Fish and Shellfish Immunology 86 (2019) 680–687

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Immunostimulation of *Cyprinus carpio* using phage lysate of *Aeromonas hydrophila*

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A R T I C L E I N F O

Keywords:
Inactivated antigen
Phage lysate
Aeromonas hydrophila
Poly $_{D,L}$ lactide-co-glycolic acid (PLGA)
Cyprinus carpio

A B S T R A C T

Over the last 50 years, various approaches have been established for the development of antigens for immunostimulation. We used phage lysate (PL), composed of inactivated antigens by the lytic bacteriophage pAh 6-c for *Aeromonas hydrophila* JUNAH strain to develop a vaccine for the prevention of *A. hydrophila infection* in *Cyprinus carpio* (common carp). We also assessed the poly $_{D,L}$ lactide-co-glycolic acid (PLGA) microparticles encapsulation method to increase the efficiency of the vaccine. Six groups of vaccines involving encapsulated by PLGA, formalin killed cells, or phage lysate at low or high concentration were prepared for intraperitoneal injection in *C. carpio*. Blood specimens and head kidney samples were collected at various time points for bacterial agglutination assay and to assess relative expression of immune-related genes interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), lysozyme C, and serum amyloid A (SAA). The vaccine groups using high dose phage lysate antigen showed significantly higher agglutination titers than all other groups at 4- and 6-weeks post vaccination (wpv), with the titer of the PLGA encapsulated vaccine group being highest from 10 wpv to the end of the experiment. The survival rate of fish immunized with the phage lysate vaccines were higher than that of fish immunized with the formailin killed cells vaccine in the challenge experiment conducted 6 wpv. Additionally, the PLGA-encapsulated high dose phage lysate antigen vaccinated groups showed the best protective efficacy in the challenge experiment 12 wpv. Vaccines using the phage lysate antigen also showed higher IL-1β and lysozyme C gene expression at 7 days post vaccination (dpv) and 2 wpv, and higher TNF-α gene expression was seen at 7 dpv. Higher SAA gene expression was seen in these groups at 1 dpv. These results suggest that phage lysate antigen has the potential to induce robust immune responses than formalin killed cells-based vaccines, and could be more effective as a novel inactivated antigen in preventing *A. hydrophila* infection in *C. carpio*.

1. Introduction

Aeromonas hydrophila is a gram-negative, rod-shaped bacterium that is widespread in freshwater habitats [1]. It is the causative agent of one of the major diseases in common carp (*Cyprinus carpio*) that leads to significant economic losses to aquaculture industry worldwide [2]. *Aeromonas hydrophila* can cause motile *Aeromonas* septicemia, which is characterized by symptoms such as hemorrhagic septicemia, infectious abnormal dropsy, exophthalmia, and fin and tail rot [3].

Various antigens of *A. hydrophila* have been developed using different approaches over the last 50 years. There is considerable research in developing genetically modified and naturally attenuated vaccines, DNA vaccines, and subunit vaccines in the field of aquaculture [4–8]. However, these approaches to produce vaccines are expensive and for economic reasons, vaccines using inactivated antigen form the mainstream in the fish industry. The inactivated antigen vaccines have

Abbreviations: colony forming unit, (CFU); days post vaccination, (DPV); formalin-killed whole-cell, (FKC); median lethal dose, (LD_{50}); micropaticles, (MPs); phosphate buffered saline, (PBS); phage lysate, (PL); poly $_{D,L}$ lactide-co-glycolic acid, (PLGA); poly(vinyl alcohol), (PVA); quantitative polymerase chain reaction, (qPCR); tryptic soy agar, (TSA); water-in-oil-in-water, (W/O/W); weeks post-vaccination, (wpv)

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https://doi.org/10.1016/j.fsi.2018.11.076
Received 25 October 2018; Received in revised form 26 November 2018; Accepted 30 November 2018
Available online 01 December 2018
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several drawbacks such as poor safety, short shelf life, weak immunogenicity, short protection duration, and uncertain immune response [9].

The inactivation of bacteria using lytic bacteriophage and its application as antigen has not been fully investigated until now. Bacterial lysate produced by lytic phage can be considered a type of antigen isolation. Because epitopes of antigen are not denatured in this method, the phage lysate can include an intact antigen without any alteration. Thus, phage lysates can induce both cellular and humoral immune response [10]. The phage lysate is composed of two components, bacteriophage particles and bacterial antigenic content. Therefore, therapeutic protection by phage particles and protective efficacy by increasing immune response mediated by antibodies or related cells can be expected. This antigen is safe for administration as all bacteria are killed by the phage and the fluid with antigen is filtered with 0.45 μm pore size membrane filter to remove intact bacteria.

Poly $_{D,L}$ lactide-co-glycolic acid (PLGA) has been previously used for controlled drug release and antigen encapsulation for vaccine administration [11,12]. The safety of PLGA was approved by the US Food and Drug Administration and has attracted attention because of its biocompatibility, biodegradability, and high stability in biological fluids and during storage [13,14]. Furthermore, entrapment in polymers can prolong drug release and enhance the therapeutic efficacy of vaccine [15,16]. The size of PLGA particles can be adjusted by controlling parameters such as molecular weight of polymer and the ratio of lactide and glycolide [17,18]. In addition, PLGA encapsulation is cheaper than other vaccine production methods, and could be easily applied in the aquatic industry.

