Bivalent vaccination of sex reversed hybrid tilapia against *Streptococcus iniae* and *Vibrio vulnificus*

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**Abstract**

*Streptococcus iniae*, a Gram-positive bacterium, and *Vibrio vulnificus*, a halophilic Gram-negative bacterium, have been associated with severe disease impacting tilapia aquaculture. Recent reports suggest that both bacteria have been associated independently and concomitantly with disease on commercial farms. Monovalent vaccines have been developed for disease control; however, the most effective delivery strategy has been via intraperitoneal (IP) injection. Due to handling stress and the cost associated with injecting each fish, a better strategy is to combine the monovalent vaccines into bivalent formulations. The objective of the present study was to test the ability of a killed bivalent *S. iniae* and *V. vulnificus* vaccine delivered by IP injection at protecting sex reversed hybrid tilapia (*Oreochromis niloticus × Oreochromis aureus*) against challenge with each bacterium, independently. In two independent trials, vaccination of tilapia with the bivalent vaccine conferred protective immunity against *V. vulnificus* and *S. iniae* as demonstrated by significant differences (*P* < 0.05) in survival curves between the sham-vaccinated and vaccinated groups. Relative percent survival values ranged from 79 to 89% for *V. vulnificus* and 69 to 100% for *S. iniae* following challenge of bivalent vaccinated fish. Use of this bivalent formulation may be a cost-effective strategy to reduce losses in tilapia co-infected with these two important bacterial pathogens.

1. Introduction

The value of tilapia (*Oreochromis* spp.) from the aquaculture sector was about $3 billion in 2008 (FAO, 2010). Tilapia culture occurs in a wide range of environments including fresh water, sea water and brackish or/and low saline waters (Paz et al., 2007; Watanabe et al., 2006). Tilapia were initially described as being more disease resistant than other species of cultured fish (Roberts and Sommerville, 1982). However, intensification of aquaculture has lead to severe disease impacting production (Shoemaker et al., 2006a). The reality in commercial tilapia production is that multiple disease agents are present (Martins et al., 2011; Soto et al., 2011) and impact fish health and production efficiency.

The impact of *Streptococcus iniae* on tilapia aquaculture has been known for more than a decade (Agnew and Barnes, 2007; Klesius et al., 2008; Shoemaker et al., 2001). *Vibrio vulnificus* has been studied for a number of years with the focus on food borne illness (Jones and Oliver, 2009) and eel aquaculture (Austin, 2010; Fouz et al., 2006). Sakata and Hattori (1988) were among the first to report *V. vulnificus* losses of 10–20% in tilapia cultured in ponds filled with saline ground water. The influence of this bacterium on brackish or low saline freshwater tilapia aquaculture appears to be emerging or re-emerging (Chen et al., 2006; Mahmud et al., 2010; Paz et al., 2007). Shoemaker et al. (2011) described a biotype 1, vsg type C, 16S rRNA type B, and vvhA type 2 *V. vulnificus* isolated from diseased hybrid tilapia (*Oreochromis niloticus × Oreochromis aureus*) cultured in an intensive water reuse system. Both *V. vulnificus* and *S. iniae* have been associated independently and concomitantly with disease on commercial tilapia farms (Soto et al., 2011; Shoemaker and Klesius, unpublished data). With the potential of each bacterium to impact human health (Baiano and Barnes, 2009; Jones and Oliver, 2009) and aquaculture production, control strategies need to be sought. This is particularly important as tilapia are often sold at live markets and prepared for cooking at home. Puncture wounds and/or injuries that occur during preparation of fish were suggested as the probable route of entry for these bacterial pathogens (Bisharat et al., 1999; Shoemaker et al., 2001).

