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Original Article

Efficacy of inactivated *Streptococcus iniae* vaccine and protective effect of β-(1,3/1,6)–glucan on the effectiveness of vaccine in red tilapia *Oreochromis niloticus* x *O. mossambicus*

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Abstract

Streptococcus iniae infections are becoming an increasing problem in aquaculture and have been reported worldwide in a variety of fish species. Our previous study showed that *S. iniae* infection in tilapia *Oreochromis* sp. and Asian sea bass *Lates calcarifer* cause serious damage in fish farm in Thailand. To prevent streptococcosis caused by *S. iniae*, a formalin-killed vaccine was applied in red tilapia *Oreochromis niloticus* x *O. mossambicus* by injection, immersion and oral vaccination. At 1 week post vaccination, levels of antibody titer and some blood parameters response to different routes of administration were significantly different. The best disease resistance was found in the group injected with vaccine plus β-(1,3/1,6)–glucan with the relative percent survival (RPS) of 95.12% followed by pure vaccine injection (RPS = 80.49%), immersion (RPS = 41.46%) and oral vaccination (RPS = 9.75%).

No difference in blood parameters of tilapia after vaccination for 4 weeks was observed. However, antibody titer of the group received vaccine plus β-(1,3/1,6)–glucan and vaccine alone were significantly higher than the other groups. RPS of fish at week 4 post vaccination showed the same trend as the highest disease resistance recorded in the group injected with vaccine plus β-(1,3/1,6)–glucan (RPS=76.00%) which significantly differ from vaccine alone (RPS=54.00%). Immersion and oral vaccination showed less effect on disease protection at week 4 post vaccination. The result from the present study indicated that formalin-killed *S. iniae* vaccine provided excellent efficacy against *S. iniae* infection in tilapia by intraperitoneal injection and β-(1,3/1,6)–glucan increased the effectiveness of vaccine produced from *S. iniae*.

Keywords: *Streptococcus iniae*, tilapia, vaccine, β-(1,3/1,6)–glucan, route of vaccination

1. Introduction

Streptococcal infections in fish were first detected among cultured rainbow trout *Oncorhynchus mykiss* in 1957 in the Shizuoka Prefecture in Japan (Hoshina *et al*., 1958). Since then, the disease has increased with outbreaks occurring in yellowtails *Seriola quinqueradiata* cultured in Japan resulting in a loss of over 10,000 ton in 1984 (Matsuyama *et al*., 1992). *Streptococcus iniae*, one of the most important streptococcal species, was first described from captive Amazon freshwater dolphins *Inia geoffrensis* (Pier and Madin, 1976). Subsequently, the bacterium was found to have spread to various cultured fish stocks, especially tilapia *Oreochromis niloticus* and the hybrid *O. niloticus* x *O. aureus* (Al-Harbi, 1994; Perera *et al*., 1994; Shoemaker *et al*., 2000), barramundi *Lates calcarifer* (Bromage *et al*., 1999) and red drum *Sciaenops ocellatus* (Eldar *et al*., 1999). In Thailand, streptococcosis caused by *S. agalactiae* and *S. iniae* in tilapia *Oreochromis* sp. and Asian sea bass *L.*

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calcarifer have been reported by our group (Suanyuk *et al*., 2005; 2008; 2010). Clinical signs of *S. iniae* infection in fish vary between species. However, the most common signs include loss of equilibrium, unilateral or bilateral exophthalmia, eye opacity, haemorrhages on bases of the fins, and darkening of skin, (Perera *et al*., 1994; Bromage *et al*., 1999; Eldar *et al*., 1999; Colorni *et al*., 2002; Suanyuk *et al*., 2010).

