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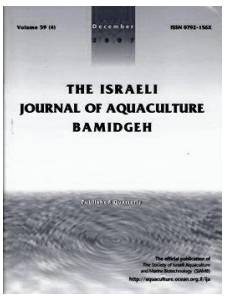

Effect of Lipopolysaccharide (LPS) and Outer Membrane Protein (OMP) Vaccines on Protection of Grass Carp (*Ctenopharyngodon idella*) against *Aeromonas hydrophila*

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Abstract

The gram-negative bacterium, *Aeromonas hydrophila*, causes high mortality and economic losses to the aquaculture industry. We investigated whether lipopolysaccharide (LPS) or outer membrane proteins (OMP) from *A. hydrophila* can enhance specific and/or non-specific immunity in grass carp (*Ctenopharyngodon idella*). Fish were injected intraperitoneally with LPS, OMP, or formalin-killed cells (FKC) from *A. hydrophila*. The control group was injected with phosphate buffered saline (PBS). All three antigens elicited strong immune responses. Respiratory burst and phagocytic activities in head kidney leukocytes and serum lysozyme activity peaked on day 21 after vaccination. Heavy chain gene transcription of immunoglobulin M and Z in the head kidney in vaccinated fish peaked on day 28. Relative percent survival was 83.3%, 72.2%, and 55.6% in the LPS, OMP, and FKC groups, respectively, but only 10% in control fish. Results suggest that LPS and OMP isolated from *A. hydrophila* can enhance specific immunity, non-specific immunity, and protection against *A. hydrophila* in fish. Thus, LPS and OMP could be important antigens for development of vaccines to control diseases caused by *A. hydrophila* in grass carp and other aquatic animals.

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Introduction

Grass carp (*Ctenopharyngodon idella*), an important aquaculture species, is commercially cultured in China for its herbivorous low protein diet and excellent growth traits. However, outbreaks of disease caused by bacteria and viruses have caused severe economic losses to the fish culture industry (Liu et al., 2010). Among grass carp pathogenic bacteria, most isolates are *Aeromonas*, universal pathogens that can infect a variety of aquatic animals (Mitchell and Plumb, 1980; Paniagua et al., 1990; Vivekanandhan and Hatha, 2002; Liu et al., 2010). Finding effective therapy is problematic because of concerns regarding harmful ecological effects associated with chemical treatments and the emergence of multiple resistance to antibiotics (Mitchell and Plumb, 1980; Vivekanandhan and Hatha, 2002).

Vaccines using whole cells and/or cell components of bacteria as immunogens can help prevent outbreaks of bacterial diseases in aquaculture (Lamers et al., 1985; Chandran et al., 2002). Lipopolysaccharide (LPS) and outer membrane proteins (OMP), components of bateria, have immunogenic properties (Hirst and Ellis, 1994; Kozinska and Guz, 2004). Common carp (*Cyprinus carpio* L.) vaccinated with crude LPS from *Aeromonas bestiarum* were better protected against disease than an unvaccinated control (Kozinska and Guz, 2004). OMP are highly immunogenic due to their exposed epitopes on the cell surface (Ebanks et al., 2005; Vazquez-Juarez et al., 2005). Vaccination with OMP from *Aeromonas veronii* induced strong antibody responses, resulting in significant protection in spotted sand bass (Vazquez-Juarez et al., 2005). Similar results were reported for immunization with major OMP from *Aeromonas hydrophila* (Swain et al., 2010) and *Aeromonas salmonicida* (Hirst and Ellis, 1994).

The purpose of this study was to investigate the effects of LPS and OMP from *A. hydrophila* on non-specific cellular and humoral parameters in grass carp and on the protective immunity of fish after experimental infection with homologous live bacteria.

