Vol. 104: 45–57, 2013
doi: 10.3354/dao02581

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published April 29

Vaccination strategies to protect goldfish *Carassius auratus* against *Aeromonas hydrophila* infection

V. Thanga Viji, K. Deepa, S. Velmurugan, M. Birdilla Selva Donio, J. Adlin Jenifer, M. Michael Babu, T. Citarasu*

Centre for Marine Science and Technology, Manonmaniam Sundaranar University Rajakkamangalam, Kanyakumari District, Tamilnadu 629502, India

ABSTRACT: Ornamental goldfish *Carassius auratus* were treated with whole cell (WC), extracellular product (ECP), outer membrane protein (OMP) and biofilm (BF) vaccines developed from the virulent *Aeromonas hydrophila* (AHV1; GenBank HQ331525.1) with and without the immunoadjuvant *Asparagus racemosus*. On various days post-vaccination (dpv), the treated fish were challenged with virulent *A. hydrophila*. These fish were monitored for survival, growth, specific bacterial reduction, and biochemical, haematological and immunological parameters. *C. auratus* attained 100% mortality within 7 d in non-vaccinated groups, whereas the vaccines helped to significantly ($p \leq 0.001$) increase survival after 25 and 50 dpv. The vaccines with immunoadjuvant (ECP_2, OMP_2 and BF_2 treatments) helped to reduce the *Aeromonas* load after the challenge, and serum albumin, globulin and protein levels were significantly ($p < 0.01$) improved in the OMP_2- and BF_2-treated groups. Haemoglobin and red blood cell counts were also significantly improved ($p < 0.05$) in the vaccinated groups compared to the control group. Additionally, haemagglutination occurred at the 1:12 dilution level in the vaccine plus immunoadjuvant-treated groups. Supplementing the vaccines with immunoadjuvant helped to improve phagocytosis to 54.07%, serum bactericidal activity to 14.6% and the albumin:globulin ratio to 7.6% in BF_2 after 50 dpv. Its positive effect significantly ($p < 0.05$) increased in vaccinated groups compared to controls. Based on the results, especially with the OMP and BF vaccines, the immunoadjuvant *A. racemosus* helped to improve the efficiency of the vaccines. This approach will aid in the development of more efficient vaccines against bacterial infections affecting the aquaculture industry.

KEY WORDS: Ornamental fish · Biofilm · Outer membrane protein · OMP · Herbal immunoadjuvants

INTRODUCTION

Ornamental fishes are rapidly gaining importance because of their aesthetic value and their immense commercial value in export trade worldwide. Approximately 90% of freshwater ornamental fishes were reported to be captive-bred (Andrews 1990). Prominent among freshwater ornamental carps cultured in India are koi carp *Cyprinus carpio* and goldfish *Carassius auratus*. While aquaculture is in the phase of rapid development and growth, intensification of fish farming often leads to the emergence of infectious and parasitic diseases. Of the pathogen-caused diseases, *Aeromonas hydrophila* and other closely related motile aeromonads hold the greatest importance for carp culturists (Daskalov 2006). *Aeromonas* sp. has been implicated in many exposed wound infections (Beaune et al. 1978). The ubiquitous, Gram-negative, and opportunistic bacterium *A. hydrophila* causes disease in several freshwater fish species such as cyprinoids (Rahman et al. 1997). *A. hydrophila* causes red fin disease, haemorrhagic septicaemia, motile aeromonad septicaemia and other infections in *C. auratus*. *A. hydrophila* infection in

*Corresponding author. Email: citarasu@gmail.com

fishes has been reported to occur occasionally in Asian countries, including China, the Philippines, Thailand and India (Ebanks et al. 2004).

Antibiotics are frequently used in aquaculture to treat larval infections. However, this practice can also result in both microbial resistance and residual accumulation in tissue, leading to overall fish immunosuppression (Van Muiswinkel et al. 1985). Fish vaccination in the aquaculture industry has been considered to be very important in reducing economic losses caused by microbial diseases (Rahman & Kawai 2000). The administration of vaccines against bacterial and viral diseases has demonstrated positive results in relation to scientific and economic approaches by reducing the chemical use (Costa 2004). Several different kinds of vaccines have been investigated and developed against *Aeromonas hydrophila*, including whole cell (WC), extracellular product (ECP), outer membrane protein (OMP), biofilm (BF) and lipopolysaccharide. Currently, however, no commercial vaccine exists. The interest in using immunostimulants, adjuvants and vaccine carriers in fish is heightened by issues of viral, bacterial, parasitic and fungal diseases that are limiting factors in culture at many fish farms, hatcheries and aquaculture stations. Newer vaccines include highly purified subunit antigens that are weakly immunogenic. Vaccine formulations often require adjuvants for increased immunological efficiency and better vaccination schedules (Ruszala-Mallon et al. 1988, Vogel 2000). Saponin-based adjuvants have the ability to modulate the cell-mediated immune system, as well as to enhance antibody production, and have the advantage that only a low dose is needed for adjuvant activity (Oda et al. 2000). Saponins are steroid or triterpenoid glycosides (Riguera 1997) that induce a strong adjuvant effect to both T-dependent and T-independent antigens, which further induce strong cytotoxic CD8+ lymphocyte responses and potentiate the response to mucosal antigens (Kensil 1996). The present study focused on the comparative efficacy of various types of vaccines (WC, ECP, OMP and BF vaccines) with herbal adjuvants against *A. hydrophila* infection in the goldfish *Carassius auratus*.

MATERIALS AND METHODS

Source of *Aeromonas hydrophila* vaccines

A highly virulent strain of *Aeromonas hydrophila* (AHV1; GenBank: HQ331525.1) was used for the entire study. It was isolated from infected *Carassius auratus* (Thanga Viji et al. 2011).

Whole cell (WC). The WC vaccine was prepared by growing AHV1 overnight in tryptic soy broth (TSB) at 20°C for 20 h followed by inactivation using 0.6% formalin. The formalin was removed by spinning the culture at 1500 × *g* (20 min) at room temperature, washing with 0.85% saline and finally re-suspending in saline to 35 mg ml^{-1} (wet weight).

Extracellular products (ECP). ECP vaccine was prepared by following the method of Adams et al. (1988) with slight modification. AHV1 was cultured in 50 ml TSB at 20°C for 18 h, with shaking at 110 rpm. The culture was subsequently centrifuged (15 000 × *g*, 10 min at 28°C), and the supernatants were removed. The supernatants were again centrifuged (25 000 × *g*, 30 min at 28°C) and filtered through 0.22 µm filters. The filtrate contained the ECP. After estimating the protein from the filtrate, they were treated with 0.6% formalin for 1 h. The formalin was washed away twice by adding 0.85% saline solution and centrifuging at 25 000 × *g* (30 min at 28°C). Finally, the ECP was re-suspended in saline solution and stored at –20°C for future vaccine studies.

Outer membrane protein (OMP). At *t* = 24 h, the AHV1 culture was harvested by centrifugation from TSB at 3000 × *g* (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). Cells were re-suspended in Tris-HCl and sonicated at 50 W for 30 s (4 times on ice). Following sonication, the suspension was mixed with Sarkosyl for solubilization of the OMP and incubated at 25°C for 30 min. After incubation, the suspension was centrifuged at 4000 × *g* (20 min), and the supernatant was collected. After centrifugation at 45 000 × *g* (45 min), the pellet was collected and stored at 20°C until it was used.

Biofilm (BF). The BF vaccine was prepared according to Azad et al. (1997), with some modifications. Briefly, AHV1 was grown on acrylic sheets suspended in TSB, and the biofilm was harvested and heat-inactivated at 90°C for 30 min. It was stored at –20°C.

Quantitative and qualitative protein analysis of AHV1 vaccines

The WC, ECP, OMP and BF supernatants obtained were re-suspended in PBS at pH 7.2, and protein was estimated according to the Bradford (1976) assay. Further, the above proteins were resolved in 10% SDS-PAGE (Laemmli 1970) to generate profiles.

Preparation of herbal immune adjuvant and experimental set-up

Asparagus racemosus tuber powders were extracted with hot water at 100°C for at least 2 h. The extracts were filtered, and the supernatants were condensed (using a rotary evaporator at 55°C), lyophilized and stored at 4°C. The extracts contained steroidal saponins possessing immunoadjuvant properties (Gautam et al. 2004).