To prevent and cure bacterial diseases, farmers usually apply antibiotics and relevant chemicals. Consequences of this practice are however detrimental and well documented. For example, residues of antibiotics have been detected both in cultured organisms and local environments, which can cause resistance to antibiotics. Vaccination is one tool which the fish farmers may use to combat disease problems. Vaccination of tilapia against *S. agalactiae* infection has been reported in Thailand (Kitancharoen *et al*., 2006; Boonyawiwat *et al*., 2007). This article aims to study the protective effect of vaccine against *S. iniae* infection in tilapia by different routes of administration. In addition, the effect of β-(1,3/1,6)–glucan on the effectiveness of *S. iniae* vaccine in red tilapia is reported in this study.

2. Materials and Methods

2. 1 Bacterium

S. iniae isolated from previous reports (Suanyuk *et al*., 2010) was used in this study. The bacterium was cultured in tryptic soy broth (TSB: Merck) and maintained on tryptic soy agar (TSA: Merck) and incubated at 35°C for 24-48 hrs.

2.2 Experimental animals

Healthy and *Streptococcus* spp. free red tilapia *O. niloticus* x *O. mossambicus* with an average weight of 7.74± 1.59 g were used as experimental animals. Fish were maintained in 100 L fiberglass tank containing 20 fish per tank. Water exchange was carried out daily at 20% level and individual tank was provided proper aeration. The temperature was maintained at 26.32±1.14, pH at 7.11±0.22 and alkalinity at 28.67±7.65 for the entire experimental duration.

2.3 Vaccine

2.3.1 Injection and immersion vaccine

S. iniae was grown aerobically in 4 L of TSB at 35°C for 24 hrs. Cells in the late stationary phase were examined for purity by spreading onto TSA and incubated at 35°C for 24 hrs. A total plate count was performed for cell enumeration and formalin was added at 1% (v/v final concentration). After 24 hrs, cells were checked for sterilization by spreading onto TSA and harvested by centrifugation at 10,000 rpm at 4°C for 10 min. The cells were then washed three times with 0.85% NaCl and diluted to $1x10^{10}$ CFU/ml using 0.1% formalin in 0.85% NaCl as a preservative and stored at 4°C until use. To produce a vaccine with adjuvant, the vaccine was emulsified in an equal volume of 2% β-(1,3/1,6)–glucan from *Saccharomyces cerevisiae* (MacroGard® AquaSol, Biorigin Scandinavia AS, Norway) to achieve final vaccine concentration of $1x10^{10}$ CFU/ml and stored at 4°C until used.

2.3.2 Oral vaccine

S. iniae was cultured as described for injection and immersion administration. After 24 hrs, cells were checked for sterilization, harvested and washed three times with 0.85% NaCl. The harvested cells were dehydrated using FreeZone® 6 liter console freeze-dry system with shell freezer (Labconco corporation, Missouri). The dehydrated cells were incorporated in feed for oral administration. Two isonitrogenous test diets were formulated to contained 30% protein, 7% fat, and 5% fiber. All diets have the same composition except the level of vaccine added to achieve 0 and 1% for control and experimental diet, respectively. The composition of the test diets is shown in Table 1.

2.4 Vaccination procedure

Red tilapia was used for comparing the degree of protection attained by fish vaccinated with different methods as follows: T1 (control) fish were hand-fed twice daily with

Table 1. Composition of experimental diets.

Ingredients	g/kg	
	Control	Treatment
Fish meal	120	120
Soybean meal	400	400
Broken rice	100	100
Palm kernel cake	140	140
Rice flour	100	90
Fish oil	20	20
Vitamin & Mineral mixed[a]	10	10
Choline chloride	6	6
Rice bran	104	104
Vaccine (Freeze dried cell)	-	10
Total	1000	1000

