, then subjected to sterility and safety tests [19]. The sterility test was performed by culturing washed bacterin on TS agar. Plates were incubated at 37°C for 24 h and examined for bacterial growth. The safety test was performed by the intraperitoneal (IP) inoculation of 20 susceptible tilapia with the prepared bacterin cells (0.1 ml). The fish were kept under observation for 2 weeks post-injection and then dead fish were subjected to necropsy for re-isolation of *Aeromonas hydrophila* using RS media. The prepared and tested vaccine was stored in the refrigerator at 4°C. Immediately before use, Formalin killed bacterial cells were washed twice with sterile saline solution and prepared to a concentration of 3 mg wet-weight/ml saline.

Vaccination experiment

Vaccination of tilapia: Fish, from group 1 served as negative control and were injected (IP) with 0.1 ml sterile saline solution. Fish of group 2 were vaccinated intraperitoneally (IP) with 0.1 ml formalin-killed *A. hydrophila* diluted in 0.1 ml sterile saline [20]. Before IP injection, fish were anaesthetized with 100 mg/L MS₂₂₂ (Tricaine methane sulfonate; Argent Chemical Laboratories, Fisheries Division). Blood samples were collected from ten fish in each group into clean dry tube at the end of the 1st, 2nd, 4th, 6th, 8th and 10th week post-vaccination (PV).

Laboratory evaluation

Blood sampling: Twenty fish were randomly collected from the vaccinated and control groups. The fish were anesthetized by immersion in water containing 0.1 ppm tricaine methane sul-

fonate (MS-222). Whole blood (0.5 ml) was collected from the caudal vein of each fish using syringes (1-ml) and 27-gauge needles that were rinsed in heparin (15 unit ml⁻¹), to determine the hematocrit values and NBT. For separation of serum, blood samples (0.5 ml) were withdrawn from the fish caudal vein, as before, and transferred to Eppendorf tubes without anticoagulant. The blood samples were centrifuged at 3000 g for 15 min and the supernatant serum was collected and stored at -20°C in screw-capped glass vials to be used for the determination of antibody titer. A further 0.5 ml blood-sample was centrifuged at 1000 g for 5 min in order to separate the plasma. The latter was stored at -20°C to be used for lysozyme activity test.

Antibody determination: Specific antibody titers, in collected sera, were determined using the bacterial agglutination test [18].

Hematocrit level: Hematocrit capillary tubes were two-third filled with the whole blood and centrifuged in a hematocrit centrifuge for 5 min and the percentage of the packed cell-volume was determined by the hematocrit tube reader [21].

Nitroblue tetrazolium activity (NBT): Blood (0.1 ml) was placed in microtiter plate wells, to which an equal amount of 0.2% NBT solution was added and incubated for 30 min at room temperature. A sample of NBT blood cell suspension (0.05 ml) was added to a glass tube containing 1 ml N, N-dimethyl formamide and centrifuged for 5 min at 3000 rpm. The supernatant fluid was measured in a spectrophotometer at 620 nm in 1 ml cuvettes [22].

Lysozyme activity: The lysozyme activity was measured using the turbidity assay. Chicken egg lysozyme (Sigma) was used as a standard and 0.2 mg/ml lyophilised *Micrococcus lysodeikticus* in 0.04 M sodium phosphate buffer (pH 5.75) was used as substrate. Fifty ml of serum was added to 2 ml of the bacterial suspension and the reduction in the absorbance at 540 nm was determined after 0.5 and 4.5 min incubation at 22°C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min [23].

