

Immunization with the *Aeromonas* OMP Provides Protection against *Aeromonas hydrophila* in Goldfish (*Carassius auratus*)

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Abstract

Aeromonas hydrophila is one of the serious pathogens in ornamental fishes causing hemorrhagic bacterial septicemia leading to inflammation and necrosis of the gastrointestinal tract, kidney, muscle, and spleen. Recent studies demonstrated that immunoproteomic vaccines provide protection against bacterial pathogens in fin fish aquaculture and the vaccines are popular due to its long lasting immunity, safety and low cost versatile characteristics. In this study we used an Outer Membrane Protein (OMP) of *Aeromonas hydrophila* as a vaccine to provide protection against the pathogen in goldfish (*Carassius auratus*). We used the extract of *Asparagus racemosus* as an adjuvant in the vaccine preparation. Survival and immunological response of the vaccinated fishes (30 and 60 days post vaccination (dpv)), were evaluated after challenge with virulent *A. hydrophila*. The vaccine treated experimental groups significantly improved ($P < 0.05$) the survival at 50% compared to the controls and had improved immunological responses including phagocytosis, albumin-globulin ratio, serum bactericidal activity, and serum lysozyme activity.

Keywords: *Aeromonas hydrophila*; Outer membrane protein; Immunoproteomic vaccines; Goldfish.

Introduction

Aeromonas hydrophila infection is a scourge of fresh and warm water fish farming worldwide. The pathogen causes motile *Aeromonas* septicemia and hemorrhagic bacterial septicemia in fishes causing significant economic losses particularly in China and India [1]. *A. hydrophila* is a ubiquitous gram-negative, rod-shaped bacterium, has wide range of host susceptibility in cat fish, common carp, goldfish and koi carp. *A. hydrophila* produces a large variety of extra cellular products (ECP) including haemolysins, aerolysin, cytotoxin and enterotoxin [2,3]. Such factors are thought to be important in the virulence of *A. hydrophila* infection in fish, which are known either as motile aeromonad septicemia [4], or hemorrhagic septicemia [5].

Currently applied treatment protocols against aquatic bacterial pathogens are rather difficult, non effective, costly and cause environmental problems. Even though antibiotics and synthetic drugs give positive effects against pathogen control, they cannot be recommended due to their bad effects such as residual effects, resistant strain developments, biomagnifications and suppression of immunity in host organisms [6].

At the global level, farmers have knowledge of the adverse effect of antibiotics and this has lead to shift to eco-friendly approaches such as use of immunostimulants, vaccine bioremediation etc. Fish vaccination in the aquaculture industry has been considered to be very important in reducing economic losses caused by the disease [7]. Several different kinds of vaccines have been investigated/developed against *A. hydrophila* including whole cell (WC), OMPs, ECPs, LPS and biofilms.

Bacterial outer membrane proteins (OMPs) play a significant role in virulence as they comprise the outermost surface in contact with host cells and are also involved in induction of immune defense factors [8]. Recently, attention has been given to OMPs as a potentially important vaccine component. OMPs are located at host-bacterial

interface and are important for host immune responses and as targets for drug therapy [9].

Oral vaccination with biofilm cells of *A. hydrophila*, elicited a significantly higher immune response and protection in carps. The better performance of the biofilm vaccine was attributed to superior antigen delivery to the lymphoid tissues as demonstrated by antigen localization using monoclonal antibodies [10]. The protective nature of bacterial biofilms was exploited for the development of an effective oral vaccine that can resist gastric destruction of epitopes, facilitating improved antigen delivery [11].

In most vaccines, adjuvants are a crucial ingredient for efficacy. Various adjuvants have been used in fishery and they induce better and more long-lasting protection than non-adjuvant vaccine. Recently the herbal immuno adjuvant *Asparagus racemosus* has been shown to improve vaccine delivery against aquatic pathogens [12]. *A. racemosus*, demonstrated significant immuno stimulatory activity particularly at the humoral level in experimental systems. Saponin is the major active immuno adjuvant compounds of *A. racemosus* and they promote peripheral lymphocyte proliferation, enhance serum antibody titer and offer safer advantages than chemical adjuvants [13]. In the present study we demonstrate that the immunoproteomic *Aeromonas* OMP vaccine in the presence of the herbal adjuvant *A. racemosus* extract could provide protection against *A. hydrophila* in ornamental goldfish (*Carassius auratus*).