[a] Vitamin and mineral mixture supplemented per kilogram feed: Thiamine (B_1) 10 mg; Riboflavin (B_2) 20 mg; Pyridoxine (B_6) 10 mg; Cyanocobalamine (B_{12}) 2 mg; Retinal (A) 4,000 IU; Cholecalciferol (D_3) 2,000 IU; Menadione sodium bisulfite (K_3) 80 mg; Folic acid 5 mg; Calcium pantothenate 40 mg; Inositol 400 mg; Niacin 150 mg; DL-alpha-tocopherol (E) 50 IU; Choline chloride 6,000 mg; Ascorbic acid (C) 500 mg; Biotin 1 mg; NaCl 0.25 g; $MgCO_3$ 3.75 g; $FeSO_4$ 0.72 g; $(CH_3COO)_2Ca \cdot 5H_2O$ 0.88 g; $ZnSO_4 \cdot 7H_2O$ 0.088 g; $MnSO_4 \cdot 4H_2O$ 0.040 g; $CuSO_4 \cdot 5H_2O$ 0.008 g; $CoCl_2 \cdot 6H_2O$ 0.00025 g; $KIO_3 \cdot 6H_2O$ 0.00075 g.

control diet to apparent satiation, T2 injected with pure vaccine by intraperitoneal injection (0.1 ml/fish) and hand-fed twice daily with control diet to apparent satiation, T3 injected with vaccine plus glucan by intraperitoneal injection (0.1 ml/fish) and hand-fed twice daily with control diet to apparent satiation, T4 immersed in water containing 10% pure vaccine for 30 sec prior to release to aquarium and hand-fed twice daily with control diet to apparent satiation, T5 hand-fed twice daily with basal diet supplemented with 1% freeze-dried vaccine to apparent satiation for 1 week followed by hand-fed twice daily with control diet to apparent satiation.

2.5 The efficacy test of vaccine

2.5.1 Effect of vaccine on antibody production

At one and four weeks post vaccination, six fish from each treatment were assayed for the presence of *S. iniae*-specific agglutinating antibody in the plasma. Experimental fish were anaesthetized with quinaldine (Coyle *et al*., 2004). Blood was then withdrawn from the caudal vein using a 1-ml syringe coated with a dry film of 1% ethylene diaminetetra-acetic acid (EDTA) and the plasma was collected by centrifugation at 3,000 rpm and 4°C for 10 min.

A two-fold dilution of plasma collected from immunized fish was made in 0.1% formalin in 0.85% NaCl. The antigen was mixed (1:1) into each dilution, left the samples at room temperature for 2 h then stored in refrigerator at 4°C. The results were read visually after 22 hrs of incubation at 4°C. The end-point titer was taken as the last well where agglutination was seen.

2.5.2 Blood parameters

Effect of vaccine on blood parameters was studied at one and four weeks post vaccination. Six fish from each treatment were used and fish blood collection was performed as described in Section 2.5.1. Haematocrit, was measured using a method of Larsen and Snieszko (1961), blood cell count was measured using a method of Supamattaya (1995), haemoglobin was measured by the method of Blaxhall and Daisley (1973), and plasma protein level was determined by the modification method of Lowry *et al*. (1951), see Supamattaya *et al*. (2000).

2.5.3 Disease resistance

At one and four weeks post vaccination, a challenge procedure was performed by intraperitoneal injection of 0.1 ml of *S. iniae* at cell concentration of approximately $5x10^7$ CFU/fish. Each experiment was conducted using triplicate tanks with 20 fish per replication. The mortalities were recorded daily for 21 days and tissue samples of fish were collected and bacterial cells isolated to confirm *S. iniae* as the cause of death. The RPS was calculated for each vaccine according to the method described below (Ellis, 1998).

$$\text{RPS} = 1 - \frac{\text{Percent vaccinate mortality}}{\text{Percent control mortality}} \times 100$$

2.6 Statistical analysis

Data from the present study was expressed as a mean with standard deviation. Significant differences were analyzed using one way ANOVA (CRD) and the differences among means using Duncan's multiple range test (Steel and Torrie, 1980). A P value of <0.05 was considered statistically significant.

