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Original Article

Growth, immune responses and protection of Nile tilapia *Oreochromis niloticus* immunized with formalin-killed *Streptococcus agalactiae* serotype Ia and III vaccines

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Abstract

The protective efficacy of formalin-killed *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) serotype Ia (GBS-Ia) and III (GBS-III) vaccines were assessed in Nile tilapia (*Oreochromis niloticus*). The fish with an average weight of 34.45± 0.08 g were immunized by intraperitoneal (i.p.) injection with 4 different formalin-killed vaccines prepared from GBS-Ia (1×10^{10} CFU/mL), GBS-III (1×10^{10} CFU/mL), and combined GBS-Ia and GBS-III in an equal volume at final concentrations 1×10^{10} CFU/mL and 2×10^{10} CFU/mL in comparison with the non-immunized control group. At 2 and 4 weeks post vaccination, no significant differences were observed ($p>0.05$) among treatments in growth performance or haemato-immunological parameters, except the increased red blood cell at 2 weeks. Significantly increased antibody titers ($p<0.05$) against GBS-Ia and GBS-III antigens were noted in the groups immunized with homologous GBS vaccines, whereas the group reacted with heterologous GBS antigen showed less antibody titer as compared with the control group. The vaccination experiment indicated that i.p. injection of Nile tilapia with formalin-killed cells prepared from GBS-Ia or GBS-III provides significant protection, with relative percent survival (RPS) value of 52.17 to 71.42%, against a challenge with the homologous serotype isolate, whereas the RPS in fish challenged with a heterologous serotype isolate varied from 20.00 to 53.57%. These results suggested that vaccines from either GBS-Ia or GBS-III have insufficient cross-protective efficacy against the other serotypes. However, a mixed vaccine produced from both GBS serotypes Ia and III provided significant protection with 65.00 to 95.66% RPS which could be an excellent vaccine to protect fish against streptococcosis caused by both GBS serotypes Ia and III.

Keywords: *Streptococcus agalactiae*, vaccine, serotype, tilapia, protection

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1. Introduction

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a Gram-positive bacterium belonging to the family Streptococcaceae. This bacterium is the leading cause of severe bacterial infection in a wide range of fish species including golden shiners *Notemigonus crysoleucas* (Robinson & Meyer, 1966), bluefish *Pomatomus saltatrix*, striped bass *Morone saxatilis,* sea trout *Cynoscion regalis* (Baya *et al*., 1990), sea bream *Sparus auratus,* wild mullet *Liza klunzingeri* (Evans *et al*., 2002), silver pomfret *Pampus argenteus* (Duremdez, Al-Marzouk, Qasem, Al-Harbi, & Gharabally, 2004), and tilapia *Oreochromis niloticus* (Salvador *et al*., 2005; Suanyuk, Fanrong, Ko, Gilbert, & Supamattaya, 2008). GBS has been shown to cause septicemia and meningoencephalitis in fish grown by aquaculture. In Thailand, GBS has caused serious damage in tilapia farming throughout the country (Jantrakajorn, Maisak, & Wongtavatchai, 2014; Suanyuk *et al*., 2008). Infected tilapia display lethargy, loss of appetite, erratic swimming, corneal opacity, exophthalmia, and hemorrhaging of the internal organs (Suanyuk, Kangheae, Khongpradit, & Supamattaya, 2005).

The taxonomic classification of GBS is conveniently based on a specific cell wall polysaccharide, which is characteristic for Lancefield's group B, and on some distinct physiological properties (Rotta, 1986). GBS can be sub-divided into ten serotypes (Ia and Ib, and II to IX) based on the composition of the capsular polysaccharide (Imperi *et al*., 2010). Only serotypes Ia and III, abbreviated here as GBS-Ia and GBS-III, have been reported causing serious damage in tilapia farming in Thailand (Dangwetngam, Suanyuk, Kong, & Phromkunthong, 2016; Rodkhum, Kayansamruaj, & Pirarat, 2011; Suanyuk *et al*., 2008; Suwannasang, Dangwetngam, Issaro, Phromkunthong, & Suanyuk, 2014). Genotyping results of GBS isolates from Thailand show as characteristics of serotype Ia that it contains genes encoding proteins Cα (*bca*) and Cβ (*bac*), three insertion sequences (IS*1381*, IS*861* and IS*Sag2*) and the group II intron GBSi1, while serotype III contains *bca*, three insertion sequences (IS*1381*, IS*Sag1*, IS*Sag2*) and the tetracycline resistance gene (*tet*M) (Suanyuk *et al*., 2008).