In this study, an inactivated vaccine candidate for *A. hydrophila* was prepared using the lytic bacteriophage pAh 6-c, which was previously isolated in our laboratory [19]. PLGA microparticles (MPs) water-in-oil-in-water (W/O/W) encapsulation method [20], was used to increase the efficiency of the vaccine. The protective efficacy of the PLGA MP-encapsulated whole-cell antigen and phage lysate vaccines were evaluated in common carp model against a direct challenge with virulent *A. hydrophila* JUNAH strain. The immunogenicity of the vaccines was assessed by agglutination test and mRNA expression analysis of the related immune genes.

2. Materials and methods

2.1. Ethics statement

All the experimental protocols were performed in accordance with the Guidelines on the Regulation of Scientific Experiments on Animals, issued by Seoul National University Institutional Animal Care and Use Committee (SNU, Republic of Korea). Anesthetizing procedure for sampling of blood and organs and euthanization of the fish were performed using tricaine methanesulfonate (MS-222).

2.2. Polymers and fish

PLGA (P1941, MW 66,000–107,000) and polyvinyl alcohol (PVA; 341584, average MW 89,000–98,000) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). A total 410 common carp (mean body weight ± SD: 10.38 ± 1.29 g) were provided by the Aquaculture Department of Kunsan University in Jeollabuk province, South Korea. The fish were acclimatized in the laboratory of the College of Veterinary Medicine of Seoul National University, Seoul, South Korea, for 20 days before commencing the experiment. They were kept in 100-L fiberglass tanks at 25 ± 2 °C and fed once a day with commercial feed (Tetra Bits Complete, Tetra). Approximately 20% of the water in each tank was changed daily.

2.3. Antigen preparation

2.3.1. Bacterial strain and bacteriophage

The *A. hydrophila* JUNAH strain, isolated from a cyprinid loach in 2009 in South Korea and stored in lyophilized condition in our laboratory, was used for this study [21]. For experiments, bacteria were cultured on tryptic soy agar (TSA; Difco, Detroit, MI, USA) at 25 °C for 24 h. A phage (pAh 6-c; Seoul National University, Seoul, Republic of Korea), which showed consistent lytic activity against *A. hydrophila* JUNAH was also stored in our laboratory. It was isolated from natural water of the Han River in May 2010 by the enrichment technique [19].

2.3.2. Preparation of formalin killed cells (FKCs)

A single colony of *A. hydrophila* JUNAH was cultured in tryptic soy broth (TSB; Difco, Detroit, MI, USA) at 25 °C for 24 h. The cultured bacteria were treated with 0.5% formalin (v/v) and maintained at 25 °C for 48 h, before being centrifuged at 10,000 × *g* for 10 min, washed twice in sterile phosphate-buffered saline (PBS), and resuspended in sterile PBS.

2.3.3. Generation of phage lysate (PL)

A. hydrophila JUNAH strain was incubated in TSB for 24 h at 25 °C with gentle agitation. The bacteria were washed twice with PBS and bacterial cell number was adjusted to 2×10^8 and 5×10^8 CFU/mL. The optimum multiplicity of infection (MOI = 0.1) of the phage, which was identified previously [19], was added. After inactivation of the bacteria, the whole fluid was filtered through a 0.45 μm pore size membrane filter and lyophilized for 48 h to remove all water. Powdered phage lysate was stored at 4 °C until use.

2.3.4. Production of PLGA encapsulated vaccine

PLGA MPs encapsulating phage lysate or FKCs were prepared with PLGA copolymer using a water-in-oil-in-water (W/O/W) double emulsion solvent evaporation technique, as previously described [10,20]. Phage lysate or FKCs were suspended in 500 μL PBS (pH 7.4), and 210 mg PLGA was dissolved in 3 mL dichloromethane. These solutions were subsequently combined and emulsified in a homogenizer (HG-15D; DAIHAN Scientific, South Korea) at 12,000 rpm for 1 min at room temperature to form the primary W/O emulsion. This was then poured into 50 mL of 4% PVA solution and homogenized at 6000 rpm for 1 min. After 2 min, an additional 50 mL of deionized water was added slowly to the suspension over the course of 30 min. The emulsion was stirred at 300 rpm for an additional 8 h to allow the organic solvent to evaporate. The resultant MPs were washed twice with PBS (pH 7.4) and centrifuged at 5000 g for 10 min. The recovered MPs were lyophilized for 48 h to preserve them for further use.

2.4. In vivo experiments

2.4.1. Vaccination

The 1300 carp were randomly divided into seven experimental groups (Table 1). Fish in the experimental groups were immunized with 0.1 mL PL, FKC, PLGA-PL or PLGA-FKC vaccine, using intraperitoneal injection. The total antigen content of the experimental groups in 0.1 mL vaccine is listed in Table 1. Control fish were injected intraperitoneally with 0.1 mL sterile PBS.

