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RESEARCH ARTICLE

Protective efficiency of an inactivated vaccine against *Streptococcus iniae* in olive flounder, *Paralichthys olivaceus*

Yong-Uk Jeong, Dharaneedharan Subramanian, Yeoung-Hwan Jang, Dong-Hwi Kim, So-Hyun Park, Kyung-il Park, Young-Don Lee, Moon-Soo Heo

Abstract. *Streptococcus iniae* is a causative agent of hemorrhagic septicemia in olive flounder, *Paralichthys olivaceus*, in Korea, resulting in serious economic losses. As a preventive measure, M VAC INIAE (Mastuken, Japan) was prepared from the *S. iniae* F2K strain and tested against the SI-36 strain prevalent on flounder fish farms on Jeju Island, Korea. F2K had a serotype of 38 (–) and SI-36 38 (+). The vaccine recognized both serotypes. It showed a very high effective immune response against *S. iniae*; the challenge test using the *S. iniae* SI-36 strain resulted in a relative percent survival (RPS) of 85.7-87.0% 2 weeks after vaccination and 71.0-80.0% 6 months after vaccination. Field vaccination and clinical challenge tests were performed at local Jeju aquafarms with *S. iniae* SI-36. These showed significantly reduced cumulative mortality when compared to the control group with RPS rates that ranged between 71-80%. Hence, the present study suggests that this vaccine showed a significant immune response against *S. iniae* and could be applied in commercial aquafarms as a therapeutic agent against β-hemolytic streptococcosis in cultured *P. olivaceus*.

Keywords: *Streptococcus iniae,* β-hemolytic streptococcosis, olive flounder, inactivated vaccine, relative percent survival

Introduction

Fish streptococcal infections have been considered to be one of the major problems associated with the worldwide intensification of aquaculture since 1970 (Baya et al. 1990, Kitao 1993, Agnew and Barnes 2007). Among *Streptococcus* infections, *Streptococcus iniae* emerged as an important fish pathogen, which was first isolated from freshwater dolphins (Pier and Madin 1976). It was reported that *S. iniae* is a causative agent of hemorrhagic septicemia that accompanies meningoencephalitis, exophthalmia, and corneal opacity in farmed fish (Roberts 2001).

By 2012, Republic of Korea farmed flounder production accounted for 70% of the world's supply (Tveteras 2012). Of this, 62% of production is derived from about 280 inland aquafarms. In Jeju

Y.U. Jeong, D. Subramanian, D.H. Kim, S.H. Park, M.S. Heo [✉]
Marine Pathogenic Microbes Lab, Department of Aquatic Biomedical Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju 690-756, South Korea
e-mail: msheo@jejunu.ac.kr

Y.-H. Jang
Jeju Special Self-Governing Province Ocean and Fisheries Research Institute, Pyoseon-myeon, Segwipo-si, Jeju 697-914, South Korea

K. Park
Department of Aquatic Life Medicine, Kunsan National University, Kunsan 573-701, South Korea

Y.D. Lee
Marine and Environmental Institute, Jeju National University, Jeju 690-814, South Korea

Y.U. Jeong and D. Subramanian contributed equally to this work.

Island alone, 24,575 mt of flounder were produced at farms covering a total of 100 ha of water. This has become one of the most important industries on this island (KOSIS 2012). Intensification of culture is probably the cause of the enhanced propagation of bacterial, viral, and/or parasitic pathogens. In May 2005, flounder cultured in cage-culture systems on Jeju Island experienced high mortality rates with bilateral exopthalmic eyes and rotten gills (Baeck et al. 2006, Jang et al. 2013).