The use of antibacterial vaccines is important in aquaculture for successful prevention or reduction of diseases. However, the protective antibodies from immune response depend on the antigenic composition of bacteria used for vaccine preparation (Klesius, Shoemaker, & Evans, 2000). Fish immunized with a vaccine prepared from a single bacterial isolate lack cross-protection against other species. Furthermore, Evans, Klesius, and Shoemaker (2004) showed that inactivated *S. iniae* vaccine does not protect tilapia against GBS infections. The immunization of rainbow trout *Oncorhynchus mykiss* with *S. iniae* serotype I vaccine resulted in an outbreak of serotype II, which is not impacted by the immune response to *S. iniae* serotype I strain that was elicited by vaccination (Bachrach, Zlotkin, Hurvitz, Evans, & Eldar, 2001).

Even though protective efficacy of a GBS vaccine produced from a single GBS isolate has been reported in cultured tilapia in Thailand (Boonyawiwat, Srisook, & Wajjwalku, 2007; Kitancharoen, Hanjavanij, & Suwannapeng, 2006), the vaccination of these fish against multiple GBS serotypes has not been done. Therefore, this study aims to assess the growth, immune responses, and protective effects in Nile tilapia, when vaccinated with formalin-killed vaccines produced from GBS-Ia and GBS-III, the major GBS serotypes isolated from infected tilapia cultured in Thailand.

2. Materials and Methods

2.1 Bacteria

Bacterial strains used in this experiment were obtained from a previous study (Suanyuk *et al*., 2008). The GBS-Ia isolate (genotype Ia-*bca*-*bac*-IS*1381*-IS*861*-GBSi1-IS*Sag2*) from the brain of infected tilapia cultured in Nakhon Si Thammarat province, and the GSB-III isolate (genotype III-4-*bca*-IS*1381*-IS*Sag1*-IS*Sag2*-*tet*M-*int*Tn) from the eye of infected tilapia cultured in Songkhla province were used in this study because these bacteria produce a high mortality in tilapia. A pure culture of the strain kept at -70°C was inoculated onto tryptic soy agar and incubated at 30°C for 24-48 hrs. Prior to use, the bacterial colonies were confirmed to be GBS by standard biochemical methods and polymerase chain reaction (Suanyuk *et al*., 2008). The Lancefield serogroup and serotype of the GBS isolates used in this study were confirmed by using a Slidex Strepto Plus (bioMérieux, France) and group B streptococci typing antisera (Denka Seiken Co. Ltd., Japan), respectively.

2.2 Experimental animals

Healthy Nile tilapia (*O. niloticus*) were obtained from a commercial fish farm and used as the experimental animals. The fish were reared to an individual weight of about 35 g in a three-ton fiberglass tank with aeration for two months. During the cultivation period, the fish were fed twice daily at 8.30 a.m. and 4.30 p.m., at 3-5% of body weight per day with a commercial feed. Test fish were sampled and examined to ensure that they were streptococcosis-free prior to use in the experiment.

2.3 Vaccine preparation

Each of GBS-Ia and GBS-III were grown aerobically in 1 L of brain heart infusion broth (BHIB) at 30°C with shaking (100 rpm) for 18 hrs. The cells were examined for purity by spreading onto brain heart infusion agar (BHIA), and incubating at 30°C for 24 hrs. Viable bacterial counts were conducted using drop-plating serial dilution onto BHIA then the bacterial cells were inactivated with 1% (v/v) formalin. After 24 hrs, the cells were checked for sterilization by spreading them onto BHIA, and harvested by centrifugation

at 10,000 rpm, at 4°C for 10 min. The cells were then washed three times with phosphate buffer saline (PBS, pH 7.4) and diluted to 1×10^{10} CFU/mL, then stored at 4°C until use. To produce a mixed vaccine, each GBS vaccine was concentrated by centrifugation at 10,000 rpm at 4°C for 10 min, and emulsified in an equal volume to achieve final vaccine concentration of each serotype either 1×10^{10} or 2×10^{10} CFU/mL, then stored at 4°C until use.

