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Efficacy of formalin-killed *Streptococcus agalactiae* vaccine in Nile tilapia (*Oreochromis niloticus* L.)

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Abstract
The formalin-killed *Streptococcus agalactiae* vaccine in Nile tilapia (*Oreochromis niloticus* L.) was evaluated in terms of its efficacy at different immersion time. The highest and lowest survival rate was recorded in T1 (93.33±4.71%) and T2 (22.31±15.68%), respectively. Fish in T1 were not experimentally challenged with the pathogenic bacterium while fish in T2 were not vaccinated but challenged with the bacterium. The relative percent survival (RPS) of vaccinated groups (T4 = 53.06±25.71%, T5 = 49.42±37.57%, T3 = 36.57±29.18% and T6 = 36.32±6.71%) showed no significant difference to each other (*p*>0.05). Treatment 4 via 1-hour immersion attained the highest level of protection against the virulent *S. agalactiae* as compared to T3 (30 minutes' immersion), T5 (90 minutes' immersion) and T6 (120 minutes' immersion). Fish in T2 to T6 already exhibited the same physical/clinical signs of bacterial infection such as corneal opacity, bleeding at the base of the fins, lethargy in swimming, erratic swimming, loss of appetite and/or difficulty in breathing.

Keywords: *Streptococcus agalactiae*, Nile tilapia, formalin-killed vaccine, relative per cent survival

1. Introduction

Nile tilapia (*Oreochromis niloticus* L.) is a hardy and most cultured freshwater fish in the world[1]. It has been contributing to the world aquaculture since the ancient Egyptian days and remains a major freshwater fish species being cultured [1]. Worldwide harvest of farmed tilapia has now surpassed 800,000 metric tons in 3,000 years ago [2]. Tilapia is more resistant to viral, bacterial and parasitic diseases than other commonly cultured fish, especially at optimum temperature for growth [2].

Streptococcus is a genus of bacteria that includes some species that cause serious diseases in a number of different hosts. A major identifying feature of *Streptococcus* is that they are Gram-positive and short rod occurring in pairs or chains[3]. The colonies in a typical agar are small, round and white[4]. *Streptococcus* species are catalase negative, non-motile, non-sporing, facultatively anaerobic and chemo-organotrophs with complex nutritional requirements and a fermentative metabolism resulting in lactic acid as major product of glucose fermentation [5].

Septicemia caused by *Streptococcus* spp. is the most severe disease problem in intensively raised tilapia[6]. Affected organs include brain, kidney and gut, among others. Most common disease signs include anorexia, exophthalmia, ascites and erratic swimming [7-10]. Recently, the tilapia-breeding industry has been hampered by outbreaks of *S. agalactiae* infection, which cause high mortality and huge economic losses. Many researchers have attempted to develop an effective *S. agalactiae* vaccines for tilapia [11].

All vertebrate animals possess an immune system which is capable of responding to large foreign molecules, called antigens, by the production of antibodies or activated white blood cells which can react with the antigens in a very specific manner to destroy them. Vaccines are preparations of antigens which, when administered to animals, induce a protective immune response without causing undesirable side effects [12].

Researches carried out in the last decade have focused on developing protective vaccines using different strategies, although limited reviews have been carried out to evaluate the efficacy of these strategies [13]. The successful bacterial vaccines that are now routinely used in aquaculture were developed largely through empirical observations and are usually based on inactivated bacteria. Despite extensive researches over many years, very few anti-viral vaccines are available and there are limited commercial vaccines against fish parasites [14].

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The fact that a small fish can be protected against an infectious disease by immersion for a few seconds in a diluted vaccine solution is one example of the remarkable ability of living organisms to cope with biological challenges [15].

Vaccines have significantly contributed to a steady decline in the mortality and morbidity that is caused by infectious diseases [16]. Vaccination mitigated the infection stress of *S. agalactiae* in tilapia and helped regulate immunity and thus decrease mortality [17].