Materials and Methods

Fish and bacteria. Grass carp (120-150 g) were purchased from a local fish farm in Tianjin, China. *Aeromonas hydrophila*, originally isolated from clinically diseased grass carp, were cultured in 100 ml Luria Bertani (LB) liquid medium with gentle agitation at 25°C for 24 h, then inactivated in 0.5% (v/v) formalin, and centrifuged at 10,000 *g* for 10 min. The sediment was washed three times with sterile phosphate buffered saline (PBS; pH 7.2) and suspended in sterile PBS at a final concentration of approximately 1.0×10^8 cells/ml.

Preparation of LPS. Lipopolysaccharide (LPS) was isolated according to the phenol-water procedure of Westphal and Jann (1965) with modification. In short, *A. hydrophila* were grown in LB at 25°C in an orbital shaker to a final concentration of OD_{600} = 5.0. Washed cells were re-suspended in distilled water and maintained at 68°C. An equal volume of 90% phenol, preheated to 68°C, was added with vigorous stirring. The mixture was kept for 10 min at 68°C, then cooled to about 10°C in an ice bath. The emulsion was centrifuged at 3000 rpm for 30 min. After centrifugation, the solution containing LPS was collected, dialyzed against distilled water for 48 h, and then centrifuged at 105,000 *g* for 3 h (Beckman Ultracentrifuge). The pellets were treated with DNase and RNase (50 μg/ml, 30°C, 30 min; Sigma) according to Westphal and Jann (1965), prior to proteinase K treatment (50 μg/ml, 65°C, 60 min; Sigma). The LPS was lyophilized and dissolved in distilled water to a final concentration of 150 μg/m and stored in aliquots at -80°C.

Preparation of OMP. Outer membrane proteins (OMP) were obtained from *A. hydrophila* as described by Biosca et al. (1993) and 0.55% (w/v) Sarkosyl solution (sodium lauryl sarkosynate) was used to dissolve inner membrane proteins. Pellets were resuspended in distilled water and the protein concentration, estimated by DC Protein Assay (BioRad), was adjusted to 150 μg/ml and stored at -20°C.

Vaccination. Before vaccination, 400 healthy grass carp (120-150 g) were acclimatized in a large tank with water temperature maintained at 24.5±0.5°C and fed a commercial feed every day for 10 days. After acclimation, fish were randomly divided into four groups of 100 fish each. Fish in three groups were intraperitoneally injected

with an aliquot of 0.1 ml LPS, OMP, or formalin-killed cells (FKC). Fish in the fourth group were injected with 0.1 ml sterile PBS (0.01 M, pH 7.4) as a control. Ten fish from each group were sampled at 0, 1, 2, 3, 4, and 5 weeks post-vaccination. Blood samples were collected from the carotid artery and allowed to clot at 4°C overnight. Serum was collected and stored at -0°C for analysis.

cDNA sample preparation. The head kidney was excised and immediately stored in liquid nitrogen for RNA extraction. Total RNA was isolated from head kidney samples with Trizol Reagent (Invitrogen). Genomic DNA in the RNA sample was digested with RNase-free DNase (Takara, Japan), incubated 15 min at 37°C. Two μg RNA from the head kidney were reverse-transcribed into cDNA using RevertAid^{TM} M-MuLV Reverse Transcriptase (Fermentas, Germany). cDNA samples were stored at -20°C before quantitative real-time PCR assay.

Isolation of viable leukocytes. Head kidney leukocytes were collected following the method described by Li et al. (2007). Briefly, the head kidney was excised aseptically, pressed through a 100-μm nylon mesh (BD, USA), and suspended in DMEM (Invitrigen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 U/ml heparin. The cell suspension was layered onto a 51/34% Percoll (GE Healthcare) and centrifuged at 400 *g* for 30 min. The leukocytes were collected, washed three times with DMEM (supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml heparin), and adjusted to 1×10^7 viable cells/ml.

