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Detection of Flavobacterium Columnare in Tissues and Pond Water using Real-Time Polymerase Chain Reaction

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Detection of *Flavobacterium columnare* in tissues and pond water using real-time polymerase chain reaction

By

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Flavobacterium columnare, a Gram-negative rod-shaped bacterium, is the causative agent of columnaris disease in a variety of fish hosts but is of particular significance to the catfish industry located in the southeastern United States. Columnaris infections are a leading cause of mortalities in catfish ponds, occurring alone or in conjunction with other diseases. Typical diagnostic methods for columnaris infections involve the use of selective media following the observation of gross signs of disease.

A real-time quantitative PCR (qPCR) assay to estimate the quantity of bacteria present in environmental and tissue samples was developed and validated. The genetic variability seen in *F. columnare* makes detection of isolates from different genomovars (genetic groups) essential to an assay for diagnostic application. Isolates from catfish generally fall into one of two different genomovars, one being virulent to catfish, while the other genomovar is thought to be largely opportunistic.

The qPCR assay described herein was designed specifically to detect *F. columnare* isolates from the two major genomovars most often associated with farm-raised catfish. The assay was shown specific to *F. columnare*, regardless of genomovar,

and demonstrated sensitivity consistent with similar qPCR assays. In addition, the assay provides quantitative information, estimating the bacterial loads in fish tissue and the environment. Two different applications of the assay are presented: (1) Estimate bacterial burden in fish tissue following immersion challenges to identify variation in transmission rates between channel and blue x channel hybrid catfish, and (2) Estimate the environmental burden of *F. columnare* in catfish ponds over the course of a single calendar year. This assay will provide an invaluable tool for researchers and diagnosticians in expanding our understanding of *F. columnare* and how it interacts with the host and environment.

DEDICATION

To my wife, Hannah, and our children; Luke, Jacob, Jonathan, and Lydia: This work is a result of the many long hours at night and on weekends away from you. Thank you for loving me enough to allow me to follow my dream. This work would not have been possible without your love, support, and encouragement.

To my parents, Gordon and Audrey, who have supported me both financially and emotionally my entire life: Thank you for encouraging me to pursue excellence and believing that I was capable of any achievement through dedication and hard work.

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LITERATURE REVIEW

1.1 Introduction

Columnaris disease was first described following an outbreak in buffalo fish, *Ictiobus bubalus* and *I. cyprinellus*, in 1917 at the U.S. Fisheries Biological Station in Fairport, Iowa. Initially, the long slender rods indicative of *Flavobacterium columnare* could be observed microscopically in wet mounts of infected tissue, but the bacteria could not be cultured. The characteristic column-like masses observed in wet mounts of infected tissue led to the adoption of *Bacillus columnaris* as a new fish pathogen (Davis 1922). Later, Ordal and Rucker (1944) placed the bacteria in the order Myxobacterales: which are long, thin gram-negative rods characterized by a creeping or flexing motility. They reported the disease causing isolates produced both fruiting bodies and microcysts, resulting in the organism being reclassified as *Chrondrococcus columnaris* (Ordal and Rucker 1944). The following year, after an analysis of its life cycle revealed the bacteria produced microcysts but no fruiting bodies, the organism was renamed *Cytophaga columnaris* (Garnjobst 1945). In 1974 the bacteria was moved from the order Myxobacterales to Cytophagales and the taxon *Flexibacter columnaris* was adopted (Buchanan and Gibbons 1974). The last reclassification occurred in 1996, when the organism was placed in the family Flavobacteriaceae as *Flavobacterium columnare* (Bernardet et al 1996).

1.2 Significance to catfish aquaculture

The catfish industry is primarily located in the southeastern United States, with Alabama, Arkansas, Louisiana, and Mississippi accounting for 53.5% of all catfish operations in the United States. These four states produced 91.5% of the total catfish sales in 2008. In 2013, Mississippi accounted for 51% of the total catfish sales in the United States (USDA/NASS 2015).

Lost revenue in the catfish industry attributed to infectious disease is the most significant hindrance to production efficiency in catfish aquaculture. The emergence of infectious disease in farm-raised catfish is largely attributed to the elevated feeding rates and increased stocking densities associated with intensive aquaculture (Hargreaves and Tucker 2004). Of the diseases associated with farm-raised catfish, the most common are the bacterial pathogens *Edwardsiella ictaluri*, causative agent of enteric septicemia of catfish (ESC), and *F. columnare* (Hawke and Khoo 2004). Columnaris infections are often mixed with infections by other bacterial species such as *Edwardsiella ictaluri*, *E. tarda*, and *Aeromonas* species (Hawke and Thune 1992). In 2009, ESC and columnaris were implicated in disease-related losses of food-size fish on more than one-third of all operations (USDA/APHIS 2010). This predilection towards mixed infections makes the economic impact of columnaris disease difficult to determine, but losses are estimated to be millions of dollars annually (Shoemaker et al 2011).

1.3 Phenotypic Description

F. columnare, the causative agent of columnaris disease, is a Gram-negative rod, typically 4-12 μm long, found throughout the world in fresh and brackish waters affecting numerous fish species (Roberts 2012). The bacteria will commonly aggregate together in

groups of long thin rods, forming columns that resemble "haystacks" when observed on tissue wet mounts. Streaking these bacteria onto a selective agar such as dilute Mueller-Hinton, modified Shieh, or *Flavobacterium columnare* Growth Media (FCGM) containing the antibiotics, neomycin and polymyxin B, will reveal yellow-orange colonies after approximately 48 hours at 30°C. Colonies may be dry and rhizoid or mucoid in appearance depending on the isolate (Bullock et al 1986). Phenotypically, *F. columnare* is characterized by rhizoid yellow/orange colonies, production of a gelatin-degrading enzyme and chondroitin lyase, binding of Congo red and growth in the presence of neomycin sulfate and polymyxin B (Griffin 1992).

1.4 Molecular Characterization of Isolates

Columnaris isolates exhibit a great degree of heterogeneity, which has prompted an array of research into their molecular nature. Serological typing of isolates to characterize differences in virulence was one of the first methods utilized to differentiate *F. columnare* (=*Chondrococcus columnaris*) into groups (Anacker and Ordal 1959). Later work used restriction fragment length polymorphisms (RFLP) of the 16S rDNA to separate isolates into three distinct genomovars (Triyanto and Wakabayashi 1999), which was later supported by Darwish and Ismaiel (2005). Arias et al (2004) used RFLP analysis of the 16S rDNA gene, sequencing of the intergenic spacer region, and amplified fragment length polymorphism (AFLP) profiling to differentiate isolates from the United States and Brazil into each of three different genomovars. The US isolates clustered into two distinct genetic groups, with the Brazilian isolates forming a third separate group.

Similarly, isolates derived from neon tetras (*Paracheirodon innesi*) from Asia clustered with other Asian isolates, separate from European and American isolates

(Michel et al 2002). A comparable study used 16S-restriction fragment length polymorphism (RFLP) for genomovar assignment of isolates obtained from wild fish (Olivarees-Fuster et al 2007). Additional confirmation of genomovar assignment was done using multilocus sequence analysis (MLSA), internal spacer region-single strand conformation polymorphism analysis (ISR-SSCP) and amplified fragment length polymorphism (AFLP). These techniques confirmed the division of the isolates into two groups that matched the initial genomovar assignment. The survey of isolates retrieved from channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), freshwater drum (*Aplodinotus grunniens*), and threadfin shad (*Dorosoma petenense)* revealed a significant correlation between host and genomovar. The majority of genomovar I isolates were found in threadfin shad (*D. petenense)* and most of the genomovar II isolates were recovered from catfish (*Ictalurid* species). (Olivares-Fuster et al 2007). Recently, RFLP analysis of partial gene sequences of the 16S rRNA gene was used to differentiate isolates into genomovars and to examine past genomovar designation (La Frentz et al 2013).

Flavobacterium columnare isolates have been shown to have significant variation in virulence to different fish species. The genetic and morphological heterogeneity of selected isolates with respect to virulence was first investigated by Thomas-Jinu and Goodwin (2004), who identified three different genomovars of *F. columnare*, but failed to demonstrate any correlation between genomovar assignment and virulence in either channel catfish or golden shiners, *Notemigonus crysoleucas*. Conversely, Shoemaker et al (2008) found that isolates that caused high mortalities in catfish fingerlings fell into a different genomovar than the less virulent isolates. Similarly, Decostere et al (1998)

demonstrated two different isolates of *F. columnare* had varying degrees of virulence in black mollies (*Poecilia sphenops*) challenged by injection and immersion. Moreover, Soto et al (2008) demonstrated that genomovar assignment of *F. columnare* isolates using pulsed field gel electrophoresis (PFGE) had a strong correlation to pathogenicity to catfish fingerlings. More recently, suppressive subtractive hybridization identified 46 genes in a virulent isolate that were absent in a nonvirulent isolate, suggesting significant genetic variation between isolates (Li et al 2010).

Flavobacterium columnare virulence factors have been investigated by researchers in efforts to determine why isolates differ in their ability to cause mortality in fish. While a number of mechanisms of virulence have been investigated, many questions remain. The ability to adhere to tissue or form biofilms has been a source of interest for researchers as a possible key to virulence. In black mollies exposed to two different *F. columnare* isolates, the more virulent isolate had significantly greater adherence to gill epithelium (Decostere et al 1999). Other research has shown adherence to tissues can vary by host (Olivares-Fuster et al 2011), although no relationship between adhesion and virulence has been determined (Suomalainen et al 2006).

The ability of *F. columnare* isolates to produce degradative enzymes has also been a focus of research. Extracellular proteases that degrade gelatin, casein, hemoglobin, fibrinogen, and elastin have been identified in *F. columnare* (Bertolini and Rohovec 1992). In addition research has shown that increased chondroitin AC lyase activity is correlated with virulence (Stringer-Roth et al 2002, Suomalainen et al 2006).

Flavobacterium columnare, like many bacterial pathogens has metabolic needs that require the acquisition of iron from the host. In a recent study, the virulence of *F.*

columnare isolates to channel catfish after growth in iron-limited media was investigated. Growth in iron-limited media resulted in differential expression of several genes related to iron transport and uptake, reducing mortality associated with a moderately virulent isolate, while lethality of a highly virulent isolate remained unchanged (Beck et al 2015). The upregulated genes involved in iron acquisition may augment iron uptake in more virulent isolates and therefore enhance their ability to initiate an infection (Beck et al 2015).

1.5 Epizootiology

To create a new table in the main body, type "Table" and press F3. Columnaris disease has been referred to as "saddleback disease" or "cotton wool disease" because of the gray areas of discoloration that sometimes occur around the base of the dorsal fin. It has also been referred to as "cigar mouth" or "mouth rot" due to the yellowish appearance inside the mouth associated with colonizing bacteria. This disease effects numerous species inhabiting both fresh and brackish waters. Ictalurid fishes and freshwater eels are considered particularly susceptible in addition to common carp, salmonids, cultured centrarchids, goldfish and tilapia (Bullock and Shotts 1986, Plumb and Hanson 2011, Roberts 2012).

Optimal water temperatures for the disease are between 25 – 32 °C. The lower threshold for disease is considered to be 15 °C, with mortality increasing with temperature (Roberts 2012, Bullock et al 1986). It has been speculated the presence of bacteria such as *Aeromonas hydrophila* and *Citrobacter freundii* may prevent the onset of disease by competing with *F. columnare* on the body surface of fish (Chowdhury and Wakabayashi 1988). Moreover, water that contains high organic matter, specifically

tannins, may have antagonistic or antimicrobial effects on some *F. columnare* isolates (Straus et al 2015).

1.6 Signs of Disease

Columnaris disease in fish often presents with white, yellow or brown necrotic lesions on the gills, skin, or fins as a result of the colonizing bacteria. Lesions may first appear as dull areas on the skin before progressing to open ulcers. The pathognomonic "saddle-back" lesion associated with columnaris infections presents as a white band that encircles the body (Bullock et al 1986). Necrotic skin lesions may have yellowish margins and necrotic gill lesions may become hemorrhagic as the disease progresses. The fins of affected fish may initially become frayed with grayish to white margins that eventually develop into necrotic lesions. Although it should be noted that during the acute stages of infection mortality may occur without any visible signs of disease (Plumb and Hanson 2011).

1.7 Diagnosis

A preliminary diagnosis of columnaris can be made through wet mount examinations of gill biopsies or skin scrapes of an affected area, looking for the characteristic flexing, gliding rod shaped bacteria. Following this presumptive diagnosis, confirmatory culture can be obtained by inoculating a swab or loop from the infected area onto a selective agar such as *Flavobacterium columnare* Growth Media (FCGM) or Ordal's media. These media are often supplemented with the antibiotics neomycin and polymyxin B to limit growth of other commensal agents that may be present in the gills

and skin. *F. columnare* will typically grow into whitish or yellow/orange colonies after approximately 48 hours at 30°C (Bullock et al 1986).

Molecular methods of confirming the presence of *F. columnare* in tissue samples have been developed as more sensitive and selective methods than standard microbiological techniques. A species-specific PCR targeting the 16S – 23S rDNA intergenic spacer sequence of the ribosomal RNA operon was developed and validated for detection of *F. columnare* in catfish tissues (Welker et al 2005). A real-time quantitative PCR is also available which targets the Chondroitin AC lyase gene. This assay offers the ability to estimate *F. columnare* quantities in different tissues (Panangala et al 2007). In addition, a loop-mediated isothermal amplification (LAMP) procedure based on the 16S ribosomal RNA gene was published as a rapid method of detection for *F. columnare* (Suebsing et al 2015). These methods are valuable research tools and offer confirmatory supplemental diagnostic support when used in conjunction with presumptive diagnosis from wet mount examination of lesions or the culture and recovery of viable organisms.

