Survival of vaccinated, feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) during natural exposure to *Flavobacterium columnare*

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A B S T R A C T

Vaccinated, feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) were cohabited with sham-vaccinated fish. Fish were exposed, under natural conditions, to *Flavobacterium columnare*, a ubiquitous bacterium associated with columnaris disease. During every time interval, the probability that a vaccinated fish would survive past time, \( t \), was greater than for sham-vaccinated fish and survivor functions were significantly different (\( p \)-value < 0.001). Overall, vaccinated fish had a 43% lower risk of death during the field trial. Overall incidence was 1.7 times greater for the sham-vaccinated (1.4%/d) as compared to the vaccinated fish (0.8%/d). Vaccination with AQUAVAC-COL (Intervet/Schering-Plough Animal Health) significantly reduced the risk of death from columnaris disease in feed-trained largemouth bass fry.

1. Introduction

*Flavobacterium* spp. are Gram-negative, filamentous, yellow-pigmented bacteria considered to be ubiquitous in the aquatic environment. Worldwide, all fish species are susceptible to infection with at least one member of this genus. One species in particular, *Flavobacterium columnare*, is an important pathogen of freshwater wild, farmed and ornamental fish. Outbreaks of columnaris disease and endemic infection with *F. columnare* have been reported from continents including North America, Europe, and Asia [1–3].

Largemouth bass (*Micropterus salmoides floridanus*; Centrarchidae), a very popular sportfish in the United States, are often reared in government hatchery programs, and then stocked to supplement wild fish populations. After the eggs obtained from broodstock are hatched, fry are stocked into ponds to feed on zooplankton and other small invertebrates. After this period of eating live food (e.g., 30–40 days at 17–24 °C) the fry are more likely to accept commercial feed, so they are harvested from the ponds, stocked into tanks for “feed training”, and provided with manufactured feed until they are the size needed for stocking into lakes and ponds. Fry may be exposed to the bacteria while they are in the pond and/or after they are moved indoors, via the water supplied to the tanks. Consequently, outbreaks of columnaris disease are very likely to occur during and after this stressful feed training period. Starvation can increase susceptibility to columnaris disease [4,5], so the fish that do not successfully feed train may be more susceptible to clinical disease. Antibiotic-medicated feed or therapeutants used by immersion can be used to control columnaris outbreaks [6–9], but have limited efficacy, and because of environmental and tissue residue concerns have extremely limited acceptability when used to prevent outbreaks [10].

Prevention of columnaris disease by vaccination is an important goal and a top priority of fish producers throughout the world [11]. Vaccine development for columnaris disease has been in progress for a number of years [e.g., [12–15]]. One result of these efforts has been the development and commercialization of a modified live columnaris immersion vaccine licensed for use in the United States for catfish [16] (AQUAVAC-COL, Intervet/Schering-Plough, *F. columnare* vaccine). This immersion vaccine, which is used at the early fry stage in catfish, might potentially be efficacious for prevention of columnaris disease in largemouth bass fry. The objective of this study was to conduct a field trial testing the efficacy of AQUAVAC-COL to enhance the survival of vaccinated feed-trained largemouth bass fry naturally exposed to *F. columnare*.

2. Methods

The vaccine trial was conducted at the Florida Bass Conservation Center’s Richloam Fish Hatchery (Webster, FL). The water for spawning broodstock and for rearing fry originated as well water, which was collected in a 1.5 acre plastic-lined water reservoir for carbon dioxide degassing and oxygenation before it was supplied...
under flow-through conditions to earthen ponds or to fiberglass culture tanks.

2.1. Pre-trial conditions, vaccination, calcein marking

Largemouth bass fry were obtained from naturally spawning broodstock that produced eggs from January 31 through February 2, 2008. On February 7th (7–9 days post-hatch (dph)), fish were immersion vaccinated with AQUAVAC-COL (Intervet/Schering-Plough) at 1.04 × 10^6 colony forming units (CFU) F. columnare/3.78 L water/20,000 fry for 2 min, then water was added to dilute vaccine to 1.04 × 10^6 CFU F. columnare/18.9 L/water/20,000 fry for an additional 30 min. Water temperature was 21.7 °C. On a per vial basis, this exposure was equivalent to using one vial of vaccine to vaccinate 100,000 fry (approximately 340 g total weight) in 18.9 L (5 gallons) of water for 2 min, then adding 75.6 L (20 gallons) of water for an additional 30 min. Sham-vaccinated fry were exposed to modified Cytophaga media and glycerol and the same environmental conditions as the vaccinated fry. During vaccination pure oxygen (O2) was added to maintain dissolved oxygen concentrations >6.0 mg/L. After vaccination, 55,000 vaccinated and 55,000 sham-vaccinated fish were stocked into each of two tilled and fertilized 0.24 ha (0.6 acre) earthen ponds.

Fish fed naturally in ponds for 33 d (38–40 dph), until March 11th, were harvested, then stocked into two 9.1 m (30′ long, 4914 L (1300 gallons) fiberglass raceways and trained to accept commercial fish feed (proprietary formulation). In general, fish that successfully accept commercial feed do so within about three days. Fry were then given an additional ten days to recover from handling stress and for those that did not successfully feed train to be culled from the two populations [17,18].

