INCREASING UNDERSTANDING OF AND DEVELOPING MANAGEMENT STRATEGIES FOR EDWARDSIELLA ICTALURI IN ORNAMENTAL FISH

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Objectives:

1. Compare the channel catfish and zebrafish strains of *E. ictaluri*
    a) at the molecular level (Mississippi State University)
    b) at the biological/serological level to assess commonalities (Mississippi State University)
2. Evaluate effectiveness of disease management strategies including the following:
    a) Optimization of vaccine design and administration (Louisiana State University)
    b) Antibiotic effectiveness (Louisiana State University)
    c) Antibiotic dosing (pharmacokinetics) (University of Florida)
    d) Potential use of commercial probiotics (University of Florida)
    e) Evaluation of naturally occurring gut anti-bacterials (bacteriocins) in zebrafish (Louisiana State University)

Anticipated benefits:

Enteric septicemia of catfish (ESC), a bacterial disease caused by *Edwardsiella ictaluri*, is a major cause of economic loss in the channel catfish industry. A variant *E. ictaluri* strain causes significant disease in zebrafish (*Danio rerio*) produced for the aquarium trade and for laboratory research. Targeted and collaborative research is needed to understand similarities and differences between these two *E. ictaluri* strains and to develop more effective management options for producers. Researchers at three institutions will collaborate to: a) determine basic molecular and biological differences and similarities between the catfish and zebrafish strains of *E. ictaluri* and b) evaluate potential effectiveness of vaccination, antibiotics, and probiotics in managing the bacterial infection.

Progress and Principal Accomplishments:

Objective 1. Compare the channel catfish and zebrafish strains of E. ictaluri

a) at the molecular level (Mississippi State University)

b) at the biological/serological level to assess commonalities (Mississippi State University)

Although previously reported host-specific to channel catfish, *Edwardsiella ictaluri* outbreaks have been reported in other aquaculture species such as tilapia and ornamental fish. The expanding host and geographic range necessitate comprehensive characterization of *E. ictaluri* isolates derived from multiple fish hosts. *Edwardsiella ictaluri* isolates from catfish (50) and ornamental fish (42) were characterized to determine genotypic and phenotypic homogeneity/ heterogeneity among isolates.

Genotyping of *E. ictaluri* isolates by rep-PCR (Repetitive Extragenic Palindromic sequence PCR) using specific primers (ERIC I & II, BOX, and GTG5) indicated differential profiles for *E. ictaluri* isolates from catfish and ornamental fish. Within groups, isolates from catfish and ornamental fish were largely clonal, with few exceptions, indicating a high degree of genetic stability among *E. ictaluri* populations within each respective industry. Amplification of *E. ictaluri*-specific virulence genes in catfish and ornamental derived *E. ictaluri* isolates indicated differences as well. Complete genomes were obtained for catfish (19) and ornamental (18) derived *E. ictaluri* isolates using the Oxford Nanopore GridIon sequencing platform.

Native plasmid profiles from ornamental and catfish derived *E. ictaluri* were consistent within groups, indicating clonality with respect to host origin (catfish or ornamental fish) and minimal interspecific/intergeneric plasmid transfer. Complete genome and plasmid sequences were screened for antibiotic resistant elements using the Comprehensive Antibiotic Resistance Database's (CARD) Resistance Gene Identifier (RGI). These analyses revealed the presence of multidrug resistant plasmids among selected antibiotic resistant catfish derived *E. ictaluri* isolates. Resistance genes in catfish derived isolates were both plasmid and genome mediated and identified the existence of two discrete multi-drug resistant plasmids carried by *E. ictaluri* isolates from catfish, one (135,268 bp) which was previously reported from *E. ictaluri,* and another (117,449 bp) reported from the closely related *E. piscicida*. No plasmid mediated antibiotic resistance was identified for any ornamental fish derived isolates.