1.8 Treatment

The approved treatments for columnaris infections have been designed to prevent or aid in the recovery of the host from an ongoing infection. Potassium permanganate ($KMnO_4$) has been used as an effective compound for the prevention and treatment of columnaris. Similarly, Perox-Aid® (35% peroxide) has been designated as an effective treatment for columnaris infections. The effectiveness of these compounds and the level of toxicity are influenced by the water chemistry and organic loads in the pond (Bruno and Woo 2011, Plumb and Hanson 2011). Copper sulfate ($CuSO_4$) has also been

investigated as a prophylactic treatment for columnaris in catfish but there are conflicting reports about its benefits (MacFarlane et al 1986, Farmer et al 2013). The chemotherapeutic, Aquaflor® has been shown to be effective at treating columnaris and ESC in catfish. The effective treatment rate is 10 mg/kg of fish for ten days and a twelve day withdrawal period following completion of treatment (Plumb and Hanson 2011).

1.9 PCR

The polymerase chain reaction (PCR) has seen broad application since its initial development by Mullis and Faloona (1987). The specificity of PCR is one of its advantages, with unique regions of an organism's genome being targeted by the assay. The application of PCR to fish diagnostics has been documented by various labs looking to distinguish between phenotypically ambiguous organisms that cause similar clinical signs in fish. In addition to PCR, the use of real-time quantitative PCR (qPCR) to quantify bacteria or other pathogens in tissues and the environment has also been employed in fish health management. Quantitative PCR assays that are precise and sensitive have been developed to detect a number of catfish pathogens, including an emergent virulent strain of *Aeromonas hydrophila*, several *Edwardsiella* species (*E. ictaluri, E. piscicida, E. piscicida*-like sp., and *E. tarda*), *Henneguya ictaluri*, and the digenetic trematode *Bolbophorus damnificus* (Griffin et al 2009, 2010, 2011, 2013, Reichley et al 2015). The qPCR assay has a high level of sensitivity when compared to other methods of detection including traditional PCR (Panangala et al 2007).

Quantitative real-time PCR allows researchers to evaluate the presence or absence of target DNA as the reaction progresses, rather than the end of the reaction as in conventional end-point PCR. In addition to this ability to monitor the reaction in "real-

time", qPCR has the added benefit of being relatively quantitative. As the PCR progresses, fluorescence increases. The cycle at which fluorescence exceeds an established threshold is called the quantification cycle (Cq). The more target DNA present at the start of the reaction, the earlier fluorescence crosses the target threshold. As such, there is an inverse relationship between Cq and the amount of starting template DNA. In short, the lower the Cq, the greater the amount of starting template. By comparing the Cq of unknown samples to concurrently run standards of known template quantity, relative quantification of target DNA can be achieved (Kubista et al 2006).

A set of established principles to guide the development of quantitative PCR assays for aquatic pathogens has been suggested in an effort to standardize methods for validation. The target gene should be carefully considered for its variability and whether other sequences from similar organisms may interfere with the assay. The clinical specificity, sensitivity, repeatability (intra-run variation) and reproducibility (inter-run variation) of the assay should be established before being incorporated into a diagnostic setting (Purcell et al 2011).

The qPCR assay described herein was validated for the detection and quantification of isolates representing the two major genomovars of *F. columnare* associated with catfish aquaculture. The assay was used in challenge studies to determine the ID_{50} dose of each genomovar and evaluate the tissue distribution of each genomovar in channel and blue x channel hybrid catfish. Also, the assay was used to evaluate bacterial concentrations in experimental catfish production ponds over the course of a single calendar year. This qPCR assay and the described applications will expand the

current body of knowledge regarding *F. columnare* and its interaction with different hosts and the environment.

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CHAPTER II

A QUANTITATIVE POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF TWO *FLAVOBACTERIUM COLUMNARE* GENOMOVARS

2.1 Introduction

Flavobacterium columnare, the causative agent of columnaris disease in fresh and brackish water fish, is a globally distributed pathogen. A Gram-negative, rod-shaped bacteria, *F. columnare* produces spreading, rhizoid, discrete yellowish/orange colonies on different selective media following 48 hour incubation at 25-30°C (Plumb and Hanson 2011). The disease was first described following an outbreak in fingerling buffalo fish (*Ictiobus* sp.), with the most notable characteristic being the column-like masses of bacteria observed on wet mounts of affected tissue (Davis 1922). The commercial catfish industry in the southeastern United States has been particularly affected by *F. columnare*, which is the second most common bacterial infection identified in catfish disease case submissions (USDA 2010).

Intraspecific genetic differences among *F. columnare* strains have been observed (Triyanto and Wakabayashi 1999; Arias et al 2004). Thomas-Jinu and Goodwin (2004) investigated the relationship between rapid amplified polymorphic DNA genomovar assignment and virulence in channel catfish *(Ictalurus punctatus*) and golden shiners *(Notemigonus crysoleucas*) and found limited correlation between genotype and

pathogenicity. Later, Soto et al (2008) investigated the relationship between pulsed field gel electrophoresis (PFGE) profiles and virulence. They found that genomovar assignment based on PFGE analysis correlated with a strains' ability to cause disease in juvenile channel catfish (*Ictalurus punctatus*), with one genomovar being more virulent than the other.

Diagnostic methods for identifying *F. columnare* can be hindered due to the slow growth of the bacteria in culture. A preliminary diagnosis can be performed by observing the characteristic "haystacks" formed by the bacteria in wet mount preparation of gills or other affected tissues. Confirmatory tests require 48 hours to produce visible colonies on selective media (FCGM, Shieh, or dilute Mueller-Hinton). Molecular detection using PCR is a faster, more specific confirmatory test than conventional biochemical assays (Welker et al 2005). A PCR has been developed to detect *F. columnare* using the 16S-23S rDNA intergenic spacer region (ISR) of the ribosomal RNA operon (Welker et al 2005). Although specific for identifying the pathogen, standard PCR is semi-quantitative and cannot give an accurate count of the bacteria present in a sample. A quantitative PCR assay amplifying the *F. columnare* chondroitin AC lyase gene has been developed to estimate the bacterial load of *F. columnare* in fish tissues (blood, gills, and posterior kidney) (Panangala et al 2007).

The current manuscript describes validation of a modified quantitative real-time PCR assay for the detection and quantification of the two major genetic groups of *F. columnare*. In addition, this methodology was used to determine the approximate ID_{50} (the dose of an infectious organism required to produce infection in 50% of the experimental subjects) concentration for *F. columnare* strains to channel catfish and

assess dose dependent bacterial loads in catfish tissues following exposure to each genetic group.

2.2 Materials and Methods

2.2.1 Bacterial strains

Flavobacterium columnare strains were chosen based on previous research that divided strains into two distinct genetic groups (Soto et al 2008), with five strains chosen from each group (Table 2.1). Genomovar A strains tend to have moderate to high virulence in channel catfish while Genomovar B strains tend to have lower virulence in channel catfish when compared to Genomovar A strains (Soto et al 2008). Strains were revived from archived cryostocks from diagnostic cases of columnaris from Alabama, Mississippi, and Louisiana. An ATCC strain of *F. columnare* was also included in the analysis. All *F. columnare* strains were cultivated at 30°C for 48 hr on *Flavobacterium columnare* growth media (FCGM) agar. Single colonies from each strain were transferred and expanded in 10-ml of FCGM broth incubated for 24 hr at 30°C while shaking at 200 revolutions per minute (rpm) (Farmer 2004). In addition to the *F. columnare* strains, five strains of environmental or taxonomically significant (strains present in the aquatic environment or related to *F. columnare* by taxonomic classification) non-target bacteria were included in the analysis to confirm specificity of the assay to *F. columnare*: *Pseudomonas aeruginosa* strain ATCC 27853, *Flavobacterium johnsoniae* ATCC 17061, *Edwardsiella tarda C07*-087, *Edwardsiella ictaluri* 93-146, and *Aeromonas salmonicida* LKAS-1 were tested to confirm the specificity of our assay. Each non-target strain was streaked onto Brain Heart Infusion (BHI) agar plates and incubated for 24 hr at 37°C (*E. tarda*, *P. aeruginosa*) or at 30°C (*A. salmonicida*, *E. ictaluri*, *F. johnsoniae*). Individual

colonies were expanded in 10-ml of BHI broth overnight at 27°C. The *Pseudomonas aeruginosa* strain was generously provided by Dr. Frank Austin of the Mississippi State University College of Veterinary Medicine. The *F. johnsoniae*, *E. tarda*, *E. ictaluri*, and *A. salmonicida* strains were obtained from the cryostock archive of the Lawrence/Karsi Research lab at the Mississippi State University College of Veterinary Medicine.

2.2.2 Growth kinetics of 94-081 and ATCC 49512

The growth kinetics of representative strains from each *F. columnare* genomovar was determined (Figure 2.1). Cultures of *F. columnare* strain 94-081, from a diseased channel catfish (*Ictalurus punctatus*) in Mississippi (Genomovar A) and *F. columnare* ATCC 49512 (CIP 103533 [TG 44/87]), from brown trout (*Salmo trutta*) fry in France (Genomovar B) were initiated by reviving cryostocks from cold storage (-80°C) and streaking them onto FCGM agar plates. After 48 hours, isolated colonies were selected and placed in 10-ml of FCGM broth for 24 hours with shaking and the procedure was repeated twice. Dilution plate counts were performed at 8, 11, 12, 13, 15, and 16 hours post inoculation into FCGM broth to evaluate growth kinetics based on culture bacterial density.

2.2.3 DNA isolation from bacterial cultures

Broth cultures (1 ml) of *F. columnare* and non-target organisms were pelleted by centrifugation at 5000 x g for 10 min at 8°C, and supernatant was removed. Genomic DNA (gDNA) was extracted from concentrated pellets using the Qiagen DNeasy Blood and Tissue Kit® following the manufacturers' suggested protocol for Gram-negative bacteria. Purified DNA was quantified using a ND-1000 Spectrophotometer

(Thermoscientific Nanodrop), diluted with Qiagen DNA hydration solution® to 100 ng/µl, and stored cryogenically for later use (-80°C).

2.2.4 Primer and probe optimization

Real-time quantitative PCR primers and probe were designed to amplify and hybridize to a sequence within the chondroitin AC lyase gene of *F. columnare* G4 (GenBank accession number AY912281; Xie, Nie, Chang, Liu, and Yao 2005). The forward primer and probe used in our study were originally developed by Panangala et al (2007). The reverse primer was designed using Primer 3 software (Rozen and Skaletsky 2000) to produce a 203 bp amplicon within the chondroitin AC lyase open reading frame. The reverse primer sequence was: 5'-GTTGTATACACATCCGAAGTTCCAT-3'. Primers and probe were synthesized commercially (Eurofins MWG Operon; Huntsville, AL), with the fluorescence resonance energy transfer probe labeled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3' end with 6-carboxy, N'-tetramethylrhodamine (TAMRA) quencher.

Primers and probe were optimized in a series of 20-µl reactions where quantities of primers (5, 10, 20 pmol/rxn) and probe (0.5, 1, 2 pmol/rxn) were independently varied, resulting in all possible combinations. Optimal primer and probe combinations were identified using gDNA isolated from both *F. columnare* ATCC 49512 and 94-081. The optimized reaction mixture consisted of 7.75 µl of PCR supermix (Applied Biosystems Environmental Master Mix v 2.0; Applied Biosystems, Carlsbad, CA), 10 pmol of each primer, 0.5 pmol of probe and 5 µl of DNA template DNA and nuclease-free water to volume.

Quantitative polymerase chain reactions were run on a Stratagene Mx 3005p platform (Agilent Technologies, Santa Clara, CA) and analyzed using the accompanying software. Reactions were performed in triplicate using the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Quantities of target DNA in unknown samples were estimated by comparison to a concurrently run standard dilution series consisting of known quantities of target DNA (purified PCR product), ranging from $1.0x10^5$ to $1.0x10^0$ copies of the target amplicon.

2.2.5 Generation of qPCR standards

Two different methods were used to purify target amplicons for use as qPCR standards. Briefly, the target PCR amplicon was generated in 20-µl reactions, consisting of 8 µl of PCR supermix (TaqMan Environmental Mastermix, 2.0; Applied Biosystems, Carlsbad, CA), 10 pmol of each primer, 5 µl of gDNA suspension and nuclease-free water to volume. As above, thermal cycling conditions consisted of 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The PCR amplicon was stained with ethidium bromide and visualized after electrophoretic migration under ultraviolet light to confirm the existence of the appropriate sized band. The product was purified using two different commercial PCR purification kits: one utilizes an enzymatic reaction to degrade unused primers and dephosphorylate free dNTPs (ExoSap-IT PCR Product Cleanup; Affymetrix USB®; Santa Clara, CA), and one uses pH manipulation and salt-precipitation to selectively bind DNA to a spin-column membrane (Qiagen QIAquick® PCR Purification Kit). PCR amplicons were quantified spectrophotometrically (Thermo Fisher Scientific Nanodrop ND-1000® Spectrophotometer, Wilmington, DE), to estimate the number of target DNA copies

present. For each method of PCR purification, serial dilutions of the PCR amplicon ($1x10^0$-$1x10^5$ copies) were analyzed in triplicate on three separate occasions to determine efficiency of PCR purification and the linear dynamic range and sensitivity of the assay, as well as assess discrepancies between purification methodologies.