Healthy goldfish *Carassius auratus* having a mean weight of 16.4 ± 1 (SD) g were purchased from J.J. Ornamental Fish Hatchery (Nagercoil, Tamil Nadu, India). They were transported to the laboratory and acclimatized in fiberglass-reinforced plastic aquaria (1000 l capacity) for 10 d to meet established laboratory conditions (10 h dark:14 h light; temperature 28 ± 2°C) and to assess their disease-free health status. They were fed with commercial feeds (Taiyo, China). After acclimatization, triplicate tanks containing 150 fish (50 fish tank^{-1}) were maintained in each treatment group (Fig. 1). The tanks had a capacity of 500 l with a flow-through system having a water flow rate of 1 ml min^{-1}. The water quality parameters included quantified dissolved oxygen at 5.2 to 7.8 µg l^{-1} and pH 7.5 to 8.1. The fish were fed ad libitum 2 to 3 times per day with formulated standard commercial pellet feed at a ratio of 10% of their body weight d^{-1}

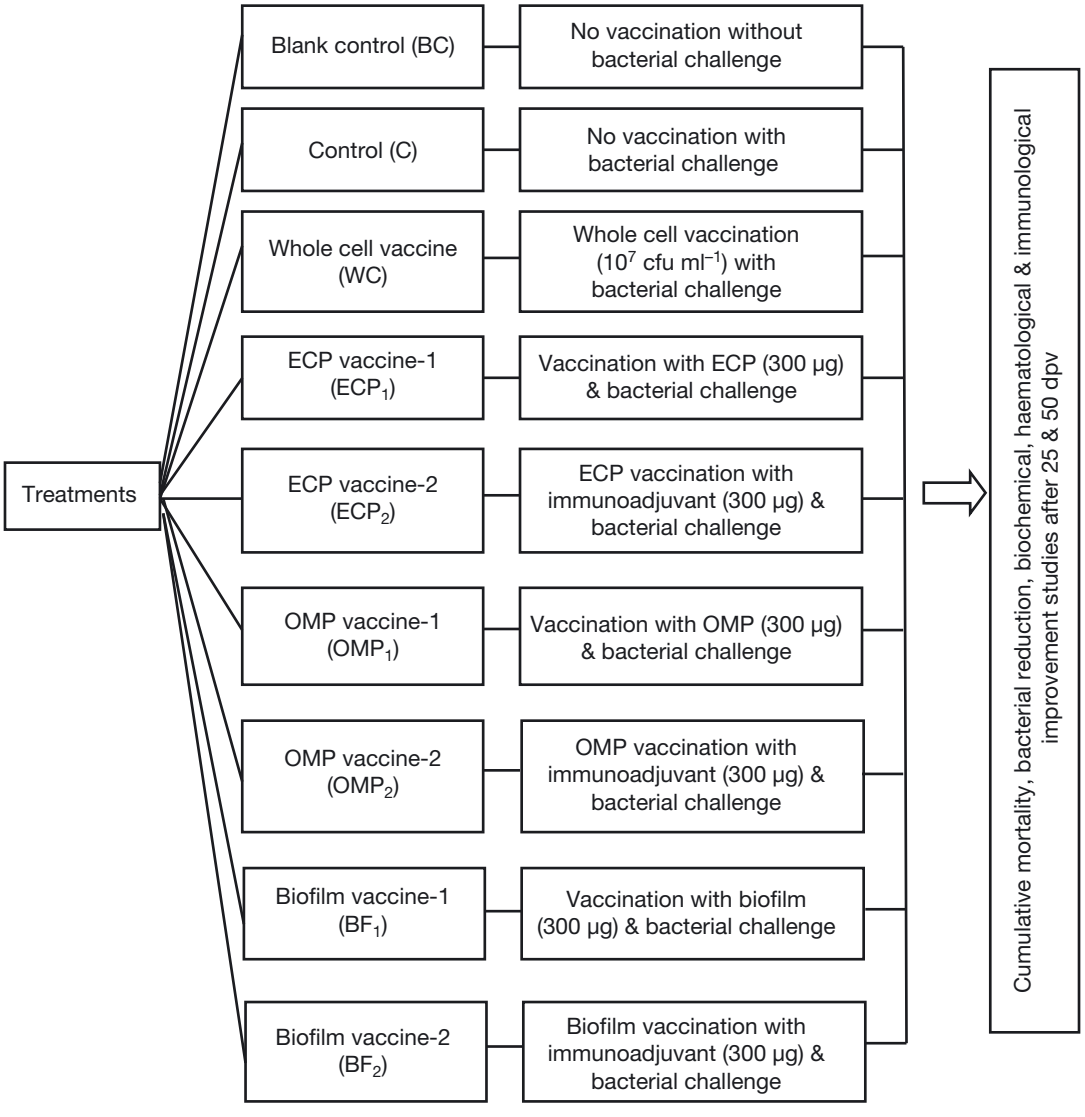

Fig. 1. Experimental set-up for vaccination strategies to protect goldfish *Carassius auratus* against *Aeromonas hydrophila* (strain AHV1) infection. dpv: days post vaccination

throughout the period of study. A partial water change was performed daily to remove waste feed and faecal matter.

Vaccine delivery and immunization

Fish were vaccinated by intraperitoneal injections twice during the culture period. The first dose was delivered on Day 1, and the second dose was delivered on Day 25 of the experimental period. The dosage deliveries were WC vaccine at 10^7 cfu ml^{-1} (50 µl fish^{-1}), and ECP, OMP and BF at 300 µg of total protein per animal. The treatment groups (ECP_2, OMP_2 and BF_2) were also immunized with equal amounts of immunoadjuvant (*Asparagus racemosus* extracts), with antigens as adjuvant. The blank control groups had no antigen delivery and no bacterial challenge. The control group injected with PBS alone (without vaccine) was subject to bacterial challenge. The experimental and control fish were fed commercial feed 3 times d^{-1}.

Challenge with virulent *Aeromonas hydrophila* after 25 and 50 d post-vaccination (dpv)

After 25 and 50 dpv, 20 fish from each group were intraperitoneally injected with 100 µl of live AHV1 at a LC_{50} dose concentration of 1×10^7 cfu fish^{-1}. Survival was observed at 6 h intervals for a maximum of 10 d, and the wet weight gain was calculated by deducting the initial weight from the final weight. The specific growth rate (SGR) was calculated by using the following formula:

$$\mathrm{SGR}\ (\%) = (\ln W_2 - \ln W_1) / (t_2 - t_1) \times 100 \qquad (1)$$

where W_2 = final weight at time t_2, and W_1 = initial weight at time t_1.

Specific bacterial count in blood and muscle

After the respective dpv period, blood was collected from the caudal vein of the challenged fish with a 1 ml plastic syringe and rinsed with anticoagulant (10% trisodium citrate), and 100 µl of blood was plated. The fish were then weighed aseptically and immersed in 50 ppm formalin solution for 5 min. They were washed thoroughly with sterilized water for 30 s to remove the remaining surface bacteria and disinfectant. The washed samples were homogenized with 5 ml of 85% sterile saline and diluted up to 10-fold. Using a sterilized pipette, a 100 µl sample was taken and poured into *Aeromonas* isolation medium. Triplicates were maintained for each sample and incubated at 37°C for 48 h.

Biochemical, haematological and immunological parameters

For the respective dpv period on Days 25 and 50, 25 randomly chosen fish from each experimental and control group were taken and anaesthetized with 50 mg MS-222 dm^{-3} of water. Blood was collected from the caudal vein using a 1 ml plastic syringe rinsed with anticoagulant. Part of the blood was transferred immediately and added to an equal volume of 10% trisodium citrate and then 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.

Saturated ammonium sulphate $(NH_4)_2SO_4$, (40%) was added to 100 µl of serum in microcentrifuge tubes, mixed well and left to stand for 1 h at room temperature. The tubes were centrifuged at 10 000 × *g* (10 min), and the supernatant (albumin fraction) from each tube was collected into separate tubes. The precipitate (globulin fraction) was then dissolved in 500 ml of distilled water. The protein content of both albumin and globulin fractions was determined by the method of Lowry et al. (1951).

Haemoglobin levels were determined by the cyanomethaemoglobin method described by Van Kampen & Zijlstra (1961). Total erythrocyte counts were performed using a haemocytometer (Hendricks 1952). The haemagglutination assay of serum samples was carried out by using a standardized method outlined by Sritunyalucksana et al. (1999) against human 'O' group RBC cells.

For the phagocytic assay, 10^7 cells of formalin-killed *Aeromonas hydrophila* were added to 100 µl of pooled blood samples in a sterile microplate and incubated for 30 min at 25°C after thorough mixing in the well. Following incubation, the blood–bacteria suspension was mixed gently, and 50 µl of this suspension was smeared on 3 glass slides. After air drying, the smears were fixed in 95% ethanol, redried and stained with May–Grunwald's Giemsa. The phagocytic cells and phagocytosed bacteria were counted (Park & Jeong 1996). For the study of serum bactericidal activity, 10 fish from each group were injected with 0.1 ml kg^{-1} body weight with live virulent *A. hydrophila* suspension (10^5 cells ml^{-1}), and blood samples were collected 10 and 90 min after

injection. Blood (100 µl) was serially diluted and plated in *Aeromonas* isolation medium (Hi Media). The albumin:globulin ratio (A:G) of the sera was calculated by following the method of Sahoo et al. (1999): the serum samples were analysed for total protein (following the dye-binding method of Bradford 1976 using bovine serum albumin as the standard), for albumin using the bromocresol green method and for globulin by subtracting the albumin value from the total protein value. Finally, the A:G ratio was calculated. Intra-agar lysozyme activity was performed against *Micrococcus luteus* culture by diluting the blood cells and delivering a drop onto the agar wells. Once the drops were allowed time to absorb into the agar, the wells were incubated upside down for 24 to 48 h at 37°C. The wells were then scored for the highest dilution of blood cells capable of lysing the test microbes.

