

IMPROVED METHODS FOR THE ISOLATION AND CHARACTERIZATION OF
FLAVOBACTERIUM COLUMNARE

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ABSTRACT

Columnaris disease, caused by the bacterium *Flavobacterium columnare*, is an economically significant problem in many warmwater fish species. Difficulties encountered in the isolation and culture of *F. columnare* have been an impediment to research on the organism and the disease it causes. The goal of this study was to improve the methods for isolation, culture, identification and maintenance of *F. columnare*. Following the evaluation of different culture media selective cytophaga agar was determined to be the optimum isolation medium, *Flavobacterium columnare* growth medium proved to be the optimum culture medium, and tryptone yeast extract agar with increased moisture was best for maintenance of cultures. Biochemical characterization of 49 *F. columnare* isolates was accomplished utilizing the method of Griffin et al. (1992), and the API ZYM system from BioMérieux (Hazelwood Missouri). Using these methods 48 of the 49 strains were presumptively identified as *F. columnare*. Ten representative *F. columnare* isolates were further characterized by the API NE system, and all isolates yielded identical phenotypes.

Molecular characterization of various strains of *F. columnare* was accomplished by random amplified polymorphic DNA analysis (reference). Strains evaluated were confirmed as *F. columnare* by polymerase chain reaction (PCR) using primers and methodology published by Bader et al (2003). The data from RAPD analysis was used to construct three groups based on similarity comparisons.

Methods for antimicrobial susceptibility testing by disk diffusion were evaluated on different formulations of dilute Mueller Hinton agar to address the problem of non distinct zones of inhibition, and to reduce the variability of resulting zone data seen when

using the published dilute Mueller Hinton formulation (Hawke and Thune 1992). The improved dilute Mueller Hinton medium formulation reduced variability by 40%, increase overall bacterial growth, and also improved the zones of inhibition by producing distinct margins.

CHAPTER 1: LITERATURE REVIEW

FLAVOBACTERIUM COLUMNARE

COLUMNARIS DISEASE

Columnaris disease, caused by the Gram-negative bacterium *Flavobacterium columnare* is one of the oldest known fish diseases in North America, and has been a significant problem in many warm water fish species for decades. The ubiquitous distribution of the organism in freshwater environments and the tendency for fish to acquire the disease after mechanical and/or environmental insults make *F. columnare* among the most common pathogens in cultured, ornamental, and wild fish populations (Shamsudin, 1994; Shotts and Starliper, 1999). The disease is distributed worldwide and wide spread throughout freshwater environments. Columnaris disease is one of the most important bacterial diseases of channel catfish, *Ictalurus punctatus*, commercially raised in the US (USDA, 2003). *Flavobacterium columnare* can infect catfish of any age, under a variety of water conditions, and during any season of the year (Griffin, 1992). Acute disease is characterized by an incubation period of less than 24 hr and the resulting mortalities occur two to three days post exposure. Mortality ranges from 10 to 100% depending on temperature (Holt et al. 1975).

HOST SUSCEPTIBILITY

Columnaris disease has been documented from at least 36 fish species throughout the world, including the commercially important species: channel catfish (*Ictalurus punctatus*); common carp (*Cyprinus carpio*); goldfish (*Carassius auratus*); eels (*Anguilla rostrata*, *A. japonica*, *A. anguilla*); tilapia (*Oreochromis spp.*); rainbow trout (*Oncorhynchus mykiss*); brown trout (*Salmo trutta*); and brook trout (*Salvelinus*

fontinalis) (Anderson and Conroy, 1969). The disease also infects important recreational species such as the largemouth bass (*Micropterus salmoides*), bluegill (*Lepomis macrochirus*), and crappie (*Pomoxis nigromaculatus*, *P. annularis*) and numerous ornamental species popular in the aquarium trade.

ECONOMIC IMPACT

Bacterial disease is the primary cause of mortality in commercially reared channel catfish, accounting for 58% of the total cases examined (9575) from 1987 to 1989 in Alabama, Mississippi, and Louisiana diagnostic laboratories (Thune, 1993). Columnaris disease is the second most prevalent bacterial disease in channel catfish accounting for approximately 23% of the total cases of bacterial etiology (Hawke and Thune, 1992). In the channel catfish industry, columnaris disease ranked second only to Enteric Septicemia of Catfish (ESC) as a cause of economic losses (USDA, 2003). Columnaris disease or mixed infections of columnaris and ESC were listed as causing the greatest economic losses on catfish farms by 70% of farmers from the four leading catfish producing states (USDA, 2003), with losses estimated in the millions of dollars. Columnaris disease can occur as the primary disease in pond or tank raised channel catfish, with mortalities as high as 50% (Plumb, 1999). Mortality rates as high as 34% have been documented in salmonid species (Williams, 1973). Columnaris outbreaks in the Melvern and Pomona Reservoir in Kansas resulted in the loss of an estimated 54,000 white bass (*Morone chrysops*) mortalities in 1997 (Jirak, personal communication).

TAXONOMY

Columnaris disease was first described by Herbert Spencer Davis in 1922 from the Mississippi River, and the causative organism was named *Bacillus columnaris*. The

causative agent was not successfully cultured and characterized until 1944 when Ordal and Rucker developed methods for the isolation and culture of the bacterium. Ordal and Rucker renamed the bacterium *Chondrococcus columnaris*. The bacterium has been renamed and reclassified several times over the years on the basis of morphological and biochemical characteristics, and has been referred to at various times as *Chondrococcus columnaris*, *Cytophaga columnaris*, and *Flexibacter columnaris* (Bernardet and Grimont, 1989). The current name *Flavobacterium columnare* was adopted following molecular characterization of archived strains (Bernardet et al. 1996).

PHENOTYPIC DESCRIPTION

Flavobacterium columnare is a long, slender, non-flagellated Gram-negative rod, 0.3 to 0.7 μm wide x 3 to 10 μm long, which exhibits gliding motility on solid surfaces. The flexing and gliding movement of individual rods can differ depending on the culture medium utilized. Colonies on cytophaga agar are flat, yellow, rhizoid, strongly adherent, and spread across solid media surfaces forming irregular margins. The bacteria form columnar aggregates on infected tissue that are often referred to as “haystacks.” The temperature range for growth of *F. columnare* is reported to be between four and 37°C with 25°C being the optimum (Amend, 1982). Growth is strictly aerobic, and the bacterium is nonhalophilic (Pacha and Ordal, 1968; Pacha and Porter, 1970). Bernardet and Grimont (1989) described the physiological characteristics of *F. columnare* as follows: strict aerobic growth; no acid produced from carbohydrates; cytochrome oxidase and catalase positive; reduces nitrate to nitrite; hydrogen sulfide is produced; cellulose, chitin, starch, esculin, and agar are not hydrolyzed; gelatin, casein, and tyrosine are hydrolyzed; arginine, lysine, ornithine are not decarboxylated; and flexirubin pigments

are produced. Chondroitin AC lyase, an enzyme produced by *F. columnare* (Griffin, 1992), degrades polysaccharides, particularly those found in cartilaginous connective tissue (Teska, 1993). The bacterium produces a capsule and the thickness of the capsule appears to be correlated with virulence. High virulence strains have a thick 120-130 nm capsule, while strains with low virulence have a thinner 80-90 nm capsule, as observed by transmission electron micrography (Decostere et al. 1998b). Production of extracellular galactosamine glycan is revealed by the absorption of congo red dye into the colony (Johnson and Chilton, 1966, McCurdy, 1969). *Flavobacterium columnare* strains are able to grow in Cytophaga broth supplemented with 0.5% NaCl, at pH values above six, and at 22°C and 25°C (Bernardet, 1989). Bertolini reported that 13 *F. columnare* isolates degraded gelatin, casein, hemoglobin, fibrinogen, and elastin (Bertolini et al. 1992). The DNA base composition of three isolates of *F. columnare* was 32 to 33 mol% G+C. The genus *Flavobacterium*, and especially *F. columnare*, secrete enzymes that can cause rapid DNA hydrolysis (Bernardet and Grimont, 1989). The presence of flexirubin-type pigments, as determined by the potassium hydroxide (KOH) method, are produced by the bacterium (Reichenbach et al. 1981). Griffin (1992) devised a method for the differentiation of *F. columnare* from other morphologically similar bacteria. The “Griffin screen” consists of five characteristics that separate *F. columnare* from other yellow pigmented Gram negative aquatic bacteria: (1) the ability to grow in the presence of neomycin sulfate and polymyxin B (2) colonies on Cytophaga agar plates typically rhizoid and pigmented pale yellow (3) production of gelatin degrading enzymes (4) binding of congo red dye to the colony (5) production of chondroitin sulfate A degrading enzymes.

STRAIN VARIABILITY

Several studies have revealed variation between isolates of *F. columnare* cultured from different hosts and different geographical regions. Warmwater and coldwater *F. columnare* isolates can be differentiated based on biochemical test results in the API 20E system® (BioMereux Vitek Hazelwood, MO) (Pyle and Shotts, 1980). Columnaris disease was separated into four groups based on serological studies by Anacker and Ordal (1959). Shamsudin and Plumb (1996) reported that four isolates of *F. columnare* from four different fish species showed uniform biochemical characteristics, but differed in their ability to grow at 15°C on media with 0.5% NaCl or at a pH of six or 10.

The proteolytic activity of *F. columnare* in vitro, suggests that the production of extracellular proteases is an important virulence mechanism of the bacterium (Song et al. 1987; Bernardet, 1989; Griffin, 1991; Bertolini and Rohovec, 1992; Teska, 1993; Plumb, 1994). Several of the extracellular proteases were characterized to have average molecular weights of 47, 40, 34, and 32 kD at day one (Bertolini et al. 1992). Only isolate (2.19) from a diseased catfish in Louisiana, had a protease with average molecular weight of 44 kD rather than the 47 kD protease (Bertolini et al. 1992). Extracellular proteases have been used to separate isolates into two groups with apparent molecular masses of 58 and 53.5 kD (group one) and 59.5, 48, and 44.5 kD (group two) (Newton et al. 1997).

There are significant differences in the virulence between different strains of *F. columnare* (Rucker et al. 1953; Bullock et al. 1986; Decostere et al. 1998b). A correlation between high virulence stains and the ability to adhere to the gills of a common carp (*Cyprinus carpio*) was found with the gill perfusion model (Decostere et al.

1998b). It is well documented that bacteria seldom rely upon one single mechanism of adherence, but that both specific and nonspecific mechanisms are often involved (Ofek and Doyle, 1994). The AJS 1 strain is a highly virulent strain which has the most affinity for gill tissue, as compared to the AJS 4 strain which is low in virulence and has a low affinity for gill tissue. Low virulence strains produce necrotic lesions on the gills and/or body surface, and often systemic infections occur. Several days elapse before mortality results from infection by low virulence strains (Rucker, 1953). High virulence strains cause death within 24 to 48 hours post exposure to the pathogen (Pacha and Ordal, 1970). Variation in the degree of virulence has also been reported between isolates of *F. columnare* isolated from salmonids (Amend, 1982).

MOLECULAR CHARACTERIZATION

The term “genomovar” has recently been introduced to denote phenotypically similar but genotypically distinct groups of bacterial isolates within a species. Polymerase chain reaction (PCR) can produce products to the more highly conserved 5S, 16S, and 23S ribosomal subunits which can potentially differentiate species and also show intraspecific differences (Wakabayashi et al. 1999). Nested PCR techniques have been developed in Japan that can be used to divide phenotypically identical strains of *F. columnare* into three distinct “genomovars” (Wakabayashi et al. 1999). Randomly amplified polymorphic DNA analysis (RAPD) has been used to show the differences between representative strains of *Photobacterium damsela* subsp. *piscicida* and *Streptococcus parauberis* as well as other aquatic bacteria (Hawke et al. 2003; Romalde et al. 1999). The RAPD technique has been used to show intraspecific genetic variation among *F. columnare* isolates from warmwater fish species (Thomas-Jinu and Goodwin,

2004). Restriction fragment length polymorphism (RFLP) has shown that a French isolate of *F. columnare* from a neon tetra, *Paracheirodon innesi* (Myers), was genetically different from the more common European and American isolates, although phenotypically it was similar except for optimal growth temperatures (Michel et al. 2002). *Flavobacterium columnare* isolates have also been characterized into two genomovars by RFLP analysis of the 16S rRNA (Wakabayashi et al. 1999).

CLINICAL SIGNS

Based on the site of infection and appearance of infected tissues, the disease has been commonly known as saddleback, fin rot, or cotton wool disease. The area of primary infection, often referred to as a saddleback lesion, can appear along the dorsal fin and extend laterally down both sides of the abdomen (Griffin, 1987). One common clinical sign of the disease is the pronounced erosion and necrosis of external tissues, with the gills often being a major site of damage (Davis, 1922). Clinical signs normally observed are; dull whitish or yellow necrotic and erosive lesions on the skin, fins, oral cavity, and/or necrosis of gill filaments. Presumptive diagnosis of columnaris disease is based on the presence of the clinical signs mentioned above and by the typical morphology of *F. columnare* in wet mounts of tissue scrapings from infected tissues. Highly virulent strains of *F. columnare* can produce death without macroscopic evidence of tissue damage (Pacha and Ordal, 1967). The bacterium is capable of entering the blood stream and is often isolated from the internal organs however internal lesions are poorly described and are often lacking (Hawke and Thune, 1992; Koski et al. 1993). The infected skin loses its natural sheen and a grey, white, or yellowish margin surrounds the focal lesion. The mouth and inner walls of the oral cavity may be covered with a

yellowish mucoid material. The bacteria multiply rapidly in infected tissues and spread quickly to surrounding areas. Columnaris disease is often thought of as an opportunistic infection with stress on the fish population being a prerequisite for infection. Stress factors such as netting, handling, and transporting fish were shown to exacerbate the incidence of infection and disease (Kumar et al. 1986).