2.4 Vaccination

Nine hundred Nile tilapia with an average weight of 34.45±0.08 g were used for comparing the growth, immune responses and degree of protection attained by fish vaccinated with the various GBS vaccines, as described below. Experimental fish were cultured in 300 L tanks filled with 180 L dechlorinated water. Five groups of fish (30 fish each × 6 replicates) were anaesthetized and i.p. injected with 0.1 mL of the different vaccines as follows: T1 (sterile PBS), T2 (1×10^{10} CFU/mL of GBS-Ia vaccine), T3 (1×10^{10} CFU/mL of GBS-III vaccine), T4 (1×10^{10} CFU/mL of each GBS-Ia+ GBS-III vaccine), and T5 (2×10^{10} CFU/mL of each GBS-Ia+ GBS-III vaccine). The fish were fed with a commercial feed to satiation twice daily at 8.30 a.m. and 4.30 p.m. The water quality parameters, temperature, total alkalinity, pH, dissolved oxygen, ammonia and nitrite were analyzed by standard methods (American Public Health Association, American Water Works Association, & Water Environment Federation, 1998; Boyd & Tucker, 1992).

2.5 Vaccine efficacy test

To evaluate the effect of GBS vaccines, the experimental fish from replications 1-3 and 4-6 were used for testing the efficacy of treatments at 2 and 4 weeks post vaccination, respectively. Growth, immune responses and protection against GBS infection at 2 and 4 weeks post vaccination were evaluated.

2.5.1 Growth performance

Fish in each tank were counted and weighed at the beginning and at the end of the experiment, following 24 hrs of feed deprivation. The weight measurements and fish counts were used for calculation of feed efficiency and specific growth rate (Hardy & Barrows, 2002; Jantrarotai, Sitasit, & Rajchapakdee, 1994). The growth performance indicators were calculated as follows:

Feed efficiency = weight gain (g) / feed consumption (g)

Specific growth rate =
[(Ln of final weight – Ln of initial weight/t
(time interval in days)] × 100

2.5.2 Haemato-immunological parameters

Five fish from each replication (15 fish/treatment) were randomly sampled and anaesthetized with 0.05 mL/L clove oil (Hamackova, Kouril, Kozak, & Stupka, 2006), and blood was collected from caudal vein/artery. Haematocrit was examined using a method of Larsen and Snieszko (1961). Haemoglobin level was measured colorimetrically by determining the formation of cyanamet-haemoglobin (Blaxhall & Daisley, 1973). Red blood cell and white blood cell were counted using a haemacytometer and a compound microscope (Supamattaya, 1995).

For the quantification of serum protein and lysozyme activity, fish blood was centrifuged at 3,000 rpm for 10 min at 4°C, and the supernatant (serum) was transferred into new microcentrifuge tube. Total serum protein was quantified colorimetrically by the method of Lowry, Rosebrough, Farr, and Randall (1951); see also Supamattaya, Ruangsri, Kiriratnikom, and Songsrichan (2000). Lysozyme activity was measured based on a turbidimetric microplate assay using *Micrococcus lysodeikticus* (Sigma) suspension as a substrate (Demers & Bayne, 1997).

2.5.3 Serum agglutination titers

Serum agglutination titers were determined using modified direct agglutination method (Roberson, 1990). Briefly, a two-fold dilution of serum collected from experimental fish (15 fish/treatment) was made in a 96 well microplate. The antigen (GBS-Ia or GBS-III vaccine) was mixed (1:1) into each dilution, the samples left at room temperature for 2 hrs, and then stored at 4°C for 22 hrs. The antibody titers were read visually and the end-point titer was taken as the last well where agglutination was seen (Suanyuk & Itsaro, 2011).