On March 24th (45 d post-vaccination), the sham-vaccinated field trial fish were calcein marked so that sham-vaccinated fish could be distinguished from vaccinated fish during the cohbitation trial [19,20]. Calcein (bis[N,N-bis(carboxymethyl)aminomethyl]fluorescein) (SE-MARK®, Western Chemical, Ferndale, WA), a fluorescent dye with excitation and emission wavelengths of 495 and 515 nm, respectively, chemically binds with alkaline earth metals such as calcium and is considered a safe and effective method for marking fish [20]. Upon binding, calcium-containing structures (e.g., jaw, scales, fin rays) fluoresce green when excited with a blue light (λ = 500 nm). The sham-vaccinated fish were exposed to calcein at a concentration of 2.5 g calcein per liter water for 4 min. Immersion in a 1.5% sodium chloride bath for 4 min prior to calcein immersion was used to increase uptake of the calcein [21] (J. Mohler, personal communication). The vaccinated fish were exposed to the same conditions as the sham-vaccinated fish except that they were not exposed to calcein.

After calcein marking, approximately 1900 sham-vaccinated fish (average body weight ± standard error (SE) = 0.94 ± 0.01 g/fish) were stocked into each of three fiberglass tanks (378.5 L) for the vaccine trial. Vaccinated fish (average body weight ± S.E. = 0.92 ± 0.03 g/fish) were also stocked into each of the same tanks for cohbitation with sham-vaccinated fish, approximately 1900 fish in each tank, for a total of approximately 3800 fish in each tank. Tank assignment for each group stocked was randomly assigned by lottery.

2.2. Vaccine trial conditions

Day 1 of the vaccine trial began two days after acclimation to the experimental tanks. Additional fish that were not feed-trained were culled during this time. Fish were observed for 44 days for signs of mortality due to a natural infection with F. columnare. Water quality was measured and recorded daily from the source water and in an individual tank if abnormal fish behavior was observed. Water quality parameters measured (with range of tolerance for largemouth bass) were water temperature (T) = 1–35 °C, dissolved oxygen (DO) = 5–20 mg/L, unionized ammonia (NH3) = <0.01 mg/L, alkalinity (ALK) = 20–500 mg/L as CaCO3, carbon dioxide (CO2) = 0–20 mg/L, and pH 6–9 [17,18,22]. Calibrated meters were used to measure T, DO, and pH. Colorimetric methods (Hach Company, Loveland, Colorado) were used to measure NH3, ALK, and CO2.

Each day at the same time, dead fish from each tank were examined under a blue light (SE-MARK detector). Employees who were blinded to the treatment assignments counted the fish and assigned them to the appropriate category. Fish were considered dead due to columnaris disease if skin scrapings and microscopic wet mounts were positive or if the erosive or necrotic skin lesions consistent with columnaris disease were apparent [2,23]. Dead fish that were thin with relatively large heads were considered starved (i.e., unsuccessfully feed-trained), and not dead due to columnaris disease. Other causes of death were fish choking while eating tank mates, being partially consumed by a cannibalistic tank mate, and jumping out of the tank. At the end of the trial, the number of fish remaining in each tank was recorded.

2.3. Data analysis

Fish considered dead due to other causes besides columnaris disease were right censored in all analyses. Survivor functions, which estimate the probability that a fish survives beyond time, t, were estimated for each tank. Median survival times, the time beyond which 50% of the fish in an exposure group were expected to survive or the time by which half of the fish had died, were also estimated. The logrank test was used to determine whether survivor functions were significantly different. The logrank test was conducted three times, once for each of the three tanks. Therefore, a Bonferroni correction of α = 0.05/3 = 0.017 was used as the cut-off to determine whether the p-value was statistically significant [24,25].

The Cox proportional hazards regression, with clustering by tank, was used to estimate the hazard ratio, or relative risk of dying for sham-vaccinated as compared to vaccinated fish. Before the Cox model was used, the “proportionality assumption” that vaccination multiplies the baseline hazard for the sham-vaccinated fish by a constant factor at any given point during the trial, was tested with an analysis of Schoenfeld residuals. After the Cox regression was estimated, smoothed hazard functions were constructed to examine how the hazard rates varied over time [24–26].

Incidence, the rate at which fish die from columnaris disease, was estimated for exposure groups in each tank. Incidence with 95% confidence intervals, was calculated as the number of deaths per total fish time at risk. Preventive fraction (PF), with 95% confidence intervals, the net proportion of all deaths in the vaccinated group that were prevented by vaccination, was estimated by 1−(incidence_vacc/incidence_unvacc) [25,27,28].

3. Results

When the 55,000 fish that had been stocked into each of the two ponds on March 11th were harvested, 33,200 unvaccinated, and 29,400 vaccinated fish were recovered. None had gross clinical signs of columnaris disease. During feed training, some sham-vaccinated fish did die with clinical signs of an F. columnare infection. However, the emaciated appearance of these fish was compatible with death primarily due to unsuccessful feed training rather than primarily due to columnaris disease. No clinical signs of columnaris disease were observed in vaccinated fish during feed training.

