



Specific Immune Response Kinetics and Mortality Patterns of Tilapia *Oreochromis niloticus* on Post-Cocktail Vaccination Period against the Infection of *Aeromonas hydrophila* and *Streptococcus agalactiae*

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ABSTRACT

Fish vaccination aims to induce a specific immune response indicated by an increase of antibodies in vaccinated fish. However, in accordance with time, the presence of antibodies will continue to decline. The purpose of this study was to determine the kinetics of specific immune response and trend mortality against *Aeromonas hydrophila* and *Streptococcus agalactiae* on tilapia following vaccination with a cocktail vaccine. Fish was vaccinated through immersion for 30 minutes in a solution of diluted vaccine. Challenge test was performed for three periods, on day 22, 50, and 78 post-vaccination, fish were challenged with single infection of *A. hydrophila* 10^8 cfu. mL⁻¹ and *S. agalactiae* 10^4 cfu. mL⁻¹ and co-infection of both bacteria by intraperitoneal. During rearing, the blood fish were taken for determining of serum antibodies, which was measured by ELISA. The results showed that the concentration of specific antibodies vaccinated fish were significantly higher than the control. The basal antibody levels of *A. hydrophila* before vaccination were higher than *S. agalactiae* with OD of 0.104 and 0.069 respectively. The maximum antibody response was reached within 70 days of the *A. hydrophila* OD= 0.264 and 56 days against *S. agalactiae* OD= 0.188. The mortality rate in the control group was significantly higher than vaccinated on all types and each challenge test period. The trend of mortality due to a single infection of *A. hydrophila* and co-infections occur more quickly than by *S. agalactiae*. Lowest mortality occurred in the vaccinated group at 50 days tested challenge.

Keywords: kinetics antibody, *Aeromonas hydrophila*, *Streptococcus agalactiae*, *Oreochromis niloticus*

1. Introduction

Immune responses existed in a fish consist of non-specific immune response (innate) and specific immune response (adaptive) (Iwama and Nakanishi, 1996). A specific immune response induction is indicated by an increased in the antibody production in the vaccinated fish. Vaccination aims to induce long-term immunity by stimulating the specific immune response

component (antibody memory) (Ellis, 1988). Vaccination can be performed by several application methods, including injection, immersion, spraying, and oral administration. Immersion method is preferably used in the aquaculture industry because it is more applicable than injection technique and produces a better protection in providing immunity than oral technique through feeding (Ellis, 1989).

Studies about vaccination in tilapia to protect it from pathogenic bacterial infection have been widely studied. There are several studies on vaccination subjected to tilapia against *Streptococcus* sp. and *Aeromonas hydrophila* (Pasnik *et al.*, 2006; Prasad and Areechon, 2010; Hardi *et al.*, 2010; Sugiani and Komarudin 2011; Taukhid and Purwaningsih, 2011; Sugiani *et al.*, 2012). The vaccination goals are the formation of specific antibodies and a positive result indicated by a high survival that will be achieved by paying attention to several terms, ie the vaccine must be immunogenic, produces a long protective time and is safe too (Sommerset *et al.*, 2005).

Along with the rearing time, the specific immune response that can protect a fish from a pathogen infection will continue to decline. Romalde *et al.* (1999) studied on toxoid from *Streptococcus* sp. and found that the protection provided by this vaccine decreased along with time. Therefore, this study aimed to analyze specific immune response kinetics and mortality patterns in tilapia vaccinated with the cocktail vaccine, then tested against single infections of *A. hydrophila* and *S. agalactiae*, and also co-infection of both pathogenic bacteria.

2. Methods

Fish

Tilapias with an average weight of 16.5 g were obtained from Research Installation of Freshwater Cultured Fish Germplasm Cijeruk. Based on verification results through microbiological test and observation during the acclimatization period, there was no symptom of characteristics that would appear when the fish is infected by motile aeromonad septicemia MAS and streptococcosis or the fish condition had been stable.

Vaccination

The cocktail vaccine tested in this study was a mixture of inactivated *A. hydrophila* whole cell monovalent vaccine and inactivated *S. agalactiae* whole cell monovalent vaccine with a formulation of 25% *A. hydrophila* and 75% *S. agalactiae*, at a concentration of 10^{11} cfu. mL⁻¹ (Sumiati *et al.*, 2012). Vaccination

was performed by immersion technique. The vaccine was diluted first with a dose of 1 mL vaccine into 10 L clean water. In the vaccination group, the fish were immersed in a diluted vaccine solution for 30 minutes and that solution was aerated at the same time. The control fish were immersed in water without the vaccine. After vaccination, the fish were reared for 4 months in a pond with a volume of 10 m³ at a density of 20 individuals. m⁻³.