DIAGNOSIS

The presence of shallow lesions with yellow or brownish discoloration on the mouth, gills, and / or fins usually indicates an infection by *F. columnare*. Presumptive diagnosis of columnaris disease is obtained by the observation of long (6-10 μm) flexing rods from a scraping of infected tissue in a wet mount at 100 to 400 X magnification. The bacteria form columnar masses on infected tissue, which are commonly referred to as “haystacks.” Definitive diagnosis of the disease can be achieved through isolation and culture of the causative bacterium, followed by identification based on biochemical testing, or molecular methods. Histopathology may provide useful information concerning the severity of the infection. We now have the ability to diagnose Columnaris disease by modern molecular methods: such as polymerase chain reaction (Bader et al. 2003) and by DNA sequence analysis (Hanson, personal communication).

MOLECULAR DIAGNOSTIC METHODS

Polymerase chain reaction (PCR) based techniques utilizing species-specific primers have been used in fish disease diagnostics for the diagnosis and epidemiology of *Flavobacterial* diseases (Toyama et al. 1996; Urdaci et al. 1998; Izumi and Wakabayashi, 2000; Wiklund et al. 2000). The first 500 nucleotides in the 5' terminus of the 16S rDNA contain enough information to allow accurate assignment of bacterial sequences to the

main lines of descent, and this terminus has been recommended as the region of interest for molecular analysis (Liesack et al. 1997). *Flavobacterium columnare* has been identified with species specific PCR primers to a portion of the 16S ribosomal RNA gene to generate unique products that can be differentiated with gel electrophoresis (Bader et al. 2003). This technique can differentiate *F. columnare* from other species of yellow pigmented, gliding bacteria, but it does not delineate various isolates of bacteria within the same species. A broad range PCR to the 16S rDNA followed by RFLP analysis and product sequencing was able to differentiate several *Flavobacterium* species including *F. columnare*, *F. psychrophilum*, *F. johnsoniae*, and *F. hibernum* (Tirola et al. 2002). Species-specific primers (Col72F and Col 1260R) have been published for PCR amplification of the ribosomal 16S rRNA gene for identification purposes (Wakabayashi et al. 1999).

ISOLATION

The primary isolation of most *Flavobacteria* is problematic, and in many cases has impeded investigations of the pathogenesis of *Flavobacterium* species (Anderson and Norton, 1991; Dalsgaard, 1993; Shotts and Starliper, 1999). In 46.5% of the *F. columnare* cases submitted to the Louisiana Aquatic Diagnostic Laboratory in 1992, the bacterium was present in a mixed infection with other pathogens such as: *Aeromonas* spp., *Edwardsiella ictaluri*, and *E. tarda* (Hawke and Thune, 1992). This poses a problem in the identification of the primary etiological agent. This along with the specialized media needed for the isolation and culture of *F. columnare* has limited the progress of research conducted on this bacterium and the disease it causes. In a typical mixed columnaris infection, the dominating *F. columnare* strain can be masked by

saprophytic species of the same genus or other genera, or the growth of *Flavobacteria* can be completely inhibited by antagonistic bacteria such as *Pseudomonas* (Tirola et al. 2002). Tirola et al. (2002) also reported that the isolation of *Flavobacteria* was unsuccessful from a number of fish samples (44%) that contained filamentous Gram-negative bacteria in microscopic examination. In a competition study, *F. columnare* failed to invade fish in the presence of *Citrobacter freundii* at an initial number approximately 100 times that of *F. columnare* in environmental water (Chowdhury and Wakabayashi, 1989). Fijan (1969) noted that when trying to isolate *F. columnare* the spreading growth or predominance of other bacteria sometimes obscures, overgrows, or prevents the formation of discrete isolated colonies. After determining the *F. columnare* minimal inhibitory concentration of polymixin B to be 1000 units/ml and neomycin to be 100 µg/ml Fijan recommended adding five µg/ml neomycin and five units/ml polymixin B to cytophaga agar to make the medium selective for *F. columnare* and selective against other inhibiting bacteria (Fijan, 1969). Hawke and Thune (1992) reported the selective media of Fijan did not inhibit many of the bacteria in mixed infections from diseased channel catfish. The formulation was improved to contain five µg/ml neomycin and 200 units/ml polymixin B, and this medium was effective in inhibiting all of the species of bacteria tested except *Flavobacterium sp.* and *Streptococcus sp.* (Hawke and Thune, 1992). Tobramycin has also been reported to be selective for *F. columnare* in Shieh medium used for the primary isolation of *F. columnare* from diseased fish (Decostere et al. 1997).

CULTURE

Flavobacterium columnare does not grow on standard bacteriological isolation media such as brain heart infusion agar, tryptic soy agar, or on standard Mueller Hinton agar, and therefore it requires specialized media for isolation, culture, and antibiotic sensitivity testing. *Flavobacterium columnare* requires a minimal growth medium with low nutritional and high moisture content. The medium is prepared for same day use which ensures the correct moisture content. Survival of *F. columnare* in water is improved by the addition of calcium, magnesium, potassium, and sodium ions (Cowdhury and Wakabayashi, 1989). The growth response of *F. columnare* was better in Chase, Shieh, and Liewes media that contain salts with the best growth in Shieh medium at 24 hours (Song et al. 1987). Holt was able to routinely culture *F. columnare* with tryptone yeast extract plus salts (TYES) medium at 25°C (Holt, 1988). Fetal bovine serum has been used as an enrichment factor to improve culture performance for *F. psychrophilum* (Michel et al. 1999), and was also shown to improve *F. columnare* culture (Fijan, 1969). More enzyme activity per mg bacterial dry weight was achieved in cytophaga broth as compared to TYES, Hsu-Shotts, and Shieh broths (Newton et al. 1997).

DISK DIFFUSION

The National Committee for Clinical Laboratory Standards (NCCLS) published the M42R document which outlines procedures for the antimicrobial susceptibility testing for aquatic bacteria by disk diffusion (NCCLS 2003). The quality control organism suggested for disk diffusion testing at 28°C is *Escherichia coli* ATCC 25922. Mueller Hinton (MH) agar is the basal medium suggested by the NCCLS for testing aquatic

bacteria in an attempt to increase the intra-laboratory and inter-laboratory reproducibility of susceptibility results (Miller et al. 2003). Dilution of standard MH medium is required for the susceptibility testing of *F. columnare* isolates, because the bacterium fails to grow on standard MH agar. Disk diffusion testing is problematic for *F. columnare* because the gliding motility of the bacterium can lead to zone irregularity and the zone margins appear "fuzzy."

MINIMAL INHIBITORY CONCENTRATION

The minimal inhibitory concentration (MIC) corresponds to the lowest concentration of a drug in a dilution series that inhibits growth of a bacterial strain. The MIC of the following drugs was determined for *F. columnare*: amoxicillin 0.06 µg/ml, oxytetracycline 0.06-0.12 µg/ml, oxolinic acid 0.06-0.12 µg/ml, norfloxacin 0.12 µg/ml, and trimethoprim > 64 µg/ml (Soltani et al. 1996). Hawke and Thune (1992) reported the MIC for *F. columnare* isolates for Romet® to be 7-15 µg/ml with disk diffusion zones ranges from 22-28 mm and the MIC for Terramycin® 0.195 µg/ml with zones of 38-40 mm.

RESISTANCE

One characteristic of *F. columnare* is resistance to neomycin and polymixin B (Fijan, 1969; Griffin, 1992; Hawke and Thune, 1992). These antibiotics are also used in isolation media to select for *F. columnare* (Fijan, 1969; Hawke and Thune, 1992). The bacterium is reported to be resistant to tobramycin at 1 µg/ml, which has also been used to supplement Shieh medium to make it selective for *F. columnare* (Decostere et al. 1997). Bernardet and Grimont (1989) reported the bacterium to have 0 mm disk diffusion zones around gentamicin (15 µg), neomycin (30 µg), kanamycin (30 µg),

polymixin B (30 µg), trimethoprim (5 µg), and actinomycin D (2.5 µg) disks. Isolates are usually sensitive to oxytetracycline and nifurpirinol, but can become resistant to ormetoprim-sulfadimethoxine (Hawke and Thune, 1992). Of 207 *F. columnare* isolates obtained from channel catfish in 1990 by the Delta Research and Extension Center, Stoneville, MS two were resistant to Terramycin® and 60 were resistant to Romet® (Johnson, 1991).

TREATMENT

An effective treatment for columnaris disease is immersion in a salt bath, or increasing and maintaining the salt level at approximately three parts per thousand (ppt) (Hawke, 2004 personal communication). The *in vitro* growth of *F. columnare* is reported to be inhibited at ten ppt NaCl, but not at five ppt and growth is best at three ppt salinity (Bernardet, 1989). In a challenge model, the adhesion of the bacteria was reduced at 3 ppt, which may explain lower mortality rates in higher salinities (Altinok and Grizzle, 2001). Because *F. columnare* primarily attacks the skin and gills the most effective treatments for columnaris disease are surface-acting disinfectants such as potassium permanganate, hydrogen peroxide, and copper sulfate (Wakabayashi, 1991). The herbicide 6,7-dihydrodipyrido (1.2a: 2, 1-c) pyrazidinium bromide (Diquat) was shown to be an effective bath treatment at 5.4 mg/L when compared to potassium permanganate 2 mg/L, chloramine-T 15 mg/L, hydrogen peroxide 75 mg/L, and copper sulfate 1 mg/L (Thomas-Jinu and Goodwin, 2004). In the USA, there are currently no drugs labeled for the treatment of columnaris disease (Thomas-Jinu and Goodwin, 2004). When drugs legal for other aquaculture uses (Terramycin® and Romet®) were administered prior to bacterial challenge, mortalities were zero in all groups (Thomas-Jinu and Goodwin,

2004). These antibiotics in the form of medicated feeds can be used to treat outbreaks of the disease, Romet® at 50 mg/kg body weight, and Terramycin® at 80 mg/kg of body weight (Hawke and Thune, 1992). The best management strategy for columnaris disease is to prevent infection by minimizing stress thus avoiding expensive antibiotic treatments and resulting withdrawal periods before the fish can be sold as a food item (Anderson and Rodgers, 1994).

OBJECTIVES

The first objective of this study is to improve the methods for the isolation, culture, and antimicrobial susceptibility testing of *Flavobacterium columnare* isolated from diseased fish. The second objective is to characterize archived strains of *F. columnare* based on conventional and molecular techniques and the third objective is to evaluate variations of dilute Mueller Hinton medium to improve antimicrobial susceptibility testing by disk diffusion methods.

CHAPTER 2: THE EVALUATION OF MEDIA FOR THE ISOLATION, MAINTENANCE, AND CULTURE OF *FLAVOBACTERIUM COLUMNARE*

INTRODUCTION

Flavobacterium columnare is the causative agent of columnaris disease, and accounts for millions of dollars of losses in aquaculture each year (USDA, 2003). The biology of the disease is poorly understood even though it is one of the oldest known fish diseases. *Flavobacterium columnare* is difficult to culture and identify, and for this reason many laboratories utilize various methods for the isolation and culture of the bacterium. Culture of the bacterium was initially accomplished on a low nutrient, low agar content media (Ordal and Rucker, 1944). A wide variety of media have been evaluated for the cultivation of *F. columnare*, and several have been adapted to serve specific purposes in the cultivation of the bacterium.

Isolation is often problematic, because the disease usually presents as a mixed infection with numerous other opportunistic bacteria. In order to inhibit the growth of the secondary bacteria, selective media must be used for the isolation of *F. columnare*. A scheme to differentiate *F. columnare* from other yellow pigmented gliding bacteria was introduced by Griffin (1992). One of Griffin's identifying characteristics was that the bacteria must be able to grow in the presence of neomycin sulfate and polymixin B (Griffin, 1992). Media made selective by the addition of these antibiotics have been suggested for the isolation of *F. columnare* from diseased tissues (Fijan, 1969; Hawke and Thune, 1992).

Culture maintenance is also a problem when working with *F. columnare*, since cultures begin to lose viability after 48 hours on cytophaga agar plates. Increasing the moisture content has been reported to extend viability of *F. columnare* cultures (Newton

et al. 1999). The moisture increase was achieved by adding one ml of sterile saline to a tube of slanted agar medium (Newton et al. 1999).

Several broth media have been suggested for the culture of *F. columnare* such as cytophaga, tryptone yeast extract, Chase, Liewes, and Shieh broths (Song et al. 1987). The growth of *F. columnare* is slow and cells often clump or auto-agglutinate in broth culture. This phenomenon results in problems with bacterial enumeration and in producing a uniform inoculum for various tests. In this study, several media will be evaluated for the isolation, maintenance, and culture of *F. columnare*.

