Vaccine 29 (2011) 5631–5637

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development of a novobiocin-resistant *Edwardsiella ictaluri* as a novel vaccine in channel catfish (*Ictalurus punctatus*)

Julia W. Pridgeon*, Phillip H. Klesius

Aquatic Animal Health Research Unit, USDA-ARS, 990 Wire Road, Auburn, AL 36832, United States

ARTICLE INFO

Article history:
Received 11 April 2011
Received in revised form 26 May 2011
Accepted 7 June 2011
Available online 24 June 2011

Keywords:
Vaccine
Edwardsiella ictaluri
Enteric septicemia of channel catfish
Novobiocin

ABSTRACT

The efficacy of a novel attenuated *Edwardsiella ictaluri* vaccine (B-50348) was determined in channel catfish (*Ictalurus punctatus*) by bath immersion and intraperitoneal (IP) injection. The vaccine was developed from a virulent strain of *E. ictaluri* (AL93-58) through selection for novobiocin resistance. When channel catfish (average weight 10 g) were IP injected with 4.2×10^6 colony-forming units (CFU) of the attenuated vaccine B-50348, no fish died. However, when the same age and size matched group of the catfish were IP injected with a lesser amount (2.4×10^6 CFU/fish) of modified live RE-33 vaccine or the AL93-58 virulent strain (2.5×10^6 CFU/fish) of *E. ictaluri*, 65% and 95% fish died, respectively. When channel catfish were challenged with AL93-58, relative percent survival values of vaccinated fish were all greater than 90% at 22, 32, and 63 days post B-50348 vaccination through intraperitoneal injection. By bath immersion, at 37 and 57 days post vaccination of B-50348, relative percent survival values were both 100% when fish were challenged by virulent *E. ictaluri* AL93-58. Our results suggest that B-50348 could be used as a novel safe and efficacious vaccine against ESC in channel catfish.

Published by Elsevier Ltd.

1. Introduction

Enteric septicemia of catfish (ESC), the most prevalent disease affecting farm-raised channel catfish, *Ictalurus punctatus*, is caused by *Edwardsiella ictaluri*, a facultative intracellular Gram-negative flagellated bacterium akin to phylogenetically related *Salmonella* [1,2]. ESC is responsible for an annual loss of $20–30 million to catfish farmers in the southeastern United States [3]. ESC is generally an acute septicemia that develops very quickly, resulting in heavy mortalities as early as 4 days after infection [1,4,5].

Initial efforts to control ESC were based on feeding the infected fish with antibiotic medicated food [6]. However, this practice is expensive and usually ineffective because sick fish normally remain off feed. Furthermore, bacterial pathogens of fish have developed resistance to antibiotics such as oxytetracycline, florfenical, and ormethoprim–sulphamethoxine [7,8]. To control ESC, traditional inactivated *E. ictaluri* as killed vaccines and attenuated *E. ictaluri* as live vaccines have been developed [8–13]. Live vaccines have been demonstrated to offer higher protection against ESC than killed vaccines [12]. Currently, a modified live *E. ictaluri* vaccine (RE-33) is registered as AquaVac-ESC in aquaculture to protect catfish against ESC. RE-33 was successfully developed by Klesius and Shoemaker [9] using rifampicin-resistant strategy. It has been demonstrated that the rifampicin-resistant RE-33 strain of *E. ictaluri* was unable to cause ESC, but was able to stimulate protective immunity in catfish [9]. However, it is not clear whether other antibiotic could also be used to attenuate *E. ictaluri* for the purpose of novel vaccine development.

