

ABSTRACT

Title of Document: DEVELOPMENT OF STANDARDIZED
ANTIMICROBIAL SUSCEPTIBILITY
TESTING METHODS AND AEROMONAS
SALMONICIDA EPIDEMIOLOGIC CUTOFF
VALUES FOR ANTIMICROBIAL AGENTS
USED IN AQUACULTURE

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Pharmacokinetics describes the time course of drug absorption, distribution, metabolism and excretion. Pharmacodynamics is the relationship between unbound drug concentration over time and the resulting antimicrobial effect. Pharmacokinetic/pharmacodynamic (PK/PD) indices quantify the relationship between pharmacokinetic parameters (i.e., area under the concentration-time curve, AUC) and microbiological parameters (i.e., minimal inhibitory concentrations, MICs), and are used to establish interpretive criteria or clinical breakpoints. The three primary PK/PD indices used are the AUC over 24 h at steady-state/MIC (AUC_{ss}/MIC), the peak concentration/MIC (C_{max}/MIC), and the percentage of time over 24 hours that the drug concentration exceeds the MIC at steady-state pharmacokinetic conditions ($T_{>MIC}$). These indices can be used to determine both appropriate dosage regimens and index magnitudes required for efficacy

and reduced antimicrobial resistance emergence. The goal of this work was to determine the relevant PK/PD index target (AUC_{ss}/MIC) for oxytetracycline (OTC) against *Aeromonas salmonicida*, causative agent of furunculosis in salmonids. To achieve this goal we first established a standardized MIC testing method for aquatic bacterial pathogens, then used this method to determine the *in vitro* susceptibility cutoff concentration (epidemiologic cutoff value) for OTC (and three other antimicrobial agents) against 217 *A. salmonicida* isolates. We conducted additional *in vivo* studies using rainbow trout to monitor achievable serum OTC concentrations in both healthy and *A. salmonicida*-challenged fish. We confirmed OTC to be highly efficacious against a susceptible *A. salmonicida* strain *in vivo*, and through pharmacokinetics studies, calculated the OTC AUC_{ss} in healthy and challenged fish to be 27.2 and 20.1 $\mu\text{g}\cdot\text{h}/\text{mL}$, respectively. The PK/PD index target reported in a neutropenic mouse model as the most applicable to the tetracyclines is an AUC_{ss}/MIC of ≥ 5 . Either of the AUC_{ss} values divided by the current epidemiologic cutoff value for *A. salmonicida* isolates (1 $\mu\text{g}/\text{mL}$) yields a product greater than this AUC_{ss}/MIC target of ≥ 5 . This work demonstrates PK/PD indices commonly used in studies in mammals to predict therapeutic efficacy can be applied in studies in fish.

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TESTING METHODS AND AEROMONAS SALMONICIDA EPIDEMIOLOGIC
CUTOFF VALUES FOR ANTIMICROBIAL AGENTS USED IN
AQUACULTURE

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Dedication

Erin, my sweet, thank you for your patience, encouragement, guidance, love and understanding.

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I would first like to recognize Dr. Renate Reimschuessel for her incredible mentorship the past 6 years. You are an amazing teacher and advisor. Thank you for your exceptional guidance, honesty, and exceptional sense of humor. I also must thank Charles Giesecker and all the other scientists, managers, and staff in the FDA - Center for Veterinary Medicine's Office of Research. Your generosity and willingness to always lend a helping hand has been very important to me, and for that I will always be grateful. I must recognize Dr. Andrew Kane for agreeing to serve as my major advisor and for your superb knack for troubleshooting and getting me and my project in the right direction. Lastly, I'd like to thank the other members of my graduate advisory committee, Drs. Ana Baya, Katherine Squibb and Jianghong Meng for their time, guidance, and for agreeing to serve on my committee.

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Chapter 1: Antimicrobial Agents Used in Aquaculture

Antimicrobial Agents Approved for Use in Aquaculture in the United States

Introduction

Antimicrobial agents have long been used to relieve pain and suffering and control infections in animals, including fish. Gutsell (1946) was the first to publish an article about using an antibiotic (sulfa) to treat bacteria in fish in the U.S. Since then, the safe and prudent prescription of efficacious drugs by veterinarians to treat aquatic animals has contributed immensely to the increased food production capacity of U.S. aquaculture. However, using antimicrobial agents is not without risk. Antimicrobial resistant pathogens and environmental bacterial species have been found in, and near fish farms where antibiotic-medicated feed has been administered (Huys et al., 2001; Guardabassi et al., 2000a; Sathiyamurthy et al., 1997; Husevag and Lunestad, 1995). Since only three antibiotics are currently available for use in U.S. aquaculture (**Table 1**), veterinarians have limited options when making treatment decisions. Continued use of the same drug(s) will likely exacerbate the very problem of resistance and may eventually diminish their utility in aquaculture. Careful *in vitro* antimicrobial susceptibility testing (AST) is required to accurately monitor the development of resistance and assist in the decision-making process of whether to administer treatment. AST and selection and prescription of the appropriate antimicrobial agent if available, is an important task of the clinician and veterinarian. Until recently, *in vitro* AST methods for the three approved antimicrobials for aquaculture, oxytetracycline (OTC), ormetoprim-sulfadimethoxine, and florfenicol, were not standardized for any aquatic bacterial species.

Table 1. Antimicrobial agents approved for use in U.S. aquaculture in poikilothermic food species. (FDA-CVM, 2007)

Drug (Manufacturer)	Species	Indication	Dosage regimen	Limitations/Comments
Oxytetracycline dihydrate (Terramycin® 200 by Phibro Animal Health)	Pacific salmon	Mark skeletal tissue	250 mg/kg/day for 4 d	--Salmon <30 g In feed as sole ration -- 7 day withdrawal time
	Salmonids	Control ulcer disease, furunculosis, bacterial hemorrhagic septicemia, and pseudomonas disease	2.5 to 3.75 g/100 lb/day for 10 d	--In mixed ration -- Water temperature not below 48.2° F --21 day withdrawal time
	Catfish	Control bacterial hemorrhagic septicemia and pseudomonas disease	2.5 to 3.75 g/100 lb/day for 10 d	--In mixed ration -- Water temperature not below 62° F --21 day withdrawal time
	Lobster	Control gaffkemia	1 g/lb medicated feed for 5 d	--In feed as sole ration --30 day withdrawal time
Oxytetracycline HCl (OxyMarine™ by Alpharma Inc., Soluble Powder-343® by Phoenix Scientific, Inc., and Terramycin-343® by Pfizer, Inc.)	Finfish fry and fingerlings	Mark skeletal tissues	200 to 700 mg oxytetracycline HCl (buffered) per liter of water for 2 to 6 h	
Sulfadimethoxine-ormetoprim (Romet-30® by Roche Vitamins, Inc.)	Salmonids	Control furunculosis	50 mg/kg/d for 5 d	--In feed --42 day withdrawal time
	Catfish	Control enteric septicemia	50 mg/kg/d for 5 d	--In feed --3 day withdrawal time
Florfenicol (Aquaflor® by Schering-Plough Animal Health Corporation)	Catfish	Control of mortality due to enteric septicemia	10 mg/kg/day for 10 consecutive d	--Veterinary Feed Directive (VFD) drug --12 day withdrawal time

Table 1. Antimicrobial agents approved for use in U.S. aquaculture in poikilothermic food species. (FDA-CVM, 2007)

Drug (Manufacturer)	Species	Indication	Dosage regimen	Limitations/Comments
	Salmonids	Control of coldwater disease	10 mg/kg/day for 10 consecutive d	--VFD drug --15 day withdrawal time
Sulfamerazine by Roche Vitamins, Inc.	Rainbow, brook, and brown trout	Control furunculosis	--10 g/100 lb/day for up to 14 d	--In feed --21 day withdrawal time --Not currently marketed

Approval applies only to the specific drug which is the subject of a new animal drug application (NADA); active ingredients from other sources (e.g. bulk drug from a chemical company or similar compounds made by companies other than those specified in the NADA) are not approved new animal drugs.