Previous reports on the bacterial diseases of cultured flounder that occurred in this area were associated with streptococcosis (Jung et al. 2004), namely *Streptococcos iniae* and *S. parauberis.* Among them, *S. iniae* is reported to be a major fish pathogen in many regions of the world. The plasminogen binding property of the pathogenic *S. iniae* strain could suggest its virulence level, which could contribute to tissue invasion and overall pathogenicity (Boyle and Lottenberg 1997). Hence, they are also zoonotic with infection in humans associated with handling and preparing infected fish (Weinstein et al. 1997). According to Jeong et al. (2006) the infection rate of *S. iniae* in Jeju Island was 46% throughout 2003, and in 2005 it occurred throughout the year with high prevalence in the summer (Nguyen et al. 2002). In particular, it was known that market size flounder over 30 cm in length were more susceptible to *S. iniae,* resulting in serious economic losses (Jeong et al. 2006). A few studies have reported on a second serotype of *S. iniae* isolated from diseased fish from Israel and the USA (Bachrach et al. 2001, Barnes et al. 2003a).

In general, the increased use of antibiotics in aquaculture has increased the resistance of *S. iniae* and prompted demands for an effective vaccine (Stoffregen et al. 1996, Shoemaker et al. 2001). Currently, several formalin-killed vaccines against β-hemolytic *S. iniae* strains are used in Korea (Cho et al. 2006, Moon et al. 2007, Shin et al. 2007). In particular, the β-hemolytic vaccine M VAC ININAE (Mastuken, Japan), which was developed with the *S. iniae* – F2K strain, is commercially available in Korea and Japan. The purpose of this study was to examine the chronic effect of serotypes of *S. iniae* in aquafarms located on Jeju, Korea in two clinical trials and to evaluate the efficacy, safety, and clinical administration of the *S. iniae* vaccine.

Material and methods

Bacterial strains and their characterization

The bacterial strains were isolated from pure cultures of infected olive flounder kidney and spleen, and they were also obtained from the Korean Collection for Type Cultures (KCTC). The strain F2K, used in the development of vaccine for *S. iniae*, was purchased from Mastuken, Japan. All bacterial strains were grown on Brain-Heart Infusion (BHI) agar (Difco, Grand Island, NY, USA) supplemented with 1.5% NaCl and 5% sheep blood (KOMED, Seoul, Korea) for 24-48 h at 25°C. Glycerol stocks of each isolated strain were prepared with 20% (v/v) glycerol in BHI broth (BD) and stored at –80°C. Isolates were cultured overnight at 20°C in BHI broth and Gram staining and motility tests were performed. General cell morphology was studied with phase contrast and scanning electron microscopy (SEM) according to standard procedures. Phenotypic characterization was carried out on all strains with an API 20E kit (BioMérieux) at 25°C, and the results were analyzed at 24 and 48 h. VITEC (BioMerieux) cards were also inoculated and allowed to incubate overnight for automated reading of reactions. Hemolysis tests were conducted in pure isolates using a 5% sheep blood agar plate (KOMED).

Vaccine preparation

The *S. iniae* vaccine was prepared with 10% neutrally-buffered formalin in a 0.3% solution on F2K strain cultures (1×10^7 CFU 100 ml^{-1}), and this was used as the inactivated *S. iniae* vaccine according to the manufacturer's instructions (M VAC INIAE, Mastuken, Japan).

Preparation of rabbit antisera

Rabbit was immunized with intravenous injection of M VAC INIAE prepared from the *S. iniae* F2K strain, twice weekly in consecutive doses of 0.2, 0.4, 0.8, and 1.0 ml. One week after the last injection, the rabbit was bled from the ear vein. Two weeks later, this immunization procedure was repeated, now with 1.0 ml doses throughout. The antisera were stored at –20°C until use.

Slide agglutination test for *S. iniae*

The test was performed by mixing a small amount of each bacterial colony with several drops of 38 (–) or 38 (+) antiserum of the *S. iniae* (F2K) developed in rabbits.

