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# Improving safety of a live attenuated *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish and evaluation of efficacy

Seong Won Nho[1], Hossam Abdelhamed[1], Attila Karsi, Mark L. Lawrence*

*Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS, USA*

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ABSTRACT

*Edwardsiella ictaluri* is a Gram-negative facultative intracellular pathogen causing enteric septicemia of channel catfish (ESC). Our recent work indicated that tricarboxylic acid cycle and one-carbon metabolism are critical pathways for *E. ictaluri* virulence. Although single and double gene deletions in these pathways resulted in safe and efficacious vaccines for use in catfish fingerlings, vaccine trials in catfish fry showed safety concerns. Therefore, we aimed to improve the safety of these mutants by constructing two triple mutant combinations. ESC-NDKL1 (Δ*gcvP*Δ*sdhC*Δ*frdA*) was constructed by introducing an in-frame deletion of *frdA* in a *gcvP-sdh* mutant. ESC-NDKL2 (Δ*gcvP*Δ*sdhC*Δ*mdh*) was constructed in a similar manner. ESC-NDKL1 strain was a better vaccine candidate compared to ESC-NDKL2, providing better safety and efficacy in catfish fry and catfish fingerlings. Field trials in earthen ponds under three vaccination conditions showed that survival was significantly higher in catfish vaccinated with ESC-NDKL1 by immersion at the fry stage, oral vaccination in ponds, and fry immersion-pond oral combination (86.74%, 81.67%, and 95.22%, respectively) compared to sham-vaccinated (42.75%), and Aquavac-ESC fry immersion vaccinated (61.51%) catfish. Our findings indicate that ESC-NDKL1 is a good candidate for further development as a vaccine for ESC.

## 1. Introduction

Channel catfish farming is the largest contributor to U.S. aquaculture production. In 2015, catfish growers in the US produced more than $361 million worth of catfish, of which $201 million (55.7%) was from Mississippi (www.nass.usda.gov). Catfish farmers reported 37% mortality rate due to enteric septicemia of catfish (ESC) (U.S. Department of Agriculture, 2011). Although antibiotics are approved for treatment of ESC (florfenicol and sulfadimethoxine/ormetoprim) and a commercial live attenuated vaccine is available (Klesius and Shoemaker, 1999), ESC is still a major threat to the catfish industry.

Live attenuated vaccines have potential for prevention of ESC. Some live attenuated vaccine candidates have been developed, including auxotrophic mutants (Lawrence et al., 1997; Thune et al., 1999) and iron-siderophore uptake mutants (Abdelhamed et al., 2013). Live attenuated *E. ictaluri* vaccines have also been developed by selecting for antibiotic resistance (Klesius and Shoemaker, 1999; Wise et al., 2015). We reported that tricarboxylic acid (TCA) cycle and one-carbon (C1) metabolism pathways contribute to *E. ictaluri* pathogenesis (Dahal et al., 2013). In particular, some TCA cycle and C1 metabolism mutants provided good protection against wild-type *E. ictaluri* infection in fingerlings, and vaccine strains with deletion of two genes in these pathways had increased safety while retaining efficacy (Dahal et al., 2014b). However, safety of vaccine candidate strains carrying two mutations in these pathways was still not optimal when tested in channel catfish fry (14 days old) (Dahal et al., 2014a).

The TCA cycle supplies intermediates and ATP for bacterial metabolism. In particular, two TCA cycle intermediates, succinate and fumarate, have protective effects against host cationic antimicrobial peptide (Barker et al., 2000). TCA cycle enzymes succinate dehydrogenase and fumarate reductase are membrane-bound enzyme complexes that are necessary for maintaining fumarate and succinate (Cecchini et al., 2002). The TCA cycle also exerts metabolic regulation over the synthesis of capsular polysaccharide by gluconeogenesis (Sadykov et al., 2010). Thus, it is not surprising that TCA cycle mutant strains have demonstrated that the pathway is related to bacterial virulence. For example, the TCA cycle in *Salmonella enterica* serovar Typhimurium is required for intracellular replication in macrophages and for full virulence in murine infection model (Yimga et al., 2006).

