수의학 박사 학위논문

Studies on improving the efficacy of fish vaccines using a novel methodology: Antigen, administration, and delivery

어류 백신의 효능 향상을 위한 새로운 방법론에 관한 연구

2020 년 2 월

서울대학교 대학원

수의학과 수의병인생물학 및 예방수의학 전공

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A Dissertation for the Degree of Doctor of Philosophy

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By

Saekil Yun

February, 2020

Major in Veterinary Pathobiology and Preventive Medicine

Department of Veterinary Medicine

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A dissertation submitted to the faculty of the Graduate School of Seoul National University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Pathobiology and Preventive Medicine

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Abstract

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Control and prevention of disease is a high priority in aquaculture, and vaccination is important to prevent outbreaks. Here, poly(d,l-lactide-co-glycolic acid) (PLGA) microparticles (MPs) approximately 36 μm in diameter were used to encapsulate and deliver *Aeromonas hydrophila* formalin-killed cells (FKC) as an antigen, and the innate and adaptive immune responses of cyprinid loaches and common carp were assessed following vaccination. The antigen was confirmed to be well encapsulated by scanning electron microscopy analysis of PLGA MP sections. Blood and head kidney specimens were collected and analyzed for bacterial agglutination activity and relative mRNA expression of immune-related genes (IL-1β, IL-10, TNF-α, lysozyme C, TGF-β, and IgM) at 2, 4, 6, and 8 weeks

post vaccination (wpv). For both fish species, the curve of antibody titer over time was shallower in the PLGA group than the FKC group. These titers in loaches and carp were very similar in the two vaccination groups until 8 and 6 wpv, respectively, but differences were subsequently noted in both species until the end of experiment. Loaches and carp were then challenged with *A. hydrophila* at 12 and 20 wpv, and 10 and 14 wpv, respectively, and relative survival rates were calculated. For both species, the PLGA groups demonstrated higher survival rates at all time points. Relative expression of IL-1β and TNF-α mRNA was significantly upregulated in the PLGA group at 2 and 4 wpv. Moreover, PLGA-MP vaccination increased relative mRNA levels of lysozyme C and IgM, which were significantly higher than those observed with FKC treatment at 2 wpv and 4, 6, and 8 wpv, respectively. In conclusion, PLGA-MP vaccines have the potential to induce longer and more potent immune responses than FKCs alone, and protect both cyprinid loaches and common carp with greater efficiency.

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 *A. 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*.

Immunization by bath immersion is likely the simplest method of fish vaccination. Although the route of immunogenicity has not been fully identified, immersion vaccination is clearly a useful labor-saving technique. In this study,

microbubble (MB) treatment was assessed for its ability to improve the efficacy of bath immersion vaccination in the cyprinid loach. MBs are commonly defined as minute particles of gas with a diameter of less than 100 μm, which generated free radicals. Here, the efficacy of MB treatment for vaccination enhancement in the cyprinid loach was assessed in direct challenge experiments using the virulent *A. hydrophila* JUNAH strain; assessments comprised agglutination titer assay and non-specific parameter analysis. Agglutination titers were high in loaches that were immunized via injection with inactivated cells (FKC group); however, non-specific immune activation parameters (e.g., lysozyme, superoxide dismutase, and phagocytic activity) were more increased in loaches that were immunized via bath immersion with MB treatment. Moreover, MB-treated loaches showed comparable survival rates, relative to loaches immunized via injection with formalin inactivated cells. Thus, higher levels of non-specific immune parameters suggest increased efficacy of this vaccine approach. Improving the effectiveness of bath immersion vaccine will increase its affordability and ease of application in aquaculture.

In this study, the methods of encapsulating antigen with a biodegradable material, strengthening immersion vaccines, and use of novel antigen were applied to enhance the effectiveness of fish vaccines against *A. hydrophila* infection. The efficacy of the methods used in this study was verified through analysis of adaptive and innate immunity and performing a challenge experiment compared to the FKC vaccine, and excellent results were achieved. Therefore, these methods will be useful to prevent diseases caused by fish pathogens. We should attempt to develop

better vaccines, considering the rising economy in aquaculture and public health of consumers.

Key words: *Aeromonas hydrophila*, encapsulation, microbubble, microparticles, phage lysate, PLGA

Student number: 2014-21933

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Abbreviations

FKC	Formalin Killed whole-Cell
MS222	tricaine MethaneSulphonate
SEM	Scanning Electron Microscopy
PLGA	Poly (d,l Lactide-co-Glycolic Acid)
FDA	Food and Drug Administration
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
MP	MicroParticle
MB	MicroBubble
NB	NanoBubble
APC	Antigen Presenting Cell
FAO	Food and Agriculture Organisation
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
PVA	Poly Vinyl Alcohol
CFU	Colony Forming Unit
LD_{50}	median Lethal Dose
RPS	Relative Percent Survival
IL	InterLeukin
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
SAA	Serum Amyloid A
PBS	Phosphate Buffered Saline
LZM	Lysozyme
SOD	Superoxide Dismutase
PL	Phage Lysate
ANOVA	Analysis Of Variance
dpv	days post vaccination
wpv	weeks post vaccination

General introduction

Control and prevention of disease is a high priority in aquaculture. However, in contrast with conditions affecting humans and other animals, an insufficient number of treatments exist for fish diseases. Vaccination is gradually being recognized as an important aspect of aquaculture, owing to its cost-effectiveness in controlling disease outbreaks (1). Nevertheless, there are two key disadvantages to vaccination. First, use of high-priced vaccines is impractical because, compared to other farming contexts such as pig or cattle rearing, the number of individuals requiring treatment in aquaculture is significantly larger. Second, the method of delivery is problematic. The majority of fish vaccines are currently delivered by injection, which is by far the most effective technique compared to oral and immersion routes. However, this approach is labor-intensive and unfeasible for small or young fish (2).

Administration of formalin-killed whole-cell (FKC) vaccines is considered the optimal strategy to control and prevent bacterial infections in aquaculture. Compared to other vaccine types, these treatments enable the delivery of highly immunogenic and protective antigens with greater convenience and economy. For these reasons, such vaccines are frequently used by many aquaculturists (3). Yet, despite their advantages, water-based FKC vaccines provide only a short period of protection (4, 5).

Immune responses induced by immersion vaccination are generally less robust

and have shorter durations than those obtained by injection (6–8). To increase the uptake of vaccines by fish mucosal tissues, thereby maximizing the efficacy of immersion vaccination, hyperosmotic immersion and ultrasound-mediated immersion have been adopted (9–13).

Poly(d,l lactide-co-glycolic acid) (PLGA) has been previously used for controlled drug release and antigen encapsulation for vaccine administration (14, 15). 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 (16, 17).

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 (18).

Microbubbles (MBs) are commonly defined as minute particles of gas with a diameter of less than 100 μm (19). MBs exhibit characteristics that differ from those of macrobubbles, which are greater than 100 μm in size. MBs have been used in aquaculture to improve the seafloor environment and water quality, as well as to reduce the rate of mortality and promote growth by enhancing available oxygen in shellfish farms (20–22).

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Literature Review

A. Biodegradable polymers for vaccines

A.1. General description

Polymeric biomaterials have been considered for antigen delivery and as a substitute for alum-based adjuvants (1). Figure I shows the application of polymeric biomaterials as a carrier of vaccine adjuvants or of antigens.

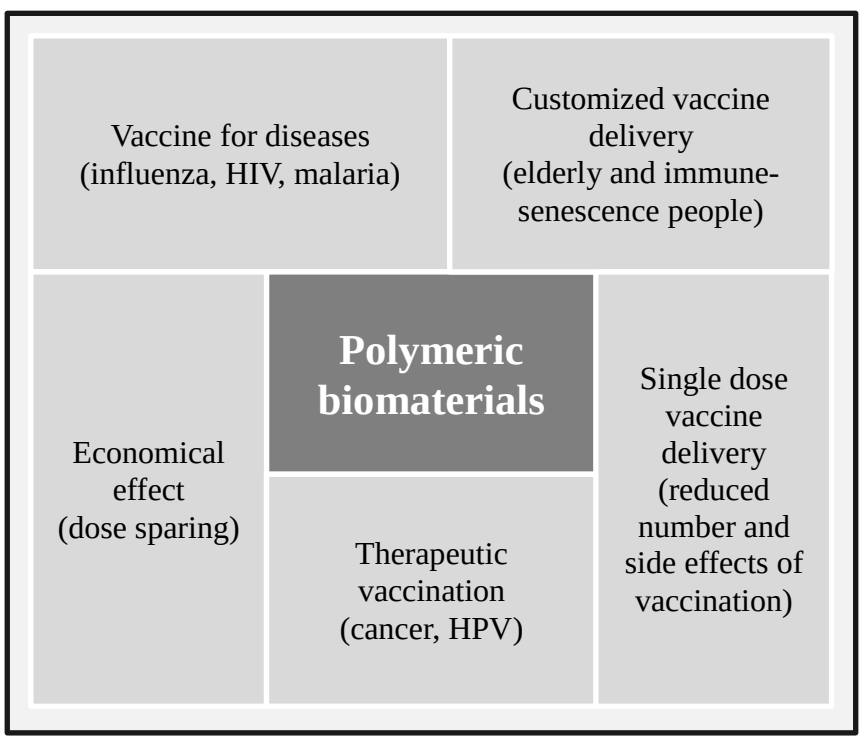


Figure I. Application of polymeric biomaterials.

It is a good strategy to induce the controlled release of a vaccine using biodegradable polymers (2). Biodegradable materials, including natural and synthetic substances, are biocompatible and interact with the biological system in vivo. In addition, they have favorable physiochemical properties, with an upper surface layer to facilitate binding and deformation for targeting certain cells. Moreover, they are easily adjustable in size and have high antigen capacity and stability in vivo.

Polymeric biomaterials can enhance immune responses through various channels. They can improve antigen stability in vivo by preventing antigen decomposition through absorption or encapsulation into the matrix of the matter (3). Further, they can enhance antigenicity by incorporating various adjuvants and antigens. In addition, they are effectively taken-in and processed by antigen-presenting cells (APCs), which enhancing cross-presentation through cytotoxic T cell immune responses mediated by major histocompatibility complex class I. Their flexibility, in terms of surface functionalization, enables active targeting of APCs through activation of endosomal toll-like receptors or surface pattern-recognition receptors, which elicit robust immune responses.

Various natural or synthetic biodegradable polymers have been investigated to apply the development of vaccines (4). Factors that impede the development of controlled-release vaccines through these polymers are as follows: i) Damage to the antigen during encapsulation; ii) Insufficient amount of antigen encapsulated; iii) Poor absorption of the antigen (5). In addition, colloidal instability can

interfere with antigen transmission clinically (6).

A.2. Microparticles and nanoparticles

Biodegradable polymers are used as a carrier for vaccine delivery, acting as an adjuvant for activating the immune system. Various forms of vaccines have been developed to be delivered with these polymers.

Biodegradable polymer particles, such as microcapsules, microspheres, and nanospheres, have been used as vaccine carriers and adjuvants. Their application involves encapsulating the antigens into small spheres or absorbing them into the sphere structures (7). The term “microcapsule” is defined as a spherical particle, with size varying from 50 nm to 2 mm, containing a core substance. In a strict sense, microspheres are spherically empty particles. However, the terms “microcapsules” and “microspheres” are often used synonymously. Encapsulation within these particles can increase the stability of the antigens, thereby efficiently delivering vaccines to APCs to stimulate immune responses (3). These particles can enhance immune responses by absorbing them into the particle or by encapsulating the antigens (8, 9).

Nanoparticles were first developed around 1970. They were initially devised as carriers for vaccines and anticancer drugs (10). To enhance tumor uptake, the strategy of drug targeting was employed, and studies focused on developing methods to reduce nanoparticle uptake by the cells of the reticuloendothelial system (11). Simultaneously, the use of nanoparticles for ophthalmic and oral

deliveries was investigated (12). Nanometer-sized polymeric nanogels or hydrogel nanoparticles are swollen networks of amphiphilic or hydrophilic polyionic polymers. Nanogels are promising multifunctional polymeric nanoparticles, with the potential to be used as delivery systems because of their unique properties, such as multivalent conjugation, high water content, and biocompatibility (13).

A.3. Poly(lactic-co-glycolic acid) (PLGA)

Numerous synthetic biopolymers, including polyesters, such as polylactides, polyglycolide, PLGA, and polycaprolactone, polyphosphazenes, and polyanhydrides have been considered for vaccine delivery. PLGA (Figure II) is a copolymer used in a host of therapeutic devices approved by the Food and Drug Administration, owing to its biodegradability and biocompatibility.

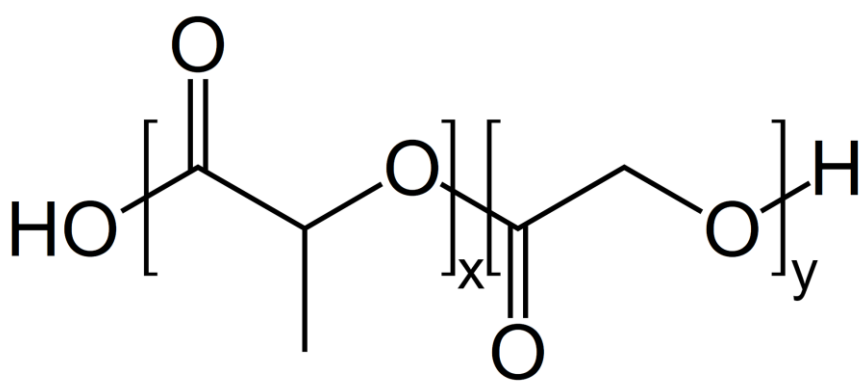


Figure II. Structure of poly(lactic-co-glycolic acid).
x=number of units of lactic acid; y=number of units of glycolic acid

PLGA is a copolymer synthesized by the random polymerization of polylactic acid and polyglycolic acid. PLGA decomposes into lactic and glycolic acid, which are biodegradable substances, and eventually eliminated from the body via the citric acid cycle (15). PLGA polymers with different stoichiometric ratios of lactic and glycolic acids and different molecular weights exhibit differences in antigen stability and release kinetics. In some studies, PLGA with monomer ratios of 85:15, 75:25, and 50:50 have degradation times of 5–6, 4–5, and 1–2 months, respectively (14). Therefore, the release kinetics of various antigens or adjuvants can be predicted. The use of PLGA with a single vaccine allows the long-term immune response to be induced without side effects (16). Vaccines using PLGAs have mainly been made in the form of microspheres, microcapsules, and nanospheres to facilitate controlled antigen delivery (1, 15, 17). Hepatitis B is a disease necessitating booster vaccination for its prevention. Li et al. formulated a single-shot hepatitis B vaccine and used PLGA microspheres as the delivery system. In their study, hepatitis B surface antigens could be successfully encapsulated and recovered from the PLGA microspheres, and a mixture of aluminum-adjuvanted and PLGA-formulated hepatitis B surface antigen could auto-boost an immune response in mice (18).

Vaccines using PLGA target the dendritic cells and release antigens over a long duration and are highly reliable and easy to process (1, 19). However, they also have limitations. The production of an acidic microenvironment can reduce antigen stability and increase hydrophobicity, and accordingly, encapsulation efficiency

and initial burst release can decrease (1, 15, 20). To overcome these limitations, magnesium hydroxide or calcium carbonate can be used (1). The characteristics of a PLGA particle as an adjuvant are complex. The process of nano- or micro-PLGA particles being absorbed into the cells has been reported (21). This PLGA particle prevents proteolytic degradation, enabling continuous antigen release (22). It can also encapsulate or absorb various adjuvants or antigens, such as bacteria (*Pseudomonas aeruginosa*, PopB), virus (hepatitis B virus, HBcAg), and parasite (malaria, SPf66) into the surface of PLGA particles (23–25). However, the clinical development of PLGA-based nanoparticles presents with several challenges, including the synthetic hydrophobic surface, low transfection efficiency (for DNA vaccines), short circulation half-life, and nonspecific tissue distribution (26–28). These limitations should be overcome through studies in the future.