Data analysis

One-way and 2-way ANOVAs were carried out using the SPSS statistics data package and Ky plot, respectively. Means were compared at the 0.05 and 0.001% level for 1-way and 2-way ANOVA, respectively.

RESULTS

Quantitative and qualitative protein analysis of vaccine candidates

The quantitative protein analysis of different vaccine candidates is outlined in Table 1. An increased protein concentration (23.54 µg) was estimated in the WC vaccines. The protein concentration was estimated as 10.06, 9.82 and 9.48 µg in OMP, BF and ECP, respectively. For qualitative analysis, numerous polypeptide bands including ~75, 65.5, 60, 55, 45, 43.7, 31.1 and 15.4 kDa were separated in the WC vaccines by SDS PAGE analysis. The bands were 60, 43.7 and 15.4 kDa in ECP, and 75, 65.5, 45 and 14.7 kDa in OMP. In BF vaccines, numerous bands were separated at molecular weights of 65.5, 45, 41.6, 38.6, 29.5 and 22.6 kDa (Fig. 2).

Table 1. Total protein quantification (±SD) from different types of vaccines for vaccination of *Carassius auratus* against *Aeromonas hydrophila* (AHV1). For each vaccine, ~300 µg protein antigen was delivered per dose

Vaccine antigen	Total protein
Whole cell vaccine (WC) (µg mg^{-1} of bacterial pellet)	23.54 ± 0.05
ECP vaccine-1 (ECP_1) (µg ml^{-1} of broth culture)	9.48 ± 0.32
OMP vaccine-1 (OMP_1) (µg ml^{-1} of bacterial pellet)	10.06 ± 0.78
Biofilm vaccine-2 (BF_2) (µg ml^{-1} of broth culture)	9.82 ± 0.25

Growth parameters during the vaccination experiment

The weight gain (mg) and SGR (%) were calculated after 25 and 50 dpv with *Aeromonas hydrophila* challenge, and the results are given in Table 2. The weight gain recorded in the control group was 0.45 mg, whereas the WC had a significantly decreased ($p < 0.05$) gain of 0.33 mg at 25 dpv. The experimental groups significantly ($p < 0.05$) differed in weight gains of 0.23, 0.57, 0.43, 0.44, 0.36 and 0.45 mg in WC, ECP_1, ECP_2, OMP_1, OMP_2, BF_1 and BF_2, respectively. We observed the 50 dpv treatment, as well as a minimum and maximum weight gain of 0.89 and 1.32 mg, which differed significantly ($p < 0.05$). An SGR of 5.53% was observed in the control group. There was little improvement observed for the SGR in other groups, except the BF_2 group (5.56%) after 25 dpv. The other groups achieved the lowest SGR of 2.77 and 3.66% in ECP_1 and WC, respectively. At 50 dpv, the SGR was 10.21% in the control group. It significantly ($p < 0.05$) decreased to 8.54% in ECP_1. Conversely, the

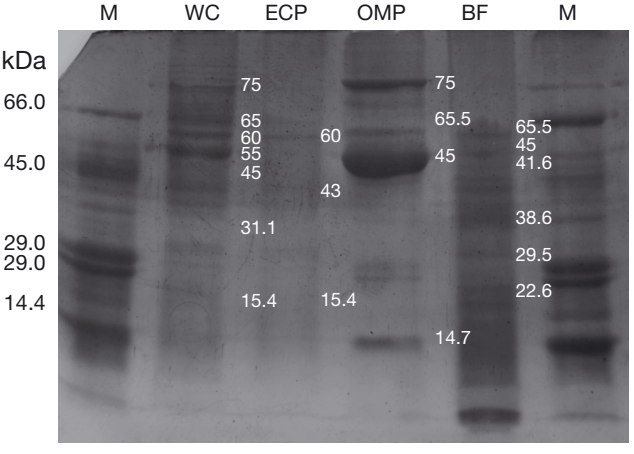

Fig. 2. Qualitative protein profile of different vaccine candidates. M: marker; WC: whole cell protein; ECP: extracellular products; OMP: outer membrane protein; BF: biofilm. Molecular weights are in kDa

Table 2. *Carassius auratus.* Growth parameters (±SD) of control vaccine-treated fish (n = 10 fish × 3 replicates) on different days post-vaccination (dpv). Within each column, values with the same superscript do not differ significantly from each other (1-way ANOVA; $p < 0.05$). SGR: specific growth rate. Treatments are described in Fig. 1

Treatment	Weight gain (mg)		SGR (%)	
	25 dpv	50 dpv	25 dpv	50 dpv
BC	0.45 ± 0.02^{a}	0.92 ± 0.02^{a}	4.85 ± 0.03^{a}	10.22 ± 0.19^{a}
C	0.51 ± 0.01^{b}	0.89 ± 0.02^{b}	5.53 ± 0.15^{b}	10.21 ± 0.03^{a}
WC	0.33 ± 0.02^{c}	0.92 ± 0.01^{a}	3.66 ± 0.02^{c}	10.23 ± 0.02^{a}
ECP_1	0.23 ± 0.01^{d}	0.76 ± 0.02^{c}	2.77 ± 0.02^{d}	8.54 ± 0.03^{b}
ECP_2	0.57 ± 0.02^{b}	1.17 ± 0.01^{d}	5.50 ± 0.02^{b}	13.2 ± 0.20^{c}
OMP_1	0.43 ± 0.01^{a}	0.96 ± 0.01^{e}	5.23 ± 0.32^{b}	10.8 ± 0.02^{a}
OMP_2	0.44 ± 0.02^{a}	1.16 ± 0.02^{d}	5.30 ± 0.26^{b}	12.84 ± 0.04^{c}
BF_1	0.36 ± 0.02^{c}	1.11 ± 0.01^{f}	3.73 ± 0.02^{c}	12.21 ± 0.03^{c}
BF_2	0.45 ± 0.03^{a}	1.32 ± 0.02^{g}	5.56 ± 0.25^{b}	14.5 ± 0.11^{d}

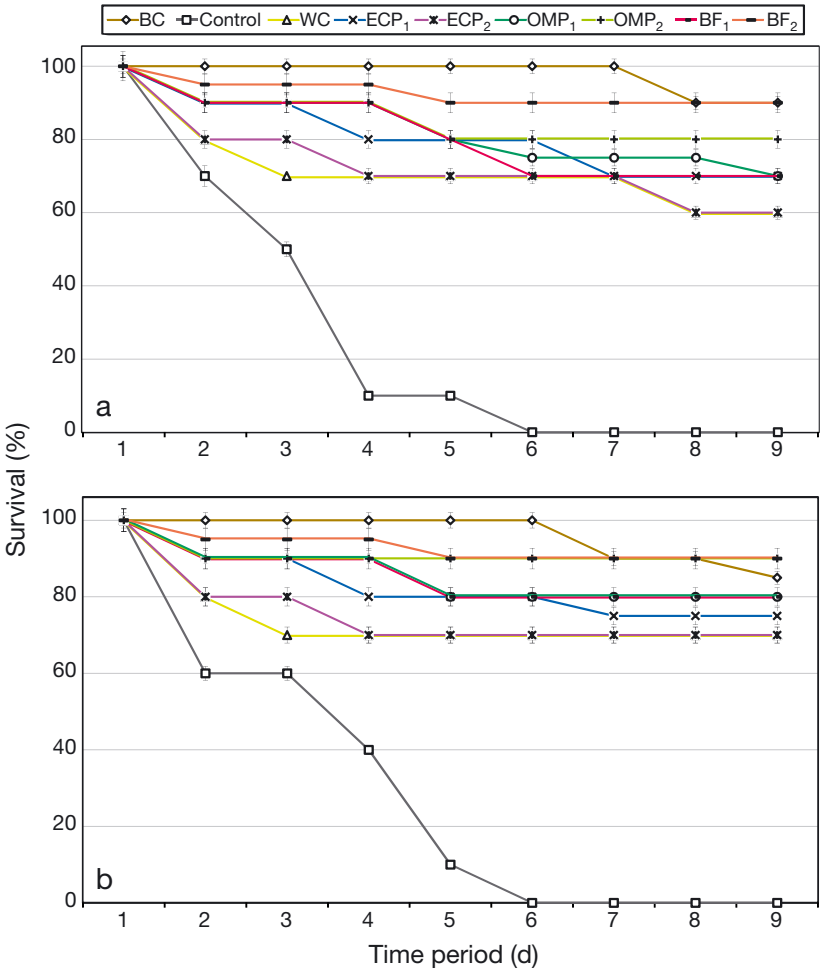

Fig. 3. *Carassius auratus.* Survival (±SE) of fish treated with different types of vaccine with and without adjuvant (n = 5 fish × 3 replicates per treatment) and challenged with AHV1 at (a) 25 d post-vaccination (dpv; values are significantly different among treatments; $F = 31.95$; $p \leq 0.001$, 2-way ANOVA) and (b) 50 dpv (values are significantly different; $F = 29.90$; $p \leq 0.001$; 2-way ANOVA). Treatments are described in Fig. 1

SGR was significantly increased to 12.21, 12.84, 13.2 and 14.5% in BF_1, OMP_2, ECP_2 and BF_2, respectively.