2.5.4 *In vivo* challenge assay

Experimental fish given various GBS vaccines were tested in triplicates for disease resistance against GBS-Ia and GBS-III at 2 and 4 weeks post vaccination. Prior to challenge, 10 fish were transferred from the stock tanks to each of fifteen 100 L challenge tanks containing 60 L dechlorinated water at temperature of 30°C. The experimental infection was carried out by i.p. injection of 0.1 mL of either GBS-Ia or GBS-III at a cell concentration of approximately 10^7 CFU/fish. Briefly, each of GBS-Ia and GBS-III were grown aerobically in BHIB at 30°C for 24 hrs. Cells were harvested by centrifugation at 10,000 rpm at 4°C for 10 min. The cells were then washed with PBS and diluted to 1×10^7 CFU/mL using PBS. The mortalities were recorded daily for 10 days. To confirm GBS as the cause of death, liver, kidney and brain tissues of infected fish were collected and streaked onto TSA. Bacterial colonies with Gram-positive cocci, catalase and oxidase negative were identified as GBS using API 20 STREP system (bioMérieux®, France). The relative percent survival (RPS)

was calculated for each GBS vaccine according to the method described by Ellis (1988).

2.6 Statistical analysis

Data from the present study is expressed as means with standard deviations. Significant differences in growth performance, haemato-immunological parameters, serum agglutinating titers and cumulative mortalities were analyzed using one way ANOVA (CRD), and differences between treatments using Duncan's multiple range test (Steel & Torrie, 1980). A P value <0.05 was considered statistically significant.

3. Results

3.1 Growth performance

No significant differences were found in average body weight, specific growth rate and feed efficiency of Nile tilapia between the treatments and the control group (Table 1). Water temperature during the experiment was 27.49±0.47°C, total alkalinity was 30.27±2.99 mg/L, pH was 6.68±0.20, dissolved oxygen was 5.63±0.74 mg/L, and ammonia and nitrite were less than 0.2 and 0.1 mg/L, respectively.

3.2 Haemato-immunological parameters

At two weeks post vaccination, fish vaccinated with the GBS vaccines produced significantly higher red blood cell than those of the control (Table 2). No significant difference in the haematocrit, haemoglobin, serum protein, white blood cell, and lysozyme activities, at two or four weeks post vaccination, were observed between the treatment groups (Tables 2 and 3).

3.3 Serum agglutinating titers

The antibody levels in Nile tilapia immunized with GBS vaccines, at two and four weeks post vaccination, are shown in Table 3. Fish immunized with GBS vaccines produced significantly higher antibodies than those in the control group (p<0.05). Significantly (p<0.05) increased antibody titers against GBS-Ia and GBS-III antigens were noted in the groups immunized with homologous GBS vaccines (1.34-3.44) whereas the groups when reacted with a heterologous GBS antigen showed lower antibody titers (0.86-1.53) (Table 3). Moreover, fish immunized with a vaccine containing GBS-Ia antigen had higher antibody titers than those immunized against GBS-III. The antibody titers for homologous GBS-Ia antigen gave titer in range of 2.52-3.44, while for homologous GBS-III antigen the corresponding range was 1.34-1.60. The mixed vaccines gave antibody titers of 2.83-3.55 against GBS-Ia antigen and 1.51-1.66 against GBS-III antigens (Table 3).

3.4 *In vivo* challenge assay

The vaccinations of Nile tilapia provided significant protection when challenged with homologous GBS serotype after two or four weeks of vaccination. The mean percent mortality and RPS of treated tilapia, following the GBS challenges, are shown in Table 4. The vaccines with formalin-killed cells protected against the homologous isolates with RPS 52.17 to 60.00 % against GBS-Ia infection, and 65.00 to 71.42 % against GBS-III infection. In contrast, when challenged with heterologous serotype the RPS was 20.00 to 52.17% against GBS-Ia infection and 20.00 to 53.57 % against GBS-III infection. However, the mixed vaccine produced from GBS serotypes Ia and III provided significant protection against both types of challenge, with RPS 80.00 to 95.66 % against GBS-Ia infection and 65.00 to 82.14 % against GBS-III infection. No significant difference in percent mortality was observed between the low (1×10^{10} CFU/mL) and the high (2×10^{10} CFU/mL) concentration of mixed vaccine (Table 4). *S. agalactiae* was re-isolated from the liver, kidney and brain of all dead fish.

