Advancing our understanding of the *Edwardsiella*

By

Stephen Ralph Reichley

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

August 2017

المنارة للاستشارات

www.manaraa.com

Copyright by

Stephen Ralph Reichley

2017

المنارة للاستشارات

www.manaraa.com

Advancing our understanding of the *Edwardsiella*

By

Stephen Ralph Reichley

Approved:

Matthew J. Griffin
(Co-Major Professor)

Mark L. Lawrence
(Co-Major Professor)

Terrence E. Greenway
(Committee Member)

Lester H. Khoo
(Committee Member)

David Wise
(Committee Member)

R. Hartford Bailey
(Graduate Coordinator)

Kent H. Hoblet
Dean
College of Veterinary Medicine

المنارة للاستشارات

www.manaraa.com

Name: Stephen Ralph Reichley

Date of Degree: August 11, 2017

Institution: Mississippi State University

Major Field: Veterinary Medical Science

Major Professors: Matthew J. Griffin and Mark L. Lawrence

Title of Study: Advancing our understanding of the *Edwardsiella*

Pages in Study 211

Candidate for Degree of Doctor of Philosophy

Diseases caused by *Edwardsiella* spp. are responsible for significant losses in wild and cultured fishes around the world. Historically, *Edwardsiella tarda* has been considered the most phenotypically and genotypically heterogeneous member of the genus. Investigations into intraspecific variability of *E. tarda* demonstrated isolates previously classified as *E. tarda* actually represent three genetically distinct yet phenotypically ambiguous groups, leading to the adoption of *E. piscicida* and *E. anguillarum* as discrete taxa. Current genomic investigations have demonstrated significant differences between these organisms. To this end, real-time quantitative PCR assays were developed to quickly and accurately detect the pathogens in pond water, fish tissue and broth culture. Additionally, whole genome sequencing was performed for representative isolates of each *Edwardsiella* spp. Furthermore, forty-seven *Edwardsiella* isolates, representing all five taxa, from different hosts and a wide temporal and geographic range were analyzed using commercial microbial identification kits, repetitive sequence-mediated polymerase chain reaction, fatty acid methyl ester analysis, antimicrobial resistance profiles, in addition to 16S, *gyrB*, *sodB* and plasmid sequencing. This analysis demonstrated key differences in gene sequences and plasmid profiles

www.manaraa.com

among these important bacterial pathogens and further supported contemporary taxonomic classifications. Additionally, a real-time multiplex PCR was developed to accurately discriminate between all *Edwardsiella* spp. affecting fish; namely *E. ictaluri*, *E. piscicida*, *E. tarda* and *E. anguillarum*. Moreover, recent publications suggest *E. piscicida* is more commonly associated with disease outbreaks in Mississippi catfish aquaculture than *E. tarda* or *E. anguillarum*. To this end, several different challenge models were evaluated. Comparative virulence was assessed, along with histopathological lesions and posterior kidney clearance rates in channel catfish (*Ictalurus punctatus*). Diagnostic case submissions suggest *E. piscicida* is more commonly associated with disease outbreaks in blue catfish (*Ictalurus furcatus*) x channel catfish hybrids compared to channel catfish. This led to investigations into the relative pathogenicity of *E. piscicida* in hybrid and channel catfish, which demonstrated a significantly lower median lethal dose ($LD_{50}$) for *E. piscicida* in hybrid catfish; an important finding given the increased production of hybrid catfish in U.S. farm-raised catfish in the southeastern United States.

المنارة للاستشارات
www.manaraa.com

DEDICATION

This dissertation is dedicated to my family. Their support, love, inspiration and understanding throughout my life and especially during the years of my PhD program made all of this possible.



www.manaraa.com

# ACKNOWLEDGEMENTS

I would like to thank Dr. Matt Griffin for his willingness to mentor me during my externship as a veterinary student and involving me then in his research projects. That experience piqued my interest in research and was the focal point of considering a PhD program. I thank you for welcoming me back to your lab two years later as a PhD student and for everything you have done for me over the past four years. I sincerely appreciate your patience with me throughout my program, the great discussions we have had, your constant sage advice and all the information you have bestowed upon me. In addition to my dissertation work, I am very appreciative for you including me in many of the other projects within your lab; these experiences greatly expanded my knowledgebase and professional network.

I would also like to thank Dr. Mark Lawrence, my co-major professor, for welcoming me into his lab and providing me office and lab space in Starkville. Your guidance and mentorship were pivotal during my PhD program and I will always be grateful to you for the opportunities you have given me. Thanks also to Dr. Lester Khoo. I appreciate you taking the chance on a veterinary student with no fish experience and your immense hospitality during my externship and throughout my PhD program. Thanks also to my other committee members Dr. Terry Greenway and Dr. David Wise. Your mentorship, instruction and friendship during my time in the Delta were much appreciated.



www.manaraa.com

I would also like to extend my sincere thanks and gratitude to the faculty and staff in the Department of Pathobiology and Population Medicine, Department of Basic Science and the Thad Cochran National Warmwater Aquaculture Center for their time and support. A special thanks to Cyndi Ware, James Steadman and Marsha Lewis for all their help in the lab. Thanks also to Dr. Graham Rosser who taught me so much along my journey and was always there for support and guidance. There were many other graduate students and post-docs who helped along the way, you know you are and I am very grateful.



المنارة للاستشارات
www.manaraa.com

TABLE OF CONTENTS

DEDICATION ........ ii

ACKNOWLEDGEMENTS ........ iii

LIST OF TABLES ........ ix

LIST OF FIGURES ........ xii

CHAPTER

I. INTRODUCTION ........ 1

1.1 Introduction ........ 1
1.1.1 U.S. catfish aquaculture ........ 1
1.1.2 Members of the *Edwardsiella* ........ 3
1.1.3 Reclassification of *Edwardsiella tarda* ........ 9
1.2 Research Objectives: ........ 14
1.2.1 Objective 1: ........ 14
1.2.2 Objective 2: ........ 14
1.2.3 Objective 3: ........ 14
1.2.4 Objective 4: ........ 14
1.2.5 Objective 5: ........ 14
1.3 Research Approaches ........ 15
1.3.1 Objective 1 Approach ........ 15
1.3.2 Objective 2 Approach ........ 15
1.3.3 Objective 3 Approach ........ 16
1.3.4 Objective 4 Approach ........ 17
1.3.5 Objective 5 Approach ........ 18
1.4 Potential Impact ........ 18
1.5 References ........ 19

II. REAL-TIME POLYMERASE CHAIN REACTION ASSAYS FOR THE DETECTION AND QUANTIFICATION OF *EDWARDSIELLA TARDA*, *EDWARDSIELLA PISCICIDA*, AND *EDWARDSIELLA PISCICIDA*-LIKE SPECIES IN CATFISH TISSUES AND POND WATER ........ 27

2.1 Introduction ........ 27
2.2 Materials and methods ........ 29



المنارة للاستشارات
www.manaraa.com

2.2.1 Bacterial cultures and isolation of genomic DNA.................29
2.2.2 Design of primer and probe sets ..........................................32
2.2.3 Generation of PCR standards ..............................................33
2.2.4 Quantitative PCR ....................................................................33
2.2.5 PCR specificity, sensitivity, repeatability, and reproducibility ....................................................................33
2.2.6 Detection of target DNA from broth culture, fish tissue, and pond water ...........................................................................34
2.2.7 Detection in experimentally infected fish .............................36
2.3 Results .................................................................................................37
2.3.1 PCR specificity, sensitivity, repeatability, and reproducibility ....................................................................37
2.3.2 Clinical sensitivity and assay variability ..............................40
2.3.3 Detection in experimentally infected fish .............................43
2.4 Discussion ...........................................................................................46
2.5 References ..........................................................................................50

III. COMPLETE GENOME SEQUENCES OF *EDWARDSIELLA ANGUILLARUM*, *EDWARDSIELLA HOSHINAE*, *EDWARDSIELLA PISCICIDA* AND *EDWARDSIELLA TARDA* ISOLATES RECOVERED FROM VARIOUS HOSTS WITH DIFFERENT GEOGRAPHIC ORIGINS ........................................................................................53

3.1 Introduction ........................................................................................54
3.2 Materials and Methods .......................................................................56
3.2.1 DNA isolation .........................................................................56
3.2.2 Sequencing .............................................................................57
3.2.2.1 *Edwardsiella anguillarum* EA181011 ............................57
3.2.2.2 *Edwardsiella anguillarum* LADL05-105 ........................58
3.2.2.3 *Edwardsiella hoshinae* ATCC 35051 .............................58
3.2.2.4 *Edwardsiella piscicida* S11-285 .....................................59
3.2.2.5 *Edwardsiella tarda* FL95-01 ..........................................59
3.2.2.6 Plasmid sequencing ........................................................60
3.2.3 Genome annotation, relatedness and intragenomic 16S heterogeneity ........................................................................61
3.3 Results .................................................................................................62
3.4 Discussion ...........................................................................................71
3.5 Acknowledgements ............................................................................76
3.6 References ..........................................................................................77

IV. ADVANCING OUR KNOWLEDGE OF THE *EDWARDSIELLA*: A COMPARATIVE PHENOTYPIC AND GENOTYPIC ANALYSIS OF *EDWARDSIELLA* SPP. ISOLATES FROM DIFFERENT HOSTS AND GEOGRAPHIC ORIGINS ......................................................................85

4.1 Introduction ........................................................................................85



المنارة للاستشارات
www.manaraa.com

4.2 Materials and Methods ............................................................88
4.2.1 Bacterial Isolates ................................................................88
4.2.2 DNA isolation.......................................................................90
4.2.3 Motility and TSI ...................................................................90
4.2.4 Microbial identification systems .........................................90
4.2.5 Fatty acid methyl ester (FAME) analysis .............................92
4.2.5.1 Preparation of bacterial isolates......................................92
4.2.5.2 Fatty acid methyl ester extraction...................................92
4.2.5.3 Gas Chromatography Conditions and Analysis ..............93
4.2.6 Antimicrobial susceptibility profiles ....................................94
4.2.7 Phylogenetic analysis ..........................................................94
4.2.8 Genetic fingerprinting .........................................................97
4.2.9 Multiplex real-time PCR .....................................................98
4.2.10 Plasmid analysis ..................................................................99
4.3 Results ........................................................................................100
4.3.1 Motility and TSI ..................................................................100
4.3.2 Microbial identification systems .......................................102
4.3.3 Fatty acid methyl ester (FAME) analysis ...........................105
4.3.4 Antimicrobial susceptibility profiles ..................................106
4.3.5 Phylogenetic analysis .........................................................110
4.3.6 Genetic fingerprinting ........................................................116
4.3.7 Multiplex PCR.....................................................................119
4.3.8 Plasmid analysis .................................................................120
4.4 Discussion...................................................................................146
4.5 Acknowledgements ...................................................................156
4.6 References .................................................................................157

V. EVALUATION OF THREE DISEASE CHALLENGE MODELS FOR *EDWARDSIELLA PISCICIDA*, *EDWARDSIELLA TARDA* AND *EDWARDSIELLA ANGUILLARUM* IN CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)...........................................................166

5.1 Introduction ...............................................................................166
5.2 Materials and Methods ..............................................................168
5.2.1 Fish ......................................................................................168
5.2.2 Bacterial Cultures ...............................................................169
5.2.3 Trial 1: Intraperitoneal injection..........................................170
5.2.4 Trial 2: Immersion exposure ...............................................170
5.2.5 Trial 3: Immersion with mucus removal .............................171
5.2.6 Trial 4: Pathology and posterior kidney clearance ..............172
5.2.7 Molecular Analysis.............................................................173
5.3 Results ........................................................................................175
5.3.1 Trial 1: Intraperitoneal injection..........................................175
5.3.2 Trial 2: Immersion exposure ...............................................177
5.3.3 Trial 3: Immersion with mucus removal .............................178
5.3.4 Trial 4: Pathology and posterior kidney clearance ..............179



المنارة للاستشارات
www.manaraa.com

5.4 Discussion....................................................................................183
5.5 References ....................................................................................188

VI. COMPARATIVE SUSCEPTIBILITY OF CHANNEL CATFISH (*ICTALURUS PUNCTATUS*), BLUE CATFISH (*ICTALURUS FURCATUS*) AND CHANNEL X BLUE HYBRID CATFISH TO *EDWARDSIELLA PISCICIDA*, *EDWARDSIELLA TARDA* AND *EDWARDSIELLA ANGUILLARUM*....................................................193

6.1 Introduction ....................................................................................193
6.2 Materials and Methods ....................................................................195
6.2.1 Fish ....................................................................................195
6.2.2 Bacterial Cultures ....................................................................195
6.2.3 Trial 1: Initial Range Test (*E. anguillarum*, *E. piscicida*, *E. tarda*) ....................................................................................196
6.2.4 Trial 2: Median Lethal Dose Refinement (*E. piscicida* and *E. tarda*) ....................................................................................197
6.2.5 Trial 3: Replicated Median Lethal Dose Determination for *E. piscicida*....................................................................................197
6.2.6 Statistical Analysis ....................................................................198
6.3 Results ....................................................................................199
6.3.1 Trial 1: Initial Range Test (*E. anguillarum*, *E. piscicida*, *E. tarda*) ....................................................................................199
6.3.2 Trial 2: Median Lethal Dose Refinement (*E. piscicida* and *E. tarda*) ....................................................................................200
6.3.3 Trial 3: Replicated Median Lethal Dose Determination for *E. piscicida*....................................................................................201
6.4 Discussion....................................................................................202
6.5 References ....................................................................................205

VII. CONCLUSION ....................................................................................208



المنارة للاستشارات
www.manaraa.com

LIST OF TABLES

2.1 Specificity of each *Edwardsiella* real-time polymerase chain reaction (qPCR) assay for the respective target. ............................................31

2.2 Real-time polymerase chain reaction primers and probes used in the current study. ........................................................................32

2.3 Mean (± the standard deviation) quantification cycles, $Log_{10}$ copy number, and relative fluorescent units for known quantities of *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda* cells in brain–heart infusion (BHI) broth, pond water, or channel catfish (*Ictalurus punctatus*) posterior kidney tissue. ..................................................................................42

2.4 Mean (± the standard deviation) quantification cycle and $Log_{10}$ copy number for channel catfish (*Ictalurus punctatus*) fingerlings experimentally challenged with *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda*. ......................................44

3.1 Associated metadata for *Edwardsiella* spp. genomes sequenced in this project (bold) and publicly available complete and draft genomes used for comparative analysis. ..........................................................57

3.2 Statistics for genomes sequenced and closed in the current study. ....64

3.3 Average nucleotide identities and (digital DNA-DNA hybridization) estimations for *Edwardsiella* genomes..............................................65

3.4 Intragenomic heterogeneity of the 16S SSU for representative *Edwardsiella* genomes sequenced in this study. ................................66

4.1 *Edwardsiella* spp. isolates analyzed in the current study. ..................89

4.2 Primers and probes used in the current study. ...................................96

4.3 Motility and triple sugar iron (TSI) analysis of the isolates used in the current study. ........................................................................101

4.4 Antimicrobial identification system results for isolates analyzed in the current study. ........................................................................103



www.manaraa.com

4.5 Mean (standard deviation) fatty acid content of *Edwardsiella* spp. analyzed in the current study. ....106

4.6 Antimicrobial susceptibility to single compounds of *Edwardsiella* spp. isolates analyzed in the current study. ....108

4.7 Antimicrobial susceptibility to combinatory compounds of *Edwardsiella* spp. isolates analyzed in the current study. ....109

4.8 16S rRNA similarity matrix between *Edwardsiella* spp. used in the current study. ....112

4.9 *gyrB* similarity matrix between *Edwardsiella* spp. used in the current study. ....114

4.10 *sodB* similarity matrix between *Edwardsiella* spp. used in the current study. ....116

4.11 Specificity of the multiplex real-time polymerase chain reaction (mPCR) assay to each respective target. ....119

4.12 Summary of open reading frames and putative functions from plasmids harvested from *Edwardsiella piscicida* isolates from different hosts and geographic origins. ....122

4.13 Summary of open reading frames and putative functions from plasmids harvested from *Edwardsiella tarda* isolates from different hosts and geographic origins. ....127

5.1 *Edwardsiella* isolates used in the current study. ....169

5.2 Real-time quantitative PCR primers and probes used for detection and quantification of *Edwardsiella* spp. in kidney biopsies. ....174

5.3 Cumulative percent mortality and $LD_{50}$ from intraperitoneal injection (Trial 1). ....176

5.4 Cumulative percent mortality from immersion exposure (Trial 2). ....177

5.5 Cumulative percent mortality from immersion exposure with mucus removal (Trial 3). ....178

5.6 Mean (range) bacterial equivalents per mg of kidney tissue from Trial 4. ....181

5.7 Kidney biopsy growth from Trial 4. ....182

6.1 Source of *Edwardsiella* isolates used for $LD_{50}$ determination. ....196




المنارة للاستشارات
www.manaraa.com

6.2 Cumulative percent mortality and $LD_{50}$ from initial range test (Trial 1)....................................................................................199

6.3 Cumulative percent mortality and $LD_{50}$ from Trial 2.......................200

6.4 Average cumulative percent mortality for *E. piscicida* in channel and hybrid catfish (Trial 3).......................................................................202



المنارة للاستشارات
www.manaraa.com

LIST OF FIGURES

1.1 Phylogenetic tree based on *sodB* sequences, constructed using the neighbor-joining method. ....................................................................10

2.1 Mean quantification cycles for known quantities of *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda* target DNA. ....................................................................39

2.2 Mean quantification cycles for known quantities of *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda* in brain–heart infusion broth, pond water, or channel catfish (*Ictalurus punctatus*) posterior kidney tissue. ....................................................................41

2.3 Nonreplicated cumulative mortality for channel catfish (*Ictalurus punctatus*) challenged with 3 different doses of *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda*..46

3.1 Sequence identity of *Edwardsiella* genomes, using *E. anguillarum* isolate LADL05-105 as the reference. Image created using the BLAST Ring Image Generator (BRIG). ....................................................................67

3.2 Sequence identity of *Edwardsiella* genomes, using *E. hoshinae* isolate ATCC 35051 as the reference. Image created using the BLAST Ring Image Generator (BRIG). ....................................................................68

3.3 Sequence identity of *Edwardsiella* genomes, using *E. ictaluri* isolate 93-146 as the reference. Image created using the BLAST Ring Image Generator (BRIG). ....................................................................69

3.4 Sequence identity of *Edwardsiella* genomes, using *E. piscicida* isolate S11-285 as the reference. Image created using the BLAST Ring Image Generator (BRIG). ....................................................................70

3.5 Sequence identity of *Edwardsiella* genomes, using *E. tarda* isolate FL95-01 as the reference. Image created using the BLAST Ring Image Generator (BRIG). ....................................................................71

4.1 Phylogenetic relationships of *Edwardsiella* spp. based on partial 16S rRNA gene sequence. ....................................................................111



المنارة للاستشارات
www.manaraa.com

4.2 Phylogenetic relationships of *Edwardsiella* spp. based on partial *gyrB* gene sequence. ....113

4.3 Phylogenetic relationships of *Edwardsiella* spp. based on partial *sodB* gene sequence. ....115

4.4 Genetic fingerprints of *Edwardsiella* spp. analyzed in the current study. ....117

4.5 Dendrogram of *Edwardsiella* spp. generated from BOX repetitive extragenic palindromic PCR profiles. ....118

4.6 Multiplex real-time PCR assay validation results. ....120

4.7 Physical maps of plasmids harvested from *E. piscicida* isolates......143

4.8 Physical maps of plasmids harvested from *E. tarda* isolates. ....144

4.9 Physical maps of plasmids harvested from *E. tarda* isolates. ....145



المنارة للاستشارات
www.manaraa.com

# CHAPTER I

# INTRODUCTION

## 1.1 Introduction

### 1.1.1 U.S. catfish aquaculture

Catfish production is the largest finfish aquaculture industry in the United States and is important to the economies of many southeastern states (Hargreaves 2002, Stankus 2010). Commercial production of channel catfish (*Ictalurus punctatus*) began in the 1960s by farmers in the Yazoo-Mississippi River floodplain, an area commonly referred to as the "Delta" (Hargreaves and Tucker 2004). Extensive growth and intensification occurred over the following decades, leading to the significant infrastructure in place today. The culture of catfish in Alabama, Arkansas, Mississippi and Texas accounts for 96% of the industry's total production with sales of food-size fish totaling $345 million in 2014. Mississippi is the top producing state with nearly 45,000 water surface acres dedicated to catfish production and sales of $190 million in 2014 (USDA 2016). The ease of spawning, efficient food conversion, tolerance of intensive culture conditions and market acceptability all contributed to the success of channel catfish aquaculture (Hargreaves 2002). However, rising production costs, increased foreign competition and more lucrative land-use alternatives, have led to a recent decline in U.S. catfish production since its peak in 2003 (Stankus 2010).



المنارة للاستشارات

www.manaraa.com

As the industry continues to mature, intensification has been necessary to maintain a profitable production model. Higher stocking rates, increased aeration and a multi-batch crop system have all been implemented to increase production efficiency. These management practices, coupled with the open-environment of pond production have increased the likelihood and propensity for infectious disease (Hawke and Khoo 2004). It is estimated that 45% of inventory losses throughout the production cycle are attributed to infectious disease (USDA 1997). The two most ruinous bacterial diseases in the catfish industry are Enteric Septicemia of Catfish (ESC), caused by *Edwardsiella ictaluri*, and Columnaris Disease, caused by *Flavobacterium columnare* (USDA/APHIS 2011). Another bacterial pathogen of note, *Edwardsiella tarda*, causes Emphysematous Putrefactive Disease of Catfish which can result in losses as high as 50% during disease outbreaks (Meyer and Bullock 1973). Historically, *E. tarda* has been perceived as a pathogen of low occurrence in catfish aquaculture. However, based on summaries of diagnostic case submissions to the Aquatic Research and Diagnostic Laboratory in Stoneville, Mississippi, there has been an increased incidence of *E. tarda* in Mississippi farm-raised catfish over the past decade (http://tcnwac.msstate.edu/publications.htm).

As a function of their more favorable production characteristics, the U.S. catfish industry has seen an increase in the production of blue catfish (*Ictalurus furcatus*) x channel catfish (*I. punctatus*) hybrid catfish. The hybrid catfish possesses many of the best traits from each parent, providing the resistance to bacterial infections like ESC from its blue parent while still maintaining quick growth rates from its channel parent (Hargreaves and Tucker 2004). At present, hybrid catfish utilization continues to



المنارة للاستشارات
www.manaraa.com

increase, accounting for an estimated 30% - 40% of total catfish production in 2014 (Li, Robinson et al. 2014).

### 1.1.2 Members of the *Edwardsiella*

For the past several decades, *Edwardsiella* has consisted of three species: *E. tarda*, *E. hoshinae* and *E. ictaluri*. There are limited reports of *E. hoshinae* from a small number of avian and reptilian hosts (Grimont, Grimont et al. 1980, Singh, Singh et al. 2004). Conversely, *E. ictaluri* is well studied and principally considered a pathogen of cultured channel catfish, causing Enteric Septicemia of Catfish (Hawke, McWhorter et al. 1981), although recent reports have implicated *E. ictaluri* in mortality events in other fish species (Soto, Griffin et al. 2012, Hawke, Kent et al. 2013).

*Edwardsiella tarda* is a Gram-negative, oxidase negative peritrichously flagellated bacteria. It was first described in the 1960s representing a collection of 37 isolates recovered from humans and animals in Brazil, Ecuador, Israel, Japan, and the United States. The biochemical profile of these isolates, designated as "Bacterium 1483-59" did not fit within any existing Enterobacteriaceae genera. Similarly, a collection of isolates designated as the "Bartholomew Group" was recovered from a hospitalized human in the United States (King and Adler 1964). Concurrently, a group of over 250 isolates from snakes, seals and humans were analyzed biochemically and serologically and designated as the "Asakusa group" within Enterobacteriaceae (Sakazaki 1965, Sakazaki 1967). Consequently, the genus *Edwardsiella* was created and these isolates were classified as a single species, *E. tarda*; "tarda" being from the Latin for slow, implying inactivity (Ewing, McWhorter et al. 1965).



المنارة للاستشارات
www.manaraa.com

*E. tarda* is widely considered one of the most globally important bacterial diseases in fish, affecting a range of economically important wild and cultured freshwater and marine fish species (Mohanty and Sahoo 2007, Xu and Zhang 2014). The pathogen is considered a zoonotic agent (Janda, Abbott et al. 1991, Janda and Abbott 1993, Mohanty and Sahoo 2007) and has been isolated from reptiles, birds and mammals on all continents, including Antarctica (Meyer and Bullock 1973, Tan and Lim 1977, VanDamme and Vandepitte 1980, Uhland, Hélie et al. 2000, Clavijo, Conroy et al. 2002, Akinbowale, Peng et al. 2006, Leotta, Piñeyro et al. 2009, Joh, Kim et al. 2011). Disease outbreaks in fish infected with *E. tarda* are typically associated with chronic losses of 5% - 10%, however mortality can be much higher and catastrophic losses can occur (Meyer and Bullock 1973, Kodama, Murai et al. 1987, Plumb and Hanson 2011).

In general, *E. tarda* is considered a warmwater pathogen. However, disease outbreaks have also occurred in temperate and coldwater fish (Meyer and Bullock 1973, Noga 2010). Environmental stressors such as high organic content, poor water quality and high water temperatures contribute to disease and outbreak severity (Uhland, Hélie et al. 2000, Plumb and Hanson 2011, Park, Aoki et al. 2012). In the late 1970s, *E. tarda* was isolated from 75% of pond water samples, 64% of pond mud samples, and 100% of frogs, turtles and crayfish in a survey of catfish ponds, with increased presence of *E. tarda* with increased water temperature (Wyatt, Nickelson et al. 1979). On olive flounder (*Paralichthys olivaceus*) farms in Japan, *E. tarda* was present in 22% - 86% of water samples (Rashid, Honda et al. 1994).

Clinical signs of *E. tarda* infection vary with location and species affected. Lesions typically manifest similar to other bacterial infections. In catfish, initial signs



المنارة للاستشارات
www.manaraa.com

typically include small, cutaneous ulcerative foci on the flanks and caudal peduncle. These progress into large, gas-filled, malodorous abscesses of the musculature (Meyer and Bullock 1973). Other clinical signs include loss of pigmentation, petechiation, exophthalmia, distention of the coelomic cavity, rectal protrusion and/or hemorrhage and opacity of the eyes (Mohanty and Sahoo 2007, Park, Aoki et al. 2012). In some cases, a hemorrhagic ulcer on the dorsal aspect of the cranium can also be present, mimicking the "hole-in-the-head" lesion commonly associated with ESC (Plumb and Hanson 2011, Khoo 2013). Internally, congested liver, spleen and kidney, along with ascites and liquefactive necrosis of the viscera is often noted (Darwish, Plumb et al. 2000, Noga 2010, Park, Aoki et al. 2012). Histologically, severe multifocal necrotizing inflammation is observed in the pro- and mesonephros, liver and spleen (Darwish, Plumb et al. 2000).

Japanese eels (*Anguilla japonica*) are another economically important fish commonly affected by *E. tarda.* In eels, *E. tarda* may display two different forms of clinical disease. The nephric form is most common, which is associated with necrotic renal foci that spread to other internal organs (Miyazaki and Egusa 1976). Alternatively, a hepatic form produces microabscesses in the liver which spreads to other organs (Miyazaki and Egusa 1976). In turbot, exophthalmia, coelomic distension and hemorrhage can be seen externally; while inflammation of the kidney, liver and spleen is often found internally (Padros, Zarza et al. 2006). In the striped bass, necrosis along the lateral line and in the gills has been noted along with epithelial hyperplasia in association with *E. tarda* infection (Herman and Bullock 1986). Similar to clinical signs in catfish, lesions in tilapia typically include loss of pigmentation, distention of the coelomic cavity



المنارة للاستشارات
www.manaraa.com

and opacity of the eyes externally with white nodules often present in the gills, kidney, liver, spleen and/or intestine (Kubota, Kaige et al. 1981).

Diagnosis of *E. tarda* is typically accomplished using traditional culture and biochemical characterization methods. *E. tarda* will readily grow on tryptic soy agar (TSA) or brain heart infusion agar (BHI) after 24-36 hr incubation at 28°C to 37°C. The bacterium is a short, motile, Gram-negative bacilli that is cytochrome oxidase negative, indole positive, producing a TSI reaction of K/A with hydrogen sulfide production (Hawke and Khoo 2004). Variations in biochemical profiles and motility of *E. tarda* isolates have been reported from different fish hosts and geographic origins (Matsuyama, Kamaishi et al. 2005, Sakai, Yuasa et al. 2009, Park, Aoki et al. 2012). Confirmatory diagnosis can also be made by species-specific PCR (Sakai, Yuasa et al. 2009, Griffin, Ware et al. 2014).

Like most bacterial infections in fish, antibiotics are the primary control measure. Reports of treatment regimens targeting *E. tarda* are sparse, however oxytetracycline, florfenicol, and oxolinic acid have all been utilized (Kusuda and Kawai 1998). During an outbreak of *E. tarda* in brook trout in Quebec, oxytetracycline at a dosage of 100 mg/kg of live fish weight, mixed with fish feed and vegetable oil resulted in complete cessation of mortality within ten days (Uhland, Hélie et al. 2000). These results were fairly consistent with a previous finding that 55 mg/kg of live fish for 10 days controlled *E. tarda* in channel catfish (Meyer and Bullock 1973). However, delivering antibiotics with feed can be challenging as fish often become inappetant when ill. Efficacy of such treatments is reliant on the fish consuming the medicated diet (Austin and Austin 1993).



المنارة للاستشارات
www.manaraa.com

Frequent and/or long-term use of medicated feeds can also lead to the emergence of antibiotic resistant strains, which have been reported for *E. tarda* (Aoki and Takahashi 1987, Stock and Wiedemann 2001, Sun, Wang et al. 2009). A survey of *E. tarda* from pond sediments, pond water and fish samples from a fish culture system revealed 78% of the recovered isolates were multi-drug resistant (Acharya, Maiti et al. 2007). These drug-resistant bacteria can pose a significant obstacle to fish health and continued production. The emergence of pathogens with multi-drug resistance has impelled the discovery and use of novel therapeutants. For example, a 1:3 combination of ormetoprim-sulfamonomethoxine (25 mg/kg/day), oxolinic acid (12.5 mg/kg/day) and miloxacin at a dose rate of 6.2 mg/kg/day was found to be effective against drug-resistant *E. tarda* infecting eels in Japan (Aoki, Kitao et al. 1989). In this instance, the novel chemotherapy was successful. However, use of antibiotics puts pressure on the microbial community and selects for antibiotic resistance, raising concerns regarding the environmental impacts of continued antibiotic use and there is no current approved antibiotic therapy labelled for *E. tarda* in foodfish in the U.S. (Evelyn 1997).

Due to environmental concerns and emergence of antimicrobial resistant bacteria, there is a growing reluctance to use antibiotics as a means of curbing bacterial infections in aquaculture (Cabello 2006). This is especially the case in developing countries where exchanges often occur between fish and humans (Miranda and Zemelman 2001). As a result, there is increased focus placed on prevention of disease. One mechanism of disease prevention is vaccination. Given the tremendous global impact of *E. tarda* on many cultured fish species, significant efforts have been made to develop an efficacious vaccine to protect against *E. tarda*. Unfortunately, a practical vaccine remains elusive,



المنارة للاستشارات
www.manaraa.com

largely on account of significant serological intraspecific variation associated with *E. tarda* (Mohanty and Sahoo 2007). However, there are reports that both atypical and typical strains of *E. tarda* share a similar O-antigen, suggesting a common vaccine targeting *E. tarda* lipopolysaccharides is within the realm of possibility (Costa, Kanai et al. 1998).

Several different types of vaccines have been developed and tested for *E. tarda*. In rohu (*Labeo rohita*) and catla (*Catla catla*), strong protection against *E. tarda* was elicited by bath immersion with an *E. tarda* bacterin. However this protection was largely dependent on the duration of the bath and age of fish (Swain, Nayak et al. 2002). Japanese flounder (*Paralichthys olivaceus*), fed a combination of formalin-killed *E. tarda* and the immunostimulant Curdlan (bacterial β-1,3-glucan) and a quillaja saponin suspension by adding the compounds to pellets every day for three weeks before challenge showed greater survival than control fish (Ashida, Okimasu et al. 1999). Additionally, a vaccine using a conserved 37kDa outer membrane protein (OMP) intraperitoneally (IP) injected demonstrated strong protection against several *E. tarda* serotypes (Kawai, Liu et al. 2004).

A double knockout *E. tarda*, deficient in genes essential for bacterial cell wall biosynthesis, was generated while investigating vaccine strategies for olive flounder (*Paralichthys olivaceus*). This vaccine provided significant protection following primary and secondary IP injections (Choi and Kim 2011). Additionally, a formalin-killed *E. tarda* immersion vaccine demonstrated over 90% relative percent survival (RPS) in olive flounder fingerlings and juveniles (Bang, Ryu et al. 2000). This vaccine was later developed and is available commercially in Korea (Park 2009). In turbot (*Scophthalmus*



المنارة للاستشارات
www.manaraa.com

*maximus*), an *E. tarda* bacterin, both in an aqueous formulation and also mixed with a non-mineral oil adjuvant, was delivered as a bath immersion and IP injection. Results showed the IP administered adjuvanted vaccine conferred 90% RPS at least six months post-vaccination (Castro, Toranzo et al. 2008). In Japanese eels (*Anguilla japonica*), intramuscular (IM) injection with a crude lipopolysaccharide (LPS) resulted in significantly higher protection than non-vaccinated eels (Salati and Kusud 1985). While showing promise in laboratory conditions, many of the aforementioned vaccine strategies have failed to garner widespread industry adoption, largely due to logistical challenges associated with their administration.

### 1.1.3 Reclassification of *Edwardsiella tarda*

Recent findings have complicated this historical literature in regards to the clinical signs, pathobiology, diagnosis and prevention of *E. tarda*. *E. tarda* was historically considered the most widespread and diverse member of the genus *Edwardsiella* (Mohanty and Sahoo 2007). In the 1990s, researchers began to note significant intraspecific genetic differences amongst *E. tarda* strains. Enzyme electrophoresis for superoxide dismustase and catalase of 144 strains of *E. tarda* revealed two distinct groups, one containing strains isolated from diseased fish and another including strains from non-diseased fish, environmental samples and human sources. Restriction fragment length polymorphism also revealed two distinct 16S rDNA ribotypes (Yamada and Wakabayashi 1998). Subsequent work identified two distinct groups of *E. tarda* based on the nucleotide sequences of an internal fragment of iron-cofactored superoxide dismutase gene (*sodB*). These groups differ from one another in their pathogenicity to fish (Figure 1.1). Their results showed that fish pathogenic *E. tarda*



المنارة للاستشارات
www.manaraa.com

grouped more closely with *E. ictaluri* whereas non-fish pathogenic *E. tarda* grouped more closely with *E. hoshinae* (Yamada and Wakabayashi 1999). Significant heterogeneity has also been demonstrated in protein profiles of different *E. tarda* strains (Panangala, Shoemaker et al. 2006).

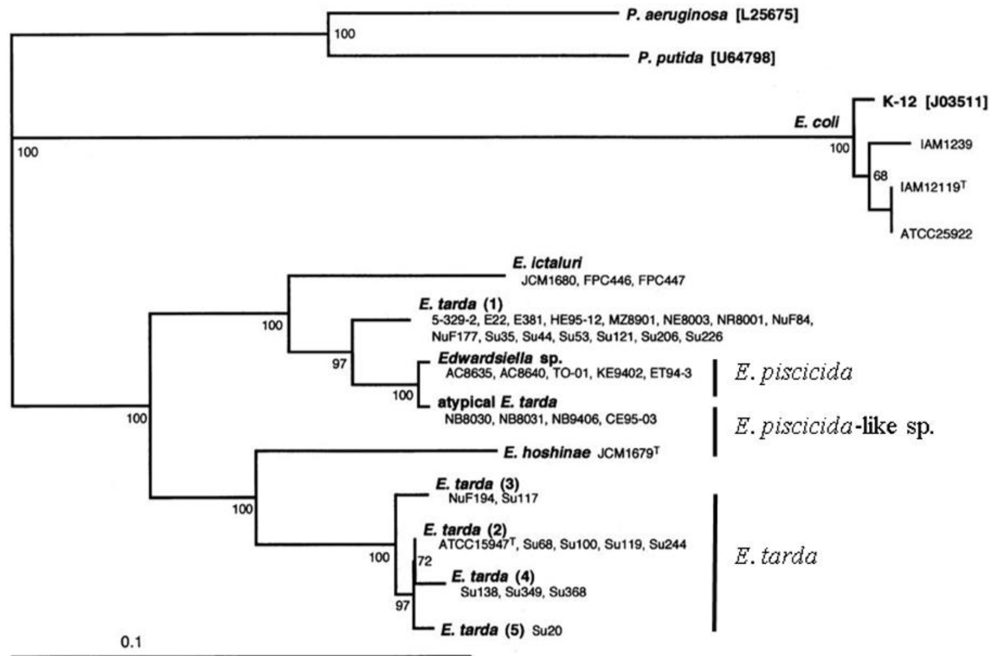


Figure 1.1 Phylogenetic tree based on *sodB* sequences, constructed using the neighbor-joining method.

Contemporary nomenclature has been added to demonstrate the recognized differences within isolates previously classified as *E. tarda*. Modified from Yamada and Wakabayashi (1999).

Additional evidence of genotypic variation within *E. tarda* was demonstrated by the marked difference in the presence and absence of fimbrial genes between fish pathogenic and non-fish pathogenic *E. tarda*. Sakai et al. (2007) targeted these differences in a PCR to detect the type 1 fimbrial gene cluster in all fish pathogenic strains tested. However, this gene cluster was not amplified in 13 of 14 non-fish



www.manaraa.com

pathogenic strains. These differences were later exploited to develop a PCR assay to differentiate between what was termed typical, motile and atypical, non-motile fish pathogenic *E. tarda* (Sakai, Yuasa et al. 2009). This work was further supported by biochemical, serological and molecular characterization of a group of 21 *E. tarda* strains from turbot in two different areas of Europe. Using randomly amplified polymorphic DNA (RAPD) analysis, significant genetic variability was seen amongst the isolates which formed two distinct clonal lineages (Castro, Toranzo et al. 2006). RAPD analysis was found by others to distinguish *E. tarda* strains originating from fish and *E. tarda* strains isolated from humans (Nucci, Silveira et al. 2002). Furthermore, two distinct groups were elucidated using repetitive extragenic palindromic-PCR (Maiti, Mandal et al. 2008, Castro, Toranzo et al. 2011).

Taking advantage of advancements in DNA sequencing technologies, higher resolution analyses were utilized to investigate the intraspecific relationships of the *Edwardsiella*. Yang et al. (2012), used genome-based phylogenetic analysis supported with multilocus sequence analysis (MLSA) of 48 distinct *Edwardsiella* strains to demonstrate *E. tarda* could be separated into two distinct genotypes (EdwGI and EdwGII). EdwGI *E. tarda* strains had high sequence similarity to *E. ictaluri* and a low sequence similarity to EdwGII. These findings were further supported by independent studies in Europe and the United States, which included a myriad of molecular techniques and identified significant variability within *E. tarda* (Abayneh, Colquhoun et al. 2012, Griffin, Quiniou et al. 2013). Consistent with earlier findings, their work revealed two distinct phylogroups. One phylogroup (ET883-like; DNA Group II) was more similar to *E. ictaluri* than the other phylogroup (*E. tarda* type strain group; DNA Group I), which



المنارة للاستشارات
www.manaraa.com

contained the *E. tarda* isolate from humans (ATCC15947). This work, supported by previous investigations into the phenotypic and genetic heterogeneity of *E. tarda*, led to the adoption of *Edwardsiella piscicida* as the fourth member of *Edwardsiella* (Abayneh, Colquhoun et al. 2013, Oren and Garrity 2013) and identified the probable existence of a fifth member of *Edwardsiella*, designated *E. piscicida*-like sp. (Griffin, Ware et al. 2014).

In subsequent studies, *E. piscicida* was identified in farm-raised catfish in the southeastern United States. A recent survey of isolates phenotypically described as *E. tarda* from diseased fish confirmed the bacterium associated with these case submissions is actually *E. piscicida*. This suggests the prevalence of *E. tarda* in farm-raised catfish is much lower than previously thought (Griffin, Ware et al. 2014). Currently, there are no unique phenotypic characteristics that allow for accurate differentiation between *E. tarda*, *E. piscicida* and *E. piscicida*-like sp. Diagnosticians and researchers must rely on molecular techniques to discriminate between these phenotypically ambiguous organisms. Real-time, quantitative PCR (qPCR) assays are becoming more common in fish disease research and diagnostics and qPCR assays are currently available for a myriad of bacterial, viral, and parasitic pathogens (Griffin, Pote et al. 2009, Bain, Cornwell et al. 2010, Soto, Bowles et al. 2010). Development of qPCR assays for the rapid detection of *E. tarda*, *E. piscicida* and *E. piscicida*-like sp. would greatly improve the diagnostic capability for these important pathogens.

Recent findings suggest that *E. tarda*, as it has been historically regarded, actually represented three genetically distinct taxa. This offers some explanation why an efficacious, broad spectrum *E. tarda* vaccine has remained elusive. Previously, an antigentic epitope may have been identified for one “strain” of *E. tarda* but was not



المنارة للاستشارات
www.manaraa.com

efficacious against another “strain” of *E. tarda*; which is likely due to investigators unknowingly working with multiple bacterial species. Appropriate identification of bacterial species may allow for proper elucidation of antigenic epitopes and may lead to more efficacious and widely-used vaccines.

The taxonomic confusion of *Edwardsiella* is further compounded by the reliance on the National Center for Biotechnology Information’s (NCBI) GenBank and the International Nucleotide Sequence Database Collaboration nomenclature when using isolates with sequence data available in these databases. As the website disclaimer reads, the NCBI taxonomy database is not an authoritative source for classification or nomenclature. The service recommends investigators consult relevant scientific literature for the most reliable and up-to-date taxonomic information. This is especially important for taxa undergoing reclassification, such as the case with *Edwardsiella* spp. At present, genomes of FL6-60 and EIB202 are identified as *E. tarda* in GenBank, however, both are actually members of *E. piscicida* (Yang, Lv et al. 2012). As a result, there is currently no complete genome for *E. tarda* in GenBank. In addition to these mislabeled genomes, countless nucleotide sequences are also misclassified. This has resulted in erroneous identification of bacterial strains and inconsistent reports of *Edwardsiella* isolates in the literature, further confounding the scientific understanding of this important genus. Whole genome sequencing and comparative genomic analyses using contemporary classification will help resolve the current confusion which exists in regards to the *Edwardsiella*.



المنارة للاستشارات
www.manaraa.com

## 1.2 Research Objectives:

### 1.2.1 Objective 1:

Development of real-time polymerase chain reaction assays for the detection and quantification of *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like species in catfish tissues and pond water.

### 1.2.2 Objective 2:

Investigations into the new taxa *Edwardsiella piscicida* and comparative genomic analysis with other *Edwardsiella* species.

### 1.2.3 Objective 3:

Comparative phenotypic and genotypic analysis of *Edwardsiella* spp. isolates from different hosts and geographic origins.

### 1.2.4 Objective 4:

Disease challenge model development and comparative susceptibility and pathology of channel catfish (*Ictalurus punctatus*) to *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like species.

### 1.2.5 Objective 5:

Comparative susceptibility of channel catfish (*Ictalurus punctatus*), blue catfish (*Ictalurus furcatus*), and channel x blue hybrid catfish to *Edwardsiella tarda*, *Edwardsiella piscicida* and *Edwardsiella piscicida*-like species.



المنارة للاستشارات
www.manaraa.com

## 1.3 Research Approaches

### 1.3.1 Objective 1 Approach

- Using previously published primer sets (Sakai, Yuasa et al. 2009, Griffin, Ware et al. 2014) real-time quantitative PCR (qPCR) assays will be developed.
- Genomic DNA from *E. tarda*, *E. ictaluri*, *E. piscicida* and *E. piscicida*-like sp., as well as closely related non-target DNA will be used to validate the sensitivity and specificity of each assay.
- qPCR assays will be validated by spiking catfish posterior kidneys, pond water, and using broth culture while ensuring correct enumeration over at least five orders of magnitude.
- This will provide a rapid method of discrimination and identification between the phenotypically ambiguous taxa; a helpful tool for additional investigations into the epidemiology and pathobiology of the *Edwardsiella* spp.

### 1.3.2 Objective 2 Approach

- Gain a better understanding of the genomic composition of *E. piscicida*, and how it compares and contrasts with other *Edwardsiella* spp.
- Utilize Next Generation Sequencing techniques to sequence, close and annotate whole genomes of representative *Edwardsiella* spp.
- Barcoded genomic DNA libraries will be prepared from the *Edwardsiella* spp. isolates using the Nextera DNA sample Prep Kit and sequenced using Illumina technology.
- Next Generation Sequencing technology will be used to generate long sequence reads, which will be corrected using Illumina data. Assembly software will be used to correct the data and produce a single contig.
- Use available tools for comparative analysis between *Edwardsiella* spp. genomes.
- Rapid Annotation using Subsystem Technology (RAST) (Aziz, Bartels et al. 2008, Overbeek, Olson et al. 2014).
- Average nucleotide identity (ANI) (Goris, Konstantinidis et al. 2007).
- Digital DNA-DNA hybridization (dDDH) (Auch, von Jan et al. 2010).



المنارة للاستشارات
www.manaraa.com

- BLAST Ring Image Generator (BRIG) (Alikhan, Petty et al. 2011).

- This will help establish the genetic characteristics of each taxa within *Edwardsiella* and provide data which can be used for a myriad of additional investigations.

### 1.3.3 Objective 3 Approach

- To further characterize these taxa, a number of phenotypic and genotypic analyses will be performed Using more than 40 *Edwardsiella* spp. isolates with broad geographical and temporal distribution.

- Phenotypic analysis will include:

  - Four commercially available microbial identification systems: API-20E (BioMerieux), BBL Crystal Enteric/Nonfermentor Identification Kit (Becton Dickinson and Company), Biolog Microbial Identification System (Biolog) and Maxtrix-Assisted Laser Desorption/ionization (MALDI) Biotyper (Bruker).

  - Motility analysis and characterization using triple sugar iron (TSI) slants.

  - Fatty acid methyl ester (FAME) analysis.

  - Antimicrobial profiles, established using the minimum inhibitory concentration (MIC) of different antimicrobial agents.

- Genotypic analysis will include:

  - Establishing phylogenetic relationships amongst the *Edwardsiella* isolates.

  - The 16S rRNA, *gyrB*, and *sodB* genes will be amplified by PCR and sequenced by Sanger sequencing. Near complete gene sequences will be aligned, compared and phylogenetic relationships established using Maximum Likelihood analysis. Sequences will be deposited in the National Center for Biotechnology Information's non-redundant nucleotide database (GenBank).

  - Using established protocols (Versalovic, Koeuth et al. 1991, Versalovic, Schneider et al. 1994, Griffin, Quiniou et al. 2013, Griffin, Ware et al. 2014) repetitive extragenic palindromic PCR (rep-PCR) will be performed on bacterial isolates to generate DNA fingerprints.



المنارة للاستشارات
www.manaraa.com

- Plasmid isolation for each isolate will be accomplished using QIAprep Spin Miniprep Kit. Isolated plasmids will then be sequenced by the Center for Computational and Integrative Biology at Massachusetts General Hospital. Resulting plasmids will then be annotated and compared for composition and alignment.

- This analysis will clearly articulate the phenotypic and genotypic characteristics of the members of *Edwardsiella* and serve as a resource for future investigations into the genus while allowing for comparisons with work done using historical taxa classification.

### 1.3.4 Objective 4 Approach

- Several different challenge models will be investigated using *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like sp. in channel catfish. These models will include bath immersion, coated feed, mucus removal, and intraperitoneal (IP) injection.

- After establishing a disease challenge model, investigations into the median lethal dose ($LD_{50}$) (Reed 1938), histopathological lesions, and posterior kidney clearance rates will be established for each of the *Edwardsiella* spp. in channel catfish.

- Briefly, for each challenge model, channel catfish will be exposed to a range of bacterial dilutions. The $LD_{50}$ will be calculated based on the cumulative mortality observed for each bacterial dose.

- Using the above information, a disease challenge model will be selected and channel catfish will be exposed to each *Edwardsiella* spp. At determined times after exposure, apparently health fish will be collected and a biopsy of the posterior kidney will be aseptically obtained. Genomic DNA will be extracted and the qPCR assay developed in Objective 2 will be used to determine the amount of target bacterial DNA copies present.

- This work will establish a disease challenge model for the *Edwardsiella* spp. in channel catfish, which can be used in future studies. Additionally, these studies will provide valuable information regarding the virulence and pathogenicity of *E. tarda*, *E. piscicida*, and *E. piscicida*-like sp. in channel catfish.



المنارة للاستشارات
www.manaraa.com

### 1.3.5 Objective 5 Approach

- Using the disease challenge model established in Objective 4, channel catfish, blue catfish, and their hybrid cross will be exposed to *E. tarda*, *E. piscicida*, and *E. piscicida*-like sp.
- The $LD_{50}$ will be calculated based on the cumulative mortality observed for each bacterial dose in each fish group (Reed 1938).
- These studies will highlight the similarities or differences in virulence and pathogenicity of the three *Edwardsiella* spp. in the different fish groups. Information obtained from these investigations will be useful for the farm-raised catfish industry, which is currently increasing utilization of hybrid catfish (Li, Robinson et al. 2014).

## 1.4 Potential Impact

This research will lead to important, practical advancements in the catfish aquaculture industry. The development of real-time quantitative PCR assays to differentiate and enumerate *Edwardsiella* species will be beneficial to researchers and diagnosticians globally. In addition, these assays, along with the phenotypic and genotypic characterization of the numerous taxa in *Edwardsiella* will provide a clear understanding of the taxonomy in this genera. Characterizing the genotypic and phenotypic profiles of *Edwardsiella* species will greatly aid in our understanding of how these pathogens differ from one another and their interactions with catfish. Generating complete and annotated genomes of each *Edwardsiella* taxa will serve as a springboard for future work focused on the epidemiology, prevention, control and treatment of these important fish pathogens. Establishing the virulence and pathogenicity differences between the *Edwardsiella* spp. in channel catfish, blue catfish, and their hybrid cross will provide valuable information to the farm-raised catfish industry.



المنارة للاستشارات
www.manaraa.com

## 1.5 References


Abayneh, T., D. J. Colquhoun and H. Sorum (2012). "Multi-locus Sequence Analysis (MLSA) of *Edwardsiella tarda* isolates from fish." Veterinary Microbiology **158**(3-4): 367-375.

Abayneh, T., D. J. Colquhoun and H. Sorum (2013). "*Edwardsiella piscicida* sp. nov., a novel species pathogenic to fish." Journal of Applied Microbiology **114**(3): 644-654.

Acharya, M., N. K. Maiti, S. Mohanty, P. Mishra and M. Samanta (2007). "Genotyping of *Edwardsiella tarda* isolated from freshwater fish culture system." Comparative Immunology, Microbiology and Infectious Diseases **30**(1): 33-40.

Akinbowale, O. L., H. Peng and M. D. Barton (2006). "Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia." Journal of Applied Microbiology **100**(5): 1103-1113.

Alikhan, N.-F., N. K. Petty, N. L. B. Zakour and S. A. Beatson (2011). "BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons." BMC Genomics **12**: 402.

Aoki, T., T. Kitao and M. Fukudome (1989). "Chemotherapy against infection with multiple drug resistant strains of *Edwardsiella tarda* in cultured eels." Fish Pathology **24**(3): 161-168.

Aoki, T. and A. Takahashi (1987). "Class D tetracycline resistance determinants of R plasmids from fish pathogens *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Pasteurella piscicida*." Antimicrobial Agents and Chemotherapy **31**(8): 1278-1280.

Ashida, T., E. Okimasu, M. Ui, M. Heguri, Y. Oyama and A. Amemura (1999). "Protection of Japanese flounder *Paralichthys olivaceus* against experimental edwarsdiellosis by formalin-killed *Edwarsdiella tarda* in combination with oral administration of immunostimulants." Fisheries Science **65**(4): 527-530.

Auch, A. F., M. von Jan, H. P. Klenk and M. Goker (2010). "Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison." Standards in Genomic Sciences **2**(1): 117-134.

Austin, B. and D. A. Austin (1993). Enterobacteriaceae representatives. Bacterial Fish Pathogens: Disease in Farmed and Wild Fish. West Sussex, England, Ellis Horwood, Limited**:** 188-226.




للاستشارات المنارة
www.manaraa.com

Aziz, R. K., D. Bartels, A. A. Best, M. DeJongh, T. Disz, R. A. Edwards, K. Formsma, S. Gerdes, E. M. Glass, M. Kubal, F. Meyer, G. J. Olsen, R. Olson, A. L. Osterman, R. A. Overbeek, L. K. McNeil, D. Paarmann, T. Paczian, B. Parrello, G. D. Pusch, C. Reich, R. Stevens, O. Vassieva, V. Vonstein, A. Wilke and O. Zagnitko (2008). "The RAST Server: rapid annotations using subsystems technology." BMC Genomics **9**: 75.

Bain, M. B., E. R. Cornwell, K. M. Hope, G. E. Eckerlin, R. N. Casey, G. H. Groocock, R. G. Getchell, P. R. Bowser, J. R. Winton, W. N. Batts, A. Cangelosi and J. W. Casey (2010). "Distribution of an invasive aquatic pathogen (viral hemorrhagic septicemia virus) in the Great Lakes and its relationship to shipping." PLoS One **5**(4): e10156.

Bang, J. D., H. Y. Ryu, C. Lee, B. S. Kim, T. X. Moon and J. Y. Ha (2000). "Effectiveness of *Edwardsiella tarda* vaccine against edwarsdiellosis of olive flounder, *Paralichthys olivaceus* in a field trial test." Bulletin of National Fisheries Research and Development Institute **58**: 40-43.

Cabello, F. C. (2006). "Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment." Environmental Microbiology **8**(7): 1137-1144.

Castro, N., A. E. Toranzo, J. L. Barja, S. Nunez and B. Magarinos (2006). "Characterization of *Edwardsiella tarda* strains isolated from turbot, *Psetta maxima* (L.)." Journal of Fish Diseases **29**: 541-547.

Castro, N., A. E. Toranzo, A. Bastardo, J. L. Barja and B. Magarinos (2011). "Intraspecific genetic variability of *Edwardsiella tarda* strains from cultured turbot." Diseases of Aquatic Organisms **95**(3): 253-258.

Castro, N., A. E. Toranzo, S. Nunez and B. Magarinos (2008). "Development of an effective *Edwardsiella tarda* vaccine for cultured turbot (*Scophthalmus maximus*)." Fish and Shellfish Immunology **25**(3): 208-212.

Choi, S. H. and K. H. Kim (2011). "Generation of two auxotrophic genes knock-out *Edwardsiella tarda* and assessment of its potential as a combined vaccine in olive flounder (*Paralichthys olivaceus*)." Fish and Shellfish Immunology **31**(1): 58-65.

Clavijo, A. M., G. Conroy, D. A. Conroy, J. Santander and F. Aponte (2002). "First report of *Edwardsiella tarda* from tilapias in Venezuela." Bulletin of the European Association of Fish Pathologists **22**: 280-282.

Costa, A. B., K. Kanai and K. Yoshikoshi (1998). "Serological characterization of atypical strains of *Edwardsiella tarda* isolated from sea breams." Fish Pathology **33**(4): 265-274.



المنارة للاستشارات
www.manaraa.com

Darwish, A., J. Plumb and J. Newton (2000). "Histopathology and pathogenesis of experimental infection with *Edwardsiella tarda* in channel catfish." Journal of Aquatic Animal Health **12**(4): 255-266.

Evelyn, T. (1997). "A historical review of fish vaccinology." Developments in Biological Standardization **90**: 3-12.

Ewing, W. H., A. C. McWhorter, M. R. Escobar and A. H. Lubin (1965). "*Edwardsiella*, a new genus of Enterobacteriaceae based on a new species, *E. tarda*." International Bulletin of Bacteriological Nomenclature and Taxonomy **15**(1): 33-38.

Goris, J., K. T. Konstantinidis, J. A. Klappenbach, T. Coenye, P. Vandamme and J. M. Tiedje (2007). "DNA-DNA hybridization values and their relationship to whole-genome sequence similarities." International Journal of Systematic and Evolutionary Microbiology **57**(Pt 1): 81-91.