Quantification of respiratory burst activity. Leukocyte respiratory burst activity was quantified by reduction of ferricytochrome C (Secombes, 1990). Briefly, 100 μl of the leukocyte suspension and an equal volume of cytochrome C (2 mg/l in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Cayman) at 1 μg/ml were placed in triplicate microtitre plates. To test specificity, another 100 μl leukocyte suspension and cytochrome C containing PMA and superoxide dismutase (SOD, Cayman) at 300 U/m were prepared in duplicate microtitre plates. Samples were mixed and incubated at room temperature 15 min. Extinctions were measured at 550 nm against a cytochrome C blank in a multiscan spectrophotometer. Readings were converted to nmoles O_2^- by subtracting the O.D. of the PMA/SOD treated supernatant from that treated with PMA alone for each fish, and converting O.D. to nmoles O_2^- by multiplying by 15.87. Final results were expressed as nmoles O_2^- produced per 10^5 leukocytes.

Quantification of phagocytosis activity. Phagocytic activity of head kidney leukocytes was determined spectrophotometrically by the method of Seeley et al. (1990). Yeast cells stained with Congo red were used as the phagocytosis target. To perform the assay, 1000 μl of the leukocyte suspension was mixed with 2000 μl Congo red stained and autoclaved yeast cell suspension (producing a yeast cell:leukocyte ratio of 20:1). The mixture was incubated at room temperature for 60 min, then 1 ml ice-cold HBSS was added and 1 ml histopaque (1.077) was injected into the bottom of each sample tube. The samples were centrifuged at 850 *g* for 5 min to separate the leukocytes from the free yeast cells. The leukocytes were harvested and washed twice with HBSS, then resuspended in 1 ml trypsin-EDTA solution (5.0 g/l trypsin, 2.0 g/l EDTA; Sigma) and incubated overnight at 37°C. Absorbance of the sample was measured at 510 nm using trypsin-EDTA as a blank.

Quantification of lysozyme activity. Serum lysozyme activity was measured using the turbidimetric method described by Hulmark (1980) with lyophilized *Micrococcus luteus* (Sigma) as the substrate. Briefly, 50 μl serum was added to a 3-ml suspension ($O.D._{570}$ = 0.3) of *M. luteus*, and the absorbance (A_0) at 570 nm was measured. After 30 min incubation at 37°C, the reaction was stopped by putting the mixture into an ice bath for 10 min. Absorbance (A) was measured at 570 nm and serum lysozyme activity (U_L) was calculated as $U_L = A(A_0 - A)$.

Real-time quantitative PCR examination of IgM and IgZ. Primer premier 5.0 software was used to design forward and reverse primers for specific grass carp IgM and IgZ heavy chain gene expression and β-actin gene (GenBank accession nos. ABD 76396, GA201421, and M25013, respectively). β-actin primers were used as a control for PCR to verify the cDNA templates. The primers that performed best in real-time amplifications

are listed in Table 1. Grass carp IgM and IgZ heavy chains and β-actin cDNA fragments were generated by RT-PCR. Amplicons were gel-purified, cloned into a pMD18-T vector, and transformed into *E. coli* strain DH5α competent cells. Cloned amplicons were confirmed by sequencing. Plasmid DNA was obtained using a commercial kit (Plasmid Mini Kit I, Omega). Serial dilutions of the resultant plasmid clones (tenfold dilutions from 10^{-1} to 10^{-8}) were used as a standard curve in each PCR run.

Table 1. Primers used for IgM and IgZ heavy chain gene expression analysis by real-time PCR.

