ABSTRACT

Title of Dissertation:	DEVELOPMENT OF METHODS TO TEST DRUG SENSITIVITY OF FISH PATHOGENIC *FLAVOBACTERIUM COLUMNARE* AND DRUG SENSITIVITY THRESHOLDS FOR *F. COLUMNARE* TO THE ANTIMICROBIAL FLORFENICOL
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Antimicrobial drugs play a key role in managing the health of fish in aquaculture. However, public health concerns about antimicrobial resistance—the ability of microorganisms to resist standard antimicrobial treatments—include the potential for antimicrobial use in aquaculture to select for resistant bacteria in and around fish farms. Recent approval of two antimicrobial drugs to treat farmed freshwater fish with infections caused by the aquatic bacteria *Flavobacterium columnare* and *F. psychrophilum* created an important need for research to monitor these bacteria for changes in antimicrobial susceptibility and to decide when the antimicrobials should be used. Therefore three studies were conducted.

The initial study optimized methods for broth microdilution testing *F. columnare* and *F. psychrophilum* and conducted a multi-laboratory standardization trial that set quality control parameters for nine antimicrobials commonly used in aquaculture, thus

creating the first standardized testing method. In the second study, we constructed frequency distributions using minimal inhibitory concentrations determined from testing 134 *F. columnare* with the standardized method. Analysis of the distributions determined the drug concentration—called an epidemiological cutoff value (ECV)—which separated the wild type isolates with no acquired or selected resistance from the non-wild type isolates. The ECV indicated 22 of 134 isolates had decreased their susceptibility to at least 1 antimicrobial. In addition, we developed a laboratory disease model with juvenile channel catfish, *Ictalurus punctatus*. We compared the virulence of three *F. columnare* isolates with wild type or three isolates with non-wild type florfenicol susceptibility using the model. We found that five isolates had similar high level virulence and were not affected by differences in florfenicol susceptibility.

Finally, we studied if decreased non-wild type florfenicol susceptibility affected the ability of the approved florfenicol treatment to control *F. columnare* infections. The approved treatment significantly reduced catfish mortality following exposure to an isolate with typical wild type florfenicol susceptibility but mortality was not reduced following exposure to an isolate with non-wild type susceptibility. Taken together, these studies provide methods and research needed to monitor *F. columnare* for changes in antimicrobial susceptibility and to rationally use florfenicol to control *F. columnare* infections.

Development of methods to test drug sensitivity of fish pathogenic *Flavobacterium columnare* and drug sensitivity thresholds for *F. columnare* to the antimicrobial florfenicol

by

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Dedication

To Heather, who encouraged me to
take the leap and then to keep
flapping my wings.

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List of Abbreviations

CPM = cumulative percent mortality

df = degrees of freedom

dpc = days post challenge

ECV = epidemiological cutoff value

FDA = Food and Drug Administration

MIC = minimal inhibitory concentration

NWT = non-wild type

P = p-value

QC = quality control

SD = standard deviation

t = t-value

U. S. = United States

WT = wildtype

Chapter 1: Introduction

Antimicrobial resistance is an important health issue due to the continuing rise of pathogenic microorganisms (bacteria, fungi, viruses and parasites) that withstand standard antimicrobial treatments. Resistance jeopardizes numerous advances made by modern human and veterinary medicine since procedures often depend on antimicrobials to prevent or treat infection. Drug-resistant microorganisms interrupt an antimicrobial's mode of action often requiring another antimicrobial that works in a different way. However, different antimicrobials are not always available, and in addition some microorganisms develop resistance to multiple drugs. Therefore, limiting antimicrobial resistance requires using these drugs as judiciously as possible.

Recommendations for judicious use include dispensing the right antimicrobial for an illness and at the discretion of a certified health professional who considers the use medically necessary (WHO, 2000). The use of antimicrobials to prevent disease, especially in food animals, is a concerning practice that can unnecessarily select for resistance (WHO, 2000, Leung et al., 2011; WHO 2014). With the goal of limiting the selection of resistance on farms, the European Union banned antimicrobial prophylaxis in food animals, opting to only allow therapeutic uses with veterinary oversight (Cogliani et al., 2011). The United States Food and Drug Administration (FDA) has recently proposed to follow suit, asking companies to voluntarily remove all approved indications of medically important antimicrobials meant to enhance growth in food animals (FDA, 2013a/b). In addition, FDA will also require veterinary oversight for all remaining therapeutic uses. By requiring veterinary oversight these countries want to ensure animals

receive appropriate therapy, i.e. one that provides an effective drug at a concentration and duration that inhibits the pathogenic microorganism and achieves a clinical cure in the infected patient (Lees and Aliabadi, 2002). Thus, clinicians should know whether or not available antimicrobials inhibit the target microorganism as determined by an antimicrobial susceptibility test.

Standard laboratory protocols for testing antimicrobial susceptibility are a key component in strategies to reduce antimicrobial resistance (Leung et al., 2011). Standardized testing provides laboratories with the same procedures and quality controls to certify results. By generating consistent results across multiple laboratories, datasets can be generated so criteria can be set to monitor for microorganisms that have developed resistance or to facilitate judicious use. Analysis of how frequently a particular microorganism has a certain antimicrobial susceptibility determines cutoff points in the test scale that define if the microorganism has decreased its susceptibility to a given antimicrobial (i.e., antimicrobial resistance). Monitoring trends in the prevalence of microorganisms with decreased susceptibility can then be compared over time and across different locations. Susceptibility tests are also compared to outcomes of clinical therapy and drug pharmacology so categories can be defined to predict the likely outcome of treatment. Clinicians use the categories to help decide when to prescribe an approved antimicrobial, facilitating prudent use of antimicrobials.

While all microorganisms (bacteria, fungi, viruses and parasites) can develop resistance, drug-resistant bacteria have created the most concern (WHO, 2014). Bacteria have very short life cycles with relatively fast mutational and/or selective processes that

allow them to quickly adapt to changes in their environment. Antimicrobials act on bacteria by inhibiting the synthesis of nucleic acids, proteins or cell walls, disrupting metabolic pathways, or altering cell membranes. Bacteria decrease their susceptibility to antimicrobials with enzymes that destroy drugs, efflux pumps that remove the drugs from the bacterial cell, modified cellular sites that drugs target and/or altered metabolic pathways (Tenover, 2006). Some bacteria intrinsically resist certain classes of antimicrobial drugs; however, resistance is often associated with mutated genes or new genes transferred from other bacteria. Although genetic mutation and gene transfer are natural processes, the overuse and misuse of antimicrobials in medicine and agriculture has influenced the emergence and spread of antimicrobial resistance (Leung et al., 2011).

As with other animal agriculture sectors, antimicrobial use in aquaculture selects for bacteria with decreased antimicrobial susceptibility (Aoki et al., 1974; Kim et al., 1993; Ho et al., 2000; Michel et al., 2005; Dung et al., 2008; Rhodes et al., 2008; Clark et al., 2009; Ishida et al., 2010). Uneaten medicated feed or treatment in open water also releases antimicrobials directly into the environment creating concerns that drug residues in sediment could alter the susceptibility of environmental bacteria (Samuelsen et al., 1991, 1992; Coyne et al., 1994; Rigos et al., 2004). Hence, the most important risk from aquaculture with regard to antimicrobial resistance is the potential for bacteria to become resistant in and around fish farms, and transfer their resistance genes to other bacteria that infect humans (Sørum et al., 2003; FAO/OIE/WHO, 2006; Maki et al., 2008; Verner-Jefferys et al., 2009).

The FDA recently approved the antimicrobials florfenicol (Aquaflor®) and oxytetracycline dihydrate (Terramycin®) for use in freshwater-reared finfish to control infections caused by the aquatic bacteria *Flavobacterium columnare* and *Flavobacterium psychrophilum* (Federal Register, 2007a/b, 2008, 2012, 2014). These bacteria are nutritionally fastidious gram-negative bacteria that are ubiquitous in freshwater infecting a wide range of wild and cultured freshwater fishes. The new drug approvals are an important improvement for U.S. aquaculture as *F. columnare* and *F. psychrophilum* are two of the most economically important diseases in the industry. In addition, Aquaflor® is the first new antimicrobial approved in U. S. for aquaculture in the last 20 years. Unfortunately, a standardized antimicrobial susceptibility test for these bacteria was not available or required during drug approval process. My first goal in this research was to standardize a susceptibility test for these fastidious bacteria.

My second goal, once I had a standardized test was to develop interpretive criteria for *F. columnare*. These criteria are integral to the test since they are the cutoff points that indicate whether isolates of the bacterium 1) have decreased susceptibility to an antimicrobial, or 2) will likely be controlled by treatment with an antimicrobial. I used the standardized test to determine the antimicrobial susceptibility patterns of *F. columnare* against antimicrobials used to treat columnaris disease as well as other antimicrobials commonly used in global aquaculture. Using the susceptibility distribution for each antimicrobial, I determined the cutoff that divides the isolates based on their susceptibility. Finally, I developed an infection model in channel catfish, *Ictalurus punctatus*, and studied whether *F. columnare* with decreased florfenicol susceptibility as indicated by the *in vitro* test can resist an actual *in vivo* treatment with Aquaflor®. This is

a key component needed to use the standard susceptibility test as a predictor of treatment so Aquaflor® can be judiciously used by the aquaculture industry.

In this chapter I provide background information about *F. columnare* and *F. psychrophilum*, their diagnostic characteristics and previous antimicrobial susceptibility testing of both species. In addition, I describe the process followed by the Clinical Laboratory Standards Institute (CLSI) to standardize antimicrobial susceptibility test methods and to develop interpretive criteria.

1.1 *Flavobacterium columnare*

Flavobacterium columnare was first described by Davis (1922) from a variety of different species of diseased freshwater fishes with gill and skin lesions. He consistently observed dirty-white or yellowish areas on some part of the fish's body (particularly on the fins) that rapidly increased in size. Once lesions emerged, the fish rapidly died. Slide scrapes immersed in a drop of water from the actively growing margin of lesions were exclusively composed of long, slender, flexible rod-shaped bacteria. Although Davis (1922) could not grow the bacteria, the easily recognizable nature of the disease with well-defined lesions and bacteria strongly suggested the bacterium caused the disease. He named the bacterium *Bacillus columnaris* because it forms columns or haystacks seen in wet preparations of gill or skin lesions.

Ordal and Rucker (1944) were the first to isolate the bacterium, renamed as *Chondrococcus columnaris*, on a low-nutrient medium, and identified it as a myxobacterium since they observed fruiting bodies and microcysts. Myxobacteria are

long thin gram-negative rods that move by a creeping or flexing motion and that have a life cycle composed of vegetative cells, fruiting bodies and/or microcysts (Bullock et al., 1986). However, Garnjobst (1945) quickly proposed changing the name to *Cytophaga columnaris* because stars/clusters formed in liquid media rather than fruiting bodies.

Later on, Leadbetter (1974) proposed naming the bacterium *Flexibacter columnaris* and also suggested that the *Flexibacter* genus also include *Cytophaga psychrophila* (syn. – *F. psychrophilum*). Bernardet and Grimont (1989) confirmed Leadbetter's proposal using DNA relatedness and phenotypic tests and also proposed changing the name of *C. psychrophila* to *Flexibacter psychrophilus*. However, they recognized the entire Cytophaga-Flexibacter-Flavobacterium phylogenetic branch had to be reorganized before their proposals could be accepted. Subsequently, Bernardet et al. (1996) studied the phylogenetic relationships of the entire group and determined based on DNA-rRNA hybridization that some *Cytophaga* and *Flexibacter* species, including *F. columnare* and *F. psychrophilum*, grouped with the *Flavobacteria*. The genus now includes all 3 species that infect fish (*Flavobacterium columnare*, *F. psychrophilum* and *F. branchiophilum*).

Fish illness from *Flavobacterium columnare*, columnaris disease (Davis, 1922; Ordal and Rucker, 1944), has become one of the most economically important bacterial diseases in the commercial production of channel catfish, *Ictalurus punctatus*, in the United States (Wagner et al., 2002). The disease is also a major problem for a wide range of other cultured fish as well as wild fish worldwide (Fish and Rucker, 1945; Borg, 1960; Wakabayaski and Egusa, 1966; Bullock et al., 1986; Bernardet, 1989). Most freshwater

fish from cold- to warmwater are susceptible to the disease usually when the fish are stressed by environmental conditions (Wakabayashi, 1991; Durburow et al., 1998). The disease remains a major problem throughout many freshwater aquaculture industries (Decostere and Haesebrouck, 1999; Pulkkinen et al., 2010) and continues to emerge for example in the US trout industry (Evenhuis et al., 2014).

Flavobacterium columnare infections are typically limited to the external surfaces (fins, gills and skin) of fish although highly virulent isolates cause mortality with no tissue damage (Pacha and Ordal, 1967). Lesions are white or yellowish patches on the gills or skin but commonly begin on the fins (Davis, 1922). Fin lesions spread out at the base of the fin. Initially, skin lesions appear as dull areas where the mucus is eroded (Durburow et al., 1998); the leading edge (margin) can be light red from inflamed skin (Bullock et al., 1986). Initial skin lesions are small but increase rapidly in size as the bacteria grows in spreading patches which can cover large portions of the body. On the dorsum, the lesions can have a characteristic saddleback appearance (Durburow et al., 1998). More chronic skin lesions have gray-blue or yellow-brown ulcerated centers where the infection has progressed through the dermis and caused muscle necrosis (Bullock et al, 1986; Durburow et al., 1998). Gill lesions appear as white or yellow eroded patches where the bacteria cause necrosis, hyperplasia and fusion of the secondary lamellae, congestion of blood vessels and/or edema (Foscarini, 1989). Systemic infections occur with limited internal pathology although the bacterium can be readily cultured from organs (Bullock et al., 1986). Reported kidney lesions from experimental infections in salmon were limited to enlargement of the Bowman's capsule in glomeruli (Pacha and Ordal, 1967).

1.2 *Flavobacterium psychrophilum*

As with *F. columnare*, Davis (1946) also first described *F. psychrophilum* infections in rainbow trout, *Oncorhynchus mykiss*, after observing sick juvenile trout in a West Virginia hatchery in 1941 and 1945 that had "peduncle disease". Beginning at the adipose fin, the trout would develop white discolored lesions that would gradually encompass the entire peduncle and caudal fin, destroying the muscles and fin in severe infections. Material scraped from the lesions always had long rod-shaped bacteria which gave "little doubt" of the lesion's cause although the bacterium could not be cultured. Shortly afterwards in 1948, an epizootic occurred among hatchery raised fingerling silver (coho) salmon, *Oncorhynchus kisutch*, in Washington State (Borg, 1960). Severe losses occurred despite low water temperatures (6-10 °C). These fish had lesions similar to those described by Davis (1946) with a long rod-shaped bacterium. Borg (1960) was able to grow the bacterium on low nutrient media and grouped them based on growth and biochemical characteristics. With pathogenicity tests Borg (1960) fulfilled Koch's postulates finding that the slow-growing low temperature group isolated from the kidneys of sick fish was the primary cause of the disease, naming it *Cytophaga psychrophila* (syn. - *Flavobacterium psychrophilum).* Infections were induced in fish held at 10 to 18 °C.

The disease is known as "Bacterial Coldwater Disease" (BCWD) since most epizootics occur at water temperatures below 15 °C (Wood, 1974). Reports of the disease were only from North American salmonids until about the mid-1980s when similar disease condition occurred in Rainbow trout fry in Europe and Japan (Cipriano and Holt, 2005; Wakabayashi, 1991; Wakabayashi et al., 1994). This form of the disease is

commonly called Rainbow Trout Fry Syndrome (RTFS, Austin and Stobie, 1991; Lorenzen et al., 1991) but it's also called fry anemia or myxobacterial infection. RTFS is usually an internal infection of fry weighing ≤ 1 gram grossly observed as weakness, pale gills, swollen abdomen, exophthalmia and/or dark coloration (Bernardet et al., 1988; Lorenzen et al., 1991; Santos et al., 1992). The fry usually have severe anemia, accumulation of fluid in the peritoneal cavity (ascites), enlarged spleens, and liver discoloration. Long rod-shaped *F. psychrophilum* are found in smears of the spleen, liver, kidney, and blood and can be readily cultured on cytophaga agar.

BCWD and RTFS cause extensive losses in salmonid aquaculture worldwide (Nematollahi et al., 2003). Although the disease occurs most frequently amongst salmonids, it also occurs in other freshwater fish (Cipriano and Holt, 2005).

1.3 Growth and Identification

As described by Bernardet and Grimont (1989), *Flavobacterium columnare* are long, slender, flexible gram negative rods that are 3 to 10 μm long and 0.3 to 0.5 μm wide although, some cells can be grow up to 10 to 25 μm long. In wet preparations the cells clearly glide with pivoting and bending. The bacterium can tolerate up to 0.5% NaCl and temperatures between 10 to 33°C (some tolerate 37°C) although optimal growth is from 20 to 25°C. A wider optimal temperature range for *F. columnare* is more likely as others have reported a higher optimum temperature, for instance, Garnjobst (1945) listed optimal growth as 25 to 30°C. On agar, the bacterium forms yellow colonies that strongly adhere. The colonies appear flat, dry, and spreading. In liquid cultures, the bacterium forms aggregates that adhere to the sides of the container. The yellow-orange color of

these bacteria is mainly derived from non-diffusible flexirubin pigment (Reichenbach et al., 1974).

Flavobacterium psychrophilum is a nonsporulating, gram-negative rod that ranges in size from 1-5 µm long by 0.3-0.5 µm wide (Bernardet and Grimont, 1989). The cells glide very slowly requiring prolonged observation, however some strains glide faster. The bacterium tolerates up to 0.5% NaCl and temperatures between 10 to 20°C. Optimal growth occurs at 15 to 18 °C with growth slowing dramatically at 6 and 22-25 °C. On agar colonies are circular with regular edges and do not adhere; however, some strains produce colonies with spreading margins. Colonies appear yellow with a smooth glossy texture.

Flavobacterium columnare and *F. psychrophilum* do not grow well on standard media such as trypticase soy, tryptic soy or brain heart infusion (Bernardet and Grimont, 1989; Daskalov et al., 1999; Cain and LaFrentz, 2007) requiring media low in nutrients. Anacker and Ordal (1955) developed one of the first recipes often referred to as "cytophaga" or "Anacker and Ordal" media. Other alternatives are tryptone yeast extract (TYE) (Fujihara and Nakatani, 1971), Shieh (Shieh, 1980) and tryptone yeast extract salts (TYES) (Holt et al., 1993). The bacteria generally grow better on media that contain salts such as Shieh and TYES (Song et al.,1988; Holt et al., 1993). Often liquid media is used to grow *F. columnare* since it adheres strongly to agar (Garnjobst, 1945).

The growth of *F. columnare* and *F. psychrophilum* varies a lot between isolates; therefore, researchers have modified the media in order to improve isolation. To prevent spreading and adherence of *F. columnare*, a specialized media was developed called

Flavobacterium columnare growth medium (FCGM), (Farmer, 2004). To improve growth of *F. psychrophilum*, the tryptone level is commonly increased (Wakabayashi and Egusa, 1974). Other improvements for *F. psychrophilum* include adding certain sugars, skimmed milk, horse serum or trace elements (Daskalov et al., 1999; Michel et al., 1999: Cepeda et al., 2004). Some have also simplified recipes (i.e. modified Shieh) to limit the number of necessary trace elements (LaFrentz and Klesius, 2009).

Several selective media use the antimicrobials polymyxin B, neomycin and/or tobramycin to selectively isolate *F. columnare* (Fijan, 1969; Decostere et al., 1997). Selective cytophaga medium uses neomycin (5 μg/ml) and polymyxin B (200 IU/ml) (Hawke and Thune, 1992), and selective Shieh medium uses tobramycin (1 μg/ml) (Decostere et al., 1997). Hsu- Shotts media incorporates gelatin along with neomycin (4 μg/ml; Bullock et al., 1986).

Many biochemical traits further characterize these bacteria (Bernardet and Grimont, 1989). However in order to speed identification, several PCR assays were developed based on specific 16S rRNA gene sequences for *F. columnare* (Toyama et al., 1996; Bader and Shotts, 1998; Bader et al., 2003; Darwish et al., 2004) and *F. psychrophilum* (Toyama et al., 1994; Urdaci et al., 1998). However, only the set developed by Darwish et al. (2004) is truly specific for *F. columnare* since the earlier sets were based on a standard strain later found to be *Flavobacterium johnsoniae* (ATCC 43622). Sensitivity and specificity were improved upon with primers that amplify the *F. columnare* 16S-23S intergenic spacer region (Welker et al., 2005). Similarly, primers

specific to the gyrase B gene improved detection of *F. psychrophilum* (Izumi and Wakabayashi, 2000).

1.4 Development of a standard susceptibility test

The standardized methods either described or developed within this dissertation followed the guidelines of the Clinical Laboratory Standards Institute (CLSI). The CLSI is a world renowned leader in developing clinical laboratory standards and guidelines. Each subject area has an expert CLSI committee that develops and approves new methods or criteria, and updates existing methods using an international consensus process.

1.4.1 Standard Methods

The CLSI recommends 3 standardized antimicrobial susceptibility testing (AST) methods: disk diffusion, broth dilution, and agar dilution. The disk diffusion technique places a paper disk over a standardized amount of the bacterium spread on the surface of an agar plate (Bauer et al., 1966). The disk is infused with a known concentration of the antimicrobial that diffuses through the agar as the bacterium grows. Disk diffusion identifies the diameter of the inhibited growth zone (zone of inhibition) around the disk. The dilution techniques grow the bacteria on agar plates or in broth tubes (macro) or wells (micro) that have different drug concentrations. Each plate, well or tube incorporates a separate antimicrobial concentration in a stepwise dilution series. A standardized amount of bacterial cells are applied to the agar surface or in the broth. Growth is assessed at a set temperature after a defined incubation period. The dilution techniques identify the minimum concentration of the antimicrobial that inhibits visible

growth of the bacterium, known as the minimal inhibitory concentration (MIC). MICs can be directly compared to the kinetics of the drug in the animal whereas inhibitory zone diameters cannot. However, zone diameters can be correlated to MICs using statistical methods (Metzler and DeHaan, 1974; Brunden et al., 1992; Bradford and Sanders, 1992).

The original set of reference protocols developed for AST of aquatic bacteria included all 3 methods described above (Alderman and Smith, 2001). These protocols brought together the standard methods developed by CLSI with modifications for aquatic bacteria. The intent was to begin developing standardized, quality controlled methods for testing the antimicrobial susceptibility of fish pathogenic bacteria so laboratories could better share data to facilitate judicious use of antimicrobials in aquaculture. Subsequent standardization trials created the first standardized disk diffusion and broth dilution susceptibility testing methods for non-fastidious aquatic bacteria that grow on normal testing media (Miller et al. 2003, 2005; CLSI 2006 a/b). The guidelines also grouped fastidious aquatic bacteria together based on similar growth requirements and recommended modifications for susceptibility testing. The recommendations give laboratories a basis to develop standard methods for the fastidious groups.

1.4.2 AST of F. columnare and F. psychrophilum

The CLSI testing guidelines for aquatic bacteria suggests Mueller-Hinton (MH) media diluted 3g/L supplemented with 5% horse or fetal calf serum for disk diffusion and broth dilution testing the fish pathogenic Flavobacteria: *F. columnare*, *F. psychrophilum* and *F. branchiophilum* (CLSI, 2006 a/b). This recommendation is based on a disk diffusion test developed for *F. columnare* (Hawke & Thune, 1992). Although some

researchers have used this media for disk diffusion of *F. columnare* (Hawke & Thune, 1992; Thomas-Jinu & Goodwin, 2004; Gaunt et al., 2010) other media are also used, particularly cytophaga (Wakabayashi & Egusa, 1966; Amin et al., 1988; Michel et al., 2002; Suomalainen et al., 2006; Kubilay et al., 2008). Alternatively, some researchers have used the agar dilution technique for *F. columnare* but on a very limited basis with cytophaga or Shieh media at different growth conditions (Soltani et al., 1995; Decostere et al., 1998).

Growing *F. columnare* on solid agar is a major drawback to both disk diffusion and agar dilution, since the bacterium prefers to grow in broth media (Garnjobst, 1945). Moreover, the gliding of *F. columnare* can distort the margins of the inhibitory zone in disk diffusion (Farmer, 2004) or can result in isolates spreading over adjacent isolates on the agar dilution plate. In order to have a more consistent method to test the susceptibility of *F. columnare*, Darwish et al. (2008) adapted the broth microdilution technique using dilute (4 g/L) Mueller-Hinton broth. This method uses a slightly more concentrated broth compared to CLSI's recommendation (3 g/L).

To test the antimicrobial susceptibility of *F. psychrophilum* researchers consistently use dilute Mueller-Hinton (MH) media supplemented as described by Hawke and Thune (1992) for disk diffusion and agar dilution testing. Incubations conditions have varied with most researchers testing at 15-18 °C and determining results at 72-96 hours (Schmidt et al., 2000; Bruun et al., 2000; Michel et al., 2003; Izumi & Aranishi, 2004; Kum et al., 2008). When compared to modified cytophaga, the growth of *F. psychrophilum* on dilute MH appeared altered in disk diffusion tests as very little growth

was observed for some isolates (Michel et al., 2003). However, the same study only observed slightly lower MICs with diluted MH when the 2 media were compared with agar dilution tests.

Some researchers have tested *F. psychrophilum* with broth microdilution although the methods have not been consistent. The broth media ranged between modified cytophaga, diluted Mueller-Hinton and full strength cation-adjusted Mueller-Hinton with temperature at 15-18 °C and incubation at 72-168 hours (Rangdale et al., 1997; Hesami et al., 2010; Del Cerro et al., 2010). As with *F. columnare, F. psychrophilum* readily grows in broth making broth dilution an attractive option for susceptibility testing; however, a standard procedure was needed. Another advantage to using broth dilution was the existing standardized method for non-fastidious aquatic bacteria. Updating the current CLSI guideline would be less labor intensive than standardizing agar dilution methods which do not have existing standard methods for aquatic bacteria. Therefore, the first objective of this research was to standardize similar broth microdilution methods for *F. columnare* and *F. psychrophilum* using dilute (4 g/L) Mueller-Hinton broth. The same testing technique was used since the bacteria have similar nutritional requirements.

1.4.3 Special considerations for AST of F. columnare and F. psychrophilum

Pure cultures of *F. columnare* or *F. psychrophilum* often clump together making it hard to get cell suspensions with a consistent concentration of 5×10^5 colony-forming units/mL required for broth microdilution testing (CLSI, 2006b; Wiegand et al., 2008). Laboratories typically grow *F. columnare* and *F. psychrophilum* in broth while stirring or shaking to get consist cell suspensions for susceptibility testing (Darwish et al., 2008,

Bruun et al., 2000). However, suspensions can be made by swabbing colonies off agar plates or diluting static broth cultures. Since some fish health laboratories may not have equipment to shake or stir during incubation, methods were needed so consistent suspensions could be made from static cultures.

Mueller-Hinton media for broth dilution testing is supplemented with Ca^{+} and Mg^{+} since these cations improve the growth of bacteria to better estimate the *in vivo* activity of antimicrobials (D'Amato et al., 1975; Nanavaty et al., 1998). Previous research suggested cations maybe unnecessary for broth dilution testing of *F. columnare* (Darwish et al., 2008). However, it was unknown if cations were necessary for broth dilution testing of *F. psychrophilum*. In addition, no one had investigated if diluting the cations will significantly affect antimicrobial susceptibility. If media with dilute cations could be used, then laboratories could simply dilute commercially prepared full strength cation-adjusted MH broth without having to replenish the cations. Therefore, the effect of different cation levels on the antimicrobial susceptibility of *F. columnare* and *F. psychrophilum* had to be characterized. In addition, the effect supplementing the broth with serum also had to be characterized since the CLSI recommends 5% serum for susceptibility testing these bacteria (CLSI, 2006).

An incubation temperature and period already existed for broth dilution testing of *F. columnare* (Darwish et al., 2008). However, no consistent incubation temperature or period existed for broth dilution testing of *F. psychrophilum*, but the growth conditions used with agar dilution provided a good reference (15-18 °C, 72-96 hours). Although *F. psychrophilum* grows optimally at 15 °C, the generation time at 18 °C is very similar

(Holt et al., 1989). Therefore, 18 °C was chosen for incubation since the existing quality control (QC) bacteria standardized for testing non-fastidious aquatic bacteria at 22 and 28 °C would grow better at the higher temperature. These bacteria are known to have stable antimicrobial susceptibility; however, we needed to test their stability at the new test conditions. Developing a *Flavobacterium* QC strain would have taken considerable effort to find strain with reliable susceptibility at 2 very different incubation temperatures. A better alternative was to develop the existing QC strains to be used with local *Flavobacterium sp.* strains developed by individual laboratories.

1.4.4 QC ranges

Standard antimicrobial susceptibility tests use QC bacteria to monitor performance. The QC bacteria are standard strains with stable susceptibility at the test conditions taken from a recognized source such as the American Type Culture Collection, ATCC (CLSI, 2008). These standard strains are tested along with target bacteria. Test performance is verified when the test results of the standard strain(s) falls within the predetermined range set for a certain antimicrobial. Out-of-range results indicate the test plates were not made with the correct drug concentrations or the operator made a mistake in the method. The cause of QC failures must be investigated and tests repeated.

QC ranges (or limits) are set with data from multi-laboratory testing trials where each lab tests the QC bacteria at a certain number of repetitions with the same media lots, drug lots and protocol. The CLSI has a guideline that clearly outlines the requirements for establishing acceptable QC ranges for susceptibility testing of veterinary bacterial

pathogens (CLSI, 2008). At least 7 laboratories from different institutions should participate in the standardization trial with at least one veterinary diagnostic laboratory. After the trial, the data are compiled and provisional QC ranges determined. The CLSI Veterinary Antimicrobial Susceptibility Testing (VAST) subcommittee reviews the ranges for addition into the guidelines. An approved range should include at least 95% of the data centered on the mode. The VAST committee has the authority to expand or shrink the ranges based on distribution of the data. QC ranges should be relatively close to the test scale cutoffs used to interpret the test, either for resistance monitoring or for predicting treatment outcome. Thus to standardize the susceptibility testing method, a standardization trial was needed whereby laboratories tested the quality control bacteria at the test conditions multiple times.

1.5 Criteria to interpret AST to monitor for drug resistance monitoring and to predict the success of drug treatments

Antimicrobial susceptibility tests are interpreted by separating the MIC or zone diameter results into categories denoted by certain cutoff points in the MIC or zone diameter scale. Two categories, wild type (WT) or non-wild type (NWT), divide the isolates based on whether they have acquired antimicrobial resistance mechanisms (Kahlmeter et al., 2003). Three categories, susceptible, intermediate or resistant, called clinical breakpoints predict the likely outcome of an antimicrobial treatment (CLSI, 2008).

Scientists track antimicrobial resistance by monitoring the prevalence of bacteria that have acquired resistance. This requires setting an MIC or zone diameter, called an

epidemiological cutoff value (ECV), which separates the WT isolates that have no resistance mechanisms from the NWT isolates (Kahlmeter et al., 2003). ECVs are determined from testing isolates of a single bacterium species. Histograms of the MIC or zone diameter distributions are then inspected for breaks that define where the WT isolates end and the NWT isolates begin (Turnidge et al., 2006). However, this convention does not work well if the WTand NWT isolate distributions overlap with no clear separation. Fortunately, statistical approaches fit curves to distribution of WT isolates so the ECV can be mathematically estimated (Kronvall, 2003; Turnidge et al., 2006).

An ECV is also used to determine clinical breakpoints; however, for this purpose it is called a "wild type cutoff". Other cutoffs are also considered that define what isolates are controlled in actual treatments (clinical cutoff) and what isolates should be controlled based on how the animal's body affects the drug, and how the drug affects the body and the bacteria (pharmacokinetic/pharmacodynamics – PK/PD – cutoff). The WT and clinical cutoffs are compared to determine the susceptible breakpoint with the PK/PD cutoff used as a weighing factor (CLSI, 2008). The **susceptible (S)** category means the antimicrobial will likely control an infection caused by the isolate. A susceptible breakpoint can include more or less than the natural distribution of WT isolates. For instance, a defined dosage regime of the antimicrobial may still control infections caused by isolates that have mechanisms that confer a low level of resistance; therefore, the susceptible breakpoint would include some of these NWT isolates.

Once the value of the susceptible breakpoint is determined, further evaluations are used to define intermediate and resistant categories (CLSI, 2008). The **intermediate (I)** category indicates treatment will only be successful if the antimicrobial concentrates at the infection site or, if available, that a higher dosing regimen is needed. Treatment of infections caused by isolates in the **resistant (R)** category will likely fail because the antimicrobial does not concentrate at the infection site to a level that inhibits the bacterium. A bacterial species that has not acquired drug resistance will only have susceptible isolates. As the bacterial population develops resistance, intermediate and resistant breakpoints are added to a particular bacterium/antimicrobial combination. However, most new antimicrobial indications for fish are existing drugs used elsewhere in veterinary and human medicine; therefore, target bacteria may already have developed or acquired resistance mechanisms.

Diagnostic laboratories use the S, I, R breakpoints to advise veterinarians in prescribing treatment, fostering judicious use of antimicrobials. Currently for aquatic bacteria, ECVs and clinical breakpoints only exist for the bacterium *Aeromonas salmonicida* (CLSI, 2010). Aquaculture needs more ECVs and clinical breakpoints for bacteria, such as *F. columnare,* that cause major diseases in aquaculture so changes in antimicrobial susceptibility can be more effectively monitored and so antimicrobials can be more effectively used.

1.6 Setting criteria to monitor *F. columnare* susceptibility changes or predict florfenicol treatment

Setting a WT cutoff (ECV) for clinical breakpoints requires testing a minimum of 100 isolates of a bacterium with a standard MIC generating susceptibility test (broth dilution or agar dilution; CLSI, 2008). The isolates should be from diverse sources to get as good a geographical and temporal separation as possible. Although no minimum requirements exist for setting an ECV, it is possible to estimate the value from as little as 30 bacterial isolates (CLSI, 2011) tested with a standard MIC or disk diffusion test. Therefore the standardized broth microdilution test satisfied requirements for both ECVs and clinical breakpoints. However research was needed on the *in vitro* susceptibility patterns of at least 100 *F. columnare* against antimicrobials approved for columnaris disease and other potential unapproved antimicrobial treatments. The resulting MIC frequency distributions and ECVs tested the null hypothesis that there are no isolates of *F. columnare* that have decreased susceptibility to antimicrobials commonly used in aquaculture.

