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Full Length Article

Immune Response of Tilapia (*Oreochromis niloticus*) after Vaccination with Autoclave-killed, Heat-killed, and Formalin-killed Whole Cell *Aeromonas hydrophila* Vaccines as Possible Serotype-independent Vaccines

Ivan Daryl C. Bactol[1], Larry V. Padilla[2] and Allan L. Hilario[3*]
[1]*Department of Biochemistry and Nutrition, College of Medicine, Pamantasan ng Lungsod ng Maynila*
[2]*Department of Biology, College of Science, Pamantasan ng Lungsod ng Maynila, Philippines*
[3]*Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines-Manila, Philippines*
[*]For correspondence: alhilario@up.edu.ph

Abstract

Nile tilapia, *Oreochomis niloticus*, is the most commonly produced and consumed fish in the Philippines. This fish is highly susceptible to *Aeromonas hydrophila* infection brought about by wastewater contamination. Since antibiotics are not routinely recommended in aqua farming, vaccination is the alternative way in preventing such bacterial infection. This study aimed to evaluate the immune response of Nile tilapia after vaccination with autoclave-killed, heat-killed and formalin-killed whole cells vaccine derived from *Aeromonas hydrophila* as possible serotype-independent vaccines. One hundred twenty Nile tilapia were randomly distributed into four tanks with corresponding treatments: normal saline solution (NSS), autoclave-killed vaccine (AKV), heat-killed vaccine (HKV), and formalin-killed vaccine (FKV) and were subjected to blood extraction 2 weeks after acclimatization and 2 weeks after vaccination. Immune response was evaluated using agglutination test. On post-vaccination, AKV had the highest mean antibody titer ($p<0.05$). Pre-vaccination antibody level was significantly different to the post-vaccination antibody levels in AKV, HKV, and FKV ($p<0.05$). This study showed that autoclave-killed, heat-killed, and formalin-killed whole cell *Aeromonas hydrophila* vaccines are possible serotype-independent whole cell vaccines that produced significant immune response in Nile tilapia.

Keywords: *Aeromonas hydrophila;* Whole cell vaccines; *Oreochromis nilocus*; Tilapia

Introduction

Aquaculture is one of the most important sectors of the Philippine agriculture. It is also one of the fastest growing sectors of food production in the world. Aquaculture, also known as aqua farming, is carried out in diverse aquatic ecosystems such as freshwater, brackish and marine waters (Food and Agriculture Organization of the United Nations, 2005). In the Philippines, freshwater aquaculture is continuously expanding as a very good source of income. Tilapia (*Oreochromis niloticus*), catfish (*Clarius macrocephalus*), carp (*Cyprinus carpio*), and mudfish (*Chana striata*) are the most commonly cultured fishes in freshwater ponds in the Philippines. Tilapia is the most produced and consumed particularly in Central Luzon (Bureau of Fisheries and Aquatic Resources, 2005).

Oreochromis niloticus is commonly known as tilapia. It has a wide range of tolerance in any environmental factors (Mjoun *et al.*, 2010). There are different varieties of tilapia, one that can reside in a low-temperature environment and another in brackish waters. Regulating the main physicochemical parameters of water such as dissolved oxygen, temperature, and pH to ideal conditions, can lead to massive productions of tilapia. Along with the growth of aquaculture, well known infectious diseases have become a problem including bacterial infection that causes size reduction, sickness, abnormalities, and death of the fish. *Aeromonas hydrophilla* that causes aeromonas infection is the most common opportunistic bacterial pathogen in freshwater fish like tilapia and can cause watery to bloody diarrhea (Yanong and Floyd, 2002; Public Health Agency of Canada, 2011).

A. hydrophila is a gram-negative, rod shaped facultative anaerobe with a size ranging from 0.3−1.0 µm wide and 1.0-3.5 µm long that has a single polar flagellum. It can produce heat labile enterotoxins that can be associated with hemolysin and cytotoxin production. They are inhabitants of aquatic ecosystems (Public Health Agency of Canada, 2011). *A. hydrophila* causes diseases in fish known as Motile Aeromonas Septicemia, Hemmorhagic Septicemia, and Ulcer Disease or Red Sore Disease. The symptoms of these diseases range from lack of appetite,

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swimming abnormalities, pale gills, bloated appearance, and skin ulcerations to sudden death in otherwise healthy fishes. *A. hydrophila* infection is a zoonotic disease. It is a disease that can be spread from animal to man and vice versa (Swann and White, 1991).