2.2.6 Sensitivity, repeatability, and reproducibility

To establish the sensitivity, linear dynamic range, repeatability, and reproducibility of the assay, serial dilutions of gDNA isolated from ATCC 49512 and 94-081 were analyzed by qPCR. Dilutions ranged from 1.0×10^7 to 1.0×10^1 copies of genomic DNA, estimated based on spectrophotometric quantification and a genome size of 3,162,432-bp for *F. columnare* ATCC 49512 (Tekedar et al 2012). Dilution series were analyzed in triplicate on three separate occasions.

In addition, 10-fold serial dilutions of known quantities of bacteria were quantified from both the ATCC 49512 and 94-081 strains. Briefly, cryostocks of the two strains were revived in 10-ml FCGM broth and grown for 12 hours (late log-phase/early lag phase) after which cultures were serially diluted 10-fold and plate counts performed. Aliquots from each dilution, ranging from $1.2x10^8$ to $1.2x10^1$ for 94-081 and $7.8x10^8$ to $7.8x10^1$ for ATCC 49512 colony forming units (CFUs) were added to a 1.5 ml microcentrifuge tube and stored at -80°C until processing. Genomic DNA was isolated as described above, and gDNA from individual aliquots were analyzed in triplicate on three separate occasions.

2.2.7 Specificity and cross-reactivity

Genomic DNA isolated from broth cultures of all *F. columnare* strains as well as non-target strains was quantified spectrophotometrically, and gDNA preparations were diluted to approximately 10 ng/µl. To test for variation in amplification from different *F. columnare* strains, as well as test for specificity of the assay against non-target organisms, 50 ng of gDNA from each strain was analyzed in triplicate.

2.2.8 Detection in broth culture and fish tissues

Biopsies (~ 25 mg) of channel catfish (*Ictalurus punctatus*) gill, liver, spleen, and posterior kidney were collected aseptically from five fingerling catfish reared for disease research at the Mississippi State University College of Veterinary Medicine in Starkville, MS, placed in 1.5 ml microcentrifuge tubes, and stored at -80°C until processing. At the time of necropsy, fish did not show any clinical signs of disease and were culture negative for *F. columnare*.

Broth cultures (10 ml) of a third isolate not used in fish challenges (*F. columnare* 155-94; Genomovar B) were grown with shaking at 200 rpm for 12 hr at 30°C. Each tissue biopsy was inoculated with 0.1 ml of *F. columnare* 155-94 culture, which was determined by standard plate counts to contain 7.42×10^7 CFU. To identify the approximate recovery and potential inhibitory effects of various catfish tissues on DNA isolation/amplification, an additional five 0.1-ml aliquots of *F. columnare* 155-94 broth culture alone were also processed. Genomic DNA was isolated from spiked tissues and broth aliquots using the DNeasy Blood and Tissue Kit ® (Qiagen, Venlo, Netherlands). . The genomic DNA from inoculated fish tissue samples, as well as broth culture aliquots, was analyzed by *F. columnare* qPCR using the reaction parameters described above. For

each plate, sample and no-template negative controls were analyzed in triplicate alongside concurrently run PCR standards consisting of serial dilutions ranging from $1.0x10^5$ to $1.0x10^0$ copies of the target amplicon.

2.2.9 Infectious Dose for *Flavobacterium columnare*

Disease challenges were conducted with *F. columnare* strain 94-081 (high virulence) and 49512 (low virulence), to determine the dose needed to infect 50% of catfish, 48 hours post-challenge. Initially, a range test was performed using challenge concentrations of $1.1x10^1$, $1.1x10^3$, $1.1x10^5$, and $1.1x10^7$ CFU/ml for 94-081 and $3.7x10^0$, $3.7x10^2$, $3.7x10^4$, and $3.7x10^6$ for 49512. Channel catfish fingerlings ($\bar{x}$= 68.2 mm; 4.6 g) were placed in nine tanks (13 fish/tank; one tank per treatment and a negative control). Challenge doses were achieved by diluting a broth culture initially grown to a concentration of ~$1.0x10^9$ CFU/ml ($1.1x10^9$ for 94-081; $3.7x10^8$ for 49512). Flow of water to each tank was suspended and the volume lowered to 10 L. Each tank received 100 ml of the dilution equating to the target dose for that tank. After 5 hours the flow of water was resumed and maintained for the duration of the experiment. Fish were observed for mortality and dead fish recorded every day for 7 days. Five fish from each tank were randomly sampled 48 hours post challenge to test for *F. columnare* infection. Gills and posterior kidneys were streaked onto dilute Mueller-Hinton agar containing 5 mg/ml of neomycin sulfate and 200 units/ml polymixin B. Gill and kidney tissues (approximately 25 mg), were then placed in separate 1.5 ml tubes and cryogenically stored (-80°C) until processing. Genomic DNA was isolated from frozen gill and kidney tissues using the Qiagen DNeasy Blood and Tissue kit. Fish displaying external/internal signs of disease were noted to compare with culture and qPCR results. The gills and

kidneys from any fish that died during the trial were also cultured to determine the proximate cause of death.

Based on the results from the range test, a second challenge was performed using more targeted doses of 94-081 to identify the median infectious dose. This second 94-081 challenge was performed as described above and consisted of challenge concentrations ranging from $1.9x10^5$, $9.6x10^4$, $1.9x10^4$, and $9.6x10^3$ CFUs No fish died or showed signs of *F. columnare* infection in the initial range test for 49512. As such, no further challenges were performed.

2.3 Results

2.3.1 Growth Kinetics

Plate counts from broth cultures of ATCC 49512 and 94-081 revealed that both isolates grew roughly at the same rate. The concentration of bacteria (CFU/ml) was determined at 8, 11, 12, 13, 15, and 16 hours post inoculation (Figure 2.1). The isolates were within one order of magnitude of each other at all time points, with both cultures entering lag phase at ~12-13 hours post inoculum.

2.3.2 qPCR Sensitivity and Specificity

Based on determined Cq values, ten-fold serial dilutions of gDNA from *F. columnare* ATCC 49512 and 94-081 were linear over six orders of magnitude ($1.0x10^6$ to $1.0x10^1$ copies), with the assay plateauing at approximately 10 copies of bacterial DNA (Figure 2.2). There were no significant differences in the sensitivity of the assay for strain 94-081 (Genomovar A) or strain ATCC 49512 (Genomovar B). Based on obtained Cq values, quantities of target DNA from the two strains were within one order of

magnitude of each other at each dilution. In addition, comparable amplification was observed from similar quantities of gDNA (50 ng) from all ten *F. columnare* strains (Table 2.2); no amplification was observed from any non-target organisms.

2.3.3 Repeatability

The assay was found to be highly repeatable and reproducible, with a SD of +/- 0.83 (Table 2.3) indicating an acceptable level of precision (Bustin et al 2009) for both strains of *F. columnare*. Samples were run in triplicate on three separate occasions to assess interrun variability. The relationship between Cq and CFU was determined from serial dilutions of 94-081 and 49512 broth cultures, using plate counts from each culture to approximate the number of CFUs in each dilution aliquot (Table 2.4).

2.3.4 Spiked Tissues

The assay detected *F. columnare* DNA from channel catfish gill, liver, spleen, and kidney biopsies spiked with 7.42×10^7 CFUs of *F. columnare* strain 155-94. The mean quantity of *F. columnare* DNA detected in spiked gill, spleen, kidney and liver demonstrated less than one order of magnitude between tissues and quantity of target DNA detected was consistent in repeat runs, demonstrating minimal interrun variability (Figure 2.4).

2.3.5 ExoSAP-IT® and QIAquick® PCR Purification

Both purification methods resulted in purified PCR product that could be amplified by the *F. columnare* qPCR assay. However, spectrophotometric quantification of the PCR products purified by enzymatic processes led to an overestimation of copies of target DNA present. There was a discrepancy of nearly one order of magnitude

between the theoretical estimations of purified PCR product and relative DNA quantities estimated by qPCR analysis, which indicated a sensitivity limit of ~100 copies of target DNA. Alternatively, serial dilutions of PCR amplicons purified using spin-column technology corresponded well with theoretical estimations based on spectrophotometric quantification, indicating a linear dynamic range of at least six orders of magnitude and a sensitivity limit of ~10 copies of target DNA, which is consistent with similar qPCR assays (Griffin et al 2009; 2011; 2013; Reichley et al 2015).

2.3.6 Infectious Dose Challenge

There was no mortality in the control tank inoculated with sterile *Flavobacterium columnare* growth media (FCGM). In experimentally infected fish, dead fish had large quantities of bacteria in tissues based on bacterial culture and qPCR results. Mortality was observed within 48 hours in the 1.9 $x10^5$ and 9.6$x10^4$ CFU treatment. Eight fish died in the 1.9$x10^5$ CFU treatment within the first 48 hours, leaving no survivors after the 48 hour sampling. Comparatively, six fish died in the 9.6$x10^4$ CFU treatment, one fish died in the 1.9$x10^4$ CFU treatment, and two fish died in the 9.6$x10^3$ CFU treatment during the 7 day trial. The qPCR estimation of target DNA present gill samples dead fish ranged from 4.4$x10^1$ to 3.8$x10^6$ copies of target DNA. Similarly, qPCR estimation of target DNA in posterior kidney if dead fish was 6.3$x10^1$ to 1.3$x10^4$ copies. Fish that survived to the end of the trial were not cultured unless external or internal signs of disease were apparent. Culture and qPCR results are presented in Table 2.5.

2.3.7 qPCR efficiency

The efficiency (E) of the qPCR was calculated from the slope of the standard curve for each run using the equation $E = 10^{(-1/slope)} - 1$. Values for all runs were within the accepted range of 90-110% (Bustin et al 2009).

2.4 Discussion

Flavobacterium columnare continues to cause significant economic losses to commercial catfish producers. An accurate method of rapid confirmation would be useful to producers and diagnosticians who base critical treatment and biosecurity decisions on the identity of the disease causing agent. Rapid identification allows for treatments to be more effective and cost efficient. The PCR primers and probe described here are specific to *F. columnare*, resulting in robust amplification from representative strains of two *F. columnare* genomovars most often associated with farm-raised catfish. The qPCR protocol described here can provide definitive diagnosis of *F. columnare* infection from infected tissues within hours, as opposed to days using traditional culture methods.

Flavobacterium columnare strains display genetic variability that has been described using various molecular methods (Arias et al 2004, Soto et al 2008). Initial research divided the species into three distinct genomovars (Triyanto and Wakabayashi 1999). Two of these are major groupings, while a third genetic group contains only a few known strains. The implications of the genetic differences between the genomovars are not completely understood, although virulence trials have shown a relationship between *F. columnare* genomovar and virulence in juvenile channel catfish (Soto et al 2008). To

our knowledge, this is the first report of a qPCR technique validated for the two primary genomovars of *F. columnare*.

The sensitivity of the qPCR assay is approximately 10 copies of target DNA. Results of qPCR analysis suggest there were no differences in detection and quantification of *F. columnare* DNA between gill, posterior kidney, and spleen tissues spiked with known quantities of *F. columnare*, and results were consistent with results from broth culture alone. It should be noted, however, the qPCR assay detected fewer copies of target DNA in liver tissue spiked with known quantities of *F. columnare*. Whether this is due to compounds present in the liver that are inhibitory to the PCR or whether this is a function of inefficiencies associated with liver tissue and the DNA isolation procedure is currently unclear.

One fish in the control treatment had one $2.1x10^2$ copies of *F. columnare* target DNA in the kidneys. No other fish in the control group had more than 5. The large quantity of bacterial DNA present in this sample compared its cohorts is unclear. Tank contamination resulting in a subclinical infection in control fish seems unlikely, although it is not outside the realm of possibility. Dead fish examined by qPCR contained between $4.4x10^1$ and $3.8x10^6$ bacteria in the gill tissue and between $6.3x10^1$ and $1.3x10^4$ bacteria in the kidney tissue. In addition, only four out of thirteen fish challenged with the highest dose, $1.9x10^5$ CFU, of 94-081 survived past 48 hours post infection. Comparatively, no fish died in the tanks challenged with ATCC 49512 at any challenge dose. Moreover, the fish challenged with ATCC-49512 did not develop any signs of columnaris disease during the challenge, even with doses as high as $1x10^7$ CFU/ml. In this current study, exposure of juvenile channel catfish to ATCC 49512 did not result in disease or death.

In summary, our results demonstrate a qPCR method that is useful for specific, quantitative detection of *F. columnare* strains from both major genomovars from catfish tissues. Our results further demonstrate this method is accurate for quantification of *F. columnare* from catfish gill, spleen, posterior kidney and liver tissues. However, when the same quantity of *F. columnare* was added to each tissue, there was significantly less *F. columnare* DNA detected in liver than other tissues ($p<.001$). While there was a significant decrease in the bacteria detected in liver tissue, the reasons for this are currently unclear. It is unknown whether this reduction is attributed to compounds in the liver inhibitory to the PCR or if components of liver tissue chemically or mechanically disrupt the DNA isolation efficiency of the Qiagen DNeasy Blood and Tissue kit®. Our data also supports previous studies that suggest differences in virulence for *F. columnare* strains 94-081 and ATCC 49512 in channel catfish. For the first time, we determined an estimated ID50 for strain 94-081 in channel catfish by immersion exposure, which will be useful for future research using our qPCR assay to quantify *F. columnare* in catfish tissues and investigate pathogenesis of columnaris disease.

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Table 2.1 *Flavobacterium columnare* isolates used in this study.

