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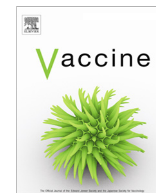

Efficacy of PLGA microparticle-encapsulated formalin-killed *Aeromonas hydrophila* cells as a single-shot vaccine against *A. hydrophila* infection

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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.

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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

Abbreviations: FKC, formalin-killed whole-cell; PLGA, poly(D,L-lactide-co-glycolic acid); MPs, microparticles; W/O/W, water-in-oil-in-water; PVA, poly(vinyl alcohol); TSA, tryptic soy agar; PBS, phosphate-buffered saline; SEM, scanning electron microscopy; CFU, colony-forming units; wpv, weeks post-vaccination; LD_{50}, median lethal dose; RPS, relative percent survival; qPCR, quantitative PCR.

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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*.

2. Materials and methods

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 °C 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.

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 °C for 24 h. Then, single colony was cultured in tryptic soy broth (TSB; Difco, Detroit, MI, USA) at 25 °C for 48 h. The cultured bacteria were treated with 0.5% formalin 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 re-suspended in sterile PBS. This suspension was adjusted to an optical density at 600 nm of 0.6 using a spectrophotometer.

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 5000*g* for 10 min. The recovered MPs were lyophilized for 48 h to preserve them for further use.

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).

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.

2.6. In vivo experiments

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.

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 °C for 24 h, with isolates being identified by PCR as previously described [24]. Vaccine efficacy was assessed by RPS using the following formula:

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