Since, the use of antibiotics is not recommended in tilapia culture, vaccination is the key in preventing bacterial infections in this species. Vaccination is the administration of antigen to a host that stimulates its adaptive immunity. Vaccines are preparations that stimulate the production of antibodies to particular antigens (Landau, 1991). It works by exposing the immune system of an animal to the part of a pathogen or the entire pathogen called antigen and then allowing time for the immune system to develop a response either by producing specific immunoglobulin or T-killer cells. Vaccination is widely practiced in aquaculture due to the growing prevalence rate of bacterial infection and antimicrobial resistance to various antibiotics. Three ways of administering vaccines can be employed such as through injection, immersion and oral vaccination (Yanong, 2008).

Oral vaccines against bacteria have proved disappointing presumably because protective antigenic determinants are destroyed in the gastrointestinal tract (Austin and Austin, 2012). Injection through anal route has been suggested as a method that would avoid the potential degradation of an orally administered vaccine in the stomach and intestine (Stoskopf, 1993).

Bacterial infections of tilapia in aquaculture would result in a decrease of its production and eventually would also lead in a decrease of income to fish farmers. Since organic farming is encouraged in aquaculture, the use of antibiotic is not practiced irrationally in treating bacterial diseases. Thus, the use of vaccines is an alternative way in treating such diseases. Different types of vaccines and the route of its administration have different effects in different fishes in terms of its antibody production. This study was conducted to determine the level of immune response of tilapia in different types of vaccines administered through anal route and the survival rate of tilapia post-vaccination after challenge with live *A. hydrophila*.

Materials and Methods

Source and Stocking of Nile Tilapia

One hundred twenty (120) healthy tilapia (*Oreochromis niloticus*) fishes with weight ranging from 50−60 grams were obtained from the Bureau of Fisheries and Aquatic Resources-National Freshwater Fisheries Technology Center, (BFAR-NFFTC, 2000), Muñoz, Nueva Ecija, Philippines. They were acclimatized in aquarium for two weeks prior to stocking. Female fishes and male fishes were cared for in separate aquarium. The fish diet was consisted of rice bran (67%) and fishmeal (33%). Five percent of the fish total body weight per day was used for their feeding requirement (BFAR-NFFTC, 2000). Aeration was provided with about 70% daily water change. Monitoring of water quality parameters such as dissolved oxygen, temperature, and pH were measured throughout the experiment to maintain the optimal condition for *O. niloticus* following the protocol as prescribed by Morales (1995). The study was approved by the Pamantasan ng Lungsod ng Maynila-University Research Center and registered to the Research Grants Administration Office of the University of the Philippines-Manila.

Experimental Set-up

Experimental set-up was divided into four groups with different treatments. Each treatment had three aquariums representing three replicates. Each aquarium had ten fishes. The four treatments (Table 1) were: autoclave-killed cell vaccine (AKV), heat-killed cell vaccine (HKV), formalin-killed cell vaccine (FKV) and normal saline solution (NSS). The fourth treatment served as the negative control group. Vaccines were derived from *Aeromonas hydrophila*.

Culture and Maintenance of Bacteria

Pure culture of *Aeromonas hydrophila* was obtained from University of the Philippines-Los Baños, National Institute of Molecular Biology and Biotechnology (BIOTECH). The bacterial culture was kept at 4°C prior to its use. Bacterial culture was sub-cultured on blood agar base supplemented with 5% of tilapia blood (Morales, 1995).

Preparation of Vaccines

Formalin-killed, autoclave-killed and heat-killed cell vaccines were prepared by inoculating live cultures of *A. hydrophila* in tryptic soy broth and incubated for 24 h at 25°C. For FKV, buffered formalin (40% w/v) was added to the broth culture at a final concentration of 0.5% (v/v) and left at 48 h at room temperature. HKV was prepared by heating the broth culture at 100°C for 30 min. AKV was prepared by autoclaving the culture media in an autoclave at 121°C and 15 pounds per square inch (psi) for 15 min. Then, the treated culture was centrifuge at 7,000x g for 30 minutes. Cell pellets were washed twice by NSS and re-suspended again in NSS. All procedures were done in sterile condition. Vaccines were kept at 4^{o}C until further use.

Sterility Test

The bacteria from the above treatments were streaked onto tryptic soy agar to test its sterility (free from living bacterial cells). If the media showed no bacterial growth, the corresponding cell solutions of *A. hydrophila* were used to make the vaccines.

Administration of Vaccines

Two weeks after acclimation period, the three prepared vaccines and NSS (control group) were administered through the anal route. Fishes were anesthetized first by

immersion in 0.06 g/L of MS-222 (Tricainemethane sulphate). To vaccinate the fishes, 1 mL syringes with a blunt-end needle was used. Approximately fifteen millimeters (~15 mm) of the needle was introduced to the anus. Each tilapia received 0.2 mL vaccine solution and 0.2 mL of NSS in control group. Then, 50 µL of pure petroleum were used to plug the anus temporarily to avoid leakage.