Griffin, M. J., L. M. Pote, A. C. Camus, M. J. Mauel, T. E. Greenway and D. J. Wise (2009). "Application of a real-time PCR assay for the detection of *Henneguya ictaluri* in commercial channel catfish ponds." Diseases of Aquatic Organisms **86**(3): 223-233.

Griffin, M. J., S. M. Quiniou, T. Cody, M. Tabuchi, C. Ware, R. C. Cipriano, M. J. Mauel and E. Soto (2013). "Comparative analysis of *Edwardsiella* isolates from fish in the eastern United States identifies two distinct genetic taxa amongst organisms phenotypically classified as *E. tarda*." Veterinary Microbiology **165**(3-4): 358-372.

Griffin, M. J., C. Ware, S. M. Quiniou, J. M. Steadman, P. S. Gaunt, L. H. Khoo and E. Soto (2014). "*Edwardsiella piscicida* identified in the Southeastern USA by *gyrB* sequence, species-specific and repetitive sequence-mediated PCR." Diseases of Aquatic Organisms **108**(1): 23-35.

Grimont, P. D., F. Grimont, C. Richard and R. Sakazaki (1980). "*Edwardsiella hoshinae*, a new species of Enterobacteriaceae." Current Microbiology **4**(6): 347-351.

Hargreaves, J. A. (2002). "Channel catfish farming in ponds: Lessons from a maturing industry." Reviews in Fisheries Science **3/4**: 499-528.

Hargreaves, J. A. and C. S. Tucker (2004). Indstury Development. Biology and Culture of Channel Catfish. C. S. Tucker and J. A. Hargreaves. San Diego, CA, Elsevier.

Hawke, J. P., M. Kent, M. Rogge, W. Baumgartner, J. Wiles, J. Shelley, L. C. Savolainen, R. Wagner, K. Murray and T. S. Peterson (2013). "Edwardsiellosis caused by *Edwardsiella ictaluri* in laboratory populations of Zebrafish Danio rerio." Journal of Aquatic Animal Health **25**(3): 171-183.



المنارة للاستشارات
www.manaraa.com

Hawke, J. P. and L. H. Khoo (2004). Infectious Diseases. Biology and culture of the channel catfish. C. S. Tucker and J. A. Hargreaves. Amsterdam, Elsevier**:** 387-443.

Hawke, J. P., A. C. McWhorter, A. G. Steigerwalt and D. J. Brenner (1981). "*Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish." International Journal of Systematic Bacteriology **31**(4): 396-400.

Herman, R. and G. Bullock (1986). "Pathology caused by *Edwardsiella tarda* in striped bass." Transactions of the American Fisheries Society **115**: 232-235.

Janda, J. M. and S. L. Abbott (1993). "Infections associated with the genus *Ewardsiella*: the role of *Edwardsiella tarda* in human disease." Clinical Infectious Diseases **17**: 742-748.

Janda, J. M., S. L. Abbott, S. Kroske-Bystrom, W. K. W. Cheung, C. Powers, R. P. Kokka and K. Tamura (1991). "Pathogenic properties of *Edwardsiella* species." Journal of Clinical Microbiology **29**(9): 1997-2001.

Joh, S.-J., M.-J. Kim, H.-M. Kwon, E.-H. Ahn, H. Jang and J.-H. Kwon (2011). "Characterization of *Edwardsiella tarda* isolated from farm-cultured eels, *Anguilla japonica*, in the Republic of Korea." Journal of Veterinary Medical Science **73**(1): 7-11.

Kawai, K., Y. Liu, K. Ohnishi and S. Oshima (2004). "A conserved 37 kDa outer membrane protein of *Edwardsiella tarda* is an effective vaccine candidate." Vaccine **22**(25-26): 3411-3418.

Khoo, L. H. (2013). Personal Communication.

King, B. M. and D. L. Adler (1964). "A previously undescribed group of Enterobacteriaceae." The American Journal of Clinical Pathology **41**(2): 230-232.

Kodama, H., T. Murai, Y. Nakanishi, F. Yamamoto, T. Mikami and H. Izawa (1987). "Bacterial infection which produces high mortality in cultured Japanese flounder (*Paralichthys olivaceus*) in Hokkaido." Japanese Journal of Veterinary Research **35**(4): 227-234.

Kubota, S., N. Kaige, T. Miyazaki and T. Miyashita (1981). "Histopathological studies on edwardsiellosis of tilapia. I. Natural Infection." Bulletin of the Faculty of Fisheries of Mie University **9**: 155-156.

Kusuda, R. and K. Kawai (1998). "Bacterial diseases of cultured marine fish in Japan." Fish Pathology **33**(4): 221-227.

Leotta, G. A., P. Piñeyro, S. Serena and G. B. Vigo (2009). "Prevalence of *Edwardsiella tarda* in Antarctic wildlife." Polar Biology **32**(5): 809-812.



المنارة للاستشارات

www.manaraa.com

Li, M. H., E. H. Robinson, B. G. Bosworth and E. L. Torrans (2014). "Growth and feed conversion ratio of pond-raised hybrid catfish harvested at different sizes." North American Journal of Aquaculture **76**(3): 261-264.

Maiti, N. K., A. Mandal, S. Mohanty and M. Samanta (2008). "Comparative analysis of genome of *Edwardsiella tarda* by BOX-PCR and PCR-ribotyping." Aquaculture **280**(1-4): 60-63.

Matsuyama, T., T. Kamaishi, N. Ooseko, K. Kurohara and T. Iida (2005). "Pathogenicity of motile and non-motile *Edwardsiella tarda* to some marine fish." Fish Pathology **40**(3): 133-135.

Meyer, F. P. and G. L. Bullock (1973). "*Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*)." Applied Microbiology **25**(1): 155-156.

Miranda, C. D. and R. Zemelman (2001). "Antibiotic resistant bacteria in fish from Concepcion Bay, Chile." Marine Pollution Bulletin **42**(11): 1096-1102.

Miyazaki, T. and S. Egusa (1976). "Histopathological studies of edwardsiellosis of the Japanese eel (*Anguilla japonica*) I. Suppurative interstitial hepatitis form." Fish Pathology **11**: 67-76.

Miyazaki, T. and S. Egusa (1976). "Histopathological studies of edwardsiellosis of the Japanese eel (*Anguilla japonica*) I. Suppurative interstitial nephritis form." Fish Pathology **11**: 33-44.

Mohanty, B. R. and P. K. Sahoo (2007). "Edwardsiellosis in fish: a brief review." Journal of Biosciences **32**: 1331-1344.

Noga, E. J. (2010). Fish Disease Diagnosis and Treatment. Ames, Iowa, Wiley-Blackwell.

Nucci, C., W. D. d. Silveira, S. d. S. Corre, G. Nakazato, S. Y. Bando, M. A. Ribeiro and A. F. P. d. Castro (2002). "Microbiological comparative study of isolates of *Edwardsiella tarda* isolated in different countries from fish and humans." Veterinary Microbiology **89**: 29-39.

Oren, A. and G. M. Garrity (2013). "List of new names and new combinations previously effectively, but not validly, published." International Journal of Systematic and Evolutionary Microbiology **63**(Pt 9): 3131-3134.

Overbeek, R., R. Olson, G. D. Pusch, G. J. Olsen, J. J. Davis, T. Disz, R. A. Edwards, S. Gerdes, B. Parrello, M. Shukla, V. Vonstein, A. R. Wattam, F. Xia and R. Stevens (2014). "The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST)." Nucleic Acids Research **42**(Database issue): D206-214.



للاستشارات

www.manaraa.com

Padros, F., C. Zarza, L. Dopazo, M. Cuadrado and S. Crespo (2006). "Pathology of *Edwardsiella tarda* infection in turbot, *Scophthalmus maximus* (L.)." Journal of Fish Diseases **29**: 87-94.

Panangala, V. S., C. A. Shoemaker, S. T. McNulty, C. R. Arias and P. H. Klesius (2006). "Intra- and interspecific phenotypic characteristics of fish-pathogenic *Edwardsiella ictaluri* and *E. tarda*." Aquaculture Research **37**(1): 49-60.

Park, S. B., T. Aoki and T. S. Jung (2012). "Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish." Veterinary Research **43**: 67.

Park, S. I. (2009). "Disease control in Korean aquaculture." Fish Pathology **44**(1): 19-23.

Plumb, J. A. and L. A. Hanson (2011). Health Maintenance and Principal Microbial Diseases of Cultured Fishes. Ames, Iowa, Blackwell Publishing, Ltd.

Rashid, M. M., K. Honda, T. Nakai and K. Muroga (1994). "An ecological study on *Edwardsiella tarda* in flounder farms." Fish Pathology **29**(4): 221-227.

Reed, L. J. (1938). "A simple method of estimating fifty per cent endpoints." American Journal of Epidemiology **27**(3): 493-497.

Sakai, T., T. Iida, K. Osatomi and K. Kanai (2007). "Detection of type 1 fimbrial genes in fish pathogenic and non-pathogenic *Edwardsiella tarda* strains by PCR." Fish Pathology **42**(2): 115-117.

Sakai, T., K. Yuasa, M. Sano and T. Iida (2009). "Identification of *Edwardsiella ictaluri* and *E. tarda* by species-specific polymerase chain reaction targeted to the upstream region of the fimbrial gene." Journal of Aquatic Animal Health **21**(2): 124-132.

Sakazaki, R. (1965). "A proposed group of the family Enterobacteriaceae, the Asakusa Group." International Bulletin of Bacteriological Nomenclature and Taxonomy **15**(1): 45-47.

Sakazaki, R. (1967). "Studies on the Asakusa group of Enterobacteriaceae (*Edwardsiella tarda*)." Japanese Journal of Medical Science and Biology **20**: 205-212.

Salati, F. and R. Kusud (1985). "Vaccine preparations used for immunization of eel *Anguilla japonica* against *Edwardsiella tarda* infection." Bulletin of the Japanese Society of Scientific Fisheries **51**(8): 1233-1237.

Singh, I. M., S. Singh, M. A. McMurphy, S. S. Crupper, F. Mills-Robertson and R. D. Applegate (2004). "Antibiotic susceptibility of *Edwardsiella hoshinae* isolated from northern bobwhite quail (*Colinus virginianus*)." Veterinary Record **155**(1): 29-29.



المنارة للاستشارات

www.manaraa.com

Soto, E., K. Bowles, D. Fernandez and J. P. Hawke (2010). "Development of a real-time PCR assay for identification and quantification of the fish pathogen *Francisella noatunensis* subsp. *orientalis*." Diseases of Aquatic Organisms **89**(3): 199-207.

Soto, E., M. Griffin, M. Arauz, A. Riofrio, A. Martinez and M. E. Cabrejos (2012). "*Edwardsiella ictaluri* as the causative agent of mortality in cultured Nile Tilapia." Journal of Aquatic Animal Health **24**(2): 81-90.

Stankus, T. (2010). "U.S. rarm-raised catfish." Journal of Agricultural & Food Information **11**(3): 176-184.

Stock, I. and B. Wiedemann (2001). "Natural antibiotic susceptibilities of *Edwardsiella tarda*, *E. ictaluri*, and *E. hoshinae*." Antimicrobial Agents and Chemotherapy **45**(8): 2245-2255.

Sun, K., H.-l. Wang, M. Zhang, Z.-z. Xiao and L. Sun (2009). "Genetic mechanisms of multi-antimicrobial resistance in a pathogenic *Edwardsiella tarda* strain." Aquaculture **289**(1-2): 134-139.

Swain, P., S. K. Nayak, A. Sahu, B. C. Mohapatra and P. K. Meher (2002). "Bath immunisation of spawn, fry and fingerlings of Indian major carps using a particulate bacterial antigen." Fish and Shellfish Immunology **13**(2): 133-140.

Tan, R. J. S. and E. W. Lim (1977). "Occurance of *Edwardsiella tarda* in the household lizard, *Gecko gecko*." Japanese Journal of Medical Science and Biology **30**: 321-323.

Uhland, F. C., P. Hélie and R. Higgins (2000). "Infections of *Edwardsiella tarda* among Brook Trout in Quebec." Journal of Aquatic Animal Health **12**(1): 74-77.

USDA (1997). Catfish NAHMS '97, Part I: Reference of 1996 U.S. catfish health production practices. Fort Collins, Colorado, Centers for Epidemiology and Animal Health.

USDA (2016). Catfish production, National Agricultural Statistics Service.

USDA/APHIS (2011). Catfish 2010, Part II: Health and Production Practices for Foodsize Catfish in the United States, 2009. Fort Collins, Colorado, Centers for Epidemiology and Animal Health.

VanDamme, L. R. and J. Vandepitte (1980). "Frequent isolation of *Edwardsiella tarda* and *Pleisiomonas shigelloides* from healthy Zairese freshwater fish: a possible source of sporadic diarrhea in the tropics." Applied and Environmental Microbiology **39**(3): 475-479.



المنارة للاستشارات
www.manaraa.com

Versalovic, J., T. Koeuth and J. R. Lupski (1991). "Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes." Nucleic Acids Research **19**(24): 6823-6831.

Versalovic, J., M. Schneider, F. J. DeBruijn and J. R. Lupski (1994). "Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction." Methods in Molecular and Cellular Biology **5**: 25-40.

Wyatt, L. E., R. Nickelson and C. Vanderzant (1979). "*Edwardsiella tarda* in freshwater catfish and their environment." Applied and Environmental Microbiology **38**(4): 710-714.

Xu, T. and X.-H. Zhang (2014). "*Edwardsiella tarda*: an intriguing problem in aquaculture." Aquaculture **431**: 129-135.

Yamada, Y. and H. Wakabayashi (1998). "Enzyme electrophoresis, catalase test and PCR-RFLP analysis for the typing of *Edwardsiella tarda*." Fish Pathology **33**(1-5).

Yamada, Y. and H. Wakabayashi (1999). "Identification of fish-pathogenic strains belonging to the genus *Edwardsiella* by sequence analysis of *sodB*." Fish Pathology **34**(3): 145-150.

Yang, M., Y. Lv, J. Xiao, H. Wu, H. Zheng, Q. Liu, Y. Zhang and Q. Wang (2012). "*Edwardsiella* comparative phylogenomics reveal the new intra/inter-species taxonomic relationships, virulence evolution and niche adaptation mechanisms." PLoS One **7**(5): e36987.



المنارة للاستشارات
www.manaraa.com

CHAPTER II

# REAL-TIME POLYMERASE CHAIN REACTION ASSAYS FOR THE DETECTION AND QUANTIFICATION OF *EDWARDSIELLA TARDA*, *EDWARDSIELLA PISCICIDA*, AND *EDWARDSIELLA PISCICIDA*-LIKE SPECIES IN CATFISH TISSUES AND POND WATER

Previously published: Reichley, S. R., C. Ware, T. E. Greenway, D. J. Wise and M. J. Griffin (2015). "Real-time polymerase chain reaction assays for the detection and quantification of *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like species in catfish tissues and pond water." Journal of Veterinary Diagnostic Investigation **27**(2): 130-139.

## 2.1 Introduction

First recognized in the late 1960s (Ewing, McWhorter et al. 1965), *Edwardsiella* are a diverse group of enteric Gram-negative bacteria, infecting a wide range of piscine, avian, reptilian, and mammalian hosts (Meyer and Bullock 1973, Hawke and Khoo 2004, Mohanty and Sahoo 2007). Until 2013, the genus consisted of only 3 taxa: *E. tarda*, *E. ictaluri*, and *E. hoshinae* (Abayneh, Colquhoun et al. 2013). There is a dearth of information regarding *E. hoshinae*, which is primarily considered a pathogen of birds and reptiles (Grimont, Grimont et al. 1980, Stock and Wiedemann 2001). By comparison, *E. ictaluri* is well studied. Principally considered a pathogen of cultured channel catfish (*Ictalurus punctatus*), *E. ictaluri* can also infect other fish species (Hawke, McWhorter et al. 1981, Plumb and Sanchez 1983, Geng, Wang et al. 2013). Recent reports have



www.manaraa.com

implicated *E. ictaluri* in mortality events outside the farm-raised catfish industry of the southeastern United States, suggesting a more cosmopolitan distribution than previously thought (Ye, Li et al. 2009, Soto, Griffin et al. 2012, Hawke, Kent et al. 2013).

*Edwardsiella tarda* is the most widespread and diverse member of the genus, infecting a wide range of hosts from a variety of ecological niches (Mohanty and Sahoo 2007). Primarily thought of as a pathogen of marine and freshwater fish, *E. tarda* has been demonstrated to have extensive genetic and phenotypic diversity. In 2012, a comparative phylogenomic study of *Edwardsiella* isolates identified 2 genetically distinct groups (EdwI and EdwII) among organisms phenotypically classified as *E. tarda* (Yang, Lv et al. 2012). The work was supported by multilocus sequence analysis of 8 different housekeeping genes, which identified 2 distinct genetic taxa (*E. tarda*–like and ET883-like) among *E. tarda* isolates from Europe and Asia (Abayneh, Colquhoun et al. 2012). Concurrently, researchers in the United States proposed the existence of 2 genetic taxa within *E. tarda*, based on the existence of 2 distinct genetic groups (DNA group I and DNA group II) from fishes in the southeastern United States (Griffin, Quiniou et al. 2013). In 2013, based on comparative phylogenomics (Yang, Lv et al. 2012), multilocus sequence analysis (Abayneh, Colquhoun et al. 2012) and DNA–DNA hybridization experiments, the taxon *E. piscicida* was adopted (Abayneh, Colquhoun et al. 2013).

A 2014 survey of *Edwardsiella* isolates from diseased catfish in Mississippi demonstrated that *E. piscicida* was more commonly associated with disease case submissions of farm-raised catfish than *E. tarda* or *E. piscicida*–like sp. (Griffin, Ware et al. 2014). Species-specific polymerase chain reaction (PCR) assays targeting the fimbrial gene cluster were developed for each individual taxa and were demonstrated specific to



المنارة للاستشارات
www.manaraa.com

their respective target organisms (Sakai, Yuasa et al. 2009, Griffin, Ware et al. 2014). In the current study, real-time PCR (qPCR) assays were developed using these established primer sets and were validated for the detection and quantification of *E. tarda*, *E. piscicida*, and *E. piscicida*–like sp. from catfish kidney tissues, pond water, and broth culture.

## 2.2 Materials and methods

### 2.2.1 Bacterial cultures and isolation of genomic DNA

The *Edwardsiella* strains used in the validation of the assays in the current study were characterized as part of an earlier study (Griffin, Ware et al. 2014) and identified by *gyrB* sequencing and species-specific PCR (Table 2.1). In addition, an *Edwardsiella hoshinae* strain (ATCC 35051), an *Escherichia coli* strain (ATCC 25922), 2 *Flavobacterium columnare* strains (94-081 and ATCC 49512), and 2 *Aeromonas hydrophila* strains (ML 09-119 and TN 97-08), including a highly virulent strain (ML 09-119) attributed to disease outbreaks in farm-raised catfish (Hossain, Waldbieser et al. 2013) were also included in the validation process. Bacteria had been maintained at –80°C in brain–heart infusion (BHI) broth (BD Diagnostic Systems) supplemented with 20% (v/v) glycerol. Frozen cultures were streaked onto Mueller–Hinton agar (BD Diagnostic Systems) plates supplemented with 5% sheep blood (blood agar plates) and allowed to incubate for 24 hr at 37°C (*E. coli*, *E. piscicida*, *E. piscicida*–like, and *E. tarda*), 24 hr at 28°C (*A. hydrophila*), or 48 hr at 28°C (*E. hoshinae*, *E. ictaluri*, and *F. columnare*). Individual colonies were picked for each isolate and expanded overnight in BHI broth (BD Diagnostic Systems) at 28°C (*A. hydrophila*, *E. hoshinae*, *E. ictaluri*, *F. columnare*) or 37°C (*E. coli*, *E. piscicida*, *E. piscicida*–like, *E. tarda)*, respectively.



المنارة للاستشارات
www.manaraa.com

Genomic DNA (gDNA) was isolated from the cultured bacteria using a commercial kit (DNeasy blood and tissue kit, Qiagen), following the manufacturer's suggested protocol for Gram-negative bacteria, resuspended in 100 µl of a commercial DNA hydration solution (DHS; Qiagen), and quantified spectrophotometrically (NanoDrop; Thermo Fisher Scientific).



المنارة للاستشارات
www.manaraa.com

Table 2.1 Specificity of each *Edwardsiella* real-time polymerase chain reaction (qPCR) assay for the respective target.

| Isolate | *E. tarda* qPCR | *E. piscicida* qPCR | *E. piscicida*–like qPCR |
|---|---|---|---|
| *Aeromonas hydrophila* | | | |
| ML 09-119 | – | – | – |
| TN 97-08 | – | – | – |
| *Escherichia coli* | | | |
| ATCC 25922 | – | – | – |
| *Edwardsiella hoshinae* | | | |
| ATCC 35051 | – | – | – |
| *Edwardsiella ictaluri* | | | |
| S97-773 | – | – | – |
| *Edwardsiella piscicida* | | | |
| MA 97-004 | – | 31.2 (0.2) | – |
| S11-285 | – | 30.7 (0.1) | – |
| LADL 97-168 | – | 31.6 (0.3) | – |
| LADL 99-462 | – | 32.0 (0.2) | – |
| S07-346 | – | 30.6 (0.1) | – |
| S07-262 | – | 30.8 (0.1) | – |
| S07-534 | – | 31.2 (0.2) | – |
| S07-275 | – | 31.2 (0.1) | – |
| S07-1019 | – | 30.9 (0.1) | – |
| S07-348 | – | 31.6 (0.2) | – |
| *Edwardsiella piscicida*–like | | | |
| LADL 05-105 | – | – | 28.2 (0.2) |
| *Edwardsiella tarda* | | | |
| ATCC 15947 | 26.7 (0.2) | – | – |
| RE-04 | 28.3 (0.1) | – | – |
| AL 98-87 | 28.3 (0.2) | – | – |
| LADL 88-209 | 27.1 (0.2) | – | – |
| FL 95-01 | 28.5 (0.2) | – | – |
| LADL 99-302 | 27.7 (0.2) | – | – |
| *Flavobacterium columnare* | | | |
| 94-081 | – | – | – |
| ATCC 49512 | – | – | – |

Analysis was performed in triplicate using approximately 50 pg of genomic DNA from each isolate. Values are reported in terms of the mean (± the standard deviation) quantification cycle (Cq) of the triplicate reactions. The user-defined baseline fluorescence threshold for Cq determination was set at 50 relative fluorescent units for all runs. Dash (–) indicates no amplification of target DNA.



المنارة للاستشارات
www.manaraa.com

### 2.2.2 Design of primer and probe sets

The development of the qPCR assays specific to *E. tarda*, *E. piscicida*, and *E. piscicida*–like sp. was based on previously published PCR primers (Sakai, Yuasa et al. 2009, Griffin, Ware et al. 2014). Oligonucleotide probes corresponding to each primer set were designed using primer design software (Primer3) (Rozen and Skaletsky 2000) and synthesized commercially (Sigma-Aldrich). Each probe was labeled with the fluorescent reporter dye, 6-carboxyfluorescein, on the 5'-end, and the quencher dye, black hole quencher-1, on the 3'-end. Sequences and other relevant information for each primer and probe set can be found in Table 2.2.

Table 2.2 Real-time polymerase chain reaction primers and probes used in the current study.

| Primer | Sequence (5'–3') | %GC | Tm (°C) |
|---|---|---|---|
| *Edwardsiella tarda* | | | |
| ET3518F | CAGTGATAAAAAGGGGTGGA | 45.00 | 57.52 |
| ET3632R | CTACACAGCAACGACAACG | 52.63 | 56.35 |
| ET3559P | AGACAACAGAGGACGGATGTGGC | 56.52 | 66.99 |
| *Edwardsiella piscicida* | | | |
| EP14529F | CTTTGATCATGGTTGCGGAA | 45.00 | 61.95 |
| EP14659R | CGGCGTTTTCTTTTCTCG | 50.00 | 59.54 |
| EP14615P | CCGACTCCGCGCAGATAACG | 65.00 | 68.31 |
| *Edwardsiella piscicida*–like | | | |
| EPL1583F | GATCGGGTACGCTGTCAT | 55.56 | 56.92 |
| EPL1708R | AATTGCTCTATACGCACGC | 47.37 | 56.62 |
| EPL1611P | CCCGTGGCTAAATAGGACGCG | 61.90 | 67.77 |

Each oligonucleotide probe was labeled with the fluorescent reporter dye, 6-carboxyfluorescein, on the 5'-end, and the quencher dye, black hole quencher-1, on the 3'-end. Melting temperatures (Tm) for oligonucleotide primers and probes were calculated using the default parameters of Primer3. GC = guanine and cytosine content; F = forward; R = reverse; P = probe.



www.manaraa.com

### 2.2.3 Generation of PCR standards

Standards for relative quantification of target DNA were generated from purified PCR products. Briefly, for each assay, target PCR amplicons were produced from gDNA isolated from *E. tarda* (ATCC 15947), *E. piscicida* (S11-285), and *E. piscicida*–like (LADL 05-105) isolates following published protocols (Griffin, Ware et al. 2014). To confirm the presence of a single, appropriately sized band, amplicons were visualized under ultraviolet light after electrophoretic passage through agarose in the presence of ethidium bromide (1 μg/ml). Band sizes were estimated by concurrent passage of a molecular weight marker (Hyperladder II; Bioline). Lastly, amplicons were purified using a commercial PCR purification kit (QIAquick; Qiagen), resuspended in DHS, and quantified spectrophotometrically (NanoDrop; Thermo Fisher Scientific).

### 2.2.4 Quantitative PCR

The 15-μl PCR reactions contained 8 μl of PCR master mix (TaqMan Environmental Mastermix; Applied Biosystems), 10 pM of each primer, 1 pM of probe, DNA template, and nuclease-free water to volume. Amplifications were performed on a qPCR system (CFX96; Bio-Rad) programmed for 1 cycle of 95°C for 15 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data collection was carried out following the 60°C annealing/extension step at the end of each cycle. For each plate, samples, as well as no-template negative controls, were run in triplicate.

### 2.2.5 PCR specificity, sensitivity, repeatability, and reproducibility

The specificity of each assay was tested against both target and non-target DNA. Genomic DNA (approximately 50 pg) from *E. tarda*, *E. piscicida*, *E. piscicida*–like, *E.*



المنارة للاستشارات
www.manaraa.com

*ictaluri*, *E. hoshinae*, *A. hydrophila*, *F. columnare*, and *E. coli* were analyzed in triplicate using the reaction conditions and thermal cycling parameters described above. Quantification cycles (Cq) for each reaction were based on a user-defined baseline threshold of 50 relative fluorescent units (RFU).

The sensitivity and linear dynamic range of each assay was determined using serial dilutions of known quantities of target DNA (purified PCR amplicons), ranging from 1 to $1 \times 10^8$ copies of target DNA per 15-µl reaction. Each dilution series was run in triplicate on 3 separate occasions to assess repeatability and reproducibility of the assay. The Cq for each reaction was based on a user-defined baseline threshold of 50 RFUs.

### 2.2.6 Detection of target DNA from broth culture, fish tissue, and pond water

The ability of each assay to detect target DNA from different substrates was evaluated. Known quantities of *E. tarda*, *E. piscicida*, and *E. piscicida*–like sp. were added to catfish kidney biopsies, pond water, or processed directly in broth culture. Initially, cryostocks of *E. tarda* (ATCC 15947), *E. piscicida* (S11-285), and *E. piscicida*–like sp. (LADL 05-105) were streaked for isolation, and individual colonies were grown in 9 ml of BHI broth for 5 hr at 37°C without agitation. After a 10-fold serial dilution, plate counts were performed in triplicate (drop-plate method) on blood agar plates using 20-µl aliquots from each dilution. Additional 20-µl aliquots from each dilution were transferred to 1.5-ml microcentrifuge tubes and stored at –80°C until processing. These cryogenically stored aliquots corresponded to aliquots used for plate counts and represented known quantities of bacteria that could be added to water samples and catfish kidney biopsies. Three aliquots from each dilution were analyzed for each substrate (pond water, catfish kidney tissue, and BHI broth) representing colony forming unit



المنارة للاستشارات
www.manaraa.com

(CFU) equivalents ranging from 1 to $1 \times 10^6$ CFUs per 20-µl aliquot. Isolation of gDNA from bacteria in broth culture was carried out using a commercial kit (DNeasy; Qiagen), following the manufacturer's suggested protocol for Gram-negative bacteria. Isolated gDNA was resuspended in 100 µl of DHS, and 5 µl of gDNA suspension from each aliquot was used in qPCR analysis. Each aliquot was run in triplicate on a plate containing no-template controls (run in triplicate) and standard positive controls (run in duplicate). Positive controls consisted of purified and quantified PCR amplicons, ranging from 5 to $5 \times 10^5$ copies of target DNA.

To evaluate the ability of the assays to detect target organisms in fish tissues, cryogenically stored aliquots of known quantities of bacteria were added to posterior kidney biopsies (approximately 25 mg) collected aseptically from channel catfish reared for disease research at the holding facility of the Thad Cochran National Warmwater Aquaculture Center (TCNWAC; Stoneville, Mississippi). Initially, catfish kidney tissues were confirmed negative for *Edwardsiella* spp. by culture and by qPCR using the assays described herein. Three aliquots from each dilution were added directly to individual kidney tissue samples prior to homogenization. Genomic DNA from spiked kidney tissues was isolated using a commercial kit (DNeasy; Qiagen), following the manufacturer's suggested protocol for animal tissues. The isolated gDNA was resuspended in 200 µl of DHS, and 5 µl of gDNA from each aliquot was used as template in qPCR analysis. As above, each aliquot was run in triplicate on a plate containing no-template controls (run in triplicate) and concurrently run standard positive controls (run in duplicate).



المنارة للاستشارات
www.manaraa.com

Similarly, to determine the ability of the assays to detect and quantify target organisms in catfish pond water, aliquots of known quantities of bacteria were added to pond water samples. Prior to the addition of bacteria, pond water used for this analysis was confirmed negative for *Edwardsiella* spp. by qPCR. Based on previously established protocols for the detection of bacteria in catfish pond water (Griffin, Mauel et al. 2011, Griffin, Goodwin et al. 2013), a water sample (20 L) was collected from a commercial catfish pond and processed within 24 hr of collection. A subsample (35 ml) of the pond water was added to a 40-ml round-bottom centrifuge tube and centrifuged at 20,000 × *g* for 10 min. The supernatant was removed, and the pellet was resuspended in 1.5 ml of nuclease-free water and transferred to a 1.8-ml microcentrifuge tube. A 20-µl aliquot from each broth culture dilution was added directly to each pellet, and DNA isolation was carried out using a commercial kit (Powersoil DNA Isolation Kit; Mo Bio), following the manufacturer's suggested protocol for wet samples. Isolated DNA was resuspended in 100 µl of elution buffer, and 5 µl was used in each individual qPCR, carried out as above. For the purposes of calculating averages throughout the study, negative reactions were assigned an RFU of 0, $Log_{10}$ starting quantities of 0.0, and Cq values of 40.0.

### 2.2.7 Detection in experimentally infected fish

The ability of the assay to detect target DNA in clinical and subclinical, experimentally infected fish was evaluated. Channel catfish fingerlings (mean weight: 21.9 g; range: 12.8–30.2 g) were obtained from the TCNWAC fish-rearing facility. For the challenge, 30 fish were placed in twenty 80-L aquaria containing 20 L of well water and held under flow-through conditions (1 L/min) with constant aeration. Bacterial cultures of *E. piscicida*, *E. piscicida*–like, and *E. tarda* were grown as described above.



المنارة للاستشارات
www.manaraa.com

Two tanks of channel catfish fingerlings were anesthetized with tricaine methanesulfonate (MS-222; Western Chemical) and injected intraperitoneally with one of the treatments (i.e., 1 dilution of 1 of the 3 bacteria): *E. piscicida* ($1.83 \times 10^5$, $1.83 \times 10^6$, and $1.83 \times 10^7$), *E. piscicida*–like ($1.33 \times 10^5$, $1.33 \times 10^6$, and $1.33 \times 10^7$), and *E. tarda* ($2.92 \times 10^5$, $2.92 \times 10^6$, and $2.92 \times 10^7$). The remaining 2 tanks were negative controls that were handled similarly but injected intraperitoneally with sterile BHI broth. For each treatment, 1 tank was designated for sampling (sampling tank) and 1 tank (mortality tank) was used to estimate the median lethal dose ($LD_{50}$) of each bacterial strain based on the number of dead fish observed for each dose (Reed 1938). The mortality tank was checked twice daily, and the number of dead fish was recorded. Apparently healthy fish ($n = 3$), with no outward signs of disease, were collected from the sampling tank at 1, 2, 5, and 7 days post-injection. In addition, dead fish observed in the sampling tanks were also collected and processed for qPCR. All sampled fish were euthanized using MS-222, and posterior kidney biopsies (approximately 25 mg) were obtained aseptically, streaked on blood agar plates, and incubated for 24 hr at 37°C to determine the presence of viable bacteria. Genomic DNA was isolated from the kidney biopsies using a commercial kit, as above. The isolated gDNA was resuspended in 200 µl of DHS, and 5 µl of gDNA was used in each individual qPCR, carried out as above.

## 2.3 Results

### 2.3.1 PCR specificity, sensitivity, repeatability, and reproducibility

Each qPCR assay demonstrated robust amplification from gDNA isolated from their respective targets, with no amplification from gDNA isolated from non-target organisms (Table 2.1). Using 10-fold serial dilutions of PCR amplicons, each assay was



المنارة للاستشارات
www.manaraa.com

linear over 8 orders of magnitude and sensitive to an estimated 5 copies of target DNA (Figure 2.1). Reactions with <5 copies of target DNA resulted in inconsistent amplification, often with no observed signal. Throughout the study, reaction efficiencies were calculated from the slope of the log-linear portion of concurrently run standards using equation 2.1 (Bustin, Benes et al. 2009) and were within the generally accepted range of 90–110% (*E. piscicida*, range: 91.3–98.0%, mean: 94.8%; *E. piscicida*–like sp., range: 94.7–105.3%, mean: 100.6%; *E. tarda*, range: 101.8–107.9%, mean: 104.4%).

$$\text{PCR efficiency} = 10^{-1/\text{slope}} - 1 \qquad (2.1)$$



المنارة للاستشارات
www.manaraa.com

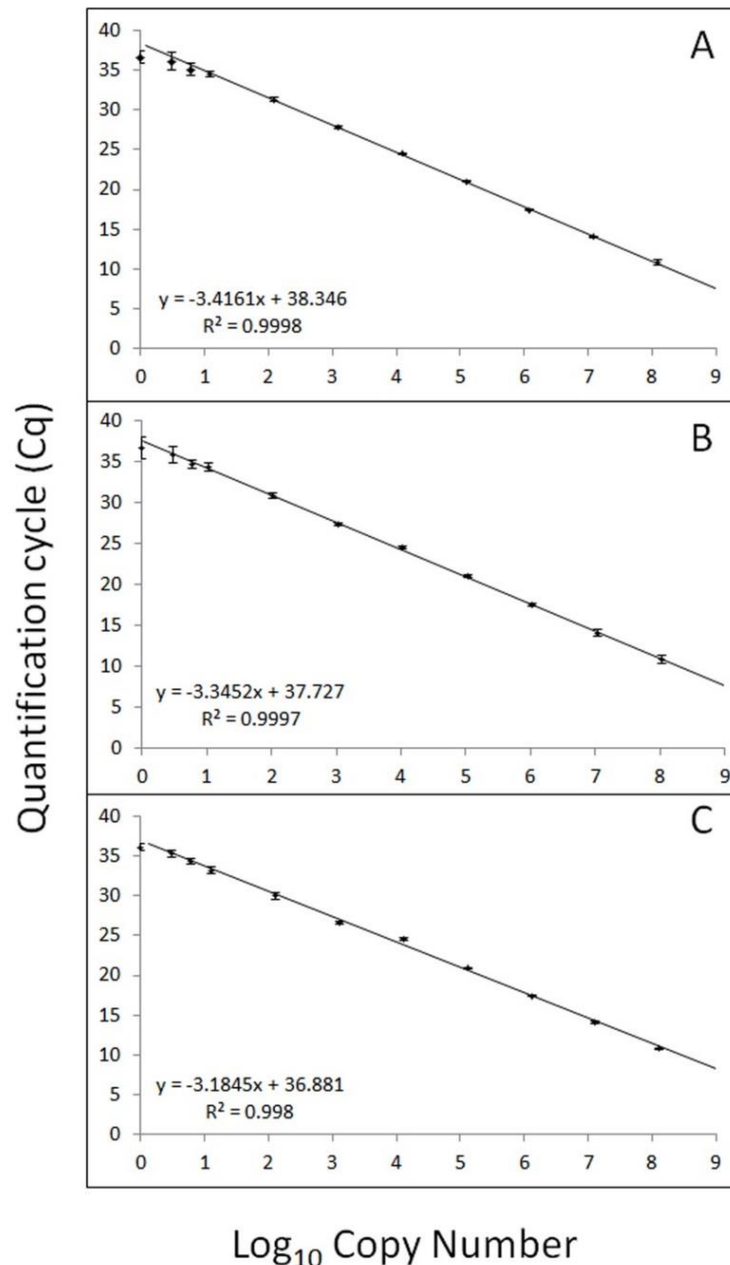


Figure 2.1 Mean quantification cycles for known quantities of *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda* target DNA.

Mean quantification cycles (Cq) for known quantities of *Edwardsiella piscicida* (**A**), *Edwardsiella piscicida*–like sp. (**B**), and *Edwardsiella tarda* (**C**) target DNA. A 10-fold dilution series of quantified purified polymerase chain reaction (PCR) product was analyzed for each assay. Error bars indicate standard deviations generated from dilution series run in triplicate on 3 separate plates. The user-defined baseline fluorescence threshold for Cq determination was set at 50 relative fluorescent units for all runs. Reaction efficiencies for each assay were calculated from the slope of the log-linear portion of concurrently run standards (PCR efficiency = $10^{-1/\text{slope}} - 1$) and were within the generally accepted range of 90–110% (*E. piscicida*: 96.2%; *E. piscicida*–like sp.: 99.0%; *E. tarda*: 107.9%).



المنارة للاستشارات
www.manaraa.com

### 2.3.2 Clinical sensitivity and assay variability

Each assay detected target DNA in gDNA preparations from approximately 100 CFU per sample from broth culture. For samples with <100 CFU, amplification was inconsistent between replicates and occasionally absent, with a proportion (44.4% for *E. tarda*; 77.7% for *E. piscicida*; 44.4% for *E. piscicida*–like sp.) of reactions from aliquots of <100 CFU per sample giving negative results. When present, amplification resulted in Cq values similar to those observed for approximately 100 CFU, demonstrating a plateau effect common near the quantifiable limits of qPCR assays. In addition, several (55.5%) reactions corresponding to aliquots of 136 CFU for *E. piscicida* were negative, suggesting this quantity was at or below the limits of the quantifiable or detectable range of the assay. Results from pond water and catfish kidney samples spiked with known quantities of target bacteria were similar to results obtained from broth culture, although kidney samples containing <100 CFU resulted in inconsistent amplification between replicates, with a proportion (44.4% for *E. tarda*; 33.3% for *E. piscicida*; 44.4% for *E. piscicida*–like sp.) of reactions giving negative results. Again, several (33.3%) reactions corresponding to aliquots of 136 CFU for *E. piscicida* were also negative. Each assay was linear over at least 5 orders of magnitude in these experiments and, under the conditions used in this study, the 3 assays had a quantifiable limit ranging from $10^3$ (*E. piscicida*) to $10^2$ (*E. piscicida*–like and *E. tarda*) CFU in kidney tissue biopsies (approximately 25 mg), pond water samples (35 ml) and broth culture (20µl; Figure 2.2; Table 2.3).



المنارة للاستشارات
www.manaraa.com

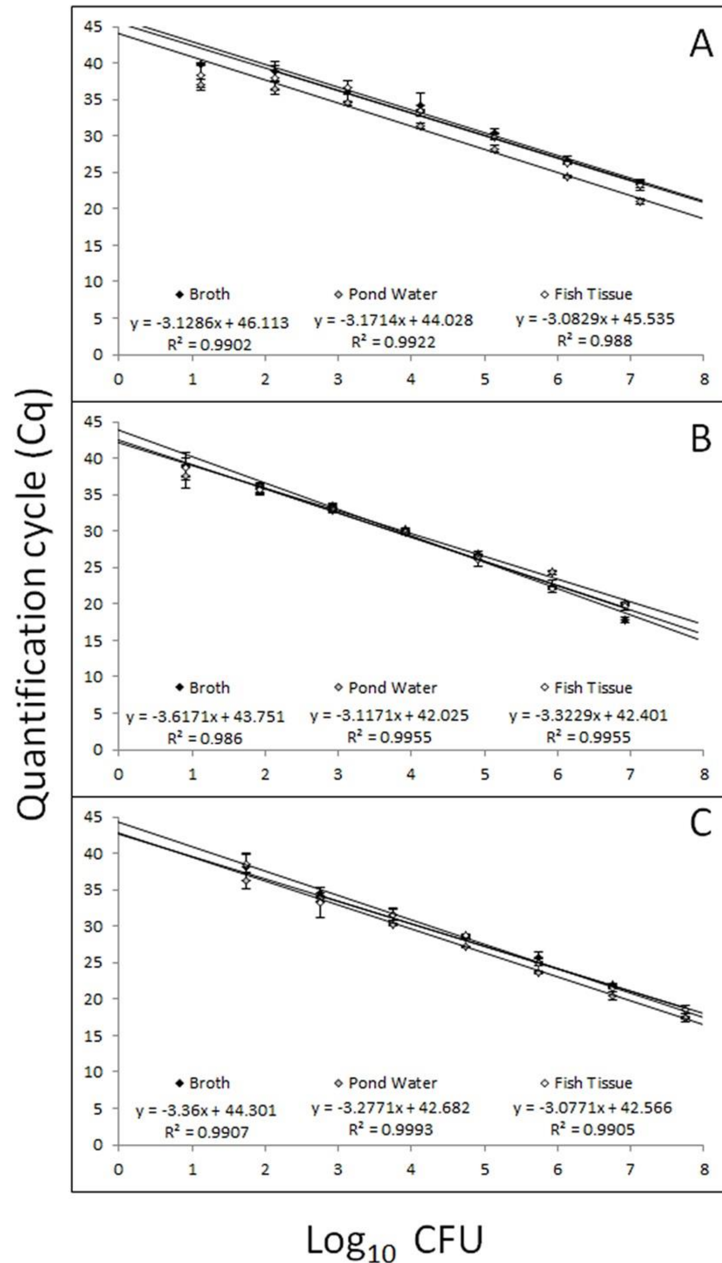


Figure 2.2 Mean quantification cycles for known quantities of *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda* in brain–heart infusion broth, pond water, or channel catfish (*Ictalurus punctatus*) posterior kidney tissue.

Mean quantification cycles (Cq) for known quantities of *Edwardsiella piscicida* (**A**), *Edwardsiella piscicida*–like sp. (**B**), and *Edwardsiella tarda* (**C**) cells in brain–heart infusion broth, pond water, or channel catfish (*Ictalurus punctatus*) posterior kidney tissue. Error bars indicate standard deviations from 3 different sample preparations. The user-defined baseline fluorescence threshold for Cq determination was set at 50 relative fluorescent units for all runs. For the purposes of plotting, reactions in which no amplification was observed were assigned Cq values of 40.



المنارة للاستشارات
www.manaraa.com

Table 2.3 Mean (± the standard deviation) quantification cycles, $Log_{10}$ copy number, and relative fluorescent units for known quantities of *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda* cells in brain–heart infusion (BHI) broth, pond water, or channel catfish (*Ictalurus punctatus*) posterior kidney tissue.

| Approximate CFU | BHI broth | | | | Pond water | | | | Kidney tissue | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cq | RFU | Copy no. | CV (%) | Cq | RFU | Copy no. | CV (%) | Cq | RFU | Copy no. | CV (%) |
| *Edwardsiella piscicida* | | | | | | | | | | | | |
| 13 | 39.9 (0.2)† | 10.4 (19.9) | 0.1 (0.3) | 198.4 | 37.0 (0.8) | 149.5 (40.1) | 1.2 (0.2) | 19.4 | 38.3 (1.7)† | 90.3 (84.0) | 0.8 (0.6) | 76.7 |
| 136 | 38.9 (1.4)† | 58.6 (54.0) | 0.6 (0.6) | 90.2 | 36.5 (0.9) | 179.8 (52.4) | 1.3 (0.3) | 21.0 | 38.0 (1.7)† | 106.4 (85.0) | 0.8 (0.7) | 80.1 |
| 1,360 | 36.1 (1.5) | 180.1 (87.7) | 1.6 (0.4) | 26.4 | 34.5 (0.3) | 293.2 (23.3) | 1.9 (0.1) | 4.9 | 36.6 (1.0) | 176.3 (56.5) | 1.4 (0.3) | 20.2 |
| 13,600 | 34.2 (1.6) | 269.0 (92.2) | 2.1 (0.5) | 21.7 | 31.4 (0.4) | 386.0 (58.5) | 2.8 (0.1) | 3.7 | 33.4 (0.2) | 353.9 (21.7) | 2.3 (0.1) | 3.0 |
| 136,000 | 30.4 (0.6) | 437.0 (49.3) | 3.2 (0.2) | 5.5 | 28.2 (0.5) | 442.3 (71.1) | 3.8 (0.2) | 4.2 | 29.9 (0.4) | 489.1 (38.0) | 3.3 (0.1) | 3.1 |
| 1,360,000 | 26.7 (0.6) | 489.1 (56.7) | 4.2 (0.2) | 3.8 | 24.4 (0.2) | 440.9 (53.9) | 4.9 (0.1) | 0.9 | 26.3 (0.2) | 511.1 (37.5) | 4.3 (0.0) | 1.1 |
| 13,600,000 | 23.4 (0.5) | 506.4 (62.0) | 5.2 (0.2) | 2.9 | 21.0 (0.4) | 409.4 (82.3) | 5.9 (0.1) | 2.1 | 23.3 (0.7) | 488.5 (10.3) | 5.2 (0.2) | 3.9 |
| *Edwardsiella piscicida*–like sp. | | | | | | | | | | | | |
| 8.3 | 38.9 (1.3)† | 55.4 (62.6) | 0.5 (0.5) | 110.6 | 37.5 (1.6)† | 125.5 (83.2) | 1.1 (0.7) | 60.9 | 38.8 (1.3) | 62.4 (64.8) | 0.5 (0.5) | 103.6 |
| 83 | 35.8 (0.7) | 206.9 (40.5) | 1.5 (0.2) | 15.2 | 36.0 (0.6) | 214.6 (30.7) | 1.6 (0.2) | 10.9 | 35.6 (0.6) | 239.7 (34.2) | 1.5 (0.2) | 12.6 |
| 830 | 33.5 (0.3) | 330.1 (26.9) | 2.2 (0.1) | 4.9 | 32.9 (0.3) | 383.1 (18.2) | 2.5 (0.1) | 3.1 | 33.0 (0.6) | 381.7 (26.8) | 2.3 (0.2) | 8.0 |
| 8,300 | 30.2 (0.3) | 447.1 (29.0) | 3.2 (0.1) | 3.2 | 29.8 (0.3) | 478.3 (40.5) | 3.4 (0.1) | 2.6 | 29.9 (0.2) | 479.1 (26.9) | 3.3 (0.1) | 2.1 |
| 83,000 | 26.9 (0.3) | 512 (41.9) | 4.2 (0.1) | 2.0 | 26.5 (0.1) | 561.2 (30.9) | 4.4 (0.0) | 0.9 | 26.0 (0.8) | 562.3 (63.0) | 4.5 (0.2) | 5.4 |
| 830,000 | 22.4 (0.9) | 537.7 (55.5) | 5.6 (0.3) | 5.0 | 24.3 (0.2) | 559.2 (39.8) | 5.0 (0.1) | 1.4 | 22.2 (0.2) | 572.2 (59.7) | 5.7 (0.1) | 1.2 |
| 8,300,000 | 17.8 (0.3) | 530.2 (42.2) | 7.0 (0.1) | 1.4 | 20.0 (0.2) | 585.4 (29.5) | 6.3 (0.1) | 1.1 | 19.6 (0.4) | 567.2 (41.8) | 6.5 (0.1) | 2.0 |
| *Edwardsiella tarda* | | | | | | | | | | | | |
| 56 | 38.3 (1.8)† | 85.0 (92.9) | 0.6 (0.6) | 107.1 | 36.2 (1.2) | 196.9 (64.3) | 1.3 (0.3) | 25.1 | 38.6 (1.4 )† | 72.0 (68.2) | 0.5 (0.4) | 95.0 |
| 560 | 34.6 (0.7) | 276.0 (36.4) | 1.8 (0.2) | 12.5 | 33.8 (0.4) | 324.8 (25.0) | 2.1 (0.1) | 6.3 | 33.3 (2.1) | 324.0 (84.3) | 2.1 (0.7) | 10.4 |
| 5,600 | 31.6 (0.8) | 382.8 (34.0 | 2.7 (0.3) | 9.5 | 30.3 (0.2) | 423.6 (32.7) | 3.1 (0.1) | 1.7 | 31.5 (0.8) | 393.7 (66.1) | 2.7 (0.3) | 9.6 |
| 56,000 | 28.6 (0.2) | 413.1 (28.8) | 3.7 (0.1) | 1.9 | 27.2 (0.1) | 438.1 (27.8) | 4.1 (0.0) | 0.9 | 28.7 (0.3) | 371.0 (52.5) | 3.6 (0.1) | 2.4 |
| 560,000 | 25.8 (0.6) | 415 (44.9) | 4.5 (0.2) | 4.0 | 23.7 (0.2) | 461.2 (30.9) | 5.1 (0.0) | 0.9 | 25.0 (0.5) | 434.9 (52.9) | 4.8 (0.2) | 3.4 |
| 5,600,000 | 22.0 (0.2) | 418.6 (24.6) | 5.7 (0.1) | 0.9 | 20.4 (0.6) | 455.0 (24.9) | 6.1 (0.2) | 2.8 | 21.5 (0.1) | 430.7 (31.2) | 5.9 (0.0) | 0.8 |
| 56,000,000 | 17.4 (0.5) | 413.7 (26.4) | 7.1 (0.2) | 2.1 | 17.5 (0.2) | 445.0 (21.0) | 7.0 (0.1) | 0.8 | 18.5 (0.6) | 410.2 (70.6) | 6.8 (0.2) | 2.8 |

The user-defined baseline fluorescence threshold for quantification cycles (Cq) determination was set at 50 relative fluorescent units (RFU) for all runs. The intra-assay coefficient of variation (CV) for the mean $Log_{10}$ copy number from 3 separate sample preparations is listed for each aliquot. Reactions in which no amplification was observed were assigned Cq values of 40, RFU values of 0, and $Log_{10}$ copy numbers of 0.0. † Amplification was not observed in all replicate reactions.



المنارة للاستشارات
www.manaraa.com

### 2.3.3 Detection in experimentally infected fish

The qPCR assays consistently amplified target DNA from apparently healthy, subclinically infected fingerlings in all experimental treatment groups up to 5 days post-injection. Large quantities of target DNA were detected from dead or moribund fish clinically infected with *E. piscicida* and *E. tarda* (Table 2.4), often equating to 4 or more orders of magnitude above the clinical sensitivity of the assay. In addition, target bacteria were confirmed by qPCR from culture in 97% (31/32 fish) of *E. piscicida* mortalities and 100% (7/7 fish) of *E. tarda* mortalities.



المنارة للاستشارات
www.manaraa.com

Table 2.4 Mean (± the standard deviation) quantification cycle and $Log_{10}$ copy number for channel catfish (*Ictalurus punctatus*) fingerlings experimentally challenged with *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda*.

| Approximate dose | Apparently healthy fish | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | | Day 2 | | Day 5 | | Day 7 | |
| | Cq | Copy no. | Cq | Copy no. | Cq | Copy no. | Cq | Copy no. |
| *E. piscicida* | | | | | | | | |
| $1.83 \times 10^5$ | 36.3 (2.2) | 1.1 (0.6) | 36.9 (3.9) | 1.5 (0.9) | 37.7 (3.5) | 0.8 (0.9) | 39.4 (0.03)† | 0.2 (0.3) |
| $1.83 \times 10^6$ | 30.4 (5.1) | 2.9 (1.4) | 35.0 (3.4) | 1.8 (0.9) | 36.1 (3.4) | 1.3 (0.8) | 39.9 (0.1) | 0.1 (0.2) |
| $1.83 \times 10^7$ | 25.9 (2.5) | 4.3 (0.7) | 25.2 (4.5) | 4.5 (1.1) | NF | NF | NF | NF |
| *E. piscicida*–like sp. | | | | | | | | |
| $1.33 \times 10^5$ | 35.9 (1.5) | 1 (0.5) | 36.6 (1.7) | 0.4 (0.3) | 38.6 (2.5) | 0.2 (0.3) | 37.9 (0.7) | 0.2 (0.3) |
| $1.33 \times 10^6$ | 31.0 (2.6) | 2.4 (0.7) | 34.0 (0.9) | 1.5 (0.3) | 35.7 (0.7) | 0.7 (0.2) | 37.8 (1.9) | 0.3 (0.3) |
| $1.33 \times 10^7$ | 26.1 (0.7) | 3.8 (0.2) | 28.2 (0.7) | 3.2 (0.2) | 31.9 (1.7) | 1.8 (0.5) | 35.1 (1.2) | 1.1 (0.4) |
| *E. tarda* | | | | | | | | |
| $2.92 \times 10^5$ | 33.0 (1.3) | 1.4 (0.4) | 36.4 (0.5) | 0.4 (0.2) | 38.3 (2.9) | 0.5 (0.7) | 39.6 (0.3)† | 0.5 (0.9) |
| $2.92 \times 10^6$ | 32.4 (4.1) | 1.6 (1.1) | 31.5 (1.0) | 1.9 (0.3) | 38.5 (2.6) | 0.8 (0.8) | 36.1 (0.3) | 1.4 (0.2) |
| $2.92 \times 10^7$ | 21.7 (1.6) | 4.9 (0.4) | 22.4 (1.9) | 4.8 (0.5) | 32.0 (2.1) | 1.8 (0.6) | 32.7 (1.1) | 0.4 (0.3) |
| | Clinically affected fish | | | | | | | |
| | Day 1 | | Day 2 | | Day 3 | | | |
| | Cq | Copy no. | Cq | Copy no. | Cq | Copy no. | | |
| *E. piscicida* | | | | | | | | |
| $1.83 \times 10^5$ | NA | NA | NA | NA | 21.9 (0.2) [*n* = 2] | 5.4 (0.1) | | |
| $1.83 \times 10^6$ | 22.8 (1.4) [*n* = 2] | 5.2 (0.4) | 21.9 (1.5) [*n* = 2] | 5.5 (0.5) | 20.4 (0.8) [*n* = 2] | 5.9 (0.2) | | |
| $1.83 \times 10^7$ | 22 (0.9) [*n* = 4] | 5.5 (0.3) | 19.8 (0.9) [*n* = 20] | 6.1 (0.3) | NF | NF | | |
| *E. piscicida*–like sp. | | | | | | | | |
| $1.33 \times 10^5$ | NA | NA | NA | NA | NA | NA | | |
| $1.33 \times 10^6$ | NA | NA | NA | NA | NA | NA | | |
| $1.33 \times 10^7$ | NA | NA | NA | NA | NA | NA | | |
| *E. tarda* | | | | | | | | |
| $2.92 \times 10^5$ | NA | NA | NA | NA | NA | NA | | |
| $2.92 \times 10^6$ | NA | NA | NA | NA | NA | NA | | |
| $2.92 \times 10^7$ | 18.9 (1.4) [*n* = 7] | 5.6 (0.4) | NA | NA | NA | NA | | |

For each treatment, posterior kidneys were sampled from apparently healthy fish ($n = 3$), with no outward signs of disease, on days 1, 2, 5, and 7. When present, posterior kidneys were also sampled from dead, clinically affected fish. Three days post-challenge, all fish had either been sampled or died in the *E. piscicida* treatment group challenged with $1.83 \times 10^7$ CFU. Dead fish were observed in only 1 *E. tarda* treatment group ($2.92 \times 10^7$ CFU). No dead fish were observed in any of the *E. piscicida*–like treatments. The user-defined baseline fluorescence threshold for Cq determination was set at 50 relative fluorescent units for all runs. Reactions in which no amplification was observed were assigned Cq values of 40 and $Log_{10}$ copy numbers of 0.0. NF = no fish left; NA = no dead or moribund fish observed. † Amplification was not observed in all replicate reactions.