In addition to WT cutoffs, clinical and PK/PD data are needed to set clinical breakpoints for florfenicol treatment of columnaris disease. Fortunately, the conditional approval process used to get florfenicol approved for columnaris disease created opportunities that make establishing clinical breakpoints achievable. First, pharmacokinetics of florfenicol in channel catfish was recently studied to support converting the approval from a conditional to an official status (Gaunt et al., 2012, 2013; Federal Register, 2012). The same research group is also considering pharmacodynamics studies on how florfenicol affects *F. columnare* (Gaunt, personal communication). Second, a columnaris disease model generated part of the substantial evidence for the conditional approval of Aquaflor® (florfenicol) to control columnaris disease in channel

catfish (Gaunt et al., 2010; Federal Register, 2007a). The model provides an alternative way to enhance the limited clinical data available from the field. Therefore, a similar disease model facilitated studying the ability of the approved florfenicol dose to control *F. columnare* infections. Disease could be induced with *F. columnare* isolates that have different antimicrobial susceptibilities near potential clinical breakpoints to test the null hypothesis that infections in channel catfish caused by an isolate with decreased susceptibility can be controlled by the approved florfenicol treatment.

A variety of different methods have been used to experimentally induce columnaris disease. In channel catfish, some columnaris disease models use a cutaneous abrasion in conjunction with a bath exposure to a single infectious *F. columnare* strain (Bader et al., 2003, 2006; Darwish and Mitchell, 2009; Darwish Et al., 2009a/b). Abrasion techniques found to effectively induce columnaris disease include rubbing a cotton gauze pad (Bader et al., 2003, 2006), branding with a hot wire (Bader et al., 2006) or scrubbing with an adhesive pad (Darwish and Mitchell, 2009; Darwish Et al., 2009a/b). However, other researchers have induced disease without a cutaneous abrasion, using only a bath exposure (Thomas-Jinu & Goodwin, 2004; Soto et al., 2008; Shoemaker et al., 2008; Gaunt et al., 2010). A very reproducible model was needed to induce disease with different strains of *F. columnare* therefore an abrasion had to be evaluated. Another important factor for the disease model was the occurrence of mortality. Most of the mortalities induced by Gaunt et al. (2010) occurred on days 3-5 which gave the fish time to eat at least some of the medicated florfenicol feed. In other columnaris disease models, most mortality occurred within 48 hours.

The ideal disease model needed to induce consistent infections in channel catfish without an abrasion that began 2 to 5 days after exposure so the fish get at least 2 to 3 florfenicol doses prior to onset of morbidity. The level of infection should be similar between the different *F. columnare* isolates we want to study. Once developed, the model can be used to test the null hypothesis that *F. columnare* isolates with decreased florfenicol susceptibility do not induce disease as effectively as isolates with typical susceptibility. These experiments were needed prior to experiments on whether the approved florfenicol treatment can control *F. columnare* infections.

1.7 Summary

Setting standard methods to test the antimicrobial susceptibility of *F. columnare* and *F. psychrophilum* will provide a considerable improvement to how aquaculturists use antimicrobials against these very important fish pathogens. Proficiency will increase between laboratories since everyone can use the same methods and quality assessment parameters. Thus, test results collected from local regions will be more comparable facilitating greater coverage in monitoring antimicrobial resistance patterns. By focusing on a test that generates an MIC, data will be more easily compared to clinical outcomes and drug kinetics facilitating the development of clinical breakpoints. This will give veterinarians more reliable data to base their decisions on when to use antimicrobials.

Focusing on interpretive criteria for *F. columnare* will standardize a test for a leading pathogen that continues to emerge in new aquaculture sectors. Effective use of florfenicol for this bacterium will contribute to reducing the reservoir of resistance

bacteria in the environment thereby limiting risks to public health and keeping antimicrobial treatments effective.

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Chapter 2: Development and precision testing of broth microdilution methods to determine the antimicrobial susceptibility of *Flavobacterium columnare* and *Flavobacterium psychrophilum*.

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2.1 Abstract

Flavobacterium columnare and *F. psychrophilum* are major fish pathogens that may require antimicrobial therapy. Choice of appropriate treatment is dependent upon determining antimicrobial susceptibility which requires validated testing methods. This paper reports on methods optimized for broth microdilution testing of *F. columnare* and *F. psychrophilum*. We developed specialized methods to make reproducible broth inoculums, and confirmed the proper incubation time and media composition. We tested the stability of potential quality control bacteria and the precision of the method. Supplementing the broth with horse serum did not improve growth which was in log phase at 48 hours for *F. columnare* and 72-96 hours for *F. psychrophilum* confirming the test should be incubated at 28°C for approximately 48 hours and at 18°C for approximately 96 hours, respectively. The most consistent susceptibility results were achieved with plain 4 g/L dilute Mueller-Hinton broth (CAMHB) supplemented with dilute calcium and magnesium. The quality control strains *Escherichia coli* (ATCC 25922) and *Aeromonas salmonicida* subsp. *salmonicida* (ATCC 33658), yielded stable minimal inhibitory concentrations (MIC) against all 7 antimicrobials tested after 30 passes at 28°C and 15 passes at 18°C. In precision tests, most MICs of the isolates agreed

100% within in one drug dilution for ampicillin, florfenicol and oxytetracycline. The agreement was lower with the ormetoprim/sulfdimethoxine combination with at least 75% agreement for all but 1 isolate. A multi-laboratory standardization trial used these methods to determine quality control limits for testing *F. columnare* and *F. psychrophilum* establishing a standardized antimicrobial susceptibility testing method for these nutritionally fastidious aquatic bacteria.

2.2 Introduction

Major advances have been made in the development of standard laboratory methods to determine the antimicrobial susceptibility of bacterial pathogens of fish. Miller et al. (2003 and 2005) conducted two multi-laboratory standardization trials to determine quality control (QC) parameters for standard disk diffusion and broth dilution testing, at 22°C and 28°C, of non-fastidious bacteria isolated from aquatic animals. Results of these trials were used to draft two Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2006a, 2006b). These guidelines provide reference methods, as well as QC parameters for testing some antimicrobials used in aquatic animal medicine.

Recently a multi-laboratory standardization trial established QC parameters for broth microdilution testing of the fish pathogenic fastidious gliding bacteria *Flavobacterium columnare* and *F. psychrophilum* (Gieseker et al., 2012). These bacteria are major pathogens of farmed fish that may require antimicrobial therapy based on susceptibility test results. Therefore, reliable susceptibility testing methods are needed to help guide clinical therapy and to monitor for changes in antimicrobial susceptibility patterns. This paper describes methods optimized to test *F. columnare* and *F.*

psychrophilum by broth microdilution. The methods were developed to facilitate a standardization trial so that a reference method could be created.

The distinctive growth features of fastidious bacteria present challenges that dictate which *in vitro* susceptibility testing method (disk diffusion, broth dilution and agar dilution) should be used. Although agar dilution has been used to test *F. psychrophilum* (Bruun et al., 2000; Michel et al., 2003; Schmidt et al., 2000), some *F. columnare* isolates glide overtop adjacent isolates spotted on the agar. In addition, the gliding rhizoid growth of *F. columnare* also disrupts Kirby-Bauer disk diffusion distorting the inhibitory zones (Farmer, 2004). Therefore, the broth dilution technique is the best option for testing both of these *Flavobacteria*, especially since they both grow well in liquid media. We choose to focus on the broth microdilution method since it is already standardized for non-fastidious aquatic bacteria (Miller, 2005; CLSI, 2006b).

The CLSI suggests Mueller-Hinton broth (MHB) diluted 1:7 (3 g/L) supplemented with 5% horse or fetal calf serum for broth dilution testing of fish pathogenic *Flavobacteria* (CLSI, 2006b). This recommendation was based on the work of Hawke and Thune (1992). However, recent research has determined plain MHB diluted 4 g/L is better for broth microdilution testing of *F. columnare* and that calcium and magnesium cations, typically added to MHB for broth dilution susceptibility testing, may not be needed (Farmer, 2004; Darwish et al., 2008). Calcium and magnesium cations are usually added to MHB since they improve the growth of bacteria to better estimate the *in vivo* activity of antimicrobials (D'Amato et al., 1975; Nanavaty et al., 1998). In preliminary testing, *F. psychrophilum* grew well in 4 g/L cation-adjusted Mueller-Hinton

broth (CAMHB) without serum (authors, unpublished results). However, further tests were needed to determine the effect of serum and cations on the growth and susceptibility of *F. columnare* and *F. psychrophilum*.

The low temperature requirement of *F. psychrophilum* presents another challenge since selected QC strains must also grow under the same test conditions. In preliminary tests, we found the two QC strains used for susceptibility testing of non-fastidious aquatic bacteria (CLSI, 2006a, 2006b), *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, grow in diluted 4 g/L CAMHB at 18°C (authors, unpublished results). *Flavobacterium psychrophilum* grows well at 15-20°C with relatively fast generation times (Pacha, 1968; Holt et al., 1989). To verify if these strains can function as QC organisms, their susceptibility to antimicrobials needed to be assessed to show they are stable.

With the goal of developing a standard broth microdilution test for *F. columnare* and *F. psychrophilum*, we optimized the method of preparing a standard cell suspension, incubation times and required media supplements. Additionally, we evaluated the test results for the existing aquatic QC strains under the modified test conditions and also performed an intra-laboratory testing trial to compare the precision of the methods among four separate operators.

2.3 Materials and Methods

2.3.1 Bacterial isolates, culture conditions and antimicrobials

Table 1 lists the 11 *F. columnare* and 13 *F. psychrophilum* isolates used in the described experiments. All of the isolates were obtained from diseased fish and were donated from laboratories in the USA, France, Netherlands, Australia, Chile and the American Type Culture Collection (ATCC). The identity of the isolates was confirmed with species-specific PCR (Darwish et al., 2004; Toyama et al., 1994). Two QC strains for standardized susceptibility testing of non-fastidious aquatic bacteria, *Eshcerichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 were used in all experiments that tested antimicrobial susceptibility with laboratory prepared broth microdilution plates.

The florfenicol (FFN) and oxytetracycline (OTC) antimicrobials used to test the effect of cations on susceptibility were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and MP Biomedicals (Santa Ana, California, USA), respectively. All antimicrobials except ormetoprim/sulfadimethoxine, used for stability testing of the potential quality control strains and for the intra-laboratory testing trial were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Stock concentrations of ormetoprim (1000 µg/mL) and sulfadimethoxine (1520 µg/mL) were purchased from Trek Diagnostic Systems, Inc. (Cleveland, Ohio, USA).

2.3.2 Effect of horse serum supplementation and incubation time on growth

Spread plate enumerations were used to evaluate *F. columnare* and *F. psychrophilum* growth in CAMHB diluted 4 g/L with or without 5% horse serum (Donor Equine Serum, Hyclone Laboratories, Logan, Utah, USA). CAMHB diluted 4 g/L was prepared by removing 8.9 mL CAMHB from commercially-prepared 11 mL CAMHB

tubes (Trek Diagnostics, Cleveland, Ohio, USA) and replacing the broth with 8.9 mL of sterile demineralized water, or replacing the broth with 8.35 mL sterile water and 0.55 mL horse serum.

Ten *F. columnare* isolates and 10 *F. psychrophilum* isolates were cultured from pure cryopreserved samples. Each isolate was subcultured twice in tryptone yeast extract salts (TYES) broth (Holt et al., 1993) incubated at 28°C for 24 hours (*F. columnare*) and 18°C for 72 hours (*F. psychrophilum*). Turbidity suspensions (0.5 McFarland) of each isolate were prepared from the second subculture. Each suspension was vortexed and the clumps were allowed to settle for 1-3 minutes. The upper, more homogeneous fraction (~2 mL) was removed, and adjusted as needed to a turbidity equivalent to a 0.5 McFarland. Fifty-five µL of this fraction was added to 11 mL of CAMHB diluted 4 g/L with or without 5% horse serum to target a bacterial concentration of 5×10^5 colony-forming units (CFU)/mL. After vortexing, the inoculated dilute CAMHB was emptied into a sterile reservoir trough, and 100 µL was pipetted into each well of a sterile 96-well plate. A separate 96-well plate was inoculated for each isolate and broth combination. *Flavobacterium columnare* plates were incubated at 28°C, while *F. psycrhophilum* plates were incubated at 18°C.

Three replicate wells were sampled (100 µL) from each 96-well plate at 0, 24, 48, 72 and 96 hours for *F. columnare* isolates and at 0, 24, 48, 72, 96, 120 and 144 hours for *F. psychrophilum*, and 10-fold serial dilutions (10^{-2} to 10^{-5}) were prepared. A 100 µL aliquot of each 10-fold dilution was spread on TYES agar and incubated at 28°C for 48 hours (*F. columnare*) and at 18°C for 96 hours (*F. psychrophilum*). Viable cell

concentrations (CFU/mL) were compared to determine whether serum supplementation improved growth and the optimal incubation time to target the log growth phase.

2.3.3 Turbidity level and inoculum volume for final target cell concentration in the test broth

To determine the McFarland turbidity and volume required to reliably achieve 5 x 10^5 CFU/mL bacterial concentrations in the broth microdilution plate wells, viable cells were enumerated directly from inoculated broth. Individual turbidity suspensions of 10 *F. columnare* isolates (0.5 McFarland) and 10 *F. psychrophilum* isolates (0.5 and 1.0 McFarland) were prepared from the second subculture as described above. Fifty-five µL, 110 µL and 165 µL were removed from each cell suspension and added to a corresponding tube of 11 mL CAMHB diluted 4 g/L. A 1:1000 dilution was prepared from each tube in sterile saline and 100 µL was spread on 3 replicate TYES agar plates. The plates were incubated at 28°C for 24 hours (*F. columnare*) and 18°C for 72 hours (*F. psychrophilum*). Afterwards, plates were counted and CFU/mL calculated.

2.3.4 Effect of Ca^{++} and Mg^{++} supplementation on susceptibility

We compared minimal inhibitory concentrations (MIC) determined with dilute broth supplemented with different levels of calcium and magnesium cations in order to confirm if cations were needed for testing and to compare MICs between dilute and full strength cation levels. The susceptibility of *F. columnare* (n=6), *F. psychrophilum* (n=6), *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 to florfenicol and oxytetracycline was tested in 4 g/L MHB supplemented with full cations (20 mg/L Ca^{++}; 2 mg/L Mg^{++}) as suggest by the CLSI for full strength CAMHB, in 4 g/L MHB

supplemented with dilute cations (4 mg/L Ca^{++}; 2 mg/L Mg^{++}) and in plain 4 g/L MHB with no cations (CLSI, 2006b).

In-house broth microdilution 96-well plates were prepared with FFN (32-0.015 µg/mL) or OTC (16-0.008 µg/mL) following CLSI guidelines (CLSI, 2006b). Mueller-Hinton broth was prepared from dehydrated media (BD Diagnostic Systems, Sparks, MD, USA) and supplemented with Ca^{++} and Mg^{++} cations using stock solutions (10 mg Ca^{++} or Mg^{++}/mL) of calcium chloride and magnesium chloride (Acros, Fair Lawn, New Jersey, USA). Separate plates were made for each drug in each broth.

To make the custom 96-well plates, the highest final drug concentration was prepared in broth media at twice the target concentration in a 50 mL centrifuge tube by adding calculated amounts of stock drug solutions (1280 µg/mL). To allow for subsequent 2-fold dilutions, double the volume needed to fill the first column of the 96-well plates was made. The highest drug concentration was then serially diluted 2-fold 11 times in the same broth media in 50 mL centrifuge tubes. Fifty µL of the appropriate concentration was added to the appropriate column of sterile 96-well plates using a multichannel pipette. The final 2 wells in the last row of the well plates were filled with plain broth; one to be left un-inoculated as a negative control and the other inoculated with cell suspension as a positive control. Plates were stored at ≤ -70°C in sterile plastic bags until used within 2 months.

Minimal inhibitory concentrations were determined using CLSI guidelines for broth microdilution testing with adaptions developed here for making *Flavobacteria* cell suspensions (CLSI, 2006b). Each isolate was subcultured twice in TYES broth (*F.*

columnare and *F. psychrophilum*) or on a blood agar plate (BAP; *E. coli* and *A. salmonicida*). Broth and agar plates were incubated at 28°C for 24 hours (*F. columnare*) and 18°C for 72 hours (*F. psychrophilum*). Separate *E. coli* ATCC 25922 and *A. salmonicida* ATCC 33658 cultures were subcultured alongside the *Flavobacterium* isolates at each incubation temperature. Five replicate cell suspensions (0.5 McFarland turbidity) were made for each isolate. Suspensions were diluted 1:50 (*Flavobacteria*) or 1:100 (*E. coli* and *A. salmonicida*) in separate 5 mL aliquots of the 3 broths. Fifty µL of each inoculated broth was added to a row of a FFN and OTC broth microdilution plate made with the corresponding broth; one row for each isolate tested. Each plate was inoculated with all 6 *F. columnare* or *F. psychrophilum* isolates, *E. coli* and *A. salmonicida*. Cell density was monitored in the broth supplemented with dilute cations with colony counts as described from 3 replicates of each bacterium using TYES (*Flavobacteria*) or BAP (QC bacteria) agar plates. The broth microdilution and colony count plates were incubated 28°C for 48 hours (*F. columnare*) and 96 hours at 18°C (*F. psychrophilum*). After incubation the broth microdilution plates were read and MIC results determined (CLSI, 2006b).

2.3.5 Stability of ATCC strains under test conditions

The susceptibility of *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 was tested after multiple consecutive subcultures to examine the stability under culture conditions developed for testing *F. columnare* and *F. psychrophilum*. Both strains were subcultured every 24 hours at 28°C and every 72-96 hours at 18°C. Susceptibility to enrofloxacin (2-0.001 µg/mL), erythromycin (128-0.06 µg/mL), florfenicol (16-0.008 µg/mL), flumequine (8-0.004 µg/mL), gentamicin (8-0.004 µg/mL),

oxytetracycline (32-0.015 µg/mL) and oxolinic acid (4-0.002 µg/mL) was examined with in-house prepared broth microdilution plates as described at 28 °C after 5, 10, 15, 20, 25 and 30 subcultures; and at 18 °C after 5, 10 and 15 subcultures.

2.3.6 Intra-laboratory precision testing

To test the precision of the *F. columnare* and *F. psychrophilum* broth microdilution methods, four operators each tested three separate bacterial suspensions of 6 *F. psychrophilum* isolates at 18°C, and three separate bacterial suspensions of 6 *F. columnare* isolates at 28°C. Tests of three separate bacterial suspensions of *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 were also included at each temperature. Susceptibility to ampicillin (0.015-32 µg/mL), oxytetracycline (0.015-32 µg/mL), florfenicol (0.008-16 µg/mL) and ormethoprim/sulfadimethoxine (0.002/0.04–4/76 µg/mL) were assayed. Custom frozen broth microdilution plates were prepared and used as described previously. All bacterial suspensions were prepared and tested on the same day.

2.4 Results

2.4.1 Broth and incubation time for optimal growth and antimicrobial susceptibility testing

Five percent horse serum did not improve the growth of *F. columnare* or *F. psychrophilum* in 4 g/L CAMHB (Figure 1 and 2). The number of viable cells for most of the isolates was higher at each time point in plain 4 g/L CAMHB. Most of the F. columnare isolates (6 of 9) appeared to be in log-phase growth by 48 hours. These isolates either had a rapid increase in cell number that peaked at 72 hours, or rapid

increase at 48 hours that remained in a relative plateau. The growth of another isolate (36413) peaked at 24 hours and stayed relatively constant. The last 2 isolates (33971 & 36417) grew slowly without a noticeable increase until 96 hours. An isolate (36434) did not grow when initially subcultured therefore it had to be excluded from the experiment.

All of the *F. psychrophilum* isolates grew in plain 4 g/L CAMHB but some did not grow in the serum supplemented broth. The number of viable cells for most of the isolates peaked at 96 hours in plain 4 g/L CAMHB then declined; however growth peaked at 72 hours for 2 isolates (36393 & 36394).

2.4.2 Inoculum volumes for final target cell concentration in the test broth

Table 2 lists the mean cell concentration (CFU/mL) and standard deviations of 10 *F. columnare* and 10 *F. psychrophilum* isolates at different McFarland cell suspensions and inoculum volumes used to prepare broth for microdilution testing. Dilute CAMHB (11mL) inoculated with either 110 µL or 165 µL of a 0.5 McFarland *F. columnare* suspension most closely achieved the initial target cell concentration of 5.0×10^5 CFU/mL. Dilute CAMHB inoculated with 55 µL of a 0.5 McFarland *F. psychrophilum* suspension or 55 µL of a 1.0 McFarland suspension most closely achieved 5.0×10^5 CFU/mL for *F. psychrophilum*.

2.4.3 Ca^{++} and Mg^{++} supplementation

Without cations the MICs of the *F. columnare* were consistently 1-2 dilutions below the MICs determined using cations (Table 3). Most of the MICs agreed between the 2 cation levels; however, some *F. columnare* did not grow in the unsupplemented

broth or in the presence of full-strength cations. All of the isolates grew in the broth supplemented with dilute cations.

Most of the *F. psychrophilum* isolates did not grow in the unsupplemented broth; however, all of the isolates grew in the cation-supplemented broths. The MICs determined for each isolate from the 2 supplemented broths agreed within 1 dilution.

The MICs of the QC bacteria agreed across the drugs and cation levels at both incubation temperatures. The cation levels had a dose-dependent effect on the oxytetracycline MICs of *A. salmonicida* subsp. *salmonicida* ATCC 33658 at 28°C and *E. coli* ATCC 25922 at 18°C.

2.4.4 QC stability

All MIC results for each drug agreed within 1 dilution for both bacteria at 28°C up to 30 subcultures and at 18°C up to 15 subcultures. Ampicillin and the 2 sulfonamide drugs were not tested because of the stability observed in the first 7 drugs tested. *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 had consistent MIC results against ampicillin and ormetoprim/sulfadimethoxine during intra-laboratory precision testing.

2.4.5 Intra-laboratory precision testing

All of the *F. columnare* MICs agreed between the operators within 1 dilution of the mode for florfenicol and oxytetracycline (Table 4). Except for one isolate, all of the ampicillin MICs also agreed. The MIC results were more variable with the potentiated sulfonamide. Four of the 6 isolates agreed within a dilution but the remaining 2 isolates

only agreed 75 % and 50 %. Both *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 had stable MICs at the conditions for testing *F. columnare*.

We observed a similar trend for *F. psychrophilum*. At least 91 % of MICs agreed between the operators within a dilution of the mode for florfenicol and oxytetracycline. Likewise most of ampicillin MICs agreed for 5 of the 6 isolates. Agreement was lower with the potentiated sulfonamide but was at least 75 %. The tested QC strains also had stable MICs at the *F. psychrophilum* test conditions.

2.5 Discussion

The broth microdilution testing procedures presented in this paper use the same test media for both *F. columnare* and *F. psychrophilum*. Supplementing the broth media with 5% horse serum was not necessary as with agar dilution and disk diffusion tests (Bruun et al., 2000; Hawke and Thune, 1992; Michel et al., 2003; Schmidt et al., 2000). Both QC bacteria currently used for non-fastidious aerobic aquatic bacteria grew well at the test conditions and had very precise MIC results similar to the QC MIC ranges established in full strength Mueller-Hinton broth (Miller et al., 2005; CSLI, 2006b). In addition, the methods proved reliable when compared between different operators.

By allowing a few minutes for the aggregated cells to settle out of suspension, we were able to make standardized cell suspensions at the standard turbidity level from statically grown cultures of *F. columnare*. Compared to standard procedures for other organisms (CLSI, 2006b) more of this suspension had to be added to the broth (1:100 dilution) to get the target cell concentration (5×10^5 CFU/mL), presumably because *F.*

columnare cells are relatively large compared to other bacteria. With this approach, we consistently prepared broth inoculums that approximated the correct number of cells.

Alternatively, cultures grown while continually mixing can likely improve the consistency of the cell suspensions. Laboratories that test *F. columnare* and *F. psychrophilum* susceptibility commonly use mixing to grow the primary cultures (Bruun et al., 2000; Darwish et al., 2008). Mixing may also improve cell viability considering cultures of *F. psychrophilum* can include many dead cells (Michel et al., 1999). Therefore, when testing these *Flavobacteria*, laboratories should first experiment with their methods to prepare the broth inoculums.

Unfortunately, *F. psychrophilum* isolates differ greatly from each other in growth rate making it hard to consistently prepare inoculums with similar cell concentration. In this study, the broth inoculum averaged the target cell concentration most closely when the suspensions at the standard turbidity were diluted in broth at the standard rate (1:200). However, in preliminary testing, we found the broth inoculums should be made in the same way as *F. columnare*. The lower dilution rate resulted in more consistent cultures, preventing better against excessively low cell numbers of slow growing isolates.

The growth of *F. columnare* sharply increased through 48 hours then slowed suggesting, as previously established, that susceptibility tests of *F. columnare* should be incubated for 48 hours at 28°C (Darwish et al., 2008). *Flavobacterium psychrophilum* growth peaked at 96 hours then sharply declined suggesting a longer incubation could impair testing results. Although the cells were growing in log phase by 72 hours, we

chose to incubate our broth microdilution tests for 96 hours to better account for slow-growing isolates.

We tested *F. psychrophilum* at 18°C since its generation time at this temperature is very similar to the established *in vitro* optimum of 15°C (Holt et al., 1989). In addition, existing QC bacteria would presumably grow better, therefore no new QC bacteria would be needed. As such, we found that drug susceptibility of both existing QC bacteria (CLSI, 2006b) were stable at both 28 and 18°C in the dilute media.

Supplementing the broth with calcium and magnesium cations works best for broth dilution testing *F. columnare* and is required for testing of *F. psychrophilum*. Cations diluted at a level similar to the dilute broth provide MIC results similar to results from test conducted in media containing full strength cations; therefore, commercially available CAMHB diluted to 4 g/L can be used. Commercial broth gives laboratories the convenience of not having to make media as well as possibly easier storage conditions and extended shelf-life.

Although full strength CAMHB was recently used for broth microdilution testing of *F. psychrophilum* (Hesami et al., 2010), we chose dilute media to have similar methods for testing both *F. columnare* and *F. psychrophilum* and to be in line with suggested media recommendations (CLSI, 2006b; Alderman and Smith, 2001). The procedures and growth conditions developed in this study along with the recently developed QC limits (Gieseker et al., 2012) provide the first standardized antimicrobial susceptibility testing method for these fastidious bacteria. We hope this method will

improve the monitoring for antimicrobial resistance and promote the judicious use of antimicrobials approved to control the fish diseases caused by these bacteria.

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2.7 Disclaimer

The views expressed in this article are those of the authors and may not reflect the official policy of the Department of Health and Human Services, the U.S. Food and Drug Administration, or the U.S. Government.

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Table 2-1. *Flavobacterium columnare* and *F. psychrophilum* isolates used to develop broth microdilution antimicrobial susceptibility testing methods.

CVM Isolate #	Original Isolate #	Host	Year Isolated	Origin	Source	Serum Supplementation	Inoculum Volume in Test Broth	Cation Supplementation
F. columnare								
22650	503-435	Channel catfish	2003	USA	Dr. Tim Santucci	+	+	+
22655	503-517	Channel catfish	2003	USA	Dr. Tim Santucci	+	+	
22683	94-060	Channel catfish	1994	USA	Dr. John Hawke	+	+	+
33677	94-082	Channel catfish	1994	USA	Dr. John Hawke	+	+	
33970	ATCC 49512	Brown trout	n/a	France	ATCC	+	+	+
33971	ATCC 49513	Black bullhead	n/a	France	ATCC	+	+	
36413	CVI unknown	unknown	1991	Netherlands	Dr. Olga Haenen	+	+	
36417	CVI 04017018	Koi	2004	Netherlands	Dr. Olga Haenen	+	+	+
36422	ALG 92-491-C	Channel Catfish	1992	USA	Dr. Joseph Newton	+	+	+
36434	JIP 07/02	Koi	2002	France	Dr. Jean-Francois Bernardet	+		
36445	M08-26807	Koi	n/a	USA	Dr. Hui-Min Hsu		+	+
F. psychrophilum								
22645	ATCC 49418	Coho salmon	n/a	USA	ATCC	+	+	+
36391	FLPS 70	Coho salmon	1981	USA	Dr. Douglas Call			
36393	FLPS 79	Coho salmon	1990	USA	Dr. Douglas Call	+	+	
36394	FLPS 80	Rainbow trout	1990	USA	Dr. Douglas Call	+	+	
36395	FLPS 85	Rainbow trout	1985	USA	Dr. Douglas Call	+	+	+
36396	FLPS 96	Ayu	1988	Japan	Dr. Douglas Call			+
36400	FLPS 69	Coho salmon	1981	USA	Dr. Douglas Call	+	+	+
36403	FLPS 74	Coho salmon	1989	USA	Dr. Douglas Call			
36408	FLPS 99	Coho salmon	1991	USA	Dr. Douglas Call	+	+	
36410	AU0205	Atlantic Salmon	2006	Chile	Dr. Ruben Avendano	+	+	+
36411	AU2706	Rainbow trout	2006	Chile	Dr. Ruben Avendano	+	+	
36429	LVDI 5/I	Common carp	1992	France	Dr. Jean-Francois Bernardet	+	+	+
36430	LVDJ XP189	Tench	1992	France	Dr. Jean-Francois Bernardet	+	+	

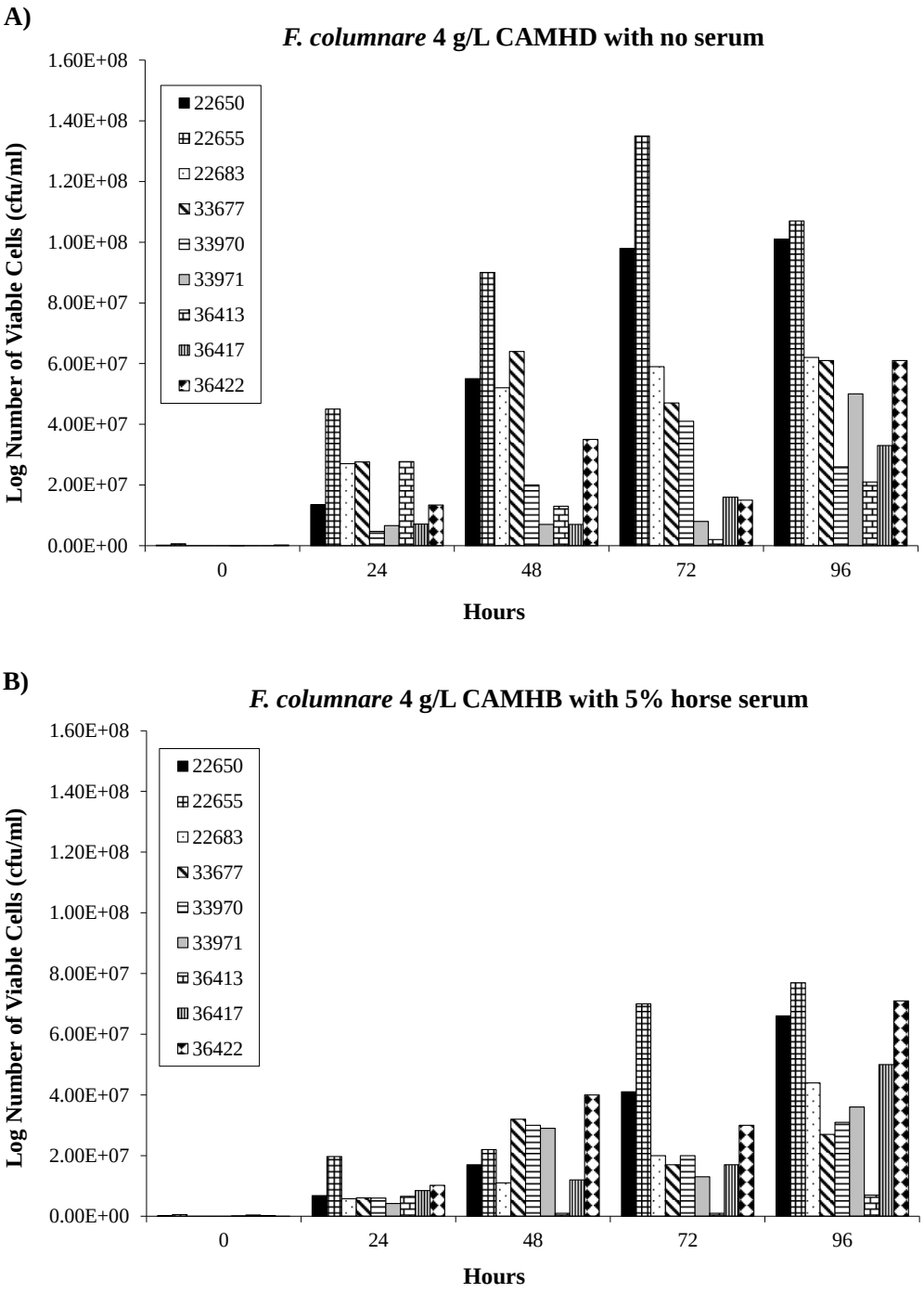

Figure 2-1. Growth of *F. columanre* (n=9) in 4 g/L dilute cation-adjusted Mueller-Hinton broth supplemented without (A) or with 5% horse serum (B).