2.4.2. Challenge experiment

All the groups (n = 30) were challenged with *A. hydrophila* JUNAH stain at 6- and 12-weeks post vaccination (wpv) with the median lethal dose (LD_{50}). LD50 was calculated using the method described by Reed & Muench, 1938 and found to be 8×10^6 CFU/fish [22]. The challenge experiment was repeated three times. Fish were anesthetized using MS-222 (100 ppm) before the challenge experiment. Fish administered with PBS only were used as controls.

Table 1
Experimental groups and quantity of the antigen used in the study.

Experimental groups	Vaccine formulation	Antigen dose (CFU/fish)
PLl	Low dose of phage lysate	2×10^8
PLh	High dose of phage lysate	5×10^8
FKC	Formalin killed cells	2×10^8
PLGA-PLl	Low dose of phage lysate encapsulated with PLGA	2×10^8
PLGA-PLh	High dose of phage lysate encapsulated with PLGA	5×10^8
PLGA-FKC	Formalin killed cells encapsulated with PLGA	2×10^8

Each group included 177 fish. Challenge experiments were performed in triplicate, and agglutination titer and qPCR analysis were performed once.

2.4.3. Clinical signs and survival analysis

After challenge experiment, clinical signs and cumulative mortalities were monitored twice a day for 2 weeks. The internal organs of dead fish were streaked onto TSA medium and incubated at 25 °C for 24 h. To confirm bacterial identify, PCR was performed on isolates as previously described [23]. Kaplan Meier survival curves were used to compare survival rates in the vaccinated groups [24].

2.4.4. Sample collection

Blood specimen (100 µL) and head kidney samples were collected from three randomly chosen fish in each group (PLl, PLh, FKC, PLGA-PLl, PLGA-PLh, and control) following anesthetization with MS-222 (100 ppm). Collection of head kidney samples was performed on 1, 3- and 7-days post vaccination (dpv), and 2, 4, 6, 8 and 10 wpv. Blood sampling was performed on 2, 4, 6, 8, 10, 12 and 14 wpv. The blood samples were transferred to microcentrifuge tubes (Eppendorf, Hamburg, Germany), and serum was collected after centrifugation at 6500 g for 10 min at 4 °C, before being stored at −20 °C until use.

2.5. Immune response assessment

2.5.1. Serum agglutination assay

The experiment was performed in microtiter plates with U-shaped wells. Serum samples were serially two-fold diluted in PBS and homologous heat-killed *A. hydrophila* (10^7 cells/mL) was added. Serum agglutination was determined by visual observation, and the endpoint titer was defined as the reciprocal of the highest dilution. This test was performed once.

2.5.2. RNA extraction and reverse transcription

Total RNA was extracted from head kidneys using TRIzol Reagent (CWBio, Beijing, China). RNA concentration and purity were assessed spectrophotometrically, which showed 260:280 ratios between 1.6 and 1.8. RNA quality was checked by electrophoresis on 1% agarose gels supplemented with 0.5 µg/mL ethidium bromide. Total RNA samples were treated with DNAse I (Promega, Madison, WI, USA) according to manufacturer's instructions to eliminate DNA contamination. PrimeScript RT Reagent Kit (TaKaRa Bio, Otsu, Japan) was used to synthesize cDNA from extracted RNA. The resulting cDNA was stored at −80 °C until use.

2.5.3. Quantitative PCR (qPCR) analysis

The gene expression of interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), lysozyme C, serum amyloid A (SAA), and housekeeping gene β-actin were analyzed with a Rotor-Gene Q instrument (QIAGEN, Hilden, Germany) following standard protocols and using the primers listed in Table 2.

The following cycling conditions were used, 95 °C for 10 min, then 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. To correct for cDNA loading variation, target gene expression was normalized to that of the housekeeping gene β-actin for all samples. To verify reaction specificity, melting curve analysis was carried out for each amplicon. Expression was analyzed using the $2^{-\Delta\Delta Ct}$ method after verifying that amplification efficiency was approximately 100%. Data for all vaccinated groups were compared with those obtained with the control samples. Each sample was processed in triplicate.

Table 2
Primers used for amplification of specific transcripts by quantitative PCR in the study.

Target		Sequence (5′–3′)	Product size (bp)	GenBank accession number
IL-1β	F*	AAGGAGGCCAGTGGCTCTGT	69	AB010701
	R*	CCTGAAGAAGAGGAGGCTGTCA		
TNF-α	F	GCTGTCTGCTTCACGCTCAA	106	AJ311800
	R	CCTTGGAAGTGACATTTGCTTTT		
Lysozyme C	F	GTGTCTGATGTGGCTGTGCT	359	AB027305
	R	TTCCCCAGGTATCCCATGAT		
SAA	F	GCAGATGGGCAGCCAAAGTA	181	AB016524
	R	GAATTACCGCGGCGAGAGA		
β-actin	F	GCTATGTGGCTCTTGACTTCGA	89	M24113
	R	CCGTCAGGCAGCTCATAGCT		

*F, forward; R, reverse.

