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Intraspecific Variability of Edwardsiella piscicida and Cross-Protective Efficacy of a Live-Attenuated Edwardsiella ictaluri Vaccine in Channel and Channel × Blue Hybrid Catfi

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Intraspecific Variability of *Edwardsiella piscicida* and Cross-Protective Efficacy of a Live-Attenuated *Edwardsiella ictaluri* Vaccine in Channel and Channel × Blue Hybrid Catfish

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Incidence and prevalence of *Edwardsiella piscicida* has increased in Mississippi farm-raised catfish in recent years. *Edwardsiella piscicida* affects mostly market-sized catfish during the final stages of the production cycle resulting in significant economic losses. The objectives of this study were to determine the genetic variability of *E. piscicida*, assess virulence in channel and hybrid catfish, and evaluate the capacity of a live-attenuated *E. ictaluri* vaccine to protect channel and hybrid catfish against heterologous *E. piscicida* isolates. This work identified five discrete *E. piscicida* lineages, along with group specific associations of several virulence related genes. In general, *E. piscicida* was shown more virulent in hybrids than channel catfish, in line with previous work. Further, a live-attenuated *E. ictaluri* vaccine was shown to confer cross-protective immunity in channel and hybrid catfish against *E. piscicida*.

DEDICATION

This thesis is dedicated in memory of Esteban J. López Porras and Luis Robles Collado who unconditionally supported and encouraged me to pursue my dreams. I would also like to dedicate this thesis to my parents Pedro J. López Baltodano and Maritza Porras Berrocal, and to my siblings Erick B. and Kendry Lopez Porras for their absolute and loving support during this journey.

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TABLE OF CONTENTS

APPENDIX

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

OVERVIEW OF THE CATFISH AQUACULTURE IN THE SOUTHEASTERN UNITED STATES AND THE IMPACTS OF *EDWARDSIELLA* SPP.

1.1 Catfish Aquaculture in the Southeastern United States

The Food and Agriculture Organization of the United Nations cites aquaculture as the fastest growing food production sector worldwide (FAO, 2016). In the U.S., channel catfish, *Ictalurus punctatus,* has been an important farmed fish species for several decades (Tucker and Robinson, 1990; Tucker and Hargreaves, 2004). The annual production of catfish in the U.S. contributes 74% of total U. S. finfish production and accounts for over ~340 million dollars (Hanson, 2019; USDA, 2019). Mississippi continues to be the largest producer in terms of water surface acres used for production (36,200 acres, 56.77% of total acreage) and total sales (207,543 million dollars, 57.58% of total sales) (USDA, 2019).

The origin of catfish farming in the U.S. can be traced back to the 1890s, with the first reports of spawning channel catfish in captivity (Tucker and Hargreaves, 2004). The oldest reports of catfish domestication in the U.S. are from the Kansas State Fish Hatchery in 1910 (Dunham and Elaswad, 2018). The first small scale catfish farms were established in Kansas from 1930 to 1940, with larger farms established in Arkansas and Mississippi in the 1940s. In the 1950s, channel catfish were shown to be the optimal ictalurid species for U.S. catfish aquaculture, as moderately

higher production could be achieved compared to other ictalurid species (Tucker and Hargreaves, 2004).

The first large commercial catfish farms were established in Alabama and Mississippi in the 1960s and in the 1970s. Catfish farming began to expand rapidly in the Delta region of Mississippi, with Mississippi assuming a lead role in the industry. As the industry expanded, production began to intensify with the adoption of fixed in-pond aerators, development of standardized diets, use of medicated feeds, optimal stocking densities, and transition from large 40-80 acre ponds to smaller 10 acre production ponds, split ponds, all of which laid the groundwork for management practices still in use today (Dunham and Elaswad, 2018; Tucker, 2019). This was also reflected in the two decades from 1982-2002, wherein water surface area was almost duplicated, yet the quantity of fish processed (an index of fish production) increased more than six-fold (Tucker and Hargreaves, 2004).

Over the past decade, adoption of complementary technologies, the use of channel (♀) (*I. punctatus*) × (♂) blue (*Ictalurus furcatus*) hybrid catfish and culture in intensive production systems, have increased production to >15,000 lbs/acre (Kumar and Engle, 2017). Hybrid catfish have demonstrated faster growth related to increased feed consumption, better feed conversion, increased survival under intensive culture conditions, higher tolerance to low oxygen, greater uniformity, and improved dress-out percentage compared to channel catfish (Bosworth et al., 2004; Green and Rawles, 2010; Dunham and Ramboux, 2014). In 2017, hybrid catfish comprised ~70% of the total U.S. catfish production (Dunham and Elaswad, 2018). The relatively superior performance of hybrids over channel catfish has contributed to the growing popularity of hybrids as a culture animal and this trend is expected to continue into the near future (Kumar and Engle, 2010; Arias et al., 2012; Torrans et al., 2015).

Despite early successes, the sustainability of U.S. aquaculture, is threatened by increased production costs, over production of seafood in general and increased incidence of infectious agents as a result of intensification. Of these factors, infectious diseases represent the greatest obstacle in maintaining the economic viability of the catfish industry. Economic losses resulting from infectious diseases are not only associated directly with mortality, but also to morbidity, poor feed conversion rates, harvesting delays, and expense of therapeutics which can be marginally effective at best (Tucker, 2019).

During the early stages of the catfish aquaculture, diseases were a relatively minor issue, but as production intensified infectious diseases became a primary limitation for increasing production yields and profitability. In the late 1970's, a Gram-negative bacterium, classified as *Edwardsiella ictaluri* was identified as the cause of large losses in commercially produced channel catfish (Hawke, 1979; Hawke et al., 1981). A closely related species *Edwardsiella tarda* was also recovered from diseased channel catfish, but research demonstrated low pathogenicity of this species and was considered highly opportunistic (Hawke and Khoo, 2004). Intragenic variability of this bacterial species was later reclassified as *Edwardsiella piscicida* which has recently emerged as a significant cause of disease and production losses in hybrid catfish (Abayneh et al., 2013; Reichley et al., 2017).

Based on diagnostic case submissions to the Thad Cochran National Warmwater Aquaculture Center's (NWAC) Aquatic Research & Diagnostic Laboratory (ARDL), there has been an increase in the number of cases of *E. piscicida* that has accompanied increased hybrid catfish production, with hybrid catfish accounting for more than 90% of *E. piscicida* diagnoses since 2013 (Griffin et al., 2019; Khoo et al., 2017, 2018).

1.2 *Edwardsiella* spp.

Members of the genus *Edwardsiella* are in the family Hafniaceae (Adeolu et al., 2016), first established by Ewing and collaborators in 1965 (Ewing et al., 1965). The genus is a group of Gram-negative enteric bacteria largely known for diseases in fish, with limited reports from humans, reptiles, amphibians, mammals and birds (Mohanty and Sahoo, 2007; Griffin et al., 2017). *Edwardsiella* spp. infect a wide variety of wild and cultured fish across a range of temperatures, salinities and environments (Mohanty and Sahoo, 2007; Griffin et al., 2017). Catfish farms in the southeastern U.S. are particularly affected, and outbreaks of *E. ictaluri* and *E. piscicida* threaten the sustainability and economic viability of these operations (Hawke et al., 1981; Wise et al., 2004).

The genus is currently comprised of five nominal species: *E. tarda*, *E. ictaluri*, *E. hoshinae*, *E. anguillarum*, and *E. piscicida* (Ewing et al., 1965; Griffin et al., 2017). *Edwardsiella tarda* is the type species, first described by Ewing et al. (1965) to accommodate a group of enteric bacteria from humans and other terrestrial animals that were not phenotypically congruous with any other known genus. *Edwardsiella tarda* was reported to cause disease in farmed channel catfish in Arkansas in 1969 (Meyer and Bullock, 1973). Since these first reports, *E. tarda* has been documented from other wild and cultured fish species and has been recognized as one of the most significant fish pathogens worldwide (Ewing et al., 1965; Mohanty and Sahoo, 2007).

In 1980, a second species, *E. hoshinae*, was described as a commensal in birds and reptiles (Grimont et al., 1980). The third species, *E. ictaluri*, was described as the etiological agent of ESC, with isolates recovered from diseased channel catfish cultured in Alabama, Georgia and Mississippi and from white catfish (*Ameiurus catus*) from Maryland (Hawke, 1979; Hawke et al., 1981). These three species comprised the genus until 2013, with the recognition of *E. piscicida*

(Abayneh et al., 2013), followed later by the description of *E. anguillarum* in 2015 (Shao et al., 2015).

In the late 1990s, researchers recognized stark intraspecific genetic differences within *E. tarda* (Yamada and Wakabayashi, 1999). These different strains were classified as typical fish pathogenic *E. tarda*, atypical fish pathogenic *E. tarda* and non-fish pathogenic *E. tarda*, which demonstrated significant genetic differences in their superoxide dismutase (*sodB*) gene, with fish pathogenic strains clustering phylogenetically with *E. ictaluri*, separate from isolates deemed non-fish pathogenic *E. tarda* from terrestrial animals and humans (Yamada and Wakabayashi, 1999). Similar relationships were reported by two independent laboratories in the U.S. and Europe, which supported the notion that fish pathogenic and fish non-pathogenic *E. tarda* represented multiple, discrete taxa (Abayneh et al., 2012; Griffin et al., 2013). This work led Abayneh et al. (2013) to re-define this genus, establishing the new species, *E. piscicida*, based on DNA-DNA hybridization and genome to genome distance calculations based on 16S rRNA gene sequences and concatenated sequence alignments of 8 housekeeping genes (*gyrB*, *mdh*, *adk*, *dnaK*, *phoR*, *metG*, *pyrG* and *aroE2*). Similarly, using a polyphasic approach consisting of DNA–DNA hybridization, average nucleic acid identity calculations from sequenced genomes and phylogenies based on 16S rRNA and concatenations of the gene set *adk*, *aroE2*, *dnaK*, *metG*, *phoR*, and *pyrG*, Shao et al. (2015) established *E. anguillarum*. It was later shown by strain-specific PCR, as well as *sodB* and *gyrB* sequencing, the isolates previously deemed typical and atypical fish pathogenic *E. tarda* were synonymous with *E. piscicida* and *E. anguillarum*, respectively (Griffin et al., 2014, 2017; Reichley et al., 2017).

1.2.1 *Edwardsiella piscicida*

Edwardsiella piscicida is a Gram-negative, rod-shaped, facultative anaerobic bacterium. The bacteria grow from 20-40°C in the presence of up to 3.0% NaCl. Optimal growth occurs at 28-30°C, forming colonies on blood agar after 24 h of incubation. Colonies are circular, slightly convex, smooth and glistening, with slight β-hemolysis under the colonies (Abayneh et al., 2013). *Edwardsiella piscicida* is indole, catalase, methyl red, lysine decarboxylase and ornithine decarboxylase positive. Motility is observed at both 25 and 37°C. The cells are negative for β-galactosidase, arginine di-hydrolase, urease, TDA, Voges Proskauer, and cytochrome-oxidase. They do not degrade gelatin, β-methyl-D-glucoside, citric acid or L-proline. The TSI reaction is K/A with H_2S production (Abayneh et al., 2013; Griffin et al., 2013).

1.2.1.1 Piscine Edwardsiellosis caused by *Edwardsiella piscicida*

Edwardsiella piscicida has been reported to cause disease in more than 20 species of fish, including channel catfish channel (♀) × blue (♂) hybrid catfish, largemouth bass (*Micropterus salmoides*), black crappie (*Pomoxis nigridis*), European eel (*Anguilla anguilla*), turbot (*Scophthalmus maximus*), barramundi (*Lates calcarifer*), Korean catfish (*Silurus asotus*), marbled eel (*Anguilla marmorata*), Japanese eel (*Anguilla japonica*), sea bream (*Evynnis japonicas*), tilapia (*Oreochromis* sp.), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and others, posing significant risks to farmed, ornamental, bait, and sport fish worldwide (Abayneh et al., 2013; Griffin et al., 2013; Camus et al., 2016, 2019; Fogelson et al., 2016; Shafiei et al., 2016; Buján et al., 2017, 2018; Griffin et al., 2017, 2019, 2020a; Loch et al., 2017; Reichley et al., 2017).

Fish infected with *E. piscicida* can display a variety of external gross clinical signs ranging from darkened skin, multifocal cutaneous petechiation, abdominal distension, and fin or skin

erosion, and swelling of tissues covering the cranium resulting in bullae formation. Rupturing of the bullae leads to an ulcerative lesion, often revealing the underlying frontal bone, similar to lesions caused by *E. ictaluri* infections hence the industry colloquialism 'Hole-in-the-Head' (Hawke et al., 1981, 2015; Bertolini et al., 1990). Common histopathological findings observed in affected fish are consistent with a generalized septicemia, with multifocal necrosis and granulomatous inflammation in the liver, spleen, and anterior and posterior kidney, with frequent Gram-negative bacilli observed associated with these lesions (Fogelson et al., 2016; Griffin et al., 2017).

1.2.1.2 *Edwardsiella piscicida* virulence-related factors

The development of disease is intrinsically associated with virulence factors carried by individual pathogens, wherein expression of these factors and their effects on the fish host are contingent on environmental (temperature, pH, etc.) and host factors (immune status, size, age, stress, diet, etc.) (Matanza and Osorio, 2018). Two of the most common virulence factors described for Gram-negative bacteria are the type III (T3SS) and type VI secretion systems (T6SS). These systems have been demonstrated essential for pathogenesis. They also are key to bacterial fitness in both intracellular and extracellular environments, when competing with other bacteria for nutrients (Yang et al., 2018).

The T3SS, also known as the injectisome, consists of a multiproteinaceous machinery that facilitates the secretion of effector proteins from the bacterial cell into the host cells (Galán & Wolf-Watz, 2006). Through this needle-like mechanism, the effector proteins manipulate host cells in several ways allowing uptake of the bacterium by the host cell where the agent can replicate and propagate infection. The T3SS effectors allow the bacterium to exploit host cell machinery for

their own benefit. The T3SS of different bacterial pathogens enable them to invade non-phagocytic cells; inhibit phagocytosis by phagocytes, to downregulate innate immunity or modulate intracellular trafficking, and establish either a survival or replication niche (Coburn et al., 2007).

Similarly, the T6SS is another delivery machinery of effector proteins found in Ed*wardsiella* species. The T6SS gene cluster encodes 13–14 conserved core components for machinery assembly and some less conserved accessory proteins and effectors related to T6SS regulation and biological functions (Records, 2011; Basler, 2015; Cianfanelli et al., 2016). The T6SSs participate in a broad variety of functions, including virulence, antibacterial activity, quorum sensing, cell-to-cell signaling and metal ion uptake (Gallique et al., 2017).

Another mechanism *Edwardsiella* species use to facilitate intracellular infection and survival is through detoxifying reactive oxygen species. To this end, genes such as *sodB* encoding superoxide dismutase and *katB* encoding catalase are essential to protect bacteria against host defenses (Han et al., 2006; Ishibe et al., 2008). In *E. ictaluri,* the virulent factors chondroitinase, urease and EacF (homologous protein of putative adhesin/hemagglutinin/ hemolysin in *Escherichia coli*) have been well characterized and given the relatedness of *E. ictaluri* to *E. piscicida* they are likely present in *E. piscicida* also, with similar functions (Cooper et al., 1996; Polyak, 2007; Booth et al., 2009). Other genes affecting *E. ictaluri* pathogenicity and virulence are *TonB* (Transport protein) and *Fur* (Ferric Update Regulator) which affect hemoglobin transportation (Santander et al., 2012).

The reservoir of potential virulence genes in *E. piscicida* may account for the reported various acute, subacute or chronic manifestations of disease in fish. The twin arginine translocation system (*Tat*), consisting of *tatABCDE,* is also considered a virulence mechanism in *Edwardsiella* spp. (De Buck et al., 2008; Wang et al., 2009). Adhesion molecules such as fimbrial protein (*FimA*)

were determined to be important virulence factors associated with fish pathogenic *Edwardsiella* spp. (Sakai et al., 2007). Two types of haemolysins (*HlyA* and *EthAB*) have been reported as factors required for invasion and penetration of *Edwardsiella* species (Chen et al., 1996; Hirono et al., 1997). Lastly, a study performed by Castro et al. (2016) showed the presence of chondroitinase, AHL-synthase, autoinducer-2 synthesis, sensor protein, and homologous genes for biosynthesis of the siderophore vibrioferrin and important to iron metabolism.

1.2.1.3 Antimicrobial Resistance of *Edwardsiella piscicida*

Abdelhamed et al. (2019) indicated that *E. piscicida* strain MS-18-199 recovered from a diseased hybrid catfish from East Mississippi was resistant to florfenicol, chloramphenicol, oxytetracycline, doxycycline, erythromycin, tetracycline, azithromycin, spectinomycin, sulfonamide, and bacitracin. This resistance was mediated by a novel plasmid containing several antimicrobial resistance-related genes, including a florfenicol efflux pump (*floR*), tetracycline efflux pump (*tetA*), tetracycline repressor protein (*tetR*), sulfonamide resistance (*sul2*), aminoglycoside O-phosphotransferase *aph*(*6*)-Id (*strB*), and aminoglycoside O-phosphotransferase *aph*(*3*)-Ib (*strA*). Similar findings were described by Liu et al. (2017), where *E. piscicida* strain EIB202 carried the multi-drug resistant IncP plasmid encoding tetracycline, streptomycin, sulfonamide and chloramphenicol resistance. Both studies demonstrated these plasmids can be transferred by both inter- and intraspecific conjugation. Furthermore, Reichley et al. (2017) described intraspecific variation for a panel of 39 antimicrobial compounds against 47 *Edwardsiella* isolates. Although no discriminatory antimicrobial compound was identified, intraspecific variation in susceptibility between *E. piscicida* isolates was more variable than other *Edwardsiella* spp.

1.3 Impacts of *Edwardsiella piscicida* on Hybrid Catfish Aquaculture

Contemporary studies, in addition to anecdotal reports from the catfish industry, have identified an emergence of *E. piscicida* in farm-raised hybrid catfish in the southeastern U. S. Out of the total cases submitted to the ARDL during the 2013-2017 period hybrid catfish cases comprised around 40% of the total submissions, and from them almost 90% were diagnosed with *E. piscicida*, with 97% of the *E. piscicida* diagnoses involving stocker or market-sized catfish (Griffin et al., 2019). This is consistent with experimental infectivity studies indicating *E. piscicida* to be up to 10 times more virulent in hybrids than in channel catfish (Reichley et al., 2017). Furthermore, *E. piscicida* tends to occur later in growing season, typically mid-to late summer, exacerbating the impact on productivity through reduced feeding and significant reductions in production. Additionally, mortalities in market-sized fish exacerbate economic losses as significant producer investments have already been incurred. The resultant losses can be particularly damaging to farm profits, not just for the mortality events itself, but also through indirect losses from reduced feeding activity, poor growth and increased feed conversion in diseased fish populations. For reasons that are unclear, *E. piscicida* does not appear to be comparatively problematic in hybrid catfish fingerling production.

The growing number of *E. piscicida* cases in hybrid catfish are troublesome given current industry trends towards increased hybrid use for catfish production. With the ongoing transition from channel catfish to hybrid catfish, the emergence of *E. piscicida* is concerning as it is the first pathogen confirmed to have increased virulence in hybrid catfish. As hybrid production expands, more research is needed to develop effective prevention strategies to mitigate losses associated with *E. piscicida* infection.