4. Discussion

This study documents the growth, immune responses and protective effects of tilapia immunized with vaccines produced from GBS serotypes Ia and III, the major serotypes

Table 1. Mean ± standard deviation body weight, specific growth rate, and feed efficiency of Nile tilapia vaccinated with the individual formalin-killed GBS, mixed formalin-killed GBS or PBS (control).

Treatment	Average body weight (g)		Specific growth rate (%/day)	Feed efficiency
	0 Week	4 Weeks	4 Weeks	4 Weeks
T1: Control	34.45±0.08[a]	48.98±2.55[a]	1.25±0.18[a]	0.37±0.04[a]
T2: GBS-Ia (1×10^{10} CFU/mL)	34 .45±0.08[a]	48.62±2.19[a]	1.22±0.17[a]	0.37±0.04[a]
T3: GBS-III (1×10^{10} CFU/mL)	34.45±0.06[a]	49.98±1.01[a]	1.33±0.08[a]	0.38±0.01[a]
T4: GBS-Ia+III (1×10^{10} CFU/mL)	34.45±0.09[a]	50.10±2.88[a]	1.33±0.22[a]	0.37±0.06[a]
T5: GBS-Ia+III (2×10^{10} CFU/mL)	34.45±0.11[a]	51.11±1.76[a]	1.41±0.12[a]	0.40±0.02[a]

Different superscripts within a column indicate a statistically significant difference (p<0.05).

Table 2. Haemato-immunological parameters of Nile tilapia vaccinated with the individual formalin-killed GBS, mixed formalin-killed GBS or PBS (control), at 2 and 4 weeks post vaccination.

Treatment	Hematocrit (%)		Hemoglobin (g/dL)		Serum protein (mg/mL)		Red blood cell ($\times 10^9$cell/mL)		White blood cell ($\times 10^7$cell/mL)	
	2 Weeks	4 Weeks	2 Weeks	4 Weeks	2 Weeks	4 Weeks	2 Weeks	4 Weeks	2 Weeks	4 Weeks
T1: Control	31.60±7.41^a	33.77±3.47^a	5.49±0.72^a	7.45±1.05^a	62.32±5.35^a	71.60±8.32^a	1.96±0.38^a	2.54±0.32^a	3.78±1.26^a	4.83±1.03^a
T2: GBS-Ia (1×10^{10} CFU/mL)	31.60±3.32^a	33.03±4.35^a	5.92±1.01^a	7.56±1.21^a	61.83±6.89^a	71.71±8.29^a	2.40±0.24^b	2.35±0.28^a	3.85±1.01^a	4.74±1.50^a
T3: GBS-III (1×10^{10} CFU/mL)	31.15±3.46^a	32.53±3.77^a	5.49±0.70^a	7.72±0.78^a	62.55±7.29^a	72.17±5.89^a	2.38±0.18^b	2.47±0.24^a	4.18±0.62^a	3.95±0.91^a
T4: GBS-Ia+III (1×10^{10} CFU/mL)	32.69±4.60^a	32.61±3.93^a	5.72±0.89^a	7.41±1.55^a	64.37±7.05^a	69.96±8.70^a	2.23+0.35^{ab}	2.58±0.39^a	4.43±0.65^a	4.06±1.26^a
T5: GBS-Ia+III (2×10^{10} CFU/mL)	32.18±3.28^a	33.47±3.40^a	5.55±0.91^a	7.04±0.95^a	61.09±10.19^a	70.60±7.08^a	2.18+0.71^{ab}	2.29±0.21^a	4.05±0.67^a	4.46±1.52^a

Different superscripts within a column indicate a statistically significant difference ($p<0.05$).

isolated from infected tilapia cultured in Thailand. In this study, no significant differences were observed in growth performance or in the haemato-immunological parameters haematocrit, haemoglobin, serum protein, white blood cell, or in lysozyme activities between treatment groups, at two or four weeks post vaccination. An exception was the red blood cell. This suggests that the GBS vaccines in the present study were safe while they slightly stimulated the innate immune responses. The results are consistent with those observed in tilapia vaccinated with a formalin-killed *S. iniae* vaccine, which induced no significant changes in the haematological parameters at 1 or 4 weeks post vaccination, although the fish that received vaccine plus β−glucan had elevated plasma protein and red blood cell relative to non-vaccinated fish (Suanyuk & Itsaro, 2011).

