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Efficacy of a vaccine against *Streptococcus parauberis* infection in starry flounder *Platichthys stellatus* Pallas

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Starry flounder, which are recently increasingly cultured in Korea, are known to highly vulnerable to *Streptococcus parauberis* infection. Five groups of starry flounder (*n*=30 for each group) were vaccinated with *S. parauberis* formalin-killed whole cells by intraperitoneal injection at a final concentration of 0, 0.01, 0.1, 1 and 10 mg fish^{-1}. Specific antibody production of 1 and 10 mg fish^{-1} administered groups significantly increased at four weeks post immunization. All vaccinated groups showed higher survival rates than a control group when five groups of fish were challenged with *S. parauberis* at a dose of 1.14×10^{4} cfu fish^{-1} and 1.14×10^{2} cfu fish^{-1}, respectively. In particular, 0.1 or higher concentrations of formalin killed bacterial cells are able to confer the fish high protection against *S. parauberis* infection.

Key words : *Streptococcus parauberis*, *Platichthys stellatus*, Vaccine, Agglutination antibody titer, Challenge test

Starry flounder, *Platichthys stellatus*, enjoy a broad geographic distribution across the North Pacific Ocean, where they can be found in marine, brackish, and freshwater influenced areas given their ability to tolerate low salinity (Orcutt, 1950; Kramer *et al*., 1995). Starry flounder aquaculture has steadily grown since starting in Korea about 5 years ago. Streptococcal infections caused by *Streptococcus* spp., *Lactococcus* spp. and *Enterococcus* spp. are of serious concern in cultured fisheries (Austin and Austin, 1999). The first *S. parauberis* infection occurred in Spanish turbot (*Scophthalmus maximus*) farms between 1993 and 1996 (Toranzo *et al*., 1994; Domenech *et al*., 1996), and since then the pathogen has continued to cause problems in aquaculture industries worldwide. In recent years, *S. parauberis* has increasingly been isolated from diseased olive flounder in Korea (Baeck *et al*., 2006; Joeng *et al*., 2006; Cho *et al*., 2007; Kang *et al*., 2007). The infection has also resulted in severe economic losses to the starry flounder aquaculture industry (Cho *et al*., 2008). Starry flounder infected with *S. parauberis* exhibit show varying disease signs, including exophthalmia, abscesses and hemorrhages around the eyes, and abdomen distention, leading to a high long-term cumulative mortality (Cho *et al*., 2008). In this study, the protection effects of a vaccine against *S. parauberis* infection were investigated. Specifically,

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starry flounder were immunized with formalin-killed cells (FKCs) of the pathogen by intraperitoneal (i.p) injection.

Materials and Methods

1. Fish and environment

Starry flounder (average body wt. = 53.1±7.1 g) were obtained from a commercial fish farm in Korea, 500 of which were maintained in 10 tanks (250 L) and provided with fresh sea water at an exchange rate of 25L h^{-1}. Their health status was examined immediately upon arrival in the aquaria and at 1 week thereafter. The fish were fed with commercial dry pellets (Jeilfeed Co., Ltd., Korea) twice daily. Water temperature (average = 20.9±0.3℃) was measured once a day during the experiment.

2. Vaccine and treatment

S. parauberis strain PH0710 (Cho *et al*., 2008), isolated from diseased starry flounder in December 2007 (Fig. 1), was used for vaccine manufacturing. The bacteria were massively cultured in Todd-Hewitt broth (1% final salt concentration; DB, USA) at 30℃ for 48 h. The bacterial culture was inactivated by adding formalin (Fluka, Germany) to a final concentration of 1% (v/v) and incubated for 24 h at room temperature. The bacterial pellet obtained by centrifugation was washed three times with sterile saline (0.85% NaCl), and concentration of bacterial suspension was adjusted to 100 mg ml^{-1} in saline. One hundred microliter of the vaccine was injected into the abdominal cavity of starry flounder (n=30 for each concentration, respectively) at concentrations of 10, 1, 0.1 and 0.01 mg $fish^{-1}$, while a control group (n=30) was injected intraperitoneally with equal volume of saline. The experiment was duplicated.

