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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.

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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 and/or 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, *vcg* 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 charr (*Salvelinus alpinus*) (Pylkko et al., 2002). A multivalent vaccine against *A. hydrophila*,

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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, 2000, 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 4×10^9 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 9.0×10^8 cfu mL^{-1}. The individual vaccines were combined 1:1 to prepare the bivalent vaccine (each fish received 2×10^8 and 4.5×10^7 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 d 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 (4×10^8 and 9×10^7 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 5.9×10^7 cfu $fish^{-1}$. A single group of 12 fish was included as mock infected 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 1.2×10^6 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.