Except for CO2 and NH3, water quality parameters remained within normal limits for largemouth bass culture for the entire
Mean water temperature was 23.4 °C (Standard Error (S.E.) = 0.2), D.O. was 13.9 (S.E. = 0.3) mg/L, alkalinity was 359.8 (S.E. = 2.4) mg/L as CaCO3, and mean pH was 7.58 (S.E. = 0.01). Carbon dioxide was between 8 and 12 mg/L until it increased to 15 mg/L on day 10, after a night time algae bloom die-off in the water reservoir. On the morning of day 12, tank water flow rate was reduced, and the tanks were aerated to dissipate the CO2. On day 13, NH3 concentration increased to approximately 1.8 mg/L because of the reduced water flow rate. Normal water flow rates were restored and NH3 concentrations returned to normal values in less than 1 h. Because it was necessary to maintain water flow rates to avoid NH3 increases, CO2 concentrations continued to increase and were between 21 and 29 mg/L until day 31 when they dropped below 20 mg/L after complete decomposition of the dead algae. CO2 concentrations then remained between 10 and 16 mg/L until the end of the trial. Throughout this period of water quality issues, fish continued to feed and did not exhibit any abnormal behavior.

The number of fish remaining in each tank was counted at the end of the trial. For the purpose of the analysis, at the beginning of the trial tank 1 contained 3620 fish, tank 2 contained 3724 fish, and tank 3 contained 3764 fish, each divided equally between vaccinated and sham-vaccinated groups.

3.1. Survivor functions

For tank 1, the median survival time for the sham-vaccinated fish was 40 days (Fig. 1). For the vaccinated fish in tank 1, the median survival time was not reached by the end of the trial. The sham-vaccinated fish had a 0.47 probability of surviving beyond the end of the trial. The vaccinated fish had a 0.68 probability of surviving beyond the end of the trial. The logrank test indicated that the two survivor curves were significantly different from each other ($\chi^2, 1$ d.f. = 148.65; $p$-value < 0.0001).

For tank 2, the median survival time for the sham-vaccinated fish was 26 days. By the end of the trial, vaccinated fish had not reached the median survival time, i.e., greater than 50% of the fish still remained. The sham-vaccinated fish had a 0.39 probability of surviving beyond the end of the trial. The vaccinated fish had a 0.56 probability of surviving beyond the end of the trial. The two survivor curves were significantly different from each other ($\chi^2, 1$ d.f. = 89.51; $p$-value < 0.0001).

Overall, survival in tank 3 was greater than in tank 1 or tank 2. Neither the sham-vaccinated or vaccinated fish reached the median survival time by the end of the trial. However, the sham-vaccinated fish reached the time by which 25% (1st quartile) of the fish would have died, 28 days, while the vaccinated fish did not reach that quartile before the end of the trial. The sham-vaccinated fish had a 0.64 probability of surviving beyond the 44 day trial period. Vaccinated fish had a 0.78 probability of surviving beyond 44 days. The two survivor curves were significantly different from each other ($\chi^2, 1$ d.f. = 101.20; $p$-value < 0.0001).

During every time interval for every tank, the probability that a vaccinated fish would survive past time, $t$, was always greater than it was for a sham-vaccinated fish (Fig. 1).

3.2. Cox proportional hazards model

Before estimating the Cox proportional hazards model, the proportional hazards assumption was tested by examining Schoenfeld residuals. This test was not statistically significant ($\chi^2 = 0.27$, 1 d.f., $p$-value = 0.60), so the Cox proportional hazards model was used. The hazard ratio for vaccination compared with no vaccination was 0.57 (Robust S.E. = 0.03; 95% C.I. = 0.51, 0.64). Vaccination had a statistically significant protective effect. Vaccinated fish had 57/100
3.3. Incidence and preventive fraction

Incidence, the rate at which fish died from columnaris disease was greater, for all tanks, for sham-vaccinated as compared to vaccinated fish (Table 1). Overall incidence was 1.7 times greater for the sham-vaccinated (1.4%/d) as compared to the vaccinated fish (0.8%/d). Confidence intervals (95%) for incidence were non-overlapping for comparison of sham-vaccinated and vaccinated fish for each tank and for the overall comparison.

The point estimates for preventive fraction, net proportion of all deaths in the vaccinated fish that were prevented by vaccination, were 0.45 (95% CI = 0.39, 0.51), 0.37 (95% CI = 0.31, 0.43), and 0.45 (95% CI = 0.37, 0.52) for tanks 1–3, respectively.

4. Discussion

In this field trial, AQUAVAC COL provided feed-trained largemouth bass fry with significant protection from mortality during natural challenge with F. columnare. Throughout the trial, in all three tanks containing cohabited sham-vaccinated and vaccinated fish, the risk of death was always higher for sham-vaccinated than for vaccinated fish. At 44 days, the death rate was 0.6%/d for sham-vaccinated fish and 0.4%/d for vaccinated fish.

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