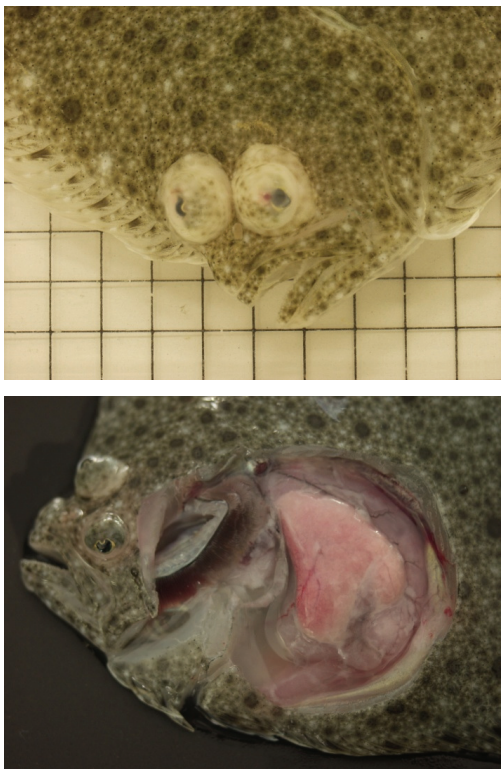

Fig. 1. The *Streptococcus parauberis* which infected starry flounder, *Platichthys stellatus* exhibit varying disease signs, including exophthalmia, abscesses and hemorrhages around the eyes (top), and abdomen distention and transparent ascites (bottom).

3. Agglutination antibody titer

Sera were collected from caudal vein of 8 fish in each experimental group at 0, 1, 2, 4, 6, 8 and 10 weeks after immunization. Agglutinating antibody titers to *S. parauberis* were measured by using the micro-agglutination test (Roberson, 1990). Briefly, 2-fold serial dilutions of each serum in sterile saline ranging from 1:20 to 1:2,560 were added to a 96 well microplate. Then, the

same amount of FKCs (A_{540}=0.8) was mixed with the serum and incubated for overnight.

4. Challenge test

To determine the efficacy of the vaccine, challenge tests were conducted at 6 weeks after vaccination. Ten fish from each group were injected intraperitoneally at the concentration of 1.14×10^4 and 1.14×10^2 cells fish^{-1} of live *S. parauberis* PH0710. Injected fish were then maintained in 40 L tanks at a water temperature of 22± 1℃ and were observed for mortalities for ten days.

5. Data analysis

Differences between antibody titers of control and vaccinated groups for each week (Week 1, 2, 4, 6, 8 and 10) was compared using one-way analysis of variance (one-way ANOVA) and Tukey Honestly Significant Difference (Tukey HSD) multiple comparison test with SPSS (version 10.1). Significance was accepted at $P<0.05$ for statistical analyses.

Results and Discussion

In general, clinical signs of *S. parauberis* infection include darkened pigment on the skin, exophthalmia, abscesses and hemorrhages around the eye and mouth, and distended abdomen. Consistent low mortalities were observed during winter, spring and summer seasons regardless of water temperatures, and mass mortalities occur in autumn (October and November) when the water temperature decreased. *Streptococcus parauberis* infection of cultured olive flounder in Korea appear to occur all year around with distinct peak during spring (March-May; 14-17℃) and fall (October-December) (Jeong *et al*., 2006). Similarly, mortalities of cultured starry flounder caused by *S. parauberis* have been shown to increase in October at a time of decreasing water temperature (Cho *et al*., 2008).

As previous studies have suggested, fish *Streptococcus* vaccines injected intraperitoneally show better efficacy than immersion (Toranzo *et al*., 1995; Klesius *et al*., 2000; Nakanish *et al*., 2002). This was why we ruled out the immersion vaccination method for this study. Overall, all vaccinated groups exhibited increased specific antibodies, although antibody titers (except for fish at one week after administration of 10 mg FKCs) were not significantly higher than the control group for the first four weeks post vaccination ($P>0.05$). Specific antibody production of 1 and 10 mg fish^{-1} administered groups increased after four weeks post immunization. In particular, significant increases of specific antibody level were observed in the group injected with 10 mg fish^{-1} at week 6 and 8, and the group injected with 1 mg fish^{-1} at week 8 (Fig. 2). However, fish immunized with low concentrations (0.1 and 0.01 mg fish^{-1}) of inactivated bacterial cells did not show significant increase in their antibody production. Specific antibody level in starry flounder against *S. parauberis* FKCs in this study appears to be lower than fish immunized with *Vibrio* spp. and the *Edwardsiella tarda* whole cell inactivated vaccine (Smith, 1988; Salati, 1988). However, specific antibody production in fish vaccinated with Gram-positive bacteria including *Enterococcus* sp. (Toranzo *et al*., 1995), *Streptococcus* sp. (Sakai *et al*., 1987 & 1989) and *S. iniae* (Cho *et al*., 2006) FKCs were similar to that observed in this study.