Fish

About 500 healthy olive flounder (mean weight of 65.5 ± 0.13 g) were obtained from commercial fish farms on Jeju Island. The fish were habituated for two weeks in a 1.5-ton tank equipped with an ultraviolet sterilizer on a closed circulatory filter, and water temperature was maintained at 22 ± 1°C prior to the experiments. Throughout the immunogenicity and challenge experiments, the fish were held under standard culture conditions and fed twice a day with a commercial pelleted diet. The fish were confirmed to be negative for *S. iniae* infection by culturing fish tissue in BHI agar (BD, USA).

Immunogenicity test

For the immunogenicity test, 500 olive flounder were selected that had not been previously exposed to antibiotics and with no traces of the *S. iniae* antibody. For vaccine inoculation, all fish were anesthetized by immersion in a tank containing 150 mg l^{-1} of tricaine methanesulfonate for 15 min and divided into two groups (n = 125, in duplicate) – the control and vaccinated groups – and each group of fish received either 100 µl of phosphate-buffered saline (PBS: 0.2 M monobasic phosphate, 0.2 M dibasic phosphate, 0.11 M sodium chloride, pH 7.4) or the vaccine intraperitoneally. To test antibody formation, serum was extracted from the tail vein of 10 fish from each PBS and vaccine-treated group starting 2 weeks after vaccination and up to 9 weeks after vaccination. The serum obtained was used for the agglutination assay (Yildirim et al. 2003). Briefly, 50 µL of serum was serially diluted in a 96-well microplate with 50 µL of PBS. After this, 50 µL of vaccine was added to each well as an antigen and incubated in a humidified chamber at 25°C for 24 h. Agglutination titers were expressed as log2 (x+1) of the reciprocal of the highest serum dilution showing visible agglutination as compared to the positive control.

The last well, in which there was only 50 mL PBS buffer, was the negative control.

Challenge experiment

For the challenge test, after two weeks of vaccination, forty fish from each vaccinated and control fish groups were injected intraperitoneally with 100 µl of the challenge strain *S. iniae*-SI-36, (1×10^7 CFU ml^{-1}, LD_{70}) and PBS, respectively. Mortalities were recorded for 2 weeks, and any clinical symptoms in survivors were noted. The cause of death and pathological symptoms were confirmed by re-isolating bacteria from kidney samples of freshly dead fish or survivors. Cumulative mortality was registered and relative percent survival (RPS) was calculated according to the formula: RPS (%) = [1 – (cumulative mortality of the vaccine-treated group/cumulative mortality of the PBS-treated group)] × 100. The vaccine was found to be efficient if the relative percent survival (RPS) rate of the vaccine-treated group was more than 60% when compared to that of PBS-treated group (Amend 1981).

First clinical trial of the vaccine at aquafarms

Vaccine efficacy was tested at five aquafarms located on the northeast (aquafarm A) and southeast (aquafarms B, C, D and E) coasts of Jeju Island (Fig.

Table 1
Phenotypic characteristics of *Streptococcus* spp. KCTC: Korean Collection for Type Cultures