Primer	*Sequence (5'–3')*
IgM-F	CGTCTACCTCCAACTCCACCAC
IgM-R	TACCGCTCTTCCACTCAGAATAAC
IgZ-F	AGCAGGAAATCCATTGTAATAAAGG
IgZ-R	ACAAAAGCATCACACTCACACAGGT
β-actin-F	CCTTCTTGGGTATGGAGTCTTG
β-actin-R	AGAGTATTTACGCTCAGGTGGG

Quantitative PCR was conducted by Chromo 4™ Continuous Fluorescence Detection from MJ Research. Amplifications were carried out at a final volume of 20 µl, containing 1 µl cDNA template, 10 µl SYBR green mix (Toyobo, Japan), 0.3 µl of each primer, and 8.4 µl ddH_2O. PCR amplification was performed in triplicate wells, using the following parameters: 3 min at 95°C, followed by 40 cycles consisting of 30 s at 94°C, 25 s at 58°C, and 25 s at 72°C. The reaction carried out without a DNA sample was used as the control. Melting curve analysis of the amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected.

Student's *t* test was performed using Microsoft Excel 2000 with $p \leq 0.05$ as the significance level. Statistical analyses were based on comparisons between the control and induction groups after IgM and IgZ heavy chain gene normalization according to the β-actin gene. Fold change was calculated as (Ts/Tn)/(Cs/Cn) where *Ts* equals the treated sample assayed for the specific gene, *Tn* equals the treated sample assayed for the normalized β-actin gene, and *Cs* and *Cn* equal the specific and normalizing genes of the calibrator PBS-injected group (Purcell et al., 2004).

Bacteria challenge. Five weeks after vaccination, 20 fish in each treatment were intraperitoneally injected with 0.1 ml of an *A. hydrophila* cell suspension prepared as follows: *A. hydrophila* was grown overnight in LB broth at 25°C, harvested by centrifugation at 850 *g* for 15 min, and washed with PBS three times. The bacteria were suspended in PBS (0.01 M, pH 7.4), adjusted to a concentration of 1.0×10^8 cfu/ml. Cumulative mortality and clinical signs were recorded daily for two weeks post-challenge and the cause of death was confirmed by re-isolating the bacteria from the liver of dead fish by conventional methods. Relative percent survival (RPS) was calculated as: RPS = (1 - %mortality in vaccinated group/%mortality in control group) × 100 (Amend, 1981).

Statistical analysis. Results are presented as average±standard error for ten fish and were compared at each time point using one-way analysis of variance (ANOVA). When overall differences were significant ($p<0.05$), Tukey's test was used to compare treatment means. Final mortality of each treated group was statistically compared with the control group using Chi-square test. Statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

Results

Respiratory burst, phagocytic, and lysozyme activity. Respiratory burst, phagocytic, and lysozyome activity in vaccinated fish was significantly higher than in the control from the second to the fourth week post-injection, then reverted to the control level in the fifth week (Fig. 1).

IgM and IgZ expression changes. The mRNA expression of IgM increased from the first week to the fourth in all fish injected with inactivated LPS, OMP, or FKC (Fig. 2). The tendency in IgZ was similar, but the increase was of a lower order of magnitude and there was no significant up-regulation at week 5, when compared to the control.