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Materials and Methods

Source of virulent *A. hydrophila* strain

Virulent strain of *Aeromonas hydrophila* was isolated in our lab, from infected goldfish (*C. auratus*). After biochemical confirmation and virulence studies, they were maintained in -80°C as glycerol stock for further studies.

Preparation of Outer Membrane Protein (OMP) vaccine

Twenty four culture of virulent *A. hydrophila* were harvested by centrifugation from Tryptic Soy Broth at 3,000 g for 20 min at 25°C. The cell pellets were washed twice in phosphate buffered saline (PBS) and once in 10 mM Tris-hydrochloride (pH 7.5). Further cells were re-suspended in Tris-HCl and sonicated at 50W, for 30s, four times on ice. After sonication the suspension was mixed with Sarkosyl for solubilization of the OMP then incubated at 25°C for 30 min. After incubation the suspension was centrifuged at 4000 g for 20 min and the supernatant was collected. After centrifugation at 45 000 g for 45 min the pellet was collected and stored at -80°C until used. The protein was quantified following the protocol of Lowry et al. [14]. The OMP proteins were resolved on a 10% SDS-PAGE [15], to generate protein profiles.

PCR amplification of OMP gene

To amplify the OMP gene from virulent *A. hydrophila*, primers were designed. The forward primer is: 5' CCC AAGCTTATG GCAGTGGTTTATGACAAA 3' and the reverse primer is 5' AACTGCAGTTAGAAGTTGTATTGCAGGGC 3'. PCR amplification was performed using 100ng bacterial genomic DNA in a 100 µl reaction mixture containing 1 X amplification buffer, 1.5 mM MgCl₂, 0.4mM dNTPs and 1 µm each primers with 5 Units of *Taq* DNA polymerase. PCR was performed in Master cycler (Eppendorf, Germany) programmed for 33 cycles, with an initial denaturation at 94°C for 5 min and each cycle with denaturation at 94°C for 30 second, annealing at 50°C for 1 min; extension at 72°C for 1 min; Final extension at 72°C for 5 min; end 4°C for 5 min. PCR amplified products were resolved in a 1% agarose gel by electrophoresis and analyzed using Gel-doc.

Immuno adjuvant

Asparagus racemosus tubers were extracted with hot water at 100°C for two hours. The extracts were filtered and the supernatant were condensed by rotary evaporator at 55°C, lyophilized and stored at 4°C. The extracts contain steroidal saponins having immunoadjuvant properties [16].

Vaccine delivery and immunization

Experimental set-up: Healthy goldfish, *Carassius auratus* having the mean weight of 30± 2.0 g were used for this immunization study. They were acclimatized and kept in quarantine tanks for a period of 10 days to assess their disease-free healthy status and fed with commercial feeds. After acclimatizing, triplicate tanks containing a total of sixty fishes (20 X 3 = 60) were maintained in each group. The tanks were of 500 l capacity, flow-through aquaria with water flow rate of 1 l/min. Vaccines were injected to the fishes, intraperitoneally once in 10 days. The OMP vaccine was injected at the rate of 2 µg/gram body weight. For immune adjuvant treated groups, 500 µg of *A. racemosus* extracts was added to the antigenic proteins. The blank control groups were unvaccinated fishes without bacterial challenge. The control groups consisted of unvaccinated fishes subjected to bacterial challenge. The experimental as well as control fishes were fed with commercial feed twice per day. The detailed vaccine protocols are given in the Figure 1.

Challenge with virulent *A. hydrophila*

On 30 and 60 days post vaccination (dpv), group of fishes were challenged with a lethal dose of *A. hydrophila* (1 X 10⁷) by injecting intramuscularly and transferring to a new 250 l aquaria. The fishes were observed for the cumulative mortality and other pathological signs for 5 days. Blood was collected from the un-challenged, challenged and moribund fishes for immunological studies.

Immunological studies

After 30 and 60 dpv fish samples were chosen from each experiment and control group, and anaesthetized with 50 mg MS-222/dm³ of water, and used for blood collection. Blood was collected from caudal vein, with 1 ml plastic syringe rinsed with anticoagulant, and a part of the blood was transferred immediately, added to an equal volume of 10% tri sodium citrate, and stored at 4°C. The remaining blood was kept at room temperature for 1 h without anticoagulant to collect the serum and stored at - 40°C.