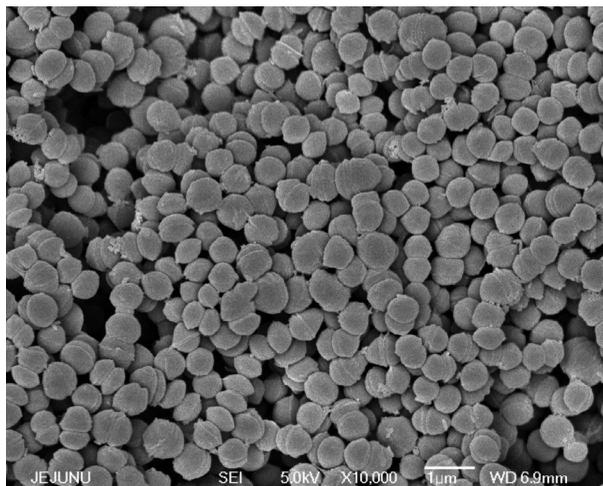| Species	*S. iniae*			*S. parauberis*	
Strain	F2K	SI-36	KCTC-3657	SP-5	KCTC-3651
Host	Olive flounder	Olive flounder	Dolphin	Olive flounder	Bovine mastitis
Gram stain	cocci	cocci	cocci	cocci	cocci
Growth at aerobic condition	+	+	+	+	+
5% CO_2	+	+	+	+	+
OF-test	+	+	+	+	+
Growth at 10°C	-	-	-	-	-
45°C	-	-	-	-	-
pH 9.6	-	-	-	-	-
Growth in 6.5% NaCl	-	-	-	-	-
40% Bile solubility test	-	-	-	-	-
0.25% Optochin sensitivity test	+	+	+	+	+
Hemolysis (Goat RBC)	β	β	β	α	γ
Hydrolysis of arginine	-	+	+	+	+
Hydrolysis of hippurate	-	-	-	+	+
Hydrolysis of esculin	+	+	+	-	-
Acid production from Inulin	-	-	-	-	-
Lactose	-	-	-	+	+
Mannitol	+	+	+	+	+
Raffinose	-	-	-	-	-
Ribose	+	+	+	-	-
Salicin	+	+	+	+	+
Sorbitol	-	-	-	-	-
Trehalose	+	+	+	+	+
Alkaline phosphatase	-	+	+	+	+
β-Galactosidase	-	-	-	+	+
α-Glucoronidase	-	-	-	-	-
α-Galactosidase	-	-	-	-	-
Pyrroidonyl arylamidase	-	+	+	+	+
VP-test	-	-	-	+	+

Figure 1. Geographical distribution of flounder farms where vaccine clinical tests were performed on Jeju Island, South Korea.

1). All farms have been practicing flounder culture for more than a decade. At each of the aquafarms, four culture ponds (two control and two vaccinated fish groups) with numbers of fish ranging from 5,700 to 10,000 and with average body weights of about 150.1 ± 0.2 g were selected. All fish in the vaccinated ponds were injected with vaccine as previously mentioned. Growth and the natural infection pattern of *S. iniae* were monitored for 20 ± 2 weeks at all the aquafarms. Necropsies were performed on all of the dead fish that exhibited β-hemolytic streptococcosis symptoms to determine whether they were infected with *Streptococcus*. Multiplex PCR diagnosis for *S. iniae* was also carried out according to Mata et al. (2004).

Second clinical trial of the vaccine at aquafarms

A second clinical trial was performed to assess the chronic efficiency of the vaccine six months after vaccination. For this, fish from aquafarms B and C were transported to the Marine Research Institute of Jeju National University, and acclimated for 4 weeks in a 1.5-ton FRP water tank equipped with an ultraviolet sterilizer in a closed circulatory filter. At the beginning of the experiment the average fish weight ranged from 410 to 700 g. In the challenge test, 100

µl (1×10^7 CFU ml^{-1}; LD$_{70}$) of SI-36 strain was injected intraperitoneally and cumulative mortality was recorded for the following 2 weeks. Vaccine efficiency was calculated by obtaining the RPS rate of both the control and vaccinated challenged fish groups. All dead fish were examined for the presence of *S. iniae* and *S. parauberis* using the multiplex PCR method.

Statistical data analysis

All data were analyzed using one-way ANOVA to test the effects of the vaccine among vaccinated and non-treated groups. A *t*-test was performed using SAS statistical software (SAS Institute, Cary, NC, USA).

Results and discussion

The isolates were strictly anaerobic, Gram-positive, sphere-shaped bacteria (Fig. 2) that grew at 37°C. The phenotypic characteristics of *Streptococcus* spp., including strains obtained from the Korean Collection for Type Cultures and the isolates from diseased fish, are listed in Table 1. Among the different strains, the F2K strain used in the production of the vaccine against *S. iniae* was compared to known KCTC-3657 and SI-36 (wild-type) strains. According to the comparison, the F2K strain showed a high similarity to the phenotypic characteristics of *S. iniae* and was distinguishable from *S. parauberis*. However, the F2K strain showed negative reactions to arginine dihydrolase, alkaline phosphatase, and pyrroidonyl arylamidase, unlike the KCTC-3657 and SI-36 strains. Therefore, the F2K strain demonstrated a different phenotypic polymorphism than that of general *S. iniae*.

