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Efficacy of Formalin-Killed, Heat-Killed and Lipopolysaccharide Vaccines Against Motile Aeromonads Infection in Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract: Formalin-killed, heat-killed and lipopolysaccharide vaccines against *Aeromonas hydrophila* and a bivalent formalin-killed vaccine against *A. hydrophila* and *A. veronei* bv. *sobria* were tested in rainbow trout (*Oncorhynchus mykiss*). The evaluation of trout fish immune response after vaccination with Aeromonads bacterins by immersion and bath challenge route was undertaken using an indirect enzyme-linked immunosorbent assay (ELISA).To test the strength of protection, the challenge process was examined using 10^5 cells of the live bacteria/ml of *A. hydrophila*. The results showed that the relative percentage of survival in the trout fish groups vaccinated by heat-killed type of vaccine were significantly higher ($P<0.05$) than that the other types of vaccines (84%). In addition, the Fish vaccinated with the bivalent vaccine of *A. hydrophila* and *A. veronii* and formalin-killed vaccine showed a high percentage of RPS (67%), while it was measured as 34% for the LPS vaccine. Thus, the bivalent and formalin-killed types of vaccines have higher RPS values compared to the LPS group.

Key words: Motile Aeromonads · Rainbow Trout · Vaccines

INTRODUCTION

Aeromonas hydrophila and other motile aeromonads, such as *A. veroneii* bv. *Sobria*, are Gram-negative bacteria and are also part of the normal flora of aquatic environment. *Aeromonas hydrophila* is known to be one of the most important bacteria associated with the diseases in marine and freshwater fishes. It can cause several health interferes in both fish and humans, including tail and skin rot and fatal hemorrhagic septicemias in fish and soft-tissue wound infection and diarrheic diseases in humans [1].

Nowadays, most of the cultured fish diseases are treated with drugs, such as antibiotics, anti-parasite, etc. The chemotherapeutic actions are particularly effective when used as early as possible and also have a wide spectrum of pathogen control. However, several difficulties are often encountered by chemotherapy. For instance, the drugs are expensive, the resistant strains of pathogens are easily induced in water and the drug residues which may deposit in fish body may introduce potential hazards to the public health as well as the environment [2]. In order to decrease the side effects of chemotherapy, using of vaccines, accompanied with good health fish managements may result in considerable disease prevention and, at the same time, increase the benefit of aquaculture production [3].

Different types of vaccines, such as whole cell, outer membrane proteins, extra-cellular proteins and lipopolysaccharides (LPS) have been investigated against *A. hydrophila* [4-6]. Injection or immersion vaccination with heat or formalin-inactivated bacterins also provides some protection against *A. hydrophila* [4, 7]. LPS is derived from Gram-negative bacteria and consists of lipid A, core polysaccharide and O-specific chain. The lipid A portion of an LPS is known as an endotoxin and is responsible for most of the immunomodulatory effects of LPS [8]. The fish immunized either intramuscularly or intraperitoneally with vaccine showed protection against challenges [9]. Moreover, agglutinating antibody was recognized in the serum of carp immunized with *A. hydrophila* bacterin following a second immersion with this vaccine. However, the fish vaccinated either orally or by immersion showed questionable protection [10].

The present study aimed to prepare formalin and heat-killed whole cells, LPS vaccines of *A. hydrophila* and a bivalent vaccine of *A. hydrophila* and *A. veronii* bv. *sobria* for direct immersion vaccination of rainbow trout.

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It also aimed to detect the fish humoral immune response by ELISA and the efficacy of the prepared vaccines by an experimental infection of the fish with *A. hydrophila* by calculating the relative percent survival.

MATERIALS AND METHODS

Animals: Rainbow trout (average weight = 20 g) were caught from a commercial fish farm (Sheshpir, Fars province, Iran). The animals were kept in 200 L plastic tanks supplied with flow-through well water at 16°C. Moreover, they were maintained under constant photoperiod conditions (12 h light / 12 h darkness) and fed with commercial trout pellets (Beyza, Iran). Before performing the manipulations, the trout were anaesthetized with 60 mg/l MS-222 (tricaine methane sulfonate, Sigma) in water.

Bacteria: Two virulent *A. hydrophila* (accession no. JF313402) and *A. veronii* bv. *sobria* (accession no. JF313414) initially isolated from dropsy and septicemic conditions of fish in Fars province, Iran were used in this study. The isolated bacteria were cultured on tryptone soya agar (Oxoid), identified biochemically [11] and by DNA sequence homology [12] and maintained as lyophilized stocks. For routine use, the cultures were grown overnight on trypticase soy agar at 25°C.