2.6. Statistical analyses

All data were analyzed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). A one-way analysis of variance (ANOVA) was used to analyze the data, followed by Duncan's multiple range test, to compare variations in immune parameters for differences at a significance level of 0.05. The mean ± standard error of the mean of assayed parameters was calculated for each group.

3. Results

3.1. Adaptive immune responses

Antibody titers of all the vaccinated groups were increased, peaked at 4 wpv, and gradually reduced until the end of the experiment. The titers in vaccine groups using high dose phage lysate antigen (PLh and PLGA-PLh) were higher than other groups at 4 and 6 wpv. The titers of the PLGA encapsulated vaccine groups were higher than the FKC or PL-only vaccine groups from 10 wpv. There were no differences in titers among PLGA-FKC, PLGA-PLl, and PLGA-PLh at 8 wpv, but the titer of the PLGA-PLh vaccine group was higher than all other groups from 10 wpv to the end of the experiment. Agglutination titers in the control groups remained at zero throughout (Fig. 1). Serum titers indicated no detectable antibodies prior to vaccination in all groups.

3.2. Survival analysis

Mortality after challenge in all vaccination groups was lower than that in the control group at both 6 and 12 wpv. In all groups, mortality began 12 h post-infection and continued up to 72 h after challenge. After 72 h, challenged fish survived for the rest of the experimental period and showed no symptoms. The survival rate of fish immunized with PLh (66.7%), PLGA-PLl (73.3%), and PLGA-PLh (70%) vaccines were higher than that of fish immunized with FKC vaccine (50%) for 6 wpv challenge experiment. However, there were no significant differences between the PLh, PLGA-PLl and PLGA-PLh groups. The difference in survival rate increased further with the FKC group for 12 wpv challenge experiment. All PLGA encapsulated groups showed a higher survival rate compared to the other groups. The survival rate of fish immunized with PLGA-PLh (63.3%) vaccinated group showed the highest protective efficacy (Fig. 2). All dead fish exhibited typical

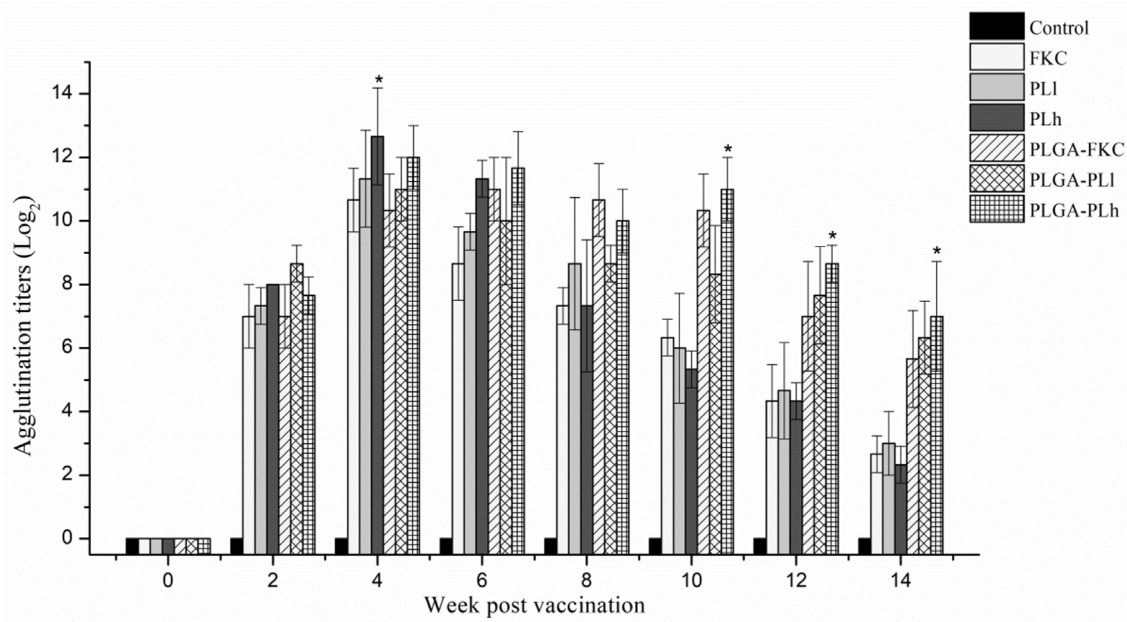

Fig. 1. Serum agglutination titers of *C. carpio* intraperitoneally administered formalin killed cells of *A. hydrophila* JUNAH strain (FKC) and its PLGA encapsulated microparticles (PLGA-FKC), low (PLl) and high (PLh) dose lysate of *A. hydrophila* JUNAH strain infected with pAh 6-c phage and its PLGA encapsulated microparticles (PLGA-PLl and PLGA-PLh), or phosphate buffered saline (Control). Bars represent mean ± standard error of the mean (n = 3). * $P < 0.05$.

clinical signs of *A. hydrophila* infection. Bacteria were isolated from these fish on TSA plates to confirm that the isolate was *A. hydrophila* using PCR method (data not shown).