B. Microbubbles and nanobubbles

B.1. General description

Microbubbles (MBs) and nanobubbles (NBs) are tiny bubbles of diameter 10–50 μm and <200 nm, respectively. MBs and NBs have been researched for various applications including industry (agriculture, fishery, etc.), life sciences, and medicine (29–32).

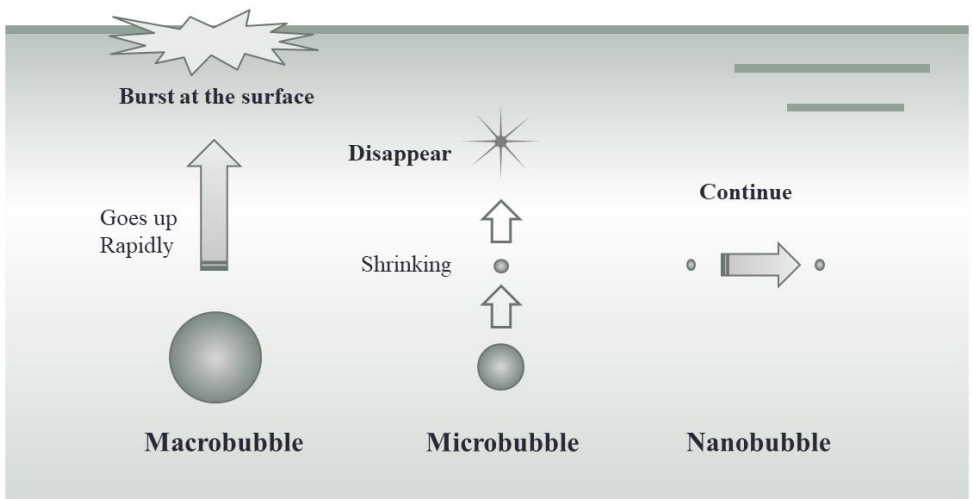


Figure III. Schematic diagram showing macro, micro and nanobubbles (36).

The existence of nanobubbles has been controversial due to some thermodynamic considerations. For example, the total free energy in a system should increase as a nanobubble is created. The nanobubble is likely to dissolve quickly into the solution due to the internal Laplace pressure (33, 34). The key

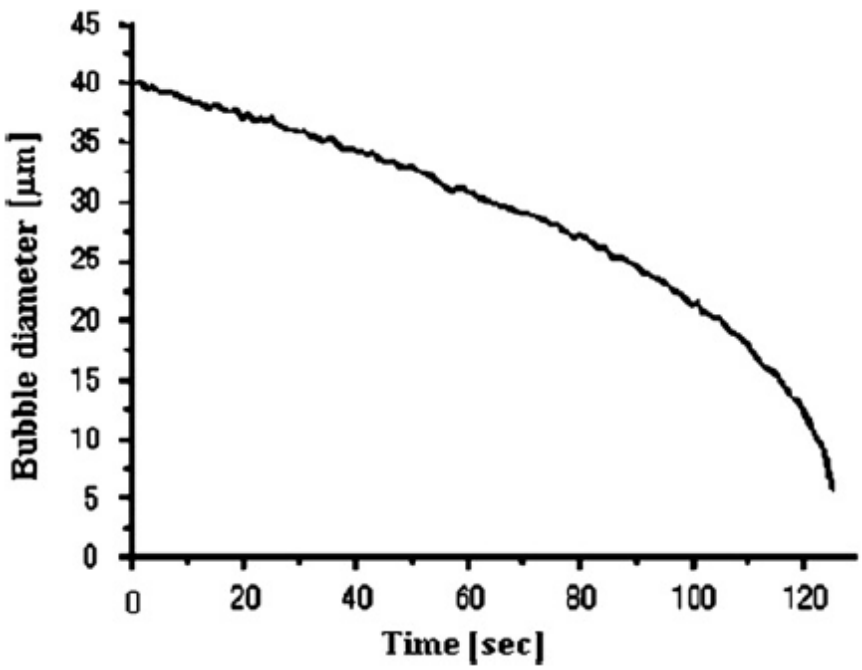
differences among macrobubbles, MBs, and NBs are shown in Figure III. MBs tend to gradually decrease in size and subsequently collapse due to the long stagnation and dissolution of interior gases into the surrounding water, whereas NBs persist to exist for months and do not burst (35). The interface of NBs consists of hard hydrogen bonds similar to those found in ice and gas hydrates. This reduces the spread rate of NBs and helps maintain the proper movement balance of NBs against high internal pressure.

B.2. Physics of micro and nanobubbles

The existence of NBs at the liquid–solid interface has been demonstrated by various techniques such as atomic force microscopy (37−41). The NBs at the liquid–solid interface have the shape of flat ellipses with a height and diameter of about 10 and 100 nm, respectively. The fact that NBs can form a large bubble by attaching to the tip end of the atomic force microscopy probe supports this (40). NBs were initially considered to have a high surface tension, meaning that as soon as NBs were formed, gases inside would be released in microseconds (42). However, the NBs can be formed freely and remain very stable for a long time in the absence of unique external environmental factors.

The most important parameters to understand the characteristics of MBs and NBs are the surface charge and bubble rising speed. In the electrophoresis experiment, MBs move in the opposite direction of the charged electrode. The surface charge of MBs is determined by the speed of MBs movement, which is

related to the value of ζ potential (43–47). MBs have slow movement and attach easily, making it easier to measure ζ potential. The ζ potential is known to be negatively charged at various pHs and measures at approximately -35 mV in distilled water. The ζ potential of MBs is confirmed to remain constant under similar water conditions regardless of their size, indicating that the amount of charge remains constant (47). Nevertheless, an increase in ζ potential was observed during the collapse as MBs were reduced in size (Figure IV).



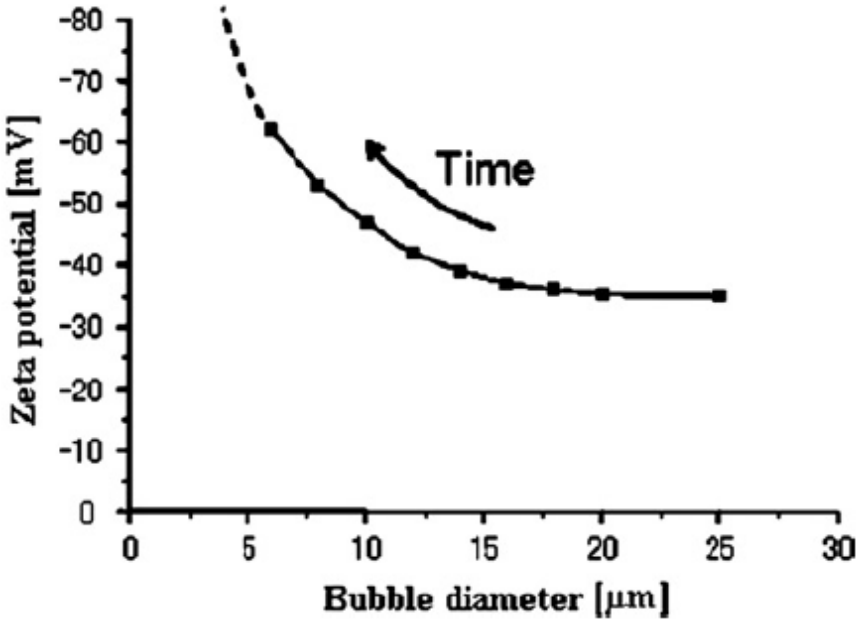


Figure IV. Changes in the size and ζ potential of microbubbles over time (36).

B.3. Generation of free radicals by collapsing microbubbles in water

According to the Young–Laplace equation, a bubble with a diameter of 1 lm at 298 Kwill have an internal pressure of 390 kPa, which is almost four times higher than the atmospheric pressure. Because the increase in pressure inside MBs is inversely proportional to their size, there is a period of very high pressure prior to MBs collapse (Figure V). If the speed of collapse of MBs is faster than the speed of sound in the water, the temperature inside the bubbles that are collapsing due to the surrounding compression can rise rapidly. The pyrolytic decomposition inside collapsing MBs can generate ⁻OH radicals and stock waves at the gas–liquid

interface (48). The rate of transfer of electrolyte ions in water is not high enough to resist the increased contraction rate of MBs. Therefore, there is a possibility of some excess ions accumulating at the gas–water interface in the final phase of the collapse process (36). As a result, ozone decomposition to produce ⁻OH can be accelerated in the case of MBs created using ozone (49). Through electron spin resonance spectroscopy, it has been proven experimentally that if MBs collapse, active oxygen will be produced (36).

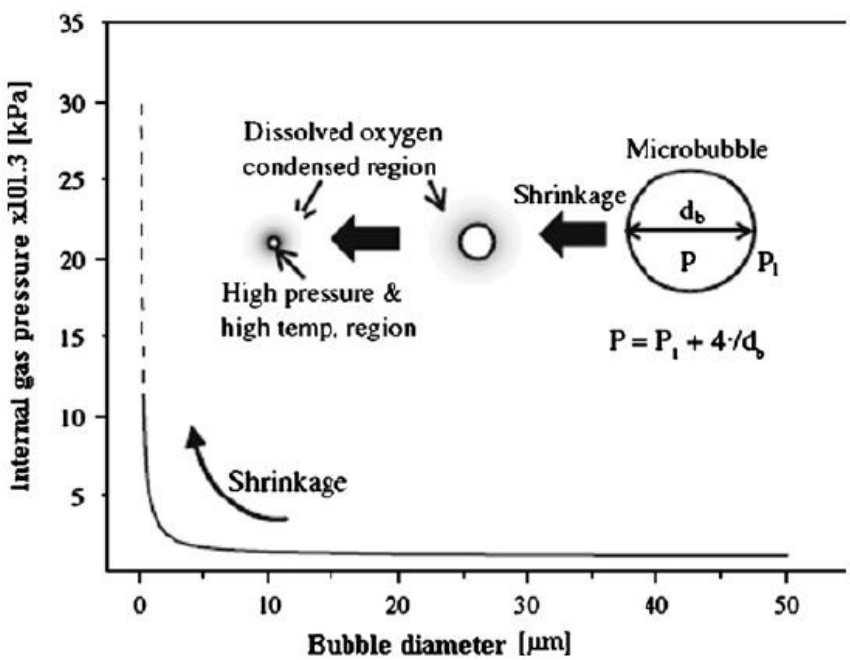


Figure V. Increase in the interior gas pressure of MBs during shrinkage (at T = 298 K, P = 100 kPa.) (50).

Young–Laplace equation:

$$P = PI + \frac{4\sigma}{d_b}$$

where P is the gas pressure, PI is the liquid pressure, r is the surface tension of the liquid and db is the bubble diameter.

C. Bacteriophage-elicited bacterial lysates

C.1. Bacteriophage biology

Bacteriophages (or phages, viruses that infect bacteria) are ubiquitous all over the world. They exist in the oceans, soil, deep sea vents, the water we drink, and the food we eat (51). Bacteriophages are harmless for all organisms, including humans, except for their target bacterial hosts (52, 53). Most bacteriophages (about 96% of those currently identified) are classified in the order of *Caudovirales*, which are tailed, have double-stranded DNA and are further classified in the families of *Siphoviridae*, *Myoviridae* and *Podoviridae* (54). There is tremendous research potential for bacteriophages because of the vast number of species and their large amount of unexplored genes. The phages are extremely diverse and can infect all bacteria on earth (55). Phages usually infect their bacterial hosts in a species or strain specific manner. They are classed into virulent or temperate phages, depending on their life cycle. Virulent phages infect bacteria using the lytic cycle. Phages inject their genes into the host bacteria and use the host's molecular machinery to replicate their progeny, which eventually release from the host through lysis of the host cell (56). Most phages use two proteins, holins and lysins, to destroy host cells. Holins penetrate the bacterial cell membrane and interact with endolysin to destroy the cell wall (57). In contrast, temperate phages infect the host bacteria through the lysogenic cycle. The phage genes remain dormant in the form of a prophage, which is replicated into the host's genome and maintained by the

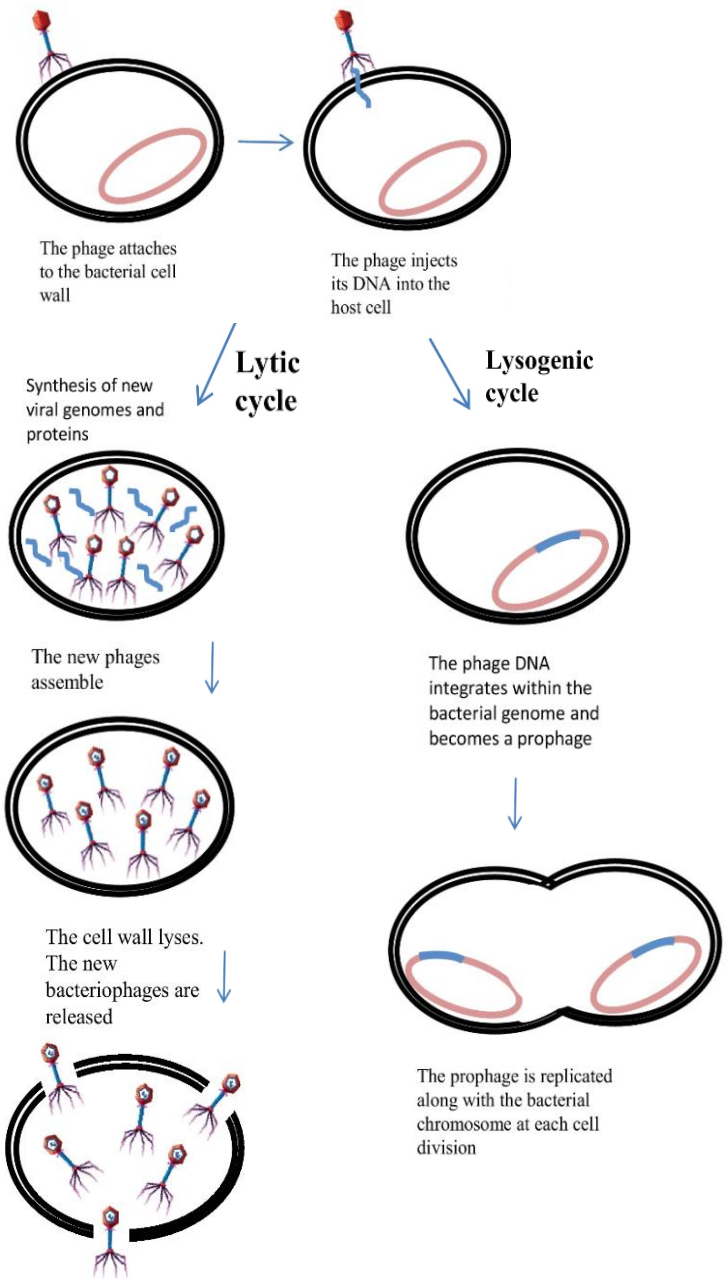


Figure VI. Overview of the main differences between the lytic and the lysogenic bacteriophage life cycle (60).

replication cycle. Temperate phages can convert to a lytic cycle by certain factors (56). Lysogeny and prophages can act as a mediator for transmitting antibiotic resistance genes or other toxin resistance genes to bacteria (58). For this reason, only lytic phages should be used in phage therapy (59). The differences between the lytic and the lysogenic life cycle are shown in Figure VI.

C.2. Bacteriophage application in aquaculture

Aquaculture is a fast-growing food production industry and could be important for feeding the future population. According to the Food and Agriculture Organisation (FAO) of the United Nations, global aquaculture production has grown from 31.1% in 2004 to 44.1% of the total production of 73.8 million tonnes of fish in 2014 (61). The possibility of using phages to treat bacterial diseases of aquacultured fish has been gaining attention. One of the first reports of this method in aquaculture is on using specific phages of *Lactococcus garvieae* to treat infected young yellowtail (*Seriola quinqueradiata*) (62). The authors analyzed the fishes' internal organs for the presence of phages, and tested *L. garvieae* isolates recovered from dead fish for phage susceptibility. All of the isolates examined remained susceptible to the phages, and phage-neutralizing antibodies were not detected in serum samples obtained from the yellowtail (62, 63).

