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Genetic and virulence diversity of Flavobacterium columnare

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GENETIC AND VIRULENCE DIVERSITY OF

FLAVOBACTERIUM COLUMNARE

By

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Flavobacterium columnare is a freshwater fish bacterium responsible for columnaris disease, the second leading cause of mortality in pond raised catfish in the southeastern United States. Pulsed-field gel electrophoresis (PFGE) is a particularly powerful tool in epidemiology and is now regarded as the gold standard for molecular typing of microorganisms. We developed methods for conducting PFGE on *F. columnare*, and determined its efficacy for characterizing *F. columnare* strains isolated from different locations in the Southeastern United States.

Virulence diversity was observed in two different immersion challenge experiments conducted with 16 different isolates in channel catfish fingerlings. A direct correlation was found between the PFGE clustered groups and virulence.

In summary, our results suggest that two genetic divisions of *F. columnare* channel catfish isolates exist, one that contains strains that are "primary" pathogens of

channel catfish (Group A), and another that are “secondary” or opportunistic pathogens of catfish (Group B).

DEDICATION

This work is dedicated to my parents, Manuel and Cecilia, and my brothers Quique and Teto. Thanks for believing in my when I lost all faith in myself.

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The author is indebted to committee Co-Advisors Dr. M.J. Mauel and M. L. Lawrence, who sacrificed time, energy, and effort to help me realize my educational goal. Their support, friendship and guidance both as advisors and as persons will always be remembered and I will always by in debt for all the help provided.

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CHAPTER I

INTRODUCTION

Columnaris disease, caused by *Flavobacterium columnare*, has been described as a globally distributed acute to chronic bacterial infection of freshwater and brackish water fishes. Many commercially important species are affected by columnaris disease, including but not limited to the channel catfish *Ictalurus punctatus*, common carp *Cyprinus carpio*, goldfish *Carassius auratus*, rainbow trout *Oncorhyncus mykiss*, Japanese eel *Anguilla japonica*, and tilapia *Oreochromis* sp. Ictalurids are often the most severely affected, but serious infections have been reported in other species as well (Plumb, 1999; Shoemaker et al., 2003). Historically, columnaris disease has been considered the second most important bacterial infection in channel catfish after *Edwardsiella ictaluri*, the causative agent of enteric septicemia of channel catfish (ESC). However, case reports from the Aquatic Diagnostic Laboratory (ADL) of the Thad Cochran National Warmwater Aquaculture Center from 1997 to 2005 have shown *F. columnare* to be the most commonly diagnosed pathogen (Bullock, 1986; NAHMS, 1997; Tucker et al. 2004; ADL, 2005).

Flavobacterium columnare is a thin gram negative rod measuring 2-10 µm in length, with flexing and gliding motility that produces yellow colonies on agar

(Bullock, et al., 1986; Darwish et al., 2004). *F. columnare* can be distinguished from other fish pathogens by its ability to grow on media containing neomycin and polymixin B or by its rhizoid pattern of growth on a low nutrient and low agar containing medium (Plumb, 1999; Griffin, 1992).

Columnaris disease was first described in 1922 by Herbert Spencer Davis, who observed the characteristic columnar masses of bacterial cells isolated from fish during a major die off in the Mississippi River. The bacterium was originally named *Bacillus columnaris*, but the nomenclature for this organism has changed over the years due to changes in phylogenetic classification. The organism had been referred to as *Chondrococcus columnaris*, *Cytophaga columnaris*, and *Flexibacter columnaris* prior to acceptance of the current name, *F. columnare* (Durborow, 1998; Thomas-Jinu and Goodwin, 2004).

Disease outbreaks are often related to environmental stress associated to high stocking densities, increased feeding rates, elevated organic loads, and high temperatures, all of which are commonly encountered problems in commercial aquaculture. Columnaris disease is characterized as an acute to chronic infection of the gills and integument, including fins. Lesions can occur along the dorsal midline and extend posterior to the dorsal fin along the lateral flanks, commonly referred to as "saddleback", but this is more the exception than the rule. Peduncular and peri-oral lesions are much more common lesions of columnaris diseases. The disease is often initiated as an external infection on the fins, body surface, or gills, which can then progress to necrotic lesions with yellowish-orange mucoid material. In some cases,

columnaris can become systemic with little or no visible pathological signs (Bullock et al., 1986; Plumb, 1999; Roberts 2001).

Molecular analysis of the 16S rDNA gene or intergenic spacer regions is the most commonly used method for analyzing the genetic diversity of *F. columnare.* Methods used have included PCR with restriction fragment length polymorphism (RFLP), PCR with sequencing, or PCR with single-strand conformation polymorphism (SSCP) (Triyanto and Wakabayashi, 1999; Arias et al., 2004; Figueiredo et al., 2005; Darwish and Ismaiel, 2005; Schneck and Caslake, 2006; Olivares-Fuster, et al., 2007). Other methods used to analyze the intraspecific diversity of *F. columnare* have included rapid amplified polymorphic DNA-PCR (RAPD-PCR) (Thomas-Jinu and Goodwin, 2004) and amplified fragment length polymorphism (AFLP) (Arias et al., 2004).

In pulsed-field gel electrophoresis (PFGE), the entire genome of an organism can be represented as a distinct pattern of DNA restriction fragments. It is a particularly powerful tool in epidemiology and is often used for typing bacterial isolates, examination of serotype prevalence and evolutionary divergence, and for identifying point sources of outbreaks (Benson and Ferrieri, 2001; Hughes and Sharp, 2001). The broad applicability, high discriminating power, and tremendous epidemiological concordance have made PFGE the gold standard of bacterial pathogen subtyping over the past twenty years (Barrett, et al., 2006). A large variety of restriction enzymes have been utilized to digest chromosomal DNA from different bacterial species to yield specific banding patterns for PFGE typing. Overall, PFGE has proven to be one of the best methods for molecular typing, with higher discriminatory power than methods

based on PCR or restriction enzyme digestion with conventional electrophoresis (Gordillo et al. 1993; Olive and Bean 1999).

In spite of the value of PFGE, only a few pathogenic bacteria of aquatic organisms, namely *Pseudomonas anguilliseptica*, *Aeromonas salmonicida, Streptococcus iniae*, *Vibrio tapetis* and *Vibrio vulnificus* have been analyzed by this technique (Weinstein et al., 1997; Castro et al., 1997; Umelo and Trust, 1998; O'hIci et al., 2000; Blanco et al., 2002; Wong, et al., 2004).

Despite the clinical and commercial significance of *F. columnare* infection, there is a paucity of information regarding the epidemiological relationships between the various bacterial isolates from the southeastern United States, which is principally a warm water aquaculture region. In addition, a better method of subtyping this pathogen can help the aquaculture industry recognize outbreaks of infection, determine the source of infection and recognize particularly virulent strains of *F. columnare*.

The specific aims of this study were to characterize *F. columnare* strains isolated from different geographic regions of the southeastern United States and to investigate the possible correlation between *F. columnare* subgroups and virulence in channel catfish fingerlings.

CHAPTER II

LITERATURE REVIEW

Catfish Industry and Catfish Diseases overview

Aquaculture as described by Landau (1992) is the large scale husbandry or rearing of aquatic organisms for commercial purposes. According to the 2005 Census of Aquaculture, the sales of fish, shellfish and related products has grown by 11.7 percent over the past seven years and has become a multi-billion dollar industry in the United States. Catfish production is the leading aquaculture industry in the United States generating over 46% of the total value of aquaculture production in the United States. This warm-water aquaculture activity has become important in the states of Mississippi, Louisiana, Arkansas, and Alabama. Mississippi led the nation with $250 million dollars in sales of aquaculture products in 2005. Arkansas, Alabama, and Louisiana had sales of aquaculture products topping $100 million. The catfish industry serves as the primary source of economic activity and employment in a number of counties of these southeastern states (Tucker et al., 2004; USDA, 2006).