Approval applies only to use of the drug for the indications and manner specified on the label.

Oxytetracycline

OTC (**Figure 1**) is a broad-spectrum antibiotic widely used in veterinary medicine, partly due to its lower order of toxicity and ability to readily distribute throughout the body and into tissues (Chambers, 2001). OTC and other tetracyclines are primarily bacteriostatic and inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome.

OTC (Terramycin[®], Phibro Animal Health) is approved by the U.S. Food and Drug Administration (FDA) as an oral antibacterial to treat many bacterial infections in animals and humans. It is also approved in aquaculture to treat furunculosis (caused by *Aeromonas salmonicida*) and pseudomoniasis in salmonids at temperatures above 9 °C, and to treat bacterial hemorrhagic septicemia and pseudomoniasis in catfish at temperatures above 16 °C (FDA-CVM, 2007). The FDA-approved dose is 2.50-3.75 g/100 lb (or 55-83 mg/kg) body weight (b.w.)/day for 10 consecutive days, and has a

withdrawal time of 21 d. OTC is also approved for treatment of gaffkemia in lobsters at a dose of 1 g/lb of feed/day for 5 consecutive days, and has a withdrawal time of 30 d. It is probably the most widely used and least expensive antibiotic for treating acute septicemias.

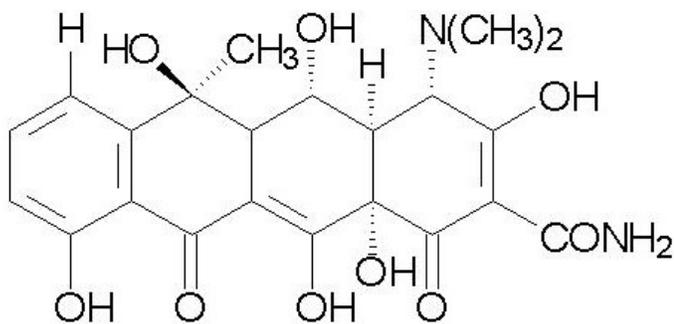


Figure 1. Molecular structure of OTC.

In June 2006, FDA's Center for Veterinary Medicine (CVM) accepted an amended product chemistry package for Phibro Animal Health's new animal drug application (NADA) #038-489 which changed the Terramycin[®] OTC formulation from the mono-alkyl (C8-C18) trimethylammonium or q-salt, to the dihydrate salt. A recent pharmacokinetics study in shrimp compared OTC-medicated feeds prepared with the q-salt form and the dihydrate form. This study showed the OTC q-salt form remained in the environment longer than the OTC dihydrate form (Reed et al., 2006). The authors suggested the q-salt form is probably more detrimental to the environment and fauna than the dihydrate form, and this was likely a strong reason for FDA's change in formulation.

An OTC HCl immersion bath treatment sponsored by Alpharma, Inc. (OxyMarine[™]) was approved by the FDA in 2003 for skeletal marking in all finfish fry and fingerlings. In 2004 and 2005 supplemental NADAs were approved for the same

indication by the FDA for OTC HCl soluble powder-343 (Phoenix Scientific, Inc.) and Terramycin-343 OTC HCl soluble powder, respectively. The FDA-approved dose is 200-700 mg/L of water for 2-6 h. Two label claims for an OTC immersion bath treatment are close to completion which address the control of mortality due to systemic columnaris infections in steelhead trout and systemic coldwater disease in freshwater-reared salmonids (Schnick, 2006).

Sulfadimethoxine-ormetoprim

Sulfadimethoxine-ormetoprim (SDM-OMP, 5:1 ratio) (**Figure 2**) is approved by the FDA as an oral antibacterial to treat furunculosis in salmonids and enteric septicemia in catfish (ESC). Under the trade name Romet-30[®], it is sponsored by Pharmaq AS (Oslo, Norway). The drug combination is also used in an extra label manner in a variety of fish species including the hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) to treat other diseases. The FDA-approved dose in salmonids is 50 mg/kg b.w./day for 5 d with a withdrawal time of 42 d. The same dose is approved in catfish with a withdrawal time of 3 d.

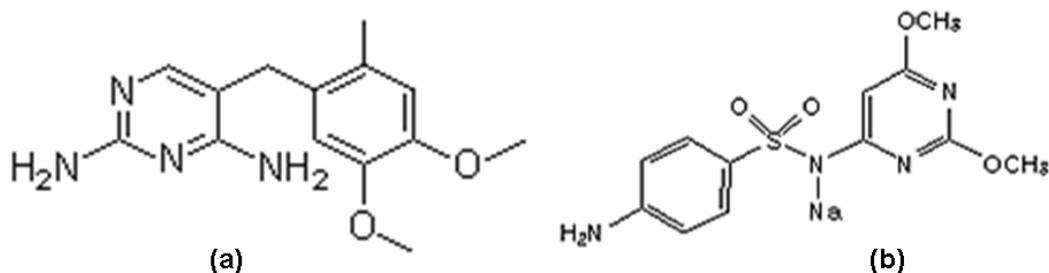


Figure 2. Molecular structure of SDM (a) and OMP (b).

Individually, SDM and OMP are active against a wide array of pathogenic microorganisms. However, when administered in combination, they work synergistically and may lower the incidence of antimicrobial resistance (Bullock et al., 1974). Sulfonamides like SDM are p-aminobenzoic acid (PABA) analogs which competitively inhibit the incorporation of PABA into folic acid. This prevents the synthesis of folic acid and subsequent bacterial growth. OMP serves as a potentiator of the anti-folate effect of sulfonamides by competitive inhibition of dihydrofolate reductase. Together they offer broad spectrum antimicrobial activity which is effective for treating infections in fish caused by *Yersinia ruckeri* (enteric redmouth disease) (Bullock and Snieszko, 1979), *A. salmonicida* (furunculosis) (Bullock et al., 1974), and *Edwardsiella ictaluri* (enteric septicemia in catfish, ESC) (Plumb et al., 1987).

Florfenicol

Florfenicol (**Figure 3**) is a broad-spectrum, primarily bacteriostatic, antibiotic with a range of activity similar to that of chloramphenicol. Florfenicol, however, does not carry the risk of inducing human aplastic anemia that is associated with chloramphenicol. Florfenicol's mode of action is through binding to the 50S ribosomal subunit and inhibition of the transpeptidyl-transferase step in protein synthesis, effective against gram-negative and gram-positive bacteria.

Florfenicol (Aquaflor[®]) is sponsored by Schering-Plough Animal Health Corporation and is approved by the FDA as an oral antibacterial to treat ESC and coldwater disease (*Flavobacterium psychrophilum*) in freshwater-reared salmonids. The FDA-approved dose is 10 mg/kg body weight/day for 10 d, and a withdrawal time of 12 d. Two additional label claims were approved by the FDA in October 2005 for

furunculosis in freshwater-reared salmonids and systemic columnaris disease in freshwater-reared salmonids and catfish (Schnick, 2006).

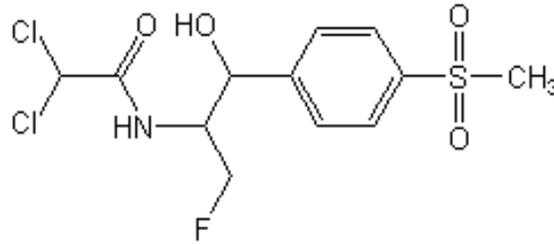


Figure 3. Molecular structure of florfenicol.