Isolate	Host	Location
Genomovar A (High Virulence)		
94-081	*Ictalurus punctatus*	Louisiana
94-078	*Ictalurus punctatus*	Louisiana
C03-133K	*Ictalurus punctatus*	Mississippi
Matt	*Ictalurus punctatus*	Mississippi
94-060	*Ictalurus punctatus*	Louisiana
Genomovar B (Low Virulence)		
ATCC 49512	*Salmo trutta*	France
155-94	*Ictalurus punctatus*	Alabama
C91-20	*Ictalurus punctatus*	Alabama
143-94	*Ictalurus punctatus*	Alabama
90-509	*Ictalurus punctatus*	Alabama

Note: Genomovar designation and virulence of isolates obtained from Soto et al (2008).

Table 2.2 Average Cq of *F. columnare* and environmentally similar strains.

Strain	Cq	Std Deviation
Genomovar A		
94-081	21.61	0.17
94-078	21.36	0.45
C03-133K	19.67	0.36
Matt	20.96	0.18
94-060	19.60	0.22
Genomovar B		
ATCC 49512	18.68	0.23
155-94	19.52	0.21
C91-20	19.66	0.25
143-94	18.32	1.13
90-509	19.29	0.25
Pseudomonas aeruginosa (ATCC 27853)	NA**	
Flavobacterium johnsoniae (ATCC 17061)	NA**	
Edwardsiella ictaluri (93-146)	NA**	
Edwardsiella tarda (C07-087)	NA**	
Aeromonas salmonicida	NA**	

Note: Strains were diluted to 10ng/µl and run in triplicate to determine average Cq.
**NA = no amplification of DNA.

Table 2.3 Assay repeatability for different genomovars**.**

94-081

Quantity (ng of gDNA)	Average Cq	SD
1.00E+00	38.99	0.33
1.00E+01	38.17	0.73
1.00E+02	35.90	0.76
1.00E+03	32.17	0.47
1.00E+04	28.69	0.39
1.00E+05	25.43	0.46
1.00E+06	20.98	0.39

49512

Quantity (ng of gDNA)	Average Cq	SD
1.00E+00	38.98	0.44
1.00E+01	37.82	0.67
1.00E+02	35.24	0.62
1.00E+03	31.43	0.53
1.00E+04	28.00	0.22
1.00E+05	24.31	0.76
1.00E+06	20.31	0.83

Note: Cq values are the mean amplification for three separate runs of gDNA.
SD: Standard Deviation

Table 2.4 Comparison of Cq and CFUs from plate counts.

49512		
CFU	**Cq**	**SD**
7.80 E+01	34.24	0.94
7.80E+02	33.30	0.73
7.80E+03	31.79	0.46
7.80E+04	28.93	0.31
7.80E+05	25.32	0.33
7.80E+06	21.76	0.40
7.80E+07	19.30	1.00
7.80E+08	15.39	0.20
94-081		
CFU	**Cq**	**SD**
1.19E+01	35.91	2.27
1.19E+02	34.96	1.01
1.19E+03	32.07	1.21
1.19E+04	28.81	1.28
1.19E+05	25.70	1.72
1.19E+06	22.13	1.15
1.19E+07	19.51	0.72
1.19E+08	15.87	0.48

Note: Cq values reported here represent the mean Cq from triplicate reactions from three separate runs

Table 2.5 Results from infectious dose challenge with *F. columnare*, 94-081.

Final Concentration in tank	Culture Results		qPCR results	
Control (FCGM Broth)	Gill	Kidney	Gill	Kidney
	NG	NG	1.18E+01	4.56E+00
	NG	NG	1.73E+01	2.49E+00
	NG	NG	1.55E+01	0.00E+00
	FC	NG	0.00E+00	2.95E+00
	NG	O	3.61E+00	2.07E+02
$9.6x10^3$	FC	NG	0.00E+00	0.00E+00
	NG	O	1.83E+01	7.59E+00
	NG	NG	2.04E+01	0.00E+00
	FC	NG	6.16E+00	7.68E+00
	FC**	FC	1.12E+02	8.01E+02
$1.9x10^4$	NG	NG	0.00E+00	1.87E+02
	NG	NG	4.42E+01	3.84E+01
	NG	NG	0.00E+00	1.20E+00
	NG	O	0.00E+00	0.00E+00
	NG	NG	1.19E+02	0.00E+00
$9.6x10^4$	NG	FC	2.76E+03	5.04E+01
	NG	NG	2.39E+01	6.52E+00
	FC	NG	5.12E+01	3.84E+00
	O	NG	3.42E+01	0.00E+00
	FC	FC	2.68E+03	2.99E+02
$1.9x10^5$*	NG	O	1.96E+00	0.00E+00
	FC	FC	5.51E+01	1.51E+02
	FC	FC	8.10E+01	2.24E+01
	FC	O	2.99E+01	3.78E+01

Note: Gill and kidney samples were taken at 48 post infection.
NG: no growth
FC: *F. columnare*
O: other bacterial species
* Only four fish survived after 48 hours.
** fungus contaminated sample

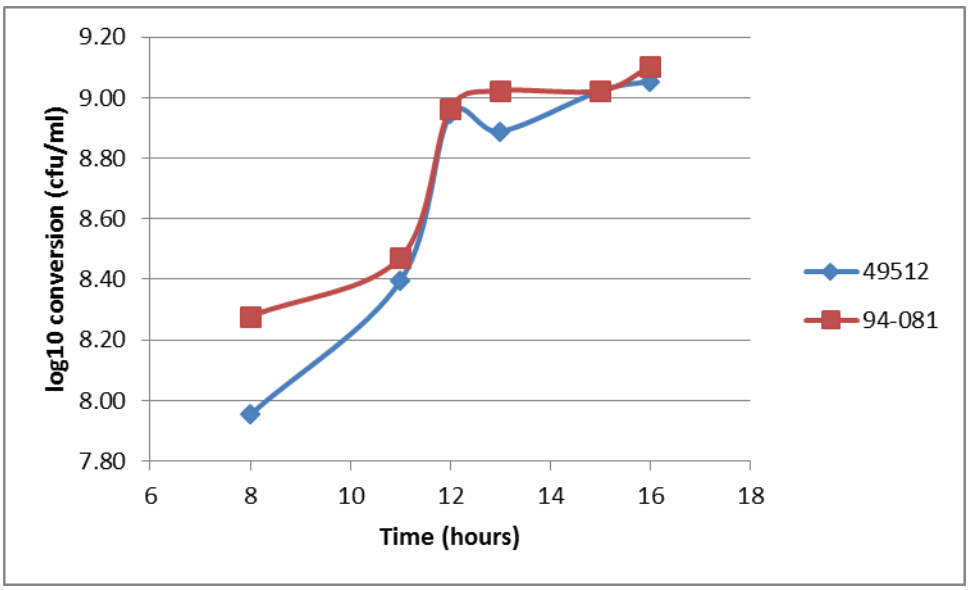

Figure 2.1 Growth kinetics of isolates from both genomovars of *F. columnare*.

Scatterplot showing the growth rates of strains from both genomovars with plate counts made to estimate bacterial concentrations (CFU/ml) in broth culture at 8, 11, 12, 13, 15, and 16 hours post inoculation.

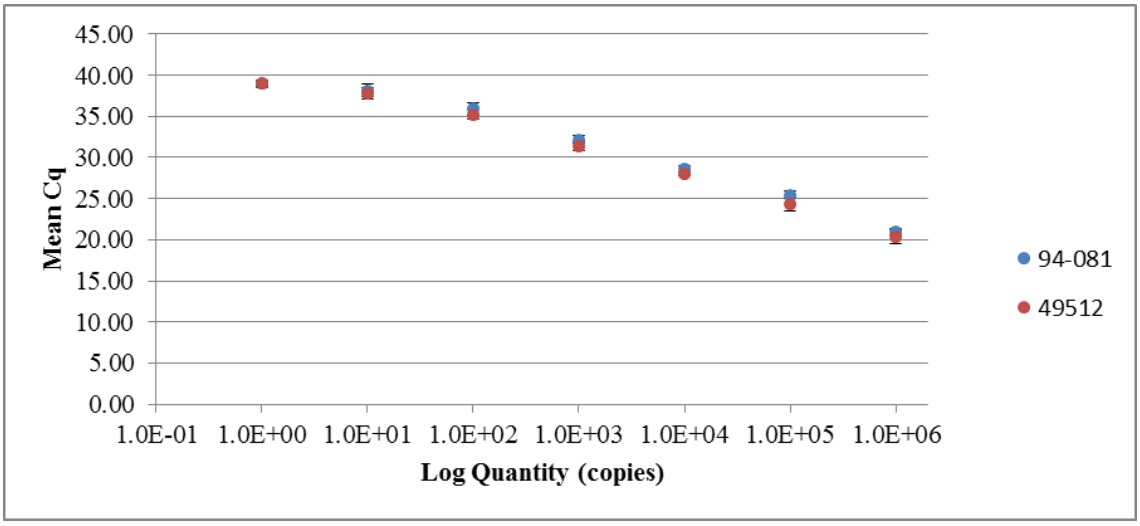

Figure 2.2 Comparison of *F. columnare* isolates from both genomovars.

Scatterplot demonstrating the relationship between mean (Cq) and log quantity of copies of gDNA. Values represent the mean of three separate runs (± SD).

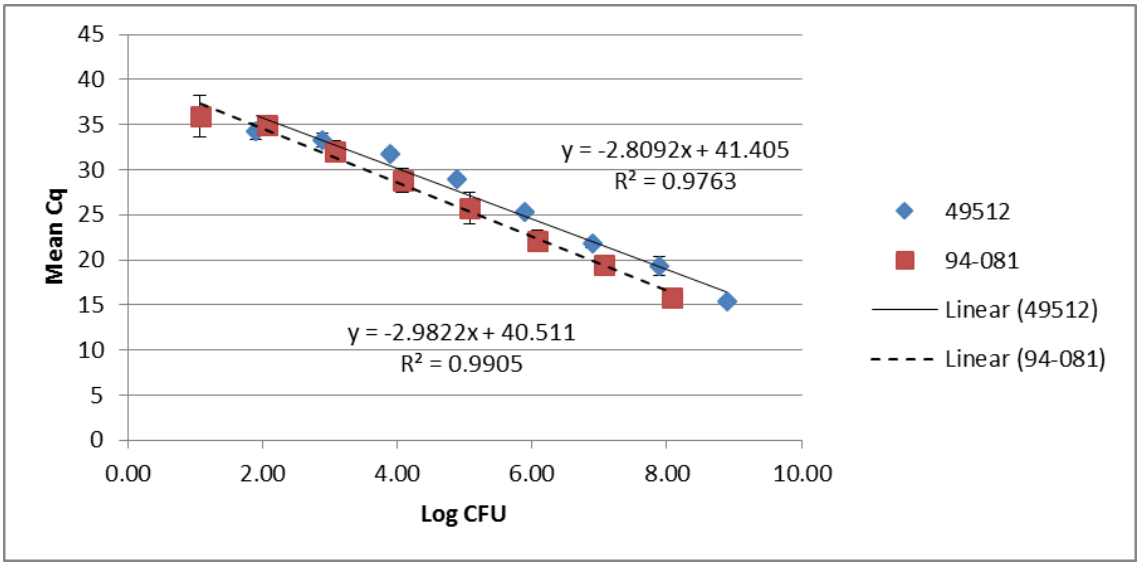

Figure 2.3 Mean Cq/log CFU of two *F. columnare* isolates.

Mean Cq for known quantities of ATCC 49512 and 94-081 on three separate sample preparations. Error bars are +/- the SD for each concentration. Samples were run in triplicate on three separate occasions.

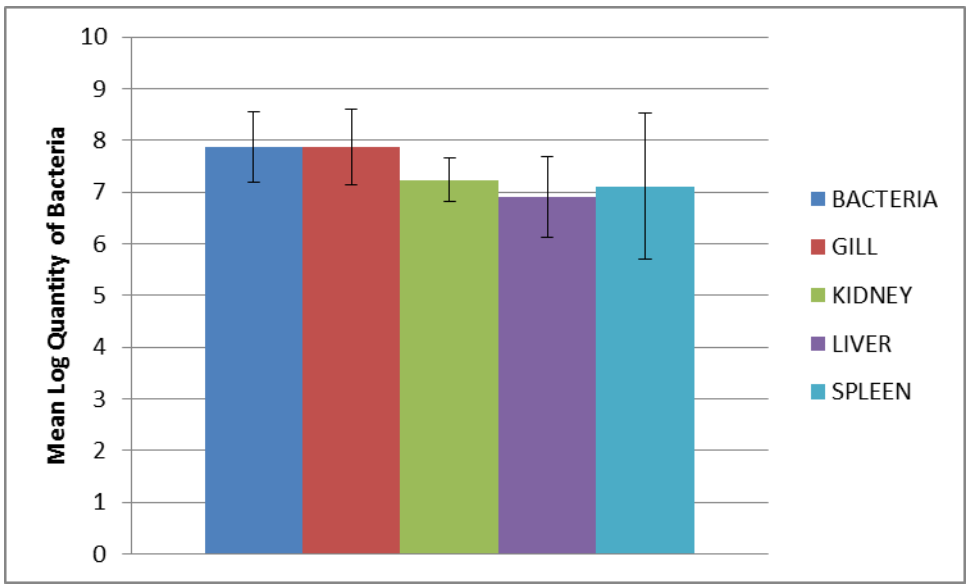

Figure 2.4 Quantity of *F. columnare* detected from a particular tissue using qPCR.

The log quantity of bacteria detected following isolation of bacterial DNA from broth or the respective tissue. Error bars are +/- the SD for the respective tissue.

