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Efficacy of a modified live *Flavobacterium columnare* vaccine in fish

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ABSTRACT

Flavobacterium columnare is an aquatic bacterium that is responsible for columnaris disease. This aquatic pathogen has a worldwide distribution and is highly infectious to both warm and cold water fish. A modified live *F. columnare* vaccine was developed by repeated passage of a virulent strain on increasing concentrations of rifampicin that resulted in attenuation. Here we report vaccination/challenge trials to evaluate efficacy and safety. In separate laboratory trials, immersion vaccination of channel catfish (*Ictalurus punctatus*) fry between 10 to 48 days post hatch (DPH) with experimental vaccine or licensed product resulted in relative percent survival (RPS) between 57–94% following challenge. Similarly, a vaccination/challenge trial using largemouth bass (*Micropterus salmoides*) fry at 10 DPH was performed using various doses of licensed product under laboratory conditions. Results demonstrated safety of the vaccine and significant protection following challenge with RPS values between 74–94%, depending on vaccine dose. Together, these trials demonstrate the vaccine administered to early life-stage channel catfish and largemouth bass is safe and reduces mortality following challenge with *F. columnare*.

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1. Introduction

Flavobacterium columnare is a common, ubiquitous aquatic bacterium that infects most species of freshwater fish [1–3]. In the cultured channel catfish (*Ictalurus punctatus*) industry it is responsible for significant economic loss (estimated at $30 million yearly) in the USA [4]. Intensive aquaculture for food [5–7] and remedial stocking programs for public use [8,9] will undoubtedly result in increased losses of fish caused by this freshwater bacterium. Early vaccination attempts consisted of formalin killed bacterins delivered with and without adjuvants that resulted in limited efficacy [10,11]. Bader et al. [12] suggested formalin destroyed important antigens and this was partly responsible for the limited effectiveness. Passive immunization studies in channel catfish demonstrated only partial protection using antiserum generated against *F. columnare* LPS or whole cell lysates [13]. In addition, past studies report fish surviving infection were immune upon re-exposure [14; Klesius and Shoemaker, unpublished]. A recent study by Pridgeon and Klesius [15] demonstrated using subtractive cDNA hybridization that 28 different genes were up-regulated 10 min post vaccination with the modified live *F. columnare* vaccine. Forty-six percent represented immune response genes mostly involved in innate and/or protective immunity following infection. Results from these studies suggest a modified live vaccine would be needed to protect early life stages of fish from columnaris disease.

In 2005, we developed a modified live vaccine against columnaris disease [16–18]. The vaccine has now been licensed by Intervet/Schering-Plough Animal Health for use in channel catfish. Attenuation of the *F. columnare* vaccine isolate by repeated passage on increasing concentrations of rifampicin showed changes of the lipopolysaccharide (LPS) profile compared to wild type isolates [19]. Western blot analysis using anti-sera generated from channel catfish immunized with the vaccine isolate (genomovar I) or from a genomovar II isolate (ALG-530) demonstrated a loss of higher molecular weight LPS bands in the vaccine isolate but cross reaction of immunodominant proteins of either type I or type II isolates was present, suggesting the core antigens are conserved.

Safety of the modified live *F. columnare* vaccine was demonstrated by conducting *in vivo* reversion back-passage using 10 day post hatch (DPH) channel catfish fry [18]. Isolation of the vaccine strain on rifampicin media from homogenized fish samples only occurred from the first fish-to-fish passage out of a total of five passages, demonstrating the vaccine strain could invade but did not revert to virulence. In addition, safety of the vaccine was shown where no mortality or signs of columnaris disease occurred in similar sized fish following immunization at 10 times the minimum protective dose [18].

This study reports laboratory efficacy in channel catfish immersion vaccinated with experimental serials or the licensed

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modified live *F. columnare* vaccine. In addition, this study reports a laboratory safety and efficacy trial of the licensed vaccine (AQUAVAC-COL™[1]) administered by immersion to largemouth bass (*Micropterus salmoides*) fry.

2. Materials and methods

2.1. Vaccine

The rifampicin modified-live *F. columnare* vaccine used in these trials was originally developed and patented by the United States Department of Agriculture-Agricultural Research Service [16,17]. Experimental vaccine serials for conducting the efficacy studies (trial 1 and 2) were prepared by Intervet Inc., at five passages from the master seed and provided frozen. The licensed vaccine AQUAVAC-COL™ was used in trials 3 and 4.

2.2. Bacteria and fish necropsy

F. columnare isolate ARS-1 (genomovar I) or ALG-00-530 (genomovar II) was grown in modified *Cytophaga* broth at 28 ± 2 °C with shaking at 100 revolutions per minute for 24 h [20,21]. Plate counts were performed to calculate the amount of bacteria used to challenge the fish as reported under each trial. *F. columnare* was confirmed by culture from caudal kidney and skin by plating on modified *Cytophaga* agar containing 1 μg ml^{-1} tobramycin [22]. *F. columnare* was identified using standard biochemical tests and fatty acid profile [23].