3.3. Immune gene expression

The PL, FKC, PLGA-PL, and PLGA-FKC vaccines affected the relative mRNA expression of immune-related genes in the head kidney of common carp in different ways. The vaccine groups (PLl, PLh, PLGA-PLl, PLGA-PLh, and PLGA-FKC) showed significantly higher IL-1β expression at 1 and 3 dpv, with higher expression in PLl and PLh vaccine groups compared to the other groups. All PLGA encapsulated groups (PLGA-FKC, PLGA-PLl, and PLGA-PLh) showed higher expression of IL-1β at 2 wpv, with the vaccines using PL antigen encapsulated by PLGA (PLGA-PLl and PLGA-PLh) showing higher expression than the FKC antigen group. The vaccine groups (PLl, PLh, PLGA-PLl, PLGA-PLh, PLGA-FKC) also showed significantly higher TNF-α expression at 1 dpv, and the vaccines using PL antigen (PLl, PLh, PLGA-PLl and PLGA-PLh) showed 2–6-fold higher expression than FKC groups (FKC and PLGA-FKC) at 7 dpv (Fig. 3). The non-PLGA encapsulated vaccine groups showed approximately 2-fold higher expression of lysozyme C than the PLGA encapsulated vaccine groups at 7 dpv, but the PLGA encapsulated vaccine groups showed higher expression than the other groups at 2 wpv. PLl and PLh vaccine groups showed higher expression of SAA than

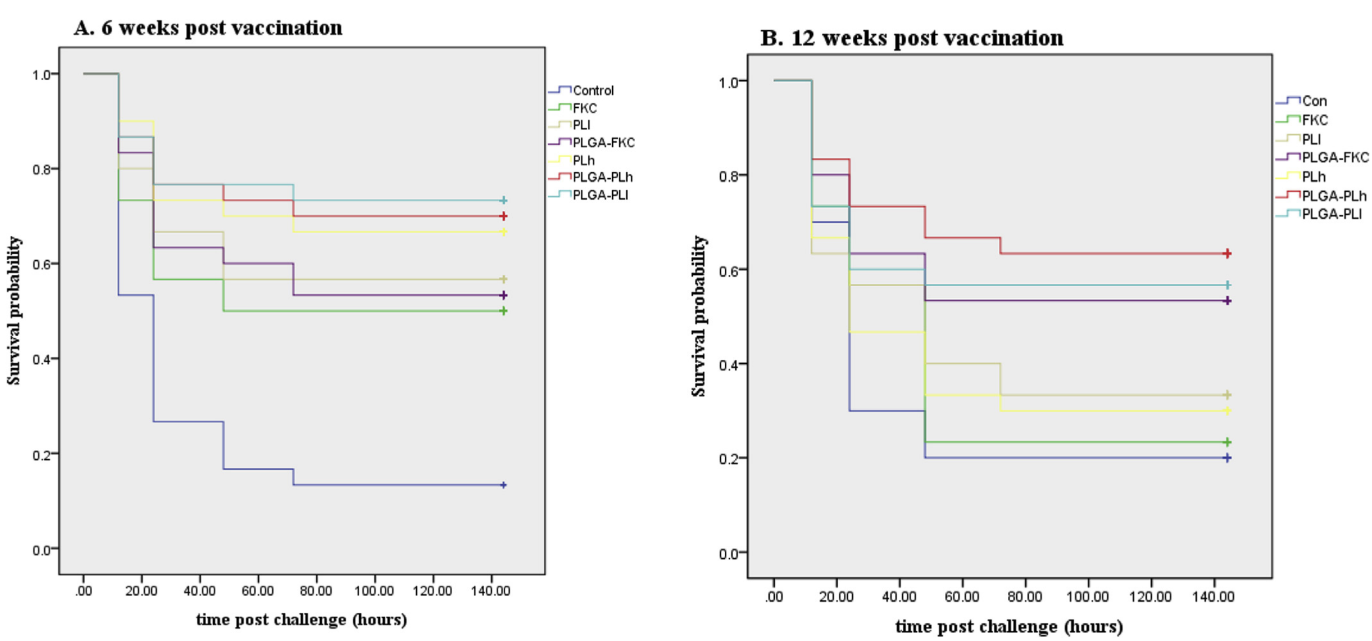

Fig. 2. Kaplan-Meier survival curve of challenge experiment on *C. carpio*. The survival differences among vaccinated groups (FKC, PLl, PLh, PLGA-FKC, PLGA-PLl, PLGA-PLh) and control are illustrated for n = 30 at 6 weeks post vaccination (A) and 12 weeks post vaccination (B).