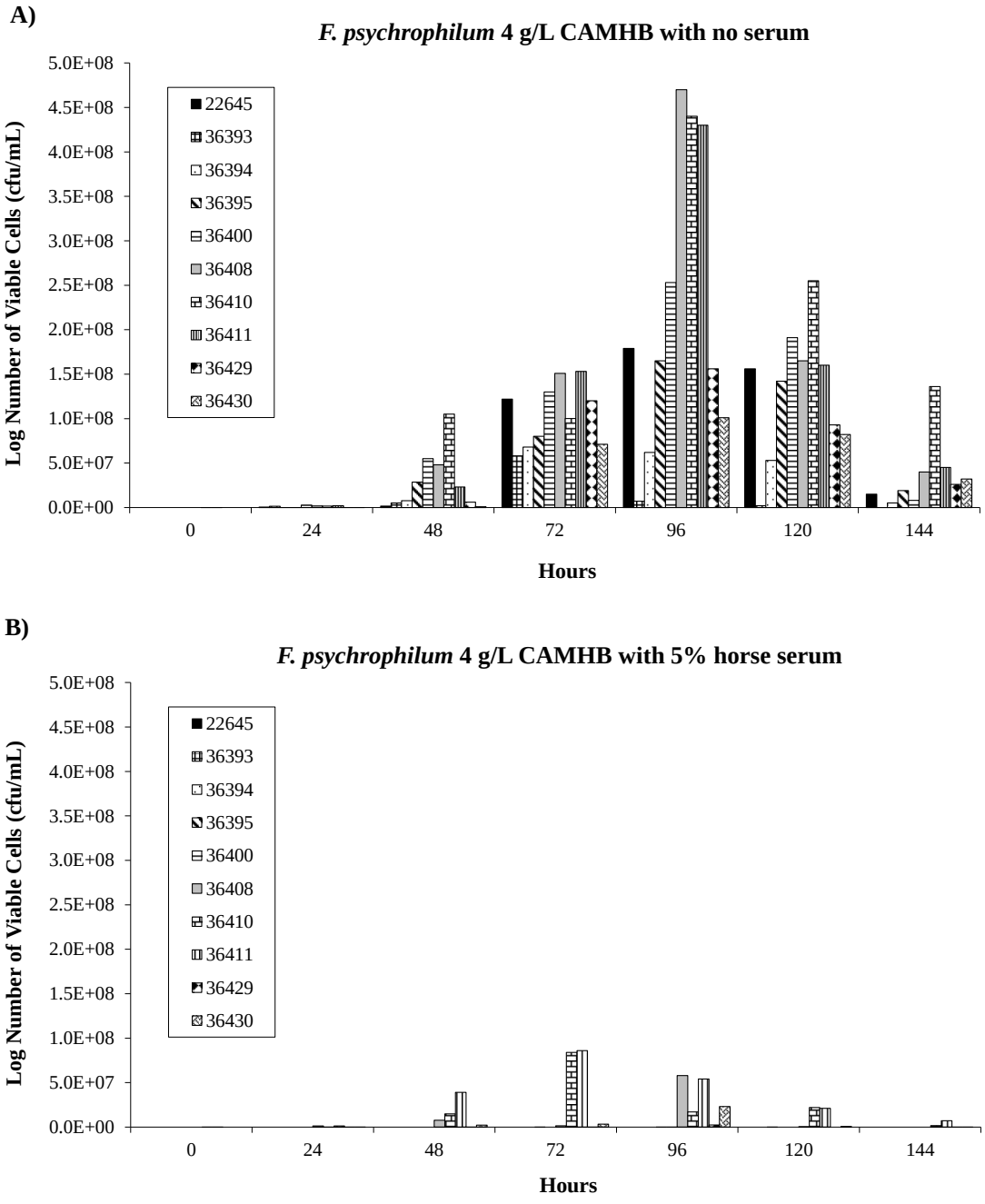

Figure 2-2. Growth of *F. psychrophilum* (n=10) in 4 g/L dilute cation-adjusted Mueller-Hinton broth supplemented without (A) or with 5% horse serum (B).

Table 2-2. Mean number of cells (CFU/mL) and standard deviations of 10 *F. columnare* and 10 *F. psychrophilum* isolates at different McFarland cell suspensions and inoculum volumes used to prepare an inoculum suspension for broth microdilution testing.

	McFarland Suspension	Inoculum Volume (µL)	Mean (CFU/mL)	Standard Deviation (CFU/mL)
F. columnare	0.5	55	3.5E+05	1.1E+05
	0.5	110*	7.4E+05	6.2E+05
	0.5	165	7.5E+05	2.6E+05
F. psychrophilum	0.5	55	6.3E+05	2.6E+05
	0.5	110	1.1E+06	4.8E+05
	0.5	165	1.5E+06	4.9E+05
	1	55	8.5E+05	2.2E+05
	1	110	1.6E+06	4.2E+05
	1	165	2.0E+06	6.6E+05

* 1 isolate had an unusually high cell concentration due most likely to technician error. Without this isolate, the mean and standard deviation were $5.7x10^5$ and $1.8x10^5$ CFU/mL, respectively.

Table 2-3. Minimal inhibitory concentrations (µg/mL) of *F. columnare*, *F. psychrophilum*, *A. salmonicida subsp. salmonicida* ATCC 33658 and *E. coli* ATCC 25922 from broth microdilution testing in 4 g/L cation-adjusted Mueller-Hinton broth supplemented with difference levels of calcium and magnesium cations.

	Oxytetracycline			Florfenicol		
Isolate	**NC**	**DC**	**FC**	**NC**	**DC**	**FC**
***F. columnare*, 28°C**						
22650	0.03	0.06-0.12	no growth	0.5-1	2	no growth
22683	0.03	0.12	0.12	0.12-2	2	2
33970	0.03	0.12	0.12-0.5	0.5	2	2
36417	2	8	8	0.5	1-2	1
36422	0.06	0.12	0.12	1-2	1	1-2
36445	no growth	0.06-0.12	no growth	no growth	1	no growth-0.25
A. salmonicida ATCC 33658	0.12	0.25-0.5	0.5	2	1	1
E. coli ATCC 25922	0.5	1	1	8-16	8	8
***F. psychrophilum*, 18°C**						
22645	no growth	0.12	0.12-0.25	no growth	1	1
36395	no growth-0.03	0.06	0.12	1	1	1
36396	no growth	0.12	0.12-0.25	no growth	1-2	2
36400	no growth	4	4-8	no growth	1	1-2
36410	no growth	8	16	no growth	2	1-2
36429	no growth	0.12	0.25	no growth	1	1-2
A. salmonicida ATCC 33658	0.12	0.12	0.5	2	1	1-2
E. coli ATCC 25922	0.25	0.5	1	16-32	16	16-32

NC=no cations, DC=dilute cations; FC=full cations

Table 2-4. Percent agreement between 4 operators in broth microdilution testing of 6 *F. columnare*, 6 *F. psychrophilum* and 2 potential QC strains with specialized conditions develop for these nutritionally fastidious *Flavobacteria*.

	Isolate	AMP	FFN	OTC	PRI
28°C, 48 hours					
	F. columnare 22650	100	100	100	100
	22655	100	100	100	100
	22683	100	100	100[b]	75
	33971	100	100	100	100
	36413	100[e]	100[c]	100	50
	36434	73[b]	100	100	100
	E. coli ATCC 25922	100	100	100	100
	A. salmonicida ATCC 33658	100[b]	100[b]	100[b]	100[b]
18°C, 96 hours	*F. psychrophilum* 36391	100[b,d]	100	100[b]	92
	36396	100	100	91[b]	83
	36400	100	100	100[b]	92
	36403	100[b]	100[b]	100[b]	91[b]
	36410	100	100	100	75
	36429	92	100	100[b]	75
	E. coli ATCC 25922	100	100[e]	92	100
	A. salmonicida ATCC 33658	92	100	92	100

[a] AMP = ampicillin, FFN = florfenicol, OTC = oxytetracycline, PRI = ormetoprim - sulfadimethoxine.

[b] Average of 11 replicates since the bacteria did not grow in one replicate.

[c] All MIC results were above the highest drug concentration tested.

[d] Some isolates had MIC at lower limit or less, presumed to agree within a dilution.

[e] Some isolates had MIC at upper limit or greater, presumed to agree within a dilution.

Chapter 3: Determination of quality control ranges to nine antimicrobials for broth microdilution susceptibility testing of *Flavobacterium columnare* and *Flavobacterium psychrophilum*.

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3.1 Abstract

A multi-laboratory broth microdilution method trial was performed to standardize the specialized test conditions required for the fish pathogens *Flavobacterium columnare* and *F. psychrophilum.* Nine laboratories tested the quality control (QC) strains *Escherichia coli* (*E. coli*) ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* (*A. salmonicida*) ATCC 33658 against 10 antimicrobials (ampicillin, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, ormetoprim / sulfadimethoxine, oxolinic acid, oxytetracycline and trimethoprim / sulfamethoxazole) in diluted (4 g/L) cation-adjusted Mueller-Hinton broth incubated at 28 and 18 ºC for 44-48 and 92-96 hours, respectively. QC ranges were set for 9 of the 10 antimicrobials. Most of the minimal inhibitory concentration (MIC) distributions (16 of 18, 9 drugs at both temperatures) for *A. salmonicida* ATCC 33658 were centered on a single median MIC ± 1 two-fold drug dilution resulting in a QC range that spanned three dilutions. More of the *E. coli* ATCC 25922 MIC distributions (7 of 16) were centered between two MIC dilutions requiring a four dilution QC range. A QC range could not be determined for *E.*

coli ATCC 25922 against 2 antimicrobials at the low temperature. These data and their associated QC ranges have been approved by the Clinical and Laboratory Standards Institute (CLSI), and will be included in the next edition of the CLSI's M49-A Guideline. This method represents the first standardized reference method for testing fish pathogenic *Flavobacterium* spp.

3.2 Introduction

The nutritionally fastidious aquatic gliding bacteria *Flavobacterium columnare* (*F. columnare*) and *F. psychrophilum* represent an important group of fish pathogens that need standard antimicrobial susceptibility testing (AST) methods. They are major fish pathogens that cause significant disease losses in freshwater aquaculture worldwide (Wakabayaski and Egusa, 1966; Bernardet, 1989; Holt et al., 1993; Wagner et al., 2002; Nematollahi et al., 2003; Pulkkinen et al., 2010). The Clinical and Laboratory Standards Institute (CLSI) provides 2 consensus-approved guidelines with reference methods and quality control (QC) criteria for disk diffusion and broth dilution susceptibility testing of non-fastidious aquatic bacteria (CLSI, 2006 a, b) based on the work of Miller et al. (2003, 2005). CLSI groups fastidious aquatic bacterial pathogens with similar growth condition requirements, and offers potential media and incubation modifications; however, no standard reference methods or QC criteria have been developed to date.

In general, three AST methods can be developed as standard CLSI reference methods: disk diffusion, broth dilution, and agar dilution. Dilution techniques (agar and broth) are preferable to disk diffusion since these tests yield a minimal inhibitory concentration (MIC) that can be directly compared to the pharmacokinetics of the drug in

the animal. However, disk diffusion is popular among fish disease diagnosticians because the method is inexpensive, easy to perform and better suits laboratories that test only a small number of isolates infrequently. Disk diffusion is not as practical for testing the fish pathogenic *Flavobacteria*, in particular *F. columnare*, since the gliding motility of the bacterium distorts the margin of the inhibitory zone (Farmer, 2004). Alternatively agar dilution, the gold standard method, has been used to test *F. psychrophilum* (Bruun et al., 2000; Schmidt et al., 2000; Michel et al., 2003), but the method is fairly labor intensive and does not lend itself nicely to *ad hoc* testing. Therefore, we determined that broth dilution, specifically broth microdilution, was the best option to develop as a standard reference AST method.

CLSI's broth dilution testing guideline for aquatic bacteria (M49-A) suggests using diluted Mueller-Hinton broth (MHB) (3 g/L) for testing *F. columnare*, *F. psychrophilum* and *F. branchiophilum*. The guide also suggests that *F. psychrophilum* and *F. branchiophilum* may need additional supplements of 5% horse or fetal calf serum, and/or NaCl (CLSI, 2006b). These recommendations were partly based on the disk diffusion methods developed by Hawke and Thune (1992) for testing *F. columnare*. However, more recent research has determined that only diluted MHB (4 g/L) is needed for broth microdilution testing of *F. columnare*, and that Ca^{++} and Mg^{++}, typically added to MHB for broth dilution susceptibility testing, may not be needed (Farmer, 2004; Darwish et al., 2008). Preliminary work at the Food and Drug Administration, Center for Veterinary Medicine, Office of Research found diluted MHB (4 g/L) yielded consistent MICs for *F. columnare* and *F. psychrophilum* isolates, as well as for both QC strains referenced in CLSI's M42-A and M49-A guidelines. We had too few isolates to test *F.*

branchiophilum adequately. We also found that Ca^{++} (4 mg/L) and Mg^{++} (2 mg/L) were needed for testing *F. psychrophilum,* and serum supplementation was not necessary for testing of *F. columnare* or *F. pyschrophilum*. Sodium chloride supplementation was not considered since *F. columnare* and *F. psychrophilum* generally do not grow in media containing more than 0.5% NaCl (Bernardet and Grimont, 1989). Ultimately, we found diluted MHB (4 g/L) with Ca^{++} (4 mg/L) and Mg^{++} (2 mg/L) was a good medium for broth dilution testing of both *F. columnare* and *F. psychrophilum* isolates.

Optimal incubation conditions for *F. columnare* and *F. psychrophilum* also needed to be established. We found that incubating *F. columnare* at 28°C for 44-48 h as recommended by the CLSI (2006b) and Darwish et al. (2008) worked well. In contrast, a slightly higher incubation temperature (18°C) and longer incubation time (96 h) than recommended by the CLSI was needed for *F. psychrophilum* (Gieseker et al., in prep.). Although 18°C is just above the recommended growth temperature of 15°C for *F. psychrophilum* (CLSI, 2006b), the bacterium grows similarly at both temperatures (Holt et al., 1989). A longer incubation time is needed as the shorter incubation times of 44-72 h, as recommended by the CLSI, yielded misleadingly low MICs in preliminary testing (Gieseker et al., unpublished).

To establish a standard AST method for fish pathogenic *Flavobacteria*, drug susceptibility testing ranges were needed for the QC strains under the altered test conditions optimized for these bacteria. Herein, we report the results of a multi-laboratory standardization trial that established MIC QC ranges of 9 antimicrobials for the QC strains, *Escherichia coli* (*E. coli* ATCC 25922; NCIMB 12210) and *Aeromonas*

salmonicida subsp. *salmonicida* (*A. salmonicida*, ATCC 33658; NCIMB 1102). CLSI's Subcommittee on Veterinary Antimicrobial Susceptibility Testing (VAST) – Aquaculture Working Group analyzed the data and proposed QC ranges to the VAST Subcommittee. The ranges presented here were unanimously accepted for inclusion in the next edition of CLSI's M49-A Guideline. These QC ranges and testing methods provide the first standard AST reference methods for the important fish pathogens *F. columnare* and *F. psychrophilum*.

3.3 Materials and Methods

The design of this study was based on CLSI guideline M37-A3 (CLSI, 2008). The QC strains, *E. coli* (ATCC 25922; NCIMB 12210) and *A. salmonicida* (ATCC 33658; NCIMB 1102), were tested by nine laboratories using broth microdilution methods developed for testing *Flavobacterium psychrophilum* and *F. columnare* (Darwish et al., 2008; Gieseker et al., *in prep*). Both QC strains were tested at 18°C (92-96 h) and 28°C (44-48 h) using three different lots of diluted (4 g/L) cation-adjusted Mueller-Hinton broth (CAMHB). All laboratories repeated each test 10 times. Each replicate had 3 panels (media lots 1, 2, & 3) (3 panels x 10 replicates = 30 replicates/laboratory for each drug/strain/temperature combination).

3.3.1 Participating Laboratories

Nine laboratories participated in the standardization trial. The laboratories were the Food and Drug Administration, Center for Veterinary Medicine, Office of Research, Laurel, Maryland, USA (FDA/CVM/OR); Animal Health Laboratory, Department of Primary Industries, Parks, Water & Environment, Launceston, Tasmania, Australia;

National Veterinary Institute, Technical University of Denmark, Frederiksberg, Denmark; Harry K. Dupree Stuttgart National Aquaculture Research Center, Stuttgart, Arkansas, USA; Mississippi State University, College of Veterinary Medicine, Stoneville, Mississippi, USA; University of Wisconsin Veterinary Diagnostic Laboratory, Madison, Wisconsin, USA; Washington Animal Disease Laboratory, Pullman, Washington, USA; Inland Aquatic Animal Health Research Institute, Department of Fisheries, Kasetsart University Campus, Jaktujak, Bangkok, Thailand; Animal Disease Diagnostic Laboratory, Purdue University, West Lafayette, Indiana, USA. All 9 laboratories completed testing for the trial. The data presented are based on 8 laboratories for *E. coli*, ATCC 25922 and 7 labs for *A. salmonicida* ATCC 33658. One laboratory admitted making mistakes on their MIC interpretations; therefore, the data from that laboratory were excluded from the analysis. In addition, another laboratory could only test *E. coli* due to import restrictions against *A. salmonicida*.

3.3.2 Broth microdilution panels and test media

Custom, commercially prepared dry-form 96-well broth microdilution panels were purchased from Trek Diagnostic Systems (Cleveland, Ohio, USA). Each well had dried residue of a separate two-fold dilution of one of 10 antimicrobials. Ten dilutions were tested for each of the following antimicrobials: ampicillin (0.03 – 16 μg/mL), enrofloxacin (0.001 – 0.5 μg/mL), erythromycin (0.25 – 128 μg/mL), florfenicol (0.12 – 64 μg/mL), flumequine (0.004 – 2 μg/mL), oxolinic acid (0.002 – 1 μg/mL), oxytetracycline (0.015 – 8 μg/mL) and ormetoprim / sulfadimethoxine (0.008/0.15 – 4/76 μg/mL). Seven dilutions were tested of gentamicin (0.03 – 2 μg/mL) and trimethoprim / sulfamethoxazole (0.008/0.15 – 0.5/9.5 μg/mL).

Three separate lots of broth were made from dehydrated powders manufactured by BBL™ (212322, lot 7331751) and Difco™ (275710, lot 8127115) (Becton-Dickinson, Sparks, Maryland, USA), and Oxoid (CM0405, lot 597351; Basingstoke, Hampshire, United Kingdom). 11-mL aliquots of each broth were prepared by Trek Diagnostics Systems. Calcium (4 mg/L) and magnesium (2 mg/L) cations were added by CLSI guidelines if the prepared media were not already supplemented.

3.3.3 Test bacteria and test conditions

American Type Culture Collection (ATCC, Manassas, Virginia, USA) reference strains of *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* ATCC 33658 were incubated at 18°C for 92-96 h and 28°C for 44-48 h in diluted (4 g/L) CAMHB.

3.3.4 Broth microdilution susceptibility testing

Tests were conducted in each laboratory over a minimum of three days with a maximum of four replicates per day. Each laboratory (besides one mentioned above) tested 10 replicates of each strain at both temperatures. Each replicate consisted of three 96-well panels; one panel for each lot of broth.

Prior to each test day, strains were subcultured twice on tryptic soy agar supplemented with 5% sheep blood. On each test day a single suspension was prepared of each strain to a predetermined turbidity measured by colorimeter (0.5 McFarland), spectrophotometer (0.08 to 0.13 at OD_{625}) or turbidimeter (20-30 NTUs).

Fifty-five µL of the cell suspension were added to each 11 mL aliquot of broth (1:200 dilution). Each 11 mL aliquot of broth was used to inoculate into a 96-well broth

microdilution panel. One hundred (100) µL of the inoculated broth was added to each well using a Sensititre® autoinoculator (Trek Diagnostic Systems, 3 labs) or a multichannel pipette (6 labs). To prevent contamination and dehydration, plastic adhesive seals were immediately attached to the panels covering the top of the wells. Within 15 minutes of inoculation, the panels were placed in their respective incubators. No more than 4 panels were stacked together to achieve a uniform temperature across the panels.

Actual cell densities were determined using a colony count procedure where two panels on each test day were arbitrarily chosen for each QC strain (one each at 18°C and 28°C). Ten µL were removed from the positive control well and placed in 10 mL sterile saline (1:1000 dilution). A 100 µl aliquot was evenly spread on a tryptic soy agar plate supplemented with 5% sheep blood. Colony count plates were incubated under the same conditions (temperature and time) as the 96-well panels, and colony-forming units (CFUs) were counted at the same time MICs were determined. Mean CFU/mL and standard deviations (SD) were calculated for each drug/strain/temperature combination across all replicates.

After the appropriate incubation period, the panel seal was removed to read panels. Panels were examined for growth in the wells, either as a pellet or turbidity. The MIC was the first well in the dilution series without growth. When interpreting results in wells with the bacteriostatic sulfonamides, growth < 80% of the pellet density in the positive control well was recorded as growth limited, indicative of a bacteriostatic effect evident as a trailing endpoint. Where this occurred, the MIC was established by identifying the well with 50% growth when compared with the positive control well. The

next highest dilution well adjacent to the 50% growth well was assumed to have approximately 80% growth; therefore the concentration in the well with 50% growth inhibition was considered the MIC.

3.3.5 Data Analysis

The data were pooled into frequency distributions by broth lot, and also by laboratory. Each drug/strain/temperature combination was analyzed separately for MIC mean, mode, median, minimum, maximum, and range.

All MIC data were entered into RangeFinder; a specialized Excel spreadsheet program that analyzes antimicrobial susceptibility testing data from CLSI standardization trials (Turnidge & Bordash, 2007). RangeFinder calculated a QC range, number of 2-fold dilutions in the range, and the percent of observations captured by the calculated range.

Frequency distributions and histograms for each drug/strain/temperature combination were used to also determine QC ranges using the median method developed by Gavan et al. (1981). Ideally, the QC ranges encompassed 95% of the observations, and spanned 3 drug concentrations centered on the mode of the distribution. If data were skewed in one direction, where the MIC frequency at the 2-fold dilution immediately adjacent to the mode was greater than or equal to 60% of MIC frequency at the mode, an additional 2-fold dilution was added to the final proposed QC range. The CLSI's VAST – Aquaculture Working Group compared the QC ranges estimated by the Gavan and RangeFinder methods to determine proposed QC ranges for each drug/strain/temperature combination. Both methods use arbitrary limits that require expert opinion.

3.3.6 Confirmation of the drug concentration on the custom dry-form broth microdilution panels

One laboratory (FDA/CVM/OR) followed the same procedures used in the standardization trial to test five subcultures of *E. coli* ATCC 25922 and five subcultures of *A. salmonicida* ATCC 33658 in undiluted CAMHB to confirm the potency of the drugs on the panels. The testing was repeated three times and each replicate was prepared on a separate day. Tests were conducted at 22°C and 28°C according to CLSI M49-A guideline. Spread plate colony counts were also prepared identically as described previously to confirm cell densities on each day testing was conducted. Drug potencies were considered acceptable if MIC test results from the two QC strains on the custom dry-form panels were within the acceptable ranges listed in the M49-A guideline.

3.4 Results and Discussion

3.4.1 Standardization trial

Using standard AST methods makes it easier for laboratories to reliably share data, monitor development of drug resistance and provide consistent clinical recommendations. Proper QC procedures with expected test results are required to confirm method performance. Miller et al., (2003, 2005) established the first standard reference disk diffusion and MIC testing methods for non-fastidious aquatic bacterial pathogens (CLSI, 2006a, b). These organisms include members of *Enterobacteriaceae* and *Vibrionaceae*, and *A. salmonicida, A. hydrophila* and other mesophilic aeromonads, *Pseudomonas* spp., *Plesiomonas shigelloides,* and *Shewanella* spp. Many fastidious aquatic bacteria are also important fish pathogens. In particular, the pathogenic gliding bacteria, *F. columnare* and *F. psychrophilum* cause disease and major economic loss for a

wide variety of freshwater aquaculture industries. Recent approval of antimicrobials in the United States to control mortality of fish infected with *F. columnare* and *F. psychrophilum* have further fueled a need for standard AST methods for these fastidious organisms. Our research directly addresses this issue and reports the results of a standardization trial to set MIC QC ranges for testing *F. columnare* and *F. psychrophilum.*

The proposed QC ranges determined by the CLSI VAST – Aquaculture Working Group were presented to the VAST subcommittee and accepted with minor revisions to be included in the next edition of the M49-A guideline for dilution susceptibility testing of bacteria isolated from aquatic animals (CLSI, 2006b). Table 1 demonstrates the lot-by-lot comparison of the MIC results from our standardization trial. No laboratory was consistently different although in some cases a laboratory had some MIC results outside of the majority (~95%) of the data distribution. A visual analysis comparing data by broth lot showed that all lots consistently agreed across the laboratories. Tables 2-5 summarize the MIC results and QC limits approved by the CLSI VAST Subcommittee for *E. coli* ATCC 25922 and *A. salmonicida* ATCC 33658. Most of the MICs observed at both temperatures for *A. salmonicida* ATCC 33658 were centered on a single median MIC ± 1 two-fold drug dilution (e.g., Fig. 1). In two cases, enrofloxacin at 18°C and ormetoprim/sulfadimethoxine at 18°C (Fig. 2), a 4 two-fold dilution range was approved since the MIC distribution was asymmetric, and had a frequency adjacent to the median that was >60% of the frequency of the median MIC. More of the approved QC ranges for *E. coli* ATCC 25922 were 4 dilutions. This was the case at 28°C for 4 of the 9 drugs, and

at 18°C for 3 of 7 drugs; in addition, because of inadequate data, QC ranges could not be established for enrofloxacin and ormetoprim/sulfadimethoxine at 18°C.

No MIC QC ranges could be set for gentamicin since a majority of laboratories observed MICs below the test range (≤0.03 μg/mL). When compared to frozen-form panels with the same drug format (performed by one laboratory, FDA/CVM/OR), gentamicin MICs from the dry-form panels were still observed to be below the test concentration range (data not shown). However, gentamicin MIC results were in range when both QC strains were tested with the dry-form panels in full strength CAMHB in this study (below) and by the manufacturer. We opted to use dry-form panels since these panels could withstand potentially longer transit times, and were shown to perform well in a similar standardization trial (Miller et al., 2005). Miller et al. (2005) also found that MIC results on dry-form and frozen-form broth microdilution panels agreed for the same QC strains.

Colony count data tracking approximated the final target concentration of approximately 5 x 10^5 CFU/mL bacteria as recommended by the CLSI (2006b). The mean CFU/mL (SD) for *A. salmonicida* ATCC 33658 was 4.0 x 10^5 CFU/mL (1.8 x 10^5) at 18°C and 4.3 x 10^5 CFU/mL (1.7 x 10^5) at 28°C. The mean CFU/mL (SD) for *E. coli* ATCC 25922 was 3.3 x 10^5 CFU/mL (1.9 x 10^5) at 18°C and 4.5 x 10^5 CFU/mL (1.6 x 10^5) at 28°C. No trends were observed between MIC results and low/high cell densities indicating the variability in the cell densities did not markedly affect MIC results.

3.4.2 Validation of drug concentrations with undiluted CAMHB

The established QC ranges from the M49-A CLSI guideline (CLSI, 2006b) for broth microdilution testing in full strength CAMHB of *A. salmonicida* ATCC 33658 and *E. coli* ATCC 25922 at 22°C (24 & 48 h) and 28°C (24 h) were used to validate the drug concentrations of the custom dry-form broth microdilution panels. All the MIC results at 22°C, were within the accepted QC ranges, and were in agreement among the replicates within ± 1 two-fold dilution except for erythromycin results for *A. salmonicida.* At 24 hours, 9 of 15 erythromycin test results were out of the accepted parameters, but by 48 hours only 2 of 15 were out of range.

All of the 28°C *A. salmonicida* erythromycin MIC results were in the QC range, but were concentrated at the lowest MIC. However, in the standardization trial the median erythromycin MIC data in diluted CAMHB for *A. salmonicida* at 18°C (96 h) was very similar to 28°C (48 h; Tables 4 & 5). At least 99.5% of the MIC data was within the same two-fold dilution range with the same median at both temperatures.

The data from this validation experiment were presented to the CLSI VAST committee concurrently with the standardization trial and the results confirmed that drug concentration ranges were valid on the dry-form panels used in the standardization trial.

3.5 Conclusions

Consistent MIC results across the laboratories showed that *E. coli* ATCC 25922 and *A. salmonicida* ATCC 33658 are reliable QC strains for broth microdilution testing at 18°C and 28°C in diluted CAMHB (4 g/L). These strains can be used for QC testing of clinical isolates of *Flavobacterium columnare* and *Flavobacterium psychrophilum.*

CLSI testing guidelines are intended to be living documents that are continually updated as new methods and approaches are developed. This newly standardized AST method for *Flavobacteria* and the MIC QC ranges provided in Table 6 will be included in the next edition of CLSI's M49-A guideline. We hope that this work as well as the work of Miller et al. (2003, 2005) will serve as helpful resources to aid in continued development of more standardized AST methods for other important aquatic bacterial pathogens.

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Table 3-1. Comparison by broth lot of MIC results across each laboratory: *Escherichia coli* ATCC 25922 incubated at 18°C for 92-96 hours. The grey shaded area denotes the CLSI-approved QC range.

Oxytetracycline *Escherichia coli* 25922 18ºC/96 hours, 8 Laboratories																											
	Lab 1			**Lab 2**			**Lab 3**			**Lab 4**			**Lab 5**			**Lab 6**			**Lab 7**			**Lab 8**			**All Labs**		
	lot 1	**lot 2**	**lot 3**	**lot 1**	**lot 2**	**lot 3**	**lot 1**	**lot 2**	**lot 3**	**lot 1**	**lot 2**	**lot 3**	**lot 1**	**lot 2**	**lot 3**	**lot 1**	**lot 2**	**lot 3**	**lot 1**	**lot 2**	**lot 3**	**lot 1**	**lot 2**	**lot 3**	**lot 1**	**lot 2**	**lot 3**
0.015																											
0.03																											
0.06																											
0.12																											
0.25	2			4			4	2		2		1	2		2	8	6	3	10	8	7	7	2	6	39	18	19
0.5	6	10	10	2	8	6	6	8	10	5	7	8	8	10	8	2	4	7		2	3	3	8	4	32	57	56
1	2			3		2				3	3	1													8	3	3
2				1	2	2																			1	2	2
4																											
8																											
N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	80	80	80
Geomean	0.50	0.50	0.50	0.54	0.66	0.76	0.38	0.44	0.50	0.54	0.62	0.50	0.44	0.50	0.44	0.29	0.33	0.41	0.25	0.29	0.31	0.31	0.44	0.33	0.39	0.45	0.45
mode	0.5	0.5	0.5	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.25	0.25	0.25	0.25	0.5	0.25	0.25	0.5	0.5
min	0.25	0.5	0.5	0.25	0.5	0.5	0.25	0.25	0.5	0.25	0.5	0.25	0.25	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
max	1	0.5	0.5	2	2	2	0.5	0.5	0.5	1	1	1	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.5	0.5	0.5	0.5	2	2	2
range	3	1	1	4	3	3	2	2	1	3	2	3	2	1	2	2	2	2	1	2	2	2	2	2	4	4	4

All lab median = 0.5

Table 3-2. MIC results for *Escherichia coli* ATCC 25922 incubated at 28 ± 2°C for 44-48 hours in diluted (4 g/L) CAMHB against 9 antimicrobials.

Antimicrobial agent	Testing Range (ug/mL)	MIC (µg/mL)			# of dilutions in QC range	% within QC range (n=239)
		Inter-Laboratory range	Median	CLSI-approved QC range		
Ampicillin	0.03–16	1–4	2	1-4	3	100.0
Enrofloxacin	0.001–0.5	0.004–0.015	0.008	0.002-0.015	4	100.0
Erythromycin	0.25–128	4–64	32	16-64	3	94.6
Florfenicol	0.12–64	1–8	4	2-8	3	99.6
Flumequine	0.004–2	0.12–0.5	0.25	0.06-0.5	4	100.0
*Ormetoprim/Sulfadimethoxine	0.008/0.15-4/76	0.06/1.19-0.5/9.5	0.25/4.75	0.12/2.38-0.5/9.5	3	97.1
Oxolinic Acid	0.002–1	0.06–0.25	0.06	0.03-0.12	3	99.6
Oxytetracycline	0.015–8	0.12–1	0.5	0.12-1	4	100.0
**Trimethoprim/Sulfamethoxazole	0.008/0.15-0.5/9.5	0.015/0.30-0.06/1.19	0.06/1.19	0.015/0.30-0.12/2.38	4	100.0 ***

*First value indicates concentration of ormetoprim; second value concentration of sulfadimethoxine
**First value indicates concentration of trimethoprim; second value concentration of sulfamethoxazole
*** n=238

Table 3-3. MIC results of *Escherichia coli* ATCC 25922 incubated at 18 ± 2°C for 92-96 hours in diluted (4 g/L) CAMHB against 9 antimicrobials.

Antimicrobial agent	Testing Range (ug/mL)	MIC (µg/mL)			# of dilutions in QC range	% within QC range (n=240)
		Inter-Laboratory range	Median	CLSI-approved QC range		
Ampicillin	0.03–16	0.25–>16	4	2-8	3	95.8
Enrofloxacin	0.001–0.5	≤0.001–0.015	0.002	No ranges proposed		
Erythromycin	0.25–128	2-32	8	4-16	3	97.5
Florfenicol	0.12–64	≤0.12–32	8	4-32	4	97.9
Flumequine	0.004–2	≤0.004–0.5	0.12	0.06-0.25	3	96.3
*Ormetoprim/Sulfadimethoxine	0.008/0.15-4/76	0.015/0.30-1/19	0.5/9.5	Ranges not accepted		
Oxolinic Acid	0.002–1	0.015–0.12	0.06	0.03-0.12	3	97.1
Oxytetracycline	0.015–8	0.25–2	0.5	0.12-1	4	97.9
**Trimethoprim/Sulfamethoxazole	0.008/0.15-0.5/9.5	≤0.008/0.15–0.12/2.38	0.03/0.59	0.015/0.30-0.12/2.38	4	97.5

*First value indicates concentration of ormetoprim; second value concentration of sulfadimethoxine
**First value indicates concentration of trimethoprim; second value concentration of sulfamethoxazole

Table 3-4. MIC results of *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 incubated at 28 ± 2°C for 44-48 hours in diluted (4 g/L) CAMHB against 9 antimicrobials.

Antimicrobial agent	Testing Range (ug/mL)	MIC (µg/mL)			# of dilutions in QC range	% within QC range (n=210)
		Inter-Laboratory range	Median	CLSI-approved QC range		
Ampicillin	0.03–16	0.12–16	0.12	0.06-0.25	3	99.0
Enrofloxacin	0.001–0.5	0.008–0.015	0.008	0.004-0.015	3	100.0
Erythromycin	0.25–128	1–16	8	4-16	3	99.5
Florfenicol	0.12–64	0.5–2	0.5	0.25-1	3	99.5
Flumequine	0.004–2	0.015–0.06	0.03	0.015-0.06	3	100.0
*Ormetoprim/Sulfadimethoxine	0.008/0.15-4/76	0.06/1.19-0.5/9.5	0.12/2.38	0.06/1.19-0.25/4.75	3	99.5
Oxolinic Acid	0.002–1	0.008–0.06	0.015	0.008-0.03	3	99.5
Oxytetracycline	0.015–8	0.12–0.5	0.12	0.06-0.25	3	99.5
**Trimethoprim/Sulfamethoxazole	0.008/0.15-0.5/9.5	≤0.008/0.15–0.12/2.38	0.06/1.19	0.03/0.59-0.12/2.38	3	99.5

*First value indicates concentration of ormetoprim; second value concentration of sulfadimethoxine
**First value indicates concentration of trimethoprim; second value concentration of sulfamethoxazole

Table 3-5. MIC results of *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 incubated at 18 ± 2°C for 92-96 hours in diluted (4 g/L) CAMHB against 9 antimicrobials.