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CHAPTER III

DISTRIBUTION OF *FLAVOBACTERIUM COLUMNARE* IN TISSUES OF CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) AND HYBRID CATFISH (*ICTALURUS PUNCTATUS* X *I. FURCATUS*) FOLLOWING IMMERSION CHALLENGE

3.1 Introduction

Flavobacterium columnare, the causative agent of columnaris disease, is a Gram-negative rod bacterium, found throughout the world in both wild and cultured fish. It is a significant disease agent in the catfish farming industry of the southeastern United States, where it causes significant losses to catfish producers. It is the second most commonly diagnosed disease agent, behind *Edwardsiella ictaluri*, which causes Enteric Septicemia of Catfish (ESC) (USDA 2010). The disease occurs primarily in the spring and fall, but has been reported year round (Hawke and Thune 1992). Infections of *F. columnare* in fish generally occur during periods of stress, or when there is high organic load in the ponds, although these conditions are not necessary for disease (Wakabayashi 1991). Clinical signs of disease usually involve yellowish-brown sores on the gills, skin or fins, which may include the "saddleback" lesion, a white band encircling the body along the dorsal flanks anterior to the dorsal fin (Roberts 2012). While these signs of disease are commonly indicative of disease, they may not be present in acute infections with rapid mortality (Plumb and Hanson 2011).

Several methods exist to identify *F. columnare*. Initial detection is typically achieved by observing the pathognomonic columnnar (haystack) formations in a wet mount of the affected tissue (Lafrentz et al 2012). This presumptive diagnosis can be confirmed using a selective culture media agar that contain antimicrobial agents (neomycin and polymyxin B) (Hawke and Thune 1992). In addition, Griffin (1992) developed a biochemical testing procedure to positively identify *F. columnare*, while a PCR assay targeting the 16S-23S rDNA intergenic spacer region has been developed to discriminate *F. columnare* from other phenotypically similar species (Welker et al 2005). Moreover, a qPCR assay was recently validated for the detection and quantification of two different *F. columnare* genomovars associated with disease in farm-raised catfish (Chapter 2).

Molecular methods have separated *F. columnare* isolates into two or three distinct genetic groups or genomovars (Triyanto and Wakabayashi 1999; Arias et al 2004; Thomas-Jinu and Goodwin 2004; Soto et al 2008). Following genetic characterization, isolates from both genomovars were used in experimental disease challenges, demonstrating a significant difference in virulence to channel catfish (Soto et al 2008). Recent studies have demonstrated hybrids to be more resistant to *F. columnare* infection than their channel catfish cohort (Arias et al 2012). This is consistent with previous work that has shown the channel (*Ictalurus punctatus*) x blue (*I. furcatus*) hybrid catfish more resistant or refractive to diseases commonly associated with the culture of channel catfish (Wolters et al 1996; Bosworth et al 2003; Griffin et al 2010). Herein, we applied a recently validated qPCR assay (Chapter 2) to estimate quantities of *F. columnare* DNA in tissues of channel and hybrid catfish following immersion challenges with strains of *F.*

columnare representing two different genomovars, one associated with high and one associated with low virulence.

3.2 Materials and Methods

3.2.1 Fish

Channel and hybrid catfish fingerlings were obtained from the USDA Warmwater Aquaculture Research Unit at the Thad Cochran Warmwater Research Center in Stoneville, Mississippi and brought to the College of Veterinary Medicine, Mississippi State University for experimental diseases challenges. Channel catfish averaged 172.6$^{\pm}$15.3 mm (mean/SD) in length and 47.9$^{\pm}$13.0 grams (mean/SD) with hybrids averaging 154.7$^{\pm}$26.2 mm (mean/SD) in length and weighing 32.7$^{\pm}$14.0 grams (mean/SD). Fish were allowed to acclimate for a period of 7 days prior to challenge.

3.2.2 Mortality trial

Fish were stocked in each of twenty-four, 40 L tanks, supplied with flow-through, dechlorinated municipal water (3.8 l/min; 25$^{\pm}$1°C) and constant aeration. Each tank was stocked with twenty channel catfish (12 tanks) or hybrid catfish fingerlings (12 tanks). There were three treatment groups for each strain of fish (4 tanks/treatment). Fish were challenged with one of two isolates of *F. columnare,* one demonstrated to be of low virulence to catfish (ATCC 49512) and one demonstrated to be of high virulence (94-081). A third group was not exposed to any bacteria to serve as negative control.

For the disease challenge, the flow of water was suspended and the volume in each tank was lowered from 30 L to 10 L. Both isolates were grown in FCGM broth with shaking at 200 rpm at 30 °C for 12 hours. One hundred mls of broth culture was added to

each respective tank, resulting in an approximate challenge dose of $4x10^6$ CFU/ml for ATCC 49512 and $2x10^5$ CFU/ml for 94-081. After 5 hours flow of water flow was resumed. The trial was maintained for 8 days, on a 12 hour light/12 dark cycle. Fish were fed to satiation daily and dead fish removed and recorded. Posterior kidneys of dead or moribund fish were cultured on dilute Mueller-Hinton agar containing 5 mg/ml of neomycin sulfate and 200 units/ml polymyxin B and incubated at 30°C for 48 hrs to confirm the presence of *F. columnare*.

3.2.3 Tissue Distribution Trial

Fish were stocked in each of twenty-four, 40 L tanks, supplied with flow-through, dechlorinated municipal water (3.8 l/min; $25^{\pm}1$°C) and constant aeration. Each tank was stocked with twenty channel catfish (12 tanks) or hybrid catfish fingerlings (12 tanks). Similar to the susceptibility trial, there were three treatment groups for each strain of fish (4 tanks/treatment). Again fish were challenged with one of two isolates of *F. columnare,* one demonstrated to be of low virulence to catfish (ATCC 49512) and one demonstrated to be of high virulence (94-081). A third group was not exposed to bacteria and served as controls. The flow to tanks was stopped and the water level was lowered to 10 L for the challenge. Both isolates were grown in FCGM broth with shaking at 200 rpm at 30 °C for 12 hours to an approximate concentration of $1.0x10^9$ CFU/ml ($1.57x10^7$ CFU/ml for 94-081; $6.67x10^6$ CFU/ml for 49512) A 100 ml inoculums of this culture was added to each tank, resulting in approximate challenge doses of $6.7x10^4$ CFU/ml for ATCC 49512 and $1.6x10^5$ CFU/ml for 94-081. The fish were exposed to the inoculums for 5 hours before water flow was resumed. The trial was maintained for 5 days, on a 12 hour light, 12 hour dark cycle and daily feeding. One fish from each tank was sampled 1,

4, 6, 12, 24, 30, 36, 48, 60, 72, 96, and 120 hrs post-challenge. Gills, liver, spleen, and posterior kidney biopsies from sampled fish were placed in individually labeled 1.5 ml micro centrifuge tubes and stored at -80°C until processing.

3.2.4 DNA Isolation

Total genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit®, following the manufacturers' suggested protocol for animal tissues and quantified spectrophotometrically (ND-1000 Spectrophotometer; Nanodrop products, Wilmington, Delaware). Genomic DNA suspensions were diluted to 10 ng/µl with DNA hydration solution® (PureGene DNA isolation Kit, QIAGEN, Limburg, Germany) and stored (-80°C) until qPCR analysis.

3.2.5 QPCR

Real-time quantitative PCR primers and probe were designed to amplify and hybridize to a sequence within the chondroitin AC lyase gene of *F. columnare* G4 (GenBank accession number AY912281; Xie et al 2005). The forward primer and probe used in our study were originally developed by Panangala et al (2007). The reverse primer was designed using Primer3 software (Rozen and Skaletsky 2000) to produce a 203 bp amplicon within the chondroitin AC lyase open reading frame. Primers and probe were synthesized commercially (Eurofins MWG Operon; Huntsville, AL), with the fluorescence resonance energy transfer probe labeled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3' end with 6-carboxy, N'-tetramethylrhodamine (TAMRA) quencher (Table 3.1). The reaction mixture consisted of 7.75 µl of PCR supermix (Applied Biosystems Environmental Master Mix v 2.0; Applied Biosystems,

Carlsbad, CA), 10 pmol of each primer, 0.5 pmol of probe and 5 µl of DNA template DNA and nuclease-free water to volume.

Quantitative polymerase chain reactions were run on a Stratagene Mx 3005p platform (Agilent Technologies, Santa Clara, CA) and analyzed using the accompanying software. Reactions were performed in triplicate using the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Quantities of target DNA in unknown samples were estimated by comparison to a concurrently run standard dilution series consisting of known quantities of target DNA (purified PCR product).

3.2.6 Statistical Analysis

Analysis of qPCR results was performed in SAS for Windows version 9.4 (SAS Institute Inc. Cary, NC, USA). Copy numbers were transformed taking the $\log_{10}$ of the copy number plus 1 to give the data a normal distribution. Separate mixed models using PROC MIXED for each tissue was used to analyze the effect of fish strain, treatment, time points, treatment and fish strain interaction, and treatment and time points interaction on log copy number. Tank identity was included as random effect with a variance component covariance structure specified. To account for the repeated measures of tanks at the different time points, a repeated statement with a spatial power law (SP(POW)) covariance structure was employed. If interaction terms were not significant, they were removed from the model. Differences in least squares means were determined for significant fixed effects. If treatment as a main effect was significant, differences in least squares means with the simulate adjustment for multiple comparisons was used to compare the treatments. If time point as a main effect was significant, differences in least

squares means (Dunnett adjustment for multiple comparisons) was used to compare each time point to the 1 hour time point. If the treatment and time point interaction was significant, the treatments were compared with one another at each time point using an lsm estimates statement with the simulate option for multiple comparisons. An alpha level of 0.05 was used to determine statistical significance.

3.3 Results

3.3.1 Susceptibility Challenge

Water leaks in both a hybrid and channel tank treated with ATCC 49512 resulted in significant losses in both tanks during the 5 hour exposure period. As such, they were removed from the analysis. All of the fish exposed to 94-081 died with the exception of 2 hybrid catfish, which were from different tanks. One of these fish cultured positive for *F. columnare*, while the other fish was culture negative. Fish from tanks exposed to 94-081 exhibited typical signs of columnaris disease: skin lesions, gill lesions, ratty fins, and some fish had a yellow tint due to the abundant amount of bacteria present. Fish started dying in both the channel and hybrid tanks 24 hours post-challenge (Figure 3.1). Comparatively, only five fish died across all ATCC 49512 tanks and no fish presented with external signs of columnaris disease (Figure 3.2). All dead and moribund fish from the 94-081 treatments were kidney culture positive for *F. columnare*, while only one fish from the ATCC 49512 treatments was kidney culture positive. No fish in either control group were culture positive for *F. columnare* and no control fish died.

For the gills, there was significant interaction ($p<0.0001$) between treatment and hour post-infection in regards to the number of copies of *F. columnare* detected. There were significantly higher *F. columnare* detected in hybrid gill tissue versus the gill tissue

of channels (p=0.0154). At 48 hours post-challenge, there were no significant differences in *F. columnare* between the 49512 treatment and the sham challenge using sterile FCGM broth, but there were significantly more copies of *F. columnare* DNA in 94-081 treatment compared to controls (p=0.0083). In addition, there were significant differences between the 49512 and 94-081 treatment, as well as the 94-081 treatment and controls over time.

For the kidneys, there were significant increases in the number of copies of *F. columnare* as the trial progressed (Figure 3.4). There were no significant differences in *F. columnare* detected in kidneys between the ATCC 49512 and 94-081 treatments (p=0.1673), nor were there significant differences between ATCC 49512 and the sham challenge using FCGM broth (p=0.5793). However, the quantity of *F. columnare* in kidneys from the 94-081 treatment was significantly higher than controls (p=0.0183). Similarly, *F. columnare* DNA detected in liver tissue of 94-081 challenged fish significantly increased as the trial progressed (p<.0001), while *F. columnare* DNA detected in spleen tissue (Figure 3.5) also increased over time, regardless of strain.

3.3.2 qPCR efficiency

The efficiency (E) of the qPCR was calculated from the slope of the standard curve for each run using the equation $E = 10^{(-1/slope)} - 1$. Values for all runs were within the accepted range of 90-110% (Bustin et al 2009).

3.4 Discussion

The preliminary challenge supported previous work that demonstrated isolate ATCC 49512 is mostly avirulent to catfish, while *F. columnare* 94-081 is highly virulent

to catfish (Soto et al 2008). Mortality occurred in both channel and hybrid catfish exposed to 94-081. Comparatively, only 3 fish (2 channels and 1 hybrid) died in the tanks challenged with ATCC 49512. The strain of catfish does not appear to affect the ability of the isolate to cause mortality.

The results from the tissue analysis reinforce the practice of sampling gill or kidney tissue when culturing *F. columnare* for diagnostics. The spleen is also an acceptable tissue to sample, but *F. columnare* was not detected in the spleen tissue to the extent that *F. columnare* was detected in gill and kidney tissue. The first 72 hours post-challenge were the most critical in determining the presence of *F. columnare* in the gill, kidney, and spleen tissues. Approximately 30-72 hours post-challenge there was a rapid increase in the quantity of bacteria detected in gill tissue in both channel and hybrid catfish exposed to 94-081, indicating rapid growth within the host tissues, possibly leading to septicemia. This rapid increase in bacterial DNA may indicate the time required for the bacteria to overwhelm the catfish immune system, and the rapid proliferation 48 hours post-challenge may indicate a point at which the fish will no longer recover, even with antibiotic intervention, on account of irreversible damage to the epithelial tissue of the gills and the onset of septicemia.