Novobiocin, also known as albamycin or cathomycin, is an antibiotic produced by the actinomycete *Streptomyces niveus*, a member of the order *Actinobacteria* [14]. Novobiocin works as an inhibitor of bacterial DNA gyrase, resulting in bacterial cell-death [15]. DNA gyrase, an ATP-dependent enzyme that acts by creating a transient double-stranded DNA break, is essential for efficient DNA replication, transcription, and recombination by catalyzing the negative supercoiling of DNA [16]. Novobiocin has also been reported to be able to eliminate plasmids from several bacterial species, such as *Escherichia coli* [17] and *Shigella sonnei* [18,19]. However, it is not clear whether novobiocin is also able to cure plasmids from *E. ictaluri*. It has been reported that *E. ictaluri* has two plasmids (pET1 and pET2) with sizes of 4807 and 5643 base pairs, respectively [20,21]. The protein homologs identified for open reading frame 1 on pET1 of *E. ictaluri* have leucine-rich repeat motifs which are components of the type III secretory systems [21]. Type III secretory systems play important roles in the pathogenesis and virulence [22]. Therefore, the objectives of this study are: (1) to determine whether novobiocin is able to cure plasmids from *E. ictaluri*; and (2) to determine whether novobiocin-resistance strategy could be used to attenuate virulent *E. ictaluri* for live vaccine development.

* Corresponding author. Tel.: +1 334 887 3741; fax: +1 334 887 2983.
E-mail addresses: Julia.Pridgeon@ars.usda.gov, juliapridgeon@yahoo.com (J.W. Pridgeon).

0264-410X/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2011.06.016

2. Materials and methods

2.1. Induction and characterization of novobiocin-resistant E. ictaluri

The virulent AL93-58 *E. ictaluri* strain was used for the induction of novobiocin resistance. Novobiocin sodium salt was purchased from Promega (Madison, WI). AL-93-58 was cultured in brain heart infusion broth (Fisher Scientific, Pittsburgh, PA) containing different concentrations of novobiocin. The initial concentration of novobiocin that allowed growth of AL93-58 was 12.5 μg/ml. After 12 passages of AL93-58 in BHI culture media containing higher concentration of novobiocin, the novel novobiocin-resistant strain of AL93-58 (B-50348) was able to grow in BHI broth containing 800 μg/ml of novobiocin. AL93-58, B-50348, and RE-33 were then grown on 5% sheep blood agar plates (Thermo Fisher Scientific Remel Products, Lenexa, KS) to check their growth. For bacterial identification, Gram-negative colonies were subjected to API-20E test (Biomérieux, Durham, NC). To understand whether novobiocin was able to cure plasmid from the parent AL93-58, plasmid DNAs were prepared from both AL93-58 and B-50348 using QIAGEN plasmid mini kit (Qiagen, Valencia, CA) and subjected to 0.7% agarose gel electrophoresis.

2.2. Virulence of B-50348 compared to AL93-58 and RE-33

To study the virulence of B-50348 compared to AL93-58 and RE-33, the three strains of *E. ictaluri* were cultured overnight in brain heart infusion broth at 27 °C. A total of twenty channel catfish (*I. punctatus*) per treatment were exposed to the three strains of *E. ictaluri* through intraperitoneal (IP) injection or bath immersion (IM). All channel catfish (Industry pool strain, USDA, ARS, Catfish Genetics Research Unit, Stoneville, MS) used in this study were raised at the USDA ARS Aquatic Animal Health Research facility located at Auburn, Alabama. To study the virulence of B-50348 by IP injection, different amounts (colony forming unit, CFU) of *E. ictaluri* in a total volume of 100 μl were injected into each catfish. To study the virulence of B-50348 by immersion, catfish were exposed to *E. ictaluri* by bath immersion at different concentrations for 1 h. The highest concentration used in bath immersion was 4.8×10^7 CFU/ml (~200-fold of the normal RE-33 vaccine dose of 2×10^5 CFU/ml [9]). Mortalities were recorded for 14 days post exposure to *E. ictaluri* and the presence or absence of *E. ictaluri* in dead fish were determined from anterior kidney samples cultured on BHI agar followed by API-20E tests (Biomérieux).