المنارة للاستشارات
www.manaraa.com

Regardless of challenge dose or isolate, viable bacteria were recovered from subclinically infected fish up to 5 days post-challenge. Of the apparently healthy, subclinically infected fish, 47% (48/102) did not exhibit bacterial growth on culture, and no viable bacteria were recovered from any fish sampled 7 days post-challenge. For each of the 3 target bacteria, negligible target DNA amplification was observed from several culture-negative fish (mean Cq = 37.2), equating to <3 CFU equivalents (Figure 2.2), below the reliable, clinical sensitivity of the assay. Similarly, amplification of target DNA from BHI-injected fish was also negligible, corresponding to quantities below the clinical and analytical sensitivity of the assay (mean Cq: *E. tarda*, 38.5; *E. piscicida*, 39.2; *E.* piscicida–like sp., 39.1).

Based on the cumulative mortalities found 7 days post-injection, the observed $LD_{50}$ for *E. piscicida* was $5.77 \times 10^5$ CFU. The $LD_{50}$ for *E. tarda* and *E. piscicida*–like sp. could not be determined. Only 17% mortality was observed in fish injected with $2.92 \times 10^7$ CFU of *E. tarda*, with no mortality seen in fish injected with $2.92 \times 10^5$ CFU or $2.92 \times 10^6$ CFU. Similarly, no mortality was observed in fish injected with *E. piscicida*–like sp. (Figure 2.3), even at doses as high as $1.33 \times 10^7$.



المنارة للاستشارات
www.manaraa.com

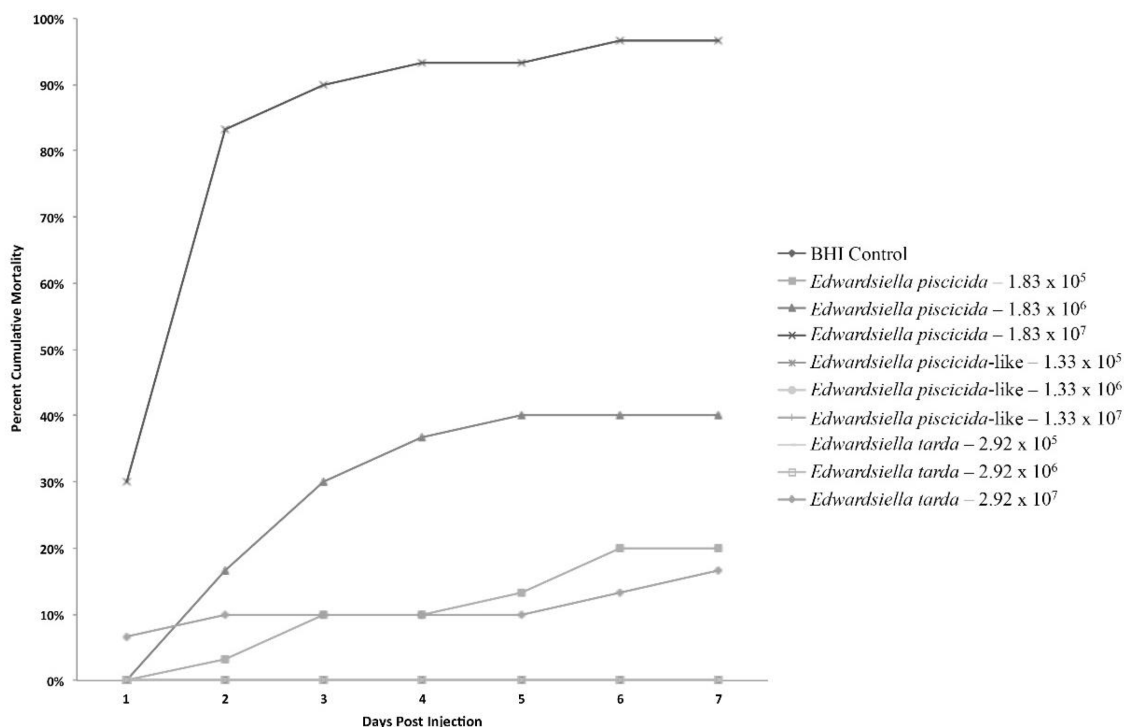


Figure 2.3 Nonreplicated cumulative mortality for channel catfish (*Ictalurus punctatus*) challenged with 3 different doses of *Edwardsiella piscicida*, *Edwardsiella piscicida*–like sp., and *Edwardsiella tarda*.

Sixty fish were challenged to each dose and distributed into 2 separate aquaria ($n$ = 30 fish/tank). Samples were collected for real-time polymerase chain reaction (qPCR) analysis from one tank (sampling tank) on days 1, 2, 5, and 7 (data presented in Table 4). No samples were collected from the second tank (mortality tank). Rather, the mortality tank was checked twice daily over the course of 7 days and dead fish recorded. The cumulative mortality observed in the mortality tank is reported.

## 2.4 Discussion

Real-time PCR assays are rapidly becoming more commonplace in fish disease work, for both research and diagnostic applications (Purcell, Getchell et al. 2011). Assays have been developed for the detection and quantification of a wide array of viral, parasitic, and bacterial fish pathogens in both fish tissues and the environment (Griffin, Pote et al. 2009, Bain, Cornwell et al. 2010, Soto, Bowles et al. 2010, Marancik and Wiens 2013). In catfish aquaculture, several assays have been developed for the detection



www.manaraa.com

and quantification of pathogens in the pond environment (Griffin, Pote et al. 2009, Griffin, Mauel et al. 2011, Griffin, Goodwin et al. 2013).

All 3 assays used in the current study were able to detect target DNA in both clinically and subclinically infected fish. While negligible amplification (Cq values ≥ 37.0) was observed from some culture-negative fish, it is assumed these high Cq values are artifacts of carryover contamination, amplification of nonspecific or background DNA, or degradation of the fluorescent probe (Burns 2008) rather than detection of nonviable organisms. While arbitrary cutoffs are not ideal (Bustin, Benes et al. 2009), this inconsistent late amplification represents target copy numbers below the clinical sensitivity of the assay. As such, it likely represents false-positives and, under the conditions described herein, Cq values of ≥37.0 in the absence of viable, cultured organisms should be considered suspect. In addition, due to differences in environmental conditions, dose effect, and other factors not accounted for during laboratory challenges, bacterial loads and qPCR results from naturally infected fish may differ from what is reported here. Future research will focus on establishing the clinical relevance of the values observed during experimental infections and how they relate to natural infections. Lastly, the data further demonstrates the limitations of using qPCR as a stand-alone diagnostic tool. Instead, qPCR is better employed as a research tool or a confirmatory test used in conjunction with identification of pathogens in histological sections or the culture and recovery of viable organisms.

At present, no distinguishing phenotypic trait has been identified to discriminate between *E. tarda*, *E. piscicida*, and *E. piscicida*–like sp. (Griffin, Quiniou et al. 2013, Griffin, Ware et al. 2014). However, the qPCR assays described herein were



www.manaraa.com

demonstrated to be highly reproducible and repeatable, with limited variability between runs. These assays provide rapid identification and differentiation of these phenotypically comparable organisms in both apparently healthy and clinically affected fish, a valuable aide for diagnostic assessments. In addition, the assays provide a means to detect and quantify these Gram-negative pathogens in catfish pond water, providing an invaluable tool for researchers and diagnosticians to evaluate the epidemiology of these organisms in cultured fish.

All surviving experimentally infected fish were culture negative by day 7, regardless of challenge dose or isolate. This is consistent with previous work investigating *E. tarda* pathogenesis in channel catfish (Darwish, Plumb et al. 2000). However, the current study demonstrated an apparent difference in virulence between *E. piscicida*, *E. piscicida*–like sp., and *E. tarda*. This variability warrants further investigation to better understand the pathogenicity of these *Edwardsiella* spp. in channel catfish.

Historically, *E. tarda* has been perceived as a pathogen of low occurrence in catfish aquaculture. However, according to summaries of diagnostic case submissions to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS (http://tcnwac.msstate.edu/publications.htm), there has been a relative increased incidence of *E. tarda* infections in farm-raised catfish in Mississippi over the last decade. Until 2013, all genetic variants of *E. tarda* were considered to be members of a genotypically diverse, yet phenotypically homogenous species. Research has demonstrated the *E. tarda* taxa is comprised of at least 2, possibly 3 phenotypically ambiguous, yet genetically distinct taxa, namely *E. tarda*, *E. piscicida*, and *E. piscicida*–



المنارة للاستشارات
www.manaraa.com

like sp. (Abayneh, Colquhoun et al. 2012, Yang, Lv et al. 2012, Abayneh, Colquhoun et al. 2013, Griffin, Quiniou et al. 2013). A survey of *E. tarda* isolates collected from diseased fish submissions to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS from 2007–2012 suggests that *E. piscicida* is more commonly associated with disease outbreaks in farm-raised catfish than *E. tarda.* A total of 44 archived isolates were examined, all of which were identified phenotypically as *E. tarda* upon initial isolation. All 44 were identified as *E. piscicida* by PCR (Griffin, Ware et al. 2014). The factors, both biotic and abiotic, that have contributed to the putative trend of increased incidence of *E. piscicida* in Mississippi aquaculture are currently unknown. However, these assays used in the current study will provide a valuable tool in identifying the aforementioned factors that may be attributing to this escalation.



المنارة للاستشارات
www.manaraa.com

## 2.5 References

Abayneh, T., D. J. Colquhoun and H. Sorum (2012). "Multi-locus Sequence Analysis (MLSA) of *Edwardsiella tarda* isolates from fish." Veterinary Microbiology **158**(3-4): 367-375.

Abayneh, T., D. J. Colquhoun and H. Sorum (2013). "*Edwardsiella piscicida* sp. nov., a novel species pathogenic to fish." Journal of Applied Microbiology **114**(3): 644-654.

Bain, M. B., E. R. Cornwell, K. M. Hope, G. E. Eckerlin, R. N. Casey, G. H. Groocock, R. G. Getchell, P. R. Bowser, J. R. Winton, W. N. Batts, A. Cangelosi and J. W. Casey (2010). "Distribution of an invasive aquatic pathogen (viral hemorrhagic septicemia virus) in the Great Lakes and its relationship to shipping." PLoS One **5**(4): e10156.

Burns, M. (2008). "Modelling the limit of detection in real-time quantitative PCR." European Food Research and Technology **226**: 1513-1524.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele and C. T. Wittwer (2009). "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments." Clinical Chemistry **55**(4): 611-622.

Darwish, A., J. Plumb and J. Newton (2000). "Histopathology and pathogenesis of experimental infection with *Edwardsiella tarda* in channel catfish." Journal of Aquatic Animal Health **12**(4): 255-266.

Ewing, W. H., A. C. McWhorter, M. R. Escobar and A. H. Lubin (1965). "*Edwardsiella*, a new genus of Enterobacteriaceae based on a new species, *E. tarda*." International Bulletin of Bacteriological Nomenclature and Taxonomy **15**(1): 33-38.

Geng, Y., K. Y. Wang, C. W. Li, S. Y. Ren, Z. Y. Zhou, X. X. Liu, X. F. Liu, W. M. Lai, X. L. Huang and D. F. Chen (2013). "Isolation and characterization of Edwardsiella ictaluri from southern catfish, *Silurus soldatovi meridionalis*, (Chen) cultured in China." Journal of the World Aquaculture Society **44**(2): 273-281.

Griffin, M. J., A. E. Goodwin, G. E. Merry, M. R. Liles, M. A. Williams, C. Ware and G. C. Waldbieser (2013). "Rapid quantitative detection of *Aeromonas hydrophila* strains associated with disease outbreaks in catfish aquaculture." Journal of Veterinary Diagnostic Investigation **25**(4): 473-481.

Griffin, M. J., M. J. Mauel, T. E. Greenway, L. H. Khoo and D. J. Wise (2011). "A real-time polymerase chain reaction assay for quantification of *Edwardsiella ictaluri* in catfish pond water and genetic homogeneity of diagnostic case isolates from Mississippi." Journal of Aquatic Animal Health **23**(4): 178-188.



المنارة للاستشارات
www.manaraa.com

Griffin, M. J., L. M. Pote, A. C. Camus, M. J. Mauel, T. E. Greenway and D. J. Wise (2009). "Application of a real-time PCR assay for the detection of *Henneguya ictaluri* in commercial channel catfish ponds." Diseases of Aquatic Organisms **86**(3): 223-233.

Griffin, M. J., S. M. Quiniou, T. Cody, M. Tabuchi, C. Ware, R. C. Cipriano, M. J. Mauel and E. Soto (2013). "Comparative analysis of *Edwardsiella* isolates from fish in the eastern United States identifies two distinct genetic taxa amongst organisms phenotypically classified as *E. tarda*." Veterinary Microbiology **165**(3-4): 358-372.

Griffin, M. J., C. Ware, S. M. Quiniou, J. M. Steadman, P. S. Gaunt, L. H. Khoo and E. Soto (2014). "*Edwardsiella piscicida* identified in the Southeastern USA by *gyrB* sequence, species-specific and repetitive sequence-mediated PCR." Diseases of Aquatic Organisms **108**(1): 23-35.

Grimont, P. D., F. Grimont, C. Richard and R. Sakazaki (1980). "*Edwardsiella hoshinae*, a new species of Enterobacteriaceae." Current Microbiology **4**(6): 347-351.

Hawke, J. P., M. Kent, M. Rogge, W. Baumgartner, J. Wiles, J. Shelley, L. C. Savolainen, R. Wagner, K. Murray and T. S. Peterson (2013). "Edwardsiellosis caused by *Edwardsiella ictaluri* in laboratory populations of zebrafish *Danio rerio*." Journal of Aquatic Animal Health **25**(3): 171-183.

Hawke, J. P. and L. H. Khoo (2004). Infectious diseases. Biology and culture of the channel catfish. C. Tucker and J. Hargreaves. Amsterdam, Elsevier**:** 387-443.

Hawke, J. P., A. C. McWhorter, A. G. Steigerwalt and D. J. Brenner (1981). "*Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish." International Journal of Systematic Bacteriology **31**(4): 396-400.

Hossain, M. J., G. C. Waldbieser, D. Sun, N. K. Capps, W. B. Hemstreet, K. Carlisle, M. J. Griffin, L. Khoo, A. E. Goodwin, T. S. Sonstegard, S. Schroeder, K. Hayden, J. C. Newton, J. S. Terhune and M. R. Liles (2013). "Implication of lateral genetic transfer in the emergence of *Aeromonas hydrophila* isolates of epidemic outbreaks in channel catfish." PLoS One **8**(11): e80943.

Marancik, D. P. and G. D. Wiens (2013). "A real-time polymerase chain reaction assay for identification and quantification of *Flavobacterium psychrophilum* and application to disease resistance studies in selectively bred rainbow trout *Oncorhynchus mykiss*." FEMS Microbiology Letters **339**(2): 122-129.

Meyer, F. P. and G. L. Bullock (1973). "*Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*)." Applied Microbiology **25**(1): 155-156.

Mohanty, B. R. and P. K. Sahoo (2007). "Edwardsiellosis in fish: a brief review." Journal of Biosciences **32**: 1331-1344.



المنارة للاستشارات

www.manaraa.com

Plumb, J. A. and D. J. Sanchez (1983). "Susceptibility of five species of fish to *Edwardsiella ictaluri*." Journal of Fish Diseases **6**: 261-266.

Purcell, M. K., R. G. Getchell, C. A. McClure and K. A. Garver (2011). "Quantitative polymerase chain reaction (PCR) for detection of aquatic animal pathogens in a diagnostic laboratory setting." Journal of Aquatic Animal Health **23**(3): 148-161.

Reed, L. J. (1938). "A simple method of estimating fifty per cent endpoints." American Journal of Epidemiology **27**(3): 493-497.

Reichley, S. R., C. Ware, T. E. Greenway, D. J. Wise and M. J. Griffin (2015). "Real-time polymerase chain reaction assays for the detection and quantification of *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like species in catfish tissues and pond water." Journal of Veterinary Diagnostic Investigation **27**(2): 130-139.

Rozen, S. and H. Skaletsky (2000). Primer3 on the WWW for general users and for biologist programmers. Methods in Molecular Biology. M. S and K. SA. Totowa, New Jersey, Humana Press. 365-386.

Sakai, T., K. Yuasa, M. Sano and T. Iida (2009). "Identification of *Edwardsiella ictaluri* and *E. tarda* by species-specific polymerase chain reaction targeted to the upstream region of the fimbrial gene." Journal of Aquatic Animal Health **21**(2): 124-132.

Soto, E., K. Bowles, D. Fernandez and J. P. Hawke (2010). "Development of a real-time PCR assay for identification and quantification of the fish pathogen *Francisella noatunensis* subsp. *orientalis*." Diseases of Aquatic Organisms **89**(3): 199-207.

Soto, E., M. Griffin, M. Arauz, A. Riofrio, A. Martinez and M. E. Cabrejos (2012). "*Edwardsiella ictaluri* as the causative agent of mortality in cultured Nile Tilapia." Journal of Aquatic Animal Health **24**(2): 81-90.

Stock, I. and B. Wiedemann (2001). "Natural antibiotic susceptibilities of *Edwardsiella tarda*, *E. ictaluri*, and *E. hoshinae*." Antimicrobial Agents and Chemotherapy **45**(8): 2245-2255.

Yang, M., Y. Lv, J. Xiao, H. Wu, H. Zheng, Q. Liu, Y. Zhang and Q. Wang (2012). "*Edwardsiella* comparative phylogenomics reveal the new intra/inter-species taxonomic relationships, virulence evolution and niche adaptation mechanisms." PLoS One **7**(5): e36987.

Ye, S., H. Li, G. Qiao and Z. Li (2009). "First case of *Edwardsiella ictaluri* infection in China farmed yellow catfish *Pelteobagrus fulvidraco*." Aquaculture **292**(1-2): 6-10.



المنارة للاستشارات

www.manaraa.com

# CHAPTER III

# COMPLETE GENOME SEQUENCES OF *EDWARDSIELLA ANGUILLARUM*, *EDWARDSIELLA HOSHINAE*, *EDWARDSIELLA PISCICIDA* AND *EDWARDSIELLA TARDA* ISOLATES RECOVERED FROM VARIOUS HOSTS WITH DIFFERENT GEOGRAPHIC ORIGINS

Portions of this chapter have previously been published:

Reichley, S. R., G. C. Waldbieser, H. C. Tekedar, M. L. Lawrence and M. J. Griffin (2015). "Complete Genome Sequence of *Edwardsiella tarda* Isolate FL95-01, Recovered from Channel Catfish." Genome Announcements **3**(3): e00682-00615.

Reichley, S. R., G. C. Waldbieser, M. Ucko, A. Colorni, L. Dubytska, R. L. Thune, M. L. Lawrence and M. J. Griffin (2015). "Complete genome sequence of an *Edwardsiella piscicida*-like species isolated from diseased Grouper in Israel." Genome Announcements **3**(4): e00829-00815.

Reichley, S. R., G. C. Waldbieser, M. L. Lawrence and M. J. Griffin (2015). "Complete genome sequence of an *Edwardsiella piscicida*-like species recovered from Tilapia in the United States." Genome Announcements **3**(5): e01004-01015.

Reichley, S. R., G. C. Waldbieser, H. C. Tekedar, M. L. Lawrence and M. J. Griffin (2016). "Complete genome sequence of *Edwardsiella piscicida* isolate S11-285 recovered from Channel Catfish (*Ictalurus punctatus*) in Mississippi, USA." Genome Announcements **4**(6): e01259-01216.

Reichley, S. R., G. C. Waldbieser, M. L. Lawrence and M. J. Griffin (2017). "Complete genome sequence of *Edwardsiella hoshinae* ATCC 35051." Genome Announcements **5**(6): e01605-01616.



المنارة للاستشارات
www.manaraa.com

### 3.1 Introduction

The genus *Edwardsiella* was first recognized in the 1960s with the description of *E. tarda* (Ewing, McWhorter et al. 1965, Meyer and Bullock 1973). Over the following decades, two additional taxa within the genus were described, *E. hoshinae* and *E. ictaluri* (Grimont, Grimont et al. 1980, Hawke, McWhorter et al. 1981). Relative to other *Edwardsiella* spp., there is limited information regarding *E. hoshinae*. This bacterium has been isolated from birds, reptiles, water, and human feces; however, its role as a human pathogen has not been established and it is not considered a zoonotic agent (Grimont, Grimont et al. 1980, Farmer and McWhorter 1984, Singh, Singh et al. 2004, Singh, Singh et al. 2013).

Comparatively, *E. tarda* and *E. ictaluri* have been implicated in epizootics in both wild and cultured fish leading to significant economic losses in global aquaculture. *E. ictaluri* is widely considered the most ruinous bacterial disease agent in catfish aquaculture worldwide (Hawke, McWhorter et al. 1981, Crumlish, Dung et al. 2002, Wise, Camus et al. 2004, Ye, Li et al. 2009, Liu, Li et al. 2010). In addition to disease associated with *E. ictaluri* in silurid culture, *E. ictaluri* has also been linked to fish kills in cultured tilapia and laboratory zebrafish colonies (Soto, Griffin et al. 2012, Hawke, Kent et al. 2013).

Similarly, *E. tarda* has been implicated in considerable economic losses in more than 20 species of commercially-important fish worldwide (Xu and Zhang 2014). Primarily considered a pathogen of marine and freshwater fishes, *E. tarda* has historically been considered the most diverse and widespread of the *Edwardsiella* species (Mohanty and Sahoo 2007, Wang, Yang et al. 2009). Recent investigations into its heterogeneity



المنارة للاستشارات
www.manaraa.com

revealed this previous classification actually encompassed three genetically distinct and phenotypically ambiguous taxa, namely *E. tarda*, *E. piscicida* and *E. anguillarum* (syn. *E. piscicida*-like sp.) (Abayneh, Colquhoun et al. 2013, Oren and Garrity 2013, Oren and Garrity 2015, Shao, Lai et al. 2015). *Edwardsiella piscicida* has since been isolated from a variety of diseased wild and cultured fish (Oguro, Tamura et al. 2014, Camus, Dill et al. 2016, Fogelson, Petty et al. 2016, Shafiei, Viljamaa-Dirks et al. 2016).

In the catfish farming region of the southeastern United States, *E. tarda* has historically been associated with emphysematous putrefactive disease of catfish, which begins as small, cutaneous lesions that can progress to deep, malodorous, putrefactive abscesses within the musculature (Meyer and Bullock 1973, Hawke and Khoo 2004). However, recent studies have demonstrated many of these cases may have been misclassified *E. piscicida*. Research has shown *E. piscicida* to be more virulent to channel catfish than *E. tarda* or *E. anguillarum* (Reichley, Ware et al. 2015). Moreover, recent molecular surveys have demonstrated that *E. piscicida* is presently more common in catfish aquaculture than either *E. tarda* or *E. anguillarum* and has been increasingly recovered from diseased farm-raised catfish in the southeastern U.S. over the past ten years (Griffin, Ware et al. 2014, Reichley, Ware et al. 2015).

Complete and draft genomes of several *Edwardsiella* isolates are available; however, several are misclassified and not in line with contemporary *Edwardsiella* systematics. While complete genome sequences for *E. tarda* have been published (Wang, Yang et al. 2009, van Soest, Stockhammer et al. 2011, Tekedar, Karsi et al. 2013), these were later determined to be *E. piscicida* (Abayneh, Colquhoun et al. 2013, Griffin, Quiniou et al. 2013, Griffin, Ware et al. 2014). The purpose of this study was to provide



المنارة للاستشارات
www.manaraa.com

complete genome sequences for representative *Edwardsiella* isolates identified in line with current taxonomic organization.

## 3.2 Materials and Methods

### 3.2.1 DNA isolation

Archived isolates for each respective *Edwardsiella* spp. (Table 3.1) were revived from cryogenic storage on Tryptic Soy Agar plates supplemented with 5% sheep blood and grown overnight at 37°C. Individual colonies were expanded overnight in 9 ml porcine Brain Heart Infusion (BHI) broth at 37°C. Bacterial cultures were pelleted by centrifugation, resuspended in 600 µl of PureGene Cell Lysis Solution (QIAGEN) supplemented with 3 µl Proteinase K (20 mg/ml) and digested overnight at 55°C. Proteins were precipitated by the addition of 200 µl PureGene Protein Precipitation Solution (QIAGEN) and pelleted by centrifugation. The supernatant was transferred to a clean 1.5 ml microcentrifuge tube and residual salts were removed by the addition of 600 µl of 100% 2-propanol. DNA was harvested by centrifugation, and the supernatant removed. The DNA pellet was then washed with 300 µl of 70% ETOH, pelleted by centrifugation, dried and resuspended in 100 µl PureGene DNA Hydration Solution (QIAGEN).



المنارة للاستشارات
www.manaraa.com

Table 3.1 Associated metadata for *Edwardsiella* spp. genomes sequenced in this project (bold) and publicly available complete and draft genomes used for comparative analysis.

| | Host | Year | Locale | Reference(s) |
|---|---|---|---|---|
| ***E. anguillarum*** **EA181011** | Grouper *Epinephelus aeneus* | 2011 | Israel | Ucko, Colorni et al. (2016) |
| ***E. anguillarum*** **LADL05-105** | Tilapia *Oreochromis* sp. | 2005 | Louisiana, USA | Griffin et al. (2013; 2014) |
| *E. anguillarum* (T) 080813 | Japanese eel *Anguilla japonica* | 2008 | Fujian, China | Shao, Lai et al. (2015) |
| ***E. hoshinae*** **ATCC 35051** | Monitor lizard *Varanus* sp. | 1980 | Chad | Grimont, Grimont et al. (1980) |
| *E. hoshinae* (T) ATCC 33379 | Puffin *Fratercula arctica* | 1980 | France | Grimont, Grimont et al. (1980) |
| *E. ictaluri* 93-146 | Channel catfish *Ictalurus punctatus* | 1978 | Alabama, USA | Williams, Gillaspy et al. (2012) |
| *E. ictaluri* (T) ATCC 33202 | Channel catfish *Ictalurus punctatus* | 1981 | Georgia, USA | Hawke, McWhorter et al. (1981) |
| ***E. piscicida*** **S11-285** | Channel catfish *Ictalurus punctatus* | 2011 | Mississippi, USA | Griffin et al. (2013; 2014) |
| *E. piscicida* (T) ET883 | European eel *Anguilla anguilla* | 1989 | Greaker, Norway | Abayneh, Colquhoun et al. (2013) |
| ***E. tarda*** **FL95-01** | Channel catfish *Ictalurus punctatus* | 1995 | Florida, USA | Griffin et al. (2013; 2014) |
| *E. tarda* (T) ATCC 15947 | Human *Homo sapiens* | 1959 | Kentucky, USA | Ewing, McWhorter et al. (1965) |

### 3.2.2 Sequencing

#### 3.2.2.1 *Edwardsiella anguillarum* EA181011

A total of 567 Mb of Pacific Biosciences (Pac-Bio) sequence (average length 6 kb) was produced from gDNA of *E. anguillarum* (syn. *E. piscicida*-like sp.) isolate EA181011. The longest 40X genome coverage of Pac-Bio reads were error-corrected with the remaining Pac-Bio data using the PBcR module within Celera Assembler v.8.2beta (Koren, Schatz et al. 2012, Koren, Harhay et al. 2013), then the longest 25X coverage of corrected Pac-Bio sequence was assembled into a single, circular chromosome. To correct variations in homopolymer lengths in the consensus Pac-Bio



المنارة للاستشارات
www.manaraa.com

data, we mapped 16X genome coverage of Ion Torrent™ sequencing reads (provided by R. Thune, Louisiana State University) to the assembled chromosome using Bowtie2 (Langmead and Salzberg 2012) and produced a consensus sequence using VarScan.v2.3.7 (Koboldt, Zhang et al. 2012).

#### 3.2.2.2 *Edwardsiella anguillarum* LADL05-105

Genomic DNA from *E. anguillarum* (syn. *E. piscicida*-like sp.) isolate LADL05-105 was sequenced using two methods: Illumina (36X coverage) and Pac-Bio (113X coverage). The PBcR module of wgs-8.2beta (Celera) (Koren, Schatz et al. 2012, Koren, Harhay et al. 2013) was used to identify the longest 40X coverage Pac-Bio reads and corrected with the remaining Pac-Bio data. The longest 25X coverage of corrected sequence was assembled into a single, circular chromosome. Illumina sequencing reads were mapped to the assembled chromosome with Bowtie2 (Langmead and Salzberg 2012) to correct variations in homopolymer lengths, and a consensus sequence was produced using VarScan.v2.3.7 (Koboldt, Zhang et al. 2012).

#### 3.2.2.3 *Edwardsiella hoshinae* ATCC 35051

Genomic DNA of *E. hoshinae* isolate ATCC 35051 was sequenced using Pac-Bio technology to a depth of 25X genome coverage (96.5 Mb). Reads ≥ 8000 bp were error-corrected with shorter Pac-Bio reads and assembled using Canu v1.0 (Berlin, Koren et al. 2015). Illumina sequences (109X coverage, minimum depth of 11X) were mapped to the Pac-Bio assembly using Burrows-Wheeler Aligner (BWA) v0.7.10-r789 (Li 2013), and base errors and insertions/deletions were corrected using Pilon v1.16 (Walker, Abeel et al. 2014) iteratively until no further base corrections were made automatically.



المنارة للاستشارات
www.manaraa.com

Overlapping sequence was identified, and the linear contig was circularized and re-linearized at a position 1 million bases distant from the original position of circularization. Illumina and Pac-Bio sequences were realigned and visualized using the Integrated Genome Viewer (Thorvaldsdottir, Robinson et al. 2013) for validation of contiguity and manual correction of assembly errors.

#### 3.2.2.4 *Edwardsiella piscicida* S11-285

Genomic DNA of *E. piscicida* isolate S11-285 was sequenced using Pac-Bio technology to 140X coverage. After correction of reads, 25X genome coverage was assembled into four contigs using Canu v1.0 (Berlin, Koren et al. 2015). Illumina sequences (30X coverage, minimum depth of 5) were mapped to the Pac-Bio assembly using BWA v0.7.10-r789 (Li 2013); base errors and insertions/deletions were corrected using Pilon v1.16 (Walker, Abeel et al. 2014) iteratively until no further base corrections were made automatically. Ribosomal RNA genes were sequenced using Sanger sequencing of cloned PCR products spanning the rRNA loci; these genes were then aligned to the genomic contigs to produce a single contig. The genome sequence was circularized and re-linearized at a position 1 M bases downstream. Illumina and Pac-Bio sequences were realigned and visualized using the Integrated Genome Viewer (Thorvaldsdottir, Robinson et al. 2013) for validation of contiguity.

#### 3.2.2.5 *Edwardsiella tarda* FL95-01

Genomic DNA of *E. tarda* isolate FL95-01 was sequenced using the Illumina (77X coverage) and Pac-Bio (88X coverage) platforms. The longest 40X coverage Pac-Bio reads were error-corrected with the remaining shorter Pac Bio reads using the PBcR



المنارة للاستشارات
www.manaraa.com

module within Celera Assembler v.8.2beta (Koren, Schatz et al. 2012, Koren, Harhay et al. 2013). The longest 25X coverage of corrected sequence was assembled into a single, circular chromosome. To correct variations in homopolymer lengths between Pac-Bio reads, Illumina sequencing reads were mapped to the assembled chromosome with Bowtie2 (Langmead and Salzberg 2012), and the Illumina-preferred consensus sequence was produced using VarScan.v2.3.7 (Koboldt, Zhang et al. 2012).

#### 3.2.2.6 Plasmid sequencing

Native plasmid DNA was harvested from all isolates using the QIAprep Spin Miniprep Kit (QIAGEN). Plasmids were separated on 0.8% agarose gel, and sizes were approximated with concurrently run standards (Supercoiled DNA Ladder, New England Biolabs). Plasmids were sequenced commercially using the complete plasmid sequencing service of the DNA Core Facility of the Center for Computational and Integrative Biology at Massachusetts General Hospital (Boston, MA, USA) and assembled using the MGH CCIB NGS *de novo* assembler UltraCycler v1.0 (Seed and Wang Unpublished). Open reading frames (ORFs) were predicted using GeneMark.hmm prokaryotic v3.25 (Besemer and Borodovsky 1999, Zhu, Lomsadze et al. 2010) and Glimmer v3.02 (Salzberg, Delcher et al. 1998, Delcher, Harmon et al. 1999). Putative function of plasmid ORFs were predicted using a BLASTX search of the National Center for Biotechnology Information (NCBI) non-redundant protein database using the Bacteria and Archaea code with e-values ≥1e-02 considered insignificant.



المنارة للاستشارات
www.manaraa.com

### 3.2.3 Genome annotation, relatedness and intragenomic 16S heterogeneity

The circularized and complete genomes of each isolate were submitted to the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) for annotation and release to GenBank. Furthermore, the genomes were submitted for Rapid Annotation using Subsystem Technology (RAST) (Aziz, Bartels et al. 2008, Overbeek, Olson et al. 2014) annotation using the Glimmer option to gather more detailed information. Publicly available complete and draft genomes of representative *Edwardsiella* strains were downloaded from NCBI (Table 3.1). Average nucleotide identities (ANI) (Goris, Konstantinidis et al. 2007) and digital DNA-DNA hybridization (dDDH) (Auch, von Jan et al. 2010) estimations were determined using online calculators (ANI: http://enve-omics.ce.gatech.edu/ani/; dDDH: http://ggdc.dsmz.de/distcalc2.php).

For each strain, ANI to other *Edwardsiella* genomes was calculated as described in Goris, Konstantinidis et al. (2007). dDDH values were estimated by dividing the sum of all identities found in high-scoring segment pairs (HSP) by the overall HSP length (dDDH Formula 2) (Auch, von Jan et al. 2010), which is independent of genome length and recommended for incomplete draft genomes. In addition, genomes sequenced in this work were compared with type strain sequences of representative *Edwardsiella* spp. available on NCBI. For unclosed genomes (ET883, ATCC 33379, ATCC 15947, ATCC 33202), pseudocontigs were created from the drafts using the default settings of CONTIGuator (Galardini, Biondi et al. 2011) and mapped to the appropriate reference genome assembled in this study. Comparisons of pseudocontigs to closed genomes were visualized using the BLAST Ring Image Generator (BRIG) (Alikhan, Petty et al. 2011). Additionally, intragenomic heterogeneity of the 16S SSU was evaluated by BLASTN



المنارة للاستشارات
www.manaraa.com

searches of SSU rRNA sequences against the complete genomes of isolates *E. anguillarum* LADL05-105, *E. hoshinae* ATCC 35051, *E. ictaluri* 93-146, *E. piscicida* S11-285 and *E. tarda* FL95-01.

### 3.3 Results

The *E. anguillarum* isolate EA181011 genome consists of one circular chromosome with 3,934,167 bp and 59.1% GC content. PGAP annotation predicted 3,476 genes encoding 3,122 proteins. RNAmmer (Lagesen, Hallin et al. 2007) predicted 8 rRNA operons. Based on comparative RAST analysis with *E. tarda* isolate FL95-01, EA181011 contains 119 unique elements, including components related to mannitol utilization, type I and type VI secretion systems, and bacteriophage P4 cluster. Isolate EA181011 does not carry any plasmids. The complete genome sequence for *Edwardsiella anguillarum* isolate EA181011 has been deposited in GenBank under the accession no. CP011364.

The *E. anguillarum* isolate LADL05-105 genome consists of one circular chromosome with 4,142,037 bp and 58.8% GC content. PGAP annotation predicted 3,686 genes encoding 3,159 proteins and 99 tRNAs. RNAmmer (Lagesen, Hallin et al. 2007) predicted 9 rRNA operons. RAST analysis revealed 150 unique subsystems in LADL05-105 compared to *E. tarda* isolate FL95-01, including elements of inositol catabolism, mannitol utilization and type VI secretion system. The LADL05-105 genome does not carry any plasmids. The complete genome sequence for *E. anguillarum* isolate LADL05-105 has been deposited in GenBank under accession no. CP011516.

The *E. hoshinae* isolate ATCC 35051 genome consists of one circular chromosome with 3,811,650 bp (56.9% GC content). PGAP annotation predicted 3,401



www.manaraa.com

genes encoding 3,204 proteins and 101 tRNAs. RNAmmer (Lagesen, Hallin et al. 2007) predicted 9 rRNA operons. RAST analysis predicted 497 subsystems with 3,526 coding sequences and 128 RNAs. No plasmids were detected in ATCC 35051. The complete genome sequence for *E. hoshinae* isolate ATCC 35051 has been deposited in GenBank under accession no. CP016043.

The *E. piscicida* isolate S11-285 genome consists of one circular chromosome of 3,923,603 bp (59.6% GC content) and 1 plasmid of 3,164 bp (48.2% GC content). PGAP annotation predicted 3,509 genes encoding 3,293 proteins. RNAmmer (Lagesen, Hallin et al. 2007) predicted 10 rRNA operons. RAST analysis predicted 497 subsystems with 3,779 coding sequences and 136 RNAs. The complete genome sequence for *E. piscicida* isolate S11-285 has been deposited in GenBank under accession no. CP016044 and its plasmid has been deposited under accession no. CP016445.

The *E. tarda* isolate FL95-01 genome consists of one circular chromosome with 3,620,701 bp and 57.3% GC content. PGAP annotation predicted 3,258 genes encoding 3,091 proteins and 101 tRNAs. RAST analysis predicted 505 subsystems with 3,318 coding sequences and 129 RNAs. Additionally, RNAmmer (Lagesen, Hallin et al. 2007) predicted 9 rRNA operons. FL95-01 does not carry any plasmids. The complete genome sequence for *E. tarda* isolate FL95-01 has been deposited in GenBank under the accession no. CP011359.

Statistics for each genome sequenced in the current study are listed in Table 3.2. The ANI and dDDH estimations for *Edwardsiella* genomes sequenced here, in addition to publicly available complete and draft genomes of representative *Edwardsiella* spp., are displayed in Table 3.3. Conservation and variability in genomic content between different



www.manaraa.com

*Edwardsiella* spp. genomes are displayed in Figures 3.1-3.5. Intragenomic 16S rDNA heterogeneity for each *Edwardsiella* spp. ranged from 0.0 - 0.6% (Table 3.4).

Table 3.2 Statistics for genomes sequenced and closed in the current study.

| | Genome Size (bp) | GC Content | Predicted Number of Genes | Predicted Number of Proteins | Predicted Number of rRNA Operons |
|---|---|---|---|---|---|
| *E. anguillarum* EA181011 | 3,934,167 | 59.1% | 3,476 | 3,122 | 8 |
| *E. anguillarum* LADL05-105 | 4,142,037 | 58.8% | 3,686 | 3,159 | 9 |
| *E. hoshinae* ATCC 35051 | 3,811,650 | 56.9% | 3,401 | 3,204 | 9 |
| *E. piscicida* S11-285 | 3,923,603 | 59.6% | 3,509 | 3,293 | 10 |
| *E. tarda* FL95-01 | 3,620,701 | 57.3% | 3,258 | 3,091 | 9 |

Number of genes and proteins were predicted using the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP). Predicted number of rRNA operons were determined by RNAmmer (Lagesen, Hallin et al. 2007).



www.manaraa.com

Table 3.3 Average nucleotide identities and (digital DNA-DNA hybridization) estimations for *Edwardsiella* genomes.

| | **EA181011** | **LADL05-105** | 080813 | **ATCC 35051** | ATCC 33379 | 93-146 | ATCC 33202 | **S11-285** | ET883 | **FL95-01** | ATCC 15947 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***E. anguillarum*** **EA181011** | - | | | | | | | | | | |
| ***E. anguillarum*** **LADL05-105** | 99.6% (95%) | - | | | | | | | | | |
| *E. anguillarum* (T) 080813 | 99.8% (98%) | 99.8% (98%) | - | | | | | | | | |
| ***E. hoshinae*** **ATCC 35051** | 83.1% (25%) | 83.1% (25%) | 83.1% (25%) | - | | | | | | | |
| *E. hoshinae* (T) ATCC 33379 | 82.8% (25%) | 82.8% (25%) | 82.8% (24%) | 99.7% (97%) | - | | | | | | |
| *E. ictaluri* 93-146 | 92.6% (50%) | 92.5% (49%) | 92.5% (50%) | 82.6% (24%) | 82.3% (24%) | - | | | | | |
| *E. ictaluri* (T) ATCC 33202 | 92.5% (49%) | 92.4% (48%) | 92.5% (49%) | 82.3% (24%) | 82.2% (24%) | 99.9% (92%) | - | | | | |
| ***E. piscicida*** **S11-285** | 94.5% (59%) | 94.4% (58%) | 94.5% (93%) | 83.0% (25%) | 82.7% (25%) | 92.2% (48%) | 92.1% (48%) | - | | | |
| *E. piscicida* (T) ET883 | 95.2% (64%) | 95.0% (61%) | 95.1% (64%) | 83.0% (24%) | 82.8% (24%) | 92.1% (48%) | 92.1% (48%) | 99.5% (93%) | - | | |
| ***E. tarda*** **FL95-01** | 83.9% (26%) | 84.0% (26%) | 83.9% (26%) | 87.4% (35%) | 87.2% (35%) | 83.1% (25%) | 82.8% (25%) | 83.4% (26%) | 83.4% (25%) | - | |
| *E. tarda* (T) ATCC 15947 | 83.6% (26%) | 83.8% (26%) | 83.4% (26%) | 87.2% (35%) | 87.2% (34%) | 82.9% (25%) | 82.8% (24%) | 83.2% (25%) | 83.2% (25%) | 99.4% (96%) | - |

*Edwardsiella* genomes sequenced in this study (bold) and publicly available complete and draft genomes of *Edwardsiella ictaluri* (93-146; CP001600.2) and *Edwardsiella piscicida* (ET883; JRGQ01000000), *Edwardsiella anguillarum* (080813; CP006664.1) and *Edwardsiella tarda* (ATCC 15947; AFJG00000000) type strains (T). Pairwise comparisons of conspecific taxa, identified as those sharing greater than 95% ANI, are highlighted in gray.



www.manaraa.com

Table 3.4 Intragenomic heterogeneity of the 16S SSU for representative *Edwardsiella* genomes sequenced in this study.

| | No. of 16S Copies in Genome | # of Differences (bp) | Dissimilarity Range |
|---|---|---|---|
| *E. anguillarum* LADL05-105 | 9 | 0 - 4 | 0.0% - 0.3% |
| *E. hoshinae* ATCC 35051 | 9 | 0 - 3 | 0.0% - 0.2% |
| *E. ictaluri* 93-146 | 8 | 0 - 3 | 0.0% - 0.2% |
| *E. piscicida* S11-285 | 10 | 0 - 6 | 0.0% - 0.4% |
| *E. tarda* FL95-01 | 9 | 0 - 9 | 0.0% - 0.6% |



المنارة للاستشارات
www.manaraa.com

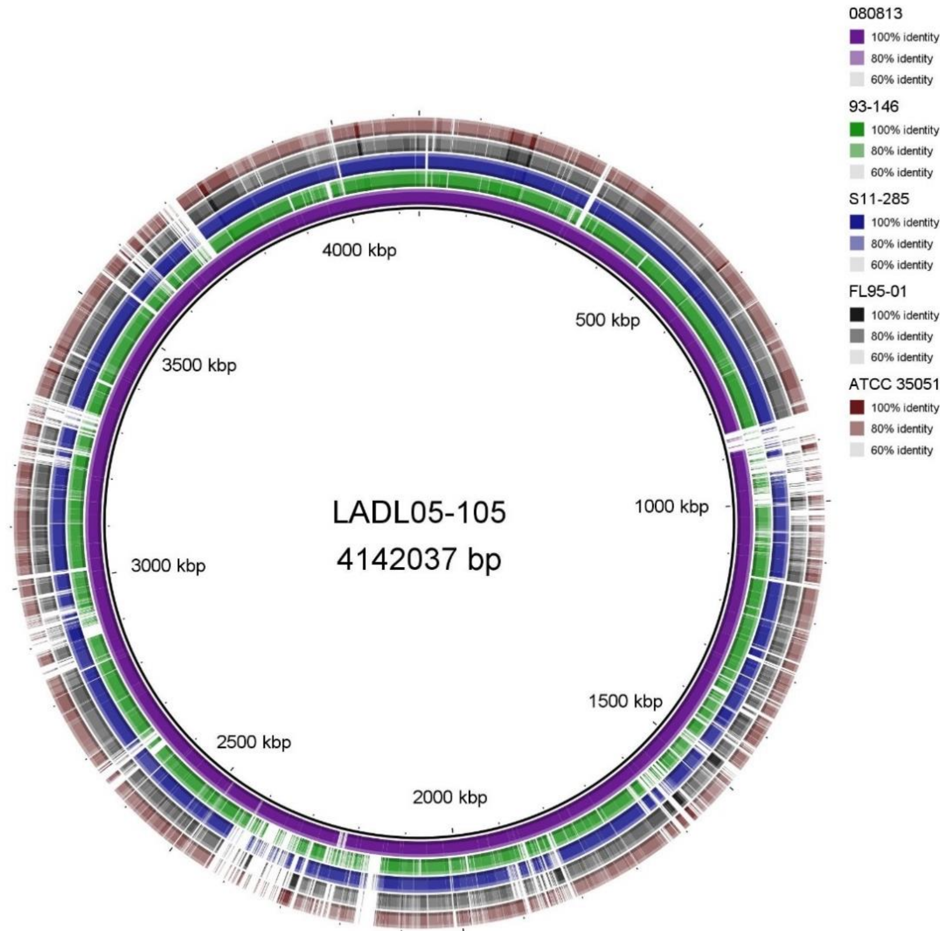


Figure 3.1 Sequence identity of *Edwardsiella* genomes, using *E. anguillarum* isolate LADL05-105 as the reference. Image created using the BLAST Ring Image Generator (BRIG).

Each ring represents a query sequence, colored to indicate the presence of hits to the reference sequence. The order of rings from inner-most to outer-most is: 080813 (*E. anguillarum*), 93-146 (*E. ictaluri*), S11-285 (*E. piscicida*), FL95-01 (*E. tarda*) and ATCC 35051 (*E. hoshinae*).



www.manaraa.com

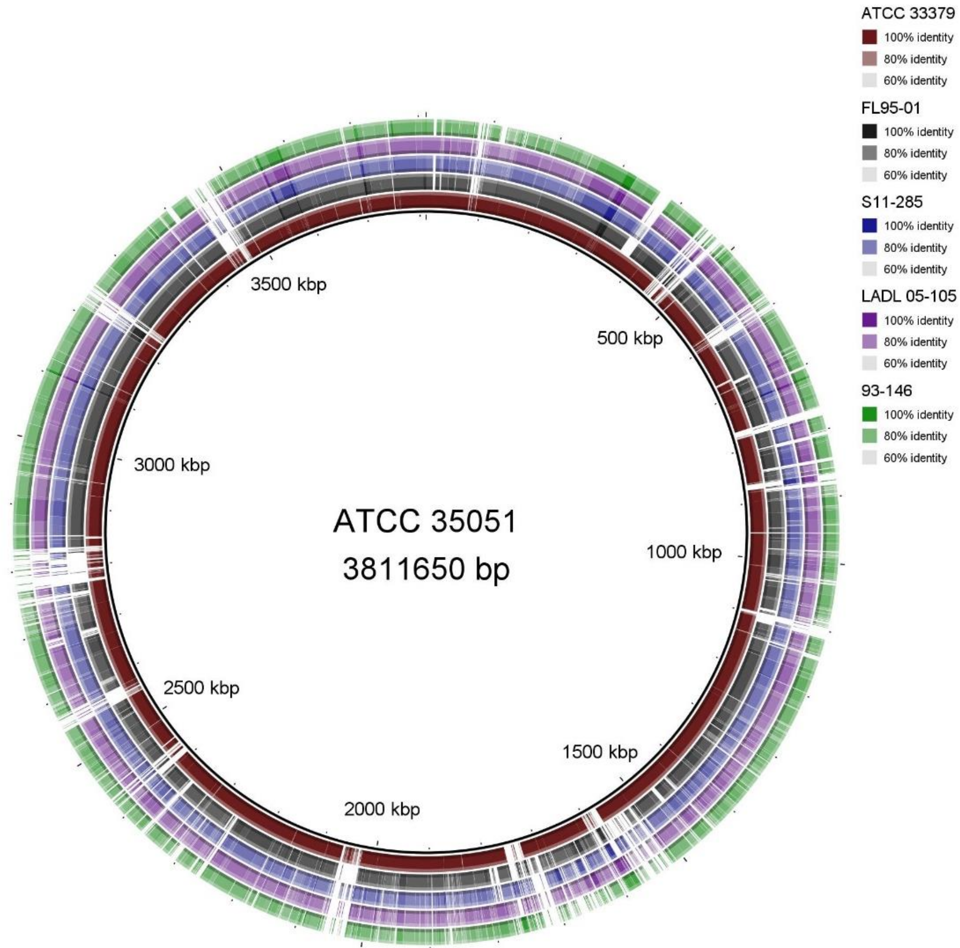


Figure 3.2 Sequence identity of *Edwardsiella* genomes, using *E. hoshinae* isolate ATCC 35051 as the reference. Image created using the BLAST Ring Image Generator (BRIG).

Each ring represents a query sequence, colored to indicate the presence of hits to the reference sequence. The order of rings from inner-most to outer-most is: ATCC 33379 (*E. hoshinae*) FL95-01 (*E. tarda*), S11-285 (*E. piscicida*), LADL05-105 (*E. anguillarum*) and 93-146 (*E. ictaluri*).



المنارة للاستشارات
www.manaraa.com

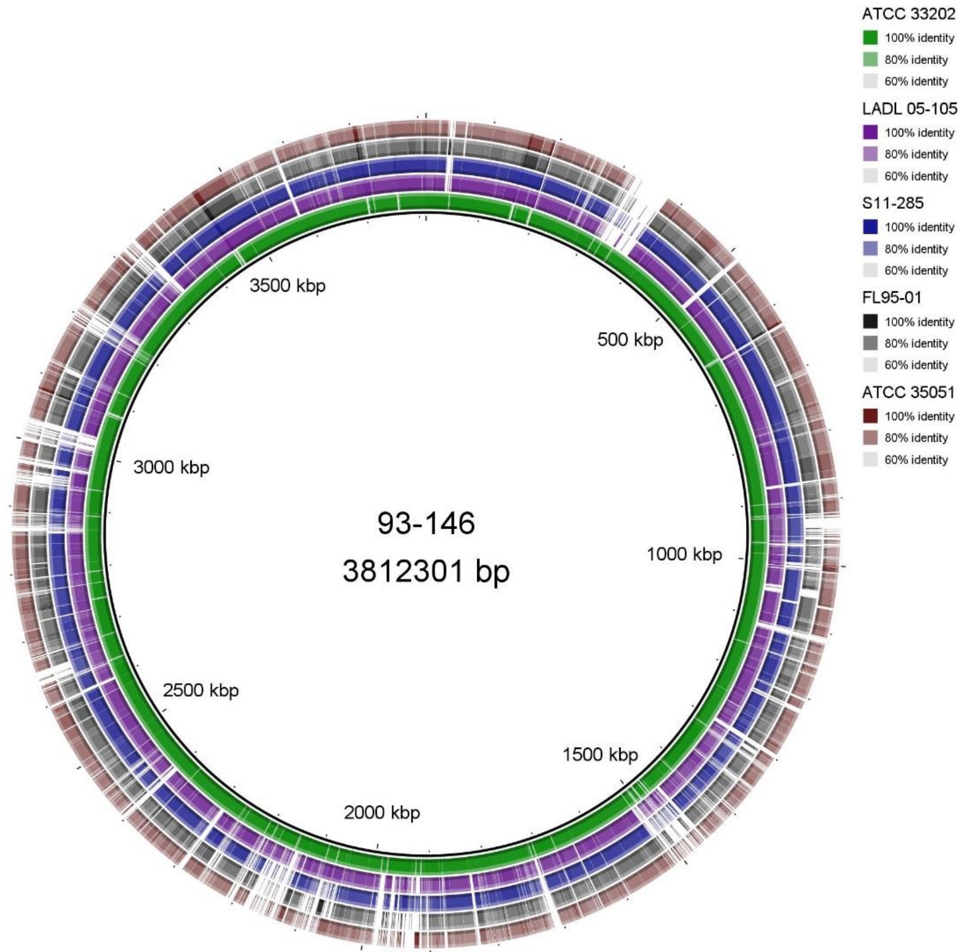


Figure 3.3 Sequence identity of *Edwardsiella* genomes, using *E. ictaluri* isolate 93-146 as the reference. Image created using the BLAST Ring Image Generator (BRIG).

Each ring represents a query sequence, colored to indicate the presence of hits to the reference sequence. The order of rings from inner-most to outer-most is: ATCC 33202 (*E. ictaluri*), LADL05-105 (*E. anguillarum*), S11-285 (*E. piscicida*), FL95-01 (*E. tarda*) and ATCC 35051 (*E. hoshinae*).



المنارة للاستشارات
www.manaraa.com

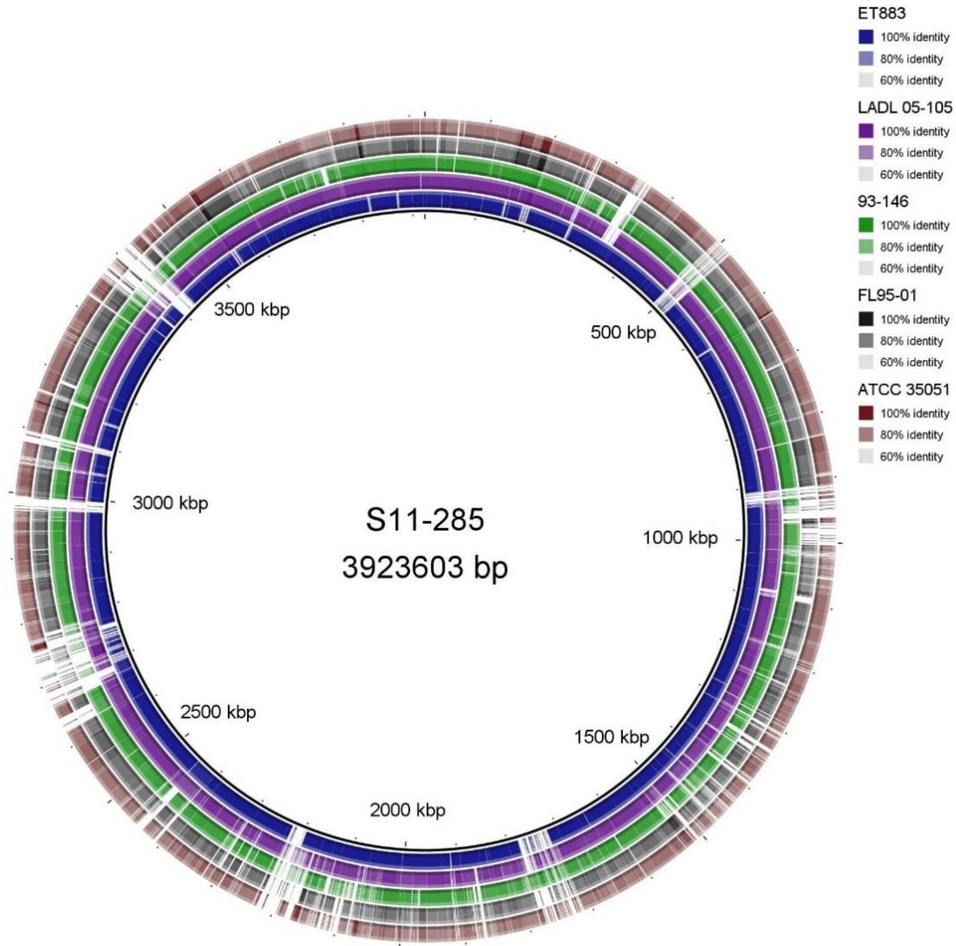


Figure 3.4 Sequence identity of *Edwardsiella* genomes, using *E. piscicida* isolate S11-285 as the reference. Image created using the BLAST Ring Image Generator (BRIG).

Each ring represents a query sequence, colored to indicate the presence of hits to the reference sequence. The order of rings from inner-most to outer-most is: ET883 (*E. piscicida*), LADL05-105 (*E. anguillarum*), 93-146 (*E. ictaluri*), FL95-01 (*E. tarda*) and ATCC 35051 (*E. hoshinae*).