Phagocytosis was performed following the method of Park and Jeong [17], using formalin killed *A. hydrophila* at the rate of 10⁷cfu/ml with 0.1 ml of blood samples of each groups. To study the serum bactericidal activity, five fishes from each group were injected with 0.1 ml/kg body weight with live virulent *A. hydrophila* suspension at the rate of 10³ cells /ml) and blood samples were collected 10 and 90 min after injection. One hundred micro liters of blood was serially diluted and plated in the aeromonas agar (Hi media, India). The albumin-globulin ratio (A-G) of the sera was calculated following the method of Sahoo et al. [18]. The serum samples were analyzed for total protein following the dye-binding method of Bradford [19], using bovine serum albumin as standard, for albumin by the bromocresol green method, and for

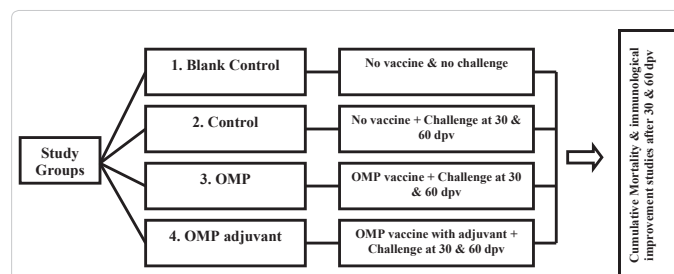


Figure 1: Experimental design for OMP vaccine preparation and delivery methods to ornamental goldfish *C. auratus*.

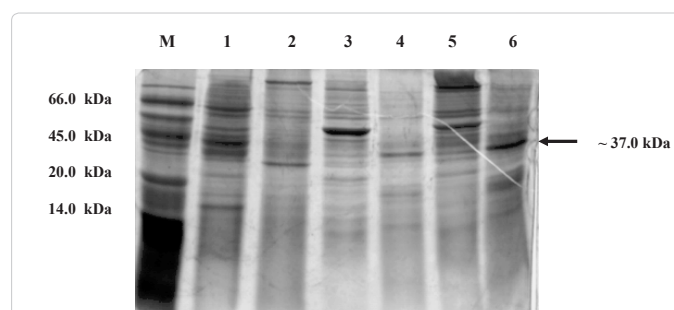


Figure 2: Outer Membrane Protein (OMP) profile of virulent *A. hydrophila* isolated from ornamental goldfish *C. auratus*. M: marker; lane 1: Virulent *A. hydrophila* whole cells isolated from Carp; lane 2: OMP of Virulent *A. hydrophila* whole cells isolated from Carp; lanes 3: *A. hydrophila* MTCC strain; Lane 4: OMP of *A. hydrophila* MTCC strain; Lane 5: Virulent *A. hydrophila* whole cells isolated from *C. auratus* and Lane 6: OMP of *A. hydrophila* isolated from *C. auratus*. The arrows indicated the corresponding OMP proteins.

globulin by subtracting the albumin value from the total protein value. Finally, the albumin-globulin ratio (A-G) was calculated. Lysozyme activity was measured according to methods described by Ellis [20]. One hundred micro liter of serum was mixed with 2 ml *Micrococcus luteus* suspension at 10^8 cfu/ml of 0.05 M sodium phosphate buffer (pH 6.2). The mixture was incubated at 25°C, and its OD was measured at 530 nm using a spectrophotometer. One unit of Lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min/ ml/ serum.

Statistical analysis

All data obtained from experiments were analyzed using one way ANOVA ($P < 0.01$ as significant level) in Statistica 6.0 computer package (Statsoft, UK). Means were also compared using SNK test.

