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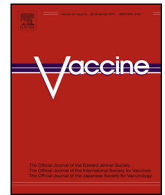

New attenuated vaccine against columnaris disease in fish: Choosing the right parental strain is critical for vaccine efficacy

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ABSTRACT

Flavobacterium columnare, the causative agent of columnaris disease, is a highly diverse species comprised by three genomovars. Genomovar II strains are more virulent toward catfishes than genomovar I isolates. The objective of this study was to compare the vaccine efficacy of avirulent mutants derived from genomovars I and II using a rifampicin-resistance strategy. First, we compared the efficacy of 13 genomovar II mutants in channel catfish (*Ictalurus punctatus*) fingerlings and identified mutant 17-23 as the best vaccine candidate based on their relative percent survival (RPS) against a highly virulent genomovar II strain (BGFS-27). In the second experiment, we vaccinated zebrafish (*Danio rerio*) with two genomovar II mutants (17-23 and 16-534) and FCRR (genomovar I mutant) followed by exposure to BGFS-27 strain. RPS values were 28.4, 20.3 and 8.1% for 17-23, 16-534, and FCRR, respectively. For experiments 3 and 4, we tested both 17-23 and FCRR in channel catfish fry and Nile tilapia (*Oreochromis niloticus*). In both experiments, vaccinated fish were divided in two groups and each challenged with either a genomovar I (ARS-1) or a II (BGFS-27) strain. Channel catfish fry vaccinated with 17-23 and FCRR followed by challenge with BGFS-27 resulted in RPS values of 37.0% and 4.4%. When fish were challenged with ARS-1, RPS values were 90.9% and 72.7% for fish vaccinated with 17-23 and FCRR, respectively. Nile tilapia vaccinated with 17-23 and FCRR followed by challenged with BGFS-27 had RPS values of 82.1% and 16.1%, respectively. When fish were challenged with strain ARS-1, RPS values were 86.9% and 75.5%. Overall, our results demonstrated that vaccination with genomovar II mutant 17-23 confers better protection in channel catfish and Nile tilapia than FCRR against columnaris disease caused by genomovar II. Both mutants were equally protective against columnaris caused by genomovar I showing that 17-23 mutant cross-protected against both genomovars.

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1. Introduction

Flavobacterium columnare is the causative agent of columnaris disease that affects wild, cultured and ornamental fish populations worldwide [1,2]. In the United States, columnaris disease is one of the leading causes of mortality in catfish farms, with in-pond mortality rates among adults and fingerlings reaching up to 60 and 90%, respectively [3]. Eradication of columnaris disease from culture settings is unlikely since this bacterium is ubiquitous in freshwater environments [4]. Sustainable aquaculture needs to maximize disease prevention and vaccination has become one of the best tools to achieve that goal. Effective vaccines are ultimately the safest prophylactic approach to mitigate the effect of infectious diseases [5]. Among the several types of vaccines available, live attenuated vaccines are those in which the pathogen has been modified and is no longer virulent to the host [6]. The use of attenuated vaccines holds tremendous potential because they present multiple immunogens while building in adjuvanticity that elicits strong humoral and cell-mediated protection [7]. In catfish aquaculture, where individual vaccination is cost-prohibitive, attenuated vaccines have the additional advantage of ease of delivery through feed or by immersion [8,9].

One of the most successful strategies used to obtain attenuated mutants in Gram-negative bacteria is by passing virulent isolates onto media containing increasing concentrations of the antibiotic rifampicin. This strategy has been successfully used to develop modified live attenuated bacterial vaccines for commercial use in cattle and fish [10–12]. Currently, a modified live *F. columnare* vaccine is available for commercial use to prevent columnaris disease under the licensed name AQUAVAC-COL^{TM} (Merk & Co., Inc.). The active ingredient in this vaccine is an avirulent rifampicin-resistant mutant of *F. columnare*, strain FCRR, derived from a genomovar I strain [13].

Recently, we have developed safe and permanently stable rifampicin-resistant mutants from highly virulent genomovar II strains [14]. Since genomovar II strains are more virulent toward

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catfishes, we hypothesized that a genomovar II-based vaccine will increase the protective effect of vaccination against columnaris disease. Therefore, the purpose of this study was to compare the efficacy of the genomovar I and II rifampicin-resistant mutants in fish species susceptible to columnaris disease. Channel catfish (*Ictalurus punctatus*), and Nile tilapia (*Oreochromis niloticus*) were chosen based on their economic relevance as food aquaculture species while zebrafish (*Danio rerio*) is cultured worldwide for both ornamental and experimental purposes.

2. Materials and methods

2.1. Fish Husbandry

Channel catfish fingerlings ($n = 540$, mean weight = 5.5 ± 0.8 g) and 21 day post hatch channel catfish fry ($n = 480$; mean weight = 0.05 ± 0.003 g) were supplied by the School of Veterinary Medicine at Auburn University and a commercial hatchery in Mississippi, respectively. Fish were transferred in aerated containers to the Aquatic Microbiology Laboratory (AML) at Auburn University. Non-sexed adult zebrafish (*Danio rerio*) ($n = 360$, mean weight = 0.45 ± 0.04 g) were purchased from Aquatica Tropicals (Plant City, FL, USA), and express shipped to AML. All male Nile tilapia juveniles ($n = 600$, mean weight = 9.4 ± 0.5 g) were obtained from E. W. Shell Fisheries Center at North Auburn Fisheries experiment Station and transported to AML in aerated containers. Upon arrival to AML, fish were stocked into 37 L glass aquaria/tanks (stocking rates are mentioned under each experiment). Fish were acclimated for at least 5 days before vaccination, and fed daily to apparent satiation with AQUAMAX Grower 100 (channel catfish fry and zebrafish) or 400 (channel catfish fingerlings and tilapia) (Purina Mills, Inc., St. Louis, MO, USA). Ten randomly selected individuals of each fish species were examined and proved culture negative for *F. columnare* prior to stocking in the tanks following standard protocols [15]. Each tank had an individual biofilter and an air stone. Water was prepared with 340 g of Marine Salt (Seachem, Madison, Georgia) diluted in 10 L of deionized water to make the primary salt stock. For tank use, 0.97 g of $CaCO_3$, 2.26 g of $NaHCO_3$, and 110 mL of the stock were mixed overnight in 55 L of deionized water. Water quality was checked daily to maintain established parameters (80 ppm alkalinity, 40 ppm hardness, 0.1 ppt salinity, 27 ± 1 °C, pH 7.8 ± 0.2 [mean ± standard error], ammonia and nitrites were non-detectable). At the time of vaccination, aquaria were assigned blindly to each treatment group. All animal protocols, including vaccination experiments, were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC number 2009-1609).