Multivalent adjuvanted vaccines (4 to 6 antigens in combination) delivered by intraperitoneal (IP) injection are commonly used in commercial Atlantic salmon (*Salmo salar*) production against bacterial and viral pathogens (Sommerset et al., 2005). Limited information is available on protective efficacy of killed bacterial vaccine antigens in combination when delivered to lower valued fish species. Li et al. (2006) demonstrated efficacy of a bivalent vaccine against *Aeromonas hydrophila* and *Vibrio fluvialis* in crucian carp (*Carassius auratus*) and efficacy was demonstrated in a bivalent vaccine against typical and atypical *Aeromonas salmonicida* in Arctic char (*Salvelinus alpinus*) (Pylkko et al., 2002). A multivalent vaccine against *A. hydrophila*,...
**Edwardsiella tarda**, and *Pseudomonas fluorescens* was demonstrated to be effective in Indian major carp (*Labeo rohita*) (Swain et al., 2007). Sun et al. (2011) administered killed *E. tarda*, *Vibrio anguillarum*, *S. iniae*, and *Vibrio harveyi* with adjuvant singly and in combinations (2, 3 or 4 antigens together) to Japanese flounder (*Paralichthys olivaceus*). The best protection was observed with a multivalent vaccine against *E. tarda* and *V. anguillarum*. Effective monovalent killed vaccines have been developed and utilized against both *S. iniae* (Eldar et al., 1997; Klesius et al., 1999, 2001; Shoemaker et al., 2010) and *V. vulnificus* (Collado et al., 2000; Shoemaker et al., 2011). The most effective delivery strategy has been via IP injection. Due to handling stress and the cost associated with injecting each fish, a better strategy would be to combine monovalent vaccines into bivalent (Bastardo et al., 2012) or multivalent formulations (Swain et al., 2007). The objective of the present study was to test the ability of a killed bivalent *S. iniae* and *V. vulnificus* vaccine delivered by IP injection at protecting sex reversed hybrid tilapia (*O. niloticus* × *O. aureus*) against challenge with each bacterium, independently.

2. Materials and methods

2.1. Fish and rearing conditions

Sex reversed F1 hybrid tilapia (*O. niloticus* × *O. aureus*) were obtained as fry from AQUASAFRA, Inc. (Bradenton, FL, USA) and used as experimental animals because these fish are commonly used in intensive production in the US. Tilapia with mean weights (±SD) of 9.7 (±2.1) g and 13.5 (±2.9) g were used in Trials 1 and 2, respectively and were acclimated for 1 week prior to vaccination. Prior to each trial, brain and head kidney tissues from 10 fish were plated onto sheep blood agar (SBA; Remel, Lenexa, KS, USA) and tryptic soy agar (TSA; Difco Laboratories, Becton Dickinson Company, Sparks, MD, USA) and incubated at 28 °C for 48 h. None of the fish sampled were culture positive for *S. iniae* or *V. vulnificus*. Following vaccination, fish were maintained in 180 L aquaria supplied with 0.5 L min⁻¹ dechlorinated municipal water and supplemental aeration was provided with air stones. Fish were fed daily (approximately 3% body weight) with Aquamax Grower (PMI Nutrition International, Inc., Brentwood, MO, USA). All procedures utilizing fish were approved by the USDA-ARS AAHRU Institutional Animal Care and Use Committee.

2.2. Bacteria and culture conditions

Virulent isolates of *S. iniae* (ARS-98-60) and *V. vulnificus* (ARS-1Br-09) were used for all portions of this study. *S. iniae* ARS-98-60 was originally isolated from hybrid striped bass (*Morone saxatilis* × *Morone chrysops*) (Klesius et al., 2000). The isolate was recovered from a frozen glycerol stock, cultured in tryptic soy broth (TSB; Difco Laboratories) for 24 h at 28 °C, and then used for bacterial challenges. *V. vulnificus* ARS-1Br-09 was originally isolated from diseased hybrid tilapia cultured in an intensive water reuse system and was characterized as a biotype 1, vcg type C, 16S rRNA type B, and vvhA type 2 isolate (Shoemaker et al., 2011). The isolate was recovered from a frozen glycerol stock and cultured in TSB supplemented with 0.5% sodium chloride (TSB + NaCl). The isolate was cultured at 28 °C for 24 h for preparation of the vaccine, and cultured at 28 °C for 5 h for bacterial challenges. For both bacterial species, the number of viable colony-forming units (cfu) mL⁻¹ were determined by spread plating 10-fold serial dilutions onto SBA using standard procedures.