The narrow host specificity of phages is a disadvantage for phage therapy. It is strain-specific rather than species-specific, which cause obstacles when treating diverse diseases with phages. However, some phages have a wide host range (64).

Another limitation is that the emergence of phage-resistant bacteria is very rapid. However, all bacterial isolates from dead fish obtained during therapy experiments were still susceptible to phages used for treatment. Furthermore, phage-resistant bacteria, which were induced *in vitro*, lacked virulence for ayu (65). Phage-neutralizing antibodies produced as a result of phage administration could be present and limit the effects of phage therapy. The neutralizing antibodies can be induced by oral administration or parenteral inoculation of phages (66). However, these neutralizing antibodies are not always present, though their presence indicates that phages can be used for therapy in fish (64, 67). A final concern about using phages as therapeutics is that phages could act as mediators of genes exchange in the environment. It is well known that some temperate phages contribute to bacterial virulence. Temperate phages with broad infectivity over species would strongly support views on antiphage therapy (68). However, this possibility is probably unlikely for therapeutic phages because of their extremely narrow host specificity.

C.3. Using phages to produce bacterial vaccines

Using phages to prepare bacterial lysates is an interesting approach as these bacterial lysates can be used as vaccines. The excellent immunogenicity of bacterial lysates is not yet clear, but it is possible that bacteriophage-mediated lysis is a more effective and gentler approach to inactivate bacteria over conventional methods. In this regard, methods such as heat-treatment, irradiation, and chemical

treatment that we use to inactivate bacteria may actually reduce antigenicity due to damage to bacterial immune epitopes (69–71). It was proposed by d'Herelle in 1916 that using gentle lysis would create an antigen with a more ideal immunogenicity (72).

C.4. Safety of bacteriophage therapy

Phages are so abundant in nature such that we are always exposed to them; thus, phages are generally considered safe for humans. This is also supported by the fact that no cases have been reported on harmful phages to human health. However, verifying a phage's safety through modern technology will be essential prior to using the phage for a treatment (74). A concern about the use of phages for treatment is that phages may affect tissues or groups of healthy microbiota that are not targets of the treatment, or may cause changes in the expression of the virulence genes of the target bacteria inducing immunological reactions in the body (74, 75). In addition, harmful substances such as endotoxins may be produced by the phage (76, 77). Phages have a very high host-specificity and can only infect certain bacteria, which means that they should not affect other bacteria in the intestinal microbiota (78). Banning the use of temperate phages will prevent the transfer of virulence genes in bacteria. The reasons for opposing the intravenous use of phages are based on the following considerations. Phages can be removed very quickly by the reticulo-endothelial system. Endotoxins could be released as phages kill bacteria. The treatment efficiency can be reduced by the production of

anti-phage antibodies in the host and can cause serious side effects such as anaphylaxis. However, anaphylaxis has not been reported in phage therapy (79).

D. References

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Chapter I

Efficacy of PLGA microparticle-encapsulated formalin-killed *Aeromonas hydrophila* cells as a single-shot vaccine against *A. hydrophila* infection

Abstract

Control and prevention of disease is a high priority in aquaculture, and vaccination is important to prevent outbreaks. Here, poly(d,l-lactide-co-glycolic acid) (PLGA) microparticles (MPs) approximately 36 μm in diameter were used to encapsulate and deliver *Aeromonas hydrophila* formalin-killed cells (FKC) as an antigen, and the innate and adaptive immune responses of cyprinid loaches and common carp were assessed following vaccination. The antigen was confirmed to be well encapsulated by scanning electron microscopy analysis of PLGA MP sections. Blood and head kidney specimens were collected and analyzed for bacterial agglutination activity and relative mRNA expression of immune-related genes (IL-1β, IL-10, TNF-α, lysozyme C, TGF-β, and IgM) at 2, 4, 6, and 8 weeks post vaccination (wpv). For both fish species, the curve of antibody titer over time was shallower in the PLGA group than the FKC group. These titers in loaches and carp were very similar in the two vaccination groups until 8 and 6 wpv, respectively, but differences were subsequently noted in both species until the end of experiment. Loaches and carp were then challenged with *A. hydrophila* at 12 and 20 wpv, and

10 and 14 wpv, respectively, and relative survival rates were calculated. For both species, the PLGA groups demonstrated higher survival rates at all time points. Relative expression of IL-1β and TNF-α mRNA was significantly upregulated in the PLGA group at 2 and 4 wpv. Moreover, PLGA-MP vaccination increased relative mRNA levels of lysozyme C and IgM, which were significantly higher than those observed with FKC treatment at 2 wpv and 4, 6, and 8 wpv, respectively. In conclusion, PLGA-MP vaccines have the potential to induce longer and more potent immune responses than FKCs alone, and protect both cyprinid loaches and common carp with greater efficiency.

Keywords: *Aeromonas hydrophila*, Aquaculture, *Cyprinus carpio*, Microparticles, *Misgurnus anguillicaudatus*, Poly(d, ,l-lactide-co-glycolic acid)

1.1. Introduction

In intensive aquaculture, single or multiple fish species are reared at high densities. Therefore, factors such as optimal husbandry, biosecurity, nutritional genetics, system management, and water quality are critical in fish farming (1). However, disease outbreaks are a substantial threat to this industry, given the presence of many pathogenic organisms in aquatic environments. The constant exposure of farmed fish to these pathogens and the use of high-density techniques by most fish farms can result in mass mortalities (2–4).

Aeromonas hydrophila is a bacterium found in aquatic environments, and is capable of causing disease in numerous fish species, including grass carp (*Ctenopharyngodon idella*) (5), channel catfish (*Ictalurus punctatus*), and tilapia (*Sarotherodon niloticus*) (6), as well as in higher vertebrates (7). Motile *Aeromonas* septicemia, a disease caused by *A. hydrophila* infection, involves symptoms such as hemorrhagic septicemia, infectious abnormal dropsy, exophthalmia, and fin and tail rot (8).

Control and prevention of disease is a high priority in aquaculture. However, in contrast with conditions affecting humans and other animals, an insufficient number of treatments exist for fish diseases. Vaccination is gradually being recognized as an important aspect of aquaculture, owing to its cost-effectiveness in controlling disease outbreaks (9). Nevertheless, there are two key disadvantages to vaccination. First, use of high-priced vaccines is impractical because, compared to

other farming contexts such as pig or cattle rearing, the number of individuals requiring treatment in aquaculture is significantly larger. Second, the method of delivery is problematic. The majority of fish vaccines are currently delivered by injection, which is by far the most effective technique compared to oral and immersion routes. However, this approach is labor-intensive and unfeasible for small or young fish (10).

Administration of formalin-killed whole-cell (FKC) vaccines is considered the optimal strategy to control and prevent bacterial infections in aquaculture. Compared to other vaccine types, these treatments enable the delivery of highly immunogenic and protective antigens with greater convenience and economy. For these reasons, such vaccines are frequently used by many aquaculturists (11). Yet despite their advantages, water-based FKC vaccines provide only a short period of protection (12, 13).

Poly(d,l-lactide-co-glycolic acid) (PLGA) has been used previously for controlled drug release and antigen encapsulation for vaccine administration (14), (15). PLGA microparticles (MPs) are prepared by the water-in-oil-in-water (W/O/W) emulsion method because of the hydrophobic characteristics of this copolymer, generally resulting in negatively charged, smooth-surfaced, spherical particles. PLGA has been approved by the US Food and Drug Administration, and has attracted attention due to its biocompatibility, biodegradability, and high stability in biological fluids and during storage (16, 17). Moreover, the degradation rate of PLGA can be modified by controlling parameters such as polymer

molecular weight and the lactide to glycolide ratio (18, 19). In addition, entrapment in polymers can prolong drug release and enhance therapeutic efficacy (20, 21).

In this study, PLGA MPs were used as a delivery system for an *A. hydrophila* FKC vaccine. The immunogenicity of this PLGA MP-encapsulated whole-cell antigen was assessed in cyprinid loaches (*Misgurnus anguillicaudatus*) and common carp (*Cyprinus carpio*) by comparing its effects to those of the FKC vaccine alone. Vaccine efficacy was evaluated by challenging fish with *A. hydrophila*.

1.2. Materials and methods

1.2.1. Polymer and fish

PLGA (P1941, MW 66,000–107,000) and poly vinyl alcohol (341584, PVA; average MW 89,000–98,000) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Three hundred healthy cyprinid loaches (mean body weight ± SD: 8.32 ± 1.23 g) and 200 common carp (mean body weight ± SD: 11.54 ± 1.91 g) were purchased from commercial fish farms in Gyeonggi and Jeollabuk provinces, South Korea, respectively. The fish were acclimatized in the laboratory of the College of Veterinary Medicine of Seoul National University, Seoul, South Korea, 20 days before commencing the experiment. They were kept in 100-L fiberglass tanks at 25 ± 2 ℃ and fed once a day with commercial feed. Approximately 20% of the water in each tank was changed daily. This study was performed in accordance with the "Guidelines on the Regulation of Scientific Experiments on Animals" by Seoul

National University Institutional Animal Care and Use Committee.

1.2.2. *Bacterial strain and preparation of FKC vaccine*

The *A. hydrophila* JUNAH strain, isolated from a cyprinid loach in 2009 in South Korea and preserved in a lyophilized condition in our laboratory, was used throughout this study (22). For experiments, bacteria were cultured on tryptic soy agar (TSA; Difco, Detroit, MI, USA) medium at 25 ℃ for 24 h. Then, single colony was cultured in tryptic soy broth (TSB; Difco, Detroit, MI, USA) at 25 ℃ for 48 h. The cultured bacteria were treated with 0.5% formalin and maintained at 25 ℃ for 48 h, before being centrifuged at 10,000g for 10 min, washed twice in sterile phosphate-buffered saline (PBS), and re-suspended in sterile PBS. This suspension was adjusted to an optical density at 600 nm of 0.6 using a spectrophotometer.

1.2.3. *Preparation of PLGA microsphere-encapsulated FKCs*

MPs encapsulating FKCs were prepared with PLGA copolymer using a W/O/W double-emulsion solvent evaporation technique, as previously described (23), with some modifications. The antigen was dissolved 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, Wonju, South Korea) at 12,000 rpm for 1 min at room temperature to form the primary W/O emulsion. This was then poured into a 50-mL 4% PVA

solution and homogenized at 6000 rpm for 1 min. After 2 min, an additional 50 mL 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 at room temperature to allow the organic solvent to evaporate. The resultant MPs were then washed with PBS (pH 7.4) twice and centrifuged at 5000g for 10 min. The recovered MPs were lyophilized for 48 h to preserve them for further use.

1.2.4. *MP size and distribution analysis*

Laser diffraction was used to measure the hydrodynamic diameter and size distribution of MPs (according to ISO 13320). The analysis was performed using an LS 13 320 instrument (Beckman Coulter, Brea, CA, USA).

1.2.5. *Scanning electron microscopy (SEM)*

The morphology of MP surfaces and sections was observed using a field-emission scanning electron microscope (Sigma; ZEISS, Cambridge, UK). To confirm the encapsulation of *A. hydrophila* cells, PLGA MPs were embedded in paraffin blocks and cut into 3-μm sections with a microtome. The sections were then mounted onto SEM stubs and coated with platinum for 180 s.

1.2.6. *In vivo experiments*

1.2.6.1. *Vaccination and challenge experiment*

The 300 loaches and 200 carp were randomly divided into three experimental

groups. Fish in the PLGA and FKC groups were administered 0.1 mL PLGA-MP or FKC-only vaccine, respectively, by intraperitoneal injection. The total antigen content in 0.1 mL vaccine was adjusted to 2×10^8 colony-forming units (CFU). Control fish were injected intraperitoneally with 0.1 mL sterile PBS. For cyprinid loaches, all three groups were challenged with *A. hydrophila* 12 and 20 weeks post-vaccination (wpv) at the median lethal dose (LD_{50}) of 5.0×10^6 CFU/fish. Common carp were challenged with the same strain 10 and 14 wpv, at the LD_{50} for this species of 1.3×10^7 CFU/fish. The challenge experiment was repeated three times.

1.2.6.2. *Clinical signs and relative percent survival (RPS)*

Clinical signs and cumulative mortalities were monitored twice a day for 2 weeks. To isolate bacteria, the internal organs of dead fish were streaked onto TSA medium and incubated at 25℃ for 24 h, with isolates being identified by PCR as previously described (24). Vaccine efficacy was assessed by RPS using the following formula:

RPS = (1 – (cumulative mortality of vaccinated group/cumulative mortality of control group)) × 100.

1.2.6.3. *Sample collection*

Using a 1-mL syringe and hypodermic needle, blood specimens were collected from the caudal veins of five randomly chosen fish in each group (PLGA, FKC, and control) following anesthetization with MS-222 (100 ppm). Blood sampling

was repeated at 2, 4, 6, 8, 10, 12, 14, 16, and 20 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 ℃, before being stored at −20 °C until use. For investigation of carp immune gene expression, head kidneys were collected from three fish in each group at 2, 4, 6, and 8 wpv. These samples were also transferred to microcentrifuge tubes and stored at −80 °C until use.

1.2.7. Immune response assessment

1.2.7.1. Bacterial agglutination test

The serum agglutination experiment was conducted in microtiter plates with 'U'-shaped wells. Sera were serially twofold diluted in PBS and the same volume of homologous heat-killed *A. hydrophila* (10^7 cells/mL) was added to each well. Plates were then kept overnight at room temperature. Agglutination activity was determined according to the lowest dilution not resulting in agglutination, and is expressed as the reciprocal of that dilution. This test was performed once.

1.2.7.2. RNA extraction and reverse transcription

Total RNA was extracted from head kidneys using TRIzol Reagent (CWBio, Beijing, China). RNA concentration and purity were quantified by spectrophotometry, 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. To eliminate DNA contamination, total RNA samples were treated with DNAse I (Promega, Madison, WI, USA), per the manufacturer's instructions. Extracted RNA was reverse transcribed to cDNA using a PrimeScript RT Reagent Kit (TaKaRa Bio, Otsu, Japan), following the manufacturer protocol. The resulting cDNA was stored at -80 ℃ until use.

1.2.7.3. *Quantitative PCR (qPCR) analysis of gene expression*

qPCR analysis of interleukin (IL)-1β, IL-10, tumor necrosis factor (TNF)-α, lysozyme C, transforming growth factor (TGF)-β, IgM, and β-actin expression was performed with a Rotor-Gene Q instrument (QIAGEN, Hilden, Germany) following standard protocols and using the primers indicated in **Table 1.1**. The following cycling conditions were used: 95 ℃ (10 min), then 40 cycles of 95 ℃ (30 s), 60 ℃ (30 s), and 72 ℃ (30 s). To correct for cDNA loading variations, target gene expression was normalized to that of the housekeeping gene β-actin for all samples. To verify reaction specificity, melt 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 the PLGA and FKC groups were compared with those obtained with the control samples. Each sample was processed in triplicate.

1.2.8. *Statistical analyses*

SPSS version 19.0 (IBM Corp., Armonk, NY, USA) was used to analyze all data.

Serum agglutination, immune gene expression and challenge data were analyzed by One-way analysis of variance was used, followed by Duncan's multiple range test, to compare variations in various immune parameters for differences at a significance level of 0.05. The mean ± standard error of the mean (SEM) of assayed parameters was calculated for each group.

1.3. Results

1.3.1. Characteristics of antigen-loaded microspheres

The FKCs were successfully encapsulated in PLGA MPs. Laser diffraction revealed that the MPs generated had a diameter of 36.83 ± 35.29 μm (mean ± SD), ranging from 10 to 40 μm. SEM confirmed the smooth and spherical nature of the PLGA MP-encapsulated FKCs **(Figure 1.1)**.