The differences in virulence between the 2 isolates in the susceptibility challenge is important in determining the pathobiology of *F. columnare* in catfish. At the end of the 8 day susceptibility trial, kidney cultures from fish exposed to ATCC 49512 were negative for *F. columnare*. The absence of *F. columnare* in kidney tissues eight days after challenge with ATCC 49512 suggests that even though ATCC 49512 is infective to catfish, the fish is able to clear the infection more readily than 94-081.

Previous research based on cumulative mortality has indicated that hybrid catfish were more resistant to *F. columnare* infections (Arias et al 2012), but cumulative mortality from the susceptibility trial was not significantly different ($p \leq 0.05$) The channel and hybrid catfish during the susceptibility trial were 100% and 97.5% after eight days. Under these challenge conditions, 94-081 resulted in nearly 100% mortality regardless of catfish strain.

The data from the individual time points suggests that both *F. columnare* isolates can initiate a general septicemia but a healthy catfish's immune system is able to overcome infection by the ATCC 49512 isolate. There was a rapid proliferation in the number of bacteria detected at 96 hours post infection in all of the treatments for both gill and kidney tissue. The reason for this spike is not clear, although at this point in the challenge only moribund fish could be sampled as they were the only fish available in the tank. It is thought, although speculative, that bacteria were being shed by these sick fish, resulting in an increase in the number of bacteria present in the tank and may have been present on the gill tissue simply as a function of being in the water with the fish.

A larger sampling size and a lower range of challenge doses might make differences between channel and hybrid catfish more perceptible and would likely minimize the contribution of outliers to our data set. However, in our challenge model there does not appear to be significant differences in susceptibility of channel and hybrid catfish to *F. columnare* isolate 94-081.

Differences in virulence factors are key in understanding the pathobiology of *F. columnare* isolates causing high losses in catfish. Our experiments demonstrate both 94-081 and ATCC 49512 are capable of infecting catfish, but that only 94-081 is capable of

causing death under these challenge conditions. This supports previous work that suggested that 94-081 was more virulent than ATCC 49512 (Soto et al 2008).

This work identifies the need to discriminate between high and low virulent isolates of *F. columnare* since isolates vary significantly based on their genomovar designation. Managers could make better decisions about how to best deal with an outbreak of columnaris if its virulence could be determined through testing. Our work also suggests, that at least for isolate 94-081, both hybrid and channel catfish are equally susceptible to infection. Future work will focus on fine tuning the assays described here in order to differentiate between different genomovars of *F. columnare*.

Table 3.1 TaqMan real-time PCR primer and probe 5'-3'sequences.

Name	**5'-3' Sequence**	**Tm(°C)**	**%GC**
Fwd	CCTGTACCTAATTGGGGAAAAGAGG	64.6	48
Rev	GTTGTATACACATCCGAAGTTCCAT	61.3	40
Probe	ACAACAATGATTTTGCAGGAGGAGTATCTGATGGG	68.2	42.9

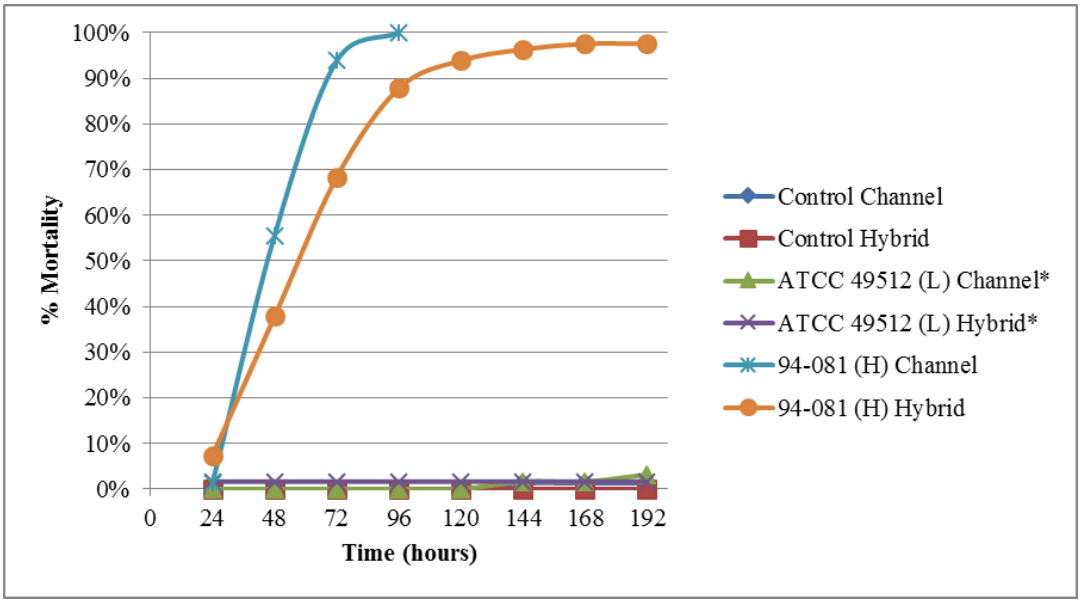

Figure 3.1 Cumulative percent mortality over time in channel and hybrid catfish exposed to two different strains of *F. columnare*.

L = low virulence
H = high virulence

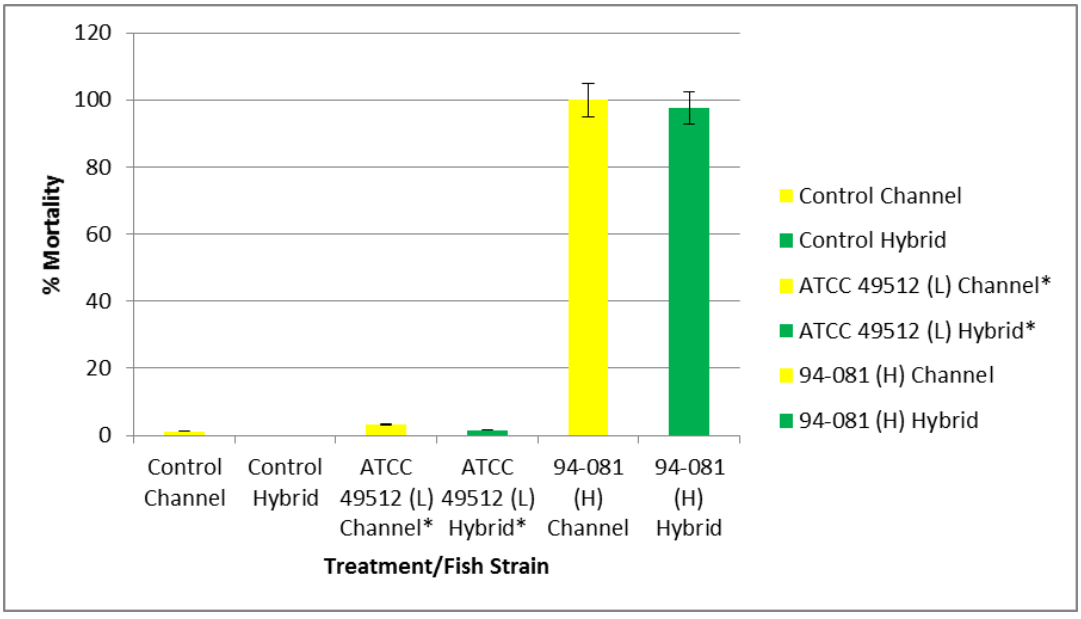

Figure 3.2 Cumulative percent mortality over time in channel and hybrid catfish exposed to two different strains of *F. columnare*.

L = low virulence
H = high virulence

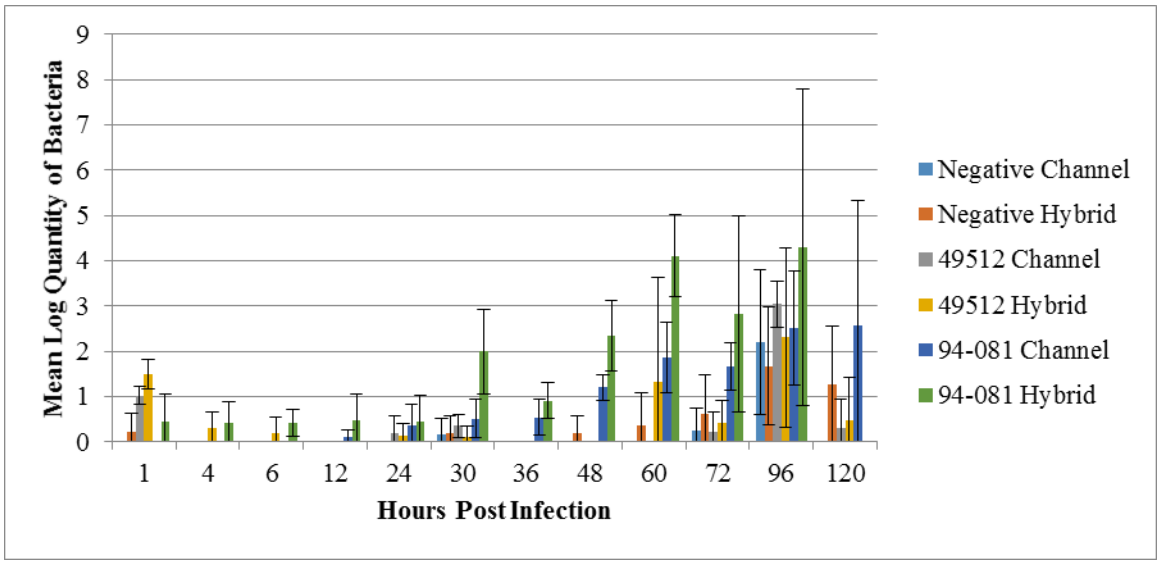

Figure 3.3 Mean (± S.D.) log quantity of *F. columnare* detected in gill tissue.

* No data for 94-081 hybrid at 120 post infection; all fish died.

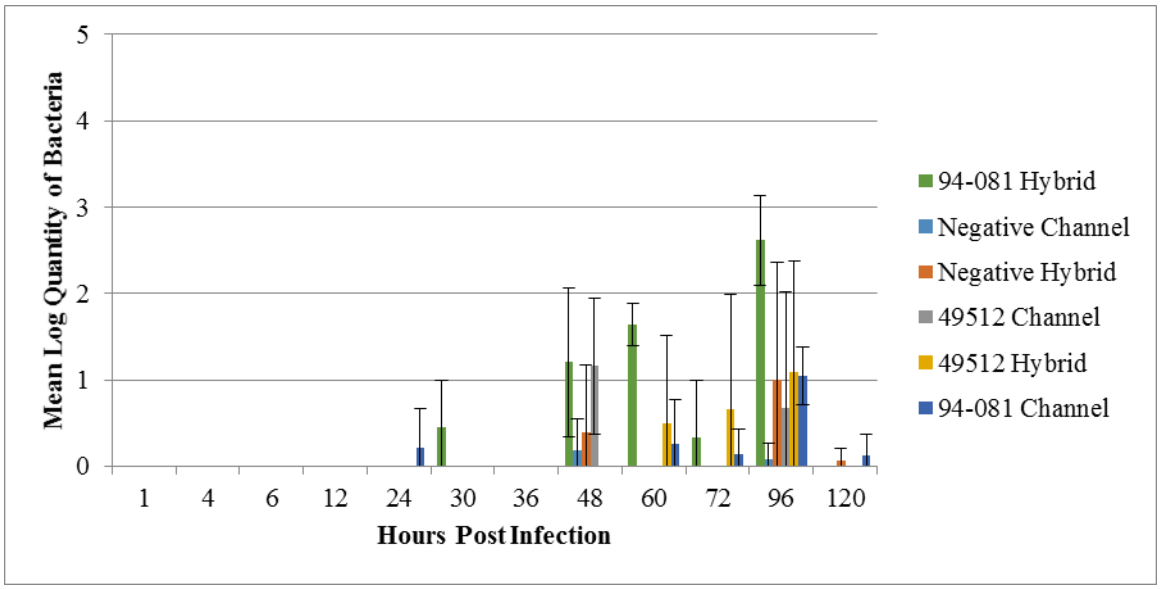

Figure 3.4 Mean (± S.D.) log quantity of *F. columnare* detected in kidney tissue

* No data for 94-081 hybrid at 120 post infection, all fish died.