Sulfamerazine

Sulfamerazine (**Figure 4**) is approved by the FDA as an oral antibacterial to treat furunculosis in trout. The FDA-approved dose is 10 g/100 lb body weight/day for up to 14 d, and has a withdrawal time of 21 d. It was last sponsored by Roche Vitamins Inc., but because many individuals were substituting a generic "sulfa drug" for sulfamerazine, Roche decided to stop manufacturing sulfamerazine (Cornell University, 2006).

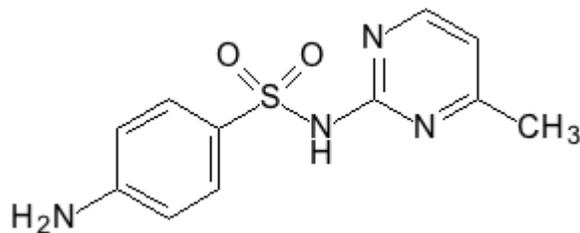


Figure 4. Molecular structure of sulfamerazine.

Other Antimicrobial Agents Used in Aquaculture Worldwide

Drug use in aquatic animal production worldwide varies dramatically. This variation occurs as a consequence of different drug approval requirements and regulatory attention. Differences in the availability and use of certain drug classes between countries can be dramatic. Japan, for example, has 29 individual or combination antibiotics approved for use in aquatic animals (Okamoto, 1992) while Canada has four and the U.S. has three available (Schnick et al., 2005).

Quinolones

Oxolinic acid (**Figure 5**) is a first generation synthetic quinolone antibiotic which possesses excellent activity against many bacterial fish pathogens, especially gram-negative organisms. It is accepted for use in aquaculture in Japan and some countries in Europe, being widely used in Norwegian aquaculture (Grave et al., 1999) to treat numerous diseases including furunculosis. Oxolinic acid is administered orally via medicated feed with a recommended dose for finfish of 12 mg/kg b.w./day for up to 7 d (EMEA, 2005). Samuelsen and Bergh (2004) showed a significantly decreased mortality in fish offered oxolinic acid-medicated feed versus the controls after a *Vibrio anguillarum* immersion challenge.

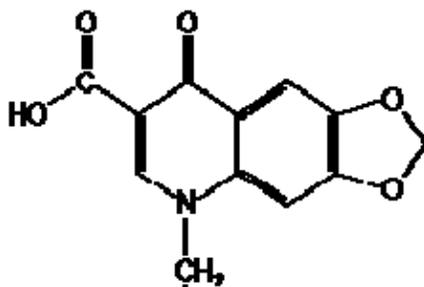


Figure 5. Molecular structure of oxolinic acid.

Flumequine (**Figure 6**), similar to oxolinic acid, is a first generation quinolone active against mainly gram-negative bacteria, and approved for use in Japan and some European countries. Flumequine inhibits DNA-gyrase (Drlica and Zhao, 1997). Vik-Mo et al. (2005) showed efficacy in laboratory trials against an experimental infection caused by *Listonella anguillarum*. Flumequine is also used in shrimp farming to combat vibriosis, however a recommended dosage has not been determined (Joint FAO/WHO Expert Committee on Food Additives, 2006).

Other quinolone antimicrobials like nalidixic acid and piromidic acid are approved in Japan, but very little is known regarding the recommended dose, pharmacokinetics, withdrawal times, or extent of their use (Jarobe et al., 1993; Uno et al., 1992; Katae et al., 1979a; Katae et al., 1979b).

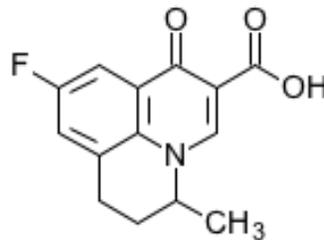


Figure 6. Molecular structure of flumequine.

Beta-lactams

Amoxicillin (**Figure 7**) is the most commonly used beta-lactam antibiotic in aquaculture worldwide. It is approved for use in Japan and some European countries (Schnick et al., 2005) to treat furunculosis and other bacterial diseases. The recommended dose is 40-80 mg/kg b.w. per day for 10 consecutive days (Roberts and Shepherd, 1997). Amoxicillin inhibits cell wall synthesis by preventing peptidoglycan

cross-linkage, thus it is primarily active against gram-positive bacteria. In mammalian medicine, amoxicillin is administered with clavulanic acid to decrease its susceptibility to degradation by beta-lactam producing bacteria and to increase the spectrum of action against gram-negative bacteria. However, this has not yet been reported in the aquaculture literature. Amoxicillin is the drug of choice within the class because it is better absorbed following oral administration, than other beta-lactam antibiotics (i.e., ampicillin).

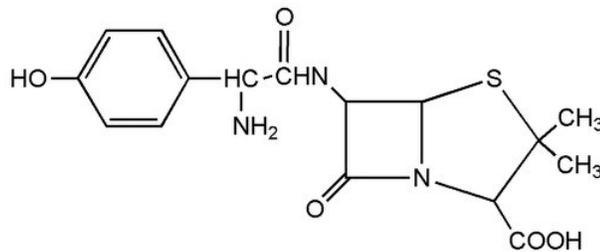


Figure 7. Molecular structure of amoxicillin.

Sulfonamides and Potentiated Sulfonamides

In addition to the frequently used SDM-OMP product, other sulfonamides and potentiated sulfonamides are approved for use overseas. Sulfamonomethoxine, sulfisozole, and SDM are all approved for use in Japanese aquaculture. Sulfamerazine is approved for use in some countries in Europe. Sulfadiazine-trimethoprim (Tribrissen[®]) and sulfamonomethoxine-OMP are used in Europe, Canada, and Japan. Romet-30[®] and these other potentiated sulfonamides have shown potent activity against several fish pathogenic species including *A. salmonicida* subsp. *salmonicida*, *V. anguillarum*, and *V. salmonicida* (Samuelsen et al., 1997; Hoie et al., 1992).

Phenicol

Florfenicol is commonly used in Japan, Canada, and Europe to treat furunculosis, columnaris and other diseases. Cyanphenicol and thiamphenicol are members of the phenicol family and are approved for use in Japan. Despite being banned from use in aquaculture producing countries in Asia and Southeast Asia, chloramphenicol has been detected in farmed shrimp imported into the European Union. Chloramphenicol is on the FDA list of prohibited substances for extra-label use in all food-producing animals due to its link to human aplastic anemia, intestinal problems and neurological reactions (FDA, 1996). The European Union established a minimum required performance limit (MRPL) for this compound of 0.3 µg/kg in tissues from food animals (EUROPA, 2007).

Pharmacokinetics of Antimicrobial Agents Commonly Used in Aquaculture in the United States

Pharmacokinetics

Pharmacokinetic data describe the rate and extent of systemic drug exposure following product administration to the target animal species. Pharmacokinetics involves the kinetics of drug absorption, distribution, metabolism, and elimination. Absorption of antimicrobial agents range from 0 to 100%, and is the process by which the compound transfers from the site of administration (intravenous, intramuscular, oral, topical, intraarterial, etc.) into the systemic circulation (central compartment) (Gerding et al., 1996). Intravenous or intramuscular administration of most antimicrobials results in a percentage bioavailability of 100%. However, absorption after oral administration is always less than 100%. Bioavailability refers only to the extent of absorption, and

provides no indication of the rapidity of absorption or the degree of protein binding.

Rates of absorption from the gastrointestinal tract can be affected by pH changes, the pKa of the drug, gastric emptying time, and contents of ingested material.