Survival after 25 and 50 dpv by AHV1 challenge

All *Carassius auratus* died (100% mortality level) within 7 d as a result of the virulent AHV1 challenge without pre-vaccination after 25 dpv. The WC and ECP_2 vaccines increased survival to 60%; the ECP_1, OMP_1 and BF_1 groups showed 70% survival; and the maximum survival of 90% was recorded in BF_2 groups after the challenge. Two-way ANOVA revealed that the survival values were significantly different from each other ($F = 31.95$; $p \leq 0.001$; Fig. 3a). The same level of survival was reflected in the vaccine-treated groups after the challenge. Immunoadjuvant-treated OMP and BF groups had the highest survival rate compared to the ECP groups. Two-way ANOVA revealed that the survival values were significantly different from each other ($F = 29.90$; $p \leq 0.001$; Fig. 3b).

Specific bacterial count after 25 and 50 dpv by AHV1 challenge

A total *Aeromonas* sp. count was performed for the specific media for blood and muscle of the challenged *Carassius auratus* after 25 and 50 dpv (Table 3). A heavier *Aeromonas* sp. load (4.3×10^5 cfu ml^{-1}) was observed in the blood of the control fish in comparison to the muscle (3.9×10^5 cfu g^{-1}). The vaccines helped to decrease the load in blood. The minimal loads observed were 1.4×10^2 and 1.6×10^2 cfu ml^{-1} in OMP_2 and BF_2 groups, respectively, after 25 dpv. The same observations were also made for the muscle. A decreased load was observed after 50 dpv in both blood and muscle. The load was 1.1×10^2 and 0.5×10^2 cfu g^{-1} in OMP_2 and BF_2 groups, respectively, in muscle.

Table 3. *Carassius auratus*. Specific *Aeromonas* count (colony-forming units [cfu], ±SD) after 25 and 50 d post-vaccination (dpv) in fish (n = 5 fish × 3 replicates) after challenge with AVH1. Treatments are described in Fig. 1

Treatment	Blood (cfu ml^{-1})		Muscle (cfu g^{-1})	
	25 dpv	50 dpv	25 dpv	50 dpv
BC	$1.3\times10^{1} \pm 0.01\times10^{1}$	$1.3\times10^{1} \pm 0.01\times10^{1}$	$1.9\times10^{1} \pm 0.10\times10^{1}$	$1.9\times10^{1} \pm 0.10\times10^{1}$
C	$4.3\times10^{5} \pm 0.06\times10^{1}$	$5.7\times10^{5} \pm 0.03\times10^{1}$	$3.9\times10^{5} \pm 0.12\times10^{1}$	$4.3\times10^{5} \pm 0.20\times10^{1}$
WC	$2.3\times10^{4} \pm 0.20\times10^{1}$	$3.6\times10^{4} \pm 0.50\times10^{1}$	$1.9\times10^{4} \pm 0.26\times10^{1}$	$1.5\times10^{3} \pm 0.40\times10^{1}$
ECP_1	$2.3\times10^{3} \pm 0.20\times10^{1}$	$5.1\times10^{3} \pm 0.10\times10^{1}$	$2.4\times10^{3} \pm 0.20\times10^{1}$	$2.6\times10^{2} \pm 0.40\times10^{1}$
ECP_2	$1.6\times10^{3} \pm 0.11\times10^{1}$	$5.5\times10^{3} \pm 0.70\times10^{1}$	$2.1\times10^{3} \pm 0.13\times10^{1}$	$1.1\times10^{2} \pm 0.30\times10^{1}$
OMP_1	$2.6\times10^{2} \pm 0.18\times10^{1}$	$4.7\times10^{2} \pm 0.11\times10^{1}$	$2.7\times10^{2} \pm 0.15\times10^{1}$	$1.3\times10^{2} \pm 0.50\times10^{1}$
OMP_2	$1.4\times10^{2} \pm 0.10\times10^{1}$	$3.7\times10^{2} \pm 0.12\times10^{1}$	$1.2\times10^{2} \pm 0.10\times10^{1}$	$1.1\times10^{2} \pm 0.30\times10^{1}$
BF_1	$2.6\times10^{3} \pm 0.20\times10^{1}$	$3.5\times10^{3} \pm 0.40\times10^{1}$	$2.4\times10^{3} \pm 0.01\times10^{1}$	$1.4\times10^{2} \pm 0.03\times10^{1}$
BF_2	$1.6\times10^{2} \pm 0.06\times10^{1}$	$1.05\times10^{2} \pm 0.01\times10^{1}$	$1.4\times10^{2} \pm 0.14\times10^{1}$	$0.5\times10^{2} \pm 0.50\times10^{1}$

Table 4. *Carassius auratus*. Biochemical parameters (±SD) of vaccine-treated fish (n = 10 fish × 3 replicates) after 25 and 50 d post-vaccination (dpv) and after challenge with AVH1. Within each column, values with the same superscript do not differ significantly from each other (1-way ANOVA; $p < 0.05$). Treatments are described in Fig. 1

Treatment	Serum albumin (µg ml^{-1})		Serum globulin (µg ml^{-1})		Serum protein (µg ml^{-1})	
	25 dpv	50 dpv	25 dpv	50 dpv	25 dpv	50 dpv
BC	221.4 ± 0.12^{a}	238.5 ± 0.05^{a}	218.2 ± 0.10^{a}	224.3 ± 0.15^{a}	424.1 ± 0.11^{a}	465.5 ± 0.15^{a}
C	239.3 ± 0.13^{b}	242.3 ± 0.20^{a}	208.3 ± 0.05^{b}	198.3 ± 0.05^{b}	436.4 ± 0.12^{b}	455.1 ± 0.15^{b}
WC	252.4 ± 0.10^{c}	259.3 ± 0.05^{b}	214.4 ± 0.15^{c}	218.6 ± 0.12^{c}	452.4 ± 0.03^{c}	464.4 ± 0.11^{a}
ECP_1	251.5 ± 0.11^{c}	258.6 ± 0.05^{b}	230.3 ± 0.13^{d}	239.3 ± 0.15^{d}	443.4 ± 0.20^{d}	480.4 ± 0.10^{c}
ECP_2	262.3 ± 0.12^{d}	247.6 ± 0.15^{c}	232.4 ± 0.05^{e}	230.5 ± 0.05^{e}	460.4 ± 0.10^{e}	487.4 ± 0.10^{d}
OMP_1	264.6 ± 0.11^{d}	271.5 ± 0.10^{d}	233.5 ± 0.10^{f}	242.4 ± 0.12^{f}	486.2 ± 0.25^{f}	490.7 ± 0.16^{e}
OMP_2	268.6 ± 0.20^{e}	275.6 ± 0.25^{d}	236.6 ± 0.15^{f}	240.5 ± 0.15^{d}	491.6 ± 0.12^{g}	494.6 ± 0.20^{f}
BF_1	252.5 ± 0.11^{c}	253.6 ± 0.15^{b}	223.6 ± 0.16^{g}	238.6 ± 0.12^{d}	486.1 ± 0.17^{f}	488.5 ± 0.15^{d}
BF_2	259.4 ± 0.10^{f}	260.5 ± 0.05^{e}	235.6 ± 0.05^{f}	240.6 ± 0.20^{d}	483.5 ± 0.08^{f}	494.7 ± 0.10^{f}

Biochemical parameter changes after 25 and 50 dpv

The biochemical parameters serum albumin, serum globulin and serum protein (µg ml^{-1}) were studied in the blood after 25 and 50 dpv following the AHV1 challenge (Table 4). A serum albumin level of 239.3 μg^{-1} was noted when no vaccination was given after 25 dpv. The level of albumin significantly ($p < 0.05$) increased to 252, 259, 262 and 268 μg^{-1} in WC, BF_2, ECP_2 and OMP_2 groups, respectively. The same significant ($p < 0.05$) differences were observed at 50 dpv. A globulin level of 208 µg was observed in the control group, whereas the experimental groups had significantly ($p < 0.05$) higher levels of globulin: 214 μg^{-1} with WC, 230 μg^{-1} with ECP_1, 233 μg^{-1} with OMP_1 and 235 μg^{-1} with BF_2. The same pattern of variations was also seen at 50 dpv. The experimental groups significantly differed ($p < 0.05$) for the globulin values. The serum protein observed was at 436.4 and 455 μg^{-1} in the controls at 25 and 50 dpv, respectively. The values significantly ($p < 0.05$) increased in the vaccinated groups. The maximum serum protein was 491 μg^{-1} with OMP_2 at 25 dpv and 494 μg^{-1} with OMP_2 and BF_2, at 50 dpv.