Recent studies proposed that bacterial success is influenced by serotypic variation (Klesius et al. 2000, Agnewa and Barnes 2007). In the present study, serological identification using *S. iniae* with antisera 38 (–) and 38 (+) was performed on *S. parauberis* strains (KCTC-3651 and SP-5) and *S. iniae* strains

Table 2
Serological characteristics of *S. iniae* and *S. parauberis*

Anti-sera	*S. iniae* strains				*S. parauberis*	
	F2K	SI-4	Sl-36	KCTC-3657	SP-5	KCTC-3651
Anti-sera to 38(–) type	+	+	+	+	-	-
Anti-sera to 38(+) type	-	+	+	+	-	-
Serotype	38(–)	38(+)	38(+)	38(+)	No type	No type

Table 3
Cumulative mortality and relative percentage survival (RPS) rate of *P. olivaceus* for 2 weeks post-vaccination with the M VAC INIAE vaccine and challenged with *S. iniae*

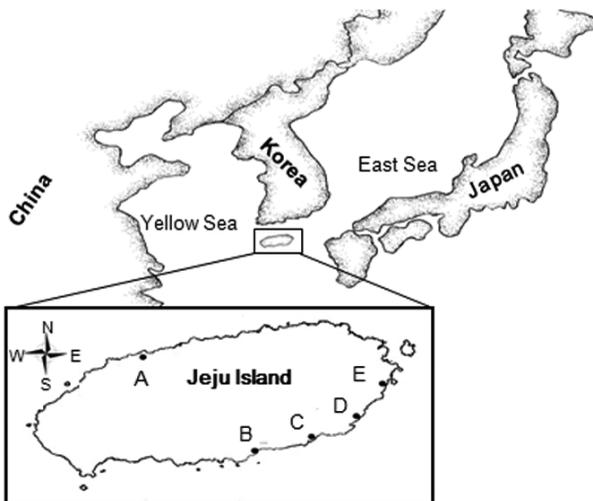| Test group	Treatment group	No. of fish (dead/ challenged)	Cumulative mortality (%)	RPS (%)
1	PBS	23/30	76.7	87.0
	Vaccinated	3/30	10.0	
2	PBS	21/30	70.0	85.7
	Vaccinated	3/30	10.0	

Figure 2. Scanning electron micrograph (SEM) of *S. iniae* strains isolated from infected olive flounder on Jeju Island, South Korea.

(F2K, KCTC-3657, SI-4 and SI-36). The results indicated that no coagulated reactions were found with any *S. parauberis* strains, while the *S. iniae* strains, such as KCTC-3657, SI-4, and SI-36, showed signs of agglutination in all 38 (–) and 38 (+) antisera, and were assigned to serotype 38 (+) (Table 2). On the other hand, strain F2K showed signs of agglutination only with the 38 (–) type antiserum; hence, it was assigned to serotype 38 (–). These results support the existence of two serotypes in *S. iniae*; the serological characters of the F2K strain indicate that it retained both 38 (–) and 38 (+)-type-specific antigens. The phenotypic polymorphism results showed that the F2K strain had many similarities to *S. iniae* serotype II, which has extremely high pathogenicity with negative arginine dehydrolase activity (Bachrach et al. 2001, Barnes et al. 2003a, Zlotkin et al. 2003). According to these studies, serotype I responded to both serotypes I and II antisera, but serotype II showed agglutination activity only with serotype II antiserum. An experiment on the removal of cell-surface protein components suggested that the antigenic determinant could only exist in serotype II, which may not be a capsule component, and, therefore, it could be a cell-surface protein (Barnes et al. 2003b). Accordingly, the high similarity of biochemical tests between the F2K and AD type strains (Barnes et al. 2003a) suggests that the F2K strain could be the same serotype as serotype II since it retains both serotype I and II antigens. Therefore, F2K is an important strain to consider when making a vaccine because the strain has a cross-reactivity to both serotypes: the 38 (–) and 38 (+) strains.

