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Efficacy of a formalin-inactivated vaccine against *Streptococcus iniae* infection in the farmed grouper *Epinephelus coioides* by intraperitoneal immunization

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ABSTRACT

Vaccination is the most effective means of preventing infectious diseases; however, few vaccines are effective against *Streptococcus iniae* (*S. iniae*) in grouper. This work presents an efficacious and safe vaccine against *S. iniae* infections in the grouper *Epinephelus coioides*. The vaccine candidate was the *S. iniae* GSI-310 strain. The vaccination was administered by intraperitoneal injection, and consisted of formalin-inactivated antigens combined with an AS-F or ISA763A adjuvant. Peripheral blood samples were collected for RT-qPCR and phagocytosis and agglutination assays. Our results indicated that immunoglobulin M (*igm*) was maximally expressed in the two vaccinated groups at 3 months post-secondary vaccination (PSV). A significant upregulation of mRNA expression for interleukin-1β (*il-1β*) and tumor necrosis factor-α (*tnf-α*) was also observed in fish treated with antigens combined with ISA763A, which peaked at 3 months PSV. In fish treated with antigens combined with AS-F, *il-1β* and *tnf-α* expression peaked at 14 days post-primary vaccination (PPV). Phagocytic activity and index increased significantly in the two vaccinated groups. Furthermore, fish in the two vaccinated groups exhibited significantly elevated agglutination titers compared to fish in the control group, in which almost no agglutination reaction was detected. In the efficacy test, the vaccinated and control groupers were treated with *S. iniae* at 1, 3, and 6 months PSV. The relative percentage survival (RPS) values of antigens with AS-F and antigens with ISA763A were both 100% at 1 and 3 months PSV; at 6 months PSV, the RPS values for these groups were 100% and 97.7%, respectively. Furthermore, the level of protection observed in the field trial closely resembled that achieved on a laboratory scale. Therefore, the proposed vaccine mixed with AS-F or ISA763A improved immune responses and provided safe and long-lasting protection in farmed groupers.

1. Introduction

Streptococcus iniae (*S. iniae*) [1], a Gram-positive bacterium, induces streptococcosis, a disease characterized by meningoencephalitis, systemic septicemia, and skin lesions [2]; this disease can lead to heavy mortality [3]. *S. iniae* affects many cultured fish species such as rainbow trout [4,5], tilapia [6,7], and grouper [8]. Of high commercial value, groupers are widely cultured in Southeast Asia. Many diseases threaten the grouper industry, and vaccination remains the most effective means of reducing mortality. Unfortunately, vaccines especially those based on recombinant antigens or inactivated pathogens are not usually able to confer protection on their own [9]. Thus, adjuvants or immunopotentiators are often administered to increase vaccine efficacy [9]. Moreover, water-based formalin-killed vaccines provide a short duration of protection [10–12]. When the same antigen preparation was administered as either an oil adjuvant or a water-soluble preparation, only the oil-based vaccine conferred protection against *Photobacterium damselae* subsp. *piscicida* [13]. Oil adjuvant is characterized by its ability to hold antigens in a stable form, thus slowing antigens processing and prolonging both tissue retention and the period of immunestimulation [14,15]. In this work,

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Seppic MONTANIDE® ISA 763A (ISA763A) or AS-F adjuvants were used in combination with inactivated bacterin. Previous research used ISA763A as a non-mineral oil emulsion in a formulation of a metabolizable adjuvant, producing satisfactory results in animals vaccinated with parasitic antigens [16]; ISA763A has also been used in fish vaccinated with bacterial antigens [17]. In contrast, AS-F is a mineral oil-based adjuvant that is not yet commercialized. Notably, the physical properties of AS-F resemble those of ISA763A.

As the first antibody produced in the immune system, IgM provides a crucial first line of defense [18,19]. IL-1β and Tnf-α are important pro-inflammatory factors, and can induce an inflammatory response by regulating the expression of other cytokines [20]. This work investigated how the tested vaccine affects immuneresponse genes by evaluating the expression of *igm*, *il-1β*, and *tnf-α* via RT-qPCR. The immuneresponse was also examined using phagocytosis and serum agglutination reaction. Finally, we evaluated the effect of the vaccine under testing on the groupers in order to determine the feasibility of using this vaccine to prevent *S. iniae* infections in this fish species.

2. Materials and methods

2.1. Fish husbandry

In total, 810 groupers were reared in three aquaria (270 fish per aquarium). The mean grouper weight was 31.1 g. Fish were reared in an indoor re-circulated seawater aquatic animal culturing system. In the field trial, 1000 fish were bred in a farm located in the outlying coastal areas of southern Taiwan. Fish were placed randomly into two artificially ponds of equal size; one pond contained 500 vaccinated groupers and the other contained 500 non-vaccinated groupers (the control group). Fish were fed twice daily with a commercially available pelleted feed.