3. Results

3.1 Efficacy test of vaccine at one week post vaccination

At one week post vaccination, antibody productions of fish vaccinated with *S. iniae* vaccine in every treatment were higher than those of the control (Table 2). The highest antibody production was observed in fish intraperitoneally injected with *S. iniae* vaccine (Table 2). The plasma protein and red blood cell of fish vaccinated with vaccine plus glucan

Table 2. Humoral antibody titers of red tilapia vaccinated with formalin-killed *S. iniae* vaccine. Titers were determined 1 and 4 week after vaccination.

Treatment	Antibody titer[1]	
	1 week	4 weeks
T1 : Control	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
T2 : Injection (pure vaccine)	1.40 ± 0.46^{c}	1.10 ± 0.35^{b}
T3 : Injection (vaccine plus glucan)	1.40 ± 0.17^{c}	1.20 ± 0.00^{b}
T4 : Immeresion (10% vaccine, 30 sec)	0.90 ± 0.30^{bc}	0.40 ± 0.35^{a}
T5 : Oral (basal diet plus 1% freeze dried vaccine)	0.70 ± 0.17^{b}	0.40 ± 0.46^{a}

[1]Log reciprocal of the greatest plasma dilution in which agglutination of *S. iniae* occurred; Means within columns not sharing the same superscript are significantly different ($p<0.05$).

Table 3. Mean blood parameters values and, in parenthesis, ranges for red tilapia immunized with *S. iniae* vaccine. Blood parameters were determined 1 week after vaccination.

Treatment	Haematocrit (%)	Haemoglobin (g/dl)	Plasma protein (mg/ml)	RBC[1] ($x10^6$ cell/mm^3)	WBC[2] ($x10^4$ cell/mm^3)
T1 : Control	26.44 ± 3.55^{ns}	6.51 ± 1.15^{ns}	43.79 ± 9.47^{a}	1.67 ± 0.43^{a}	4.10 ± 1.13^{ns}
	(23.08-32.39)	(5.22-8.18)	(31.07-53.23)	(0.99-2.16)	(2.13-5.03)
T2 : Injection (pure vaccine)	26.25 ± 3.06^{ns}	6.78 ± 1.32^{ns}	45.57 ± 3.31^{ab}	2.12 ± 0.54^{ab}	4.69 ± 1.08^{ns}
	(21.69-29.27)	(4.83-8.02)	(41.90-49.90)	(1.41-3.07)	(3.18-6.08)
T3 : Injection (vaccine plus glucan)	24.88 ± 5.93^{ns}	6.88 ± 1.20^{ns}	52.15 ± 3.09^{b}	2.73 ± 0.69^{b}	4.72 ± 2.03^{ns}
	(19.18-31.75)	(5.22-8.72)	(48.90-57.73)	(1.96-3.76)	(2.65-8.23)
T4 : Immeresion (10% vaccine, 30 second)	25.03 ± 1.64^{ns}	7.50 ± 1.36^{ns}	46.26 ± 3.90^{ab}	1.85 ± 0.38^{a}	4.55 ± 1.29^{ns}
	(22.22-26.25)	(5.53-9.46)	(40.90-50.57)	(1.40-2.46)	(3.05-6.68)
T5 : Oral (basal diet plus 1% freeze dried vaccine)	26.53 ± 3.61^{ns}	7.27 ± 0.65^{ns}	45.18 ± 6.76^{ab}	1.69 ± 0.48^{a}	4.17 ± 1.02^{ns}
	(20.83-30.26)	(6.58-8.25)	(37.07-57.07)	(1.22-2.40)	(2.30-5.28)

[1] RBC; Red blood cell, [2] WBC; White blood cell, Means within columns not sharing the same superscript are significantly different ($p<0.05$).

were significantly higher than those of the control. No significant difference in the haematocrit, haemoglobin, and white blood cell was observed between vaccinated and non-vaccinated fish (Table 3).