1.4 Prevention and Control of *Edwardsiella piscicida*

Control of infectious diseases is one of the most important goals in global aquaculture. Moving forward, prevention of diseases in farmed fish for human consumption will likely require a polyphasic approach, consisting of a combination of different management strategies including high biosecurity standards, proactive health management, high quality feeding, effective immunization, and antimicrobial stewardship.

In the catfish industry, control of enteric bacterial diseases is mostly limited to feed restriction during outbreaks or administration of medicated feeds. While feed restriction can effectively reduce the spread of pathogens via the fecal-oral route of transmission, this strategy negatively affects fish growth due to lost feed days (Wise et al., 2004). An alternative approach to control disease is through medicated feed. Medicated feeds have been shown to be effective, but are expensive, and as outbreaks progress, feeding activity is significantly reduced, decreasing the efficacy of medicated feeds (Tucker and Robinson, 1990). Additionally, the increased use of antimicrobials in medicated feeds can increase the incidence of antibiotic resistance, and may select for more virulent strains, both limiting effectiveness of medicated treatments and increasing morbidity and mortality associated with disease (Cabello, 2006). As a result of these limitations, the most practical and cost-effective means to combat infectious diseases is through prevention. In this regard, vaccination provides an effective means to protect fish against various viral and bacterial pathogens (Embregts and Forlenza, 2016; Ma et al., 2019).

Researchers at NWAC have developed a live-attenuated *E. ictaluri* vaccine that demonstrates exceptional protection against *E. ictaluri* in channel and hybrid catfish (Wise et al., 2015a, 2015b; Aarattuthodiyil et al., 2020). This orally delivered vaccine is coupled with a mechanized delivery system capable of delivering measured doses of vaccine with feed. The

vaccine, as well as the oral delivery platform, has been proven to be highly effective in experimental and commercial field trials, resulting in significantly improved yield, feed efficiency, and survival in hybrid and channel catfish (U.S. Patent# 8999319) (Wise et al. 2015a, 2020; Peterson et al., 2016; Greenway et al., 2017; Chatakondi, et al. 2018; Aarattuthodiyil et al. 2020). Production analysis of commercial field vaccination trials indicates ESC increased gross sales by $3,750/ha/acre which in theory can be used to estimate the cost of ESC in catfish fingerlings (Kumar et al., 2019; Wise et al., 2020).

Similarly, preliminary data generated from the same research group suggests evidence of cross immunoreactivity between *E. piscicida* and *E. ictaluri*. Channel and hybrid catfish challenged with *E. piscicida* demonstrated improved survival over naïve cohorts, when exposed to the virulent wildtype *E. ictaluri* isolate S97-773. In a subsequent trial, channel and hybrid catfish immunized with the live, attenuated *E. ictaluri* vaccine were protected against infection following exposure to *E. piscicida* isolate S11-285 (Griffin et al., 2020b). These findings suggest *E. piscicida* and *E. ictaluri* share similar protective immunogenic epitopes, indicating the *E. ictaluri* vaccination platform also has utility in reducing economic losses associated with *E. piscicida* in hybrid catfish. Given the increased incidence and prevalence of *E. piscicida* associated with hybrid catfish production, it is serendipitous that the newly developed *E. ictaluri* vaccine also provides protection against *E. piscicida.* In theory, this negates the need to develop an *E. piscicida*-specific vaccine, saving public agencies and private industry significant time and financial investment.

While the *E. ictaluri* vaccine shows promise, these studies involved only a single strain of *E. piscicida* (S11-285). The effectiveness of the *E. ictaluri* vaccine to protect against heterologous *E. piscicid*a strains has not been evaluated. Molecular analysis of archived *E. piscicida* isolates suggests a greater genetic heterogeneity than observed amongst *E. ictaluri* isolates (Griffin et al.,

2011, 2013, 2014; Reichley et al., 2017). At present, the biological implications of this variability are unknown and the pathogenicity of discrete *E. piscicida* lineages in channel and hybrid catfish are unresolved. More research is required to determine if the current ESC vaccine provides adequate cross-protection against all possible *E. piscicida* variants or if this protection is limited to a few select, closely related strains. A wider phenotypic and molecular characterization of *E. piscicida* will aid the development of a more effective and economically pragmatic pathogen-targeted management strategies to limit the impacts of *E. piscicida* on catfish aquaculture.

1.5 Objectives

The wide host range and virulence displayed by *E. piscicida* make it a potentially devastating pathogen in catfish aquaculture. With the emergence of *E. piscicida* in hybrid catfish, development of effective pathogen-specific control strategies to reduce economic losses has become an industry priority. Previous work has shown a live, attenuated *E. ictaluri* vaccine (Wise et al., 2015b) is protective against *E. piscicida* strain S11-285 from farm-raised catfish in Mississippi (Aarattuthodiyil et al., 2020; Griffin et al., 2020b). The purpose of this study was to 1) Establish the genetic diversity of *E. piscicida* from diagnostic case submissions to the ARDL in Stoneville, MS and determine virulence associations of *E. piscicida* variants in channel and hybrid (♀ channel x ♂ blue) catfish; 2) Evaluate the cross-protective effects of a live, attenuated *E. ictaluri* vaccine to protect channel and hybrid catfish against heterologous *E. piscicida* challenges.

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CHAPTER II

MULTILOCUS SEQUENCE ANALYSIS, PLASMID PROFILING AND VIRULENCE GENE PROFILING OF *EDWARDSIELLA PISCICIDA* ISOLATES FROM MISSISSIPPI CATFISH AQUACULTURE WITH AN ASSESSMENT OF VIRULENCE IN CHANNEL AND CHANNEL × BLUE HYBRID CATFISH

2.1 Introduction

Catfish is one of the most important farm-raised fish species in the United States, accounting for the majority of total U.S. aquaculture production. With total sales of $360 million in 2018 (USDA NASS, 2017), catfish aquaculture is also one of the most important agricultural commodities of several southern states. Mississippi is the largest catfish producer in the U.S., with 36,200 water surface acres used for catfish production (56.77% of the total U.S. water surface acres) with total sales exceeding 200 million dollars (57.58% of total sales in U.S.) (USDA, 2019).

Over the past decade, catfish aquaculture has been transitioning from producing almost exclusively channel catfish to also producing channel (*Ictalurus punctatus*) (♀) × blue (*Ictalurus furcatus*) (♂) hybrid catfish (Russo et al., 2009). Hybrid catfish are superior to channel catfish in several production parameters, including increased resistance to several important infectious diseases, namely enteric septicemia of catfish caused by *Edwardsiella ictaluri*, columnaris disease caused by *Flavobacterium columnare*, and proliferative gill disease caused by *Henneguya ictaluri* (Wolters et al., 1996; Arias et al., 2012; Bosworth et al., 2013). However, coupled with this

increase in hybrid production has been an increase in piscine edwardsiellosis, caused by *Edwardsiella piscicida* (Khoo et al., 2017, 2018).

The increase in *Edwardsiella piscicida* cases is reflected in the diagnostic case summaries from the Aquatic Research and Diagnostic Laboratory (ARDL) in Stoneville, MS. From 2013-2017, hybrids made up ~40% of total diagnostic submissions to the ARDL yet accounted for >90% of *E. piscicida* diagnoses, which supports research data indicating increased virulence of *E. piscicida* in hybrid catfish (Reichley et al., 2018). The emergence of *E. piscicida* in hybrid catfish is worrisome given current industry trends towards increased use of hybrid catfish in intensive catfish production. Furthermore, the increased pathogenicity of *E. piscicida* in hybrids, and adverse effects mainly on market-sized fish, result in pronounced economic losses as significant producer investments have been made that cannot be recovered (Khoo et al., 2017, 2018; Griffin et al., 2019).

The *Edwardsiella* genus was established by Ewing and collaborators in 1965 as a member of the family *Enterobacteriaceae*. Recently, the *Edwardsiella* have been reassigned to the family *Hafniaceae* (Ewing et al., 1965; Adeolu et al., 2016). This genus is comprised of Gram-negative pathogens that predominantly infect wild and farm-raised fish. While primarily known for the diseases they cause in fish, Edwardsiella spp. have also been reported to infect a broad range of animal clades such as reptiles, amphibians, birds, and mammals, including humans. Edwardsiella spp. have been isolated from a variety of temperatures, salinity, and environments globally. (Mohanty and Sahoo, 2007; Leotta et al., 2009; Griffin et al., 2017; Miniero Davies et al., 2018).

From 1981-2013, the *Edwardsiella* genus consisted of three discrete taxa: *E. tarda*, *E. hoshinae* and *E. ictaluri*. Research in the late 1990s to early 2000s revealed significant genetic variability among *E. tarda* isolates recovered from different hosts and locations, suggesting

isolates identified as *E. tarda* were polyphyletic groups genetically diverse but phenotypically cryptic (Yamada and Wakabayashi, 1999; Matsuyama et al., 2005; Castro et al., 2006; Maiti et al., 2009; Griffin et al., 2013). These different genotypic *E. tarda* variants were commonly classified either as typical fish pathogenic *E. tarda*, atypical fish pathogenic *E. tarda*, or fish-nonpathogenic *E. tarda* isolated mostly from humans and other mammals (Yamada and Wakabayashi, 1999; Matsuyama et al., 2005; Sakai et al., 2007; Reichley et al., 2017). The different categories of *E. tarda* isolates were revealed to be polyphyletic groups by analysis of the *sodB* and Type 1 Fimbrial gene cluster, virulence gene profiles, and repetitive sequence mediated PCR (Yamada and Wakabayashi, 1999; Sakai et al., 2007; Wang et al., 2011; Nakamura et al., 2013). In 2012, a study by Abayneh et al. (2012) revealed similar polyphyletic origins of *E. tarda* in Europe based on multi-locus sequence analysis of several housekeeping genes. Similar results were shown by Griffin et al. (2013) investigating *E. tarda* in the United States. These studies eventually led to the re-definition of typical fish pathogenic *E. tarda* as *E. piscicida* (Abayneh et al., 2013; Griffin et al., 2014, 2017; Reichley et al., 2017). Similarly, what was previously deemed atypical fish pathogenic *E. tarda* (syn. *E. piscicida*-like sp.) was later described as *E. anguillarum* (Griffin et al., 2014; Shao et al., 2015; Reichley et al., 2017). This genus currently consists of five nominal species: *E. tarda*, *E. hoshinae*, *E. ictaluri*, *E. piscicida*, and *E. anguillarum* (Griffin et al., 2017).

Edwardsiella piscicida is a Gram-negative, rod-shaped and facultative anaerobe bacterium, with optimum temperature for growth at 28-30°C, forming colonies on blood agar after 24 h of incubation, however, growth can occur at 25°C and 37°C (Abayneh et al., 2013). *Edwardsiella piscicida* has been reported to cause disease in more than 28 species of fish, including channel catfish, hybrid catfish, largemouth bass (*Micropterus salmoides*), European eel (*Anguilla anguilla*), Turbot (*Scophthalmus maximus*), Korean catfish (*Silurus asotus*), Marbled eel (*Anguilla*

marmorata), Japanese eel (*Anguilla japonica*), Sea bream (*Evynnis japonica*), and posing potential risks to other farmed, ornamental, baitfish, and sport fish species worldwide (Abayneh et al., 2013; Griffin et al., 2014; Camus et al., 2016; Fogelson et al., 2016; Shafiei et al., 2016; Buján et al., 2017; Reichley et al., 2017).

A study by Griffin et al. (2014) revealed that isolates from catfish aquaculture in the southeastern United States previously classified as *E. tarda* were actually *E. piscicida*, the emergence of which was later tied to increased hybrid production (Griffin et al., 2019). Further work by Reichley et al. (2017) revealed important intraspecific variation among *E. piscicida* isolates from a variety of fish hosts in phenotypic characteristics, plasmid profiles, antibiograms, and genetic variability among *Edwardsiella* spp. isolates from different fish hosts and geographic origins. Moreover, studies performed by Wang et al. (2011) and Castro et al. (2016) showed differences in virulence-related genes carried by isolates formerly classified as typical fish pathogenic *E. tarda*. The aim of the present study was to determine the genetic variability among *E. piscicida* isolates recovered from farm-raised catfish in Mississippi and associated virulence in channel and hybrid catfish fingerlings.

2.2 Materials and Methods

2.2.1 Bacterial isolates

A total of 158 *E. piscicida* isolates (Table 2.1), identified by *Edwardsiella* spp. multiplex qPCR (Reichley et al., 2015; Griffin et al., 2019) were used in this study. These isolates largely originated from channel and hybrid catfish cases submitted to the Aquatic Research and Diagnostic Laboratory (ARDL) at the Thad Cochran National Warmwater Aquaculture Center (NWAC), Stoneville, MS, and cryopreserved at -80°C. Additional isolates were recovered from other freshwater fish species and identified in previous work (Griffin et al., 2013). Isolates were revived

on Mueller-Hinton II Agar (BBL™, Becton Dickinson and Company) plates supplemented with 5% defibrinated sheep blood (MHBA) and incubated for 24 h at 37°C (Reichley et al., 2015). Individual colonies from each isolate were expanded in 9 mL of porcine brain heart infusion broth (BHIb) (Bacto; Becton, Dickinson and Company). After 24 h of growth, 1 mL was subsampled and stored cryogenically (-80°C, 15% glycerol) for further studies.

2.2.2 DNA Extraction

All archived isolates were initially revived on MHBA and incubated for 24 h at 37°C (Abayneh et al., 2013; Griffin et al., 2013;). Individual colonies from each isolate were expanded in 5 mL BHIb at 37°C for 24 h. Aliquots (2 mL) of overnight culture were pelleted by centrifugation at 15,000 × g for 5 min. Genomic DNA (gDNA) was isolated from each bacterial pellet using a commercial DNA extraction kit (Gentra Puregene DNA isolation kit; Qiagen) following the manufacturer's protocol for Gram-negative bacteria. Isolated gDNA was resuspended in 100 µL of Puregene DNA hydration solution (DHS) and quantified spectrophotometrically (NanoDrop 2000; Thermo Fisher Scientific). Resuspended gDNA was diluted with DHS to achieve a final concentration of ~10 ng/µL and stored at 4°C until further use.

2.2.3 Repetitive sequence mediated PCR analysis

Initially, all 158 *E. piscicida* isolates were screened by repetitive sequence mediated PCR (rep-PCR) using the Enterobacterial Repetitive Intergenic Consensus (ERIC) I&II primers. Isolates were divided into four subsets. Each subset was analyzed by rep-PCR using the ERIC I&II primers (Table 2.2) (Versalovic et al., 1991, 1994) and modified protocols outlined by Griffin et al. (2013). Briefly, analysis consisted of 13 µL of iQ™ Supermix (2×) (Bio-Rad Laboratories, Inc.; Hercules, CA), 20 pmol (ERIC I and II), ~50 ng of gDNA template and nuclease-free water

to volume. Amplifications were performed on a C1000 Touch thermal cycler (Bio-Rad Laboratories, Inc.) with the following settings: initial denaturation at 95°C for 10 min; then 35 cycles at 95°C for 1 min, 40°C for 1 min, and 72°C for 5 min; then 35 cycles of 95°C for 1 min, 55°C for 1 min; final extension at 72°C for 5 min. Aliquots of each amplification reaction mixture (12 µL each) and the molecular weight standard HyperLadder™ 50bp (Bioline; Meridian Life Sciences; Memphis, TN) were passed through a 2% (weight/volume) agarose gel in the presence of ethidium bromide (0.5 mg/mL) and visualized under UV light. Visibly distinct bands were manually annotated and genetic fingerprints were analyzed using the Quantity One software v. 4.6.5 (Bio-Rad Laboratories) to calculate Dice coefficients and dendrograms were generated from Dice matrices based on the unweighted pair-group method using arithmetic averages (UPGMA). From these initial reactions, a subset of 39 isolates (Table 2.7) representing the main phylogroups identified for each subset were chosen for further molecular analysis. Rep-PCR using the ERIC I&II primers was then repeated for this subset. In addition, these 39 isolates were also analyzed using the BOX and $(GTG)_5$ rep-PCR primers using the protocols outlined above (Table 2.2).

2.2.4 Multilocus Sequence Analysis (MLSA)

Isolated gDNA of select *E. piscicida* representative isolates (n=39) was used for MLSA analysis. The housekeeping genes *phoU* (Phosphate-specific transport system accessory protein), *pgi* (Glucose-6-phosphate isomerase) and *gyrB* (DNA gyrase subunit B) were amplified by end-point PCR following the procedures described by Griffin et al. (2013). Amplification reactions (50 µL) consisted of 25 µL 2× Phusion™ Flash High-Fidelity PCR Master Mix (Thermo Scientific™), 20 pmol of each primer (Table 2.3), 20 ng of gDNA and nuclease-free water to volume. Amplifications were performed on a C1000 Touch thermal cycler with the following settings: initial denaturation at 98°C for10 s; 40 cycles: 98°C for 10 s, 55°C for 5 s, 72°C for 15 s; final

extension at 72°C for 1 min. Amplicons were visualized under UV light after electrophoretic migration through a 0.8% agarose gel containing ethidium bromide (0.5 mg/mL) and then purified using QIAquick™ Gel Extraction Kit (Qiagen). The purified products were sequenced directly using the corresponding external and internal *gyrB* sequencing primers (Table 2.3). Purified PCR products were processed by Eurofins on an ABI 3730xl DNA analyzer (Applied Biosystems). Contiguous sequences were assembled using the corresponding chromatograms in Geneious Prime® 2020.1.1 (Biomatters, Ltd., Auckland, New Zealand, 2019). Additional sequences from isolates described in Table 2.4 were retrieved from the National Center for Biotechnology Information (NCBI) database and included in this MLSA. *Edwardsiella anguillarum* ET080813 was used as outgroup.

2.2.5 Phylogenetic Analysis

Phylogenetic analysis for rep-PCR fingerprints is described above. For the MLSA, gene sequences for each gene (*gyrB*, *pgi*, *phoU*) were individually aligned and concatenated in Geneious Prime® (version 2020.1). The best fit substitution model for the dataset was chosen by jModelTest 2.1.10 using the Bayesian Information Criterion (BIC) for each gene dataset (Darriba et al., 2012). The chosen models were: GTR+G for *gyrB*, HKY+I for *pgi*, and K8 for *phoU*. Phylogenetic trees were calculated from concatenated DNA sequence alignments by Bayesian inference using MrBayes v3.2.6. with posterior probability distributions generated using the models selected previously, with four chains running, simultaneously, for 1000000 generations, and every 100th tree sampled, until convergence was achieved (Ronquist and Huelsenbeck, 2003).

2.2.6 Virulence-Related Factors Screening

Representative *E. piscicida* isolates (n=39) were screened for the presence of virulence-related genes using primers initially described by Wang et al. (2011) and Castro et al. (2016), in addition to several new primer sets developed specifically for this study (Table 2.5). Final amplification reaction (25 µL) consisted of 13 µL of Econotaq PLUS GREEN 2X Master Mix (Lucigen Corporation, Middleton, WI, USA), 10 pmol of each primer, 20 ng of gDNA, and nuclease-free water to volume. Virulence-related genes were amplified individually using the following settings: initial denaturation at 95°C for 5 min; 35 cycles: 95°C for 1 min, (annealing temperatures (T_m) are listed in Table 2.5)°C for 1 min; final extension at 72°C for 1 min and a final extension at 72°C for 5 min. 10 µL of each amplification reaction were passed through a 1% agarose gel containing ethidium bromide (0.5 mg/mL) and visualized under UV light.