Historically, infectious diseases were responsible only for a small percentage of fish losses. The high stocking and feeding rates associated with commercial production, in conjuction with the widespread practice of a multiple-batch cropping systems, lack of

biosecurity, limited treatment options, have all contributed to the development of infectious diseases to become the primary limiting factor in catfish production. However disease outbreaks are not uncommon even on well-run facilities (Tucker et al., 2004). In 2005, the Thad Cochran Warm Water Aquaculture Center, located in Stoneville, MS, reported that the bacterial diseases enteric septicemia of catfish (ESC) and columnaris disease dominated the numbers of producer submitted cases. Examined as a single disease, ESC accounted for 14.3% of cases, but in combination with other agents was diagnosed in 31.1% of cases (30.7% in 2004). Alone, columnaris accounted for 20.8% of cases, but in combination with other pathogens, columnaris was present in 49.4% of all cases (40.9% in 2004), making it the most common disease seen by the ADL. Reports from the ADL since 1997 have columnaris as the most common pathogen encountered in channel catfish aquaculture. Columnaris is rapidly becoming the most important pathogen in the cultured catfish industry in the US (Shoemaker et al., 2003; ADL, 2005).

Taxonomic classification

Columnaris disease was first described in 1922 by Herbert Spencer Davis, who observed the characteristic columnar masses of bacterial cells isolated from affected gills and fins during a major die off of fish in the Mississippi River, at Fairport, Iowa. The bacterium itself was not isolated, but it was described in wet mounts and named *Bacillus columnaris.* The nomenclature for this organism has changed over the years due to improvements in phylogenetic classification methodologies. Initially included in

the order Myxobacterales, and the bacteria was renamed *Chondrococcus columnaris* by Ordal and Rucker (1944). One year later, Garnjobst (1945) placed the organism in the genus Cytophaga and suggested *Cytophaga columnaris* as the new name. Buchanan and Gibbons (1974) observed that the columnaris bacterium produced neither fruiting bodies nor microcysts, therefore it was removed from the Myxobacterales, and placed in the order Cytophagales, and renamed *Flexibacter columnaris.* Bernardet and Grimont, in 1989, presented DNA phylogenetic and phenotypic characterization to justify retaining the name *Flexibacter columnaris.* Bernardet et. al (1996) re-described *F. columnaris* and renamed the organism that causes columnaris in freshwater fish *Flavobacterium columnare*. The genus *Flavobacterium*, yellow pigmented bacteria, contains three additional fish pathogens that affect salmonids: *F. psychrophilum, F. branchiophilum,* and *F. aquatile.* (Bullock, 1986; Bernardet and Grimont, 1989; Bernardet et al., 1996; Durborow, 1998).

Phenotypic Description

Flavobacterium columnare, the causative agent of columnaris disease, is a gram negative rod that measures 2 to 10 um long and about 0.5 um in diameter. It is motile by gliding, produces yellow colonies on agar, decomposes several polysaccharides (but not cellulose), and has been described as a facultative anaerobe and chemoorganotroph. Most of the isolates appear as spreading, rhizoid, discrete colonies with yellow centers that adhere tightly to the media and form irregular margins in the media surface (Figure 1).

Yellow colored bacterial colonies are a result of flexirubin pigments. In wet mount preparations, the organisms may be seen to arrange themselves into columnar masses, commonly referred as "haystack formations" (Figure 2).

The organism has a temperature range of 4-37° C, with an optimal temperature range from 25 to 30° C. (Durborow, 1998; Plumb, 1999; Roberts, 2001). Griffin (1992) developed a simple procedure for identifying this bacterium. The procedure takes advantage of five biochemical or cultural characteristics that in combination are believed to be unique to *F. columnare*. These characteristics are: 1) the ability to grow in the presence of neomycin sulfate and polymixin B; 2) the production of typical thin, rhizoid, yellowish colonies; 3) the ability to degrade gelatin; 4) the ability to bind congo red; and 5) the production of chondroitin lyase (Griffin, 1992). Biophysical and biochemical characteristics of *F. columnare,* according to Plumb (1999) are listed in Table 1.

Table 1. Biophysical and biochemical characteristics of *F. columnare* (Plumb, 1999).

Characteristic	*Flavobacterium columnare*
Cell morphology	Long, Gram-negative rod
Cell size (um)	0.3-0.5 X 3-10
Yellow pigmented colony	+
Motility	Gliding
Flexirubin pigment	+
Binds Congo Red	+
Resistant to neomycin sulfate, polymixin B	+
Chondroitin lyase	+
α- nitrophenyl-β-D-galactopymanoride	-
Growth on peptone	+
Glucose source of carbon	-
Acid from carbohydrates	-
Degradation of Gelatin Casein Starch Tyrosine	 + - - -
Urease	?
H_2S	+
Nitrate reduced	-
Catalase	+
Cytochrome oxidase	+
Optimum growth at (°C)	25-30
Growth tolerance (°C)	10-37
G + C content (mol%)	32-37
Habitat	Freshwater (saprophytic)

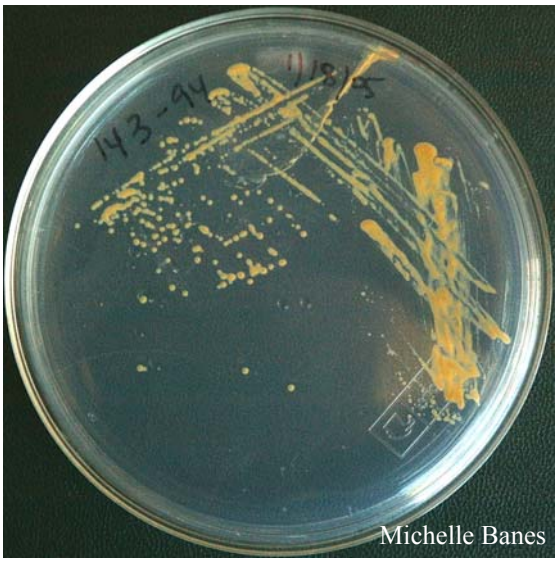


Figure 1. *F. columnare* isolate 143-94 in *F. columnare* growth medium (FCGM) agar.

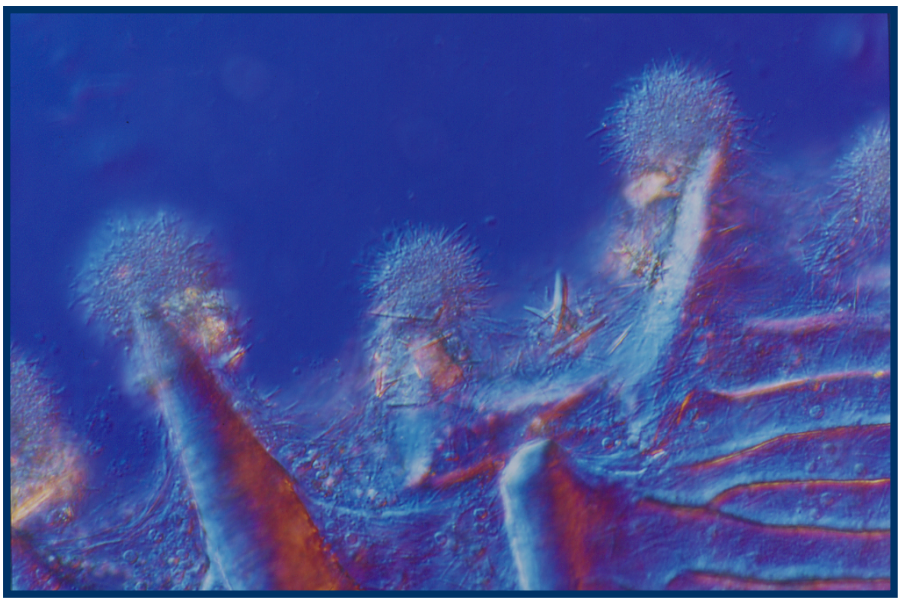


Figure 2. Columnar masses of *Flavobacterium columnare* colonizing the scale of a Tilapia nilotica (*Oreochromis niloticus*) and acquiring "haystack" formation.