Drug distribution refers to the transfer of drug from one location to another within the body and influences the systemic concentration. Usually, the lesser amount of drug distributed the greater amount present in the systemic concentration, and vice versa. Distribution is independent of the mode of administration. The rate and extent of distribution is determined by the rate of delivery to tissues (i.e., blood perfusion rate), ability of the drug to pass from the systemic circulation to tissue sites (i.e., diffusion), and affinity of the drug to proteins in plasma, serum or tissues (i.e., binding or bioavailability).

The liver is the primary organ where enzymatic metabolism occurs. Metabolic reactions include oxidation, reduction, and hydrolysis (Phase I CYP450 enzymes) and glucouronidation, methylation, and sulfation (Phase II conjugative enzymes). These reactions result in more polar compounds which help facilitate their elimination.

Drug elimination is defined as the irreversible removal of drug from the body by all routes of elimination. The kidney is the main excretory organ for the removal of metabolic waste products (and drugs). The degree of lipid solubility and extent of ionization in blood determines how much drug will be excreted by the kidneys. In humans, the kidneys receive approximately 25% of the cardiac output which is the same as the liver. Biliary excretion by the liver of products of biotransformation is another route of elimination present in almost all vertebrates. In addition, the bile facilitates the absorption of ingested lipids and serves as a major route for cholesterol elimination.

Oxytetracycline

For many years researchers have investigated the absorption, distribution, metabolism, and elimination profiles of OTC in various fish and shellfish species (Uno et al., 2006; Reed et al., 2006; Chen et al., 2004; Rigos et al., 2004; Wang et al., 2004; Coyne et al., 2004b; Bernardy et al., 2003; Haug and Hals, 2000; Abedini et al., 1998; Doi et al., 1998; Du et al., 1997).

Absorption. Tetracyclines are known for their poor absorption from the gastrointestinal (GI) tract, with OTC categorized as intermediate meaning when the stomach is empty 60-80% of an oral dose is absorbed. OTC absorption in mammals is decreased in the presence of dairy products, calcium, magnesium, and iron or zinc salts in the gastrointestinal tract. Divalent and trivalent cations bind OTC decreasing its antimicrobial activity (Lunestad and Samuelsen, 2001). This can be particularly important in aquaculture when OTC is administered to fish in seawater where cation levels are high (Barnes et al., 1995). Unavoidable contact with seawater occurs when OTC surface-coated feed pellets are offered. Contact can also occur in the upper GI tract of marine teleost fishes that characteristically drink seawater continuously to compensate for water loss. Encapsulation of OTC in the feed pellet may avoid direct drug-cation interaction, however, for absorption to take place the drug must be in the liquid form, thus mixing with cation-rich fluid in the gut is unavoidable.

Few researchers have reported absorption half-lives ($T_{1/2\alpha}$) of OTC in fish following non-intravenous routes of administration. Wang et al. (2001) reported a $T_{1/2\alpha}$ of 2.3 h in orally administered black seabream.

Bjorklund and Bylund (1990) showed absorption of OTC in freshwater fish was faster at higher temperatures. At 16 °C, the maximum plasma concentration (C_{\max}) was reached after only 1 h ($2.1 \pm 0.5 \mu\text{g/mL}$), while at 10 °C and 5 °C the C_{\max} was reached after 12 h ($5.3 \pm 1.7 \mu\text{g/mL}$) and 24 h ($3.2 \pm 1.8 \mu\text{g/mL}$), respectively. Bjorklund and Bylund explained the effect of temperature was due to the fact that fish are poikilothermic, that is their internal temperature varies, often matching the ambient temperature of the immediate environment (Bjorklund and Bylund, 1990). Further, an increased ambient and internal temperature should correlate with increased gastric emptying and metabolism in poikilothermic fish species. Therefore, for many farmed fish species, the withdrawal times, based on temperature dependent residue levels, are determined in degree days (Alderman, 2000). For instance 150° days for OTC would represent a withdrawal period of 15 d at 10 °C or of 10 d at 15 °C.

Distribution. Few studies have investigated the distribution phase of OTC, with regard to the diffusion of OTC from the systemic circulation into fish tissues and body spaces (peripheral compartments). These researchers primarily used the intravenous route of administration. Black et al. (1991) calculated a rapid distribution half-life of only 0.9 h after a single intravenous bolus dose in rainbow trout. Similarly, Rigos et al. (2003a) calculated a distribution half-life of only 2 h in gilthead sea bream. Interestingly, there does not appear to be much of a difference in OTC distribution half-lives in fish and those reported in humans (Gerding et al., 1996). The ability of OTC to rapidly distribute into the tissues and body spaces where a given pathogen may be targeted, along with its affordability, is part of the justification for its historically widespread use in aquaculture.

Metabolism. Tetracyclines are not metabolized *in vivo*, but rather are excreted predominantly unchanged in the urine (50-80% of the given dose) (Gerding et al., 1996). Oka et al. (1989) showed that tetracyclines photodecomposed easily in an aqueous solution comparable to a fish pond. Interestingly, Halling-Sorensen et al. (2002) reported other known OTC degradative products to have antimicrobial activity at levels close to that of their parent compounds. These compounds have largely been ignored in quantitative pharmacokinetics studies due to the minimal metabolism *in vivo* of tetracyclines, but may need to be considered in future studies.

Elimination. Results of studies on the elimination phase of OTC from the muscle after oral administration show an obvious decrease in the elimination half-life ($T_{1/2\beta}$) in higher water temperatures. In muscle, $T_{1/2\beta}$ values ranged from 600 h in brook trout at 7 °C (Herman et al., 1969) to 46 h in perch at 20 °C (Wang et al., 2004). Similarly, reported values of $T_{1/2\beta}$ in plasma, serum, and whole blood follow the same temperature-dependent trend as observed in muscle. Haug and Hals (2000) calculated a $T_{1/2\beta}$ of 578 h in plasma of arctic char held at 6 °C in freshwater. Whereas Rigos et al. (2004) calculated a $T_{1/2\beta}$ of 21 h in plasma of sea bass held at 22 °C in saltwater. Poikilothermy is again the probable cause of piscine ambient temperature-dependent changes in OTC elimination. We can find a few inconsistencies in the literature, however. Jacobsen (1989) calculated an estimated $T_{1/2\beta}$ of 48 h from a study conducted in rainbow trout held at 12 °C. This value is more than 400 h less than that observed in another study conducted in rainbow trout held at 11 °C (Abedini et al., 1998). Despite these inconsistencies, and drastic differences in $T_{1/2\beta}$, mandatory temperature-specific

withdrawal times (**Table 1**) have been established for orally administered OTC (FDA-CVM, 2007).

Sulfadimethoxine-ormetoprim

Pharmacokinetic parameters have been determined individually for SDM and OMP in rainbow trout (Droy et al., 1990; Kleinow and Lech, 1988) and channel catfish (Michel et al., 1990; Squibb et al., 1988) using intravenous and oral administration. Pharmacokinetic investigations of the two drugs (Romet[®]) administered orally together have been conducted in hybrid striped bass (Bakal et al., 2004), Atlantic salmon (Samuelson et al., 1995), channel catfish (Milner et al., 1994), chinook salmon (Walisser et al., 1990), and rainbow trout (Droy et al., 1989). As with many different pharmacokinetic assessments in fish species, researchers have used several different water temperatures making direct comparisons of data problematic. Temperature-related differences have significant impacts on absorption and excretion kinetics even within the same species (Borgan et al., 1981).