Haematological parameter changes after 25 and 50 dpv

Haemoglobin had a blank control value of 8.43 mg dl^{-1} with no vaccination when AHV1 challenge was initiated. After the challenge, the haemoglobin level was drastically decreased to 7.17 mg dl^{-1}. The experimental vaccines helped to significantly ($p < 0.05$) improve the haemoglobin levels to 8.05, 8.60, 9.24 and 9.42 mg dl^{-1} with ECP_2, OMP_1, OMP_2 and BF, respectively, after 25 dpv. At 50 dpv, the haemoglobin level was similar to those at 25 dpv. A red blood cell (RBC) count of 0.86 ($\times 10^{6}$ mm^{-3}) was observed in the control fish after 25 dpv, and this significantly ($p < 0.05$) increased to 1.32, 1.34, 1.35 and 1.42 ($\times 10^{6}$ mm^{-3}) with WC, OMP_1, BF_2 and OMP_2, respectively. At 50 dpv, haemoglobin increased to a maximum level of 1.48 mg dl^{-1} in the BF_2 groups due to the

Table 5. *Carassius auratus*. Haematological parameters (±SD) of vaccine-treated fish (n = 10 fish × 3 replicates per treatment) after 25 and 50 d post-vaccination (dpv) and after challenge with AVH1. Within each column, values with the same superscript do not differ significantly from each other (1-way ANOVA; $p < 0.05$). Hb: haemoglobin; RBC: red blood cells. Treatments are described in Fig. 1

Treatment	Hb (mg dl^{-1})		RBC (× 10^6 mm^{-3})	
	25 dpv	50 dpv	25 dpv	50 dpv
BC	8.43 ± 0.03^a	8.82 ± 0.04^a	1.02 ± 0.050^a	1.05 ± 0.060^a
C	7.17 ± 0.02^b	7.66 ± 0.04^b	0.86 ± 0.002^b	0.84 ± 0.004^b
WC	7.44 ± 0.04^b	7.83 ± 0.02^b	1.32 ± 0.050^c	1.36 ± 0.050^c
ECP_1	7.72 ± 0.02^b	7.92 ± 0.03^b	1.20 ± 0.003^d	1.26 ± 0.030^d
ECP_2	8.05 ± 0.03^c	8.17 ± 0.05^c	1.24 ± 0.030^e	1.28 ± 0.020^d
OMP_1	8.60 ± 0.03^d	9.04 ± 0.04^d	1.34 ± 0.067^c	1.38 ± 0.007^c
OMP_2	9.24 ± 0.04^e	9.64 ± 0.04^e	1.42 ± 0.090^f	1.57 ± 0.002^e
BF_1	8.83 ± 0.03^d	9.17 ± 0.02^d	1.24 ± 0.040^e	1.28 ± 0.004^d
BF_2	9.42 ± 0.03^e	9.80 ± 0.01^e	1.35 ± 0.031^c	1.48 ± 0.001^f

immunoadjuvant effects (Table 5). The haemagglutinin assay revealed that the agglutination took place at a dilution of 1:4 with WC and 1:8 with ECP1, ECP2, OMP_1 and BF_1, respectively. The immunoadjuvant helped to increase the titre value, and agglutination occurred at the 1:12 dilution level for OMP_2 and BF_2 (Table 6).

Immunological parameter changes after 25 and 50 dpv

The percentage of phagocytic activity showed a significant variance ($p < 0.01$) between the control and vaccine-treated goldfish following the AHV1 challenge. In the control group, around 18% of the *Aeromonas hydrophila* cells were phagocytosed, and the percentage increased significantly ($p < 0.01$) to 44.1, 45.05, 46.05 and 47.08% in WC, BF_1, ECP_2 and BF_2 groups, respectively. After 50 dpv, WC, OMP_2, BF_1 and BF_2 groups showed significant increases ($p < 0.01$) in the phagocytic activity to a level of more than 50% (Fig. 4). The fish serum bactericidal activity for 25 and 50 dpv is shown in Fig. 5. A serum bactericidal activity of 3.88% was observed in the control groups, whereas the ECP_1, OMP_2 and BF_2 groups had significantly higher ($p < 0.01$) serum bactericidal activities of 7.82, 11.2 and 13.4%, respectively, after 25 dpv. The fish vaccinated with the immunoadjuvant displayed more than 10% difference in serum bactericidal activity from the

Table 6. *Carassius auratus*. *In vitro* haemoagglutinin assay of vaccine-treated fish blood with human 'O' group red blood cells after 25 and 50 d post-vaccination (dpv) and after challenge with AVH1. +: agglutination occurred; −: agglutination did not occur; +ive: positive; −ive: negative. Treatments are described in Fig. 1

Treatment	Dilution	Haemoagglutination activity	
		25 dpv	50 dpv
BC	−ive control	+	+
	+ive control	−	−
	1:1	−	−
C	−ive control	+	+
	+ive control	−	−
	1:1	−	−
WC	−ive control	+	+
	+ive control	−	−
	1:1	+	+
	1:2	+	+
	1:4	−	−
ECP_1	−ive control	+	+
	+ive control	−	−
	1:1	+	+
	1:2	+	+
	1:4	−	+
	1:8	−	−
ECP_2	−ive control	+	+
	+ive control	−	−
	1:1	+	+
	1:2	+	+
	1:4	+	+
	1:8	−	+
OMP_1	−ive control	+	+
	+ive control	−	−
	1:1	+	+
	1:2	+	+
	1:4	+	+
	1:8	−	+
OMP_2	−ive control	+	+
	+ive control	−	−
	1:1	+	+
	1:2	+	+
	1:4	+	+
	1:8	+	+
	1:12	−	+
BF_1	−ive control	+	+
	+ive control	−	−
	1:1	+	+
	1:2	+	+
	1:4	+	+
	1:8	−	+
BF_2	−ive control	+	+
	+ive control	−	−
	1:1	+	+
	1:2	+	+
	1:4	+	+
	1:8	+	+
	1:12	−	+

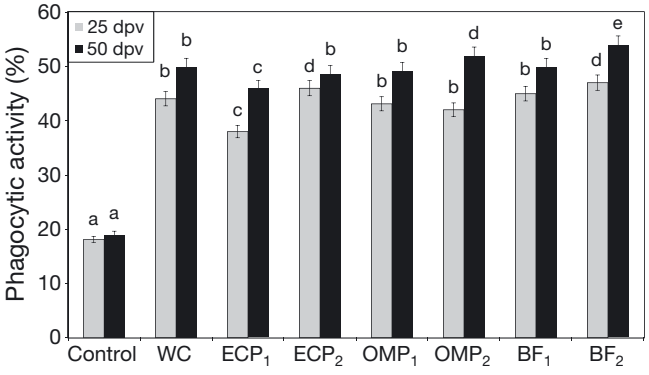

Fig. 4. *Carassius auratus.* Phagocytic activity (±SE) of fish treated with different types of vaccine with and without adjuvant (n = 5 fish × 3 replicates per treatment) and challenged with virulent AHV1 after 25 and 50 d post-vaccination (dpv). Values with the same superscript do not differ significantly from each other ($p < 0.01$; 1-way ANOVA). Treatments are described in Fig. 1

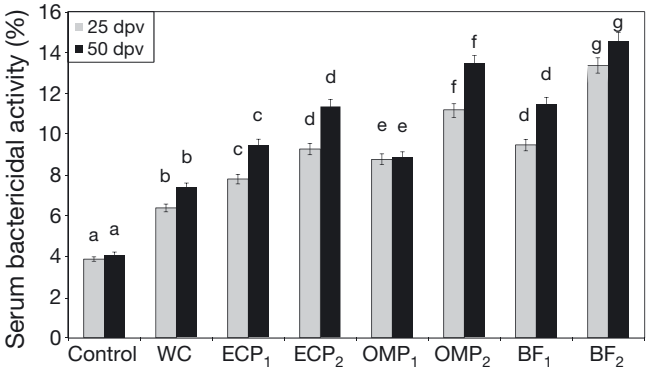

Fig. 5. *Carassius auratus.* Serum bactericidal activity (±SE) of fish treated with different types of vaccine with and without adjuvant (n = 5 fish × 3 replicates per treatment) and challenged with virulent AHV1 after 25 and 50 d post-vaccination (dpv). Values with the same superscript do not differ significantly from each other ($p < 0.01$; 1-way ANOVA). Treatments are described in Fig. 1

control, and were significantly ($p < 0.01$) different at 50 dpv. The A:G ratios were 1.3 and 1.7% in the control groups at 25 and 50 dpv, respectively. These ratios significantly ($p < 0.01$) increased in the experimental groups, and the maximum ratios of 5.5 and 7.6%, respectively, were recorded in the BF_2 groups at 25 and 50 dpv (Fig. 6). The intra-agar lysozyme activity of the blood serum of vaccinated fish performed against AHV1 is given in Table 7 for 25 and 50 dpv. The zone of inhibition was found to be 4.59 mm in the control group at 10^{-1} dilution at 25 dpv. Two-way ANOVA revealed that the intra-agar lysozyme activity significantly ($F = 52.33$; $p \leq 0.001$) increased in vaccinated groups when compared to the control group. A similar outcome was reflected at 50 dpv. The control group had a 5.44 mm zone of inhibition. This zone significantly ($F = 31.78$; $p \leq 0.001$) increased to 12.67, 14.77, 15.7 and 18.77 mm with WC, BF_2, ECP_1 and OMP_2, respectively, in 10^{-1} dilutions.