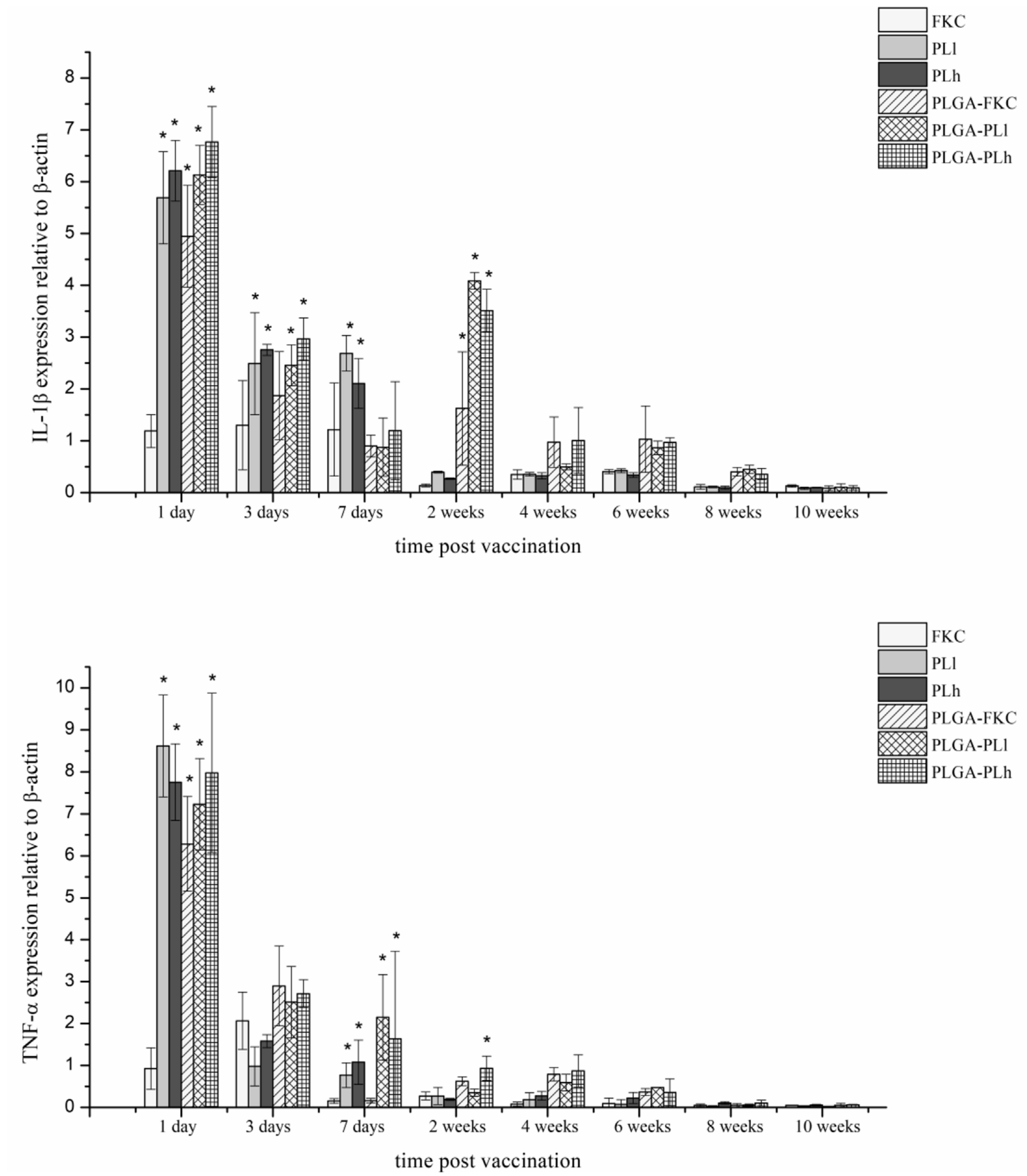

Fig. 3. Relative mRNA expression of pro-inflammatory factors IL-1β and TNF-α in the head kidneys of *C. carpio* intraperitoneally administered formalin killed cells of *A. hydrophila* JUNAH strain (FKC) and its PLGA encapsulated microparticles (PLGA-FKC), low (PLl) and high (PLh) dose of *A. hydrophila* JUNAH strain lysate infected with pAh 6-c phage and its PLGA encapsulated microparticles (PLGA-PLl and PLGA-PLh), or phosphate buffered saline (Control). Bars represent mean ± standard error of the mean (n = 3). * $P < 0.05$.

the other groups (Fig. 4).

4. Discussion

Most vaccines against bacterial fish diseases are based on inactivated bacteria, as it can be applied inexpensively, and are generally recognized to induce strong immunity [25,26]. In this study, a novel inactivated phage lysate vaccine candidate against *A. hydrophila* was developed. Few studies have applied this method of using phage lysate against bacterial diseases. Phage lysate is expected to contain a variety of intact antigens, as the phage decomposes bacteria in a specific and gentle manner [27–29]. PLGA encapsulation method for production of vaccines was also applied in this study. PLGA are biodegradable particles readily taken up by antigen-presenting cells and facilitate activation of the immune system [30]. In previous studies, the PLGA encapsulated vaccine showed longer and more effective performance compared to the FKC antigen-only vaccine [20]. Here, we demonstrate that an inactivated vaccine using phage lysate antigen of the *A. hydrophila* JUNAH strain, can provide highly efficient protection against *A. hydrophila* infection. We also demonstrate the possibility of applying the PLGA encapsulation method to these antigens.