2.2. Bacterial culture and inactivation of bacteria

The *S. iniae* GSI-310 strain was cultured on tryptic soy agar (TS) with 1.5% NaCl (TSN) at 25 °C for 48 h. Colonies were subcultured onto brain heart infusion agar with 1.5% NaCl (BHIN) at 25 °C for 24 h. The cell concentration was then adjusted to an optical density of 3 (OD_{610} = 3; 10^{10} CFU/mL). The formalin-inactivated whole-cell vaccine was prepared as in Tsai et al. [21] with slight modifications. Following inactivation, the bacterial pellet was re-suspended at a final concentration of 10^{10} CFU/mL in PBS containing 0.01% formalin. By incubating the aqueous bacterin on a blood agar plate at 25 °C for 48 h, we confirmed that the bacterial cells were completely inactivated.

2.3. Virulence test (LD_{50})

S. iniae was used in the experimental infection of groupers to determine the LD_{50} dosage. Eight groups of 20 fish were tested. Through the serial dilution method, different concentrations of cells (10^{-1} to 10^{-7}) or sterile PBS were intraperitoneally injected into groupers. Next, 10 μL of different concentrations of bacteria were cultured onto TSN agar at 25 °C for 24–48 h. Notably, each cell concentration was run in duplicate. Mortalities were recorded daily for 14 days after challenge. *S. iniae* was re-isolated from the liver, spleen, kidney, and brain of moribund and dead groupers after challenge.

2.4. Vaccination procedures

The vaccine was prepared by mixing aqueous bacterin with the ISA763A or AS-F adjuvant on the day of vaccination, and contained either a 5:5 (v/v) ratio of aqueous bacterin (10^{10} CFU/mL) to AS-F adjuvant, or a 6:4 (v/v) ratio of aqueous bacterin (10^{10} CFU/mL) to ISA763A. Farmed groupers in the control group were intraperitoneally injected with 0.1 mL of PBS as a mock immunization. At 14 days post-primary vaccination (PPV), identical booster immunizations were performed in the vaccinated groups. In the laboratory aquarium system, experimental fish were divided into three groups of 270, and fish in each group received i.p. injections consisting of the following: Group 1, 0.1 mL of antigens mixed with ISA763A; Group 2, 0.1 mL of antigens mixed with AS-F; Group 3, 0.1 mL of PBS. In the field trial, 500 groupers were intraperitoneally injected with 0.1 mL of antigens mixed with ISA763A; the control group of 500 groupers received no treatment. Vaccine efficacy was monitored by examining fish using the methodology described for the laboratory challenge test (see below).

2.5. Challenge trials

Groupers from the vaccinated and control groups were challenged by i.p. injection with 0.1 mL of a bacterial suspension of live *S. iniae* GSI-310 strain (10^8 CFU per fish) at 1, 3, and 6 months post-secondary vaccination (PSV). Challenged groupers were recorded daily for 3 weeks. All dead fish were examined to confirm the re-isolation of the inoculated strain from internal organs by streaking directly onto TSN agar plates. Bacterial colonies were examined using PCR (as described in Zlotkin et al. [22]) to determine whether they belonged to the *S. iniae* GSI-310 strain.

2.6. Leukocyte isolation

Peripheral blood from 10 groupers was collected in EDTA-containing tubes, diluted 1:3 (v/v) in PBS (145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4, pH 7.5), and overlaid on a layer formed by 3 mL of Histopaque-1077 (Sigma). Gradients were centrifuged at 400 × *g* at 25 °C for 30 min. Cells at the interface were harvested, washed in PBS, and then re-suspended in 1 mL of L15 medium.

2.7. RT-qPCR

Total RNA was extracted from peripheral blood leukocytes (PBLs) by using TRIzol reagent (Invitrogen). Single-strand cDNA was synthesized from 1 μg of total RNA using 200 U of M-MLV reverse transcriptase (Promega), based on the manufacturer's protocol. Reactions were performed at 42 °C for 90 min and terminated by heating at 85 °C for 10 min. The expressions of *igm* [23], *il-1β*, and *tnf-α* [24] in PBLs were evaluated using RT-qPCR. *β-Actin* [25] served as the internal control. RT-qPCR was performed using iQ SYBR Green Supermmix (Bio-Rad Laboratories), and each sample was run in triplicate. The optimal annealing temperature for all primers was determined using the thermal gradient feature of the CFX96 system (Bio-Rad Laboratories). PCR conditions were 95 °C for 3 min, followed by 40 two-step cycles, each consisting of 95 °C for 10 s and 59 °C for 30 s. Amplification was detected using SYBR Green fluorescence during the step at 59 °C, and reaction specificity was monitored by melt curve analysis. Results were analyzed using the CFX Manager Software package (Bio-Rad Laboratories). Relative expression was calculated using the "delta-delta method" formula $2^{-[\Delta CP\cdot sample - \Delta CP\cdot control]}$, where 2 represents the perfect PCR efficiency; statistical significance was calculated using the Volcano plot.

2.8. Phagocytosis analysis

PBLs at a density of 10^7 cells/mL were cultured on coverslips in L15 medium containing 20% FBS at 27 °C for 5 h; 10^7 CFU/mL bacteria were then seeded on coverslips and incubated at 27 °C