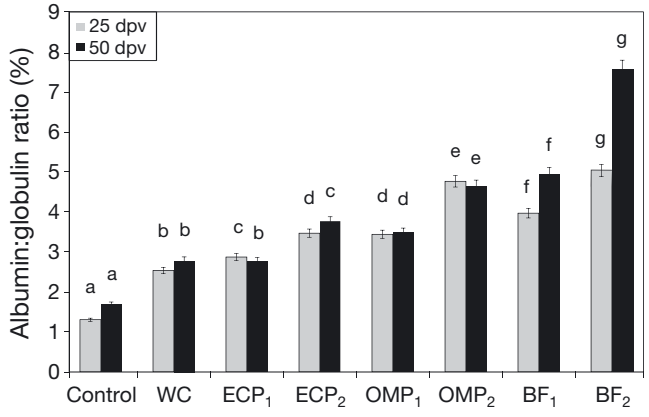

Fig. 6. *Carassius auratus.* Albumin:globulin ratio (±SE) of fish treated with different types of vaccine with and without adjuvant (n = 5 fish × 3 replicates per treatment) and challenged with virulent AHV1 after 25 and 50 d post-vaccine (dpv). Values with the same superscript do not differ significantly from each other ($p < 0.01$; 1-way ANOVA). Treatments are described in Fig. 1

Table 7. *Carassius auratus.* Zone formation (mm, ± SD) of intra-agar lysozyme activity of the blood serum of fish treated with vaccines (n = 10 fish × 3 replicates per treatment) and challenged with AHV1 after 25 and 50 d post-vaccination (dpv). All values are significantly different among treatments (2-way ANOVA: $F = 52.33$ [25 dpv]; $F = 31.78$ [50 dpv]; $p \leq 0.001$). Treatments are described in Fig. 1

Treatment	10^{-1} dilution		10^{-2} dilution		10^{-3} dilution	
	25 dpv	50 dpv	25 dpv	50 dpv	25 dpv	50 dpv
C	4.59 ± 0.03	5.44 ± 0.04	4.81 ± 0.04	4.33 ± 0.10	2.55 ± 0.03	3.63 ± 0.07
WC	12.08 ± 0.12	12.67 ± 0.08	12.02 ± 0.15	11.50 ± 0.03	8.34 ± 0.02	9.85 ± 0.01
ECP_1	14.26 ± 0.05	15.70 ± 0.11	14.38 ± 0.01	13.87 ± 0.01	8.07 ± 0.03	8.57 ± 0.08
ECP_2	14.83 ± 0.05	15.48 ± 0.15	15.36 ± 0.12	14.85 ± 0.12	9.28 ± 0. 01	9.77 ± 0.01
OMP_1	15.46 ± 0.05	17.46 ± 0.15	11.76 ± 0.02	11.26 ± 0.01	8.24 ± 0.02	9.45 ± 0.01
OMP_2	17.84 ± 0.02	18.77 ± 0.01	16.18 ± 0.08	15.67 ± 0.04	10.24 ± 0.01	11.75 ± 0.06
BF_1	13.46 ± 0.01	14.05 ± 0.02	13.07 ± 0.01	12.56 ± 0.08	7.36 ± 0.14	8.17 ± 0.12
BF_2	14.25 ± 0.03	14.77 ± 0.02	14.37 ± 0.12	13.86 ± 0.02	9.25 ± 0.04	10.76 ± 0.01

DISCUSSION

During the last 20 yr, vaccination has been established as a preventive method against various bacterial pathogens in aquaculture (Sommerset et al. 2005). Our work concentrated on traditional vaccine candidates, including WC and ECP, and the modern approaches of OMP and BF vaccines with a safe herbal adjuvant. A higher amount of total protein (23.54 µg) is present in the WC vaccine due to the numerous polypeptides ranging in molecular weight from 15 to 65 kDa. The WC vaccines helped to improve fish survival and other parameters when compared to the control. *Aeromonas hydrophila* aroA is an attenuated strain that has been assessed as a live vaccine in rainbow trout *Oncorhynchus mykiss*, which showed higher and long-lasting immunity (Vivas et al. 2005). The elicitation of high and long-lasting immune responses by live bacterial vaccines has been related to the persistence of the vaccine in the tissues (Marsden et al. 1996). The ECP of AHV1 consists of a few polypeptides ranging from 30 to 45 kDa and has higher virulence. In our previous study (Thanga Viji et al. 2011), the ECP of this strain had strong haemolytic and proteolytic activities and caused 100% mortality in *Carassius auratus*. *A. hydrophila* isolated from oyster produced higher levels of toxins such as haemolysin and cytotoxin (Tsai et al. 1997). Nieto & Ellis (1991) fractionated ECP of *A. hydrophila* to produce a purified polypeptide toxin of 15.5 kDa which was 300 times more toxic than the crude ECP. OMP contains 4 polypeptides ranging from 15 to 65 kDa, and the BF contains numerous protein bands. In our previous work (Thanga Viji et al. 2012), a prominent OMP with a molecular weight of 37 kDa was extracted from 3 different strains of *A. hydrophila.* The OMPs of *A. hydrophila* are rather heterogeneous in most of the strains but a 36 kDa protein is common to most strains. Khushiramani et al. (2008) isolated 3 to 4 high-intensity OMP bands from 40 strains of *A. hydrophila* that ranged from 25 to 45 kDa. Asha et al. (2004) studied the expression of antigenic BF from *A. hydrophila* and identified about 15 protein bands that ranged from 10 to 15 kDa.

The SGR as well as the weight gain was not reflected after 25 dpv in vaccinated *Carassius auratus*, whereas the groups treated with vaccine plus immunoadjuvant achieved significant growth after 50 dpv. This may be the immune boosting effect of immunoadjuvants, and the herbal immunoadjuvant-like *Asparagus racemosus* helps to increase the antigen uptake and thus improves the immune system. Saponin-based adjuvants have the ability to modulate the cell-mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity (Cohen & Bioterrorism 2001). Relative to the vaccines which were delivered alone, the booster dose helped to reduce the cumulative mortality to 10% after Day 10 following the challenge in the present study. Recently, attention has been given to immunoproteomic vaccines such as bacterial OMP (Maji et al. 2006) and BF vaccines (Azad et al. 1999) to confer immune responses against bacterial and viral pathogens. The OMP vaccines helped to decrease the cumulative mortality to 30% and the OMP with adjuvant to 20% after *Aeromonas hydrophila* challenge (Thanga Viji et al. 2012). Fang et al. (2000) showed significant protection against 2 isolates of *A. hydrophila* in blue gourami *Trichogaster trichopterus* (75 and 87.5% relative percent survival, RPS) immunized with a recombinant 43 kDa OMP. The 37 kDa OMP of *A. hydrophila* was immunogenic in rohu carp (Khushiramani et al. 2007). Among the different types of vaccine treatment in the present study, the *C. auratus* group treated with OMP and BF with adjuvant showed higher immunogenic performance including survival and decreased specific bacterial load. Similarly, *C. auratus gibelio* vaccinated with formalin-killed cells of *A. hydrophila* with the adjuvant *Apis mellifera* propolis showed an enhanced RPS of 67.8% after challenge with *A. hydrophila* (Chu 2006). The adjuvant could enhance immune response by increasing the activity of leukocytes and plasmocytes as well as by speeding up the production of specific antibodies (Williams et al. 1989).

The immunoadjuvant helps to improve albumin, globulin and protein levels in the serum levels when combined with ECP, OMP and BF. Herbal immunostimulants such as *Lonicera japonica* and *Ganoderma lucidum* also help to increase the immunoglobulin level in *Oreochromis niloticus* against *Aeromonas hydrophila* challenge. Rairakhwada et al. (2007) found that the globulin content was significantly enhanced in levan-fed common carp fingerlings. Misra et al. (2006) reported that total serum protein content increased significantly in fish fed with dietary doses of β-glucan.