In the immunogenicity test against *S. iniae* (F2K) the agglutination titer rapidly increased from $2^{2.7}$ to 2^4 from week 2 to 4 two weeks after the vaccination

Table 4
Number of fish and cumulative mortality rates for each clinical test group reared 6 months at 5 flounder aquafarms on Jeju Island, Korea

Aquafarm	Group	No. of fish	Average body weight (g)	No. of dead fish caused by*			Total # of fish examined	Cumulated mortality (%)	Total dead fish
				S. iniae	*S. parauberis*	Other disease**			
A	Control	6,400	165	0	3	26	55	7.6	448
	Vaccinated	6,100	165	0	7	38	61	5.7	547
B	Control	3,338	176	0	4	40	53	13.6	465
	Vaccinated	3,289	165	3	2	64	87	16.6	348
C	Control	10,000	165	1	16	137	177	6.2	502
	Vaccinated	9,229	165	4	13	139	175	6.2	505
D	Control	9,000	125	0	20	59	116	12.5	1,128
	Vaccinated	8,663	125	0	7	73	118	27.5	2,371
E	Control	5,669	125	0	156	28	303	36.8	2,095
	Vaccinated	5,700	125	0	143	52	297	38.4	2,187

*Examination was carried out on ≤ 10 dead fish randomly selected on each day.
**Other diseases: Edwardsiellosis, Vibriosis, Flexibacteriosis, Ichtyobodiosis, Scuticociliatosis.

treatment, reaching a maximum of $2^{4.6}$ at week 8, and then declining to $2^{4.5}$ at week 9 post-vaccination (Fig. 3). These results indicate that the immune system of the olive flounder generate the specific antibody and has positive immunogenicity against *S. iniae* (F2K).

The practical protection effect was determined through challenge tests, which were performed twice 2 weeks after vaccination. Fish that had been artificially infected with S-36 started to die on the fourth day after injection and presented typical signs of *Streptococcus* infection, such as exophthalmia (pop-eye), hemorrhaging in the eye, ascitic fluid in the intra-peritoneal cavity, and a darkened surface. Furthermore, the cumulative mortality rates of PBS-treated groups 1 and 2 were 76.7 and 70.0%, respectively, while the cumulative mortality rate of vaccine-treated groups 1 and 2 was only 10.0% (Table 3). Mortality of fish in the control groups initiated from 4-5% at day 4 then sharply increased to about

60% on day 7, while in the vaccine treated groups it was less than 10% during the same period (Fig. 4). The efficiencies of the vaccine (RPS) in groups 1 and 2 were 87.0 and 85.7%, respectively (Table 3). The protection results suggest that single administration of the vaccine showed similar efficacies to the results Moon et al. (2007) and Cho et al. (2006) reported regarding testing the efficacies of vaccines against *S. iniae*. Culture re-isolation of the challenge strain confirmed that the fish were infected by the challenged strain and not by other pathogenic fish bacteria (data not shown). Therefore, the vaccine challenge test results indicated that fish can develop specific antibodies against *S. iniae* (SI-36) when the vaccine was administered to *P. olivaceus*.