The general objective of this study was to evaluate the efficacy of formalin-killed *S. agalactiae* vaccine in Nile tilapia (*O. niloticus* L.). Specifically, the study aimed to: (1) determine the best immersion time of Nile tilapia to the inactivated vaccine; (2) compare the per cent survival of vaccinated and non-vaccinated Nile tilapia; (3) compare the vaccine efficiency of various immersion time; and (4) compare the bacterial resistance of vaccinated and non-vaccinated Nile tilapia.

2. Materials and Methods
2.1. Experimental fish
Nile tilapia FaST strain (approximately 20g) were obtained from the Freshwater Aquaculture Center (FAC), Central Luzon State University (CLSU). The experimental fish were maintained and conditioned in glass aquaria (12 × 24 × 12 inches) for 10 days. The fish were fed with commercial diet at 7% feeding rate with three times feeding frequency (9:00 am, 1:00 pm and 3:00 pm). The fish were provided with good environment by siphoning out the waste and uneaten feeds, water replacement and provision of aerator.

2.2. Preparation of *S. agalactiae*
Pure culture of *S. agalactiae* was obtained from the research study "Epidemiology of *Streptococcus* spp. in Farmed Nile tilapia (*Oreochromis niloticus* L.) in Lubao, Pampanga." The identity of the isolates was confirmed by 16S rDNA sequencing. The bacterium was sub-cultured in Trypticase Soy Agar (TSA) plates. About 2-3 colonies were suspended in 5 mL Trypticase Soy Broth (TSB). After 18-24 hours of incubation, the bacterial suspension was adjusted to 10^8 cells/mL using McFarland turbidity standard.

2.3. Preparation of vaccine
The vaccine was prepared according to Klesius *et al.* [18]. *S. agalactiae* was cultured in TSB and it was incubated at 15 °C for 3-5 days. The cultures were treated with 3% formalin at 15 °C for 24 h. The bacterial suspension was centrifuged at 10,000 rpm for 20 min. The supernatant was concentrated 10 folds. The final concentration was 1×10^8 cells/mL.

2.4. The protocol of vaccination
A completely randomized design (CRD) was used in setting-up the glass aquaria. The study employed two controls and four treatments with three replications (Table 1). Each aquarium was stocked with 10 pieces of healthy and conditioned tilapia with approximate individual weight of 20 g. The experimental fish were immersed in aerated 3 L *S. agalactiae* suspension for 30, 60, 90 and 120 minutes. The immersion of fish in positive control was done in 3 L sterile distilled water (Table 1).

Table 1: Description of controls and treatment that were used in the experiment.

T1 (Negative control)	Non-vaccinated and unchallenged tilapia
T2 (Positive control)	Non-vaccinated and challenged tilapia
T3	Vaccinated tilapia via 30 minutes' immersion
T4	Vaccinated tilapia via 60 minutes' immersion
T5	Vaccinated tilapia via 90 minutes' immersion
T6	Vaccinated tilapia via 120 minutes' immersion

2.5. Challenge test
Two weeks after vaccination, each fish was intraperitoneally injected with 0.1 mL of *S. agalactiae* preparation. The control groups were injected with the same volume of sterile distilled water. The fish were fed with a commercial diet at 7% feeding rate with three times feeding frequency. The aquaria were provided with aerators and good husbandry was followed until the end of the experiment.

2.6. Data gathered
Clinical signs of disease, morbidity and mortality were recorded daily for four weeks and the experiment was terminated when 100% morbidity or mortality has occurred among the challenged groups. Dead or moribund fish were necropsied, and smears from skin, liver and kidney were serially diluted (10^{-2} dilution) and spread aseptically on TSA plates. The plates were incubated at 37 °C for 18-24 hours [19]. Mortalities were valid if the recovered colonies have similar morphological characteristics compared to the known bacterium that was used in the experiment.