Challenge Test of Fishes

Two weeks after anal vaccination, ten fishes in each replicate in both vaccinated and unvaccinated groups were challenged against *A. hydrophila* (challenge test). Each fish was anesthetized first before intramuscular infection of 0.1 mL of bacterial suspension of *A. hydrophila* with 1 x 10^8 colony forming unit per milliliter (cfu/mL). Dead fishes were collected and recorded within 14 days post-challenge. The level of protection of each vaccine was calculated using the formula for Relative Percent Survival (RPS), which was proposed by Amend (1981). The formula for RPS is given below:

$$\text{RPS} = \left(1 - \frac{\%\ Treatment\ Mortality}{\%\ Control\ Mortality}\right) \times 100\%$$

Fish Serum Collection

Ten fishes in each replicate were extracted of blood prior to administration of vaccine, prior to challenged test and two weeks after the challenged test. Fishes were anesthetized before it is subjected to blood extraction. Blood samples were collected from each fish using 1 mL syringe with 25-G needle attached. Needle was inserted at the ventral midline portion of the fish tail. The needle was directed slightly cranially and dorsally until it encountered the vertebral bodies. Approximately, 0.1 mL of blood was collected from each fish. Collected blood was stored in Eppendorf tube and was left overnight to clot. Clot rimming done and centrifuged. The serum was collected for measuring antibody using agglutination test.

Agglutination Test

Antibody of each treatment was measured quantitatively using two-fold serial dilution in microtiter plate with 96 "U" bottom wells with sterile PBS as diluent. Using a micropipette, 25 µL of PBS diluent was dropped in each well except in the first well. Same amount of serum was dropped in the first well. A total of 25 µL of serum was transferred to the second well and mixed well. Then, 25 µL of solution in second well was transferred to the third well until it reached the last well. Equal volumes of autoclaved-killed *A. hydrophila* bacterial cells with a density of 1.7 at 600 nm were added to each well and were gently mixed. Microtiter plate was covered then incubated overnight at room temperature. It was examined for agglutination. Negative patterns, indicating that immunoreaction had not taken place, showed condensed flocculation particles with a cross-packing structure at the bottom of the microtiter plate. On the other hand, positive patterns, indicating that immunoreaction had occurred, showed an expanded agglutination pattern of particles (McPherson and Pincus, 2011). Titers were expressed as the reciprocal of highest dilution. The term used to describe the concentration of antibody in serum is "titer", which is the reciprocal of the highest dilution producing a definite agglutination reaction (South East Asian Fisheries Development Center, 2001).

Statistical Analysis

Differences among mean antibody titer of *O. niloticus* with different type of vaccines and differences in antibody titer among different type of vaccines at different periods of serum collection (pre-vaccination, post-vaccination and 2 weeks after challenge test) were subjected to test of statistical significance using one-way Analysis of Variance. Pairwise Tukey test was used to evaluate if there were significant differences between treatments and between different types of vaccines at different periods of serum collection. The relative percent survival was tested for significance using the chi-square. Significance was set at $p<0.05$.

Results

Agglutination Test

The pre-vaccination mean titers of all groups were comparable with one another ($p>0.05$). The post-vaccination mean titer was highest in AKV group and lowest in FKV group. The post-vaccination mean titers of AKV (9.33), HKV (8.80), and FKV (7.08) groups were significantly higher that the mean titer of the NSS (2.96) group ($p<0.05$). The post-vaccination mean titers of AKV, HKV, and FKV groups were not significantly different from one another ($p>0.05$). The post-challenge mean titer was highest in HKV group and lowest in FKV group. The post-challenge mean titers of AKV, HKV and FKV groups were not significantly different from one another ($p>0.05$). The post-challenge mean titers of HKV (9.27), AKV (7.68), and FKV (6.27) groups were significantly higher that the mean titer of the NSS (2.74) group ($p<0.05$). Similarly, the post-challenge mean titers of HKV, AKV, and FKV groups were not significantly different from one another ($p>0.05$). The mean titers of FKV were notably the lowest in both the post-vaccination and post-challenge time points. The post-vaccination mean titers of AKV, HKV and FKV groups slightly decreased on post-challenge levels but the differences were not significant (Table 2).

Relative Percent Survival

The relative percent survival of the AKV and HKV groups were 90% while the FKV was insignificantly lower at

86.67% (*p>0.05*). The RPS for the AKV, HKV, and FKV groups were higher than in NSS group with a RPS of 73.34%. However, the differences were not significant (*p>0.05*) (Table 3).