Results and Discussion

Outer Membrane Protein (OMP)

The outer membrane protein (OMP) profiles of the virulent *A. hydrophila* isolated from infected carp as well as goldfish and the non-virulent strain from Microbial Type Culture Collection (MTCC) are shown in Figure 2. The OMP profile revealed that, there is a polypeptide having a molecular weight of around 37 kDa in all the three *A. hydrophila* strains and among these strains, the *A. hydrophila* which was isolated from goldfish had high expression of OMP protein. As goldfish had high expression of OMP protein we used bacteria isolated from goldfish to isolate OMP in our study. The PCR amplification of the OMP gene revealed that, the product was successfully amplified at 1008 bp in the genomic DNA isolated from *A. hydrophila* isolated from goldfish. Unfortunately there is no amplification found in the MTCC and isolates of Carp (Figure 3). Quin et al. [21] isolated three carbohydrate reactive OMP (CR-OMP) having the molecular weight of 43, 40 and ~ 14 kDa from the *A. hydrophila* strain A6 by affinity chromatography. OMPs act as an adhesin and contribute to the virulence. An immunoreactive antigen of the outer membrane protein of *A. hydrophila* was isolated from goldfish, *C. auratus* by Maji et al. [22]. Also Khushiramani et al. [9] isolated 3 to 4 high intensity OMP bands from 40 strains of *A. hydrophila* had the range between 25 to 45 kDa.

OMP Vaccines and Challenge

The fishes from 30 and 60 dpv control groups were succumbed to death 100 % within 5 days after pathogenic *A. hydrophila* challenge (Figure 4a & b). The percentage of cumulative mortality was decreased significantly ($P < 0.01$) in the experimental vaccines treated groups. After 30 dpv, the OMP vaccines helped to decrease the cumulative mortality to 40 %, the OMP with adjuvant decreased the cumulative mortality 30 % after *A. hydrophila* challenge. The same manner was reflected in the 60 dpv (Figure 4b), the immunoadjuvant help to decrease the cumulative mortality of 20%. Recently attention has been given to the immunoproteomic vaccines such as bacterial outer membrane proteins (OMP) [23], vaccine to favour highly immune responses against the bacterial as well as viral pathogens. Adjuvant can be used to improve the immune response to vaccine antigens for several different purpose, including increasing the immunogenicity of weak antigens, enhancing the speed and duration of the immune response, modulating antibody activity, specificity, isotope (or) subclass distribution, stimulating cell mediated immunity, enhancing immune responses in immunologically immature or senescent individuals and decreasing the dose of antigen competition in combination vaccines [24]. *Asparagus racemosus* has been used as a good adjuvant in human

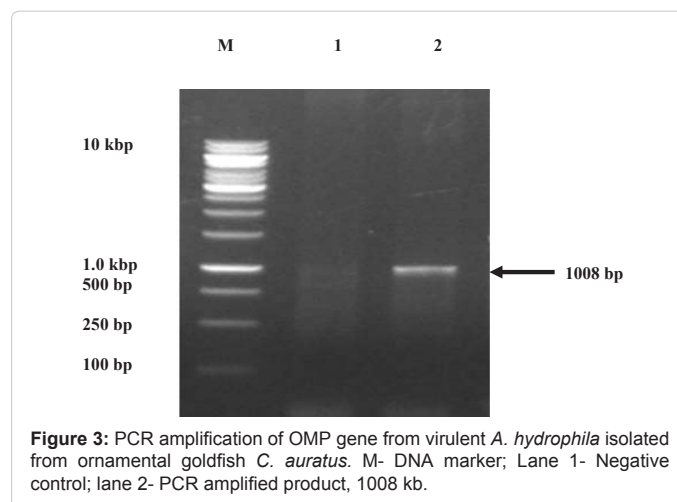


Figure 3: PCR amplification of OMP gene from virulent *A. hydrophila* isolated from ornamental goldfish *C. auratus*. M- DNA marker; Lane 1- Negative control; lane 2- PCR amplified product, 1008 kb.