المنارة للاستشارات
www.manaraa.com

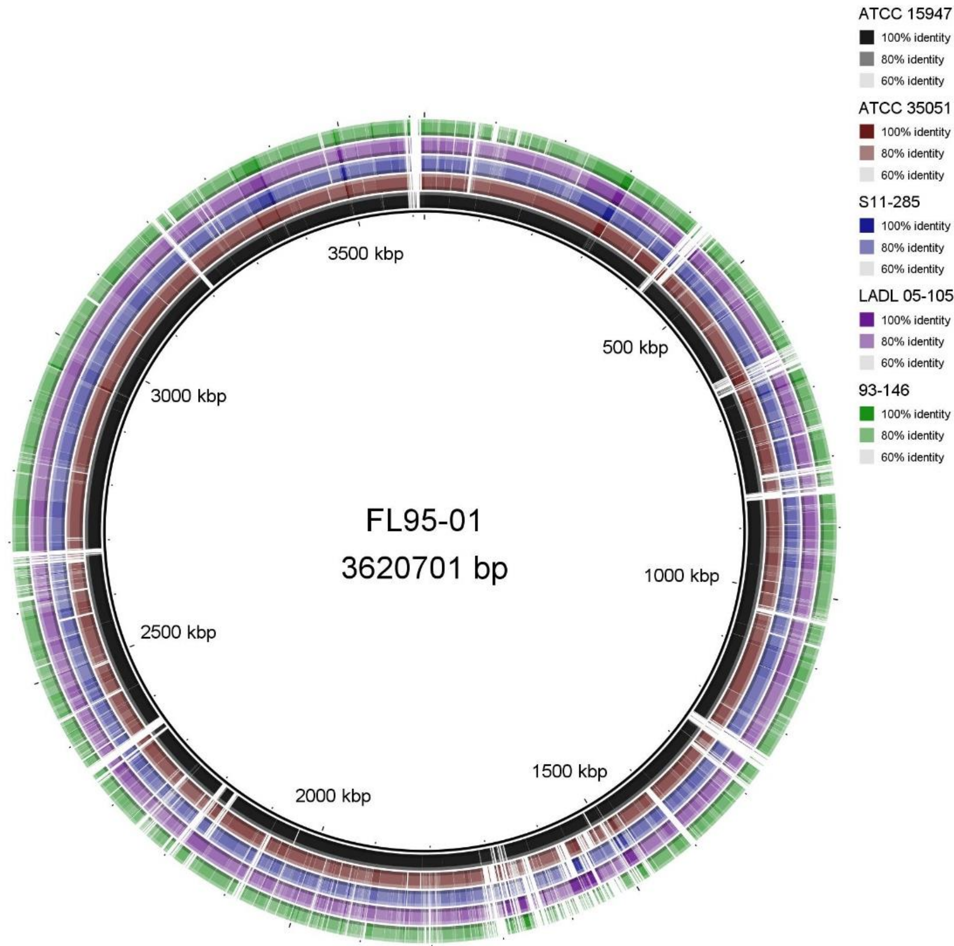


Figure 3.5 Sequence identity of *Edwardsiella* genomes, using *E. tarda* isolate FL95-01 as the reference. Image created using the BLAST Ring Image Generator (BRIG).

Each ring represents a query sequence, colored to indicate the presence of hits to the reference sequence. The order of rings from inner-most to outer-most is: ATCC 15947 (*E. tarda*), ATCC 35051 (*E. hoshinae*), S11-285 (*E. piscicida*), LADL05-105 (*E. anguillarum*) and 93-146 (*E. ictaluri*).

### 3.4 Discussion

Reductions in economic constraints of next-generation DNA sequencing technology, as well as more resolute and user friendly methods of analyses, has led to increased utilization of genomic tools in bacterial identification and classification. These technologies have aided in the determination of bacterial species, although they can be



www.manaraa.com

impacted by genome size and are often reliant on unverified publicly available datasets (Clarridge 2004, Konstantinidis, Ramette et al. 2006). As these databases continue to grow, the definition of a bacterial species needs to be revisited as antecedent classifications schemes, largely based on pheno-biochemical characteristics, often fail to differentiate between phenotypically ambiguous yet genetically discrete taxa. Further confounding this issue is the misplaced reliance on the National Center for Biotechnology Information's (NCBI) GenBank and the International Nucleotide Sequence Database Collaboration nomenclature as taxonomic authorities when identifying isolates from disease outbreaks or those used in research studies.

This is especially important for taxa undergoing reclassification, such as the *Edwardsiella* spp. When using GenBank to identify a bacterial isolate, many scientists rely on comparisons of partial 16S rRNA sequence data to publicly accessible databases, in spite of the fact these databases are not authoritative sources for classification or nomenclature. The NCBI website provides a disclaimer stating as such and recommends investigators consult relevant scientific literature for the most reliable and up-to-date taxonomic information. Numerous studies have demonstrated the limitations of 16S rRNA sequences for determining species and confusion still exists regarding its use for this purpose. Research into the inter- and intraspecies variation amongst some groups of bacteria has demonstrated that while 16S rRNA sequences are appropriate to establish relationships between genera or well-resolved taxa, they lack sufficient resolution to discriminate between recently diverged species (Fox, Wisotzkey et al. 1992, Turenne, Tschetter et al. 2001, Clarridge 2004).



www.manaraa.com

Identifications based on 16S rRNA sequences can also be misleading when employing an arbitrary similarity cutoff of 98% - 99%, as has been previously suggested (Kim, Oh et al. 2014). This cutoff is contradictory to previous reports that demonstrated strains with ≥ 97% similarity may or may not belong to the same species (Stackebrandt and Goebel 1994). Still other publications report acceptable divergence cutoffs for species delimitation ranging from 0.5% - 1.5% (Janda and Abbott 2002, Clarridge 2004), depending on genus. Further complicating the utility of universal cutoffs is the presence of intragenomic 16S heterogeneity exceeding 1% for some species. While intragenomic 16S heterogeneity in the *Edwardsiella* is not this extreme, values range from 0% - 0.6% which supports intraspecific variation (0.1% - 0.7%) reported for the some *Edwardsiella* spp. in previous studies (Janda and Abbott 2002, Griffin, Quiniou et al. 2013). This supports recent assertions that 16S SSU sequencing has limited utility for species delineation within *Edwardsiella* (Griffin, Quiniou et al. 2013, Griffin, Ware et al. 2014, Griffin, Reichley et al. 2016). Taken collectively, establishment of a universal 16S rRNA similarity/dissimilarity cutoff for species delineation for all bacterial genera is unlikely.

Alternatively, in instances when 16S rRNA is insufficient to differentiate closely related congeners, alternative reference genes with greater taxonomic resolution should be considered (Woo, Lau et al. 2008). For example, the single-copy *gyrB* gene, present in all bacteria, has been proposed as a more reliable marker to discern between closely related bacterial species within *Enterobacteriaceae*, including the *Edwardsiella* (Dauga 2002, Griffin, Quiniou et al. 2013, Griffin, Ware et al. 2014, Griffin, Reichley et al. 2016). Likewise, *sodB*, *rpoB* and *dnaK* have all been proposed as suitable alternatives to



المنارة للاستشارات
www.manaraa.com

16S rRNA sequencing for identification and classification of unknown bacteria (Yamada and Wakabayashi 1999, Dahllöf, Baillie et al. 2000, Stepkowski, Czaplinska et al. 2003).

With the rapidly expanding dataset of closed genomes, it is prudent for researchers to ensure accurate and appropriate bacterial identification prior to releasing genomes and amend existing taxonomic errors present in databases. This issue has been previously raised for other genera and should apply to *Edwardsiella* as well (Beaz-Hidalgo, Hossain et al. 2015). Historically, bacterial strains exhibiting more than 70% DNA-DNA hybridization (DDH) estimations were considered members of the same species (Wayne, Brenner et al. 1987). While this 70% DDH standard is pragmatic and generally applicable, it has recently received significant criticism, largely due to the difficulty in implementation, the large phenotypic variation within many named prokaryote taxa, inability to generate archival data and failure to recognize genomic evolution and ecological adaptation (Cohan 2002, Gevers, Cohan et al. 2005). This has led many in the scientific community to question the utility of DDH in light of recent developments in genomic sequencing technologies and bioinformatics, while citing the need for a revalidation of the definition of bacterial species. At present, no universal criteria have been agreed upon (Konstantinidis, Ramette et al. 2006, Gao and Gupta 2012).

Average nucleotide identity and genome-to-genome sequence comparison have recently been introduced as alternatives to DNA-DNA hybridization, with notable advantages. Average nucleotide identity (ANI) is a robust measure of the genetic and evolutionary distance between bacterial species and is not affected by lateral transfer or recombination of genes; thus providing advantages over DDH (Konstantinidis and Tiedje



المنارة للاستشارات
www.manaraa.com

2005, Gao and Gupta 2012). Using 28 bacterial strains from 7 different genera, Goris, Konstantinidis et al. (2007) demonstrated a 95% ANI corresponds to the traditional cut-off point of 70% DDH for species delineation.

Similarly, genome-to-genome sequence comparison is another tool that has been shown to be valuable in reconstructing trees in both *Archaea* and *Bacteria* (Wolf, Rogozin et al. 2001). This tool is advantageous over traditional DDH because it is *in silico* which allows for the reuse of genomes indefinitely. It also works well for draft genomes and has been suggested to outperform ANI (Auch, von Jan et al. 2010). Digital DDH (dDDH) performed *in silico* using sequence-based genome blast distance phylogeny strongly correlates with wet-lab DDH and provides increased accuracy (Meier-Kolthoff, Auch et al. 2013).

Each of the genomes presented in the current study adhere to established guidelines (>70% dDDH and >95% ANI) for species delineation by genome-to-genome comparison (Goris, Konstantinidis et al. 2007, Auch, von Jan et al. 2010). This is an important finding, as it highlights the need for more robust discriminatory methods to differentiate between *E. anguillarum, E. piscicida* and *E. tarda*. The inability to discriminate amongst these *Edwardsiella* spp. by phenotypic characteristics using common commercial phenotypical and/or biochemical identification systems, coupled with the limited discriminatory power of 16S rRNA sequence comparison, can result in erroneous classification and has led to ambiguous reporting within the literature (Kim, Kang et al. 2014, Zhou, Geng et al. 2014, Lu, Wang et al. 2015, Mo, Zhou et al. 2015). ANI and dDDH results from this study support previous research (Griffin, Ware et al. 2014) and demonstrates *E. anguillarum* and *E. piscicida* are more closely related to *E.*



المنارة للاستشارات
www.manaraa.com

*ictaluri*, resulting in a clustering of fish pathogenic *Edwardsiella*, separate from *E. hoshinae* and *E. tarda*, which are now generally recognized to be associated with reptilian, avian and mammalian hosts; although sporadic reports of *E. tarda*, as it is currently defined, still exist.

Consistent identification and classification of subject organisms lays the foundation for infectious disease research and fosters more reliable reporting among different laboratories and within the scientific literature. The collection of closed genomes reported herein were submitted to GenBank in line with contemporary taxonomic nomenclature consistent with current *Edwardsiella* systematics. These genomes will better facilitate proper taxonomic assignment and minimize erroneous classifications of *Edwardsiella* isolates in future research. Moreover, these closed genomes will assist in further studies investigating the biology of these important bacteria and help researchers gain a better understanding of their interactions in the environment and within different hosts.

### 3.5 Acknowledgements

Sequencing was performed at the USDA, ARS, Genomics and Bioinformatics Research Unit, Sheron Simpson and Brian Scheffler; and Mississippi State University Institute for Genomics, Biocomputing, and Biotechnology, Chuan-Yu Hsu.



المنارة للاستشارات
www.manaraa.com

### 3.6 References


Abayneh, T., D. J. Colquhoun and H. Sorum (2013). "*Edwardsiella piscicida* sp. nov., a novel species pathogenic to fish." Journal of Applied Microbiology **114**(3): 644-654.

Alikhan, N.-F., N. K. Petty, N. L. B. Zakour and S. A. Beatson (2011). "BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons." BMC Genomics **12**: 402.

Auch, A. F., M. von Jan, H. P. Klenk and M. Goker (2010). "Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison." Standards in Genomic Sciences **2**(1): 117-134.

Auch, A. F., M. von Jan, H. P. Klenk and M. Göker (2010). "Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison." Standards in Genomic Sciences **2**.

Aziz, R. K., D. Bartels, A. A. Best, M. DeJongh, T. Disz, R. A. Edwards, K. Formsma, S. Gerdes, E. M. Glass, M. Kubal, F. Meyer, G. J. Olsen, R. Olson, A. L. Osterman, R. A. Overbeek, L. K. McNeil, D. Paarmann, T. Paczian, B. Parrello, G. D. Pusch, C. Reich, R. Stevens, O. Vassieva, V. Vonstein, A. Wilke and O. Zagnitko (2008). "The RAST Server: rapid annotations using subsystems technology." BMC Genomics **9**: 75.

Beaz-Hidalgo, R., M. J. Hossain, M. R. Liles and M. J. Figueras (2015). "Strategies to avoid wrongly labelled genomes using as example the detected wrong taxonomic affiliation for aeromonas genomes in the GenBank database." PLoS One **10**(1): e0115813.

Berlin, K., S. Koren, C. S. Chin, J. P. Drake, J. M. Landolin and A. M. Phillippy (2015). "Assembling large genomes with single-molecule sequencing and locality-sensitive hashing." Nature Biotechnology **33**(6): 623-630.

Besemer, J. and M. Borodovsky (1999). "Heuristic approach to deriving models for gene finding." Nucleic Acids Research **27**(19): 3911-3920.

Camus, A., J. Dill, A. McDermott, N. Hatcher and M. Griffin (2016). "*Edwardsiella piscicida*-associated septicaemia in a blotched fantail stingray *Taeniura meyeni* (Mueller & Henle)." Journal of Fish Diseases **39**(9):1125-1131.

Clarridge, J. E., 3rd (2004). "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases." Clinical Microbiology Reviews **17**(4): 840-862.

Cohan, F. M. (2002). "What are bacterial species?" Annual Review of Microbiology **56**: 457-487.




المنارة للاستشارات
www.manaraa.com

Crumlish, M., T. T. Dung, J. F. Turnbull, N. T. N. Ngoc and H. W. Ferguson (2002). "Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam." Journal of Fish Diseases **25**: 733-736.

Dahllöf, I., H. Baillie and S. Kjelleberg (2000). "*rpoB*-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity." Applied and Environmental Microbiology **66**(3): 3376-3380.

Dauga, C. (2002). "Evolution of the gyrB gene and the molecular phylogeny of *Enterobacteriaceae*: a model molecule for molecular systematic studies." International Journal of Systematic and Evolutionary Microbiology **52**: 531-547.

Delcher, A. L., D. Harmon, S. Kasif, O. White and S. L. Salzberg (1999). "Improved microbial gene identification with GLIMMER." Nucleic Acids Research **27**(23): 4636-4641.

Ewing, W. H., A. C. McWhorter, M. R. Escobar and A. H. Lubin (1965). "*Edwardsiella*, a new genus of Enterobacteriaceae based on a new species, *E. tarda*." International Bulletin of Bacteriological Nomenclature and Taxonomy **15**(1): 33-38.

Farmer, J. J. and A. C. McWhorter (1984). Genus X. *Edwardsiella*. Bergey's Manual of Systematic Bacteriology. N. R. Krieg and J. G. Holt. Baltimore, MD, Williams and Wilkins.

Fogelson, S. B., B. D. Petty, S. R. Reichley, C. Ware, P. R. Bowser, M. J. Crim, R. G. Getchell, K. L. Sams, H. Marquis and M. J. Griffin (2016). "Histologic and molecular characterization of *Edwardsiella piscicida* infection in largemouth bass (*Micropterus salmoides*)." Journal of Veterinary Diagnostic Investigation **28**(3): 338-344.

Fox, G. E., J. D. Wisotzkey and J. Peter Jurtshuk (1992). "How close is close: 16s rRNA sequence identity may not be sufficient to guarantee species identity." International Journal of Systematic Bacteriology **42**(1): 166-170.

Gao, B. and R. S. Gupta (2012). "Microbial systematics in the post-genomics era." Antonie Van Leeuwenhoek **101**(1): 45-54.

Gevers, D., F. M. Cohan, J. G. Lawrence, B. G. Spratt, T. Coenye, E. J. Feil, E. Stackebrandt, Y. V. d. Peer, P. Vandamme, F. L. Thompson and J. Swings (2005). "Re-evaluating prokaryotic species." Nature Reviews Microbiology **3**: 733-739.

Goris, J., K. T. Konstantinidis, J. A. Klappenbach, T. Coenye, P. Vandamme and J. M. Tiedje (2007). "DNA-DNA hybridization values and their relationship to whole-genome sequence similarities." International Journal of Systematic and Evolutionary Microbiology **57**(Pt 1): 81-91.



المنارة للاستشارات
www.manaraa.com

Griffin, M. J., S. M. Quiniou, T. Cody, M. Tabuchi, C. Ware, R. C. Cipriano, M. J. Mauel and E. Soto (2013). "Comparative analysis of *Edwardsiella* isolates from fish in the eastern United States identifies two distinct genetic taxa amongst organisms phenotypically classified as *E. tarda*." Veterinary Microbiology **165**(3-4): 358-372.

Griffin, M. J., S. R. Reichley, T. E. Greenway, S. M. Quiniou, C. Ware, D. X. Gao, P. S. Gaunt, R. P. Yanong, D. B. Pouder, J. P. Hawke and E. Soto (2016). "Comparison of *Edwardsiella ictaluri* isolates from different hosts and geographic origins." Journal of Fish Diseases **39**(8): 947-969.

Griffin, M. J., C. Ware, S. M. Quiniou, J. M. Steadman, P. S. Gaunt, L. H. Khoo and E. Soto (2014). "*Edwardsiella piscicida* identified in the Southeastern USA by *gyrB* sequence, species-specific and repetitive sequence-mediated PCR." Diseases of Aquatic Organisms **108**(1): 23-35.

Grimont, P. D., F. Grimont, C. Richard and R. Sakazaki (1980). "*Edwardsiella hoshinae*, a new species of Enterobacteriaceae." Current Microbiology **4**(6): 347-351.

Hawke, J. P., M. Kent, M. Rogge, W. Baumgartner, J. Wiles, J. Shelley, L. C. Savolainen, R. Wagner, K. Murray and T. S. Peterson (2013). "Edwardsiellosis caused by *Edwardsiella ictaluri* in laboratory populations of Zebrafish *Danio rerio*." Journal of Aquatic Animal Health **25**(3): 171-183.

Hawke, J. P. and L. H. Khoo (2004). Infectious Diseases. Biology and culture of the channel catfish. C. S. Tucker and J. A. Hargreaves. Amsterdam, Elsevier**:** 387-443.

Hawke, J. P., A. C. McWhorter, A. G. Steigerwalt and D. J. Brenner (1981). "*Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish." International Journal of Systematic Bacteriology **31**(4): 396-400.

Janda, J. M. and S. L. Abbott (2002). "Bacterial Identification for Publication: When Is Enough Enough?" Journal of Clinical Microbiology **40**(6): 1887-1891.

Kim, K. I., J. Y. Kang, J. Y. Park, S. J. Joh, H. S. Lee and Y. K. Kwon (2014). "Phenotypic traits, virulence-associated gene profile and genetic relatedness of *Edwardsiella tarda* isolates from Japanese eel *Anguilla japonica* in Korea." Letters in Applied Microbiology **58**(2): 168-176.

Kim, M., H. S. Oh, S. C. Park and J. Chun (2014). "Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes." International Journal of Systematic and Evolutionary Microbiology **64**(Pt 2): 346-351.



المنارة للاستشارات
www.manaraa.com

Koboldt, D. C., Q. Zhang, D. E. Larson, D. Shen, M. D. McLellan, L. Lin, C. A. Miller, E. R. Mardis, L. Ding and R. K. Wilson (2012). "VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing." Genome Research **22**(3): 568-576.

Konstantinidis, K. T., A. Ramette and J. M. Tiedje (2006). "The bacterial species definition in the genomic era." Philosophical Transactions of the Royal Society B **361**(1475): 1929-1940.

Konstantinidis, K. T. and J. M. Tiedje (2005). "Genomic insights that advance the species definition for prokaryotes." Proceedings of the National Academy of Sciences of the University States of America **102**(7): 2567-2572.

Koren, S., G. P. Harhay, T. P. Smith, J. L. Bono, D. M. Harhay, S. D. Mcvey, D. Radune, N. H. Bergman and A. M. Phillippy (2013). "Reducing assembly complexity of microbial genomes with single-molecule sequencing." Genome Biology **14**: R101.

Koren, S., M. C. Schatz, B. P. Walenz, J. Martin, J. T. Howard, G. Ganapathy, Z. Wang, D. A. Rasko, W. R. McCombie, E. D. Jarvis and M. P. Adam (2012). "Hybrid error correction and de novo assembly of single-molecule sequencing reads." Nature Biotechnology **30**(7): 693-700.

Lagesen, K., P. Hallin, E. Rødland, H. Stærfeldt, T. Rognes and D. Ussery (2007). "RNammer: consistent annotation of rRNA genes in genomic sequences." Nucleic Acids Research **35**(9): 3100-3108.

Langmead, B. and S. L. Salzberg (2012). "Fast gapped-read alignment with Bowtie 2." Nature Methods **9**(4): 357-359.

Li, H. (2013). "Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM." arXiv **1303.3997v2 [q-bio.GN]**.

Liu, J. Y., A. H. Li, D. R. Zhou, Z. R. Wen and X. P. Ye (2010). "Isolation and characterization of *Edwardsiella ictaluri* strains as pathogens from diseased yellow catfish *Pelteobagrus fulvidraco* (Richardson) cultured in China." Aquaculture Research **41**(12): 1835-1844.

Lu, J. F., W. N. Wang, G. L. Wang, H. Zhang, Y. Zhou, Z. P. Gao, P. Nie and H. X. Xie (2015). "*Edwardsiella tarda* EscE (Orf13 protein) is a Type III Secretion System-secreted protein that is required for the injection of effectors, secretion of translocators, and pathogenesis in fish." Infection and Immunity **84**(1): 2-10.

Meier-Kolthoff, J. P., A. F. Auch, H.-P. Klenk and M. Göker (2013). "Genome sequence-based species delimitation with confidence intervals and improved distance functions." BMC Bioinformatics **14**(1): 60.



المنارة للاستشارات
www.manaraa.com

Meyer, F. P. and G. L. Bullock (1973). "*Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*)." Applied Microbiology **25**(1): 155-156.

Mo, Z.-Q., L. Zhou, X. Zhang, L. Gan, L. Liu and X.-M. Dan (2015). "Outbreak of *Edwardsiella tarda* infection in farm-cultured giant mottled eel *Anguilla marmorata* in China." Fisheries Science **81**(5): 899-905.

Mohanty, B. R. and P. K. Sahoo (2007). "Edwardsiellosis in fish: a brief review." Journal of Biosciences **32**: 1331-1344.

Oguro, K., K. Tamura, J. Yamane, M. Shimizu, T. Yamamoto, T. Ikawa, K. Ohnishi, S. Oshima and M. Imajoh (2014). "Draft genome sequences of two genetic variant strains of *Edwardsiella piscicida*, JF1305 and RSB1309, isolated from olive flounder (*Paralichythys olivaceus*) and Red Sea bream (*Pagrus major*) cultured in Japan, respectively." Genome Announcements **2**(3): e00546-00514.

Oren, A. and G. M. Garrity (2013). "List of new names and new combinations previously effectively, but not validly, published." International Journal of Systematic and Evolutionary Microbiology **63**(9): 3131-3134.

Oren, A. and G. M. Garrity (2015). "List of new names and new combinations previously effectively, but not validly, published." International Journal of Systematic and Evolutionary Microbiology **65**(7): 2017-2025.

Overbeek, R., R. Olson, G. D. Pusch, G. J. Olsen, J. J. Davis, T. Disz, R. A. Edwards, S. Gerdes, B. Parrello, M. Shukla, V. Vonstein, A. R. Wattam, F. Xia and R. Stevens (2014). "The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST)." Nucleic Acids Research **42**(Database issue): D206-214.

Reichley, S. R., C. Ware, T. E. Greenway, D. J. Wise and M. J. Griffin (2015). "Real-time polymerase chain reaction assays for the detection and quantification of *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like species in catfish tissues and pond water." Journal of Veterinary Diagnostic Investigation **27**(2): 130-139.

Reichley, S. R., G. C. Waldbieser, H. C. Tekedar, M. L. Lawrence and M. J. Griffin (2015). "Complete Genome Sequence of *Edwardsiella tarda* Isolate FL95-01, Recovered from Channel Catfish." Genome Announcements **3**(3): e00682-00615.

Reichley, S. R., G. C. Waldbieser, M. Ucko, A. Colorni, L. Dubytska, R. L. Thune, M. L. Lawrence and M. J. Griffin (2015). "Complete genome sequence of an *Edwardsiella piscicida*-like species isolated from diseased Grouper in Israel." Genome Announcements **3**(4): e00829-00815.



المنارة للاستشارات
www.manaraa.com

Reichley, S. R., G. C. Waldbieser, M. L. Lawrence and M. J. Griffin (2015). "Complete genome sequence of an *Edwardsiella piscicida*-like species recovered from Tilapia in the United States." Genome Announcements **3**(5): e01004-01015.

Reichley, S. R., G. C. Waldbieser, H. C. Tekedar, M. L. Lawrence and M. J. Griffin (2016). "Complete genome sequence of *Edwardsiella piscicida* isolate S11-285 recovered from Channel Catfish (*Ictalurus punctatus*) in Mississippi, USA." Genome Announcements **4**(6): e01259-01216.

Reichley, S. R., G. C. Waldbieser, M. L. Lawrence and M. J. Griffin (2017). "Complete genome sequence of *Edwardsiella hoshinae* ATCC 35051." Genome Announcements **5**(6): e01605-01616.

Salzberg, S. L., A. L. Delcher, S. Kasif and O. White (1998). "Microbial gene identification using interpolated Markov models." Nucleic Acids Research **26**(2): 544-548.

Seed, B. and H. Wang (Unpublished).

Shafiei, S., S. Viljamaa-Dirks, K. Sundell, S. Heinikainen, T. Abayneh and T. Wiklund (2016). "Recovery of *Edwardsiella piscicida* from farmed whitefish, *Coregonus lavaretus* (L.), in Finland." Aquaculture **454**: 19-26.

Shao, S., Q. Lai, Q. Liu, H. Wu, J. Xiao, Z. Shao, Q. Wang and Y. Zhang (2015). "Phylogenomics characterization of a highly virulent *Edwardsiella* strain ET080813(T) encoding two distinct T3SS and three T6SS gene clusters: Propose a novel species as *Edwardsiella anguillarum* sp. nov." Systematic and Applied Microbiology **38**(1): 36-47.

Singh, B. R., V. Singh, N. Ebibeni and R. K. Singh (2013). "Antimicrobial and herbal drug resistance in enteric bacteria isolated from faecal droppings of common house lizard/gecko (*Hemidactylus frenatus*)." International Journal of Microbiology **2013**: 340848.

Singh, I. M., S. Singh, M. A. McMurphy, S. S. Crupper, F. Mills-Robertson and R. D. Applegate (2004). "Antibiotic susceptibility of *Edwardsiella hoshinae* isolated from northern bobwhite quail (*Colinus virginianus*)." Veterinary Record **155**(1): 29-29.

Soto, E., M. Griffin, M. Arauz, A. Riofrio, A. Martinez and M. E. Cabrejos (2012). "*Edwardsiella ictaluri* as the causative agent of mortality in cultured Nile Tilapia." Journal of Aquatic Animal Health **24**(2): 81-90.

Stackebrandt, E. and B. M. Goebel (1994). "Taxonomic note: a place for DNA-DNA reassociation and 16s rRNA sequence analysis in the present species definition in bacteriology." International Journal of Systematic Bacteriology **44**(4): 846-849.



المنارة للاستشارات

www.manaraa.com

Stepkowski, T., M. Czaplinska, K. Miedzinska and L. Moulin (2003). "The variable part of the *dnaK* gene as an alternative marker for phylogenetic studies of rhizobia and related alpha *Proteobacteria*." Systematic and Applied Microbiology **26**(4): 483-494.

Tekedar, H. C., A. Karsi, M. L. Williams, S. Vamenta, M. M. Banes, M. Duke, B. E. Scheffler and M. L. Lawrence (2013). "Complete genome sequence of channel catfish gastrointestinal septicemia isolate *Edwardsiella tarda* C07-087." Genome Announcements **1**(6): e00959-00913.

Thorvaldsdottir, H., J. T. Robinson and J. P. Mesirov (2013). "Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration." Briefings in Bioinformatics **14**(2): 178-192.

Turenne, C. Y., L. Tschetter, J. Wolfe and A. Kabani (2001). "Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species." Journal of Clinical Microbiology **39**(10): 3637-3648.

Ucko, M., A. Colorni, L. Dubytska and R. L. Thune (2016). "*Edwardsiella piscicida*-like pathogen in cultured grouper." Diseases of Aquatic Organisms **121**(2): 141-148.

van Soest, J. J., O. W. Stockhammer, A. Ordas, G. V. Bloemberg, H. P. Spaink and A. H. Meijer (2011). "Comparison of static immersion and intravenous injection systems for exposure of zebrafish embryos to the natural pathogen *Edwardsiella tarda*." BMC Immunology **12**: 58.

Walker, B. J., T. Abeel, T. Shea, M. Priest, A. Abouelliel, S. Sakthikumar, C. A. Cuomo, Q. Zeng, J. Wortman, S. K. Young and A. M. Earl (2014). "Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement." PLoS One **9**(11): e112963.

Wang, Q., M. Yang, J. Xiao, H. Wu, X. Wang, Y. Lv, L. Xu, H. Zheng, S. Wang, G. Zhao, Q. Liu and Y. Zhang (2009). "Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches." PLoS ONE **4**(10).

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr and H. G. Truper (1987). "Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics " International Journal of Systematic Bacteriology **37**(4): 463-464.

Williams, M. L., A. F. Gillaspy, D. W. Dyer, R. L. Thune, G. C. Waldbieser, S. C. Schuster, J. Gipson, J. Zaitshik, C. Landry, M. M. Banes and M. L. Lawrence (2012). "Genome sequence of *Edwardsiella ictaluri* 93-146, a strain associated with a natural channel catfish outbreak of enteric septicemia of catfish." Journal of Bacteriology **194**(3): 740-741.



المنارة للاستشارات
www.manaraa.com

Wise, D. J., A. C. Camus, T. E. Schwedler and J. S. Terhune (2004). Health Management. Biology and culture of channel catfish. C. S. Tucker and J. A. Hargreaves. Amsterdam, Elsevier**:** 444-502.

Wolf, Y. I., I. B. Rogozin, N. V. Grishin, R. L. Tatusov and E. V. Koonin (2001). "Genome trees constructed using five different approaches suggest new major bacterial clades." BMC Evolutionary Biology **1**(1): 8.

Woo, P. C. Y., S. K. P. Lau, J. L. L. Teng, H. Tse and K.-Y. Yuen (2008). "Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories." Clinical Microbiology and Infection **14**(10): 908-934.

Xu, T. and X.-H. Zhang (2014). "*Edwardsiella tarda*: an intriguing problem in aquaculture." Aquaculture **431**: 129-135.

Yamada, Y. and H. Wakabayashi (1999). "Identification of fish-pathogenic strains belonging to the genus *Edwardsiella* by sequence analysis of *sodB*." Fish Pathology **34**(3): 145-150.

Ye, S., H. Li, G. Qiao and Z. Li (2009). "First case of *Edwardsiella ictaluri* infection in China farmed yellow catfish *Pelteobagrus fulvidraco*." Aquaculture **292**(1-2): 6-10.

Zhou, Y., Y. Geng, K.-Y. Wang, X.-L. Huang, D.-F. Chen, X. Peng, Z.-J. Zhong and Z.-L. Chen (2014). "*Edwardsiella tarda* infection in cultured Ya-fish, *Schizothorax prenanti*, in China." Aquaculture Research **47**(7): 2349-2354.

Zhu, W., A. Lomsadze and M. Borodovsky (2010). "Ab initio gene identification in metagenomic sequences." Nucleic Acids Research **38**(12): e132.



المنارة للاستشارات
www.manaraa.com

# CHAPTER IV

# ADVANCING OUR KNOWLEDGE OF THE *EDWARDSIELLA*: A COMPARATIVE PHENOTYPIC AND GENOTYPIC ANALYSIS OF *EDWARDSIELLA* SPP. ISOLATES FROM DIFFERENT HOSTS AND GEOGRAPHIC ORIGINS

## 4.1 Introduction

The *Edwardsiella* genus was first recognized in the 1960s to describe a group of isolates that did not fit within any known group of *Enterobacteriaceae*. Initially referred to simply as "bacterium 1483-1459," this group included representatives of the "Bartholomew" group first isolated from a human patient with enteric fever and acute gastroenteritis (King and Adler 1964) and possessed many phenotypic similarities to the "Asakusa" group reported from snakes in Japan (Sakazaki and Murata 1962, Sakazaki 1965, Sakazaki 1967). Based on phenotypic differences between the 1483-1459 strains and other groups of *Enterobacteriaceae*, the genus was designated *Edwardsiella* and the species *tarda* was adopted to represent this previously undescribed group (Ewing, McWhorter et al. 1965).

Prior to 2013, the genus consisted of only 3 taxa: *E. tarda*, *E. ictaluri* and *E. hoshinae* (Abayneh, Colquhoun et al. 2013), which represented a diverse group of Gram-negative bacteria infecting a wide range of piscine, reptilian, avian and mammalian hosts (Mohanty and Sahoo 2007). There are limited reports of *E. hoshinae* from a small number of avian and reptilian hosts (Grimont, Grimont et al. 1980, Singh, Singh et al.



المنارة للاستشارات
www.manaraa.com

2004). Conversely, *E. ictaluri* is well-studied and is the causative agent of enteric septicemia of catfish (ESC) in catfish aquaculture in the southeastern United States (Hawke, McWhorter et al. 1981). Although predominantly considered a pathogen of US farm-raised channel catfish, reports have implicated *E. ictaluri* in mortality events in catfish aquaculture in Asia (Crumlish, Dung et al. 2002, Yuasa, Kholidin et al. 2003, Ye, Li et al. 2009, Suanyuk, Rogge et al. 2014) and Pangasius catfish imported into the Caribbean (Phillips, Reichley et al. 2016). Moreover, *E. ictaluri* was recently reported from mortality events in tilapia pond-culture in Central America, laboratory populations of zebrafish in the United States and wild populations of ayu (*Plecoglossus altivelis*) in Japan (Sakai, Kamaishi et al. 2008, Soto, Griffin et al. 2012, Hawke, Kent et al. 2013).

Comparatively, *Edwardsiella tarda* is cited as the causative agent of edwardsiellosis in fish and has been reported from over 20 fish species across seven continents (Hawke and Khoo 2004, Mohanty and Sahoo 2007). It has also been isolated from reptiles, birds and mammals, and it has moderate zoonotic potential in young, elderly and immune-compromised individuals (Sharma, Kaura et al. 1974, Tan and Lim 1977, Nucci, Silveira et al. 2002, Mohanty and Sahoo 2007, Leotta, Piñeyro et al. 2009, Wang, Yan et al. 2012). Despite its wide host range, *E. tarda* has mostly been implicated in disease outbreaks in cultured fish and is one of the most important bacterial pathogens in global aquaculture (Kodama, Murai et al. 1987, Castro, Toranzo et al. 2006, Xu and Zhang 2014).

Primarily viewed as a pathogen of marine and freshwater fish, *E. tarda* has extensive phenotypic and genetic diversity. In 2012, a comparative phylogenomic study demonstrated isolates phenotypically identified as *E. tarda* comprised two genetically



www.manaraa.com

distinct, polyphyletic groups (Yang, Lv et al. 2012). This work was supported by concurrent investigations utilizing multilocus sequence analysis of *E. tarda* isolates in Asia and Europe, as well as genotypic and phenotypic analysis of *E. tarda* isolates from fish in the United States (Abayneh, Colquhoun et al. 2012, Griffin, Quiniou et al. 2013). These studies concluded that isolates historically classified as *E. tarda* actually represented three genetically distinct, yet phenotypically indistinguishable species. Further phenotypic characterization, DNA-DNA hybridization and phylogenetic analysis led to the adoption of *E. piscicida* as a fourth member of the genus in 2013 (Abayneh, Colquhoun et al. 2013). Expanding on these analyses, polyphasic phenotypic and genomic characterization of *Edwardsiella* isolates from diseased eels led to the addition of a fifth species of *Edwardsiella*, *E. anguillarum*, in 2015 (Shao, Lai et al. 2015).

Previous research documenting phenotypic and genotypic diversity of *E. tarda* resulted in multiple generalized designations to account for the extensive intraspecific variability (Yamada and Wakabayashi 1999, Matsuyama, Kamaishi et al. 2005, Castro, Toranzo et al. 2006, Sakai, Iida et al. 2007, Wang, Wang et al. 2011, Xu and Zhang 2014). As a result, isolates of *E. tarda* primarily fell into one of three different categories: 1) typical motile fish pathogenic *E. tarda;* 2) atypical non-motile fish pathogenic *E. tarda*; and 3) fish non-pathogenic *E. tarda* (Yamada and Wakabayashi 1999, Nakamura, Takano et al. 2013). The recent segregation of *E. tarda* into three discrete taxa suggests these designations likely correspond with this separation (Griffin, Ware et al. 2014, Shao, Lai et al. 2015). The purpose of the current study was to utilize routine phenotypic and genotypic analyses, coupled with popular microbial identification systems and confirmatory methods, to evaluate current bacterial identification procedures for



المنارة للاستشارات
www.manaraa.com

differentiating the *Edwardsiella*. Furthermore, an ancillary goal of this work was to link historical records of different *E. tarda* categories to current phylogenomic assignments with contemporary taxonomic nomenclature.

## 4.2 Materials and Methods

### 4.2.1 Bacterial Isolates

Isolates of *E. anguillarum*, *E. hoshinae*, *E. ictaluri*, *E. piscicida* and *E. tarda* were obtained from collaborators and various biological collections and expanded in porcine brain heart infusion broth (Bacto™, Becton Dickenson and Company) (BHIb) at optimal growth temperatures for each isolate (37°C: *E. anguillarum*, *E. hoshinae*, *E. piscicida*, *E. tarda*; 28°C: *E. ictaluri*). Aliquots of these broth cultures were stored cryogenically (-80°C) with 15% v/v glycerol. A panel of 47 representative isolates from 10 countries and 19 host species, isolated over a 47 year period, were chosen for analysis (Table 4.1). Of note, isolate Edwardsiella 9.1 was isolated during the original description of emphysematous putrefactive disease in channel catfish (*Ictalurus punctatus*; Meyer and Bullock 1973). For all phenotypic analyses, cryostocks of archived isolates were revived by isolation streaking on Mueller-Hinton II Agar (BBL™, Becton Dickinson and Company) supplemented with 5% defibrinated sheep blood (Hemastat Laboratories, Dixon CA) and grown for 24 (*E. anguillarum*, *E. hoshinae*, *E. piscicida*, *E. tarda*) or 48 (*E. ictaluri)* hr at optimal temperatures for each respective isolate.



المنارة للاستشارات
www.manaraa.com

Table 4.1 *Edwardsiella* spp. isolates analyzed in the current study.

| Isolate | Species | Host | Geographic Origin | Year of Isolation |
|---|---|---|---|---|
| EA181011 | *E. anguillarum* | White grouper | Israel | 2011 |
| LADL05-105 | *E. anguillarum* | Tilapia | Louisiana, USA | 2005 |
| 43472 | *E. anguillarum* | Blue striped grunt | Maryland, USA | 2003 |
| 43664 | *E. anguillarum* | Striped bass | Maryland, USA | 1994 |
| 43473 | *E. anguillarum* | Tilapia | Maryland, USA | 1997 |
| 43659 | *E. anguillarum* | Tilapia | Maryland, USA | 1998 |
| 43651 | *E. anguillarum* | Tilapia | Maryland, USA | 1999 |
| ATCC35051 | *E. hoshinae* | Monitor | Chad | 1978 |
| 11-149A | *E. ictaluri* | Zebrafish | Florida, USA | 2011 |
| S97-773 | *E. ictaluri* | Channel catfish | Mississippi, USA | 1997 |
| RUSVM-1 | *E. ictaluri* | Tilapia | W. Hemisphere | 2012 |
| PB 07-309 | *E. piscicida* | Smallmouth bass | Arkansas, USA | 2007 |
| NFAVS-1 | *E. piscicida* | Largemouth bass | Florida, USA | 2014 |
| F373.2 | *E. piscicida* | Turbot | France | 2012 |
| HL1 | *E. piscicida* | Turbot | Holland | 2006 |
| HL25 | *E. piscicida* | Turbot | Holland | 2006 |
| HL32 | *E. piscicida* | Turbot | Holland | 2006 |
| WFE1 | *E. piscicida* | Fluke | Japan | 2002 |
| S11-285 | *E. piscicida* | Channel catfish | Mississippi, USA | 2011 |
| C1490 | *E. piscicida* | Largemouth bass | New York, USA | 2014 |
| CMT 8211-1 | *E. piscicida* | Rainbow trout | North Carolina, USA | 2014 |
| REDS 81911-E | *E. piscicida* | Rainbow trout | North Carolina, USA | 2014 |
| RBR8 | *E. piscicida* | Turbot | Portugal | 2008 |
| SC 09-03 | *E. piscicida* | Smallmouth bass | South Carolina, USA | 2009 |
| ACC69 | *E. piscicida* | Turbot | South Europe | 2005 |
| CAQ 8.10 | *E. piscicida* | Turbot | Spain | 2009 |
| CAQ 10.10 | *E. piscicida* | Turbot | Spain | 2009 |
| CAQ 39 | *E. piscicida* | Turbot | Spain | 2009 |
| A15-02670 | *E. piscicida* | Blotched fantail stingray | Georgia, USA | 2015 |
| 43628 | *E. piscicida* | Koi | Maryland, USA | 2000 |
| 43662 | *E. piscicida* | Seatrout | Maryland, USA | 1988 |
| 43644 | *E. piscicida* | Striped bass | Maryland, USA | 1994 |
| 43475 | *E. piscicida* | Striped bass | Pennsylvania, USA | 1996 |
| 43658 | *E. piscicida* | Striped bass | Pennsylvania, USA | 1996 |
| 43468 | *E. piscicida* | Striped bass | Maryland, USA | 1999 |
| 43656 | *E. piscicida* | Striped bass | Maryland, USA | 2000 |
| Edwardsiella 9.1 | *E. tarda* | Channel catfish | Arkansas, USA | 1969 |
| Edwardsiella 9.2 | *E. tarda* | Channel catfish | West Virginia, USA | 1977 |
| Edwardsiella 9.3 | *E. tarda* | Flounder | Virginia, USA | 1984 |
| Edwardsiella 9.4 | *E. tarda* | Channel catfish | Georgia, USA | 1979 |
| FL95-01 | *E. tarda* | Channel catfish | Florida, USA | 1995 |
| 070720-1 3A | *E. tarda* | Tilapia | Michigan, USA | 2007 |
| 070720-1 2HLDOM | *E. tarda* | Tilapia | Michigan, USA | 2007 |
| 43657 | *E. tarda* | Bottlenose dolphin | Maryland, USA | 2000 |
| 43650 | *E. tarda* | Hooded seal | Maryland, USA | 2004 |
| 43627 | *E. tarda* | Tilapia | Pennsylvania, USA | 2000 |
| 43663 | *E. tarda* | Toadfish | Maryland, USA | 1988 |



المنارة للاستشارات
www.manaraa.com

### 4.2.2 DNA isolation

Cryostocks were revived as above, and individual colonies were expanded for 24-48 hr in static BHIb at appropriate temperatures for each isolate. Cultures were pelleted by centrifugation and genomic DNA (gDNA) was isolated using a commercial DNA isolation kit following the manufacturer's suggested protocols for Gram-negative bacteria (Gentra® Puregene® DNA isolation kit; QIAGEN). Isolated gDNA was resuspended in 100 µl of DNA hydration solution (DHS, Gentra® Puregene® DNA isolation kit; QIAGEN), quantified spectrophotometrically (NanoDrop 2000, ThermoFisher Scientific), diluted with DHS to a final concentration of 10 ng/µl and cryogenically stored (-80°C) until further use.

### 4.2.3 Motility and TSI

Individual colonies of *Edwardsiella* sp. isolates were stabbed into motility medium (Difco, 1998) and evaluated for motility after 48 hr at 37°C (*E. anguillarum*, *E. hoshinae*, *E. piscicida*, *E. tarda*) or 28°C (*E. ictaluri*). Glucose, sucrose and lactose fermentation, in addition to hydrogen gas and/or hydrogen sulfide production in triple sugar iron medium (TSI; Oxoid LTD), was determined using similar incubation conditions.

### 4.2.4 Microbial identification systems

The commercial API-20E system (BioMerieux) was used for all bacterial species in accordance with the manufacturer's instructions. Briefly, after incubation (24 hr at 37°C for *E. anguillarum*, *E. hoshinae*, *E. piscicida*, *E. tarda*; 48 hr at 28°C for *E. ictaluri*), all reagents were added and a seven digit profile number was generated. Profile



المنارة للاستشارات
www.manaraa.com

numbers were submitted to BioMerieux for microbial identification. Additionally, isolates were analyzed using the Biolog Microbial Identification System (Biolog) according to manufacturer's instructions. In short, isolates were streaked from archived cryostocks on Biolog Universal Growth (BUG) agar with 5% sheep blood (Biolog). After 24 hr at 28°C, colonies were picked and added to the inoculating fluid (Biolog) to reach 92% - 98% transmittance (%T). Gen III microplates were inoculated and incubated at 28°C for 24 hr, after which reactions were read and identification performed using OmniLog® Data Collection software (Biolog).

Lastly, bacterial isolates were subjected to the BBL Crystal Enteric/Nonfermentor Identification Kit (Becton Dickinson and Company). Cryostocks were streaked for isolation on Mueller-Hinton II Agar (BBL™; Becton Dickinson and Company) supplemented with 5% defibrinated sheep blood (Hemastat Laboratories, Dixon CA). Individual colonies were picked using a sterile toothpick and resuspended in inoculating fluid to achieve 0.5 McFarland standard turbidity before addition to the assay panel. Panels were incubated at 28°C for 24 - 48 hr, and reactions were visualized and recorded.

A commercial matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker MALDI-TOF Biotyper LT) was used for bacterial identification and generation of peptide mass spectral profiles. Bacterial colonies were applied to a spot on the MALDI-TOF target plate and overlaid with freshly made 70% formic acid and α-cyano-4-hydroxycinnamic acid matrix solution following the manufacturer's recommended protocol. The spectra were captured in positive linear mode in a mass range of 2 - 20 kDa with a laser frequency of 60 Hz (IS1: 20 kV; IS2: 18 kV; lens: 6 kV; extraction delay time: 100 ns). Spectra were acquired in automatic mode



المنارة للاستشارات
www.manaraa.com

by accumulating a maximum of 240 profiles (6 × 40 laser shots from different positions of the target spot). Bacterial identification was performed using default settings of the software provided with the Bruker MALDI-TOF system. A score value >2 indicated highly probable bacterial genus and species identification. The peptide spectra were collected and analyzed using Flexanalysis software (Bruker).

### 4.2.5 Fatty acid methyl ester (FAME) analysis

#### 4.2.5.1 Preparation of bacterial isolates

The 47 *Edwardsiella* spp. isolates used for fatty acid methyl ester (FAME) extraction analysis were grown under identical conditions. Frozen stock from each isolate was streaked onto sheep blood agar (SBA; Remel, Lenexa, KS) and incubated for 24 h at 28ºC. Following incubation, an average of 35 mg of bacteria (wet weight) were harvested from each plate by carefully scraping the surface of the agar plate. Bacterial cells were placed into individual Pyrex glass tubes, centrifuged for 1 min at 5,250 x *g* and then used for the fatty acid extraction.

#### 4.2.5.2 Fatty acid methyl ester extraction

Bacteria were saponified by adding 1.0 ml of Saponification Reagent (150 ml of deionized distilled water combined with 150 ml of HPLC grade methanol and 45 grams of sodium hydroxide [certified ACS]) to each of the tubes. Each tube was then immediately vortexed for 5-10 s and boiled for 5 min in water at 100ºC. The tubes were vortexed for 10 s then boiled for 25 min at 100ºC. Samples were then methylated by the addition of 2.0 ml Methylation Reagent (162.5 ml of 6.0N hydrochloric acid with 137.5



المنارة للاستشارات
www.manaraa.com

ml of HPLC grade methanol), vortexed for 10 s, then immediately cooled on ice for 10 min.

Following methylation, FAMEs were extracted by the addition of 1.25 ml Extraction Solvent (200 ml of HPLC grade hexane combined with 200 ml of HPLC grade methyl-tert-butyl ether) to each sample. The samples were immediately loaded into a circular rotator and centrifuged for 10 min at 3,000 x *g* to ensure adequate combination of sample and Extraction Solvent. The tubes were centrifuged for 1 min at 5,250 x *g* to separate extraction waste from the sample. The bottom phase was removed and discarded from each of the samples using a long tip Pasteur pipette. Three ml of Base Wash (5.4 grams of sodium hydroxide diluted in 45 ml of distilled water) was added to the top phase of each sample and then immediately centrifuged for 5 min at 3,000 x *g*. The samples were then held upright at room temperature to complete the separation between the bottom and top phases. The top phase (100 µL) from each sample was removed and transferred into a glass vial (National Scientific Target Vials C4011-1) fitted with 100 µl glass insert with polymer feet and lid.

#### 4.2.5.3 Gas Chromatography Conditions and Analysis

For gas chromatography, an HP-Ultra-2 column with a length of 25 mm, diameter of 0.200 mm and a film of 0.33 µm was used. The RCLIN6 method was used for the chromatography; the temperature was increased at 28ºC/min from 170ºC to 288ºC, the split ratio was 40:1, and the total run was 6.23 min. Following gas chromatography, samples were analyzed using the Sherlock Microbial Identification System (MIS) RCLIN6 6.2 library.



المنارة للاستشارات
www.manaraa.com

### 4.2.6 Antimicrobial susceptibility profiles

The minimum inhibitory concentration (MIC) of 39 different antimicrobial agents was determined for all 47 *Edwardsiella* spp. isolates for potential identification of specific inherent resistance/susceptibility that could be exploited to differentiate among the *Edwardsiella* spp. MICs were evaluated using the Sensititre® GN4F and AVIAN1F plate formats (Trek Diagnostic System) using the manufacturer's suggested protocol. *Escherichia coli* ATCC25922 was used as the quality control strain. Inocula were prepared by suspending individual colonies in sterile distilled water to a 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 (provided by the manufacturer) and incubated (24 hr at 37°C for *E. anguillarum*, *E. hoshinae*, *E. piscicida*, *E. tarda*; 48 hr at 28°C for *E. ictaluri*). Following incubation, plates were checked visually and MIC values were defined as the lowest drug concentration exhibiting no visible growth.

### 4.2.7 Phylogenetic analysis

Three different gene targets were chosen for amplification and sequencing to link historical *E. tarda* isolates to contemporary phylogenomic assignments. Primers used for amplification and sequencing of the 16S rRNA, *gyrB* and *sodB* genes are listed in Table 4.2. Amplification reactions (50 µl) were performed using 43 µl of Platinum® High Fidelity PCR SuperMix (Invitrogen), 20 pmol of each primer, ~50 ng of gDNA and nuclease-free water to volume. Amplifications were performed using a C1000 Touch thermal cycler (Bio-Rad Laboratories, Inc.). For 16S rRNA and *gyrB*, the following cycling conditions were used: 3 min denaturation at 94°C; 45 cycles of 30 sec at 94°C, 30



المنارة للاستشارات
www.manaraa.com

sec at 52°C, 2 min at 68°C; and 7 min extension at 68°C. For *sodB* the following cycling conditions were used: 2 min denaturation at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 42°C, 30 sec at 72°C; and 7 min extension at 72°C. Amplicons were visualized with UV light after electrophoretic passage through a 0.8% agarose gel containing ethidium bromide (0.5 µg/ml), excised, and purified using Qiaquick™ columns (QIAGEN). Purified PCR products were sequenced commercially using the same primers employed to generate the amplicons (Eurofins Genomics, Louisville, KY). Contiguous sequences were assembled, and ambiguous base calls were manually determined from corresponding chromatograms using Geneious v10.0.7 (Biomatters, Ltd.) (Kearse, Moir et al. 2012).



المنارة للاستشارات
www.manaraa.com

Table 4.2 Primers and probes used in the current study.

| Primer | Sequence (5'-3') | Source |
|---|---|---|
| | **16S sequencing** | |
| 27F | GAGTTTGATCCTGGCTCAG | Rainey et al. (1996) |
| 1525R | AGAAAGGAGGTGATCCAGCC | |
| | ***gyrB* sequencing** | |
| GyrB630F | GGATAACGCGATTGACGAAG | Griffin et al. (2014) |
| GyrB1245R | ATCRTCYTTCATGGTCGARA | |
| GyrB2198F | TAAAGACGATGAGGCGATGG | |
| GyrB2540R | GCCGTGARCAAARTCRAA | |
| | ***sodB* sequencing** | |
| E1F | ATGTCRTTCGAATTACCTGC | Yamada and Wakabayashi (1999) |
| 497R | TCGATGTARTARGCGTGTTCCCA | |
| | **Repetitive extragenic palindromic PCR** | |
| BOX | CTACGGCAAGGCGACGCTGACG | Versalovic et al. (1991, 1994) |
| ERIC I | ATGTAAGCTCCTGGGGATTCAC | Versalovic et al. (1991, 1994) |
| ERIC II | AAGTAAGTGACTGGGGTGAGCG | Versalovic et al. (1994) |
| $GTG_5$ | GTGGTGGTGGTGGTG | Versalovic et al. (1994) |
| | **Multiplex real-time PCR** | |
| *E. tarda* | | |
| ET3518F | CAGTGATAAAAAGGGGTGGA | Reichley et al. (2015) |
| ET3632R | CTACACAGCAACGACAACG | |
| ET3559P | AGACAACAGAGGACGGATGTGGC | |
| *E. piscicida* | | |
| EP14529F | CTTTGATCATGGTTGCGGAA | Reichley et al. (2015) |
| EP14659R | CGGCGTTTTCTTTTCTCG | |
| EP14615P | CCGACTCCGCGCAGATAACG | |
| *E. anguillarum* | | |
| EA1583F | GATCGGGTACGCTGTCAT | Reichley et al. (2015) |
| EA1708R | AATTGCTCTATACGCACGC | |
| EA1611P | CCCGTGGCTAAATAGGACGCG | |
| *E. ictaluri* | | |
| EI481F | ACTTATCGCCCTCGCAACTC | Griffin et al. (2011) |
| EI658R | CCTCTGATAAGTGGTTCTCG | |
| EI561P | CCTCACATATTGCTTCAGCGTCGAC | |



www.manaraa.com

Sequences from 16S rRNA, *gyrB* and *sodB* were trimmed and aligned using the Multiple Sequence Comparison by Log-Expectation (M.U.S.C.L.E.; Edgar 2004) application of MEGA v6 (Tamura, Stecher et al. 2013), and pairwise sequence similarities were determined. Moreover, *sodB* sequences of *Edwardsiella* spp. were compared to *sodB* sequences of typical motile fish pathogenic *E. tarda* (GenBank AB009853); atypical non-motile fish pathogenic *E. tarda* (GenBank AB009584); and fish non-pathogenic *E. tarda* (GenBank AB009850) (Yamada and Wakabayashi 1999). Bayesian Inference Criterion identified the Kimura 2-parameter model with gamma distribution (16S rRNA), Tamura-Nei model with gamma distribution (*gyrB*) and Tamura 3-parameter model with gamma distribution (*sodB*) as the best-fit nucleotide substitution model for Maximum Likelihood analysis (Nei and Kumar 2000). All positions containing gaps and missing data were eliminated. The final trees were constructed from 1000 bootstrap replicates. Sequences for 16S rRNA, *gyrB* and *sodB* genes were deposited in GenBank.