Challenge test

The antigen sources were *A. hydrophila* AHL0905-2 isolate inoculated on Trypticase Soy Agar (TSA) medium and *S. agalactiae* N14G isolate inoculated on Brain Heart Infusion Agar (BHIA) medium. The challenge tests were performed on three experimental groups, ie group one on day 22 after vaccination, group two on day 50 after vaccination, and group three on day 78 after vaccination. The challenge tests were performed with single bacterial infections of *A. hydrophila* 10^7 cfu. mL⁻¹ and *S. agalactiae* 10^4 cfu. mL⁻¹, and the co-infection of both pathogenic bacteria with a composition ratio of 1:1. The fish were injected via intraperitoneal route at a dose of 0.1 mL and were then reared for 14 days after the challenge test.

Preparation of blood sample

Serum samples were collected from 4 fish in each group (vaccination and control). Blood samplings were performed before vaccination and every two weeks after vaccination during the rearing period. Blood was taken without using anti-coagulant, then performed centrifugation and the obtained serum was stored in a freezer at a temperature of -20 °C prior to use for the next test.

Antibody level

Evaluation of specific immune response kinetics (antibody levels) of the vaccinated tilapia was performed through indirectenzyme-linked immunosorbent assay (ELISA) method adopting a method described by Pasnik *et al.* (2005) and Sugiani *et al.* (2014). Antigens used to coat microtiter plate wells were the sonicated *A. hydrophila* or *S. agalactiae* whole

cells with each concentration of $10 \mu\text{g. mL}^{-1}$, using 96 microtiter plate wells. Well was coated with $50 \mu\text{L}$ *A. hydrophila* or *S. agalactiae* antigen diluted in $50 \mu\text{L}$ carbonic-bicarbonate buffer 0.1 M (pH 9.6) with three replications, incubated overnight at a temperature of 4°C and rinsed three times with 0.01 M phosphate buffer saline containing 0.05% Tween-20 (PBS-T). It was then covered with 3% bovine serum albumin(BSA), re-rinsed with PBS-T. Serum from each experimental group at a volume of $100 \mu\text{L}$ was diluted at a ratio of $1:50$ and was transferred to the microtiter plate well, incubated at a temperature of 25°C for an hour and re-rinsed. Specific antibody immunoglobulin rabbit anti-tilapia at a volume of $100 \mu\text{L}$ ($1:200$) was added into the microtiter plate, incubated for an hour at a temperature of 25°C , then re-rinsed. Peroxidase conjugated goat anti rabbit IgG was diluted at a ratio of $1:5000$, then added at a volume of $100 \mu\text{L}$ into each well, incubated at a temperature of 25°C for an hour and re-rinsed with PBS-T. Onestep ultra TMB-ELISA $100 \mu\text{L}$ was then added, incubated for 20 minutes at a temperature of 25°C and the reaction stopped by adding $1 \text{ M H}_2\text{SO}_4$ 100

μL . The microtiter plate was incubated at a temperature of 25°C for 5 minutes in a dark space and optical density (OD) was recorded at an absorbance of 450 nm in ELISAreader.

Statistical analysis

Data of antibody levels in the form of optical density (OD) values from the serum of vaccinated tilapia and control groups and mortality patterns were analyzed through descriptive statistic and were presented in charts.

3. Results and Discussion

The specific immune responses of tilapia from the vaccinated group and the control group were measured using ELISA, subjected to *A. hydrophila* and *S. agalactiae* antigens. The presence of specific antibodies was indicated by an increased in the antibody production in the vaccinated fish. Antibody production kinetics in the vaccinated fish group and the control subjected to *A. hydrophila* and *S. agalactiae* antigens are presented in Figure 1.