Antimicrobial agent	Testing Range (ug/mL)	MIC (µg/mL)			# of dilutions in QC range	% within QC range (n=210)
		Inter-Laboratory range	Median	CLSI-approved QC range		
Ampicillin	0.03–16	0.12–1	0.12	0.06-0.25	3	98.1
Enrofloxacin	0.001–0.5	0.004–0.015	0.008	0.004-0.03	4	100.0
Erythromycin	0.25–128	4–16	8	4-16	3	100.0
Florfenicol	0.12–64	0.5–2	0.5	0.25-1	3	99.5
Flumequine	0.004–2	0.03–0.12	0.03	0.015-0.06	3	98.1
*Ormetoprim/Sulfadimethoxine	0.008/0.15-4/76	0.06/1.19-0.25/4.75	0.06/1.19	0.03/0.59-0.25/4.75	4	99.5
Oxolinic Acid	0.002–1	0.008–0.06	0.015	0.008-0.03	3	99.5
Oxytetracycline	0.015–8	0.12–1	0.12	0.06-0.25	3	99.5
**Trimethoprim/Sulfamethoxazole	0.008/0.15-0.5/9.5	0.015/0.30–0.06/1.19	0.03/0.59	0.015/0.30-0.06/1.19	3	100.0

*First value indicates concentration of ormetoprim; second value concentration of sulfadimethoxine
**First value indicates concentration of trimethoprim; second value concentration of sulfamethoxazole

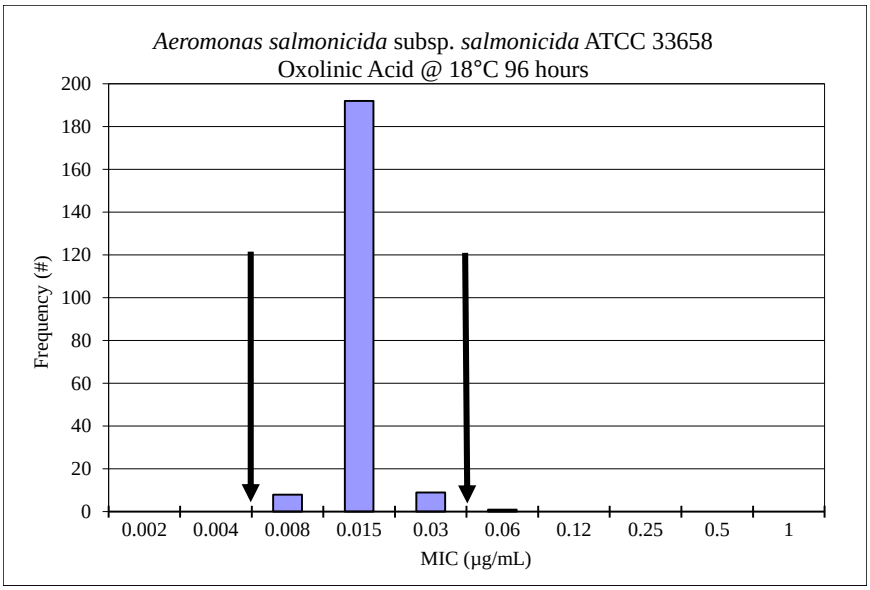

Figure 3-1. MIC frequency distribution of *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 against oxolinic acid, incubated at 18 °C for 96 hours. Black arrows indicate the 3 dilution CLSI-approved MIC QC range.

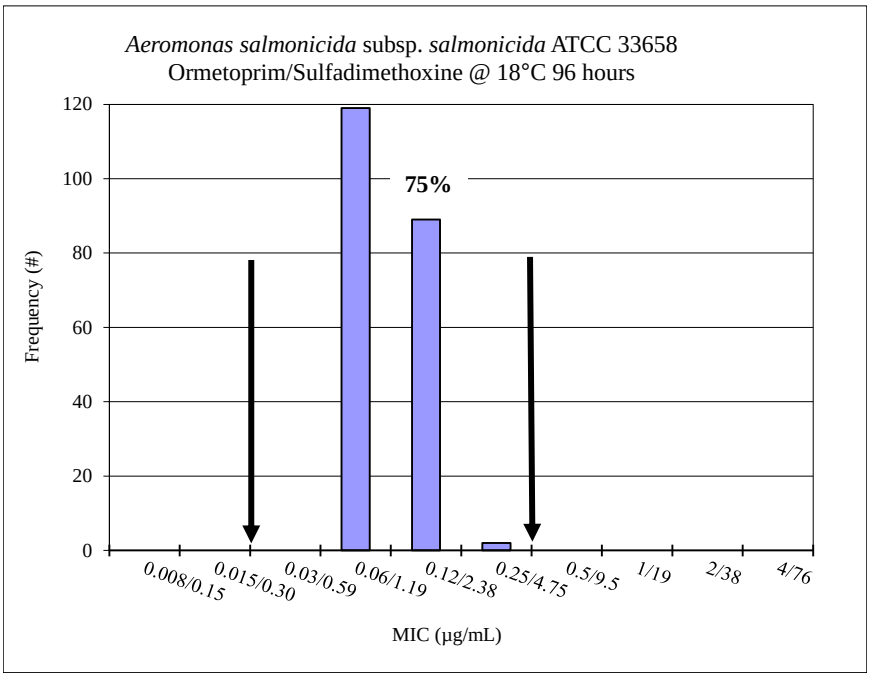

Figure 3-2. MIC frequency distribution of *Aeromonas salmonicida* ATCC 33658 against ormetoprim/sulfadimethoxine, incubated at 18 °C for 96 hours. Black arrows indicate the CLSI-approved MIC QC range. A 4-dilution MIC range was approved since the number of data points at 0.12/2.38 µg/mL was 75% of the data points in the mode, 0.06/1.19 µg/mL.

Table 3-6. Summary of CLSI approved MIC QC ranges (µg/mL) for broth dilution susceptibility testing in dilute 4 g/L CAMHB.

Antimicrobial agent	*Escherichia coli* ATCC 25922		*Aeromonas salmonicida* ATCC 33658	
	28 °C, 44-48 h	18 °C, 92-96 h	28 °C, 44-48h	18 °C, 92-96 h
Ampicillin	1 - 4	2 - 8	0.06 - 0.25	0.06 - 0.25
Enrofloxacin	0.002 - 0.015	No ranges proposed	0.004 - 0.015	0.004 - 0.03
Erythromycin	16 - 64	4 - 16	4 - 16	4 - 16
Florfenicol	2 - 8	4 - 32	0.25 - 1	0.25 - 1
Flumequine	0.06 - 0.5	0.06 - 0.25	0.015 - 0.06	0.015 - 0.06
*Ormetoprim/Sulfadimethoxine	0.12/2.38 - 0.5/9.5	Ranges not accepted	0.06/1.19 - 0.25/4.75	0.03/0.59 - 0.25/4.75
Oxolinic Acid	0.03 - 0.12	0.03 - 0.12	0.008 - 0.03	0.008 - 0.03
Oxytetracycline	0.12 - 1	0.12 - 1	0.06 - 0.25	0.06 - 0.25
**Trimethoprim/Sulfamethoxazole	0.015/0.3 - 0.12/2.38	015/0.3 - 0.12/2.38	0.03/0.59 - 0.12/2.38	0.015/0.3 - 0.06/1.19

*First value indicates concentration of ormetoprim; second value concentration of sulfadimethoxine
**First value indicates concentration of trimethoprim; second value concentration of sulfamethoxazole

Chapter 4: Wild-type cutoff values calculated using a standardized broth microdilution susceptibility testing method for *Flavobacterium columnare*

Gieseker, C M, Crosby, T C, Woods, L C. In Preparation.

4.1 Abstract

The gliding aquatic bacterium *Flavobacterium columnare* causes columnaris disease, a common problem for wild and farmed freshwater fish worldwide. Recently, a broth microdilution method was standardized to test the susceptibility of *F. columnare* against antimicrobials commonly used in aquaculture. We used the new method to generate minimal inhibitory concentrations (MICs) for 134 *F. columnare* isolates using commercial frozen broth microdilution plates with diluted (4 g/L) cation-adjusted Mueller-Hinton broth at 28°C for 44-48 hours. We constructed MIC frequency distributions to calculate epidemiological cutoff values (ECVs) for 10 antimicrobials, which separate the wild type (WT) isolates from the non-wild type (NWT) isolates that have acquired or selected antimicrobial resistance. ECVs were calculated for ampicillin, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, ormetoprim-sulfadimethoxine, oxolinic acid, oxytetracycline, and trimethoprim-sulfamethoxazole. We observed a clear bimodal division in the MIC frequency distribution of ampicillin, florfenicol, flumequine, and oxytetracycline. The distributions of enrofloxacin, erythromycin, and oxolinic acid were right-skewed toward higher MICs suggesting the

NWT isolates overlapped the WT isolates. Twenty-two isolates with apparent resistance, as indicated by decreased drug susceptibility relative to estimated cutoff values, were found for ampicillin, enrofloxacin, erythromycin, florfenicol, flumequine, oxolinic acid, and oxytetracycline. Of these isolates, 13 had decreased susceptibility to a single drug class, 3 isolates to 2 drug classes, and 6 isolates to 3 or more drug classes. These MIC frequency distributions and provisional ECVs provide data needed to set epidemiological cutoff values to monitor for the development of drug resistance among *F. columnare*.

4.2 Introduction

Flavobacterium columnare is a gram negative gliding bacterium ubiquitous in freshwater environments that belongs to the family Flavobacteriaceae in the bacterial phylum group *Cytophaga-Flavobacterium-Bacteroides* (Bernardet et al., 2002). The bacterium causes columnaris disease in a large array of wild and cultured freshwater fish throughout the world (Wakabayaski and Egusa, 1966; Bernardet, 1989; Pulkkinen et al., 2010). Columnaris disease starts as dull whitish or yellowish patches on the gills or skin that rapidly expand (Davis, 1922). Biopsies or scrapes of the lesions show golden long, slender, flexible rod-shaped bacterial cells that aggregate into columns or piles. The disease was first discovered from freshwater fishes in the Midwestern United States by Davis (1922) followed by isolation of the bacterium by Ordal and Rucker (1944). As commercial aquaculture has grown, so have losses from *F. columnare*, for example, the bacterium is a leading disease problem in United States (U. S.) catfish (Wagner et al., 2002) and Finnish trout industries (Pulkkinen et al., 2010).

In response to columnaris disease, researchers have identified potential antimicrobial treatments by testing certain *F. columnare* isolates for their susceptibility to various antimicrobials (Wakabayashi and Egusa, 1966; Amin et al., 1988; Hawke and Thune, 1992; Soltani et al., 1995; Decostere et al., 1998; Michel et al., 2002; Thomas-Jinu and Goodwin, 2004b; Suomalainen et al., 2006; Kubilay et al., 2008; Gaunt et al., 2010). Generally, *F. columnare* have decreased susceptibility to aminoglycosides and polymyxins while they are generally susceptible to penicillins, macrolides, phenicols, tetracyclines, and nitrofurans. Against folate inhibitors, *F. columnare* range widely in their susceptibility. Unfortunately, these generalizations are based on a low number of isolates tested with a wide variety of methods that differed in media, temperature, and/or incubation conditions. Moreover, no consistent method has been used to interpret the isolates' susceptibility test results.

Recently, a broth microdilution method for antimicrobial susceptibility testing of *F. columnare* was standardized to improve the ability of veterinarians to effectively treat columnaris disease and for researchers to monitor the emergence of antimicrobial resistance (Gieseker et al., 2012). The method uses the broth dilution technique since *F. columnare* grows better in liquid media (Garnjobst, 1945). Broth dilution tests determine the minimal inhibitory concentration (MIC) of a particular antimicrobial against the targeted bacteria. Expected quality control (QC) MIC ranges were established so tests could be validated (CLSI, 2014).

Now that a standard susceptibility test exists for *F. columnare*, criteria, called epidemiological cutoff values (ECVs), are needed so that the test can be interpreted

correctly and used to monitor the development of antimicrobial resistance. The first step in setting an ECV for a broth dilution test is to construct MIC frequency distributions for each bacterium-antimicrobial combination (Turnidge and Paterson, 2007; CLSI, 2011). Analysis of the distributions, with visual inspection and/or statistics (Kronvall et al., 2003; Turnidge et al., 2006;), determines the MIC that separates the wild-type (WT) isolates from the non-wild-type (NWT) isolates with acquired or selected antimicrobial resistance mechanisms.

Our purpose here was to develop provisional ECVs using MIC frequency distributions of *F. columnare*, using the new standard broth microdilution technique. We collected over 100 *F. columnare* isolates from different locations, times, and fish species and tested them against 10 antimicrobials. These antimicrobials include drugs approved for *F. columnare* and unapproved drugs that may be used illegally or in countries with limited regulatory oversight. We then used a specialized Excel spreadsheet program (ECOFFinder) to analyze the frequency distribution to determine wild-type cutoff valves based on the methods developed by Turnidge et al. (2006).

4.3 Materials and Methods

4.3.1 Bacterial isolates and antimicrobials

One hundred and thirty-four *F. columnare* isolates were donated from laboratories in the United States, France, Netherlands, Singapore, and Belgium (Table 1). The 134 isolates represent 120 unique isolate numbers with 14 duplicate numbers that probably derived from the same bacterial clone (2 numbers repeated 3 times; 10 numbers repeated 2 times). The identity of the isolates was confirmed by polymerase chain reaction (PCR)

of the 16S rRNA gene using the methods and species-specific primers described by Darwish et al. (2004). The QC strains *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 were used to validate the antimicrobial susceptibility testing results.

4.3.2 Antimicrobial susceptibility testing

All broth microdilution tests followed CLSI guideline VET04-A2 (CLSI, 2014). The susceptibility of *F. columnare* against 10 antimicrobials was determined using commercially prepared, custom frozen broth microdilution plates (Trek Diagnostic Systems, Cleveland, Ohio). Each round bottom 96-well plate was filled with 50 µL of one drug at double the final test concentration or left empty as a positive control for cell growth. The wells were pre-filled with 10 two-fold dilutions of antimicrobials: ampicillin (0.06-32 µg/mL), enrofloxacin (0.002-1 µg/mL), erythromycin (0.5-256 µg/mL), florfenicol (0.25-128 µg/mL), flumequine (0.015-8 µg/mL), oxytetracycline (0.06-32 µg/mL), oxolinic acid (0.008-4 µg/mL), and ormetoprim/sulfadimethoxine (0.015/0.03-8/152 µg/mL), or 7 two-fold dilutions of gentamicin (0.12-8 µg/mL) and trimethoprim/sulfamethoxazole (0.015/0.3-1/19 µg/mL). The last 2 wells in the final row were left empty, filled only with the bacterial inoculum (positive control). Plates were received frozen and stored at ≤ -70° C until used. Seven rounds of testing were done to test each *F. columnare* isolate once. The QC strains *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 were tested in each round.

Prior to testing, each *F. columnare* isolate was subcultured twice in tryptone yeast extract salts (TYES) broth (Holt et al., 1993) starting from a frozen culture stored at ≤ -

70° C. Frozen *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 were also subcultured twice on tryptic soy agar plates with 5% sheep blood. Broth and agar plates were incubated at 28°C for 24 hours. A single suspension was prepared of each isolate using the second subculture. Cultures of *F. columnare* were vortex mixed and held from 1-3 minutes to allow bacterial clumps to settle. The upper, more homogeneous fraction (approximately 2 mL) was removed, and adjusted as needed with sterile saline to a standard turbidity (0.5 McFarland) using a colorimeter (Hach Company, Loveland, Colorado, USA). Separate suspensions of *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 were prepared as specified by CLSI (CLSI, 2014). Each bacterial suspension was diluted in 11 mL 4 g/L cation-adjusted Mueller-Hinton broth (CAMHB); 1:200 (*F. columnare*) and 1:100 (*E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658). Fifty µL of inoculated CAMHB was pipetted into each well, a separate plate for each isolate. Prior to sealing and incubating the plates, 10 µL of inoculum was removed from a positive control, diluted 1:1000 in sterile saline and 100 µL of the dilution was spread on TYES (*F. columnare*) or BAP agar plate (*E. coli*, *A. salmonicida* subp. *salmonicida*) to monitor for a targeted cell density of approximately 5×10^5 colony-forming units (CFU)/mL. The broth microdilution and colony count plates were incubated at 28°C for 44-48 hours. Broth microdilution plates and colony count plates were read and MICs determined as specified in CLSI VET04 (CLSI, 2014). MICs were defined as the lowest drug concentration that prevented visible growth (or a growth reduction of ≥ 80% for sulfonamides) of the bacteria.

We retested *F. columnare* isolates that had an MIC below the original dilution range tested for erythromycin (n=58) and oxytetracycline (n=35). Frozen broth microdilution 96-well plates were prepared with 11 dilutions of erythromycin (0.002-2 µg/mL) or oxytetracycline (0.002-2 µg/mL) following CLSI guidelines (CLSI, 2014) with 4 g/L CAMHB (Becton, Dickinson and Company, Sparks, Maryland). Separate plates were made for each drug.

The highest final drug concentration was prepared in broth media at twice the target concentration. To allow for subsequent 2-fold dilutions, double the volume needed to fill the first column of the 96-well plates was made in a 50 mL centrifuge tube. The highest drug concentration was then serially diluted 2-fold 11 times in the same broth media in 50 mL centrifuge tubes. Fifty microliters of the appropriate concentration was added to the appropriate column of sterile 96-well plates using a multichannel pipette. The wells in the last column (1 positive control/isolate) and the lowest well in the second to last column (negative control) were only filled with broth. Plates were stored at ≤ -70°C until used. A multichannel pipette was used to inoculate each row with a separate isolate. Multiple plates were used to test each *F. columnare* isolate once. Every plate had 6 *F. columnare* isolates and the 2 quality control bacteria.

We tabulated the MIC data by concentration into frequency distributions for each antimicrobial in a format similar to DeClercq et al. (2013) to facilitate comparison, and also calculated MIC median and geomean. Our work here and that of DeClercq et al. (2013) are the only 2 studies that have tested the susceptibility of a relatively large and

diverse isolate population of *F. columnare*. Declercq et al. (2013) used 3 g/L CAMHB, whereas we used 4 g/L (CLSI, 2014).

4.3.3 Determination of provisional ECVs

The MIC count data was entered into ECOFFinder; a specialized Excel spreadsheet program that fits a log-normal distribution to MIC frequency distributions in order to determine ECVs (Turnidge et al., 2006). ECOFFinder calculated 4 ECVs that include 95.0, 97.5, 99.0, and 99.9 % of the isolates in the susceptible population. The ECV is the highest MIC of the WT population of a bacterial species that has typical antimicrobial susceptibility. Any isolates with an MIC above the ECV presumably have developed decreased susceptibility and are termed NWT.

4.4 Results

Both QC strains, *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subp. *salmonicida* ATCC 33658, produced MICs within the approved ranges for all antimicrobials tested except gentamicin, for which no QC ranges exist (Chapter 4: Gieseker et al., 2012).

MICs of most *F. columnare* isolates were grouped together in the distribution at the lower range (Table 2). A few NWT isolates had higher MICs that skewed the distribution to the right for all drugs except gentamicin and the sulfonamides. The geomean for each drug was typically higher than the median due to the right-skewed distribution. The isolate MICs were more widely distributed for both potentiated

sulfonamides, with MICs concentrated in 4 drug concentrations that had a relatively even unimodal MIC distribution out to 5 and 7 drug concentrations.

A few isolates were clearly less susceptible to the drugs ampicillin, florfenicol, flumequine, and oxytetracycline with MICs separated from the WT population by at least 1 two-fold dilution. In comparing the different ECVs (Table 3), the 99.9% cutoff value most clearly separated the less susceptible WT isolates. The primary population overlapped the less susceptible isolates in the enrofloxacin, erythromycin, and oxolinic acid MIC distributions; therefore, we used the 99.9% cutoff value to identify the less susceptible isolates (Table 2).

Table 4 lists the NWT *F. columnare* isolates with decreased antimicrobial susceptibility. Twenty-two of the 134 isolates had elevated MICs for at least 1 of the antimicrobials. Three of these isolates exhibited decreased susceptibility to 2 drug classes and 6 isolates to 3 or more drug classes. The majority of these NWT isolates from channel catfish (50%) or koi (27%).

4.5 Discussion

We studied the antimicrobial susceptibility patterns of *F. columnare* against 10 antimicrobials with a broth microdilution method that used 4 g/L dilute cation-adjusted Mueller-Hinton broth. Our results provide MIC frequency distributions and provisional ECVs to be considered by CLSI's Subcommittee for Veterinary Antimicrobial Susceptibility Testing to set official ECVs. The MIC distributions clearly show some *F. columnare* isolates have decreased susceptibility to antimicrobials commonly used in

aquaculture. In general, our results agree with the work of Darwish et al. (2008) and DeClercq et al. (2013) who also studied the antimicrobial susceptibility patterns of *F. columnare* using similar, but non-standardized broth microdilution methods.

To our knowledge, this is the first report of *F. columnare* isolates with elevated MICs against florfenicol. These isolates were clearly separated from the majority of wild-type isolates suggesting the isolates have decreased susceptibility to florfenicol (Turnidge and Paterson, 2007). An ECV of ≤ 4 μg/mL best separated the NWT isolates from the main WT isolate group. This ECV agrees with recent reports, since all *F. columnare* tested had florfenicol MICs ≤ 2 μg/mL (Darwish et al., 2008; Declercq et al., 2013).

Declercq et al. (2013) found two *F. columnare* isolates with elevated MICs to chloramphenicol which is in the same class as florfenicol. We did not test chloramphenicol; however, our work together with DeClercq et al (2013) suggests some *F. columnare* could exhibit decreased susceptibility to both drugs. Bacteria with the enzyme chloramphenicol acetyltransferase selectively inactivates only chloramphenicol (Schwarz et al., 2004), whereas the floR gene mediates the specific efflux of both chloramphenicol and florfenicol (Butaye et al., 2003). Less specific multidrug efflux proteins also export chloramphenicol and/or florfenicol. All of our phenicol-tolerant isolates with decreased phenicol susceptibility and those reported by Declercq et al. (2013) had elevated MICs against multiple drugs suggesting a non-specific resistance mechanism that could include both phenicols; however, the mechanism needs to be investigated.

We also found multiple *F. columnare* with decreased susceptibility to ampicillin and erythromycin, with MICs above the provisional ECVs. The WT and NWT isolates were clearly separated for ampicillin, but overlapped for erythromycin. Some isolates had decreased susceptibility to only ampicillin while others included multiple drugs. All NWT isolates with respect to erythromycin also had decreased susceptibility to other drug classes. Therefore, some of these isolates appear to have a single resistance mechanism specific to beta-lactams while others have non-specific or multiple mechanisms. Although previous broth microdilution testing found *F. columnare* with elevated MICs against ampicillin or erythromycin (Darwish et al., 2008; DeClercq et al., 2013), this is the first definitive report of multiple *F. columnare* isolates from various sources with elevated MICs against these antimicrobials.

As recently reported by Declercq et al. (2013), we also observed individual isolates with decreased susceptibility to some or all of the three quinolones and/or oxytetracycline (DeClercq et al., 2013). The WT and NWT MIC distributions overlapped for enrofloxacin and oxolinic acid. The provisional ECVs split the bimodal distributions of flumequine and oxytetracycline MICs. As is common practice in setting MIC breakpoints, these provisional ECVs may need adjustment or additional testing so they can most accurately delineate the WT isolate population (Turnidge and Paterson, 2007).

We also found unimodal distributions for the aminoglycoside gentamicin and the 2 folate inhibiting combinations suggesting the isolates have not acquired resistance. These distributions are comparable to those reported by DeClercq et al. (2013) using a similar broth microdilution method. However, disk diffusion and agar dilution testing

suggests some *F. columnare* have decreased susceptibility to aminoglycosides and/or folate inhibitors (Wakabayashi and Egusa, 1966; Hawke and Thune, 1992; Soltani et al., 1995; Decostere et al., 1998; Michel et al., 2002; Thomas-Jinu and Goodwin, 2004b; Suomalainen et al., 2006; Kubilay et al., 2008). It is unclear whether these previous reports are indeed true. Experimental infections using two *F. columnare* isolates (PB-02-41 & AL-94-203) which showed decreased susceptibility (based on disk diffusion tests) to the ormetoprim/sulfadimethoxine (Romet®) were controlled by 50 mg/kg Romet® fed once daily for 15 days prior to bacterial challenge (Thomas-Jinu and Goodwin, 2004 a/b). Therefore, the particular disk diffusion method used to test isolates PB-02-41 and AL-94-203 did not appear to accurately predict resistance or, the decreased susceptibility indicated by the disk diffusion test was still within a level that the treatment could cure.

Our results confirm that some ornamental fish species are a source of NWT *F. columnare* with decreased susceptibility to multiple drugs (Declercq et al., 2013); however, channel catfish grown for food also carry these organisms. All three NWT channel catfish isolates were collected in the same year from the same region. Preliminary pulse-field gel electrophoresis with an adapted method for *F. columnare* (Soto et al., 2008) showed two of the three catfish isolates were clonal.

Our testing results and provisional ECVs provide the necessary data to allow the establishment of official ECVs for this pathogen. Although they provide a general indication of susceptibility, ECVs should not be used to guide therapy since they are based solely on *in vitro* data and do not consider *in vivo* effects. However, ECVs do define the MIC distribution of WT isolates that are integral, along with

pharmacokinetic/pharmacodynamic data and clinical outcome data, to setting clinical breakpoints (CLSI, 2008, 2011). Therefore, our results in combination with existing pharmacokinetic data (Gaunt et al., 2012, 2013), data generated on pharmacodynamics, and on treatment outcomes will help foster clinical breakpoints for *F. columnare*.

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4.7 References

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Table 4-1. *Flavobacterium columnare* isolates used for broth microdilution antimicrobial susceptibility testing.

Isolate	Host	Year Isolated	Collection Location	Source
ATCC 23463	Chinook salmon	n/a	USA	ATCC
S03-423	Channel catfish	2003	USA	Tim Santucci
S03-427	Channel catfish	2003	USA	Tim Santucci
S03-435	Channel catfish	2003	USA	Tim Santucci
S03-439	Channel catfish	2003	USA	Tim Santucci
S03-480	Channel catfish	2003	USA	Tim Santucci
S03-517	Channel catfish	2003	USA	Tim Santucci
S03-521	Channel catfish	2003	USA	Tim Santucci
S03-523	Channel catfish	2003	USA	Tim Santucci
S03-531	Channel catfish	2003	USA	Tim Santucci
S03-532	Channel catfish	2003	USA	Tim Santucci
S03-554B	Channel catfish	2003	USA	Tim Santucci
S03-559	Channel catfish	2003	USA	Tim Santucci
S03-574	Channel catfish	2003	USA	Tim Santucci
S03-578	Channel catfish	2003	USA	Tim Santucci
S03-579	Channel catfish	2003	USA	Tim Santucci
88-173	Channel catfish	1988	USA	John Hawke
94-060	Channel Catfish	1994	USA	Mark Lawrence
94-081	Channel Catfish	1994	USA	Attila Karsi
94-082	Channel catfish	1994	USA	John Hawke
94-141	Channel catfish	1994	USA	John Hawke
94-147	Channel catfish	1994	USA	John Hawke
95-132	Channel catfish	1995	USA	John Hawke
96-511	Rainbow trout	1996	Unknown	John Hawke
97-376	Hybrid striped bass	1997	USA	John Hawke
ATCC 49512	Brown trout	1987	France	ATCC
ATCC 49513	Black bullhead	1987	France	ATCC
3-5-1991	Unknown	1991	Neatherlands	Olga Haenen
CVI 08012761	Barramundi	2008	Neatherlands	Olga Haenen
CVI 07014696	Koi	2007	Neatherlands	Olga Haenen
L90-640	Channel Catfish	1990	USA	Priscilla Barger
L90-629	Channel Catfish	1990	USA	Priscilla Barger
L90-629	Channel Catfish	1990	USA	Attila Karsi
ALG 92-491-C	Channel Catfish	1992	USA	Priscilla Barger
Dickerson 1	Channel Catfish	1995	USA	Priscilla Barger
Evans	Channel Catfish	1995	USA	Mark Lawrence
143-94	Channel Catfish	1994	USA	Priscilla Barger
155-94	Channel Catfish	n/a	Unknown	Priscilla Barger
JIP 02/06 (1)	Betta	2006	Singapore	Jean-Francois Bernardet
M09-35447	Cisco	2009	USA	Hui-Min Hsu
M08-26807	Koi	2008	USA	Hui-Min Hsu
M08-26885	Bluegill	2008	USA	Hui-Min Hsu
080925-1-26fcF	Chinook salmon	2008	USA	Thomas Loch
081016-1-7fcM	Coho salmon	2008	USA	Thomas Loch
20075A	Splake	n/a	USA	Hui-Min Hsu
CVI 04017018	Koi	2004	Neatherlands	Olga Haenen
09013931	Koi	2009	Neatherlands	Olga Haenen
10009061-1	Koi	2010	Belgium	Olga Haenen
10012931	Koi	2010	Neatherlands	Olga Haenen
10012573-2	Koi	2010	Neatherlands	Olga Haenen
S06-258	Catfish	2006	USA	Pat Gaunt
S06-251	Channel catfish	2006	USA	Pat Gaunt
S06-337	Channel catfish	2006	USA	Pat Gaunt
S07-889	Catfish	2007	USA	Pat Gaunt
S07-684	Catfish	2007	USA	Pat Gaunt

S07-685	Catfish	2007	USA	Pat Gaunt
S08-520	Catfish	2008	USA	Pat Gaunt
S08-541	Catfish	2008	USA	Pat Gaunt
S08-543	Catfish	2008	USA	Pat Gaunt
S09-158	Channel catfish	2009	USA	Pat Gaunt
S09-162	Channel catfish	2009	USA	Pat Gaunt
S09-382	Channel catfish	2009	USA	Pat Gaunt
S10-239	Hybrid catfish	2010	USA	Pat Gaunt
S09-319	Catfish	2009	USA	Pat Gaunt
PB02-41	Koi	2002	USA	Andy Goodwin
n/a	Channel catfish	n/a	USA	Pat Gaunt
LDA39 H4927	Black bullhead	1998	France	Jean-Francois Bernardet
JIP 17/01	Koi	2001	France	Jean-Francois Bernardet
JIP 14/00	Neon tetra	2000	Singapore	Jean-Francois Bernardet
90-059	Channel Catfish	n/a	n/a	Mark Lawrence
1191-B	Channel Catfish	n/a	n/a	Mark Lawrence
C003133K	Channel Catfish	2003	USA	Mark Lawrence
C91-20	Channel Catfish	1991	n/a	Mark Lawrence
091006-1-9fcF	Chinook salmon	2009	USA	Thomas Loch
101007-1 5 Fc Male	Chinook salmon	2010	USA	Thomas Loch
101007-1 49 Fc	Chinook salmon	2010	USA	Thomas Loch
101012-1 55 Fc	Chinook salmon	2010	USA	Thomas Loch
111005-1 30 Fc Male	Chinook salmon	2011	USA	Thomas Loch
111025-1 34 Fc Male	Chinook salmon	2011	USA	Thomas Loch
111025-1 6 Fc Female	Chinook salmon	2011	USA	Thomas Loch
120210-1 FHM-URCF	Fathead minnow	2012	USA	Thomas Loch
90-106	Channel Catfish	1990	USA	Attila Karsi
92-002	Channel Catfish	1992	USA	Attila Karsi
94-078	Channel Catfish	1994	USA	Attila Karsi
S09-157	Channel catfish	2009	USA	Pat Gaunt
S09-177	Channel Catfish	2009	USA	Attila Karsi
S09-194	Channel Catfish	2009	USA	Attila Karsi
C-066	Channel Catfish	2010	USA	Attila Karsi
C-068	Channel Catfish	2010	USA	Attila Karsi
C-069	Channel Catfish	2010	USA	Attila Karsi
C-074	Channel Catfish	2010	USA	Attila Karsi
CB10-151	Steelhead Trout	2010	USA	Attila Karsi
S13-814	Channel catfish	2013	USA	Pat Gaunt
S07-452	Channel catfish	2007	USA	Pat Gaunt
S07-455	Channel catfish	2007	USA	Pat Gaunt
S07-457	Channel catfish	2007	USA	Pat Gaunt
S09-108	Channel catfish	2009	USA	Pat Gaunt
S09-153	Channel catfish	2009	USA	Pat Gaunt
S10-302	Channel catfish	2010	USA	Pat Gaunt
S10-502	Channel catfish	2010	USA	Pat Gaunt
S11-222	Channel catfish	2011	USA	Pat Gaunt
S11-311	Channel catfish	2011	USA	Pat Gaunt
S11-492	Channel catfish	2011	USA	Pat Gaunt
S12-598	Channel catfish	2012	USA	Pat Gaunt
S13-109	Channel catfish	2013	USA	Pat Gaunt
S13-182	Channel catfish	2013	USA	Pat Gaunt
S13-492	Channel catfish	2013	USA	Pat Gaunt
S13-517	Channel catfish	2013	USA	Pat Gaunt
S13-529	Channel catfish	2013	USA	Pat Gaunt
S13-533	Hybrid catfish	2013	USA	Pat Gaunt
S13-541	Channel catfish	2013	USA	Pat Gaunt
S13-543	Hybrid catfish	2013	USA	Pat Gaunt
S13-549	Hybrid catfish	2013	USA	Pat Gaunt
S13-554	Channel catfish	2013	USA	Pat Gaunt
S13-562	Hybrid catfish	2013	USA	Pat Gaunt
Bio-Med	Channel catfish	1996	USA	Cova Arias
Grizzle	Channel catfish	2000	USA	Cova Arias
ALM-05-53	Channel catfish	2005	USA	Cova Arias
ALM-05-39	Threadfin shad	2005	USA	Cova Arias
ALM-05-28	Blue catfish	2005	USA	Cova Arias

Table 4-2. Frequency distributions, median, geomean and number of the 134 isolates with on-scale MICs. Red numbers are the isolates with an MIC above the 99.9 % cutoff value listed in Table 3. * MIC values given are only the ormetoprim or trimethoprim concentrations. Erythromycin and oxytetracycline have wider MIC ranges due to re-testing with laboratory-prepared plates.