2.3. Vaccine preparation and administration

2.3.1. Trial 1

The modified *S. iniae* bacterin and killed *V. vulnificus* vaccines were prepared according to Klesius et al. (1999; 2000) and Shoemaker et al. (2011), respectively. Briefly, *S. iniae* (ARS-98-60) was cultured in TSB for 72 h at 28 °C and the culture was then treated for 24 h with 0.3% formalin. The formalin-treated culture was centrifuged at 7000 × g for 30 min. The cell-free supernatant was concentrated 20-fold using a 2 kDa spiral concentrator (EMD Millipore Corporation, Billerica, MA, USA), filtered sterilized (0.2 μm), and used to resuspend the cells (original plate count yielded 4 × 10⁸ cfu mL⁻¹). *V. vulnificus* (ARS-1Br-09) was grown at 28 °C in 500 mL TSB + NaCl for 24 h prior to adding 1% (v/v) formalin to inactivate the cells the original plate count yielded 9.0 × 10⁸ cfu mL⁻¹. The individual vaccines were combined 1:1 to prepare the bivalent vaccine (each fish received 2 × 10⁸ and 4.5 × 10⁷ cfu/fish of *S. iniae* and *V. vulnificus*, respectively). Sex reversed hybrid tilapia with a mean weight of 9.7 (±2.1) g were used as experimental animals. Seventy five fish were vaccinated intraperitoneally (IP) with 100 μL of the bivalent vaccine and an equal number of fish were sham vaccinated IP with 100 μL of sterile TSB + NaCl. Following vaccination, the two groups of fish were held in individual 180 L aquaria until bacterial challenge with *V. vulnificus* or *S. iniae* at 75 or 76 dpv post vaccination (dpv), respectively.

2.3.2. Trial 2

The individual *S. iniae* and *V. vulnificus* vaccines prepared as described above were concentrated two-fold and then combined 1:1 to prepare the bivalent vaccine for Trial 2. This process was used so the delivered dose of each antigen would be equivalent to the original vaccine dose for each antigen (4 × 10⁸ and 9 × 10⁷ cfu/fish of *S. iniae* and *V. vulnificus*, respectively). Briefly, 40 mL of the killed *V. vulnificus* vaccine was centrifuged at 4000 × g for 20 min, 20 mL of the supernatant was discarded, and then the cells were resuspended into the remaining supernatant. The same process was carried out using the modified *S. iniae* bacterin vaccine. The concentrated bivalent vaccine was then delivered to tilapia with a mean weight of 13.5 (±2.9) g. Fifty fish were vaccinated IP with 100 μL of the bivalent vaccine, and an equal number of fish were sham vaccinated IP with 100 μL of sterile TSB. Following vaccination, the two groups of fish were held in individual 180 L aquaria until bacterial challenge with *V. vulnificus* or *S. iniae* at 109 or 76 dpv, respectively.

2.4. Bacterial challenges

2.4.1. Trial 1

At 75 dpv, duplicate groups of 12–13 tilapia from the vaccinated and sham vaccinated tanks were challenged with *V. vulnificus*. Fish were challenged by IP injection with 100 μL containing *V. vulnificus* at a concentration of 5.9 × 10⁷ cfu fish⁻¹. A single group of 12 fish was included as mock injected controls and was injected IP with 100 μL of sterile TSB + NaCl. Following challenge, fish were maintained in 57 L aquaria filled with 40 L of static water containing 1.5 g sea salt (Crystal Sea® marine mix; Marine Enterprises International, Baltimore, MD) L⁻¹ with temperature of 28+2 °C maintained by aquarium heaters. Each day half of the tank water volume was drained, re-filled, and sea salt was added to maintain a concentration of 1.5 g L⁻¹. Microbial isolation from at least 50% of the dead fish from each tank was accomplished by inoculating samples of the brain onto SBA.