1.3.2. Immune response assessment

1.3.2.1. Adaptive immune responses

Serum titers indicated no detectable antibodies prior to vaccination in all groups. In the cyprinid loach vaccination groups, agglutination titers had increased at 2 wpv. These titers peaked at 4 wpv and slowly decreased until the end of the experiment. Antibody titers in the common carp vaccination groups were also increased at 2 wpv, before peaking at 6 wpv and slowly decreasing until the experiment's conclusion. For both loaches and carp, the PLGA group exhibited a shallower titer curve than the FKC group. Titers were very similar in the two

vaccinated groups until 8 and 6 wpv in loaches and carp, respectively. After these points, differences were discernible in both fishes until the end of the experiment. Agglutination titers in the control groups of both species remained at zero throughout **(Figure 1.2)**. The titers of PLGA MPs group were significantly higher than the FKCs group in both loach and carp from 10 weeks to the end of experiments.

1.3.2.2. *RPS and mortality after challenge*

Mortality after challenge in both vaccination groups was lower than that in the control group at 12 and 20 wpv (loaches) and 10 and 14 wpv (carp). In all groups, mortality began 18 h post-infection, and continued up to 72 h after challenge. After this point, the surviving fish remained alive for the rest of the experiment and showed no symptoms. In the loach PLGA and FKC groups, mean RPS values were 65.22 and 17.39% at 12 wpv, and 45 and 10% at 20 wpv, respectively. In the carp PLGA and FKC groups, these values were 77.78 and 55.56% at 10 wpv, and 54.54% and 18.18% at 14 wpv, respectively **(Table 1.2)**. All dead fish exhibited typical clinical signs of A. hydrophila infection. Bacteria isolated from these fish on TSA plates were confirmed to be *A. hydrophila* by PCR (data not shown).

1.3.2.3. *Immune gene expression*

The PLGA-MP and FKC vaccines affected the relative mRNA expression of immune-related genes in the carp head kidney in different ways. Carp in the PLGA

group showed significantly higher IL-1β and TNF-α expression 2 and 4 wpv, but no significant differences were noted 6 and 8 wpv. TGF-β and IL-10 expression did not significantly differ between the two vaccine groups at any time point **(Figure 1.3)**. Lysozyme C mRNA expression was significantly higher in the PLGA group than the FKC group at 2 wpv. IgM mRNA levels demonstrated an increasing trend from 2 to 6 wpv, with expression being higher in the FKC group 2 wpv, but greater in the PLGA group 4, 6, and 8 wpv **(Figure 1.4)**.

1.4. Discussion

Historically, vaccines have been produced by inactivation. Inactivated vaccines represent a large proportion of total vaccine use in aquaculture (25), as they can be applied in this industry inexpensively. For the efforts of increasing the efficacy of vaccine, combined vaccine (26, 27) and adjuvant (28) were widely used. However, this combined vaccine has its disadvantages such as antigen competition. Adjuvants can lead to side effects such as inflammation at the injection site, intra-abdominal adhesions, pigmentation and granulomas (29). It can also cause immediate appetite reduction in Atlantic salmon (30). The cases of autoimmunity induced by intraperitoneal injection of adjuvant oil have also been reported (31, 32).

Biodegradable particles are receiving increased attention owing to their remarkable properties as carriers of low-molecular-weight drugs and macromolecules, including proteins, peptides, and genes. Such materials provide improved biocompatibility and enable easy encapsulation of drugs or vaccines (33).

They are readily taken up by antigen-presenting cells and facilitate activation of the immune system (34). In the present study, the immunological effects of PLGA MP-encapsulated *A. hydrophila* FKCs as an antigen delivery system were investigated. In the present research, we paid close attention to PLGA MP size because a large number of FKCs (the chosen antigen) needed to be encapsulated inside each particle. As whole bacterial cells (up to 1 μm) were used, the MPs had to be relatively large in order to remain in the body for a long period, releasing their antigen payload. One advantage of PLGA MPs is that they can act as an adjuvant for induction of cell-mediated immunity, stimulating cytotoxic T lymphocyte responses (35) and cytokine production (35, 36). These properties also facilitate the generation of antibodies in the humoral immune response.

SEM revealed the morphology and distribution pattern of PLGA MPs, the mean diameter of which was 36.83 ± 35.29 μm **(Figure 1.1)**, ranging from 10 to 40 μm. MPs over 50 μm in diameter presented a problem when injecting fish, as together, these large particles obstructed the bore of the hypodermic needle. To eliminate this shortcoming, a 50-μm mesh was applied as a filter. To confirm that the A. hydrophila is encapsulated inside the particles clearly, the particles were embedded with paraffin and sectioned with microtome by 3 μm. This section was observed by SEM and the antigen was found on the surface of the section of PLGA MPs in SEM analysis **(Figure 1.1)**.

Bacterial agglutination titers showed that the PLGA-MP vaccine resulted in a longer-term immune response than administration of FKCs alone. In both carp and

loaches, agglutination titers were noticeably higher from 8 wpv in the PLGA groups than the FKC groups. Relative survival rates were calculated following bacterial challenge. Loaches were challenged in two experiments, performed 12 and 20 wpv. The PLGA vaccine-treated groups showed RPS values (65.22% at 12 wpv and 45% at 20 wpv) significantly higher than those of the FKC-only groups. For carp, the two challenge experiments were carried out 10 and 14 wpv. Similarly, RPS in the PLGA groups (77.78% at 10 wpv and 54.54% at 14 wpv) was significantly higher than that recorded for the FKC-only groups. Here, we have therefore shown that use of PLGA MPs resulted in a more effective vaccine than administration of FKCs alone.

Teleost fish have a complex immune system, comprising innate (involving lysozymes, the complement system, immunocytes, and cytokines) and adaptive immunity (including antibody production and lymphocyte activity) (37). Macrophages, lymphocytes, granulocytes, and dendritic, mast, and epithelial cells, and include ILs, TNFs, TGFs, interferons, and chemokines are sources for cytokines (38). IL-1β is an early-response pro-inflammatory cytokine. This stimulates immune responses by activating lymphocytes or inducing the release of other cytokines that subsequently activate macrophages, natural killer cells, and lymphocytes (39). In the present study, relative IL-1β and TNF-α mRNA levels were significantly upregulated in the PLGA group at 2 and 4 wpv. Moreover, expression of the anti-inflammatory cytokines TGF-β and IL-10 did not significantly differ between the two vaccinated groups. Lysozymes are crucial

molecules in innate immune defense in fish, playing a key role in preventing infection with exogenous pathogens (40), and IgM constitutes the most important component of adaptive humoral immunity in teleost fish (41, 42). Here, the PLGA-MP vaccine upregulated relative lysozyme C and IgM mRNA levels, which were significantly higher in this group at 2 wpv and 4, 6, and 8 wpv, respectively, than in the FKC-only group. For a certain period after vaccination, fish treated with FKCs alone may exhibit stronger IgM mRNA expression than those administered a PLGA-MP vaccine, owing to the latter's encapsulation of antigens and, potentially, delivery of lower antigen quantities. However, the PLGA-MP vaccine tested here resulted in higher IgM mRNA expression after 4 wpv. These results suggest that, compared to FKCs alone, PLGA MP-based vaccines may induce more potent immune responses of greater duration, protecting against A. hydrophila infection.

In this study, PLGA MPs encapsulating *A. hydrophila* FKCs were evaluated for their efficacy as an antigen delivery system for fish vaccination. These MPs demonstrated the potential to bring about longer and more robust immune responses than FKC-only vaccines, and more efficiently prevent infection in both cyprinid loaches and common carp. Notably, immunization via PLGA MPs activated both innate and adaptive immunity against *A. hydrophila* infection.

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Table 1.1. Primers used for amplification of specific transcripts by quantitative PCR.

Target*		Sequence (5′ to 3′)	Product size (bp)	GenBank accession number
IL-1β	F	AAGGAGGCCAGTGGCTCTGT	69	AB010701
	R	CCTGAAGAAGAGGAGGCTGTCA		
IL-10	F	GCTGTCACGTCATGAACGAGAT	132	AB110780
	R	CCCGCTTGAGATCCTGAAATAT		
TNF-α	F	GCTGTCTGCTTCACGCTCAA	106	AJ311800
	R	CCTTGGAAGTGACATTTGCTTTT		
Lysozyme C	F	GTGTCTGATGTGGCTGTGCT	359	AB027305
	R	TTCCCCAGGTATCCCATGAT		
TGF-β	F	ACGCTTTATTCCCAACCAAA	97	AF136947
	R	GAAATCCTTGCTCTGCCTCA		
IgM	F	GATGCCCCGATTGGCTATGA	181	AB004105
	R	GGGTCATCGGTTCACCCTTT		
β-actin	F	GCTATGTGGCTCTTGACTTCGA	89	M24113
	R	CCGTCAGGCAGCTCATAGCT		

*F, forward; R, reverse.

Table 1.2. Mean cumulative mortality and relative percent survival (RPS) after *Aeromonas hydrophila* challenge of cyprinid loaches and common carp treated with *A. hydrophila* formalin-killed cells (FKCs) in poly(D,L-lactide-co-glycolic acid) microparticles (PLGA MPs), FKCs alone, or phosphate-buffered saline (PBS).

Species	Wpv	Group	Mean survival** (%)	Mean RPS (%)
Cyprinid loach	12	PLGA MPs	73.33 ± 3.33 *	65.22
		FKCs	36.67 ± 6.67	17.39
		PBS	23.33 ± 3.33	-
	20	PLGA MPs	63.33 ± 8.82 *	45
		FKCs	40 ± 5.77	10
		PBS	33.33 ± 3.33	-
Common carp	10	PLGA MPs	86.67 ± 6.67 *	77.78
		FKCs	73.33 ± 6.67	55.56
		PBS	40 ± 13.33	-
	14	PLGA MPs	66.67 ± 6.67 *	54.54
		FKCs	46.67 ± 11.55	18.18
		PBS	26.67 ± 13.33	-

Wpv, Weeks post-vaccination; RPS, relative percent survival; *significant differences ($P<0.05$) in survival rate compare between PLGA and FKC group; **means ± standard errors of the mean from three independent experiments.

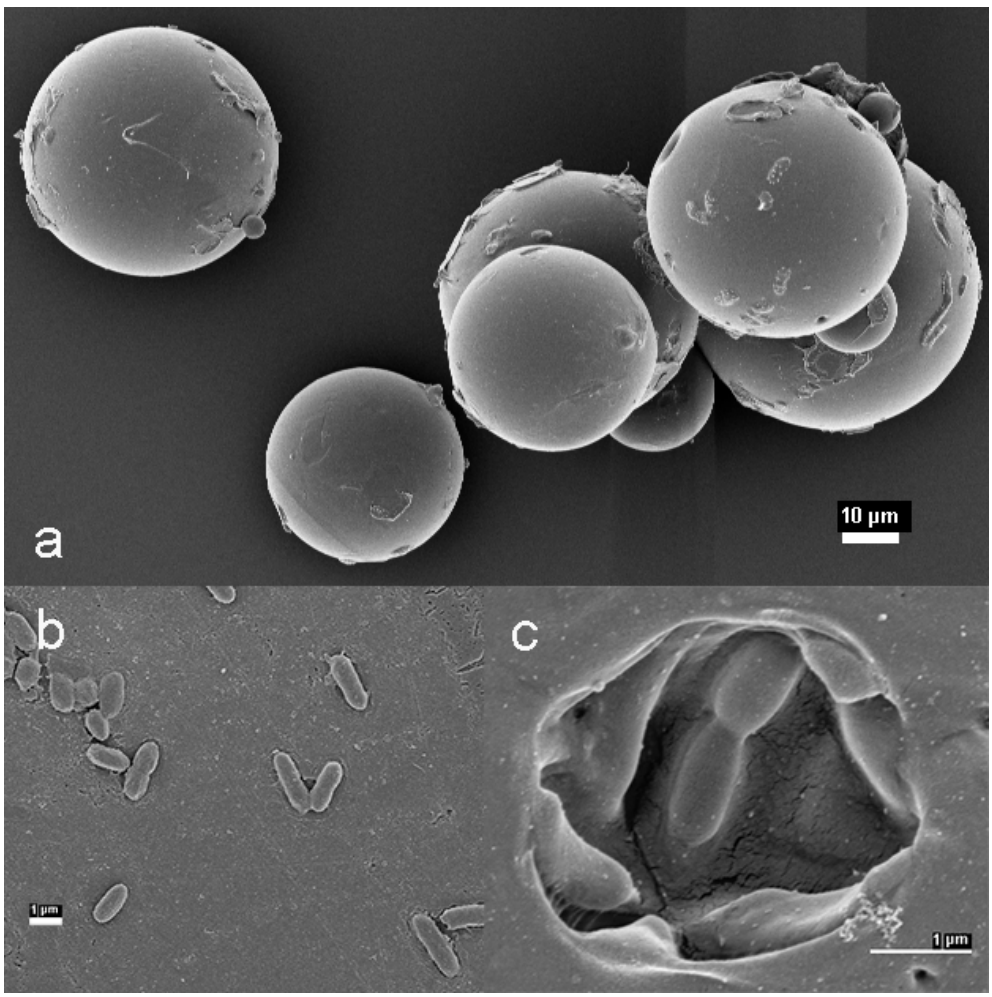

Figure 1.1. Scanning electron micrograph of poly(D,L-lactide-co-glycolic acid) microparticles encapsulating *Aeromonas hydrophila* formalin-killed cells (a, bar = 10 µm). Scanning electron micrograph of sectioned poly(D,L-lactide-co-glycolic acid) microparticles (bar = 1 µm). *Aeromonas hydrophila* cells (b) and the particle surface (c) are shown.

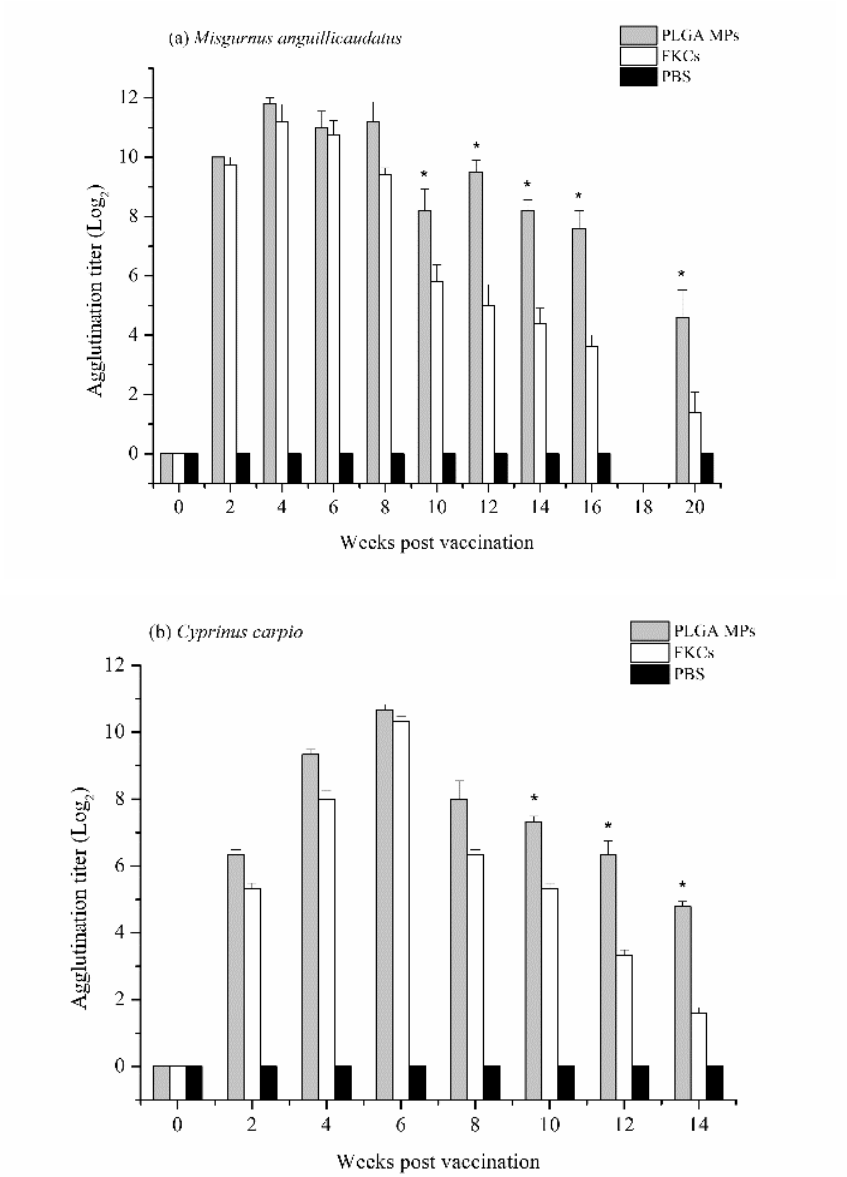

Figure 1.2. Serum agglutination titers of *Misgurnus anguillicaudatus* (a) and *Cyprinus carpio* (b) having been intraperitoneally administered *Aeromonas hydrophila* formalin-killed cells (FKCs) in poly(D,L-lactide-co-glycolic acid) microparticles, FKCs alone, or phosphate-buffered saline. Bars represent means ± standard errors of the mean (n = 3). *P < 0.05.