Absorption. Bakal et al. (2004) provided the most complete investigation into the absorption of SDM and OMP when administered in combination. After IP injection in hybrid striped bass at a ratio of 5:1, SDM to OMP, the $T_{1/2\alpha}$ was 5.4 h and 0.7 h respectively. After oral administration the $T_{1/2\alpha}$ was 3.9 h and 0.2 h respectively. The slower absorption rate after IP administration compared to oral administration may be attributable to the increased surface area enhanced by the peristaltic movements of the GI tract. Also SDM is more soluble in an acidic environment which could allow for more rapid uptake of the drug from the stomach.. Bakal et al. also calculated a low bioavailability (4.6%) for SDM indicating poor absorption from the GI tract of hybrid

striped bass. Although the 5:1 ratio of SDM-OMP is commonly used in feed formulations, Bakal et al. found it does not represent the actual drug ratio found in the plasma or serum of animals.

Due to the differing absorption rates described above, this drug combination does not exist in a constant ratio within the animal. To complicate the issue even further, *in vitro* susceptibility determinations are typically conducted using a ratio of 20:1 (Miller et al., 2005) which has been proven to be the optimal ratio of synergism of sulfonamides and their potentiators (Mandell and Sande, 1990).

Bakal et al. (2004) proposed using the total amount of the drug actually absorbed (area under the concentration-time curve, AUC) in the ratio calculation. This allowed the more close approximation of the average ratio of the drugs in the animal. After oral administration in the hybrid striped bass, Bakal et al. calculated a ratio of 2.14:1 (SDM:OMP) based on the AUC for each compound. The relationship of these *in vivo*-derived ratios to the ratios used in *in vitro* susceptibility testing is still unclear. How *in vivo*-derived ratios may be used to help predict therapeutic efficacy for a pathogen with a different MIC ratio also remains to be seen.

Samuelsen et al. (1995) aimed to investigate the bioavailability and pharmacokinetics of SDM and OMP in combination in Atlantic salmon held in 10 °C seawater. They showed a considerably higher bioavailability of 39% for SDM and 89% for OMP. Compared to the 4.6% calculated by Bakal et al. (2004) in hybrid striped bass held in 16-17 °C freshwater, this exhibits the inconsistency of data present in the literature.

Distribution. Several researchers have investigated the distribution into tissues of SDM and OMP when administered alone in aquatic animal species. Kleinow and Lech (1988) and Squibb et al. (1988) reported comprehensive pharmacokinetics, low protein binding, and wide distribution of radiolabelled SDM following oral and intravenous administration in rainbow trout and channel catfish, respectively. However, only Samuelsen et al. (1995) monitored rainbow trout tissues for the presence of the two compounds after simultaneous administration. They found the SDM and OMP volumes of distribution at steady state to be 0.39 and 2.48 L/kg, respectively. This suggests a much wider distribution of OMP into the tissues outside the plasma than what was seen with SDM. The distribution volume for SDM was similar to that reported previously in experiments involving the single compound, 0.422 L/kg (Kleinow and Lech, 1988), 0.622 L/kg (Squibb et al., 1988), and 0.40 L/kg (Michel et al., 1990). Considerably higher distribution volumes have been reported by researchers for OMP, 4.854 in rainbow trout (Droy et al., 1990) and 5.503 L/kg in catfish (Plakas et al., 1990). Samuelsen et al. (1995) also noted the kidney had the highest OMP concentration of any organ tested. In salmonids, the kidney contains cells rich in melanin (melanomacrophages). They postulated OMP may bind to the melanin in these cells and could explain the high concentration and prolonged $T_{1/2\beta}$ of OMP in kidney compared to the other organs.

Metabolism. Uno et al. (1993) showed both SDM and sulfamonomethoxine were metabolized by rainbow trout to the N_4 -acetylated conjugate which was the major metabolite, and the N_1 -glucuronide conjugate and N_4 -acetyl- N_1 -glucuronide double conjugate which were present in lesser amounts. Kleinow et al. (1992) monitored SDM

and N₄-acetylated SDM in several tissues and plasma of rainbow trout. They found 20 h after administration the majority of compound in the plasma was the parent SDM. Conversely, N₄-acetylated SDM predominated in the bile. Slightly higher levels of N₄-acetylated SDM were found in the liver. These findings agree with Squibb et al. (1988) who reported that approximately 90% of the biliary SDM occurred as N₄-acetylated SDM in catfish. These findings also support the view that liver enzymes are instrumental in the extraction of the N₄-acetylated SDM compound from the parent SDM.

Plakas et al. (1990) found several unidentified OMP metabolites in the urine of catfish.

Elimination. Samuelsen et al. (1995) showed a fairly rapid elimination for both SDM and OMP, as the elimination half-life, T_{1/2β}, was 9.9 and 25.6 h, respectively. In channel catfish T_{1/2β} for SDM and OMP was found to be 12.6 and 12.8 h (Michel et al., 1990; Squibb et al., 1988), whereas Droy et al. (1990; 1989) calculated T_{1/2β} for SDM and OMP in rainbow trout to be 16.1 and 17.5 h, respectively.

Florfenicol

Florfenicol is a relatively new antimicrobial agent to aquaculture, when compared to OTC and SDM-OMP which have been used for decades in aquaculture worldwide. Researchers only began studying the pharmacokinetics of florfenicol in fish in the mid-1990's. Pharmacokinetics investigations of florfenicol have been conducted in Atlantic salmon (Horsberg et al., 1996; Horsberg et al., 1994; Martinsen et al., 1993), cod (Samuelsen et al., 2003), channel catfish (Wrzesinski et al., 2006), rainbow trout (Pinault et al., 1997), and koi and three spot gourami (Yanong and Curtis, 2005).

Absorption. Horsberg et al. (1996), Martinsen et al. (1993), and Samuelsen et al. (2003) showed florfenicol absorption after oral administration to be fast and complete with T_{max} and bioavailability values of 6 h and 99%, 10.3 h and 96.5%, and 7 h and 91%, respectively. After oral administration Yanong et al. (2005) estimated a $T_{1/2\alpha}$ in koi and three-spot gourami to be 1.4 h and 0.6 h, respectively. After intramuscular administration they calculated a $T_{1/2\alpha}$ of 3.5 and 0.1 h, respectively.

Distribution. Virtually identical volumes of distribution at steady state were observed by Horsberg et al. (1996) (1.12 L/kg) and Martinsen et al. (1993) (1.32 L/kg) in Atlantic salmon, and Samuelsen et al. (2003) (1.1 L/kg) in cod. These values indicate florfenicol distributes throughout the body in both species and suggests tissue concentration may be similar to those found in plasma.

Metabolism. Metabolism studies of florfenicol have identified florfenicol amine as the major metabolite in muscle tissue although florfenicol parent is more predominant in skin (FDA-CVM, 2005). Florfenicol amine lacks antibacterial activity, but serves as the marker residue. From 48 h after administration and throughout a study by Horsberg et al. (1996) florfenicol amine was found in higher concentrations in Atlantic salmon plasma than florfenicol. Samuelsen et al. (2003) noted a considerable difference in the $T_{1/2\beta}$ of florfenicol after oral administration in cod (39 h), when compared to that observed by Horsberg et al. in Atlantic salmon (14.7 h). A negligible temperature difference was cited as a possible explanation however, no florfenicol amine was detected in either plasma or tissues of cod. The apparent lack of this metabolic pathway in cod may have contributed to the slower elimination of florfenicol and its amine in cod than in Atlantic salmon. However, such a difference in $T_{1/2\beta}$ has not been shown in

previous studies with cod and other antimicrobial agents (Samuelsen et al., 2000; Elema et al., 1994; Rogstad et al., 1993).

Metabolism of florfenicol presumably occurs in the liver but this has not been shown experimentally.