We used the same number of bacteria for FKC and PL vaccine preparation in this study. Previous PLGA vaccine studies used FKC as an antigen, and it was difficult to encapsulate large quantities of antigen into PLGA particles. However, with the phage lysate it was easier to encapsulate of high concentrations of antigen. Therefore, the experiments were conducted using two antigen concentrations of the phage lysate.

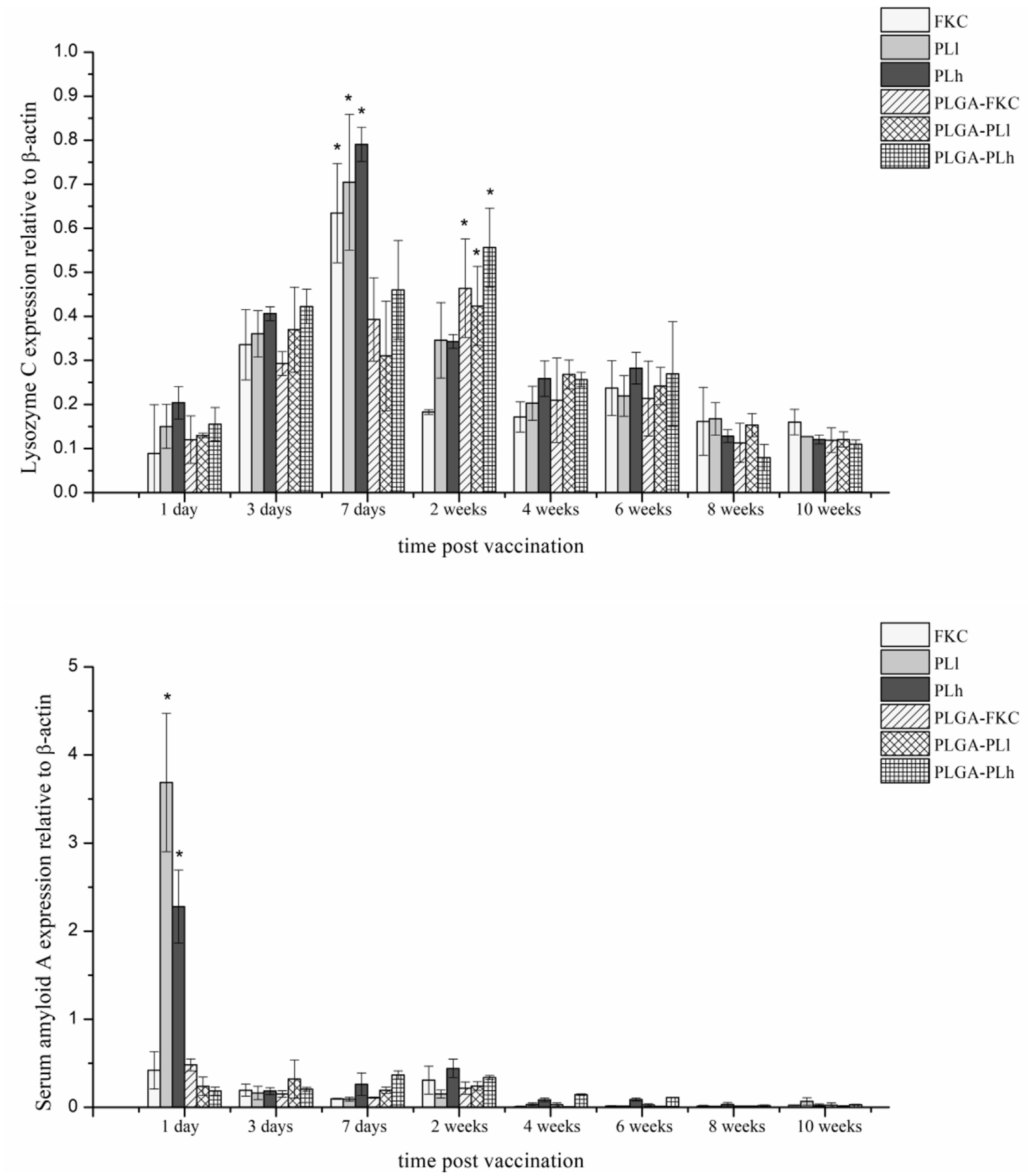

Fig. 4. Relative expression of lysozyme C and serum amyloid A (SAA) transcripts in the head kidneys of *C. carpio* intraperitoneally administered formalin killed cells of *A. hydrophila* JUNAH strain (FKC) and its PLGA encapsulated microparticles (PLGA-FKC), low (PLl) and high (PLh) dose of *Aeromonas hydrophila* JUNAH strain lysate infected with pAh 6-c phage and its PLGA encapsulated microparticles (PLGA-PLl and PLGA-PLh), or phosphate buffered saline (Control). Bars represent mean ± standard error of the mean (n = 3). * $P < 0.05$.