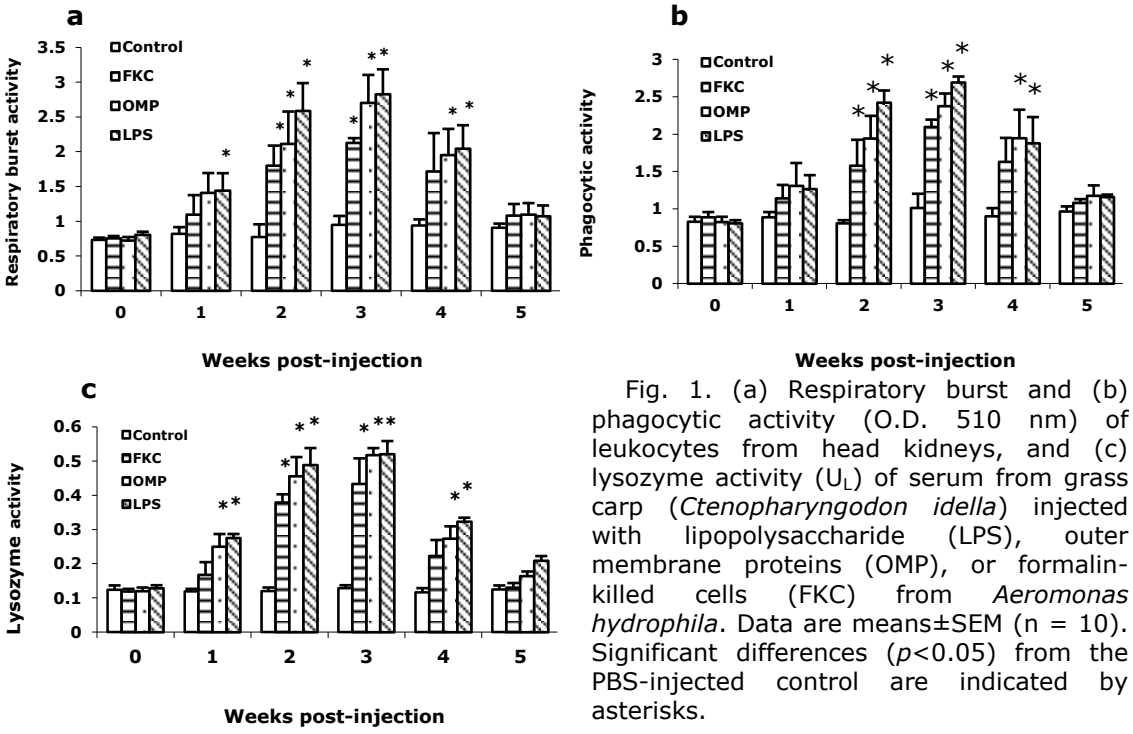

Fig. 1. (a) Respiratory burst and (b) phagocytic activity (O.D. 510 nm) of leukocytes from head kidneys, and (c) lysozyme activity (U_L) of serum from grass carp (*Ctenopharyngodon idella*) injected with lipopolysaccharide (LPS), outer membrane proteins (OMP), or formalin-killed cells (FKC) from *Aeromonas hydrophila*. Data are means±SEM (n = 10). Significant differences (p<0.05) from the PBS-injected control are indicated by asterisks.

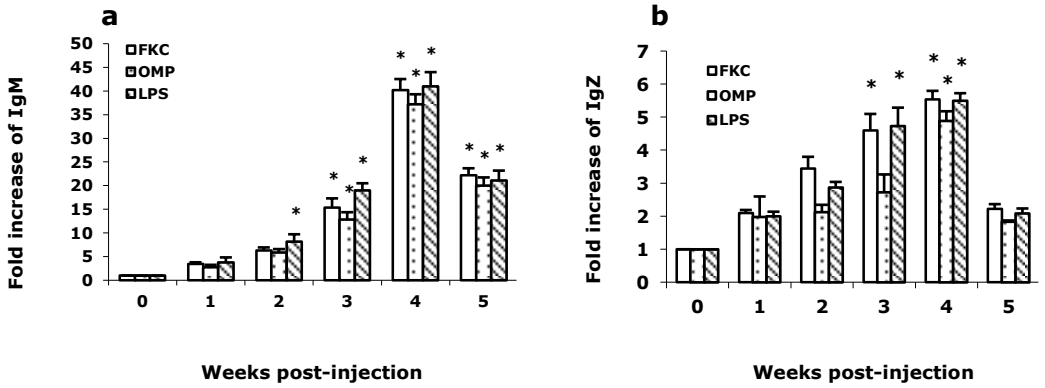

Fig. 2. Quantitative real-time PCR of grass carp (a) IgM and (b) IgZ heavy chain gene expression after stimulation with lipopolysaccharide (LPS), outer membrane proteins (OMP), or formalin-killed cells (FKC) of *Aeromonas hydrophila*, compared to a PBS-injected control. Melting curve analyses were performed and single specific melting peaks were observed, including amplification specificity (data not shown). Data are means±SEM (n = 10). Significant differences (p<0.05) from the control are indicated by asterisks.