At 76 dpv, duplicate groups of 13 fish from the vaccinated and sham vaccinated tanks were challenged with *S. iniae* as described by Shoemaker et al. (2010). Fish were challenged by injection IP with 100 μL volume containing *S. iniae* at a concentration of 1.2 × 10⁸ cfu fish⁻¹. A single group of 13 fish was included as mock infected controls and fish were injected IP with 100 μL of sterile TSB. Following challenge, fish were maintained in 57 L aquaria supplied with 26±2 °C flow through fresh water (0.5 L min⁻¹). Challenged fish were monitored for 14 d post challenge and microbial isolation was completed on at least 50% of the dead fish per tank.
2.4.2. Trial 2

The challenge conditions were the same as that in Trial 1 (i.e., *S. iniae* in freshwater and *V. vulnificus* in static salt water) with duplicate groups of 10 fish from the vaccinated and sham vaccinated tanks. A single group of 10 sham vaccinated fish was utilized as mock infected control fish. *S. iniae* challenge was administered at 76 dpv and each fish was challenged with 1.85 × 10^5 cfu fish^-1_. The *V. vulnificus* challenge was administered at 109 dpv and each fish was challenged with 3.9 × 10^7 cfu fish^-1_.

2.5. Agglutinating antibody titer

Blood was collected from the caudal vasculature of individual fish prior to and post challenge (Trial 2) using a non-heparinized syringe with 25-gauge needle to obtain serum for antibody titer determination. Serum was collected following centrifugation of clotted blood at 3000 × g for 10 min and stored frozen at −20 °C until analysis. Agglutinating antibody titer was determined with a microagglutination assay using each antigen independently following the method of Klesius et al. (2000) and Shoemaker et al. (2011) for *S. iniae* and *V. vulnificus*, respectively. Briefly, *V. vulnificus* was grown for 8 h in TSB + NaCl at 28 °C and killed with 1% (v/v) formalin. *S. iniae* was grown in TSB for 24 h at 28 °C and killed with 1% (v/v) formalin. The killed bacteria were then centrifuged at 4000 × g for 15 min and the resulting cell pellets were resuspended and washed in phosphate buffered saline (PBS, pH 7.2). Following three washes, the bacteria were adjusted to an optical density of 0.6 at 540 nm in PBS. Round bottom 96-well microtiter plates were initially plated with 25 μL of PBS (one for the *V. vulnificus* antigen and one for the *S. iniae* antigen). In each well of the first row, 25 μL of serum was added and mixed. Following mixing, two-fold serial dilutions were made. After this, 25 μL of either the *V. vulnificus* or *S. iniae* cell suspension was added to each well. Positive and negative control sera were included on each plate as assay controls. Plates were covered and incubated overnight (18 h) at 22 ± 2 °C. The agglutination endpoint was determined as the final dilution where visible cell agglutination was observed. Titers were reported as the reciprocal of the highest serum dilution showing visible agglutination.

2.6. Statistical analyses

Survivor fractions were calculated using the product limit (Kaplan–Meier) method. For each bacterial challenge in both trials, survival curves for the vaccinated and sham-vaccinated groups were compared using a log-rank test (Mantel–Cox). The data for replicate tanks were pooled because the survival curves of the replicate tanks were not significantly different following log-rank tests. Survival data were analyzed and graphically represented using GraphPad Prism (version 5.03, GraphPad Software, Inc., La Jolla, CA, USA). Relative percent survival (RPS) values of 79 and 69% were observed for vaccinated fish following challenge with *V. vulnificus* and *S. iniae*, respectively. *V. vulnificus* and *S. iniae* were reisolated from the brain of 91% (10/11) and 93% (13/14) of the examined fish, respectively. There were no mortalities in the mock infected control group.