MATERIALS AND METHODS

MEDIA FORMULATIONS

Table 2.1 Formulations of various media evaluated for the primary isolation, culture and maintenance of *F. columnare* cultures.

Ingredient: g/l	Medium				
	Cytophaga	DMH	FCGM	Hsu-Shotts	Shieh
Tryptone	0.5		8.0	2.0	
Peptone					5.0
Beef extract	0.2	0.43			
Gelatin				3.0	
Casein		2.5			
Yeast extract	0.5		0.8	0.5	
Starch		0.2			
Na-acetate	0.2				0.01
MgSO ₄ (7H ₂ O)			1.0		0.3
CaCl ₂ (2H ₂ O)			0.74	0.3	0.0067
NaCl			5.0		
Sodium Citrate			1.5		

Table Continued

Ingredient: g/l	Cytophaga	DMH	FCGM	Hsu-Shotts	Shieh
Glucose					1.0
Na pyruvate					0.1
Citric acid					0.01
BaCl ₂ H ₂ O					0.01
K ₂ HPO ₄					0.1
KH ₂ PO ₄					0.05
FeSO ₄ 7H ₂ O					0.001
NaHCO ₃					0.005
Agar	9.0	9.0	9.0	10.0	10.0

EVALUATION OF ISOLATION MEDIA

Bacteria

Combinations of the aquatic bacteria *Aeromonas hydrophilia*, *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Streptococcus difficilis*, and *Flavobacterium columnare* were used to simulate a mixed infection of *F. columnare* from an external lesion. The five species of aquatic bacteria were obtained from the archived strain repository of the Louisiana Aquatic Diagnostic Laboratory (LADL) Louisiana State University. Strains were archived at -70°C in aliquots of BHI broth with 15% glycerol. Aliquots from archived strains were streaked onto blood agar plates except for *F. columnare*, which was cultured on Cytophaga agar. All cultures were incubated at 28°C for 24 hours. The cultures were checked for purity and subcultures were incubated for an additional 24 hours at 28°C. The five cultures were used to make bacterial suspensions that were adjusted to a McFarland #2 optical density standard (Difco, Detroit, MI). A mixed

culture was created by combining one ml of each suspension in a sterile test tube and vortexing until a uniform suspension was achieved. This mixed bacterial suspension was used to inoculate the various media to evaluate their suitability as primary isolation media for *F. columnare*.

Media

Five different media were evaluated with and without added antibiotics in this study; Cytophaga agar, Shieh agar, Hsu – Shotts agar, *Flavobacterium columnare* Growth Media (FCGM), and dilute Mueller Hinton agar (Ordal et al. 1944; Shieh, 1980; Bullock et al. 1986; Hawke and Thune, 1992; Cooper personal communication, 2003). Formulations of the various media are listed in Table 2.1. For selective media, the antibiotics neomycin and polymixin B were added to the media after it had cooled below 50°C. The concentrations of antibiotics, five µg/ml neomycin sulfate and 200 units/ml polymixin B, were based on the levels published by (Hawke and Thune, 1992). The antibiotics were selected because of previous use in primary isolation media for *F. columnare* (Hawke and Thune, 1992). The media was poured to a volume of 20 ml per sterile petri plate.

Standardized Streaking Method

A standardized dilution streaking method was used to evaluate the ability of the various media to allow formation of isolated colonies of *F. columnare*. A standard inoculum was created by saturating a sterile cotton swab with an inoculum from the standardized mixed culture suspension. Plates were inoculated by first swabbing the top 1/3 of the plate with the mixed suspension. A sterile wire inoculating loop was used to make three streaks across the heavily inoculated portion of the plate with a standard

dilution streaking technique. After flaming the loop, three more streaks were made with a reduction of coverage area each time. This process was repeated a third time after flaming. This was done in triplicate for each medium. The media evaluated were scored positive or negative for the presence of isolated *F. columnare* colonies, which were confirmed by light microscopy. Isolated colonies were sub-cultured to ensure purity.

EVALUATION OF MAINTENANCE MEDIA

Bacteria

Isolate LADL-97-376 was the test strain used to evaluate media for the ability to maintain viable cultures over time. *Flavobacterium columnare* growth medium (FCGM) broth was used to culture the bacteria for the inoculum, and cells were pelleted by centrifugation in an Avanti J-25 automatic refrigerated centrifuge (Beckman Palo Alto, CA) at 2900 RPM. For a standard inoculum, 100 µl of a bacterial suspension adjusted to a McFarland #2 density standards was added per replicate for all media tested.

Media

Six media were evaluated in triplicate for the maintenance of *F. columnare* cultures. The five media listed previously were evaluated along with the addition of tryptone yeast extract (TYE) agar (4.0 g tryptone, 0.4 g yeast extract, 10.0 g agar per liter) (Garnjobst, 1945). Tryptone yeast extract agar was not used as an isolation media because there are no reports of it being used as an isolation media in the literature. All media were prepared by adding 20 ml of agar media to a 50 ml tube, autoclaving, and allowing the media to solidify at a 45° angle. Two ml of sterile 0.85% saline was added to each tube to maintain high moisture content. Following inoculation, all slants were incubated at 28°C until viability was lost in all test replicates. Viability was tested by

removing some of the growth on the slant with a wire loop and streaking on an agar plate of the same test medium to eliminate any nutritional shock. To confirm the culture had lost viability, the slants that tested non-viable were sub-cultured for three consecutive days. If no positive growth occurred in any of these subcultures, the bacteria on the slant were considered to be no longer viable.

EVALUATION OF BROTH CULTURE MEDIA

Four media were tested; dilute Muller Hinton, Cytophaga, Shieh, and FCGM. Tryptone yeast extract broth was not tested in this study because Song et al. (1989) reported growth of *F. columnare* in the medium to be limited when compared to Shieh medium. Hsu-Shotts medium was not included because it is published as an isolation medium. The performance of broth media was evaluated by determining the maximum number of colony forming units (CFU)/ml produced following 24 hours of incubation at 28°C, and by absorbance at 600 nm. Bacteria were enumerated by measuring absorbance and performing colony counts following serial dilutions by the drop plate method (Herigstad et al. 2001). The four media were tested in triplicate and absorbances were measured with a spectrophotometer (Beckman DU 640 Palo Alto, CA), which measures the density of the culture by light absorbance at 600 nm. Cultures were grown in sterile 50 ml centrifuge tubes containing 40 ml of test broth per tube. All tubes were inoculated with 200µl from a bacterial suspension adjusted to a McFarland #5 standard of *F. columnare* (97-376) and incubated in an orbital shaker (New Brunswick Edison, NJ) at 28°C and 200 RPM for 24 hours. After 24 hours incubation, each culture was removed and vortexed to ensure that all bacterial cells were in suspension. The absorbance was determined with a Beckman DU 640 spectrophotometer set on vis function at 600 nm.

Each test was run in triplicate. The colony counts were determined by making serial ten-fold dilutions in wells of a 96 well microtiter plate (Thermo Lab Systems Franklin, MA). Serial ten-fold dilutions were made by diluting 20 μ l of sample in 180 μ l of sterile distilled water. Drops containing 20 μ l samples of the 10^{-5} , through 10^{-10} dilutions were placed on agar plates. The samples were allowed to absorb into the agar, and then the plates were incubated at 28°C for 24 hours. Colonies were counted in each dilution and the cfu/ml calculated.

Statistical Analysis

Statistics were performed on the data that resulted from the evaluation of broth culture media study. Statistical analysis system (SAS) software (version 8.2) was used to indicate significant differences between growth measurements of the media at the confidence level of 95%. The raw data growth measurements of absorbance (600 nm) and log transformed cfu/ml from each media in replicates were used for the statistical analysis procedure. A global linear model (GLM) was utilized to evaluate the growth of the *F. columnare* isolate in each media. The similarity between analysis of variance (ANOVA), analysis of covariance (ANCOVA), and regression has lead to these procedures to be sub-grouped under the global linear model procedure (Norman and Streiner, 2000). The GLM was used to calculate the sum of squares and mean square for the dependent variables (absorbance and log cfu/ml) for each of the four media. A Scheffe's post hoc test was then performed to indicate significant differences between the means of each of the four media. The Scheffe's test did this by allowing pair wise comparisons between the means of each media to be made.

RESULTS

EVALUATION OF ISOLATION MEDIA

Non-selective media failed to consistently produce isolated *F. columnare* colonies. Selective media performed better in all instances, with selective cytophaga agar (SCA) producing numerous well isolated, pure *F. columnare* colonies in all replicates (Figure 2.4). The selective Shieh agar also performed well with three of three plates yielding colonies containing *F. columnare*, however upon microscopic evaluation two of the three plates did not yield pure cultures (Fig. 2.5). The same problem occurred with selective Hsu-Shotts medium, which allowed other bacteria to be associated with the *F. columnare* in most of the colonies (Fig. 2.6). Selective FCGM and dilute Mueller Hinton media performed poorly as isolation media and none of the plates produced isolated *F. columnare* colonies (Fig. 2.7 and Fig. 2.8).



Figure 2.1 Isolated colonies of *Flavobacterium columnare* on selective cytophaga agar following a mixed inoculum.



Figure 2.2 Isolated colonies of *Flavobacterium columnare* on a selective Shieh agar following a mixed inoculum.



Figure 2.3 Isolated colonies of *Flavobacterium columnare* on selective Hsu-Shotts agar following a mixed inoculum.

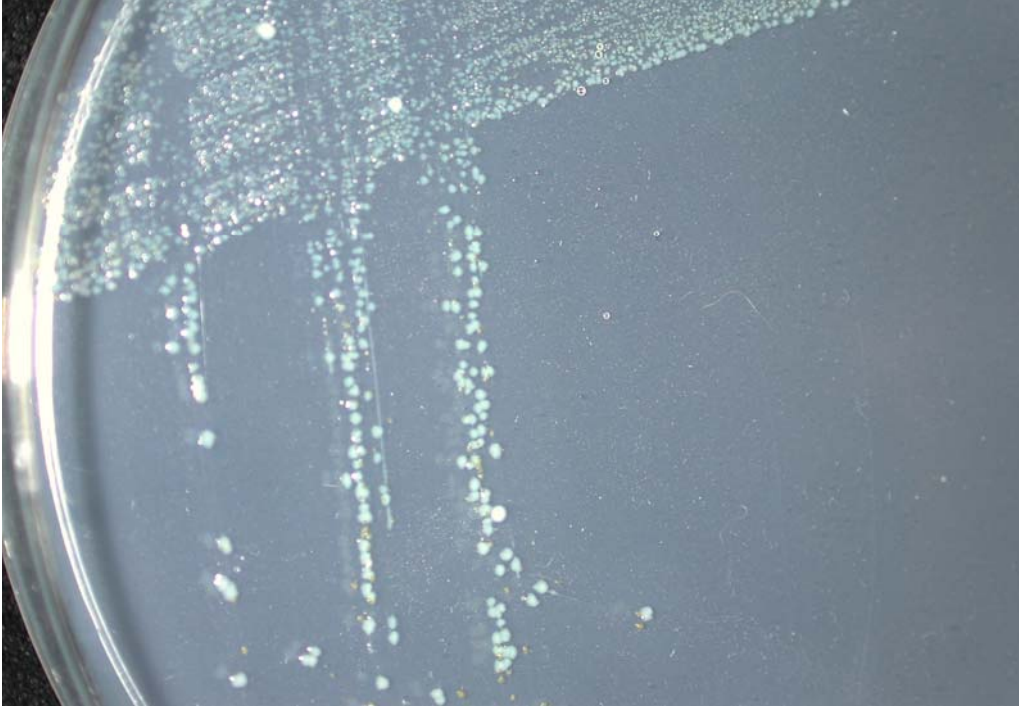


Figure 2.4 Failure to obtain isolated colonies of *Flavobacterium columnare* on selective *Flavobacterium columnare* growth medium from a mixed inoculum. Note: colony morphology does not match that of *Flavobacterium columnare*.



Figure 2.5 Failure to obtain isolated colonies of *Flavobacterium columnare* on dilute Mueller Hinton agar from a mixed inoculum. Note: colony morphology does not match that of *Flavobacterium columnare*.

EVALUATION OF MAINTENANCE MEDIA

All six media maintained viability of *F. columnare* a minimum of 22 days. The first non-viable culture was observed on day 23 on FCGM, and the last viable culture was detected on day 84 post-inoculation on TYE. The average for all media tested was 30 days post inoculation. Cultures on FCGM lost viability at 23 days post inoculation. Shieh medium cultures were the second to lose viability at day 32 and viability was lost on Hsu-Shotts medium at day 34. One replicate of cytophaga medium, and one replicate of dilute Mueller Hinton medium lost viability on day 56, but both had a replicate that was negative on day 45. The TYE medium outperformed the other tested media in the maintenance of *F. columnare* cultures, with all replicates still viable on day 78. One replicate on TYE medium lost viability 83 days post inoculation.