2.2.7 Antimicrobial Susceptibility Profiling

Minimal inhibitory concentrations (MIC) were determined using AVIAN1F plate formats (Trek Diagnostic System) following the manufacturer's suggested protocol. *Escherichia coli* ATCC 25922 was used as the quality control strain. Each inoculum was prepared by suspending individual colonies in sterile distilled water to achieve 0.5 McFarland-standard turbidity; 30 µL of the suspension was added to 11 mL of cation-adjusted Mueller-Hinton broth (Sigma-Aldrich), and 50 µL of the inoculum was added to each well. Plates were covered with an adhesive seal and incubated 24 h at 37°C. Following incubation, plates were checked visually, and MIC values were recorded, where MIC values were defined as the lowest drug concentration exhibiting no visible bacterial growth. Antibiotic sensitivity tests were also performed for selected representative isolates by disk diffusion assays using the Aquaflor® (florfenicol), Romet® (5:1

sulfadimethoxine/ormetoprim), and Terramycin® (oxytetracycline) following Hudzicki's recommendations (2009).

2.2.8 Plasmid Profiling and Sequencing

Individual colonies recovered from archived cryostocks of selected *E. piscicida* isolates (n=39) were expanded as above in BHIb cultures. Aliquots (3 mL) of expanded cultures were concentrated by centrifugation (17,000 × g; 5 min) and native plasmids harvested using the QIAprep® Spin Miniprep Kit (Qiagen; Hilden, Germany). A total of 50 µL of resuspended plasmid DNA was electrophoresed through agarose gels (0.8%), in the presence of ethidium bromide (0.5 mg/mL) and concurrently run standards (Supercoiled DNA Ladder; New England BioLabs).

High molecular weight DNA was isolated from all isolates shown to carry plasmids and used in long read sequencing. Sequencing libraries were barcoded using the Rapid Barcoding Kit (RBK004; Oxford Nanopore Technologies [ONT], Oxford, UK), pooled and run on v9.4.1 ONT flow cells using the GridION platform. Samples were demultiplexed in real-time using the ONT MinKNOW high accuracy basecaller. Sequence FASTQ files were trimmed using NanoFilt (https://github.com/wdecoster/nanofilt) to remove 100 bp from each end, and filtered to obtain sequences with minimum length of 1000 bp. Genomic contigs were assembled using Canu v1.8 (Koren et al., 2017) and consensus sequence errors were corrected using Medaka (https://github.com/nanoporetech/medaka). To validate circularized genomes, overlapping sequence at the contig ends was removed, 1 Mb at the end of the genomic contig was moved to the 5' end, then long reads were realigned to the contig with minimap2 (https://github.com/lh3/minimap2/blob/master/cookbook.md). The alignments were visualized in Integrated Genomics Viewer (Thorvaldsdóttir et al., 2013) to validate continual read coverage

across the junction. Plasmid DNA sequences were analyzed using the Glimmer plugin for Open reading frames (ORFs) prediction in Geneious Prime® (version 2020.1) and the gene prediction program GeneMark.hmm prokaryotic (version 3.25) (Besemer and Borodovsky 1999; Zhu et al. 2010). Putative function of plasmid ORFs were predicted using a BLASTX search of the NCBI non-redundant protein database using the Bacteria and Archaea code, with e-values ≥1e-04 considered insignificant hits.

2.2.9 Infectivity Trials: Initial Passage

All fish used in this study were reared indoors for disease research at the Thad Cochran National Warmwater Aquaculture Center rearing facility located on the campus of the Mississippi State University Delta Research and Extension Center in Stoneville, MS. All animal handling procedures were performed in compliance with the Mississippi State University Institutional Animal Care and Use Committee. To account for differences cryogenic storage times, all isolates used in infectivity trials were passed through channel catfish prior to challenge. Isolates were revived from cryopreservation on MHBA and incubated for 24 h at 28°C. Individual colonies of each isolate were expanded in 9 mL of BHIb for 18 h at 28°C. Channel catfish fingerlings (~10 g) were injected intracoelomically with 0.1 mL of dilute (1:20000) BHIb culture approximating an exposure dose of ~$1x10^4$ colony-forming units (CFU) per fish. After 48 h, fish were euthanized by an overdose of tricaine methanesulfonate (MS-222) (300 mg/mL bath) and kidneys cultured on MHBA. Aerobic cultures were incubated for 24 h at 28°C and individual colonies expanded in BHIb as described above. Aliquots (15% v/v glycerol) of passed isolates were then cryopreserved at -80°C (15% glycerol) until infectivity trials.

2.2.10 Infectivity Trials

Two infectivity trials were performed using two representative isolates from MLSA clades 1-4 and one isolate from MLSA Clade 5. Channel catfish ($\bar{x}$ initial weight = 10.4 g) and hybrid catfish ($\bar{x}$ = 10.8 g) fingerlings were transferred from the rearing facility to 80-L aquaria containing 22 L of aerated well water (~25°C) exchanged at a rate of 1 L/min. Channel and hybrid catfish (20 fish/aquaria) were distributed into six treatments, with 5 replicate aquaria/treatment. Treatments corresponded to each of the five *E. piscicida* MLSA clades and one control group (Figure 2.1). Fish were acclimated for two weeks prior to challenge. For infectivity challenges, passed isolates were revived from cryogenic storage by isolation streaking on MHBA as above and individual colonies in 9 mL of BHIb for 18 h at 28°C. Bacterial cultures were diluted (1:2000) to achieve target doses equating the approximate median lethal dose for *E. piscicida* (Table 2.6; Reichley et al., 2015; 2017). Delivered doses were estimated by standard plate count techniques on triplicate blood agar plates using the drop-plate method (Herigstad et al., 2001). Feed was withheld 24 h prior to challenge. Fish from each replicate were anesthetized with MS-222 (100 mg/mL) and injected intracoelomically with 0.1 mL of dilute culture. Sham control fish were handled similarly but were exposed with 0.1 ml sterile BHIb. Fish were monitored twice daily for 15 days and mortality recorded. Posterior kidney from dead fish was aseptically cultured on MHBA to confirm bacterial presence.

2.2.11 Statistical Analyses

Cumulative mortality between *E. piscicida* MLSA clades and among fish type (hybrid and channel catfish) was analyzed by two-way ANOVA, followed by Tukey's post-hoc test ($\alpha = 0.05$). Analyses were performed using GraphPad Prism 8 (Graphpad Software, Inc; San Diego, CA).

2.3 Results

2.3.1 Genetic Fingerprinting

Initial screening of the 158 *E. piscicida* isolates by ERIC rep-PCR revealed multiple discrete genetic clusters (Figure 2.2). A subset of 39 *E. piscicida* isolates representing the predominant clusters from each data set were selected based on initial screening and used for further molecular characterization. Rep-PCR using ERIC I&II, BOX, and $(GTG)_5$ primers revealed well supported genetic clusters, groupings were inconsistent and isolate placement within clusters differed for each primer set (Figure 2.3).

2.3.2 Multilocus Sequence Analysis (MLSA)

Bayesian inference analysis of concatenated housekeeping genes (*gyrB*, *pgi*, *phoU*) revealed the 39 *E. piscicida* isolates fell into five discrete phyletic groups (Figure 2.4), with isolates from Mississippi farm-raised catfish present in all five clades. MLSA clades 1 and 2 were largely populated by isolates recovered from either channel or hybrid catfish from Mississippi, while MLSA clade 4 was exclusively comprised of *E. piscicida* isolates from Mississippi catfish. MLSA clade 3 was comprised predominantly of isolates from Asia and only two isolates from catfish fell into this group. MLSA clade 5 was underrepresented with just two isolates, one from Asia and another from Mississippi. MLSA groupings were most consistent with phyletic groupings based on $(GTG)_5$ fingerprinting (Table 2.7).

2.3.3 Virulence-Related Genes Screening

The presence of virulence related genes, as determined by PCR, loosely correlated with MLSA grouping (Table 2.8). MLSA Clade1 and 3, as well as the majority of isolates from MLSA clade 2, were PCR positive for the *PefC* gene, encoding for an outer membrane usher protein.

Similarly, most MLSA 2 isolates were positive for *Eta1,* thought to be associated with putative bacterial adhesins. Furthermore, all isolates from MLSA clades 3 and 4 were positive for the *Invasin* genes. All 39 isolates were positive for the *VgrG*-1 gene, which encodes a structural and secretor protein of a T6SS system, as described in previous studies (Zheng and Leung, 2007). Comparably, the T6SS gene *VgrG-2 was* only present in MLSA 1 and 2 isolates. Similarly, one CRISPR system-related genes were limited to MLSA 1 and 5 clades.

2.3.4 Antimicrobial Susceptibility Profiles

Kirby-Bauer disc diffusion assessments of antimicrobial susceptibility revealed the majority of the 39 *E. piscicida* isolates analyzed were susceptible to Aquaflor®, Romet®, and Terramycin®. One isolate from MLSA clade 1 was resistant to Romet®. Comparably, one isolate from MLSA clade 2 demonstrated intermediate resistance to Romet, with another MLSA 2 isolate resistant to Terramycin® (Table 2.9). The MICs of different antimicrobial compounds were tested for all 39 *E. piscicida* isolates from catfish in the present study, resulting in a wide range of intraspecific variation for oxytetracycline, tetracycline, amoxicillin, and penicillin (Table 2.10). However, no discriminatory antimicrobial compound was identified among the clades. For many of the antimicrobials tested the MICs for different isolates within each *E. piscicida* clade were largely consistent. The antimicrobial susceptibility profile of the isolate S07-348 generated from this analysis was consistent with the putative antibiotic resistance function of plasmid-carried open reading frames (ORFs) (Figure 2.6, and Table A.1 in appendix).

2.3.5 Plasmid Profiling

There were marked variability in plasmid content and organization among *E. piscicida* isolates (Figure 2.5). Of the 39 *E. piscicida* isolates analyzed in the study, 17 carried plasmids, 5

of which carried two (Figure 2.5) plasmids. Physical maps of sequenced plasmids are presented (Figure 2.6). Predicted plasmid associated genes were associated with plasmid replication and structural maintenance, T6SS virulence-related factors, toxin–antitoxin (TA) systems and antimicrobial resistance, which correlated with MIC and Kirby-Bauer data (Tables 2.9 and 2.10). Summaries of predicted proteins for each *E. piscicida* plasmid are presented in Appendix Table 2.1.

2.3.6 Infectivity Trial

Pooled cumulative mortality from treatments was greater ($p < 0.001$) in hybrid catfish than in channel catfish (Figure 2.7). Tukey's post-hoc multiple comparisons revealed no significant differences in mortality among MLSA groups in hybrid catfish. However, mortality in channel catfish exposed to MLSA clade 5 was significantly higher ($p < 0.05$) than other MLSA groups (Figure 2.8).

2.4 Discussion

Catfish aquaculture is a significant economic driver in the southeastern United States, particularly Mississippi and Alabama. Historically, farm-raised catfish has been focused on production of channel catfish. However, recent industry trends have shifted towards production of hybrid catfish. While initially hybrid catfish were largely refractive to diseases that plagued channel catfish production, as hybrid production has intensified, infectious agents have emerged with a predisposition to hybrids. A survey of diagnostic submissions to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS, from 2013 to 2017 evinced an emergence of *E. piscicida* within Mississippi catfish aquaculture attributed to increased hybrid production (Griffin et al., 2019). Previous work has revealed *E. ictaluri* in catfish aquaculture is largely clonal with limited

genetic variations among field isolates from disease outbreaks (Griffin et al., 2011; Aarattuthodiyil et al., 2020). Comparably, *E. piscicida* from catfish are largely variable (Griffin et al., 2014; Reichley et al., 2017), although the role of this genetic diversity in *E. piscicida* virulence in channel and hybrid catfish is unknown.

Previous work has demonstrated the utility of rep-PCR methods for intraspecific typing among *Edwardsiella* species (Wang et al., 2011; Griffin et al., 2013, 2014, 2016; Reichley et. al., 2017). The current study supports these previous studies indicating *E. piscicida* from catfish aquaculture is genetically heterologous based on rep-PCR profiles, although clustering based on these profiles were inconsistent among primer sets. Some *E. piscicida* isolates were placed into different cluster depending on the set of primers used, for example, using the ERIC I&II, BOX, $(GTG)_5$ primer sets showed in total 6, 5, and 4 different genetic clusters, respectively (Figure 2.3). While rep-PCR is a useful tool for rapid assessments of genetic diversity among bacterial isolates, the method lacks resolution, repeatability and portability of more resolute methods like sequencing. Further, rep-PCR does not lend itself to inclusion of isolates that are not on hand and its utility can be compromised when analysis includes of large datasets requiring digital manipulation of gel images. Comparably, MLSA analysis contributes to the construction of large, sharable datasets and affords inclusion of isolates from across the globe through publicly accessible databases (Glaeser et al., 2015).

The MLSA supported rep-PCR data evincing the presence of significant genetic variability among *E. piscicida* isolates from catfish, revealing five discrete phyletic groups (Figure 2.4). Assignment of isolates to genetic groups by MLSA disagreed with rep-PCR using ERIC I&II and BOX primers, but it was largely in-line with diversity assessment using the $(GTG)_5$ primers.

Edwardsiella piscicida isolates recovered from farm-raised catfish were diverse, with representative isolates present in all five MLSA groupings. MLSA Clade 4 comprised of isolates recovered from farm-raised catfish. Similarly, MLSA Clades 1 and 2 also consisted predominantly of catfish isolates. Isolates from Europe were similarly diverse, with representative isolates present in MLSA Clades 1 and 3. Comparably, MLSA profiles of *E. piscicida* isolates from various fish hosts in Asia fell largely in MLSA Clade 3, which includes the *E. piscicida* type strain ET-883. One Asian isolate joined MS isolate S11-534 in Clade 5. It is unknown whether the overrepresented *E. piscicida* MLSA clades reflect the true prevalence of those clades on MS catfish farms or is merely a function of sampling and arbitrary submission of disease case submissions to the ARDL. Moreover, these results suggest isolates originating from Asia may be more clonal than isolates from Europe and the U.S. This could be attributed to Asian countries largely exporting fish and fish products. Congruously, the increased diversity observed in European and US isolates may be a function of increased globalization and the transboundary trafficking of aquaculture production by net-importers. Further epidemiological investigations are warranted to determine the prevalence and incidence of *E. piscicida* MLSA clades in catfish and other global aquaculture industries, as well as the health and economic implications of this genetic plasticity.

The majority of the virulence-related genes initially investigated by Wang et al. (2011) and Castro et al. (2016) were present in the *E. piscicida* isolates from catfish isolates. The conserved presence of these gene targets across all *E. piscicida* MLSA clades offers insight into the pathogenicity of these isolates in catfish, contrary to reports indicating environmental *Edwardsiella* spp. isolates lack many virulence-related genes and incomplete Type III (T3SS) and Type VI (T6SS) secretion systems (Leung et al., 2019). While the majority of virulence related genes were present, there were differences in some factors associated with MLSA clades.

The *VgrG* gene typically indicates the presence of the T6SS (Cianfanelli et al., 2016). In the current study, two *VgrG* orthologue genes were exclusively present in *E. piscicida* MLSA Clades 1 and 2, while only one *VgrG* orthologue was detected in MLSA clades 3, 4, and 5. This suggests the presence of two different T6SS present in MLSA Clades 1 and 2. The *VgrG* genes encode outer components of the T6SS apparatus which secretes effector proteins of the T6SS, playing a crucial role in different stages of bacterial pathogenesis (Pukatzki et al., 2007). Further studies are needed to elucidate the biological significance of this T6SS redundancy in some *E. piscicida* strains.

Likewise, clade specific variability was also observed for *cse1* and *cse2*, which were only found in MLSA clade 1 and 5. The *cse1* and *cse2* are homologous to genes found in the CRISPR Type I-E system, hypothesized to play a role in pathogenicity in some *Escherichia coli* strains (García-Gutiérrez et al., 2015). The *PefC* gene, detected in *E. piscicida* isolates from MLSA clades 1, 2, and 5, is part of an operon coding for different proteins that form a fimbrial structure playing an important role during adhesion process of infection. *PefC* genes has homology with *PapC* and *FaeD* genes which encode outer membrane proteins required for the biosynthesis of P and K88 fimbriae of *E. coli*, respectively, related to tissue tropism and virulence (Cantey et al., 1999). The *Eta1* gene, homologous of a putative bacterial adhesin, detected only in MLSA clade 2, has been described as a very important factor of virulence during host colonization, adhesion, and systemic dissemination (Sun et al., 2012). *Intimin/Invasin*, found only in groups III and IV, is another gene associated with the adhesion process in atypical *E. tarda* isolates and its homologous proteins are associated with production of the attaching and effacing lesion in the gastrointestinal tract by different *E. coli* pathotypes (Cookson et al., 2007). *Invasin* found only in the same MLSA clade 3, is a virulence related factor of the inverse autotransporter protein family to which *intimin* also

belongs to and it plays essential roles in hemolytic activity, biofilm formation, adhesion, internalization, and pathogenicity of *E. tarda* (Dong et al., 2013). These differences in MLSA clades carrying genes encoding proteins with crucial roles during the attachment and invasion processes and harboring a second T6SS may indicate that edwardsiellosis dynamics induced by different *E. piscicida* MLSA clades could vary in infection timing, tissue-tropism, and pathology during natural disease outbreaks. Further studies are needed to establish whether there are differences in terms of pathogenesis induced by different *E. piscicida* MLSA clades using infection models that resemble more those seen in natural disease outbreaks.

All told, 10 unique plasmids were identified from analyzed *E. piscicida* isolates. The discovery of a multitude of genes encoding plasmid-mediated proteins putatively related to plasmid integration and excision, mobilization, replication, and stability indicates *E. piscicida* isolates from catfish aquaculture possess the machinery to facilitate perpetuation in diverse environments. While the economic impacts of *Edwardsiella* spp. on catfish aquaculture are well documented, the consequences of *Edwardsiella*–associated plasmids disseminated within the industry are presently unclear. This work highlights a knowledge gap in our understanding of plasmid-trafficking in catfish aquaculture.

While plasmids were only detected in <50% of analyzed isolates, it is important to note that the methods employed here may be limited in their ability to extract very large plasmids or plasmids with low copy numbers. Although 17 of the 39 *E. piscicida* carried plasmids, there was no correlation between plasmid presence and MLSA clade. The presence of the plasmid genes tetracycline resistance transcriptional repressor *TetR* and tetracycline efflux MFS transporter *Tet(A)* were associated with antibiotic resistance observed in S07-348 isolate, which showed resistance to Terramycin® (oxytetracycline) by the disk diffusion method and high MICs for

oxytetracycline and tetracycline. Similarly, antimicrobial susceptibility profiles were variable within groups and no discriminatory antibiotic agent was identified for any of the MLSA clades. This variability was observed mostly in the antimicrobials oxytetracycline, tetracycline, amoxicillin, and penicillin. Oxytetracycline is one of the most widely used antimicrobials in aquaculture (Seyfried et al., 2010), providing a selective pressure that may have driven an emergence of resistance among some isolates.

Cumulative mortality for all pooled treatments demonstrated increased mortality in hybrid versus channel catfish. This corroborates previous studies reporting increased virulence of *E. piscicida* in hybrid catfish (Reichley et al., 2017). Even though significant genetic variability was observed among *E. piscicida* isolates, there was no difference in virulence among MLSA groups in hybrids. Comparatively, there was increased mortality in channel catfish exposed to the MLSA clade 5 compared to other MLSA groups. Based on our data there might be virulence-related factors that confer to this group more virulence in channel catfish. However, MLSA group 5 appears to be underrepresented among catfish isolates. Further investigations using additional representative isolates from MLSA group 5 are warranted.