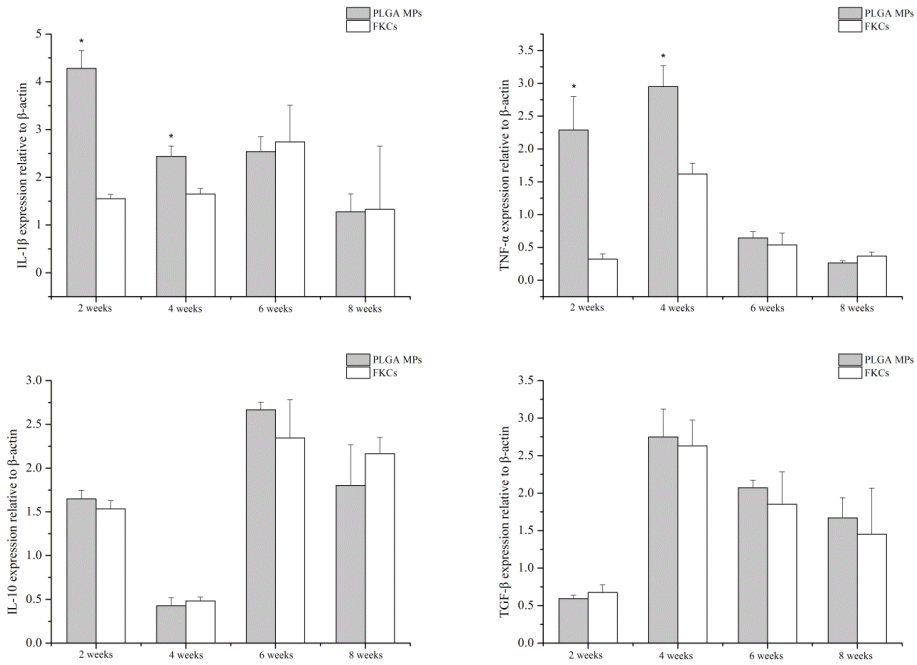

Figure 1.3. Relative mRNA expression of pro-inflammatory (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10 and TGF-β) in the head kidneys of *Cyprinus carpio* intraperitoneally administered *Aeromonas hydrophila* formalin-killed cells (FKCs) in poly(D,L-lactide-co-glycolic acid) microparticles, FKCs alone, or phosphate-buffered saline. Bars represent means ± standard errors of the mean (n = 3). *P < 0.05.

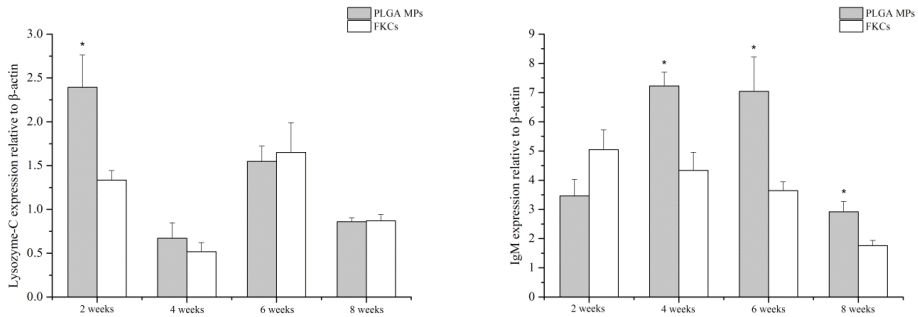

Figure 1.4. Relative mRNA expression of lysozyme C and IgM in the head kidneys of *Cyprinus carpio* intraperitoneally administered *Aeromonas hydrophila* formalin-killed cells (FKCs) in poly(D,L-lactide-co-glycolic acid) microspheres, or FKCs alone. Bars represent means ± standard errors of the mean (n = 3). *P < 0.05.Relative mRNA expression of pro-inflammatory

Chapter II

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

Abstract

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*.

Key words: *Aeromonas hydrophila*, *Cyprinus carpio*, Inactivated antigen, Phage lysate, Poly(d,l lactide-co-glycolic acid)

2.1. 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). *A. 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 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.

2.2. Materials and methods

2.2.1. 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).

2.2.2. 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.

2.2.3. *Antigen preparation*

2.2.3.1. *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).

2.2.3.2. *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.

2.2.3.3. *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.

2.2.3.4. *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.

2.2.4. *In vivo experiments*

2.2.4.1. *Vaccination*

The 1,300 carp were randomly divided into seven experimental groups **(Table**

2.1). 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 2.1**. Control fish were injected intraperitoneally with 0.1 mL sterile PBS.

2.2.4.2. *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}). LD_{50} 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.

2.2.4.3. *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).

2.2.4.4. *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.

2.2.5. Immune response assessment

2.2.5.1. 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.

2.2.5.2. 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.

2.2.5.3. *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 2.2**.

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.

2.2.6. *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.

2.3. Results

2.3.1. 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 **(Figure 2.1)**. Serum titers indicated no detectable antibodies prior to vaccination in all groups.

2.3.2. 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 **(Figure 2.2)**. All dead fish exhibited typical 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).

2.3.3. *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 **(Figure 2.3)**. 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 the other groups **(Figure 2.4)**.

2.4. 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.

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.

2.5. References

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Table 2.1. 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.

Table 2.2. Primers used for amplification of specific transcripts by quantitative PCR in the study.

Target		Sequence (5′ to 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.

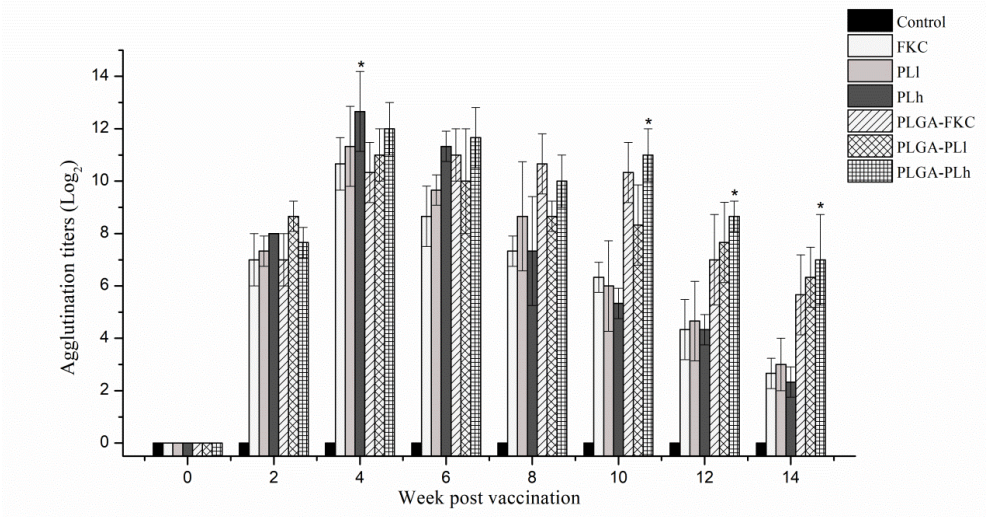

Figure 2.1. Serum agglutination titers of *C. carpio* intraperitoneally administered formalin killed cells of *A. hydrophila* JUNAH strain (FKC) and its PLGA encapsulated microparticles (PLGA-FKC), low (PLl) and high (PLh) dose lysate of *A. hydrophila* JUNAH strain infected with pAh 6-c phage and its PLGA encapsulated microparticles (PLGA-PLl and PLGA-PLh), or phosphate buffered saline (Control). Bars represent mean ± standard error of the mean (n = 3). * $P < 0.05$.

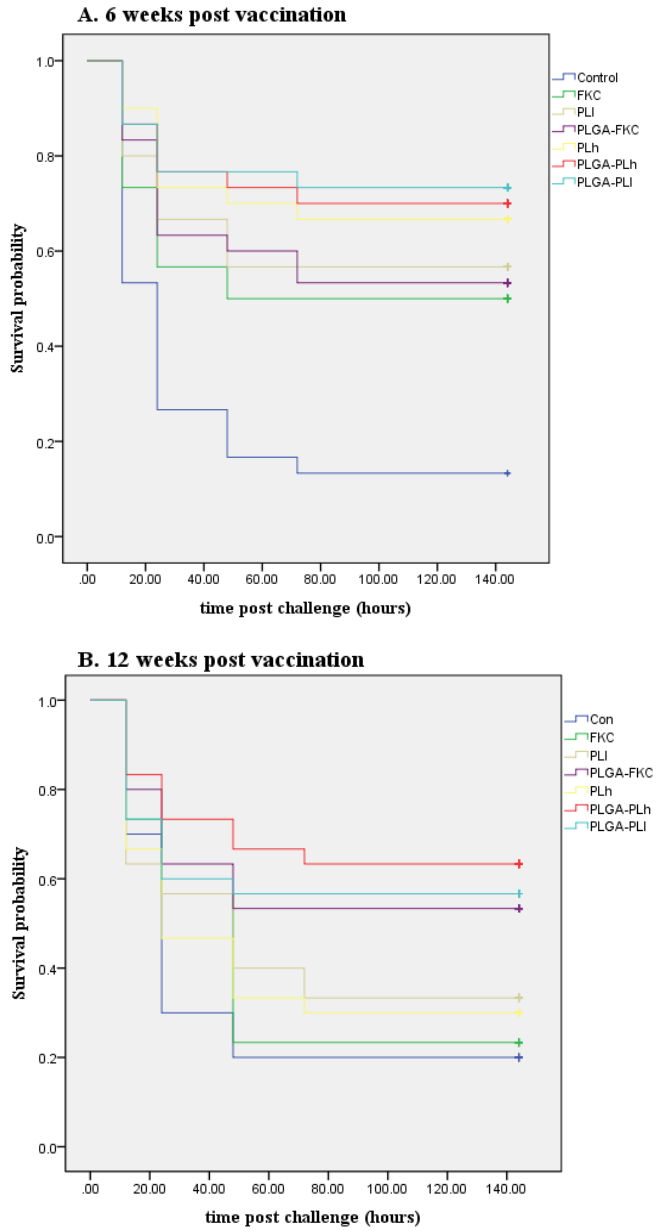

Figure 2.2. Kaplan-Meier survival curve of challenge experiment on *C. carpio*. The survival differences among vaccinated groups (FKC, PLl, PLh, PLGA-FKC, PLGA-PLl, PLGA-PLh) and control are illustrated for n = 30 at 6 weeks post vaccination (A) and 12 weeks post vaccination (B).

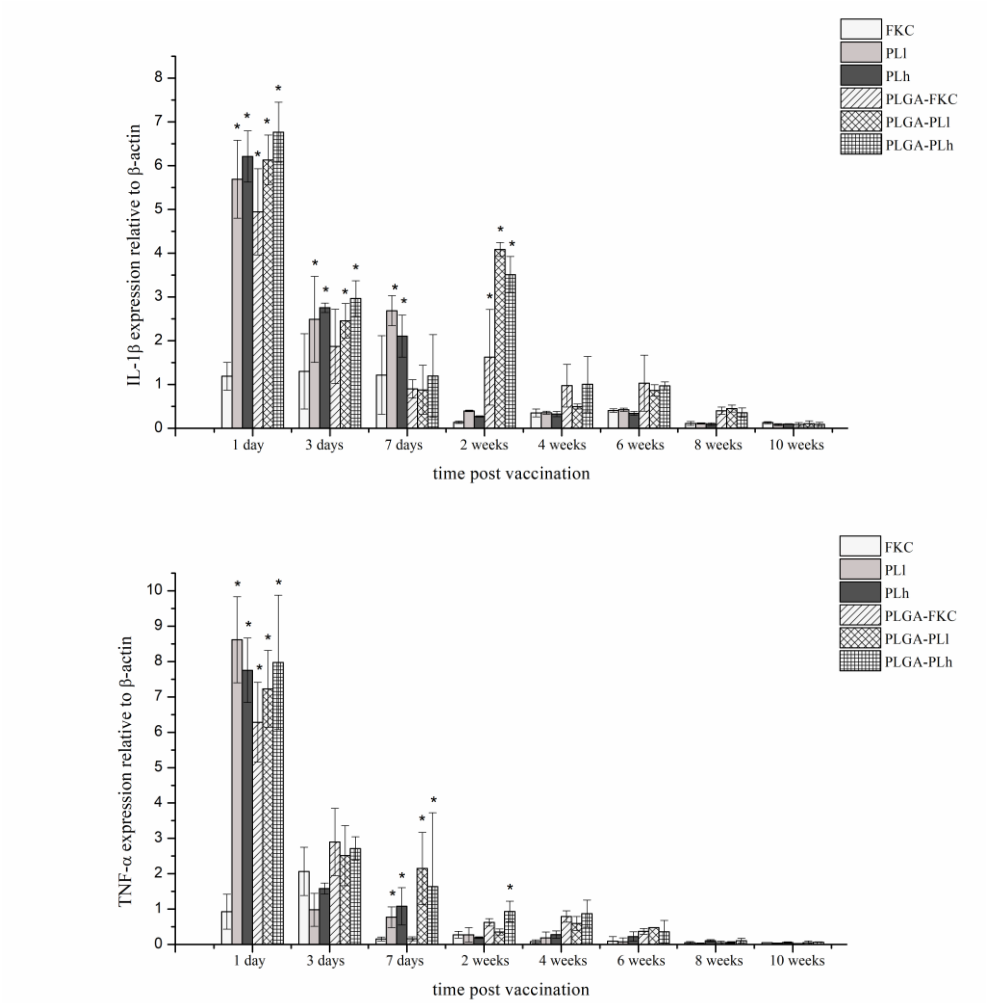

Figure 2.3. Relative mRNA expression of pro-inflammatory factors IL-1β and TNF-α in the head kidneys of *C. carpio* intraperitoneally administered formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC) and its PLGA encapsulated microparticles (PLGA-FKC), low (PLl) and high (PLh) dose of *A. 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). * $P < 0.05$.