Epizootiology

Columnaris disease, also known as "Saddleback disease", or "Fin rot", is one of the most common bacterial infections seen in freshwater fish aquaculture. It has been described as a globally distributed infection of freshwater and brackish water fishes. Many commercially important species are affected by columnaris, including but not limited to channel catfish *Ictalurus punctatus*, common carp *Cyprinus carpio*, goldfish *Carassius auratus*, rainbow trout *Oncorhyncus mykiss*, Japanese eel *Anguilla japonica*, and tilapia *Oreochromis* sp. Ictalurids are often the most severely affected, but heavy infections have been reported in other species as well. According to Plumb (1999), no wild or cultured freshwater fish, including ornamental fish in aquaria, are totally resistant to columnaris (Noga, 1996; Plumb, 1999; Shoemaker et al., 2003).

Transmission of *F. columnare* can be indirect via the environment or by cohabitation with carrier fish shedding the bacterium. Direct transmission can result from contact with infected fish. Although it can survive in the aquatic environment and mud, fish serve as the primary reservoir (Bullock, 1986; Noga, 1996; Welker, et al., 2005).

The disease may occur as a primary or secondary infection and clinically has been described as having a chronic, acute, or peracute onset (Roberts, 2001). Several factors play an important role in the development of the disease. Water quality related factors include high mineral content in the water (hardness), low salinity, low dissolved oxygen, organic pollution and high nitrite. Additional factors associated with outbreaks include physical injury (net damage), starvation, crowded conditions, and repeated and

rough handling (Soskopf, 1993; Noga, 1996; Plumb, 1999; Decostere et al., 1999; Altinok and Grizzle, 2001; Shoemaker et al., 2003). Stoskopf (1993) reported that columnaris disease is usually more pathogenic at temperatures above 15° C, and that mortality and acuteness of disease onset typically increase with temperature. Shoemaker et al. (2003) demonstrated that deprivation of feed for at little as 7 days reduced innate resistance of catfish to *F. columnare*. Although columnaris is diagnosed year round in channel catfish aquaculture, during the late spring and early fall there is an increase in the number of diagnosed cases. This could be due to the water quality and management changes present during the summer months.

Different virulence factors have been described for *F. columnare*, but virulence mechanisms are in generally poorly characterized and more research in this area is needed for a complete understanding of the pathogenesis of the disease. Newton et al. (1997) partially characterized 23 proteases derived from channel catfish raised in the southeastern United States. Proteases are believed to be important virulence factors in the development of columnaris diseases. Two of the most studied degradative enzymes are chondroitin AC lyase and hyaluronidase which degrade chondroitin sulfates A and C, and hyaluronic acid respectively. Hyaluronan and chondroitin are components of the extracellular matrix, thus suggesting that this enzyme plays an important role in the severity of the invasion, necrosis and tissue destruction associated with *F. columnare* (Newton, et al., 1997; Stringer-Roth, et al., 2002; Xie, et al., 2005). Suomalainen et al. (2006) found that this enzyme was significantly related to strain virulence at 25° C when tested in vivo using rainbow trout, *Oncorhynchus mykiss*. Lipopolysaccharide

(LPS) and capsular polysaccharide has been demonstrated to play important roles in columnaris pathogenesis by contributing to the structural integrity of the bacteria and protecting them from host immune defenses (Vinogradov et al., 2003; Bader et al., 2005; Zhang et al., 2006).

Columnaris is often diagnosed in association with one or more other pathogenic organisms. According to the ADL, in 2006 columnaris diseases singly accounted for 13.7% of cases, but in combination with other pathogens, *F. columnare* was present in 68.4% of all cases (49.4% in 2005), making it the most common disease seen by the ADL. ESC and columnaris were diagnosed together in 14.8% of case submissions. The incidence of these two diseases have remained relatively consistent over the past 9 years, where on average ESC was diagnosed in 37.2% and columnaris in 44.1% of all cases.

According to Chowdhury and Wakabayashi (1989) and Plumb (1999), the survival and infectivity of *F. columnare* declines in the presence of other species of bacteria. In vitro testing by researchers found that the survival of *F. columnare* was reduced in presence of either *A. hydrophila* or *C. freundii*, and in the case of in vivo infection experiments, *F. columnare* infection did not occur in the presence of competitive bacteria.

Clinical Signs

Columnaris disease is characterized as an acute to chronic infection of the gills, the integument (including the fins), and oropharynx (Noga 1996; Plumb 1999; Roberts

2001). The severity of the disease is dependent on the virulence of the strain, and environmental factors such as water quality and other stressors. *F. columnare* can act both as a primary pathogen or a secondary opportunistic pathogen. Lesions usually begin externally on body surfaces and gills, and tend to vary with the fish species. Commonly in catfish peduncular and peri-oral lesions are present causing detrimental effects to fish health. Skin lesions usually develop into hemorrhagic- necrotic erosions and ulcers, which may present a whitish or yellowish coloration (due to masses of pigmented bacteria), surrounded by a ring of inflamed skin. Lesions on the gills are usually less common, but when present are more severe and detrimental to the health of the fish. Gill lesions begin at the tip of the lamella and colonize all the way to the gill arch, usually producing necrotic lesions. As the bacteria colonize the skin and gills, they can penetrate deeper tissues and become systemic, predisposing the fish to secondary infections like aeromoniasis and saprolegniasis (Bullock, 1986; Stoskopf, 1993; Noga 1996; Plumb, 1999; Roberts, 2001). The importance of systemic columnaris infections is poorly understood, and only Hawke and Thune (1992) have reported the presence of inflammatory changes (swelling) of the posterior kidney in some cases of columnaris diseases.

Diagnosis

A rapid, presumptive diagnosis of columnaris disease can be given after observing typical lesions and long, thin, rods (0.5 to 1.0 X 4 to 10 µm) in wet mounts preparations made from scrapings of gill, or skin lesions. Organisms are most numerous

in the spreading margins or expanding lesions. One important characteristics of this gram negative rod is its flexing and gliding pattern of motility and the formation of "haystacks" in wet mounts allowed to stand 5-10 minutes following preparation (Noga, 1996; Plumb 1996). According to Roberts (2001) microscopic examination of tissue lesions can reveal epidermal spongiosis and necrosis, with ulceration and extension of the necrosis into the dermis. Hyperaemia and hemorrhages are often seen at the periphery of the lesions. In some cases, the long thin rods can be observed in the tissues better visualized with Giemsa than Gram or Hematoxylin and Eosin stains.

Culture of the bacteria is possible and different media have been described for the routine culture. Common bacteriological isolation media such as Blood Agar, Brain-Heart infusion, and tryptic soy agar will not support the growth of this pathogen. *F. columnare* requires specialized, fresh media, with low nutritional and high moisture content. Selective media containing antibiotics can help reduce the presence of contaminating bacteria. Griffin (1992) recommended the use of Polimixin B and neomycin sulfate as two options. Farmer (2004) described and compared different media for isolation, maintenance and broth culture growth. According to his research, the low nutrient content of selective Cytophaga agar (SCA) gave the best results for inhibiting other bacteria and promoting the growth of *F. columnare* in agar media. Farmer also reported that *Flavobacterium columnare* growing media (FCGM)has faster growth, higher yields of cells, and seemed to prevent the clumping of cells, thus giving the best results for broth culture. Recommended temperatures for this bacteria vary from 25-30° C for 48 hours (agar) or 24 hours (broth).

Definitive diagnosis can be achieved by biochemical testing (as shown in table 1), and by molecular methods. Several molecular methods have been described for the definitive diagnosis of *F. columnare*. Several researchers have focused on the sequence of the 16S rRNA and the16S-23S rDNA intergenic spacer region for the construction of species-specific PCR primers to identify and discern *F. columnare* from other related and common water bacteria (Toyama et al., 1996; Tiirola et al., 2002; Bader et al, 2003, Darwish et al., 2003; Welker et al., 2005). Researchers have also developed serological techniques, such as an indirect ELISA and indirect fluorescent antibody test (IFA) for the detection of humoral antibody responses against *F. columnare*, which are useful tools to determine natural exposure to the organism and for rapid diagnosis (Shoemaker et al., 2005; Panangala, 2006).

Management of Columnaris disease

Good management practices are desirable in any aquaculture operation and form the basis of all diseases prevention programs. Ideally, fish should never be handled when in weakened condition or under environmental stress. Since aquaculture practices are inherently stressful, every attempt should be made to minimize the impacts of stress through proper management of water quality parameters, feeding practices, and stocking densities among others.