Elimination. With the exception of the longer $T_{1/2\beta}$ of cod described above, florfenicol elimination is quite rapid compared to most other antimicrobial agents used in aquaculture. Martinsen et al. (1993) and Horsberg et al. (1996) calculated $T_{1/2\beta}$ in Atlantic salmon to be 12.2 and 14.7 h, respectively. The rapidity of absorption and subsequent elimination in most species, coupled with the general lack of potential of antimicrobial resistance impacting human health (due to the scant use of structurally related compounds thiamphenicol and chloramphenicol) has contributed to the attractiveness of florfenicol in aquaculture.

Antimicrobial Susceptibility Testing

Current Status of Methods

When veterinarians decide whether or not to treat fish with antimicrobial agents, they must consider the anticipated pharmacokinetics of the antimicrobial agent in the target fish species under the given conditions (i.e., water temperature, salinity, hardness). They must also choose an antimicrobial agent that is effective against the disease. A well-controlled and standardized antimicrobial susceptibility test (AST) is the best means to obtain this information. A critical component of an AST method is its ability to accurately predict a clinical outcome following treatment based on the AST result. In other words, does a likely susceptible *in vitro* AST result automatically mean therapy will be efficacious? Conversely, does a likely resistant *in vitro* test result mean therapy will

not be efficacious? The purpose of an *in vitro* test is not to mimic *in vivo* conditions, rather provide reproducible results which can be used to predict clinical outcome (CLSI/NCCLS, 2007b).

Three reproducible AST methods are currently available for use in veterinary medicine. Agar and broth dilution susceptibility tests result in a minimal inhibitory concentration (MIC) for a single bacterial isolate and provide the most clinical relevance, where the MIC may be directly related to an achievable tissue or plasma concentration *in vivo*. Disk diffusion susceptibility tests yield diameters of the zone of inhibition which provide no correlation with achievable concentrations *in vivo*, but are simple and very affordable tests to run. In recent years, the E-test has gained popularity as a simple diffusion-based susceptibility test resulting in an MIC shown to yield virtually identical results as the more traditional broth microdilution tests (Luber et al., 2003).

Prior to 2001 aquatic animal disease researchers commonly used AST methods and clinical breakpoint values (susceptible, intermediate, and resistant) developed in their own laboratories. Different methods and breakpoint values prevented accurate inter-laboratory comparisons and correlation to clinical cases. In 2001 a draft set of protocols for AST methods for aquatic bacterial pathogens was published by Alderman and Smith (2001). These protocols were adopted by many fish health laboratories (Smith, 2005), however there were no standardized AST methods with quality control (QC) organisms and parameters. It was not until 2003 that a Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards, NCCLS) disk diffusion AST method was standardized and QC organisms and parameters established (CLSI/NCCLS, 2006a; Miller et al., 2003). Shortly thereafter in 2005, a CLSI/NCCLS

broth microdilution AST method was also standardized and QC parameters established (**Chapter 3**) (CLSI/NCCLS, 2006b; Miller et al., 2005). These standardized methods apply only to non-fastidious aquatic pathogens (Group 1 bacteria) that grow well on and in unsupplemented Mueller-Hinton media. These pathogens include members of *Enterobacteriaceae*, non-psychrophilic (grow best at temperatures >20°C) *Aeromonas* spp., *Pseudomonas* spp., *Plesiomonas shigelloides*, *Shewanella* spp., and non-obligate halophilic members of *Vibrionaceae*.

Interpretive Criteria

How does one decide if an isolate is susceptible, resistant, or intermediate based on a single AST result? The data obtained from the test, MIC or zone diameter, must be interpreted based on potential clinical effect. These interpretive criteria or breakpoints are determined by a number of considerations.

MIC clinical breakpoint values are determined from three main sources of information (CLSI/NCCLS, 2007a):

1. Pharmacokinetic and/or pharmacodynamic studies of the antimicrobial agent in the target animal species used to determine the likelihood of achieving a concentration of the drug at the target site.
2. Historical clinical outcomes are correlated with MICs of clinical isolates evaluated.
3. The antimicrobial agent's MIC distribution for the pathogen is examined to develop epidemiologic cutoffs.

Zone diameters from disk diffusion tests can also be used to interpret an isolate's level of susceptibility. Zone diameter clinical breakpoints are determined primarily from

large AST data distributions where each isolate's diameter of zone of inhibition is plotted on the x-axis and its MIC is plotted on the y-axis. Regression analysis has often been used to suggest appropriate zone diameter breakpoint values, but is dependent upon a fairly even distribution of organisms at each MIC tested, particularly in the range of the intermediate MIC ± 2 to 3 two-fold dilutions (Fuchs et al., 2002). With many of the newer antibiotics (i.e., florfenicol) however, resistant organisms are rare, and the MIC distribution is heavily weighted toward very susceptible MICs, resulting in an unreliable regression line. To overcome this problem the error rate-bounded method of Metzler and DeHaan (1974) as modified by Brunden et al. (1992) is commonly used to select disk diffusion clinical breakpoints, and has been accepted by the CLSI (CLSI/NCCLS, 2007a).

Historically in the U.S., the CLSI as an independent standard-setting organization, reviewed data submissions and approved breakpoint values. These breakpoint values were often used by FDA's Center for Veterinary Medicine (CVM) on the product label. Since clinical breakpoints for animal drugs affect safety and effectiveness of the antimicrobial, CVM makes the final decision about the breakpoint used in an approved product. Agreement upon breakpoint values between the two organizations is desirable but not obligatory.

By definition a clinical breakpoint is the classification of projected clinical outcome of patient treatment based on the causative microorganism's *in vitro* response to an antimicrobial agent relative to the exposure to that agent that is attainable using the labeled dose regimen for the target animal species for that type of infection and infecting organism (CLSI/NCCLS, 2007a). Thus, the bacterial species- and target animal species-

specificity of these values provides one a level of reliability in these values. It is also important to note that interpretive criteria apply only if the laboratory has conducted AST according to specific standardized methods.

Currently, there are no universally accepted clinical breakpoint values available for any antimicrobial agent or fish pathogen in any fish species. Reimschuessel et al. (2005) published a searchable database (Phish-Pharm) from hundreds of research publications that investigate the pharmacokinetic profiles of numerous compounds in multiple fish species and water conditions. Meta-analyses of data taken from this database, along with *in vitro* MIC and zone diameter distributions for a given bacterial pathogen (Miller and Reimschuessel, 2006; Smith and Hiney, 2005; Coyne et al., 2004a; Tsoumas et al., 1989) can be used to help determine clinical breakpoints in aquaculture. Additional investigations are also needed to determine actual pharmacokinetic/ pharmacodynamic (PK/PD) index targets (i.e., $T_{>MIC}$, AUC/MIC, C_{max}/MIC) for a given drug against a bacterial pathogen in order to predict clinical efficacy and prevent the emergence of resistance.

The studies described in this dissertation are the foundation for developing such breakpoints for the fish pathogen *A. salmonicida*. This work required developing standardized AST methods for aquatic pathogens. These were used to develop epidemiologic cutoff values from AST data distributions. New microbiological and high-performance liquid chromatography methods were developed to detect OTC in fish serum. These methods were used to provide pharmacokinetic and pharmacodynamic data for two OTC feed formulations. In addition, pharmacokinetic data was obtained from

both infected and healthy fish to correlate with clinical outcome and the susceptible MIC epidemiologic cutoff value.