EVALUATION OF BROTH CULTURE MEDIA

All test media produced growth that was measurable by both absorbance and the bacteria were enumerated by drop plate dilution methods. The broth culture growth data is presented in Table 2.2. *Flavobacterium columnare* cultures have been reported to auto-agglutinate, and form clumps of cells rather than being evenly dispersed throughout the broth medium. Cultures of *F. columnare* grown in dilute Mueller Hinton broth produced clumps, but through a series of vortexing and shaking, the bacterial cells were suspended evenly before growth measurements were taken. Of the four media tested, FCGM broth produced the highest absorbance and colony forming units (cfu)/ml, and had significantly higher growth than any other media tested. Absorbances ranged from 0.0701 to 0.2383, and the cfu/ml ranged from 7.3×10^6 to 3.6×10^9 .

Table 2.2 Growth of *F. columnare* in various broth media at 28°C for 24 hours. Data is presented as absorbance at 600nm, and by colony forming units per ml.

Test Medium	Absorbance (600nm)			Mean Abs	Colony counts (cfu/ml)			Mean cfu/ml
DMH*	0.0701	0.0899	0.0975	0.0858	7.3X10 ⁶	1.6X10 ⁷	1.6X10 ⁷	1.3X10 ⁷
Cytophaga*	0.1703	0.1658	0.1579	0.1646	6.0X10 ⁷	5.0X10 ⁷	8.0X10 ⁷	6.3X10 ⁷
Shieh*	0.2183	0.2061	0.1992	0.2078	3.0X10 ⁸	2.6X10 ⁸	3.8X10 ⁸	3.1X10 ⁸
FCGM*	0.2382	0.2383	0.2367	0.2377	9.7X10 ⁸	3.6X10 ⁹	2.0X10 ⁹	2.2X10 ⁹

- * Indicates significance at P = 0.05
- Proc GLM and Scheffe's test was used to analyze the data

DISCUSSION

EVALUATION OF ISOLATION MEDIA

The mixed inoculum was intended to mimic a mixed infection that might be encountered in a natural columnaris infection. However, in natural infections the bacteria in open lesion are rarely found at the same concentration. Also the species of contaminating bacteria could vary from one location to another. For this reason, further evaluation of the media in the diagnostic laboratory would provide useful information. The selectivity of the antibiotics and their ability to inhibit the aquatic bacteria tested was known from previous studies. The effect of additional nutrient in some of the media seemed to boost the growth of non-columnaris bacteria and made the media less selective. The low nutrient content of SCA seemed to give a growth advantage to *F. columnare* and nutritionally inhibit some of the other aquatic bacteria. Also the high moisture content of SCA allowed the *F. columnare* to glide away from the other bacteria allowing them to be more easily isolated. The effect of the levels of nutrient and salt in various media on antibiotic activity is not known but more nutrient rich media seemed inferior in inhibiting non-columnaris bacteria.

EVALUATION OF MAINTENANCE MEDIA

The results show that *F. columnare* cultures can be maintained well past 30 days when additional moisture is added to the tubes. The viability of *F. columnare* typically does not extend beyond 48 hours on a standard cytophaga agar plate. By maintaining high moisture content, cultures can persist beyond the time at which an agar plate culture normally loses viability. The formation of sphaeroplasts signal the loss of viability in *F. columnare* cultures, and this feature could be used to predict when viability of cultures was waning. These results also show that simple low nutrient media out perform more complex high nutrient media for culture maintenance. This could in part be to the slow growth of the bacterium in these media which result in lower levels of metabolic toxins being produced, and ultimately extending the time before sphaeroplast formation.

EVALUATION OF BROTH CULTURE MEDIA

The growth of *F. columnare* cultures can be problematic, because of the strict nutrient requirements, relatively slow growth, and clumping of cells. *Flavobacterium columnare* growth medium (FCGM) had significantly faster growth and higher yields of cells than any other media tested. *Flavobacterium columnare* growth medium also seemed to prevent the clumping of cells seen in the past with other media. This could be due in part to the addition of several salts to the basal medium; however the reason for lack of cell clumping in the media is not fully understood. It could be due to changes in the surface charge of the outer membrane of the bacteria. The reason this feature is significant is the improved ability to enumerate the bacterium from broth cultures. This will greatly improve standardization of suspensions in research applications.

CHAPTER 3: BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF REPRESENTATIVE STRAINS OF *FLAVOBACTERIUM COLUMNARE*

INTRODUCTION

The ubiquitous distribution of the bacterium *Flavobacterium columnare* in fresh water environments and the tendency for fish to acquire the disease after mechanical or environmental insult makes *F. columnare* among the most described pathogens in cultured, ornamental, and wild fish populations (Shamsudin and Plumb, 1996; Shotts and Starliper, 1999). Columnaris disease accounts for millions of dollars of economic losses each year in commercial aquaculture, the aquarium trade, and in wild fish. A major constraint in *F. columnare* research and diagnostics has been the lack of the ability to distinguish these organisms from other phenotypically similar yellow pigmented bacteria (Paster et al. 1985). Several studies have revealed variation between isolates of *F. columnare* cultured from different hosts and geographical regions. Variability also exists in the pathogenicity of columnaris isolates (Decostere et al. 1999). It has been reported that some strains of *F. columnare* can be differentiated based on biochemical, serological, and molecular analysis (Anacker and Ordal, 1959; Pyle and Shotts, 1980; Wakabayashi et al. 1999). Because of the intraspecific variation, it has been suggested that there may be more than one strain of *F. columnare* that can cause disease in susceptible hosts. In this study, 49 isolates of *F. columnare*, obtained from 12 species of fish and several different geographic regions, were characterized and evaluated for variability. The strains were characterized biochemically by the Griffin screen (Griffin, 1992) and the API NE, and API ZYM systems available from BioMereaux/Vitek (Hazelwood MO). The API 20E system was not included in this study due to the lack of positive results in a preliminary study. Bader et al. (2003) published a polymerase chain reaction (PCR) protocol that has

been proposed as a method to identify *F. columnare* isolates. The PCR primers were designed to the integral spacer region of the 16S rRNA small subunit which is the target sequence for species specific identification of many bacteria including the yellow-pigmented bacteria. Random Amplified Polymorphic DNA (RAPD) analysis has been used to characterize isolates of some species of bacteria and to identify different genotypes (Romalde et al. 1999; Hawke et al. 2003; Thomas-Jinu and Goodwin, 2004). A single oligonucleotide primer in a PCR reaction is used in RAPD analysis to characterize genetic polymorphisms. The primer anneals to the template DNA where the template sequence matches the primer and then synthesizes a PCR product or several products of varying size. The products are then loaded into an agarose gel and subjected to electrophoresis which separates the products based on size. Staining with ethidium bromide allows visualization of a banding profile. The banding profiles are used to compare the genetic polymorphisms of bacterial strains and can be analyzed to reveal similarities and dissimilarities between isolates. Using these techniques we hope to differentiate strains into groups and gain knowledge about the intraspecific variation of *F. columnare* isolates.

STRAIN LIST

The strains of *F. columnare* used in the study as well as their hosts, state, and season isolated are listed in the Table 3.1. Bacterial strains were suspended in 0.85% saline, supplemented with 20% glycerol, and were frozen at -70°C in 1 ml aliquots until needed for analysis.

Table 3.1 Archived *F. columnare* isolates listed by strain number, fish species from which they were isolated, state of origin, and season isolated from diseased fish.

Strain Number	Fish Species	State	Season
LADL ¹ -88-173	Channel Catfish	Louisiana	Summer
LADL-92-002	Channel Catfish	Louisiana	Winter
LADL-93-002	Channel Catfish	Louisiana	Winter
LADL-94-060	Channel Catfish	Louisiana	Spring
LADL-94-078	Channel Catfish	Louisiana	Spring
LADL-94-081	Channel Catfish	Louisiana	Spring
LADL-94-082	Channel Catfish	Louisiana	Spring
LADL-94-104	Channel Catfish	Louisiana	Spring
LADL-94-140	Channel Catfish	Louisiana	Spring
LADL-94-141	Channel Catfish	Texas	Spring
LADL-94-147	Channel Catfish	Louisiana	Spring
LADL-95-132	Channel Catfish	Louisiana	Spring
LADL-95-255	Channel Catfish	Louisiana	Summer
LADL-96-511	Rainbow Trout	Unknown	Winter
LADL-96-513	Rainbow Trout	unknown	Winter
LADL-97-323	Largemouth Bass	Louisiana	Summer
LADL-97-374	Channel Catfish	Louisiana	Fall
LADL-97-376	Hybrid Striped Bass	Florida	Fall
LADL-01-089	Common Carp	Louisiana	Summer
LADL-01-093	Channel Catfish	Louisiana	Summer
LADL-01-100	White Crappie	Louisiana	Summer
LADL-02-063	White Crappie	Louisiana	Summer
LADL-02-176	Common Carp	Louisiana	Fall
LADL-02-185	Yellow Perch	Minnesota	Winter
LADL-03-061	Channel Catfish	Louisiana	Summer
LADL-03-124	Blue Catfish	Louisiana	Summer
LADL-04-046	Channel Catfish	Louisiana	Spring
LADL-04-060	Tilapia	Florida	Spring
LADL-04-066	Largemouth Bass	Louisiana	Spring
LADL-04-076	Bluegill	Louisiana	Summer
PB ² -2	Unknown	Arkansas	Unknown
PB-2-02	Unknown	Arkansas	Unknown
PB-7	Unknown	Arkansas	Unknown
PB-12199	Channel Catfish	Arkansas	Unknown
PB-10121	Unknown	Arkansas	Fall
PB-F. col	Unknown	Arkansas	Unknown
PB-10121-2	Unknown	Arkansas	Fall
AL ³ -94-203	Hybrid Catfish	Alabama	Unknown
PB-02-12	Fathead Minnow	Arkansas	Winter
PB-02-41	Koi	Arkansas	Spring
PB-02-51	Golden Shiner	Arkansas	Spring
PB-02-97	Koi	Illinois	Spring
PB-02-110	Golden Shiner	Arkansas	Spring
PB-04-02	Platy	Arkansas	Spring
PB-04-23	Koi	Washington	Winter
LV ⁴ -359-01	Channel Catfish	Arkansas	Unknown
LV-152-02	Channel Catfish	Arkansas	Unknown
LV-339-01	Channel Catfish	Arkansas	Unknown
LV-345-01	Channel Catfish	Arkansas	Unknown

Footnotes below

Footnotes:

¹ Louisiana Aquatic Diagnostic Laboratory, Louisiana State University

² Fish Disease Laboratory, University of Arkansas at Pine Bluff

³ Southeastern Cooperative Fish Disease Laboratory, Auburn University

⁴ Cooperative Extension Service, Laboratory, Lake Village, Arkansas

Channel Catfish (*Ictalurus punctatus*)

Rainbow Trout (*Oncorhynchus mykiss*)

Largemouth Bass (*Micropterus salmoides*)

Hybrid Striped Bass (*Morone saxatilis* X *M. chrysops*)

Common Carp (*Cyprinus carpio*)

Koi (*Cyprinus carpio*)

White Crappie (*Pomoxis annularis*)

Yellow Perch (*Perca flavescens*)

Blue Catfish (*Ictalurus furcatus*)

Tilapia (*Tilapia mossambique*)

Bluegill (*Lepomis microchirus*)

Hybrid Catfish (*Ictalurus punctatus* X *I. furcatus*)

Fathead Minnow (*Pimephales promelas*)

Golden Shiner (*Notemigonus crysoleucas*)

Platy (*Xiphophorus maculatus*)

MATERIALS AND METHODS

BIOCHEMICAL CHARACTERIZATION

Griffin Screen

All isolates were initially identified by the method of Griffin (1992), which uses five characteristics of *F. columnare* to differentiate it from other gram negative long flexing rods. To conform, the bacterium must be shown to satisfy the following requirements: produces flat, spreading, yellow, and rhizoid colonies on Cytophaga agar; grows in the presence of neomycin and polymixin B, production gelatin degrading enzymes; colonies must bind congo red dye; produces chondroitin sulfate A degrading enzymes. The first two points of the screen were tested on cytophaga and selective cytophaga agar. Colonies of *F. columnare* are flat, yellow, rhizoid, and are adherent to the agar surfaces. The presence of the enzyme gelatinase was tested by the hydrolysis of a gelatin substrate. The ability of the colonies to bind congo red dye was tested by

flooding 24 hr cultures with five ml of congo red dye. The dye was allowed to bind for one min followed by rinsing with distilled water. The results were recorded immediately. The test for the presence of a chondroitinase enzyme was performed according to an unpublished protocol by Cooper (personal communication). Briefly, FCGM agar was supplemented with 2.5 ml of a solution of chondroitin sulfate A at 10mg/ml to 17.5 ml of agar. The FCGM agar was melted and allowed to cool to below 50°C before the chondroitin was added so that no denaturation would occur. The plates were then poured and allowed to cool. The chondroitin supplemented agar plates were inoculated by making a single streak with a sterile cotton swabs saturated with a standardized suspension of bacteria, and were incubated for 48 hours at 28°C. Plates were then developed using two ml of a 4% bovine albumin solution per plate and allowed to incubate at room temperature for five min. The plates were then acidified by adding three ml of 1N HCL, and allowed to develop for 15 min. Positive results were scored when distinct zones of clearing occurred near bacterial growth. Cloudy areas existed where the chondroitin sulfate A was not degraded. Isolates were also tested for the presence of a flexirubin type pigments using the potassium hydroxide (KOH) method outlined in Bergey's Manual of Determinative Bacteriology 9th Edition (1994). Briefly, one drop of 3% KOH was added to 48 hour cultures on cytophaga agar slants and observing a change in pigment from yellow to brown.