2.3. Channel catfish vaccination and challenge – trial 1

In trail 1, 2000 channel catfish fry (NWAC-103) at 7-10 DPH were obtained from Dr. William Wolters (USDA-ARS, Catfish Genetics Research Unit, Stoneville, MS) and housed in a single tank. Prior to vaccination, 5 pools of ten fry each (50 fry total) were homogenized and pools of five fry were plated on modified *Cytophaga* agar with tobramycin as described above. The homogenized pools were culture negative for *F. columnare*. At 48 DPH, 400 fish (mean weight = 1.5 g fish^{-1}) were immersion vaccinated in a 2L bath at 28 ± 2 °C containing the experimental serial at 1×10^6 CFU ml^{-1} or 5×10^6 CFU ml^{-1}) for 2 min followed by an additional 13 min in the vaccine bath diluted 2-fold (4L total) with water. An additional 800 fish were sham vaccinated with *Cytophaga* broth containing 10% glycerol (v:v) in the same manner. Following vaccination, fish in each treatment group were fed 3–4% body weight daily. Aquaria were supplied with flow-through dechlorinated water at a rate of approximately 0.5 L min^{-1}. Water flow rate was checked and adjusted daily to ensure proper water exchange. Water temperature was maintained by a central heater at 26 ± 2 °C. Water was continuously aerated and a photoperiod of 12:12 h light:dark was used.

Fourteen days prior to challenge, 3 tanks of 50 fish (mean weight = 2.58 g fish^{-1}) from each treatment group were transferred to 57 L aquarium and taken off feed [20,21]. At 57 days post vaccination, each tank of fish (9 tanks) were cohabitation challenged with 3 dead fish obtained 24 h after intraperitoneal challenge injection with 50 μl containing 1×10^9 CFU ml^{-1} virulent *F. columnare* (strain ARS-1; genomovar I). The dead fish utilized for cohabitation exhibited typical signs of columnaris disease including saddleback and gill lesions. A subsample of the dead fish were cultured and *F. columnare* was recovered from these fish. Following removal of dead fish at 24 h, feeding was resumed. Three tanks of 50 mock-infected control fish were also included in the trial. Fish in each tank were monitored for 21 d following challenge. Dead fish were cultured as described above to re-isolate *F. columnare.*

2.4. Channel catfish vaccination and challenge – trial 2

Approximately 3000, channel catfish fry (NWAC-103 strain) at 1 DPH were supplied by Dr. William Wolters (USDA-ARS, Catfish Genetics Research Unit, Stoneville, MS) and housed in a single tank. Prior to vaccination, 50 fry were examined as described above and were culture negative for *F. columnare*. At 7 DPH, approximately 600 fry (mean weight ~ 0.035 g fry^{-1}) were immersed in 113 ml of the vaccine bath for 2 min at the following doses 4×10^5, 4×10^6, 1×10^7 or 1×10^8 CFU ml^{-1}. Following the 2 min exposure, the vaccine bath was diluted 2-fold (by addition of an equal volume of water) and the fish held for 13 min, giving a total immersion time of 15 min. After vaccination, the fry were removed from the vaccine bath and placed into 57 L aquaria in their respective treatment groups until challenge. Sham vaccinated fish were immersed in media (modified *Cytophaga* broth containing 10% glycerol (v:v) at the same dilution rate for 15 min. Following vaccination, fry were fed at 3–4% body weight daily at time of swim-up (1 day following vaccination).

Both vaccinated and sham vaccinated fish were challenged following feed deprivation as in trial 1 [20,21]. Briefly, feed was withheld for 8 d prior to challenge to increase the susceptibility of fish to columnaris disease. At 51 DPH, fish (mean weight = 1.65 g fish^{-1})from each vaccine treated group were split into three tanks. Each replicate tank contained 64–100 fish. In addition, the 168 sham vaccinated fish were equally split into 3 replicate tanks (56 fish/tank). Three dead fish from an intraperitoneal challenge (50 μl of 1×10^9 CFU ml^{-1}) with virulent *F. columnare* (strain ARS-1; genomovar I) were added to each aquarium (as described above). Following removal of dead fish after 24 h, feeding was resumed. Three tanks of 56 mock-infected control fish were also included in the trial. Fish in each tank were monitored for 21 d following challenge. Dead fish were cultured as described above to re-isolate *F. columnare.*

2.5. Channel catfish, vaccination and challenge – trial 3

Prior to experimentation, 10 pools of 5 fry each (mean weight ~ 0.049 g fry^{-1}) at 13 DPH were cultured to verify the fish were free of *F. columnare* as described above. In this trial, 22.8 grams of fish (~400, 15 DPH fry) were immersion vaccinated in 1 L water containing 1×10^7 CFU ml^{-1} vaccine for 2 min following dilution to 2L for an additional 13 min. Total immersion time was 15 min. Another group of fish (22.8 grams of fish) was sham vaccinated with media as above. The vaccinated or sham vaccinated fry were fed fry starter 3–4 times daily at ~3–4 percent body weight per day and were housed in 57 L aquaria until time of challenge. At 12 days prior to challenge, fish in each group were weighed (mean weight = 1.47 g fish^{-1} and the sham vaccinated fish were marked with SE-Mark [9,24,25]. Briefly, fish were immersed in 4 L of water containing 1.5% NaCl for 4 min to enhance the uptake of SE-Mark. Following the brief salt bath, fish were transfer to 0.5 L of a 1% solution of SE-mark that was combined with 2.5 L water for an immersion exposure of 1.5 h to mark the fish (sham vaccinates). The vaccinated group was immersed into 1.5 % NaCl (4L) for 4 min followed by immersion in 3 L water for 1.5 h to mimic marking. At time of challenge (55 days post vaccination), 25 sham vaccinates and 25 vaccinates were combined and cohabitation immersion challenged in 3 L of water

[1] The use of trade, firm or corporate names in this publication is for the information and convenience of the reader, such use does not constitute an endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service for any product or service or to the exclusion of other products that may be suitable.