Chapter 2: Antimicrobial Resistance Mechanisms and Susceptibility Testing of Aquatic Isolates

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Introduction

Advances in aquatic animal medicine have increased the number of antimicrobial therapeutants available for use in aquaculture. Antimicrobials used in worldwide aquaculture are listed in **Table 2**. Some of these compounds are used almost exclusively in aquatic animal medicine, while others are also important in human medicine. With antimicrobial use comes the risk of exposed bacterial populations becoming resistant to those drugs. Concerns that fish pathogens and environmental microorganisms could become resistant and then transfer their resistance genes to human pathogens have been expressed by a number of agencies and researchers (FDA-CVM, 2003; Angulo, 2000; Moll et al., 1999; Aoki, 1997; Kruse and Sorum, 1994). Several studies have identified antimicrobial resistant microorganisms in human and veterinary medicine (Schroeder et al., 2002a; Schroeder et al., 2002b; Threlfall et al., 2000), in addition to environmental isolates acquired from aquaculture settings (Huys et al., 2001; Guardabassi et al., 1999; Nygaard et al., 1992; McPhearson et al., 1991). Other studies have identified transmissible resistance genes in aquatic pathogens (Petersen et al., 2000; Guardabassi et

al., 2000b; Rosser and Young, 1999; Adams et al., 1998; Dahlberg et al., 1997). The resistance genes from aquatic isolates have similar genetic sequences as a number of resistance genes found in mammalian isolates. The mechanism of action of the aquatic resistance genes also parallel those found in mammalian isolates.

Table 2. Antimicrobial agents used in global aquaculture.

Antimicrobial agent	Bacterial disease controlled
Amoxicillin	Furunculosis, rickettsial infection, coldwater disease
Ampicillin	Coldwater disease, streptococcosis
Bicozamycin	
Chloramphenicol	Bacterial fin erosion, carp erythrodermatitis, columnaris, enteric redmouth, furunculosis, haemorrhagic septicaemia, pasteurellosis, ulcerative dermatitis, vibriosis
Chlortetracycline	Coldwater disease, saltwater columnaris
Clindamycin	Bacterial kidney disease
Doxycycline	Mycobacteriosis, streptococcosis
Erythromycin	Bacterial kidney disease, streptococcosis
Florfenicol	Furunculosis
Flumequine	Furunculosis, vibriosis, enteric redmouth
Furanace	Bacterial fin erosion, coldwater disease, columnaris, gill disease, haemorrhagic septicaemia, vibriosis
Furazolidone	Carp erythrodermatitis, furunculosis, vibriosis
Josamycin	
Kanamycin	Bacterial fin erosion, haemorrhagic septicaemia, mycobacteriosis, vibriosis
Kitasamycin	
Lincomycin	
Minocycline	Mycobacteriosis
Myroxacin	
Nalidixic acid	
Nitrofurantoin	Vibriosis
Novobiocin	
Oleandomycin	
Oxolinic acid	Columnaris, enteric redmouth, furunculosis, haemorrhagic septicaemia, vibriosis

Table 2. Antimicrobial agents used in global aquaculture.

Antimicrobial agent	Bacterial disease controlled
Oxytetracycline	Acinetobacter disease, bacterial fin erosion, carp erythrodermatitis, coldwater disease, columnaris, edwardsiellosis, emphysematous putrefactive disease, enteric redmouth, enteric septicaemia, furunculosis, gill disease, haemorrhagic septicaemia, redpest, salmonid blood spot, saltwater columnaris, streptococcosis, ulcerative dermatitis, pseudomonas disease, gaffkemia
Penicillin dihydrostreptomycin	
Penicillin G	Bacterial kidney disease
Piromidic acid	
Rifampin	Mycobacteriosis
Spiramycin	Bacterial kidney disease
Streptomycin	Haemorrhagic septicaemia, mycobacteriosis
Sulfadiazine-trimethoprim	Enteric redmouth, furunculosis, <i>Plesiomonas shigelloides</i> infection, vibriosis
Sulfadimethoxine-ormetoprim	Furunculosis, enteric septicemia of catfish
Sulfamerazine	Bacterial kidney disease, coldwater disease, columnaris, enteric redmouth, furunculosis, haemorrhagic septicaemia
Sulfamethazine, sodium salt	Bacterial kidney disease, coldwater disease, columnaris, furunculosis, vibriosis
Sulfamonomethoxine	
Sulfamonomethoxine-ormetoprim	
Sulfisoxazole	Bacterial kidney disease, coldwater disease, columnaris, enteric redmouth, furunculosis, haemorrhagic septicaemia
Sulfonamides	Nocardiosis
Tetracycline	Carp erythrodermatitis, columnaris, furunculosis, streptococcosis
Thiamphenicol	
Tiamulin	Enteric redmouth

Information obtained from Austin and Austin (1987) and Schnick (2006).

To reduce the potential of selecting for resistant bacteria in the aquatic environment, it is essential to use antimicrobial agents judiciously (National Aquaculture Association Board of Directors, 2003). The objective of antimicrobial therapy in all animals should be to provide a drug that is biologically active against the target pathogen,

at the appropriate concentration at the site of infection for the appropriate length of time (Lees and Aliabadi, 2002). In other words, appropriate antimicrobial chemotherapy involves optimizing dosing regimes (pharmacokinetics) with antibacterial activity at the site of infection (pharmacodynamics). There are great challenges to conducting pharmacokinetic and pharmacodynamic (PK/PD) studies in the aquatic animal, as evidenced by the limited amount of data in this area. Nevertheless, the basic principles of judicious use of antimicrobials apply to aquatic animal medicine, just as it applies to veterinary medicine in general, and to human medicine (National Aquaculture Association Board of Directors, 2003; AVMA Executive Board, 2002).

When using antimicrobials it is essential to ascertain that the drug being considered will be effective in treating a particular disease causing agent. This may require antimicrobial susceptibility testing (AST) of the microorganism in question. Such testing is well standardized for mammalian isolates in protocols such as those described by the National Committee for Clinical Laboratory Standards (NCCLS). Conducting AST on bacteria from the aquatic environment is not well established and is complicated by the fact that most of the isolates from aquatic animals, and their environment, require or prefer growth conditions different from those isolates obtained from homeothermic hosts. Further, researchers have diverged from the NCCLS standardized AST protocols when evaluating the antimicrobial susceptibility of aquatic microflora. Many of these deviations include studies which have inappropriately used NCCLS-approved interpretive criteria (susceptible and resistant breakpoints) for testing at 35°C when testing isolates at 22°C. Other studies have incubated isolates for a time that was 'growth dependent'. This indicates that there was no uniform incubation time for the isolates tested. Another

inappropriate practice has been using arbitrary zone diameters, or zones of inhibition of any substantial size, as the criteria for determining if an organism is resistant or susceptible to a given drug. The usefulness of such data is questionable and also negates the ability to reliably compare data between laboratories. In other words, a poorly controlled antimicrobial susceptibility test, based on laboratory-specific methods could cause the attending veterinarian to prescribe antimicrobial agents that are clinically ineffective.