Biochemical Testing With the API System

The API system BioMerieux Vitek (Hazelwood, MO) was used to characterize ten representative strains of *F. columnare*. Ten representative isolates were chosen based on their hosts and geographic locations to ensure strain diversity, the strain number

of the isolates tested were as follows: LADL 88-173, 96-511, 97-323, 97-376, 01-100, 02-063, 02-176, 02-185, 03-061, and 03-124. The strains were isolated from eight different fish hosts, and represented four geographic locations. The NE strips (BioMerieux Vitek) were incubated at 28°C for 24 hours. Inoculation, addition of reagents, and scoring of the results was done according to the manufacturer's protocol. The enzyme activity of the ten strains was determined with the API ZYM system. The inoculation and addition of reagents for the API ZYM kits was also done according to the manufacturer's protocol. The strips were incubated for five hours at 28°C.

MOLECULAR CHARACTERIZATION

DNA Extraction

Deoxyribonucleic acid (DNA) was extracted and purified by a modified phenol chloroform isoamyl alcohol (PCI) method (Marmur, 1961). Briefly, cells from 40 ml FCGM cultures were centrifuged in an automatic refrigerated centrifuge (Beckman Avanti J-25) at 4000 X g for 10 minutes at 10°C. The supernatant was discarded and five ml of buffer (10 mM Tris pH 8.0) was added to resuspend the cells. This was followed by the addition of 500 µl 10% sodium diosulfate (SDS) to the buffered solution to lyse the cells and release the DNA. The DNA was treated with 10 µl of 50 µg/µl Rnase A and incubated at 37°C for 30 min to degrade the RNA. Fifty µl of 20 mg/µl Proteinase K was added to the solution and incubated at 37°C for 1 hour. This step was added to degrade the proteins in the culture. Cultures of *F. columnare* produce high levels of polysaccharides, and removal of polysaccharides was accomplished by adding 1.8 ml of 5 M NaCl and mixing by inversion. Finally, 1.5 ml of NaCl Cetyltrimethyl Ammonium Bromide (CTAB) solution was added and the solution mixed. The CTAB binds to and

forms a complex with both polysaccharides and residual proteins. Samples were then incubated at 65°C for 20 min, and five ml of phenol chloroform-isoamyl alcohol was added to each sample and mixed. The suspension was then centrifuged at 6000 X g for 10 min at 4°C (Beckman Avanti J-25). The top layer was removed and saved, and the lower discarded. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the sample and mixed followed by centrifugation at 6000 X g for 10 min at 4°C. The top layer was saved and the lower discarded. A volume of three M sodium acetate pH 5.2 was added and the samples mixed. Then 2 ½ times the sample volume of absolute ethanol was added and mixed. The DNA was then spooled on a glass rod and rinsed with 70% ethanol. The samples were resuspended in one ml of 10 mM Tris-HCl pH 8.0, and stored at 4°C until ready for analysis. The boil prep outlined by Bader et al. (2003) was also attempted; however it yielded low levels of lower quality DNA. For this reason the phenol chloroform extraction was used for all samples. All samples were analyzed spectrophotometrically (Beckman DU 640) to determine the DNA concentration, protein level, and RNA level. Samples were diluted to a final concentration of 50 µg/µl for the PCR and RAPD analysis to standardize the DNA volume per reaction.

Polymerase Chain Reaction

All strains were analyzed by Polymerase Chain Reaction (PCR) with the primers of Bader et al. (2003). The primer sequences were as follows: FvpF1 5' GCC CAG AGA AAT TTG GAT 3', and FvpR1 5' TGC GAT TAC TAG CGA ATC C 3'. This procedure was done first to confirm the identity of isolates as *F. columnare*. Initial trials were done according to the protocol by Bader et al. (2003), and resulted in some variation between isolates when replicates were analyzed. This could have been in part due to

pipetting error, or variation in the amount of DNA per reaction. Once the reaction mixture was modified by the use of a pre-optimized RAPD PCR bead in each reaction, and standardizing the DNA concentration to 50 µg/µl, no variation was observed. The isolates were placed in two groups because the thermocycler would only accommodate 24 reactions at a time. Group one consisted of extracted DNA from isolates 1-24 and group two consisted of DNA extracted from isolates 25-49. The reaction mixture consisted of one pre-optimized RAPD PCR bead (Amersham Biosciences Piscataway, NJ), 14 µl molecular grade nuclease free water (Fisher Fair Lawn, NJ), one µl template DNA, five µl primer FvpF1, and five µl primer FvpR1 for a final volume of 25 µl per reaction. A Perkin Elmer Gene Amp 2400 thermocycler was used for the PCR reactions. RAPD analysis beads are composed of dATP, dCTP, dGTP, dTTP, BSA, AmpliTaq, Stoffel fragment, and buffer. The thermocycler was programmed at 95°C for 30 min for denaturation, then 25 cycles of 95°C for 30 sec, 59°C for 30 sec, 72°C for one min, followed by a final extension of 72°C for seven min and indefinite hold at 4°C. The PCR products were subjected to electrophoresis on a single 1% agarose gel subjected to electrophoresis for one hour at 65 volts and stained with five µg/ml ethidium bromide to visualize the targeted 1,192 bp product. The amplified products were loaded into wells of a 2% agarose gel and DNA bands visualized with five µg/ml ethidium bromide.

RAPD Analysis

The DNA from the extraction described above was also used in the Random Amplified Polymorphic DNA (RAPD) analysis. The RAPD analysis kit is available from Amersham Biosciences (Piscataway, NJ) and contains reaction beads, six different primers, and control DNA. The analysis was performed according to the manufacturer's

protocol. Briefly, the reactions were performed in 25 µl volumes containing a pre-optimized RAPD bead, five µl of five pmol/µl primer (25 pmol total), 18 µl molecular grade water (Fisher), and one µl of temp DNA 50 µg/µl. The contents were then mixed gently to dissolve the bead completely. The thermocycler (Perkins Elmer Gene Amp 2400) was programmed as follows: five min at 95°C for denaturation, followed by 45 cycles of one min at 95°C, one min at 40°C, and two min at 72°C. This was followed by a final extension of eight min at 72°C and an indefinite hold at 4°C. Primer five was used in this study because work done by Thomas-Jinu and Goodwin, (2004) showed that primer five gave numerous products with good separation to allow for profile analysis. The primer sequence was as follows: 5' AAC GCG CAA C 3'. Isolates were amplified in two groups because only 24 reactions could be completed in the thermocycler at one time; the first group contained isolates 1-24 and the second group 25-49. The amplified products were loaded into wells of a 2% agarose gel and subjected to electrophoresis for five hours at 36 volts and DNA bands visualized with five µg/ml ethidium bromide. The results were then compared and the isolates separated and grouped based on their individual banding profiles. A second RAPD analysis was done to confirm reproducibility of banding patterns. The resulting banding patterns allowed to the strains to be organized into four different groups Type I, II, III, and non-conforming. The banding profiles were analyzed using the Quantity One imaging and analysis program (BioRad Hercules, CA), and similarity comparisons were produced. Statistical analysis of the similarity of the banding profiles was based on the Dice coefficient and unweighted paired group method using arithmetic averages also known as weighted

average linking (UPGAMA). This analysis was chosen because it gives the best clustering and is affected the least by outliers.

RESULTS

BIOCHEMICAL CHARACTERIZATION

Griffin Screen

Of the strains tested, only two isolates did not pass all points of the Griffin screen. Isolates 95-255 and 03-067 were positive for growth in the presence of neomycin and polymixin B and for flat yellow rhizoid colonies, but were negative for chondroitinase, gelatinase, and congo red binding. Isolate 95-255 was excluded from the rest of the studies, because the original archived sample lost viability. Isolate 03-067 was used as a representative of yellow pigmented gliding bacteria that do not conform to the Griffin screen.

API Systems

All strains of *F. columnare* produced the profile 0441455 in the API NE system. The positive reactions were as follows: esculin hydrolysis, D-glucose utilization, L-arabinose utilization, potassium gluconate, capric acid, malic acid, and citrate. The resulting enzyme profiles generated in the API ZYM system were 0400030000-3000000000 at five hours for all isolates. The positive reactions were as follows: esterase (C4), leucine arylamidase, and an acid phosphatase.

MOLECULAR CHARACTERIZATION

Polymerase Chain Reaction Analysis

The PCR results were very consistent and reproducible once the reaction mixture was modified and the amount of DNA per reaction was standardized. Of the 49 isolates

tested, only strain 03-067 failed to produce a PCR product and this isolate was also negative for several points of the Griffin screen. The species specific 1192 kb PCR product was visualized in the agarose gel for the other 48 isolates tested indicating that the isolates were indeed *F. columnare*. This technique proved to be a useful identification tool that is less time consuming than the Griffin screen. Figures 3.1, 3.2 and 3.3 show the PCR results of all 49 strains.

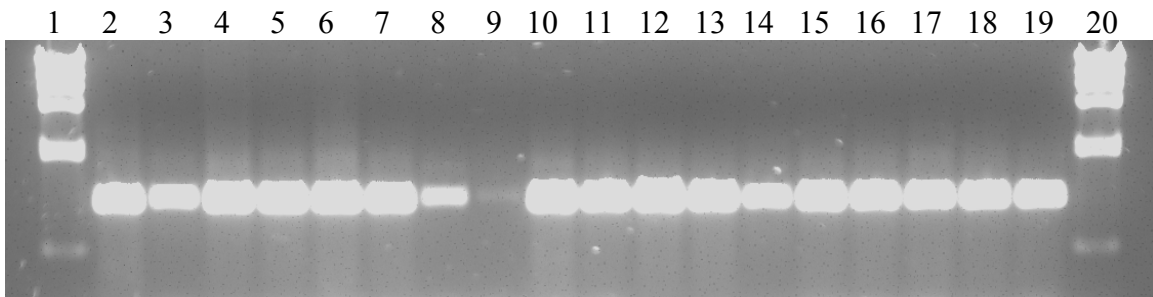


Figure 3.1 Agarose gel of PCR products amplified from *F. columnare* isolates using primers of Bader et al. (2003). Lane 1 λ Hind III ladder, Lane 2 LADL-88-173, Lane 3 LADL-92-002, Lane 4 LADL-93-002, Lane 5 LADL-94-060, Lane 6 LADL-94-078, Lane 7 LADL-94-081, Lane 8 LADL-94-082, Lane 9 LADL-94-104, Lane 10 LADL-94-140, Lane 11 LADL-94-141, Lane 12 LADL-94-147, Lane 13 LADL-95-132, Lane 14 LADL-96-511, Lane 15 LADL-96-513, Lane 16 LADL-97-323, Lane 17 LADL-97-374, Lane 18 LADL-97-376, Lane 19 LADL-01-089, Lane 20 λ Hind III ladder.

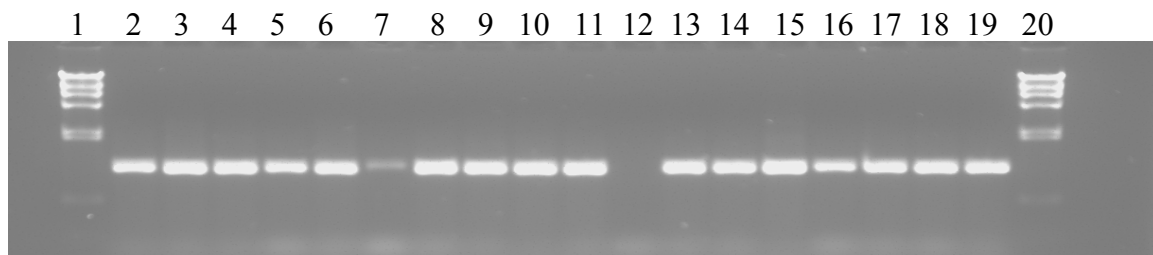


Figure 3.2 Agarose gel of PCR products amplified from *F. columnare* isolates using primers of Bader et. al (2003). Lane 1 λ Hind III ladder, Lane 2 LADL-01-093, Lane 3 LADL-01-100, Lane 4 LADL-02-063, Lane 5 LADL-02-176, Lane 6 LADL-02-185, Lane 7 LADL-03-061, Lane 8 LADL-03-124, Lane 9 LADL-04-046, Lane 10 LADL-04-060, Lane 11 LADL-03-067, Lane 12 PB-7, Lane 13 PB-2, Lane 14 PB-2-02, Lane 15 PB-12199, Lane 16 PB-10121, Lane 17 PB-10121-02, Lane 18 AL-94-203, Lane 19 PB-02-12, Lane 20 λ Hind III ladder.