Vaccination of red tilapia with *S. iniae* vaccine provided significant levels of protection in all treatments challenged with *S. iniae* after 1 week of vaccination. The survival rate, mean percent mortality and RPS of *S. iniae* vaccinated and non-vaccinated tilapia following *S. iniae* challenge are shown in Figure 1 and 2. Fish given vaccine plus glucan by injection had RPS of 95.12% followed by pure vaccine injection with RPS of 80.49%. Immersion and oral vaccination provided RPS of 41.46 and 9.75%, respectively (Figure 2).

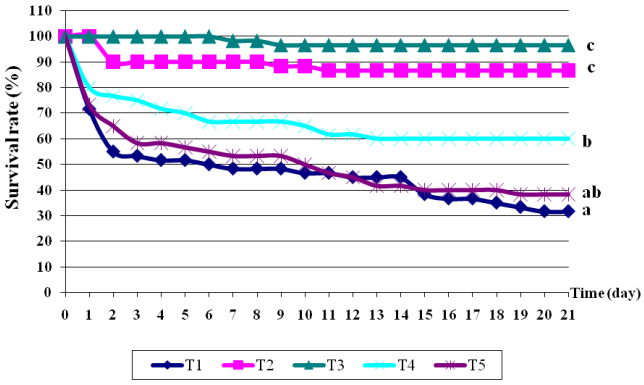

Figure 1. Survival rate of red tilapia challenged with *S. iniae* (1 week post vaccination). Value with different letters are significantly different ($p<0.05$).

3.2 Efficacy test of vaccine at four weeks post vaccination

At four weeks post vaccination, antibody production of fish vaccinated by injection with *S. iniae* pure vaccine and vaccine plus glucan were significantly higher ($p<0.05$) than those of the fish vaccinated by immersion, oral and non vaccinated control fish (Table 2). Similar levels of the heamatocrit, haemoglobin, plasma protein and red and white blood cell of fish immunized via different routes ($p<0.05$) after 4 weeks with *S. iniae* vaccine was observed (Table 4).

Vaccination of red tilapia with *S. iniae* vaccine provided significant levels of protection in every treatment challenged after 4 weeks with *S. iniae*. The survival rate, mean percent mortality and RPS of *S. iniae* vaccinated and non-vaccinated tilapia following *S. iniae* challenge are shown in Figure 3 and 4. Fish given vaccine plus glucan by injection had a RPS of 76.00% which is higher than those vaccinated with pure vaccine with RPS of 54.00%. Immersion and oral vaccination provided less protection with RPS of -4.00 and -8.00%, respectively (Figure 4).

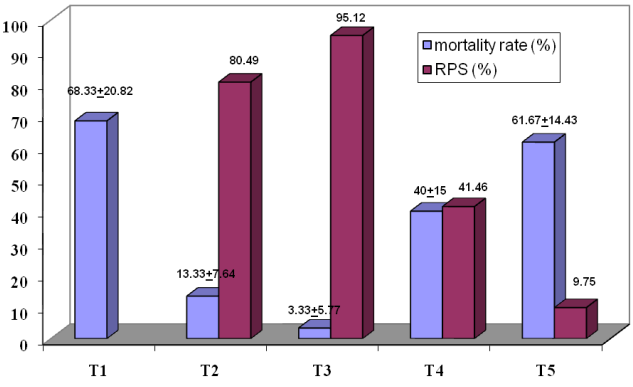

Figure 2. Mortality rate and relative percent survival of red tilapia immunized with formalin - killed *S. iniae* vaccine and challenged 1 week after vaccination.

Table 4. Me an blood parameters values and, in parenthesis, ranges for red tilapia immunized with *S. iniae* vaccine. Blood parameters were determined week 4 after vaccination.