### 4.2.8 Genetic fingerprinting

Repetitive extragenic palindromic PCR (rep-PCR) fingerprinting was performed on isolates using previously published primer sets (Table 4.2) and modifications to existing protocols (Versalovic, Koeuth et al. 1991, Versalovic, Schneider et al. 1994, Griffin, Quiniou et al. 2013, Chou, Griffin et al. 2014, Griffin, Ware et al. 2014). Briefly, 50 µl reactions comprising 25 µl of IQ Supermix (BioRad; Hercules, CA), 20 pmol (ERIC I & II) or 40 pmol (ERIC II; BOX; $GTG_5$) of primer, 10 ng of DNA template and nuclease-free water to volume. Amplifications were performed on a C1000 Touch thermal cycler (Bio-Rad Laboratories, Inc.) with the following temperature profiles:



المنارة للاستشارات
www.manaraa.com

BOX, ERIC II, ERIC I&II, 1 cycle at 95°C for 10 min; 5 cycles of 95°C for 1 min, 40°C for 1 min, and 72°C for 5 min; and 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 5 min; $GTG_5$, 1 cycle at 95°C for 10 min; 45 cycles of 95°C for 1 min, 46°C for 1 min, and 72°C for 3 min with a final extension at 72°C for 10 min. Aliquots of each amplification reaction (10 µl each) and a molecular weight standard (Hyperladder 50 bp; Bioline) were electrophoresed through a 1.5% (wt/vol) agarose gel with ethidium bromide (0.5 µg/ml) and visualized under ultraviolet light. Genetic fingerprints generated by the BOX primer were analyzed using Quantity One software v. 4.6.9 (Bio-Rad Laboratories, Inc.). Band sizes were estimated by comparison with concurrently run standards, and distinct bands were manually annotated to calculate Dice coefficients and generate a dendrogram based on the unweighted pair-group method using arithmetic averages (UPGMA).

### 4.2.9 Multiplex real-time PCR

A real-time, multiplex polymerase chain reaction (mPCR) specific to *E. anguillarum, E. ictaluri*, *E. piscicida* and *E. tarda* was developed based on previously published primers, probes and protocols (Sakai, Yuasa et al. 2009, Griffin, Quiniou et al. 2013, Reichley, Ware et al. 2015). Primers and probes (Table 4.2) were synthesized commercially (Eurofins MWG; Louisville, KY); each probe was labeled with a fluorescent reporter dye (*E. anguillarum*, Texas Red; *E. ictaluri*, 5-HEX; *E. piscicida*, 6-FAM; *E. tarda*, Cy5) on the 5'-end and appropriate quencher dye (black hole quencher-1 for HEX and 6-FAM; black hole quencher-2 for Texas Red and Cy5) on the 3'-end. The 25-µl reaction contained 12 µl of PCR master mix (TaqMan Environmental Mastermix 2.0, Applied Biosytems), 5 pM of each primer, 0.5 pM of each probe, DNA template and



المنارة للاستشارات
www.manaraa.com

nuclease-free water to volume. Amplifications were performed on a CFX96 thermal cycler (Bio-Rad Laboratories, Inc.) with the following temperature profile: 1 cycle of 95°C for 15 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data collection occurred following the 60°C annealing/extension step at the end of each cycle.

Specificity of the mPCR assay was tested against both target and non-target *Edwardsiella* gDNA. Additionally, the specificity and sensitivity of the assay was tested using serial ten-fold dilutions of target gDNA, supplementing each reaction with an equal mixture of ~10,000 copies of each non-target *Edwardsiella* sp. gDNA to ensure large quantities of non-target DNA did not impair reaction efficiency. Samples, as well as no-template controls, were run in triplicate using the reaction conditions above. Each plate was run in triplicate on three separate occasions to assess the repeatability and reproducibility of the assay. Quantification cycles (Cq) for each reaction were based on a user-defined baseline threshold of 50 relative fluorescent units (RFUs).

### 4.2.10 Plasmid analysis

For all isolates, plasmid DNA was harvested from 3 ml of expanded BHIb cultures using the QIAprep Spin Miniprep Kit (QIAGEN). Plasmids were identified by separation on a 0.8% agarose gel. When present, plasmid sizes were approximated with concurrently run standards (Supercoiled DNA Ladder, New England Biolabs). Harvested plasmids were submitted to the complete plasmid sequencing service of the DNA Core Facility of the Center for Computational and Integrative Biology at Massachusetts General Hospital (Boston, MA, USA) for sequencing. Open reading frames (ORFs) were predicted using GeneMark.hmm prokaryotic v3.25 (Besemer and Borodovsky 1999, Zhu, Lomsadze et al. 2010) and Glimmer v3.02 (Salzberg, Delcher et al. 1998, Delcher,



المنارة للاستشارات
www.manaraa.com

Harmon et al. 1999). 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-02 considered insignificant.

## 4.3 Results

### 4.3.1 Motility and TSI

The motility and TSI results for each isolate are listed in Table 4.3. The *E. hoshinae* isolate and all *E. piscicida* isolates were motile; motility was also observed from the three *E. ictaluri* isolates, although dispersion was not as widespread. Observed motility of *E. anguillarum* and *E. tarda* isolates varied by isolate. All *Edwardsiella* spp. isolates tested positive for glucose fermentation. No hydrogen sulfide production was observed in the *E. hoshinae* or *E. ictaluri* isolates; production from *E. anguillarum* isolates was weak. All *E. piscicida* and *E. tarda* isolates were positive for hydrogen sulfide production. Gas production was present in 6/7 *E. anguillarum*, 1/1 *E. hoshinae*, 0/3 *E. ictaluri*, 25/25 *E. piscicida* and 10/11 *E. tarda* isolates.



المنارة للاستشارات
www.manaraa.com

Table 4.3 Motility and triple sugar iron (TSI) analysis of the isolates used in the current study.

| Isolate | Motility | TSI |
|---|---|---|
| ***E. anguillarum*** | | |
| EA181011 | - | K/A + gas + H2S (weak) |
| LADL05-105 | + | K/A + gas + H2S (weak) |
| 43472 | + (weak) | K/A + gas + H2S (weak) |
| 43664 | + (weak) | K/A + H2S (weak); no gas |
| 43473 | + | K/A + gas + H2S (weak) |
| 43659 | + | K/A + gas + H2S (weak) |
| 43651 | + | K/A + gas + H2S (weak) |
| ***E. hoshinae*** | | |
| ATCC35051 | + | A/A +gas |
| ***E. ictaluri*** | | |
| 11-149A | + (weak) | K/A |
| RUSVM-1 | + (weak) | K/A |
| S97-773 | + (weak) | A/A |
| ***E. piscicida*** | | |
| PB 07-309 | + | K/A + gas |
| NFAVS-1 | + | K/A + gas +H2S |
| F373.2 | + | K/A + gas +H2S |
| HL1 | + | K/A + gas +H2S |
| HL25 | + | K/A + gas +H2S |
| HL32 | + | K/A + gas +H2S |
| WFE1 | + | K/A + gas +H2S |
| S11-285 | + | K/A + gas +H2S |
| C1490 | + | K/A + gas +H2S |
| CMT 8211-1 | + | K/A + gas +H2S |
| REDS 81911-E | + | K/A + gas +H2S |
| RBR8 | + | K/A + gas +H2S |
| SC 09-03 | + | K/A + gas +H2S |
| ACC69 | + | K/A + gas +H2S |
| CAQ 8.10 | + | K/A + gas +H2S |
| CAQ 10.10 | + | K/A + gas +H2S |
| CAQ 39 | + | K/A + gas +H2S |
| A15-02670 | + | K/A + gas +H2S |
| 43628 | + | K/A + gas +H2S |
| 43662 | + | K/A + gas + H2S (weak) |
| 43644 | + | K/A + gas +H2S |
| 43475 | + | K/A + gas +H2S |
| 43658 | + | K/A + gas +H2S |
| 43468 | + | K/A + gas +H2S |
| 43656 | + | K/A + gas +H2S |
| ***E. tarda*** | | |
| *Edwardsiella* 9.1 | + | K/A + gas +H2S |
| *Edwardsiella* 9.2 | + | K/A + gas +H2S |
| *Edwardsiella* 9.3 | + | K/A + gas +H2S |
| *Edwardsiella* 9.4 | + | K/A + gas +H2S |
| FL95-01 | + | K/A + gas +H2S |
| 070720-1 3A | + | K/A + gas +H2S |
| 070720-1 2HLDOM | + | K/A + gas +H2S |
| 43657 | - | K/A + gas +H2S |
| 43650 | + | K/A + gas +H2S |
| 43627 | + | K/A + gas +H2S |
| 43663 | - | K/A + H2S |



المنارة للاستشارات
www.manaraa.com

### 4.3.2 Microbial identification systems

The API 20E system correctly identified all PCR-confirmed *E. tarda* and *E. hoshinae* isolates with ≥99% confidence. The three *E. ictaluri* isolates from three different fish hosts all produced API codes in line with previous reports (Soto, Griffin et al. 2012, Hawke, Kent et al. 2013), which resulted in an identification of *E. coli* with a 52.7% confidence (CL). Of the *E. piscicida* isolates tested, 64% (16/25) were identified as *E. tarda* (CL: 96.7% to 99.9%). The remaining 36% (9/25) of *E. piscicida* isolates produced codes that were non-definitive as they represented multiple species. Similarly, 29% (2/7) of *E. anguillarum* isolates produced non-definitive ambiguous codes. Of the remaining *E. anguillarum* isolates, 4/7 (57%) were identified as *E. tarda* (CL: 96.7% - 99.4%) and 1/7 (14%) was identified as *Vibrio parahaemolyticus* (CL: 53.2%). API 20E results are consistent with those reported previously for *Edwardsiella* (Yamada and Wakabayashi 1999, Alcaide, Herraiz et al. 2006, Castro, Toranzo et al. 2006) and can be found in Table 4.4.



المنارة للاستشارات

www.manaraa.com

Table 4.4 Antimicrobial identification system results for isolates analyzed in the current study.

| Isolate | API 20E | | | Biolog | | BBL Crystal Nonfermentor | | | MALDI-TOF | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Code | ID | CL | ID | CL | Code | ID | CL | ID | CS |
| ***E. piscicida*** | | | | | | | | | | |
| PB 07-309 | 6364000* | | | *E. tarda* | 65% | 2403010113 | *E. tarda* | 99.9% | *E. tarda* | 2.23 |
| NFAVS-1 | 6764000* | | | *E. tarda* | 81% | 2002010113 | *E. tarda* | 99.5% | *E. tarda* | 2.12 |
| F373.2 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 87% | 2003010113 | *E. tarda* | 99.2% | *E. tarda* | 2.18 |
| HL1 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 83% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.25 |
| HL25 | 4344000 | *E. tarda* | 99.4% | *E. ictaluri* | 67% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.21 |
| HL32 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 58% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.24 |
| WFE1 | 4764000 | *E. tarda* | 96.7% | *E. tarda* | 59% | 2003100113 | *E. tarda* | 98.7% | *E. tarda* | 2.23 |
| S11-285 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 95% | 2403110113 | *E. tarda* | 99.9% | *E. tarda* | 2.25 |
| C1490 | 6764000* | | | *E. tarda* | 68% | 2002000113 | *E. tarda* | 99.9% | *E. tarda* | 2.13 |
| CMT 8211-1 | 6764000* | | | *E. tarda* | 86% | 2003000113 | *E. tarda* | 99.7% | *E. tarda* | 2.24 |
| REDS 81911-E | 4764000 | *E. tarda* | 96.7% | *E. tarda* | 58% | 2003100113 | *E. tarda* | 98.7% | *E. tarda* | 2.21 |
| RBR8 | 6564000* | | | *E. ictaluri* | 62% | 2003000113 | *E. tarda* | 99.7% | *E. tarda* | 2.26 |
| SC 09-03 | 4764000 | *E. tarda* | 96.7% | *E. tarda* | 94% | 0403010113 | *E. tarda* | 99.9% | *E. tarda* | 2.20 |
| ACC69 | 6564000* | | | *E. tarda* | 62% | 2003000113 | *E. tarda* | 99.7% | *E. tarda* | 2.18 |
| CAQ 8.10 | 6565000* | | | *E. ictaluri* | 80% | 2002000113 | *E. tarda* | 99.9% | *E. tarda* | 2.22 |
| CAQ 10.10 | 4564000 | *E. tarda* | 97.4% | *E. ictaluri* | 81% | 2003000113 | *E. tarda* | 99.7% | *E. tarda* | 2.18 |
| CAQ 39 | 4544000 | *E. tarda* | 99.9% | *E. ictaluri* | 62% | 2002000113 | *E. tarda* | 99.9% | *E. tarda* | 2.25 |
| A15-02670 | 4344000 | *E. tarda* | 99.4% | *E. tarda* | 81% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.24 |
| 43628 | 4764000 | *E. tarda* | 96.7% | *E. tarda* | 83% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.24 |
| 43662 | 6364000* | | | *E. tarda* | 83% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.24 |
| 43644 | 6764000* | | | *E. tarda* | 74% | 2003100113 | *E. tarda* | 98.7% | *E. tarda* | 2.18 |
| 43475 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 62% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.28 |
| 43658 | 4744000 | *E. tarda* | 99.4% | *E. ictaluri* | 88% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.14 |
| 43468 | 6744000 | *E. tarda* | 99.4% | *E. ictaluri* | 69% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.23 |
| 43656 | 4344000 | *E. tarda* | 99.4% | *E. ictaluri* | 67% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.17 |
| ***E. anguillarum*** | | | | | | | | | | |
| EA181011 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 70% | 2002010113 | *E. tarda* | 99.5% | *E. tarda* | 2.15 |
| LADL05-105 | 4344100 | *V. parahaemolyticus* | 53.2% | *E. ictaluri* | 68% | 2403014113 | *E. tarda* | 99.9% | *E. tarda* | 2.17 |
| 43472 | 6744100 | *E. tarda* | 99.4% | *E. tarda* | 76% | 2003114113 | *B. gladioli* | 94.4% | *E. tarda* | 2.31 |
| 43664 | 4764000 | *E. tarda* | 96.7% | *E. hoshinae* | 69% | 2003114113 | *B. gladioli* | 94.4% | *E. tarda* | 2.29 |
| 43473 | 6744100 | *E. tarda* | 99.4% | *E. ictaluri* | 76% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.23 |
| 43659 | 6745100* | | | *E. ictaluri* | 97% | 2003114113 | *B. gladioli* | 94.4% | *E. tarda* | 2.20 |
| 43651 | 6345100* | | | *E. ictaluri* | 86% | 2003114113 | *B. gladioli* | 94.4% | *E. tarda* | 2.26 |
| ***E. hoshinae*** | | | | | | | | | | |
| ATCC35051 | 4744120 | *E. hoshinae* | 99.9% | *E. hoshinae* | 98% | 0443014013 | *E. hoshinae* | 99.9% | *E. hoshinae* | 2.26 |
| ***E. ictaluri*** | | | | | | | | | | |
| 11-149A | 4004000 | *E. coli* | 52.7% | *E. ictaluri* | 97% | 2003010023# | | | *E. ictaluri* | 2.31 |
| RUSVM-1 | 4004000 | *E. coli* | 52.7% | *E. ictaluri* | 72% | 2002000103# | | | *E. ictaluri* | 2.02 |
| S97-773 | 4004000 | *E. coli* | 52.7% | *E. ictaluri* | 70% | 2002000113 | *E. tarda* | 78.6% | *E. ictaluri* | 2.32 |
| ***E. tarda*** | | | | | | | | | | |
| Edwardsiella 9.1 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 62% | 2002000113 | *E. tarda* | 99.9% | *E. tarda* | 2.33 |
| Edwardsiella 9.2 | 4744000 | *E. tarda* | 99.4% | *E. ictaluri* | 67% | 0403110013 | *E. tarda* | 99.9% | *E. tarda* | 2.33 |
| Edwardsiella 9.3 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 94% | 0402000013 | *E. tarda* | 99.9% | *E. tarda* | 2.29 |
| Edwardsiella 9.4 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 71% | 2402000013 | *E. tarda* | 99.9% | *E. tarda* | 2.49 |
| FL95-01 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 96% | 2002010113 | *E. tarda* | 99.5% | *E. tarda* | 2.39 |
| 070720-1 3A | 6744000 | *E. tarda* | 99.4% | *E. tarda* | 72% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.43 |
| 070720-1 2HLDOM | 6744000 | *E. tarda* | 99.4% | *E. tarda* | 72% | 2002000113 | *E. tarda* | 99.9% | *E. tarda* | 2.26 |
| 43657 | 6744000 | *E. tarda* | 99.4% | *E. tarda* | 96% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.34 |
| 43650 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 96% | 2003110113 | *E. tarda* | 70.3% | *E. tarda* | 2.34 |
| 43627 | 4744000 | *E. tarda* | 99.4% | *E. tarda* | 96% | 2003010113 | *E. tarda* | 99.2% | *E. tarda* | 2.47 |
| 43663 | 6744000 | *E. tarda* | 99.4% | *E. tarda* | 94% | 2003010113 | *E. tarda* | 99.2% | *E. tarda* | 2.30 |

CL = Confidence Level; CS = Confidence Score; *unacceptable profile in API, multiple species ID possible; #profile not in BBL database, unable to provide an ID.



www.manaraa.com

The Biolog Microbial Identification System identified all study isolates as members of *Edwardsiella* (*E. hoshinae*, *E. ictaluri* or *E. tarda*) (Table 4.4) with various levels of confidence. The *E. hoshinae* isolate (CL: 98%) and the *E. ictaluri* isolates (CL: 70% - 97%) were both correctly identified. *E. tarda* isolate *Edwardsiella* 9.2 was identified as *E. ictaluri* (CL: 67%). All other *E. tarda* isolates (10 of 11; 91%) were identified as *E. tarda*, in agreement with PCR results (CL: 62% - 96%). Similarly, *E. piscicida* isolates were identified as either *E. tarda* (17 of 25; 68%; CL: 58% - 95%) or *E. ictaluri* (8 of 25; 32%; 62% - 88%). The *E. anguillarum* isolates also generated multiple codes, resulting in identifications of *E. ictaluri* (4/7; 57%; 68% - 97%); *E. tarda* (2/7; 29%; CL: 70% - 76%); or *E. hoshinae* (1/7; 14%; CL: 69%).

The BBL Crystal Enteric/Nonfermentor Identification Kit also correctly identified the *E. hoshinae* isolate (CL: 99.9%) and all *E. tarda* isolates (CL: 70.3% - 99.9%). Of the three *E. ictaluri* isolates, only one (S97-773, isolated from diseased catfish) produced a code present in the BBL database, which identified it as *E. tarda* (78.6%). The *E. piscicida* isolates produced a variety of codes, all resulting in an identification of *E. tarda* from the BBL database with confidence levels ranging between 70.3% and 99.9%. Of the seven *E. anguillarum* isolates, four (57%) produced identical codes, which resulted in an identification of *Burkholderia gladioli* (CL: 94.4%). The remaining three *E. anguillarum* isolates produced similar codes resulting in an identification of *E. tarda* (CL: 70.3% - 99.9%). BBL Crystal codes are consistent with those reported previously for *Edwardsiella* (Griffin, Quiniou et al. 2013, Griffin, Ware et al. 2014) and listed in Table 4.4.



المنارة للاستشارات
www.manaraa.com

The MALDI identification score for each isolate, based on the Bruker Biotyper RTC v. 3.1 and microbial peptide mass spectra database V5.0.0.0, (Bruker Daltonics, Billerica, MA), is displayed in Table 4.4. Bruker MALDI-TOF correctly identified all the *E. tarda, E. ictaluri* and *E. hoshinae* isolates examined with an identification score above 2.0. All *E. piscicida* and *E. anguillarum* isolates tested were identified as *Edwardsiella tarda* with a score above 2.0. However, unique species-specific peptide mass peaks (*m*/*z*) at 8793, 7629 and 4252 were observed in the spectral profiles for *E. piscicida*, *E. anguillarum* and *E. tarda*, respectively.

### 4.3.3 Fatty acid methyl ester (FAME) analysis

The major fatty acid constituents of the *Edwardsiella* isolates were 14:0, 16:0, 17:0 cyclo, summed feature 3 (16:1 w7c/16:1 w6c, 16:1 w6c/16:1 w7c) and summed feature 8 (18:1 w7c, 18:1 w6c). Fatty acid analysis results are displayed in Table 4.5.



المنارة للاستشارات
www.manaraa.com

Table 4.5 Mean (standard deviation) fatty acid content of *Edwardsiella* spp. analyzed in the current study.

| Fatty acid | *E. hoshinae** | *E. ictaluri* | *E. piscicida* | *E. anguillarum* | *E. tarda* |
|---|---|---|---|---|---|
| **12:0** | **1.80** | **1.62** (0.08) | **0.97** (0.18) | **1.05** (0.16) | **2.45** (0.18) |
| **13:0** | **2.21** | **0.41** (0.15) | **1.23** (0.49) | **0.67#** (0.18) | **0.69** (0.22) |
| **14:0** | **9.41** | **11.15** (0.40) | **15.06** (1.13) | **14.10** (1.01) | **11.22** (0.98) |
| **16:0** | **22.51** | **28.29** (2.07) | **26.65** (1.49) | **30.83** (1.91) | **29.24** (0.85) |
| **17:0 cyclo** | **11.12** | **8.84** (3.91) | **21.47** (5.02) | **15.07** (3.11) | **13.83** (2.79) |
| **17:0** | **1.79** | **0.48** (0.17) | **1.24** (0.36) | **0.72** (0.37) | **0.86** (0.27) |
| **18:1 w9c** | **1.14** | **1.57** (0.16) | **1.47** (0.22) | **1.36** (0.16) | **1.52** (0.12) |
| **18:0** | **1.24** | **1.65** (0.44) | **1.12** (0.21) | **1.10** (0.17) | **1.28** (0.16) |
| **19:0 cyclo w8c** | **0.61** | **2.02** (1.44) | **1.12** (0.50) | **1.37** (0.40) | **1.52** (0.44) |
| **Summed Feature 2** | **4.00** | **4.57** (0.52) | **4.83** (0.46) | **4.54** (0.35) | **4.35** (0.26) |
| **Summed Feature 3** | **27.40** | **31.07** (3.58) | **15.68** (5.70) | **22.89** (4.29) | **23.71** (3.43) |
| **Summed Feature 5** | **0.90** | **1.28** (0.08) | **1.10** (0.21) | **0.85** (0.16) | **0.80** (0.06) |
| **Summed Feature 8** | **11.75** | **5.67** (1.97) | **5.99** (1.01) | **4.54** (0.56) | **7.61** (1.09) |

* standard deviation cannot be calculated, only 1 *E. hoshinae* isolate was analyzed.
# fatty acid was only present in 5 of 7 *E. anguillarum* isolates analyzed.

### 4.3.4 Antimicrobial susceptibility profiles

The minimal inhibitory concentration of 39 antimicrobial compounds was tested for all 47 *Edwardsiella* isolates in the current study, resulting in a range of intraspecific and interspecific variation for each antimicrobial compound (Table 4.6 and 4.7). However, no discriminatory antimicrobial compound was identified. For many of the carbapenems, cephalosporins and macrolides, the MICs for different isolates within each *Edwardsiella* species were largely consistent. Greater variation amongst MICs were



www.manaraa.com

present for aminoglycosides and tetracyclines. Susceptibility of *E. piscicida* isolates to amoxicillin was more variable than the other *Edwardsiella* spp. with MICs ranging from ≤0.5 to 4 mg/L. Similarly, the resistance patterns of *E. anguillarum* isolates to penicillin displayed a greater degree of intraspecific variation than the other *Edwardsiella* spp. All *Edwardsiella* isolates exhibited a MIC of ≤0.5 mg/L for florfenicol. *E. anguillarum*, *E. hoshinae* and *E. ictaluri* isolates displayed MICs for oxytetracycline ranging from ≤0.5 to 2 mg/L; in comparison, *E. piscicida* and *E. tarda* oxytetracycline MIC ranged from ≤0.5 to 16 mg/L.



المنارة للاستشارات
www.manaraa.com

Table 4.6 Antimicrobial susceptibility to single compounds of *Edwardsiella* spp. isolates analyzed in the current study.

| **Antibiotic** | **Taxon** | **Number of Strains with MIC (mg/l) of** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **≤0.5** | **1** | **2** | **4** | **8** | **16** | **32** | **64** | **128** | **≥256** |
| AMINOGLYCOSIDES | | | | | | | | | | | |
| **Amikacin** | all strains | | | | 47 | | | | | | |
| **Gentamicin** | all strains | 36 | 11 | | | | | | | | |
| **Neomycin** | all strains | | 47 | | | | | | | | |
| **Spectoinomycin** | all strains | | | | 39 | | 6 | 1 | | 1 | |
| **Streptomycin** | all strains | 45 | | | | | | 1 | | | 1 |
| **Tobramycin** | all strains | | 47 | | | | | | | | |
| CARBAPENEMS | | | | | | | | | | | |
| **Doripenem** | all strains | 47 | | | | | | | | | |
| **Ertapenem** | all strains | 47 | | | | | | | | | |
| **Imipenem** | all strains | 47 | | | | | | | | | |
| **Meropenem** | all strains | 47 | | | | | | | | | |
| CEPHALOSPORINS | | | | | | | | | | | |
| **Cefapime** | all strains | 46 | | 1 | | | | | | | |
| **Cefazolin** | all strains | 30 | | 15 | 2 | | | | | | |
| **Ceftazidime** | all strains | 46 | | 1 | | | | | | | |
| **Ceftiofur** | all strains | 47 | | | | | | | | | |
| **Ceftriaxone** | all strains | 47 | | | | | | | | | |
| MACROLIDES | | | | | | | | | | | |
| **Erythromycin** | all strains | | | 1 | 3 | 43 | | | | | |
| **Tylosin tartrate** | all strains | | | | | | | | | | |
| PENCILLINS | | | | | | | | | | | |
| **Amoxicillin** | *E. anguillarum* | 1 | 6 | | | | | | | | |
| | *E. hoshinae* | 1 | | | | | | | | | |
| | *E. ictaluri* | 3 | | | | | | | | | |
| | *E. piscicida* | 1 | 9 | 11 | 4 | | | | | | |
| | *E. tarda* | 10 | 1 | | | | | | | | |
| **Ampicillin** | all strains | | | | 47 | | | | | | |
| **Penicillin** | *E. anguillarum* | | | 1 | 3 | 2 | 1 | | | | |
| | *E. hoshinae* | 1 | | | | | | | | | |
| | *E. ictaluri* | 2 | | 1 | | | | | | | |
| | *E. piscicida* | | | | 4 | 10 | 11 | | | | |
| | *E. tarda* | | | 6 | 3 | 2 | | | | | |
| **Piperacillin** | all strains | | | | | 46 | | | | 1 | |
| QUINOLONES | | | | | | | | | | | |
| **Ciprofloxacin** | all strains | 46 | 1 | | | | | | | | |
| **Enrofloxacin** | all strains | 45 | 1 | | | | | | | | |
| **Levofloxacin** | all strains | 47 | | | | | | | | | |
| TETRACYCLINES | | | | | | | | | | | |
| **Minocycline** | *E. anguillarum* | 1 | | | 3 | 3 | | | | | |
| | *E. hoshinae* | 1 | | | | | | | | | |
| | *E. ictaluri* | 3 | | | | | | | | | |
| | *E. piscicida* | 5 | | 10 | 6 | 1 | 3 | | | | |
| | *E. tarda* | 11 | | | | | | | | | |
| **Oxytetracycline** | *E. anguillarum* | 2 | 4 | 1 | | | | | | | |
| | *E. hoshinae* | | | 1 | | | | | | | |
| | *E. ictaluri* | 3 | | | | | | | | | |
| | *E. piscicida* | 10 | 8 | 2 | | 1 | 4 | | | | |
| | *E. tarda* | 2 | 6 | 1 | | | 2 | | | | |



www.manaraa.com

Table 4.6 (continued)

| Antibiotic | Taxon | Number of Strains with MIC (mg/l) of | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ≤0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | ≥256 |
| **Tetracycline** | *E. anguillarum* | 2 | 5 | | | | | | | | |
| | *E. hoshinae* | 1 | | | | | | | | | |
| | *E. ictaluri* | 2 | 1 | | | | | | | | |
| | *E. piscicida* | 18 | 2 | 1 | | | 4 | | | | |
| | *E. tarda* | 6 | 3 | | | | 2 | | | | |
| OTHER | | | | | | | | | | | |
| **Aztreonam** | all strains | 46 | | | | | | 1 | | | |
| **Clindamycin** | all strains | 2 | | 1 | 12 | 32 | | | | | |
| **Florfenicol** | all strains | 47 | | | | | | | | | |
| **Nitrofurantoin** | all strains | | | | | | 46 | | | 1 | |
| **Novobiocin** | all strains | 5 | 3 | 3 | 6 | 30 | | | | | |
| **Sulphadimethoxine** | all strains | | | | | | 4 | | 1 | | 42 |
| **Sulphathiazole** | all strains | | | | | | 3 | | 1 | 1 | 42 |
| **Tigecycline** | all strains | 43 | | 4 | | | | | | | |

Minimal inhibitory concentrations (MICs) were obtained by the broth microdilution method using the Sensititre® GN4F and AVIAN1F plates, following the manufacturer's protocol.

Table 4.7 Antimicrobial susceptibility to combinatory compounds of *Edwardsiella* spp. isolates analyzed in the current study.

| Antibiotic | Taxon | Number of Strains with MIC (mg/l) of | | |
|---|---|---|---|---|
| | | ≤4/2 | 8/4 | ≥16/8 |
| Ampicillin/sublactam | all strains | 47 | | |

| Antibiotic | Taxon | Number of Strains with MIC (mg/l) of | | | |
|---|---|---|---|---|---|
| | | ≤0.5/9.5 | 1/19 | 2/38 | ≥4/76 |
| Trimethoprim/sulfamethoxazole | all strains | 26 | | 7 | 14 |

| Antibiotic | Taxon | Number of Strains with MIC (mg/l) of | | | |
|---|---|---|---|---|---|
| | | ≤8/2 | 16/2 | 32/2 | ≥64/2 |
| Ticarcillin/clavulanic acid | all strains | 47 | | | |

| Antibiotic | Taxon | Number of Strains with MIC (mg/l) of | | | | |
|---|---|---|---|---|---|---|
| | | ≤8/4 | 16/4 | 32/4 | 64/4 | ≥128/4 |
| Piperacillin/tazobactam | all strains | 47 | | | | |

Minimal inhibitory concentrations (MICs) were obtained by the broth microdilution method using the Sensititre® GN4F and AVIAN1F plates, following the manufacturer's protocol.



المنارة للاستشارات
www.manaraa.com

### 4.3.5 Phylogenetic analysis

Within groups, partial 16S rRNA sequences (1,062 bp) displayed high intraspecific similarity (99.90% - 100%). However, 16S rRNA had low discriminatory power among *Edwardsiella* congeners, with 99.15% - 99.91% interspecific similarity among them (Figure 4.1, Table 4.8). Conversely, *gyrB* (1,800 bp) and *sodB* (461 bp) displayed high discriminatory power among *Edwardsiella* congeners (84.02% - 95.88% and 83.95% - 97.16%, respectively); while at the same time maintaining high intraspecific similarity (99.47% - 100% and 99.72% - 100%, respectively; Figures 4.2 and 4.3; Tables 4.9 and 4.10). *E. anguillarum* and *E. piscicida* shared the highest similarity with one another, with 95.88% at *gyrB* and 97.16% at *sodB*. Conversely, *E. hoshinae* and *E. ictaluri* were the most divergent, with 84.02% identity at *gyrB* and 83.95% at *sodB*.



المنارة للاستشارات
www.manaraa.com

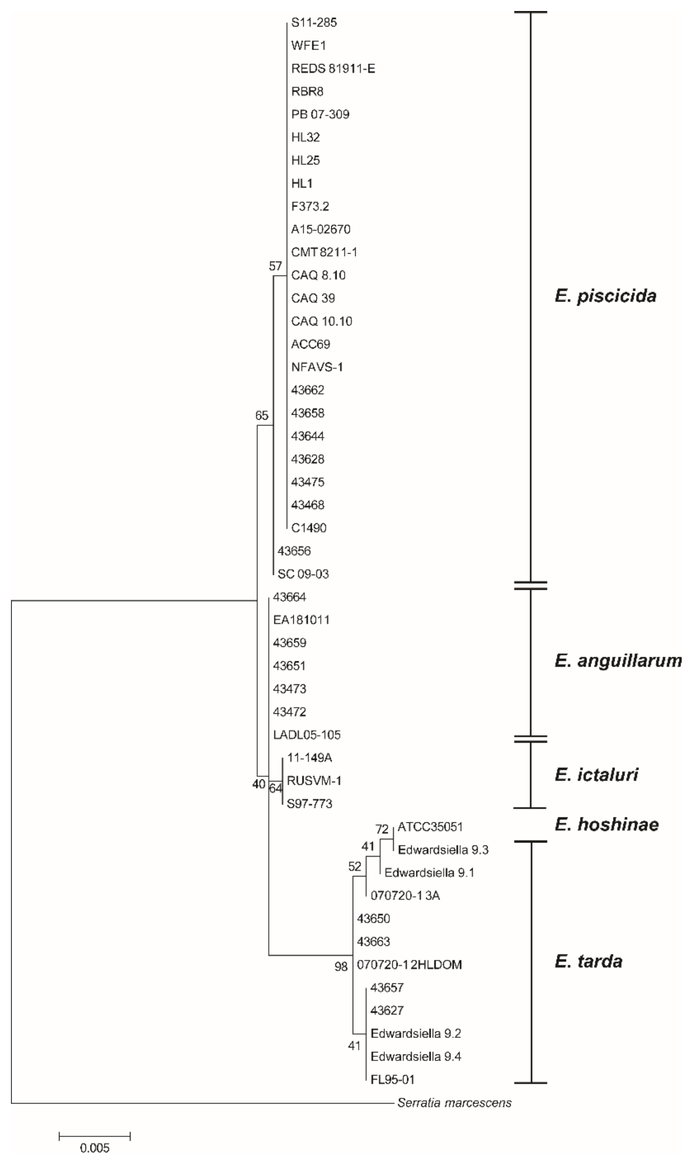


Figure 4.1 Phylogenetic relationships of *Edwardsiella* spp. based on partial 16S rRNA gene sequence.

Phylogenetic relationships of *Edwardsiella* spp. analyzed in the current study. Relatedness was inferred from the maximum likelihood method based on 1,062 bp of 16S rRNA gene sequence and rooted at *Serratia marcesens*. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) is shown next to the branches.



www.manaraa.com

Table 4.8 16S rRNA similarity matrix between *Edwardsiella* spp. used in the current study.

| | **EA** | **EH** | **EI** | **EP** | **ET** |
|---|---|---|---|---|---|
| **EA** | 100.00 | | | | |
| **EH** | 99.15 | 100.00 | | | |
| **EI** | 99.91 | 99.25 | 100.00 | | |
| **EP** | 99.72 | 99.05 | 99.63 | 99.99 | |
| **ET** | 99.34 | 99.81 | 99.26 | 99.24 | 99.90 |

Values represent the percent similarity across 1,062 bp of the 16S locus. EA = *E. anguillarum*; EH = *E. hoshinae*; EI = *E. ictaluri*; EP = *E. piscicida*; ET = *E. tarda*.



المنارة للاستشارات
www.manaraa.com

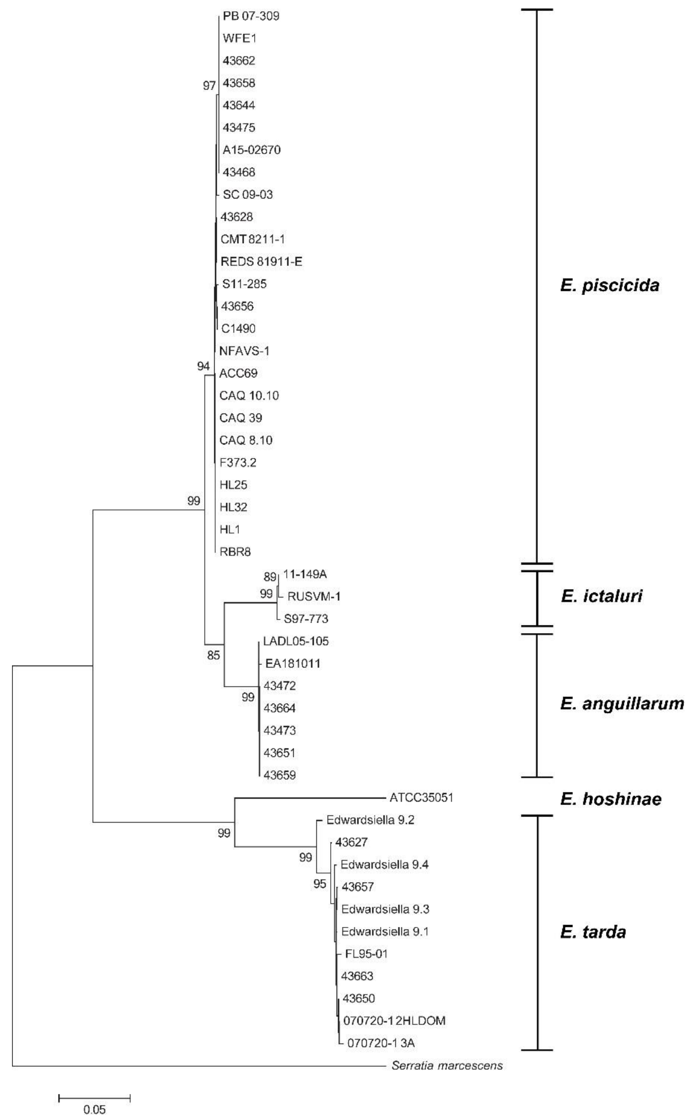


Figure 4.2 Phylogenetic relationships of *Edwardsiella* spp. based on partial *gyrB* gene sequence.

Phylogenetic relationships of *Edwardsiella* spp. analyzed in the current study. Relatedness was inferred from the maximum likelihood method based on 1,800 bp of *gyrB* gene sequence and rooted at *Serratia marcesens*. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) is shown next to the branches.



www.manaraa.com

Table 4.9 *gyrB* similarity matrix between *Edwardsiella* spp. used in the current study.

| | **EA** | **EH** | **EI** | **EP** | **ET** |
|---|---|---|---|---|---|
| **EA** | 99.94 | | | | |
| **EH** | 84.05 | 100.00 | | | |
| **EI** | 94.61 | 84.02 | 99.73 | | |
| **EP** | 95.88 | 84.72 | 94.82 | 99.78 | |
| **ET** | 85.02 | 88.86 | 84.70 | 85.81 | 99.47 |

Values represent the percent similarity across 1,800 bp of the *gyrB* locus. EA = *E. anguillarum*; EH = *E. hoshinae*; EI = *E. ictaluri*; EP = *E. piscicida*; ET = *E. tarda.*



المنارة للاستشارات
www.manaraa.com

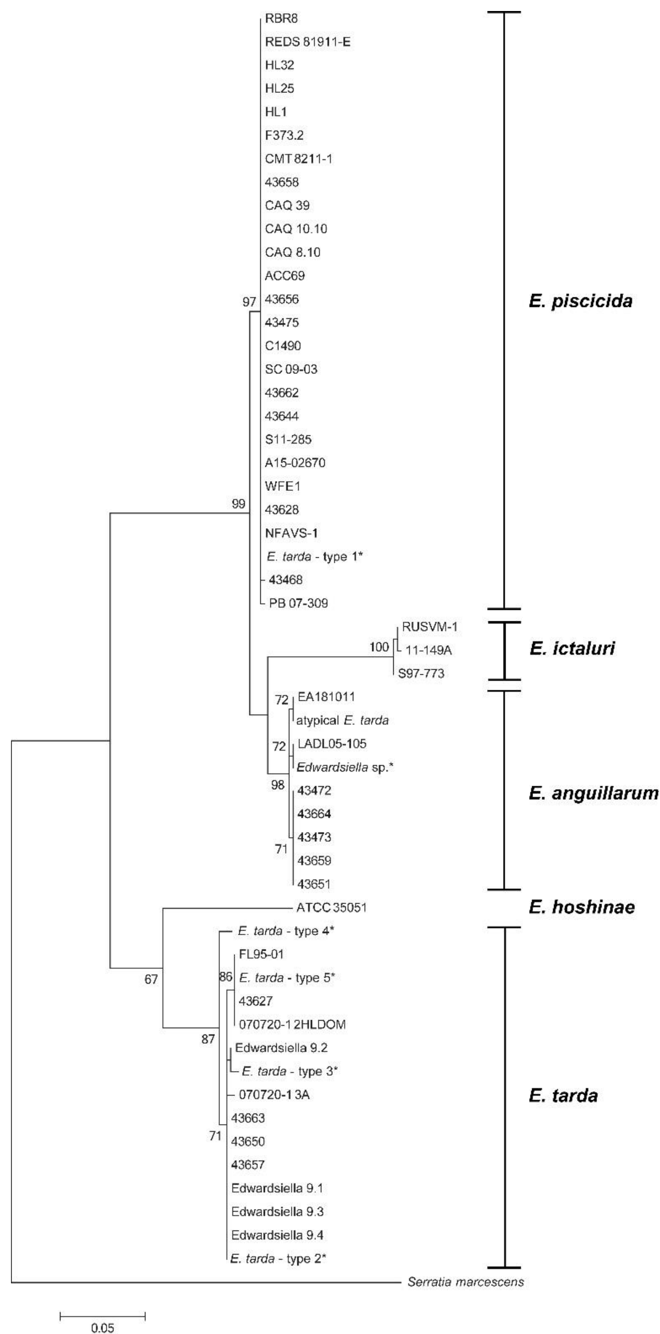


Figure 4.3 Phylogenetic relationships of *Edwardsiella* spp. based on partial *sodB* gene sequence.

Phylogenetic relationships of *Edwardsiella* spp. analyzed in the current study and isolates described by Yamada and Wakabayashi 1999. Relatedness was inferred from the maximum likelihood method based on 461 bp of the *sodB* gene sequence and rooted at *Serratia marcesens*. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *isolate described by Yamada and Wakabayashi 1999.



المنارة للاستشارات
www.manaraa.com

Table 4.10 *sodB* similarity matrix between *Edwardsiella* spp. used in the current study.

| | EA | EH | EI | EP | ET |
|---|---|---|---|---|---|
| **EA** | 99.81 | | | | |
| **EH** | 86.86 | 100.00 | | | |
| **EI** | 92.56 | 83.95 | 99.81 | | |
| **EP** | 97.16 | 86.99 | 92.39 | 99.97 | |
| **ET** | 88.54 | 91.38 | 86.16 | 89.12 | 99.72 |

Values represent the percent similarity across 461 bp of the *sodB* locus. EA = *E. anguillarum*; EH = *E. hoshinae*; EI = *E. ictaluri*; EP = *E. piscicida*; ET = *E. tarda*.

### 4.3.6 Genetic fingerprinting

Similar to the phylogenetic analysis, rep-PCR profiles for *Edwardsiella* spp. isolates formed five distinct clusters representing the five taxa of *Edwardsiella,* regardless of primer set. Of the four primer sets evaluated, the BOX and $GTG_5$ primers demonstrated the least amount of intraspecific variability (Figure 4.4), with the BOX primer generating the most consistent patterns within groups. UPGMA analysis based on the BOX primer placed these five clusters within two larger phylogroups. In line with previous reports, *Edwardsiella piscicida*, *E. anguillarum* and *E. ictaluri* formed one cluster, and the other group contained *E. tarda* and *E. hoshinae* isolates. The genetic profiles of *E. anguillarum*, *E. ictaluri* and *E. piscicida* all shared greater than 90% similarity within their respective taxa. The profiles of *E. tarda* isolates demonstrated the greatest intraspecific variability, with only 60% – 96.4% similarity amongst isolates (Figure 4.5).



www.manaraa.com

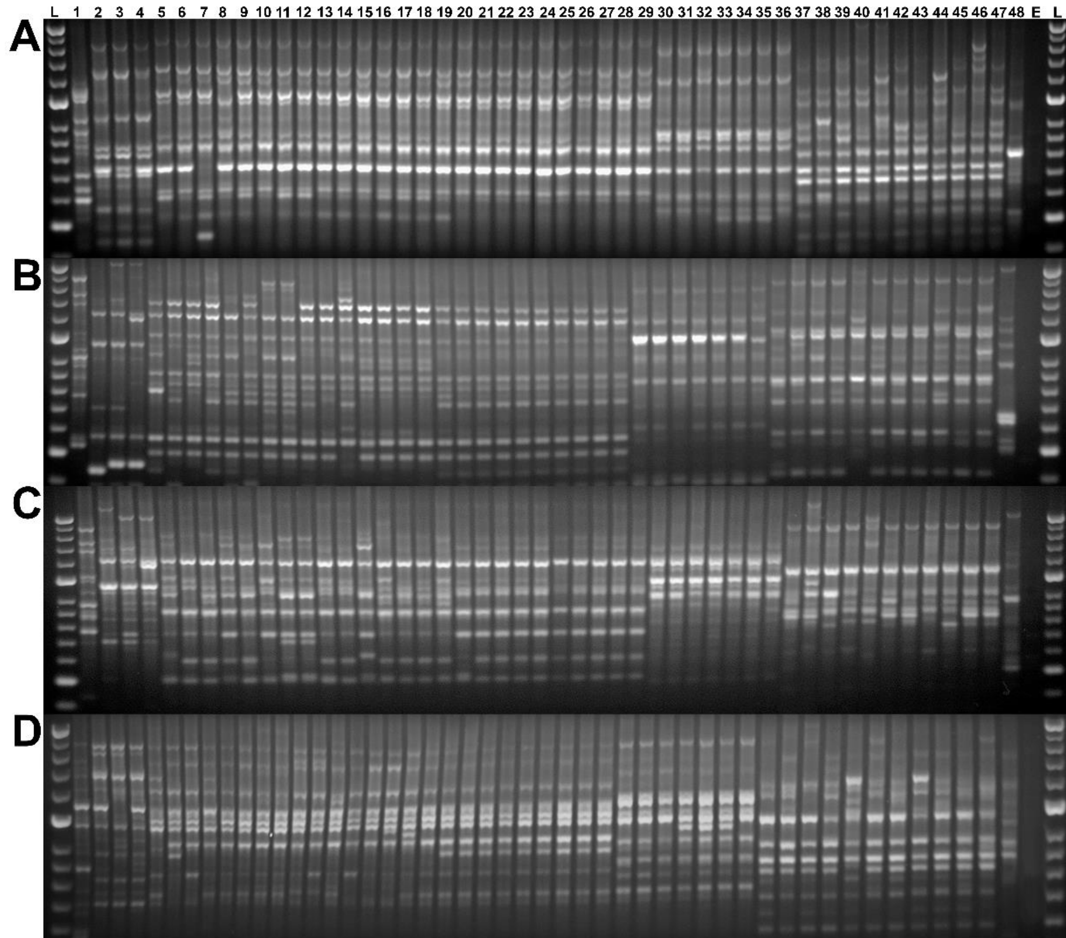


Figure 4.4 Genetic fingerprints of *Edwardsiella* spp. analyzed in the current study.

Repetitive extragenic palindromic PCR amplification of gDNA from *Edwardsiella hoshinae* (Lane 1), *E. ictaluri* (Lanes 2-4), *E. piscicida* (Lanes 5-29), *E. anguillarum* (Lanes 30-36) and *E. tarda* (Lanes 37-47); using E. coli as an outlier (ATCC 25922, Lane 48), no template control (Lane E) and concurrently run standards (Hyperladder 50bp; Lane L). Genetic profiles were generated using A) Box; B) ERIC I&II; C) ERIC II; and D) $GTG_5$ primers.



المنارة للاستشارات
www.manaraa.com

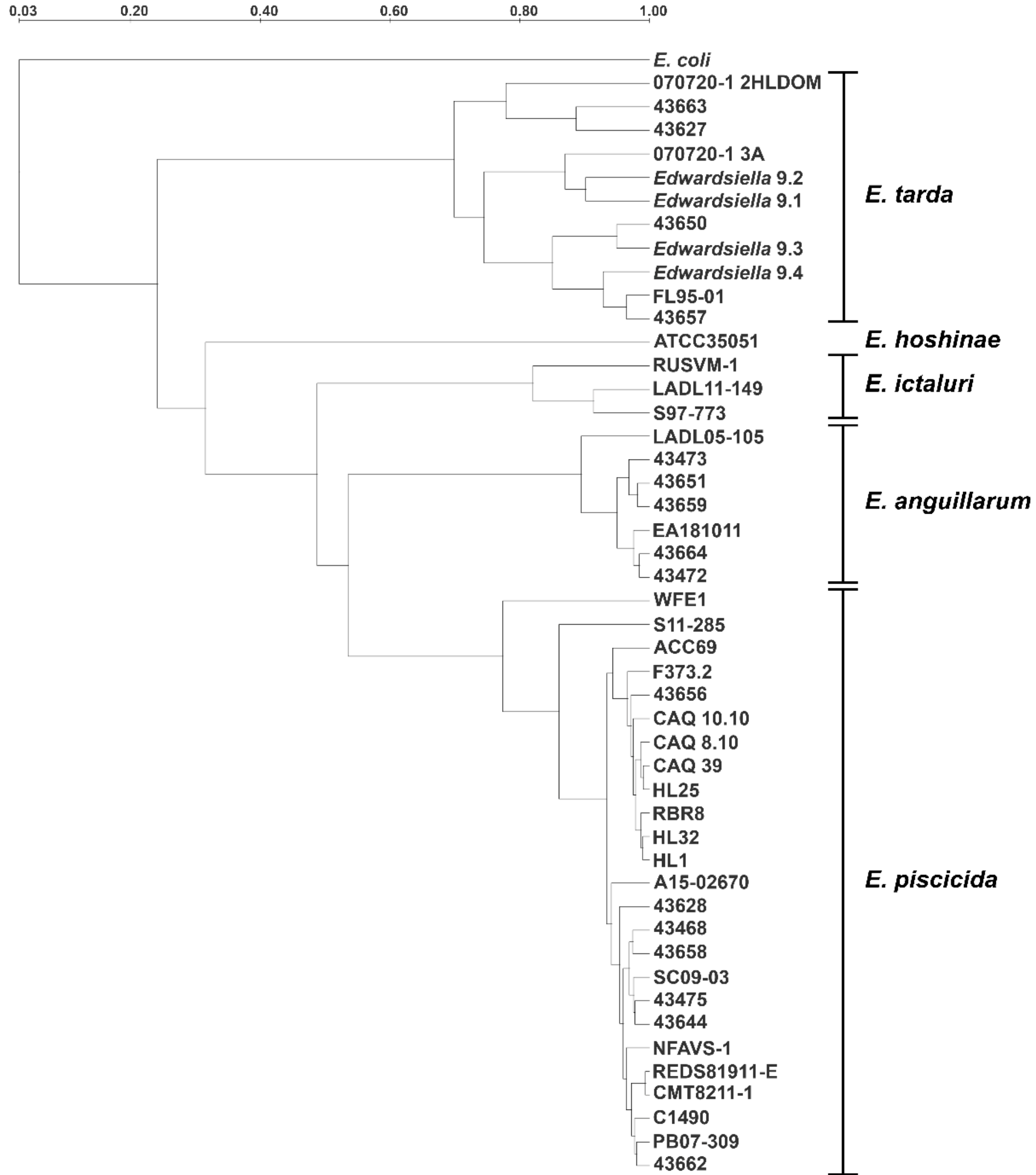


Figure 4.5 Dendrogram of *Edwardsiella* spp. generated from BOX repetitive extragenic palindromic PCR profiles.

Dendrogram of *Edwardsiella* spp. generated from BOX repetitive extragenic palindromic PCR profiles using unweighted pair-group arithmetic average (UPGMA) cluster analysis rooted at *E. coli* (ATCC 25922). Dice coefficients are displayed above the dendrogram.



المنارة للاستشارات
www.manaraa.com

### 4.3.7 Multiplex PCR

The mPCR assay was repeatable and reproducible, with linear dynamic ranges covering at least 5 orders of magnitude. Disproportionately large quantities of non-target DNA had no marked effect on amplification efficiency; dilution curves and amplification plots were comparable when run with each *Edwardsiella* spp. target gDNA alone or in the presence of non-target gDNA (Table 4.11; Figure 4.6) with a quantifiable limit of ~100 copies of target DNA. Reaction efficiencies were calculated using equation 4.1 (Bustin, Benes et al. 2009) from the slope of the log-linear portion of the serial ten-fold dilutions for each *Edwardsiella* sp. and were within the generally accepted range of 90% - 110%.

$$\text{PCR efficiency} = 10^{-1/\text{slope}} - 1 \qquad (4.1)$$

Table 4.11 Specificity of the multiplex real-time polymerase chain reaction (mPCR) assay to each respective target.

| | *E. anguillarum* gDNA alone | *E. ictaluri* gDNA alone | *E. piscicida* gDNA alone | *E. tarda* gDNA alone | All *Edwardsiella* spp. gDNA mixed together |
|---|---|---|---|---|---|
| *E. anguillarum* | 23.28 (0.07) | - | - | - | 23.21 (0.09) |
| *E. ictaluri* | - | 22.61 (0.10) | - | - | 22.49 (0.05) |
| *E. piscicida* | - | - | 22.63 (0.15) | - | 22.50 (0.09) |
| *E. tarda* | - | - | - | 22.93 (0.13) | 22.50 (0.12) |

Values are reported in terms of the mean (standard deviation) quantification cycle (Cq). The user-defined fluorescence threshold for Cq determination was set at 50 relative fluorescent units. Dash ( - ) indicates no amplification of DNA.



المنارة للاستشارات
www.manaraa.com

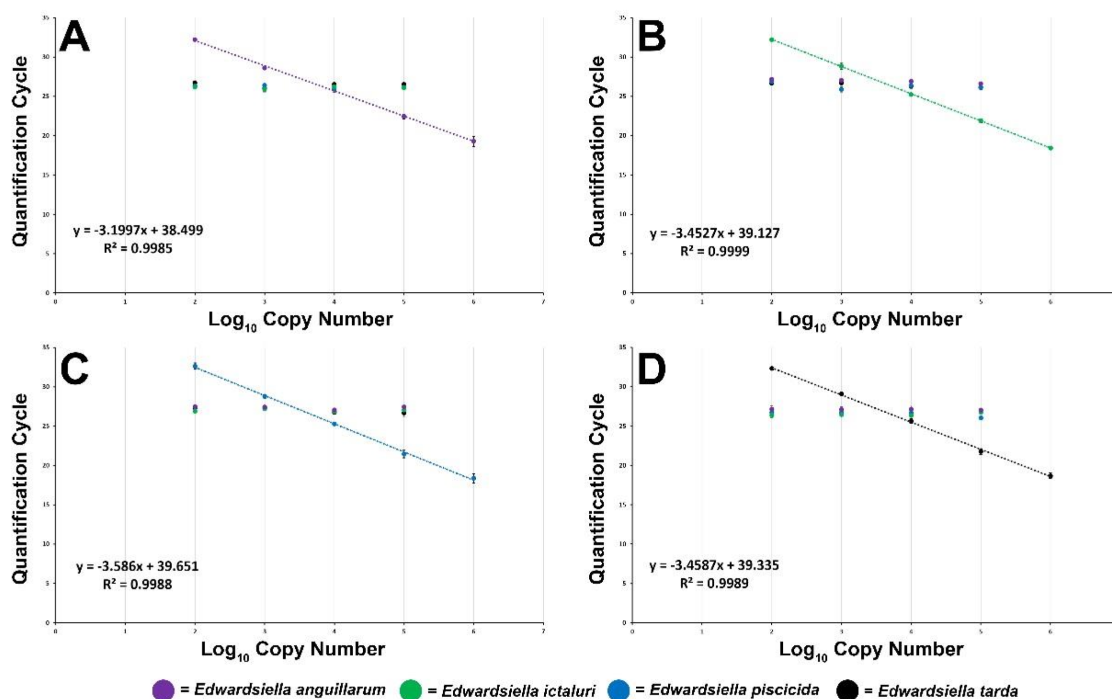


Figure 4.6 Multiplex real-time PCR assay validation results.

Mean quantification cycles (Cq) for known serial 10-fold dilutions of A) *E. anguillarum*; B) *E. ictaluri*; C) *E. piscicida*; and D) *E. tarda*. Dilution series for each assay was performed in the presence of an equal mixture of ~10,000 copies of each non-target *Edwardsiella* sp. gDNA. Error bars indicate standard deviations generated from samples run in triplicate on 3 separate plates. The user-defined baseline threshold for Cq determination was set at 50 relative fluorescent units for all runs.

