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Development of three-valent vaccine against streptococcal infections in olive flounder, *Paralichthys olivaceus*

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ABSTRACT

Streptococcus iniae and *S. parauberis* types I and II are major bacterial pathogens affecting the olive flounder, *Paralichthys olivaceus*. With the introduction of formalin-killed vaccine for *S. iniae*, recent epidemiological studies have indicated that *S. parauberis* infections are becoming increasingly severe and frequent in the olive flounder farming industry. Here, the formalin-killed bacterins were used to develop a three-valent vaccine against three streptococcal infections, and its efficacy was assessed in laboratory- and field-challenge trials. In the laboratory-challenge test, it obtained 75%, 75% and 90% relative percent survival (RPS) values in vaccinated olive flounders challenged with *S. iniae*, *S. parauberis* type I, and *S. parauberis* type II, respectively. For the field-challenge trial, the three-valent vaccine was administered to over 100,000 olive flounders, which were compared to non-injected control fish. An RPS of 74% was observed when *S. parauberis* infection naturally occurred at 11 fish farms. Serum antibody measurements using olive flounder immunoglobulin-specific antibodies indicated that immunized fish had significantly higher serum antibody levels than control fish up to 6 months post-vaccination. These results demonstrate that the three-valent vaccine using formalin-killed bacterins could effectively protect olive flounders against three major streptococcal infections.

Statement of relevance: The olive flounder, *P. olivaceus*, is an economically important fish species in Northeast Asia and the most valuable fish species in South Korea, corresponding to 56.5% of the total mariculture production in 2010 (Baeck et al., 2006). However, streptococcal infections often break out among farmed olive flounders subjected to certain conditions (e.g., high stocking density, poor water quality and high water temperature), leading to mass mortality and severe economic losses (Baeck et al., 2006; Cho et al., 2008) Antibiotic treatments are usually recommended to address such outbreaks, but the prevalence of multi-drug-resistant bacteria means that antibiotic compounds are not always able to eradicate the infections (Creeper and Buller, 2006).

The Gram-positive bacteria, *Streptococcus iniae* and *S. parauberis*, cause streptococcosis and have severe effects on aquaculture of the olive flounder, barramundi, channel catfish, European sea bass, rainbow trout, striped bass, tilapia, turbot and yellow tail (Gudmundsdóttir and Björnsdóttir, 2007; Han et al., 2011). In the olive flounder, fish infected with *S. iniae* usually show severe pathological changes, such as darkening, exophthalmia, rectal hernia, abdominal distension, ascites and congestion of intestinal organs, whereas fish infected with *S. parauberis* rarely exhibited any remarkable pathological finding except for darkening of the skin (Kim et al., 2006).

Some vaccines have been reported to prevent *S. iniae* infection, showing high relative percent survival (RPS) among olive flounders in laboratory trials; such vaccines are now commercially available (Cheng et al., 2010a). However, recent epidemiological studies have suggested that *S. parauberis* infection has increased and become more severe and frequent as much as infections of *S. iniae*, which was previously considered the major bacterial pathogen among olive flounder (Park et al., 2012; Park, 2009; Perera et al., 1998). *S. parauberis* can be divided into two serotypes (types I and II) based on serological testing of a distinctive capsular polysaccharide layer (Bromage and Owens, 2002; Plant and Lapatra, 2011). Since the two serotypes possess specific antigenic characteristics, a vaccine should be able to address both types in order to effectively prevent *S. parauberis* infection.

The present study was undertaken to develop a three-valent vaccine against both serotypes of *S. parauberis* plus *S. iniae* using formalin-killed vaccine preparation method. The efficacy of this vaccine against the major streptococcal infections of olive flounder was calculated as RPS and conducted in both laboratory trials and large-scale

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field trial. This vaccine will be highly useful and more advantageous since the use of this does not require several antibiotics which means safer food production and more sustainable environment.

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1. Introduction

The olive flounder, *Paralichthys olivaceus*, is an economically important fish species in Northeast Asia (Cheng et al., 2010a; Kanai et al., 2009; Park et al., 2012) and the most valuable fish species in South Korea, corresponding to 56.5% of the total fisheries production in 2010 (Park et al., 2012). However, streptococcal infections often break out among farmed olive flounders subjected to certain conditions (e.g., high stocking density, poor water quality and high water temperature), leading to mass mortality and severe economic losses (Cheng et al., 2010a; Cheng et al., 2010b; Park et al., 2012). Antibiotic treatments are usually recommended to address such outbreaks, but the prevalence of multi-drug-resistant bacteria means that antibiotic compounds are not always able to eradicate the infections (Park et al., 2009).

The Gram-positive bacteria, *Streptococcus iniae* and *S. parauberis*, cause streptococcosis and have severe effects on aquaculture of the olive flounder, barramundi, channel catfish, European sea bass, rainbow trout, striped bass, tilapia, turbot and yellow tail (Bromage and Owens, 2002; Domeénech et al., 1996; Kusuda et al., 1976; Lahav et al., 2004; Nho et al., 2009, Shin et al., 2006, Shoemaker et al., 2001; Zlotkin et al., 1998a, b). Several pathological studies have indicated that the clinical symptoms of streptococcosis can vary according to the host, geographical distribution and infecting pathogen (Cho et al., 2008; Creeper and Buller, 2006; Evans et al., 2000; Lee et al., 2007; Perera et al., 1998; Won et al., 2010). In the olive flounder, fish infected with *S. iniae* usually show severe pathological changes, such as darkening, exophthalmia, rectal hernia, abdominal distension, ascites and congestion of intestinal organs, whereas fish infected with *S. parauberis* rarely exhibited any remarkable pathological finding except for darkening of the skin (Lee et al., 2007; Nguyen et al., 2001a; Nguyen et al., 2001b).