Antimicrobial	**No. of *F. columnare* with MIC (µg/ml)**													# in range	median	geomean	out of range	
	0	0.01	0.02	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64				<	>
Ampicilin				2	29	92				1	1	1	1			127	0.25	0.23	2 (<0.03)	6 (>16)
Enrofloxacin	1	30	86	5	1	2	1	2								128	0.015	0.015	1 (<0.001)	5 (>0.5)
Erythromycin	0	0			1	4	42	45	29	2	1	2	1		3	130	0.5	0.5		
Florfenicol							1	3	77	45	3		1	3	1	134	1	1.45		
Flumequine				2	104	14	2		5	1	1					129	0.06	0.08		5 (>4)
Gentamicin							1	16	49	40	24					130	1	1.46		6 (>4)
Oxolinic acid			1	27	86	3	4	2	2	2						125	0.12	0.12		7 (>2)
Oxytetracycline			3	106	8	2		2	1	2	1	1				126	0.06	0.08		5 (>16)
Sulfadimethoxine / ormetoprim*			1	2	17	43	46	21	4							134	0.5	0.37		
Sulfamethoxazole / trimethoprim*			1	21	47	34	29									132	0.12	0.18	1 (<0.008)	1 (>0.5)

Table 4-3. ECVs estimated with the program COFinder (Turnidge et al., 2006) using the frequency distributions in Table 2.

	Estimated ECVs based on percent of data covered			
Antimicrobial	95.0%	97.5%	99.0%	99.9%
Ampicilin	0.25	0.25	0.25	0.25
Enrofloxacin	0.03	0.03	0.03	0.03
Erythromycin	1	2	2	4
Florfenicol	2	2	2	4
Flumequine	0.12	0.12	0.12	0.12
Gentamicin	4	8	8	16
Oxolinic acid	0.25	0.25	0.25	0.25
Oxytetracycline	0.12	0.12	0.12	0.12
Sulfadimethoxine / ormetoprim	1	2	2	4
Sulfamethoxazole / trimethoprim	0.5	1	1	2

Table 4-4. NWT *Flavobacterium columnare* isolates with decreased antimicrobial susceptibility based on provisional ECVs.

Isolate Number	Fish Host	Origin	Year Isolated	Phenotype
S03-554B	Catfish	United States	2003	Amp, Enro, Ery, Flor, Flum, Otc, Oxo
S03-559	Catfish	United States	2003	Amp, Enro, Ery, Flor, Flum, Otc, Oxo
CVI 07014696	Koi	Netherlands	2007	Amp, Enro, Ery, Flor, Flum, Otc, Oxo
3-5-1991	Unknown	Netherlands	1991	Amp, Enro, Ery, Flor, Flum, Oxo
S03-517	Catfish	United States	2003	Amp, Ery, Flor, Flum, Otc, Oxo
JIP 14/00	Neon Tetra	Singapore	2000	Amp, Enro, Flum, Otc, Oxo
CVI 08012761	Barramundi	Netherlands	2008	Enro, Flum, Otc, Oxo
10009061-1	Koi	Belgium	2010	Enro, Flum, Otc, Oxo
10012931	Koi	Netherlands	2010	Enro, Flum, Otc, Oxo
S03-480	Catfish	United States	2003	Enro, Flum, Oxo
09013931	Koi	Netherlands	2009	Enro, Flum, Oxo
S13-814	Channel Catfish	United States	2013	Enro, Oxo
S07-455	Channel Catfish	United States	2007	Flum, Oxo
95-132	Catfish	United States	1995	Amp
S09-194	Channel Catfish	United States	2009	Amp
111025-1 6 Fc Female	Chinook salmon	United States	2011	Flum
Dickerson 1	Channel Catfish	United States	1995	Flum
CVI 04017018	Koi	Netherlands	2004	Otc
M09-35447	Cisco	United States	2009	Otc
JIP 17/01	Koi	France	2001	Otc
155-94	Channel Catfish	Unknown	1994	Oxo
ALM-05-28	Blue catfish	United States	2005	Oxo

Amp = ampicillin, Enro = enrofloxacin, Ery = erythromycin, Flor = florfenicol, Flum = flumequine; Otc = oxytetracycline, Oxo = oxolinic acid.

Chapter 5: Development of a columnaris disease model and comparison of infectivity among *F. columnare* isolates that differ in their florfenicol susceptibility.

Gieseker, C M, Hasbrouck, N R, Evans, E R, Crosby, T C, Stine, C B, Rodriguez, L R, Reimschuessel, R, Woods, L C. In Preparation.

5.1 Abstract

We exposed channel catfish *Ictalurus punctatus* to *Flavobacterium columnare* to develop a laboratory model of columnaris disease. Fish were immersed in a static water bath of approximately 10^6 colony-forming units of *F. columnare* for 2 or 4 hours at 28 °C. Afterwards, the fish were held in observation tanks with a constant water flow at 26 °C where the fish were fed and observed for disease signs. An initial experiment compared the ability of 3 abrasion techniques to induce infections. We induced consistent infections without abrasion, using only crowding and bath immersion. Additional experiments compared the effect of different exposure times, fish densities, bacterial concentrations, temperatures and ammonia levels in the bath immersion on the onset and intensity of infection. Shorter exposure times reduced the intensity of the infections but did not slow onset. Lowering the bacterial exposure delayed onset slightly; however, lowering the density had no effect. Neither exposing at 26 °C instead of 28 °C nor binding ammonia slowed onset or reduced intensity of the infections. Once optimized, we used the model to compare the ability of 6 different *F. columnare* isolates to induce infections. Five of the six isolates induced a cumulative percent mortality ≥ 87%. The

remaining isolate did not induce any infection. The model and virulent *F. columnare* isolates provide materials and methods needed to study whether *in vitro* florfenicol susceptibility can accurately predict if an approved florfenicol dose can control the disease.

5.2 Introduction

Death due to columnaris disease caused by the nutritionally fastidious gliding bacterium *Flavobacterium columnare* is a major problem in many freshwater aquaculture industries (Davis, 1922; Fish and Rucker, 1945; Bernardet, 1989). In the United States (U.S.), columnaris disease is a leading pathogen in the channel catfish industry and is emerging in the rainbow trout industry (Wagner et al., 2002; Evenhuis et al., 2014). The importance of the disease to the U.S. industry motivated the development of drugs for this disease that resulted in the recent approval of the antimicrobials florfenicol (Aquaflor®) and oxytetracycline (Terramycin®) to control mortality in freshwater-reared fish (Federal Register, 2007, 2008, 2012). Because of these approvals and concern about aquaculture's role in antimicrobial resistance following antimicrobial use (FAO/OIE/WHO, 2006), a testing procedure was standardized to determine the antimicrobial susceptibility of *F. columnare* (Chapter 4: Gieseker et al., 2012). With a standard susceptibility testing protocol, all laboratories can use the same methods to monitor the development of antimicrobial resistance or to recommend antimicrobials for treatment.

In order to appropriately use susceptibility test results when prescribing treatment, veterinarians need clinical breakpoints. These breakpoints are particular drug

concentration cutoffs that define categories which predict the likely outcome of an approved antimicrobial treatment. Setting clinical breakpoints requires field effectiveness and susceptibility testing data that correlate the antimicrobial susceptibility of the bacterial isolate causing the infection with the treatment outcome. Unfortunately, limited field effectiveness data is available for columnaris disease. However, a laboratory study on the effectiveness of florfenicol to control experimentally induced *F. columnare* infections generated substantial evidence for the recent approval of Aquaflor® in the U.S. (Gaunt et al., 2010).

A model similar to that used by Gaunt et al. (2010) would be a good way to enhance the effectiveness data needed to set clinical breakpoints. The model induced *F. columnare* infections by crowding catfish in a bath exposure to a single infective isolate of *F. columnare*. About half of the untreated fish developed infections and died mostly 3 to 5 days after exposure to the bacterium. Another columnaris disease model causes a similar time course of mortality, but with lower survival ($\leq$ 20 %) since they abraded the catfish prior to exposure (Darwish and Mitchell, 2009; Darwish et al., 2009). When compared, abrasion clearly lowers survival of catfish exposed to *F. columnare*; however, significant mortality was still induced without abrasion with a time course within 2 days (Bader et al., 2003, 2006). Abrasion or mucus removal also makes channel catfish more vulnerable to infection by different isolates (Soto et al., 2008). Therefore an abrasion may be necessary to get consistent infections between different *F. columnare* isolates to study the ability of the approved florfenicol treatment to control infections caused by isolates with susceptibility near potential breakpoints.

This report describes 6 experiments to develop a columnaris disease model in our laboratory. We used methods similar to Gaunt et al. (2010) to compare 3 types of abrasion techniques to induce infections. We also tested the effect of different exposure duration, fish density, bacterial concentration, temperature and ammonia level in the bath immersion on the onset and intensity of infection. Lastly, we compared the ability of 6 different *F. columnare* isolates to induce infections under the model's conditions. The isolates varied in their susceptibility to the antimicrobial florfenicol.

5.3 Materials & Methods

5.3.1 Bacteria and culture conditions

Table 1 lists the *F. columnare* isolate used in experiments (1-4) to develop the disease model and in the disease induction experiments (5-6). All experiments to develop a *F. columnare* infection model in our laboratory used a single *F. columnare* isolate (Postive control) supplied by Dr. Patricia Gaunt (Mississippi State University). Gaunt et al. (2010) used this isolate to study the effectiveness of florfenicol (Aquaflor®, Merck Animal Health) to control *F. columnare* infections induced in channel catfish, *Ictalurus punctatus*. Our experiments to compare the ability of different *F. columnare* isolates to induce disease were supplied by Dr. Mark Lawrence and Dr. Tim Santucci (Mississippi State University). The 3 *F. columnare* isolates with typical wild type susceptibility have been compared to other *F. columnare* isolates in laboratory challenges of juvenile fingerling channel catfish (Soto et al., 2008). Isolate WT-3 induced low mortality and grouped with other non-virulent isolates based on pulsed-field gel electrophoresis

(PFGE). Isolate WT-1 and WT-2 cause high mortality and grouped with other virulent isolates.

The identity of the isolates was confirmed by PCR of the 16S rRNA gene using the methods and species-specific primers described by Darwish et al. (2004). Prior to each disease model or infection induction experiment, each *F. columnare* isolate was subcultured twice in Shieh broth (Shieh, 1980) starting from a frozen culture kept at ≤ -70 °C in 20% glycerol. The second subculture was used to make suspensions adjusted with sterile saline to a 1.0 McFarland turbidity measured with a colorimeter (Hach Company, Loveland, Colorado, U.S.). Fifteen milliliters of the suspension was inoculated into sterile flasks containing 2 L of Shieh broth. The flasks were incubated at 28°C for approximately 72 hours while stirring at 100 rpms. Afterwards, the flasks were stirred and amount of inoculum specified by the experiment (see below) removed. While still stirring, 1 mL of broth was removed from the flasks and diluted ten-fold seven times (10^{-1} to 10^{-7}) in sterile saline. The 10^{-5} to 10^{-7} dilutions (100 µL) were spread on separate Shieh agar plates incubated at 28°C and colonies counted after 44-48 hours.

5.3.2 Fish and rearing conditions

Channel catfish (3-4 inch) were purchased from a commercial catfish farm (Osage Beach, Missouri) for experiments 1 to 3 and 2-3 inch channel catfish were obtained from the USDA Catfish Genetics Unit (Stoneville, Mississippi) for experiments 4 to 6. Gross necropsy, skin scrapes, gill biopsies and histopathology were conducted on 5 fish to assess their health status at the time of arrival. Afterwards the catfish were reared in our laboratory under standard procedures in 240 L aquaria constantly supplied with

supplemental aeration and 26±1 °C conditioned well water flowing at approximately 1-2 L/min. The fish were fed commercial catfish feed (Zeigler Bros., Gardners, Pennsylvania) at 2% body weight once daily 5 days/week and grown to approximately 20 g prior to each experiment.

All of the experiments used aquaria supplied with conditioned freshwater from a 50 gallon head tank and supplemental aeration. A digital temperature controller (Love Controls®, Michigan City, Indiana) maintained the temperature in the head tank. The aquarium systems consisted of four 240 L (60 gallon) tanks or eight 80 L (20 gallon) tanks. The 80L aquarium systems housed the fish prior to the exposure for acclimation and after the exposure for observation. Water flowed at 26±1 °C at a rate of approximately 500 mL/min.

The bath exposure tanks were individual 240 L or 80 L aquaria located in a separate systems from those used for acclimation and observation. Each tank was divided into 3 equal compartments with a slotted acrylic divider that allows the compartments to share water. Water flowed into the tank at approximately 28 °C. After all of the fish were moved into the exposure tank, the water flow was stopped and temperature maintained with 100 or 200 watt aquarium heaters (Aquarium Systems, Sarrebourg, France). Each exposure started when the water flow was stopped, water level lowered, heater turned on and broth inoculum added.

A water quality monitoring system continuously recorded the pH, DO and temperature in representative 240 L and 80 L tanks. Water samples collected from the exposure tanks and from the observation tanks were tested for ammonia and nitrite using

the Nessler and Diazotization spectrophotometric methods, respectively (Hach Inc., Loveland, Colorado). Water samples were taken from the exposure tanks both before and after exposure.

5.3.3 Acclimation

Fish were placed in the observation tanks at least 2 weeks prior to each disease model or disease induction experiment to acclimate to the tank conditions. Groups of 10 fish were moved from the holding tanks into the observations tanks. The fish were fed commercial catfish feed (Cargill, Inc., Franklinton, Louisiana or, Zeigler Bros., Gardners, Pennsylvania) at 2% body weight once daily 7 days/week. A commercial laboratory found no detectable levels (limit of detection) of the antimicrobial oxytetracycline (< 5.68 ppm) in the Cargill feed (Central Analytical Laboratories, Eurofins Scientific, Metairie, Louisiana). No detectable levels of the antimicrobials florfenicol (< 0.001 ppm), chloramphenicol (<0.0003 ppm), thiamphenicol (< 0.001 ppm), oxytetracycline (< 0.1 ppm), sulfadimethoxine (< 0.01 ppm) and ormetoprim (< 0.01 ppm) were found in the Zeigler feed. The same lot of feed used during acclimation was used in the corresponding experiment.

5.3.4 Development of the disease model

5.3.4.1 Experiment 1: comparison of abrasion techniques

We compared the effect of 3 abrasion techniques on inducing *F. columnare* infections on channel catfish using methods similar to Gaunt et al. (2010). This experiment used 90 fish acclimated in nine 80 L observation tanks (10 fish/tank). The observation tanks were randomly assigned to an abrasion technique (3 tanks/technique).

Working with 1 observation tank at a time, the fish were removed and anaesthetized individually in 1 g tricaine methanesulfonate (Western Chemical, Ferndale, Washington) in approximately 6 L tank water (~20 ppt). Once sedated, the fish were either abraded with a motorized tool (Gieseker et al., 2006), dental cotton (Bader et al., 2003) or not abraded (Gaunt et al., 2010). Fish abraded with the motorized tool were lightly scraped similar to a close shave above the lateral line between the posterior margin of the dorsal fin and the anterior margin of the adipose fin. The abrasion width was approximately 1 cm wide from two passes from back to front, and approximately 3 to 4 cm in length following the procedure. Fish abraded with sterile dental cotton were lightly rubbed in the same area above the lateral line as with the tool abrasion. The cotton was passed twice back to front using the end of the dental cotton. The cotton was turned over between passes. The fish (including those not abraded) were allowed to recover from anesthesia (in fresh water) before being placed in the exposure tank. To minimize variations in the procedure, only one person performed all of the abrasions.

All of the fish were exposed in one 240 L exposure tank in a separate compartment for each abrasion technique at 28 °C. After adding all of the fish, the water was lowered to 60 L and sampled (5 mL) for a bacterial count. 300 mL of an approximately 72 hour *F. columnare* broth culture was added spread evenly across the 3 compartments. The exposure ended promptly after 4 hours by moving the fish into 9 observation tanks (10 fish/tank) at 26 °C randomized between the treatments (1 treatment/tank). Immediately after moving the fish, the exposure tank water was sampled (5 mL) again for another bacterial count. The abrasions and exposures created the following treatment groups:

Group 1.1: no abrasion, 4 hour exposure,

Group 1.2: tool abrasion, 4 hour exposure, and

Group 1.3: cotton abrasion, 4 hour exposure.

The bacterial count water samples were diluted tenfold (10^{-1} to 10^{-4}) in sterile saline. One hundred µL of the 10^{-3} and 10^{-4} dilutions were spread on separate Shieh agar plates incubated at 28°C and colonies counted after 44-48 hours. The fish were observed, fed and necropsied as described below for 12 days. On the final day all remaining fish were sacrificed and necropsied.

5.3.4.2 Experiment 2: 2 versus 4 hour exposure

The first experiment induced a high level of infection and found an abrasion was not necessary to induce consistent infections. In this experiment the same methods without an abrasion were used to compare between exposing the fish for 2 or 4 hours. We also replicated the 4 hour exposure using an 80L exposure tank to test if the model could be scaled down.

This experiment used 90 fish acclimated in nine 80 L observation tanks (10 fish/tank). After acclimation, 60 fish were moved from the observation tanks into a 240 L exposure aquarium (30 fish in 2 of 3 compartments). Another 30 fish were moved into an 80L exposure aquarium (10 fish/compartment). All of the fish added to each compartment were randomly selected from the 9 observation tanks. To start the

exposures, we lowered the water in exposure tanks, sampled the water for a pre-exposure bacterial count (as in Experiment 1) and added the *F. columnare* broth culture:

240 L aquaria: 40 L water, 200 mL broth and

80 L aquaria: 20 L water, 100 mL broth.

After 2 hours at 28 °C, 30 fish were removed from 1 compartment in the 240 L exposure tank and placed into 3 randomly assigned observation tanks (10 fish/tank) at 26 °C. At 4 hours the remaining fish in the 240 L exposure tank were moved to another 3 observation tanks (10 fish/tank) and water sampled for a post-exposure bacterial count. The fish in the 80 L exposure were also moved to 3 additional observation tanks after 4 hours. The exposures created the following treatment groups:

Group 2.1: 240 L aquaria, 2 hour exposure,

Group 2.2: 240 L aquaria, 4 hour exposure, and

Group 2.3: 80 L aquaria, 4 hour exposure.

The fish were observed, fed, and necropsied as described for 10 days. All remaining fish were sacrificed and necropsied on the final day.

5.3.4.3 Experiment 3: Variance in fish density and bacterial concentration

This experiment tested if reducing the fish density and/ or bacterial concentration in the bath exposure tank would slow the onset of infection to allow more time to feed the

fish in future experiments. We exposed for 4 hours since a 2 hour exposure (experiment 2) induced mortality below our target level.

The experiment used 90 fish divided into 2 trials. After acclimation, the first trial exposed 30 fish (30 fish/treatment) with reduced density in the exposure (Group 3.1) and 30 fish with reduced bacteria (Group 3.2). The second trial exposed 30 fish with reduced density and reduced bacteria (Group 3.3). The fish were randomly moved into 80 L exposure tanks (10 fish/compartment), 1 for each treatment group. After all fish were moved, we lowered the water level, sampled the water for pre-exposure bacterial count and *F. columnare* broth culture added in the following volumes:

Group 3.1: 30 L water, 150 mL *F. columnare* culture,

Group 3.2: 20 L water, 50 mL *F. columnare* culture, and

Group 3.3: 30L water, 75 mL *F. columnare* culture.

After 4 hours at 28 °C, 10 fish in each exposure tank compartment were randomly moved into 3 corresponding observation tanks at 26 °C, and water sampled for a post-exposure bacterial count. The fish were observed, fed, and necropsied as described (Group 3.1 and 3.2: 11 days; Group 3.3: 9 days). On the final the day the remaining fish were necropsied.

5.3.4.4 Experiment 4: Effect of binding ammonia and/or removing temperature stress to slow the onset of infection

Total ammonia levels increased dramatically during the static bath exposures. The unionized ammonia levels rapidly rose to near critical levels presumably creating stress. This experiment tested if removing ammonia and/or reducing the slight temperature increase during the exposure would slow the onset of the infection.

Catfish were bath exposed to *F. columnare* (isolate 36490) for 2 hours in an 80 L aquarium as described in order to test if using an ammonia detoxifying solution used in aquariums will 1) reduce the ammonia level and 2) slow the onset of the infection. In addition, we also investigated if removing the slight temperature increase during the exposure would slow the infection with or without the ammonia detoxifying solution. We used a 2 hour exposure to be in-line with the methods used in experiments 5 and 6.

104 fish were acclimated for 2 weeks. To begin exposure, the fish were randomly moved into five 80-L exposure tanks. After all fish were moved, the water level was lowered to 24 L (12 L for the negative control fish), tank heaters turned on, water samples collected for a bacterial count and water quality, and 7.9 mL of ammonia detoxifying solution (Ammo-Lock®) added as assigned. The treatment groups were as follows:

Group 4.1: 8 fish, Temperature increase (28 °C water) and no ammonia reducer (negative control – not exposed to *F. columnare*);

Group 4.2: 24 fish, Temperature increase (28 °C water) and no ammonia reducer (positive control – same as previous exposures);

Group 4.3: 24 fish, Temperature increase (28 °C water) and ammonia reducer;

Group 4.4: 24 fish, No temperature increase (26 °C water) and ammonia reducer;

Group 4.5: 24 fish, No temperature increase (26 °C water) and no ammonia reducer.

To begin the exposure, 180 mL of a 72-hour *F. columnare* broth culture was added to each exposure tank. 60 mL of un-inoculated broth media was added to the negative control tank. After 2 hours, the fish in the negative control tank were moved into 1 observation tank at 26 °C and the fish in the other exposure tanks were randomly moved into 3 corresponding observation tanks (9 fish/tank, n=12) also at 26 °C. After moving the fish, we sampled the exposure tank water for bacteria counts and to test water quality. Water samples collected before and after exposures were analyzed for ammonia with the indophenol method in addition to Nessler method as stated above (Hach Inc., Loveland, Colorado). The fish were observed, fed, and necropsied for 4 days. On the final the day, the remaining fish were necropsied.

5.3.4.5 Experiments 5 and 6: Disease induced by F. columnare that vary in their sensitivity to florfenicol

We used the disease model to compare the ability of 6 *F. columnare* isolates to induce infections with a 2 hour bath exposure. We chose the shorter exposure to possibly improve observing mortality differences between the positive control and the test isolates. Two experiments were performed; Experiment 5 tested 3 *F. columnare* isolates with typical wild type susceptibility to the antimicrobial florfenicol based on laboratory testing and Experiment 6 tested 3 *F. columnare* isolates with decrease non-wild type susceptibility to florfenicol.

Each experiment used 150 fish (300 fish total). The experiments only differed in the *F. columnare* isolates tested. After acclimating 2 weeks, 30 fish were moved into the first of five 80 L exposure tanks; 2 fish from each of the 15 observation tanks. After these fish were moved, the water level was lowered to 20 L, pre-exposure water samples collected for a bacterial count and water quality, tank heaters turned on, and 150 mL of broth culture added. Preliminary experiments determined that 150 mL of broth culture best approximated the target cell concentration of 10^6 CFU/mL across all of the isolates. The remaining 4 groups of 30 fish were moved into corresponding exposure tanks. Movements and exposures were staggered by 20 minutes to allow time for fish movement after each exposure. The 5 treatment groups were as follows:

Experiment 5

Group 5.1: un-inoculated broth as a negative control,

Group 5.2: Positive control isolate,

Group 5.3: *F. columnare* isolate WT-1,

Group 5.4: *F. columnare* test isolate WT-2, and

Group 5.5: *F. columnare* test isolate WT-3.

Experiment 6

Group 6.1: un-inoculated broth as a negative control,

Group 6.2: Positive control isolate,

Group 6.3: *F. columnare* isolate NWT-1,

Group 6.4: *F. columnare* test isolate NWT-2, and

Group 6.5: *F. columnare* test isolate NWT-3.

A completely randomized design was used with 3 observation tanks for each of the 5 exposure scenarios (negative control, positive control and 3 test isolates). A table of random assignments was generated, with the restriction that no more than 2 replicates of each exposure in 1 of the 3 flow-through aquarium systems. Unblinded collaborators assigned the randomization and moved the fish from the exposure to observation tanks. The unblinded collaborators did not participate in observations following exposure.

After a 2 hour static bath exposure at 28 °C, the fish were removed in a rotating sequence from each compartment in the exposure tank in groups of 10 with a bucket filled with water from the receiving observation tank. One exposure tank was moved at a time into the 3 corresponding observation tanks (10 fish/tank) at 26 °C. The water in the exposure tanks was sampled collected for a bacterial count and water quality immediately after moving the fish. The fish were observed, fed, and necropsied as described for 14 days. On the final day the remaining fish were necropsied. The bacterial counts were performed as described in Experiment 1. The water quality samples tested for ammonia and nitrite using spectrophotometric methods (Hach Inc., Loveland, Colorado).

5.3.5 Observations/Feeding/Necropsy

The fish were observed in the morning (prior to feeding) and afternoon for brown to yellowish lesions typical of columnaris disease (Durborow, 1998) on the skin, gills

and/or fins. We set criteria to determine if a fish was moribund during the course of the experiments to develop the disease model. Moribund fish were defined as having met 2 of 3 criteria: 1) lethargy, 2) listing unable to maintain vertical position and 3) have significant skin lesions. Dead fish were collected and moribund fish removed and sacrificed with an overdose of tricaine methanesulfonate (500-1000 ppm).

In Experiments 1-3, the fish were fasted the day of the exposure and subsequently fed commercial catfish feed (Cargill, Inc., Franklinton, Louisiana) at 2% body weight once daily 7 days/week until the end of the experiment. In Experiment 3, the feed was re-pelleted at described below. The last group in Experiment 3 (Group 3.3) was offered feed 4 hours after exposure to test if medicated feed could be given at an earlier time point. All fish in Experiments 4-6 were fed re-pelleted commercial catfish feed (Zeigler Bros., Gardners, Pennsylvania) at 2% body weight 4 hours after exposure and subsequently fed once daily 7 days/week until the final day of the experiment. The re-pelleted feed was pulverized, 20% filtered water added, re-pelleted with a laboratory pellet mill (California Pellet Mill, Crawfordsville, Indiana), and dried at 32 °C for 4 hours. The re-pelleted feed was developed for a future drug effectiveness trial that will use the disease model described here. The feed amounts were adjusted based on the number of fish remaining in the tank. Feed consumption was assessed as 0, 25, 50, 75 or 100 percent approximately 15 min. after feeding.

Moribund and dead fish were necropsied and sampled for microbiology to confirm infections. Each necropsy noted the date of collection, tank number, weight and fork length. Wet preparations of skin lesions (or normal skin if no lesions are present) and

a gill biopsy were analyzed. Microbiology samples were taken from skin (just anterior to the dorsal fin or lesion if present), posterior kidney and spleen. Samples were cultured on Shieh agar (Shieh, 1980) and incubated at 28±1°C for approximately 48 hours. Yellow, rhizoid colonies strongly adhered to the agar were considered *F. columnare*. Representative *F. columnare* were frozen at -80°C. Some non-*F. columnare* isolates were identified using the Biolog® system (Biolog, Inc., Hayward, California). Skin, gill, liver, spleen, and posterior kidney samples were collected for histopathology from representative fish, fixed in 10 % neutral buffered formalin, and stained with hematoxylin and eosin. Select tissues were special stained with the methods Gram, Periodic Acid Schiff (PAS), and Grocott's Methenamine Silver (GMS) to better visual the bacteria and confirm a fungal infection of the eye.

5.3.6 Calculations and Statistics

Each sacrificed moribund fish was considered a death in order to calculate cumulative percent mortality for each experimental group. Fish left in the tanks because they minimally meant our moribund criteria always died by the next observation period (≤ 12 hours). The cumulative percent mortality at Day 14 post-exposure to each isolate was modeled for the disease induction experiments using a generalized linear model (GLIMMIX) procedure available in SAS assuming a binomial distribution and logit link. The model included isolate as a fixed effect. The estimated mean cumulative percent mortality for each disease induction experiment was calculated for each isolate along with 95% confidence intervals.

The experimental protocol was approved by the Animal Care and Use Committee at the Office of Research, Center for Veterinary Medicine, U.S. Food and Drug Administration, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (2011) and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

5.4 Results

Table 2 lists the average weight and length of the fish, exposure duration, and *F. columnare* concentration at the end of the exposure for all of the experiments. The size of the fish was kept consistent by using different batches of fish for each experiment. The disease induction trials used the same batch of fish therefore the fish were larger in the second trial. Table 3 lists the ammonia and nitrite levels in the exposure and observation tanks during experiments to develop the disease model (Experiments 1-3) and to compare how different isolates induce disease (Experiments 5-6). Table 4 lists the ammonia and nitrate levels in Experiment 4 that tested if removing the temperature and/or ammonia stress would slow the onset of infection.

5.4.1 Development of the disease model

5.4.1.1 Experiment 1: comparison of abrasion techniques

The cumulative percent mortality (CPM) was consistent between the 3 abrasion techniques (Fig. 1). The abrasion with the motorized tool rapidly caused all of the fish to be moribund or dead within 1 day post-challenge (dpc). However, the cotton abrasion and non-abrasion also caused a similar level of moribund or dead fish by 2 dpc. The skin

lesions were associated with the motorized tool abrasion but not the dental cotton abrasion. The skin lesions observed on the fish abraded with dental cotton or fish not abraded were often associated with fins although many were randomly located.

5.4.1.2 Experiment 2: 2 versus 4 hour exposure

Reducing the exposure time to 2 hours only slowed the onset of infection slightly but the CPM was lower compared to the 4 hour exposure (43% and 70 % respectively for exposures using a 240 L tank). When scaled down with less fish and a smaller 80 L exposure tank, the infection onset and CPM was similar to the larger exposure conditions (Fig. 2). The fish became moribund or died mostly 1-2 dpc when exposed for 4 hours and 2 dpc when exposed 2 hours.

5.4.1.3 Experiment 3: Variance in fish density or bacterial concentration, or both

Challenging the fish with fewer bacteria delayed the onset of disease signs slightly but not the CPM (Fig. 3). A majority of the fish did not appear moribund 1 dpc but died overnight. Lowering the fish density in the challenge bath did not delay the disease onset. When combined, less fish density and fewer bacteria also did not delay the disease.

5.4.1.4 Experiment 4: Effect of binding ammonia and/or removing temperature stress on the onset of infection

Increasing the temperature slightly to 28 °C during the exposure resulted in a lower CPM compared with exposures at 26 °C (54% versus 75 %, respectively; Fig. 4).

The ammonia binding solution did not lower the CPM either with or without the temperature increase during the exposure. Onset of infection was slightly delayed by holding fish at 28 °C without binding ammonia.

5.4.1.5 Experiments 5 and 6: Disease induced by F. columnare that vary in their sensitivity to florfenicol

One isolate (WT-3) with typical (wild type) florfenicol susceptibility did not induce infection (0% CPM). The other 5 *F. columnare* isolates (with typical or decreased florfenicol susceptibility) induced infections statistically similar to the positive control isolate used to develop the disease model. Table 5 lists the estimated CPM and 95% confidence intervals for each treatment group. All of the infectious isolates and the positive control induced a CPM ≥ 87 % (Fig. 5 and 6).

5.4.2 Gross, Microbiological and Histological Observations

Gross observations. Most of the moribund and dead fish examined had long filamentous bacteria on their gills and/or skin. These bacteria were often aggregated into columns that had a haystack appearance diagnostic of *F. columnare* (Fig. 7A). Skin lesions were often dull circular areas with a thin red margin on the circumference (Fig. 7B). Aside from lesions formed on the motorized tool abrasion in Experiment 1, the skin lesions typically originated at the base of the fins, but were also randomly located on the body surface. None of the surviving fish had lesions.

Microbiological observations. *Flavobacterium columnare* were isolated from skin, posterior kidney, and spleen of most moribund or dead fish (Table 6). The

occurrence of *F. columnare* on the skin of survivors varied between the experiments. The bacterium remained on the skin of survivors in Experiments 1 -3 (50, 100, and 75 %, respectively), but was not isolated in Experiments 5 and 6. Internal bacteria were only isolated from the kidney and spleen in Experiment 2.

We also consistently isolated cream-colored round colonies (not *F. columnare*) on Shieh media with smooth margins from the skin, kidney, and spleen of the fish in the disease induction experiments. This colony was also noted in earlier experiments but not consistently recorded. The colony was equally prevalent on the skin of moribund and surviving fish suggesting the bacteria were part of the normal skin flora. Representative colonies identified as *Aeromonas hydrophila, A. veronii, A. bestiarum, A. sobria* and *Plesiomonas shigelloides*. Internally these bacteria were more prevalent in the moribund fish versus the survivors, suggesting the bacteria entered following *F. columnare*.

Histological observations. The moribund fish had focal skin and gill lesions. On the skin, the epidermis was gone with bacteria in the dermal connective tissue causing necrosis and edema (Fig. 8A). More advanced lesions penetrated through the dermis into the muscle causing focal necrosis and mild inflammation. Some fish, usually near death or already dead, had more advanced lesions encompassing large areas of necrotic skin and muscle. A Gram stain clearly demonstrated the bacteria in the dense connective tissue layer of the dermis and in the fascia of the muscle of the advanced lesions (Fig. 8B).

Gill lesions ranged from mild hypertrophy of the gill lamellae to severe hyperplasia of lamellae with focal areas of necrosis (Fig. 8C). A Gram stain showed the

bacteria in the connective tissue of the gill arch. On occasion, the bacteria also infected the eye eroding the epidermis of the cornea and caused necrosis, edema, inflammation in the connective tissue and choroid rete (Fig. 8D). Some eyes were also co-infected with fungus as confirmed with PAS and GMS stains.

Internally, in moribund fish, the lymphatic tissue of the splenic white pulp was depleted assumedly because lymphocytes were being mobilized into the blood (Fig. 9A). Many inflammatory cells, particularly monocytes, were in the blood vessels. Some of the vessels were also severely dilated in the spleen, liver and posterior kidney (Fig. 9B). A Gram stain of the internal organs did not show any bacteria.

Angular cyst-like pockets with clear lumens were also seen in the spleen in a few moribund and surviving fish (Fig. 9C). The tissue surrounding these pockets appeared to be compressed somatic tissue. In a few cases the pocket was filled with non-descript material perhaps of a parasite or dying somatic tissue (Fig. 9D). Except for the cyst-like pockets, all of the tissues from the surviving fish appeared normal.