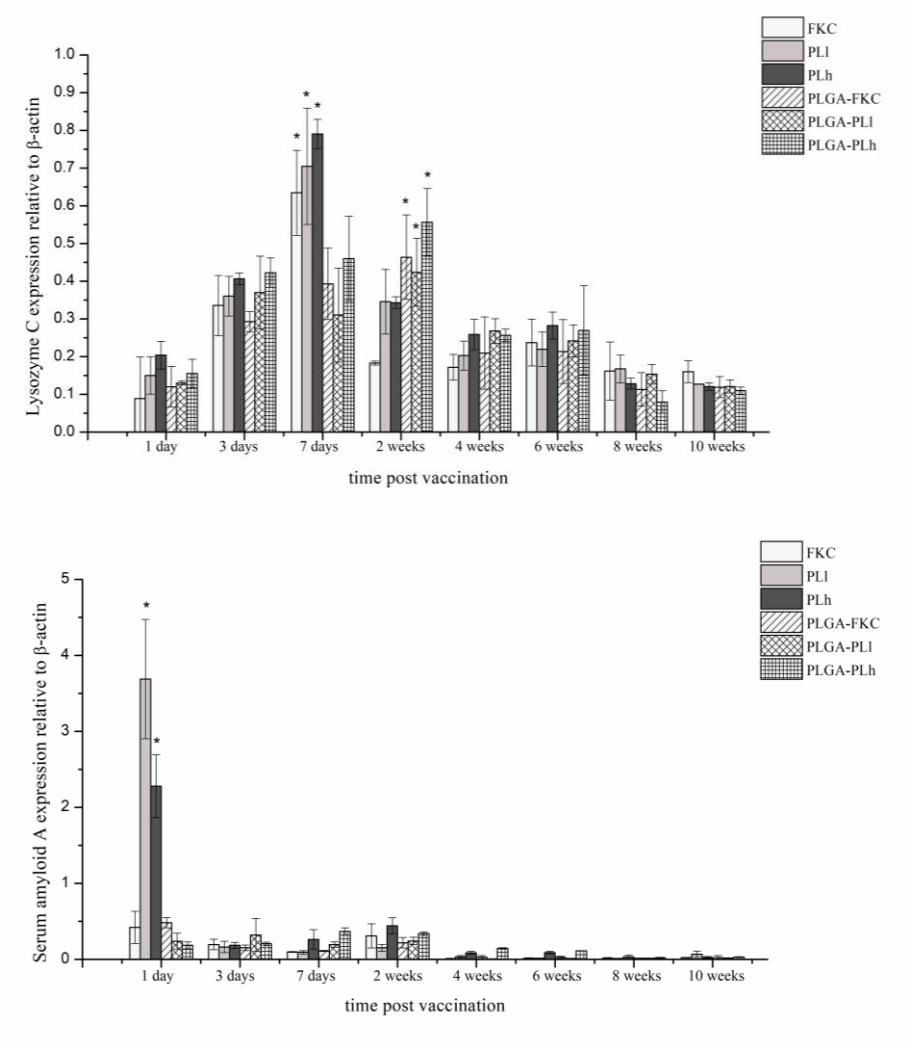

Figure 2.4. Relative expression of lysozyme C and serum amyloid A (SAA) transcripts in the head kidneys of *C. carpio* intraperitoneally administered formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC) and its PLGA encapsulated microparticles (PLGA-FKC), low (PLl) and high (PLh) dose of *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). * $P < 0.05$.

Chapter III

Enhanced bath immersion vaccination through microbubble treatment in the cyprinid loach

Abstract

Immunization by bath immersion is likely the simplest method of fish vaccination. Although the route of immunogenicity has not been fully identified, immersion vaccination is clearly a useful labor-saving technique. In this study, microbubble (MB) treatment was assessed for its ability to improve the efficacy of bath immersion vaccination in the cyprinid loach. MBs are commonly defined as minute particles of gas with a diameter of less than 100 μm, which generated free radicals. Here, the efficacy of MB treatment for vaccination enhancement in the cyprinid loach was assessed in direct challenge experiments using the virulent *Aeromonas hydrophila* JUNAH strain; assessments comprised agglutination titer assay and non-specific parameter analysis. Agglutination titers were high in loaches that were immunized via injection with inactivated cells (FKC group); however, non-specific immune activation parameters (e.g., lysozyme, superoxide dismutase, and phagocytic activity) were more increased in loaches that were immunized via bath immersion with MB treatment. Moreover, MB-treated loaches showed comparable survival rates, relative to loaches immunized via injection with formalin inactivated cells. Thus, higher levels of non-specific immune parameters

suggest increased efficacy of this vaccine approach. Improving the effectiveness of bath immersion vaccine will increase its affordability and ease of application in aquaculture.

Key words: *Aeromonas hydrophila*, Bath immersion, Cyprinid loach, Formalin-inactivated cells, Microbubbles, Vaccine

3.1. Introduction

Aquaculture is an important component of a fishery for food, according to the Food and Agriculture Organization of the United Nations. Loss reduction is critical for maintaining stable food production. A variety of factors affect aquaculture, such as optimal husbandry, biosecurity, nutrition genetics, system management, and water quality; however, disease outbreaks are a critical factor in aquaculture industry losses (1−4).

For bacterial and parasitic diseases, there are many available chemotherapeutants. These antibiotics and parasiticides are effective for disease treatments; however, they can cause damage to the aquatic environment and may lead to problems associated with antibiotic resistance. Vaccination is gradually gaining acceptance as an important component of aquaculture, based on its effectiveness in controlling disease outbreaks (5). A few aspects must be considered to overcome the disadvantages involved in vaccination. First, high-priced vaccines are difficult to be applied economically, compared to those used in other farming industries, such as pig or cattle rearing. Second, the administration method influences the use of the vaccine; the syringe injection method is an effective immunostimulation technique compared to oral and immersion routes, but is labor-intensive, thus requiring a greater financial investment. The route of immunogenicity of the immersion vaccine has not been fully identified, and host antigen absorption is affected by various conditions (e.g., vaccine concentration,

immersion time, animal size, adjuvant use, antigenic form, and water temperature) (6). However, immersion vaccination is clearly a useful labor-saving technique.

Microbubbles (MBs) are commonly defined as minute particles of gas with a diameter of less than 100 μm (7). MBs exhibit characteristics that differ from those of macro bubbles, which are greater than 100 μm in size. MBs have been used in aquaculture to improve the seafloor environment and water quality, as well as to reduce the rate of mortality and promote growth by enhancing available oxygen in shellfish farms (8−10). These bubbles rise slowly to the surface and remain in solution for an extended period; moreover, they can aid in gas dissolution into solution (11). In MBs, surface tension causes high internal pressure, which can cause the gas to dissolve in the surrounding water, resulting in condensation and collapse (7); free radicals are generated when the bubbles are collapsed, and these are useful for decomposition of organic chemicals and for wastewater treatment processes (12).

Immune responses induced by immersion vaccination are generally less robust and have shorter durations than those obtained by injection (13−15). To increase the uptake of vaccines by fish mucosal tissues, thereby maximizing the efficacy of immersion vaccination, hyperosmotic immersion and ultrasound-mediated immersion have been adopted (16−20). The free radicals produced by microbubbles could be able to stimulate the mucosal surfaces of fish, such as in the preceding methods, to increase the uptake of antigens (21, 22). In the present study,

MB treatment was used to enhance the efficacy of bath immersion vaccination; the efficacy of MB-enhanced vaccination was evaluated in a cyprinid loach model through the direct challenge with the virulent *Aeromonas hydrophila* JUNAH strain. The immunogenicity of the vaccines was assessed by agglutination test and the activities of lysozyme (LZM), superoxide dismutase (SOD), and phagocytosis.

3.2. Materials and methods

3.2.1. *Ethics statement*

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

3.2.2. *Animals*

A total of 700 cyprinid loaches (*Misgurnus anguillicaudatus*, mean body weight ± standard deviation: 8.28 ± 1.85 g) were purchased from commercial fish farms in Gyeonggi province, Republic of Korea. The fish were acclimatized in the laboratory of the College of Veterinary Medicine of SNU (Seoul, Republic of Korea) for 30 days before commencing the experiment. Fish were kept in 200-L fiberglass tanks at 25 ± 2 °C and fed twice per day with commercial feed (Tetra Bits Complete, Tetra, USA). Approximately 30% of the water in each tank was changed

daily.

3.2.3. Vaccine preparation

Aeromonas hydrophila JUNAH strain, stored in the lyophilized condition in our laboratory was used for this study. The bacterial strain was isolated from a cyprinid loach in 2009, in the Republic of Korea (23). The bacteria were recovered from the lyophilized condition and sub-cultured in tryptic soy broth (Difco, Detroit, MI, USA) at 25 °C for 24 h. The cultured bacterial cells were harvested and suspended in phosphate buffered saline (PBS), then treated with 0.5% formalin (v/v) and maintained at 25 °C for 48 h. The cells were washed twice and resuspended in PBS.

3.2.4. Vaccination

The 700 loaches were randomly divided into 10 experimental groups, immunized with 3 different methods. Immersion-vaccinated groups (IM1, IM2, and IM3) were bath immersion-vaccinated for 1 h with formalin-inactivated *A. hydrophila* cells (1×10^9 colony-forming units (CFU)/mL). The groups, immersion-vaccinated with MB-treatment (MB1, MB2, MB3, MB4, and MB5), were immersion-vaccinated for 1 h with formalin-inactivated *A. hydrophila* cells (1×10^9 CFU/mL); concurrent treatment with MBs was performed. The MBs were generated by using a homogenizer (HG-15D; DAIHAN Scientific, Republic of Korea) at a rate of 6000 rpm, and continuously supplied into vaccinated water tanks. The formalin-inactivated cells group (FKC) was immunized with 0.1 mL of

formalin-inactivated *A. hydrophila* cells (2×10^8 CFU/fish), through intraperitoneal injection. Control fish were treated with microbubbles for 1 h. Vaccinated fish were immediately returned to their rearing tanks.

IM1 and MB1 groups were immersion-vaccinated only 1 time. IM2 and MB2 groups were booster-vaccinated once, 1 week after initial vaccination. IM3 and MB3 groups were booster-vaccinated twice, at 1-week intervals. The MB4 group was booster-vaccinated 3 times, at 1-week intervals. The MB5 group was booster-vaccinated twice, at 1-week intervals, then booster-vaccinated again at 6 weeks post-vaccination (wpv). Experimental groups and corresponding schedules of vaccination are listed in **Table 3.1**.

3.2.5. *Sample collection*

Blood specimens for agglutination titer, LZM, and SOD analysis were collected from the caudal vein of three randomly chosen fish in each group (IM1, IM2, IM3, MB1, MB2, MB3, MB4, MB5, FKC, and control) after they were anesthetized with MS-222 (300 ppm). For measurement of phagocytic activity, a syringe with anticoagulant was used to collect specimens. For the agglutination assay, samples were collected at 2, 4, 6, 8, and 10 wpv; for the immune response assessment, samples were collected at 1, 2, 3, and 4 wpv. Blood specimens for agglutination, LZM activity, and SOD activity assays were transferred to microcentrifuge tubes (Eppendorf, Hamburg, Germany); serum was collected after centrifugation at 6500 g for 10 min at 4 °C. Then, the collected serum was stored at −20 °C until use.

Leukocytes from whole blood were separated using the Ficoll gradient method described by Noble et al. (24). Cell viability was evaluated using the trypan blue exclusion test and cell concentration was determined using a hemocytometer. Harvested cells were adjusted to 1×10^6 cells/mL for use in the assay.

3.2.6. *Serum agglutination assay*

The assay was conducted in microtiter plates with U-shaped wells. Serum samples (n = 3) were serially two-fold diluted in PBS and homologous heat-killed *A. hydrophila* (10^7 cells/mL) was added. Serum agglutination activity was determined on the basis of the lowest dilution without agglutination and is expressed as the reciprocal of that dilution. This test was performed once.

3.2.7. *Non-specific immune parameters*

LZM and SOD activities were measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). One unit of lysozyme activity was defined as the amount of lysozyme that caused a reduction in absorbance of 0.001 unit per min at 530 nm; one unit of SOD was defined as the amount required to reduce the rate of xanthine reduction by 50% in a 1-mL reaction system. The optical density for SOD activity was measured at 550 nm. Assessment of phagocytic activity in whole blood was performed using a commercial kit (CytoSelect™ 96-Well Phagocytosis Assay, Cell Biolabs, San Diego, CA, USA). *Escherichia coli* was used as a substrate and the absorbance was measured at

450 nm.

3.2.8. *Challenge experiment*

The experimental groups (n = 30) were challenged with *A. hydrophila* JUNAH stain at 4 wpv with the median lethal dose by intraperitoneal injection. Fish were anesthetized using MS-222 (300 ppm) before the challenge experiment. Cumulative mortalities and clinical signs were monitored twice per day for 2 weeks. The head kidneys of dead fish were streaked onto tryptic soy agar, and isolated bacteria were identified by polymerase chain reaction (PCR) method as previously described (25). Vaccine efficacy was assessed by relative percent survival (RPS) using the following formula:

RPS = (1 – (cumulative mortality of vaccinated group/cumulative mortality of control group)) × 100.

3.2.9. *Statistical analysis*

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.

3.3. Results

3.3.1. *Safety of microbubble treatment*

MB treatment was performed for 3 h in a group of fish (n = 10) that were observed for 2 weeks to verify the safety of the free radicals generated by the collapse of MBs; notably, no symptoms or mortality occurred in those fish. These findings suggested that no safety issues would be encountered in the present study because 1 h of vaccination was used in this safety assay.

3.3.2. *Antibody titers*

The MB groups showed tendencies for higher titers than the IM groups. The MB1 group showed significantly higher titers than the IM1 group at 2 and 4 wpv. A higher number of vaccinations was associated with higher antibody titer in both IM groups and MB groups. However, the MB3 group, which underwent booster-vaccinated 3 times, and the MB4 group, which underwent booster-vaccinated 4 times, showed similar antibody titers. The MB5 group was designed to ensure that antibody titer is induced again upon booster-vaccination following a delay after initial vaccination. The antibody titer of the MB5 group peaked at 4 wpv and then decreased; however, it increased again at 8 wpv after vaccination at 6 wpv. Throughout the experimental period, the FKC group showed higher titers than the other groups. Agglutination titers in the control groups remained at zero throughout the experimental period, as shown in **Figure 3.1**. Serum titers indicated no detectable antibodies prior to vaccination in all groups.

3.3.3. *Non-specific immune response*

In the analysis of LZM activity, MB groups (MB1, MB2, and MB3) showed significantly higher activity than IM groups (IM1, IM2, and IM3) at 2, 3, and 4 wpv. IM and MB groups showed no significant differences in the first week; however, the MB groups showed increased LZM activity after 2 wpv, which peaked at 3 wpv and then decreased. Group MB3 showed a significantly higher level of LZM activity than group FKC at 3 and 4 wpv. LZM activity in Group MB2 also consistently increased and was higher than that of group FKC at 4 wpv **(Figure 3.2)**. The serum SOD activity of the MB groups was significantly greater than that of the IM groups throughout the experimental period; it peaked at 1 wpv, then slowly decreased. The MB groups showed similar levels of SOD activity to those of the FKC group during the first week. Thereafter, the MB groups showed significantly greater levels of SOD activity; after 3 wpv, the levels were maintained in a stable manner **(Figure 3.3)**. The phagocytosis indexes of the MB groups were higher than those of IM groups throughout the experiment. The phagocytosis index of the MB1 group peaked at 2 wpv, then slowly decreased; however, the phagocytosis index of the MB2 and MB3 groups were sustained throughout the experiment. These groups showed lower phagocytosis indexes than that of the FKC group at 1, 2, and 3 wpv; However, the MB2 and MB3 groups showed higher phagocytosis indexes than that of the FKC group at 4 wpv, because the phagocytosis index of the FKC group decreased **(Figure 3.4)**.