Treatment options for columnaris disease are limited and often ineffective. Potassium permanganate is commonly used at a dose of 2 mg/l (5.4 pounds per acre-foot) or at a higher concentration if the water organic load is high. Copper sulfate (0.5

ppm) is also a common treatment option used against columnaris. However, neither treatment is approved by the FDA and their effectiveness is challenged by several diagnosticians (Durborow et al., 1998; Plumb, 1999; Camus, 2007). According to Plumb (1999), the most effective way to treat this disease is a combination of potassium permanganate and Oxytetracycline-medicated feed. Noga (1996) speculates that the potassium permanganate treatment could stimulate anorexic fish to eat the antibiotic feed. However, the use of oxytetracycline is not approved for treatment of columnaris disease in catfish or other species and falls under extralabel use. Suomalainen et al (2005) has reported the use of high concentrations of salt and low pH, as another treatment option. These conditions significantly reduced the numbers of viable bacterial cells following in vitro exposures. Studies conducted by Thomas-Jinu and Goodwin (2004) compared common treatments for columnaris and reported that antibiotic treatment with oxytetracycline or a combination of sulfadimethoxine and ormetropin (Romet ®) in feed prior to a bacterial challenge resulted in no mortality. The authors demonstrated that the most effective bath treatment was achieved using Diquat, a herbicide labeled for aquatic use. Immersion treatments with chloramine-T, potassium permanganate, hydrogen peroxide and copper sulfate showed some reduction in mortality, but was not effective against acute columnaris. In 2007, U.S. Food and Drug Administration (FDA) approved the conditional approval of Aquaflor®-CA1 (florfenicol) Type A medicated article for the control of mortality in catfish due to columnaris disease associated with *Flavobacterium columnare*. Aquaflor®-CA1 is the first FDA conditionally approved drug for columnaris disease in catfish. Aquaflor®-

CA1 is also the first drug that FDA has conditionally approved under the Minor Use and Minor Species (MUMS) Animal Health Act. The potential for an effective vaccine exists, but has not been developed to the point of common usage by catfish farmers.

Flavobacterium columnare diversity

Several investigators have focused their studies on *F. columnare* heterogeneity. Shamsudin and Plumb (1996) characterized morphological, biochemical, and physiologically different isolates of *F. columnare*. They reported that some of the morphological characteristics differ between strains (mainly colony morphology), but the biochemical characteristics varied little among the isolates. This study also demonstrated minor physiological variation between isolates such as the ability to grow at 15°C, on media with 0.5% NaCl, at pH 6 or less, or at pH 10 or higher. Studies by Decostere et al. (1998) also illustrated variations in temperature growth ranges among isolates recovered from tropical and temperate fish species.

Decostere et al. (1998), (1999a), (1999b), Decostere (1999), and Thomas- Jinu and Goodwin (2004) all demonstrated differences in virulence among strains of *F. columnare*. These studies have reported variation in virulence of *F. columnare* when infecting different fish species (channel catfish (*Ictalurus punctatus*), black mollies (*Poecilia sphenops*), common carp (*Cyprinus carpio* L.), golden shiners (*Notemigonus crysoleucas*)) by intramuscular injection or immersion exposure methods. Research comparing lipopolysacharide and protein profiles from different strains of *F. columnare*

again demonstrated variation in virulence among isolates (Newton et al., 1997; Stringer-Roth et al., 2002; Zhang et al., 2006).

The advent of molecular methods such as restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), polymerase chain reaction (PCR), end-label sequencing, DNA-DNA hybridization, rapid amplified polymorphic DNA, and amplified fragment length polymorphism have given researchers valuable tools to identify and analyze the genetic variability of *F. columnare.* The above mentioned tools have provided a better understanding of the intraspecies genotypic diversity of *F. columnare* isolates cultured from a large number of hosts from various geographical regions (Bader et al., 1998; Triyanto and Wakabayashi; 1999, Michel et al., 2002; Thomas-Jinu and Goodwin, 2004; Arias, et al., 2004; Figueiredo et al., 2005; Darwish and Ismaiel, 2005; Schneck and Caslake, 2006; Olivares-Fuster, et al., 2007).

Pulsed-Field Gel Electrophoresis

In pulsed-field gel electrophoresis (PFGE), the entire genome of an organism can be represented as a distinct pattern of DNA restriction fragments, and it is a particularly powerful tool in epidemiology. PFGE is often used for typing bacterial isolates, examination of serotype prevalence, evolutionary divergence, and for identifying point sources of outbreaks (Benson and Ferrieri, 2001; Hughes and Sharp, 2001). The broad applicability, high discriminating power, and tremendous epidemiological concordance of PFGE has made it the gold standard of bacterial

pathogen subtyping over the past twenty years (Barrett, et al., 2006). PFGE is essentially the comparison of large genomic DNA fragments after digestion with a restriction enzyme. A large variety of restriction enzymes have been utilized in the digestion of many different bacteria, and their specific banding patterns have been defined by PFGE. Overall, PFGE has proven to be a superior method for molecular typing when compared to PCR and restriction enzyme digestion with conventional electrophoresis (Gordillo et al. 1993; Olive and Bean 1999).

Only a few pathogenic bacteria of aquatic organisms, namely *Pseudomonas anguilliseptica*, *Aeromonas salmonicida, Streptococcus iniae*, *Vibrio tapetis* and *Vibrio vulnificus* have been analyzed by PFGE (Weinstein et al., 1997; Castro et al., 1997; Umelo and Trust, 1998; O'hIci et al., 2000; Blanco et al., 2002; Wong, et al., 2004).

CHAPTER III

MATERIAL AND METHODS

Bacterial strains

Our study included 30 clinical isolates of *Flavobacterium columnare* obtained from channel catfish cultured in the southeastern United States from 1989 to 2006 and the ATCC-49512 isolate from France. Isolates were identified by both morphology and by PCR using species-specific primers (Welker et al. 2005) (Table 2).

The 20 µl PCR reaction was composed of 0.2 µM of each primer, 200 µM of dNTPs, 1.5 mM $MgCl_2$, 0.5U of *Taq* DNA polymerase and 1X buffer B (Promega Corporation, Madison, WI), and approximately 100 ng of template DNA. Cycling conditions consisted of an initial denaturation step of 5 min at 94°C, followed by 30 cycles of 30s at 94°C, 45s at 56°C, and 60s at 72°C; with a final extension step of 10 min at 72°C, on an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The PCR product was subjected to electrophoresis on a 1.2% agarose gel and stained with Gelstar® nucleic acid stain (Cambrex, East Rutherford, NJ).

Table 2. *Flavobacterium columnare* isolates examined in this study.

Isolate	Host	Location	Year
Dickerson	Channel catfish	Georgia	1995
ATTC 49512	Brown trout	France	1989
94-060	Channel catfish	LSU	1994
CO3 133K	Channel catfish	Starkville, MS	2003
94-081	Channel catfish	LSU	1994
C00 84-4	Channel catfish	Starkville, MS	2000
94-078	Channel catfish	LSU	1994
1191-B			
155-94			1994
C56-1	Channel catfish	Starkville, MS	2006
EVANS 2	Channel catfish	Georgia	1995
ALG9249-1C		Georgia	
92-002	Channel catfish	Louisiana	1992
MATT	Channel catfish	Stoneville, MS	2006
S05-79	Channel catfish	Stoneville, MS	2005
1-19-05 #2	Channel catfish	Stoneville, MS	2005
90-509			1990
90-497	Channel catfish	MS	1990
C91-20	Channel catfish	MS	1991
L90-659	Channel catfish	MS	1990
L90-629	Channel catfish	MS	1990
143-94	Channel catfish	Louisiana	1994
90-252	Channel catfish	MS	1990
90-106	Channel catfish	MS	1990
523-03	Channel catfish	Stoneville, MS	2003
S06-177	Channel catfish	Stoneville, MS	2006
S03-487	Channel catfish	Stoneville, MS	2003
521-03	Channel catfish	Stoneville, MS	2003
S03-579	Channel catfish	Stoneville, MS	2003
S04-280	Channel catfish	Stoneville, MS	2004
S05-233	Channel catfish	Stoneville, MS	2005