3.2. Trial 2

Similar to Trial 1, vaccination of tilapia with the bivalent vaccine conferred protective immunity against *V. vulnificus* and *S. iniae* as demonstrated by significant differences (P < 0.05) in survival curves between the sham-vaccinated and vaccinated groups (Fig. 2). For the *V. vulnificus* challenge, the sham-vaccinated fish had a 55% probability of surviving beyond the end of the trial, while the vaccinated fish had a 95% probability of surviving. Relative percent survival (RPS) values of 79 and 69% were observed for vaccinated fish following challenge with *V. vulnificus* and *S. iniae*, respectively. *V. vulnificus* and *S. iniae* were reisolated from the brain of 100% (6/6) and 100% (10/10) of the examined fish, respectively. There were no mortalities in the mock infected control group.

3.3. Antibody response

Prior to bacterial challenges in Trial 2, vaccinated fish exhibited mean agglutination antibody titers of 6.7 ± 2.0 and 10.4 ± 1.1 against *S. iniae* and *V. vulnificus*, respectively (Table 1). These antibody titers were significantly (P < 0.05) higher than those of the sham vaccinated fish which exhibited mean agglutinating antibody titers of 0.3 ± 0.3 and 1.0 ± 0.6 against *S. iniae* and *V. vulnificus*, respectively. Post challenge titers increased 30–100 fold compared to pre-challenge levels but there were no significant differences between the vaccinated and sham vaccinated fish regardless of antigen used in the assay (Table 1).
that are potentially protective, including enolase, glyceraldehyde-3-phosphate dehydrogenase, and fructose-bisphosphate aldolase (LaFrentz et al., 2011). Tilapia immunized with the monovalent V. vulnificus vaccine generate antibodies specific for both protein and carbohydrate antigens of the bacterium, some of which are likely protective antigens (LaFrentz and Shoemaker, unpublished data). The antibody titers observed in the present study were lower than what is typically observed following immunization with the monovalent S. iniae (Whittington et al., 2005) and V. vulnificus (Shoemaker et al., 2011) vaccines. Whether this is a result of combining the monovalent vaccines together is not known but may warrant further investigation. Nikoskelainen et al. (2007) suggested the potential for antigenic competition and other immunological processes that could negatively affect antibody responses against multivalent vaccines. Regardless of the low antibody titers, robust protection was observed and specific antibody titers against V. vulnificus and S. iniae post-challenge were similar to that observed with the monovalent V. vulnificus (Shoemaker et al., 2011) and S. iniae vaccines (Whittington et al., 2005).

A few studies have indicated that certain combinations of different bacteria in multivalent vaccines can enhance protection compared to a monovalent vaccine (Amend and Johnson, 1984; Midtlyng et al., 1996; Hoel et al., 1997; Sun et al., 2011). Sun et al. (2011) suggested that the presence of E. tarda and V. anguillarum induced a co-immunostimulatory effect that enhanced protection. Amend and Johnson (1984) indicated a similar response when combining A. salmonicida and Renibacterium salmoninarum as a bivalent vaccine in salmonids. Interestingly, this potential adjuvant effect has been associated with different Vibrio spp. (Hoel et al., 1997; Midtlyng et al., 1996). Although we did not include single vaccination groups in the present study, the results suggest that V. vulnificus and S. iniae together may induce a synergistic immune effect as RPS values in the present study (Trial 2, using original vaccine composition) are higher than values obtained by V. vulnificus vaccination only (89% versus 60–73%; Shoemaker et al., 2011) and S. iniae vaccination only (100% versus 72–95%; Klesius et al., 2006), although 100% RPS has been documented with the S. iniae vaccine (Shoemaker et al., 2006b, 2010).

In summary, immunization of hybrid tilapia with a bivalent vaccine comprised of S. iniae and V. vulnificus was demonstrated to confer significant levels of protection against both bacterial pathogens. The use of this vaccine formulation may provide a cost-effective strategy to reduce losses in tilapia co-infected with these two important bacterial pathogens.