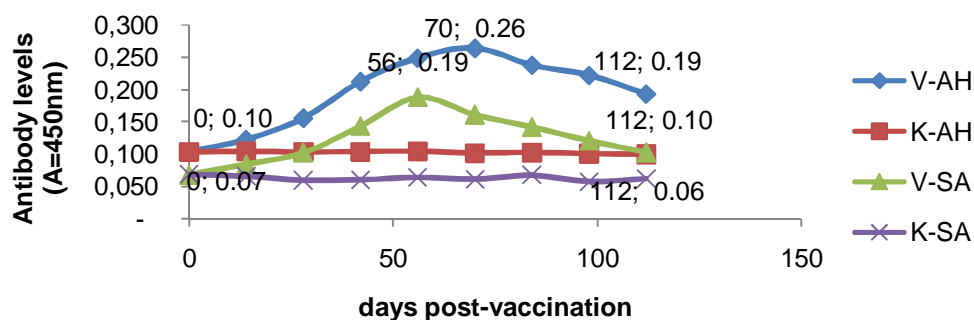


Figure 1. Specific antibodies kinetics of tilapia on post-vaccination period against *A. hydrophila* and *S. agalactiae* antigens. V-AH = the antibody of the vaccinated tilapia with coating *A. hydrophila*; K-AH = the antibody of the control fish with coating *A. hydrophila*; V-SA = the antibody of the vaccinated tilapia with coating *S. agalactiae*; K-SA = the antibody of the control fish with coating *S. agalactiae*.

The basal antibody levels of tilapia against *A. hydrophila* were higher compared to *S. agalactiae*, with OD values were 0.104 and 0.069, respectively. The antibody titer of the vaccinated fish serum was higher than that on control. The antibody concentration of the fish on post-vaccination in the vaccinated group continued to increase, against both *A. hydrophila* and *S. agalactiae*. The maximum immune responses were reached on day 56 for *S. agalactiae* with an OD value of 0.188 and day 70 against *A. hydrophila* with an OD value of 0.264 (Figure 1). This condition occurred allegedly because the characteristics of the two antigens were different, *A. hydrophila* is Gram negative bacteria group having complex structures compared to *S. agalactiae* as Gram positive bacteria group having a simpler structure. Antigenic determinants (epitopes) owned by *A. hydrophila*

are more complex consisting of lipopolysaccharide (LPS), outer membrane protein (OMP), α and β hemolysin, aerolysin, enterotoxin ACT, ALT and AST, protease, and RNase. Amonabactin, enterotoxin, and cytoxine are other virulence factors in *Aeromonas* species (Angka *et al.*, 1995; Perez *et al.*, 2002; Alavandi and Ananthan, 2003; Poobalane, 2007). Those substances are virulence factors and several virulence factors are immunogenic.

Based on the cumulative mortality rate of the experimental fish after the challenge test, the mortality patterns of the fish in each challenge test period with single bacterial infections of *A. hydrophila* and *S. agalactiae*, and co-infection of both bacteria could be observed. The mortality patterns of tilapia in the vaccinated group after the challenge test are presented in Figure 2.