The intracoelomic injection model used in this study was initially validated by Reichley (2017) and demonstrated the injection model produced the most consistent results using MLSA Clade 1 isolate S11-285 (Reichley et al., 2015; 2017; Griffin et al., 2020). It is recognized the intracoelomic injection model does not accurately mimic the natural route of infection and artificial inductions of infection by injection may increase virulence. However, immersion and oral models of infection have been unable to induce disease in channel and hybrid catfish under experimental conditions (Reichley 2017), which limits investigations elucidating virulence and pathogenic mechanisms among various field *E. piscicida* isolates. As a result, this model may produce biased

mortality results among MLSA groups. Further, the injection model supersedes normal modes of pathogenesis during the first stages of infection, precluding *E. piscicida* from interacting with external mucosal surfaces of the skin and gills, or the gastrointestinal tract. Moreover, injection limits the ability to evaluate different stages of pathogenesis, such as colonization, adhesion, and dissemination as well as differences in the onset of the disease.

The current study established the genetic variability of *E. piscicida* isolates from farm-raised catfish in Mississippi. The biological implications of this diversity are unknown and further research is required to determine the role of these variants in potential disease outbreaks. Aspects of disease including outbreak dynamics (e.g. acute, subacute, chronic) as well host susceptibility (age/size) may vary by MLSA group.

Previous studies have indicated a genetic variability amongst *E. tarda* isolates that led to categorize them into the new species *E. piscicida* and *E. anguillarum* (Abayneh et al., 2013; Shao et al., 2015). It is remarkable this genetic variability as observed in this study exists in a geographical region such as Mississippi with two main largescale farmed fish, hybrid and channel catfish. A different scenario seems to be for *E. piscicida* isolates from Asia, which according to this study are more clonal complex. Furthermore, this intraspecific genetic variability described in *E. piscicida* isolates differs from the genetic structure of *E. ictaluri* isolates from the same region, which have been described to be more conserved (Griffin et al. 2011; Aarattuthodiyil et al., 2020).

2.5 Tables

Table 2.1 *Edwardsiella piscicida* isolates used in this study. Most isolates were recovered from cases submitted to the ARDL. Isolates were previously identified as *E. piscicida* by a multiplex qPCR (Reichley et al. 2015; Griffin et al., 2018). *Edwardsiella piscicida* recovered from other freshwater fish species were also included (Griffin et al., 2013, 2014). LMB = Largemouth bass.

Isolate	Host	Isolate	Host	Isolate	Host	Isolate	Host	Isolate	Host	Isolate	Host
LADL97-168	Channel	S11-295	Hybrid	S14-309	Channel	S16-344	Hybrid	S17-132	Hybrid	S17-460	Hybrid
LADL99-462	Channel	S11-310	Channel	S14-384	Hybrid	S16-407	Hybrid	S17-244	Hybrid	S17-470	Hybrid
MA97-004	Tilapia	S11-508	Hybrid	S14-431	Hybrid	S16-408	Hybrid	S17-294	Hybrid	S17-513	Hybrid
S07-1004	Blue	S11-509	Channel	S15-83	Hybrid	S16-409	Hybrid	S17-295	Hybrid	S17-527	Hybrid
S07-1019	Blue	S11-534	Hybrid	S15-96	Channel	S16-417	Hybrid	S17-297	Hybrid	S17-529	Hybrid
S07-1094	Channel	S11-551	Hybrid	S15-102	Hybrid	S16-418	Hybrid	S17-321	Hybrid	S17-540	Hybrid
S10-67	Hybrid	S11-552	Channel	S15-197	LMB	S16-419	Hybrid	S17-327	Hybrid	S17-541	Hybrid
S07-262	Channel	S11-553	Channel	S15-225	Hybrid	S16-435	Hybrid	S17-332	Hybrid	S17-562	Hybrid
S07-275	Channel	S11-616	Hybrid	S15-250	Hybrid	S16-463	Hybrid	S17-335	Hybrid	S17-563	Hybrid
S07-276	Channel	S11-632	Channel	S15-341	Hybrid	S16-464	Hybrid	S17-338	Hybrid	S17-564	Hybrid
S07-346	Channel	S11-680	Channel	S15-573	Hybrid	S16-465	Hybrid	S17-340	Hybrid	S17-631	Hybrid
S07-347	Channel	S11-688	Channel	S16-51	Channel	S16-466	Hybrid	S17-341	Channel	S17-655	Hybrid
S07-348	Channel	S12-272	Channel	S16-119	Hybrid	S16-488	Hybrid	S17-342	Hybrid	S17-656	Hybrid
S07-356	Channel	S12-273	Channel	S16-124	Hybrid	S16-567	Hybrid	S17-383	Hybrid	S17-671	Hybrid
S07-357	Channel	S12-281	Hybrid	S16-132	Channel	S16-572	Hybrid	S17-384	Hybrid	S17-672	Channel
S07-358	Channel	S12-307	Channel	S16-138	Hybrid	S16-591	Hybrid	S17-385	Hybrid	S17-673	Hybrid
S07-534	Channel	S12-378	Hybrid	S16-182	Hybrid	S16-592	Hybrid	S17-386	Hybrid	S17-674	Hybrid
S07-907	Channel	S12-408	Channel	S16-190	Hybrid	S16-631	Hybrid	S17-397	Hybrid	S17-676	Hybrid
S08-209	Channel	S12-419	Hybrid	S16-200	Hybrid	S16-668	Hybrid	S17-399	Hybrid	S17-677	Hybrid
S10-279	Channel	S12-420	Hybrid	S16-201	Hybrid	S16-717	Hybrid	S17-410	Hybrid	S17-722	Hybrid
S10-430	Hybrid	S13-156	Hybrid	S16-202	Hybrid	S16-728	Hybrid	S17-413	Hybrid	S17-731	Hybrid
S10-512	Hybrid	S13-370	Hybrid	S16-221	Hybrid	S16-730	Hybrid	S17-423	Hybrid	S17-77	Hybrid
S11-62	Channel	S13-469	Hybrid	S16-278	Hybrid	S16-731	Hybrid	S17-424	Hybrid	S17-85	Channel
S11-159	Channel	S13-636	Channel	S16-279	Hybrid	S16-739	Hybrid	S17-441	Hybrid		
S11-222	Channel	S13-640	Hybrid	S16-285	Hybrid	S17-36	Hybrid	S17-442	Hybrid		
S11-233	Channel	S13-826	Channel	S16-292	Hybrid	S17-59	Hybrid	S17-443	Hybrid		
S11-285	Channel	S14-264	Hybrid	S16-293	Hybrid	S17-114	Hybrid	S17-449	Hybrid		

Table 2.2 Primers used for repetitive bacterial DNA elements-based polymerase chain reaction (rep-PCR).

Primer	Sequence (5′-3′)	*Tm* (°C)	Reference
BOX	CTACGGCAAGGCGACGCTGACG	52	Versalovic et al., 1994
ERIC I	ATGTAAGCTCCTGGGGATTCAC	52	Versalovic et al., 1994
ERIC II	AAGTAAGTGACTGGGGTGAGCG	52	Versalovic et al., 1994
$(GTG)_5$	GTGGTGGTGGTGGTG	40	Versalovic et al., 1994

Table 2.3 Genes and sequencing primers used for multilocus sequence analysis (MLSA).

Gene	Primer	Sequence (5′-3′)	Source	Product length (bp)
gyrB				
	GyrB630F	GGATAACGCGATTGACGAAG	Griffin et al., 2014	1670
	GyrB1425F	ATGACCCGTACGCTGAACA		
	GyrB1949R	GGAGAGCATCTTGTCGAAGC		
	GyrB2540R	GCCGTGARCAAARTCRAA		
pgi				
	PgiF	ATATCCGCACCCAGGTAATG	Griffin et al., 2013	651
	PgiR	TGTCAGCAGCTGTTCCAGAT		
phoU				
	PhoF	ATATCCGCACCCAGGTAATG	Griffin et al., 2013	588
	PhoR	TGTCAGCAGCTGTTCCAGAT		

Table 2.4 Sequences form *E. piscicida* isolates deposited in the NCBI database were retrieved and used in the MLSA. *E. anguillarum* ET080813 was used as an outgroup.

Isolate	Geographic origin	Host	GenBank Assembly No.	Reference
JF1305	Japan	*Paralichythys olivaceus*	ASM71115v1	Oguru et al., 2014
JF1307	Japan	*Paralichythys olivaceus*	ASM317517v1	Sugiura et al., 2018
JF1411	Japan	*Paralichythys olivaceus*	ASM317519v1	Sugiura et al., 2018
ET-1	South Korea	*Paralichythys olivaceus*	ASM335424v1	NA
ETW41	South Korea	Eel pond water	ASM207583v1	NA
EIB202	China	*Scophthalmus maximus*	ASM2086v1	Wang et al., 2009
FL6-60	USA	Striped bass	ASM14630v1	Griffin et al., 2014
ET883	Norway	*Anguilla anguilla*	ASM80451v1	Abayneh et al., 2013
MS-18-199	USA	Hybrid Catfish	ASM415332v1	Abdelhamed
PPD130/91	China	*Hyphessobrycon eques*	JACQ00000000	Shao et al., 2015
ACC35.1	Europe	*Scophthalmus maximus*	ASM189620v1	Buján et al., 2017
C07-087	USA	*Ictalurus punctatus*	ASM34856v1	Tekedar et al., 2013
MA97-004	USA	Tilapia	ASM307473v1	Griffin et al., 2014
ET080813	China	*Anguilla japonica*	ASM26476v2	Shao et al., 2015

Table 2.5 Set of primers used in the present study for screening of virulence-related genes. Length of the products are listed.

Target Gene	Primers and Sequences (5′-3′)	*Tm* (°C)	Length (bp)	Source
ethA	ethAF: TGCTGGCTAACCCCAACGGCATCAC	56.7[a]	1250	Wang et al., 2011
	ethAR: GATCCCGCCCCAGTAGGTGTGGTTG	69.4[a]		Wang et al., 2011
chon	chonF: ACCCGGCCTACGCTAAAGA	59.3[a]	874	Wang et al., 2011
	chonR: GGAACGGCAAACTGGAACA	57.8[a]		
sodB	sodBF: ATGTCATTCGAATTACCTGC	53.1[a]	578	Wang et al., 2011
	sodBR: TCGATGTAATAAGCGTGTTCCCA	59.6[a]		
katB	katBF: CAACGCGGACCTTGACCTTGCTCTA	66.7[a]	645	Wang et al., 2011
	katBR: AGTCAGGGAGGTTCCCAGGCTATTG	65.6[a]		
fimA	fimAF: CCGCTGTGAGTGGTCAGGCAA	64.6[a]	481	Wang et al., 2011
	fimAR: ATGGTGAACGGGCTGGTCGCGTTG	69.4[a]		
evpP	evpPF: TCCCGTCTATGCCTGGTT	56.0[a]	1459	Wang et al., 2011
eseC	eseCF: CAGTCGCAGCACGATCACCCACAGA	69.2[a]	1287	Wang et al., 2011
	eseCR: CGCGCCGTCCATTAGGCTGCCGATA	70.7[a]		
eseC-eseE	eseER: CAGCATCACATCCGTCAGGCGTCGT	69.3[a]	2296	Wang et al., 2011
mukF	mukFF: CTTAACCGCTTTACCAGCGAGTTGG	64.3[a]	795	Wang et al., 2011
	mukFR: ATACTCCTCAAACTCCAAATCGGGC	62.8[a]		
ureG	ureGF: ATGAAAAAAATTATTCGTGTCGGCA	62.8[a]	1500	Wang et al., 2011
	ureGR: TCACTTTATTTCGCTGTGTGTAAAT	57.6[a]		
tatA	tatAF: CGGTATCAGCATTTGGCAGTTGTTG	63.6[a]		Wang et al., 2011
	tatAR: TTCTTCACTTCAGGCTGCTGCTCAA	64.7[a]	228	
tatB	tatBF: TAGTGAACTGCTGCTGGTAATGGTC	62.5[a]	486	Wang et al., 2011
	tatBR: TCGTGTGAGGTGGAAGGAGAAGATG	63.7[a]		
tatC	tatCF: GTATCCTGGTGGTATTCCTGGC	59.8[a]	547	Wang et al., 2011
	tatCR: GTCAGCAGCATGCCAACAACGAAG	65.8[a]		
tatD	tatDF: AGCGCGTCAGGCGGGATTAAATG	65.6[a]	696	Wang et al., 2011
	tatDR: TGGCGGGCATTGTTTTCAGTCAC	64.3[a]		
tatE	tatEF: TTACCAAGCTTCTGGTCATTGC	58.8[a]	183	Wang et al., 2011
	tatER: ATTCTTTATTCTCTACCCGGGGCGC	64.4[a]		
cds1	chond1: TCTCCACCCATAATGCCACG	62.4[a]	435	Castro et al., 2016
	chond2: CAAACGGCGTCGTGTAGTCG	64.5[a]		
edwI	Q1-1: ATCCGCAGCATCGAATGGCT	62.4[b]	360	Castro et al., 2016
	Q1-2: GAAGGATAACGATGTGGTGT	58.4[b]		
luxS	Q2-1: CTCTGGGATGCTCCGCTGAT	64.5[b]	310	Castro et al., 2016
	Q2-2: ATCACCGTATTCGATCTGCG	60.4[b]		
qseC	Q3-1: CAGCAGTAGCAGGATCACCA	62.4[b]	260	Castro et al., 2016
	Q3-2: ATGGACGTATGCTGCTCAAC	60.4[b]		
pvsA	pvsA1: CTGGAGCAGTACCTCGACGG	66.6[b]	313	Castro et al., 2016
	pvsA2: CGATGCTGCGGTAGTTGATC	62.4[b]		
pvsB	pvsB1: GATGTTCATCACCATCACCC	60.4[b]	217	Castro et al., 2016
	pvsB2: GCTTTGCAGCAGGTATTTCA	58.4[b]		
pvsD	cds3-1: GCTCAATGAAGACTTTCGTC	58.4[b]	747	Castro et al., 2016
	cds3-2: GTCCGCAGGTTGTTTTTGCT	60.4[b]		
pvuA	recept1: AGCGTCATCAGCAACCAGCA	62.4[b]	420	Castro et al., 2016
	recept2: GCTGCTGATATAGGTGTCGG	62.4[b]		
VgrG-1	VgrG1F: CTCTATCCGGGCCTGCATG	64.5[b]	600	This study
	VgrG1R: GCCTATTATGCCGAGCTGGT	62.4[b]		
VgrG-2	VgrG2F: GCGATTGCGTTCCATGAAGG	62.4[b]	800	This study
	VgrG2R: TCTGTTGCCCCTGTGATGAC	62.4[b]		
cse1	cse1F: CCGTTTGATGGGGGCAAAAC	60.3[b]	650	This study
	cse1R: GTGCCGCTTCTGCTTATTGG	59.9[b]		
cse2	cse2F: CCTTTTCCTGCCTGCCGTAA	60.6[b]	501	This study
	cse2R: AACCGCAAGCCTCACAGC	61[b]		
PefC	PefCF: CAAGCGCTCATTCAACGACC	62.4[b]	500	This study
	PefCR: CCTGGAGTAAGCAGCTGGAC	64.5[b]		
Eta1	RT-F1: CAGGAAAGTGATTGGTGGC	56.5[b]	160	Sun et al., 2012
	RT-R1: TCCAATACTCCTTCTCGGTGC	59.5[b]		
Intimin/Invasin	IntF: TCCACAGCGGACCTACTGTA	60[b]	650	This study
	IntR: GAATCGACCCGTTACAGGCA	60.1[b]		
Invasin	InvAF: AACACCACCTCTCGGTTAGC	59.7[b]	700	This study
	InvAR: CAGCCGGGGTCAACTATACG	60.2[b]		

Table 2.6 Representative *E. piscicida* isolates used in infectivity trial. Estimated *E. piscicida* doses (Colony Forming Units [CFU] per gram of fish) administered to hybrid and channel catfish. Two separate challenges were performed using representative isolates of each of the MLSA groups. Isolate S11-534 was used twice due to it was the only representative isolate of the respective group.

Isolate	MLSA Clade	Host	Estimated Dose (CFU/g)
Challenge 1			
LADL99-462	MLSA 1	Channel catfish	1.23×10^4
S16-51	MLSA 2	Channel catfish	1.63×10^4
S11-233	MLSA 3	Channel catfish	1.43×10^4
S08-209	MLSA 4	Channel catfish	1.71×10^4
S11-534	MLSA 5	Hybrid catfish	1.09×10^4
Challenge 2			
S11-285	MLSA 1	Channel catfish	9.78×10^3
S17-335	MLSA 2	Hybrid catfish	7.93×10^3
S13-636	MLSA 3	Channel catfish	8.49×10^3
LADL97-168	MLSA 4	Channel catfish	1.04×10^4
S11-534	MLSA 5	Hybrid catfish	7.45×10^3

Table 2.7 Comparative results of genetic clusters obtained by the different genotyping methods multilocus sequence typing (MLSA), and rep-PCR using different primers ($[GTG]_5$, BOX, and ERIC I&II).

Isolate	MLSA	$(GTG)_5$	BOX	ERIC I&II
S07-1019	1	2	5	5
S12-281	1	2	5	6
S10-512	1	2	5	6
S15-83	1	2	1	5
S12-408	1	2	4	6
S14-264	1	2	5	6
S12-420	1	2	5	6
S16-36	1	2	5	5
S10-67	1	2	5	6
S11-285	1	2	5	5
S11-509	1	2	5	6
S15-225	1	2	5	6
S07-534	1	2	3	5
S16-730	1	2	5	6
LADL 99-462	1	3	5	5
S07-348	2	4	3	3
S15-341	2	4	3	2
S14-431	2	4	5	2
S16-119	2	4	4	3
S15-96	2	4	2	2
S16-278	2	4	3	3
S17-410	2	4	3	3
S17-541	2	4	2	2
S07-262	2	4	2	3
S16-51	2	4	3	3
MA 97-004	2	4	3	3
S17-335	2	4	2	4
S13-370	2	3	4	5
S16-567	2	4	3	4
S11-233	3	3	2	4
S13-636	3	3	2	4
S15-197	4	3	4	1
S17-540	4	1	4	4
S16-465	4	1	1	4
S07-275	4	1	2	4
S08-209	4	1	4	4
S07-346	4	3	2	1
LADL 97-168	4	1	3	4
S11-534	5	4	4	4

Table 2.8 Results of molecular screening of virulence-related genes in *E. piscicida* isolates. Each MLSA is represented by total isolates used in this study.