Treatment	Haematocrit (%)	Haemoglobin (g/dl)	Plasma protein (mg/ml)	RBC[1] ($x10^6$ cell/mm^3)	WBC[2] ($x10^4$ cell/mm^3)
T1 : Control	26.63±9.27^{ns} (12.09-35.29)	7.40±2.16^{ns} (3.47-9.42)	65.89±6.43^{ns} (56.22-74.04)	1.99±0.54^{ns} (1.14-2.78)	5.26±1.91^{ns} (2.65-7.40)
T2 : Injection (pure vaccine)	27.69±5.46^{ns} (19.57-36.00)	7.92±1.14^{ns} (6.39-9.54)	63.64±14.25^{ns} (52.04-89.67)	2.39±0.33^{ns} (1.92-2.94)	4.65±0.71^{ns} (3.48-5.45)
T3 : Injection (vaccine plus glucan)	27.00±6.54^{ns} (17.14-35.87)	7.08±1.43^{ns} (4.52-8.53)	69.64±11.31^{ns} (57.67-80.95)	2.36±0.41^{ns} (1.72-2.90)	5.76±1.14^{ns} (3.90-7.18)
T4 : Immeresion (10% vaccine, 30 second)	33.84±7.07^{ns} (28.41-47.62)	8.39±1.35^{ns} (6.39-10.04)	57.55±7.43^{ns} (49.49-68.95)	2.41±0.39^{ns} (2.00-2.90)	4.02±1.39^{ns} (2.20-5.68)
T5 : Oral (basal diet plus 1% freeze dried vaccine)	26.96±5.50^{ns} (22.22-34.02)	7.78±0.88^{ns} (6.46-8.80)	56.58±4.58^{ns} (49.49-61.85)	2.26±0.34^{ns} (1.90-2.87)	4.41±1.35^{ns} (2.90-6.38)

[1] RBC; Red blood cell, [2] WBC; White blood cell, Means within columns not sharing the same superscript are significantly different ($p<0.05$).

4. Discussions

S. iniae has great impact on the Asian sea bass and tilapia cultivation in Thailand. Presently, streptococcosis occurs in various aquaculture areas throughout the country. Studies on the application of streptococcosis vaccines are necessary to assess preventive measures against the damage caused by this disease. In this study, potency testing revealed that the vaccine is safe with no mortality or disease signs were observed in fish vaccinated with *S. iniae* vaccine within 10 days post vaccination (data not shown). In general, a vaccine must simulate that natural defense in producing immunity, but it must not produce clinical illness. Killed or inactivated vaccines are usually safer than live vaccines, but even these can produce unwanted side effect (Ellis, 1998).

Effective vaccines have been developed against *S. iniae* for delivery by injection (Eldar *et al*., 1997; Klesius *et al*., 1999; 2000). Findings of the present study indicate that injection of formalin-killed *S.iniae* vaccine provides higher level of protection than those of immersion and oral vaccination. Eldar *et al*. (1997) reported that vaccination of rainbow trout by intraperitoneal injection of a formalin killed bacterin of *S. iniae* resulted in antibody production in vaccinated fish. After challenging with a virulent strain of *S. iniae*, unvaccinated fish had a mortality rate greater than 50% compared to vaccinated fish with a mortality rate less than 5%.

β-glucan are structural polysaccharides in the cell walls of most yeasts (Manner *et al.* 1973; Robertsen *et al.*, 1990; Chen and Ainsworth 1992), brown algae (Dalmo and Seljelid, 1995; Dalmo *et al.*, 1995) and fungi (Brown and Gordon, 2003). The present study showed that intraperitoneal injection of vaccine plus glucan enhanced better protection than those of pure vaccine. This may be explained by the fact that β-glucan activated both specific and non-specific immunity such as increasing the specific antibody

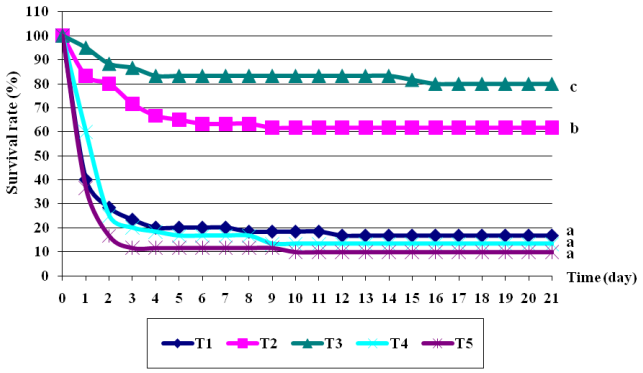

Figure 3. Survival rate of red tilapia challenged with *S. iniae* (4 weeks post vaccination). Value with different letters are significantly different ($p<0.05$).