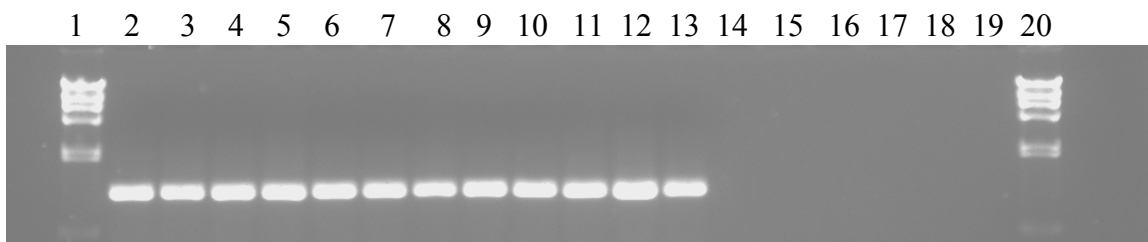


Figure 3.3 Agarose gel of PCR products amplified from *F. columnare* isolates using primers of Bader et al. (2003). Lane 1 λ Hind III ladder, Lane 2 PB-02-41, Lane 3 PB-02-51, Lane 4 PB-02-97, Lane 5 PB-02-110, Lane 6 PB-04-02, Lane 7 LV-339-01, Lane 8 LV-345-01, Lane 9 LV-359-01, Lane 10 LV-152-02, Lane 11 PB-F. col, Lane 12 LADL-04-066, Lane 13 LADL-04-067, Lane 14 negative control, Lane 20 λ Hind III ladder.

Random Amplified Polymorphic DNA Analysis

Random Amplified Polymorphic DNA analysis (RAPD) proved to be a good DNA fingerprinting technique to identify genetic polymorphisms and enabled strain differentiation among isolates of *F. columnare*. The banding profiles produced provided good discrimination and allowed many of the strains to be placed into three groups. Twenty isolates were classified as Type I profiles (Fig. 3.4), 13 were Type II profiles (Fig. 3.5), eight were Type III (Fig. 3.6), and seven isolates were deemed non-conforming to any of the previous three groups (Fig. 3.7). There was some variation within the Type I and III groups but strains were at least 75% related when analyzed. The Type II group had more variation, but strains in this group were still at least 40% related. In the non-conforming group each strain was less than 25% related to any other strain analyzed. The non conforming group contained two cold water strains, the columnaris-like isolate 03-067, and four warm-water *F. columnare* isolates. All strains in this group were presumptively identified as *F. columnare* by the Griffin screen and confirmed by PCR except for 03-067, which was used as representative columnaris-like bacteria along with the *E. coli* control from the kit.

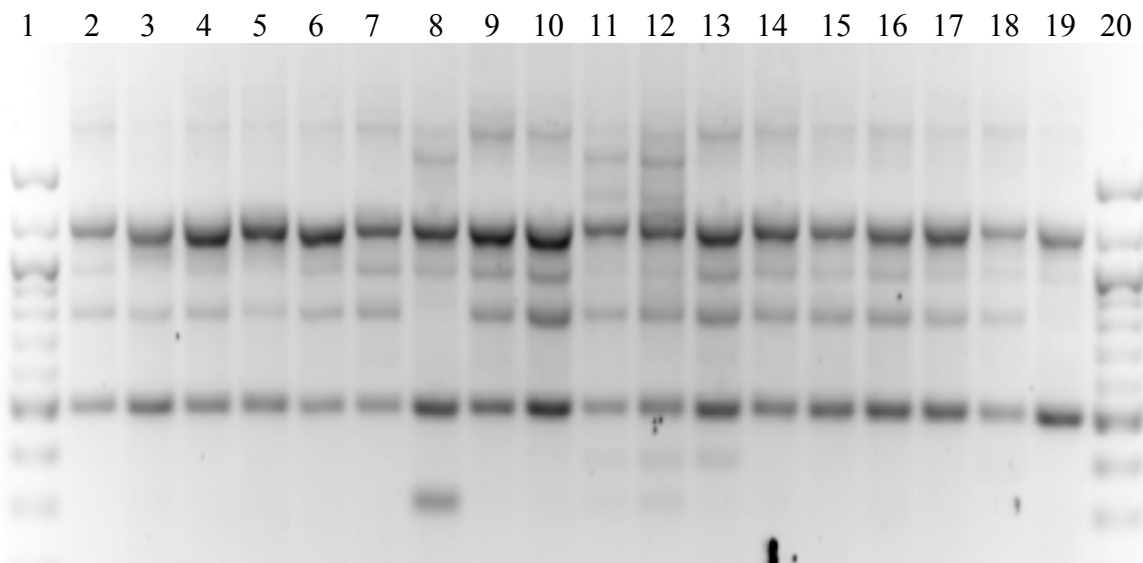


Figure 3.4 Agarose gel of RAPD PCR products for Type I *F. columnare* isolates. Lane 1 100 base pair ladder, Lane 2 LADL-88-173, Lane 3 LADL-94-060, Lane 4 LADL-94-078, Lane 5 LADL-94-081, Lane 6 LADL-94-082, Lane 7 LADL-94-147, Lane 8 LADL-95-132, Lane 9 LADL-97-374, Lane 10 LADL-01-093, Lane 11 LADL-02-063, Lane 12 LADL-03-061, Lane 13 LADL-03-124, Lane 14 PB-2, Lane 15 PB-10121, Lane 16 LV-339-01, Lane 17 LADL-04-046, Lane 18 LADL-04-066, Lane 19 LADL-04-076, Lane 20 100 base pair ladder.

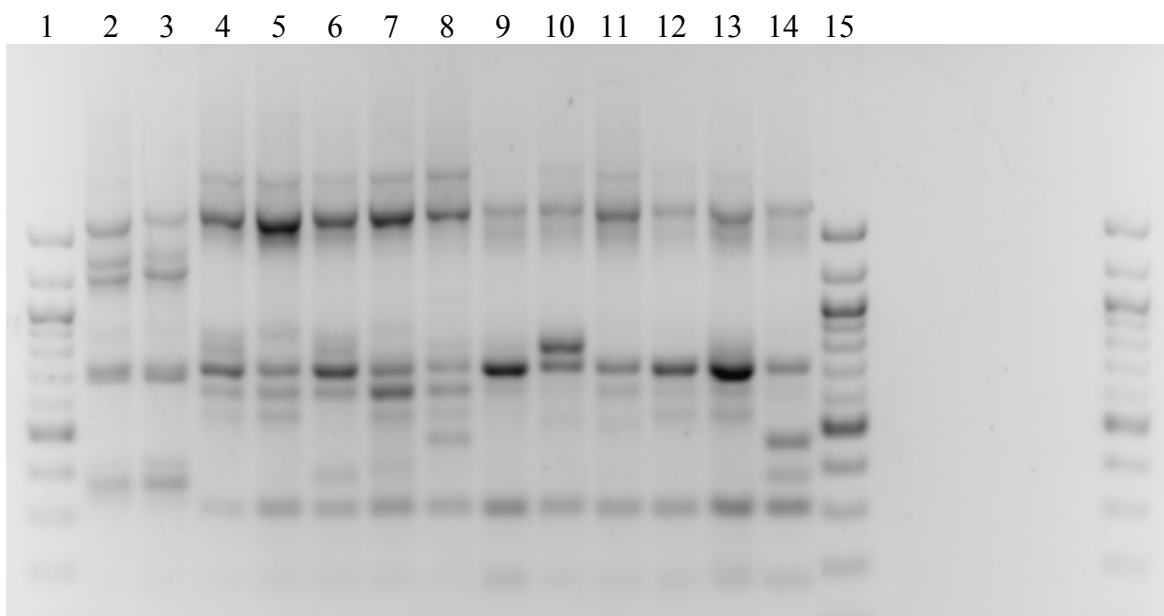


Figure 3.5 Agarose gel of RAPD PCR products for Type II *F. columnare* isolates. Lane 1 100 base pair ladder, Lane 2 LADL-94-104, Lane 3 LADL-94-140, Lane 4 LADL-97-376, Lane 5 LADL-02-176, Lane 6 LADL-02-185, Lane 7 PB-02-12, Lane 8 PB-02-41, Lane 9 PB-02-51, Lane 10 PB-02-097, Lane 11 PB-02-110, Lane 12 LV-152-02, Lane 13 PB-F. col, Lane 14 PB-04-23, Lane 15 100 base pair ladder.

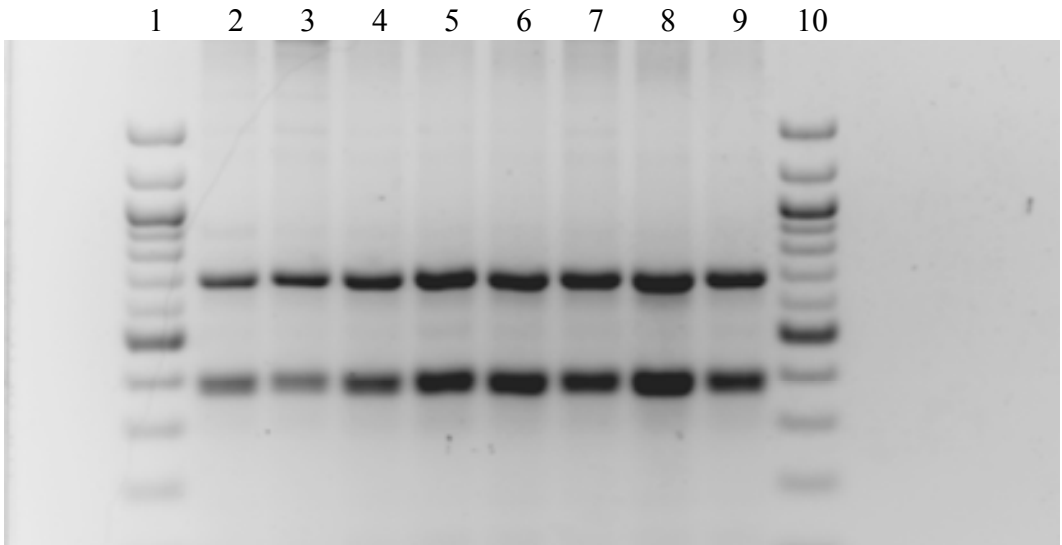


Figure 3.6 Agarose gel of RAPD PCR products for Type III *F. columnare* isolates. Lane 1 100 base pair ladder, Lane 2 LADL-92-002, Lane 3 LADL-94-141, Lane 4 LADL-97-323, Lane 5 LADL-01-089, Lane 6 PB-7, Lane 7 PB-12199, Lane 8 AL-94-203, Lane 9 PB-04-02, Lane 10 100 base pair ladder.

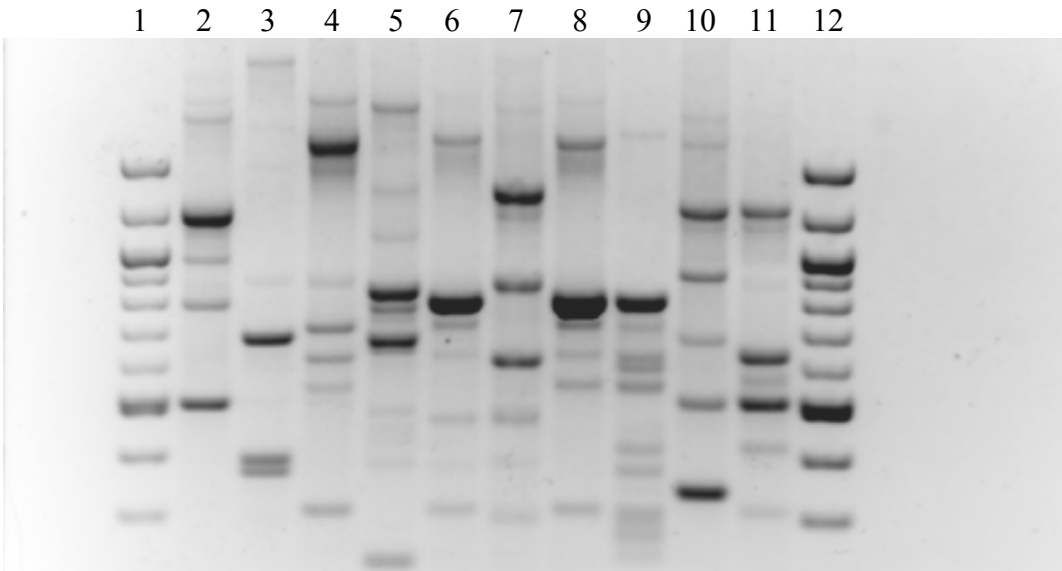


Figure 3.7 Agarose gel of RAPD PCR products for Non-Conforming *F. columnare* isolates, Lane 2 is a representative isolate from Type I and Lane 3 is a representative isolate from Type II. These were added for visual comparison. Lane 4 LADL-93-002, Lane 5 LADL-96-511, Lane 6 LADL-96-513, Lane 7 LADL-01-100, Lane 8 LADL-03-067, Lane 9 LV-345-01, Lane 10 LV-359-01, Lane 11 *E. coli* control, Lane 12 100 base pair ladder.