Whole genome analysis revealed discrete genomic differences of *E. ictaluri* among host groups, including the presence of a putative unique phage elements in ornamental fish derived isolates. Type 4 Secretion System (T4SS) elements present in both catfish and ornamental isolates were identified and confirmed. Three different published MLST schemes for the *Edwardsiella* were assessed for ability to determine intraspecific differences among *Edwardsiella* isolates from different hosts, all revealing two different phyletic lineages for catfish and ornamental derived isolates. Signal assessment of individual gene components from each MLST approach identified optimal MLST gene targets for delineating intraspecific variability among *E. ictaluri* strains. These data evinced catfish and ornamental fish-derived isolates represent two discrete phyletic lineages. Clonality of these groups indicates a high degree of genetic stability among *E. ictaluri* isolates within these two respective industries.

Colony morphology, motility, biochemical and enzymatic profiles of *E. ictaluri* isolates from catfish and ornamental fish species were comparable. Regarding growth characteristics, ornamental derived *E. ictaluri* isolates in porcine BHI broth auto-aggregated to the bottom of the culture tube, while catfish derived isolates produced more turbid growth. The protein profiles (whole cell lysate representing structural proteins and lipopolysaccharides indicating surface proteins) for these host-derived isolates revealed by SDS-PAGE and stained with either Coomassie or silver stain, respectively, were mostly similar. Serological profiling to determine antigenicity of these identified proteins is ongoing.

Susceptibility of *E. ictaluri* isolates against approved antibiotics (Aquaflor®, Terramycin®, and Romet®) in catfish aquaculture by disc diffusion revealed variable degrees of susceptibility, especially for antibiotic resistant catfish isolates, which correlated with the presence of multi-drug resistant plasmids. Ornamental fish derived isolates were 'susceptible/ responsive' to all three antibiotics indicating a lack of acquired resistance, consistent with genomic data. The Minimal Inhibitory Concentration (MICs) of 18 antimicrobial agents, as determined using the Sensititre™ AVIAN1F plate, indicated differential susceptibility of isolates against the tested antimicrobial agents. Antibiotic resistant, catfish derived isolates demonstrated resistance to multiple compounds, which loosely correlated with the presence of MDR plasmids.

These results demonstrate isolates from catfish and ornamental fish represent discrete clonal populations. This work identified the existence of two, discrete multidrug resistant plasmids carried by antibiotic resistant *E. ictaluri* isolates from catfish aquaculture. Comparably, native plasmids among ornamental isolates were unique to the ornamental isolates and harbored no recognized antibiotic resistance genes. The high degree of genetic homogeneity of ornamental isolates indicates management practices (probiotics; vaccines; antimicrobial regimes) deemed affective against ornamental strains should be broadly applicable within the industry.

Objective 2. Evaluate effectiveness of disease management strategies including the following:

a) Optimization of vaccine design and administration (Louisiana State University)

A rifampicin resistant mutant strain of *Edwardsiella ictaluri* was developed using the parent strain LADL 11-194 isolated from zebrafish. This mutant was developed by following the procedures outlined in a patent for a modified live E. ictaluri vaccine in channel catfish. E. ictaluri was serially passaged on BHI agar supplemented with increasing concentrations of rifampicin, starting with 5µg/mL, increasing by increments of 10µg/mL, until reaching a final concentration of 320µg/mL. (Klesius et al. 2000). Attenuation of the rifampicin resistant mutant strain was confirmed by laboratory challenge of naïve zebrafish.

Live attenuated mutant strains of E. ictaluri developed in our lab at LSU, ΔureG and ΔesrC , were prepared for inclusion in the vaccine study.

Preliminary challenge trials with the virulent parent strains were unsuccessful with the initial population of zebrafish we obtained from Florida. The conclusion was the members of the population were either survivors of a previous E. ictaluri outbreak or were vaccinated with a

commercial vaccine. A second population of zebrafish was ordered. LSU has requested a NCE to complete outstanding work.

Objective 1b) Antibiotic effectiveness (Louisiana State University)

Antibiotic effectiveness trials will be conducted concurrently with the vaccine trials. Antibiotics have been ordered to incorporate into medicated feeds. LSU has requested a NCE to complete outstanding work.