3.3.4. Survival analysis

The cumulative mortality was observed after the challenge experiment at 4 wpv **(Figure 3.5)**. In all groups, mortality began at 6 h post-infection and continued through 72 h after challenge. The observation was performed for 2 weeks, and the surviving fish remained alive for the rest of the experiment; they showed no symptoms after 72 h. The FKC group showed higher RPS (43.48%), relative to that of the control group. The survival rates of both IM and MB groups also tended to increase as the number of vaccinations increased. The MB1 group showed a higher survival rate (26.09%) than the IM1 group (13.04%); two or more vaccinations led to higher survival (MB2 (52.17%), MB3 (56.52%) and MB4 (52.17%)) than that observed in the FKC group. There were no notable differences among the groups (MB2, MB3, and MB4) that showed higher survival rates than the FKC group **(Figure 3.5)**. All fish that died during the challenge experiment exhibited typical clinical signs of A. hydrophila infection. The isolated bacteria were confirmed to be A. hydrophila by using the PCR method (data not shown).

3.4. Discussion

Currently, injection of inactivated vaccines is used in the mainstream aquaculture industry, as inactivated vaccines can be produced inexpensively, and are generally recognized to induce stronger immunity than other vaccination methods (e.g., oral vaccines and immersion vaccines) (26, 27). The immersion

method is not widely used because its effects are weaker than those of injection vaccines. However, the immersion method, unlike the injection vaccine approach, can greatly reduce the manpower required for the vaccination process. This provides a competitive advantage in that it allows easy vaccination without the aid of experts. The purpose of this study was to improve the effectiveness of the bath immersion technique using MB treatment. Notably, MB treatment is used as a clean and efficient method to purify drinking water and wastewater, and has attracted increasing research interest in recent years (26); moreover, it has physical and chemical properties that can modify fish skin. The size of MBs is gradually reduced as it rises and subsequently collapses because of the dissolution of interior gases into the surrounding water (7). The temperature inside the bubbles at the time of collapse is > 5000 K; thus, free radicals (e.g., superoxide and hydroxyl) are created (12, 28, 29, 30, 31). These may be involved in the initiation of free radical chain reactions, which may damage cells (32). Cell membrane-dependent functions are affected by membrane fluidity and physical state, both of which are determined by the membrane lipid acyl chain profile; notably, acyl chains are transformed by free radicals (33). External free radicals could affect the mucous cell membrane of the fish in a manner similar to that of hyperosmotic pretreatment, which affects osmotic pressure in the cell membrane, as well as in a manner similar to that of ultrasound treatment, which widens the intercellular space (34, 35). Using these features of MB, this study was intended to confirm the possibility of improving the effectiveness of the bath immersion technique. Further studies are needed to reveal

the mechanism by which MB treatment affects the absorption of antigens.

After immunization, fish that were injection-vaccinated with inactivated bacteria showed increased agglutination titer against A. hydrophila. The MB groups showed higher antibody titers than IM groups. These results are similar to those of prior studies that used hyperosmotic pretreatment to accelerate the uptake of antigens (36). During all experimental periods, the FKC group showed higher titer than that of all other groups, peaking at 4 wpv then gradually decreasing; this was similar to the findings of our previous study (37). In general, high antibody titers are considered to have high protection capabilities. However, specific serum antibody titers are not always related to the level of protection (38, 39). Thus, further research is needed to investigate the underlying mechanism.

The levels of important immunological parameters, such as LZM, SOD, and phagocytic activities in fish were measured, compared to control fish. LZM is an enzyme that splits peptidoglycan in the bacterial cell wall and plays an important role in natural defense mechanisms (40). In higher vertebrates, this enzyme is speculated to contribute to opsonization, immune response potentiation, and antiviral activity (41). SOD is a primary antioxidant defense enzyme generated in response to oxidative stress. Phagocytic cells also play an important role in antibacterial defense mechanisms (42). LZM activity was significantly higher in immunized groups, indicating that the vaccine elicited a non-specific immune response (43). Wang et al. reported that SOD activities also significantly increased in Chinese breams immunized with the Omp38 recombinant protein (44). In

assessments of all parameters, the FKC group showed the highest levels in the first week, but MB2 and MB3 groups both showed the highest levels at 4 wpv. The method of MB treatment is expected to induce non-specific immune responses, as MB-treated groups showed higher levels than all IM groups in assessments of LZM, SOD, and phagocytic activities. Superoxide anion, a type of free radical generated by MBs, is converted rapidly into H_2O_2, which freely cross cell membranes (45) and reacts with thiols to form disulfide bonds (46). In addition, H_2O_2 has been reported to participate in many cellular processes, such as cell growth, stem cell renewal, cell death, cell senescence, and cell migration, as well as immune responses (47−51). This bath immersion vaccine enables vaccination with multiple treatments at low cost, such that it is possible to induce greater non-specific immune responses than with the original FKC vaccine.

Agglutination titers of the MB2 and MB3 groups were lower than those of the FKC group during this experimental period, but non-specific immune parameters were significantly higher. These non-specific immune parameters, as well as antibody titers, are critical to protection from diseases (52, 53). Immunization by immersion is likely to be the simplest method of vaccination (54). Moreover, if the effectiveness of bath immersion vaccine can be improved through a variety of approaches, it could be more broadly applied in the prevention of fish diseases.

In this study, MBs were used to enhance the efficacy of bath immersion vaccine against *A. hydrophila* infection. The MB-treated groups, MB3 and MB4, showed higher survival rates that were comparable to those of the FKC group; those groups

also showed high levels of non-specific parameters. This suggests that MB-treated bath immersion vaccination groups may be effective than an immersion vaccinated only groups, and in some respects, it may be equally or more effective to FKC injection vaccination. Therefore, consideration of its application in a fish farm may be useful, according to the results of this study. Through research involving more diverse techniques, the efficacy of the bath immersion vaccine may be improved, which could lead to the provision of an affordable and easy to apply vaccine for aquaculture.

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Table 3.1. Experimental groups and immunization schedule.

Experimental groups	Methods	Vaccination (weeks)						
		0	1	2	3	4	5	6
FKC	IP[a]	Treated						
IM1	IM[b]	Treated						
IM2	IM	Treated	Treated					
IM3	IM	Treated	Treated	Treated				
MB1	MB[c]	Treated						
MB2	MB	Treated	Treated					
MB3	MB	Treated	Treated	Treated				
MB4	MB	Treated	Treated	Treated	Treated			
MB5	MB	Treated	Treated	Treated				Treated

Formalin-inactivated cells (FKC) group was vaccinated with 2 x 10^8 CFU/fish of *Aeromonas hydrophila* inactivated cells by intraperitoneal injection.

Immersion-vaccinated groups (IM1, IM2 and IM3) and immersion-vaccinated with microbubble-treated groups (MB1, MB2, MB3, MB4 and MB5) groups were bath-immersed with 1 x 10^9 CFU/L of *A. hydrophila* inactivated cells.

[a]IP, intraperitoneal injection; [b]IM, immersion; [c]MB, immersion with microbubble treatment.

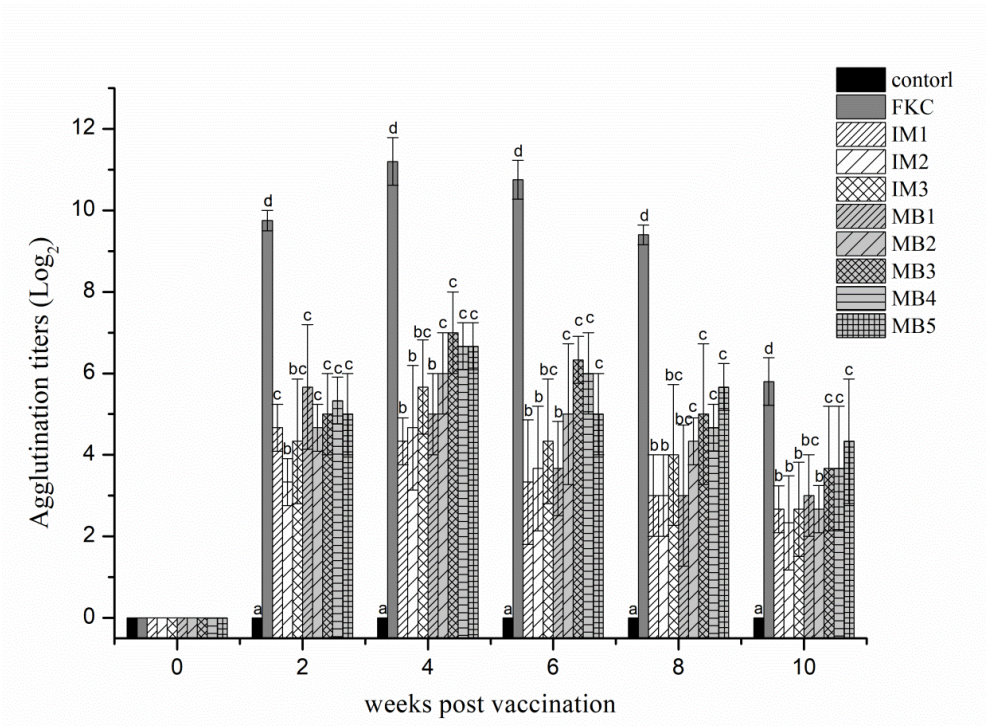

Figure 3.1. Serum agglutination titers of cyprinid loach vaccinated formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC) by intraperitoneal injection, and bath immersion of *A. hydrophila* JUNAH strain (IM1, IM2, and IM3), bath immersion of *A. hydrophila* JUNAH strain with microbubble treatment (MB1, MB2, MB3, MB4, and MB5), or microbubble treatment without antigens (Control). Bars represent mean ± standard error of the mean (n = 3). Different letters above the bars represent the statistical significance ($p < 0.05$) among different groups at the same time point.

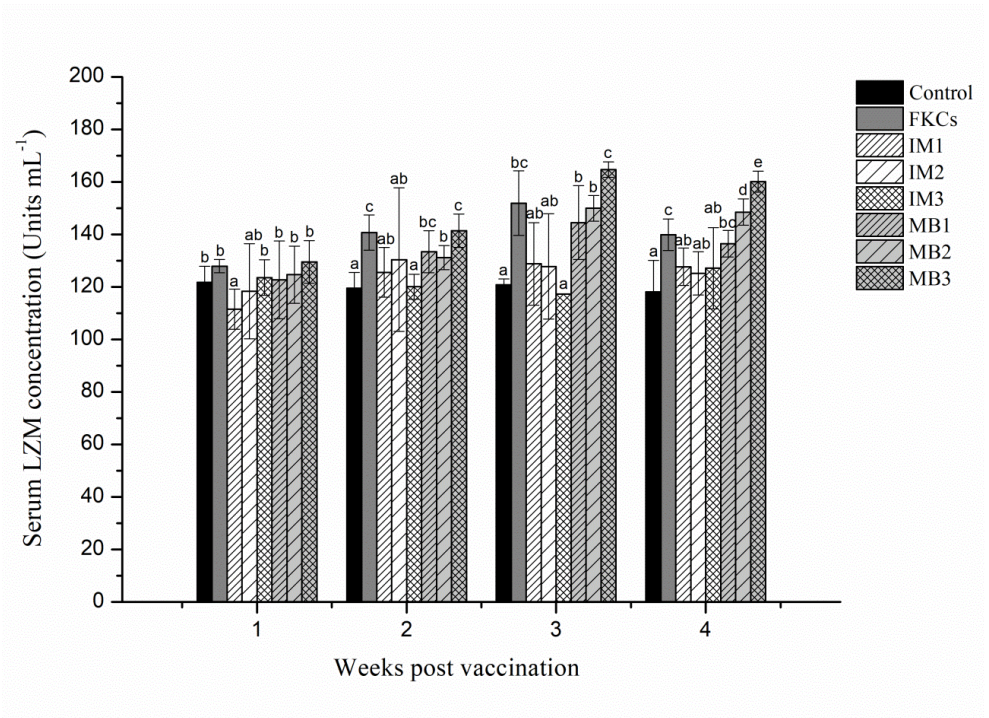

Figure 3.2. The activity of lysozyme in the blood of cyprinid loach, intraperitoneally administered formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC), vaccinated by bath immersion method (IM1, IM2 and IM3), vaccinated by bath immersion method with microbubble treatment (MB1, MB2 and MB3), or microbubble treatment without antigens (Control). Bars represent mean ± standard error of the mean (n = 3). Different letters above the bars represent the statistical significance ($p < 0.05$) among different groups at the same time point.

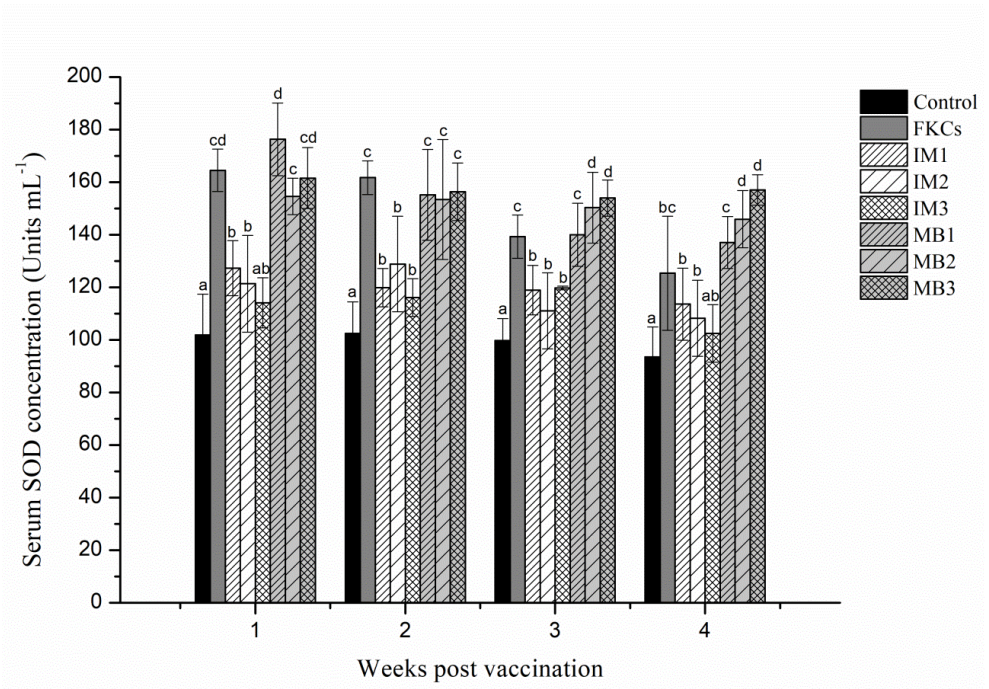

Figure 3.3. The activity of superoxide dismutase in the blood of cyprinid loach, intraperitoneally administered formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC), vaccinated by bath immersion method (IM1, IM2 and IM3), vaccinated by bath immersion method with microbubble treatment (MB1, MB2 and MB3), or microbubble treatment without antigens (Control). Bars represent mean ± standard error of the mean (n = 3). Different letters above the bars represent the statistical significance ($p < 0.05$) among different groups at the same time point.