DISCUSSION

BIOCHEMICAL CHARACTERIZATION

Griffin Screen

The Griffin screen proved useful in the presumptive identification of *F. columnare* isolates from other yellow pigmented bacteria. Fifty isolates were tested and 48 passed all points of the screen with the addition of the flexirubin pigment test. The screen was able to detect the columnaris-like isolate and differentiate it from the other *F. columnare* isolates. The chondroitinase and congo red absorption test were the two tests that separated the two non-*F. columnare* isolates from the other isolates, however all points of the screen must be tested to result in an accurate identification. The Griffin screen is time consuming and an in depth procedure that requires several reagents, which makes it less suitable for diagnostic purposes. The Griffin screen was able to accurately identify 48 of the 48 strains confirmed by PCR as *F. columnare*

API Systems

The ten *F. columnare* isolates tested produced consistent results in the API NE and API ZYM systems, indicating the API systems have the ability to consistently identify *F. columnare* isolates by biochemical testing. Unfortunately closely related organisms such as *Flavobacterium psychrophilum* or *F. johnsoniae* were not used for comparison. This should be examined further in the future. The strains tested represented eight different fish species and four different states. Two cold water isolates and eight warm water isolates were analyzed, and the API NE profiles were consistent across all isolates. Pyle and Shotts (1980) reported that coldwater and warmwater isolates produced different biochemical phenotypes in the API 20E system, however we did not

observe this in the API 20E or NE system. Pyle and Shotts grew the bacteria in Ordal's broth, and the strip was inoculated with the broth culture and this could have led to differences in reactions they noted. This could indicate that the biochemical tests incorporated on the API NE strip are more suitable for identification of the *F. columnare* species. The API NE system is much faster than the Griffin screen, with several positive reactions that seem to give a consistent acceptable code that can be used for identification of *F. columnare* in the future. The APIZYM system produced only three positive reactions which indicate the organism has little enzyme activity. More reactions would have been desirable to consider this as an identification tool. Because of the slow somewhat fastidious growth of *F. columnare*, longer incubation times may have improved the sensitivity of the tests. Our results show that *F. columnare* isolates yield the same phenotype regardless of the fish species or geographic origin.

MOLECULAR CHARACTERIZATION

Polymerase Chain Reaction Analysis

The PCR identified 48 isolates as *F. columnare* and confirmed that isolate 03-067 was not *F. columnare*. The PCR results were in agreement with the Griffin screen results on every isolate tested. The DNA isolation procedure was more time consuming than the boil prep suggested by Bader et al. (2003), but the DNA obtained from the phenol chloroform extraction was of higher quality and volume. The consistency of the PCR results were improved by the PCI DNA isolation procedure, standardization of 50 µg/µl template DNA per reaction, and the pre-optimized PCR beads (Amersham Sciences). Species-specific primers developed for the identification of *F. columnare* isolates, that produce products that could be sequenced, would provide more detailed information on

strain differentiation. This approach is being examined by Dr. Larry Hanson at Mississippi State University.

Random Amplified Polymorphic DNA Analysis

The RAPD results show that there are several different *F. columnare* strains that can be identified by RAPD analysis. This analysis also shows that there is probably not one defined character such as fish species or geographic location that is consistent with these groups, and that it is more than likely a multifactorial effect that leads to the different genomovars (Table 3.3). Sequence analysis would be one way to confirm and define these groups. RAPD grouping was reported to be correlated with virulence to specific fish species (Thomas-Jinu and Goodwin, 2004). It would be interesting to incorporate a challenge experiment to evaluate strain virulence by group. The RAPD analysis delineated different strains of *Flavobacterium columnare* from phenotypically identical strains, and can be used to provide additional strain information beyond identification. This technique could be useful as an identification marker for challenge strains where the possibility of contamination by resident *F. columnare* is possible.

CHAPTER 4: IMPROVED MEDIUM FOR ANTIBIOTIC SUSCEPTIBILITY TESTING FOR *FLAVOBACTERIUM COLUMNARE*

INTRODUCTION

The control of bacterial disease is one of the great challenges in aquaculture, and columnaris disease ranks as one of the most important diseases in warmwater aquaculture. One method for the control of bacterial disease is by antibiotic treatment. There are no treatments approved by the U. S. Food and Drug Administration for the treatment of columnaris disease in any fish species. Thomas-Jinu and Goodwin (2004) reported seven of 17 test isolates were resistant to Romet®. Previous studies by Johnson (1991) indicated a high degree of resistance in isolates of *F. columnare* from catfish farms. Studies by Hawke and Thune (1992) indicated a low level of antibiotic resistance in *F. columnare* strains. These examples clearly demonstrate the need for accurate sensitivity data to ensure that the etiological agent is sensitive before recommending antibiotic therapy. In aquatic diagnostic laboratories, disk diffusion has been the most widely used susceptibility method but many labs have developed their own methods for testing isolates. The guidelines set by the Aquaculture Working Group of the Subcommittee on Veterinary Antimicrobial Susceptibility Testing of the NCCLS suggest using Mueller Hinton (MH) agar as the basal medium for disk diffusion sensitivity testing. Group 3 of the NCCLS M42-R document contains the gliding, flexing, and yellow-pigmented Gram-negative bacteria, and recommends that they be tested on dilute Mueller Hinton agar (DMH) formulated with 3g MH broth and 9g agar per liter. Hawke and Thune (1992) recommended the addition of five percent fetal calf serum to the basal DMH agar as a susceptibility test medium. Michel et al. (1999) reported no significant difference between fetal calf and equine serum when used as an enriching factor for the culture of

Flavobacterium psychrophilum. Dilute Mueller Hinton agar agar has been utilized in some labs for sensitivity testing of *F. columnare* strains, but disk diffusion testing is problematic with plate to plate variation in zone size and indistinct or “fuzzy” margins of the resulting zones of inhibition (Fig 4.1).

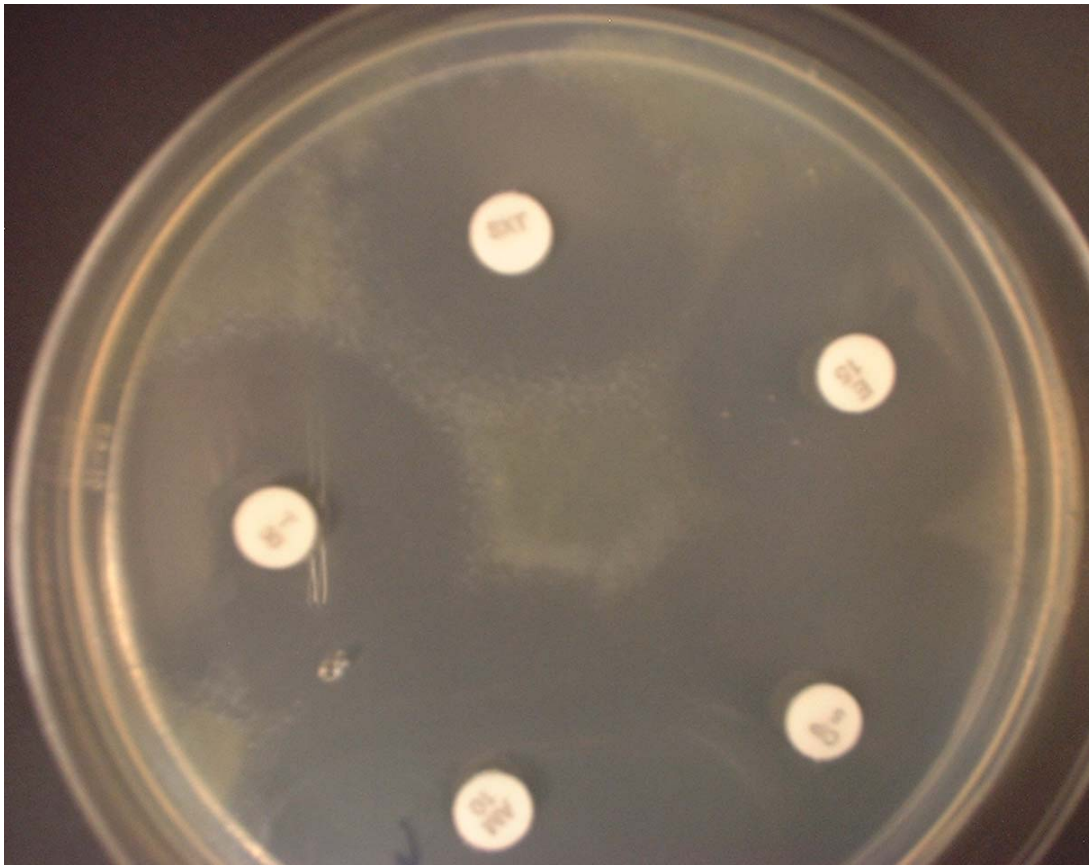


Figure 4.1 Antimicrobial susceptibility disk diffusion plate of a representative *F. columnare* isolate on dilute MH agar. Note the poor growth of the bacterium, and the “fuzzy” appearance of zone margins.

Thomas-Jinu and Goodwin (2004) stated that isolates that appeared resistant to Romet® by disk diffusion on DMH agar were susceptible when challenged and treated. This could be explained by errors in the sensitivity testing procedure, or in the interpretation of the zones of inhibition. For use in disk diffusion susceptibility testing procedures, DMH medium was formulated with different levels of MH broth and agar per

liter, and with the addition of fetal calf or equine serum to improve the growth of *F. columnare* and the resulting zone size consistency and uniformity around antibiotic susceptibility disks.

MATERIALS AND METHODS

BACTERIAL STRAINS

Escherichia coli (ATCC 25922), *F. columnare* (ATCC 23463), and ten clinical *F. columnare* isolates were tested by disk diffusion. The identity of the isolates was confirmed by PCR Bader et al. (2003), and by the Griffin screen (Griffin 1992). All isolates were frozen in 0.85% saline with 20% glycerol at -70°C until needed for the study.

DEVELOPMENT OF A SUSCEPTIBILITY TEST MEDIUM

To insure uniformity, one lot of each of five antimicrobial agents, one lot of Mueller Hinton medium, and one lot of equine serum was used in all disk diffusion test evaluations. The antimicrobial disks (BBL) chosen were Sulfamethoxazole : trimethoprim (SXT 25 µg), Sulfadimethoxine : ormetoprim (PRI-MOR 25 µg), Oxolinic acid (OA 2 µg), Oxytetracycline (T 30 µg), Florfenicol (FFC 30 µg). The basal MH broth used was (Difco) lot # 3126187, and the degranulated agar used was (Difco) lot # 3265229. The concentrations of MH broth evaluated were 3g, 3.5g, 4g, and 5g per liter. The agar concentrations that were evaluated were 9g, 12g, 15g, and 17g per liter. Varying the concentrations of MH broth was done to determine the optimum amount of nutrients for *F. columnare* growth. The varying agar levels were evaluated for their effects on zone appearance by limiting the gliding motility of the bacterium. Each medium was evaluated for thickness of growth, distinct zones, and conventional zone

margins. Once the optimum concentration of broth to agar was determined, 5% equine serum lot # ANE18713 (Hyclone Logan, Utah) was evaluated and compared to fetal calf serum as a growth supplement for *F. columnare* cultures on MH agar plates (Hawke and Thune, 1992).

PREPARATION OF INOCULUM

The inoculum was prepared in 40 ml of *Flavobacterium columnare* growth medium (FCGM) broth in 50 ml tubes inoculated by cotton swab from Cytophaga agar plates. The *F. columnare* suspensions were subjected to centrifugation in a refrigerated centrifuge at 2900 RPM for 10 min at 10°C. Pelleted cells were resuspended in five ml sterile saline. The centrifugation was repeated and the pelleted cells resuspended in sterile saline to a density equivalent of a McFarland #2 standard. The cell density was measured with a colorimeter (BioMerieux Vitek, Hazelwood, MO), which uses light dispersion to determine the density of a suspension. The surface of the test medium was inoculated by completely covering the surface of the plate with bacteria by streaking with a cotton swab saturated with bacteria from the standardized suspension. Following inoculation the plate was allowed to incubate at room temperature for five minutes prior to the addition of disks. A 12 disk dispenser (BBL Sparks, MD) was used to dispense five antimicrobial disks spaced equidistantly on each plate. The plates were incubated at 28°C for 48 hours with inhibition zone diameters being measured and recorded at 24 and 48 hours. No more than four plates were placed in a stack for incubation.

RESULTS

Maximum growth *F. columnare* occurred on the medium containing four grams of Mueller Hinton broth per liter. Isolates grew well on all concentrations of agar, but the best zone definition resulted on the medium containing 17 grams of agar per liter. The higher agar concentration seemed to limit the bacterium's gliding motility, and yielded the most distinct zone margins. The optimum media formulation was determined to contain 4 grams MH broth and 17 grams agar per liter. This medium gave the highest bacterial growth and allowed for better definition of zones of inhibition. Equine serum improved the growth of *F. columnare* cultures on dilute MH agar, and was not observationally different from fetal calf serum. Because of availability and cost of equine serum compared to fetal calf serum it was determined to be a suitable enrichment factor for the improved medium. Bacterial growth appeared heavier and had more distinct yellowish color on media containing serum, and this improved the readability of the disk diffusion zones represented in Fig 4.2, 4.3, and 4.4.