### 4.3.8 Plasmid analysis

Twenty-one (45%) of the *Edwardsiella* spp. isolates carried plasmids. Summaries of open reading frames and the putative functions of their predicted proteins from *E. piscicida* and *E. tarda* plasmids can be found in Tables 4.12 and 4.13. Physical maps of isolated plasmids are available in Figures 4.7 – 4.9. Each of the three *E. ictaluri* isolates carried two plasmids, consistent with previous reports for these isolates (Griffin, Reichley et al. 2016). Nine of the *E. piscicida* isolates (F373.2, HL1, HL25, HL32, RBR8, ACC69, CAQ 8.10, CAQ 10.10 and CAQ 39) recovered from turbot between 2005 and 2012 in



المنارة للاستشارات
www.manaraa.com

Europe shared an identical plasmid of 3,782 bp. Four additional *E. piscicida* isolates (PB 07-309, S11-285, SC 09-03 and 43644) from various hosts carried plasmids of different sizes and compositions. The 3,164 bp plasmid identified in isolate S11-285 was in agreement with previous reports (Reichley, Waldbieser et al. 2016). Several *E. tarda* isolates carried two plasmids; 070720-3A (2,241 bp, 6,544 bp), Edwardsiella 9.1 (4,102 bp, 4,067 bp), Edwardsiella 9.3 (2,328 bp, 3,189 bp) and Edwardsiella 9.4 (6,920 bp, 65,317 bp). Additionally, *E. tarda* isolate Edwardsiella 9.2 carried one plasmid of 27,938 bp. No plasmids were detected in any of the *E. anguillarum* isolates or *E. hoshinae* isolate ATCC 35051, consistent with previous reports (Reichley, Waldbieser et al. 2015, Reichley, Waldbieser et al. 2015, Reichley, Waldbieser et al. 2017).



www.manaraa.com

Table 4.12 Summary of open reading frames and putative functions from plasmids harvested from *Edwardsiella piscicida* isolates from different hosts and geographic origins.

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| F373.2<br>HL1<br>HL25<br>HL32<br>RBR8<br>ACC69<br>CAQ 8.10<br>CAQ 10.10<br>CAQ 39 | Group - 1 | 493..1134 (+) | RloB superfamily; hypothetical protein of unknown function; most similar to hypothetical protein [*Klebsiella pneumoniae*]; GenBank WP_048991273.1 | 115/202 (57%) | 4.00E-77 |
| | Group - 2 | 1152..1337 (+) | hypothetical protein of unknown function; most similar to hypothetical protein G972_04939 [*Escherichia coli* UMEA 3355-1]; GenBank EQZ07072.1 | 37/51 (78%) | 7.00E-13 |
| | Group - 3 | 1298..1453 (-) | replication initiation factor; most similar to replication initiation protein [*Enterobacter cloacae*]; GenBank WP_048210874.1 | 34/56 (61%) | 1.00E-10 |
| | Group - 4 | 1915..2091 (-) | Rop superfamily; RNA polymerase; most similar to RNA polymerase [*Edwardsiella tarda*]; GenBank WP_035607970.1 | 40/56 (71%) | 5.00E-09 |
| | Group - 5 | 3013..499 (+) | P-loop NTPase superfamily; hypothetical protein of unknown function; most similar to hypothetical protein [*Klebsiella pneumoniae*]; GenBank WP_048991271.1 | 215/419 (51%) | 8.00E-155 |
| PB 07-309 | PB07-309-1 | 677..1144 (+) | hypothetical protein of unknown function; most similar to hypothetical protein [*Enterobacteriaceae*]; GenBank WP_039023474.1 | 127/151 (84%) | 1.00E-89 |
| | PB07-309-2 | 1328..1483 (-) | hypothetical protein of unknown function; most similar to replication initiation protein [*Enterobacter*]; GenBank WP_052684121.1 | 29/42 (69%) | 9.00E-10 |



www.manaraa.com

Table 4.12 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| PB 07-309 | PB07-309-3 | 1946..2242 (-) | hypothetical protein of unknown function; most similar to hypothetical protein [*Edwardsiella tarda*]; GenBank WP_034166090.1 | 24/36 (67%) | 1.00E-08 |
| | PB07-309-4 | 2744..279 (-) | hypothetical protein of unknown function; most similar to hypothetical protein [*Escherichia coli*]; GenBank WP_052266651.1 | 170/192 (89%) | 1.00E-122 |
| S11-285 | S11-285-1 | 10..606 (+) | hypothetical protein of unknown function; most similar to hypothetical protein [*Edwardsiella tarda*]; GenBank WP_034166090.1 | 140/147 (95%) | 2.00E-91 |
| | S11-285-2 | 879..1529 (+) | hypothetical protein of unknown function; most similar to hypothetical protein [*Pectobacterium carotovorum*]; GenBank WP_039520268.1 | 181/216 (84%) | 4.00E-133 |
| | S11-285-3 | 1596..2534 (-) | Replicase superfamily; replicase protein most similar to replicase [*Enterobacteriaceae*]; GenBank WP_038870790.1 | 257/308 (83%) | 2.00E-179 |
| SC 09-03 | SC09-03-1 | 397..702 (-) | hypothetical protein of unknown function; most similar to hypothetical protein [uncultured bacterium]; GenBank AET87465.1 | 101/101 (100%) | 8.00E-65 |
| | SC09-03-2 | 734..880 (-) | hypothetical protein of unknown function; most similar to Resolvase (plasmid) [*Citrobacter freundii*]; GenBank AKJ18929.1 | 47/48 (98%) | 4.00E-25 |
| | SC09-03-3 | 968..1096 (+) | PFL_4706 family; conjugal transfer protein most similar to hypothetical protein, partial [*Bacillus cereus*]; GenBank WP_000306031.1 | 43/43 (100%) | 3.00E-21 |



www.manaraa.com

Table 4.12 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| SC 09-03 | SC09-03-4 | 1152..2417 (-) | MFS superfamily; tetracycline resistance protein most similar to tetracycline resistance protein A [*Laribacter hongkongensis*]; GenBank AAW83817.1 | 421/421 (100%) | 0.0 |
| | SC09-03-5 | 2421..3128 (+) | TetR superfamily; tetracycline repressor protein most similar to tetracycline repressor protein TetR [*Plesiomonas* sp. ZOR0011]; GenBank WP_047706566.1 | 202/203 (99%) | 2.00E-133 |
| | SC09-03-6 | 3133..3270 (+) | plasmid replication protein most similar to replication protein C [*Candidatus snodgrassella* sp. T4_34144]; GenBank AFV98734.1 | 46/46 (100%) | 3.00E-24 |
| | SC09-03-7 | 3439..3879 (+) | Rep_3 superfamily; plasmid replication protein most similar to MULTISPECIES: hypothetical protein [*Chlamydia*]; GenBank WP_030122376.1 | 145/146 (99%) | 1.00E-100 |
| | SC09-03-8 | 4037..4915 (-) | P-loop_NTPase superfamily; replication protein most similar to RepA [*Chlamydia suis*]; GenBank AAR96040.1 | 292/292 (100%) | 0.0 |
| | SC09-03-9 | 4944..5270 (-) | hypothetical protein of unknown function; most similar to hypothetical protein [*Gulbenkiania indica*]; GenBank WP_055434597.1 | 107/108 (99%) | 2.00E-52 |
| | SC09-03-10 | 5267..5593 (-) | PemK superfamily; growth inhibitor protein most similar to putative PemK-like protein [*Chlamydia trachomatis* RC-F(s)/852]; GenkBank AGR96051.1 | 108/108 (100%) | 1.00E-70 |



المنارة للاستشارات
www.manaraa.com

Table 4.12 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| SC 09-03 | SC09-03-11 | 5590..5814 (-) | DUF3018 superfamily; hypothetical protein of unknown function most similar to MULTISPECIES: hypothetical protein [Proteobacteria]; GenBank WP_043149934.1 | 74/74 (100%) | 9.00E-46 |
| | SC09-03-12 | 5881..8553 (-) | Relaxase superfamily; mobilization protein most similar to mobilization protein [*Enterobacter* sp. GN02768]; GenBank WP_047353284.1 | 890/890 (100%) | 0.0 |
| | SC09-03-13 | 8543..8857 | mobilization protein; most similar to mobilization protein B [*Aeromonas salmonicida* subsp. *salmonicida*]; GenBank AIM49702.1 | 104/104 (100%) | 6.00E-68 |
| | SC09-03-14 | 9149..9526 (+) | mobilization protein; most similar to MobC [*Chlamydia suis*]; GenBank AAR96037.1 | 124/125 (99%) | 2.00E-59 |
| | SC09-03-15 | 9539..10222 (+) | mobilization protein; most similar to MULTISPECIES: protein mobD [Bacteria]; GenBank WP_009873358.1 | 227/227 (100%) | 6.00E-148 |
| | SC09-03-16 | 10215..10853 (+) | mobilization protein; most similar to MULTISPECIES: protein mobE [Bacteria]; GenBank WP_009873359.1 | 212/212 (100%) | 8.00E-117 |
| | SC09-03-17 | 10850..11602 (+) | hypothetical protein of unknown function; most similar to MULTISPECIES: OfxX fusion product [Bacteria]; GenBank WP_009873360.1 | 250/250 (100%) | 2.00E-128 |



www.manaraa.com

Table 4.12 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| 43644 | 43644-1 | 184..594 (+) | hypothetical protein of unknown function; most similar to hypothetical protein [*Escherichia coli*]; GenBank WP_044864692.1 | 129/136 (94%) | 5.00E-84 |
| | 43644-2 | 854..1417 (+) | DnaK domain; molecular chaperone involved in posttranslational modification and protein turnover; most similar to hypothetical protein [*Enterobacteriaceae*]; GenBank WP_001749520.1 | 159/187 (85%) | 1.00E-112 |
| | 43644-3 | 1452..1934 (-) | hypothetical protein of unknown function; most similar to hypothetical protein [*Enterobacteriaceae*]; GenBank WP_000043122.1 | 134/151 (89%) | 2.00E-90 |
| | 43644-4 | 2915..3358 (-) | hypothetical protein of unknown function; most similar to hypothetical protein [*Edwardsiella tarda*]; GenBank WP_034166090.1 | 142/147 (97%) | 2.00E-95 |



www.manaraa.com

Table 4.13 Summary of open reading frames and putative functions from plasmids harvested from *Edwardsiella tarda* isolates from different hosts and geographic origins.

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.1 Plasmid 1 | E9.1 p1-1 | 3..293 (-) | Hypothetical protein; most similar to hypothetical protein [*Klebsiella pneumoniae*]; GenBank WP_072039985.1 | 57/66 (86%) | 5.00E-31 |
| | E9.1 p1-2 | 1183..1626 (-) | DNA replication regulator SLD3; most similar to hypothetical protein (plasmid) [*Edwardsiella ictaluri*]; GenBank ALT06052.1 | 117/123 (95%) | 3.00E-74 |
| | E9.1 p1-3 | 2024..2287 (+) | Hypothetical protein; most similar to MULTISPECIES: hypothetical protein [*Enterobacteriaceae*]; GenBank WP_016947370.1 | 46/69 (67%) | 4.00E-14 |
| | E9.1 p1-4 | 2582..3274 (-) | Hypothetical protein; most similar to hypothetical protein [*Erwinia tasmaniensis*]; GenBank WP_012443038.1 | 148/212 (70%) | 1.00E-102 |
| | E9.1 p1-5 | 3302..3586 (+) | Hypothetical protein; most similar to hypothetical protein [*Erwinia tasmaniensis*]; GenBank WP_012443037.1 | 34/76 (45%) | 1.00E-08 |
| | E9.1 p1-6 | 3595..3774 (+) | Hypothetical protein; most similar to hypothetical protein [*Erwinia pyrifoliae*]; GenBank WP_014539854.1 | 23/40 (58%) | 1.00E-07 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.1 Plasmid 2 | E9.1 p2-1 | 75..1634 (+) | MbeD_MobD superfamily; DNA polymerase most similar to DNA polymerase [*Escherichia coli*]; GenBank WP_042011586.1 | 328/491 (67%) | 0 |
| | E9.1 p2-2 | 1783..2268 (+) | DUF3018 superfamily; hypothetical protein of unknown function most similar to hypothetical protein WC7_00264 [*Citrobacter* sp. KTE151]; GenBank EOQ51299.1 | 112/161 (70%) | 3.00E-82 |
| | E9.1 p2-3 | 2268..2519 (+) | Hypothetical protein; most similar to MULTISPECIES: hypothetical protein [*Enterobacteriaceae*]; GenBank WP_016151475.1 | 27/75 (36%) | 2.00E-08 |
| | E9.1 p2-4 | 3694..4065 (-) | MobC superfamily; mobilization protein most similar to Mobilization protein mbeC [*Erwinia amylovora*]; GenBank WP_015056330.1 | 102/124 (82%) | 1.00E-66 |
| Edwardsiella 9.2 | E9.2-1 | 27925..1000 (+) | DNA_BRE_C superfamily; integrase most similar to MULTISPECIES: integron integrase [Bacteria]; GenBank WP_000845048.1 | 337/337 (100%) | 0 |
| | E9.2-2 | 1209..1553 (+) | DUFF3330 and EAL superfamily; hypothetical protein most similar to hypothetical protein, partial [*Escherichia coli*]; GenBank WP_050586177.1 | 114/114 (100%) | 3.00E-78 |



www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.2 | E9.2-3 | 1679..2239 (+) | Ser_Recominbase superfamily; transposase most similar to MULTISPECIES: transposase [Proteobacteria]; GenBank WP_000147567.1 | 185/186 (99%) | 3.00E-118 |
| | E9.2-4 | 2242..5208 (+) | DDE_Tnp_Tn3 superfamily; transposase most similar to transposase [*Escherichia coli*]; GenBank WP_063112526.1 | 987/988 (99%) | 0 |
| | E9.2-5 | 5617..5856 (-) | Hypothetical protein of unknown function; most similar to MULTISPECIES: hypothetical protein [Proteobacteria]; GenBank WP_008168813.1 | 74/79 (94%) | 3.00E-44 |
| | E9.2-6 | 5944..7305 (+) | Replication protein; most similar to RepA replication protein [*Pseudomonas aeruginosa*]; GenBank WP_011270176.1 | 452/453 (99%) | 0.00E+00 |
| | E9.2-7 | 7462..7848 (-) | Partition protein; most similar to ParC [*Pseudomonas alcaligenes*]; GenBank WP_011178358.1 | 127/128 (99%) | 3.00E-87 |
| | E9.2-8 | 7882..8109 (-) | ParG superfamily; chromosome partitioning protein most similar to MULTISPECIES: chromosome partitioning protein ParB [Proteobacteria]; GenBank WP_011178357.1 | 73/73 (100%) | 8.00E-43 |
| | E9.2-9 | 8125..8763 (-) | P-loop_NTPase superfamily; peptide transporter most similar to MULTISPECIES: peptide transporter [Proteobacteria]; GenBank WP_008166580.1 | 212/212 (100%) | 4.00E-135 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.2 | E9.2-10 | 8919..9188 (-) | Hypothetical protein of unknown function; most similar to MULTISPECIES: hypothetical protein [*Enterobacteriaceae*]; GenBank WP_049854846.1 | 74/74 (100%) | 2.00E-43 |
| | E9.2-11 | 9185..10138 (-) | BsuBI Pst RE superfamily; endoneuclease most similar to MULTISPECIES: hypothetical protein [*Enterobacteriaceae*]; GenBank WP_023277455.1 | 317/317 (100%) | 0 |
| | E9.2-12 | 10135..11604 (-) | SAM-dependent methyltransferase; most similar to MULTISPECIES: SAM-dependent methyltransferase [Proteobacteria]; GenBank WP_043496682.1 | 485/489 (99%) | 0 |
| | E9.2-13 | 11614..12183 (-) | SER_Recominbase superfamily; recombinase most similar to hypothetical protein [*Escherichia coli*]; GenBank WP_023277464.1 | 189/189 (100%) | 2.00E-120 |
| | E9.2-14 | 12452..13279 (-) | DnaQ like superfamily; DNA polymerase III subunit epsilon most similar to DNA polymerase III subunit epsilon [*Enterobacter cloacae*]; GenBank WP_059385974.1 | 275/275 (100%) | 0 |
| | E9.2-15 | 13560..13784 (+) | Hypothetical protein; most similar to hypothetical protein [*Escherichia coli*]; GenBank WP_032334003.1 | 73/74 (99%) | 7.00E-44 |
| | E9.2-16 | 13820..15991 (-) | Relaxase superfamily; DNA topoisomerase most similar to hypothetical protein G759_04906 [*Escherichia coli* HVH 98 (4-5799287)]; GenBank ESJ97390.1 | 720/723 (99%) | 0 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.2 | E9.2-17 | 15988..16482 (+) | Mobilization protein; most similar to MULTISPECIES: mobilization protein [Proteobacteria]; GenBank WP_008168810.1 | 114/114 (100%) | 2.00E-49 |
| | E9.2-18 | 16520..17413 (-) | KfrA_N superfamily, hypothetical protein most similar to mucin, partial [*Enterobacter asburiae*]; GenBank KJW94424.1 | 46/47 (98%) | 3.00E-22 |
| | E9.2-19 | 17631..18065 (-) | MerR-DNA bind superfamily; regulatory protein most similar to MULTISPECIES: mercuric resistance operon regulatory protein [Gammaproteobacteria]; GenBank WP_000429836.1 | 144/144 (100%) | 3.00E-101 |
| | E9.2-20 | 18137..18487 (+) | MerT superfamily; mercuric transporter most similar to MULTISPECIES: mercury transporter [*Enterobacteriaceae*]; GenBank WP_001309999.1 | 116/116 (100%) | 4.00E-65 |
| | E9.2-21 | 18501..18776 (+) | HMA superfamily; mercuric transport protein periplasmic component most similar to mercuric transport protein periplasmic component [*Escherichia coli*]; GenBank WP_062859448.1 | 66/66 (100%) | 9.00E-39 |
| | E9.2-22 | 18773..19234 (+) | MerC superfamily; mercury transport protein MerC most similar to MULTISPECIES: mercury transporter MerC [*Enterobacteriaceae*]; GenBank WP_000082530.1 | 152/153 (99%) | 2.00E-92 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| | E9.2-23 | 19286..20980 (+) | Pyr_redox_dim superfamily; mercury(II) reductase most similar to MULTISPECIES: mercury(II) reductase [Gammaproteobacteria]; GenBank WP_000105636.1 | 564/564 (100%) | 0 |
| | E9.2-24 | 20998..21360 (+) | HTH_MerR-SF superfamily; transcrptional regulator most similar to transcriptional regulator MerD [*Shigella sonnei*]; GenBank CSP82243.1 | 61/61 (100%) | 3.00E-52 |
| | E9.2-25 | 21357..21593 (+) | MerE superfamily; mercury resistance protein most similar to MerE protein, partial [*Escherichia coli* MS 145-7]; GenBank EFO55297.1 | 78/78 (100%) | 9.00E-36 |
| Edwardsiella 9.2 | E9.2-26 | 21590..22297 (+) | EAL superfamily; EAL domain protein most similar to EAL domain-containing protein [*Escherichia coli*]; GenBank WP_000204523.1 | 234/235 (99%) | 2.00E-146 |
| | E9.2-27 | 22336..24051 (+) | RVE superfamily; transposase most similar to MULTISPECIES: transposase [Bacteria]; GenBank WP_000935451.1 | 571/571 (100%) | 0 |
| | E9.2-28 | 24054..25046 (+) | P-loop NTPase superfamily; transposase most similar to MULTISPECIES: transposition protein TniB [Enterobacterales]; GenBank WP_009485925.1 | 330/330 (100%) | 0 |
| | E9.2-29 | 25015..25515 (-) | NAT_SF superfamily; B-acetyltransferase most similar to MULTISPECIES: N-acetyltransferase [Bacteria]; GenBank WP_000376623.1 | 166/166 (100%) | 5.00E-119 |



www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.2 | E9.2-30 | 25643..26509 (-) | Pterin-binding superfamily; dihydropteroate synthase most similar to dihydropteroate synthase [*Enterobacter cloacae*]; GenBank AIC76511.1 | 288/288 (100%) | 0 |
| | E9.2-31 | 26476..26823 (-) | EamA superfamily; transporter protein most similar to QacEdelta1 multidrug exporter [*Citrobacter freundii*]; GenBank WP_053764310.1 | 115/115 (100%) | 3.00E-72 |
| | E9.2-32 | 26956..27081 (+) | DUF1010 superfamily; hypothetical protein most similar to MULTISPECIES: hypothetical protein [Gammaproteobacteria]; GenBank WP_031969044.1 | 41/41 (100%) | 3.00E-20 |
| | E9.2-33 | 27354..27938 (-) | EamA superfamily; efflux transporter most similar to QacG quaternary ammonium resistance protein [*Pseudomonas aeruginosa*]; GenBank BAT21089.1 | 177/179 (99%) | 4.00E-111 |
| Edwardsiella 9.3 Plasmid 1 | E9.3 p1-1 | 1..372 (+) | Hypothetical protein of unknown function; most similar to hypothetical protein (plasmid) [*Edwardsiella ictaluri*]; GenBank ALT06052.1 | 25/36 (69%) | 4.00E-09 |
| | E9.3 p1-2 | 1295..1459 (-) | Hypothetical protein of unknown function; most similar to MULTISPECIES: hypothetical protein [*Enterobacter*]; GenBank WP_048702272.1 | 25/51 (49%) | 6.00E-10 |
| Edwardsiella 9.3 Plasmid 2 | E9.3 p2-1 | 245..688 (+) | SLD3 superfamily; hypothetical protein of unknown function; most similar to hypothetical protein (plasmid) [*Edwardsiella ictaluri*]; GenBank ALT06052.1 | 138/147 (94%) | 3.00E-93 |



www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.3 Plasmid 2 | E9.3 p2-2 | 713..1006 (-) | Hypothetical protein of unknown function; most similar to hypothetical protein [*Escherichia coli*]; GenBank WP_024261689.1 | 26/34 (76%) | 9.00E-05 |
| | E9.3 p2-3 | 1488..1751 (-) | Plasmid_stabil superfamily; plasmid stabilization protein most similar to hypothetical protein [*Escherichia coli*]; GenBank WP_000624147.1 | 55/87 (63%) | 6.00E-30 |
| | E9.3 p2-4 | 1751..2158 (-) | Hypothetical protein of unknown function; most similar to hypothetical protein [*Klebsiella oxytoca*]; GenBank WP_004099074.1 | 86/130 (66%) | 1.00E-43 |
| | E9.3 p2-5 | 2515..2805 (+) | Hypothetical protein of unknown function; most similar to MULTISPECIES: hypothetical protein [*Enterobacteriaceae*]; GenBank WP_023237786.1 | 65/95 (68%) | 4.00E-39 |
| Edwardsiella 9.4 Plasmid 1 | E9.4 p1-1 | 5861..746 (-) | Pyocin_S and HMHc superfamilies; colicin most similar to hypothetical protein [*Edwardsiella tarda*]; GenBank WP_071526397.1 | 522/525 (99%) | 0 |
| | E9.4 p1-2 | 1000..1422 (+) | Hypothetical protein most similar to hypothetical protein [*Edwardsiella tarda*]; GenBank WP_035600447.1 | 137/140 (98%) | 6.00E-99 |
| | E9.4 p1-3 | 1726..1917 (+) | Rop superfamily; RNA polymerase most similar to RNA polymerase [*Edwardsiella tarda*]; GenBank WP_035599702.1 | 63/63 (100%) | 3.00E-24 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.4 Plasmid 1 | E9.4 p1-4 | 1914..2231 (+) | Hypothetical protein most similar to hypothetical protein [*Edwardsiella tarda*]; GenBank WP_035599705.1 | 86/86 (100%) | 2.00E-43 |
| | E9.4 p1-5 | 2221..2520 (+) | HDc superfamily; RelE toxin most similar to addiction module toxin RelE [*Edwardsiella tarda*]; GenBank WP_035599708.1 | 98/99 (99%) | 2.00E-65 |
| | E9.4 p1-6 | 2530..4062 (-) | MbeD_MobD superfamily; nuclease most similar to nuclease [*Edwardsiella tarda*]; GenBank WP_071526362.1 | 510/510 (100%) | 0 |
| | E9.4 p1-7 | 4052..4375 (-) | MobC superfamily; mobilization protein most similar to mobilization protein [*Edwardsiella tarda*]; GenBank WP_035599715.1 | 107/107 (100%) | 1.00E-71 |
| | E9.4 p1-8 | 5357..5509 (-) | Lysis_col superfamily; lysis protein most similar to lysis protein [*Edwardsiella tarda*]; GenBank WP_071526395.1 | 50/50 (100%) | 6.00E-28 |
| Edwardsiella 9.4 Plasmid 2 | E9.4 p2-1 | 65166..460 (+) | Hypothetical protein most similar to hypothetical protein [*Shigella sonnei*]; GenBank WP_072106162.1 | 193/203 (95%) | 7.00E-87 |
| | E9.4 p2-2 | 1354..1824 (-) | IS5 family tranposase; most similar to IS5 family transposase [*Edwardsiella piscicida*]; GenBank WP_071890340.1 | 127/143 (89%) | 3.00E-84 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| | E9.4 p2-3 | 2185..2811 (-) | DDE_Tnp_Tn3 and P-loop NTPase superfamilies; transposase most similar to Tn3 family transposase, partial [*Klebsiella pneumoniae*]; GenBank OKC90594.1 | 137/164 (84%) | 7.00E-93 |
| | E9.4 p2-4 | 2812..3087 (-) | Transposase most similar to hypothetical protein AE36_03165 [*Klebsiella pneumoniae* BIDMC 61]; GenBank KDH23851.1 | 91/91 (100%) | 2.00E-59 |
| | E9.4 p2-5 | 4327..4458 (-) | Transposase most similar to Transposase [*Edwardsiella tarda* FL6-60]; GenBank ADM40880.1 | 31/32 (97%) | 3.00E-13 |
| Edwardsiella 9.4 Plasmid 2 | E9.4 p2-6 | 4567..4872 (-) | Hypothetical protein most similar to hypothetical protein [*Edwardsiella ictaluri*]; GenBank WP_049640101.1 | 38/92 (41%) | 2.00E-11 |
| | E9.4 p2-7 | 5381..6076 (+) | Hypothetical protein most similar to hypothetical protein [*Edwardsiella ictaluri*]; GenBank WP_041730357.1 | 136/234 (58%) | 2.00E-67 |
| | E9.4 p2-8 | 6132..6812 (+) | PapD_N superfamily; flagellar-assembly chaperone protein most similar to hypothetical protein [*Edwardsiella ictaluri*]; GenBank WP_035609284.1 | 130/216 (60%) | 4.00E-81 |
| | E9.4 p2-9 | 9206..9943 (+) | EAL superfamily; domain-encoding protein most similar to REC domain-containing phosphodiesterase [*Arcobacter nitrofigilis*]; GenBank | 72/230 (31%) | 6.00E-24 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| | E9.4 p2-10 | 10082..10699 (-) | P-loop NTPase superfamily; partitioning protein most similar to plasmid partition protein A (plasmid) [*Yersinia enterocolitica* LC20]; GenBank AHM76714.1 | 65/202 (32%) | 4.00E-27 |
| | E9.4 p2-11 | 11033..11428 (-) | Transposase most similar to transposase [*Edwardsiella piscicida*]; GenBank WP_069579582.1 | 125/131 (95%) | 5.00E-85 |
| | E9.4 p2-12 | 11512..11628 (-) | Hypothetical protein most similar to hypothetical protein EC12741_0935 [*Escherichia coli* 1.2741]; GenBank EIG81351.1 | 26/26 (72%) | 6.00E-08 |
| Edwardsiella 9.4 Plasmid 2 | E9.4 p2-13 | 11695..12087 (+) | Transposase most similar to transposase [*Edwardsiella tarda*]; GenBank WP_005293073.1 | 100/110 (91%) | 3.00E-54 |
| | E9.4 p2-14 | 12268..12852 (-) | Transposase most similar to transposase [*Edwardsiella tarda*]; GenBank WP_035600927.1 | 181/196 (92%) | 1.00E-110 |
| | E9.4 p2-15 | 13636..14151 (+) | Y2_Tnp superfamily; IS91 family transposase most similar to IS91 family transposase [*Edwardsiella hoshinae*]; GenBank WP_024524995.1 | 170/171 (99%) | 1.00E-118 |
| | E9.4 p2-16 | 16506..17552 (-) | IncFII_repA superfamily; replication protein most similar to replication protein [*Edwardsiella tarda*]; GenBank WP_035599321.1 | 297/310 (96%) | 0 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| | E9.4 p2-17 | 17982..18548 (-) | Restriction endonuclease-like superfamily; hypothetical protein most similar to hypothetical protein [*Edwardsiella tarda*]; GenBank WP_068872228.1 | 156/171 (91%) | 1.00E-102 |
| | E9.4 p2-18 | 18769..19386 (-) | Fin0_conjug_rep superfamily; conjugal transfer protein most similar to hypothetical protein [*Edwardsiella hoshinae*]; GenBank WP_024524787.1 | 187/206 (91%) | 7.00E-74 |
| | E9.4 p2-19 | 25445..27649 (-) | TraD_N and P-loop NTPase superfamily; conjugal transfer protein most similar to conjugal transfer protein TraD [*Edwardsiella hoshinae*]; GenBank WP_024524784.1 | 714/734 (97%) | 0 |
| Edwardsiella 9.4 Plasmid 2 | E9.4 p2-20 | 27736..28254 (-) | PRK13741 superfamily; hypothetical protein most similar to hypothetical protein [*Edwardsiella hoshinae*]; GenBank WP_024524783.1 | 164/172 (95%) | 3.00E-106 |
| | E9.4 p2-21 | 32467..33006 (-) | conjugal transfer protein TrbB most similar to type-F conjugative transfer system pilin assembly thiol-disulfide isomerase TrbB [*Edwardsiella tarda*]; GenBank WP_035599587.1 | 177/179 (99%) | 3.00E-115 |
| | E9.4 p2-22 | 32987..33220 (-) | TraQ superfamily; conjugal transfer protein TraQ most similar to conjugal transfer protein TraQ [*Edwardsiella tarda*]; GenBank WP_068872072.1 | 77/77 (100%) | 2.00E-48 |
| | E9.4 p2-23 | 33231..33983 (-) | Thioredoxin-like superfamily; conjugal transfer protein TraF most similar to conjugal transfer protein TraF [*Edwardsiella tarda*]; GenBank WP_035599547.1 | 249/250 (99%) | 4.00E-174 |



www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.4 Plasmid 2 | E9.4 p2-24 | 35842..36504 (-) | TrbC_Ftype superfamily; conjugal transfer protein TrbC most similar to type-F conjugative transfer system pilin assembly protein TrbC [*Edwardsiella tarda*]; GenBank WP_035599590.1 | 189/190 (99%) | 4.00E-135 |
| | E9.4 p2-25 | 37522..38157 (+) | PRK13738 superfamily; conjugal transfer protein TraW most similar to conjugal transfer pilus assembly protein TraW [*Edwardsiella tarda*]; GenBank WP_035599550.1 | 203/211 (96%) | 7.00E-150 |
| | E9.4 p2-26 | 38159..38530 (-) | TrbI_Ftype superfamily; conjugative transfer protein TrbI most similar to type-F conjugative transfer system protein TrbI [*Edwardsiella tarda*]; GenBank WP_068872079.1 | 120/123 (98%) | 1.00E-81 |
| | E9.4 p2-27 | 41171..41560 (-) | Hypothetical protein most similar to hypothetical protein [*Salmonella enterica*]; GenBank WP_000870324.1 | 51/123 (41%) | 2.00E-17 |
| | E9.4 p2-28 | 41689..42255 (-) | TraV superfamily; conjugal transfer protein TraV most similar to type IV conjugative transfer system protein TraV [*Edwardsiella tarda*]; GenBank WP_068872083.1 | 184/188 (98%) | 2.00E-104 |
| | E9.4 p2-29 | 42467..42835 (-) | Hypothetical protein most similar to hypothetical protein [*Edwardsiella hoshinae*]; GenBank WP_035370753.1 | 111/122 (91%) | 5.00E-65 |
| | E9.4 p2-30 | 44235..44966 (-) | TraK superfamily; conjugal transfer protein TraK most similar to conjugal transfer protein TraK [*Edwardsiella tarda*]; GenBank WP_035599563.1 | 237/243 (98%) | 2.00E-160 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.4 Plasmid 2 | E9.4 p2-31 | 44953..45519 (-) | TraE superfamily; conjugal transfer protein TraE most similar to type IV conjugative transfer system protein TraE [*Edwardsiella tarda*]; GenBank WP_068872092.1 | 188/188 (100%) | 2.00E-136 |
| | E9.4 p2-32 | 45537..45842 (-) | TraL superfamily; conjugal transfer protein TraL most similar to conjugal transfer protein TraL [*Edwardsiella tarda*]; GenBank WP_035599570.1 | 101/101 (100%) | 5.00E-69 |
| | E9.4 p2-33 | 45858..46205 (-) | TraA superfamily; conjugal transfer protein TraA most similar to conjugal transfer protein TraA [*Edwardsiella tarda*]; GenBank WP_035599572.1 | 114/115 (99%) | 2.00E-41 |
| | E9.4 p2-34 | 46277..46411 (-) | TraY superfamily; conjugal transfer protein most similar to hypothetical protein [*Edwardsiella tarda*]; GenBank WP_074428760.1 | 44/44 (100%) | 1.00E-23 |
| | E9.4 p2-35 | 47561..48301 (-) | TraT superfamily; conjugal transfer protein TraT most simiar to conjugal transfer protein TraT [*Edwardsiella hoshinae*]; GenBank WP_024524762.1 | 231/246 (94%) | 1.00E-150 |
| | E9.4 p2-36 | 49183..49656 (+) | Lysozyme-like superfamily; lytic transglycosylase most similar to lytic transglycosylase [*Edwardsiella tarda*]; GenBank WP_035599583.1 | 155/157 (99%) | 7.00E-112 |
| | E9.4 p2-37 | 50161..51066 (-) | DUF1738 superfamily; DNA primase most similar to DNA primase [*Edwardsiella tarda*]; GenBank WP_035599585.1 | 299/301 (99%) | 0 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.4 Plasmid 2 | E9.4 p2-38 | 52448..52879 (-) | PsiB superfamily; plasmid SOS inhibition protein B most similar to plasmid SOS inhibition protein B [*Edwardsiella hoshinae*]; GenBank WP_024524757.1 | 138/143 (97%) | 4.00E-98 |
| | E9.4 p2-39 | 54953..55195 (-) | DUF905 superfamily; hypothetical protein most similar to hypothetical protein [*Salmonella enterica*]; GenBank WP_070795100.1 | 70/80 (88%) | 7.00E-47 |
| | E9.4 p2-40 | 55248..55793 (-) | RPA_2b-aaRSs_0BF-like superfamily; single-stranded DNA-binding protein most similar to single-stranded DNA-binding protein [*Edwardsiella hoshinae*]; GenBank WP_024524755.1 | 171/181 (94%) | 6.00E-83 |
| | E9.4 p2-41 | 56080..56337 (-) | Hypothetical protein most similar to hypothetical protein [*Edwardsiella hoshinae*]; GenBank WP_071991033.1 | 61/68 (90%) | 1.00E-35 |
| | E9.4 p2-42 | 56622..57050 (-) | Antirestrict superfamily; antirestriction protein most similar to MULTISPECIES: antirestriction protein [*Edwardsiella*]; GenBank WP_024524753.1 | 138/142 (97%) | 4.00E-100 |
| | E9.4 p2-43 | 57499..57918 (-) | DUF1380 superfamily; adenine-specific methyltransferase most similar to adenine-specific methyltransferase [*Edwardsiella tarda*]; GenBank WP_035600999.1 | 130/139 (94%) | 6.00E-79 |



المنارة للاستشارات
www.manaraa.com

Table 4.13 (Continued)

| Plasmid Source | ORF | Location (+/-) | Conserved domain; putative product; function | Alignment and identity | E-value |
|---|---|---|---|---|---|
| Edwardsiella 9.4 Plasmid 2 | E9.4 p2-44 | 57976..58656 (-) | AdoMet_Mtases superfamily; DNA methylase most similar to DNA methylase [*Edwardsiella tarda*]; GenBank WP_035601001.1 | 216/226 (96%) | 7.00E-163 |
| | E9.4 p2-45 | 59017..59358 (-) | Hypothetical protein most similar to MULTISPECIES: hypothetical protein [*Enterobacteriaceae*]; GenBank WP_023321981.1 | 75/111 (68%) | 4.00E-50 |
| | E9.4 p2-46 | 59351..59710 (-) | Plasmid_stab_B superfamily; plasmid stability protein most similar to plasmid stability protein [*Enterobacter kobei*]; GenBank WP_063434128.1 | 90/119 (76%) | 6.00E-57 |
| | E9.4 p2-47 | 59736..60692 (-) | NBD_sugar-kinase HSP70_actin superfamily; recombinase most similar to MULTISPECIES: recombinase [Enterobacterales]; GenBank WP_043135399.1 | 268/318 (84%) | 0 |
| | E9.4 p2-48 | 61096..61734 (+) | PHA02518 superfamily; partitioning protein ParA most similar to plasmid partitioning protein ParA (plasmid) [*Raoultella ornithinolytica*]; GenBank ALD82414.1 | 199/212 (94%) | 2.00E-135 |
| | E9.4 p2-49 | 61734..62027 (+) | Hypothetical protein most similar to CopG family transcriptional regulator [*Serratia* sp. 506_PEND]; GenBank WP_075202068.1 | 94/97 (97%) | 2.00E-61 |
| | E9.4 p2-50 | 62136..62936 (-) | DNA_BRE_C superfamily; resolvase most similar to resolvase [*Edwardsiella tarda*]; GenBank WP_050979670.1 | 237/260 (91%) | 5.00E-168 |
| | E9.4 p2-51 | 63665..64606 (-) | Hypothetical protein most similar to hypothetical protein [*Klebsiella pneumoniae*]; GenBank WP_032448293.1 | 207/300 (69%) | 5.00E-140 |



المنارة للاستشارات
www.manaraa.com

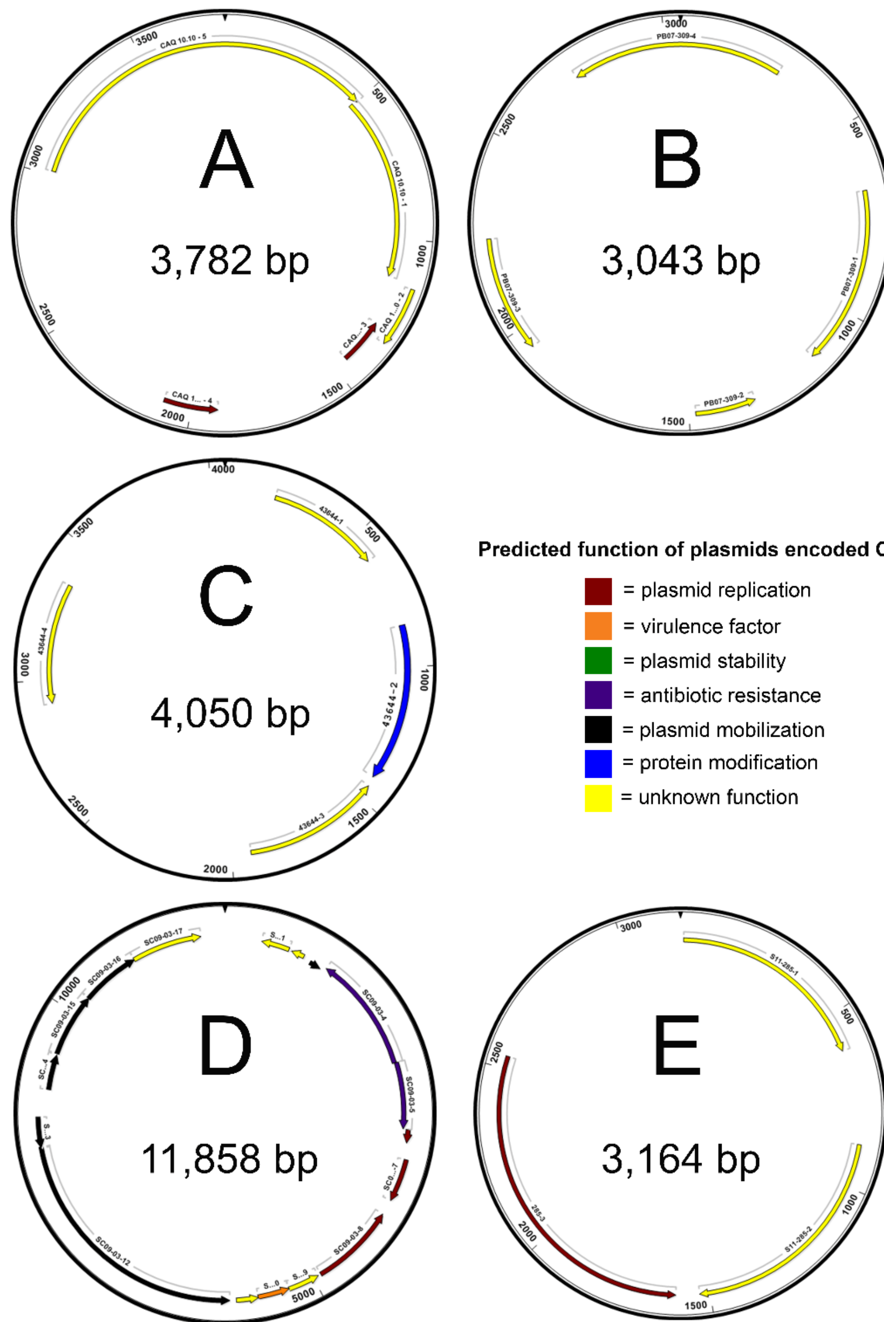


Figure 4.7 Physical maps of plasmids harvested from *E. piscicida* isolates.

Physical maps of complete nucleotide sequences of plasmids harvested from *E. piscicida* isolates A) Group; B) PB 07-309; C) 43644; D) SC 09-03; and E) S11-285. Maps indicate locations of predicted open reading frames (ORFs), which are color-coded according to predicted function. Predicted products and putative function of ORFs is provided in Table 4.12.



www.manaraa.com

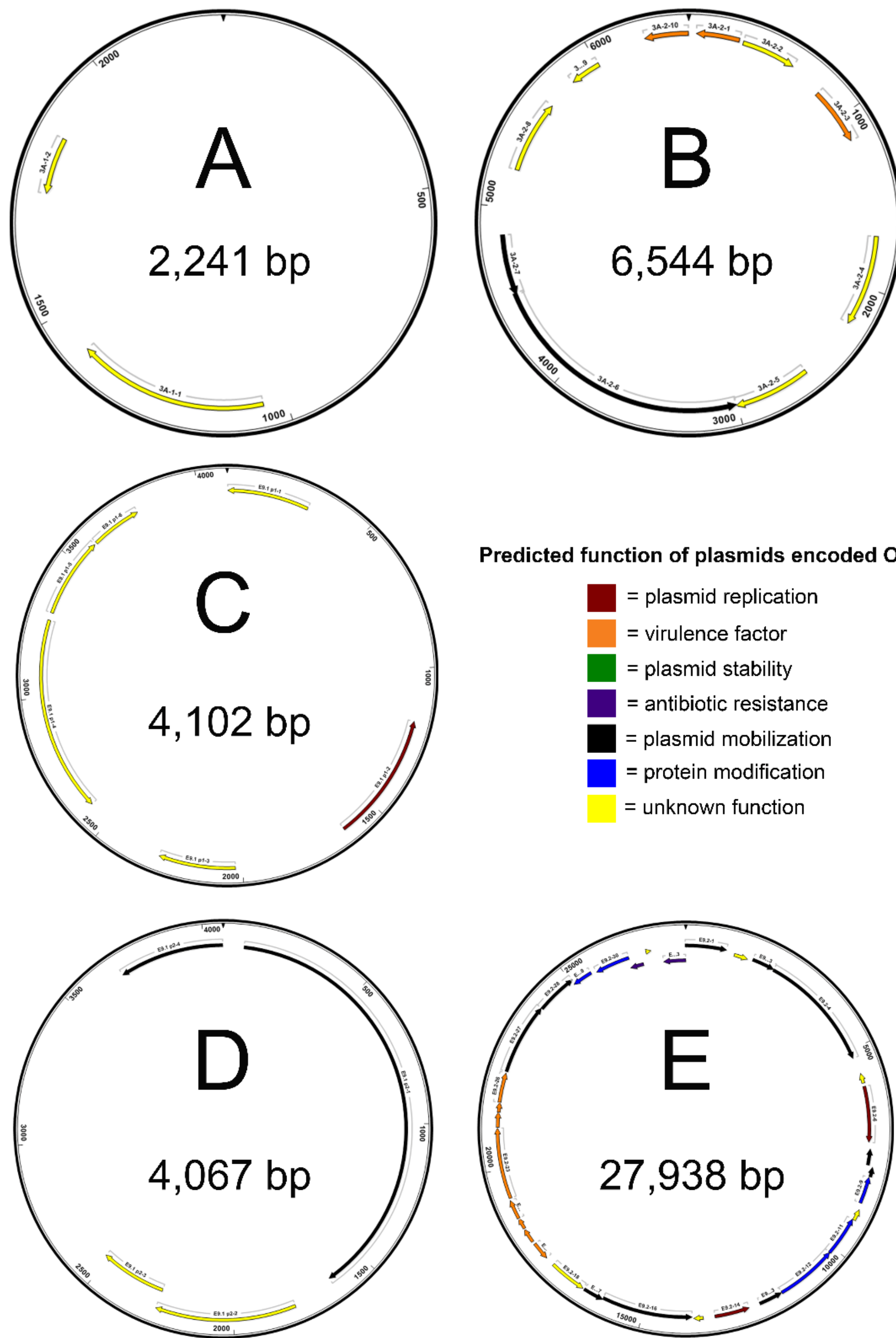


Figure 4.8 Physical maps of plasmids harvested from *E. tarda* isolates.

Physical maps of complete nucleotide sequences of plasmids harvested from *E. tarda* isolates A) and B) 070720-1 3A; C) and D) Edwardsiella 9.1; and E) Edwardsiella 9.2. Maps indicate locations of predicted open reading frames (ORFs), which are color-coded according to predicted function. Predicted products and putative function of ORFs is provided in Table 4.13.



المنارة للاستشارات
www.manaraa.com

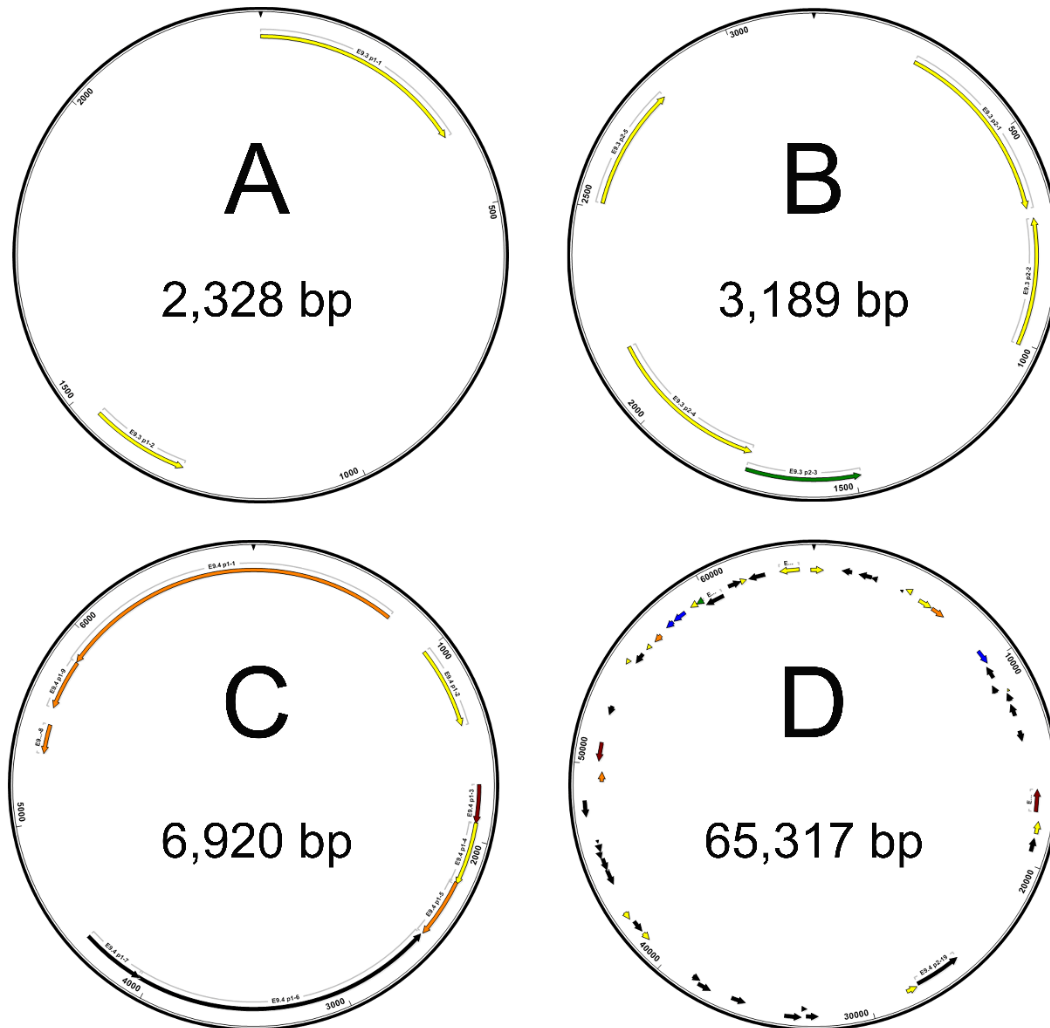


Figure 4.9 Physical maps of plasmids harvested from *E. tarda* isolates.

Physical maps of complete nucleotide sequences of plasmids harvested from *E. tarda* isolates A) and B) Edwardsiella 9.3; C) and D) Edwardsiella 9.4. Maps indicate locations of predicted open reading frames (ORFs), which are color-coded according to predicted function. Predicted products and putative function of ORFs is provided in Table 4.13.



المنارة للاستشارات
www.manaraa.com

## 4.4 Discussion

Since 1981 the *Edwardsiella* genus has been comprised of three taxa: *E. tarda*, *E. hoshinae* and *E. ictaluri*. More recent investigations into the phenotypic and genotypic variation of *E. tarda* have led to the recognition that isolates previously classified as *E. tarda* actually represent three distinct taxa: *E. tarda*, *E. piscicida* and *E. anguillarum*. In light of these findings, the current study was intended to characterize the five *Edwardsiella* species using common phenotypic and genotypic analyses and demonstrate the importance of updating microbial identification systems to reflect contemporary taxonomy.

Previous work demonstrated variations in biochemical profiles of *Edwardsiella* isolates from different fish hosts and geographic origins (Matsuyama, Kamaishi et al. 2005, Sakai, Yuasa et al. 2009, Park, Aoki et al. 2012). The work described here is consistent with these previous studies, with extant intraspecific phenotypic variation within some groups. This is not surprising, given the broad diversity of fish hosts, broad geographic distribution and wide temporal range of these isolates. Marked inter- and intraspecific variation was also present in fatty acid content; however no discriminatory fatty acid was identified, similar to previous findings (Griffin, Quiniou et al. 2013). Although conventional phenotypic methods are user-friendly and relatively inexpensive, certain groups of bacteria are difficult to identify using conventional techniques, specifically rare isolates or isolates with ambiguous profiles (Woo et al. 2008).

The four microbial identification systems analyzed in this study all correctly identified *Edwardsiella* taxa recognized and validated for each respective system. However, at present, none of four databases associated with the systems used here



المنارة للاستشارات
www.manaraa.com

recognize *E. anguillarum* or *E. piscicida*. The increasing use of molecular techniques and growing number of new bacterial taxa identified using genomic technology poses a problem for phenotype database management, resulting in prokaryote databases that lag behind evolving systematics (McMeekin, Baranyi et al. 2006). Moreover, commercial test panel configurations are relatively constant over time, and as new species are defined, more appropriate discriminatory metabolic phenotypic tests may not be present in current test panel arrangements (Janda and Abbott 2002). Furthermore, many microbial identification databases still consider 16S rRNA as the gold standard for taxon identification, the limitations of which are discussed below.

Within the species formerly classified as *E. tarda* (*E. tarda*, *E. piscicida* and *E. anguillarum*), no distinct phenotypic pattern emerged amongst API 20E and BBL Crystal codes. In addition, no confirmative identifying profile was apparent using the Biolog Microbial identification system. It is worth noting, however, intraspecific variation in phenotypic characteristics were noted within *Edwardsiella* species. This is consistent with previous work that did not identify a discriminatory metabolic fingerprint to differentiate among different *E. tarda* phylogroups (Griffin, Quiniou et al. 2013), suggesting that isolates identified phenotypically as *E. tarda*, regardless of identification system employed, require supplemental molecular confirmation. In light of these findings, and given the rapidly increasing number of representative *Edwardsiella* genomes available, further work establishing a discriminatory metabolic profile for each *Edwardsiella* sp. is warranted.

Similarly, the role of 16S rRNA sequence for differentiation of the *Edwardsiella* species has recently been called into question (Griffin, Quiniou et al. 2013, Griffin, Ware



المنارة للاستشارات
www.manaraa.com

et al. 2014, Griffin, Reichley et al. 2016). The utility of 16S rRNA for bacterial identification has long been a topic of debate, largely due to the high percentage of sequence similarity between some closely related species, the lack of a definitive intraspecific dissimilarity value and absence of universal guidelines (Fox, Wisotzkey et al. 1992, Janda and Abbott 2002, Clarridge 2004). Moreover, some organisms possess rRNA genes in multiple heterogeneous copies, complicating the differentiation between closely related species because intragenomic heterogeneity in some species can exceed 1% (Huang 1996, Janda and Abbott 2007). The intragenomic heterogeneity among *Edwardsiella* spp. 16S rRNA sequences ranges from 0.0 - 0.6% (Reichley, unpublished data).

As a result of these limitations, high SSU sequence identity (>99%) does not always imply accuracy in microbial identification to the species level, as previously described for other genera, such as *Aeromonas*, *Bacillus*, *Bordetella*, *Burkholderia*, *Campylobacter*, *Enterobacter*, *Mycobacteria*, *Neisseria*, *Pseudomonas* and *Streptococcus* (Turenne, Tschetter et al. 2001, Janda and Abbott 2007). This is important to note because many contemporary studies still rely on partial 16S rRNA sequences for molecular confirmation of bacterial identification, often citing 16S rRNA sequences deposited in the National Center for Biotechnology Information's (NCBI) GenBank and the International Nucleotide Sequence Database (Nilsson, Ryberg et al. 2006, Wragg, Randall et al. 2014). These databases are non-peer reviewed and generally accept any listed name and sequence that is submitted. This can pose a problem when attempting to identify unknown microorganisms because erroneous identification can occur if archived sequences are inaccurate or misclassified (Clarridge 2004). This is further complicated by



المنارة للاستشارات
www.manaraa.com

SSU searches where inconsistent sequence ends, ambiguous entries, pseudogaps and insertions can result in misleading sequence matches (Turenne, Tschetter et al. 2001).

Alternatives to using 16S rRNA sequences for appropriately discriminating between closely related congeners has been demonstrated for several prokaryote genera, including *Edwardsiella* (Dauga 2002, Griffin, Ware et al. 2014). Although 16S sequencing is useful in identifying unknown isolates to genus, the discriminatory power significantly diminishes at the species level, especially in closely related species (Woo, Lau et al. 2008). In these instances, alternative reference genes should be considered. The single-copy *gyrB* gene, encoding the ATPase domain of DNA gyrase, is essential for DNA replication and is present in all prokaryotes. It contains conserved motifs that facilitate the development of genera-specific or family specific primers (Huang 1996). The *gyrB* gene has been used to explore the diversity of a wide range of bacteria and is more resolute than 16S rRNA in differentiating closely related members of the *Enterobacteriaceae*, including the *Edwardsiella* (Dauga 2002, Griffin, Ware et al. 2014). The utility of *gyrB* in *Edwardsiella* classification and identification has been demonstrated (Griffin, Ware et al. 2014, Griffin, Reichley et al. 2016), and the work reported here further supports the use of *gyrB* over 16S rRNA as an appropriate marker for discrimination of *Edwardsiella* species.

Similar to *gyrB*, the iron-cofactored superoxide dismutase gene (*sodB*) has high discriminatory power amongst the *Edwardsiella*. Prior to the segregation of *E. tarda* and the identification of *E. piscicida* and *E. anguillarum* as discrete taxa, an internal fragment of *sodB* was used to distinguish between fish pathogenic and fish non-pathogenic *E. tarda* (Yamada and Wakabayashi 1999). This work raised questions whether fish



المنارة للاستشارات
www.manaraa.com

pathogenic *E. tarda* and fish non-pathogenic *E. tarda* type strain from humans (ATCC 15947) were truly monophyletic. The present analysis of *sodB* sequences found similar groupings and allowed for correlation between these historical analyses and contemporary nomenclature.