Survival rate was computed using the formula:

$$\text{Survival rate} = \frac{\text{total number of fish survived}}{\text{total number of fish stocked}} \times 10$$

Vaccine efficacy was calculated as the relative per cent of survival (RPS):

$$RPS = \left[1 - \left(\frac{\text{percentage (\%) mortality in vaccinated fish}}{\text{percentage (\%) mortality in control}}\right)\right] \times 100$$

2.7. Statistical analysis
Survival rate and RPS were transformed using Arcsin before the data were analyzed using statistical test. One-way Analysis of Variance (ANOVA) was used for testing the significant differences among means; Tukeys test was used for the comparison of means.

3. Results and Discussion
3.1. Survival rate and relative per cent of survival (RPS)
Two weeks after the vaccination of the experimental fish via immersion, the tilapias were intraperitoneally challenged with 0.1 mL of *S. agalactiae* (T3-T6). The fish in the negative control (T1) was injected with the same volume of sterile distilled water. Presented in Table 2 are the average weight, survival rate and RPS of tilapia in the non-vaccinated and vaccinated groups. The average weight of tilapia used in the experiment ranged from 16.15-17.34 g and statistical analysis revealed no significant difference between initial weights across non-vaccinated and vaccinated groups when compared

(p>0.05). The highest survival rate was recorded in T1 (93.33±4.71%) followed by T4 (70.43±12.67%), T5 (64.81±24.98%), T3 (54.62±14.40%), T6 (51.48±6.43%) and lastly T2 (22.31±15.68%). The result was expected because fish in T1 were not experimentally challenged with the pathogenic bacterium and fish in T2 were not vaccinated but challenged with the bacterium. Groups that were vaccinated (T3-T6) had significantly higher survival rate when compared to non-vaccinated and challenged group (T2) ($p<0.05$). The results revealed that the RPS of vaccinated groups (T4 = 53.06±25.71%, T5 = 49.42±37.57%, T3 = 36.57±29.18%, T6 = 36.32±6.71%) showed no significant difference to each other ($p>0.05$). Treatment 4 via 1-hour immersion attained the highest level of protection against the virulent *S. agalactiae*.

Table 2: The average body weight (g), mean per cent survival (%) and relative per cent survival (%) of the experimental fish in the non-vaccinated and vaccinated groups.

Treatment	Average Weight (g)	Survival Rate (%)	RPS (%)
T1 (- Control)	17.34±0.71[a]	93.33±04.71[a]	-
T2 (+ Control)	16.86±0.34[a]	22.31±15.68[c]	-
T3	16.76±0.52[a]	54.62±14.40[b]	36.57±29.18[a]
T4	16.18±0.59[a]	70.43±12.67[b]	53.06±25.71[a]
T5	16.23±0.98[a]	64.81±24.98[b]	49.42±37.57[a]
T6	16.15±0.20[a]	51.48±06.43[b]	36.32±06.71[a]

Note: T1 (Negative control) = Non-vaccinated and unchallenged, T2 (Positive control) = Non-vaccinated and challenged, T3 = Vaccinated tilapia via 30 minutes' immersion, T4 = Vaccinated tilapia via 60 minutes' immersion, T5 = Vaccinated tilapia via 90 minutes' immersion, T6 = Vaccinated tilapia via 120 minutes' immersion.

Immersion vaccination works on the ability of mucosal surfaces to recognize pathogens they get in contact with. When fish are immersed in water containing the diluted vaccine, the suspended antigens from the vaccine may be adsorbed through the skin, across the gill membrane, and/or by ingestion [20, 21]. Specialized cells, such as antibody-secreting cells, present in the skin and gill epithelium will be activated to protect the fish from exposure to the live pathogen at a later stage, which will respond by producing antibody [20, 21] and systemic immune response builds up [22]. Warm water species may be required 1-3 weeks to develop antigen [23].