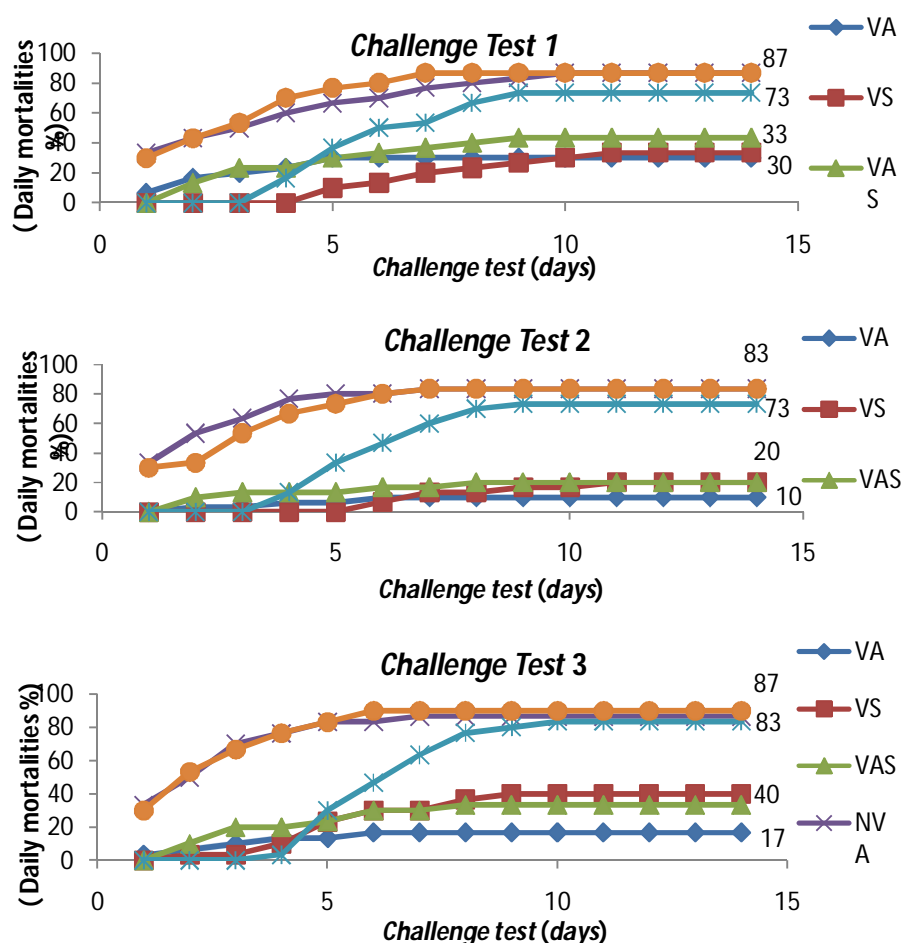


Figure 2. The mortality patterns of tilapia vaccinated with the cocktail vaccine after challenged by single infections of *A. hydrophila* and *S. agalactiae*, and co-infection of both bacteria on the challenge test period 1, 2 and 3. V = vaccinated group, NV = non-vaccinated group, A = *A. hydrophila*, S = *S. agalactiae*, AS = Co-infection of *A. hydrophila* and *S. agalactiae*

Infection patterns of those two bacteria were different, the single infection of *A. hydrophila*, and co-infection caused acute infections while *S. agalactiae* caused sub-acute infection. The mortality rate on each challenge test group was different. The observations of mortality patterns of tilapia challenged on day 22, 50, and 78 after vaccination, were conducted for 14 days. On all challenge test periods, the mortalities of the control fish had occurred since 24 hours after the challenge test with the single infection of *A. hydrophila* and co-infection. The mortality reached about 30% and continued to increase until day 9 with an average mortality rate of 87%, and that occurred on all challenge test periods. On the vaccinated group, the mortality of the fish occurred starting from day 2 with a range about 13% for each challenge test group, and continued to occur until day 7. Mortality rates of the vaccinated fish varied, the highest mortalities caused by the single infection of *A. hydrophila* and co-infection occurred on day 22 of the challenge test (30 and 43%), followed by day 78 of the challenge test (17 and 40%). The lowest mortalities occurred on day 50 with mortality rates of 10 and 20%, respectively.

The mortality of the fish on the challenge test group with the single infection of *S. agalactiae* began to occur on day 4 both on the vaccinated and the control groups on all challenge test periods, except on the challenge test day 50, the mortality of the vaccinated group began to occur on day 6. The mortality on the vaccinated group was lower than that on the control group for each challenge test period ($P < 0.05$). The lowest mortality rate of the fish in the challenge test group with the single infection of *S. agalactiae* occurred on the challenge test on day 50 (20%), followed by day 22 (33%), and the highest on day 78 with a mortality rate of 40%.

Kinetics of the fish antibodies against *A. hydrophila* have been reported by several researchers. Results of this study were in line with the study conducted by Bastardo *et al.* (2012) who obtained that the basal antibody concentration of rainbow trout vaccinated with bivalent vaccine against *A. hydrophila* was higher than that on *Lactococcus garviae* with OD values of 0.101 and 0.028, respectively. Similarly, results of the study by Dehghani *et al.* (2012) obtained the basal antibody concentration of rainbow trout against *A. hydrophila* at a value of 0.15. As reported by previous studies, in this study, it could be known that the basal antibody level in tilapia against *A. hydrophila* was 0.104. A high concentration of the fish antibody against *A.*

hydrophila in this study was suspected caused by the fish had been exposed to antigens before vaccination treatments. Ismail *et al.* (2010) also stated that tilapia that had experienced physical contact naturally with *A. hydrophila* had antibody titer value at log 2.

Results of the study by Vinay *et al.* (2013) stated that the OD value of rohu *Labeo rohita* in the control group against *A. hydrophila* ranged 0.05-0.07, while the antibody titer OD of the fish exposed to free cells of the bacteria ranged 0.10-0.13 and the antibody titer OD of the fish vaccinated with biofilm ranged 0.40-0.67. Those are very natural, because naturally, *A. hydrophila* is the normal microflora of the fish, this species is also widely distributed in almost all waters in the world and is able to adapt on aquatic environments with wide ranges of humidity, turbidity, pH, salinity, and temperature (Swann and White, 1991). Therefore, the fish body will respond responsively against *A. hydrophila*, so antibody concentrations of the experimental fish serum detected will be higher. Vaccination aims to induce the long-term immunity by stimulating the component of the specific immune response that is an antibody. The antibody formation was affected by several factors, such as temperature, vaccine dose, vaccine administration method, age, the fish weight and antigen properties (Ellis, 1988).