In situ DNA isolation and PFGE

F. columnare strains were grown on *Flavobacterium columnare* growth medium (FCGM) agar plates at 30°C for 2 days. Cells were harvested, resuspended in 5 ml of FCGM broth, and incubated in a shaking incubator for 24 hrs at 30°C to obtain final optical density at 600 nm (OD_{600}) of 0.7. Genomic DNA was prepared following the CHEF Genomic DNA Plug Kits Instruction Manual (Bio-Rad) with some modifications. Cells were harvested by centrifugation at 10000 x g for 10 min at 4°C, the supernatant was discarded, and bacterial pellet resuspended in 0.3 ml of cell suspension buffer (BioRad). The cell suspension was mixed with an equal volume of 2% (w/v) low-melting-point agarose (BioRad). Solidified plugs were incubated in lysis buffer containing lysozyme (BioRad)) for 3 hours at 37°C. The original lysis buffer was replaced with additional buffer containing Proteinase K (BioRad)), and the plugs were incubated at 56°C for 48 hrs. Plugs were washed 4 times with Wash Buffer (BioRad) (2^{nd} and 3^{rd} washes with PMSF). *Mlu*I and *Pme*I (New England BioLabs) were used for restriction endonuclease digestion according to the manufacturer's instructions. DNA fragments were resolved by pulsed field-gel electrophoresis (PFGE) using a CHEF Mapper System (Bio-Rad).

Electrophoresis of digested samples was performed through 1% pulsed field certified agarose (BioRad) under the following conditions: running time 36 hours, temperature 14°C, voltage gradient 6 V, initial pulse time 1.17 s, final pulse time, 10.30 s, included angle 120º. Gels were stained with 0.5 mg of ethidium bromide, and DNA was visualized by UV transillumination.

PFGE pattern analysis

Initially, 5 *F. columnare* isolates were screened by PFGE using enzymes *Mlu*I, *Bam*HI, *Pme*I, and *Pac*I to determine which restriction endonucleases produced the most informative DNA profile comparisons.

Macrorestriction patterns (MRPs) were analyzed both visually and by computer-aided methods. Bionumerics (Applied Maths, Inc.) version 3 software was used to normalize the DNA fragment migration distances relative to those of the lambda ladder and small DNA marker (New England BioLabs). Isolates with a DNA band pattern differing by ≥ 1 band were defined to have a distinct PFGE profile.

The similarities between MRPs were expressed by Pearson coefficient correlation. Clustering by the unweighted pair group method using arithmetic averages (UPGMA) was used for dendrogram construction.

Experimental infection of fish

The first experimental infection study was conducted to determine the virulence of 16 different *F. columnare* isolates grouped by PFGE. The chosen isolates were clustered in Group A (90-106, 1191-B, 94-060, 94-081, C03133K, C0084-04, Matt, S05-79), and group B (90-509, C91-20, 143-94, 92-002, ATTC-49512, EVANS, ALG-92491, and C56-1). The challenge method used by Thomas-Jinu and Goodwin (2004) was followed with some variations. Briefly, bacteria for challenges were prepared by plating previously frozen isolates of *F. columnare* on FCGM agar plates for 48 hours at 30° C and transferring colonies to 800 ml of FCGM broth in a shaking incubator for 24

hours at 30° C. The OD_{600} of the FCGM broth culture was diluted to 0.8 using a spectrophotometer (Thermo Spectronic, Madison, WI, USA) to ensure a consistent concentration of bacteria for challenges.

A week prior to experimental infections, channel catfish fingerlings were transferred into 37-L experimental tanks (15/tank), which were supplied with flow-through carbon-filtered municipal water at approximately 25°C. For experimental infections, water flow was stopped, and water level was dropped to 10 L of water in each tank. One-hundred mL of bacterial culture was added directly to the water in each tank, with four replicate tanks for each bacterial strain. Four control tanks of 15 fish were exposed to sterile FCGM broth. After a 5-h exposure with constant aeration, water flow was restored in each tank to gradually remove bacteria. Feed was restricted throughout the experiment. The water temperature was maintained at approximately 25°C, and light was controlled on a 12 hour cycle. Fish were held for 8 days and observed for clinical signs and mortality. Posterior kidney samples of all moribund and dead fish were cultured on FCGM agar, and the identity of the bacteria isolated from at least three fish per isolate from different tanks was evaluated and confirmed by PFGE.

A second experimental challenge was performed to determine whether compromises in the fish skin/mucus barrier would increase the pathogenicity of *F. columnare* isolates. Four different *F. columnare* isolates were compared, two from group A (94-081 and Matt) and two from group B (143-94 and C56-1), and three different skin treatments (abraded, skin mucus removal, and un-abraded fish) were

applied. The bath immersion protocol followed during the first experimental challenge was repeated with some variations.

Thirty minutes before bacterial challenge, fish were sedated in a separate tank containing approximately 100 µg/ml tricaine methane sulphonate (Argent Chemical Labs, Redmond, WA, USA) (MS222). After sedation, 180 fish were abraded with a sterile scalpel in the dorsolateral abdomen. Abrasions were approximately 2 cm in length and penetrated through the epidermis and dermis. Skin mucus was removed from another 180 fish by dragging a paper towel twice, back and forth, along the lateral line and dorsal portions of the abdomen. A third group of 180 fish received no skin treatment and were considered the un-abraded controls. Following skin treatment, fish were returned to their respective treatment tank and allowed to recover from the anesthetic prior to challenge. Three tanks containing 15 fish each served as controls and were sham exposed to uninoculated FCGM broth as previously described.

Statistics

Percent mortalities from immersion bath experiments were compared using the General Linear Model utility of the SAS v. 9.1 software package, and statistical differences between groups were determined by the least significant difference test at $P < 0.01$.

CHAPTER IV

RESULTS

PCR

The primer pair FCISRFL and FCISRR1 reported by Welker et al. (2005) yielded the expected amplified fragment based on the 16S-23S rDNA intergenic spacer region (ISR) of the ribosomal RNA operon from all 31 isolates included in the study. ISR amplicons from *F. columnare* ranged in size from 500 to 550 bp, as reported by Welker (2005).

PFGE

Based on the initial screening of restriction endonucelases, *Mlu*I and *Pme*I were selected for genome similarity comparisons. Due to the clarity of the banding profile, *Mlu*I was chosen for the fish challenge trial.

PFGE of *F. columnare* chromosomal DNA digested with *Pme*I yielded 11-17 fragments in the 32.08 – 243.07 kb range, while *Mlu*I digested DNA yielded patterns of 11-19 fragments in the 24.49 – 192.51 kb range. The PFGE patterns on the same isolate for each restriction endonuclease were found to be stable and reproducible on at least three separate gels. All isolates showed a high degree of genetic diversity, with unique

PFGE patterns resulting from both *Mlu*I or *Pme*I digestion. PFGE DNA profiles produced by nine of the isolates using the restriction endonucleases *Mlu*I (Fig. 3A) and *Pme*I (Fig. 3B) are provided.

Clustering of the *Mlu*I restriction patterns divided the isolates into two groups (designated A and B), with 75.37% and 86.30% similarity, respectively, within the groups (Fig. 4). Between the two groups, there was 63.97 % similarity. No distinctive geographical correlation was found between the clustered subgroups. Clustering of the strains using *Pme*I restriction patterns showed an overall 82.38 % similarity index (data not shown). Again, the isolates were clustered into two groups with 83.57 % and 84.56 % similarity. No clear geographical correlation was found with *Pme*I.