The effectiveness of vaccination is primarily dependent on the amount of antigen taken up by fish[24]. It improves the adsorption of antigens through gill tissues, skin and intestines and promotes their retention in the lymphoid tissues providing a slow depot release [25]. Several factors which might influence this uptake were considered; the length of immersion time [26], because smaller, younger fish may have immature immune systems and hyperosmotic immersion can result to possible gill damage [25, 27].

The formalin-killed whole cell suspensions are very easy and inexpensive to manufacture [28]. Vaccination through immersion is an effective method for mass vaccination, especially of small fish [29]. Its main advantage is that it is cost effective because a large number of fish can be vaccinated at the same time without individual handling. As such, it is less likely to cause mortality during vaccination and it impacts less stress-related immune suppression [13, 29]. This vaccination strategy is less used compared to the injectable vaccines [13].

Prolonged immersion may offer greater protection [30]. According to Baba *et al*. [31], two hours immersed in the vaccine is more effective, but this present study revealed that 30 minutes' immersion was already enough to improve the survival of the challenged fish. Giordano *et al*. [32] reported that Nile tilapia immunized with the *S. agalactiae* vaccine could increase antibodies against *S. agalactiae*. Increased antibodies are also often associated with an increase in the RPS [33]. Within 3-7 days, acute *Streptococcus* infections in fish induce 50-70% mortality rates [4, 3]. The study of Pasnik *et al*. [35] showed that, per cent survival of tilapia vaccinated with Group B *Streptococcus* (GBS) vaccine was 67%, 62% and 49%, while the control or non-vaccinated fish was 16%, 16% and 4%.

Mortalities of fish vaccinated by intraperitoneal (IP) injection and by multiple puncture/immersion at 14 days after challenge were 35 and 40%, respectively, while those for non-vaccinated control fish or fish vaccinated by immersion was 80% [29]. Previous studies showed that whole-cell inactivated *S. agalactiae* vaccines could provide protection to tilapia (weight >20 g), with a RPS of 35-41.46% [35]. It also displayed good protection for 30 g tilapia by IP injection (RPS = 96.88%), immersion (RPS = 67.22%) and oral administration (RPS = 71.81%) at 15 d post-vaccination[36].

Today the majority of the fish vaccines are delivered by injection, which is by far the most effective method compared to the oral and immersion route [15]. Injection of formalin-killed vaccine provides higher level of protection than those of immersion and oral vaccination [37]. Immersion administration of these same vaccines failed to provide high levels of adaptive immune protection [38].

Unfortunately, vaccines are usually not able to confer protection on their own; especially those vaccines based on recombinant antigens or inactivated pathogens[25]. Protection using immersion methods may not last long and a second vaccination may be required [39].

3.2. Challenge test

Within 24 hours (1 day) of challenge with *S. agalactiae*, some of the tilapia in T2-T6 already exhibited one of the physical/clinical signs of bacterial infection such as corneal opacity, bleeding at the base of the fins, lethargy in swimming, erratic swimming, loss of appetite and/or difficulty in breathing (Table 3). In a study conducted by Giordano *et al*. [32], the Nile tilapia challenged with *S. agalactiae* showed alterations in behaviour and similar clinical signs such as anorexia, lethargy, erratic swimming, exophthalmia (bulging of eyes) and ascites (fluid internal organs). Macroscopically, skin hemorrhage, splenomegaly (enlargement of spleen), hepatomegaly (enlargement of liver) with organ paleness and visceral adherences were observed. One day after the challenge test, the first mortality was recorded in T1 and T2. The death of fish in T1 was not attributed to biological agent such as bacteria because of the absence of physical and/or clinical sign of infection. In the case of dead fish in T2, the cause of death was related to the injected bacterium because of the observed clinical signs of *S. agalactiae* infection. In the vaccinated fish, the first mortality

was recorded after 48 hours' challenge. The physical/clinical signs of infection worsen as the time progresses especially in the case of T2. Majority of the experimental fish in T2 showed 4-5 signs of bacterial infection. Highest mortality coefficients were observed in days 1-2 after inoculation (accumulated mortality 44.4%), and a second peak of mortality occurred at days 6-7. No further mortality was recorded after 16 days of challenge test. In total, 65 fish were necropsied, and smears from kidney, liver and skin were serially diluted and spread aseptically on TSA plates. In the challenged groups (T2-T6), the bacterium was recovered in the kidney of the necropsied fish with 100% recovery. Per cent recovery from the liver ranged from 25.00% in T5 to