There are contra indications in antibody kinetics of tilapia against *S. agalactiae* with some similar studies. Results of the study conducted by Pasnik *et al.* (2005) showed that tilapia vaccinated through injection with inactivated *S. agalactiae* vaccine had the maximal antibody concentration on day 90 post-vaccination with an OD value of 0.192. On the other hand, the study by Sukenda *et al.* (2015) explained that the antibody titer value produced by vaccination through injections of *S. agalactiae* ECP, whole cells and their combination in tilapia continued to decline after 28 days post-vaccination. In this study, the maximum antibody was detected on day 56 post-vaccination by immersion with an OD value of 0.188.

Several factors suspected to become the causes of differences in results of studies conducted were the fish size and the working seed concentration used as the vaccine material. Pasnik *et al.* (2005) used tilapia with an average weight of 45.5 g while the experimental fish used in the study by Sukenda *et al.* (2015) were smaller with a weight range of 20-25 g. The fish size very affects the protection level of a vaccine against the target pathogen. Johnson *et al.*

(1982) reported that salmon with a weight of 4 g vaccinated with *Vibrio anguillarum* and *Yersinia ruckeri* through immersion gave a longer protection than those on 1 and 2 g. Another factor is the working seed concentration of the used *S. agalactiae* vaccine. The working seed concentration of *S. agalactiae* vaccine used by Sukenda et al. (2015) followed results of the study conducted by Pasnik et al. (2005) that was 10^9 cfu. mL⁻¹, while isolates used in those studies were different. That difference in isolate was suspected of giving different protection levels when the fish were exposed by the target pathogen. It should be known in the vaccine manufacturer that the optimum concentration has to be known first. Vaccination aims to induce the long-term immunity by stimulating the component of the specific immune response that is the antibody. The antibody formation was affected by several factors, such as temperature, vaccine dose, vaccine administration method, age, the fish weight and antigen properties (Ellis, 1988).

Specific antibody kinetics of tilapias vaccinated against *A. hydrophila* and *S. agalactiae* obtained during the rearing period correlated with the protection after conducted the challenge test with homolog antigens. On the early immunity induction period, antibodies produced were not so much so when there was an antigen exposure, the fish were not maximal in protecting themselves against an infection. This indicated by higher mortality percentages on the first challenge test period compared to the next periods. The maximum protection occurred on the second phase, indicated by the lowest mortality numbers among other two groups. On the third phase of the challenge test, although the number of antibodies had begun to decrease, but the fish were still able to protect themselves against co-infection of *A. hydrophila* and *S. agalactiae*. It made the protection value after conducted co-infection becoming the highest from those challenged on day 50. These results were in line with several studies that were ever conducted (Pasnik et al., 2005; Ibrahim et al., 2008; Abdel-Hadi et al., 2009; Prasad and Areechon, 2010).

There are several factors that determine vaccination outcomes, including antigen source, production method, inactivation method, concentration, dose, application method, and the addition of adjuvant (Brown, 1978; Johnson et al., 1982; Ellis, 1989; Thuvander et al., 1994; Pretto-Giordano et al., 2010). Ostland et al. (2008) studied that the antibody production against an antigen usually took several weeks although this depended on fish species and water temperature,

where T helper cells and B cells that recognize foreign molecules of an antigen, will induce specific immune responses of a known antigen.

Thuvander et al. (1987) explained that vaccination on rainbow trout juvenile with *Vibrio anguillarum* bacterin triggered a high protective immunity level and it lasted long at least for 46 weeks. Pasnik et al. (2005) analyzed that tilapia vaccinated with ECP and whole cells of *S. agalactiae* through injection until day 90 still provided a protection up to 62%. Plumb (1984) explained that a higher antibody titer could not always be interpreted to provide a high protection or immunity level as well. But a thing that could be studied from results of this study was vaccination on tilapia through immersion with a combined cocktail vaccine of *A. hydrophila* and *S. agalactiae* could induce the specific immune response against homolog antigens. Kinetics of the antibody production resulted from that cocktail vaccine reached the peaks at different times. This indicated that specific immune response resulted by the body would be different on different antigens.