Previous studies have shown that *S. iniae* and *S. parauberis* can be distinguished from each other by their hemolytic activities, biochemical test results, and genetic properties (Nho et al., 2009; Shin et al., 2006; Romalde et al., 1999). In addition, their mortality rates differed: in a laboratory test, 100% of olive flounders injected with 4×10^9 cells of *S. iniae* were dead within a week, whereas those infected with 4×10^9 cells of *S. parauberis* showed 74% mortality over the course of 15 days (Nho et al., 2009).

Some vaccines have been reported to prevent *S. iniae* infection, showing high relative percent survival (RPS) among olive flounders in laboratory trials; such vaccines are now commercially available (Cheng et al., 2010a; Klesius et al., 2000; Shin et al., 2007a, b; Shoemaker et al., 2010). However, recent epidemiological studies have suggested that *S. parauberis* infections have become more severe and frequent like infections of *S. iniae*, which was previously considered the major bacterial pathogen among olive flounder (Baeck et al., 2006; Jeong et al., 2006; Kang et al., 2007; Kim et al., 2006; Nho et al., 2009; Shin et al., 2006). *S. parauberis* can be divided into two serotypes (types I and II) based on serological testing of a distinctive capsular polysaccharide layer (Han et al., 2011; Kanai et al., 2009). Since the two serotypes possess specific antigenic characteristics, a good vaccine should be able to address both types in order to effectively prevent *S. parauberis* infection.

Therefore, the present study was undertaken to develop a three-valent vaccine against both serotypes of *S. parauberis* plus *S. iniae* using formalin-killed vaccine preparation method. The efficacy of this vaccine against the major streptococcal infections of olive flounder was calculated as RPS and serum antibody measurements in both laboratory trials and large-scale field trial.

2. Materials and methods

2.1. Bacteria and culture conditions

To investigate the distribution of the Korean serotypes of *S. parauberis*, 122 strains isolated from diseased olive flounder in Jeju, South Korea from 2003 to 2009 (Nho et al., 2009) except 2007 were subjected to agglutination tests for differentiation of types I and II. Among them, strains P45 and Namhae45 were selected as representative of *S. parauberis* types I and II, respectively and were used to investigate the three-valent vaccine (Nho et al., 2009). For *S. iniae*, Jeju45 strain was selected as the representative for evaluating the vaccine (Shin et al., 2007a, b). All strains were routinely cultured at 25 °C for 24 h with tryptone soya agar (TSA; Oxoid, Hampshire, England) or tryptone soya broth (TSB; Oxoid) supplemented with 2% (w/v) NaCl (to generate TSA-2 and TSB-2, respectively).

2.2. Agglutination test

Strains P45 (types I) or Namhae45 (types II) were cultured in TSB-2 and inactivated with 1% (v/v) formalin to produce antisera for the agglutination test (Kanai et al., 2009). Rabbits (one per strain) were immunized four times with formalin-killed P45 or Namhae45 at intervals of two weeks. One milliliter of the formalin-killed P45 or Namhae45 was mixed with equal volumes of Freund's complete adjuvant (Sigma, St. Louis, MO) for the first immunization, and then with Freund's incomplete adjuvant (Sigma) for the subsequent immunizations. One week after the final immunization, sera were collected and used for agglutination tests. Briefly, U-bottomed 96-well plates were separately coated with 100 μl of each *S. parauberis* strain (1×10^{11} colony forming units (CFU)/ml), which had been grown in TSB-2 and inactivated with 1% (v/v) formalin. Then, 100 μl of diluted rabbit serum (1:250) directed against P45 or Namhae45 was added to triplicate wells. The plates were incubated at 25 °C for 2 h, and then overnight at 4 °C. A positive reaction was recorded when agglutination was observed in wells containing anti-type I or -II serum and killed bacterins.

2.3. Vaccine formulation

The three-valent vaccine contained formalin-killed bacterins of *S. iniae* (F2K-2), *S. parauberis* type I (M4Y) and *S. parauberis* type II (M5E). These strains were kindly provided by Kyoritsu Seiyaku Corporation (Japan). To produce the vaccine, the three strains were cultured separately in TSB-2 at 25 °C until optical density (O.D.) 1.0 at 600 nm (early stationary phase), and then treated with 0.3% (v/v) formalin at 4 °C for 48 h with slow agitation. The inactivated cultures were separately centrifuged at 4500 ×*g* for 20 min, washed three times with phosphate-buffered saline (PBS, pH 7.2), and resuspended in PBS at 1×10^{10} CFU/ml. Finally, the individual bacterial suspensions were mixed equally and kept at 4 °C until use.

2.4. Fish, vaccination and challenge with S. iniae, S. parauberis type I, and S. parauberis type II

To evaluate the vaccine's efficacy under artificial laboratory conditions, we purchased naïve olive flounders with an approximate weight of 50 g from a commercial fish farm in South Korea. The fish were acclimated in an aerated 1000-L fiber-reinforced plastic tank for four weeks at 26 °C. After measuring the weight of flounders (approximately 52 g), the fish were distributed to seven 200-L plastic tanks ($N = 25$ fish/

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