85.71% in T2. Meanwhile, bacterium recovery in the skin of necropsied fish was from 16.66% (T3) to 61.90% (T2) (Table 4). Kidney samples appear to produce the most reliable/reproducible sampling results [40, 41]. In fish the anterior kidney is one of the most prominent hematopoietic organs and it is likely that phagocytes directed towards the periphery originate from the anterior kidney at least for a substantial part [42]. The injected and recovered *S. agalactiae* colonies were characterized as yellow, small in size, round in shape, entire margin, convex elevation and smooth in texture. The recovery of the injected *S. agalactiae* has served as a proof that the same bacterium is the one responsible for the recorded mortality and observed clinical signs of infection.

Table 3: Clinical signs of infection, onset of infection and mortality of the experimental fish in non-vaccinated and vaccinated groups.

Treatment	Clinical Signs of Infection	Onset of Clinical Sign of Infection	Onset of Mortality
T1 (- Control)	No clinical abnormalities	---	Day 1
T2 (+ Control)	Corneal opacity, lethargy in swimming, erratic swimming, bleeding at the base of the fins, difficulty in breathing, loss of appetite	Day 1	Day 1
T3	Corneal opacity, lethargy in swimming, erratic swimming, bleeding at the base of the fins, difficulty in breathing, loss of appetite	Day 1	Day 2
T4	Corneal opacity, lethargy in swimming, erratic swimming, bleeding at the base of the fins, difficulty in breathing, loss of appetite	Day 1	Day 2
T5	Corneal opacity, lethargy in swimming, erratic swimming, bleeding at the base of the fins, difficulty in breathing, loss of appetite	Day 1	Day 2
T6	Corneal opacity, lethargy in swimming, erratic swimming, bleeding at the base of the fins, difficulty in breathing, loss of appetite	Day 1	Day 2

Note: T1 (Negative control) = Non-vaccinated and unchallenged, T2 (Positive control) = Non-vaccinated and challenged, T3 = Vaccinated tilapia via 30 minutes' immersion, T4 = Vaccinated tilapia via 60 minutes' immersion, T5 = Vaccinated tilapia via 90 minutes' immersion, T6 = Vaccinated tilapia via 120 minutes' immersion.

Table 4: Number of dead fish (per cent recovery is in parenthesis) wherein the bacterium was recovered in the kidney, liver and skin.

Treatment	Kidney	Liver	Skin	Total Mortality (no.)
T1 (- Control)	---	---	---	2
T2 (+ Control)	21 (100.00)	18 (85.71)	13 (61.90)	21
T3	12 (100.00)	7 (58.33)	2 (16.66)	12
T4	8 (100.00)	3 (37.50)	2 (25.00)	8
T5	8 (100.00)	2 (25.00)	2 (25.00)	8
T6	14 (100.00)	6 (42.85)	3 (21.42)	14

Note: T1 (Negative control) = Non-vaccinated and unchallenged, T2 (Positive control) = Non-vaccinated and challenged, T3 = Vaccinated tilapia via 30 minutes' immersion, T4 = Vaccinated tilapia via 60 minutes' immersion, T5 = Vaccinated tilapia via 90 minutes' immersion, T6 = Vaccinated tilapia via 120 minutes' immersion.

4. Conclusion
The results revealed that 1-hour vaccination (T4) via immersion could provide the best protection of the fish against the virulent *S. agalactiae*. Immersion vaccination is a cost effective method for mass vaccination, especially on small fish. Therefore, formalin-killed *S. agalactiae* vaccine can be used in the prevention of diseases and mortalities.

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