Significantly increased humoral antibodies against GBS-Ia and GBS-III antigens were found with homologous GBS vaccinations as compared with the control group, indicating that tilapia produce highly specific circulating humoral agglutinins. Moreover, fish immunized with a vaccine containing GBS-Ia antigen had higher antibody titers than those immunized with GBS-III vaccine. Different GBS antigenic determinants such as Cα (*bca*) and Cβ (*bac*) may lead to different antibody production. Cα is a surface expressed antigenic determinant capable of eliciting protective antibody mediated immunity (Madoff, Michel, & Kasper, 1991) and Cβ is known to bind to the Fc region of human immunoglobulin A (Jerlström, Chhatwal, & Timmis, 1991). Our previous study supports this hypothesis because GBS-Ia contains genes encoding proteins Cα and Cβ, while GBS-III contains only Cα (Suanyuk *et al*., 2008). In this study, each treatment group had comparatively low humoral antibody activity against the heterologous GBS antigen, suggesting that the serotype is an important determinant. Although GBS-Ia and GBS-III share some common antigens, each may not be able to stimulate the fish immune system against both GBS serotypes. Conjugate vaccines generated with GBS types IV and VII capsular polysaccharide induced serotype specific antiserum and provided survival of > 90% of newborn pups challenged with homologous serotype (Paoletti & Kasper, 2002). In *S. pneumoniae*, the differences between serotypes appear more significant complement resistance than the differences between strains (Melin, Trzciński, Meri, Käyhty, & Väkeväinen, 2010b). Pnuemococcal isolates of certain serotypes were particularly resistant to complement deposition and opsonophagocytic killing (Melin *et al*., 2009, 2010a). High concentrations of polysaccharide-specific antibodies are required for opsonophagocytic killing of the complement resistant *S. pneumoniae* strain (Melin *et al*., 2010b).

At four weeks post vaccination, slightly decreased antibody titer of fish immunized with vaccines containing homologous GBS antigen was noted in comparison with the level at two weeks. The decreased antibody titer was correlated with the RPS of fish challenged with homologous GBS serotype. Moreover, RPS of fish immunized for four weeks with GBS vaccine is higher than 60% against homo-

Table 3. Lysozyme activities and antibody levels of Nile tilapia vaccinated with the individual formalin-killed GBS, mixed formalin-killed GBS or PBS (control), at 2 and 4 weeks post vaccination.

Treatment	Lysozyme (μg/mL)		Antibody titre[1,2]			
	2 Weeks	4 Weeks	2 Weeks		4 Weeks	
			GBS-Ia antigen	GBS-III antigen	GBS-Ia antigen	GBS-III antigen
T1: Control	10.06±0.99^{a}	9.98±1.24^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
T2: GBS-Ia (1×10^{10} CFU/mL)	10.34±1.44^{a}	9.82±1.06^{a}	3.44±0.28^{c}	0.86±0.41^{b}	2.52±0.56^{c}	0.88±0.35^{b}
T3: GBS-III (1×10^{10} CFU/mL)	10.07±1.29^{a}	9.73±1.56^{a}	1.53±0.48^{b}	1.34±0.65^{c}	1.10±0.35^{b}	1.60±0.36^{c}
T4: GBS-Ia+III (1×10^{10} CFU/mL)	10.50±1.90^{a}	9.95±1.82^{a}	3.42±0.19^{c}	1.55±0.58^{c}	2.83±0.48^{c}	1.51±0.40^{c}
T5: GBS-Ia+III (2×10^{10} CFU/mL)	10.41±1.85^{a}	9.84±1.58^{a}	3.55±0.13^{c}	1.66±0.66^{c}	2.83±0.59^{c}	1.60±0.31^{c}

[1] Log_{10} reciprocal of the greatest serum dilution in which agglutination with different serotype of GBS occurred;
[2] Zero values indicate no agglutination occurred; Different superscripts within a column indicate a statistically significant difference ($p<0.05$).

Table 4. Evaluation of the protection afforded Nile tilapia against challenges with the GBS-Ia or GBS-III by injection vaccinations with formalin-killed vaccines.