Therefore, the current work confirms what was defined as typical motile fish pathogenic *E. tarda* is synonymous with *E. piscicida*. *E. piscicida* isolates in the current study share 99.8% -100% similarity at *sodB* to typical motile fish pathogenic *E. tarda* isolates described by Yamada and Wakabayashi (1999) (GenBank AB009853). Similarly, *sodB* sequence analysis showed that atypical non-motile fish pathogenic *E. tarda* are *E. anguillarum,* while isolates identified as *E. tarda* in the current study were found to be synonymous with fish non-pathogenic *E. tarda* (including the *E. tarda* type strain from humans, ATCC 15947). This agrees with previous genomic assessments demonstrating high genome sequence homology (>97%) between the typical motile (NUF806) and atypical non-motile (FPC503) *E. tarda* strains characterized by Matsuyama et al. (2005) and the new species *E. piscicida* and *E. anguillarum*, respectively (Nakamura, Takano et al. 2013, Shao, Lai et al. 2015).

Repetitive extragenic palindromic PCR (rep-PCR) fingerprinting is another common molecular technique used to estimate the relative degrees of similarity between bacterial isolates (Versalovic, Koeuth et al. 1991, Versalovic, Schneider et al. 1994). The rep-PCR analysis in this study produced distinctive banding patterns for each member of the *Edwardsiella*, with some intraspecific variation. This variation was anticipated and congruent with previous research (Griffin, Quiniou et al. 2013, Griffin, Ware et al. 2014,



المنارة للاستشارات
www.manaraa.com

Griffin, Reichley et al. 2016), demonstrating the ability of rep-PCR to distinguish among *Edwardsiella* spp.

Plasmid analysis revealed slightly more than half of the bacterial isolates analyzed carry at least one native plasmid. Plasmid content included several predicted genes associated with replication, antibiotic resistance and virulence, although this content varied by group and by isolate. The plasmids harvested from *E. ictaluri* isolates in this study supported previous characterization (Griffin, Reichley et al. 2016). The three *E. ictaluri* plasmids are similar in size; however, they differ in composition and arrangement, which is likely a function of being isolated from different fish hosts in different geographic locales.

It is not surprising that nine of the *E. piscicida* isolates (F373.2, HL1, HL25, HL32, RBR8, ACC69, CAQ 8.10, CAQ 10.10 and CAQ 39) all carry an identical plasmid because these cultures were recovered from a single fish host (turbot) in Europe during a short temporal range (2005 - 2012). This plasmid encodes a replication initiation factor and RNA polymerase, along with several hypothetical proteins. The plasmid harvested from Edwardsiella 9.1, the isolate used in the description of *E. tarda* in channel catfish (Meyer and Bullock 1973), encoded a DNA polymerase, mobilization protein and several hypothetical proteins.

Plasmids from remaining isolates all vary in size, composition and arrangement. This is attributed to the diversity of fish hosts, geographic origins and year isolated. Of note, 6 of the 13 (46%) remaining plasmids harvested encode mobilization proteins, nucleases, transposases and various resistance genes. *Edwardsiella piscicida* isolate SC 09-03 recovered from a smallmouth bass in South Carolina, USA carries an 11,858 bp



www.manaraa.com

plasmid with several ORFs encoding genes related to tetracycline resistance. During MIC analysis, SC 09-03 demonstrated resistance to the highest concentrations of tetracycline, oxytetracycline and minocycline analyzed in the current study. Plasmids from *E. tarda* isolates Edwardsiella 9.2 and Edwardsiella 9.4, recovered from channel catfish in the United States, contain ORFs encoding transposases and conjugal transfer proteins. In addition, the 27,938 bp plasmid from Edwardsiella 9.2 contains ORFs encoding for mercury resistance.

It is important to note the methods employed here may be limited in their ability to isolate very large plasmids or plasmids of low copy number. For example, multidrug resistance plasmids belonging to the IncA/C family are widely distributed among enterobacterial isolates (Fricke, Welch et al. 2009) and have been reported from some *E. ictaluri* isolates from farm-raised channel catfish in the southeastern United States (Welch, Evenhuis et al. 2009, LaFrentz, Welch et al. 2011). The IncA/C plasmids are usually very large and typically present in low copy numbers. While an IncA/C type plasmid was not observed in any of these isolates, future studies employing more robust techniques suitable for the harvest of very large and/or low copy number plasmids are warranted.

Real-time, quantitative PCR (qPCR) assays are becoming more common in fish disease research and diagnostics. Assays are currently available for a plethora of bacterial, viral and parasitic fish pathogens (Purcell, Getchell et al. 2011). Previous research validated qPCR assays for the detection and quantification of *E. anguillarum*, *E. piscicida* and *E. tarda* in broth culture, pond water and catfish tissue (Reichley, Ware et al. 2015). The real-time, multiplex PCR developed and validated in this study



المنارة للاستشارات
www.manaraa.com

demonstrated appropriate specificity, sensitivity, reproducibility and repeatability to reliably discriminate among *E. anguillarum, E. ictaluri*, *E. piscicida* and *E. tarda* (Bustin, Benes et al. 2009). In addition, the presence of large quantities of non-target DNA had no measurable effect on PCR efficiency suggesting this assay could also have application as a research tool for environmental DNA (eDNA) assessments in aquaculture systems similar to other qPCR assays (Griffin, Pote et al. 2009, Griffin, Mauel et al. 2011, Griffin, Goodwin et al. 2013). Because no distinguishing phenotypic character has been identified to date for *E. anguillarum, E. piscicida* and *E. tarda*, this assay serves as a rapid method of confirmatory identification for all *Edwardsiella* species infecting fish.

Lastly, matrix assisted laser desorption/ionization time of flight (MALDI-TOF) is an emerging technology for microbial identification. MALDI-TOF generates protonated ions and uses time of flight to generate a peptide mass fingerprint for each sample (Singhal, Kumar et al. 2015). It can be used for rapid microbial identification from a pure culture, dramatically improving time to identification (Dixon, Davies et al. 2015). The use of MALDI-TOF MS for species and subspecies identification has been reported in several different bacteria (Barbuddhe, Maier et al. 2008, Dieckmann, Helmuth et al. 2008, Seibold, Maier et al. 2010).

Initially, MALDI-TOF classified all *E. anguillarum* and *E. piscicida* isolates as *E. tarda*. This was expected, because *E. anguillarum* and *E. piscicida* are not currently recognized by the microbial peptide mass spectra database v5.0.0.0 (Bruker Daltonics). However, observation of individual spectral profiles revealed discriminatory peaks were present for each *Edwardsiella* spp. Thus, in spite of deficiencies in the current microbial



www.manaraa.com

database, MALDI-TOF can reliably discriminate among the five current *Edwardsiella* taxa, including the species formerly classified as *E. tarda*.

Molecular typing methods described here were all in agreement with taxonomic assignments for all isolates. Despite the lack of a discriminatory metabolic or phenotypic character, MALDI-TOF correlated with multiplex PCR, *gyrB, sodB* and rep-PCR identifications and classifications. While molecular confirmation of suspect *Edwardsiella* isolates is ideal in terms of generating archival data for comparison in future studies, MALDI-TOF offers a reliable, cost effective alternative for clinical laboratories that require rapid, reliable identification.

Another significant finding that resulted from the current research is the confirmation that Edwardsiella isolate 9.1, which is from the original description of emphysematous putrefactive disease in catfish aquaculture in the 1970s, as well as other suspected *E. tarda* isolates from catfish aquaculture in the early 1980s, are factually *E. tarda*. Recent molecular surveys suggest *E. piscicida* is far more common in United States catfish aquaculture than *E. tarda*, and it was suspected these original isolates and other reports of *E. tarda* in fish prior to the adoption of *E. piscicida* may have been misclassified (Griffin, Ware et al. 2014). Although this may be the case in some instances, it does not hold true for all historical isolates. Comparing archived *sodB* sequences from previous reports to data generated here, *E. tarda* as it is currently defined still occasionally causes disease outbreaks in fish (Yamada and Wakabayashi 1999, Griffin, Quiniou et al. 2013, Griffin, Ware et al. 2014, Reichley, Ware et al. 2015, Shao, Yuan et al. 2016).



www.manaraa.com

Proper identification of bacterial isolates is the foundation on which clinical diagnostics and infectious disease research is built. Consistent taxonomic assignment of bacteria facilitates the definition of host-microbe relationships, the development of therapeutic and preventative strategies and it is the cornerstone of epidemiological investigations (Janda and Abbott 2002). This is especially true for the *Edwardsiella*, because different members of the genus demonstrate varying degrees of pathogenicity to different hosts (Matsuyama, Kamaishi et al. 2005, Abayneh, Colquhoun et al. 2013, Reichley, Ware et al. 2015). The methodologies described here provide reliable methods of identification of the *Edwardsiella* species and are consistent with current taxonomic schemes. Moreover, the zoonotic potential of *E. tarda* and the variable pathogenicity of *E. anguillarum*, *E. piscicida* and *E. tarda* in different hosts makes proper identification of isolates recovered from fish and aquaculture systems extremely important.

*E. tarda* plays an important role in zoonotic infections and is one of the principal pathogens acquired from fish and shellfish, including ornamental pet fish (Vandepitte, Lemmens et al. 1983, Javier 2012, Haenen, Evans et al. 2013). The clinical disease that manifests in humans infected with *E. tarda* may be associated with necrotic skin lesions, gastroenteritis, and in severe cases, a septicemia leading to osteomyelitis, meningitis or cholecystitis (Gilman, Madasamy et al. 1971). At present, the zoonotic potential of *E. anguillarum* and *E. piscicida* are unknown, and it is unclear if these previous reports are in reference to *E. tarda* as it is currently defined or one of the newly recognized species. Consistent methods of identification in line with contemporary systematic nomenclature will limit ambiguity in such reports moving forward. Therefore, it is imperative that nomenclature consistency is applied across different laboratories and throughout different



المنارة للاستشارات
www.manaraa.com

countries. The limitations of databases such as GenBank and the unverified taxa classifications associated with submissions further emphasizes the importance for researchers and diagnosticians to remain attentive to current literature.

### 4.5 Acknowledgements

The author gratefully acknowledges Cynthia Ware, James Steadman, Terrence E. Greenway, Patricia S. Gaunt, Julio C. García, Benjamin R. LeFrentz, Anil Thachil, Geoffrey C. Waldbieser, Cynthia B. Stine, Cova R. Arias, Thomas Loch, Timothy J. Welch, Rocco C. Cipriano, Lester H. Khoo, David J. Wise, Mark L. Lawrence, Matt J. Griffin, Al Camus, D. Barbara Petty, Roy Yanong and Ronald Thune for generously sharing bacterial isolates and performing some of the analyses that were performed in the current study.



www.manaraa.com

## 4.6 References

Abayneh, T., D. J. Colquhoun and H. Sorum (2012). "Multi-locus Sequence Analysis (MLSA) of *Edwardsiella tarda* isolates from fish." Veterinary Microbiology **158**(3-4): 367-375.

Abayneh, T., D. J. Colquhoun and H. Sorum (2013). "*Edwardsiella piscicida* sp. nov., a novel species pathogenic to fish." Journal of Applied Microbiology **114**(3): 644-654.

Alcaide, E., S. Herraiz and C. Esteve (2006). "Occurrence of *Edwardsiella tarda* in wild European eels *Anguilla anguilla* from Mediterranean Spain." Diseases of Aquatic Organisms **73**: 77-81.

Barbuddhe, S. B., T. Maier, G. Schwarz, M. Kostrzewa, H. Hof, E. Domann, T. Chakraborty and T. Hain (2008). "Rapid identification and typing of *Listeria* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry." Applied Environmental Microbiology **74**(17): 5402-5407.

Besemer, J. and M. Borodovsky (1999). "Heuristic approach to deriving models for gene finding." Nucleic Acids Research **27**(19): 3911-3920.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele and C. T. Wittwer (2009). "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments." Clinical Chemistry **55**(4): 611-622.

Castro, N., A. E. Toranzo, J. L. Barja, S. Nunez and B. Magarinos (2006). "Characterization of *Edwardsiella tarda* strains isolated from turbot, *Psetta maxima* (L.)." Journal of Fish Diseases **29**: 541-547.

Chou, L., M. J. Griffin, T. Fraites, C. Ware, H. Ferguson, N. Keirstead, J. Brake, J. Wiles, J. P. Hawke, M. T. Kearney, R. G. Getchell, P. Gaunt and E. Soto (2014). "Phenotypic and genotypic heterogeneity among *Streptococcus iniae* isolates recovered from cultured and wild fish in North America, Central America and the Caribbean islands." Journal of Aquatic Animal Health **26**: 263-271.

Clarridge, J. E., 3rd (2004). "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases." Clinical Microbiology Reviews **17**(4): 840-862.

Crumlish, M., T. T. Dung, J. F. Turnbull, N. T. N. Ngoc and H. W. Ferguson (2002). "Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam." Journal of Fish Diseases **25**: 733-736.



المنارة للاستشارات
www.manaraa.com

Dauga, C. (2002). "Evolution of the gyrB gene and the molecular phylogeny of *Enterobacteriaceae*: a model molecule for molecular systematic studies." International Journal of Systematic and Evolutionary Microbiology **52**: 531-547.

Delcher, A. L., D. Harmon, S. Kasif, O. White and S. L. Salzberg (1999). "Improved microbial gene identification with GLIMMER." Nucleic Acids Research **27**(23): 4636-4641.

Dieckmann, R., R. Helmuth, M. Erhard and B. Malorny (2008). "Rapid classification and identification of *Salmonellae* at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry." Applied Environmental Microbiology **74**(24): 7767-7778.

Dixon, P., P. Davies, W. Hollingworth, M. Stoddart and A. MacGowan (2015). "A systematic review of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry compared to routine microbiological methods for the time taken to identify microbial organisms from positive blood cultures." European Journal of Clinical Microbiology and Infectious Diseases **34**: 863-876.

Edgar, R. C. (2004). "MUSCLE: multiple sequence alignment with high accuracy and high throughput." Nucleic Acids Research **32**(5): 1792-1797.

Ewing, W. H., A. C. McWhorter, M. R. Escobar and A. H. Lubin (1965). "*Edwardsiella*, a new genus of Enterobacteriaceae based on a new species, *E. tarda*." International Bulletin of Bacteriological Nomenclature and Taxonomy **15**(1): 33-38.

Fox, G. E., J. D. Wisotzkey and J. Peter Jurtshuk (1992). "How close is close: 16s rRNA sequence identity may not be sufficient to guarantee species identity." International Journal of Systematic Bacteriology **42**(1): 166-170.

Fricke, W. F., T. J. Welch, P. F. McDermott, M. K. Mammel, J. E. LeClerc, D. G. White, T. A. Cebula and J. Ravel (2009). "Comparative genomics of the IncA/C multidrug resistance plasmid family." Journal of Bacteriology **191**: 4750-4757.

Gilman, R., M. Madasamy, E. Gan, M. Mariappan, C. Davis and K. Kyser (1971). "*Edwardsiella tarda* in jungle diarrhoea and a possible association with *Entamoeba histolytica*." Southeast Asian Journal of Tropical Medicine and Public Health **2**(2): 186-189.

Griffin, M. J., A. E. Goodwin, G. E. Merry, M. R. Liles, M. A. Williams, C. Ware and G. C. Waldbieser (2013). "Rapid quantitative detection of *Aeromonas hydrophila* strains associated with disease outbreaks in catfish aquaculture." Journal of Veterinary Diagnostic Investigation **25**(4): 473-481.



المنارة للاستشارات
www.manaraa.com

Griffin, M. J., M. J. Mauel, T. E. Greenway, L. H. Khoo and D. J. Wise (2011). "A Real-Time Polymerase Chain Reaction Assay for Quantification *of Edwardsiella ictaluri* in Catfish Pond Water and Genetic Homogeneity of Diagnostic Case Isolates from Mississippi." Journal of Aquatic Animal Health **23**(4): 178-188.

Griffin, M. J., L. M. Pote, A. C. Camus, M. J. Mauel, T. E. Greenway and D. J. Wise (2009). "Application of a real-time PCR assay for the detection of *Henneguya ictaluri* in commercial channel catfish ponds." Diseases of Aquatic Organisms **86**(3): 223-233.

Griffin, M. J., S. M. Quiniou, T. Cody, M. Tabuchi, C. Ware, R. C. Cipriano, M. J. Mauel and E. Soto (2013). "Comparative analysis of *Edwardsiella* isolates from fish in the eastern United States identifies two distinct genetic taxa amongst organisms phenotypically classified as *E. tarda*." Veterinary Microbiology **165**(3-4): 358-372.

Griffin, M. J., S. R. Reichley, T. E. Greenway, S. M. Quiniou, C. Ware, D. X. Gao, P. S. Gaunt, R. P. Yanong, D. B. Pouder, J. P. Hawke and E. Soto (2016). "Comparison of *Edwardsiella ictaluri* isolates from different hosts and geographic origins." Journal of Fish Diseases **39**(8): 947-969.

Griffin, M. J., C. Ware, S. M. Quiniou, J. M. Steadman, P. S. Gaunt, L. H. Khoo and E. Soto (2014). "*Edwardsiella piscicida* identified in the Southeastern USA by *gyrB* sequence, species-specific and repetitive sequence-mediated PCR." Diseases of Aquatic Organisms **108**(1): 23-35.

Grimont, P. D., F. Grimont, C. Richard and R. Sakazaki (1980). "*Edwardsiella hoshinae*, a new species of Enterobacteriaceae." Current Microbiology **4**(6): 347-351.

Haenen, O., J. Evans and F. Berthe (2013). "Bacterial infections from aquatic species: potential for and prevention of contact zoonoses." Revue scientifique et technique (International Office of Epizootics) **32**(2): 497-507.

Hawke, J. P., M. Kent, M. Rogge, W. Baumgartner, J. Wiles, J. Shelley, L. C. Savolainen, R. Wagner, K. Murray and T. S. Peterson (2013). "Edwardsiellosis caused by *Edwardsiella ictaluri* in laboratory populations of Zebrafish *Danio rerio*." Journal of Aquatic Animal Health **25**(3): 171-183.

Hawke, J. P. and L. H. Khoo (2004). Infectious Diseases. Biology and culture of the channel catfish. C. S. Tucker and J. A. Hargreaves. Amsterdam, Elsevier**:** 387-443.

Hawke, J. P., A. C. McWhorter, A. G. Steigerwalt and D. J. Brenner (1981). "*Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish." International Journal of Systematic Bacteriology **31**(4): 396-400.



المنارة للاستشارات
www.manaraa.com

Huang, W. M. (1996). "Bacterial diversity based on type II DNA topoisomerase genes." Annual Review of Genetics **30**: 79-107.

Janda, J. M. and S. L. Abbott (2002). "Bacterial identification for publication: when is enough enough?" Journal of Clinical Microbiology **40**(6): 1887-1891.

Janda, J. M. and S. L. Abbott (2007). "16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls." Journal of Clinical Microbiology **45**(9): 2761-2764.

Javier, S. (2012). "*Edwardsiellosis*, an emerging zoonosis of aquatic animals." Biohelikon: Immunity & Diseases **1**(1): 1-2.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Ashton, P. Meintjes and A. Drummond (2012). "Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data." Bioinformatics **28**(12): 1647-1649.

King, B. M. and D. L. Adler (1964). "A previously undescribed group of Enterobacteriaceae." The American Journal of Clinical Pathology **41**(2): 230-232.

Kodama, H., T. Murai, Y. Nakanishi, F. Yamamoto, T. Mikami and H. Izawa (1987). "Bacterial infection which produces high mortality in cultured Japanese flounder (*Paralichthys olivaceus*) in Hokkaido." Japanese Journal of Veterinary Research **35**(4): 227-234.

LaFrentz, B. R., T. J. Welch, C. A. Shoemaker, J. D. Drennan and P. H. Klesius (2011). "Modified live *Edwardsiella ictaluri* vaccine, AQUAVAC-ESC, lacks multidrug resistance plasmids." Journal of Aquatic Animal Health **23**: 195-199.

Leotta, G. A., P. Piñeyro, S. Serena and G. B. Vigo (2009). "Prevalence of *Edwardsiella tarda* in Antarctic wildlife." Polar Biology **32**(5): 809-812.

Matsuyama, T., T. Kamaishi, N. Ooseko, K. Kurohara and T. lida (2005). "Pathogenicity of motile and non-motile *Edwardsiella tarda* to some marine fish." Fish Pathology **40**(3): 133-135.

McMeekin, T. A., J. Baranyi, J. Bowman, P. Dalgaard, M. Kirk, T. Ross, S. Schmid and M. H. Zwietering (2006). "Information systems in food safety management." International Journal of Food Microbiology **112**(3): 181-194.

Meyer, F. P. and G. L. Bullock (1973). "*Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*)." Applied Microbiology **25**(1): 155-156.

Mohanty, B. R. and P. K. Sahoo (2007). "Edwardsiellosis in fish: a brief review." Journal of Biosciences **32**: 1331-1344.



المنارة للاستشارات
www.manaraa.com

Nakamura, Y., T. Takano, M. Yasuike, T. Sakai, T. Matsuyama and M. Sano (2013). "Comparative genomics reveals that a fish pathogenic bacterium *Edwardsiella tarda* has acquired the locus of enterocyte effacement (LEE) through horizontal gene transfer." BMC Genomics **14**: 642.

Nei, M. and S. Kumar (2000). Molecular Evolution and Phylogenetics. New York, Oxford University Press.

Nilsson, R. H., M. Ryberg, E. Kristiansson, K. Abarenkov, K. H. Larsson and U. Koljalg (2006). "Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective." PLoS One **1**: e59.

Nucci, C., W. D. d. Silveira, S. d. S. Corre, G. Nakazato, S. Y. Bando, M. A. Ribeiro and A. F. P. d. Castro (2002). "Microbiological comparative study of isolates of *Edwardsiella tarda* isolated in different countries from fish and humans." Veterinary Microbiology **89**: 29-39.

Park, S. B., T. Aoki and T. S. Jung (2012). "Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish." Veterinary Research **43**: 67.

Phillips, A. C., S. R. Reichley, C. Ware and M. J. Griffin (2016). "*Edwardsiella ictaluri* infection in Pangasius catfish imported from West Bengal into the Southern Caribbean." Journal of Fish Diseases: doi:10.1111/jfd.12552.

Purcell, M. K., R. G. Getchell, C. A. McClure and K. A. Garver (2011). "Quantitative polymerase chain reaction (PCR) for detection of aquatic animal pathogens in a diagnostic laboratory setting." Journal of Aquatic Animal Health **23**(3): 148-161.

Reichley, S. R., G. C. Waldbieser, M. L. Lawrence and M. J. Griffin (2015). "Complete genome sequence of an *Edwardsiella piscicida*-like species recovered from Tilapia in the United States." Genome Announcements **3**(5): e01004-01015.

Reichley, S. R., G. C. Waldbieser, M. L. Lawrence and M. J. Griffin (2017). "Complete genome sequence of *Edwardsiella hoshinae* ATCC 35051." Genome Announcements **5**(6): e01605-01616.

Reichley, S. R., G. C. Waldbieser, H. C. Tekedar, M. L. Lawrence and M. J. Griffin (2016). "Complete genome sequence of *Edwardsiella piscicida* isolate S11-285 recovered from Channel Catfish (*Ictalurus punctatus*) in Mississippi, USA." Genome Announcements **5**(6): e01259-01216.

Reichley, S. R., G. C. Waldbieser, M. Ucko, A. Colorni, L. Dubytska, R. L. Thune, M. L. Lawrence and M. J. Griffin (2015). "Complete genome sequence of an *Edwardsiella piscicida*-like species isolated from diseased Grouper in Israel." Genome Announcements **3**(4): e00829-00815.



المنارة للاستشارات
www.manaraa.com

Reichley, S. R., C. Ware, T. E. Greenway, D. J. Wise and M. J. Griffin (2015). "Real-time polymerase chain reaction assays for the detection and quantification of *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like species in catfish tissues and pond water." Journal of Veterinary Diagnostic Investigation **27**(2): 130-139.

Sakai, T., T. Iida, K. Osatomi and K. Kanai (2007). "Detection of type 1 fimbrial genes in fish pathogenic and non-pathogenic *Edwardsiella tarda* strains by PCR." Fish Pathology **42**(2): 115-117.

Sakai, T., T. Kamaishi, M. Sano, K. Tensha, T. Arima, T. Iida, T. Nagai, T. Nakai and T. Iida (2008). "Outbreaks of *Edwardsiella ictaluri* infection in ayu *Plecoglossus altivelis* in Japanese rivers." Fish Pathology **43**(4): 152-157.

Sakai, T., K. Yuasa, M. Sano and T. Iida (2009). "Identification of *Edwardsiella ictaluri* and *E. tarda* by species-specific polymerase chain reaction targeted to the upstream region of the fimbrial gene." Journal of Aquatic Animal Health **21**(2): 124-132.

Sakazaki, R. (1965). "A proposed group of the family Enterobacteriaceae, the Asakusa Group." International Bulletin of Bacteriological Nomenclature and Taxonomy **15**(1): 45-47.

Sakazaki, R. (1967). "Studies on the Asakusa group of Enterobacteriaceae (*Edwardsiella tarda*)." Japanese Journal of Medical Science and Biology **20**: 205-212.

Sakazaki, R. and Y. Murata (1962). "The new group of the Enterobacteriaceae, the Asakusa group." Japanese Journal of Bacteriology **17**: 616.

Salzberg, S. L., A. L. Delcher, S. Kasif and O. White (1998). "Microbial gene identification using interpolated Markov models." Nucleic Acids Research **26**(2): 544-548.

Seibold, E., T. Maier, M. Kostrzewa, E. Zeman and W. Splettstoesser (2010). "Identification of *Francisella tularensis* by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry: fast, reliable, robust, and cost-effective differentiation on species and subspecies levels." Journal of Clinical Microbiology **48**(4): 1061-1069.

Shao, J., J. Yuan, Y. Shen, R. Hu and Z. Gu (2016). "First isolation and characterization of *Edwardsiella tarda* from diseased Asian swamp eel, *Monopterus albus* (Zuiew)." Aquaculture Research **47**(11): 3684-3688.



المنارة للاستشارات
www.manaraa.com

Shao, S., Q. Lai, Q. Liu, H. Wu, J. Xiao, Z. Shao, Q. Wang and Y. Zhang (2015). "Phylogenomics characterization of a highly virulent *Edwardsiella* strain ET080813(T) encoding two distinct T3SS and three T6SS gene clusters: Propose a novel species as *Edwardsiella anguillarum* sp. nov." Systematic and Applied Microbiology **38**(1): 36-47.

Sharma, V. K., Y. K. Kaura and P. Singh (1974). "Frogs as carriers of *Salmonella* and *Edwardsiella*." Antonie van Leeuwenhoek **40**: 171-175.

Singh, I. M., S. Singh, M. A. McMurphy, S. S. Crupper, F. Mills-Robertson and R. D. Applegate (2004). "Antibiotic susceptibility of *Edwardsiella hoshinae* isolated from northern bobwhite quail (*Colinus virginianus*)." Veterinary Record **155**(1): 29-29.

Singhal, N., M. Kumar, P. K. Kanaujia and J. S. Virdi (2015). "MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis." Frontiers in Microbiology **6**: 791.

Soto, E., M. Griffin, M. Arauz, A. Riofrio, A. Martinez and M. E. Cabrejos (2012). "*Edwardsiella ictaluri* as the causative agent of mortality in cultured Nile Tilapia." Journal of Aquatic Animal Health **24**(2): 81-90.

Suanyuk, N., M. Rogge, R. Thune, M. Watthanaphiromsakul, N. Champhat and W. Wiangkum (2014). "Mortality and pathology of hybrid catfish, *Clarias macrocephalus* (Gunther) x *Clarias gariepinus* (Burchell), associated with *Edwardsiella ictaluri* infection in southern Thailand." Journal of Fish Diseases **37**(4): 385-395.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar (2013). "MEGA6: Molecular Evolutionary Genetics Analysis version 6.0." Molecular Biology and Evolution **30**(12): 2725-2729.

Tan, R. J. S. and E. W. Lim (1977). "Occurance of *Edwardsiella tarda* in the household lizard, *Gecko gecko*." Japanese Journal of Medical Science and Biology **30**: 321-323.

Turenne, C. Y., L. Tschetter, J. Wolfe and A. Kabani (2001). "Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species." Journal of Clinical Microbiology **39**(10): 3637-3648.

Vandepitte, J., P. Lemmens and L. De Swert (1983). "Human *Edwardsiellosis* traced to ornamental fish." Journal of Clinical Microbiology **17**(1): 165-167.

Versalovic, J., T. Koeuth and J. R. Lupski (1991). "Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes." Nucleic Acids Research **19**(24): 6823-6831.



المنارة للاستشارات
www.manaraa.com

Versalovic, J., M. Schneider, F. J. DeBruijn and J. R. Lupski (1994). "Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction." Methods in Molecular and Cellular Biology **5**: 25-40.

Wang, X., M. Yan, Q. Wang, L. Ding and F. Li (2012). "Identification of *Edwardsiella tarda* isolated from duck and virulence genes detection." African Journal of Microbiology Research **6**(23): 4970-4975.

Wang, Y. M., Q. Y. Wang, J. F. Xiao, Q. Liu, H. Z. Wu and Y. X. Zhang (2011). "Genetic relationships of *Edwardsiella* strains isolated in China aquaculture revealed by rep-PCR genomic fingerprinting and investigation of *Edwardsiella* virulence genes." Journal of Applied Microbiology **111**(6): 1337-1348.

Welch, T. J., J. Evenhuis, D. G. White, P. F. McDermott, H. Harbottle, R. A. Miller, M. Griffin and D. Wise (2009). "IncA/C plasmid-mediated florfenicol resistance in the catfish pathogen *Edwardsiella ictaluri*." Antimicrobial Agents and Chemotherapy **53**(2): 845-846.

Woo, P. C. Y., S. K. P. Lau, J. L. L. Teng, H. Tse and K.-Y. Yuen (2008). "Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories." Clinical Microbiology and Infection **14**(10): 908-934.

Wragg, P., L. Randall and A. M. Whatmore (2014). "Comparison of Biolog GEN III MicroStation semi-automated bacterial identification system with matrix-assisted laser desorption ionization-time of flight mass spectrometry and 16S ribosomal RNA gene sequencing for the identification of bacteria of veterinary interest." Journal of Microbiological Methods **105**: 16-21.

Xu, T. and X.-H. Zhang (2014). "*Edwardsiella tarda*: an intriguing problem in aquaculture." Aquaculture **431**: 129-135.

Yamada, Y. and H. Wakabayashi (1999). "Identification of fish-pathogenic strains belonging to the genus *Edwardsiella* by sequence analysis of *sodB*." Fish Pathology **34**(3): 145-150.

Yang, M., Y. Lv, J. Xiao, H. Wu, H. Zheng, Q. Liu, Y. Zhang and Q. Wang (2012). "*Edwardsiella* comparative phylogenomics reveal the new intra/inter-species taxonomic relationships, virulence evolution and niche adaptation mechanisms." PLoS One **7**(5): e36987.

Ye, S., H. Li, G. Qiao and Z. Li (2009). "First case of *Edwardsiella ictaluri* infection in China farmed yellow catfish *Pelteobagrus fulvidraco*." Aquaculture **292**(1-2): 6-10.



المنارة للاستشارات
www.manaraa.com

Yuasa, K., E. B. Kholidin, N. Panigoro and K. Hatai (2003). "First isolation of *Edwardsiella ictaluri* from cultured striped catfish *Pangasius hypophthalmus* in Indonesia." Fish Pathology **38**(4): 181-183.

Zhu, W., A. Lomsadze and M. Borodovsky (2010). "Ab initio gene identification in metagenomic sequences." Nucleic Acids Research **38**(12): e132.



المنارة للاستشارات

www.manaraa.com

CHAPTER V

EVALUATION OF THREE DISEASE CHALLENGE MODELS FOR *EDWARDSIELLA PISCICIDA*, *EDWARDSIELLA TARDA* AND *EDWARDSIELLA ANGUILLARUM* IN CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

## 5.1 Introduction

The genus *Edwardsiella* was first recognized in the 1960s with the description of *E. tarda* (Ewing, McWhorter et al. 1965, Meyer and Bullock 1973). Subsequently, two additional *Edwardsiella* taxa were described in the early 1980s, namely *E. hoshinae* and *E. ictaluri* (Grimont, Grimont et al. 1980, Hawke, McWhorter et al. 1981). Limited information is available regarding *E. hoshinae*. While the species has been isolated from birds, reptiles, water and human feces, its role as a human pathogen has not been established, and at present it is not considered a zoonotic agent (Grimont, Grimont et al. 1980, Farmer and McWhorter 1984, Singh, Singh et al. 2004, Singh, Singh et al. 2013).

In contrast, much is known regarding *E. tarda* and *E. ictaluri*, both of which have been implicated in epizootics leading to significant losses in cultured and wild fish. *Edwardsiella ictaluri* is widely considered one of the most ruinous bacterial disease agents in catfish aquaculture worldwide (Hawke, McWhorter et al. 1981, Crumlish, Dung et al. 2002, Wise, Camus et al. 2004, Ye, Li et al. 2009, Liu, Li et al. 2010). Furthermore,



www.manaraa.com

*E. ictaluri* has also been linked to fish kills in cultured tilapia and laboratory zebrafish colonies (Soto, Griffin et al. 2012, Hawke, Kent et al. 2013).

Similarly, *E. tarda* has been associated with considerable economic losses in more than 20 species of commercially-important fish worldwide (Xu and Zhang 2014). Primarily regarded as a pathogen of marine and freshwater fishes, *E. tarda* has historically been considered the most diverse and widespread of the *Edwardsiella* species (Mohanty and Sahoo 2007, Wang, Yang et al. 2009). Recent investigations into its heterogeneity revealed this previous classification actually encompassed three genetically distinct, yet phenotypically ambiguous taxa: *E. tarda*, *E. piscicida* and *E. anguillarum* (syn. *E. piscicida*-like sp.) (Abayneh, Colquhoun et al. 2013, Oren and Garrity 2013, Griffin, Ware et al. 2014, Oren and Garrity 2015, Shao, Lai et al. 2015). *Edwardsiella piscicida* has since been isolated from a variety of diseased wild and cultured fish (Oguro, Tamura et al. 2014, Camus, Dill et al. 2016, Fogelson, Petty et al. 2016, Shafiei, Viljamaa-Dirks et al. 2016, Bujan, Toranzo et al. 2017).

Farm-raised catfish is the largest aquaculture industry in the United States and is a vital component of the economies of several southern states (Hargreaves 2002, Stankus 2010, USDA 2014). Sales of food-size fish totaled $345 million in 2014, with Alabama, Arkansas, Mississippi and Texas accounting for 96% of the industry's total production. Mississippi is the top producing state with nearly 45,000 water surface acres dedicated to catfish production and sales of $190 million in 2014 (USDA 2016). In the catfish farming region of the southeastern United States, *E. tarda* has been associated with emphysematous putrefactive disease of catfish, which begins as small, cutaneous lesions that can progress to deep, malodorous, putrefactive abscesses within the musculature



www.manaraa.com

(Meyer and Bullock 1973, Hawke and Khoo 2004). However, recent studies demonstrated that many of these recent cases should be classified as *E. piscicida*, which is more virulent to channel catfish than *E. tarda* or *E. anguillarum* (Reichley, Ware et al. 2015). Furthermore, molecular surveys demonstrated *E. piscicida* is presently more common in catfish aquaculture than *E. tarda*, and it has been increasingly recovered from diseased farm-raised catfish in the southeastern U.S. over the past ten years (Griffin, Ware et al. 2014, Reichley, Ware et al. 2015).

The research described herein evaluated three different disease challenge models with isolates originally classified as *E. tarda* but reclassified in accordance with current *Edwardsiella* systematics. Moreover, the pathogenicity, histopathological lesions and posterior kidney clearance rates were determined in channel catfish exposed to *E. anguillarum, E. piscicida* and *E. tarda* exposed by intraperitoneal injection.

## 5.2 Materials and Methods

### 5.2.1 Fish

Fish used in this study were reared for disease research at the Thad Cochran National Warmwater Aquaculture Center. Prior to challenge, fish were maintained in 2,000 L tanks with 1,000 L of well water (~26°C) under flow-through conditions (4 L/min) with supplemental aeration. For passage and experimental infectivity studies, fish were held in 80 L aquaria containing 22 L of well water and held under flow-through conditions (1 L/min) with constant aeration. Prior to challenge, fish were acclimated to a water temperature of ~30°C over 72 hr and feed was withheld. Following challenge, fish were maintained in well water (~30°C) under flow-through conditions with constant aeration and monitored twice daily for morbidity and mortality.



المنارة للاستشارات
www.manaraa.com

### 5.2.2 Bacterial Cultures

Archived cryostocks of *Edwardsiella* spp. isolates (Table 5.1) were revived by isolation streaking on Mueller-Hinton II Agar (BBL; Becton Dickinson and Company) plates supplemented with 5% defibrinated sheep blood (Hemastat Laboratories, Dixon CA) and incubated for 24 hr at 37°C. Individual colonies of each isolate were expanded (37°C; 200 rpm; Excella E24, New Brunswick Scientific) in 9 ml of porcine brain-heart infusion broth (BHIb) (Bacto; Becton Dickinson and Company) and passed through channel catfish fingerlings (n=3/isolate) on two successive 48 hr passages. To accomplish this, fish were IP injected with bacteria (*E. anguillarum*: 5 x $10^6$ CFU; *E. piscicida*: 1.5 x $10^6$ CFU; *E. tarda*: 6 x $10^6$ CFU) and euthanized after 48 hr by an overdose of buffered MS-222. Isolates were recovered from the posterior kidney on Mueller-Hinton II Agar (BBL; Becton Dickinson and Company) plates supplemented with 5% defibrinated sheep blood (Hemastat Laboratories, Dixon CA), expanded as above and the procedure was repeated. After the second 48 hr passage, individual colonies were expanded as above and stored cryogenically (-80°C; 15% v/v glycerol) until challenge.

Table 5.1 *Edwardsiella* isolates used in the current study.

| Isolate* | Species | Host | Location |
|---|---|---|---|
| LADL05-105 | *E. anguillarum* | *Oreochromis* sp. | Louisiana, USA |
| S11-285 | *E. piscicida* | *Ictalurus punctatus* | Mississippi, USA |
| FL95-01 | *E. tarda* | *Ictalurus punctatus* | Florida, USA |

*Isolates have previously been described by: Griffin, Quiniou et al. (2013); Griffin, Ware et al. (2014); Reichley, Ware et al. (2015).



المنارة للاستشارات
www.manaraa.com

For each challenge, cryostocks of passed isolates were revived, and individual isolates were expanded overnight (37°C; 200 rpm) in 9 ml of BHIb. Following overnight incubation, 300 µl of each culture was added to 250 ml of sterile BHIb and expanded overnight (37°C; 200 rpm). Cultures were then diluted to achieve approximate targeted doses for each isolate. Enumeration of bacteria for all studies was accomplished using serial dilution and colony counts on triplicate drop plates using 20 µl (Herigstad, Hamilton et al. 2001).

### 5.2.3 Trial 1: Intraperitoneal injection

For each *Edwardsiella* sp., fingerling channel catfish (mean 17.3g, ±6.4g) were stocked into 80 L tanks containing 22 L of well water (30 total tanks; 3 isolates; 9 dilutions/isolate; 3 control tanks; 10 fish/tank). After acclimation, fish were anesthetized with tricaine methanesulfonate (MS-222) and intraperitoneally (IP) injected with 3-fold serial dilutions of each bacteria ranging from $10^5$ to $10^8$ (*E. piscicida* and *E. tarda*) or $10^4$ to $10^8$ (*E. anguillarum*) CFU per fish. Three tanks of ten fish were handled similarly but injected with sterile BHIb to serve as non-infected controls. Fish were monitored twice daily for 7 days, and the number of dead fish was recorded. The median lethal dose ($LD_{50}$) was calculated using methods described by Reed (1938).

### 5.2.4 Trial 2: Immersion exposure

For each *Edwardsiella* sp., fingerling channel catfish (mean 36.1g, ±13.1g) were stocked into 80 L tanks containing 22 L of well water (27 total tanks; 3 isolates; 8 dilutions/isolate; 3 control tanks; 10 fish/tank). After acclimation, water flow into each tank was stopped and the water level was lowered to 10 L. Serial 3-fold dilutions of each



المنارة للاستشارات
www.manaraa.com

bacteria were added to appropriate tanks with exposure doses ranging from $10^4$ to $10^8$ CFU/ml. Aeration was maintained throughout the exposure. After 4 hr, water flow was resumed and water level was returned to 22 L. Fish in the remaining three tanks were handled similarly, but sterile BHIb was added to the tanks to serve as non-infected controls. Fish were monitored twice daily for 7 days and the number of dead fish was recorded.

### 5.2.5 Trial 3: Immersion with mucus removal

For each *Edwardsiella* sp., fingerling channel catfish (mean 17.4g, ±2.9g) were stocked into 80 L tanks containing 22 L of well water (25 total tanks; 3 isolates; 8 dilutions/isolates; 1 control tank; 10 fish/tank). After acclimation, water flow into each tank was stopped and the water level was lowered to 10 L. Fish were removed from the tank, anesthetized with tricaine methanesulfonate (MS-222) and the mucus coat was manually removed from both lateral aspects of each fish with a dry paper towel. Fish were returned to the tank, recovered from anesthesia, and serial 3-fold dilutions of each bacteria were added to appropriate tanks with exposure doses ranging from $10^4$ to $10^8$ CFU/ml (*E. piscicida* and *E. tarda*) or $10^4$ to $10^7$ (*E. anguillarum*). Aeration was maintained throughout the exposure. After 4 hr, water flow was resumed and water level was returned to 22 L. Fish in the remaining tank were handled similarly, but sterile BHIb was added to the tank to serve as non-infected controls. Fish were monitored twice daily for 7 days and the number of dead fish was recorded.



المنارة للاستشارات
www.manaraa.com

### 5.2.6 Trial 4: Pathology and posterior kidney clearance

Two different *in vivo* studies were conducted to evaluate the histopathological changes and posterior kidney clearance rates in channel catfish exposed to *E. anguillarum, E. piscicida* and *E. tarda* by intraperitoneal injection. In the first study, the histopathological changes and posterior kidney clearance rates were evaluated at doses approximating the calculated $LD_{50}$ from Trial 1. For each bacterial dose (*E. piscicida*, 3.4 x $10^5$ CFU; *E. tarda*, 3.0 x $10^7$ CFU; *E. anguillarum*, 4.3 x $10^8$ CFU), 30 fingerling channel catfish (mean 14.5g ±8g) were stocked into two 80 L tanks containing 22 L of well water (8 total tanks; 3 isolates; 2 tanks/dose; 2 control tanks; 30 fish/tank); one tank was used for posterior kidney clearance sampling, and one tank was used for histopathology sampling. Two tanks of control fish were handled similarly but injected with sterile BHIb to serve as non-infected controls. At days 1, 3, 5, 7, 9 and 11 post-injection, three fish were arbitrarily sampled from the tanks designated for histopathology sampling. Fish were euthanized by an overdose of MS-222, a midline incision was made into the coelomic cavity, the right operculum was excised, the caudle peduncle was removed and the fish were preserved in 10% neutral buffered formalin until routine processing at a later date.

On the same day, three fish were arbitrarily sampled from the tanks designated for posterior kidney clearance sampling, euthanized by an overdose of MS-222 and kidney biopsies were obtained aseptically using necropsy instruments treated with DNAse/RNAse (DNA Away™ Surface Decontaminant, Thermo Fisher Scientific) between fish. Kidney biopsies were streaked on blood agar plates and incubated for 24 hr at 37°C to determine the presence of viable bacteria. Tissue biopsies were individually



المنارة للاستشارات
www.manaraa.com

placed in microcentrifuge tubes and stored at -80°C until processed for qPCR analysis. Fish were monitored twice daily for 11 days and the number of dead fish was recorded.

As a result of the initial pathology and clearance experiment, a second study was performed. In the second study, qPCR estimations of bacterial tissue concentrations and kidney touch cultures were performed on the same fish used for histological analysis. To better evaluate differences in persistence between different *Edwardsiella* spp., fish were injected with comparable doses of each bacteria. As above, 30 fingerling channel catfish (27.5 g ±2.8g) were stocked into an 80 L tank containing 22 L of well water and held under flow through conditions for each *Edwardsiella* sp. After acclimation, fish were anesthetized with MS-222 and IP injected with approximately the same dose for each *Edwardsiella* sp. (*E. piscicida*, 1.9 x $10^6$ CFU; *E. tarda*, 1.8 x $10^6$ CFU; *E. anguillarum*, 2.5 x $10^6$ CFU). One tank of 30 fish was handled similarly but injected with sterile BHIb. At 1, 3, 7, 14 and 21 days post-injection (dpi), three fish were arbitrarily sampled from each tank, euthanized by an overdose of MS-222 and a biopsy (~25mg) was aseptically obtained from the posterior kidney. Fish were then preserved in 10% neutral buffered formalin until routine processing at a later date. Posterior kidney biopsies were streaked on blood agar plates and placed in microcentrifuge tubes for qPCR analysis. Fish were monitored twice daily for 21 days for morbidity and mortality and the number of dead fish was recorded.

### 5.2.7 Molecular Analysis

Genomic DNA (gDNA) was isolated from kidney biopsies, and qPCR analysis was performed using previously published primers, probes and amplification protocols (Reichley, Ware et al. 2015). Briefly, gDNA from kidney biopsies was isolated using a



المنارة للاستشارات
www.manaraa.com

commercial kit (DNeasy blood and tissue kit, Qiagen Inc., Valencia, CA) following the manufacturer's suggested protocol for animal tissues. Isolated gDNA was resuspended in 200 µl of DNA hydration solution (DHS; PureGene DNA hydration solution, Qiagen Inc., Valencia, CA), and 5 µl of gDNA from each aliquot was used as template in qPCR analysis. The 15-µl PCR reactions contained 8 µl of PCR master mix (TaqMan Environmental Mastermix 2.0, Applied Biosystems, Foster City, CA), 10 pM of each primer, 1 pM of probe, DNA template and nuclease-free water to volume. Amplifications were performed on a qPCR system (CFX96, Bio-Rad Laboratories, Hercules, CA) programmed for 1 cycle of 95°C for 15 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data collection was conducted following the 60°C annealing/extension step at the end of each cycle. Samples, as well as no-template negative controls, were run in triplicate and standards were run in duplicate. Primers and probes used are listed in Table 5.2.

Table 5.2 Real-time quantitative PCR primers and probes used for detection and quantification of *Edwardsiella* spp. in kidney biopsies.

| Primer/Probe | Sequence (5'–3') |
|---|---|
| *E. tarda* | |
| ET3518F | CAGTGATAAAAAGGGGTGGA |
| ET3632R | CTACACAGCAACGACAACG |
| ET3559P | AGACAACAGAGGACGGATGTGGC |
| *E. piscicida* | |
| EP14529F | CTTTGATCATGGTTGCGGAA |
| EP14659R | CGGCGTTTTCTTTTCTCG |
| EP14615P | CCGACTCCGCGCAGATAACG |
| *E. anguillarum* | |
| EA1583F | GATCGGGTACGCTGTCAT |
| EA1708R | AATTGCTCTATACGCACGC |
| EA1611P | CCCGTGGCTAAATAGGACGCG |

Primers and probes described by Reichley, Ware, et al. (2015). Each oligonucleotide probe was labeled with the fluorescent reporter dye, 6-carboxyfluorescein, on the 5'-end, and the quencher dye, black hole quencher-1, on the 3'-end. F = forward primer; R = reverse primer; P = probe.



المنارة للاستشارات
www.manaraa.com

## 5.3 Results

### 5.3.1 Trial 1: Intraperitoneal injection

Cumulative percent mortality and calculated median lethal doses for this trial are listed in Table 5.3. High cumulative mortality was observed in most of the fish groups injected with *E. piscicida*, with less than 50% mortality only observed in doses of 3.3 x $10^5$ CFU and below. Conversely, less than 50% mortality was observed in *E. tarda* doses less than 3.6 x $10^7$ CFU and in all doses of *E. anguillarum*. In BHIb injected fish, 7% cumulative mortality was observed. The $LD_{50}$ for IP injection of *E. piscicida* and *E. tarda* in fingerling channel catfish was 2.1 x $10^4$ CFU/g and 3.5 x $10^6$ CFU/g, respectively. Median lethal dose for *E. anguillarum* could not be established because the maximum cumulative mortality observed was only 30%.



المنارة للاستشارات
www.manaraa.com

Table 5.3 Cumulative percent mortality and $LD_{50}$ from intraperitoneal injection (Trial 1).

| | **Dose (CFU/fish)** | **Cumulative Percent Mortality** | **Median Lethal Dose (CFU/g)** |
|---|---|---|---|
| | 7.2E+08 | 100% | |
| | 2.4E+08 | 100% | |
| | 8.0E+07 | 100% | |
| | 2.7E+07 | 100% | |
| *E. piscicida* | 8.9E+06 | 90% | 2.1E+04 |
| | 3.0E+06 | 90% | |
| | 9.9E+05 | 100% | |
| | 3.3E+05 | 40% | |
| | 1.1E+05 | 20% | |
| | 9.8E+08 | 90% | |
| | 3.3E+08 | 80% | |
| | 1.1E+08 | 90% | |
| | 3.6E+07 | 40% | |
| *E. tarda* | 1.2E+07 | 0% | 3.5E+06 |
| | 4.0E+06 | 0% | |
| | 1.3E+06 | 0% | |
| | 4.5E+05 | 0% | |
| | 1.5E+05 | 0% | |
| | 3.8E+08 | 0% | |
| | 1.3E+08 | 0% | |
| | 4.2E+07 | 10% | |
| | 1.4E+07 | 0% | |
| *E. anguillarum* | 4.7E+06 | 0% | NC |
| | 1.6E+06 | 0% | |
| | 5.2E+05 | 30% | |
| | 1.7E+05 | 0% | |
| | 5.8E+04 | 10% | |
| BHI Control | N/A | 7% | N/A |

For each dose, channel catfish fingerlings were stocked (n=10 fish/tank) into discrete tanks. Fish were monitored for morbidity and mortality twice daily for 7 days and the number of dead fish was recorded. N/A: not applicable, sterile BHI. NC: not calculated, 50% mortality was not observed.



المنارة للاستشارات
www.manaraa.com

### 5.3.2 Trial 2: Immersion exposure

Eight out of ten (80%) of fish exposed to 2.8 x $10^8$ CFU/ml of *E. tarda* died the day of exposure. No mortality was observed in fish exposed to lower doses of *E. tarda* or to *E. piscicida*, *E. anguillarum* or BHI controls. Cumulative percent mortality for this trial is listed in Table 5.4.

Table 5.4 Cumulative percent mortality from immersion exposure (Trial 2).

| | Dose (CFU/ml) | Cumulative Percent Mortality |
|---|---|---|
| *E. piscicida* | 3.48E+08 | 0% |
| | 1.16E+08 | 0% |
| | 3.92E+07 | 0% |
| | 1.31E+07 | 0% |
| | 4.35E+06 | 0% |
| | 1.45E+06 | 0% |
| | 4.83E+05 | 0% |
| | 1.61E+05 | 0% |
| | 5.37E+04 | 0% |
| *E. tarda* | 2.76E+08 | 80% |
| | 9.20E+07 | 0% |
| | 3.11E+07 | 0% |
| | 1.04E+07 | 0% |
| | 3.45E+06 | 0% |
| | 1.15E+06 | 0% |
| | 3.83E+05 | 0% |
| | 1.28E+05 | 0% |
| | 4.26E+04 | 0% |
| *E. anguillarum* | 1.36E+08 | 0% |
| | 4.53E+07 | 0% |
| | 1.53E+07 | 0% |
| | 5.10E+06 | 0% |
| | 1.70E+06 | 0% |
| | 5.67E+05 | 0% |
| | 1.89E+05 | 0% |
| | 6.30E+04 | 0% |
| | 2.10E+04 | 0% |
| BHI Control | N/A | 0% |

For each dose, channel catfish fingerlings were stocked (n=10 fish/tank) into discrete tanks. Fish were monitored for morbidity and mortality twice daily for 7 days and the number of dead fish was recorded.



www.manaraa.com

### 5.3.3 Trial 3: Immersion with mucus removal

Mortality was only observed in two *E. piscicida* treatment groups, 5.2 x $10^7$ CFU/ml and 1.7 x $10^7$ CFU/ml. This groups had cumulative mortality of 70% and 30%, respectively. No mortality was observed in fish exposed to lower doses or fish exposed to 1.55 x $10^8$ CFU/ml. One out of ten (10%) fish died from the groups exposed to 2.6 x $10^7$ CFU/ml, 8.6 x $10^6$ CFU/ml and 3.2 x $10^5$ CFU/ml of *E. anguillarum*. No mortality was observed in fish exposed to *E. tarda* or the BHIb. Cumulative percent mortality for this trial is listed in Table 5.5.

Table 5.5 Cumulative percent mortality from immersion exposure with mucus removal (Trial 3).

| | Dose (CFU/ml) | Cumulative Percent Mortality |
|---|---|---|
| *E. piscicida* | 1.55E+08 | 0% |
| | 5.17E+07 | 70% |
| | 1.72E+07 | 30% |
| | 5.75E+06 | 0% |
| | 1.92E+06 | 0% |
| | 6.39E+05 | 0% |
| | 2.13E+05 | 0% |
| | 7.10E+04 | 0% |
| *E. tarda* | 1.75E+08 | 0% |
| | 5.83E+07 | 0% |
| | 1.94E+07 | 0% |
| | 6.47E+06 | 0% |
| | 2.16E+06 | 0% |
| | 7.19E+05 | 0% |
| | 2.40E+05 | 0% |
| | 7.99E+04 | 0% |
| *E. anguillarum* | 7.78E+07 | 0% |
| | 2.59E+07 | 10% |
| | 8.64E+06 | 10% |
| | 2.88E+06 | 0% |
| | 9.60E+05 | 0% |
| | 3.20E+05 | 0% |
| | 1.07E+05 | 0% |
| | 3.56E+04 | 0% |
| BHI Control | N/A | 0% |

For each dose, channel catfish fingerlings were stocked (n=10 fish/tank) into discrete tanks. Fish were monitored for morbidity and mortality twice daily for 7 days and the number of dead fish was recorded.



المنارة للاستشارات
www.manaraa.com

### 5.3.4 Trial 4: Pathology and posterior kidney clearance

Histologically, a putative dose response was noted in the histologic lesions observed during the first study, in which fish were administered doses equating to the calculated median lethal dose of each *Edwardsiella* sp. Interstitial nephritis of the anterior and posterior kidney, hepatitis, splenitis and enteritis were observed in all treatment groups with cellular damage. In addition, submucosal edema of the gastrointestinal tract, congestion of the spleen and nephrosis were observed in a majority of the fish injected with *E. tarda*. In fish injected with *E. anguillarum*, necrotizing pancreatitis was observed beginning the day after injection and continued variably throughout the remainder of the study. The LD50 doses for *E. tarda* and *E. anguillarum* were several orders of magnitude higher than that for *E. piscicida*.

In the second study with fish receiving $10^6$ CFU of each *Edwardsiella* sp., disease progression varied between individual fish and between different bacterial groups. In general, changes noted across all treatment groups included interstitial nephritis with mononuclear infiltrates in both the anterior and posterior kidney, splenitis, hepatitis and enteritis. Micro- and macrovesicular hepatocellular vacuolation was consistently observed across all fish groups at all sampling time points. In fish injected with *E. piscicida,* splenitis and enteritis was typically more severe, with moderate to severe lymphoid depletion and a more acute onset relative to other treatment groups. No significant microscopic lesions were observed in the brain tissue for any fish examined.

For the first clearance study wherein fish were exposed to doses equating to the approximate median lethal dose for each *Edwardsiella* sp., *E. piscicida* was only detected by qPCR at 1 and 3 dpi, whereas *E. tarda* and *E. anguillarum* were detected as late as 11



المنارة للاستشارات
www.manaraa.com

dpi (Table 5.6). Growth from kidney biopsies was observed from the majority of fish up to 7 dpi. There was variable growth from *E. tarda* and *E. anguillarum* injected fish 9 dpi. No growth was observed from any treatment group 11 dpi (Table 5.7). Comparably, in fish challenged with equivalent exposure doses for each bacteria, bacterial equivalents per mg of kidney tissue were most numerous one day after injection and decreased as the challenge progressed. *E. piscicida* was detected by qPCR out to 21 dpi; whereas *E. tarda* and *E. anguillarum* were detected by qPCR at 14 dpi but not 21 dpi (Table 5.6). Bacterial growth on blood agar plates was observed from all samples 1 dpi and a majority of samples 3 dpi. However, no bacterial growth was observed from biopsies from 7 dpi and beyond (Table 5.7).