Vaccines

Preparation of Formalin and Heat-Killed Whole Cell Vaccines: In order to prepare the bacterins, each bacterial isolate was inoculated separately into tryptic soy broth (TSB) and incubated for 24 h at 25°C. Formalin (40% w/v) was added to the broth culture at a final concentration of 0.5% (V/V) and left 48 hrs at room temperature. In case of bivalent formalin-killed vaccine formulation, equal portion of each bacterin was added to constitute one volume of the vaccine. Besides, the heat killed vaccine was prepared by heating the broth culture for 30 min at 100°C. The inactivated cells were counted with the hemocytometer (1×10^8 cells/ml) for all the isolates. After that, the bacterins were tested for their sterility (free from the living cells) by streaking them onto trypticase soy agar which showed no growth.

LPS Extraction: LPS was prepared by following the method of Kido *et al.* [13]. A 1.5 ml overnight culture of bacterial cells was centrifuged. Then, the bacterial pellet was suspended in 100 µl of tri-ethylamine (TAE) buffer and mixed with 200 µl of alkaline solution containing 3 g of SDS, 0.6 g of trizma base and 6.4 ml of 2 M NaOH in 100 ml of H_2O. Afterwards, the mixture was heated at 60°C for 70 min and mixed with phenol-chloroform (1:1, v/v), centrifuged at 16,000 ×g for 10 min and the supernatant was mixed with 200 µL of H_2O and 50 µL of 3 M sodium acetate (pH 5.2). LPS was precipitated by adding 2 volumes of ethanol. The final precipitation of LPS was dissolved in 50 µL of H_2O and the concentration of LPS was about 2 mg/ml.

Vaccination and Experimental Challenge: Vaccination of rainbow trouts was performed with LPS, formalin killed and heat killed vaccines of *A. hydrophila* and a bivalent formalin killed vaccine of *A. hydrophila* and *A. veronii* bv. *sobria* by direct immersion route [7]. Groups of 10 rainbow trout fish with 2 replicates were used for each type of vaccination in addition to the control group in each fiberglass tank (a total of 100 fish). The fish were immersed for 1 min in diluted vaccine in a separate vaccine glass aquarium (1 volume of vaccine to 10 volumes of aquarium water = 10^7 cells/ml). Vaccine dose for LPS was calculated as 2 mg/ml. After the vaccination, the fish were washed and returned to their original holding tanks. The control group's fish were kept untreated until the end of the vaccination process. Blood samples from the vaccinated and the control fish were taken from the caudal vein by plastic syringes just before the immunization and 3 weeks after the end of the vaccination process. Bath challenge was applied to the fish as follows: *A. hydrophila* was inoculated in 500 ml of tryptic soy broth for 24 h at 25°C. Also, the cultures (1 volume) were added to 10 volumes of a tank water to gain 10^5 cells of the live bacteria/ml. The challenge process persisted for 15 min for both the vaccinated and the control fish. Then, the fish were transferred to their original tanks and observed for one-week post challenge for any clinical abnormalities and mortalities. Afterwards, isolation of the pathogen from the kidney tissues and cultivation onto specific media from the moribund fish of each group were undertaken. Post challenge mortalities were recorded in both the vaccinated and the control fish. Moreover, the level of protection was calculated according to the following formula [14].

Relative level of protection or Relative Percent Survival (RPS) = 1 – (*Percent of immunized mortality / Percent of control mortality*) × 100 %

Enzyme Linked Immunosorbent Assay (ELISA): The evaluation of the trout fish immune response after vaccination with Aeromonas bacterins by immersion and bath challenge route was done using the indirect-ELISA

according to Loghothetis and Austin [4]. Polystyrene 96-well plate (Linbro) was coated with 100 µl of the antigens. Serum dilutions were prepared serially from 1:200 to 1:25 in PBS and 0.1 ml of each serum dilution was added to duplicate wells of vertical rows in the microdilution plates. Rabbit anti-trout immunoglobulin was diluted in PBS containing 0.5% Tween at 0.5% bovine serum albumin. The substrate used was 2,2'- azino-bis (3-ethyl benzen thiazoline-6-suphonic acid) diammonium salt (ABTS) in 100 mM citiric phosphate, pH 4.2 and 2.5 mM hydrogen peroxide was added. The optical density (O.D) was measured at 490nm using an automated micro plate reader (Bio-Tek). Pooled positive and negative control sera were also included.

Statistical Analysis: All values were given as mean ± S.D., which were means with 95% confidence intervals. Statistical differences between the parameters were tested using paired sample t-test, at the 5% level of significance. All the statistical analyses were performed through the SPSS statistical software (Version 11.5).

RESULTS AND DISCUSSION

Antibody titer was measured using the ELISA three weeks after the vaccination. As shown in Table 1, the antibody titers reached high level in all the vaccinated fish comparing with control group.