Experimental infection

Clinical signs of columnaris disease (Bullock et al., 1986; Plumb, 1999; and Roberts 2001) were found in the challenged fish. Typical columnaris signs seen included "saddleback" lesions, fin necrosis and skin ulceration with yellowish discoloration (Fig. 5). Final percent mortalities from the first bath immersion experiment for the 16 *F. columnare* strains tested are shown in Fig. 6. Overall, the strains that were clustered into group A by *Mlu*I restriction pattern (90-106, 1191-B, 94-060, 94-081, C03133K, C0084-04, Matt, and S05-79) were more virulent in channel catfish fingerlings, with an average percent mortality of 60.8% after 8 days. By contrast, group B isolates (90-509, C91-20, 143-94, 92-002, ATTC-49512, EVANS, ALG-92491, and C56-1) had an average percent mortality of 8.5%. These results

indicate that the isolates of group A are significantly more virulent to healthy un-abraded channel catfish fingerlings than group B isolates ($P < 0.01$). *F. columnare* was reisolated from the kidneys of the morbid fish, and a complete PFGE profile analysis was performed (Figure 7). Results demonstrated that, in every case, the same *F. columnare* strains that were used for the experimental infections were isolated from the kidneys of dead fish. None of the control fish died.

In the second experimental challenge, higher mortality rates were found in the abraded and skin mucus removal treatments than in the un-abraded treatment (Figure 8). Group A isolates (94-081 and MATT) were found to cause average percent mortalities of 90, 87, and 100% in the un-abraded, skin mucus removal and abraded fish treatments, respectively. Group B isolates (143-94 and C56-1) caused average mortalities of 20, 86, and 66%, in the un-abraded, skin mucus removal, and abraded fish treatments, respectively. Overall percent mortality was significantly lower in the un-abraded treatment. No significant difference was found between the abraded and skin mucus removal treatment ($P<0.01$) (Figure 9). When compared as PFGE-subgroups, only un-abraded fish exposed to PFGE Group-B isolates, showed significantly lower percent mortalities when compared to the other treatments. There was not a significant difference in percent mortalities between un-abraded, skin mucus removal and abraded fish when exposed to PFGE-Group A isolates (Figure 10).

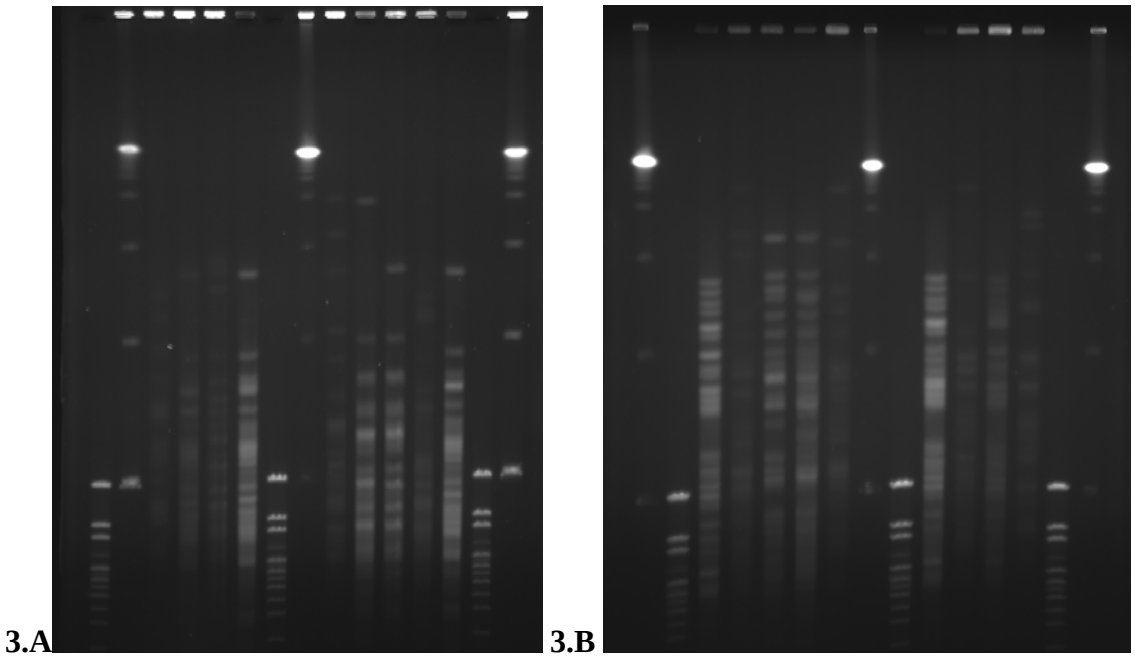


Figure 3. PFGE profiles of *Flavobacterium columnare* strains. (A) *Mlu*I digestion, Lanes: 1, 7, and 14, 8-48 kb PFGE molecular size marker, 2, 8, 15, Lambda Ladder molecular size marker, Lane 3, S05-79, Lane 4, 90-106, Lane 5, 90-252, Lane 6, 143-94, Lane 9 L90-629, Lane 10, L90-659, Lane 11, C91-20, Lane 12, 90-497, Lane 13, 90-509. (B) *Pme*I digestion, Lanes: 1, 8, 15, Lambda Ladder molecular size marker, Lane 2, 9, and 14, 8-48 kb PFGE molecular size marker, Lane 3, 90-509, Lane 4, 90-497, Lane 5, C91-20, Lane 6, L90-659, Lane 7, L90-629, Lane 10, 143-29, Lane 11, 90-252, Lane 12, 90-106, Lane 13, S05-79.

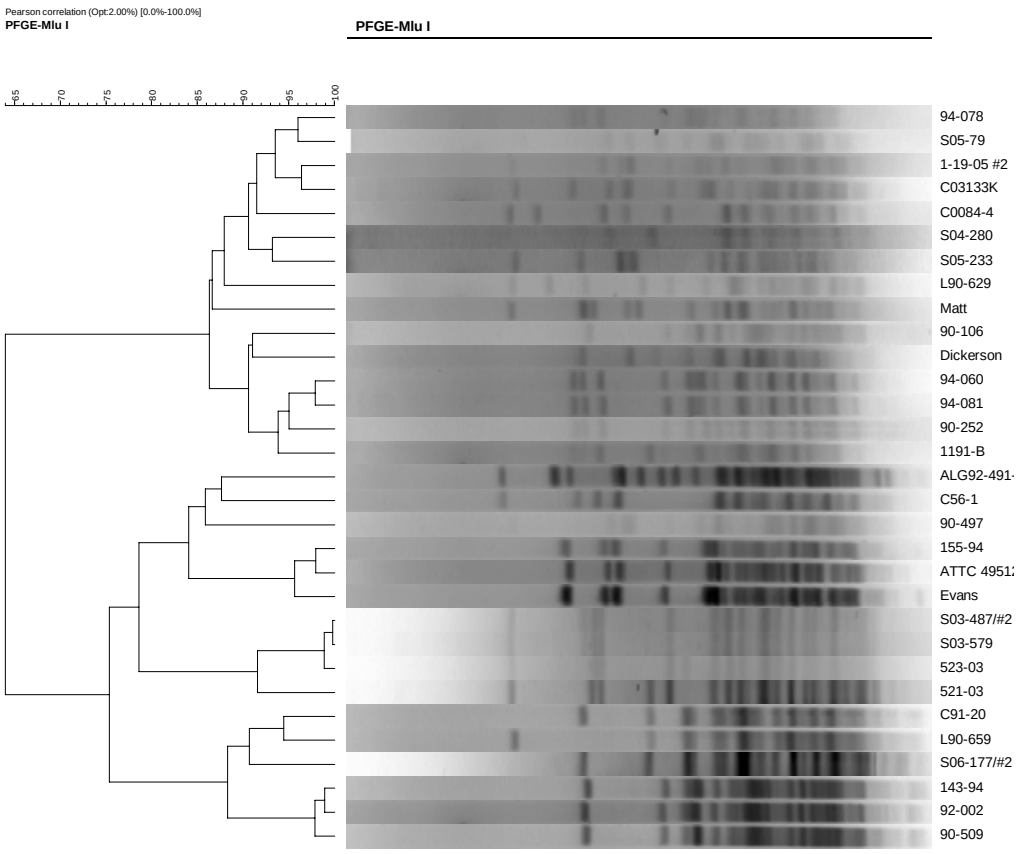


Figure 4. UPGMA dendrogram describing PFGE-based genetic similarity of *F. columnare* strains digested with *Mlu*I.