Objective 1c) Antibiotic dosing (pharmacokinetics) (University of Florida)
Our pharmacokinetic (PK) study determined the population PK of enrofloxacin and florfenicol after oral and bath dosing in giant danio (*Devario aequipinnatus*), a closely related proxy for zebrafish (*Danio rerio*). Leveraging latest population PK estimation algorithms allowed us to estimate both the population mean PK parameters and their between subject variability with good precisian for the destructive sampling datasets (i.e. each fish only contributed one blood and one muscle sample). Drug concentrations were determined by sensitive, specific and precise LC-MS/MS assays.

Informed by a pilot PK study, we employed optimal design methodology to propose informative sampling times for the subsequent main PK studies of both compounds. The final models were simultaneously estimated over all data (i.e. pilot and main study) and over both routes of dosing (for enrofloxacin) to get the most consistent parameter estimates. The predictive performance of the estimated models was evaluated and confirmed by visual predictive checks. Specifically, the models adequately captured the central tendency and the between subject variability of the observed concentrations. This qualified these population PK models for Monte Carlo simulations.

Plasma protein binding information was obtained from the literature across a range of animal species. Florfenicol binding was very consistent across species. A range of unbound fractions has been published and was used for enrofloxacin in our Monte Carlo simulations.

The efficacy of quinolones (enrofloxacin) and of florfenicol best correlates with the fAUC0-24h/MIC. For quinolones, two published PK/PD target values were employed based on decades of PK/PD data on quinolones in mice and humans (2, 8, 12, 17). Several of these studies reflect treatment of ‘normal’ or severe infections. Depending on the usage of enrofloxacin in fish (i.e. either for prophylaxis or treatment), it can be argued that the lower (i.e. easier to achieve) fAUC0-24h/MIC target of 30 may be more applicable than the higher target value of 75. The latter is more difficult to achieve. The choice of the target will depend on the severity of infection and the type of the primary infecting pathogen.

The PK/PD breakpoint was defined as the highest MIC with a probability of PK/PD target attainment of at least 90% following standard practice. These breakpoints ranged from 0.125 to 0.5 mg/L for an oral dose of 8 mg/kg enrofloxacin. These PK/PD breakpoints would have been approximately 2-fold higher, had we used the in part smaller PK/PD target values for enrofloxacin against E. coli in broiler chicken (26). Given that the fAUC/MIC targets of 30 and

75 are based on very substantial amounts of data across numerous fluoroquinolone in mice and humans, we employed the latter targets.

When enrofloxacin was dosed via a bath (at 10 mg/L for 5 h), the PK/PD breakpoints ranged from 0.016 to 0.0625 mg/L and were thus approximately 4-8 fold lower than the breakpoints for oral dosing. For florfenicol, the PK/PD breakpoints were 2 to 4 mg/L, depending on the fAUC/MIC target employed.

We integrated the AUCs from 0 to 24 h, since the current studies and Monte Carlo simulations only simulated a single dose. For multiple dosing scenarios, these PK/PD breakpoint will be (slightly) higher, depending on the total daily dose. If fish are actually infected, longer treatment durations are likely necessary to cure an infection.

In summary, this study defined the population PK of enrofloxacin and florfenicol in giant danio (*Devario aequipinnatus*), a proxy for zebrafish. The highest MICs with a good probability of successful treatment (or prophylaxis) of an infection ranged from 2 to 4 mg/L for 60 mg/kg oral florfenicol, from 0.125 to 0.5 mg/L for 8 mg/kg oral enrofloxacin, and from 0.016 to 0.0625 mg/L for bath dosing (10 mg/L for 5 h) of enrofloxacin.

Objective 1d) Potential use of commercial probiotics (University of Florida)

This study was done to determine if probiotic use in aquaculture resulted in changes in community structure of gastrointestinal microflora of fish. A second objective was to evaluate whether *E. ictaluri* titers were reduced in fish treated with probiotics.
E. ictaluri isolates from Florida facilities (archived at MSU) were sent to two different commercial probiotics manufacturers for laboratory assessment of their probiotic bacterial strains. Both companies performed in lab experiments to assess inhibition by their strains of probiotics, and both companies identified probiotic products that worked to inhibit *E. ictaluri*. These products were sent to the UF IFAS Tropical Aquaculture Laboratory with administration guidelines.