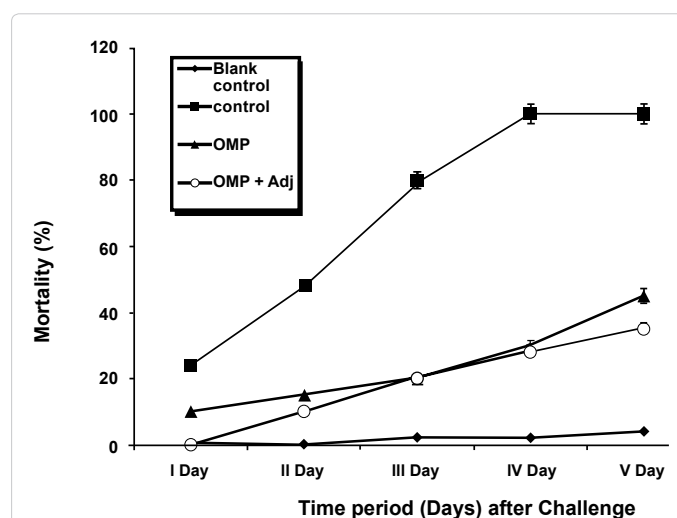


Figure 4a: Cumulative mortality (%) of OMP vaccine treated goldfish *C. auratus* against virulent *A. hydrophila* challenge after 30 dpv.

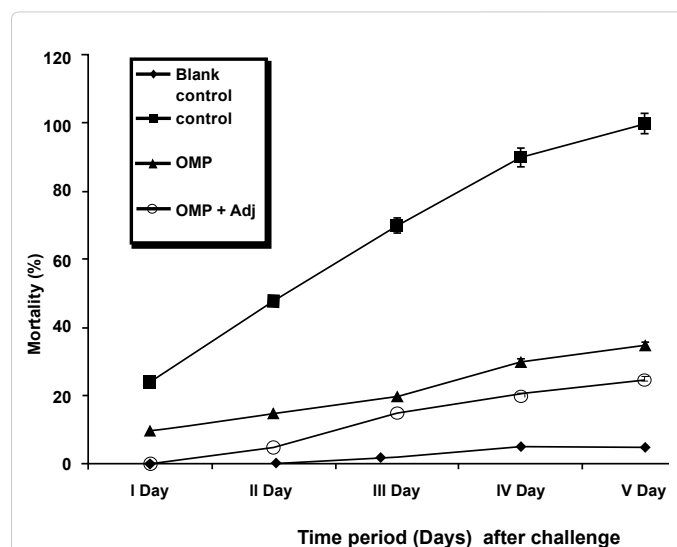


Figure 4b: Cumulative mortality (%) of OMP vaccine treated goldfish *C. auratus* against virulent *A. hydrophila* challenge after 60 dpv.

diseases [25], and give an efficient and sustained immunostimulation to improve immunogenicity of weak or low dose of antigens. The OMP vaccines helped to decrease the cumulative mortality to 30 % and the OMP with adjuvant had to 20 % after *A. hydrophila* challenge. Among the different period of vaccinations, the efficacy increased when the prolonged period of vaccine treatment was done. The immunoadjuvant potential of *A. racemosus* aqueous root extract was evaluated by [16], in *Bordetella pertussis* immunized with diphtheria, tetanus, pertussis (DTP) vaccine, and resulted significant increase in antibody titers. Anbarasu et al. [26], found that formalin inactivated vaccines were superior to heat killed preparations, especially when the bacterins were injected with adjuvants. Thune and Plumb [27], found that both sac fry and swim-up fry vaccinated by immersion in sonicated polyvalent bacterin were protected against challenge with homologous bacteria, indicating an early onset of immunocompetence in channel catfish. Also humoral and protective responses of oral administration of an *Aeromonas hydrophila* biofilm vaccine in three species of carp, catla (*Catla catla* Ham.), rohu (*Labeo rohita* Ham.), and common carp (*Cyprinus carpio* Lin.) were studied by Azad et al. [10] and got higher antibody production against the pathogenic challenge.

Immunological Studies

Immunological responses were analyzed from the serum and blood cells of the 30 and 60 dpv vaccinated goldfish after *A. hydrophila* challenge (Table 1). The percentage of phagocytic activity showed a significant variance ($P<0.01$) between the control and vaccine treated goldfish after *A. hydrophila* challenge. In the unvaccinated, control group, around 46% of the *A. hydrophila* cells was phagocytosed and the percentage was drastically decreased to 28% when no vaccination was given after challenge. The experimental vaccines groups significantly ($P<0.01$) increased the percentage of phagocytosis compared to the control group (Table 1). Similar results were observed in 60dpv after *A. hydrophila* challenge. Increased value of A- G ratio in vaccine treated groups indicated immune responses due to vaccine and adjuvant. The A-G ratio increased in vaccinated fishes in both 30 dpv and 60 dpv compared to unvaccinated fishes. The herbal adjuvant *A. racemosus* helped to boost the immune system along with OMP vaccines. There was a higher A-G ratio observed with prolonged vaccine treatments. Our study demonstrated that the OMP vaccines helped to improve the immunological parameters in the presence of the immunoadjuvants. The immunoadjuvant *A. racemosus* in vaccines may activate the antigen presenting cells (e.g. macrophages) to produce cytokines which