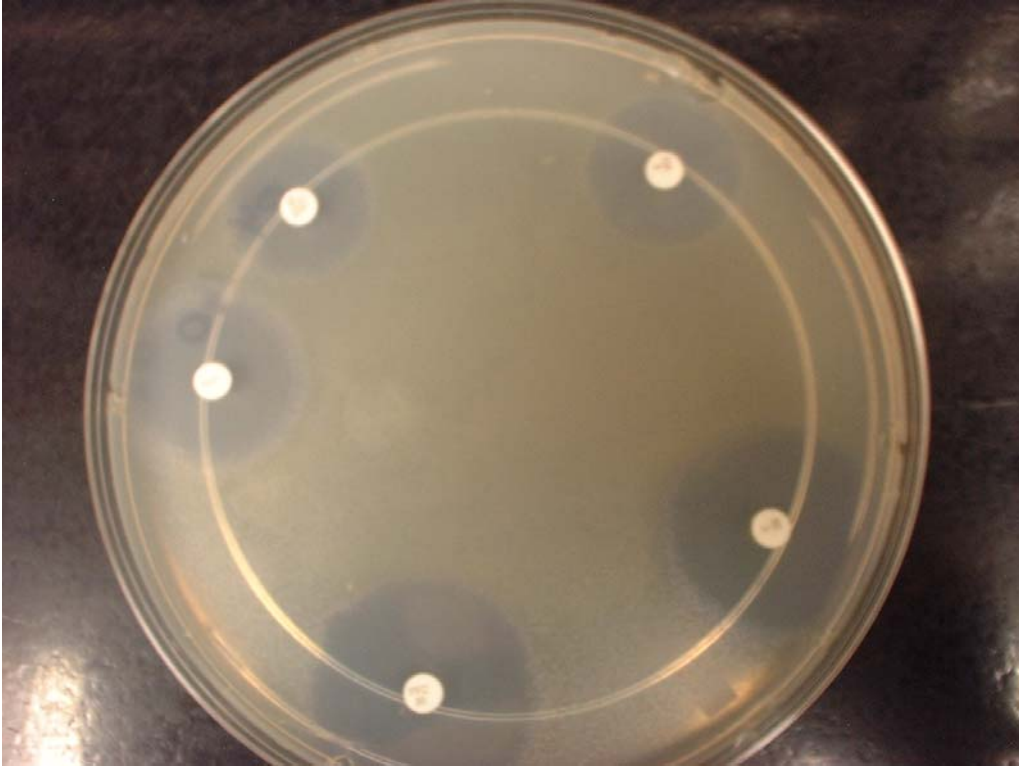


Figure 4.2 Antimicrobial susceptibility disk diffusion plate of a representative *F. columnare* isolate on improved Dilute Mueller Hinton agar with 5% equine serum.

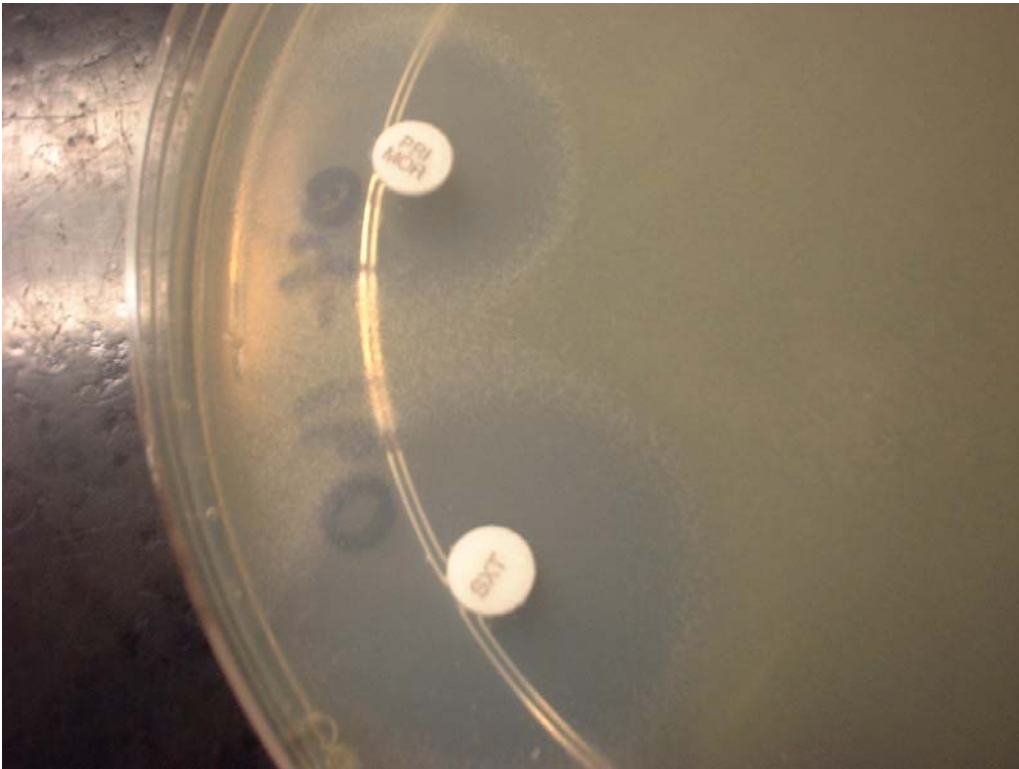


Figure 4.3 Antimicrobial susceptibility disk diffusion plate of a representative *F. columnare* isolate on improved Dilute Mueller Hinton agar with 5% equine serum.

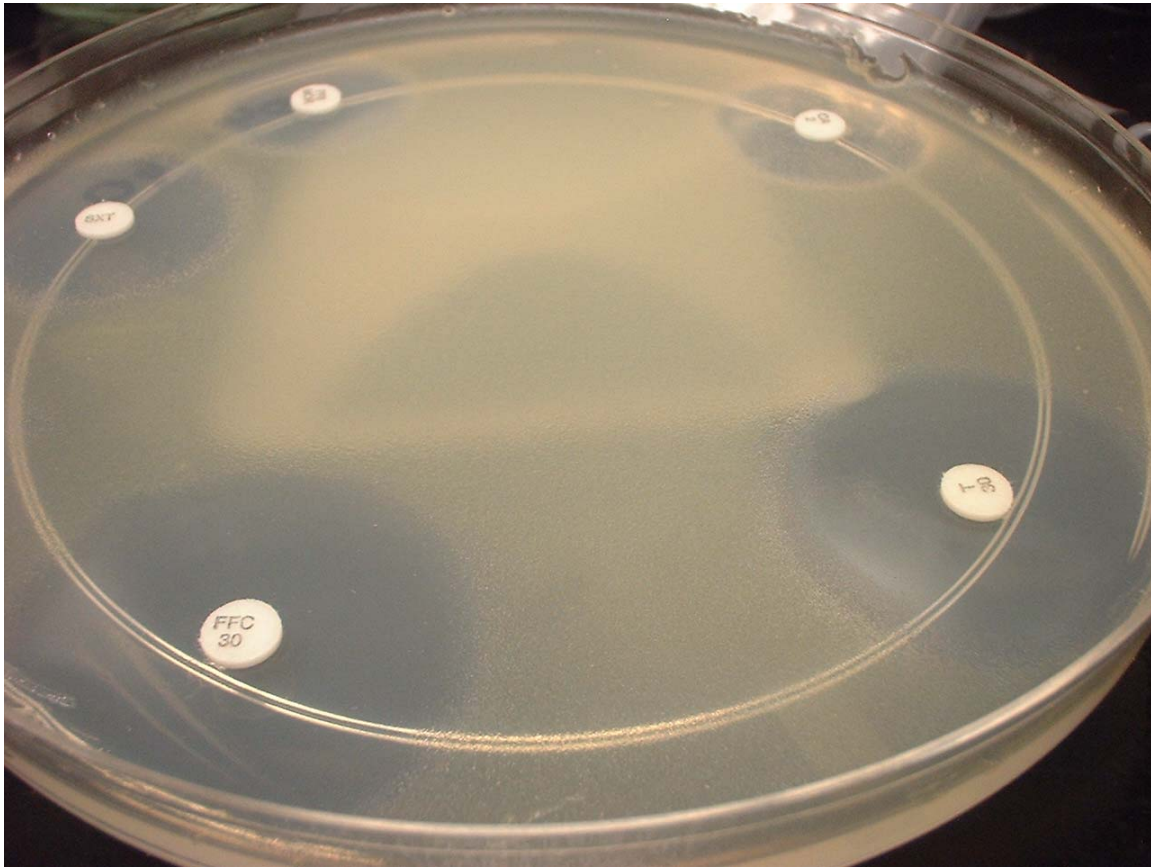


Figure 4.4 Antimicrobial susceptibility disk diffusion plate of a representative *F. columnare* isolate on improved Dilute Mueller Hinton agar with 5% equine serum.

The final improved medium composition is as follows: 4g MH broth, 17g agar, 50 ml equine serum, per 1 liter of distilled water. This improved medium was then tested for variation between isolates and between replicates. The test strains used to evaluate the variation in zone diameter for disk diffusion testing on the improved dilute Mueller Hinton agar were the ten isolates listed above with the addition of two ATCC strains. The three main antibiotics tested were Sulfamethoxazole : trimethoprim (SXT), Sulfadimethoxine : ormetoprim (PRI-MOR), and Oxytetracycline(T30). The data was collected and the ranges and averages determined for the original dilute MH and for the improved dilute MH agar. Zone diameter data for the *F. columnare* isolates tested is

given in table 4.1. The potential quality control strain ATCC 25922 *E. coli* was also tested using the improved DMH medium and the range and averages were determined to be 26-27 mm (26.2 mm) for SXT, 18-21 mm (18.8 mm) for PRI-MOR, and 21-22 mm (21.4 mm) for T-30.

Table 4.1 Resulting zone diameters for eleven *F. columnare* isolates subjected to antimicrobial susceptibility testing by disk diffusion on DMH and Improved DMH media.

Drug	SXT 25 (Sulfamethoxazole trimethoprim)		PRI-MOR 25 (Sulfadimethoxine ormetoprim)		T-30 (Oxytetracycline)	
	A	B	A	B	A	B
Medium						
Range (mm)	15-32	23-30	13-28	20-27	28-42	30-35
Mean (mm)	22	27	18.8	24	36.4	33.3

A= DMH medium

B= Improved DMH medium

DISCUSSION

The standard formula of dilute Mueller Hinton agar (3g broth, 9g agar, 50 ml fetal calf serum, 1 liter distilled water), was modified to improve overall growth and zone determination in disk diffusion testing of *F. columnare* isolates. The improved dilute Mueller Hinton agar recommended is four grams per liter MH broth, 17 grams per liter agar, 50 ml equine serum per liter. The potential quality control strain ATCC *E. coli* was able to grow and yield consistent and acceptable zone diameter data on the improved DMH medium. However, to compare this data to data resulting on standard DMH media more research must be completed to evaluate the interactions that could result from lowering the nutrient level and adding 5% equine serum. Also the relationship to zone size and efficacy of the drug *in vivo* is yet to be determined. This medium gave the highest growth observed in this study, while also improving the disk diffusion zone results. Zone variability was reduced by 40% when the improved medium was

compared to the original DMH agar. Variability was reduced because of the improved growth of the bacterium, which made measuring the zone diameters more exact and reproducible with distinct zones of inhibition.

CHAPTER 5. CONCLUSIONS

Selective Cytophaga agar produced isolated colonies and outperformed the other tested media for the isolation of *F. columnare* from a mixed bacterial suspension. Media modified by additional moisture content by slanting the agar in a tube and adding sterile saline was able to maintain cultures beyond the time at which an agar plate culture loses viability. Simple low nutrient media performed better than the more complex high nutrient media for culture maintenance, with tryptone yeast extract medium maintaining viability for 78 days. Of the broth media evaluated, *Flavobacterium columnare* growth medium had significantly higher growth than any other media tested, producing the highest absorbance values and colony forming units per ml. Forty eight of 49 *F. columnare* isolates were presumptively identified by the Griffin screen, this technique proved useful for the identification of *F. columnare* isolates from other yellow pigmented bacteria. Ten representative *F. columnare* isolates were subjected to the API NE and ZYM systems and produced consistent results, indicating the API systems ability to identify *F. columnare* isolates by biochemical phenotype. However, strains of *F. columnare* could not be differentiated by biochemical characterization. The polymerase chain reaction method of Bader et al. (2003) was used to confirm the identity of 48 of 49 archived *F. columnare* isolates. Examination of *F. columnare* strains by Random Amplified Polymorphic DNA analysis (RAPD) produced several different banding patterns “fingerprints”. Attempts to relate demographic characters such as fish host, geographic location or season of occurrence were unsuccessful. No single common relationship was seen among members of groups it appears that there is a multifactoral effect that leads to the different genomovars. Variations of dilute Mueller Hinton agar

(DMH) were evaluated to improve the results of disk diffusion testing of *F. columnare* isolates. The improved DMH agar formula was found to be four grams per liter MH broth, 17 grams per liter agar, 50 ml equine serum per liter. This medium improved growth of the bacterium and also improved the disk diffusion zone results, by reducing zone size variability by 40% when compared to the original proposed medium. Several avenues for future research were opened by this study. An immersion challenge would provide useful information for correlation of RAPD group with virulence as was reported by Thomas-Jinu and Goodwin (2004). The challenge model could also be used to evaluate the accuracy of antimicrobial susceptibility data produced on improved dilute Mueller Hinton agar. Future work on determination of minimal inhibitory concentration (MIC) of the various drugs would be beneficial in this regard. This would allow comparisons to be made from disk diffusion plates and MIC to a controlled *in vivo* environment which may help determine breakpoints for drug susceptibility values.

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