5.5 Discussion

We successfully developed a model of columnaris disease in channel catfish. The experimental infections caused a rapid onset of disease following a 2 or 4 hour bath exposure to a known infective isolate of *F. columnare*. As intended, the model is very similar to the columnaris disease model used to generate evidence for the recent U.S. approval of Aquaflor® to control mortality in channel catfish (Gaunt et al., 2010). Similar to Gaunt et al. (2010), only crowding and exposure to the bacterium in a static

bath was needed to induce infection. Although both models were developed with the same *F. columnare* isolate at a similar concentration and exposure duration, the models differed in intensity and onset of infections. Our model had a faster onset with a higher percent mortality. The primary differences between the models were the hardness of the water, the fish, and the temperature in the exposure bath.

We used filtered well water which was relatively soft (30 ppm as CaCO3) as opposed to the unfiltered pond water used by Gaunt et al. (2010) which had about 10 times more hardness. Gaunt et al. could not induce *F. columnare* infections in channel catfish using their well water which had hardness similar to their pond water but less organic matter (Gaunt, personal communication). High water hardness and organic matter enhances the survival of *F. columnare* (Fijan, 1968). However, *F. columnare* grows relatively well in a wide range of water hardness including the moderately soft water used here (Chowdury and Wakabayashi, 1988; Cai et al., 2013). Of the salts that contribute to water hardness, *F. columnare* is most sensitive to calcium at high levels (200 to 2000 ppm); however, no threshold was determined from 20 to 200 ppm (Chowdury and Wakabayashi, 1988). More recent research reports similar growth in 12 to 360 ppm calcium salts but significantly more biofilm at 360 ppm (Cai et al., 2013). Therefore, calcium could contribute to the difference in the onset and intensity of the infections between the models. High calcium levels in the water used by Gaunt et al. (2010) could limit growth of the bacterium requiring organic matter and/or cause biofilms to form on tank surfaces therefore slowing attachment and growth on the fish.

Our model uses larger catfish (~20 to 40 g) than Gaunt et al. (~8 g), although we maintained a similar density in the exposure and observation tanks. Another *F. columnare* infection model induced a similar rate and intensity of infection without abrasion as we describe with fish of similar size (~30 g; Bader et al., 2006). However, the model used a different *F. columnare* isolate at a higher dose (10^8 CFU/mL) and a shorter duration (15 min.). Therefore, it is possible that larger juvenile catfish are more susceptible to infection, possibly being more affected by the handling and crowding required with laboratory models. Crowding is known to predispose fish to attachment and infection of *F. columnare* (Wakabayashi, 1991).

Our second disease induction experiment demonstrated the importance of consistently crowding the fish in the exposure bath. Although the fish were bigger relative to the first experiment, we did not adjust the water level in the bath to maintain a similar density of fish (~ 30 g fish/ L water). The average density in the exposure bath in the first experiment was 36 g fish/ L water whereas in the second experiment the density averaged 57 g fish/ L water. Consequently, the infections developed faster compared to the first trial clearly suggesting that the increased crowding influenced the infection rate.

We had to change fish suppliers during the study and found the catfish from the new supplier were more susceptible to infection. A shorter 2 hour exposure induced the same onset and intensity of infection as when the fish from the original vendor were exposed 4 hours. Separate families of channel catfish can have different susceptibility to columnaris disease (LaFrentz et al., 2012; Beck et al., 2012; Peatman et al., 2013).

Therefore, it is important to confirm the duration of exposure and/or inoculum concentration when working with fish from a different source.

In order to induce a slight temperature stress, we raised the water temperature in our exposure 2 degrees. However, the warmwater catfish actually appeared to benefit for the temperature increase as we observed a slight delay in the onset of infections and less mortality. Therefore we used the temperature increase in the disease model.

We also tried to bind the ammonia in the exposure tank in order to remove any stress from the elevated ammonia levels detected at the end of exposure. A proprietary chemical clearly bound the ammonia; however, in error, we exceeded the target dose by a factor of approximately 2.5. The overdose did not appear to cause toxicity since we did not observe any enhanced sensitivity to the infections. Binding ammonia during the exposure did not slow the onset of infections or reduce mortality; therefore, the binding agent was not added to the disease model.

Five of the 6 *F. columnare* isolates compared for their ability to induce infections in our disease model caused a similar high level of infection. However, an isolate known to have low virulence did not induce any infection (Soto et al., 2008). Although 3 of the virulent isolates had decreased non-wild type florfenicol susceptibility, their ability to induce infections was not impaired. Similar results occurred when 3 different strains of the closely related bacterium, *Flavobacterium psychrophilium*, were injected into rainbow trout, *Oncorhynchus mykiss* (Bruun et al., 2003). Each strain was highly virulent although they differed greatly in their oxytetracycline susceptibility (MIC = 0.25, 4 or 8 µg/mL). In some strains of pathogenic bacteria, decreased antimicrobial susceptibility has

correlated with a decreased virulence in animal infection models (Magnúsdóttir et al., 2000; Chen et al., 2010); however, in other pathogenic bacteria decreased susceptibility does not associate with decreased virulence (Johnson et al., 2012).

The 2 *F. columnare* isolates (94-060 and C003133K) with typical wild type florfenicol susceptibility are known to be virulent based on previous challenges of channel catfish (Soto et al., 2008). To our knowledge, this is the first time that virulence of the 3 non-wild type isolates (S03-517, S03-554B and S03-559) has been characterized. Other than isolates S03-517 and S03-554B, the virulent isolates all have unique PFGE patterns when analyzed with the method developed by Soto et al., (2008) using the *mlu*I and *pas*I restriction enzymes (data not shown).

Our study developed a repeatable disease model that begins by acclimating juvenile channel catfish (18-46 g) to the observation tanks and feed at low density (~2.5-6 g fish/ L) in 26 °C water for at least 2 weeks prior to bacterial exposure. To induce *F. columnare* infections, the catfish are put into an exposure tank at a crowded density (~30 g fish/ L water) in water at 28 °C, and then approximately 10^6 CFU/mL of a virulent *F. columnare* isolate is added. After 2 or 4 hours, we move the catfish back into the observation tanks at the same conditions used for the acclimation. The fish readily eat the feed fed during acclimation as early as 4 hours after exposure therefore future studies on drug effects can begin treatment at this time. We also developed a 3-point scale to consistently assess when the fish are moribund and should be removed from the tank. This disease model and virulent *F. columnare* isolates provide materials and method to

study whether *in vitro* florfenicol susceptibility can accurately predict if an approved florfenicol treatment can control columnaris disease in channel catfish.

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5.7 Disclaimer

The views expressed in this paper are those of the authors and may not reflect the official policy of the Department of Health and Human Services, the U.S. Food and Drug Administration, or the US Government.

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Table 5-1. *Flavobacterium columnare* isolate used to develop the disease model and isolates compared for the ability to induce disease and associated minimal inhibitory concentration (MIC) against florfenicol. (WT = wild type; NWT = non-wild type)

Designation	Isolate #	Host	Year Isolated	Collection Location	MIC (µg/mL)
Positve control	not given	Channel catfish	n/a	USA	2
WT-1	94-060	Channel catfish	1994	USA	2
WT-2	C003133K	Channel catfish	2003	USA	2
WT-3	C91-20	Channel catfish	1991	USA	2
NWT-1	S03-517	Channel catfish	2003	USA	32
NWT-2	S03-554B	Channel catfish	2003	USA	64
NWT-3	S03-559	Channel catfish	2003	USA	32

Table 5-2. Average size of the fish used in each experiment. Also reported is the duration of each exposure and *F. columnare* concentration at the end of the exposure.

Experiment	Avg. weight (g)	Avg. length (cm)	Exposure Time (hour)	*F. columnare* at end of exposure (CFU/mL)
Development of Disease model				
Exp. 1 – abrasion techniques	22.8	12.9	4	4.0E+06
Exp. 2 – 4 versus 2 hour exposure				
60 gallon tank	21.3	12.6	2	not sampled
60 gallon tank	23.3	12.5	4	2.3E+06
20 gallon tank	21.8	12.3		2.2E+06
Exp. 3 – Variance in fish density and bacterial concentration				
Fewer bacteria	22.2	12.4		4.7E+05
Lower fish density	17.9	12.1	4	2.2E+06
Fewer bacteria and lower density	24.7	13.1		6.7E+04*
Disease Induction				
Exp. 5 - highly FFN susceptible *F. columnare*				
negative control, media only	30.7	13.8		0.0E+00
postive control, 36490	21.3	12.7		1.0E+06
WT-1	23.0	13.0	2	5.3E+05
WT-2	21.8	12.8		1.9E+06
WT-3	23.7	13.0		2.0E+06
Exp. 6 - poorly FFN susceptible *F. columnare*				
negative control, media only	46.1	15.4		0.0E+00
postive control, 36490	35.4	14.4		2.1E+06
NWT-1	39.0	14.9	2	1.4E+06
NWT-2	35.9	14.5		7.2E+05
NWT-3	34.8	14.3		8.2E+05

*Note: The tank water was released prior to taking the colony count samples, therefore only the residual water at the bottom of the tank could be sampled.

Table 5-3. Ammonia and nitrite levels measured in the exposure and observation tanks during Experiments 1-3 to develop the model and Experiments 4-5 to compare how different *F. columnare* induce disease.

Experiment	Ammonia (Nessler)				Nitrate			
	Exposure tanks			Obs.	Exposure tanks			Obs.
	0 h	2 h	4 h	Tanks*	0 h	2 h	4 h	Tanks*
Development of Disease model								
Exp. 1 – abrasion techniques	0.037		2.070	0.049	0.0026		0.0067	0.0029
Exp. 2 – 4 versus 2 hour exposure								
60 gallon tank	0.024	1.299	1.847	0.113	0.0018	0.0060	0.0091	0.0038
20 gallon tank	0.012	1.342	2.205		0.0031	0.0062	0.0124	
Exp. 3 – Variance in fish density and bacterial concentration								
Fewer bacteria	0.198		1.719		0.0057		0.0067	
Lower fish density	0.187		1.523	not measured	0.0048		0.0061	not measured
Fewer bacteria and lower density	0.181		0.426		0.0019		0.0023	
Disease Induction								
Exp. 4 - highly FFN susceptible *F. columnare*								
negative control, media only	0.199	1.379			0.0013	0.0027		
postive control, 36490	0.227	2.242			0.0018	0.0056		
WT-1	0.19	1.864		0.088	0.0024	0.0028		0.0033
WT-2	0.142	1.976			0.0016	0.0037		
WT-3	0.144	1.802			0.0015	0.0042		
Exp. 5 - poorly FFN susceptible *F. columnare*								
negative control, media only	0.548	2.106			0.0074	0.0107		
postive control, 36490	0.278	2.83			0.0069	0.0169		
NWT-1	0.326	2.823		0.313	0.0071	0.0171		0.0078
NWT-2	0.206	2.108			0.0052	0.0173		
NWT-3	0.263	2.109			0.0054	0.0151		

* Data was pooled since we only monitored represenative tanks that did not include all treatments.

Table 5-4. Ammonia and nitrite levels (mg/L) measured in the exposure and observation tanks when temperature and/or ammonia levels varied in the bath challenges (Experiment 6).

Treatment	Nitrite		Ammonina (salicylate)		Ammonia (indophenol) monochloramine		Ammonia (indophenol) free ammonia (NH_3 + NH_4)		Unionized ammonia	
	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h
Control, non-exposed, no temperature increase, no ammonia reducer	0.002	0.003	0.07	0.15	0.03	0.02	0.08	0.22	0.00055	0.01518
Temperature increase, no ammonia reducer	0.003	0.003	0.11	0.2	0.03	0	0.14	0.26	0.00084	0.0156
No temperature increase, no ammonia reducer	0.004	0.003	0.9	0.19	0.03	0.03	0.08	0.2	0.00048	0.012
Temperature increase, ammonia reducer	0.004	0.002	0.38	0.17	0.02	0.03	0	0.01	0	0.00069
No temperature increase, ammonia reducer	0.003	0.002	0.32	0.14	0.02	0.02	0	0	0	0

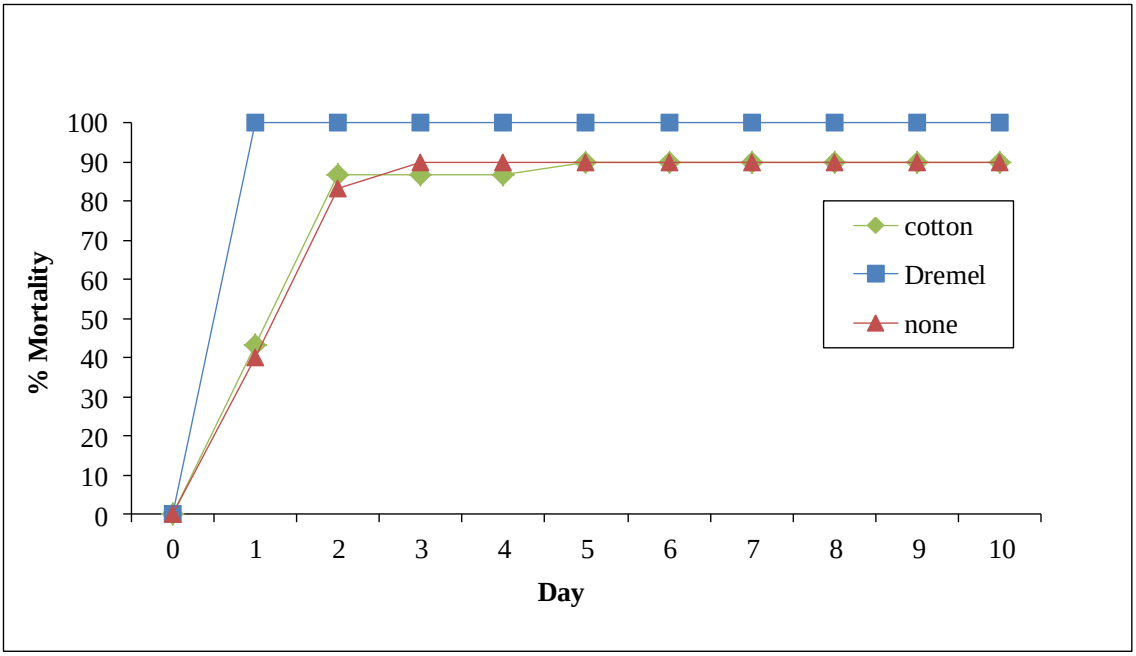


Figure 5-1. Percent mortality of catfish experimentally infected with *F. columnare* using 1 of 3 abrasion techniques and a 4 hour static bath exposure.

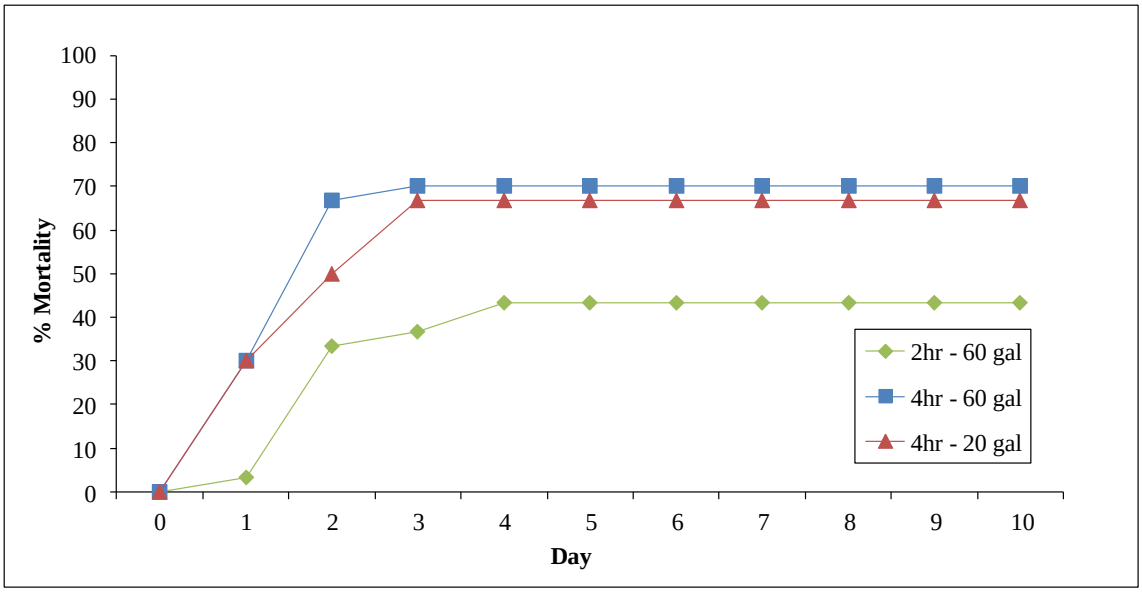


Figure 5-2. Percent mortality of catfish experimentally infected with *F. columnare*. A repeat of the 4 hour non-abrasion exposure in a 60 gallon exposure tank, the 4 hour exposure scaled to a 20 gallon tank and a 2 hour exposure in a 60 gallon tank.

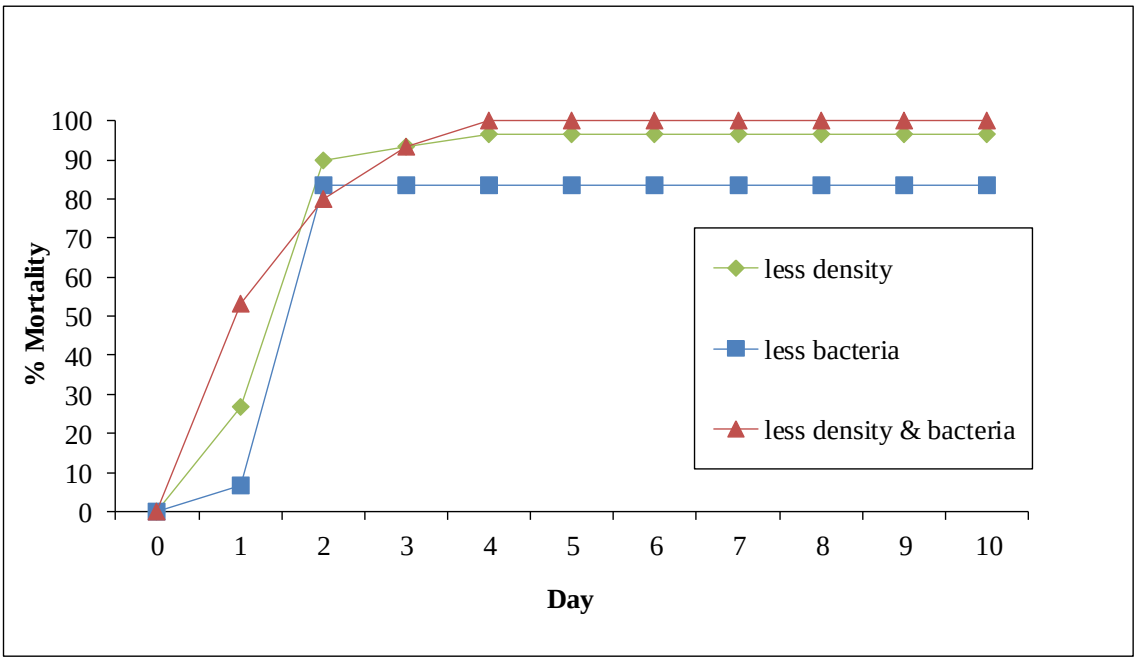


Figure 5-3. Percent mortality of catfish experimentally infected with *F. columnare* comparing exposures with less fish density, less bacteria, or both less density and less bacteria in the 4 hour bath exposure.

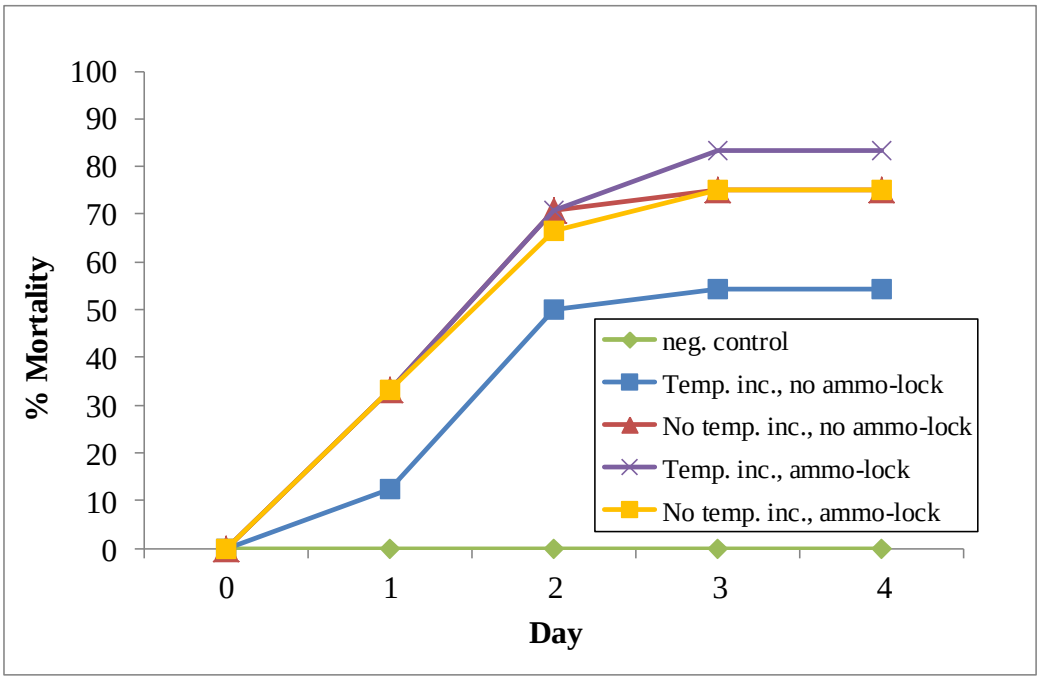


Figure 5-4. Percent mortality of catfish experimentally infected with *F. columnare* varying the temperature and ammonia-binding solution in the 2 hour exposure.

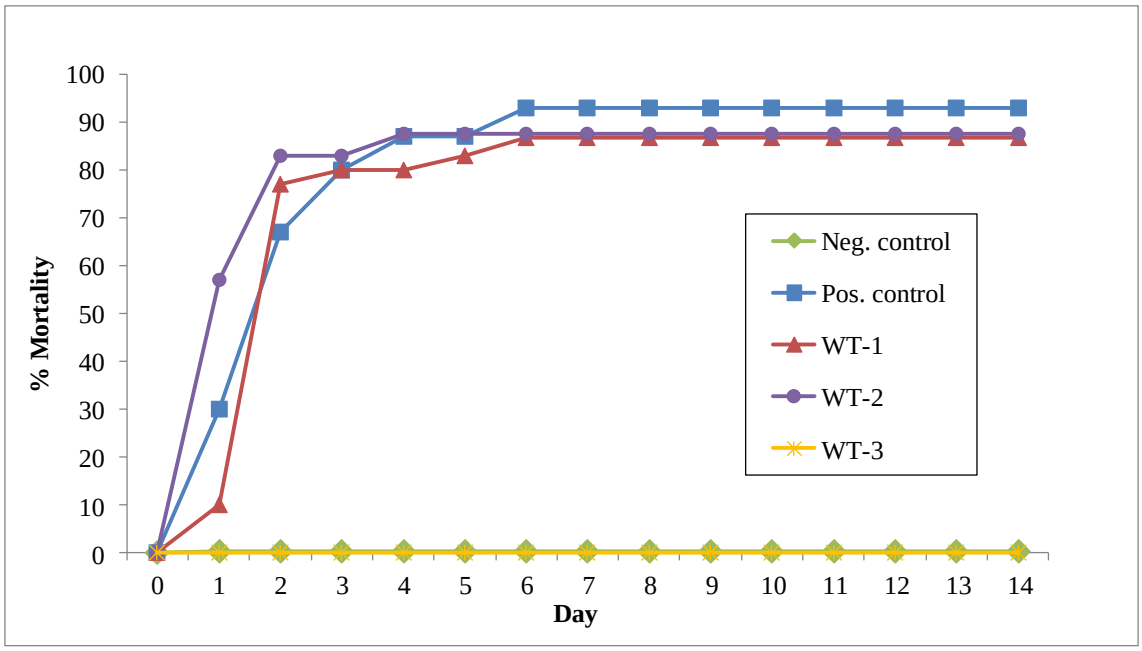


Figure 5-5. Percent mortality of catfish experimentally infected with *F. columnare* (2 hour exposure at 28 °C, ~10^6 CFU/mL) comparing 3 isolates with high susceptibility to florfenicol based on antimicrobial susceptibility testing.

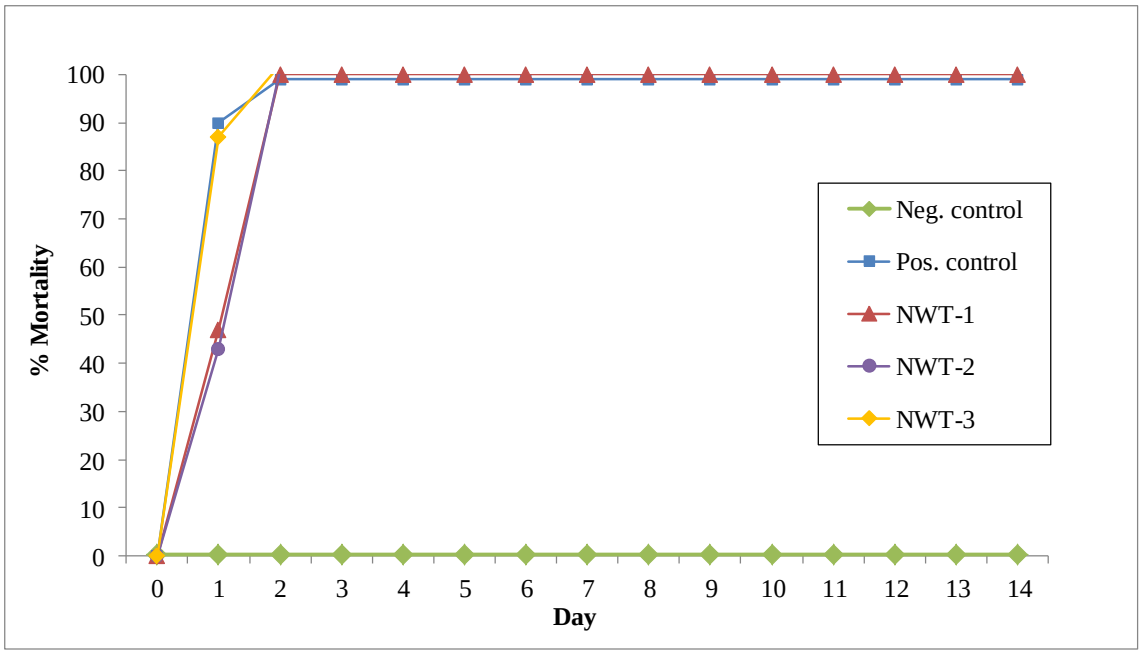


Figure 5-6. Percent mortality of catfish experimentally infected with *F. columnare* (2 hour exposure at 28 °C, ~10^6 CFU/mL) comparing 3 isolates with low susceptibility to florfenicol based on antimicrobial susceptibility testing.

Table 5-5. Least squares mean cumulative percent mortality at day 14 for both trials of the disease induction experiment.

Trial	Exposure treatment (~ 10^6 CFU/mL)	Estimated % Mortality	95% Confidence Interval
Phase I - *F. columnare* highly susceptible to florfenicol	Negative control - plain broth	0.0 (SE =0.01)	(0.0, 100)
	Positive control isolate	93.3 (SE = 4.01)	(76.9, 98.3)
	Isolate WT-1	86.7 (SE = 5.47)	(69.4, 94.9)
	Isolate WT-2	86.7 (SE = 5.47)	(69.4, 94.9)
	Isolate WT-3	3.3 (SE = 2.89)	(0.5, 20.2)
Phase I - *F. columnare* poorly susceptible to florfenicol	Negative control - plain broth	0.0 (SE =0.00)	(0.0, 0.0)
	Positive control isolate	100 (SE = 0.00)	(100, 100)
	Isolate NWT-1	100 (SE = 0.00)	(100, 100)
	Isolate NWT-2	100 (SE = 0.00)	(100, 100)
	Isolate NWT-3	100 (SE = 0.00)	(100, 100)

SE = standard error of least squares estimated mean

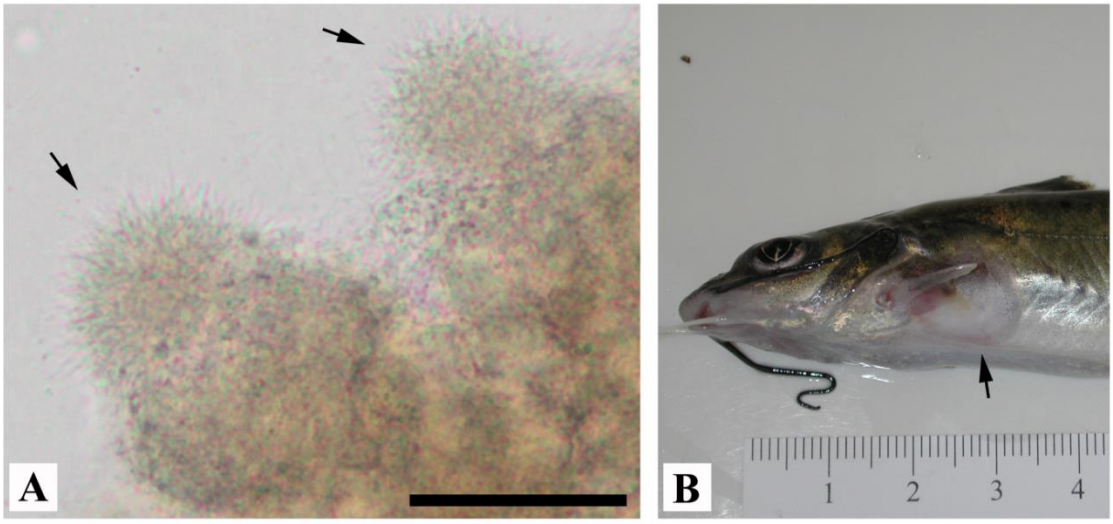


Figure 5-7. Gross observations of *F. columnare* infection. (A) Wet preparation of aggregate columns (arrows) of *F. columare* (scale bar = 50 µm); (B) Spreading skin lesion with depigmented appearance around a pectoral fin with a thin red margin (arrow).

Table 5-6. Condition of the fish at necropsy and percent of *F. columnare* colonies and contaminating cream colonies isolated from the skin, kidney or spleen.

Experiment #	Disease condition at necropsy	N	F. columnare colonies			Cream colonies		
			skin	kidney	spleen	skin	kidney	spleen
1	Moribund/Dead	41	100	100	98			
	Survivors	6	50	0	0			
2	Moribund/Dead	22	100	95	95			
	Survivors	9	100	22	33			
3	Moribund/Dead	30	97	90	93			
	Survivors	4	75	0	0			
4 - FFN susceptible isolates	Moribund	45	98	84	76	100	64	76
	Survivors	48	0	0	0	89	14	11
5 - FFN resistant isolates	Moribund	50	94	80	84	100	68	74
	Survivors	9	0	0	0	100	22	44

Microbiology was not collected in experiment 6.

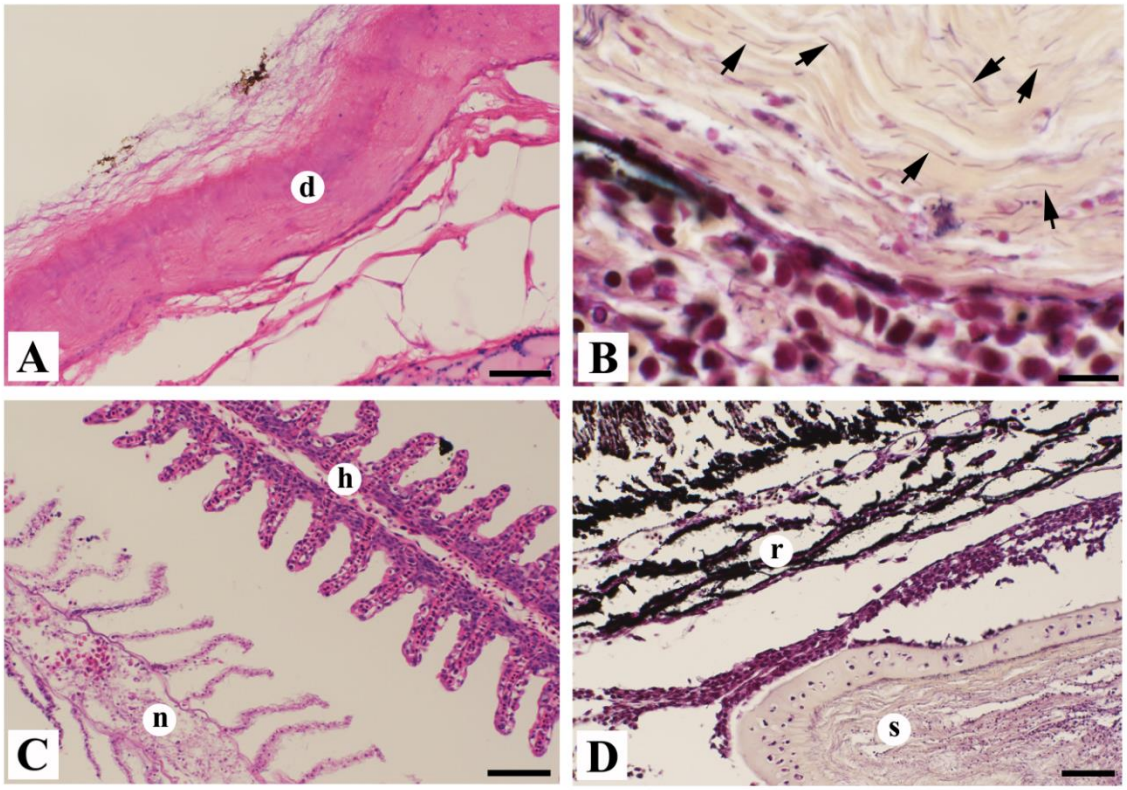


Figure 5-8. Skin, gill and eye changes associated with *F. columnare* infection. (A) Skin missing its epidermis with dermal connective tissue (d) frayed and necrotic at the surface. Underlying muscle has mild focal inflammation (arrow) (20x); (B) Gram stain of the dermal connective tissue infected with *F. columnare* (arrows) and underlying blood and inflammatory cells in the hypodermis (40x); (C) Gill filaments with hypertrophic (h) and necrotic (n) lamellae; (D) Gram stain of *F. columnare* infecting the sclera (s) of the eye with inflammation and edema (40x). The retina (r) is also seen with edema, hemorrhage and inflammation.