4. Discussion

The advantage of an effective multivalent vaccine is that a single dose containing multiple antigens may be administered, thus making injection vaccination more cost effective in low value fish species. Results of this study demonstrate that administration of a bivalent vaccine comprised of S. iniae and V. vulnificus to tilapia stimulated protective immune responses against both bacterial pathogens. In Trial 1, the bivalent vaccine contained each antigen at an equal concentration (i.e., half the amount of antigen as the original formulation) that induced protective immunity against V. vulnificus and S. iniae with RPS of 79 and 69%, respectively. In the second trial, the individual vaccines were concentrated two-fold prior to being mixed together so that the delivered dose of each antigen would be equivalent to the original vaccine composition. Immunization of tilapia with the concentrated bivalent vaccine resulted in stronger protection against both pathogens with RPS values of 89 and 100% following challenge with V. vulnificus and S. iniae, respectively.

Tilapia immunized with the bivalent vaccine generated antibodies specific for both bacterial pathogens and these were likely involved in the protection observed. Previous research has demonstrated that specific antibodies are important for protection of tilapia against S. iniae (LaFrentz et al., 2011; Shelby et al., 2002) and V. vulnificus (LaFrentz and Shoemaker, unpublished data). An immunoproteomic approach utilizing antibodies from tilapia immunized with the monovalent S. iniae vaccine identified numerous proteins that are potentially protective, including enolase, glyceraldehyde-3-phosphate dehydrogenase, and fructose-bisphosphate aldolase (LaFrentz et al., 2011). Tilapia immunized with the monovalent V. vulnificus vaccine generate antibodies specific for both protein and carbohydrate antigens of the bacterium, some of which are likely protective antigens (LaFrentz and Shoemaker, unpublished data). The antibody titers observed in the present study were lower than what is typically observed following immunization with the monovalent S. iniae (Whittington et al., 2005) and V. vulnificus (Shoemaker et al., 2011) vaccines. Whether this is a result of combining the monovalent vaccines together is not known but may warrant further investigation. Nikoskelainen et al. (2007) suggested the potential for antigenic competition and other immunological processes that could negatively affect antibody responses against multivalent vaccines. Regardless of the low antibody titers, robust protection was observed and specific antibody titers against V. vulnificus and S. iniae post-challenge were similar to that observed with the monovalent V. vulnificus (Shoemaker et al., 2011) and S. iniae vaccines (Whittington et al., 2005).

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In summary, immunization of hybrid tilapia with a bivalent vaccine comprised of S. iniae and V. vulnificus was demonstrated to confer significant levels of protection against both bacterial pathogens. The use of this vaccine formulation may provide a cost-effective strategy to reduce losses in tilapia co-infected with these two important bacterial pathogens.

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Table 1
Agglutinating antibody titers of sex reversed hybrid tilapia vaccinated or sham vaccinated with a bivalent vaccine (●) or sham-vaccinated (○).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antigen</th>
<th>Pre-challenge</th>
<th>Post-challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range (N)</td>
<td>Mean titer* (±SE)</td>
</tr>
<tr>
<td>Sham vaccinated</td>
<td>S. iniae</td>
<td>0–4 (15)</td>
<td>0.3±0.3a</td>
</tr>
<tr>
<td></td>
<td>V. vulnificus</td>
<td>0–8 (15)</td>
<td>1.0±0.6a</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>S. iniae</td>
<td>0–32 (15)</td>
<td>6.7±2.0b</td>
</tr>
<tr>
<td></td>
<td>V. vulnificus</td>
<td>4–16 (15)</td>
<td>10.4±1.1b</td>
</tr>
</tbody>
</table>

* Titers were reported as the reciprocal of the highest serum dilution showing visible agglutination. Mean titers with different superscripts indicate a significant difference (P<0.05).
References