The objective of the current study was to improve safety of the TCA cycle and C1 metabolism mutants by developing vaccine candidate strains containing three gene mutations in these pathways. Safety and

* Corresponding author.
*E-mail address:* lawrence@cvm.msstate.edu (M.L. Lawrence).
[1] These authors have contributed equally to this study.

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**Table 1**
Bacterial strains and plasmids.

| Strain | Relevant Characteristics | References |
|---|---|---|
| *Edwardsiella ictaluri* | | |
| 93–146 | Wild type; pEI1$^+$; pEI2$^+$; Col$^r$ | (Lawrence et al., 1997) |
| *EiΔgcvPΔsdhC* | 93–146 derivative; pEI1$^+$; pEI2$^+$; Col$^r$; *ΔgcvP ΔsdhC* | (Dahal et al., 2014a) |
| *EiΔgcvPΔsdhCΔfrdA* | 93–146 derivative; pEI1$^+$; pEI2$^+$; Col$^r$; *ΔfrdAΔgcvP ΔsdhC* | This study |
| *EiΔgcvPΔsdhCΔmdh* | 93–146 derivative; pEI1$^+$; pEI2$^+$; Col$^r$; *ΔgcvP ΔsdhCΔmdh* | This study |
| *Escherichia coli* | | |
| CC118 λ*pir* | Δ(*ara-leu*); *araD*; Δ*lacX74*; *galE*; *galK*; *phoA20*; *thi-1*; *rpsE*; *rpoB*; *argE*(Am); *recAl*; λ*pir*R6K | (Herrero et al., 1990) |
| SM10 λ*pir* | *thi*; *thr*; *leu*; *tonA*; *lacY*; *supE*; *recA*; :RP4-2-Tc:Mu; Km$^r$; λ*pir*R6K | (Miller and Mekalanos, 1998) |
| S17-1 λ*pir* | *RP4-2 (Km:Tn7, Tc:Mu-1), ΔuidA3:pir$^+$, recA1, endA1, thi-1,hsdR17, creC510* | (Metcalf et al., 1994) |
| Plasmids | | |
| pMEG-375 | 8142 bp, Amp$^r$, Cm$^r$, *lacZ*, R6K *ori*, *mob incP*, *sacR sacB* | (Dozois et al., 2003) |
| p*EiΔfrdA* | 10,242 bp, Δ*frdA*, pMEG-375 | (Dahal et al., 2013) |
| p*EiΔmdh* | 8981 bp, Δ*mdh*, pMEG-375 | (Dahal et al., 2013) |

vaccine efficacy of the new vaccine candidates was determined in catfish fingerlings and fry in the laboratory and earthen ponds. These findings demonstrate potential of one of these vaccine candidates with mutations in *sdhC*, *frdA*, and *gcvP* (named ESC-NDKL1) for immersion vaccination of catfish fry for commercial production. A pond trial further indicated that combining the immersion vaccination with an oral booster can improve efficacy of ESC-NDKL1.

## 2. Materials and methods

### 2.1. Ethics statement

All fish experiments were conducted under a protocol (15-043) approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University.

### 2.2. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. ictaluri* was grown at 30 °C using brain heart infusion (BHI) broth and agar (Difco, Sparks, MD). *Escherichia coli* was grown at 37 °C using Luria-Bertani (LB) broth and agar (Difco). Cloning of DNA fragments into pMEG-375 was conducted in *E. coli* CC118 λ*pir*, and conjugal transfers of recombinant plasmids derived from pMEG-375 into *E. ictaluri* were conducted using *E. coli* SM10 λ*pir* or S17-1 λ*pir* as the donor strain. Ampicillin was used at 100 μg/ml to maintain pMEG-375 and its derivatives, and colistin was used at 12.5 μg/ml for counter selection against *E. coli* donor strains following conjugation.