To reduce risks of the vaccine using phage lysate antigen, we performed two safety measures. First, to eliminate the possibility of unlysed intact bacteria from causing disease, we filtered the phage lysate using 0.45 μm pore size membrane filter. Second, there is a risk of the presence of exotoxins in the phage lysate. Despite the bacterial washing steps, there is the potential to produce exotoxin by bacteria during the lysis process. *A. hydrophila* has been reported to produce exotoxins, potential virulence factors such as cytolysin, hemolysin (aerolysin), cytotoxic enterotoxin, and a cholera toxin-like factor [31–35]. Therefore, an experiment was conducted to assess mortality by exotoxin in the phage lysate. However, the intraperitoneal administration of the highest concentration of phage lysate used in this study did not result in mortality of the fish (data not shown), suggesting the absence of exotoxin in our antigen preparation. The toxin of bacteria is a factor that inhibits stability in the development of vaccines. Future research will require studying methods of reducing or eliminating bacterial toxins in phage lysate vaccines.

The humoral and cell-mediated immune response elicited by vaccine candidates play an important role in protection. In the bacterial agglutination test, in the early stage of 4 wpv, the PLh group had a better immune response than the other groups. However, at 8 wpv, the PLGA encapsulated vaccine groups showed a higher immune response than the other groups, while 10 wpv the PLGA-PLh group had the highest titer until the end of the experiment. Overall, the group vaccinated with PLGA-PLh showed the highest and long-term immune response over time. The challenge experiment was performed 6 and 12 wpv and assessed using Kaplan-Meier survival analysis. At 6th week of challenge, PLh, PLGA-PLl, and PLGA-PLh groups showed higher survival rates than the other vaccine groups. There were little differences among these groups, but PLGA-PLh group showed the most effective protection from *A. hydrophila* infection, suggesting that the phage lysate possessed highly conserved cross-reactive antigens and the PLGA vaccine resulted in a more effective vaccine.

Teleost fish have a complex immune system, comprising of innate (lysozymes, the complement system, immunocytes, and cytokines) and adaptive (antibody production and lymphocyte activity) immunity [36]. IL-1β and TNF-α are pro-inflammatory cytokines, mainly investigated in fish. Cytokines are modulators of immune response related to both innate and adaptive immune systems [37]. IL-1β stimulates immune responses by activating lymphocytes or inducing the release of other cytokines that subsequently activate macrophages, natural killer cells, and lymphocytes [38]. TNF-α induces the inflammatory response by regulating the expression of other cytokines, including IL-1β [39,40]. In the present study, relative transcript levels of IL-1β were significantly upregulated in all vaccine groups (PLl, PLh, PLGA-FKC, PLGA-PLl, and PLGA-PLh) at 1 and 3 dpv. Only PL vaccine groups (PLl and PLh) were significantly upregulated at 7 dpv, but PLGA vaccine groups (PLGA-FKC, PLGA-PLl, and PLGA-PLh) were higher than other vaccine groups at 2 wpv. Relative transcript levels of TNF-α were similar to IL-1. All vaccine groups (PLl, PLh, PLGA-FKC, PLGA-PLl, and PLGA-PLh) showed higher expression at 1 dpv. The PL vaccine groups (PLl, PLh, PLGA-PLl, and PLGA-PLh) showed higher expression at 2 wpv. At the beginning of the experiment, the PL and PLGA groups had higher gene expression than the FKC group, and in the second week, PLGA vaccine groups had higher gene expression. However, vaccine groups using PL antigen showed higher expression in the PLGA vaccine groups. Thus, the PL antigens caused a stronger immune response than the existing FKC vaccine, and PLGA encapsulation further improved the efficacy of the PL antigen. Lysozymes are crucial molecules in innate immune defense in fish, preventing infection from exogenous pathogens [41]. FKC, PLl, and PLh vaccinated groups significantly upregulated transcripts of lysozyme C at 7 dpv, but PLGA vaccine groups upregulated the gene expression at 2 wpv. SAA belongs to a highly conserved group of apolipoproteins, and it plays an important role in the early phase of the innate immune response in counteracting infection and taking part in inflammatory regulation [42]. Relative mRNA expression of SAA was significantly upregulated in PLl and PLh vaccine groups at 1 dpv. These results suggest that the PL antigen could induce stronger immune response than FKC vaccine in providing protection against *A. hydrophila* infection.

In this study, the vaccine using PL antigen and PLGA encapsulation were evaluated for their efficacy as antigen delivery systems for fish vaccination. A vaccine using PL antigen should consider the existence of exo- and endotoxins produced by the bacteria during vaccine development. In addition, there are limitations to the production of PL, because specific lytic bacteriophage should be isolated for effective PL generation. Nevertheless, the vaccines studied here demonstrated the potential to cause more robust immune responses than PLGA-FKC or FKC vaccines, and more effectively prevent *A. hydrophila* infection in *C. carpio*. The application of phage lysate could be an alternative for developing novel potent inactivated antigen in fish.

Conflicts of interest

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

This research was supported by Cooperative Research Program for Agriculture Science and Technology Development (Supportive managing project of Center for Companion Animals Research) by Rural Development Administration (PJ013985032018), and by Global Ph.D Fellowship Program through the National Research Foundation of Korea funded by the Ministry of Education (NRF-2015H1A2A1029732).

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