2.3. Vaccination of catfish with B-50348 followed by challenge with AL93-58

The B-50348 experimental vaccine was cultured in BHI broth at 27 °C at 125 rpm overnight before vaccination. Catfish (11 ± 3 g) were vaccinated either by IP injection or by IM exposure. Vaccination by IP was conducted in three trials and each trial had two groups of fish (control or vaccinated, 20 fish per treatment). In trial I, two vaccination doses (1.38×10^6 and 1.84×10^6 CFU per fish) were used and vaccinated fish were challenged at 22 days post vaccination (dpv). In both trial II and trial III, vaccination dose was 8.67×10^6 CFU/fish and fish were challenged at 32 and 63 days post vaccination, respectively. For immersion vaccination, two trials were conducted. In both trials, channel catfish (20 fish per treatment) were bath immersed in water containing approximately 2.7×10^7 CFU/ml of *E. ictaluri* B-50348 for 1 h. In trial I, catfish were challenged at 37 days post vaccination. In trial II, catfish were challenged at 57 days post vaccination. As sham-vaccination controls, channel catfish were either IP injected with BHI or IM in BHI. Mortalities were recorded for 14 days post challenge of AL93-58. Results of experimental challenge were presented as relative percent of survival (RPS) as described previously [9]. RPS was calculated according to the following formula [23]:

$$\text{RPS} = \left\{1 - \left(\frac{\text{vaccinated mortality}}{\text{control mortality}}\right)\right\} \times 100$$

2.4. Minimum effective vaccination dose of B-50348 in channel catfish

To determine the minimum effective vaccination dose of B-50348 by IP, six doses (1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 CFU/fish) of B-50348 were IP injected to channel catfish. For each treatment group, 50 fish were used. To determine the minimum vaccination concentration of B-50348 by immersion, three concentrations (1×10^5, 1×10^6, and 1×10^7 CFU/ml) were used to immerse channel catfish for 1 h. As sham-vaccination control, fish were injected or bath immersed with tryptic soy broth. At 28 days post vaccination, fish were challenged by virulent parent isolate AL93-58 at dose of 1.0×10^5 CFU/fish through IP injection. Mortalities were recorded for 14 days post challenge and the presence or absence of *E. ictaluri* in dead fish was determined as described earlier. Results of *E. ictaluri* AL93-58 challenge were presented as RPS.

2.5. Enzyme-linked immunosorbent assay (ELISA)

For ELISA experiments, serum from B-50348 IP vaccinated fish or sham IP vaccinated fish were collected at 15-, 30-, and 60-dpv (pre-challenge). The vaccination dose for B-50348 was 2.2×10^5 CFU/fish. Five fish per treatment at each time point were used to collect serum. Antibody titer against *E. ictaluri* was determined using the method described previously [24]. Briefly, ELISA plates (Falcon FAST Plate 3931, Part 1, Becton Dickinson, Lincoln Park, NJ, USA) were coated with 500 μg μl^{-1} of the *E. ictaluri* AL93-58 antigen [25] for 10 min with shaking (200 rev min^{-1}). After coating, the plates were drained, shaken dry, and washed with phosphate buffered saline (PBS) containing 0.3% Tween 20. Catfish serum to be assayed was diluted 1:10 in PBS then added in 0.1 ml amounts to duplicate wells of a microtiter plate. Serum samples collected from catfish at 30 days post infection of virulent *E. ictaluri* AL 93-58 were used as positive controls. PBS without any serum added was used as negative control. The plates were incubated 30 min at 25 °C with shaking (200 rev min^{-1}) and washed four times with PBS containing 0.3% Tween. Monoclonal peroxidase-conjugated anti-catfish IgG heavy chain antibody [25] was used to detect catfish antibody. This conjugate was diluted 1:2000 in PBS and added to each well in 0.1 ml amounts, incubated for 30 min, and washed as previously described. Finally, each well of the microtiter plate received 0.1 ml of the peroxidase substrate (*O*-phenylenediamine-hydrogen peroxide, 4 mg, Sigma). Incubation was conducted in the dark for 10 min. The reaction was stopped by applying 0.05 ml of 2 N HCl per well. The optical density (OD) was read with a Dynatech ELISA plate reader (Chantilly, VA, USA) at 490 nm.

2.6. Statistical analysis

All statistical analyses were performed using SigmaStat 3.5 software (Systat Software, Inc., Point Richmond, CA). Differences in antibody titers were analyzed with Student's *t*-test. The significance level was defined as $P < 0.05$.