5. A

5. B

Figure 5. Typical clinical signs of columnaris disease present in channel catfish fingerlings in the current study resulting from experimental immersion challenge. (A), skin and fin necrosis with yellowish coloration, and (B), saddle back lesion and fin rot.

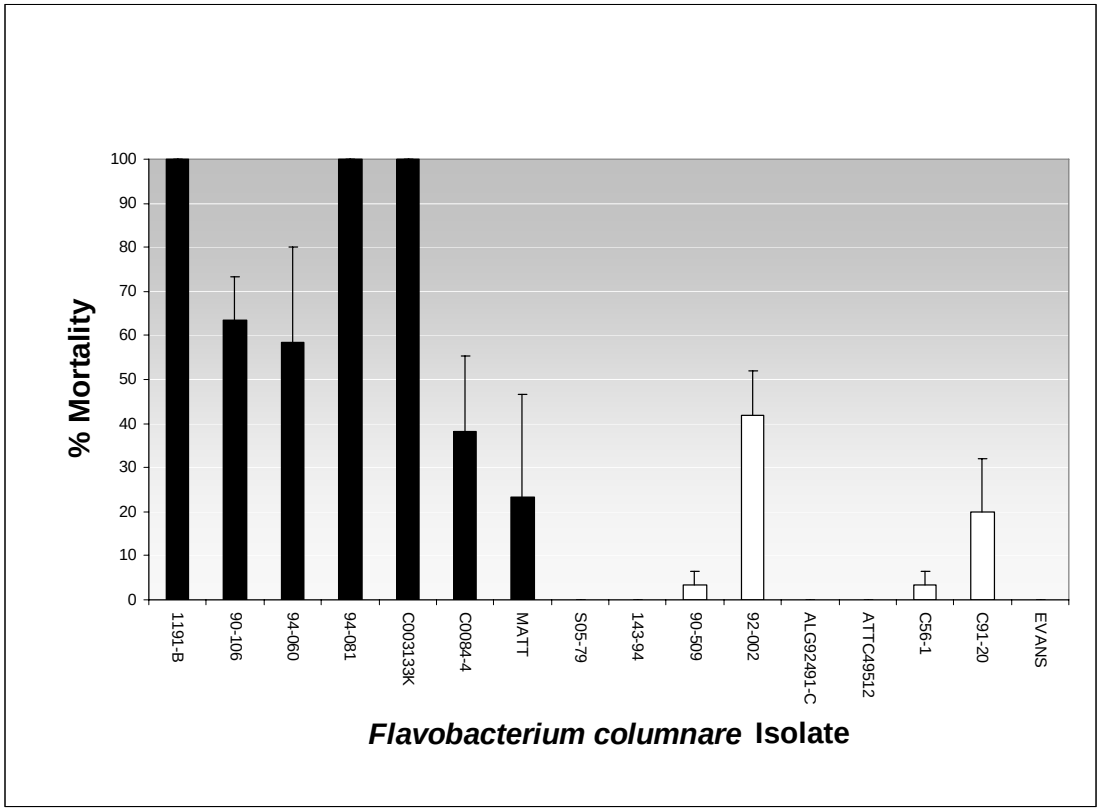


Figure 6. Percent mortalities caused by *F. columnare* isolates in channel catfish fingerlings following experimental infection by bath immersion.

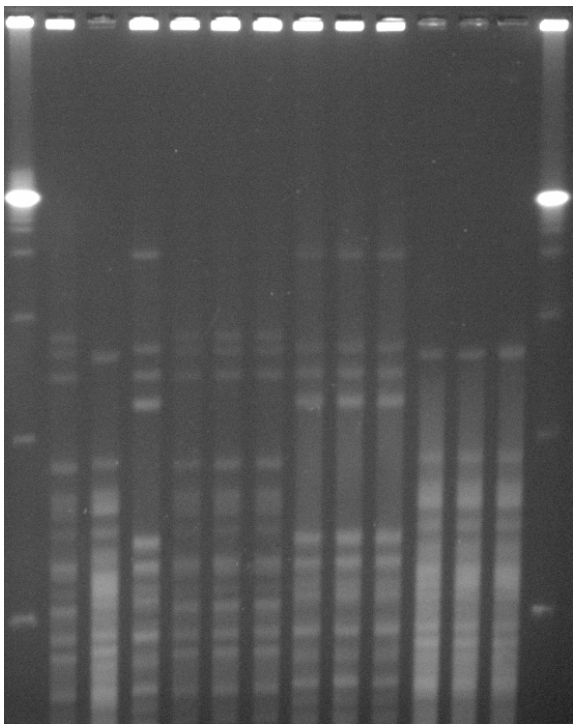
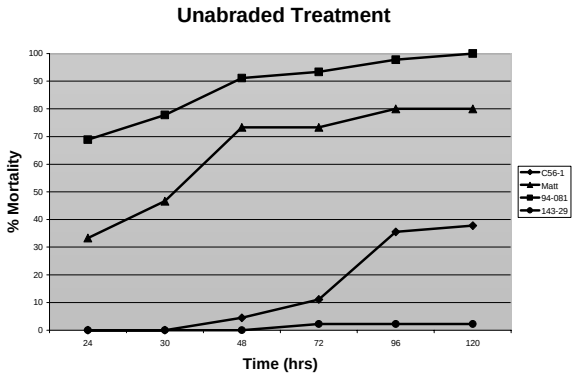
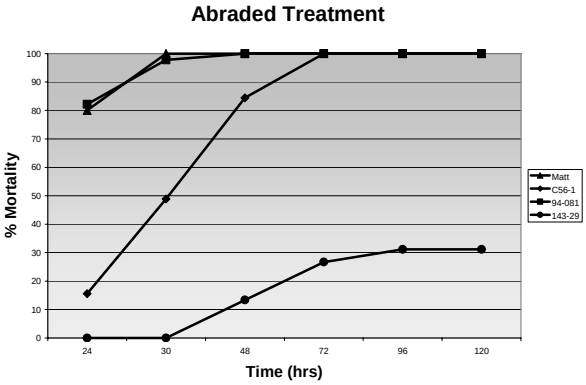


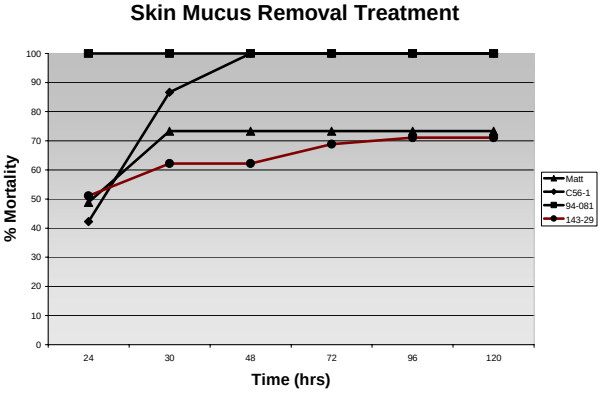
Figure 7. *Mlu*I PFGE profiles of *F. columnare* isolates before and after recovery from experimentally infected channel catfish fingerlings. Lanes 1 and 14, Lambda Ladder molecular size marker; Lane 2, original 94-081 isolate; Lane 3, original 143-94 isolate; Lane 4, original C56-1 isolate; Lanes 5, 6, and 7, recovered 94-081 isolate; Lanes 8, 9, and 10, recovered C56-1 isolate; Lanes 11, 12, and 13, recovered 143-94 isolate.



8.A



8.B



8.C.

Figure 8. Percent mortalities of channel catfish fingerlings after bath immersion with four different isolates of *F. columnare* following three different skin treatments. (A) Un-abraded treatment, (B) abraded treatment, (C) skin mucus removal treatment.