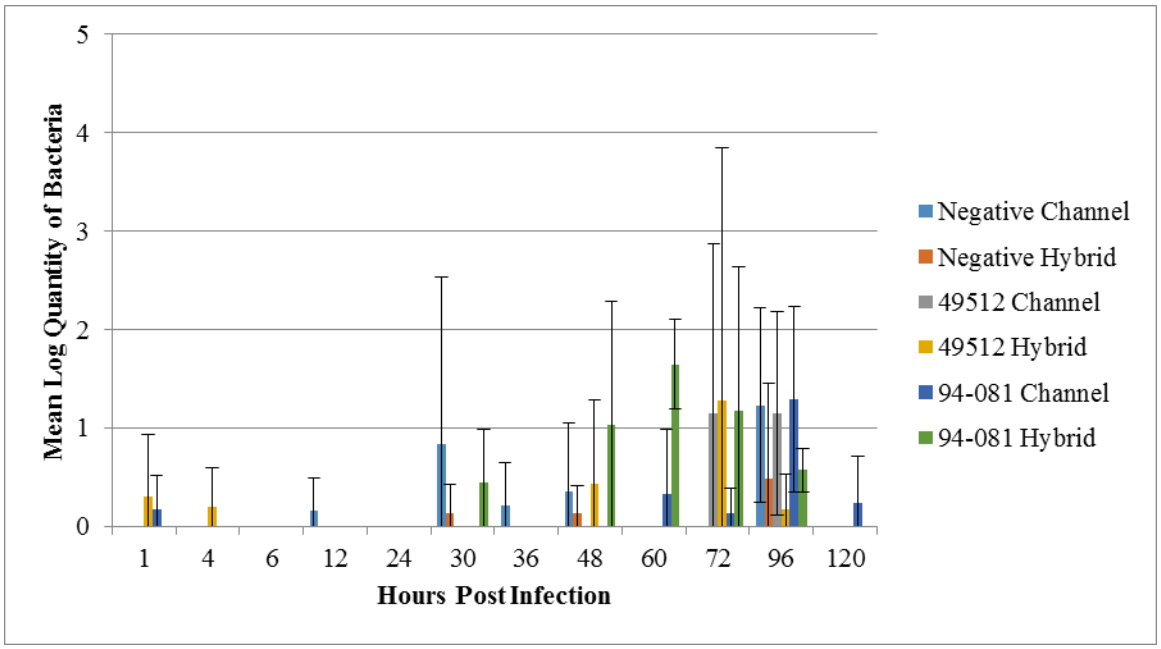

Figure 3.5 Mean (± S.D.) quantity of *F. columnare* detected in spleen tissue

Error bars are +/- the SD for the hours post infection.
* No data for 94-081 hybrid at 120 post infection; all fish died.

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CHAPTER IV

APPLICATION OF A QUANTITATIVE POLYMERASE CHAIN REACTION ASSAY FOR ESTIMATION OF *F. COLUMNARE* CONCENTRATIONS IN POND WATER

4.1 Introduction

Flavobacterium columnare, the causative agent of columnaris disease, is a gram-negative rod considered ubiquitous in most aquatic environments. Disease outbreaks often occur when fish are stressed from crowding, netting, or handling (Plumb and Hanson 2011). *Flavobacterium columnare* affects numerous fresh and brackish water species of fish, but infections are particularly severe in cultured catfish (Plumb and Hanson 2011). In Mississippi, where catfish is cultured extensively, columnaris disease ranks second only behind *Edwardsiella ictaluri*, the causative agent of Enteric Septicemia of Catfish (ESC) (USDA 2010).

The extent to which *F. columnare* affects catfish makes its detection, management, and prevention a priority for producers. Detection based on traditional microbiological techniques involve wet mount observation of haystack formations on affected tissue. This presumptive diagnosis is confirmed by culture and growth of yellowish/orange colonies on low nutrient media such as Shieh agar, often supplemented with the antimicrobial agents' tobramycin, polymyxin B and neomycin (Decostere et al 1997). A polymerase chain reaction (PCR) has been developed targeting the 16S-23S

rDNA intergenic spacer region of the ribosomal RNA operon of *F. columnare* to serve as a more sensitive method of detection (Welker et al 2005). More recently, a real-time PCR probe-based assay was developed to quantify *F. columnare* in gill, kidney, and blood samples (Panangala et al 2007).

The use of real-time PCR to quantify bacteria in environmental samples was shown be effective at detecting fish pathogens in river water (Hallett and Bartholomew 2006) as well as commercial catfish ponds (Griffin et al 2009; 2011; 2013). The use of DNA isolation kits specifically designed to target and remove compounds inhibitory to the PCR has allowed researchers to accurately detect pathogenic DNA from a variety of samples, including catfish pond water, which is high in tannic and humic inhibitory compounds (Griffin et al 2011, 2013, Strepparava et al 2014, Reichley et al 2015).

We recently described validation of a qPCR assay for the detection and quantification of *F. columnare* from the two major genomovars associated with farm-raised catfish (Chapter 2, 3). In this current study, we apply this qPCR method to detect and quantify *F. columnare* in pond water, following 18 experimental production ponds over the course of a single calendar year.

4.2 Materials and Methods

4.2.1 qPCR Assay

Real-time quantitative PCR primers and probe were designed to amplify and hybridize a sequence within the chondroitin AC lyase gene of *F. columnare* G4 (GenBank accession number AY912281; Xie et al 2005). The assay produces a 203 bp amplicon within the chondroitin AC lyase open reading frame. Primers and probe were synthesized commercially (Eurofins MWG Operon; Huntsville, AL), with the

fluorescence resonance energy transfer probe labeled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3' end with 6-carboxy, N'-tetramethylrhodamine (TAMRA) quencher.

Quantitative polymerase chain reactions were run on a Stratagene Mx 3005p platform (Agilent Technologies, Santa Clara, CA) and analyzed using the accompanying software. Reactions were performed in triplicate using the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Quantities of target DNA in unknown samples were estimated by comparison to a concurrently run standard dilution series consisting of known quantities of target DNA (purified PCR product) ranging from $1x10^5$ to $1x10^5$ copies.

4.2.2 Limit of sensitivity, specificity, and repeatability

Water (20 liters) was collected from three catfish aquaculture ponds on the Mississippi Agricultural and Forestry Experiment Station facility in Stoneville, MS. These pond water samples (collected in January) were processed according to previously established protocols for quantification of bacterial DNA in pond water samples (Griffin et al 2011, 2013, Reichley et al 2015). Briefly, 40-ml subsamples from each pond were transferred 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 suspended in 1.5 ml of nuclease-free water and transferred to a 1.8-ml microcentrifuge tube. Total genomic DNA was isolated using the Mo Bio UltraClean DNA isolation kit. Using the validated *F. columnare* qPCR primers and protocols described above, these pond water samples were determined to be *F. columnare* qPCR negative.

These pond water samples were then used to evaluate the sensitivity of the assay in environmental samples. Broth cultures of strains ATCC 49512 and 94-081, representing the two major genomovars of *F. columnare*, were grown to approximately $1.0x10^9$ CFU/ml, confirmed by plate counts on *F. columnare* growth media (FCGM). Ten-fold serial dilutions were made of each strain, and 100 µl aliquots from each dilution (n = 3) ranging from 10^8 to 10^3 CFU were added directly to individual pellets obtained from concentrating 40 ml samples from the three *F. columnare* negative ponds. Two DNA isolation kits were compared for their ability to isolate total DNA from pond water for quantification by qPCR: MO BIO Ultra Clean Soil® kit and MO BIO Powersoil® kit.

4.2.3 Pond Water Sampling

Pond water samples (1 l) were collected from eighteen catfish ponds at the pond facility of the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS. These ponds were part of a continuous production study and were maintained according to standard industry practices (Hargreaves and Tucker 2004) over the course of the sampling period. Ponds were sampled monthly from November 2012 to November 2013. Pond water was taken from the surface of each pond using 1 l containers. After water was collected, containers were shaken to evenly distribute the contents, and 35 ml of water was concentrated by centrifugation at 20,000 x g for 10 minutes. The supernatant was decanted and the pellet suspended in 1 ml of distilled water prior to transfer to a 1.5 ml microcentrifuge tube. The pellet was then concentrated again in a table top centrifuge at 10,000 x g for 1 minute. The supernatant was removed and total DNA was isolated from the pellet using the MO BIO Ultra Clean® kit following the manufacturer's

suggested protocol. qPCR was performed on each sample as described to estimate the concentration of *F. columnare* present in the pond.

4.3 Results

4.3.1 Sensitivity and reproducibility

Genomic DNA isolation from serial dilutions of ATCC 49512 and 94-081 was performed twice using both the Ultra Clean® and the Powersoil® kit. Based on qPCR estimations of *F. columnare* DNA, the amount of ATCC 49512 or 94-081 bacteria added to each aliquot were within a one order of magnitude of each other at each dilution. The assay was linear over six orders of magnitude regardless of DNA isolation kit (Table 4.1). The sensitivity limit of the assay was approximately 10^3 CFU for both strains in spiked samples from all three ponds using both DNA isolation kits. The limit of sensitivity of broth cultures from both strains was also approximately 10^3 CFUs (25 CFU/ml) per sample using both DNA isolation kits. Samples that contained $<10^3$ CFUs (25 CFU/ml) had inconsistent amplification in replicates, suggesting the quantifiable range of the assay is $>10^3$ CFUs (25 CFU/ml). Although the Ultra Clean® and Powersoil® kits detected similar numbers of target copies of DNA, the Ultra Clean® kit had a higher degree of variability (Figure 4.1, Figure 4.2). In particular, there was more inter/intra-plate variation in Cq values obtained from dilutions of pond water with strain 94-081.

4.3.2 Detection in pond water

Pond water sampling began in November of 2012 and concluded in November 2013. *Flavobacterium columnare* was not detected in pond water samples until April of 2013 when two ponds were found positive for *F. columnare*. At this time, total DNA

detected was less than 10 copies per 35 ml for these two ponds. This was also the first month since sampling began in November 2012 that the maximum air temperature for the sample day was greater than 25 °C (27.2 °C). The highest air temperatures occurred during sampling in June, July, and August with temperatures being 33.9, 32.8, and 33.3 °C, respectively. The highest quantities of bacteria detected in individual ponds occurred in May and October, with the highest average *F. columnare* burden among all ponds observed in October (Figure 4.3, Figure 4.4).

4.3.3 qPCR efficiency

The efficiency (E) of the qPCR was calculated from the slope of the standard curve for each run using the equation $E = 10^{(-1/slope)} - 1$. Values for all runs were within the accepted range of 90-110% (Bustin et al 2009).

4.4 Discussion

The versatility of qPCR has led to the development of assays to detect aquatic pathogens from tissues and the environment (Griffin et al 2009, 2011, 2013 Marancik and Wiens 2013, Reichley et al 2015). The qPCR procedure described here is an accurate method for quantification of both major genomovars of *F. columnare* (Soto et al 2008) from pond water. The qPCR accurately quantified DNA from strain 94-081, a highly virulent catfish strain, and ATCC 49512, a strain that is less virulent to catfish. The assay also had good reproducibility, as demonstrated by analysis of serial dilutions from pond water on separate plates. Interestingly, sensitivity of the assay was similar in detecting *F. columnare* DNA from pond water and from broth culture ($\geq 10^3$ CFU).

The UltraClean® and PowerSoil® kits were compared in our experiments to determine if there was a difference in the ability of the kits to purify DNA from *F. columnare* in pond water. The SD calculated from Cq values of individual serial dilutions was ≤ 3.61 using UltraClean® and ≤ 1.81 for PowerSoil® kits, suggesting a slightly greater degree of variability in DNA isolation using the UltraClean® kit. However, the precision for our assay is consistent with those seen in similar assays detecting fish pathogens from pond water (Griffin et al 2011, Griffin et al 2013, Reichley et al 2015). Compared to the UltraClean® kit, the PowerSoil® kit contains an additional step using Inhibitor Removal Technology® (IRT), a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins (MO BIO Laboratories 2013). This step theoretically allows for better detection by removal of PCR inhibitors. The UltraClean® kit was chosen for our monthly survey of ponds because it was less expensive and less labor intensive, yet it retained good precision in detection of *F. columnare* from pond water. However, it is worthy of note that since the completion of our qPCR validation and pond water survey, MO BIO has discontinued the UltraClean® soil DNA kit in favor of the PowerSoil DNA isolation kit.

Flavobacterium columnare is distributed throughout the world and considered ubiquitous in aquatic environments, although the prevalence of *F. columnare* in the environment is poorly understood (Mohammed and Arias 2014). There were 18 samples for each month (except for November 2013); however, only 19.7% (46 out of 234) of the samples were positive for *F. columnare*. These positive samples occurred from April through November of 2013. Water temperature, pH, and dissolved organic matter may

have contributed to the lack of detection for some months. Previous research has revealed that tannins can have antimicrobial properties at sufficient concentrations and that dissolved calcium can also affect survival rates of *F. columnare* (Straus et al 2015).

There were no outbreaks of columnaris disease in any of the ponds during our sampling period. The application of our assay to ponds at the Thad Cochran Warmwater Aquaculture facility in Stoneville, MS revealed that *F. columnare* may not be detected from ponds during typical winter months., or is below the detectable limits of our assay. Pond water testing revealed a biphasic pattern of bacteria detected by the assay. This data suggests that *F. columnare* may reside in different areas within the pond, perhaps in the sediment at times when conditions do not favor its growth. Increases in water temperature may encourage the growth of bacteria while leading to increased susceptibility of the fish. An outbreak of disease would have allowed us to see what levels of bacteria were present in the water and if they matched culture results from fish. The numbers of *F. columnare* in pond water prior to start of mortality might give clues that could be used to predict an outbreak of columnaris. Future research examining the levels of *F. columnare* detected from water samples during an outbreak of disease and what happens to the bacterial numbers present in water following treatment would be useful to researchers. Interestingly, the *F. columnare* genome sequence indicates potential for strains to utilize nitrate as an electron acceptor for anaerobic metabolism (Tekedar et al 2012). Thus, anaerobic pond sediments could be another reservoir for *F. columnare*. Another survey for *F. columnare* could focus on fewer ponds with more sampling points, examining water and sediment.

The use of qPCR adapted for environmental water samples can serve as a useful research tool to investigate environmental quantities of F. columnare. In the current study, we showed that a qPCR designed for fish tissues could be adapted to detect F. columnare in pond water. The quantifiable limit in pond water was approximately 25 CFU/ml for either the MO BIO UltraClean® or PowerSoil® kits. The results from our survey indicate that F. columnare may fluctuate throughout the year due to environmental factors such as water temperature. Use of this assay could provide insight into what factor(s) favor the growth of the bacteria in commercial catfish ponds and when conditions are favorable for an outbreak of columnaris.

Table 4.1 Comparison of MO BIO Soil DNA Isolation kits.