These two commercial aquaculture probiotics were tested at two different zebrafish production facilities. Production parameters (fish growth, density, and size) and microbiome (changes in bacterial flora within the gastrointestinal tract) were measured in male and female fish from control ponds and ponds treated with one of the two probiotics. This study aimed to determine whether probiotics altered the natural gut microbiome of male and female zebrafish.

Each farm had two experimental groups, commercial Probiotic A and commercial Probiotic B, and a control group with no probiotics added (control ponds). Each treatment included 3 pond replicates, resulting in a total of 9 ponds at each farm. All probiotics were dosed as per manufacturer's recommendations. Probiotic A was dosed at 4 grams per 10,000 gallons once weekly. Probiotic B was dosed 40 grams per 10,000 gallons twice weekly. Larval fish were collected the day the ponds were stocked in February and March of 2020. Three ponds, one for each experimental group, were stocked every two weeks. Juvenile zebrafish were collected from all 9 ponds in June at Farm 1 and July at Farm 2. Adult zebrafish were collected at the end of July at Farm 1 and mid-August at Farm 2. Zebrafish were dissected for gill, skin, feces, and

intestine sample. Microbiome analysis on fecal material was successfully completed and analyzed by DNA sequencing.

(*Note: we have also extracted the skin microbiome (not originally planned in the proposal) to assess the frequency of presence/absence of *E. ictaluri*. This information will also be provided as soon as analysis is complete.)

We were able to detect more than a thousand different genera of bacterial species in the gut of zebrafish. Unexpectedly, very few zebrafish in the population had evidence for the presence of *E. ictaluri* in the gut. There were only two samples which contained *Edwardsiella* species collected from Farm 1 and one sample collected from Farm 2. This low-detection frequency was surprising, and there was not enough data to draw conclusions about efficacy of the probiotic. The genus *Edwardsiella* is either not abundant in the gastrointestinal system of zebrafish or is very low in abundance at the two farms. As a Plan B, we have turned to PCR-based detection on the skin, working under the assumption that the bacteria are present at higher levels in this tissue (more exposed to the water and outside environment). These data will be completed this month and are expected to reveal novel insight into efficacy of the probiotic treatments. We have noted that there are differences in *E. ictaluri* between the skin and pond water and have observed both "present" and "absent" hits on fish skin.

Despite challenges detecting *Edwardsiella* in the gut, we learned a great deal about the impacts of probiotics treatments in cultured fish. Zebrafish treated with Probiotic A and B did not differ in their weights or density over the 5-7 month study at either pond, suggesting probiotics do not affect metrics related to mass production. In addition, the zebrafish larval microbiome was distinct from the adult gut microbiome, suggesting that transfer from the hatching facility into the ponds for grow-out shapes the microbiome over time. We did not observe any difference in the microbiome diversity between male and female zebrafish collected from the same ponds. Intriguingly fish treated with Probiotic B differed in their microbiome profile compared to fish from the control pond, while Probiotic A did not significantly alter the composition of the microbiome. Relative proportions of *Firmicutes* and *Fusobacteria* were altered with Probiotic B. *Firmicutes* is considered to have a negative effect on the microbiome and is related to adverse health. Probiotic B reduced the proportion of "bad" microbes in the gut of fish at Farm A but increased the proportion of Firmicutes at Farm B, suggesting the response to probiotics may be partially dependent upon microenvironmental factors at a given fish farm. We are continuing to compile data on pathogenic bacteria in the gut.

In summary, this was the first study conducted to determine the effects of probiotic use on the gastrointestinal microbiome of cultured ornamental fish. Taken together, our data are significant because they demonstrate the use of probiotics in aquaculture setting may not affect the growth of fish but can rather modify the host microbiota. We also demonstrate that the concentration of probiotic used in the dosing regimen are important in achieving microbiome shifts in the gastrointestinal system as Probiotic A (a lower concentration) did not influence the species richness. Additional studies can now be conducted to evaluate frequency of probiotic treatment administration or fish-specific and, potentially, pathogen specific responses.