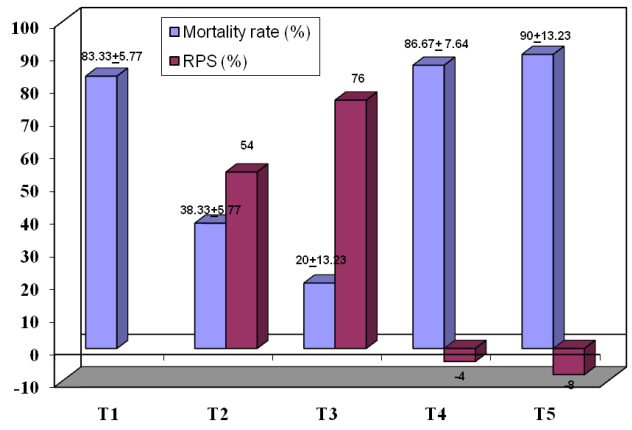

Figure 4. Mortality rate and relative percent survival of red tilapia immunized with formalin - killed *S. iniae* vaccine and challenged 4 week after vaccination.

secreting cells and specific humoral immune response (antibody production) (Siwicki *et al*., 2004), activating the element of the non-specific defense mechanisms, including macrophages, neutrophils, thrombocytes, complement and lysozyme (Matsuyama *et al*., 1992; Jørgensen *et al*., 1993) and enhancement of fish resistance to bacterial infections (Robertsen *et al*., 1990; Matsuyama *et al*., 1992).

In this study, immersion vaccination provided lower protection than those given by injection. Killed vaccines are generally administered by injection in order to achieve good efficacy. Evans *et al*. (2004) demonstrated that bath immunization of tilapia resulted in RPS values that were two times lower than those achieved with intraperitoneal vaccination.

Although the injected vaccination provides excellent protection, they are labor-intensive and usually induce stress. A potential mass delivery strategy is oral administration via feed (Vandenberg, 2004) which has been used successfully against a variety of bacterial diseases (Lillehaug, 1989; Plumb and Vinitnantharat, 1994; Kawai and Hatamoto, 1999; Park *et al*., 2001). In this study, attempt with oral vaccination using killed *S. iniae* vaccine has been unsuccessful. Oral vaccine in the present study was prepared by the addition of bacterial cells to feed without a carrier or protective coating. This might lead to the damage of antigen during digestive process. Improvement or modification of bacterin incorporated in fish feed such as the use of alginate microparticles containing formalin-killed bacterin (Romalde *et al*., 2004) or Oralject™ technology which relies on the temporary reduction of the digestive processes by administration of antiproteases and membrane permeability enhancers in combination with the vaccine (Vandenberg, 2004; Shoemaker *et al*., 2006) may permit the antigen to escape digestive hydrolysis and have enhanced vaccine component uptake.

In summary, formalin-killed *S. iniae* vaccine provided excellent efficacy against *S. iniae* infection in tilapia by intraperitoneally injection method and β-(1,3/1,6)–glucan increased the effectiveness of vaccine produced from *S. iniae* in fish. Immersion and oral vaccination showed less effect on disease protection at week 4 post vaccination suggesting that increasing immersion time, vaccine concentration or booster vaccination may be needed to increase protection. Further studies on vaccination of *S. iniae* vaccine in tilapia under field condition need to be investigated.

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