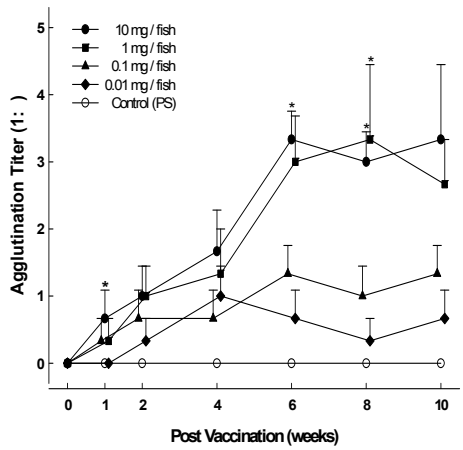

Fig. 2. Changes in agglutination titers of starry flounder, *Platichthys stellatus* Pallas at 10 (●), 1 (■), 0.1 (▲) and 0.01 (◆) mg fish^{-1} and control (physiological saline, PS; ○) after immunization by intraperitoneal injection. Data are represented as mean+S.E. (n=10). *, indicate significant differences ($P < 0.05$) between the groups. The same letter indicates no significant differences between groups.

When fish were intraperitoneally injected with *S. parauberis* (1.14×10^4 cfu fish^{-1}) at 6 weeks post immunization, the cumulative mortality of control group was 100% for 10 days, whereas only 0-30% mortality was recorded in the vaccinated groups. Cumulative mortality was 80% in the control and 0-10% in the vaccinated groups for injection dose of 1.14×10^2 cfu fish^{-1} (Table 1). Although agglutinating antibody titers observed in this study were relatively low, the vaccine still conferred protection against *S. parauberis*. Previous studies have revealed that antibody titer does not always correlate with survival rate after pathogenic challenges. For example, Toranzo *et al*. (1995) reported that i.p. vaccination with FKCs of *Enterococcus* sp. protected turbot from enterococcal infection, although the antibody titer in the serum was less than 1:2. Also, Elder *et al*. (1997) described that antibody titer of rainbow trout was 1:1 at 6 weeks post immunization with *S. iniae* FKCs, although fish survival was approximately 90% after being challenged by the pathogen.

Table 1. Comparative analysis of *Streptococcus parauberis* vaccine (formalin killed cells) trial via intraperitoneal injection

Challenge Dose (cfu/fish)	Immunogen Dose (mg/fish)	Daily Mortality (fish number) Post injection (days)										Cumulative Mortality (%)
		1	2	3	4	5	6	7	8	9	10	
1.14×10^4	10	0	0	0	0	0	0	1	0	0	0	10
	1	0	0	0	0	0	0	0	0	0	0	0
	0.1	0	0	0	0	0	0	0	1	0	0	10
	0.01	0	0	0	0	1	1	1	0	0	0	30
	Control	0	0	1	3	3	1	1	0	1	0	100
1.14×10^2	10	0	0	0	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0	0	0	0
	0.1	0	0	0	0	0	0	0	0	0	0	0
	0.01	0	0	0	1	0	0	0	0	0	0	10
	Control	0	0	0	1	1	2	3	1	0	0	80

In general, it is accepted that antibody production of fish against streptococci is more affected by proteins than by polysaccharides (Elder *et al*., 1995; Bercovier *et al*., 1997). However, despite the fact that streptococcal capsular polysaccharide is very weakly antigenic, polysaccharides are most important for their virulence, and their specific anti-capsular antibodies play a vital role in resistance to streptococcal diseases (Wessels *et al*., 1987; Sorensen and Herichsen, 1984; Eldar *et al*., 1997; Shutou *et al*., 2007a&b). Moreover, because the extracellular polysaccharide capsule of a human pathogen *S. pneumonia* inhibits the host's complement activity, neutrophil phagocytosis and bacterial death by neutrophil extracellular traps (Kim *et al*., 1999; Melin *et al*., 2009; Hyams *et al*., 2010a&b), systemic antibodies to the capsular polysaccharide have essential role in protection against invasive pneumococcal disease (Malley, 2010). Therefore, even small amounts of capsular polysaccharides contained in a vaccine could trigger production of polysaccharide-derived antibodies, and induce serum components, which attack bacterial cell surfaces (Shutou *et al*., 2007a&b).

As described above, the reason why starry flounder exhibiting relatively low antibody titers could be protected against *S. parauberis* may be because the fish could produce capsular polysaccharide-derived antibodies, which may induce cellular immunity. This study suggests that the use of a whole cell inactivated vaccine is very effective in preventing *S. parauberis* infection and thereby lowers the mortality of starry flounder. Research on antigenicity of *S. parauberis* and cellular immune responses in Starry flounder is currently being carried out in our laboratory.

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