Gene	MLSA 1	MLSA 2	MLSA 3	MLSA 4	MLSA 5
ethA	15/15	14/14	2/2	7/7	1/1
chon	15/15	14/14	2/2	7/7	1/1
katB	15/15	14/14	2/2	7/7	1/1
fimA	15/15	14/14	2/2	7/7	1/1
evpP	15/15	14/14	2/2	7/7	1/1
eseC	15/15	14/14	2/2	7/7	1/1
eseE	15/15	14/14	2/2	7/7	1/1
mukF	15/15	14/14	2/2	7/7	1/1
ureG	0/15	0/14	0/2	0/7	0/1
tatA	15/15	14/14	2/2	7/7	1/1
tatB	15/15	14/14	2/2	7/7	1/1
tatC	15/15	14/14	2/2	7/7	1/1
tatD	15/15	14/14	2/2	7/7	1/1
tatE	15/15	14/14	2/2	7/7	1/1
Q1	15/15	14/14	2/2	7/7	1/1
Q2	15/15	14/14	2/2	7/7	1/1
Q3	15/15	14/14	2/2	7/7	1/1
pvsA	15/15	14/14	2/2	7/7	1/1
recept	15/15	14/14	2/2	7/7	1/1
VgrG1	15/15	14/14	0/2	0/7	0/1
VgrG2	15/15	14/14	0/2	0/7	0/1
cse1	15/15	0/14	0/2	0/7	1/1
cse2	15/15	0/14	0/2	0/7	1/1
PefC	15/15	13/14	0/2	0/7	1/1
Eta1	0/15	12/14	0/2	0/7	0/1
Intimin/Invasin	0/15	0/14	2/2	7/7	0/1
Invasin	0/15	0/14	2/2	7/7	0/1

Table 2.9 Antimicrobial susceptibility patterns of *Edwardsiella piscicida* isolates identified by the disk diffusion assay. Breakpoints of the disk diffusion assay used to determine antimicrobial susceptibility of *E. piscicida* isolates are described.

Level of Susceptibility	Antimicrobial	MLSA 1	MLSA 2	MLSA 3	MLSA 4	MLSA 5
Susceptible ≥ 20 mm						
	Aquaflor®	15/15	14/14	2/2	7/7	1/1
	Romet®	14/15	13/14	2/2	7/7	1/1
	Terramycin®	15/15	12/14	2/2	7/7	1/1
Intermediate 11-19 mm						
	Aquaflor®	0/15	0/14	0/2	0/7	0/1
	Romet®	0/15	1/14	0/2	0/7	0/1
	Terramycin®	0/15	0/14	0/2	0/7	0/1
Resistant ≤ 20						
	Aquaflor®	0/15	0/14	0/2	0/7	0/1
	Romet®	1/15	0/14	0/2	0/7	0/1
	Terramycin®	0/15	2/14	0/2	0/7	0/1

Table 2.10 Minimal inhibitory concentrations (MICs) of single antimicrobials in *Edwardsiella piscicida* isolates analyzed in the present study.

		No. of strains with MIC (mg/liter):									
Antibiotic (range, mg/liter)	Taxon	≤0.5	1	2	4	8	16	32	64	128	≥256
Enrofloxacin (0.12–2)	All strains	39									
Gentamicin (0.5–8)	All strains	39									
Ceftiofur (0.25–4)	All strains	39									
Neomycin (2–32)	All strains		39								
Erythromycin (0.12–4)	All strains						39				
Oxytetracycline (0.25–8)	MLSA 1	1	11	3			1				
	MLSA 2	3	9				1				
	MLSA 3	1	1								
	MLSA 4		6	1							
	MLSA 5		1								
Tetracycline (0.25–8)	MLSA 1	2	11	2			1				
	MLSA 2	4	7	1			1				
	MLSA 3	1	1								
	MLSA 4		6	1							
	MLSA 5		1								
Amoxicillin (0.25–16)	MLSA 1		3	12	1						
	MLSA 2		7	5	1						
	MLSA 3			2							
	MLSA 4		3	4							
	MLSA 5	1									
Spectinomycin (8–64)	All strains					39					
Sulfadimethoxine (32–256)	All strains										39
Florfenicol (1–8)	All strains		39								
Sulfathiazole (32–256)	All strains										39
Penicillin (0.06–8)	MLSA 1					12	2				
	MLSA 2				1	11	1				
	MLSA 3					1	1				
	MLSA 4				2	4	1				
	MLSA 5			1							
Streptomycin (8–1,024)	All strains	39									
Novobiocin (0.5–4)	All strains					39					
Tylosin tartrate (2.5–20)	All strains							39			
Enrofloxacin (0.12–2)	All strains	39									
Clindamycin (0.5–4)	All strains					39					

2.6 Figures

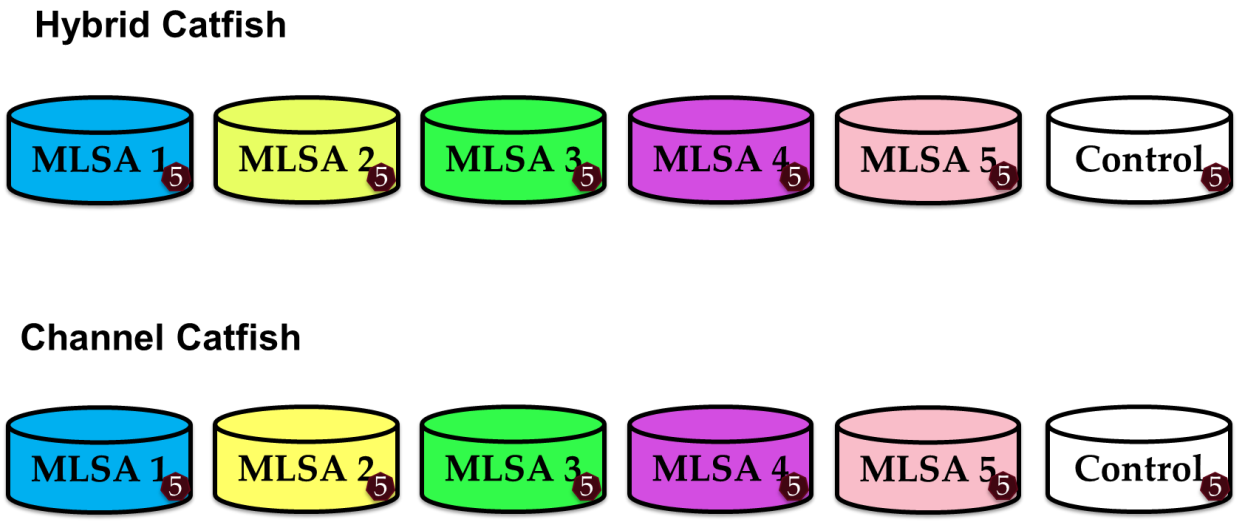

Figure 2.1 Schematic for infectivity trial. Hybrid and channel catfish were challenged by respective *E. piscicida* variants. Control groups were exposed to sterile BHI broth. Each treatment consisted of 5 replicates stocked with 20 fish/aquaria for each *E. piscicida* MLSA clade. Mortality was recorded twice daily for fifteen days post-challenge.

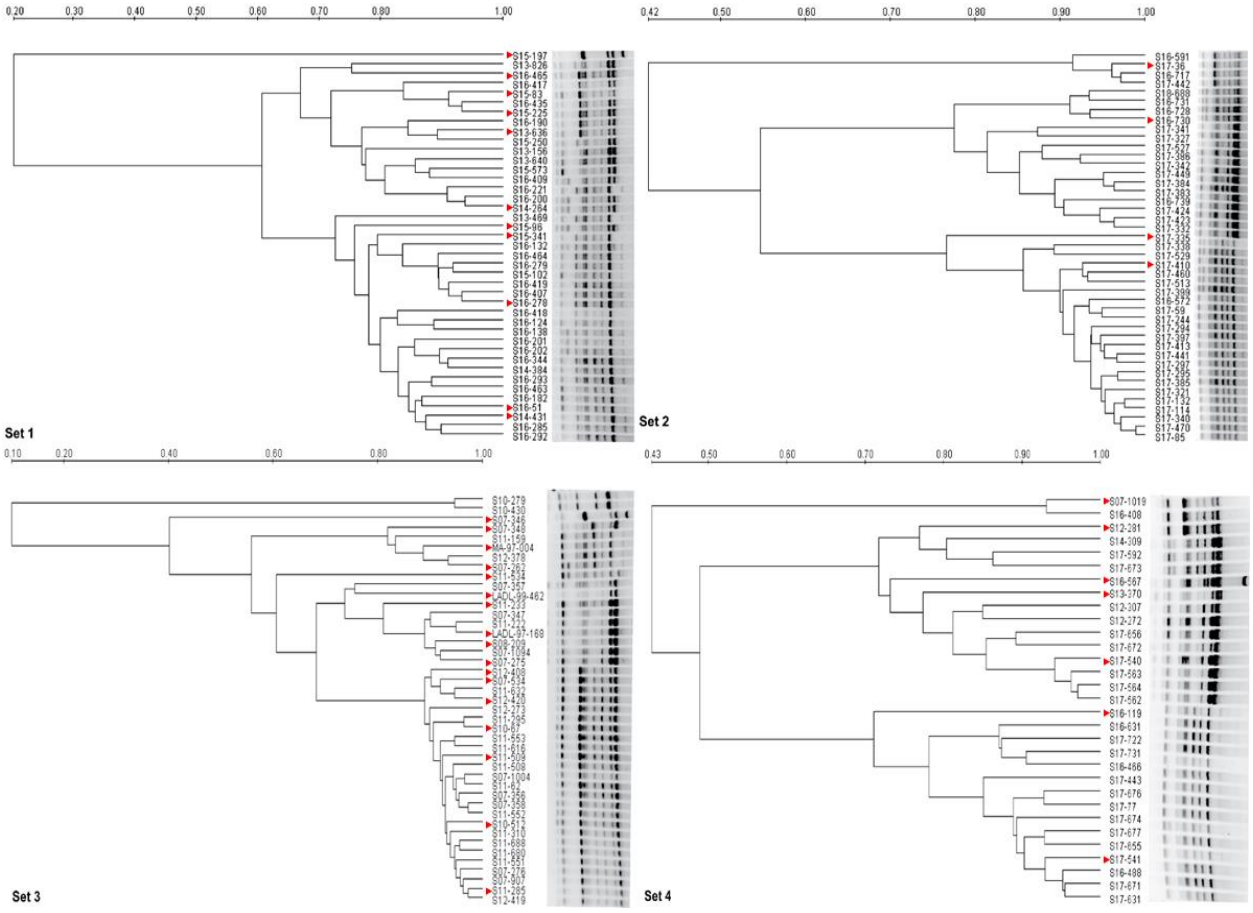

Figure 2.2 Genetic fingerprints generated by rep-PCR. A total of 158 *E. piscicida* isolates were initially screened ERIC I & II rep-PCR. Dendrograms were generated from Dice coefficient similarity matrices based on the unweighted pair group method with arithmetic mean (UPGMA). Representative isolates (red triangle) were chosen for further analysis.

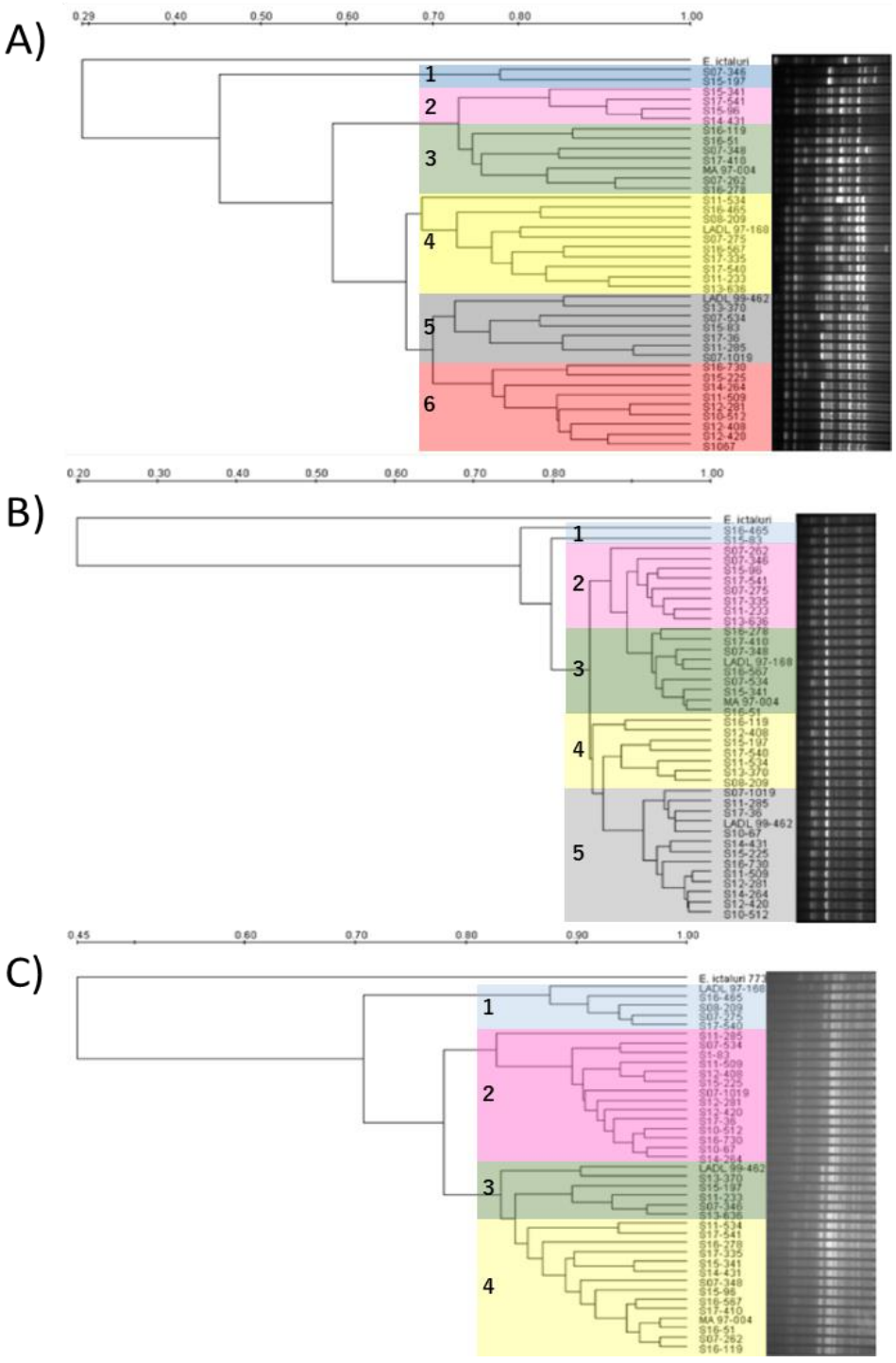

Figure 2.3 Genetic fingerprints generated by rep-PCR. Dendrograms were generated from Dice coefficient similarity matrices based on the unweighted pair group method with arithmetic mean (UPGMA). Rep-PCR profiles were generated using the (A) ERIC I and II, (B) BOX, and (C) $(GTG)_5$ primer sets. *E. ictaluri* S93-773 isolate was used as outgroup. Color coded represent main clusters identified for each rep-PCR.

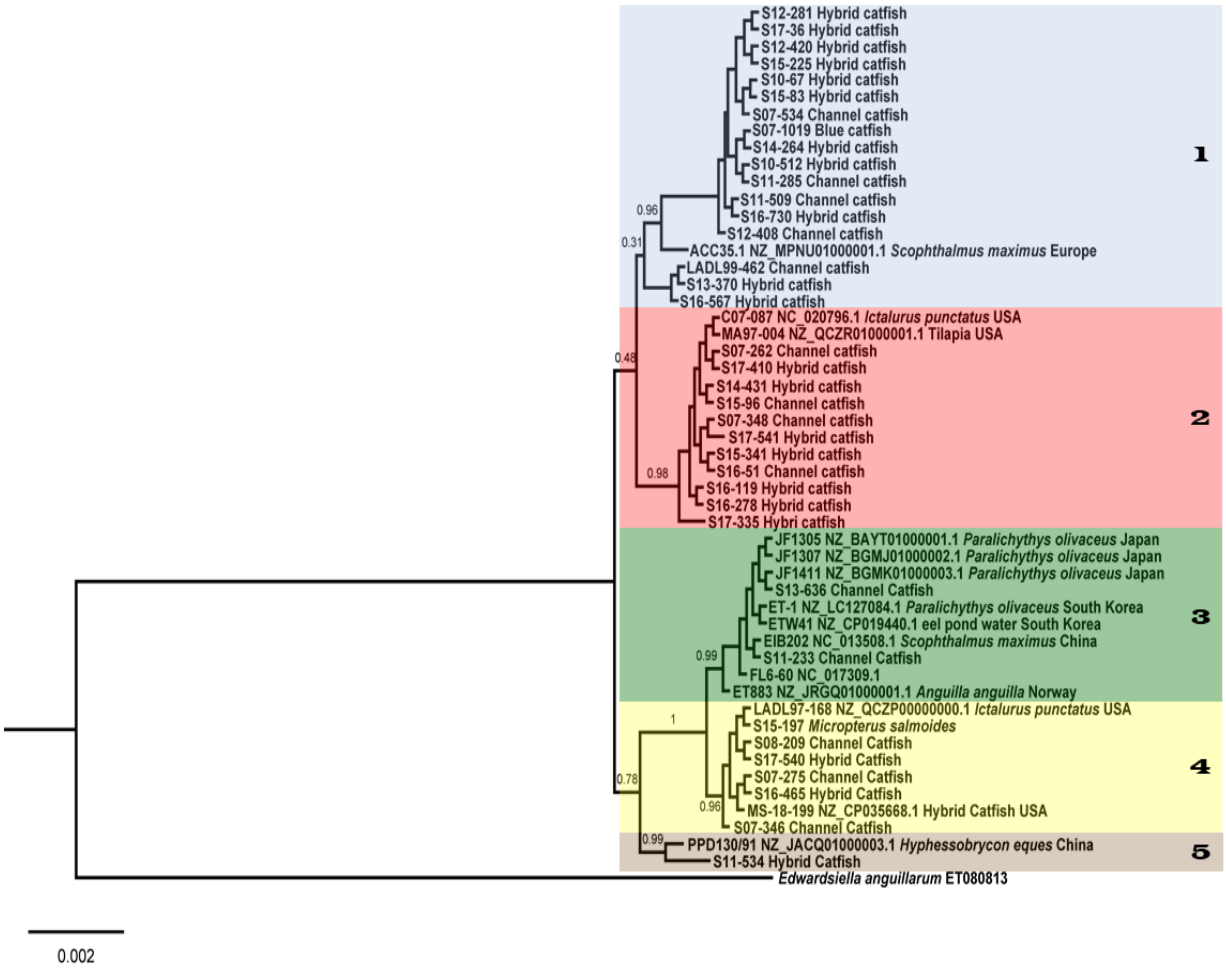

Figure 2.4 Phylogenetic relationships of 39 *E. piscicida* isolates recovered from channel catfish, hybrid catfish and other freshwater fish species. Phylogenetic tree was constructed based on Bayesian inference using concatenated alignment of *gyrB*, *pgi*, and *phoU* gene sequences and rooted at *E. anguillarum* ET080813. Sequences retrieved from the NCBI database were included in the MLSA. NCBI sequences references, host, and location are presented.