The results of clinical trials carried out in five flounder aquafarms on Jeju Island using the *S. iniae* vaccine are illustrated in Table 4. The average weights of the fish in each clinical trial ranged from 125 to 176 g, and the evaluation group had
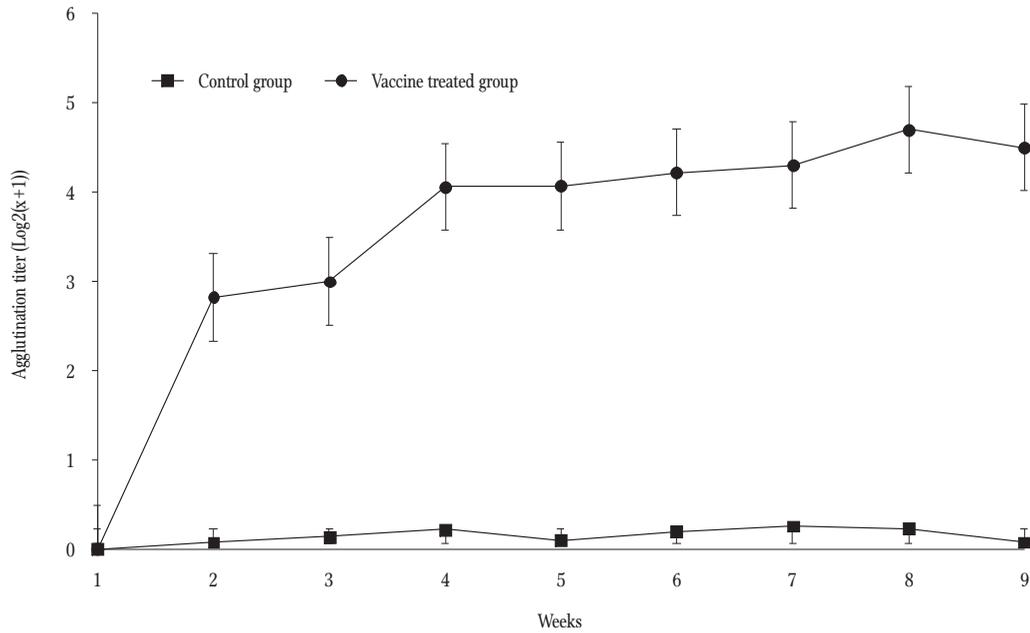
Figure 3. Serum antibody response in agglutination titers against *S. iniae* from olive flounder (*P. olivaceus*) injected with the M VAC INIAE vaccine. Control fish were treated with physiological saline. Closed squares, control group; closed circle, vaccine-treated group.
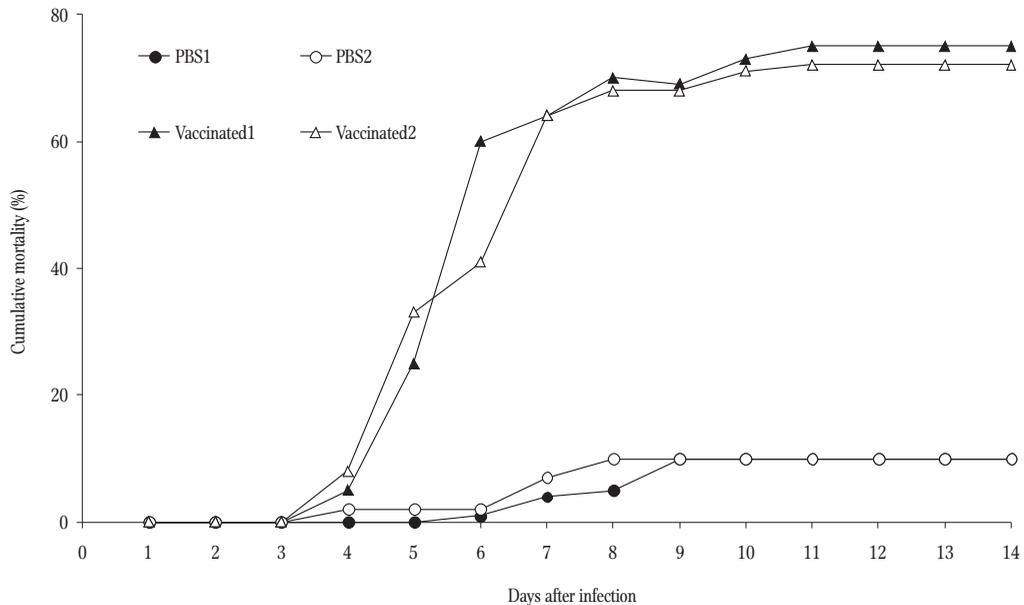