Results of this study demonstrated that the protection level of tilapia on the infection of *A. hydrophila* was higher compared to those on *S. agalactiae* and co-infection after the challenge test using LD₅₀ dose. This was suspected to relate to differences on characteristics, antigenicities, and virulence levels of those antigens. *A. hydrophila* is Gram negative bacterium, while *S. agalactiae* belongs on Gram positive bacterium. According to Williams (2003) virulence components of Gram positive bacteria are their exotoxins (ECP and other toxins), on the contrary with Gram negative bacteria, LPS (endotoxin) is more virulent. Moreover, Pretto-Giordano et al (2010) stated that factors that could cause an immunogenicity of an antigen were complex chemical compounds of an antigen and epitope or antigenic determinant. *A. hydrophila* has multivalent determinants that are outer membrane protein F (OmpF) 47 kDa, thermostable hemolysis protein 50 kDa derived from ECP, lipopolysaccharide 31-38 kDa and S-layers or Paracrystalline surface protein 52 kDa. *S. agalactiae* only has cell surface protein that provides a protective immunity. Sugiani (2012) analyzed that *A. hydrophila* at a high dose (LD₁₀₀) is more deadly on tilapia compared to *S. agalactiae*. For *S. agalactiae* applies the opposite, in a low dose (LD₅₀), this species is more virulent compared to *A. hydrophila* on tilapia.

Bacteria as antigens used as sources of vaccine materials in this study were composed of

protein compounds that had different levels and sizes on each compiler structure. The protein profiles of bacteria can be used as a reference in determining the immunogenic potential of a vaccine, because each protein has different immunogenic capabilities. In addition, protein profiles can be used as identification characters to see strains of bacteria, as conducted by Korkoca and Boynukara (2003).

Based on results of the study by Sugiani (2012) there were differences in protein bands resulted by *A. hydrophila* and *S. agalactiae* whole cell vaccine inactivated by formaldehyde. The characterization of *A. hydrophila* protein using SDS-PAGE resulted 17 protein bands, including 143.72; 119.57; 110.51; 94.39; 72.57; 58.81; 51.57; 34.77; 31.30; 28.18; 25.36; 22.83; 19.50; 15.80; 15.00; 13.50; and 11.53 kDa. For the same strain, Purwaningsih (2013) analyzed *A. hydrophila* monovalent vaccine demonstrating protein bands sizing 103.48, 83.76, 72.76, 64.70, 53.62, 51.16, 45.50, 35.98, 27.79, 24.14, 22.50, 16.58, 14.74, 12.50 and 7.29 kDa. On the other hand, protein bands of *S. agalactiae* in the study by Sugiani (2012) were 10 bands that were 111.86; 83.42; 79.09; 58.98; 54.45; 34.61; 23.20; 18.74; 17.77; and 15.97 kDa.

Stuart (1999) analyzed that an immunogenic antigen molecule had a size more than 60 kDa. According to Perez *et al.* (2002) stated that acetylcholinesterase protoxin that was the protein molecule sizing 45 kDa owned by *A. hydrophila* could induce the immune response of trout. Poobalan (2007) analyzed that a protein of *A. hydrophila* with a size of 50 kDa was a molecule that was very immunogenic. Amrullah *et al.* (2014) analyzed that ECP protein toxin of *S. agalactiae* sizing 89 kDa could increase specific and non-specific immune responses on tilapia.

A long protective immunity generated by cocktail vaccination in tilapia indicated that it might not need re-vaccination (booster) for the rearing of the fish with a rearing period that was similar to the rearing period in this study that was about four months. However, if vaccination is conducted at nursery until grow-out period with a rearing duration up to eight months or for the rearing of the broodstock candidate with a rearing period for 6-14 months, so before the primary antibody heading to the decline phase, booster should be performed, so the fish is expected to be able to increase immune responses through the formation of the secondary antibody, so those antibodies can provide a protection when there is a similar antigen exposure during the rearing period.

4. Conclusion

In conclusion, the basal antibody level in the fish before vaccination against *A. hydrophila* was higher than that against *S. agalactiae*. *A. hydrophila* and *S. agalactiae* bacterins cocktail vaccination could induce and increase the production of specific antibodies in tilapia, and the maximum antibody responses were reached within 70 days against *A. hydrophila* and 56 days against *S. agalactiae*. The lowest mortality rates were obtained on day 50 of the challenge test period in the vaccinated tilapias against *A. hydrophila*, *S. agalactiae*, and co-infection.

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