2.2. Bacterial strains and growth conditions

Previously generated and characterized rifampicin-resistant mutants of *F. columnare* genomovar I [13] and II [14] were used in the following experiments. Briefly, mutants were generated by successive passes on culture medium with increasing concentrations of rifampin (from 50 μm/mL to 300 μg/mL). Only one mutant from genomovar I was available (FCRR) while 8 genomovar II mutants were included in the study. All *F. columnare* strains used in this study were stored at −80 °C as glycerol stocks and were cultured on modified Shieh (MS) agar [14] or in MS broth with shaking at 100 rpm at 28 °C for 24 h. Plate counts of each strain (in triplicates) were carried out to calculate the average number of colony forming units per milliliter (CFU/mL) of bacteria used to vaccinate or to challenge fish as reported under each experiment.

Table 1
Comparison of the vaccine efficacy of rifampicin-resistant mutants obtained from different genomovar II parent strains. Vaccinated fish were challenged with virulent genomovar II strain BGFS-27 (challenge dose of 1.6×10^7 CFU/mL). Results from channel catfish vaccination (Experiment 1) are shown as cumulative percent survival and RPS. Within a column, different superscript letters means significant difference ($p < 0.05$).

Parent strain[a]	Mutant strain	Vaccination dose (CFU/mL)	Cumulative percent mortality (mean ± SE)	RPS
AL-CC-11	11-131	7.0×10^6	27.0 ± 6.8^{a}	49.1
	11-132	9.1×10^6	$39.0 \pm 5.0^{b,c}$	26.4
AL-CC-15	15-132	2.0×10^7	$34.0 \pm 13.3^{b,c}$	27.3
	15-133	2.5×10^7	28.0 ± 8.6^{a}	49.1
AL-CC-16	16-532	1.7×10^7	$34.0 \pm 13.3^{b,c}$	32.0
	16-534	2.1×10^7	30.0 ± 7.7^{a}	40.0
AL-CC-17	17-13	2.1×10^7	$36.0 \pm 14.2^{b,c}$	32.1
	17-23	1.7×10^7	24.0 ± 5.7^{a}	54.7
	Control	–	53.0 ± 8.6^{c}	–

[a] See Olivares-Fuster and Arias [14] reference for complete details on the parent strains.

2.3. Mutant safety and stability

The *in vitro* stability of genomovar II mutant 17-23 had already been tested [14] but its *in vivo* stability was assayed by inoculating five channel catfish fingerlings (of approximately 15 cm in length) with *ca.* 10^5 CFU/mL of 17-23. Fish were inoculated by intraperitoneal injection and monitored for 2 days for signs of disease. After 2 days, fish were euthanized with 300 ppm of tricaine methanesulfonate, and 17-23 mutant was recovered onto MS supplemented with 300 μg/mL of rifampicin. The newly recovered isolate was used as inoculum for the second round of intraperitoneal injections. This iterative process was repeated six times. No mortalities or sings of columnaris disease were observed. In addition, and to confirm the safety of 17-23 by immersion, 30 channel catfish fingerlings (equally divided into 3 replicates) were immersed in *ca.* 10^8 CFU/mL of 17-23. This experiment was repeated twice. No mortalities or signs of columnaris disease were observed

2.4. Comparison between vaccine efficacies of genomovar II mutants – Experiment 1

Channel catfish fingerlings ($n = 540$) were vaccinated with Genomovar II mutants (Table 1) by immersion following previously described protocols [16]. Briefly, fish (15 fish/tank; 4 replicates per treatment) were vaccinated in a 2 L bath at room temperature (25 °C) containing the experimental vaccine (see Table 1 for each individual dose). Control treatment fish (15 fish/tanks; 4 replicates) were sham vaccinated using MS broth as inoculum in the vaccination bath. After 30 min in the vaccination bath, fish were returned to their tanks and maintained under normal husbandry conditions. At 28 days post-vaccination, fish were challenged by immersion with genomovar II strain BGFS-27, a highly virulent strain of *F. columnare* as per Shoemaker et al. [17]. Fish were returned to their tanks and monitored twice a day for signs of disease. Moribund and dead fish were promptly removed from the tanks. *F. columnare* was isolated from the dead fish by plating on MS agar medium containing 1 μg/mL tobramycin [18]. Putative *F. columnare* colonies were presumptively identified based on their pigmentation and characteristic colony morphology on agar plates and selected colonies were confirmed by specific PCR [19].

2.5. Zebrafish vaccination – Experiment 2

A total of 336 adult zebrafish were vaccinated by immersion in a 2 L bath for 30 min with the following treatments: 17-23, 16-534 and FCRR at concentrations of 7.8×10^6, 1.5×10^6 and

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