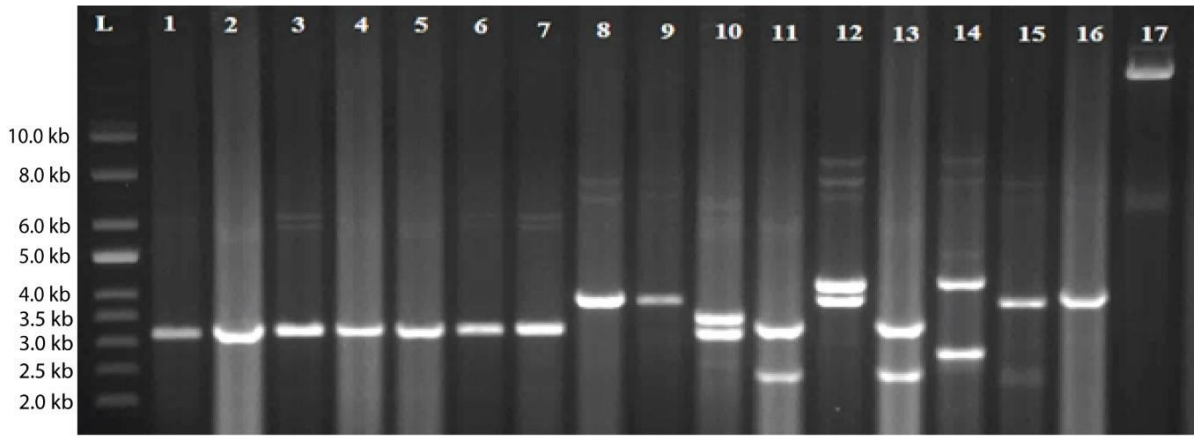

Figure 2.5 Native plasmid profiles from 17 plasmid-carrying *E. piscicida* isolates. Lane designations are as follows: L = Supercoiled DNA ladder; lanes 1=S11-285; 2 = S11-509; 3 = S10-512; 4 = S10-67; 5 = S12-420; 6 = S17-335; 7 = S15-341; 8 = S12-281; 9 = LADL 99-462; 10 = S16-278; 11 = S16-51; 12 = S14-431; 13 = S15-96; 14 = S08-209; 15 = S07-1019; 16 = LADL 97-168; 17 = S07-348.

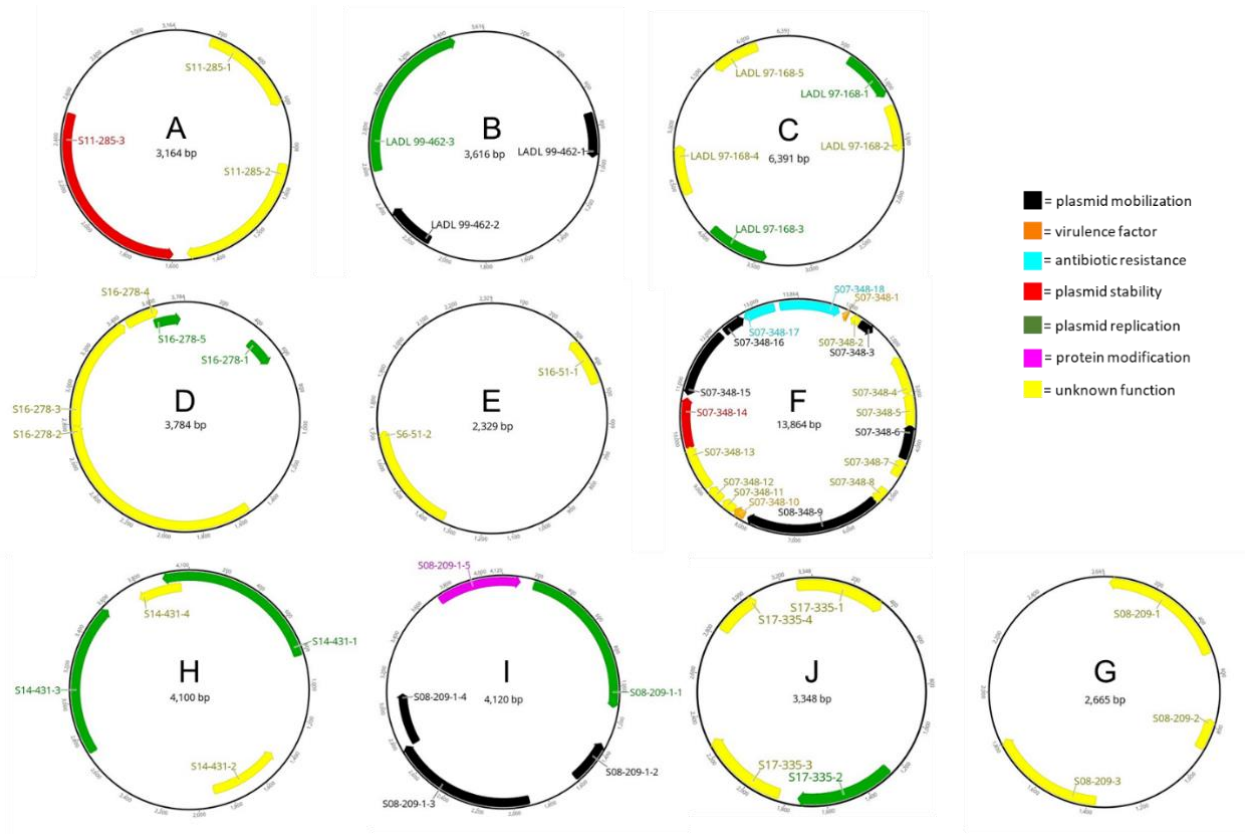

Figure 2.6 Physical maps of 10 unique native plasmids harvested and sequenced from *E. piscicida* isolates. Isolates S11-285, S11-509, S10-512, S10-67, S12-420, S16-51, S15-96, S12-281, and S07-1019 (A); LADL99-462 (B); LADL97-168 (C); S15-341, S16-278, and S14-431 (D); S16-51 and S15-96 (E); S07-348 (F); S09-208 (G); S14-431 (H); S08-209 (I); S17-335 (J). Maps indicate locations of predicted open reading frames (ORFs), which are color coded according to predicted function.

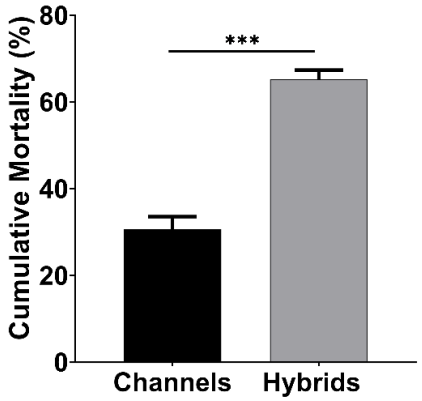

Figure 2.7 Pooled cumulative mortality by fish genotype. Mortality in hybrid catfish was significantly higher than channel catfish ($p < 0.001$).

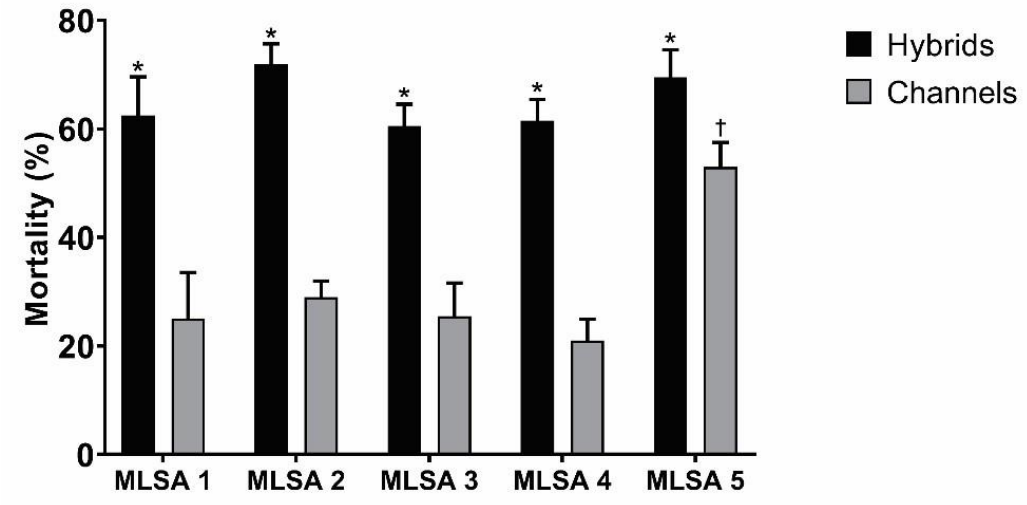

Figure 2.8 Cumulative mortality of channel and hybrid catfish exposed to different *E. piscicida* MLSA groups. Within MLSA groups, (*) indicates significant differences ($p < 0.05$) between channel and hybrid catfish. Within channel and hybrid catfish, (†) indicates significant differences between MLSA groups ($p < 0.05$). Interaction fish genotype × *E. piscicida* MLSA clades was not significant ($p > 0.1$).

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CHAPTER III

EVALUATION OF CROSS-PROTECTIVE EFFICACY OF A LIVE-ATTENUATED *EDWARDSIELLA ICTALURI* VACCINE AGAINST HETEROLOGOUS *EDWARDSIELLA PISCICIDA* ISOLATES IN CHANNEL AND CHANNEL × BLUE HYBRID CATFISH

3.1 Introduction

Aquaculture is the fastest growing food production industry and one of the most important sources of protein for human consumption worldwide, contributing significantly to the growth and stabilization of the economies (FAO, 2016). Based on World Bank (2013) and Food and Agriculture Organization of the United Nations (FAO) (2016) it is estimated aquaculture industry growth will continue globally over the next decade. However, despite this promising future, infectious diseases remain a significant factor threatening the sustainability of global aquaculture. The nature of the aquatic environment, where aquacultured animals and their pathogens (obligate and opportunistic) are in constant interaction, host and intensive production conditions play critical roles in the emergence and establishment of disease.

Outbreaks of infectious diseases in aquaculture species can result in mortality rates up to 100% and pose major threats to the profitability and sustainability of the industry. Another important aspect to consider and highlighted by international organizations like FAO, World Organization for Animal Health (OIE), and the World Health Organization (WHO), is the

excessive use of antimicrobials in animal production systems. In aquaculture, antimicrobials are widely used to combat bacterial diseases resulting in the emergence of antibiotic resistant strains in some industries (Dung et al., 2009). These selective pressures have created reservoirs of drug-resistant bacteria and trafficking of resistance genes in bacterial fish pathogens in the aquatic environment (Heuer et al., 2009). This has led to new genetic variants or pathotypes among common pathogens that display different behavior in terms of virulence and resistance to antimicrobials commonly used in aquaculture (WHO, 2006; Cabello et al., 2013; FAO, 2016).

Edwardsiella spp. are a group of gram-negative enteric pathogens of the family Hafniaceae responsible for significant bacterial diseases negatively impacting aquaculture on a global scale (Griffin et al., 2017). While mostly known for the diseases they cause in wild and cultured fishes across a range of temperatures, salinities and environments, they have also been reported from reptiles, amphibians, birds and mammals, including humans (Mohanty and Sahoo, 2007; Adeolu et al., 2016; Griffin et al., 2017). The genus is comprised of five species, *E. tarda*, *E. hoshinae*, *E. ictaluri*, *E. piscicida*, and *E. anguillarum*.

Edwardsiella piscicida (formerly many *E. tarda* cases in fish) is a rod-shaped, facultative anaerobic bacterium (Abayneh et al., 2013) and the etiological agent of edwardsiellosis in fish and considered an emerging disease issue in global aquaculture (Griffin et al., 2014; Buján et al., 2018). There are reports of *E. piscicida* causing disease in more than 28 fish species around the world (Griffin et al., 2020a) and is presently considered a significant bacterial pest in channel (*Ictalurus punctatus*) × blue (*Ictalurus furcatus*) hybrid catfish production systems in the U.S. catfish aquaculture (Griffin et al., 2019).

Similarly, *Edwardsiella ictaluri* is one of the most important pathogens in the U.S. farm-raised catfish industry and the causative agent of enteric septicemia of catfish (ESC). Losses

attributed to ESC are estimated to exceed $50 million annually due to lost feed days, treatment expenditures and direct losses due to high mortality (Russo et al., 2009). Over the past decade, the U.S. catfish industry has transitioned from producing almost exclusively channel catfish to also producing channel × blue hybrids. Hybrids have shown improved performance in different production traits compared to channel catfish (Dunham and Elaswad, 2018). Correlating with increased hybrid production has been an increase in the number of *E. piscicida* diagnoses at the Aquatic Research and Diagnostic Laboratory (ARDL) at the Thad Cochran National Warmwater Aquaculture Center (NWAC) in Stoneville, MS. Hybrids made up ~40% of total diagnostic submissions to the ARDL, yet accounted for >90% of *E. piscicida* diagnoses, which corroborates research data that indicates increased virulence of *E. piscicida* in hybrid catfish (Reichley et al., 2018). The emergence of *E. piscicida* in hybrid catfish is worrisome given current industry trends towards increased hybrid production. Further, since *E. piscicida* affects mostly market-sized fish, the consequent economic losses are of greater concern given significant foregone producer investment (Griffin et al., 2019; Khoo et al., 2017, 2018; Kumar et al., 2019a).

In the previous study (chapter II), a Multilocus Sequence Analysis (MLSA) method was used to genotype *E. piscicida* isolates recovered from hybrid and channel catfish farmed in Mississippi. In this work, at least five discrete *E. piscicida* MLSA groups were identified, revealing important differences between MLSA-groups in the presence of virulence-related factors.

Several studies have shown the benefits of vaccine candidates to prevent disease by inducing long term immune-protection against not only the target pathogens, but also cross-protective immunity against closely related agents with commonly shared antigens (Salonius et al., 2005; Ma et al., 2019). In addition to improved production efficiency through disease prevention, another benefit of vaccines is decreased reliance on antimicrobials. Efficacious

vaccines against bacterial pathogens serve a pivotal role in mitigating dependence on antimicrobials in animal agriculture, particularly in aquaculture, which has significant impact in ecological systems and human health (WHO, 2006; Morrison and Saksida, 2013; Hoelzer et al., 2018).

Recently, NWAC researchers developed a live-attenuated *E. ictaluri* vaccine that offers exceptional protection against ESC in channel and hybrid catfish under experimental conditions and in commercial field trials (Wise et al., 2015; Peterson et al., 2016; Greenway et al., 2017; Chatakondi et al., 2018; Kumar et al., 2019b; Wise et al., 2020; Aarattuthodiyil et al., 2020). Further experimental evidence indicates that the live attenuated *E. ictaluri* vaccine also provides protection in channel and hybrid catfish against at least one *E. piscicida* isolate (Griffin et al., 2020b). The aim of the current study was to build upon the work of Griffin et al. (2020b) and evaluate the efficacy of a live-attenuated *Edwardsiella ictaluri* to protect channel and hybrid catfish against heterologous *E. piscicida* genetic variants.

3.2 Materials and Methods

3.2.1 Experimental Trial 1: Evaluation of cross-protection of a live-attenuated *Edwardsiella ictaluri* vaccine against heterologous *Edwardsiella piscicida*

3.2.1.1 *Edwardsiella* spp.

Five *Edwardsiella piscicida* isolates, described previously (Chapter II), were chosen for analysis representing unique MLSA lineages, in addition to a wild-type *E. ictaluri* isolate. Isolates were obtained from the archival collection of Dr. Matt Griffin at NWAC and are listed in Table 3.1.

3.2.1.2 Fish

Hybrid (~9.8 g/fish) and channel catfish (~12.1 g/fish) were reared indoors for disease research at NWAC. Fish were transferred from rearing tanks to 80-L aquaria (20 fish/aquaria) containing 22 L of aerated well water exchanged at a rate of 1 L/min and acclimated for 2 weeks prior to vaccination. Each group of channel and hybrid catfish were subdivided into vaccinated and non-vaccinated subgroups, resulting in seven treatments (including unexposed controls), with three replicates per treatment (Figure 3.1). Fish were maintained at 25ºC throughout testing.

3.2.1.3 Vaccination

A live-attenuated *E. ictaluri* vaccine developed by Wise et al. (2015) was used in this study to vaccinate channel and hybrid catfish. A cryopreserved vaccine serial (V19-BHP 050219), produced from a master seed stock following previously established protocols (Greenway et al., 2017), was thawed at room temperature. A total of 3 mL was taken from the thawed 50 mL vaccine serial and expanded in 6 L brain heart infusion broth (BHIb) at 28°C in a shaker incubator at 225 rpm for 18 h. Viable bacteria were determined by standard plate counts using the drop-plate method (Herigstad et al., 2001). Plate counts were performed on Mueller-Hinton II agar plates supplemented with 5% sheep blood incubation at 28°C for 48 h (Herigstad et al., 2001). Fish were deprived of feed for at least 24 h pre-vaccination. Vaccine was administered by stopping the flow of water to each aquarium of the designated vaccinated treatments and adding 100 mL of the cultured vaccine to the water, targeting a dose of ~1.58×10^7 CFU/mL. After 1 h, water flow was resumed. Non-vaccinated fish were sham vaccinated by exposing fish to 100 mL of sterile BHIb for 1 h under same static conditions. Following vaccination fish were fed once daily to satiation.

3.2.1.4 Initial passage

To account for differences in length of cryogenic storage for each isolate, an *E. piscicida* isolate representing each of the five different phyletic groups, in addition to *E. ictaluri* isolate S97-773, were revived from cryopreservation on Mueller-Hinton II agar plates supplemented with 5% defibrinated sheep blood (MHBA) and incubated for 24 h (*E. piscicida*) or 48 h (*E. ictaluri*) at 28°C. For each isolate, individual colonies were expanded in 9 mL of BHIb at 200 rpm for 24 h at 28°C. Each isolate was then passed through channel catfish fingerlings (~10.6 g/fish; 5 fish per isolate). Fish were injected intracelomically with 0.1 mL of diluted (1:2000) broth culture, targeting doses of ~1×10^4 CFU/fish. After 48 h, fish were euthanized by an overdose of tricaine methanesulfonate (MS-222), and isolates were aseptically recovered from kidneys, cultured on Mueller–Hinton II agar plates supplemented with 5% defibrinated sheep blood and incubated for 24 h at 28°C and individual colonies were expanded as above and stored at -80°C (15% glycerol) until immunization trials.

3.2.1.5 Infectivity Trial

Passed isolates were revived from cryostorage by isolation streaking on MHBA and incubated for 24 h at 28°C. Individual colonies of each isolate were expanded overnight in 9 mL of BHIb as previously described. Bacterial cultures were diluted (1:2000) to achieve target doses of approximately 1.5×10^4 CFU/g (Table 3.2) and delivered doses were determined by standard plate counts as above. Fish were deprived of feed 24 h for challenge. After 30-days post-vaccination, fish from all six *Edwardsiella* spp. treatments, both vaccinated and non-vaccinated fish, were anesthetized with MS-222 (100 mg/mL bath) and injected intracelomically with 0.1 mL of dilute broth culture of each isolate in line with previous studies (Reichley et al., 2015; Reichley et al., 2017). Control groups were handled similarly but received a sham injection of 0.1 mL of

sterile BHIb. Fish were monitored twice daily for 14 days, and mortality was recorded. The posterior kidney from dead fish was cultured aseptically on Mueller–Hinton II agar plates supplemented with 5% defibrinated sheep blood to confirm the presence of *Edwardsiella* spp.

3.2.2 Experimental Trial 2: Evaluation of Cross-protection of *Edwardsiella piscicida* against Challenge with *Edwardsiella ictaluri* Wild-Type

3.2.2.1 Fish

Hybrid catfish (~10.8 g/fish) and channel catfish (~10.2 g/fish) were transferred from rearing tanks to 80-L aquaria (20 fish/aquaria) containing 22 L of aerated well water exchanged at a rate of 1 L/min. Fish were acclimated for 2 weeks prior to immunization with *E. piscicida* isolates. Channel catfish and hybrid catfish groups were divided into five treatments (1 treatment/3 replicates; 1 replicate/20 fish) corresponding to five *E. piscicida* isolates representing each MLSA groups (Table 3.1); negative (10 fish/replicate; 3 replicates) and positive (ESC) (10 fish/replicate; 3 replicates) control groups for both channel and hybrid catfish (Figure 3.2).