Approximate CFU	**Cq**	**SD**
MO BIO Ultra Clean®		
1.07E+03	39.35†	0.85
1.07E+04	36.40†	2.82
1.07E+05	32.72	3.11
1.07E+06	28.41	3.36
1.07E+07	25.27	3.09
1.07E+08	22.61	3.61
MO BIO Powersoil®		
1.13E+03	37.78†	1.81
1.13E+04	33.43	1.55
1.13E+05	29.93	1.39
1.13E+06	25.93	1.27
1.13E+07	22.56	1.26
1.13E+08	18.54	1.07

Note: Fluorescence threshold was set at 100 for analysis.
qPCR from broth culture samples not included in averages.
Cq: Mean amplification for ATCC 49512 and 94-081.
DNA isolations were done twice for each isolate and kit.
SD: Standard deviation
†At least one replicate in the dilution had no detectable Cq.

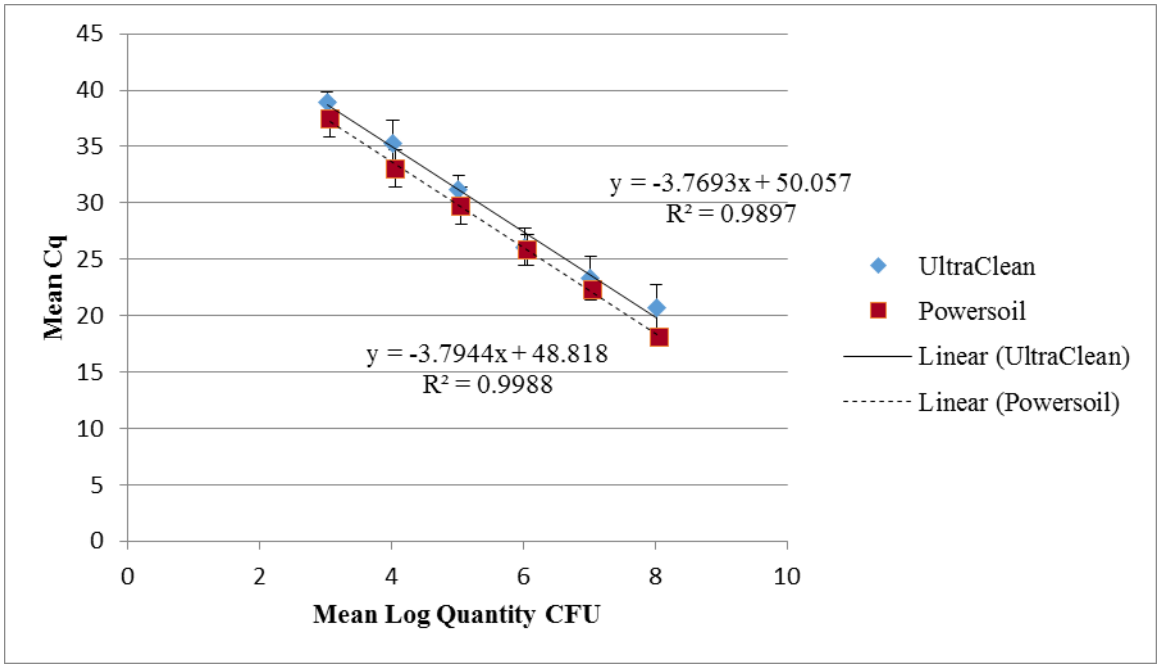

Figure 4.1 Correlation between mean bacterial concentrations and mean qPCR cycle thresholds for *F. columnare* ATCC 49512 DNA isolated by UltraClean® and PowerSoil® kits from pond water

Error bars are the standard deviation for that dilution.

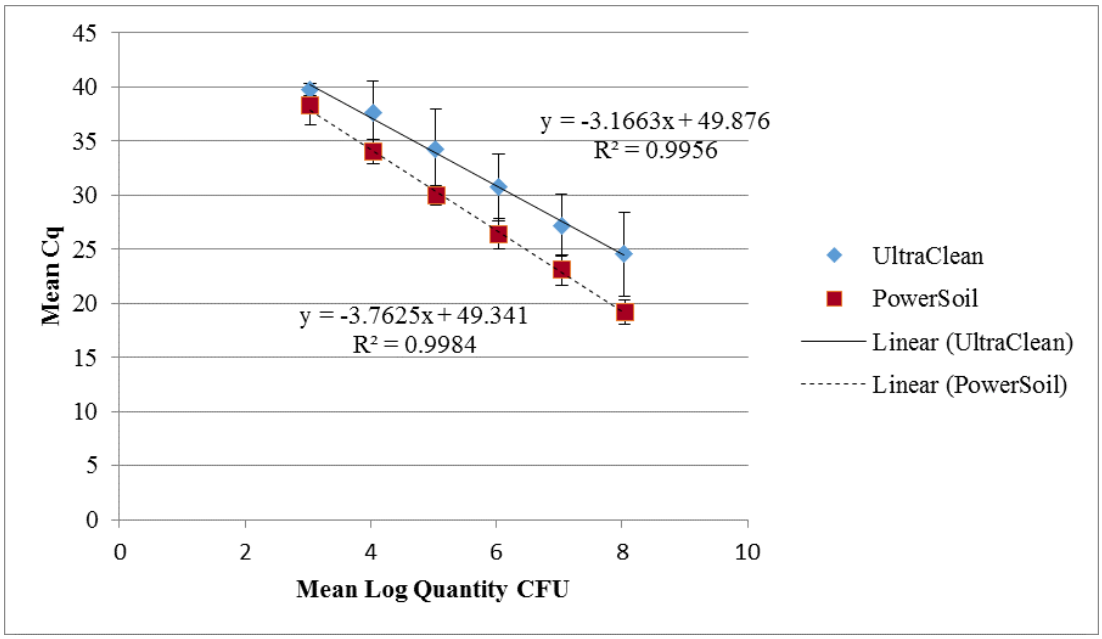

Figure 4.2 Correlation between mean bacterial concentrations and mean qPCR cycle thresholds for *F. columnare* 94-081. DNA isolated by UltraClean and PowerSoil kits from pond water

Error bars are the standard deviation for that dilution.

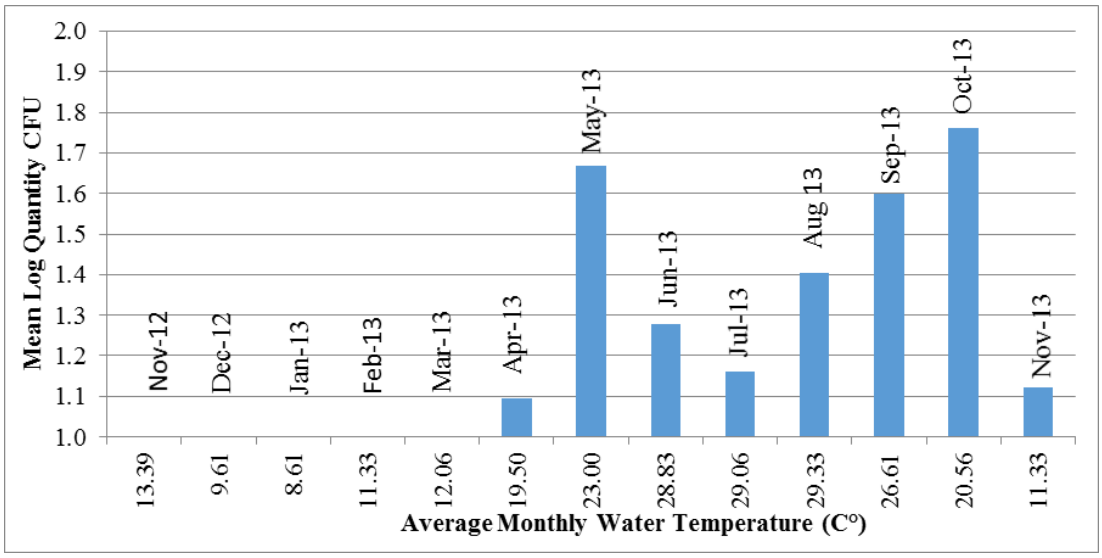

Figure 4.3 Mean log bacterial concentration of *F. columnare* in catfish ponds for each month during the sampling period

Average monthly temperatures from the Thad Cochran National Warmwater Aquaculture Center diagnostic lab.

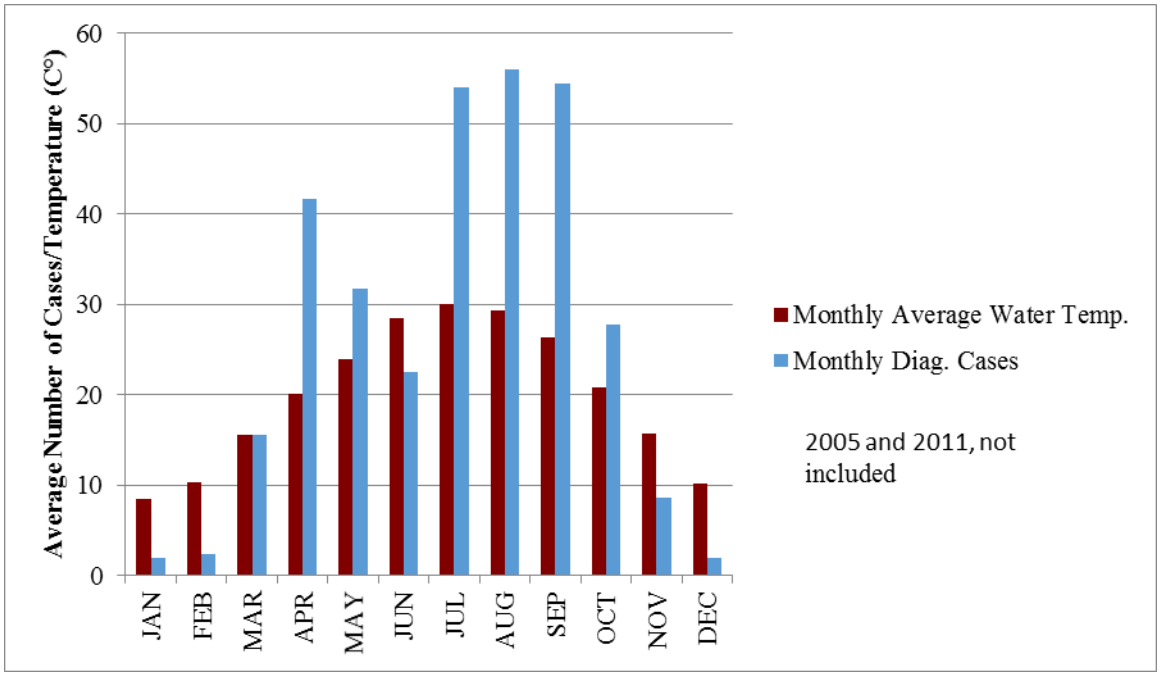

Figure 4.4 Thad Cochran Warmwater Aquaculture diagnostic lab average monthly cases of columnaris and average water temperatures (C°) for 2001 – 2014.

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CHAPTER V

CONCLUSION

Flavobacterium columnare remains a significant pathogen in aquaculture despite efforts by researchers to better understand the pathogen and determine better methods of treatment. It is of particular concern in the Southeastern United States where catfish aquaculture is impacted annually by morbidity and mortality associated with outbreaks of columnaris disease. Current methods of detection require an initial diagnosis by observing characteristic signs followed by confirmation of the pathogen using selective media. The isolates belonging to *F. columnare* constitute a genetically diverse group with the same phenotypic characteristics. While they may be phenotypically indistinguishable, molecular methods have allowed researchers to separate isolates into different genomovars based on these genetic profiles.

The quantitative real–time PCR developed as a result of our research detects isolates from both genomovars of *F. columnare*. Detecting both genomovars is essential since one isolate may cause high mortalities and another may cause limited mortality in catfish. The assay described in our research was highly specific and very sensitive for both genomovars in accordance with similar assays developed for other bacterial pathogens. Molecular methods of detection are advantageous for positively identifying *F. columnare* since it grows slower than many environmentally similar organisms.

The mechanisms of pathogenesis involved in columnaris disease have been studied but many questions remain to be answered by researchers. *F. columnare* is considered an opportunistic pathogen generally causing disease following exposure to environmental or mechanical stressors. The response of the catfish's immune system to different *F. columnare* isolates has not been well documented to date. The research presented in this study indicates that less virulent catfish isolates may cause a general septicemia that is cleared by the host. Further research is needed to clarify these results and identify what mechanisms lead to increased virulence in catfish.

The nature of *F. columnare* within the host environment has not been well studied although it is generally considered ubiquitous. The quantity of bacteria present in ponds during different times of the year and how environmental factors may influence the presence of bacteria has not been documented by researchers. The qPCR assay initially developed to detect *F. columnare* present in tissue samples was validated for pond water samples. The sensitivity of the assay with water samples was comparable to that with tissue samples. The application of our assay to ponds at the Thad Cochran Warmwater Aquaculture facility in Stoneville, MS revealed that *F. columnare* may not be detected from ponds during typical winter months. Pond water testing revealed a biphasic pattern of bacteria detected by the assay. This data suggests that *F. columnare* may reside in different areas within the pond, perhaps in the sediment at times when conditions do not favor its growth.

The research presented in this study has proven the usefulness of qPCR as a research tool to study *F. columnare*. The information presented here represents just the beginning of potential areas to research various aspects into the nature of *F. columnare*.

Applications of this research tool may lead to advances in the early diagnosis of columnaris disease and insight into the complex dynamic that exists in a commercial catfish pond between pathogen and host.