Objective 1e) Evaluation of naturally occurring gut anti-bacterials (bacteriocins) in zebrafish (Louisiana State University)

For this study, 20 pond cultured individuals were sacrificed upon receipt. Five different gram negative bacteria were consistently isolated from the gut contents of the zebrafish and identified via API20E testing*: Moellerella wisconsensis*, *Pseudomonas aeruginosa, Aeromonas hydrophila, Aeromonas caviae, and Aeromonas sobria* were identified from the cultures. We will further confirm the identity of these isolates using MALDI-TOF and evaluate their antibacterial activity in vitro. LSU has requested a NCE to complete outstanding work.

Impacts:

- Better understanding of biological differences and similarities between bacterial strains of *E. ictaluri* from catfish compared to those from ornamentals will help guide disease management options for producers and fish health managers
- More accurate antibiotic dosing regimens for florfenicol and enrofloxacin will help producers treat diseased fish and minimize economic losses
- Vaccines and probiotic bacteria designed specifically to combat *E. ictaluri* isolates from the zebrafish industry can help promote "good bacteria" and thus may help prevent disease and minimize economic losses.

Publications, Manuscripts, or Papers Presented:

Publications in Print

Johnson, Divya, "Phenotypic and genotypic characterization and comparison of *Edwardsiella ictaluri* isolates derived from catfish and ornamental fish species" (2021). MS Thesis. Mississippi State University, Mississippi State, MS USA.

Presentations

Johnson, D., M. J. Griffin, E. T. Woodyard, L. H. Khoo, G. C. Waldbieser, R. P. E. Yanong, J. P. Hawke and S. Aarattuthodi. Phenotypic and genotypic characterization and comparison of *Edwardsiella ictaluri* isolates derived from catfish and ornamental fish species. AFS-FHS Summer Student Seminar Series. Oral Presentation. Online. June 2021. Link.

Brammer-Robbins, E.M., E. K. Freeman, A. S. Kanarek, J. H. Bisesi, E. J. Cassiano, Q. M. Tuckett, R.P.E. Yanong, C. J. Martyniuk. 2021. Evaluating probiotic treatments in the ornamental fish aquaculture industry: implications for managing *Edwardsiella ictaluri* outbreaks and fish microbiota. International Association for Aquatic Animal Medicine, Virtual Conference. Online. May 2021.

Vorbach, B.S., J. Bulitta, J. Zhou, Y. Lang, and R.P.E. Yanong. Pharmacokinetics and pharmacodynamics of enrofloxacin and florfenicol in the giant danio (*Devario aequipinnatus*) following oral and bath administration. International Association for Aquatic Animal Medicine, Virtual Conference. Poster Session. Online. May 2021.

Johnson, D., M. J. Griffin, L. H. Khoo, G. C. Waldbieser, and S. Aarattuthodi. Biological, molecular and serological characterization of *E. ictaluri* isolates in catfish and ornamental fish species. 46th annual meeting of Mississippi Chapter of the American Fisheries Society. February 12-14, 2020 in Gulfport, MS.

Johnson, D., M. J. Griffin, L. H. Khoo, G. C. Waldbieser, and S. Aarattuthodi. Molecular characterization of *Edwardsiella ictaluri* isolates and efficacy of E. ictaluri vaccine to protect channel catfish fingerlings against the field isolates. 45th Annual meeting of Mississippi Chapter of American Fisheries Society. February 20-22, 2019 in Jackson, MS.

Results at a Glance:

- New knowledge of how *Edwardsiella ictaluri* isolates from zebrafish differ from channel catfish isolates will help ornamental fish farmers better control the disease.
- Enrofloxacin and florfenicol drug studies using a closely related species, the giant danio, will help producers more accurately dose medicated feeds for effective disease treatment.
- Probiotics added to pond water will shift the intestinal bacterial communities of zebrafish, can enhance numbers of “good bacteria” and may help reduce losses from *E. ictaluri.*