3.2.2.2 Immunization with Edwardsiella piscicida

Representative *E. piscicida* isolates from each MLSA group used in Experiment 1 were revived from cryopreservation on MHBA plates and incubated for 24 h at 28°C. Individual colonies of each isolate were expanded in 9 mL of BHIb for 24 h at 28°C with shaking (200 rpm). Following overnight incubation, 1 mL of each culture was used to seed 650 mL of sterile BHIb and expanded 18 h at 28°C with shaking (200 rpm). Fish were deprived of feed 24 h pre-immunization. Delivered doses were determined by plate counts using the drop-plate method. Immunizing doses were administered to vaccinated groups (3 aquaria/isolate) by suspending water flow and adding 200 mL of undiluted broth culture to each respective aquarium. After 1 h, water flow was resumed. Non-immunized fish received 200 mL of sterile BHIb. For a 30-days period

fish were kept at a constant temperature of 25°C and fed once daily to satiation. Fish were checked twice daily for mortality and if present, dead fish were recorded and removed.

3.2.2.3 Infectivity Trial

After thirty-days post-exposure to *E. piscicida* isolates by immersion, fish from all five MLSA and positive control (ESC) treatments were exposed to wild-type *Edwardsiella ictaluri* S97-773 isolate. *Edwardsiella ictaluri* S97-773 was revived from cryopreservation on MHBA and incubated for 48 hours at 28°C. An individual colony was expanded in 9 mL of BHIb at 200 rpm for 24 h at 28°C. Following overnight incubation, two separate Erlenmeyer flasks containing each one 2000 mL of sterile BHIb were inoculated separately with 1 mL of the starter culture and growth at 225 rpm for 18 h at 28°C. After this period, expanded cultures from these flasks were combined and total bacterial counts were performed from this mixture. Fish were deprived of feed 24 h pre-challenge. Challenges were conducted by stopping the water flow (60 min static exposure) and adding 100 mL of the virulent culture to each aquaria to deliver a final dose of ~5.98×10^6 CFU/mL Viable bacteria in the expanded culture was determined as described previously and used to estimate exposure dose. The negative and positive control replicates received 100 mL of sterile BHIb. Fish were monitored twice daily for 15 days for morbidity and mortality.

3.2.3 Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8 (Graphpad Software, Inc). Survival curves for each treatment from hybrid and channel catfish groups were analyzed by Kaplan-Meier survival estimations and group differences were analyzed by the log-rank test. For all statistical analyses, p-values < 0.05 were considered significant. Vaccine efficacy reported as

relative percent survival (RPS) (Equation 2.1), was determined for all the treatments, as they had statistically significant lower mortalities than the non-vaccinated groups (Amend, 1981).

$$\text{RPS} = 1 - \left[\frac{\%\text{mortality of vaccinated treatment}}{\%\text{mortality of non-vaccinated treatment}}\right] \times 100 \quad (2.1)$$

3.3 Results

3.3.1 Experimental Trial 1

All vaccinated groups received an immunizing dose of ~1.58×10^7 CFU/mL of *E. ictaluri* 340X2 serial V19-BHP 050219. Challenge doses for each heterologous *E. piscicida* are presented in Table 3.2. No mortality was observed in non-vaccinated groups during the pre-challenge period, but mortality was recorded in vaccinated groups, in hybrids ranged from 11.7% to 25% and in channels from 3.3% to 15%. The live-attenuated *E. ictaluri* vaccine induced significant protection against all virulent *E. piscicida* genetic variants in both hybrids and channels that survived vaccination (Figure 3.3). Relative percent survival ranged from 54.7% to 77.8% in vaccinated hybrids and 80.5% to 100% in vaccinated channels (Table 3.2). RPS of pooled vaccinated treatments was 64.3% in hybrids and 94.3% in channels (Figure 3.4). Pairwise comparisons of survival curves between vaccinated and non-vaccinated groups were significant ($p < 0.05$) for all treatment groups.

3.3.2 Experimental Trial 2

No mortality was observed in the thirty-day post-immunization period following *E. piscicida* exposure. Delivered doses for each isolate are presented in Table 3.3. Immersion exposure for all *E. piscicida* heterologous isolates conferred a low level of cross-protective

immunity against *E. ictaluri* S93-773 wild-type infection (Figure 3.5). Following challenge there was 100% mortality in non-immunized channel and hybrid catfish. All fish immunized by bath immersion with heterologous *E. piscicida* had significantly improved survival compared to non-immunized controls (Table 3.3). Pooled survival in hybrids previously exposed to *E. piscicida* was 34.7% compared to 0% for naïve fish. Similarly, pooled survival in channel catfish immunized by bath immersion with heterologous *E. piscicida* isolates prior to exposure to *E. ictaluri* was 26.7% compared to 0% in naïve controls (Figure 3.6).

3.4 Discussion

Given the nature of intensive production settings in aquaculture, wherein fish are in constant interaction with the aquatic environment and exposed to a wide range of potential pathogens, the risk of infectious disease is high. This risk increases when environmental or production related factors (e.g. increased temperatures, high stocking densities, poor water quality, etc.) favor pathogens or induce a state of stress and immunosuppression in the host (Pulkkinen et al., 2010). Mucosal sites of the skin, gills or digestive tract are typically the first sites where host and pathogens interact, and tissue tropism usually plays an important role in attachment, colonization, and the invasion processes. Therefore, the mucosal immunity generated and induced at these sites is extremely important in order to neutralize these stages of the disease process. In this regard, studies have described the potential and capacity of vaccines to be used to promote a competent immune response at the mucosal level via immersive, oral, or injectable delivery (Plant and La Patra, 2011; Salinas, 2015; Soto et al., 2015).

Several studies have investigated the efficacy of bacterial vaccines to confer cross-protective immunity against other bacterial pathogens closely related to the initial target (Lillehaug et al., 1990; Hoel et al., 1998). Vaccines with the ability to elicit cross-protective immune

responses are highly desirable in aquaculture as a primary prophylactic measure. In addition to reducing economic losses, efficacious vaccines also mitigate antibiotic use, meliorating selective pressures that result in environmental reservoirs of antibiotic resistance (Midtlyng et al., 2011).

Previous work has indicated the live-attenuated *Edwardsiella ictaluri* vaccine developed by Wise et al. (2015) also protects channel catfish against *E. piscicida* isolate S11-285 (Griffin et al., 2020b), although the efficacy against heterologous *E. piscicida* isolates was unknown. Given the considerable genetic heterogeneity amongst *E. piscicida* isolates described in previous work (Chapter II), a study investigating the cross-protective potential of a live-attenuated *E. ictaluri* vaccine against heterologous *E. piscicida* variants was prudent.

This current work indicates the live-attenuated *E. ictaluri* vaccine induces a cross-protective immune response in both hybrid and channel catfish against all tested *E. piscicida* genetic variants recovered from Mississippi catfish aquaculture. Relative percent survival for hybrid catfish fingerlings ranged from 54.7-77.8% compared to 80.5-100% in channels. Overall, the vaccine afforded less protection against *E. piscicida* than *E. ictaluri*, and this protective effect was more pronounced in channels compared to hybrid catfish. This can be expected, as the vaccine is derived from an *E. ictaluri* wild-type strain. It stands to reason a vaccine would provide better protection against conspecific wild-type strains versus congeners. Despite this, the vaccine-induced protective response elucidated in hybrid and channel catfish against all heterologous *E. piscicida* genetic variants suggests the vaccine will have efficacy in the field, where exposure levels are drastically lower than the doses employed in these controlled laboratory challenges.

Within the *Edwardsiella, E. ictaluri* and *E. piscicida* are closely related species (Abayneh et al., 2013; Griffin et al., 2014, 2017; Shao et al., 2015). As a result, it is expected they may carry common antigens with sufficient epitopic conservation to induce a cross-protective immunological

memory in hybrids and channels catfish. This phenomenon has been described in similar studies investigating cross-protective immunity in fish vaccines (Poobalane et al., 2010; Wang et al., 2013). In Experiment 1, post-vaccination, pre-challenge losses were inconsistent with other studies (Wise et al., 2015; Peterson et al., 2016; Greenway et al., 2017; Chatakondi et al., 2018; Aarattuthodiyil et al., 2020; Griffin et al., 2020b). This response could be attributed to an elevated vaccine dose used to ensure an adequate immune response in hybrids, which are typically less susceptible to *E. ictaluri.* Further, the vaccine was delivered by bath immersion rather than the oral vaccination procedures defined by Wise et al. (2015), which could alter the safety and efficacy of the target immunization dose.

In spite of low-level post-vaccination mortality, there was significantly improved survival ($p<0.001$) in fish immunized with the live-attenuated *E. ictaluri* vaccine and subsequently challenged with heterologous *E. piscicida* isolates, consistent with previous studies (Griffin et al., 2020b). Similarly, there was improved survival in fish immunized with *E. piscicida* and subsequently challenged with wild-type *E. ictaluri* ($p<0.001$), in line with previous work (Griffin et al., 2020b). The *E. ictaluri* induced mortality in control groups was 100%, suggesting an extremely high exposure dose. Still, previous exposure to *E. piscicida* heterologous isolates by bath immersion resulted in improved survival. These experimental trials support the hypothesis there is sufficient epitopic conservation between *E. ictaluri* and *E. piscicida* to support cross-protective immunity by a live-attenuated *Edwardsiella ictaluri* in both hybrid and channel catfish. Further studies are needed to elucidate this.

In the current study, protection afforded by the *E. ictaluri* vaccine against heterologous *E. piscicida* challenge was significantly higher in channel catfish than in hybrid catfish ($p < 0.001$). This could be attributed to hybrids' increased susceptibility to *E. piscicida* (Reichley et al., 2017),

however, other factors such as variability in fish size and age between channel and hybrids could have played a role.

The live-attenuated *E. ictaluri* vaccine has been in use for several years to vaccinate channel and hybrid catfish against *E. ictaluri* on catfish farms in Mississippi (Kumar et al., 2019). Anecdotal reports from the industry imply reduced incidence of *E. piscicida* in commercially raised vaccinated hybrids and preliminary laboratory investigations indicate the *E. ictaluri* vaccine was protective against *E. piscicida* isolate S11-285, which has been shown to cause increased mortality in hybrid catfish (Reichley et al., 2018). Similar trends were observed in other genetic variants of *E. piscicida*, where mortality is increased in hybrid catfish compared to channel catfish cohorts (Chapter II). A serendipitous benefit of this live-attenuated *E. ictaluri* vaccine is the cross-protective efficacy against heterologous *E. piscicida* isolates, which may negate the need for significant research investment to develop an *E. piscicida*-specific vaccine. Furthermore, the live-attenuated *E. ictaluri* vaccine has already overcome many of the regulatory hurdles for licensing and distribution on commercial farms, offering a readily available solution to an emerging problem in hybrid catfish production (Griffin et al., 2018, 2020b).

At present, the live-attenuated *Edwardsiella ictaluri* vaccine is delivered on commercial operations using an oral delivery platform, which allows for in-pond vaccine delivery. Recent work evaluating this approach in channel catfish fingerlings, orally vaccinated approximately 40–50 days post-stocking resulted in significant improvements in survival, feed conversion ratio, feed fed, and total yield (Wise et al., 2019). This oral delivery method has been evaluated in hybrid catfish under laboratory conditions, revealing a similar level of protection (Chatakondi et al., 2018). In the current study, the live-attenuated *E. ictaluri* vaccine was delivered by immersion to ensure uniform delivery to channel and hybrid cohorts and establish proof of concept that the *E.*

ictaluri vaccine can infer an adequate cross-protective immune response against heterologous *E. piscicida* isolates. An immersion model of vaccine evaluation was chosen based on previous work where hybrid feeding activity in tanks was reduced compared to channel catfish cohorts (Griffin et al., 2020b).

Comparably, the level of protection against *E. ictaluri* provided by previous bath immersion with heterologous *E. piscicida* isolates was markedly lower than in previous work (Griffin et al., 2020b). While a protective effect was observed, reduced survival can be attributed to an extreme response of naïve fish to a high challenge dose which resulted in 100% mortality in the naïve channel and hybrid groups within 5 and 7 days, respectively. Another aspect that may contribute to reduced protection is the inability to induce infection by immersion exposure. This method of inoculation does not induce reliable and reproducible disease and does not reflect infection dynamics observed during natural epizootics. Limited disease transmission by immersion exposure could be related to poor pathogen invasion of the fish host. Consequently, antigen uptake maybe restricted, which in turn reduces antigen processing resulting in inadequate protection against an extreme *E. ictaluri* exposure dose. Still, survival was significantly improved in fish that were immunized by *E. piscicida* immersion bath. Further, compared to channel catfish, survival of fish immunized with *E. piscicida* was greater in hybrid catfish ($p < 0.05$). This observation could simply be related to decrease susceptibility of hybrid catfish to *E. ictaluri* infection (Wolters et al., 1996) or dynamics of *E. piscicida* infection in channel catfish is such that the cross protective immune response against *E. ictaluri* infection is limited. Regardless, this work supports previous indications there is sufficient epitopic conservation among *E. ictaluri* and *E. piscicida* to provide cross-protective immunity against both pathogens using a live-attenuated *E. ictaluri* vaccine.

With increased adoption of hybrid catfish in intensive production systems (Kumar et al., 2016; Kumar and Engle, 2017) coupled with the increased susceptibility of hybrids to *E. piscicida*, identification of effective control measures against Edwardsiellosis in hybrids is critical for industry sustainability. The current study supports previous work demonstrating the live, attenuated *E. ictaluri* vaccine is highly effective in protecting hybrid catfish against subsequent *E. ictaluri* infections (Chatakondi et al., 2018). The cross-protective efficacy demonstrated herein is a fortuitous added benefit, suggesting channel or hybrid catfish immunized with *E. ictaluri* also receive some level of protection against *E. piscicida*. The impacts these findings will have on a commercial scale are presently unknown, but recent industry-scale vaccine trials in hybrid catfish have yielded estimated net economic benefits of vaccinating hybrid catfish with the live-attenuated *E. ictaluri* vaccine to be $6,145/ha. It is thought this benefit is multifactorial and associated with minimizing economic losses to both *E. ictaluri* and *E. piscicida* in hybrid production through administration of the ESC vaccine (Kumar et al., 2019b; Griffin et al., 2020b).

Despite an increasing incidence and prevalence of *E. piscicida* in hybrid catfish production systems, an *E. piscicida*-specific vaccine may be unnecessary as inferred from the current study. While this vaccine platform requires further optimization to capitalize on these findings, the multivalent nature of the live-attenuated *E. ictaluri* vaccine has the potential to significantly minimize impact of *E. piscicida* in both channel and hybrid stocks. The cross-protective efficiency of the *E. ictaluri* vaccine revealed here has the potential to significantly improve catfish health, improving production efficiency while simultaneously reducing industry reliance on costly medicated feeds.

3.5 Tables

Table 3.1 *Edwardsiella* spp. isolates used in this study. Each *E. piscicida* isolate was classified into a specific MLSA clade in a previous study (Chapter II).

Species	Isolate	*E. piscicida* MLSA Clade	Host	Year of Isolation
E. piscicida	S11-285	MLSA 1	Channel catfish	2011
E. piscicida	S17-335	MLSA 2	Hybrid catfish	2017
E. piscicida	S11-233	MLSA 3	Channel catfish	2011
E. piscicida	S08-209	MLSA 4	Channel catfish	2008
E. piscicida	S11-534	MLSA 5	Hybrid catfish	2011
E. ictaluri	S97-773	-	Channel catfish	1997

Table 3.2 Estimated exposure doses (Colony Forming Units [CFU] per gram of fish) administered to vaccinated and non-vaccinated hybrid and channel catfish. Fish were challenged with *E. piscicida* MLSA representative isolates and *E. ictaluri* wild-type 30-days post-vaccination. Probability of survival (%) determined by Kaplan-Meier estimations; relative percent survival (RPS) calculated according to Amend (1981).

Fish Group	Challenge Group (Isolate)	Estimated Dose (CFU/g)	Survival (%) Vaccinated	Survival (%) Naive	RPS (%)
Hybrid Catfish					
	MLSA 1 (S11-285)	1.49×10^4	82.6	21.7	77.8
	MLSA 2 (S17-335)	1.34×10^4	62.3	16.7	54.7
	MLSA 3 (S11-233)	1.68×10^4	67.4	16.7	60.9
	MLSA 4 (S08-209)	1.85×10^4	80	15	76.5
	MLSA 5 (S11-534)	1.62×10^4	59.6	10	55.1
	E. ictaluri (S97-773)	1.09×10^4	95.7	0	95.8
Channel Catfish					
	MLSA 1 (S11-285)	1.21×10^4	100	65	100
	MLSA 2 (S17-335)	1.09×10^4	91.2	55	80.5
	MLSA 3 (S11-233)	1.36×10^4	100	53.3	100
	MLSA 4 (S08-209)	1.49×10^4	100	45	100
	MLSA 5 (S11-534)	1.32×10^4	94.8	23.3	93.3
	E. ictaluri (S97-773)	8.82×10^3	98.2	0	98.2

Table 3.3 Estimated immunizing doses (Colony Forming Units [CFU] per gram of fish) administered to hybrid and channel catfish immunized by bath exposure to *E. piscicida*. Thirty days post immunization, fish were challenged by bath immersion with wild-type *E. ictaluri* S93-773 (~5.98×10^6 CFU/mL). Kaplan-Meier estimated 15-day survival (%) are presented.

Fish Group	**Immunizing group (Isolate)**	**Estimated Immunizing Dose (CFU/mL)**	**Survival (%)**
Hybrid Catfish			
	MLSA 1 (S11-285)	1.92×10^7	36.7
	MLSA 2 (S17-335)	2.42×10^7	26.7
	MLSA 3 (S11-233)	2.52×10^7	41.7
	MLSA 4 (S08-209)	2.58×10^7	43.3
	MLSA 5 (S11-534)	2.34×10^7	25
	Sham	-	0
Channel Catfish			
	MLSA 1 (S11-285)	1.92×10^7	18.3
	MLSA 2 (S17-335)	2.42×10^7	25
	MLSA 3 (S11-233)	2.52×10^7	28.3
	MLSA 4 (S08-209)	2.58×10^7	33.3
	MLSA 5 (S11-534)	2.34×10^7	28.3
	Sham	-	0

3.6 Figures

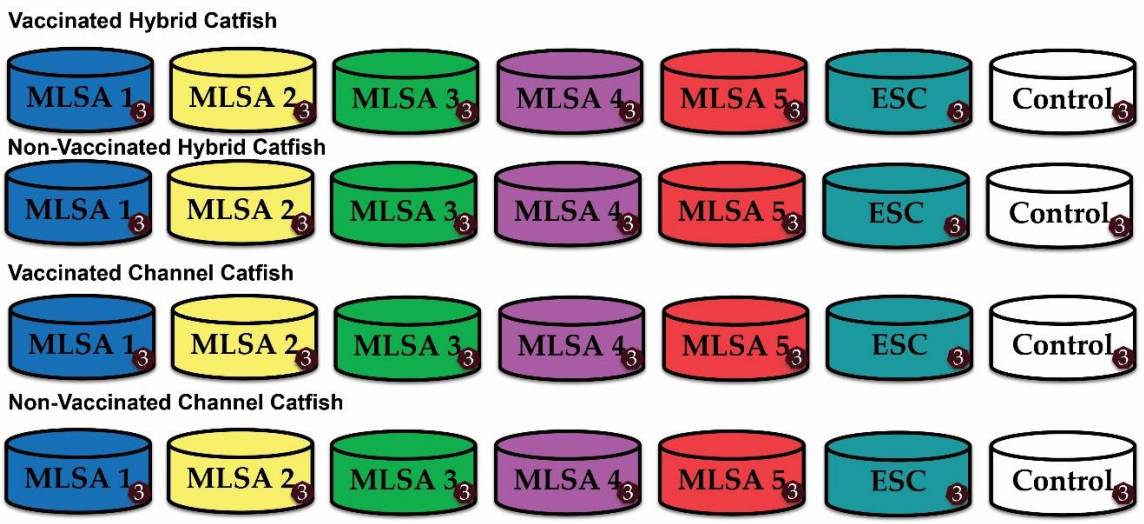

Figure 3.1 Schematic for Experimental trial 1. Vaccinated groups were immunized with a live-attenuated *Edwardsiella ictaluri* vaccine (Wise et al., 2015). Thirty days post vaccination both groups, vaccinated and non-vaccinated, were challenged with *E. piscicida* isolates representing each MLSA and an *E. ictaluri* wild-type isolate (ESC). Each treatment consisted of 3 replicates aquaria (20 fish/aquaria).