In *Carassius auratus*, haemoglobin (9.79 g dl^{-1}) and haematocrit (28.90%) values improved compared to the controls (Harikrishnan & Balasundaram 2008). In the present study, the level of haemoglobin and RBC increased compared to the control groups, and the maximum levels were increased in the adjuvant-treated groups. Sahu et al. (2007) reported that WBC and RBC counts were higher in *Labeo rohita* finger-

lings fed *Mangifera indica* kernel when compared to controls. In our experiment, the fish were also treated without vaccines, and low levels of RBC (0.8×10^6 mm^{-3}) were recorded after 25 and 50 dpv. The low level of RBC caused losses of body fluids and oxygen carrying capacity, which eventually led to mortality. In infected untreated fish, decreased RBC, haematocrit and haemoglobin concentrations indicated that RBCs were destroyed by leukocytosis with subsequent erythroblastosis (Hawk et al. 1954). The haemoagglutinin assay in our work revealed that the vaccinated groups react even at the lowest dilution, and the adjuvant-treated groups were agglutinated at dilutions of 1:12 in ECP_2, OMP_2 and BF_2. This may be due to the increased antibody production including the albumin, globulin and protein levels in the blood serum of the adjuvant-treated groups. The immunoadjuvant potential of *Asparagus racemosus* aqueous root extract was evaluated in experimental animals immunized with diphtheria, tetanus, pertussis (DTP) vaccine; the immunized animals had a significantly increased level in antibody titres against *Bordetella pertussis* challenge (Gautman et al. 2004).

The prolonged period of vaccination (50 d in the present study) is responsible for higher immunological improvement compared to the shorter period (25 d) of vaccination. The immunological parameters of phagocytosis serum bactericidal activity, A:G ratio and intra-agar lysozyme activity were increased by the vaccines. The immunoadjuvant helped to significantly enhance the immunological activities along with the vaccines. The phagocytic activity was also increased in experimental groups as compared to the control fish. The immunoadjuvant helped to enhance the phagocytic activity over 1.5 times. The BF vaccines with immunoadjuvants were more highly immunogenic than other vaccine preparations. The BF antigens had enhanced uptake and longer retention compared to that of free-cell vaccine (Azad et al. 1997). The extracts of 4 Chinese herbs (*Rheum officinale, Andrographis paniculata, Isatis indigotica, Lonicera japonica*) increased phagocytosis of white blood cells of crucian carp (Chen & Kusuda 1996). Tatefuji et al. (1996) reported that the compounds of propolis could enhance macrophage mobility and spreading. Due to the active nature of saponins, the herbal immunoadjuvant *Asparagus racemosus* may greatly improve the induction of major histocompatibility complex class I-restricted CD81 cytotoxic T lymphocyte responses.

Azad et al. (2000) administered *Aeromonas hydrophila* BF vaccine to 3 species of carp (*Catla catla*, *Labeo rohita* and *Cyprinus carpio*) at different doses and for varying periods of time. Among the 3 carp species, catla produced the highest antibody and protective response, followed by rohu and common carp after 15 and 20 dpv. In our study, the serum bactericidal activity was increased in the vaccines without adjuvant and increased more than 2 times in the adjuvant-treated groups compared to the control group. The IgY edible antibody, produced with the herbal adjuvant *Asparagus racemosus,* is a good vaccine candidate for improving serum bactericidal activity against white-spot symdrome virus (WSSV) infection in *Penaeus monodon* (Kumaran et al. 2010). Herbal immunostimulants also enhanced the serum bactericidal and lysozyme activity against *Vibrio harveyi* infection in grouper *Ephinephelus tauvina* (Sivaram et al. 2004, Punitha et al. 2008). The increased A:G ratio in vaccine-treated groups in our study indicated higher immune responses. The highest A:G ratio was observed in immunoadjuvant-treated groups compared to the other groups. The prolonged time post-vaccinations also helped to improve the A:G ratio. The herbal adjuvant *A. racemosus* helped to boost the immune system along with OMP and BF vaccines. 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. Cuesta et al. (2005) investigated the propolis on the innate immune responses of gilthead seabream and found that it has limited immunostimulatory effects. Total lysozyme is a measurable humoral component of the non-specific defence mechanism, and while reports on modulation of lysozyme activity in fishes are rare, increased values have been recorded by various authors after activation of the immune system with immunomodulants (Engstad & Robertson 1993, Siwicki et al. 1994) and by feeding 4 different Chinese herbs (*Rheum officinale, Andrographis paniculata, Isatis indigotica, Lonicera japonica*; Chen & Kusuda 1996). The intra-agar lysozyme activity was higher in the vaccinated groups, and the maximum level observed showed a 2-fold increase in the immunoadjuvant plus BF-treated groups. The herbal adjuvant *A. racemosus* enhanced lysozyme activity in tilapia fed low (0.1%) and medium (0.5%) doses of herbs (Yin et al. 2006). *A. racemosus* was also responsible for higher lysozyme activity, along with *Aeromonas hydrophila* OMP in *C. auratus* (Thanga Viji et al. 2012) and higher immune responses in *P. monodon* treated with anti-WSSV IgY, which was produced from *A. racemosus* extracts (Kumaran et al. 2010). We conclude that the herbal immunoadjuvant *A. racemosus* improved the immunoadjuvant activity

of different vaccines (WC, ECP, OMP and BF) and improved the immunological enhancement in *C. auratus*. *A. racemosus* was found to be highly promising as a safe and low-cost adjuvant in fish vaccines against *Aeromonas* sp. infection.

Acknowledgements. V.T.V. gratefully acknowledges the UGC-Networking Resource Centre in Biological Sciences (NRCBS), School of Biological Sciences, Madurai Kamaraj University, India. We dedicate this article to our late Professor, M. Peter Marian, Former Head, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, India.

LITERATURE CITED

Adams A, Auchinachie N, Bundy A, Tatner MF, Horne MT (1988) The potency of adjuvanted injected vaccines in rainbow trout (*Salmo gairdneri* Richardson) and bath vaccines in Atlantic salmon (*Salmo salar* L.) against furunculosis. Aquaculture 69:15–26

Andrews C (1990) The ornamental fish trade and fish conservation. J Fish Biol 37:53–59

Asha A, Nayaka DK, Shankara KM, Mohan CV (2004) Antigen expression in biofilm cells of *Aeromonas hydrophila* employed in oral vaccination of fish. Fish Shellfish Immunol 16:429–436

Azad IS, Shankar KM, Mohan CV (1997) Evaluation of an *Aeromonas hydrophila* biofilm for oral vaccination of carp. In: Flegel TW, Macrae IH (eds) Diseases in Asian aquaculture III. Fish Health Section, Asian Fisheries Society, Manila, p 181–186

Azad IS, Shankar KM, Mohan CV, Kalita B (1999) Biofilm vaccine of *Aeromonas hydrophila* — standardization of dose and duration for oral vaccination of carps. Fish Shellfish Immunol 9:519–528

Azad IS, Shankar KM, Mohan CV, Kalita B (2000) Uptake and processing of biofilm and free-cell vaccines of *Aeromonas hydrophila* in Indian major carps and common carp following oral vaccination — antigen localization by a monoclonal antibody. Dis Aquat Org 43: 103–108

Beaune J, Llorca G, Gonin A, Bran Y, Fleurette J, Neidhart JP (1978) Phlegmon nécrotique de la main, point de départ d'une septicémie due à *Aeromonas hydrophila.* Démonstration de l'origine ichtyologique de l'infection. Nouv Presse Med 7:1206–1207

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Chen CF, Kusuda RA (1996) Preliminary study on the adjuvant activity of henbane in oral vaccination of grass carp (*Ctenopharyngodon idellus*). J Huazhong Agric Univ 15: 157–161

Chu W (2006) Adjuvant effect of propolis on immunization by inactivated *Aeromonas hydrophila* in carp (*Carassius auratus gibelio*). Fish Shellfish Immunol 21:113–117

Cohen J, Bioterrorism ME (2001) Vaccines for biodefense: a system in distress. Science 294:498–501

Costa AB (2004) Estratégias para o estudo de bactérias potencialmente patogênicas na piscicultura. In: Cyrino JEP, Urbinati EC, Fracalossi DM, Castagnolli N (eds) Tópicos especiais em piscicultura de água doce tropical intensiva. Tec Art, São Paulo, p 387–404

Cuesta A, Rodri A, Esteban MA, Meseguer J (2005) *In vivo* effects of propolis, a honeybee product, on gilthead sea bream innate immune responses. Fish Shellfish Immunol 18:71–80

Daskalov H (2006) The importance of *Aeromonas hydrophila* in food safety. Food Contr 17:474–483

Ebanks RO, Dacancy A, Goguen M, Pinto DM, Ross NW (2004) Differential proteomic analysis of *Aeromonas hydrophila* outer membrane proteins in response to low iron and *in vivo* growth conditions. Proteomics 4: 1074–1085