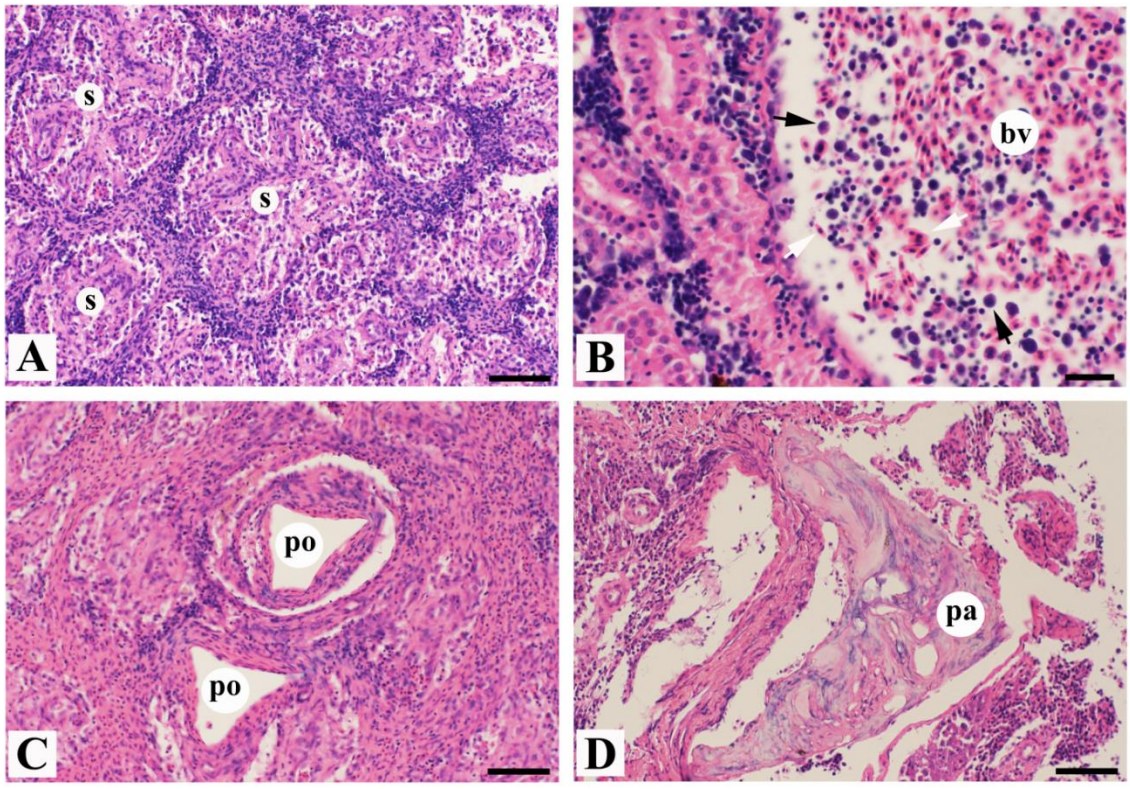


Figure 5-9. Spleen and kidney changes associated with *F. columnare* infection. (A) Splenic corpuscles (s) of the white pulp depleted of lymphatic cells with edema seen as white space in the tissue (20x); (B) Dilated blood vessel (bv) in the posterior kidney filled with many blood (white arrows) and lymphatic cells (black arrows) (40x); (C) Angular cyst-like pockets (po) in the spleen (20x); (D) Cyst-like pocket filled with possible parasite (pa) or decaying somatic tissue (20x).

Chapter 6: Effect of differences in florfenicol susceptibility on the ability of an approved treatment to control *Flavobacterium columnare* infections in channel catfish, *Ictalurus punctatus*.

Gieseker, C M, Hasbrouck, N R, Crosby, T C, Evans, E R, Stine, C B, Parker, A J, Reimschuessel, R, Woods, L C. In Preparation.

6.1 Abstract

We experimentally infected channel catfish *Ictalurus punctatus* with either *Flavobacterium columnare* isolate 94-060 or isolate S03-559, then treated the fish with an approved florfenicol treatment. The isolates differed in their florfenicol susceptibility. Our goal was to determine if the approved treatment could protect catfish from infections caused by a *F. columnare* (S03-559) which had tested as less susceptible to florfenicol than a wild type (WT) *F. columnare* such as isolate 94-060. One hundred eighty catfish were exposed to either: 1) *F. columnare* isolate 94-060, 2) *F. columnare* isolate S03-559, or 3) uninoculated media (negative control) in a 2 hour static bath at 28±1º C. After exposure, the fish were randomly distributed among 6 tanks per exposure (10 fish/tank, 18 tanks total) and held at 25±1º C to observe for moribund fish. We fed the fish an approved dose (10 mg drug/kg fish body weight) of florfenicol-medicated catfish feed or a non-medicated feed beginning 4 hours post-exposure once a day. After 10 days, all of the fish were fed the non-medicated feed for an additional 8 days. Any moribund fish that had at least 2 of 3 defined signs (lesions, lethargy, and listing) was sacrificed and necropsied. We conducted 3 blinded replicate experiments with 540 catfish. The

medicated feed successfully protected fish from infections induced with the WT isolate 94-060 but not isolate S03-559. The cumulative mortality of fish exposed to isolate 94-060 was 16 % for the fish fed the medicated feed and 83 % for the fish fed the non-medicated feed. The mortality of fish exposed to the isolate S03-559 was 82 % when treated versus 90% without treatment. This data shows significantly improved survival if catfish infected with susceptible isolate 94-060 are treated with a 10-day dose of 10 mg/kg dose florfenicol. Survival did not improve among catfish infected with the less susceptible isolate S03-559. Our results will help set clinical breakpoints so veterinarians can used the results of a standardized susceptibility test to interpret if florfenicol treatment will control *F. columnare* in channel catfish.

6.2 Introduction

In the United States (U.S.), columnaris disease caused by the nutritionally fastidious gliding bacterium *Flavobacterium columnare* is a leading pathogen in the channel catfish industry and is emerging in the rainbow trout industry (Wagner et al., 2002; Evenhuis et al., 2014). In 2007, the U.S. Food and Drug Administration (FDA) conditionally approved the use of the drug Aquaflor® (active ingredient = 50 % florfenicol) to control columnaris disease in farmed channel catfish *Ictalurus punctatus* under the Minor Species Minor Use Act of 2004 (Federal Register, 2007). Aquaflor® is new to the U.S. aquaculture industry as of 2005 when it was originally approved to control enteric septicemia of channel catfish (Federal Register, 2005). Recently the U.S. FDA officially approved the use of florfenicol to treat columnaris disease expanding it to include all freshwater-reared finfish and adding a dosage range of 10-15 mg drug /kg

body weight (Federal Register, 2012, 2014). These new approvals will increase the use of Aquaflor® to treat columnaris disease. Therefore, the industry must use this antimicrobial as prudently as possible to limit the possibility that *F. columnare* isolates develop decreased susceptibility to florfenicol.

In order to facilitate prudent use, an antimicrobial susceptibility test was standardized for *F. columnare* (Chapter 4: Gieseker et al., 2012).The standardized method provides laboratories with the same methods and quality control measures to monitor for changes in susceptibility among *F. columnare* isolates. Criteria to interpret test results to predict treatment outcomes are still needed. These criteria, called clinical breakpoints, help guide veterinarians to decide which antimicrobial to use. Clinical breakpoints are particular drug concentrations that categorize the results of standardized susceptibility testing based on the likely success of treatment (susceptible, intermediate, or resistant). Isolates in the susceptible category are likely controlled by the treatment while resistant isolates are not. Treatment may control isolates in the intermediate category, but in some cases a higher dosage may be necessary.

Setting clinical breakpoints requires field effectiveness data and *in vitro* susceptibility testing data with pharmacokinetic /pharmacodynamic data used as an interpreting factor (CLSI, 2008). Recent tests of 134 *F. columnare* with the standardized susceptibility test method found a small group of isolates that have decreased susceptibility to florfenicol (Chapter 4: Gieseker et al., 2012). Wild type (WT) isolates (with typical susceptibility) were inhibited by ≤ 4 µg/mL florfenicol. Five non-wild type (NWT) isolates required ≥ 16 µg/mL florfenicol before growth was inhibited. These

florfenicol concentrations could represent potential susceptible and resistant cutoff points that could potentially define clinical breakpoints. However, such cutoffs must be correlated with the outcome of treatment from clinical use in the field. Only minimal field effectiveness data is available for columnaris disease which has not been correlated with the standardized susceptibility test.

Fortunately, a columnaris disease model that experimentally infected channel catfish with *F. columnare* generated effectiveness data for the recent approval of Aquaflor® in the U.S. (Gaunt et al., 2010). A similar model would be a good way to study whether infections induced with *F. columnare* with decreased florfenicol susceptibility withstand treatment with the approved dose of Aquaflor®. To facilitate this study, we developed a columnaris disease model with channel catfish similar to the one used by Gaunt et al. (2010) and tested the ability of 6 *F. columnare* to induce infections (Chapter 5). Two isolates with typical (WT) florfenicol susceptibility were highly virulent along with 3 isolates with decreased (NWT) susceptibility. In the present study we infected catfish with either a wild type *F. columnare* isolate or a non-wild type isolate, and then treated the infected fish with the approved dose of florfenicol. The results provide critical evidence on effectiveness of Aquaflor® treatment of *F. columnare* with various florfenicol susceptibilities.

6.3 Materials & Methods

6.3.1 Experimental design

Three replicate experiments used a randomized block design in which 3 separate aquarium systems each had 6 observation tanks (18 tanks total) randomly assigned to a set of 3 exposures:

1) un-inoculated broth,

2) *F. columnare* isolate (94-060) with WT florfenicol susceptibility, or

3) *F. columnare* isolate (S03-559) with decreased NWT susceptibility.

The 2 tanks assigned to each exposure were fed either: 1) non-medicated fish feed or 2) florfenicol-medicated feed at 10 mg/kg for 10 days. Treatments began 4 hours post-exposure. After 10 days all of the fish were fed non-medicated feed for another 8 days.

6.3.2 Fish and rearing conditions

We obtained a single batch of 540 juvenile channel catfish (mean weight = 3.6 g, SD = 2.4; mean length = 6.7 cm, SD = 1.3) from the USDA Catfish Genetics Unit (Stoneville, Mississippi). The catfish were maintained in 240 L aquaria constantly supplied with supplemental aeration and 26±1 °C freshwater flowing at approximately 1-2 L/min. The fish were fed commercial catfish feed with at least 35 % protein (Zeigler Bronze, Zeigler Bros., Gardners, PA) at 2% body weight once daily 5 days/week and grown to approximately 20g prior to the experiments. The mean weight (SD) prior to the 3 replicate experiments weighed in groups of 10 fish was 16.8 g (2.7), 24.8 g (3.8) and 30.2 g (2.9), respectively.

The aquaria were fed conditioned well water from a 50-gal head tank. The calcium, magnesium and sodium levels in the water just prior to the study were 16, 12 and 18 mg/L, respectively. The water hardness estimated using the calcium and magnesium levels was 89.2 (as mg/L CaCO3). A digital temperature controller controlled the hot water input into the head tank and float valves controlled the cold water input. Water was delivered by gravity flow, controlled by a valve, for each aquarium. Each tank also had supplemental aeration. A water quality monitoring system (Water Management Technologies, Baton Rouge, Louisiana) continuously recorded the dissolved oxygen (DO), pH and temperature in representative 240 L and 80 L tanks. Water samples collected from the observation and exposure tanks were tested for ammonia and nitrite using the Nessler and Diazotization spectrophotometric methods, respectively (Hach Inc., Loveland, Colorado).

The observation tanks consisted of 3 separate systems with eight 80 L aquaria fed water at 26±1 °C at a rate of approximately 500 ml/min. These tanks housed the fish 14 days prior to the exposure (acclimation) and 18 days after the exposure (treatment/observation). The mean water quality values (SD) during the acclimation period for the entire study were temperature = 25.7 °C (0.4), DO = 6.9 mg/L (0.6) and pH = 7.3 (0.2). Ammonia and nitrite were not monitored as historically the levels in our aquaria with continuous water flow are well below the toxicity thresholds for unionized ammonia (0.05 mg/L) and nitrite (0.10 mg/L; Francis-Floyd et al., 1990). The mean water quality values (SD) post-exposure for the entire study were temperature = 25.5 °C (0.4), DO = 7.0 mg/L (0.6), pH = 7.4 (0.3), ammonia = 0.08 mg/L (0.07) and nitrite = 0.008 mg/L (0.008).

The bath exposure tanks were part of one system with four 240 L aquaria fed water at 28±1 °C at about 1-2 L/min. Each tank (n=3) was divided into 3 equal compartments with a slotted acrylic divider that allowed the compartments to share water. The water flowed until the start of each exposure. Afterwards, 100-watt aquarium heaters (Aquarium Systems, Sarrebourg, France) maintained the water temperature. After shutting off the water flow, the water level was lowered to maintain a fish density of 30 g body weight/L water. The water level was the same in each exposure tank calculated from the average fish weight measured when the fish were moved for acclimation, the number of fish in the exposure tank (n=60), and divided by the fish density. The water levels in the exposure tanks for the 3 replicate experiments were 33.5, 50 and 60 L, respectively. The mean temperature, DO and pH (SD) from 1 representative exposure tank for the entire study was 27.8 °C (0.9), 4.0 mg/L (0.9) and 6.9 (0.3), respectively.

6.3.3 Custom Catfish Feed

The custom feed was made using methods of Li et al. (1993) adapted from previous drug effectiveness and pharmacokinetic studies (Gaunt et al., 2010, 2012, 2013). Each batch of custom feed was made using the same lot of commercial fish feed as a base (Zeigler Bronze, Ziegler Bros., Gardiner, PA). This was the same type of feed used to grow the fish prior to the study. A commercial laboratory found no detectable levels (limit of detection) of the antimicrobials florfenicol (< 0.100 ppm), oxytetracycline (< 0.100 ppm), ormetoprim (< 0.100 ppm) or sulfadimethoxine (< 0.100 ppm) in the base feed (Central Analytical Laboratories, Eurofins Scientific, Metairie, Louisiana).

The feed was pulverized with a mini grinder (Retsch, Haan, Germany). Approximately 10L of pulverized feed filled a 16 quart shell of a laboratory V-Blender (Kelly-Paterson, East Stroudsburg, PA). The V-Blender mixed the dry powder for 20 minutes with a combination of tumble mixing (25 rpm) and an internal intensifier bar (3500 rpm). With the V-Blender drive running, 2 L of purified water (20%) was added through a siphon tube and the mixture blended for an additional 10 minutes. Afterwards, the mixture was pelleted with a CL5 laboratory pellet mill (California Pellet Mill, Crawfordsville, IN) using 3/32" pelleting die. The pellets were then spread on racks and dried with a commercial food dehydrator (Cabela's, Sidney, NE) at 32 °C (90 °F) for 4 hours. The dried feed pellets were mixed, sampled for testing (below) and placed in plastic storage container and held at 4 °C until used.

Separate batches of non-medicated and medicated feed were made prior to each of the 3 replicate experiments. The non-medicated feed was always made first. The medicated feed was made by weighing the ground feed (~10 L) and adding 500 mg florfenicol/kg of feed (454 g florfenicol/ ton) in the V-Blender shell just prior to mixing. All of the components of the V-blender, pellet mill and food dehydrator were washed following each feed preparation session. After each replicate experiment, all of these components were further washed in a commercial glassware washer. Preliminary experiments verified the florfenicol was evenly distributed in the medicated feed and that the drug was stable in the feed for at least 2 months when held at 4 °C (tested 7, 14, 35 and 63 days after manufacture).

Each batch of non-medicated or medicated feed was tested for florfenicol concentration using the methods of Hayes (2013) by a commercial laboratory (Lancaster Lab, Eurofins Scientific, Portage, MI). No florfenicol was found in any of the non-medicated control feed. The average florfenicol concentration (ppm) from 3 replicate tests for each experiment was 502, 500, and 513 respectively. A commercial forage laboratory tested the moisture, protein, fat, fiber, ash, calcium, and phosphorus of the custom prepared feeds (Cumberland Analytical Laboratories, Hagerstown, MD). The mean (SD) moisture level was 11.1 (2.1) %. The other mean (SD) levels as % dry matter were as follows: protein = 38.7 (0.2), fiber = 6.9 (1.0), fat = 8.7 (0.1), ash = 9.0 (0.4), calcium = 1.8 (0.04), and phosphorus = 1.2 (0.03).

6.3.4 Bacteria and culture conditions

Dr. Mark Lawrence and Dr. Tim Santucci (Mississippi State University) supplied the 2 *F. columnare* isolates used in the bath exposures. The isolates were collected from channel catfish in the U.S. in 1994 (94-060) or 2003 (S03-559). We confirmed the identity of the isolates with *F. columnare* specific PCR (Darwish et al., 2004; Welker et al., 2005). The minimal inhibitory concentration (MIC) against florfenicol, determined with recently standardized testing methods (CLSI, 2014), was 2 µg/mL for isolate 94-060 and 32 µg/mL for isolate S03-559. Previous experiments found both isolates are highly virulent to channel catfish *Ictalurus punctatus* in our challenge model (Chapter 5).

To prepare the broth inoculums for each experiment, each isolate was subcultured twice in Shieh broth (Shieh, 1980) at 28 °C for 24 hours starting from frozen cultures kept at ≤ -70 °C in 20% glycerol. The second subculture was adjusted with sterile saline

to a 1.0 McFarland turbidity measured with a colorimeter (Hach Company, Loveland, Colorado, USA). Fifteen mL of each suspension was inoculated into a corresponding sterile flask containing 2 L of Shieh broth. An additional 2 L flask of Shieh broth was not inoculated. The flasks were incubated at 28°C for approximately 72 hours while stirring at 100 rpms. Afterwards, the flasks were stirred and amount of inoculum removed (Experiment 1 = 251 mL, Experiment 2 = 375 mL and Experiment 3 = 450 mL). Preliminary experiments found 150 mL of the inoculum in 20 L of exposure tank water best approximated the target level of 10^6 CFU/mL *F. columnare* in the bath exposure. Therefore, we calculated the inoculum volume by multiplying the 150 mL inoculum/20 L water ratio by the water level in the exposure tank.

6.3.5 Fish Movements and Exposure

Two weeks prior to each experiment, 180 fish in groups of 10 were netted from the holding tanks, weighed and added to 1 of the 18 observation tanks. The fish were fed the custom non-medicated feed at 2% body weight once daily 7 days/week.

Just prior to exposure, 3 groups of 20 fish were moved from the observation tanks into the 3 compartments of an exposure tank. A randomization list (nQuery, Statistical Solutions, Cork, Ireland) assigned the fish from the observation tanks to an exposure tank compartment. After all fish were moved the water flow was stopped, the water level lowered, tank temperature measured, tank heaters turned on, water sampled for a bacterial count and for water quality (pre-inoculum ammonia and nitrite), and the broth inoculum added (un-inoculated broth). The process was repeated for the 2 other exposure

tanks (2 *F. columnare* inoculums). The start times of the fish movements and exposures were staggered 20-30 minutes to allow time for post-exposure movements and samples.

Each exposure was 2 hours at approximately 28 °C. To end each exposure, 10 fish were moved from each exposure tank, weighed in a bucket of water and gently poured into the receiving observation tank. A separate bucket for moving fish was assigned to each observation tank. A randomization list (nQuery, Statistical Solutions, Cork, Ireland) assigned 2 observation tanks (non-medicated or medicated feed) in each block to 1 of the 3 exposure tanks. Unblinded collaborators generated the assignments and moved the fish. The unblinded collaborators did not participate in observations following exposure.

Immediately after removing the fish, the exposure tank water was sampled for a bacterial count and water quality (post-inoculum ammonia and nitrite). The colony count samples were diluted ten-fold (10^{-1} to 10^{-4}) in sterile saline. One hundred µL of the 10^{-3} and 10^{-4} dilutions were spread on separate Shieh agar plates incubated at 28°C and *F. columnare* colonies counted after 44-48 hours. The water quality samples were tested for ammonia and nitrite using methods listed above immediately following each exposure. Table 1 lists the mean ammonia and nitrite values for the treatment groups just prior to and immediately after each exposure and the *F. columnare* levels at the end of each exposure. No *F. columnare* was cultured from the bath exposure water sampled just prior to adding the broth inoculum.

6.3.6 Observations/Feeding/Necropsy

The fish were observed at least twice a day in the morning (prior to feeding) and afternoon for brown to yellowish lesions typical of columnaris disease (Durborow, 1998)

on the skin, gills and/or fins and to assess their health status (healthy, moribund or dead). Healthy fish had either no clinical signs of disease, or early signs not meeting the criteria for moribund. Moribund fish were defined as meeting 2 of 3 criteria: 1) lethargy, 2) listing unable to maintain vertical position and 3) have significant skin lesions defined as 1 or more lesions ≥ 1 cm. Dead fish were collected and moribund fish removed and sacrificed with an overdose of tricaine methanesulfonate (500-1000 ppm).

The fish were fed either custom non-medicated or florfenicol-medicated fish feed at 2% body weight based on the weight measured when the fish were moved post-exposure. Once daily feeding began 4 hours after exposure and subsequently in the morning 7 days/week until the final day of the experiment. Each day the feed was weighed for each observation tank from an assigned feed bottle. The amount of feed given was adjusted based on the number of fish remaining in the tank after removing any dead fish. Prior to each experiment, an unblinded collaborator filled the bottles with either non-medicated or florfenicol-medicated feed based on the randomization list used to move the fish post-exposure. The fish were fed from these bottles for 10 days. Afterwards, the bottles were filled with non-medicated feed and feeding continued until the final day. Feed consumption was assessed as 0, 25, 50, 75 or 100 percent approximately 15 min. after feeding. In order to track feeding, the number of fish in the tank was multiplied by the feed score then averaged within treatments and across experiments to calculate an average daily feed score for each treatment for the entire study.

Each day post-exposure, up to 3 moribund fish/observation tank were examined with a full necropsy. Each necropsy noted the date of collection, tank, weight and fork length, and classified the condition as dead, moribund or healthy (no clinical signs). The external appearance was noted and lesions described. We prepared and analyzed wet preparations of skin (lesions if present) and a gill biopsy. Gills, skin, posterior kidney, and spleen were cultured on non-selective Shieh agar (Shieh, 1980) and incubated at 28±1°C and observed at approximately 24 and 48 hours. Yellow, rhizoid colonies strongly adhered to the agar were considered *F. columnare*. Representative colonies of *F. columnare* and other bacteria were frozen at -80°C. Skin, gill, liver, spleen, and posterior kidney samples were collected for histopathology from representative fish, fixed in 10 % neutral buffered formalin, and stained with hematoxylin and eosin. Select histopathology slides were stained with Gram's method to characterize bacteria and Masson's trichrome method to view the connective tissue in the splenic corpuscles.

All other dead and moribund fish were examined with an abbreviated necropsy. We noted the date, tank, weight, fork length and condition. Only the gills and skin were cultured on selective Shieh medium containing 5 µg/mL neomycin and 200 units/mL polymyxin B (Hawke and Thune, 1992) and incubated at 28±1°C and observed at approximately 24 and 48 hours.

Representative isolates from each treatment group were re-identified with *F. columnare* specific PCR (cited above). The relatedness of representative *F. columnare* from each treatment group was compared with pulsed-field gel electrophoresis using the *mlu*I restriction enzyme (PFGE; Soto et al., 2008). We also re-tested the florfenicol

susceptibility of *F. columnare* collected from the fish with in-house frozen broth microdilution 96-well plates prepared following CLSI guidelines with 11 dilutions of florfenicol (64-0.06 µg/mL) (CLSI, 2014) with 4 g/L CAMHB (Becton, Dickinson and Company, Sparks, Maryland).

6.3.7 Statistics

Each sacrificed moribund fish was considered a death in order to calculate cumulative percent mortality for each experimental group. In previous experiments to develop our columnaris disease model, catfish which were not sacrificed because they only minimally met the moribund criteria always died by the next observation period (≤ 12 hours). The cumulative percent mortality at Day 18 was calculated post-exposure to each treatment was modeled using a generalized linear mixed model (GLIMMIX) procedure available in SAS, assuming a binomial distribution and using a logit link. The model included isolate, feed type (non-medicated vs. medicated), and the isolate by feed type interaction as fixed effects. Since trials were pooled, trials and blocks nested within trials were included as random effects. The isolate by feed type interaction was tested at $\alpha = 0.05$. The estimated mean cumulative percent mortality for each treatment group was calculated from back-transformed least squares mean estimates along with 95% confidence intervals.

The experimental protocol was approved by the Animal Care and Use Committee at the Office of Research, Center for Veterinary Medicine, U.S. Food and Drug Administration, and all procedures were conducted in accordance with the principles

stated in the Guide for the Care and Use of Laboratory Animals (2011) and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

6.4 Results

6.4.1 Mortality

The fish exposed to *F. columnare* isolate 94-060 with WT florfenicol susceptibility or to NWT isolate S03-559, and then fed non-medicated feed had a cumulative mortality of 83 % and 90 %, respectively (Fig. 1). Similarly, the fish exposed to the isolate S03-559 and treated with medicated feed had 82 % cumulative mortality. In contrast, the cumulative mortality was only 16 % for the fish exposed to isolate 94-060 and treated with medicated feed. None of the non-exposed fish fed either non-medicated or medicated feed became moribund or died.

The fish died from 1 to 18 days post-challenge (dpc) with most deaths occurring from 1 to 4 dpc. Both non-medicated treatment groups had a similar onset of illness. Mortality following challenge with the wild type isolate 94-060 from 1 to 4 dpc was 11, 54, 81, and 81 %. Mortality following challenge with the NWT isolate S03-559 from 1 to 4 dpc was 26, 81, 88, and 90 %.

The onset of illness was very different between the 2 medicated treatment groups. Mortality from 1 to 4 dpc following challenge with NWT isolate S03-559 was 9, 43, 61, and 76 %. Although the mortality was similar to the non-medicated groups, florfenicol treatment appeared to slow the rate of infection on day 3 and 4 dpc. On the other hand, mortality of the fish challenged with the WT isolate 94-060 was 1, 6, 6, and 12 %. After

day 4, the fish had no signs of infection until 12 dpc when 1 fish in the second experiment became moribund. Another fish in the same tank became moribund at the end of the experiment at 18 dpc. The delayed mortality occurred again in the third experiment with 1 fish becoming moribund at 18 dpc.

The percent mortality for the unexposed, non-medicated group was not significantly different from the medicated group (df = 40, t = -0.13, P = 0.9006). Likewise, the percent mortality for the S03-559 exposed, non-medicated group was not significantly different from the medicated group (df = 40, t = 1.51, P <= 0.1378), indicating the bacterium was apparently not affected by the treatment. However, the percent mortality for the 94-060 exposed, non-medicated group was significantly higher than the medicated group (df = 40, t = 8.17, P < 0.0001), indicating the treatment was effective.

6.4.2 Fish and Feeding

Mean weight (SD) of the fish in the 3 replicate experiments was 25.9 g (9.8), 32.6 g (12.7), and 42.0 g (14.1) respectively. Mean length (SD) was 13.3 cm (1.7), 13.7 cm (1.7), and 14.9 cm (1.6) respectively.

Most of the treatment groups ate well when offered food 4 hours after exposure. Only 2 of 54 observation tanks (18/experiment) only ate 75 % of the feed whereas all of the other tanks ate all of the feed. The 2 non-exposed treatment groups (non-medicated and medicated) ate most of their feed throughout the study; the weighted average ranged from 3.8 to 4.0. Feeding rates dropped on day 1 amongst the fish exposed to isolate 94-060 and fed non-medicated feed, and both treatment groups exposed to isolate S03-559

(non-medicated and medicated). Surviving fish in these groups resumed eating at least 75 % of the feed within 4 to 10 days post challenge, after we removed the last moribund fish.

The fish exposed to isolate 94-060 and fed medicated feed only ate about half of the feed on day 1 but began eating at least 75 % of the feed by day 3. Otherwise these fish ate all of the feed throughout most of the study with a daily weighted average that ranged from 3.4 to 3.8.

6.4.3 Gross lesions

Most of the moribund fish examined with a full necropsy had long rod-shaped bacteria on their gills and/or skin. These bacteria aggregated into columns that had a haystack appearance diagnostic of *F. columnare* (Fig. 2A). Skin lesions often began at the base of the fins, appearing as dull circular areas with a thin red outer margin (Fig. 2B). More advanced infections had multifocal skin lesions. Some fish became moribund without skin lesions but had pale necrotic area in the gill filaments. Some of these fish had a small skin lesion on the outside margin of the operculum on the side near the pale area. None of the surviving fish had lesions. Internally, some moribund fish had enlarged spleens that were either pale or dark in color.

6.4.4 Histopathology

The moribund fish had focal skin and gill lesions. Skin lesions were missing epidermis with bacteria in the dermal connective tissue causing necrosis and edema (Fig. 3A). Focal necrosis was just under some lesions but did not extend further into the muscles. Occasionally the muscles also had mild hemorrhage and inflammation. On

occasion, the bacteria also infected the eye and nares eroding epidermis and causing necrosis, edema, and inflammation in the connective tissue and muscles. Gill lesions ranged from mild hypertrophy of the gill lamellae to severe hyperplasia (Fig. 3B) that fused lamellae. Heavily infected gills also had focal areas of necrosis with long rod-shaped gram negative bacteria present.

Internally, the spleens of moribund fish had mild to severe depletion of lymphatic white pulp that matched the severity of lesions observed in the gills and skin (Fig. 3C). Many inflammatory cells, particularly monocytes, were in congested blood vessels of the spleen, liver, and posterior kidney. No bacteria were seen in the organs or congested blood vessels. Some moribund and surviving fish had angular cyst-like structures with clear lumens in their spleens. Occasionally the pockets contained non-descript tissue most likely of a parasite (Fig. 3D), and thus these structures are likely an incidental finding. Except for the structures, all of the tissues from the surviving fish appeared normal.

6.4.5 Microbiology

The *F. columnare*-like isolates sampled from the fish re-identified as *F. columnare* by PCR. Most moribund fish examined with a full necropsy had *F. columnare* on or in their skin, gills, posterior kidney, and spleen regardless of exposure isolate or type of feed (Table 2). The bacterium was also present on skin and gills of moribund fish examined with an abbreviated necropsy.

We did not find *F. columnare* on any of the surviving unexposed or exposed fish given non-medicated feed. We did culture a few *F. columnare* colonies on the gills of 2

unexposed, medicated fish. We also cultured *F. columnare* from some survivors in both exposed, medicated groups. One of 15 (7%) surviving fish exposed to isolate S03-559 had *F. columnare* on its gills. Sixteen of 78 (20 %) fish that survived exposure to isolate 94-060 following treatment had *F. columnare* on their skin and 15 of these 78 (19%) fish had *F. columnare* on its gills. One of these fish also had *F. columnare* in its posterior kidney in addition to its skin and gills.

We also consistently isolated other bacteria (round colonies with smooth margins) from the skin and gills of most of the fish, regardless of treatment. These bacteria were equally prevalent on the skin of moribund and surviving fish. We cultured fewer of these bacteria on the skin and gills of fish examined with an abbreviated necropsy because the selective isolation media inhibited their growth. Internally, we cultured a lower prevalence of these bacteria from the spleen and kidneys of the unexposed and exposed non-medicated fish. In general, the prevalence in the internal organs was similar except for the exposed medicated fish, which had less of these bacteria in the survivors versus the moribund fish.

The 2 *F. columnare* used in this study had different PFGE patterns (Fig. 4). *F. columnare* isolates collected from the fish formed two main groups with each having the same pattern as the original exposure isolate. The single *F. columnare* isolate cultured from an unexposed fish had the same pattern as the wild type isolate 94-060. When re-tested with the standardized susceptibility test, the *F. columnare* isolated from the fish post-exposure all had the same MIC of 1 µg/mL regardless of treatment group.

6.5 Discussion

Treatment of channel catfish with 10 mg florfenicol/kg body weight once daily for 10 days following exposure to a *F. columnare* isolate with typical WT florfenicol susceptibility (isolate 94-060) significantly reduced the cumulative percent mortality as compared to fish not treated following the exposure. Based on florfenicol susceptibility, isolate 94-060 grouped together with most *F. columnare* (n=129) tested with a recently standardized antimicrobial susceptibility test (Chapter 4: Gieseker et al., 2012). The minimal inhibitory florfenicol concentration of the isolate (MIC = 2 µg/mL) was in the upper part of the group's MIC frequency distribution which had a median of 1 µg/mL that ranged from 0.25 to 4 µg/mL. In contrast, the approved treatment did not reduce mortality following exposure to NWT *F. columnare* isolate S03-559 which had decreased florfenicol susceptibility (MIC = 32 µg/mL). This isolate was 1 of 5 *F. columnare* isolates that had an MIC ≥ 16 µg/mL clearly separate from most of the *F. columnare* isolates.

Both *F. columnare* isolates induced an onset and intensity of disease similar to our experiments to develop the model and to another published columnaris disease model (Bader et al., 2003, 2006). However, infections developed faster and with greater mortality than the infection model used to study Aquaflor® treatment's ability to control columnaris disease (Gaunt et al., 2010). Due to the faster onset of our infections, we started feeding our fish 4 hours after exposure. All of the fish ate immediately after exposure (day 0). Therefore all of our fish had at least 1 feeding prior to the time some fish stopped eating on the day after exposure (day 1). The treatment schedule used by Gaunt et al. (2010) resulted in a similar number of doses prior to mortality. They started

treatment 1 day after exposure and their fish began to die on day 2 with most dying from day 3 to 5.

We included two unexposed treatment groups (medicated or non-medicated) to identify any possible influence of our custom feed. The feeds had no unwanted antimicrobials and, based on general nutrient levels, had the same composition as the base commercial feed. Both groups ate well and had no mortality indicating the feed had no influence on the results other than delivering the antimicrobial. The feeds were made using the same methods used to study the efficacy and pharmacokinetics following single and multiple 10 mg/kg doses (Gaunt et al., 2010, 2012, 2013).