Challenge test with A. hydrophila. Fish were challenged with *A. hydrophila* five weeks after immunization, and cumulative mortality was registered for the following two weeks (Fig. 3). Mortality occurred 24 h after challenge. Relative percent survival was high in the LPS and OMP groups, 83.3% and 72.2%, respectively, and lower in the FKC group (55.6%). In the PBS-injected control groups, 90% of the fish died after challenge with live bacteria. Symptoms of congestion and ulcers were observed in all diseased fish, and *A. hydrophila* was isolated from the liver of dying individuals.

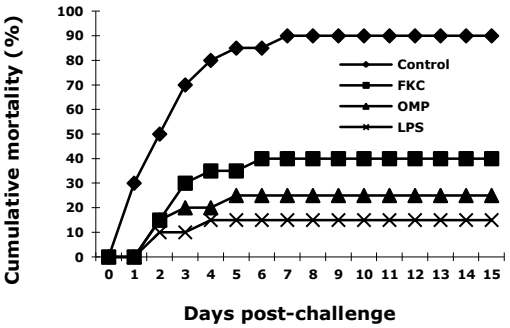

Fig. 3. Cumulative mortality (%) of vaccinated grass carp after artificial infection with *Aeromonas hydrophila*.

Discussion

Enhancement of the immune system seems to be the most promising method of preventing diseases in fish as the nonspecific immune system is the first line of defense against invading pathogens, and is more important for low aquatic animals than for mammals (Harikrishnan et al., 2009). In this study, the immunostimulating effects of LPS and OMP were seen in the significantly enhanced respiratory burst, phagocytic, and lysozyme activity, and IgM and IgZ in grass carp vaccinated with LPS or OMP from *A. hydrophila*.

Respiratory burst, phagocytic, and lysozyme activity are responsible for early activation of the inflammatory response before antibody production and play an important role in the innate defense mechanism of fish (Harikrishnan et al., 2009; Nayak, 2010). Innate immune responses of fish increase significantly after vaccination, achieving high resistance to bacterial infection (Harikrishnan et al., 2009; Swain et al., 2010). In this study, LPS and OMP enhanced respiratory burst, phagocytic, and lysozyme activity in grass carp. Together with the relative percent survival of the vaccinated groups after challenge, these results suggest that LPS and OMP can induce beneficial effects in the host, such as non-specific activation of macrophages and enhanced disease protection. Likewise, in earlier studies, fish treated with immunostimulants had increased phagocytic, respiratory burst, and lysozyme activity (Yin et al., 2009; Wu et al., 2010).

There is considerable information on the development of specific antibody responses of fish vaccinated against bacterial disease, and significant protection correlates with high levels of antibodies (Xu et al., 2009; Ardo et al., 2010). Although there are differences among fish with different genetic backgrounds, the levels of specific antibodies obtained in fish after vaccination usually correlates well with survival following challenge (Xu et al., 2009; Ardo et al., 2010). In our study, quantitative real-time PCR showed that IgM and IgZ heavy chain gene expression was significantly induced following injection of LPS, OMP, or FKC. The up-regulation of both IgM and IgZ heavy chain gene transcripts was dramatic in the LPS group and remained high throughout the experiment. Together with relative percent survival, this suggests that humoral immunity is important in protecting grass carp against *A*. *hydrophila*.

In the present study, grass carp immunized with LPS, OMP, or FKC of *A*. *hydrophila* had higher immune response levels and survival rates than the PBS-injected control, suggesting that they can boost the level of phagocytic and humoral immune responses, enhancing protection against disease. The high survival obtained in fish vaccinated with LPS and OMP suggests that these can be considered for development of vaccines against *A*. *hydrophila* in grass carp and other aquatic animals. Further studies should be performed to distinguish mechanisms in immune responses after stimulation by LPS and OMP of *A*. *hydrophila*.

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