### 2.3. Construction of in-frame deletion mutants

To construct new triple mutation combinations, Δ*frdA* and Δ*mdh* mutations were added separately to the *EiΔgcvPΔsdhC* mutant strain (Dahal et al., 2013) using plasmids p*EiΔfrdA* and p*EiΔmdh* (Table 1). p*EiΔfrdA* and p*EiΔmdh* were mobilized into double mutant *EiΔgcvPΔsdhC* by conjugation (Karsi and Lawrence, 2007). The recipient bacteria were spread on BHI agar containing colistin and ampicillin, and positive colonies were streaked on BHI agar with 5% sucrose and 0.35% mannitol, which selects for loss of pMEG-375 with the *sacB* gene. Deleted regions were amplified from the resulting ampicillin sensitive colonies and confirmed by sequencing. The two triple mutants were designated ESC-NDKL1 (*EiΔgcvPΔsdhCΔfrdA*) and ESC-NDKL2 (*EiΔgcvPΔsdhCΔmdh*).

### 2.4. Safety and efficacy of the ESC-NDKL1 and ESC-NDKL2 in catfish

Vaccine safety in specific pathogen free (SPF) catfish fry (3.17 ± 0.05 cm, 335.92 ± 20.02 mg) and fingerlings (7.75 ± 0.08 cm, 4500 ± 14.07 mg) was determined for the two triple mutants compared to commercial live attenuated vaccine Aquavac-ESC (Klesius and Shoemaker, 1999). Virulence and vaccine efficacy trials were conducted as described previously (Abdelhamed et al., 2013; Dahal et al., 2013). One treatment group was used as a sham control. 14-day old catfish fry were stocked into 20 tanks at a rate of 40 fish/tank, and three-month-old catfish fingerlings were stocked into 20 tanks at a rate of 25 fish/tank. Fish were fed twice a day with a commercial catfish feed (Rangen, Inc., Buhl, ID). The fry experiment included four replicates per treatment, and the fingerling experiment included three replicates per treatment. Experiments were conducted in 40-L tanks supplied with flow-through water (1 L/min) at 25 °C throughout. For immersion vaccination, the water level in each tank was lowered to 10 L, and 100 mL of overnight bacteria culture (adjusted to appropriate dose using optical density at 600 nm ($OD_{600}$)) was added to each tank in the corresponding groups. Vaccination doses were approximately $6.0 \times 10^6$ CFU/ml water and $4.5 \times 10^7$ CFU/ml water for catfish fry and fingerlings, respectively. After 1 h, water flow was restored to each tank, and fish were maintained as usual. Bacteria were flushed from tanks within 40–60 min. Mortalities were recorded daily for 21 days. *E. ictaluri* was confirmed as cause of death by culturing trunk kidney swabs on BHI agar from each mortality. At 21 days post-vaccination, vaccinated and sham control groups were immersion exposed to *E. ictaluri* strain 93–146 (approximately $3.8 \times 10^7$ CFU/ml water), and fish mortalities were monitored daily for 21 days.

### 2.5. Vaccine efficacy of ESC-NDKL1 in catfish ponds

Approximately 6000 17 day-old specific pathogen free (SPF) catfish fry were stocked into five tanks (1200/tank) supplied with flow-through dechlorinated water (1-L/min). Water temperature was maintained at 25–26 °C. The five tanks corresponded to five treatment groups (fry immersion, pond oral, fry immersion-pond oral combination, Aquavac ESC fry immersion, and sham-vaccinated). After two days acclimation, catfish fry (19 days post-hatch) in three treatment groups (fry immersion, fry immersion-pond oral, and Aquavac-ESC fry immersion) were vaccinated by bath immersion (approximately $4 \times 10^7$ CFU/ml of water for 1 h) in the rearing tanks. Vaccine was gradually removed by restoring water flow with complete removal in approximately 40–60 min. Fry in the sham-vaccinated were exposed to an equivalent volume of brain heart infusion (BHI) broth. The tanks were observed daily for mortalities.

Five earthen ponds (0.0485 ha each, with an average depth of 1.5 m) located at the South Farm Aquaculture Research Facility at Mississippi State University were prepared by draining and drying four weeks prior to stocking. The ponds were then filled with groundwater. Three weeks before stocking, the ponds were fertilized with Perfect Pond Plus Fertilizer (Alabama, USA), and dissolved oxygen was