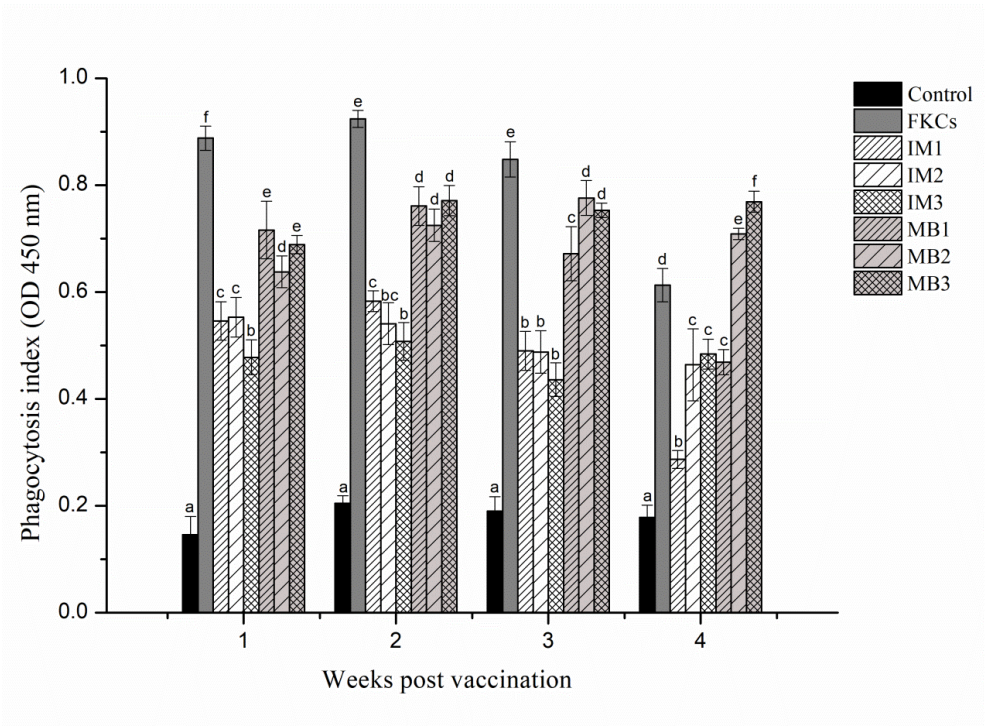

Figure 3.4. The activity of phagocytosis in the blood of cyprinid loach, intraperitoneally administered formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC), vaccinated by bath immersion method (IM1, IM2 and IM3), vaccinated by bath immersion method with microbubble treatment (MB1, MB2 and MB3), or microbubble treatment without antigens (Control). Bars represent mean ± standard error of the mean (n = 3). Different letters above the bars represent the statistical significance ($p < 0.05$) among different groups at the same time point.

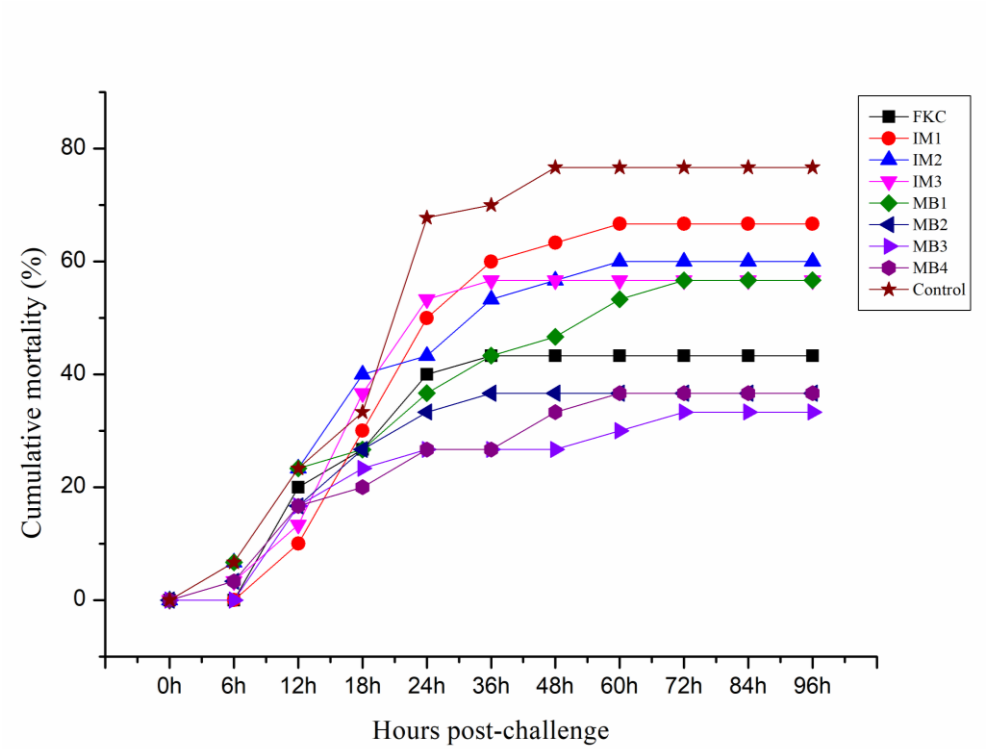

Figure 3.5. Cumulative mortality curve of challenge experiment on cyprinid loach, intraperitoneally administered formalin killed cells of *Aeomonas hydrophila* JUNAH strain (FKC), vaccinated by bath immersion method (IM1, IM2 and IM3), and vaccinated by bath immersion method with microbubble treatment (MB1, MB2, MB3, and MB4).

GENERAL CONCLUSION

For enhancing the efficacy of fish vaccines, various materials and methods were considered in the researches. At first, poly(d,l-lactide-co-glycolic acid) MPs encapsulating *Aeromonas hydrophila* FKCs were developed and evaluated. PLGA microparticles (MPs) approximately 36 μm in diameter were formed *A. hydrophila* formalin-killed cells (FKC) was encapsulated as an antigen. We observed that innate and adaptive immune responses of cyprinid loaches and common carp were increased than control group.

Secondly, we considered that the vaccine using phage lysates (PLs) antigen and PLGA encapsulation. It 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*.

Lastly, microbubbles (MBs) were used to enhance the efficacy of bath immersion vaccine against *A. hydrophila* infection. The MB-treated groups, MB3 and MB4, showed higher survival rates that were comparable to those of the FKC group; those groups also showed high levels of non-specific parameters. This

suggests that MB-treated bath immersion vaccination groups may be effective than an immersion vaccinated only groups, and in some respects, it may be equally or more effective to FKC injection vaccination.

Based on these results, the methods we used in the researches (PLGA encapsulation, Phage lysates and microbubble treatment) showed the possibility of enhancing the efficacy of original fish vaccines. These vaccines longer the its protective effect and enhance the own efficacy. In addition, the vaccines were designed to provide of an affordable and easy to apply vaccine for aquaculture. Therefore, the applications could be alternatives for developing novel potent vaccines in fish.

국문초록

어류 백신의 효능 향상을 위한 새로운 방법론에 관한 연구

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수산업에서 질병의 제어와 예방 아주 높은 우선순위를 가지며, 백신은 이 질병의 발생을 예방할 중요한 수단이다. 여기서, 대략 36 마이크로미터 크기의 poly(d,l-lactide-co-glycolic acid) (PLGA) microparticles (MPs)을 사용하여 *Aeromonas hydrophila* formalin-killed cells (FKC)을 항원으로 캡슐화 하였으며, 미꾸라지와 잉어에서 선천 및 적응 면역 반응을 평가하였다. PLGA MP 절편의 주사 전자 현미경 분석을 통해 항원은 잘 캡슐화 된 것을 확인 하였다. IL-1β, IL-10, TNF-α, lysozyme C, TGF-β, 그리고 IgM의 면역 관련 유전자의 상대적 mRNA 발현과 세균 응집 활성의 분석을 위해 혈액 및 두신 샘플을 수집하였다. 두 어종 모두에서 항체 역가 곡선은 PLGA 그룹이 FKC 그룹보다 완만하였다. 미꾸라지와 잉어의 항체 역가는 각각 8주와 6주를 기점으로 실험 종료에 가까워질수록 FKC 그룹

과의 차이가 벌어지기 시작하였다. 이후 두 어종은 각각 12주와 20주 그리고 10주와 14주에 *A. hydrophila* 균주를 공격접종 하였으며, 상대 생존률을 계산하였다. 두 종 모두에서 PLGA 그룹이 모든 실험 시점에서 높은 생존률이 나타났다. IL-1β와 TNF-α의 mRNA는 PLGA 그룹에서 2주와 4주에 유의미하게 상향 조절되었다. 더욱이 PLGA-MP 백신접종은 lysozyme C와 IgM의 mRNA 유전자 발현량이 각각 2주와 4주 그리고 6주와 8주에서 유의미하게 높게 나타나는 것을 확인하였다. 결론적으로 PLGA MP 백신은 FKC 백신보다 더 길고 강한 면역 반응을 유도할 가능성이 있으며, 미꾸라지와 잉어의 질병을 예방하는데 큰 효과가 있을 것으로 보인다.

지난 50년 동안 면역자극을 위한 항원의 개발을 위해 다양한 접근법들이 만들어져 왔다. 우리는 *A. hydrophila* JUNAH의 용해성 박테리오파아지인 pAh 6-c를 이용하여 불활화한 항원으로 구성되어 있는 파아지 용해물을 사용하여 잉어에서 *A. hydrophila* 감염을 예방하는 백신을 개발하고자 하였다. 앞선 연구에서 사용된 PLGA를 이용하여 항원을 캡슐화하여 백신으로 사용하였으며, 이를 선천면역과 적응면역 반응을 측정하여 효과는 평가하였다. 항체 역가 실험에서는 PLGA를 사용한 그룹이 캡슐화 없이 단독의 항원인 FKC나 phage lysate를 사용한 그룹보다 높은 항체가를 보여 주었다. 그러나 같은 PLGA 그룹 내에서도 phage lysate를 사용한

그룹이 FKC를 캡슐화한 그룹보다 높은 항체가를 나타내었다. TNF-α, IL-1β, lysozyme C, 그리고 SAA의 유전자 발현 또한 PLGA 그룹이 높은 발현량을 보였으며, phage lysate를 사용한 그룹이 더 높은 발현량을 보였다. 이러한 결과는 종합적으로 phage lysate가 FKC 보다 강도 높은 면역 반응을 유도할 잠재력이 있음을 보여준다.

침지법을 통한 면역강화는 어류 백신접종법 중 가장 쉬운 방법으로 보인다. 면역원성을 제공하는 경로가 아직 완전히 밝혀지지는 않았음에도 침지 백신법은 노동력 절감을 위한 아주 용이한 방법임에는 틀림이 없다. 본 연구에서는 마이크로버블 처리가 잉어에서 침지 백신의 효율을 증가시키는 능역이 있음을 평가 하고자 한다. 마이크로버블은 약 100 마이크로미터 미만의 직경을 가지는 작은 가스 입자로 정의 하고는 한다. 여기서는 백신의 효율 증진을 위한 마이크로버블 처리의 효율을 잉어에서 항체가와 면역인자 분석을 통해 평가하고자 한다. 항체가에서는 FKC를 복강 주사한 그룹이 침지 그룹들 보다 높게 나타났지만, lysozyme, superoxide dismutase, 그리고 phagocytosis 활성은 오히려 마이크로버블을 처리한 침지그룹에서 더 증가하는 것을 확인 하였다. 더욱이 마이크로버블 처리 그룹의 미꾸라지는 FKC 주사 그룹과 비교했을 때 비슷한 생존율을 보였다. 비특이성 면역 파라미터의 높은 수준은 이 백신법이 효력 증가를 시킬 수 있음을 보여준다.

지금까지의 연구들에서 생분해성 물질을 이용한 항원의 캡슐화 방법, 침지 백신의 효과 증진을 위한 방법, 새로운 항원의 적용을 통해 *A. hydrophila* 감염에 대한 어류백신의 효과를 향상시키고자 하였다. 본 연구에서 사용된 방법론들은 선천 면역과 적응 면역 반응의 분석과 공격실험을 통해서 기존의 FKC 백신과 비교하고자 하였으며, 훌륭한 결과는 보여 주었다. 그러므로 이 방법론들은 어류 질병의 예방을 위해 가치가 있을 것으로 보인다. 앞으로 우리는 수산업이라는 특성을 고려한 더 나은 백신의 개발과 공중 보건을 위해 노력해야 할 것이다.

Key words: *Aeromonas hydrophila*, microbubble, Microparticles, Poly(d,l lactide-co-glycolic acid) (PLGA), 잉어, 미꾸라지, 파아지 용해물, 수산양식

Student number: 2014-21933

List of published articles

In progress

1. **Saekil Yun**, Sib Sankar Giri, Hyoun Joong Kim, Sang Geun Kim, Sang Wha Kim, Se Jin Han, Jun Kwon, Woo Taek Oh, Se Chang Park. Vaccination of fish against *Aeromonas hydrophila* infections using the novel approach of transcutaneous immunization with dissolving microneedle patches. Fish and Shellfish Immunology. 2019.
2. **Saekil Yun**, Sib Sankar Giri, Hyoun Joong Kim, Sang Geun Kim, Sang Wha Kim, Se Jin Han, Jun Kwon, Woo Taek Oh, Se Chang Park. Effect of plasma activated water on the hatching rate of dormant cysts of brine shrimp, *Artemia salina*. Aquaculture. 2019.

2019

1. **Saekil Yun**, Sib Sankar Giri, Hyoun Joong Kim, Sang Geun Kim, Sang Wha Kim, Jung Woo Kang Se Jin Han, Jun Kwon, Woo Taek Oh, Cheng Chi, Jin Woo Jun, Se Chang Park. Enhanced bath immersion vaccination through microbubble treatment in the cyprinid loach. Fish and Shellfish Immunology. 2019(91), 12-18. https://doi.org/10.1016/j.fsi.2019.05.021.
2. **Saekil Yun**, Jin Woo Jun, Sib Sankar Giri, Hyoun Joong Kim, Cheng Chi, Sang Geun, Kim, Sang Wha Kim, Jung Woo Kang, Se Jin Han, Jun Kwon, Woo Taek Oh, Se Chang Park. Immunostimulation of *Cyprinus carpio* using phage lysate of *Aeromonas hydrophila*. Fish and Shellfish Immunology. 2019(86), 680-687.
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2. Hyoun Joong Kim, Jin Woo Jun, Sib Sankar Giri, **Saekil Yun**, Sang Guen Kim, Se Chang Park. Ostreid Herpesvirus-1μVar infection in bay scallop (*Argopecten irradians*) hatcheries in Korea. Marine Biotechnology Conference (MBC) 2019. Shizuoka, Japan, 2019.

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Acknowledgments

In February 2020, I finished all my doctoral degree courses and graduated from school. It's just one end, but there will be a bunch of new beginnings. Therefore, I believe that there's a long way to go. There have been so many funny, happy and sometimes sad things that have happened, but this is the most self-fulfilling moment of my entire life. It would not have been possible without infinite supports of my family members and advisor, professor Se Chang Park, to finish a doctoral degree.

Most of all, I would like to express my gratitude to Professor Se Chang Park who guided me. The teaching style and ideas of the professor have influenced me so much and will become a great part of my life. Many thanks to the professor who's trying to make a great researcher, not just the hand down the knowledge and skills, I was able to become a good researcher.

Without infinite support from the family members, I couldn't have done my research. I always feel grateful to father, mother, father-in-law, mother-in-law, and my wife, Chae-Young and son, Ethan.

The people who helped the most in my researches were probably colleagues who worked with me. They have helped me with so many things,

and the discussions with them were the source of creative ideas. I offer special thanks to the lab colleagues. Dr. Sib Sankar Giri, Prof. Jin Woo Jun, Hyoun Joong Kim, Sang Guen Kim, Prof. Cheng Chi, Jeong Woo Kang, Jun Kwon, Se Jin Han, Woo Taek Oh are all very grateful.

I met professor Seung-jun Lee of Seowon University through microneedle research. I am always thankful that he always helped me with difficult requests with a smile. It would have been impossible to start the microneedle research without his help. I hope he is always healthy and keeps a good relationship between me and him.

I was very lucky that I got noticed about the global Ph.D. fellowship (GPF) program of the national research foundation (NRF) in 2015. I believe that the five-year scholarship has enriched my research work, which has led to the quality of my research. Thank you to all of the members of the national research foundation.

I thank Professor Byung Chun Lee, Dr. Ji Hyung Kim, Dr. Jin Woo Jun, and Dr. Jee Eun Han, the committee for this thesis. Based on the detailed comments and advice, I was able to organize the final steps of my doctoral degree successfully.

I also would like to express my sincere thanks to everyone who has helped me with my researches. I will always reward their faith by performing honest and reliable research.

This work was supported by the Ministry of Education, through the Brain Korea 21 Program for Veterinary Science, Seoul National University and the Research Institute for Veterinary Science, Seoul National University, and by Global Ph.D. Fellowship Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015H1A2A1029732).

In the fall of 2019

Saekil, Yun.