can activate lymphocytes producing specific antibodies. Numerous studies have reported the contribution of adjuvant to immune response in fishes [28]. Most of studies demonstrated that adjuvant could enhance immune response through increasing activity of leucocyte and plasmocyte as well as speeding up production of specific antibody [29]. Cuesta et al. [30], had investigated the propolis on the innate immune responses of gilthead sea bream, and found that it has limited immunostimulatory effects although intraperitoneal administration was more effective than dietary intake. The results of this work found that the propolis has immunostimulatory function and were consistent with others. Tatefuji et al. [31], reported that the compounds of propolis could enhance macrophage mobility and spreading. Due to the saponins active nature of the herbal immuno adjuvant *A. racemosus* it may be greatly improve the induction of major histocompatibility complex (MHC) class I-restricted CD81 cytotoxic T lymphocyte (CTL) responses.

Serum bactericidal activity also significantly ($P<0.01$) improved than the control group due to immune enhancement given by vaccines. The activity increased more than four times in all vaccine treated groups. Serum Lysozyme activity of 23.75 and 26 IU/ml were observed in zero vaccination group after 30dpv and 60dpv respectively. The activity increased significantly ($P<0.01$) as 35.75 and 38 IU/ml in OMP and OMP with adjuvant respectively of 30 dpv groups. Similar results were observed in the serum lysozyme activity of 60 dpv. IgY edible antibody raised with the herbal adjuvant *A. racemosus* as good vaccine candidate for improving serum bactericidal as well as lysozyme activity against WSSV infection in *P. monodon* [12]. The herbal immunostimulants also enhance the serum bactericidal and lysozyme activity against *V. harveyi* infection in grouper (*Ephinephelus tauvina*) [32, 33].

Conclusion

The active principles of the *A. racemosus* help to improve immunity during vaccination. OMP vaccine combined with the herbal adjuvant from *A. racemosus* is effective in providing protection against *A. hydrophila* infection in goldfish (*C. auratus*). The *Aeromonas* OMP antigenic protein could be used as a potential vaccine to control *A. hydrophila* in fishes.

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Treatments	Immunological parameters							
	Phagocytosis (%)		Albumin Globulin ratio		Serum Bactericidal Activity (%)		Serum Lysozyme Activity (IU/ml)	
	30 dpv	60 dpv	30 dpv	60 dpv	30 dpv	60 dpv	30 dpv	60 dpv
Blank Control	46.74 ^a ± 1.43	47.88 ^a ± 3.33	3.33 ± 0.04 ^a	2.99 ± 0.05 ^a	6.34 ± 0.25 ^a	5.97 ± 0.64 ^a	34.7 ± 1.5 ^a	37.9 ± 1.5 ^a
Control	28.33 ^b ± 2.03	32.65 ^b ± 1.76	1.07 ± 0.05 ^b	1.33 ± 0.02 ^b	4.06 ± 0.04 ^b	3.55 ± 0.01 ^b	23.75 ± 2.33 ^b	26.55 ± 1.09 ^b
OMP	48.73 ^a ± 2.33	51.34 ^c ± 3.98	4.27 ± 0.59 ^c	5.07 ± 0.9 ^c	9.77 ± 0.95 ^c	11.23 ± 1.23 ^c	35.95 ± 2.59 ^a	39.02 ± 1.45 ^c
OMP+ Adj	54.63 ^c ± 1.69	55.78 ^d ± 3.0	5.08 ± 0.84 ^d	7.2 ± 0.55 ^d	13.65 ± 1.03 ^d	15.65 ± 2.89 ^d	38.50 ± 2.22 ^c	43.22 ± 2.98 ^d

Table 1: Immunological studies of OMP vaccine treated goldfish *C. auratus* against virulent *A. hydrophila* challenge after 30 and 60 dpv. Values with the same superscript do not differ significantly from each other ($P<0.01$).

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