Engstad RE, Robertson B (1993) Recognition of yeast wall glucan by Atlantic salmon (*Salmo salar*). Dev Comp Immunol 17:319–330

Fang HM, Ling KC, Ge R, Sin M (2000) Enhancement of protective immunity in blue gourami, *Trichogaster trichopterrus* (Pallas), against *Aeromonas hydrophila* and *Vibrio anguillarum* by *A. hydrophila* major adhesion. J Fish Dis 23:137–145

Gautam M, Diwanay S, Gairola S, Shinde Y, Patki P, Patwardhan B (2004) Immunoadjuvant potential of *Asparagus racemosus* aqueous extract in experimental system. J Ethnopharmacol 91:251–255

Harikrishnan R, Balasundaram C (2008) *In vitro* and *in vivo* studies of the use of some medicinal herbals against the pathogen *Aeromonas hydrophila* in goldfish. J Aquat Anim Health 20:165–176

Hawk PB, Oser BL, Summerson WH (1954) Practical physiological chemistry. The Blakiston Company, Philadelphia, PA

Hendricks LJ (1952) Erythrocytes counts and hemoglobin determinations for two species of sucker, genus *Catostomus*, from Colorado. Copeia 1952:265–266

Kensil CR (1996) Saponins as vaccine adjuvants. Crit Rev Ther Drug Carrier Syst 13:1–55

Khushiramani R, Girisha SK, Karunasagar I, Karunasagar I (2007) Cloning and expression of an outer membrane protein ompTS of *Aeromonas hydrophila* and study of immunogenicity in fish. Protein Expr Purif 51:303–307

Khushiramani R, Girisha SK, Bhowmick PP, Karunasagark I (2008) Prevalence of different outer membrane proteins in isolates of *Aeromonas* species. World J Microbiol Biotechnol 24:2263–2268

Kumaran T, Babu MM, Selvaraj T, Dhas SA, Citarasu T (2010) Production of anti WSSV IgY edible antibody using herbal immunoadjuvant *Asparagus racemosus* and its immunological influence against WSSV infection in *Penaeus monodon*. J Aquacult Feed Sci Nutr 2:1–5

Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685

Lowry OH, Rosenberough NJ, Farr AL, Randal RJ (1951) Protein measurement with folinphenol reagent. J Biochem 193:265–275

Maji S, Mali P, Joardar SN (2006) Immunoreactive antigens of the outer membrane protein of *Aeromonas hydrophila*, isolated from goldfish, *Carassius auratus* (Linn.). Fish Shellfish Immunol 20:462–473

Marsden MJ, Vaughan LM, Foster TJ, Secombes CJ (1996) A live (Delta aro A) *Aeromonas salmonicida* vaccine for furunculosis preferentially stimulates T-cell responses

relative to B-cell responses in rainbow trout (*Oncorhynchus mykiss*). Infect Immun 64:3863–3869

Misra CK, Das BK, Mukherjee SC, Phalguni P (2006) Effect of long term administration of dietary β-glucan on immunity, growth and survival of *Labeo rohita* fingerlings. Aquaculture 255:82–94

Nieto TP, Ellis AE (1991) Heterogeneity of extra-cellular proteases produced by different isolates of *Aeromonas hydrophila* and *A. sobria* pathogenic for fish. J Fish Dis 14:229–235

Oda K, Matsuda H, Murakami T, Katayama S, Ohgitani T, Yoshikawa M (2000) Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants. Biol Chem 381:67–74

Park KH, Jeong HD (1996) Enhanced resistance against *Edwardsiella tarda* infection in tilapia (*Oreochromis niloticus*) by administration of protein-bound polysaccharide. Aquaculture 143:135–143

Punitha SMJ, Babu MM, Sivaram V, Shankar VS and others (2008) Immunostimulating influence of herbal biomedicines on non-specific immunity in grouper *Epinephelus tauvina* juvenile against *Vibrio harveyi* infection. Aquacult Int 16:511–523

Rahman MH, Kawai K (2000) Outer membrane proteins of *Aeromonas hydrophila* induce protective immunity in goldfish. Fish Shellfish Immunol 10:379–382

Rahman MH, Kawai K, Kusuda R (1997) Virulence of starved *Aeromonas hydrophila* to cyprinid fish. Fish Pathol 32: 163–168

Rairakhwada D, Pal AK, Bhathena ZP, Sahu NP, Jha A, Mukherjee SC (2007) Dietary microbial levan enhances cellular non-specific immunity and survival of common carp (*Cyprinus carpio*) juveniles. Fish Shellfish Immunol 22:477–486

Riguera R (1997) Isolating bioactive compounds from marine organisms. J Mar Biotechnol 15:187–193

Ruszala-Mallon V, Lin YI, Durr FE, Wang BS (1988) Low molecular weight immunopotentiators. Int J Immunopharmacol 10:497–510

Sahoo PK, Mohanty J, Mukherjee SC (1999) The three imunomodulators on haemotological parameters and immunity level in rohu (*Labeo rohita*) fingerlings. J Aquacult Trop 14:127–135

Sahu S, Das BK, Pradhan J, Mohapatra BC, Mishra BK, Sarangi N (2007) Effect of *Magnifera indica* kernel as a feed additive on immunity and resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings. Fish Shellfish Immunol 23:109–118

Sivaram V, Babu MM, Citarasu T, Immanuel G, Murugadass S, Marian MP (2004) Growth and immune response of juvenile greasy groupers (*Epinephelus tauvina*) fed with herbal antibacterial active principle supplemented diets against *Vibrio harveyi* infections. Aquaculture 237:9–20

Siwicki AK, Anderson DP, Rumsey GL (1994) Dietary intake of immunostimulants by rainbow trout affects nonspecific immunity and protection against furunculosis. Vet Immunol Immunopathol 41:125–139

Sommerset I, Krossoy B, Biering E, Frost P (2005) Vaccines for fish in aquaculture. Expert Rev Vaccines 4:89–101

Sritunyalucksana K, Sithisarn P, Withayachumnarnkul B, Flegel TW (1999) Activation of prophenoloxidase, agglutinin and antibacterial activity in haemolymph of the black tiger prawn, *Penaeus monodon*, by immunostimulants. Fish Shellfish Immunol 9:21–30

Tatefuji T, Izumi N, Ohta T, Arai S, Ikeda M, Kurimoto M (1996) Isolation and identification of compounds from Brazilian propolis which enhance macrophage spreading and mobility. Biol Pharm Bull 19:966–970

Thanga Viji V, Michael Babu M, Velmurugan S, Kumaran T, Anand SB, Gunasekaran P, Citarasu T (2011) Virulence factors and molecular cloning of outer membrane protein (OMP) gene from virulent *Aeromonas hyrophila* isolated from infected gold fish *Carassius auratus.* Bangladesh J Microbiol 28:70–75

Thanga Viji V, Michaelbabu M, Anand SB, Gunasekaran P, Citarasu T (2012) Immunization with the *Aeromonas* OMP provides protection against *Aeromonas hydrophila* in goldfish (*Carassius auratus*). J Microb Biochem Technol 4:45–49

Tsai GJ, Tsai FC, Kong ZL (1997) Effects of temperature, medium composition, pH, salt and dissolved oxygen on haemolysin and cytotoxin production by *Aeromonas hydrophila* isolated from oyster. Int J Food Microbiol 38: 111–116

Van Kampen E, Zijlstra WG (1961) Standardization of hemoglobinometry 11. The hemiglobincyanide method. Clin Chim Acta 6:538–544

Van Muiswinkel WB, Anderson DP, Lamers CHJ, Egberts E, Van Loon JJA, Ijssel JP (1985) Fish immunology and fish health. In: Manning MJ, Tatner MF (eds) Fish immunology. Academic Press, London, p 1–8

Vivas J, Razquin B, López-Fierro P, Villena AJ (2005) Modulation of the immune response to an *Aeromonas hydrophila* aroA live vaccine in rainbow trout: effect of culture media on the humoral immune response and complement consumption. Fish Shellfish Immunol 18: 223–233

Vogel FR (2000) Improving vaccine performance with adjuvants. Clin Infect Dis 30(Suppl 3):S266–S270

Williams DL, Yaeger RG, Pretus HA, Browder IW, McNamee RB, Jones EL (1989) Immunization against *Trypanosoma cruzi*: adjuvant effect of glucan. Int J Immunopharmacol 11:403–410

Yin G, Jeney G, Racz T, Xu P, Jun X, Jeney Z (2006) Effect of two Chinese herbs (*Astragalus radix* and *Scutellaria radix*) on non-specific immune response of tilapia, *Oreochromis niloticus.* Aquaculture 253:39–47

Editorial responsibility: John Austin, Oldendorf/Luhe, Germany

Submitted: June 11, 2012; Accepted: January 29, 2013
Proofs received from author(s): March 31, 2013