Challenge after vaccination: Fish from both groups (n = 90), with average weight of 10±2.3

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Table 2. Hematological and immunological parameters of new season tilapia from the 1st till the end of the 10th week post vaccination (PV) (Mean ± SE)

Period PV	Treatment	Antibody titer	HCV	NBT	Lysozyme activity
1 week	Control	2.00 ^{Ba} ±0.00	30.33 ^{Aa} ±1.45	0.176 ^{Aa} ±0.07	8.40 ^{Aa} ±0.71
	Vaccinated	4.00 ^{Ab} ±0.58	30.63 ^{Ab} ±2.09	0.242 ^{Ab} ±0.06	8.08 ^{Aa} ±0.98
2 week	Control	2.33 ^{Ba} ±0.33	31.33 ^{Aa} ±2.40	0.199 ^{Ba} ±0.01	8.10 ^{Aa} ±0.67
	Vaccinated	5.33 ^{Aab} ±0.88	32.40 ^{Aab} ±1.34	0.266 ^{Ab} ±0.01	9.12 ^{Aa} ±0.30
4 week	Control	2.33 ^{Ba} ±0.33	29.67 ^{Aa} ±0.88	0.186 ^{Ba} ±0.03	8.30 ^{Aa} ±1.15
	Vaccinated	5.67 ^{Aab} ±0.67	33.90 ^{Aab} ±3.88	0.281 ^{Aab} ±0.04	9.33 ^{Aa} ±0.67
6 week	Control	2.33 ^{Ba} ±0.33	30.00 ^{Aa} ±2.08	0.194 ^{Ba} ±0.08	8.33 ^{Aa} ±0.49
	Vaccinated	6.67 ^{Aa} ±0.33	35.00 ^{Aa} ±2.52	0.310 ^{Aab} ±0.05	9.43 ^{Aa} ±0.30
8 week	Control	2.00 ^{Ba} ±0.58	30.67 ^{Aa} ±1.76	0.174 ^{Ba} ±0.02	8.37 ^{Aa} ±0.31
	Vaccinated	6.67 ^{Aa} ±0.33	35.33 ^{Aa} ±1.20	0.354 ^{Aa} ±0.10	9.60 ^{Aa} ±0.38
10 week	Control	2.33 ^{Ba} ±0.33	30.00 ^{Aa} ±1.73	0.190 ^{Ba} ±0.02	8.20 ^{Aa} ±0.61
	Vaccinated	6.00 ^{Aa} ±0.00	35.00 ^{Aa} ±2.89	0.345 ^{Aa} ±0.03	9.30 ^{Aa} ±0.12

Capital letter: compare between treatments within the same period; Small letter: compare between the periods within the same treatment.

g, were randomly obtained from each treatment and were used for challenge test at the end of the 6th, 8th and 10th weeks post-vaccination (30 fish/challenge). Fish, of each group, were divided into 3 equal groups and reared in a separate glass aquarium (50 × 60 × 70 cm). Fish was IP inoculated with 0.5 ml (10⁸ bacteria cells ml⁻¹) of culture suspension of the reference pathogenic strains of *Aeromonas hydrophila* [24]. Mortality was recorded and the dead fish subjected to necropsy for bacterial re-isolation. The relative level of protection (RLP) among the vaccinated and challenged fish was determined [25] using the following equation: RLP% = 100-(% stimulated mortality ÷ percent mortality in control group) × 100.

Statistical analysis: One way and two-way analyses of variance (ANOVA) were carried. Also, Duncan's Multiple Range Test [26] was used to determine differences among treatments (mean at significance level of P<0.05). Standard errors were also estimated. Analysis was carried out using the SAS package [27].

Results

As shown in **Table 2**, the antibody titer of vaccinated new-season tilapia showed significant higher values than unvaccinated group at all periods. The hematocrit and lysozymes activity values showed, a non significant increased in comparison with unvaccinated group at all periods PV. The NBT showed significant increased in comparison with unvaccinated group at all

periods except one week PV. The relative level of protection of vaccinated tilapia after challenge infection was 66.66, 63.63, 49.99% at the 6th, 8th and 10th week PV.

Discussion

One of the constraints facing tilapia culture in Egypt is the low temperature during the winter season, which leads to low survival. Cold temperatures also adversely affect food fish production, as hatcheries fail to produce fry to stock in the ponds. From December to March, water temperatures range from 9-20°C. Breeding activities stop at temperature below 20°C. Fish, especially in shallow ponds, are vulnerable to low temperatures. From April to December, water temperatures range from 25°C to 35°C, is the most suitable for rearing tilapia [28]. However, since spawning begins in April, seeds are not normally available until June-July, thus limiting the length of the production season.