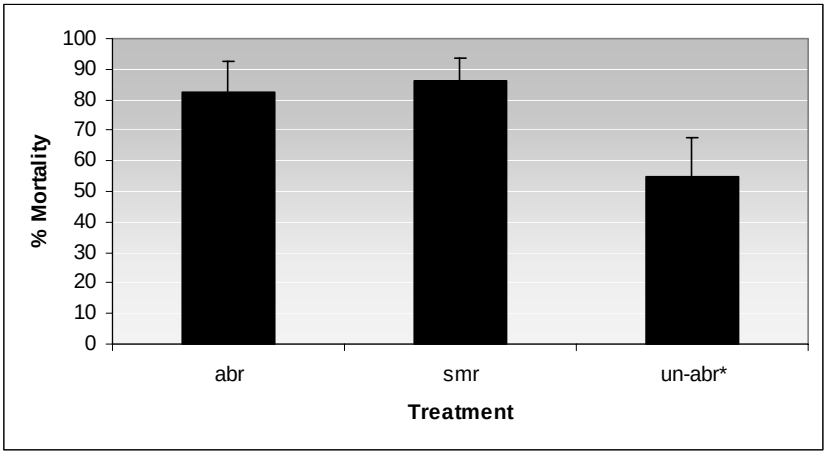


Figure 9. Overall average percent mortalities from four *F. columnare* isolates in channel catfish fingerlings following immersion challenge. Abr = skin abrasion smr = skin mucus removed, un-abr = unabraded. * represents statistically significant difference in percent mortality ($p<0.01$).

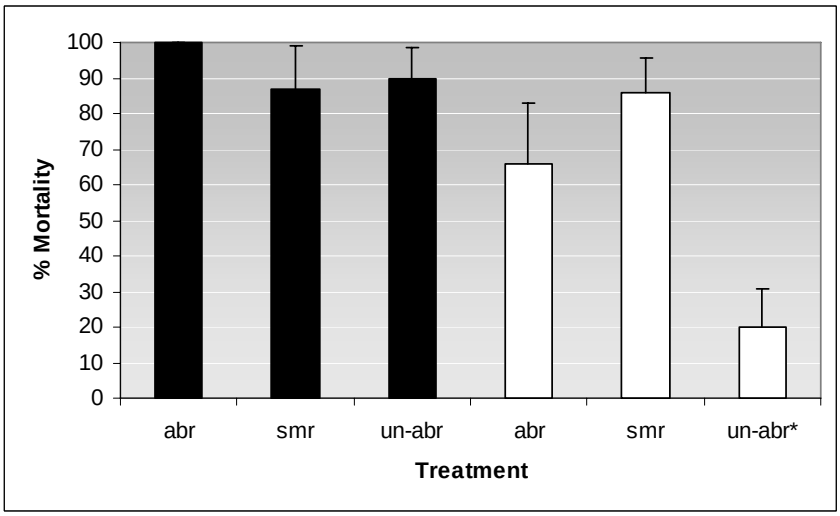


Figure 10. Average percent mortalities of channel catfish fingerlings after bath immersion with *F. columnare* isolates belonging to PFGE-Group A and PFGE- Group B. Abr = skin abrasion smr = skin mucus removed, un-abr = unabraded. Black = PFGE Group A, White = PFGE Group B. * represents statistically significant difference in percent mortality ($p<0.01$).

CHAPTER V

DISCUSSION

Due to *F. columnare*'s role as one of the most significant pathogens affecting the channel catfish aquaculture industry, a thorough understanding of its distribution, pathogenicity, and physical and genetic variation is necessary. This information is important for determining the amount of variation between strains to enable vaccine development and identification of virulence factors. In addition, if *F. columnare* strains vary in pathogenic potential and/or host predilection, this genetic information could allow the development of diagnostic tests to predict the pathogenic potential of *F. columnare* isolated from catfish ponds.

PFGE is a valuable typing method that has been used for the epidemiological investigation of several human pathogenic bacteria, including *Listeria monocytogenes*, *Escherichia coli*, and *Mycobacterium* sp. (Isumiya, et al., 1997**;** Autio, et al., 1999; Hughes et al., 2001). It has also been used for the fish pathogens *Pseudomonas anguilliseptica*, *Lactococcus garvieae*, and *A. salmonicida* (Garcia, et al., 2000; Blanco, 2002; Kawanishi, et al., 2005). In our study, PFGE proved to be a useful technique for distinguishing intraspecific genetic variation among *F. columnare* isolates recovered from southeastern United States catfish aquaculture facilities.

Although both *Mlu*I and *Pme*I restriction patterns clustered *F. columnare* strains into two major genetic groups, *Mlu*I restriction patterns revealed a greater degree of variation between *F. columnare* strains than *Pme*I restriction patterns. Therefore, we used *Mlu*I as our primary method for *F. columnare* PFGE analysis. Using *Mlu*I restriction analysis, group A included 48.39 % (15) of the strains analyzed, and group B included 51.61% (16). Strains in group A can be further divided into two subgroups, one containing 9 strains, and the other containing 6 strains. Similarly, group B strains can be divided into two subgroups, one containing 6 strains and the other containing 10 strains.

Flavobacterium columnare and *Edwardsiella ictaluri* are the most common pathogens affecting channel catfish aquaculture in the Southeastern United States; thus, a widespread distribution of this pathogen among aquaculture facilities exists (Tucker et al., 2004). Isolates from Louisiana, Georgia, Stoneville, MS, and Starkville, MS were analyzed, and no clear geographic correlation was observed between the two subgroups. One explanation for this could be the high exchange of channel catfish fingerlings between farms and research stations. Interestingly, all four of the Stoneville, MS isolates from 2003 (523-03, 521-03, S03-487, and S03-579) clustered together with more than 90% similarity, suggesting a common ancestor among these isolates. Because the focus of the current study was on *F. columnare* isolates affecting channel catfish aquaculture, isolates from other fish species (except for strain ATCC 49512) were not included. Therefore, direct comparison of the genetic groups identified in the current study cannot be compared to previously reported *F. columnare* genetic groups

using 16S RNA (Triyanto and Wakabayashi, 1999) or RAPD (Thomas-Jinu and Goodwin, 2004), but future studies using PFGE to analyze *F. columnare* from other fish species should allow a direct comparison.

Studies have shown that some *F. columare* isolates are more pathogenic than others (Decostere et al., 1998; Thomas-Jinu and Goodwin, 2004). Genetic grouping of *F. columnare* by RAPD analysis showed stronger correlation with the fish species of origin than with virulence (Thomas-Jinu and Goodwin, 2004). For the first time, results from the current experimental challenge suggest a direct correlation between genetic groups of *F. columnare* and pathogenicity to channel catfish fingerlings. This suggests two genetic divisions of *F. columnare* channel catfish isolates exist, one that contains strains that act as primary pathogens of channel catfish (group A), and another that act as secondary opportunistic pathogens (group B) only causing significant mortalities when skin damage provides a portal of entry. However, there is variation in the virulence of individual strains within each group, with three strains in group A causing less than 40% mortality (one strain causing no mortalities) and one strain from group B causing more than 40% mortality. Therefore, genetic analysis of *F. columnare* by PFGE may not allow complete prediction of virulence for an individual strain, but it will allow classification of strains into either group A or B, which would allow classification as either a high or low risk for being a primary *F. columnare* pathogen.

The second experimental challenge strongly supported conclusions from the first. As in the first experimental challenge, isolates belonging to group A showed a higher degree of virulence than isolates clustered in Group B, especially when un-

abraded fingerlings were challenged. On the other hand, once the skin/mucus barrier of fish was compromised, all of the isolates caused high mortality rates. In particular, the mortality rates of the Group B strains increased dramatically. These results support the conclusion that Group B strains tend to be opportunistic pathogens, requiring host compromise to cause mortality, while other (Group A) strains can act as primary pathogens and cause high levels of mortality in un-abraded fish. Interestingly, mucus removal was just as effective as skin abrasion in causing increased mortalities, indicating that mucus is an important barrier against *F. columnare* infection.

Conclusion

In conclusion, our data demonstrates that PFGE is a useful, reliable and reproducible molecular technique for genetically fingerprinting *F. columnare*. PFGE also re-illustrated the high heterogeneity of this aquatic microorganism. The results of the experimental immersion challenges showed a correlation between the two genetic groups and virulence, indicating that PFGE is a potentially useful tool for determining whether *F. columnare* isolates are more likely to be a primary or secondary pathogen. This information may allow producers to determine how aggressive their treatment strategy needs to be during a columnaris outbreak. In addition, future vaccine development research for columnaris disease should focus on antigens expressed by the more pathogenic group A.

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