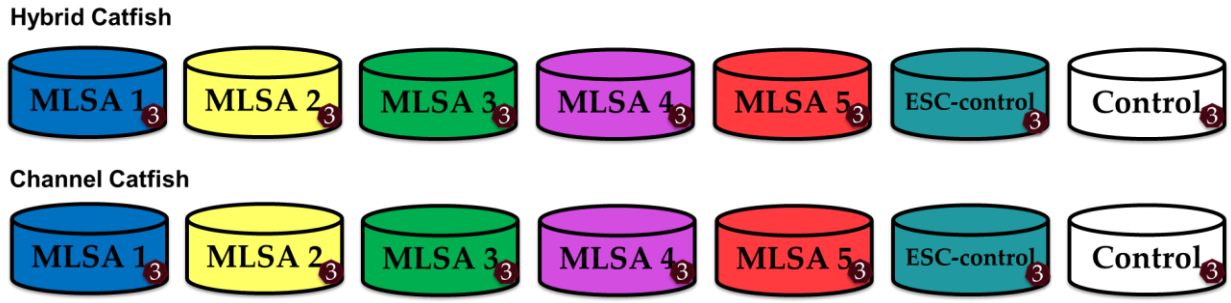

Figure 3.2 Schematic for experimental trial 2. Channel and hybrid catfish were immunized by respective *E. piscicida* variants. Positive (ESC-control) and negative control (Control) groups, were sham-exposed to sterile BHI broth. Thirty-days post-immunization all *E. piscicida* exposed fish as well as positive controls were challenged with *E. ictaluri* wild-type S97-773. Each treatment consisted of 3 replicates stocked with 20 fish/aquaria for *E. piscicida* MLSA group, and 10 fish/aquaria for positive and negative controls.

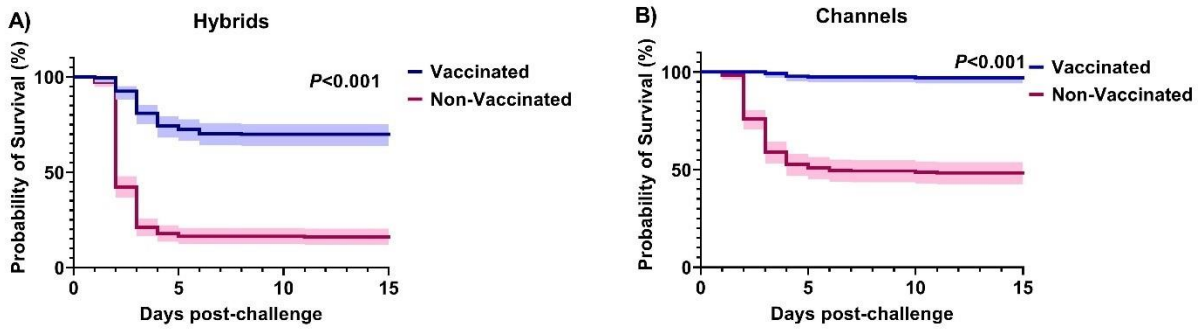

Figure 3.3 Kaplan-Meier analysis of survival for vaccinated and non-vaccinated hybrid (A) and channel (B) catfish exposed to *E. piscicida.* Thirty days post-vaccination with a live-attenuated *Edwardsiella ictaluri* vaccine hybrid and channel catfish were challenged with heterologous *E. piscicida* isolates representing five discrete phyletic groups. Survival was significantly improved in vaccinated channel and hybrid catfish ($p < 0.001$). Mean pooled survival curves and 95% confidence intervals (shaded) are presented.

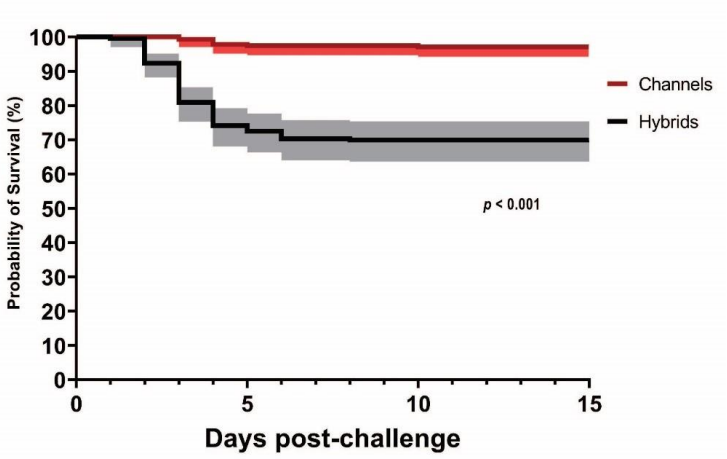

Figure 3.4 Kaplan-Meier survival estimations of pooled hybrid and channel catfish immunized with the live-attenuated *Edwardsiella ictaluri* vaccine and challenged with heterologous *E. piscicida* isolates. Mean pooled survival curves and 95% confidence intervals (shaded) are presented. Survival was significantly higher in vaccinated channel than hybrid catfish ($p < 0.001$).

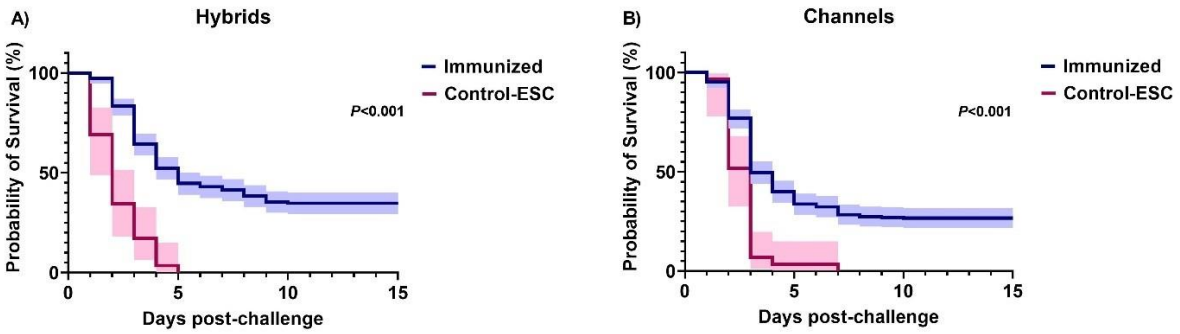

Figure 3.5 Kaplan-Meier survival estimations for hybrid (A) and channel (B) catfish immunized with heterologous *E. piscicida* isolates and challenged with wild-type *E. ictaluri* (S97-773). Mean pooled survival curves and 95% confidence intervals (shaded) are presented.

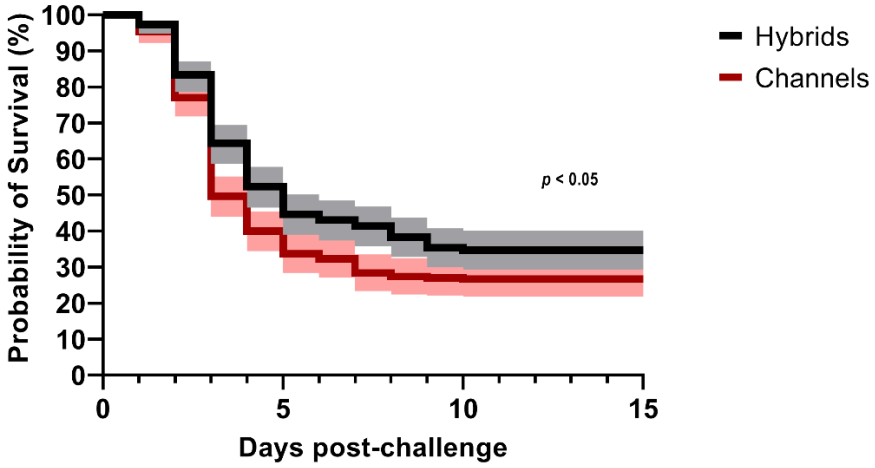

Figure 3.6 Kaplan-Meier survival estimations of pooled hybrid and channel catfish immunized with heterologous *E. piscicida* isolates and challenged with wild-type *E. ictaluri* (S97-773). Mean pooled survival curves and 95% confidence intervals (shaded) are presented. Difference was statistically different between both groups ($p < 0.05$).

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APPENDIX A

CONTINUED TABLES

Table A.1 Summary of open reading frames and putative functions from plasmids recovered from *E. piscicida* isolates from channel and hybrid catfish from Mississippi. Conserved domains and putative product/function of plasmid encoded ORFs were predicted using BLASTX. Physical maps of complete nucleotide sequences of plasmids collected from *E. piscicida* are showed in Figure 2.6.

Plasmid Source	ORF	Location (+/-)	Conserved domain; putative product; function	Alignment and identity	E-value
S11-285 S11-509 S10-512 S10-67 S12-420 S16-51 S15-96 S12-281 S07-1019	S11-285-1	879-1,529	Hypothetical protein. GenBank Sequence ID: WP_069579691.1	147/147(100%)	2e-100
	S11-285-2	879-1,529	Hypothetical protein. GenBank Sequence ID: WP_071890403.1	216/216(100%)	1e-159
	S11-285-3	1,596-2,522	primase C-terminal domain-containing protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_069579693.1	307/308(99%)	0.0
LADL 99-462	LADL 99-462-1	727-963 (+)	MobC family plasmid mobilization relaxosome protein [*Edwardsiella ictaluri*] GenBank Sequence ID: WP_015396717.1	78/78(100%)	4e-49
	LADL 99-462-2	2,109-2,363 (+)	mobilization relaxase [*Edwardsiella piscicida*] GenBank Sequence ID: PVD73727.1	84/84(100%)	4e-28
	LADL 99-462-3	2,584-3,462 (-)	primase C-terminal domain-containing protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_109579606.1	291/292(99%)	0.0
LADL 97-168	LADL 97-168-1	592-1,077 (+)	helix-turn-helix domain-containing protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_109745712.1	161/161(100%)	3e-112
	LADL 97-168-2	1,171-1,605 (+)	hypothetical protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_109745715.1	143/144(99%)	7e-103
	LADL 97-168-3	3,404-3,982 (-)	helix-turn-helix domain-containing protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_109745713.1	191/192(99%)	2e-137
	LADL 97-168-4	4,383-4,847 (+)	hypothetical protein [*Edwardsiella piscicida*] GenBank Sequence ID: ATV90675.1	154/154(100%)	3e-111
	5		hypothetical protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_109745714.1	147/147(100%)	8e-101
S16-278 S15-341 S14-431	S16-278-1	493-1,134 (+)	RloB superfamily protein [*Edwardsiella piscicida*] GenBank Sequence ID: ATV90670.1	212/213(99%)	7e-154
	S16-278-2	1,152 - 1,337 (+)	hypothetical protein [*Edwardsiella piscicida*] GenBank Sequence ID: ATV90671.1	60/61(98%)	4e-3
	S16-278-3	1,298-1,463 (-)	replication initiation factor [*Edwardsiella piscicida*] GenBank Sequence ID: ATV90672.1	53/54(98%)	9e-32
	S16-278-4	1,915-2,091 (-)	Rop superfamily RNA polymerase [*Edwardsiella piscicida*] GenBank Sequence ID: ATV90673.1	57/58(98%)	2e-19
	S16-278-5	3,013-499 (+)	P-loop NTPase superfamily protein [*Edwardsiella piscicida*] GenBank Sequence ID: ATV90674.1	421/422(99%)	0.0

Table (continued)

Plasmid Source	ORF	Location (+/-)	Conserved domain; putative product; function	Alignment and identity	E-value
S16-51 S15-96	S16-51-1	299-463 (-)	hypothetical protein [*Edwardsiella* tarda] GenBank Sequence ID: AWH59678.1	53/54(98%)	1e-28
	S16-51-2	1,333-1,704 (+)	hypothetical protein, partial [*Edwardsiella* tarda] GenBank Sequence ID: AWH59677.1	123/123(100%)	6e-74
S07-348	S07-348-1	891-1019 (-)	MULTISPECIES: type VI secretion protein [Bacteria] GenBank Sequence ID: WP_001260374.1	42/42(100%)	9e-22
	S07-348-2	1,074-1,259 (-)	hypothetical protein HMPREF9543_03549 [Escherichia coli MS 146-1] GenBank Sequence ID: EFK89631.1	61/61(100%)	3e-26
	S07-348-3	1,285-1,590 (+)	MULTISPECIES: tyrosine-type recombinase/integrase [Bacteria] GenBank Sequence ID: WP_009873361.1	101/101(100%)	9e-66
	S07-348-4	2,243-2,995 (-)	MULTISPECIES: hypothetical protein [Bacteria] GenBank Sequence ID: WP_009873360.1	250/250(100%)	9e-130
	S07-348-5	2,992-3,630 (-)	MULTISPECIES: hypothetical protein [Bacteria] GenBank Sequence ID: WP_009873359.1	212/212(100%)	5e-118
	S07-348-6	3,623-4,306 (-)	MULTISPECIES: protein mobD [Bacteria] GenBank Sequence ID: WP_009873358.1	227/227(100%)	3e-149
	S07-348-7	4,319-4,675 (-)	protein mobC [Klebsiella pneumoniae] GenBank Sequence ID: WP_134366923.1	117/118(99%)	4e-55
	S07-348-8	4,991-5,302 (+)	mobilization protein B [Aeromonas salmonicida subsp. salmonicida] GenBank Sequence ID: AIM49702.1	103/103(100%)	5e-68
	S07-348-9	5,292-7,964 (+)	relaxase/mobilization nuclease domain-containing protein [Enterobacter hormaechei] GenBank Sequence ID: WP_058670912.1	889/890(99%)	0.0
	S07-348-10	8,031-8,255 (+)	MULTISPECIES: antitoxin MazE family protein [Proteobacteria] GenBank Sequence ID: WP_043149934.1	74/74(100%)	1e-46
	S07-348-11	1,978-2,091 (+)	MULTISPECIES: type II toxin-antitoxin system PemK/MazF family toxin [Bacteria] GenBank Sequence ID: WP_020915708.1	74/74(100%)	4e-45
	S07-348-12	8,576-8,902 (+)	MULTISPECIES: hypothetical protein [Bacteria] GenBank Sequence ID: WP_009873353.1	108/108(100%)	3e-53
	S07-348-13	8,931-9,809 (+)	MULTISPECIES: AAA family ATPase [Bacteria] GenBank Sequence ID: WP_009873352.1	292/292(100%)	0.0
	S07-348-14	9,790-10,782 (+)	MULTISPECIES: plasmid replication [Bacteria] GenBank Sequence ID: WP_009873351.1	330/330(100%)	0.0
	S07-348-15	10,835-12,214 (+)	MULTISPECIES: transposase [Bacteria] GenBank Sequence ID: WP_009873366.1	459/459(100%)	0.0
	S07-348-16	12,291-12,746 (+)	MULTISPECIES: IS200/IS605 family transposase [Bacteria] GenBank Sequence ID: WP_009873365.1	151/151(100%)	2e-110
	S07-348-17	13,404-12,769 (-)	MULTISPECIES: tetracycline resistance transcriptional repressor TetR [Enterobacterales] GenBank Sequence ID: WP_047706566.1	211/211(100%)	2e-141
	S07-348-18	13,510-835 (+)	MULTISPECIES: tetracycline efflux MFS transporter Tet(A) [Enterobacterales] GenBank Sequence ID: WP_047706567.1	221/221(100%)	0.0

Table (continued)

Plasmid Source	ORF	Location (+/-)	Conserved domain; putative product; function	Alignment and identity	E-value
S08-209	S08-209-1-1	21-517 (+)	hypothetical protein [Salmonella enterica] GenBank Sequence ID: WP_080077697.1	109/165(66%)	7e-77
	S08-209-1-2	772-894 (-)	hypothetical protein [*Edwardsiella* tarda] GenBank Sequence ID: AWH59744.1	20/27(74%)	0.047
	S08-209-1-3	1,367-1,810 (-)	hypothetical protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_109745714.1	142/147(97%)	3e-96
S14-431	S14-431-1	4,929-829 (-)	RNA-directed DNA polymerase, partial [Escherichia coli] GenBank Sequence ID: WP_099374019.1	177/271(65%)	5e-128
	S14-431-2	1,455-1,898 (-)	hypothetical protein [*Edwardsiella piscicida*] GenBank Sequence ID: ATV90669.1	141/147(96%)	8e-96
	S14-431-3	2,704-3,582 (-)	primase C-terminal domain-containing protein [Klebsiella pneumoniae] GenBank Sequence ID: WP_048333045.1	250/292(86%)	3e-178
	S14-431-4	3,779-4,051 (-)	hypothetical protein SEEH4319_12017 [Salmonella enterica subsp. enterica serovar Heidelberg str. RI-11-014319] GenBank Sequence ID: KDS08109.1	47/83(57%)	5e-26
S08-209 (4120 bp)	S08-209-2-1	183-1,124 (+)	primase C-terminal domain-containing protein [*Edwardsiella* tarda] GenBank Sequence ID: WP_109610003.1	312/313(99%)	0.0
	S08-209-2-3	1,347-1,601 (-)	mobilization relaxase [*Edwardsiella piscicida*] GenBank Sequence ID: PVD73727.1	84/84(100%)	4e-28
	S08-209-2-3	1,906-2,757 (+)	mobilization relaxase [*Edwardsiella* tarda] GenBank Sequence ID: PVD68506.1	284/284(100%)	0.0
	S08-209-2-4	2,747-3,082 (+)	MobC family plasmid mobilization relaxosome protein [*Edwardsiella ictaluri*] GenBank Sequence ID: WP_015396717.1	111/111(100%)	3e-74
	S08-209-2-5	3,726-106 (+)	GNAT family N-acetyltransferase [Salmonella enterica subsp. enterica] GenBank Sequence ID: EDU9078998.1	167/167(100%)	7e-124
S17-335	S17-335-1	3,276-371 (+)	hypothetical protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_109745714.1	147/147(100%)	8e-101
	S17-335-2	1,258-1,743 (+)	helix-turn-helix domain-containing protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_109745712.1	161/161(100%)	3e-112
	S17-335-3	1,837-2,271 (+)	hypothetical protein [*Edwardsiella piscicida*] GenBank Sequence ID: WP_109745715.1	143/144(99%)	7e-103
	S17-335-4	2,834-3,060	hypothetical protein [*Edwardsiella ictaluri*] GenBank Sequence ID: AOX48525.1	33/38(87%)	3e-22