Figure 4. Changes in the cumulative mortality rate of olive flounder (*P. olivaceus*) 2 weeks post-vaccination with the M VAC INIAE vaccine, challenged with *S. iniae*. PBS 1 – phosphate-buffered saline test group 1, PBS 2 – phosphate-buffered saline test group 2.

approximately 5,600 to 10,000 fish. However, no effect of the vaccine was observed at any of the aquafarms. At each aquafarm, cumulative mortality ranged from 5 to 38% depending on the aquafarm and with no difference between the vaccinated and control groups. It is believed that the main reason the vaccine had no effect is attributed to the very low

occurrence of *S. iniae* during the experiment. Indeed, the multiplex PCR assay designed to detect *S. iniae* and *S. parauberis* showed that *S. iniae* were diagnosed in only 3 and 4 vaccinated fish at aquafarms B and C, respectively. In the control group, only 1 dead fish showed *S. iniae* infection at aquafarm C. In contrast to *S. iniae* infection, high prevalence of *S.*

Table 5
Cumulative mortality and relative percent survival (RPS) of olive flounder (*P. olivaceus*) challenge with *S. iniae* (SI-36) 6 months after vaccination at aquafarms B and C

Aquafarm	Treatment group	Challenge period	No. of fish (dead/challenged)	Cumulative mortality (%)	RPS (%)
B	Control	2 weeks	75/100	75.0	80.0
	Vaccinated		15/100	15.0	
C	Control	2 weeks	82/125	66.0	71.00
	Vaccinated		20/104	19.2	

parauberis was noted at all aquafarms during the experiment, suggesting that the vaccine does not have a protective effect against *S. parauberis*. Therefore, the efficacy of the vaccine M VAC INIAE was not consistent at all aquafarms as high levels of other bacterial and parasitic diseases such as edwardsiellosis, vibriosis, flexibacteriosis, and scuticociliatosis were also causative agents of dead fish at the aquafarms (Table 4).

Due to low prevalence of *S. iniae* at the aquafarms, more than 200 fish immunized or non-treated at aquafarms B and C, respectively, were challenged with *S. iniae* (SI-36) 6 months after vaccination and monitored for mortality rates and RPS for a 2-week period (Table 5). During the experiment, the non-treated fish group started to die on the sixth day after injection at both aquafarms, showing typical symptoms of streptococcosis, with exophthalmia, hemorrhaging in the eye, ascitic fluid in the intraperitoneal cavity, and a darkened surface. The cumulative mortalities of the vaccinated and non-treated groups at aquafarm B were 15.0 and 75.0%, respectively. Similarly, at aquafarm C cumulative mortality rates in the test and control groups were 19.2 and 66.0%, respectively. RPS rates were 80% at aquafarm B and 71% at aquafarm C. Re-isolating the challenge strain in dead fish confirmed that the vaccine could be a homogeneous strain. More than 80% of dead fish showed typical symptoms of streptococcosis in the control group, while vaccinated fish exhibited only a 20% mortality

rate caused by disease in each aquarium (data not shown).

Hence, the results of this study permit concluding that the vaccine M VAC INIAE has a protective effect on the wild type *S. iniae* (SI-36) in olive flounders on Jeju, and immunity against *S. iniae* lasted for at least 6 months. However, the application of the vaccine to control *S. iniae* in the olive flounder on Jeju should be done based on the prevalence of the disease.

Author contributions. Y-U.J. designed the experiment and methods; all of the authors did all the experiment procedures; D.S. statistically analyzed the data, prepared the manuscript, and conducted the literature review; Y-D. L and M-S. H reviewed the article.

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