Treatment	2 Weeks		4 Weeks	
	Mortality rate (%)	RPS (%)[1]	Mortality rate (%)[1]	RPS (%)[1]
	Challenged with GBS-Ia			
T1: Control	76.67±11.55^{c}	-	66.67±15.28^{c}	-
T2: GBS-Ia (1×10^{10} CFU/mL)	36.67±5.77^{b}	52.17	26.67±11.55^{bc}	60.00
T3: GBS-III (1×10^{10} CFU/mL)	36.67±15.28^{b}	52.17	53.33±37.86^{bc}	20.00
T4: GBS-Ia+III (1×10^{10} CFU/mL)	3.33±5.77^{a}	95.66	13.33±23.09^{ab}	80.00
T5: GBS-Ia+III (2×10^{10} CFU/mL)	10.00±14.14^{a}	86.96	6.67±5.77^{a}	90.00
	Challenged with GBS-III			
T1: Control	93.33±5.77^{c}	-	66.67±15.28^{c}	-
T2: GBS-Ia (1×10^{10} CFU/mL)	43.33±15.28^{b}	53.57	53.33±15.28^{bc}	20.00
T3: GBS-III (1×10^{10} CFU/mL)	26.67±11.55^{ab}	71.42	23.33±5.77^{ab}	65.00
T4: GBS-Ia+III (1×10^{10} CFU/mL)	16.67±15.28^{a}	82.14	16.67±15.28^{a}	75.00
T5: GBS-Ia+III (2×10^{10} CFU/mL)	16.67±5.77^{a}	82.14	23.33±23.09^{ab}	65.00

[1]RPS (Relative percent survival) = [1 – (% mortality of vaccinates/% mortality of controls)] x 100.
Different superscripts within a column indicate statistically significant differences ($p<0.05$).

logous GBS infection indicating that our GBS vaccines conferred protection against GBS up to four weeks. Pasnik, Evans, and Klesius (2005) reported that percent survival of tilapia vaccinated with GBS vaccine was 67, 62 and 49% while the control fish was 16, 16 and 4% after 47, 90 and 180 days post vaccination, respectively. Further investigation of the duration of protective efficacy in tilapia immunized with vaccine produced from different GBS serotypes is required for understanding the fish immune system following the vaccination.

Experimentally, bacterial and parasitic vaccines have provided cross-protection against both homologous and heterologous serotypes in some cases (Cypriano & Ruppenthal, 1987; Swennes, Findly, & Dickerson, 2007; Xu, Klesius, & Panangala, 2006). The present study reveals that the fish vaccinated with formalin-killed cells prepared from either GBS-Ia or GBS-III are well protected against the homologous isolate, whereas the RPS values in challenges with heterologous serotype were quite low, from 20.00 to 53.57%. This finding indicated that our vaccines had insufficient cross-protective efficacy against the other serotype. This is consistent with prior observations on the protection of Japanese flounder *Paralichthys olivaceus* immunized with formalin-killed *S. parauberis* serotypes I or II, that proved to be effective against the homologous isolates with RPS 100%, whereas in challenges with the heterologous isolate the RPS values varied from 0 to 100% (Mori & Fukuda, 2012). However, Osman, Mohamed, Rahman,

and Soliman (2009) report that a polyvalent vaccine provide higher efficacy against more than one type of bacteria. Our results corroborate the above study and show that the mixed vaccine provided significant protection with RPS 65.00 to 95.6% against either GBS-Ia or GBS-III infections, suggesting that a mixed GBS vaccine could protect tilapia against streptococcosis caused by GBS infections.

In summary, formalin-killed vaccines prepared from GBS serotype Ia or III provided significant protection against challenges with homologous serotypes, whereas the protective efficacy against heterologous GBS serotype was poor, indicated insufficient cross-protective efficacy. However, the mixed vaccine produced from both GBS serotypes Ia and III provided significant protection, and could be used as an excellent vaccine to protect fish against streptococcosis caused by GBS infections in Thailand. Further studies on growth, hemato-immunological parameter and cross-protection to different GBS subserotypes, and on the application of mixed GBS vaccine in tilapia under field conditions need to be investigated.

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