Development of freeze-dried bivalent fish vaccine product with skimmed milk as stabiliser

Desy Sugiani1,*, Angela Mariana Lusiastuti1, Tuti Sumiati1, Setiadi Setiadi1, Uni Purwaningsih1, Lila Gardenia1, Taukhid2, Murwantoko3, Alim Isnansetyo3, and Desy Putri Handayani3

1Research Center for Veterinary Science, Research Organization for Health, National Research and Innovation Agency, Indonesia
2Research Center for Fisheries, Research Organization for Earth and Maritime, National Research and Innovation Agency, Indonesia
3Department of Fisheries, Faculty of Agriculture, Gadjah Mada University, Indonesia

Abstract. Bacterial vaccines have been tested for their effectiveness in protecting fish from potential virulent diseases. The current vaccine preparations are mostly in liquid form, which affects prices due to an increase in the cost of shipment, the stability of liquid products also has limitations in long-term storage. This study aims to develop a method of preparation of bivalent vaccine through freeze dry method with skimmed milk as a filler for optimization of the vaccine products. The preparation of bivalent vaccine by freeze dry method can reduce 96.67% of total weight compared to vaccines in liquid form. The quality test showed that bivalent vaccine products by freeze dry method with skimmed milk filler containing bacteria Aeromonas hydrophila and Streptococcus agalactiae are passed the sterility and viability test. The application of the vaccine can provide a positive response to increase the antibody titer and other hematological parameter responses and increase the survival rate of fish. The development of freeze-dried vaccine products can optimize fish vaccines as a recommended product in fish disease management.

1 Introduction

The extensive use of antibiotics to treat bacterial diseases in aquaculture has led to antibiotic resistance to various types of fish pathogens. Therefore, an alternative method to prevent and control these pathogens is needed in the aquaculture industry. Fish vaccination has become a standard protocol in aquaculture activities. Vaccination stimulates the immune system and increases fish's resistance to certain types of pathogens to decrease disease outbreaks [10, 12].

Streptococcosis and motile aeromonad septicemia (MAS) are major diseases in tilapia aquaculture, causing mass mortality with significant economic losses [7]. Streptococcus agalactiae is a pathogen that causes streptococcosis [9]. Co-infection outbreaks between S. agalactiae and other pathogens in tilapia contribute to higher mortality and economic losses [6]. Improved awareness of the pathology and pathogenesis of co-infections can help control the outbreaks. The incidence of Streptococcus outbreaks in tilapia is influenced by the
severity of infection and the geographical coverage of outbreaks, which increased as water quality changed due to global warming, polluted environment, and river acidification [8].

The development of a bivalent vaccine to control Streptococcus and Aeromonas infections in red hybrid tilapia shows good results [7]. The current vaccine preparations are mostly in liquid form, which affects prices due to an increase the cost of shipment, the stability of liquid products also has limitations in long-term storage. This study aims to develop a method of preparation of bivalent vaccine through freeze dry method with skimmed milk as a filler for optimization of the vaccine products.

2 Material and method

2.1 Fish and bacterial strains

Tilapia fingerlings of 15 ± 2 g were obtained from a Bogor-West Java fish farmer. The fish were kept at 29 ± 3°C and fed twice a day with commercial feed that contains high protein. The fish were anaesthetised with MS-222 prior to injection and blood sampling (ethical considerations). Streptococcus agalactiae and Aeromonas hydrophila used local isolate collections.

2.2 Vaccine preparation

Vaccine preparation used the dry culture method in agar media, A. hydrophila bacteria were grown in TSA, and S. agalactiae cultured in BHIA, all colonies diluted into sterile saline (NaCl 0.845%), those bacterial cultures inactivated with 3% (v/v) of neutral buffer formalin (NBF). The inactivated vaccine stock solution were added with 5% skim milk, mixed thoroughly to homogenize, frozen at -20°C for at least 24 hours, and continued by the process of freeze dried method in Cool-safe dryers until the product became a dry powder.

2.3 Sterility and viability test

A viability (inactivation) test was conducted to determine the growth ability of bacteria in the vaccine on selective agar media. A total of 0.1 ml of vaccine was inoculated on TSA, BHIA, and Shauten agar and stored at 28-30°C for 24 to 72 hours based on the characteristics of A. hydrophila and S. agalctiae incubations period [11]. Streptococcus agalactiae entered a period of exponential growth and peaked near stability between 48 hours and 72 hours [5].

2.4 Safety and efficacy tests

A safety test was conducted on tilapia (Oreochomis niloticus) by intra peritoneal (IP) injection of 0.1 mL vaccine per fish and with physiological saline as control. Clinical symptoms and fish mortality were observed for 3 days, and reisolation of bacteria A. hydrophila and S. agalactiae from the treated fish. Vaccines are safe if no mortality and active bacteria colonies obtained from reisolation are similar to the vaccine isolates.

2.5 NBT Assay

The production of oxygen free radicals from the phagocytosis process in the blood evaluated by nitro blue tetrazolium (NBT) stain method and analysed at 540 nm wavelength with a spectrophotometer [1].
2.6 Antibody titer analysis

Antibody titers were measured by direct agglutination of the antigen-antibody test. The agglutination test was performed in a 96-well microtiter with a 'U' shaped well bottom. Vaccinated and non-vaccinated fish serum as positive control were exposed respectively to *A. hydrophila* and *S. agalactiae*, and serially two-fold diluted. Titer values were scored in log 2.