The signs and external erosions on the skin and gills of the moribund fish using our disease model were consistent with typical signs of columnaris disease (Davis, 1922; Foscarini, 1989; Pacha and Ordal, 1967; Durburow et al., 1998). The lesions typically started at the base of the fins, particularly on the pectoral and anal fins. Scrapes of the lesions had a long rod-shaped bacterium that formed columnar aggregates typical of *F. columnare* (Durburow et al., 1998). Skin lesions were missing epidermis with long rod-shaped bacteria in the dermal connective tissue, similar to saddle-back lesions *F. columnare* causes on young Atlantic salmon *Salmo salar* (Morrison et al., 1981). The *F. columnare* also affected the gill epithelium causing the cells to enlarge. In more chronically affected fish, the gill epithelial cells proliferated causing occlusion and clubbing, often associated with necrosis of the secondary lamellae. These changes presumably impaired the ability of oxygen to diffuse from water to blood (Foscarini, 1989).

Most of the moribund fish consistently had skin lesions; however, some became moribund with minimal signs of infection. These fish had some gill necrosis that was sometimes associated with small skin lesions on edge of the operculum. Highly virulent strains of *F. columnare* may cause mortality by causing systemic infections that enter through the gills without causing skin lesions (Pacha and Ordal, 1967). However, we did not consistently observe moribund fish lacking skin lesions suggesting these fish were part of the natural variation in the pattern of lesion production.

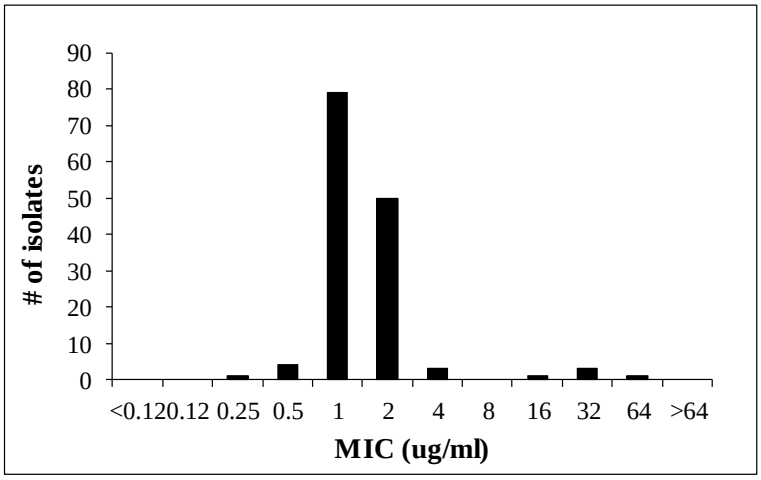
The moribund fish clearly had systemic infections because *F. columnare* was isolated from the spleen and/or posterior kidney. The spleen was also consistently enlarged and dark. The most notable histological changes were depletion of the lymphatic white pulp in the spleen and the vasculitis observed in congested blood vessels. Chen et al. (2011) found similar changes in farmed channel catfish with an acute septicemia caused by *Streptococcus iniae*. Their findings included severe inflammation in the spleen with reduced lymphoid tissue in the white pulp and blood vessels congested with many inflammatory cells. We did not observe inflammation in the spleen of our fish but did note lymphocyte depletion.

Our PFGE results clearly show that the *F. columnare* isolate added to the exposure bath was the same isolate collected back from the fish. We isolated other non-*F. columnare* bacteria; however, these bacteria did not appear to be primary pathogens as they were equally present on the moribund and surviving fish. Conversely, *F. columnare* was highly prevalent both in and outside of the moribund fish whereas it was not isolated from most surviving exposed fish. Interestingly, the non-wild type isolate S03-559 did

not retain its decreased florfenicol susceptibility when re-isolated from the fish. The isolate had to be regrown multiple times to get an uncontaminated culture; therefore its susceptibility may have changed as subsequent generations of the isolate were cultured on the microbiological media.

Determining clinical breakpoints begins with defining the susceptible MIC breakpoint. Infections caused by any *F. columnare* in the susceptible category should be controlled by treatment. Our results suggest setting the susceptible florfenicol breakpoint at ≤ 2 µg/mL, similar to the provisional wild type cutoff determined from testing 134 *F. columnare* using our standardized testing method (Chapter 4: Gieseker et al., 2012). The cutoffs comprising 99.0 and 99.9 % of the wild type isolates were 2 and 4 µg/mL, respectively. Recent susceptibility testing with a similar unstandardized susceptibility test of another 100 *F. columnare* isolates had florfenicol MICs ≤ 2 µg/mL (DeClercq et al., 2013). Since these potential clinical and wild type cutoffs agree, official breakpoints can be set without further influence from a cutoff based on PK/PD data (CLSI, 2008). However, the pharmacokinetics of florfenicol in channel catfish following the same treatment further supports our provisional breakpoint. The florfenicol concentration in the blood maintained a steady state at 2.5 µg/mL during the 10 day treatment (Gaunt et al., 2013).

Three of the 129 *F. columnare* isolates with wild type florfenicol susceptibility based on recent tests with the standardized method had a MIC of 4 µg/mL (Chapter 4: Gieseker et al., 2012).



When possible, standard-setting organizations (e.g. CLSI) avoid setting a susceptible clinical breakpoint that splits the wild type MIC distribution (CLSI 2008). Considering that MICs inherently vary by ± 1 drug dilution (Turnidge and Patterson, 2007), these few *F. columnare* may still be controlled by the approved 10 mg/kg florfenicol treatment. However, it may be best to have these isolates in the intermediate category which suggests they may be controlled by a higher dose. The US FDA recently increased the maximum daily dose of Aquaflor® to provide a dosage range of 10-15 mg/kg (Federal Register, 2012; 2014). This higher dose would presumably increase the peak and steady state blood concentrations, therefore increasing the likelihood that treatment at the higher dose would control *F. columnare* isolates in the upper part of the wild type MIC distribution. However, little knowledge is publicly available on the pharmacokinetics of the 15 mg/kg dose in fish over the treatment period; therefore, it is not clear how much the drug serum concentration would increase compared to 10 mg/kg dose (Reimschuessel et al., 2005).

We clearly show the approved 10 mg/kg florfenicol treatment fails to control infections caused by the *F. columnare* (isolate S03-559) with decreased florfenicol susceptibility (MIC = 32 µg/mL). The amount of florfenicol needed to inhibit the isolate is well above the peak blood concentration following a single 10 mg/kg dose of florfenicol (7.6 µg/mL) in channel catfish *Ictalurus punctatus* and 20 mg/kg dose in Korean catfish *Silurus asotus* (9.6 µg/mL), and is an order of magnitude higher than steady state blood concentrations of the approved 10 mg/kg dose treatment (Park et al., 2006; Gaunt et al., 2012; Gaunt et al., 2013). Based on this data, the approved treatment will likely fail to inhibit any of the 5 *F.columnare* isolates found in recent testing with a florfenicol susceptibility ≥ 16 µg/mL (Chapter 4: Gieseker et al., 2012). Hence these isolates appear to define a resistant susceptibility breakpoint for the standardized broth microdilution testing of *F. columnare*.

Our effectiveness data reported here and the florfenicol MIC frequency distribution from recent *in vitro* testing provide some of the necessary data needed to set clinical breakpoints for the recently standardized *in vitro* test (Chapter 4: Gieseker et al., 2012; CLSI, 2014). Setting breakpoints will give prescribers the ability to interpret test results needed to judiciously use the approved florfenicol product to control columnaris disease.

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6.7 Disclaimer

The views expressed in this paper are those of the authors and may not reflect the official policy of the Department of Health and Human Services, the U.S. Food and Drug Administration, or the US Government.

6.8 References

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Table 6-1. Ammonia and nitrite levels in the tanks at the beginning (0 h) and end (2h) of the bath exposures, and *F. columnare* level at the end of the exposure.

Experiment	Treatment	Ammonia (mg/L)		Nitrite (mg/L)		*F. columnare*
		0 h	2 h	0 h	2 h	(CFU/mL)
1	Negative control	0.16	1.36	0.003	0.013	0
	Isolate 94-060	0.22	2.69	0.004	0.012	3.5×10^6
	Isolate S03-559	0.27	2.81	0.004	0.014	1.9×10^6
2	Negative control	0.19	1.18	0.008	0.016	0
	Isolate 94-060	0.20	2.61	0.005	0.016	2.6×10^6
	Isolate S03-559	0.19	2.65	0.009	0.012	1.0×10^6
3	Negative control	0.18	1.28	0.009	0.014	0
	Isolate 94-060	0.19	2.84	0.006	0.020	8.3×10^6
	Isolate S03-559	0.18	3.11	0.007	0.013	1.3×10^6

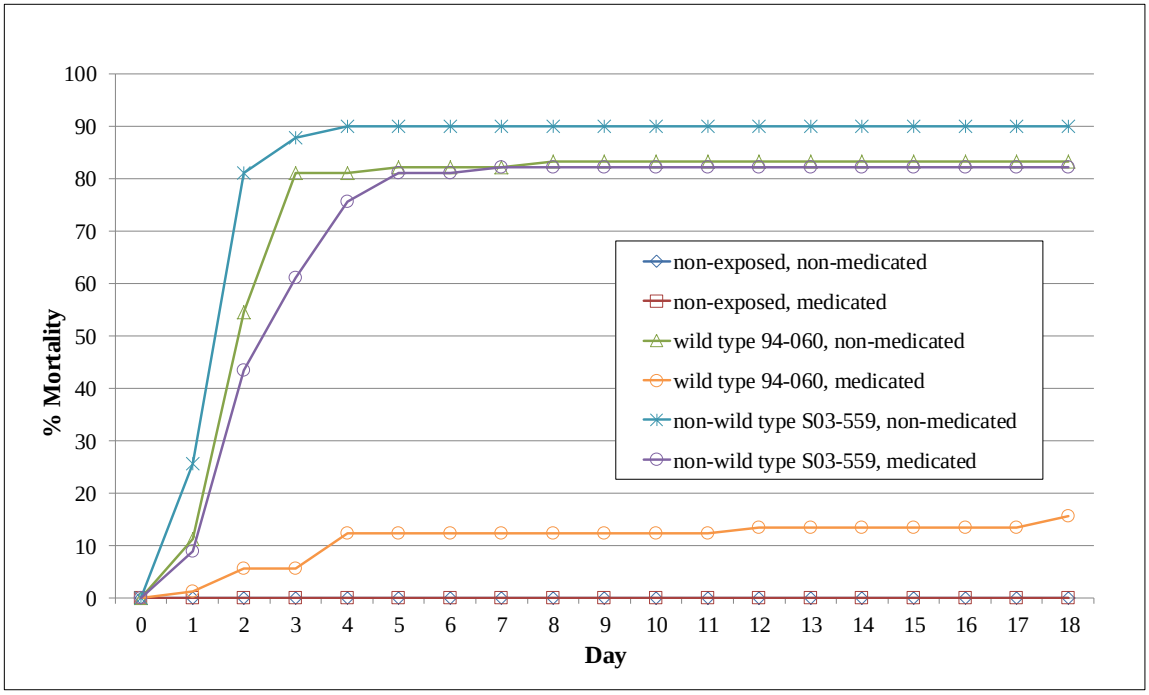

Figure 6-1. Cumulative percent survival of channel catfish challenged with *F. columnare* then treated with 10 mg florfenicol/kg body weight once daily for 10 days. The fish were non-exposed, exposed to an isolate with high florfenicol susceptibility or exposed to an isolate with poor florfenicol susceptibility.

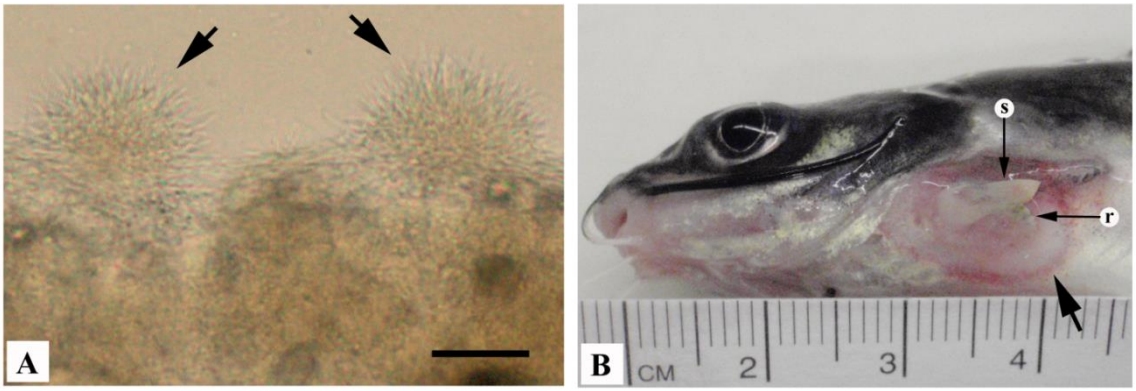

Figure 6-2. (A) Wet preparation of a skin lesion with aggregates of *F. columnare* (arrows, scale bar = 20 µm); (B) Circular skin lesion around pectoral fin of a channel catfish. The fin at the lesion's center has eroded spine (s) and fin rays (r). Skin has a dull appearance with a thin red margin (arrow).

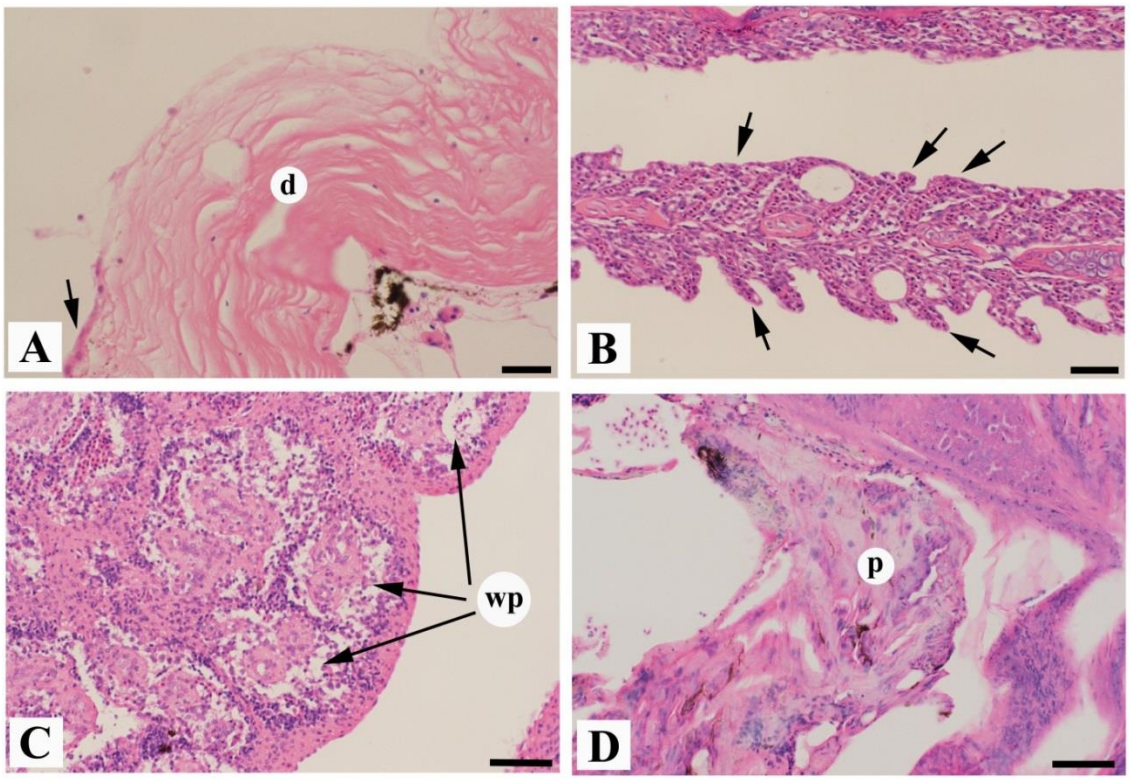

Figure 6-3. Skin, gill and spleen changes caused by *F. columnare* infection. (A) Dermal connective (d) tissue of the skin missing most of its epidermis (arrow, scale bar = 20 µm); (B) Gill filament with hyperplastic epithelium occluding the spaces between secondary lamellae (arrows, scale bar = 20 µm); (C) Spleen with white pulp (wp) deplete of lymphatic cells (arrows, scale bar = 50 µm);); (D) Amorphous tissue of a possible parasite in cyst-like pockets observe in the spleen (p, scale bar = 100 µm).

Table 6-2. Percent of channel catfish with *F. columnare* or non-*F. columnare* colonies isolated from the skin, gill, posterior kidney or spleen. Separate results are given for the full and abbreviated necropsies.

Treatment	Necropsy (F/A)*	n	% *F. columnare*				% other colony types			
			skin	gill	kidney	spleen	skin	gill	kidney	spleen
Unexposed, non-medicated feed										
Survivors	F	28	0	0	0	0	100	100	25	18
Survivors	A	61	0	0			66	41		
Unexposed, medicated feed										
Survivors	F	27	0	0	0	0	93	96	22	19
Survivors	A	62	0	3			69	69		
Wild type isolate 94-060, non-medicated feed										
Moribund	F	50	100	98	98	94	96	96	34	22
Moribund/Dead	A	26	92	96			54	65		
Survivors	F	14	0	0	0	0	100	100	29	0
Wild type isolate 94-060, medicated feed										
Moribund	F	11	100	100	100	82	100	100	64	55
Survivors	F	27	22	19	7	0	96	93	4	22
Survivors	A	51	20	20			49	31		
Non-wild type isolate S03-559, non-medicated feed										
Moribund	F	44	98	98	98	95	93	95	36	36
Moribund/Dead	A	37	97	62			81	38		
Survivors	F	9	0	0	0	0	100	100	44	22
Non-wild type isolate S03-559, medicated feed										
Moribund	F	53	96	96	94	90	98	98	34	35
Moribund/Dead	A	21	100	71			81	57		
Survivors	F	15	0	7	0	0	100	100	0	7

* F = Full necropsy; A = Abbreviated necropsy

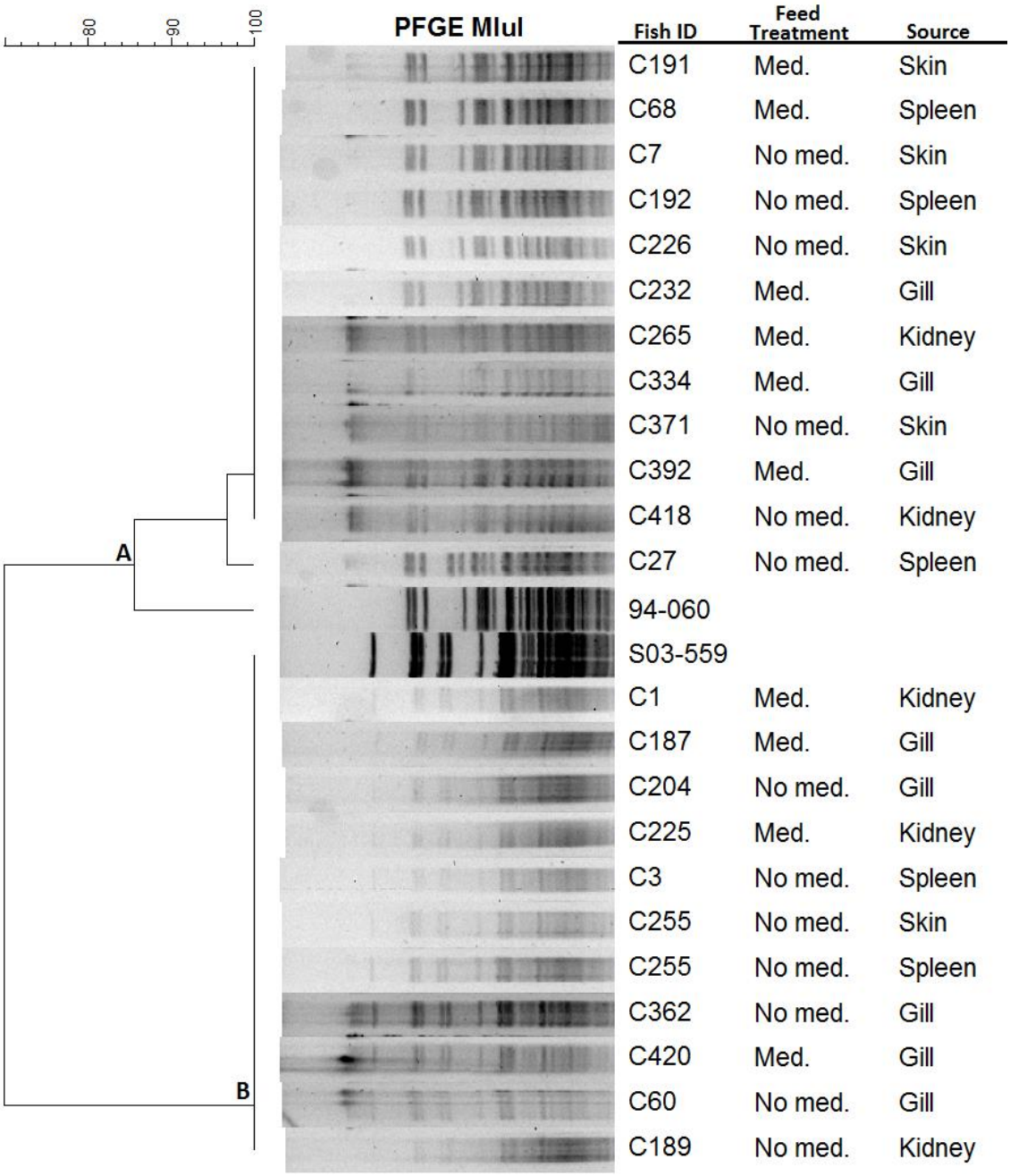

Figure 6-4. Dendrogram of PFGE patterns of *F. columnare* isolates collected from the catfish after bath exposure to *F. columnare* isolate 94-060 or isolate S03-559. Isolates C7, C27, C68, C191, C192, C226, C232, C265, C371, C392 and C418 were isolates from fish exposed to isolate 94-060. Isolates C1, C3, C60, C187, C189, C204, C225, C255, C362, and C420 were isolated from fish exposed to S03-559. Isolate C334 was isolated from the unexposed medicated treatment group.

Chapter 7: Discussion and Summary

The use of antimicrobials in farmed fish to control infections caused by the aquatic bacterial pathogens *Flavobacterium columnare* and *Flavobacterium psychrophilum* created an important need for reliable test methods and for research to guide therapy. Standard antimicrobial susceptibility testing is one key strategy to monitor the development of antimicrobial resistance, and it is essential that laboratories all use the same methods and interpretive criteria (Leung et al., 2011). Up to now, no test method had been standardized for the nutritionally fastidious *F. columnare* and *F. psychrophilum*. In addition, there was very little data regarding changes in antimicrobial susceptibility of *F. columnare* nor data regarding decreased florfenicol susceptibility that could limit treatment of this common, but fastidious pathogen.

7.1 Standardized Testing

The research described in this dissertation developed test methods and adaptions specific for the unique growth characteristics of these two yellow-pigmented gliding bacteria. Both *F. columnare* and *F. psychrophilum* grow well in liquid media; therefore, broth dilution was the best test to standardize. Fortunately, when we began there was already a basis for developing a broth method since researchers had recently determined 4 g/L dilute Mueller-Hinton media worked well for broth microdilution testing *F. columnare* (Farmer et al., 2004; Darwish et al., 2008). However, some aspects of the media were not known, such as the effects of supplementing the broth with cations, as typically done for routine susceptibility tests, or adding serum as done to enhance growth in previous disk diffusion and agar dilution tests (Hawke and Thune, 1992; Bruun et al.,

2000; Michel et al., 2003). In the end, we needed to add dilute cations so the method would work for *F. psychrophilum* but found that serum should not be added since growth either stayed the same or actually declined.

By far, the biggest challenge was making consistent cell suspensions. The precision of susceptibility tests relies on testing a similar number of bacteria each time (Wiegand et al., 2008). Most *F. columnare* isolates adhere strongly to agar; therefore, suspensions are best made from broth cultures. In addition, *F. psychrophilum* isolates vary a lot in their growth rate and cultures can have a lot of dead cells that make turbidity readings used to standardize the suspension inaccurate (Michel et al., 1999). Fortunately, our adaptations for making cell suspensions from static broth cultures worked well for *F. columnare*; however, we still get a lot of variation with *F. psychrophilum*. Therefore, additional work will be needed to improve the consistency of *F. psychrophilum* suspensions.

To validate our method, we conducted a multi-laboratory trial. The trial developed quality control (QC) ranges for a standard strain of *Escherichia coli* and *Aeromonas salmonicida* subsp. *salmonicida* against 9 antimicrobial drugs commonly used in aquaculture. Laboratories could test these strains alongside *F. columnare* or *F. psychrophilum* to validate if the drug concentrations were correct or if a mistake was made in setting up the test. Moreover, the QC ranges allow laboratories to make their own plates when their needs prevent buying commercial plates. QC bacteria provide a means to validate the drug concentrations in laboratory prepared plates, made in a 96-well format or 8-well strips, using the VET04 guideline (CLSI, 2014).

In collaboration with other members of the CLSI Aquaculture Working Group (within the Veterinary Antimicrobial Susceptibility Testing Subcommittee), sections specific for broth microdilution testing of *F. columnare* and *F. psychrophilum* (based on chapter 2) were recently added to the latest edition of the VET04 guideline (CLSI, 2014a). A separate supplement for performance standards was also created which includes the quality control ranges developed in chapter 3 (CLSI 2014 b). With standard methods in place, the data developed in chapters 4-6 will help develop interpretive criteria to complete standardized testing of *F. columnare* so researchers and clinicians can evaluate test results in the same manner.

7.2 Monitoring for antimicrobial resistance

Our research clearly shows there are *F. columnare* isolates with decreased susceptibility to multiple antimicrobials used in aquaculture. The antimicrobials we tested represent 7 drug classes: 3 quinolones, 2 potentiated sulfonamides, and 1 representative each from the beta-lactam, macrolide, aminoglycoside, phenicol and tetracycline classes. Twenty-two of our 134 (17%) *F. columnare* isolates had susceptibility above our provisional epidemiological cutoff values (ECVs). Most notable were *F. columnare* isolates with decreased susceptibility to florfenicol, erythromycin and ampicillin. All previous research that tested the florfenicol susceptibility of *F. columnare* reported isolates with relatively low susceptibility similar to our wild type (WT) isolate group (Michel et al, 2002; Suomalainen et al 2006a; Darwish et al., 2008, Gaunt et al., 2010; Declercq et al., 2013). Our finding about florfenicol is especially important since some of

the non-wild type (NWT) isolates came from channel catfish farmed in the US (Federal Register, 2007a/b, 2012, 2014)

The multiple isolates we found with NWT susceptibilities to ampicillin and erythromycin also confirmed recent broth microdilution testing that found a single *F. columnare* isolate with decreased ampicillin or erythromycin susceptibility (Darwish et al., 2008; Declercq et al. 2013). All previous *F. columnare* tested by disk diffusion or agar dilution reported all isolates as highly sensitive to erythromycin. Of 3 studies that tested *F. columnare* against ampicillin by disk diffusion (Amin et al., 1988; Michel et al., 2002; Suomalainen et al 2006a), one study did report some isolates that were "slightly sensitive" to ampicillin (Amin et al., 1988).

Nine of the 22 isolates we found with decreased antimicrobial susceptibility had elevated minimal inhibitory concentrations (MICs) to more than 1 antimicrobial drug class. Our isolates with decreased erythromycin and florfenicol susceptibility all classified as NWT against multiple drugs representing 4 to 5 drug classes. Whereas decreased ampicillin susceptibility was singular in some cases and multi-drug in others. As recently reported from testing with a similar but unstandardized broth microdilution method, we also found decreased susceptibility to enrofloxacin, flumequine and/or oxolinic acid (all quinolones), or oxytetracycline that was also either singular or multi-drug (Declercq et al., 2013). Five of the isolates with non-typical phenotypes in our study were also in the study by Declercq et al. (2013). The phenotypes of these isolates were very consistent between the 2 studies which provide strong evidence validating the test and these isolate's NWT susceptibilities.

Future research should investigate the genetic origins of these non-typical phenotypes. In chapter 4 some MIC frequency distributions had clearly separate WT and NWT isolates but these groups overlapped for other drugs. Therefore, if certain genes only associate with the NWT phenotypes, then those genotypes could help confirm that the ECVs are set on the correct drug concentration. Secondly, discovering genotypes could also lead to faster antimicrobial susceptibility tests. A major challenge is the time it takes to run susceptibility tests, especially for slow growing bacteria from cold-blooded animals like fish. If genotype can reliably predict NWT antimicrobial phenotypes, then whole genome sequencing could speed up the time it takes to give aquatic veterinarians susceptibility results. Thirdly, if resistance genes are present then, whether they are located on chromosomal DNA or mobile elements such as plasmids should be investigated. Suomalainen et al. (2006b) did not find plasmids in 7 *F. columnare* isolates collected from Finnish fish disease outbreaks and 1 type strain; however, plasmids are found in *F. psychrophilum* (Madsen and Dalsgaard, 2000). Recent broth microdilution testing with Anacker and Ordal broth recently found *F. psychrophilum* with elevated MICs against flumequine and oxolinic acid which were associated with mutations in the quinolone resistance determining region of the *gyrA* gene (Shah et al., 2012). However, no plasmid-mediated *qnr* genes were found. A consistent method for testing the different isolates will help research target which isolates to investigate by sequencing.

Additional *F. columnare* testing by other laboratories using our standard method is needed so the Aquaculture working group can incorporate some inter-laboratory variation into the provisional ECVs. Dr. Turnidge, who is on the CLSI VAST committee, has advised that including at least some inter-laboratory testing is desired for setting

official ECVs. The similarity in results between our study and the one by Declercq et al. (2013) suggests the standard method should have good precision between laboratories.

7.3 Judicious use of florfenicol for columnaris disease

It is important to note that decreased susceptibility as determined by an ECV does not necessarily mean that the bacterium cannot be inhibited by treatment (CLSI, 2011). For example, an isolate with low-level resistance can still be inhibited as long as treatment can achieve an effective drug concentration at the infection site for the appropriate duration. In regards to aquaculture, some scientists suggest laboratories advise clinicians on whether to initiate treatment based on if ECVs classify isolates as WT or NWT (Smith, 2008). This approach provides clinicians with the best information available until enough data on clinical outcomes and drug kinetics develops to set clinical breakpoints. However, fish with curable infections from a bacterium with low level resistance might go untreated, allowing the bacterium to persist and possibly develop a higher level of resistance. Therefore, our work to supplement limited clinical data with an experimental disease model is a very justifiable approach to move along setting interpretive clinical breakpoints for aquaculture.

In developing our disease model, we found that some *F. columnare* with decreased florfenicol susceptibility have an increased virulence. Other studies of pathogenic bacteria in animal infection models have associated decreased antimicrobial susceptibility with decreased virulence (Magnúsdóttir et al., 2000; Chen et al., 2010). All 5 of our *F. columnare* isolates induced a similar level of mortality regardless of differences in susceptibility. Similar results were found in a study that intraperitoneally

injected rainbow trout, *Oncorhynchus mykiss*, with 1 of 3 *F. psychrophilum* strains that had different oxytetracycline susceptibility (Bruun et al., 2003). All 3 isolates killed most fish even though they had different oxytetracycline MICs (0.25, 4 and 8 µg/mL).

We have shown in chapter 6 that experimental infections with non-wild type *F. columnare* (florfenicol MIC = 32 µg/mL) are not controlled by treatment with 10 mg/kg dose of florfenicol given once daily for 10 days. Since broth microdilution results usually vary by ± 1 drug dilution (Turnidge and Patterson, 2007), the same florfenicol treatment will likely fail to control infections caused by any of the 5 of isolates found in chapter 4 with a florfenicol MIC between 16 and 64 µg/mL. Similarly, Bruun et al. (2003) found a 100 mg/kg dose of oxytetracycline for 10 days failed when rainbow trout were infected with *F. psychrophilum* strains with low susceptibility (MIC_{OTC} = 4 or 8 µg/mL) as determined by an unstandardized agar dilution test (Bruun et al., 2000).The treatment prevented deaths when trout were infected with a strain with high susceptibility (MIC_{OTC} = 0.25 µg/mL).

Unexpectedly, our isolate with non-wild type florfenicol susceptibility did not retain its elevated MIC when re-isolated from the moribund fish after exposure. The isolate had to be sub-cultured multiple times to re-purify it; therefore, the multiple growth cycles could have changed its susceptibility. The results of chapter 6 support our original test; however, more research is needed to determine if the decreased susceptibility varies and what parameters of storage could affect this. Future studies to evaluate this could include infecting catfish with the isolates recovered from the challenge fish to test

whether their change in susceptibility makes the isolates susceptible to florfenicol treatment.

In addition to florfenicol, clinicians also use oxytetracycline to control mortalities caused by *F. columnare* in rainbow trout (Pulkkinen et al., 2010; Federal Register, 2008). Therefore, future studies with our disease model should study if differences in OTC susceptibility affect the approved treatment. LaFrentz et al. (2012) recently developed a columnaris disease model with rainbow trout fry to investigate virulence. The model uses a bath immersion without abrasion similar to our catfish model. Therefore, methods already exist to help adapt our model to trout or to possibly facilitate a collaboration. Further study with channel catfish is also valuable since it would help veterinarians responsibly use OTC when requirements are meant for extra-label use.

The MIC frequency distributions and provisional ECVs determined in chapter 4 meet most requirements for setting ECVs for *F. columnare*. In addition, the comparison of drug effectiveness between 2 *F. columnare* isolates with different florfenicol susceptibility, in combination with the MIC distributions, offers good evidence that should expedite setting clinical breakpoints.

7.4 Conclusion

In summary, the studies reported here have standardized an antimicrobial susceptibility test specific for 2 nutritionally fastidious gliding bacteria that continue to be important disease problems in many aquaculture industries (Wagner et al., 2002; Evenhuis et al., 2014). This work has already stimulated additional research as a multi-

laboratory testing trial recently used our methods to generate proposed ECVs for *F. psychrophilum* (Smith et al., 2014). Hopefully, our research will stimulate continued improvements in monitoring for changes in antimicrobial susceptibility among these pathogens on individual farms and regions. Overall, we hope our research will help improve the prudent use of antimicrobials in aquaculture for the benefit of public health by limiting development of antimicrobial resistance and by helping the veterinarians effectively manage fish health so the aquaculture industry can provide people with healthy sources of protein.

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