Discussion

Agglutination test is a simple and easy assay to perform. It does not require special equipment. The reciprocal of the highest dilution giving a positive reaction is known as the titer. It provides a measure of the amount of the antibody in the serum of vaccinated organisms or hosts (Tizard, 1995). *Oreochromis niloticus* was subjected to blood extraction prior to vaccination, 2 weeks after vaccination and 2 weeks after the challenge test. Table 2 shows that test organism, tilapia (*O. niloticus*), mounted a significant immune response after anal immunization from the three vaccine preparations. The autoclave and heat-killed vaccines gave the highest titers in the post-vaccination and post-challenge respectively. The formalin-killed vaccine gave the lowest titers in both time points.

The increase in antibody level may be due to the immune cells that can be found in the epithelium of the gut. According to Rombout *et al*. (2014), all immune cells necessary for a local immune response are abundantly present in the gut mucosa of teleost. The local immune response can be monitored after intestinal immunization, which in this study was introduced anally. The results were similar in the study conducted by Grabowski *et al*. (2004) in which the primary antibody response initially peaked 2 weeks after immunization and subsided 4 weeks after immunization. The decrease in antibody titer two weeks after the challenge test may be due to stress and waning of immunologic memory and response (Magnadottir, 2010). There are also many reports regarding the protective role of fish specific agglutinating antibodies (Ibrahem *et al.*, 2008). These antibodies play an important role in protecting fish against bacterial infections like *A. hydropjhila* (Kwon *et al.*, 2005).

Although post-challenge test did not show significant result despite higher survival of vaccinated tilapia, this study provided insight in the possible strategy to prevent bacterial infection and prevention of increasing antimicrobial resistance. There are many reasons why such whole cell vaccines may mount a good immune response but do poorly with post-challenge. One study indicates that there is no correlation between protection and level of serum specific antibody (Klesius *et al.*, 2000). Others report that antibody titer and RPS among the intraperitoneal and intramuscular vaccinates did not correlate. According to Magnadottir (2010), different stress inducers could greatly influence fish immune response. Blood extraction of fishes is a stressful procedure, which involved handling and transporting from one tub to another tub. These stressors can weaken the immune response of the fish.

Table 1: The types of vaccine and whole bacterial cell concentration of *Aeromonas hydrophila* used in the different groups

Groups	Type of Vaccine	Bacterial Cell Concentration
AKV	Autoclave-killed	10^9 cells/mL
HKV	Heat-killed	10^9 cells/mL
FKV	Formalin-killed	10^9 cells/mL
NSS	Normal Saline Solution	

Table 2: Mean Titer of antibodies against *Aeromonas hydrophila* in vaccinated and unvaccinated tilapia (*Oreochromis niloticus*) at different time points

Groups	Pre-vaccination	Post-vaccination	Post-challenge	P-value
NSS	2.83	2.96	2.74	>0.05*
AKV	2.93	9.33	7.68	<0.05**
HKV	2.71	8.80	9.27	<0.05**
FKV	2.62	7.08	6.27	<0.05**

*Pre-vaccination vs. post-vaccination vs. post-challenge; **Pre-vaccination vs. post-vaccination

Table 3: Relative Percent Survival of *O. niloticus* after challenge test with *Aeromonas hydrophila*-derived vaccines

Groups	Relative Percent	P-value
NSS	74.34	
AKV	90.00	>0.05*
HKV	90.00	>0.05*
FKV	86.67	>0.05*

This study also has similar results with the study of Dehghani *et al*. (2012). Although the present study showed superior antigenicity of whole cell vaccines prepared by attenuating live bacterial cells with physical methods like heat and autoclaving, Dehghani *et al.* (2012) report that formalin-killed and heat-killed *A. hydrophila* are equally antigenic in red trout. However, this study showed that formalin-killed whole cell vaccine has lower antigenicity. The formalin-killed vaccine has the lowest antibody level as compared with other types of vaccines and at different time points. This may be due to the cross-linking properties of formalin that may have resulted in reduced antigenicity (Grabowski *et al*., 2004).

Conclusion

This study showed that whole cell vaccines derived from *Aeromonas hydrophila* attenuated by autoclaving, formalin and heat-treatments produced significant immune response using agglutination test. While relative percent survival was higher in vaccinated tilapia than the unvaccinated tilapia (*Oreochromis niloticus*), the difference was not significant. This study provided an easy and inexpensive alternative to produce vaccines against opportunistic bacterial infection in farmed fishes like tilapia. Further studies on vaccination in different teleost fishes are highly encouraged and should consider the virulence of the bacteria used in post-challenge test and the route of vaccination.

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