3 Result and discussion

The preparation of bivalent vaccine by freeze dry method can reduce 96.67% of total weight compared to vaccines in liquid form. The dry weight of *A. hydrophila* bacterial cells is 0.35 grams, the dry weight of *S. agalactiae* bacterial cells is 0.41 grams, and skim milk 5 grams. Freeze dried products in powder form, as shown in Figure 1.

Vaccines under freeze-dried conditions can improve the viability of bacterial cells. However, the protectant, rehydration medium, freezing temperature, and initial cell concentration influence it. Bacteria are sensitive to freeze-dried methods and their viability without the protectant. Supplementation with 10% skimmed milk in a rehydration medium improved the viability of the cells [15].

Tilapia injected intra peritoneally with vaccines recorded zero mortality within 24 hours post injection, normal swimming movements, good response to feed, and no inflammation or ulceration symptoms in the injection area. Behavioral changes and damage to the injection site usually occur if there is a toxic effect of formalin killed compounds to the fish. The sterility and viability test results showed no bacterial outgrowth on the media throughout the predetermined incubation period (Table 1).

The results showed that vaccines can improve the ability of phagocytic cells to fight antigens. Optical density (OD) value of vaccinated fish is significantly different (P<0.05) compared to the control. However, the best effect on NBT value was the bivalent vaccine challenged with *S. agalactiae* when compared to *A. hydrophila* protection and control. NBT values ranged from 0.203-0.596 on day one after treatment and increased significantly on the...
3rd, 6th, 9th, and 12th days after vaccination to *S. agalactiae*. The NBT value is presented in Figure 2.

**Table 1.** Data of sterility and viability test of freeze dried bivalent vaccines inactivated with formalin

<table>
<thead>
<tr>
<th>Media Culture</th>
<th>Incubation periods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td>TSA (Tryptic Soy Agar)</td>
<td>-</td>
</tr>
<tr>
<td>BHIA (Brain Heart Infusion Agar)</td>
<td>-</td>
</tr>
<tr>
<td>Shauten Agar</td>
<td>-</td>
</tr>
</tbody>
</table>

Remarks: (-) negative as no contamination/none bacterial growth

**Fig. 2.** NBT-assay of vaccinated tilapia used bivalent vaccine with stabilizer

Hosts use phagocytosis activity as a protective response to eliminate a foreign agent. Phagocyte cells will rapidly migrate to the infection site to engulf and destroy the invasive pathogens [14]. The effect of this defense process leads to the release of oxygen free radicals. Tilapia could increase the expression of inflammatory factors in antimicrobial immunity against *S. agalactiae* infection due to the presence of the scavenger receptor CD36 [4]. *Streptococcus agalactiae* bacteria can multiply in the bodies of tilapia and reach a peak number in each tissue at 24 hours post-infection, and *S. agalactiae* is detected mainly in the blood phagocytosed by phagocytes (especially macrophages) and some red blood cells, and attached to the inner wall of blood vessels [2].

**Fig. 3.** Antibody titer levels of tilapia vaccinated with bivalent freeze-dried vaccines of *A. hydrophila* and *S. agalactiae*.
Antibody titer levels of the bivalent vaccine with stabilizer showed a significant difference compared to the control (P<0.05). The results indicated that antibody titer levels increased significantly in response to the challenged test with *S. agalactiae* bacteria, with the highest value of 6 (log-2) at week two post-vaccination compared with protection against *A. hydrophila* at week 1 to week 3. The control group had the lowest antibody levels among the treatments (Figure 3).

Application of the vaccine by injection was safe for the fish. 2 weeks after injection, no mortality was recorded in either group, and no abnormalities were observed through the observation period to responses of the inactivated *S. agalactiae* whole cell vaccine (SAIV) prepared and compounded with either of the two adjuvants [13]. Vaccination treatment can stimulate a higher serum antibody titer than the control [3].

![Fig. 4. RPS rate of tilapia vaccinated with bivalent freeze-dried vaccines of A. hydrophila and S. agalactiae](#)

The highest RPS value was reached by the group of vaccinated fish with bivalent vaccine and challenged by *S. agalactiae* (72.73%), as shown in Figure 4. The RPS value from the challenged test on the vaccinated and unvaccinated fish groups as controls showed a significant difference (P<0.05). The challenged test used the LD50 dose of *S. agalactiae*, which was 10^4 CFU ml^-1, and for *A. hydrophila* was 10^7 CFU ml^-1, with a challenge test period of about 3 weeks. A low RPS result for *A. hydrophila* is due to the unstable virulence of *A. hydrophila*. Therefore, the initial LD50 target becomes more lethal.

Protein-based stabilizers in vaccine products as a vaccine base for oral application can protect the surface of bacterial cells through the digestive tract. The current fish vaccine development has proven to be an excellent response to the application of oral vaccination. Oral administration of nano vaccines for tilapia can induce innate and adaptive immune responses. The relative protection level reached 76.31% after being challenged with 10^6 CFU ml^-1 [16].

4 Conclusion

The development of freeze-dried vaccine products can optimize fish vaccines as a recommended product in fish disease management. This product is safe, sterile, viable, increases the antibody titers, and protects the fish from bacterial infections (*Aeromonas hydrophila, Streptococcus agalactiae*).

References

15. L. Yang, Y. Ma, Y. Zhang, Biol. 34, 4 (2007)