Figure 1 shows the antibody levels in sera from fish exposed to the prepared antigens in four manners. The four types of vaccines caused a significant elevation of anti *A. hydrophila* antibodies after 21 days post-immunization compared to the control fish. The O. Ds of LPS and the bivalent vaccines were significantly lower ($P<0.05$) than that of formalin and heat-killed vaccine types. Nevertheless, There were no significant differences ($P>0.05$) between O.Ds of the formalin and the heat-killed vaccines. Also, no significant difference was observed between the fish immune response immersed in LPS and the bivalent vaccines.

The efficacy of each type of vaccination was determined by the relative percent survival (RPS) through the application of challenge test (Bath challenge route) with the virulent strain of *A. hydrophila* (Fig. 2). The challenge results (Table 1) showed that the relative percentage of survival in trout fish groups vaccinated by heat-killed type of vaccine was significantly higher ($P<0.05$) than that of the other types of vaccines (84%). Moreover, the Fish vaccinated with the bivalent vaccine of *A. hydrophila* and *A. veronii* and formalin-killed vaccine showed an equally high percentage of RPS (67%) and for the LPS vaccine, the RPS was 34%. Thus, the bivalent and formalin-killed types of vaccines have higher RPS values compared to the RPS value of the LPS group.

During the last two decades, protection of the fish against infectious diseases, such as hemorrhagic septicemic disease due to *A. hydrophila*, has grown extremely. According to a great number of reports, the effectiveness of vaccination was dependent on the route of administration and was significantly higher when vaccination was carried out through injection [4, 15-18]. The collections of aquatic vaccines are delivered by injection, which is by far the most effective method compared to oral or immersion methods. However, it is labor intensive, expensive and not practical for large numbers of fish under 20 g.

Attempts to develop novel oral and immersion delivery methods have resulted in varying degrees of success. Nevertheless, Bath immunization, where the water level is lowered, offers the advantage of zero handling stress but the disadvantage of requiring a large number of vaccines [19].

Commonly, in the aquaculture industry fish is exposed to immersion vaccination with a concentrated solution for a short period of time. Prolonged immersion, however, may suggest a greater level of protection. Prolonged immersion has been considered in rainbow trout [20, 21]. The present study investigated the short-term exposure of the immersion method in the rainbow trout. This method may be decrease the exposure to stressing conditions.

Baba *et al.* [22] suggested better protection against *A. hydrophila* among the carp which were vaccinated with crude lipopolysaccharide (LPS) rather than whole-cell formalin killed vaccine. Besides, Immunization of the carp with 10 and 20 ìg/ml of LPS solution induced an effective immune protection. In the present study, 2 mg/ml of LPS solution was used for bath immunization (1 min); however, it didn't induce an effective protection.

Injection of LPS alone and along with FCS (fetal calf serum) into the brown trout, channel catfish, eels, turbot, carp and rainbow trout created superior antibody titer against *S. typhimurium, E. ictaluri, E. tarta, cytophaga-like bacteria, A. bestiarum* and *F. psychrophilum* [22-27]. Similar results have been obtained by Collado *et al.* [15] who administered numerous vaccine formulations and lipopolysaccharides to eels by injection and immersion methods and reported the highest protection against *V. vulnificus* by injection.

Table 1: Optical density, relative percent survival, mortality and survival percentages of Trout fish vaccinated with different types of vaccines with consequent challenge

Type of vaccine	Route of vaccination	Route of challenge	OD	% of mortality	% of survival	RPS
Heat – killed	Immersion	Bath	1.10	10	90	84%
Formalin – killed	Immersion	Bath	1.06	20	80	67%
LPS	Immersion	Bath	0.92	40	60	34%
Bivalent	Immersion	Bath	0.84	20	80	67%
Control	Immersion	Bath	0.15	60	40	-

RPS: Relative percent survival

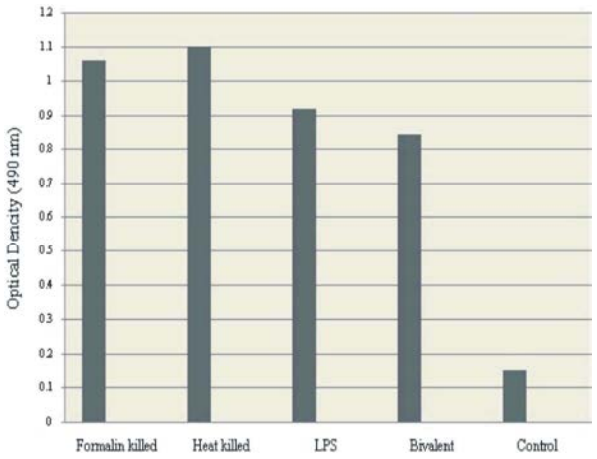

Fig. 1: Antibody titer of vactinated Trout fish groups the control group three weeks after vaccination, measured by the ELISA