المنارة للاستشارات
www.manaraa.com

Table 5.6 Mean (range) bacterial equivalents per mg of kidney tissue from Trial 4.

| | | | **Study 1** | | | |
|---|---|---|---|---|---|---|
| **Dose (CFU)** | **Day 1** | **Day 3** | **Day 5** | **Day 7** | **Day 9** | **Day 11** |
| ***E. piscicida*** | | | | | | |
| 3.4E+05 | 828.4 (0.2-3177.6) | 16.3 (0-62.7) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) |
| ***E. tarda*** | | | | | | |
| 3.0E+07 | 1.2E+04 (7.4E+03-1.9E+04) | 3214.6 (124.0-8289.6) | 281.0 (27.4-899.5) | 719.4 (1.5-2062.4) | 4.9 (0-15.6) | 0.8 (0-2.7) |
| ***E. anguillarum*** | | | | | | |
| 4.3E+08 | 1.1E+04 (5.6E+03-2.1E+04) | 1.2E+04 123.3-2.7E+04) | 1.4E+05 (1.4E+04-2.4E+05) | 1.1E+04 (500.8-3.5E+04) | 641.8 (0-2172.8) | 30.5 (0-83.5) |
| **BHI** | | | | | | |
| | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) |

| | | | **Study 2** | | |
|---|---|---|---|---|---|
| | **Day 1** | **Day 3** | **Day 7** | **Day 14** | **Day 21** |
| ***E. piscicida*** | | | | | |
| 1.9E+06 | 7.2E+03 (53.8-1.5E+04) | 110.7 (4.5-229.6) | 71.0 (1.8-144.5) | 17.3 (0-62.4) | 8.7 (0-36.6) |
| ***E. tarda*** | | | | | |
| 1.8+06 | 727.4 (412.0-1035.7) | 55.0 (0-207.0) | 13.0 (0-39.2) | 14.7 (0-64.3) | 0 (0-0) |
| ***E. anguillarum*** | | | | | |
| 2.5E+06 | 6.6E+04 (1.6E+03-1.8E+05) | 441.1 (0-958.6) | 391.9 (70.1-1044) | 10.5 (0-45.3) | 0 (0-0) |
| **BHI** | | | | | |
| | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) |



المنارة للاستشارات
www.manaraa.com

Table 5.7 Kidney biopsy growth from Trial 4.

| Study 1 | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Dose (CFU)** | **Fish** | **Day 1** | **Day 3** | **Day 5** | **Day 7** | **Day 9** | **Day 11** |
| ***E. piscicida*** | | | | | | | |
| | Fish 1 | + | + | + | + | - | - |
| 3.4E+05 | Fish 2 | + | + | + | + | - | - |
| | Fish 3 | + | + | - | + | - | - |
| ***E. tarda*** | | | | | | | |
| | Fish 1 | + | + | + | + | + | - |
| 3.0E+07 | Fish 2 | + | + | + | + | - | - |
| | Fish 3 | + | + | + | - | - | - |
| ***E. anguillarum*** | | | | | | | |
| | Fish 1 | + | + | + | + | + | - |
| 4.3E+08 | Fish 2 | + | + | + | + | + | - |
| | Fish 3 | + | + | + | + | - | - |
| **BHI** | | | | | | | |
| | Fish 1 | - | + | - | + | - | - |
| | Fish 2 | - | - | - | - | - | - |
| | Fish 3 | - | - | - | - | - | - |

| Study 2 | | | | | | |
|---|---|---|---|---|---|---|
| **Dose (CFU)** | **Fish** | **Day 1** | **Day 3** | **Day 7** | **Day 14** | **Day 21** |
| ***E. piscicida*** | | | | | | |
| | Fish 1 | + | + | - | - | - |
| 1.9E+06 | Fish 2 | + | + | - | - | - |
| | Fish 3 | + | - | - | - | - |
| ***E. tarda*** | | | | | | |
| | Fish 1 | + | - | - | - | - |
| 1.8+06 | Fish 2 | + | + | - | - | - |
| | Fish 3 | + | - | - | - | - |
| ***E. anguillarum*** | | | | | | |
| | Fish 1 | + | + | - | - | - |
| 2.5E+06 | Fish 2 | + | + | - | - | - |
| | Fish 3 | + | + | - | - | - |
| **BHI** | | | | | | |
| | Fish 1 | - | - | - | - | - |
| | Fish 2 | - | - | - | - | - |
| | Fish 3 | - | - | - | - | - |

+ : bacterial growth observed on blood agar plate after 24 hr incubation at 37°C
- : no bacterial growth observed on blood agar plate after 24 hr incubation at 37°C



المنارة للاستشارات
www.manaraa.com

## 5.4 Discussion

Recent studies have revealed bacteria previously classified as *E. tarda* actually encompass three genetically distinct, yet phenotypically ambiguous taxa: *E. tarda*, *E. piscicida* and *E. anguillarum* (Abayneh, Colquhoun et al. 2013, Shao, Lai et al. 2015). Subsequent analysis demonstrated *E. piscicida* is present in the southeast United States and is more commonly associated with disease outbreaks in U.S. farm-raised catfish than *E. tarda* or *E. anguillarum* (Griffin, Ware et al. 2014). Furthermore, *E. piscicida* has relatively increased pathogenicity in channel catfish (Reichley, Ware et al. 2015). The median lethal doses calculated in the current study further support previously findings, demonstrating *E. piscicida* may pose a greater risk to channel catfish than *E. tarda*. *E. anguillarum* has consistently failed to induce mortality over 30% in channel catfish fingerlings when injected with doses as high as $10^7$ CFU/fish (Reichley, Ware et al. 2015) and $10^8$ CFU/fish (the current study).

The immersion challenge performed herein failed to induce notable mortality associated with *E. piscicida*, *E. tarda* and *E. anguillarum* infection. These results differ from previously published reports of immersion exposure to *E. tarda* inducing 70% - 80% mortality in channel catfish 3 - 5 dpi (Wiedenmayer, Evans et al. 2006). Other researchers abraded channel catfish prior to immersion exposure to evaluate histopathological findings; however, the mortality associated with this challenge model was not reported (Darwish, Plumb et al. 2000). Given this study took place prior to the recognition of *E. piscicida* as a distinct taxa, it is possible the strain used was *E. piscicida* rather than *E. tarda* as it is currently defined. Regretfully, the absence of accompanying



المنارة للاستشارات
www.manaraa.com

archived molecular data for the isolates used in previous studies precludes comparisons to current *Edwardsiella* systematics.

Interestingly, on the day of exposure, 80% mortality was observed in the group of fish exposed to $10^8$ CFU/ml of *E. tarda*. However this acute mortality, occurring within hours of infection, is unlikely due to infectious septicemia but rather attributed to other factors, such as poor water quality or possibly the presence of an *E. tarda* endotoxin or other extracellular products. Previous work has shown extracellular products are associated with *E. tarda* pathogenicity in fish; however, these studies took place prior to the recognition of *E. piscicida* and *E. anguillarum* and the identity of the isolates in relation to current taxonomic classification is not known (Suprapto, Nakai et al. 1995, Das, Pattnaik et al. 2001).

In the present study, mucus removal from fish prior to immersion was performed on both lateral aspects of the fish manually using a paper towel. This method induced variable mortality in fish exposed to *E. piscicida* and *E. anguillarum*. *E. tarda* mortality was acute with most mortality occurring in the first 36 - 48 hrs. Comparatively, the IP injection challenge model produced results consistent with previous findings (Reichley, Ware et al. 2015) and provided sufficient mortality for *E. piscicida* and *E. tarda* $LD_{50}$ calculation. The negligible mortality observed after IP injection of *E. anguillarum* was similar to previous reports, suggesting this species poses minimal risk to channel catfish under these experimental conditions. Similar to the other studies in the current work, *E. tarda* mortality was acute with most mortality occurring in the first 36 - 48 hrs.

Posterior kidney clearance rates observed here demonstrate an acute presence after injection with diminishing numbers of bacteria for *E. piscicida*, *E. tarda* and *E.*



المنارة للاستشارات
www.manaraa.com

*anguillarum* over two weeks. After two weeks, negligible amounts of *E. piscicida* were present, suggesting infection by these *Edwardsiella* spp. may not readily lead to chronically infected or latent carriers. Additionally, cultures from kidney biopsies resulted in variable growth after 7 dpi with no growth 11 dpi and on, consistent with previous reports of *E. tarda* in channel catfish (Darwish, Plumb et al. 2000).

Histopathological lesions noted in this study are consistent with those previously reported from experimentally infected channel catfish and resembled changes typically seen in septicemias resulting from other similar Gram-negative bacteria (Darwish, Plumb et al. 2000, Reimschuessel 2008). Numerous reports describe the pathology of *E. tarda* in fish; however, these were performed prior to the recognition that bacteria classified at the time as *E. tarda* actually represented three genetically distinct taxa: *E. tarda*, *E. piscicida* and *E. anguillarum* (Miyazaki and Egusa 1976, Kubota, Kaige et al. 1981, Herman and Bullock 1986, Darwish, Plumb et al. 2000, Uhland, Hélie et al. 2000, Padros, Zarza et al. 2006, Park, Aoki et al. 2012).

Histopathological lesions in the present study were primarily located in the anterior and posterior kidney, liver and spleen. Interstitial nephritis as well as necrotizing hepatitis and splenitis were all consistent with lesions in catfish reported by Darwish, Plumb et al. (2000). Splenitis and nephritis have also been described from *E. tarda* infections in turbot, largemouth bass, brook trout and rainbow trout (Francis-Floyd, Reed et al. 1993, Uhland, Hélie et al. 2000, Padros, Zarza et al. 2006, Řehulka, Marejková et al. 2012). In previous reports, distinct granulomas were variably described from the kidney of *E. tarda*-infected fish (Francis-Floyd, Reed et al. 1993, Padros, Zarza et al. 2006); these lesions were not observed in the present study which may be due to host



المنارة للاستشارات
www.manaraa.com

differences, duration of infection or other factors. Splenitis and nephritis are commonly associated with infection by Gram-negative bacteria and have been described from fish infected with *Edwardsiella ictaluri* and *Aeromonas hydrophila* (Miyazaki and Plumb 1985, Crumlish, Thanh et al. 2010).

Dorsocranial ulceration and meningoencephalitis reported from infection with *Edwardsiella ictaluri* were not noted in the present study; however, these lesions typically do not appear until weeks after intial exposure and are more indicative of a chronic infection (Newton, Wolfe et al. 1989). It should be noted the gross lesions associated with this chronic form of enteric septicemia of catfish (ESC) have been reported in fish infected with *E. piscicida* (Plumb and Hanson 2011, Khoo 2013). In the present study, lesions from fish injected with higher doses resulted in more severe and prevalent lesions, demonstrating a dose response. The submucosal edema of the gastrointestinal tract observed in fish injected with $10^7$ CFU of *E. tarda* and necrotizing pancreatitis observed in fish injected with $10^8$ CFU of *E. anguillarum* are likely attributed to the high bacterial doses, which are unlikely to be encountered naturally.

This is the first study investigating the pathology of molecularly confirmed *E. tarda*, *E. piscicida* and *E. anguillarum* isolates in channel catfish, and it lays the foundation for future work investigating the pathogenicity of these bacteria in catfish. Moreover, this work identifies minimal pathogenicity of these bacteria by immersion exposure, suggesting infection caused by these species may be more opportunistic in nature. Lastly, the negligible mortality associated with *E. tarda* and *E. anguillarum* challenges supports previous assertions that these are pathogens of minimal concern to channel catfish aquaculture (Griffin, Ware et al. 2014, Reichley, Ware et al. 2015). As



www.manaraa.com

such, future research endeavors would benefit from focusing on the biology, management, treatment and prevention of *E. piscicida*.



المنارة للاستشارات

www.manaraa.com

## 5.5 References


Abayneh, T., D. J. Colquhoun and H. Sorum (2013). "*Edwardsiella piscicida* sp. nov., a novel species pathogenic to fish." Journal of Applied Microbiology **114**(3): 644-654.

Bujan, N., A. E. Toranzo and B. Magarinos (2017). "Draft genome sequence of *Edwardsiella piscicida* strain ACC35.1 isolated from diseased turbot (*Scophthalmus maximus*) in Europe." Genome Announcements **5**(7).

Camus, A., J. Dill, A. McDermott, N. Hatcher and M. Griffin (2016). "*Edwardsiella piscicida*-associated septicaemia in a blotched fantail stingray *Taeniura meyeni* (Mueller & Henle)." Journal of Fish Diseases **39**(9):1125-1131.

Crumlish, M., T. T. Dung, J. F. Turnbull, N. T. N. Ngoc and H. W. Ferguson (2002). "Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam." Journal of Fish Diseases **25**: 733-736.

Crumlish, M., P. C. Thanh, J. Koesling, V. T. Tung and K. Gravningen (2010). "Experimental challenge studies in Vietnamese catfish, *Pangasianodon hypophthalmus* (Sauvage), exposed to *Edwardsiella ictaluri* and *Aeromonas hydrophila*." Journal of Fish Diseases **33**(9): 717-722.

Darwish, A., J. Plumb and J. Newton (2000). "Histopathology and pathogenesis of experimental infection with *Edwardsiella tarda* in channel catfish." Journal of Aquatic Animal Health **12**(4): 255-266.

Das, B. K., P. Pattnaik, G. Murjani and S. C. Mukherjee (2001). "*Edwardsiella tarda* endotoxin as an immunopotentiator in singhi, *Heteropneustes fossilis* fingerlings." Indian Journal of Experimental Biology **39**: 1311-1313.

Ewing, W. H., A. C. McWhorter, M. R. Escobar and A. H. Lubin (1965). "*Edwardsiella*, a new genus of Enterobacteriaceae based on a new species, *E. tarda*." International Bulletin of Bacteriological Nomenclature and Taxonomy **15**(1): 33-38.

Farmer, J. J. and A. C. McWhorter (1984). Genus X. *Edwardsiella*. Bergey's Manual of Systematic Bacteriology. N. R. Krieg and J. G. Holt. Baltimore, MD, Williams and Wilkins.

Fogelson, S. B., B. D. Petty, S. R. Reichley, C. Ware, P. R. Bowser, M. J. Crim, R. G. Getchell, K. L. Sams, H. Marquis and M. J. Griffin (2016). "Histologic and molecular characterization of *Edwardsiella piscicida* infection in largemouth bass (*Micropterus salmoides*)." Journal of Veterinary Diagnostic Investigation **28**(3): 338-344.




المنارة للاستشارات
www.manaraa.com

Francis-Floyd, R., P. Reed, B. Bolon, J. Estes and S. McKinney (1993). "An epizootic of *Edwardsiella tarda* in largemouth bass (*Micropterus salmoides*)." Journal of Wildlife Diseases **29**: 334-336.

Griffin, M. J., C. Ware, S. M. Quiniou, J. M. Steadman, P. S. Gaunt, L. H. Khoo and E. Soto (2014). "*Edwardsiella piscicida* identified in the Southeastern USA by *gyrB* sequence, species-specific and repetitive sequence-mediated PCR." Diseases of Aquatic Organisms **108**(1): 23-35.

Grimont, P. D., F. Grimont, C. Richard and R. Sakazaki (1980). "*Edwardsiella hoshinae*, a new species of Enterobacteriaceae." Current Microbiology **4**(6): 347-351.

Hargreaves, J. A. (2002). "Channel catfish farming in ponds: lessons from a maturing industry." Reviews in Fisheries Science **3/4**: 499-528.

Hawke, J. P., M. Kent, M. Rogge, W. Baumgartner, J. Wiles, J. Shelley, L. C. Savolainen, R. Wagner, K. Murray and T. S. Peterson (2013). "Edwardsiellosis caused by *Edwardsiella ictaluri* in laboratory populations of Zebrafish *Danio rerio*." Journal of Aquatic Animal Health **25**(3): 171-183.

Hawke, J. P. and L. H. Khoo (2004). Infectious Diseases. Biology and culture of the channel catfish. C. S. Tucker and J. A. Hargreaves. Amsterdam, Elsevier**:** 387-443.

Hawke, J. P., A. C. McWhorter, A. G. Steigerwalt and D. J. Brenner (1981). "*Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish." International Journal of Systematic Bacteriology **31**(4): 396-400.

Herigstad, B., M. Hamilton and J. Heersink (2001). "How to optimize the drop plate method for enumerating bacteria." Journal of Microbiological Methods **44**: 121-129.

Herman, R. and G. Bullock (1986). "Pathology caused by *Edwardsiella tarda* in striped bass." Transactions of the American Fisheries Society **115**: 232-235.

Khoo, L. H. (2013). Personal Communication.

Kubota, S., N. Kaige, T. Miyazaki and T. Miyashita (1981). "Histopathological studies on Edwardsiellosis of tilapia. I. Natural Infection." Bulletin of the Faculty of Fisheries of Mie University **9**: 155-156.

Liu, J. Y., A. H. Li, D. R. Zhou, Z. R. Wen and X. P. Ye (2010). "Isolation and characterization of *Edwardsiella ictaluri* strains as pathogens from diseased yellow catfish *Pelteobagrus fulvidraco* (Richardson) cultured in China." Aquaculture Research **41**(12): 1835-1844.



المنارة للاستشارات
www.manaraa.com

Meyer, F. P. and G. L. Bullock (1973). "*Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*)." Applied Microbiology **25**(1): 155-156.

Miyazaki, T. and S. Egusa (1976). "Histopathological studies of Edwardsiellosis of the Japanese eel (*Anguilla japonica*) I. Suppurative interstitial nephritis form." Fish Pathology **11**: 33-44.

Miyazaki, T. and J. A. Plumb (1985). "Histopathology of *Edwardsiella ictaluri* in channel catfish, *Ictalurus punctatus* (Rafinesque)." Journal of Fish Diseases **8**: 389-392.

Mohanty, B. R. and P. K. Sahoo (2007). "Edwardsiellosis in fish: a brief review." Journal of Biosciences **32**: 1331-1344.

Newton, J. C., L. G. Wolfe, J. M. Grizzle and J. A. Plumb (1989). "Pathology of experimental enteric septicaemia in channel catfish, *Ictalurus punctatus* (Rafinesque), following immersion-exposure to *Edwardsiella ictaluri*." Journal of Fish Diseases **12**: 335-347.

Oguro, K., K. Tamura, J. Yamane, M. Shimizu, T. Yamamoto, T. Ikawa, K. Ohnishi, S. Oshima and M. Imajoh (2014). "Draft genome sequences of two genetic variant strains of *Edwardsiella piscicida*, JF1305 and RSB1309, isolated from olive flounder (*Paralichythys olivaceus*) and Red Sea bream (*Pagrus major*) cultured in Japan, respectively." Genome Announcements **2**(3): e00546-00514.

Oren, A. and G. M. Garrity (2013). "List of new names and new combinations previously effectively, but not validly, published." International Journal of Systematic and Evolutionary Microbiology **63**(9): 3131-3134.

Oren, A. and G. M. Garrity (2015). "List of new names and new combinations previously effectively, but not validly, published." International Journal of Systematic and Evolutionary Microbiology **65**(7): 2017-2025.

Padros, F., C. Zarza, L. Dopazo, M. Cuadrado and S. Crespo (2006). "Pathology of *Edwardsiella tarda* infection in turbot, *Scophthalmus maximus* (L.)." Journal of Fish Diseases **29**: 87-94.

Park, S. B., T. Aoki and T. S. Jung (2012). "Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish." Veterinary Research **43**: 67.

Plumb, J. A. and L. A. Hanson (2011). Health Maintenance and Principal Microbial Diseases of Cultured Fishes. Ames, Iowa, Blackwell Publishing, Ltd.

Reed, L. J. (1938). "A simple method of estimating fifty per cent endpoints." American Journal of Epidemiology **27**(3): 493-497.



المنارة للاستشارات
www.manaraa.com

Řehulka, J., M. Marejková and P. Petráš (2012). "Edwardsiellosis in farmed rainbow trout (*Oncorhynchus mykiss*)." Aquaculture Research **43**: 1628-1634.

Reichley, S. R., C. Ware, T. E. Greenway, D. J. Wise and M. J. Griffin (2015). "Real-time polymerase chain reaction assays for the detection and quantification of *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like species in catfish tissues and pond water." Journal of Veterinary Diagnostic Investigation **27**(2): 130-139.

Reimschuessel, R. (2008). General Fish Histopathology. Fish Diseases. J. C. Eiras, H. Segner, T. Wahli and B. G. Kapoor. India, Science Publishers. **Vol 1:** 1-40.

Shafiei, S., S. Viljamaa-Dirks, K. Sundell, S. Heinikainen, T. Abayneh and T. Wiklund (2016). "Recovery of *Edwardsiella piscicida* from farmed whitefish, *Coregonus lavaretus* (L.), in Finland." Aquaculture **454**: 19-26.

Shao, S., Q. Lai, Q. Liu, H. Wu, J. Xiao, Z. Shao, Q. Wang and Y. Zhang (2015). "Phylogenomics characterization of a highly virulent *Edwardsiella* strain ET080813(T) encoding two distinct T3SS and three T6SS gene clusters: propose a novel species as *Edwardsiella anguillarum* sp. nov." Systematic and Applied Microbiology **38**(1): 36-47.

Singh, B. R., V. Singh, N. Ebibeni and R. K. Singh (2013). "Antimicrobial and herbal drug resistance in enteric bacteria isolated from faecal droppings of common house lizard/gecko (*Hemidactylus frenatus*)." International Journal of Microbiology **2013**: 340848.

Singh, I. M., S. Singh, M. A. McMurphy, S. S. Crupper, F. Mills-Robertson and R. D. Applegate (2004). "Antibiotic susceptibility of *Edwardsiella hoshinae* isolated from northern bobwhite quail (*Colinus virginianus*)." Veterinary Record **155**(1): 29-29.

Soto, E., M. Griffin, M. Arauz, A. Riofrio, A. Martinez and M. E. Cabrejos (2012). "*Edwardsiella ictaluri* as the causative agent of mortality in cultured Nile Tilapia." Journal of Aquatic Animal Health **24**(2): 81-90.

Stankus, T. (2010). "U.S. rarm-raised catfish." Journal of Agricultural & Food Information **11**(3): 176-184.

Suprapto, H., T. Nakai and K. Muroga (1995). "Toxicity of extracellular products and intracellular components of *Edwardsiella tarda* in the Japanese Eel and flounder." Journal of Aquatic Animal Health **7**: 292-297.

Uhland, F. C., P. Hélie and R. Higgins (2000). "Infections of *Edwardsiella tarda* among Brook Trout in Quebec." Journal of Aquatic Animal Health **12**(1): 74-77.

USDA (2014). 2013 Census of Aquaculture, National Agricultural Statistics Service. **3**.



للاستشارات المنارة

www.manaraa.com

USDA (2016). Catfish production, National Agricultural Statistics Service.

Wang, Q., M. Yang, J. Xiao, H. Wu, X. Wang, Y. Lv, L. Xu, H. Zheng, S. Wang, G. Zhao, Q. Liu and Y. Zhang (2009). "Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches." PLoS ONE **4**(10).

Wiedenmayer, A. A., J. J. Evans and P. H. Klesius (2006). "Experimental *Edwardsiella tarda* infection in nonabraded channel catfish *Ictalurus punctatus* by immersion." Fisheries Science **72**: 1124-1126.

Wise, D. J., A. C. Camus, T. E. Schwedler and J. S. Terhune (2004). Health Management. Biology and culture of channel catfish. C. S. Tucker and J. A. Hargreaves. Amsterdam, Elsevier**:** 444-502.

Xu, T. and X.-H. Zhang (2014). "*Edwardsiella tarda*: an intriguing problem in aquaculture." Aquaculture **431**: 129-135.

Ye, S., H. Li, G. Qiao and Z. Li (2009). "First case of *Edwardsiella ictaluri* infection in China farmed yellow catfish *Pelteobagrus fulvidraco*." Aquaculture **292**(1-2): 6-10.



المنارة للاستشارات

www.manaraa.com

# CHAPTER VI

# COMPARATIVE SUSCEPTIBILITY OF CHANNEL CATFISH (*ICTALURUS PUNCTATUS*), BLUE CATFISH (*ICTALURUS FURCATUS*) AND CHANNEL X BLUE HYBRID CATFISH TO *EDWARDSIELLA PISCICIDA*, *EDWARDSIELLA TARDA* AND *EDWARDSIELLA ANGUILLARUM*

## 6.1 Introduction

Catfish production is the largest finfish aquaculture industry in the United States and an important component of the economies of many southeastern states (Hargreaves 2002, Stankus 2010). The catfish aquaculture industry in the U.S. began primarily with propagation of channel catfish (*Ictalurus punctatus*). Other catfish species, including blue catfish (*Ictalurus furcatus*), have not been popular with the aquaculture industry due to slow maturation rates, poor feed conversion and poor captive spawning success (Graham 1999). However, research has demonstrated blue catfish are more resistant than channel catfish to several important diseases, including enteric septicemia of catfish (Wolters, Wise et al. 1996), channel catfish virus (Silverstein, Bosworth et al. 2008) and proliferative gill disease (Bosworth, Wise et al. 2003, Griffin, Camus et al. 2010). As a result, blue catfish X channel catfish hybrid fish were developed to capitalize on the favorable traits of both channel and blue catfish. In the early stages of industry development, technical issues prevented widespread utilization and adoption of hybrid



المنارة للاستشارات
www.manaraa.com

catfish as a culture animal (Hargreaves and Tucker 2004). However, over time and with considerable effort, these technical obstacles have diminished in scale, and hybrid utilization has increased to an estimated 30% - 40% of total catfish production in the United States (Li, Robinson et al. 2014).

*Edwardsiella tarda* is an enteric, zoonotic, Gram-negative bacteria and one of the most important bacterial diseases in wild and cultured fish globally (Mohanty and Sahoo 2007, Xu and Zhang 2014). Recent investigations into the phenotypic and genotypic variation of *E. tarda* have led to the reclassification of bacterial isolates previously considered *E. tarda.* This reorganization of the genus segregated *E. tarda* into three distinct taxa: *E. tarda*, *E. piscicida and E. anguillarum* (Yang, Lv et al. 2012, Abayneh, Colquhoun et al. 2013, Griffin, Quiniou et al. 2013, Shao, Lai et al. 2015).

A survey of isolates previously classified as *E. tarda* recovered from disease outbreaks in cultured catfish in the southeastern USA revealed all those isolates to be *E. piscicida* (Griffin, Ware et al. 2014). Furthermore, summaries of diagnostic case submissions to the Aquatic Research and Diagnostic Laboratory (ARDL) in Stoneville, Mississippi suggest an increased incidence of *E. piscicida* outbreaks in farm-raised catfish in Mississippi over the past decade, with a putative trend towards increased incidence in hybrid catfish. Meanwhile, investigations have demonstrated differences in median lethal dose amongst *E. tarda, E. piscicida* and *E. anguillarum* in channel catfish, suggesting the threat *E. tarda* and *E. anguillarum* pose to channel catfish is minimal (Reichley, Ware et al. 2015). The work described herein investigated the comparative susceptibility of channel catfish, blue catfish and their hybrid cross to molecularly confirmed isolates of *E. tarda*, *E. piscicida* and *E. anguillarum*.



المنارة للاستشارات
www.manaraa.com

## 6.2 Materials and Methods

### 6.2.1 Fish

All fish in this study were produced and housed for disease research at the rearing facility of the Thad Cochran National Warmwater Aquaculture Center. Prior to challenge, genetic groups of fish (channel catfish, blue catfish, interspecific hybrid cross) were maintained separately in 2,000 L tanks with 1,000 L of well water (~26°C) under flow-through conditions (4 L/min) with supplemental aeration. For infectivity studies, fish were held in 80 L aquaria containing 22 L of well water and held under flow-through conditions (1 L/min) with constant aeration. Prior to challenge, fish were acclimated to a water temperature of ~30°C over 72 hr and feed was withheld. Following challenge, fish were fed daily to satiation and monitored for morbidity and mortality.

### 6.2.2 Bacterial Cultures

Cryostocks of *Edwardsiella* spp. isolates (Table 6.1) archived at -80°C were revived on Mueller-Hinton II Agar (BBL; Becton Dickinson and Company) plates supplemented with 5% defibrinated sheep blood (Hemastat Laboratories, Dixon CA) and incubated for 24 hr at 37°C. Individual colonies of each isolate were expanded (37°C; 200 rpm, Excella E24, New Brunswick Scientific) in 9 ml of porcine brain-heart infusion broth (BHIb) (Bacto; Becton Dickinson and Company). Isolates were passed through channel catfish fingerlings (n=3/isolate) using two successive 48-hr passages. Briefly, fish were injected intraperitoneally with 100 µl of overnight broth culture. After 48 hr, fish were euthanized by an overdose of MS-222, and isolates were recovered by kidney culture, expanded as above, and the procedure was repeated. After the second 48 hr passage, individual colonies were cultured and expanded as above and stored



المنارة للاستشارات
www.manaraa.com

cryogenically (-80°C; 15% v/v glycerol) until use for challenges. For each challenge, cryostocks of passaged isolates were revived, and individual isolates were expanded overnight (37°C; 200 rpm, Excella E24, New Brunswick Scientific) in 9 ml of BHIb. Following overnight incubation, 300 ul of each culture was added to 250 ml of sterile BHIb and expanded overnight (37°C; 200 rpm, Excella E24, New Brunswick Scientific). Cultures were then diluted to achieve estimated targeted doses for each isolate. Enumeration of bacteria for all studies was accomplished using serial dilution and colony counts on triplicate drop plates using 20 µl (Herigstad, Hamilton et al. 2001).

Table 6.1 Source of *Edwardsiella* isolates used for $LD_{50}$ determination.

| Isolate* | Species | Host | Location |
|---|---|---|---|
| LADL05-105 | *E. anguillarum* | *Oreochromis* sp. | Louisiana, USA |
| S11-285 | *E. piscicida* | *Ictalurus punctatus* | Mississippi, USA |
| FL95-01 | *E. tarda* | *Ictalurus punctatus* | Florida, USA |

*Isolates have previously been described by: Griffin, Quiniou et al. (2013); Griffin, Ware et al. (2014); Reichley, Ware et al. (2015).

### 6.2.3 Trial 1: Initial Range Test (*E. anguillarum*, *E. piscicida*, *E. tarda*)

For each *Edwardsiella* spp., ten fingerling channel catfish (mean 18.9g, ±4.3g), blue catfish (mean 21.6g, ±5.3g) and hybrid catfish (mean 19.8g, ± 6.9g) were stocked into seven tanks (63 tanks; 3 isolates; 3 fish genetic groups/isolate; 7 tanks/isolate; 10 fish/tank). Following 1 week acclimation, fish in each tank were anesthetized with tricaine methanesulfonate (MS-222) and injected intraperitoneally (IP) with 10-fold serial dilutions of each bacteria, ranging from $10^7$ to $10^1$ CFU. For each fish strain, fish in the remaining tank were handled similarly but injected with sterile BHIb to serve as non-



www.manaraa.com

infected controls. Fish were monitored twice daily for 21 days, and the number of dead fish was recorded. The posterior kidney from all dead fish was cultured aseptically on Mueller-Hinton II Agar (BBL; Becton Dickinson and Company) plates supplemented with 5% defibrinated sheep blood (Hemastat Laboratories, Dixon CA).

### 6.2.4 Trial 2: Median Lethal Dose Refinement (*E. piscicida* and *E. tarda*)

The IP median lethal dose ($LD_{50}$) in channel, blue and hybrid catfish was determined for *E. piscicida* and *E. tarda* using 3-fold serial dilutions. For each isolate, ten channel catfish (mean 22.6g, standard deviation 4.3g), blue catfish (mean 25.4g, standard deviation 7.1g) and hybrid catfish (mean 19.5g, standard deviation 5.5g) were placed into six tanks (36 tanks; 2 isolates; 3 fish genetic groups /isolate; 6 tanks/isolate; 10 fish/tank). Fish were handled as above and injected IP with 3-fold serial dilutions ranging from $10^6$ to $10^3$ CFU for *E. piscicida* and $10^7$ to $10^4$ CFU for *E. tarda*. A single tank of 10 sham-injected (BHIb) fish served as non-infected control for each fish genetic group. Fish were monitored twice daily for 21 days, and the number of dead fish was recorded. The posterior kidney from all dead fish was aseptically cultured on Mueller-Hinton II Agar (BBL; Becton Dickinson and Company) plates supplemented with 5% defibrinated sheep blood (Hemastat Laboratories, Dixon CA).

### 6.2.5 Trial 3: Replicated Median Lethal Dose Determination for *E. piscicida*

Given the importance of channel catfish and hybrid catfish production to the U.S. catfish industry, the $LD_{50}$ of *E. piscicida* to both channel and hybrid catfish was further investigated at three different doses with replication. Challenge conditions were consistent with the experiments described above. For each dose, channel catfish



www.manaraa.com

fingerlings (mean 29.1g, standard deviation 7.9g) and hybrid fingerlings (mean 27.6g, standard deviation 7.6g) were stocked (n=20 fish/tank) into four tanks (24 tanks; 1 isolate; 3 doses; 2 fish genetic groups/dose; 4 tanks/dose; 20 fish/tank). Bacterial cultures were diluted to achieve treatment doses equating to $4.17 \times 10^4$, $1.25 \times 10^5$ and $3.75 \times 10^5$ CFU/fish. A single tank of 20 sham-injected (BHIb) fish served as non-infected control for each fish genetic group. Because previous experiments demonstrated limited mortality after 10 days post-challenge, the current experimental challenge was terminated after 14 days. Fish were monitored for morbidity and mortality twice daily, and the number of dead fish was recorded. The posterior kidney from all dead fish was aseptically cultured on Mueller-Hinton II Agar (BBL; Becton Dickinson and Company) plates supplemented with 5% defibrinated sheep blood (Hemastat Laboratories, Dixon CA).

### 6.2.6 Statistical Analysis

For all challenges, median lethal dose ($LD_{50}$) was calculated based on cumulative mortality data (Reed 1938). Mortality data from the replicated $LD_{50}$ dose determination for *E. piscicida* in channel and hybrid catfish were analyzed by ANOVA using the PROC mixed function in Statistical Analysis System version 9.3 (SAS Institute, Cary, North Carolina). For significance testing, data were transformed prior to analysis using equation 6.1 (Anscombe 1948).

$$p' = arcsin \sqrt{\frac{X+3/8}{n+3/4}} \tag{6.1}$$



المنارة للاستشارات
www.manaraa.com

## 6.3 Results

### 6.3.1 Trial 1: Initial Range Test (*E. anguillarum*, *E. piscicida*, *E. tarda*)

Cumulative percent mortality for each fish group and bacterial dose over the 21-day trial is presented in Table 6.2. Bacteria were recovered from posterior kidneys of 100% (90/90), 100% (21/21) and 88% (7/8) of dead fish challenged with *E. piscicida*, *E. tarda and E. anguillarum*, respectively.

Table 6.2 Cumulative percent mortality and $LD_{50}$ from initial range test (Trial 1).

| | | **Cumulative Percent Mortality** | | |
|---|---|---|---|---|
| | Dose (CFU/fish) | Channel Catfish | Hybrid Catfish | Blue Catfish |
| *E. piscicida* | 4.67E+07 | 100% | 100% | 100% |
| | 4.67E+06 | 100% | 100% | 100% |
| | 4.67E+05 | 30% | 100% | 90% |
| | 4.67E+04 | 0% | 40% | 50% |
| | 4.67E+03 | 0% | 10% | 0% |
| | 4.67E+02 | 0% | 20% | 0% |
| | 4.67E+01 | 0% | 0% | 0% |
| | **$LD_{50}$** | **$4.77 \times 10^5$ CFU/g** | **$1.84 \times 10^4$ CFU/g** | **$2.75 \times 10^4$ CFU/g** |
| *E. tarda* | 3.67E+07 | 90% | 50% | 100% |
| | 3.67E+06 | 30% | 10% | 10% |
| | 3.67E+05 | 0% | 0% | 0% |
| | 3.67E+04 | 0% | 0% | 0% |
| | 3.67E+03 | 0% | 0% | 0% |
| | 3.67E+02 | 0% | 0% | 0% |
| | 3.67E+01 | 0% | 0% | 0% |
| | **$LD_{50}$** | **$4.39 \times 10^6$ CFU/g** | **$1.46 \times 10^7$ CFU/g** | **$4.73 \times 10^6$ CFU/g** |
| *E. anguillarum* | 1.50E+07 | 20% | 10% | 30% |
| | 1.50E+06 | 0% | 10% | 0% |
| | 1.50E+05 | 0% | 0% | 0% |
| | 1.50E+04 | 0% | 0% | 10% |
| | 1.50E+03 | 0% | 0% | 0% |
| | 1.50E+02 | 0% | 0% | 0% |
| | 1.50E+01 | 0% | 0% | 0% |
| | **$LD_{50}$** | **NC** | **NC** | **NC** |
| BHI Control | N/A | 0% | 0% | 0% |

For each dose, channel catfish fingerlings, hybrid fingerlings and blue fingerlings were stocked (n=10 fish/tank) into discrete tanks. Fish were monitored for morbidity and mortality twice daily for 21 days and the number of dead fish recorded. NC: not calculated, 50% mortality was not observed.



المنارة للاستشارات
www.manaraa.com

For this initial range test, the approximate $LD_{50}$ of *E. piscicida* was 4.77 x $10^5$ CFU/g in channel catfish, 1.84 x $10^4$ CFU/g in hybrid catfish and 2.75 x $10^4$ CFU/g in blue catfish. Approximate $LD_{50}$ of *E. tarda* was 4.39 x $10^6$ CFU/g in channel catfish, 1.46 x $10^7$ CFU/g in hybrid catfish and 4.73 x $10^6$ CFU/g in blue catfish. The highest mortality observed for all doses of *E. anguillarum* was 30%, precluding $LD_{50}$ calculations.

### 6.3.2 Trial 2: Median Lethal Dose Refinement (*E. piscicida* and *E. tarda*)

Cumulative percent mortality for each fish group and bacterial dose is presented in Table 6.3. Bacteria were recovered from posterior kidneys of 100% of dead fish challenged with *E. piscicida* (111/111) and *E. tarda* (16/16).

Table 6.3 Cumulative percent mortality and $LD_{50}$ from Trial 2.

| | | **Cumulative Percent Mortality** | | |
|---|---|---|---|---|
| | Dose (CFU/fish) | Channel Catfish | Hybrid Catfish | Blue Catfish |
| *E. piscicida* | 2.50E+06 | 100% | 100% | 100% |
| | 8.33E+05 | 100% | 100% | 100% |
| | 2.78E+05 | 60% | 100% | 100% |
| | 9.26E+04 | 10% | 80% | 70% |
| | 3.09E+04 | 0% | 20% | 70% |
| | 1.03E+04 | 0% | 0% | 10% |
| | **$LD_{50}$** | **9.38 x $10^3$ CFU/g** | **2.81 x $10^3$ CFU/g** | **1.05 x $10^3$ CFU/g** |
| *E. tarda* | 2.33E+07 | 50% | 20% | 40% |
| | 7.77E+06 | 20% | 10% | 20% |
| | 2.59E+06 | 0% | 0% | 0% |
| | 8.63E+05 | 0% | 0% | 0% |
| | 2.88E+05 | 0% | 0% | 0% |
| | 9.59E+04 | 0% | 0% | 0% |
| | **$LD_{50}$** | **8.48 x $10^6$ CFU/g** | **NC** | **NC** |
| BHI Control | N/A | 0% | 0% | 0% |

For each dose, channel catfish fingerlings, hybrid fingerlings and blue fingerlings were stocked (n=10 fish/tank) into discrete tanks. Fish were monitored for morbidity and mortality twice daily for 21 days and the number of dead fish recorded. NC: not calculated, 50% mortality was not observed.

The approximate $LD_{50}$ of *E. piscicida* was 9.38 x $10^3$ CFU/g in channel catfish, 2.81 x $10^3$ CFU/g in hybrid catfish and 1.05 x $10^3$ CFU/g in blue catfish. Comparatively, the approximate $LD_{50}$ of *E. tarda* was 8.48 x $10^6$ CFU/g in channel catfish. Median lethal



المنارة للاستشارات
www.manaraa.com

dose of *E. tarda* in hybrid and blue catfish could not be calculated as 50% mortality was not achieved in these fish groups.

### 6.3.3 Trial 3: Replicated Median Lethal Dose Determination for *E. piscicida*

Mortality significantly increased with dose ($p<0.0001$), and mortality in hybrid catfish was significantly higher than mortality in channel catfish ($p<0.0001$) at each dose (Table 6.4). Cumulative percent mortality for *E. piscicida* in each fish group after fourteen days can be found in Table 4. Bacteria consistent with challenge isolates were recovered from posterior kidneys from 100% (219/219) of dead fish challenged with *E. piscicida*. One control channel catfish died, but no growth was observed from posterior kidney culture for this fish. The $LD_{50}$ for *E. piscicida* calculated from this replicated study was $1.29 \times 10^5$ CFU/g in channel catfish fingerlings and $2.22 \times 10^4$ CFU/g in hybrid catfish fingerlings.



www.manaraa.com

Table 6.4 Average cumulative percent mortality for *E. piscicida* in channel and hybrid catfish (Trial 3).

| | **Average Cumulative Percent Mortality** | | | |
|---|---|---|---|---|
| | **Arcsine Transformed** | | **Non-Transformed** | |
| Dose (CFU/fish) | Channel | Hybrid | Channel | Hybrid |
| 3.75E+05 | 0.691 | 1.342 | 41% | 96% |
| 1.25E+05 | 0.392 | 1.095 | 14% | 79% |
| 4.17E+04 | 0.219 | 0.700 | 4% | 41% |
| BHI | 0.345 | 0.134 | 10% | 0% |
| | | PSE | p-Value | |
| | Fish | 0.0414 | <0.0001 | |
| | Dose | 0.0507 | <0.0001 | |
| | Fish x Dose | 0.0717 | 0.2936 | |

For each dose, channel catfish fingerlings and hybrid fingerlings were stocked (n=20 fish/tank) into four discrete tanks, respectively. Fish were monitored for morbidity and mortality twice daily for 14 days and the number of dead fish recorded. For significance testing, data were arcsine transformed prior to analysis using equation 6.1 (Anscombe 1948). PSE = pooled standard error.

## 6.4 Discussion

Farm-raised catfish is an important agricultural commodity in the southeastern United States, particularly in Mississippi, which is responsible for more than half of national gross catfish sales (Stankus 2010, USDA 2016). Infectious disease is one of the major challenges for farm-raised catfish, accounting for approximately 45% of inventory loss throughout the production cycle. Of the losses associated with infectious disease, approximately 60% are attributable to bacterial infections (USDA 1997), with the majority of disease related losses attributed to *Edwardsiella ictaluri* and *Flavobacterium columnare* (USDA/APHIS 2011).

In addition to these important bacteria, diagnostic case submissions to the Aquatic Research and Diagnostic Laboratory (ARDL), located at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, Mississippi, indicate an increased incidence of *E. tarda* infections over the past decade. However, identification of these isolates is based on phenotypic characterization and, at present, a discriminatory



www.manaraa.com

phenotypic test to differentiate *E. tarda* and *E. piscicida* has yet to be established. Molecular surveys have identified the majority of bacterial isolates from catfish aquaculture in the southeastern United States originally classified as *E. tarda* to actually be members of the recently described taxa *E. piscicida* (Griffin, Ware et al. 2014). Moreover, previous research, which was supported in the work reported here, demonstrates *E. piscicida* is more virulent in channel catfish than *E. tarda* (Reichley, Ware et al. 2015).

In the current study, the initial IP $LD_{50}$ range test resulted in minimal mortality associated with *E. anguillarum* in channel, hybrid or blue catfish, even at doses as high as 1.50 x $10^7$ CFU/fish. These results support previous research suggesting *E. anguillarum* is a pathogen of minimal concern to U.S. farm-raised catfish (Reichley, Ware et al. 2015). Ucko, Colorni et al. (2016) suggest members of *E. anguillarum* are of more importance to piscine hosts in the marine and brackish water environments. Furthermore, the refined median lethal dose study demonstrated an $LD_{50}$ for *E. tarda* at least three orders of magnitude higher than *E. piscicida* in the channel catfish, blue catfish and channel x blue catfish hybrids evaluated, indicating *E. tarda* is unlikely to be a significant threat to catfish aquaculture.

Anecdotal reports from industry and analysis of the diagnostic case submissions to the ARDL reveal the majority of *E. piscicida* isolates are recovered from hybrid catfish, despite hybrid catfish accounting for a minority of diagnostic case submissions (Khoo 2017). Findings from the current study corroborate these diagnostic case trends. Across all experimental trials, hybrids and blue catfish demonstrated increased susceptibility to *E. piscicida* over channel catfish, with calculated $LD_{50}$'s consistently one



www.manaraa.com

order of magnitude lower for *E. piscicida* in hybrids compared to channels. The replicated *E. piscicida* $LD_{50}$ study (Trial 3) demonstrated a significant increase in hybrid mortality compared to channel catfish at all administered doses ($p<0.0001$). These results, coupled with the increasing utilization of hybrid catfish in the U.S. catfish aquaculture industry, may explain the increased incidence of *E. piscicida* in U.S. catfish aquaculture in recent years.

Hybrid catfish continue to gain popularity in U.S. catfish aquaculture due to favorable production characteristics such as increased growth, tolerance to crowding and resistance to several pathogens typically problematic in channel catfish culture. However, as hybrid catfish culture expands, the potential for the emergence of pathogens of increased pathogenicity to hybrid catfish may also increase. To our knowledge, *E. piscicida* is the first bacterial pathogen to demonstrate increased pathogenicity in hybrid catfish compared to channel catfish. These results, coupled with trends in diagnostic case submissions and anecdotal industry reports, suggest *E. piscicida* is an emergent threat to hybrid catfish production and warrants further study.



المنارة للاستشارات
www.manaraa.com

## 6.5 References


Abayneh, T., D. J. Colquhoun and H. Sorum (2013). "*Edwardsiella piscicida* sp. nov., a novel species pathogenic to fish." Journal of Applied Microbiology **114**(3): 644-654.

Anscombe, F. J. (1948). "The transformation of poisson, binomial and negative-binomial data." Biometrika **35**(3/4): 246-254.

Bosworth, B. G., D. J. Wise, J. S. Terhune and W. R. Wolters (2003). "Family and genetic group effects for resistance to proliferative gill disease in channel catfish, blue catfish and channel catfish x blue catfish backcross hybrids." Aquaculture Research **34**: 569-573.

Graham, K. (1999). A review of the biology and management of blue catfish. Catfish 2000: proceedings of the international ictalurid symposium, Bethesda, Maryland, American Fisheries Society.

Griffin, M. J., A. C. Camus, D. J. Wise, T. E. Greenway, M. J. Mauel and L. M. Pote (2010). "Variation in susceptibility to *Henneguya ictaluri* infection by two species of catfish and their hybrid cross." Journal of Aquatic Animal Health **22**(1): 21-35.

Griffin, M. J., S. M. Quiniou, T. Cody, M. Tabuchi, C. Ware, R. C. Cipriano, M. J. Mauel and E. Soto (2013). "Comparative analysis of *Edwardsiella* isolates from fish in the eastern United States identifies two distinct genetic taxa amongst organisms phenotypically classified as *E. tarda*." Veterinary Microbiology **165**(3-4): 358-372.

Griffin, M. J., C. Ware, S. M. Quiniou, J. M. Steadman, P. S. Gaunt, L. H. Khoo and E. Soto (2014). "*Edwardsiella piscicida* identified in the Southeastern USA by *gyrB* sequence, species-specific and repetitive sequence-mediated PCR." Diseases of Aquatic Organisms **108**(1): 23-35.

Hargreaves, J. A. (2002). "Channel catfish farming in ponds: lessons from a maturing industry." Reviews in Fisheries Science **3/4**: 499-528.

Hargreaves, J. A. and C. S. Tucker (2004). Indstury Development. Biology and Culture of Channel Catfish. C. S. Tucker and J. A. Hargreaves. San Diego, CA, Elsevier.

Herigstad, B., M. Hamilton and J. Heersink (2001). "How to optimize the drop plate method for enumerating bacteria." Journal of Microbiological Methods **44**: 121-129.

Khoo, L. H. (2017). Personal Communication.




www.manaraa.com

Li, M. H., E. H. Robinson, B. G. Bosworth and E. L. Torrans (2014). "Growth and feed conversion ratio of pond-raised hybrid catfish harvested at different sizes." North American Journal of Aquaculture **76**(3): 261-264.

Mohanty, B. R. and P. K. Sahoo (2007). "Edwardsiellosis in fish: a brief review." Journal of Biosciences **32**: 1331-1344.

Reed, L. J. (1938). "A simple method of estimating fifty per cent endpoints." American Journal of Epidemiology **27**(3): 493-497.

Reichley, S. R., C. Ware, T. E. Greenway, D. J. Wise and M. J. Griffin (2015). "Real-time polymerase chain reaction assays for the detection and quantification of *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like species in catfish tissues and pond water." Journal of Veterinary Diagnostic Investigation **27**(2): 130-139.

Shao, S., Q. Lai, Q. Liu, H. Wu, J. Xiao, Z. Shao, Q. Wang and Y. Zhang (2015). "Phylogenomics characterization of a highly virulent *Edwardsiella* strain ET080813(T) encoding two distinct T3SS and three T6SS gene clusters: propose a novel species as *Edwardsiella anguillarum* sp. nov." Systematic and Applied Microbiology **38**(1): 36-47.

Silverstein, P. S., B. G. Bosworth and P. S. Gaunt (2008). "Differential susceptibility of blue catfish, *Ictalurus furcatus* (Valenciennes), channel catfish, *I. punctatus* (Rafinesque), and blue x channel catfish hybrids to channel catfish virus." Journal of Fish Diseases **31**: 77-79.

Stankus, T. (2010). "U.S. rarm-raised catfish." Journal of Agricultural & Food Information **11**(3): 176-184.

Ucko, M., A. Colorni, L. Dubytska and R. L. Thune (2016). "*Edwardsiella piscicida*-like pathogen in cultured grouper." Diseases of Aquatic Organisms **121**(2): 141-148.

USDA (1997). Catfish NAHMS '97, Part I: Reference of 1996 U.S. catfish health production practices. Fort Collins, Colorado, Centers for Epidemiology and Animal Health.

USDA (2016). Catfish production, National Agricultural Statistics Service.

USDA/APHIS (2011). Catfish 2010, Part II: Health and production practices for foodsize catfish in the United States, 2009. Fort Collins, Colorado, Centers for Epidemiology and Animal Health.

Wolters, W. R., D. J. Wise and P. H. Klesius (1996). "Survival and antibody response of channel catfish, blue catfish, and channel catfish female × blue catfish male hybrids after exposure to *Edwardsiella ictaluri*." Journal of Aquatic Animal Health **8**(3): 249-254.



المنارة للاستشارات
www.manaraa.com

Xu, T. and X.-H. Zhang (2014). "*Edwardsiella tarda*: an intriguing problem in aquaculture." Aquaculture **431**: 129-135.

Yang, M., Y. Lv, J. Xiao, H. Wu, H. Zheng, Q. Liu, Y. Zhang and Q. Wang (2012). "*Edwardsiella* comparative phylogenomics reveal the new intra/inter-species taxonomic relationships, virulence evolution and niche adaptation mechanisms." PLoS One **7**(5): e36987.



المنارة للاستشارات

www.manaraa.com

# CHAPTER VII

# CONCLUSION

Diseases caused by *Edwardsiella* spp. are responsible for significant losses in many important wild and cultured fish species around the world. Recent investigations of the genotypic and phenotypic variability of *Edwardsiella tarda* led to the understanding that bacteria historically classified as *E. tarda* actually represented three genetically distinct, yet phenotypically ambiguous species: *E. tarda, E. piscicida* and *E. anguillarum*. To better understand this genus, whole genome sequencing was performed on representative isolates of *E. anguillarum*, *E. hoshinae*, *E. piscicida* and *E. tarda*. These analyses demonstrated significant genetic differences between these phenotypically similar taxa. The genomes closed as part of this dissertation will better facilitate proper taxonomic assignment and minimize erroneous classifications of *Edwardsiella* isolates in future research. Additionally, these closed genomes will assist in further studies investigating the biology of these important bacteria and help researchers gain a better understanding of their interactions in the environment and within different hosts.

Expanding on the genomic differences identified amongst the *Edwardsiella* spp., qPCR assays were developed for rapid identification and to estimate relative abundance of *E. tarda*, *E. piscicida* and *E. anguillarum* in fish tissue and pond water. These assays were then reassessed in a multiplex format, which offers a convenient, cost-effective and practical means to differentiate amongst the members of *Edwardsiella* affecting fish (*E.*



المنارة للاستشارات
www.manaraa.com

*ictaluri*, *E. piscicida*, *E. tarda* and *E. anguillarum*) in a single PCR. Genomic differences were further explored by evaluating the 16S rRNA, *gyrB* and *sodB* sequences. The limitations of relying on partial 16S rRNA sequences for isolate classification are detailed throughout the dissertation and the advantages of using the single copy genes *gyrB* and *sodB* in addition to 16S rRNA were demonstrated. Additionally, sequencing of the *sodB* gene linked contemporary classifications to historical designations. This work confirmed isolates previously defined as typical, motile, fish pathogenic *E. tarda* are synonymous with *E. piscicida*; atypical, non-motile fish pathogenic *E. tarda* are conspecific with *E. anguillarum*, while *E. tarda* as it is currently defined is congruent with fish non-pathogenic *E. tarda*.

One obstacle that has prevented segregation of the distinct congeners within the group of isolates historically classified as *E. tarda* is the lack of a distinguishing phenotypic characteristic. The increasing use of molecular techniques and growing number of new bacterial taxa identified in the post-genomic age poses a problem for phenotype database management, resulting in prokaryote databases that lag behind evolving systematics. Furthermore, commercial test panel configurations are relatively constant over time, and as new species are defined, more appropriate discriminatory metabolic phenotypic tests may not be present in current test panel arrangements. However, data presented in this dissertation revealed discriminatory peaks were present for each *Edwardsiella* spp. using MALDI-TOF methodology. This demonstrates that in spite of deficiencies in current phenotypic databases, MALDI-TOF offers a reliable, cost effective alternative for clinical laboratories that require rapid, reliable identification.



المنارة للاستشارات
www.manaraa.com

Furthermore, catfish production is the largest aquaculture industry in the United States and is an important component of the economy in many southeastern states. Research has demonstrated *E. piscicida* is more commonly associated with disease in Mississippi farm-raised catfish than *E. tarda*. However, one important finding culminating from this work is the confirmation that *Edwardsiella* isolate 9.1 from the original description of emphysematous putrefactive disease in catfish aquaculture in the 1970s, as well as other suspected *E. tarda* isolates from catfish aquaculture in the early 1980s, are factually *E. tarda*. This would suggest that *E. piscicida* is an emerging pathogen within the catfish industry, as historical isolates associated with catfish culture were not merely misclassified *E. piscicida*.

To further explore *E. piscicida*, *E. tarda* and *E. anguillarum* in catfish, several disease models were evaluated, along with histopathological changes and posterior kidney clearance rates. Furthermore, anecdotal reports from the catfish industry, coupled with diagnostic case submissions to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS, suggest *E. piscicida* to be an emerging pathogen in channel (♀) x blue (♂) hybrid catfish. Given the increasing popularity of hybrid catfish, research into the comparative pathogenicity of *E. piscicida*, *E. tarda* and *E. anguillarum* in channel, blue and hybrid catfish was warranted. Comparative challenges demonstrated increased pathogenicity of *E. piscicida* in hybrid and blue catfish over channel catfish. Conversely, *E. anguillarum* and *E. tarda* appear to be of minimal concern in U.S. farm-raised catfish, with $LD_{50}$'s nearly 2-3 orders of magnitude higher than *E. piscicida*.

The work presented in this dissertation has advanced our understanding of the genus *Edwardsiella*. The findings from this work will provide valuable insight for the



المنارة للاستشارات
www.manaraa.com

U.S. catfish farmers and provides the groundwork for additional investigations into prevention, control and treatment for these important bacterial pathogens.



المنارة للاستشارات
www.manaraa.com