Among the major problems that faces fish culture in Egypt, two important challenges should be solved, 1st making fry available throughout the year, especially during and immediately after the winter season, which due to the effects of cold temperatures and associated secondary bacterial infections negatively impacts tilapia growth and production and fry survival. The second challenge is to recover the time (winter and early spring) lost, when there is no investment in fish production due to shortages of fry.

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Immunization plays an important role in the control of infectious diseases of man and animals. Bacterins and vaccines are used extensively. Immunization of fish proved to be effective for disease control, only on laboratory level [29].

The present study aimed to evaluate the response of tilapia fry reared in new season to the aeromonas vaccine. The antibody titer of vaccinated new-season tilapia showed significant higher values than unvaccinated group at all periods. Catfish (*Clarias lazera*) which were IP immunized by *A. hydrophila* bacterin showed the maximum antibody titer at 3rd and 4th week PV [30]. The immunized fish were protected against challenge using virulent strain of *A. hydrophila*, since the synthesized antibodies were produced against the injected antigen and resulted in a protection rate after challenge infection where survival rate was 66.66, 63.63, 49.99% at the 6th, 8th and 10th week post vaccination. Nearly similar results were reported by other study [20]. On the other hand, other study mentioned that the oral vaccine can be efficiently employed in the culture system to overcome the infectious diseases [31].

The hematocrit and lysozymes activity values showed, a non significant increased in comparison with unvaccinated group at all periods PV. The NBT test is used to determine the respiratory burst activity, especially of neutrophils and monocytes. The NBT showed significant increased in comparison with unvaccinated group at all periods except one week PV. Earlier investigator reported that high phagocytic ability in gilthead sea bream (*S. aurata* L.) specimens given a mixture of two inactivated bacteria [32]. Another finding reported a marked increase in the number of lymphocytes and macrophages around the activated melanomacrophage centers in the kidney together with a maximal splenic response in the form of activated melanomacrophage centers with marked increase in macrophages and lymphocytes together with proliferation of hematopoietic elements around the splenic sinuses [30]. The activation of mononuclear phagocytic cells and the melanomacrophages might play a role in the antibody release to the circulation and also increased the hematocrit and lysozymes activity and NBT values in vaccinated group in comparison with unvaccinated group at all periods post vaccination. Similar studies showed that 10 days after

immunization with a polyvalent vaccine at a concentration 1×10^8 CFU/mL, there was an increase on erythrocytes, leukocytes, thrombocytes and circulating lymphocytes production [33].

The *Aeromonas hydrophila* challenge infection of the vaccinated tilapia resulted in the highest mortality in the non-vaccinated control group, followed by the vaccinated control. The relative level of protection of vaccinated tilapia after challenge infection was higher in the new-season tilapia at the 6th week PV. A significant reduction in fish mortality was reported after challenge with various pathogens [34, 35]. Low mortalities were observed in Atlantic salmon vaccinated against *Aeromonas salmonicida* [36], in turbot vaccinated against *Enterococcus* sp. [37], in yellowtail challenged with *Enterococcus seriolocida* [38] and in swordtails, rosy barbs and black tetras challenged with *Aeromonas hydrophila* or *Pseudomonas fluorescence* and fed beta-glucans [39].

It may be concluded that, vaccination of new season tilapia against *A. hydrophila* increase the resistance to such infection and consequently improve the survival and economic outcome. The injection route may be reliable for the immunization of small number of highly prized fish; but is difficult to be applied on a large scale. So, other routes of vaccination should be investigated to select an alternative method to be used on a large scale. Further studies to improve the survival of overwintering tilapia fish through the use of immunostimulants and probiotics together with implementing the vaccination program is recommended.

Acknowledgements

We would like to express our deep thanks and appreciation to World Fish Center, Abbsaa, Egypt for supporting our research idea and providing Nile tilapia fish and facilities for this experiment

Disclosure of conflict of interest

None.

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