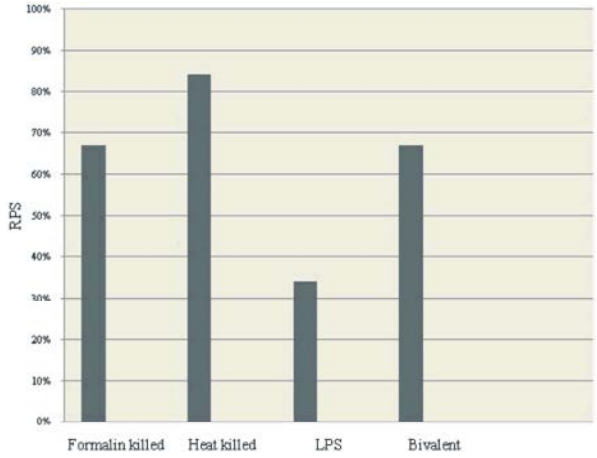

Fig. 2: Relative percent of the rainbow trout vacinated with different types of vaccines

The minor immunity created by the immersion method might be due to the fact that the gills and the skin of the trout cannot take up sufficient quantities of LPS to inducing immunity. In addition, Huising *et al.* [28] reported that the gills and the skin of the fish carp only marginally obtained LPS-DTAE in the direct immersion method.

Baba *et al.* [22] explained that while bath immune stimulation induced an effective immune defense against *A. hydrophila*, oral administration did not give any protection. This may be attributed to the possible damage of much of the LPS during its passage through the digestive tract of the carp. Similar results were also observed in turbot when LPS was administrated through the oral route [29]. Guttvik *et al.* [30] reported that Atlantic salmon fries receiving LPS-coated feed did not display any quantifiable amount of specific antibodies and survival against *A. salmonicida.*

Our results showed that though the LPS group created admissible titer of specific antibodies, the RPS displayed lower protection for this vaccinated group compared to the three other groups.

Anbarasu *et al*. [31] found that formalin inactivated vaccines were superior to heat killed preparations, especially when the bacterins were injected by adjuvants. Prasad and Areechon [32] showed that the vaccines prepared from formalin-killed *A. hydrophila* cells can induce humoral immune response and well protect red tilapia against a virulent *A. hydrophila*. In addition, Osman *et al*. [33] used the monovalent and polyvalent vaccine prepared from formalizing the whole culture of *Aeromonas* spp. (*A. hydrophila, A. sobria* and *A. caviae) and P. fluorescens* in Tilapia fish through the immersion route for 30 min and orally for 7 days. They showed that the polyvalent vaccine prepared from the bacterins of *A. hydrophila*, *A. sobria*, *A. caviae* and *P. fluorescens* through the immersion route have had higher efficacy in comparison to the other types of vaccines.

There are many contradictory reports regarding the protective role of fish specific agglutination antibody. Some showed that there is no correlation between protection and the level of serum specific antibodies [22, 34]. Others, on the other hand, have reported that these antibodies play a major role in protecting the fish against some infections [35-37]. The presented serological data and the results of the Relative Percent Survival (RPS) in the heat-killed and the formalin-killed types of vaccines confirmed the correlation between protection and the level

of serum humoral antibodies in protecting the fish against *A. hydrophila* infection. However, in the group vaccinated with the LPS vaccine through the immersion route, no correlation was found between protection and the level of serum specific antibodies.

Motile aeromonads are one of the most taxonomically and antigenically diverse groups of bacteria pathogenic to fish. The amount of antigenic diversity inherent within this group is especially expressed within H and O somatic antigens. Ewing and Betty [38] described 12 O-antigen groups and 9 H-antigen groups. Each group was further divided into a number of additional serotypes. Chodyniecki [39] also found a high degree of antigenic diversity among the strains of motile aeromonads obtained from the same population of fish and even from different organs of the same fish. Monovalent bacterins were originally prepared against *A. hydrophila*; however, these vaccines only provided acceptable levels of protection against challenge with a homologous bacterium. In fact, the fish did not show any immunized responses to the infection by heterologous strains of *A. hydrophila* [40]. Although some strains of motile aeromonads have common somatic antigens [41-43], it has been consistently demonstrated that a monovalent antiserum could agglutinate only a small percentage of the total isolates examined.

In the present study the efficacy of the formalin and heat-killed whole cells, LPS of *A. hydrophila* and bivalent vaccine of *A. hydrophila* and *A. veronii* bv. *sobria* for direct immersion vaccination of rainbow trout was investigated. The results showed that the monovalent heat-killed vaccine and, secondly, monovalent and bivalent formalin-killed vaccines offered a good immunity against the homologous virulent *A. hydrophila* strain.

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