A major factor contributing to the lack of interlaboratory reproducibility of AST results of bacterial pathogens isolated from aquatic species has been the lack of a standardized testing method including quality control (QC) organisms and QC ranges for AST of isolates requiring temperatures lower than those already standardized by the NCCLS (CLSI/NCCLS, 2007b). In addition to QC parameters, resistant and susceptible breakpoints need to be defined for the bacterial pathogens that are unique to aquatic animal species, whose growth preferences have not already been addressed by the current NCCLS methods (CLSI/NCCLS, 2007b). In other words, an isolate determined to be susceptible using breakpoints established by testing isolates from a warm-blooded animal at 35°C, does not necessarily correlate with clinical efficacy in the aquatic animal host. While several studies have been conducted to determine the susceptibility of aquatic bacterial pathogens to antimicrobial agents few attempts have been made to determine the criteria for defining resistance in the aquaculture setting. Bruun et al. (2000) tried to define resistance based on three criteria: minimum inhibitory concentration (MIC) values alone; PK data and MIC values; and an “*in vivo* resistance” definition, which asked if it was possible to treat an infection under similar conditions to those found on an

aquaculture farm? These three basic definitions are the major components, discussed in the NCCLS guideline M37-A (CLSI/NCCLS, 2007a). The NCCLS document gives a thorough explanation of each criteria, and specific examples of the types of data needed for determining resistant, intermediate, and susceptible phenotypes in a given group of bacteria. These criteria involve first identifying the appropriate testing conditions and then identifying the appropriate QC organisms and corresponding QC ranges. Using appropriate QC strains, the *in vitro* activity of the drug against the target pathogen is then determined for several hundred bacterial pathogens. In addition, the target animal PK-PD data is generated and is correlated with the results of clinical studies. When analyzed in its entirety these components are then used in developing interpretive criteria for bacterial pathogens in the targeted animal species, including aquaculture species. In aquaculture, research is needed to determine resistant, intermediate, and susceptible breakpoints for AST of bacterial isolates that grow at lower temperatures. Such information will help determine the efficacy of different antimicrobials for susceptible aquatic isolates, and will facilitate the clinician's choice of an appropriate antimicrobial. This data will also help researchers and diagnosticians monitor the development of resistant microorganisms in the aquatic environment.

Antimicrobial Resistance in the Aquatic Environment

Why is there a need to monitor changes in resistance?

Antimicrobial agents are known to accumulate in the environment (i.e., sediments, water column). This accumulation arises from various sources including uneaten medicated feed and from urine and fecal excretions. Repeated exposure of aquatic microflora to an agent has been shown to increase the likelihood of selecting for a

bacterial population that has acquired resistance characteristics. Several studies have shown an increase in the number of resistant bacteria obtained from the sediments, the water column, and animal species following antimicrobial treatment (Petersen et al., 2002; Chee-Sanford et al., 2001; Guardabassi et al., 2000a; Guardabassi et al., 1999; DePaola et al., 1995; Nygaard et al., 1992; Sandaa et al., 1992; McPhearson et al., 1991).

There is also concern that antimicrobial resistance genes can be transferred from aquatic microflora to human pathogens (Angulo, 2000; Garrett et al., 1997). For example, Sorum (1998) described the *in vitro* transfer of a resistance or R-plasmid from an atypical *Aeromonas salmonicida* isolate to *Escherichia coli*, *Salmonella enterica* serovar Enteritidis, and *S. enterica* ser. Typhimurium. Thus, concern has also been voiced regarding terrestrial farm antimicrobial usage (Padungton and Kaneene, 2003; Schroeder et al., 2002a).

Conversely, an increase in resistance in resident bacterial populations in the aquatic setting has occurred following exposure to drugs from human sources such as hospital sewage. Huys et al. (2001) studied the relationship between antimicrobial “tolerance” and taxonomic diversity among oxytetracycline-resistant heterotrophic bacterial populations found in hospital sewage and in freshwater aquaculture water samples. They found several “tolerant” genera in both sites tested, however the isolates originating from the hospital sewage site exhibited higher frequencies of “tolerance” to two of the drugs tested, ampicillin and kanamycin.

In order to reduce the introduction of antimicrobials into receiving waters from aquaculture farms, some commercial suppliers of aquaculture products, university researchers, and fish farmers are developing innovative intensive indoor closed culture

systems for some fish species traditionally raised in outdoor pond or cage settings (Losordo et al., 1999; Losordo et al., 1998; Timmons and Losordo, 1994). These recirculating systems reduce the potential for fish to be exposed to drugs and pathogens commonly found in agricultural and urban runoff.

Resistance Mechanisms and Associated Genes Identified in Aquatic Bacteria

In the last 20 years, several studies have demonstrated that similar antimicrobial resistance genes are present in bacterial isolates from the aquatic environment and from warm-blooded animals (Petersen et al., 2002; Schmidt et al., 2001a; Kim and Aoki, 1993). Genes that code for antimicrobial resistance may be acquired in one of three ways: plasmids or transposons, integrons, or by chromosomal mutation. Resistance genes have been identified in many aquatic pathogens including *A. salmonicida* (Schmidt et al., 2001a; Schmidt et al., 2001b; Oppegaard and Sorum, 1994), *Yersinia ruckeri* (Klein et al., 1996), and *Vibrio salmonicida* (Sorum et al., 1992) which are the etiological agents of furunculosis, enteric redmouth, and coldwater vibriosis, respectively.

Acquisition of resistance genes via R-plasmids, has been shown to occur in several fish pathogens including *A. salmonicida* (Schmidt et al., 2001b), *A. hydrophila*, *V. anguillarum*, *Pseudomonas fluorescens*, *Edwardsiella tarda* (Aoki, 1988), *Photobacterium damsela* subsp. *piscicida* (Magarinos et al., 1992) and *Y. ruckeri* (De Grandis and Stevenson, 1985). Plasmids are known to be reservoirs of resistance genes to drugs such as tetracycline, trimethoprim, sulfonamides, and chloramphenicol, used in the aquatic environment (Lewin, 1992). The transfer of R-plasmids is thought to play a major role in the horizontal transfer of resistance in the fish farming environment. These conjugal elements have been found to contain resistance genes encoding resistance to

virtually all antimicrobial agents used in aquaculture (Tran and Jacoby, 2002; Aoki, 1997; Roberts, 1996).

The genes associated with antimicrobial resistance code for a number of different resistance mechanisms (Lewin, 1992). Many of the genes identified in aquatic isolates encode for efflux proteins (i.e., *tet* genes). Efflux proteins, possessed by both Gram-negative and Gram-positive organisms, are energy-dependent membrane associated proteins which export some antimicrobials, including tetracyclines, out of the cell (Roberts, 1996). Schmidt et al. found that 30% of oxytetracycline-resistant aeromonads tested carried one or two of the five tetracycline resistance genes that were examined (Schmidt et al., 2001a). Three of these genes, *tetA*, *tetE*, and *tetD* encode for proteins involved in the efflux pump. Most tetracycline resistance genes in aquatic isolates such as *A. hydrophila*, *E. tarda*, *V. salmonicida*, and *P. piscicida* have been found on plasmids or transposons (Guardabassi et al., 2000b; Sorum et al., 1992; Aoki and Takahashi, 1987). Transposons are mobile genetic elements capable of site-specific recombination with assistance from transposase enzymes.

Other gene complexes encoding for plasmid-mediated antimicrobial resistance in aquatic pathogens include *dhfr*, *sul*, and the chloramphenicol transferase (CAT) enzymes. Studies of *dhfr* have shown that the gene is present on plasmids and transposons (Adrian and Klugman, 1997; Huovinen et al., 1995). An R-plasmid associated class I integron containing the *dhfrI* gene (encodes resistance to trimethoprim) was found in *Acinetobacter* spp. isolated from the aquatic environment (Petersen et al., 2000), and *A. salmonicida* (Schmidt et al., 2001b). Trimethoprim acts as an inhibitor of the enzyme dihydrofolate reductase, which is an essential enzyme in folic acid synthesis. A defect in

the *dhfr* gene results in an altered target enzyme dihydrofolate reductase, thereby reducing its affinity for trimethoprim, which leads to reduced susceptibility to the drug. Similarly, a defect in the genes *sulI* or *sulIII*, encoding the enzyme dihydropteroate synthase results in an altered affinity for the sulfonamides. Sulfonamides inhibit the enzyme dihydropteroate synthase, blocking the formation of dihydropteroic acid from para-aminobenzoate and dihydropteroate (Smith and Lewin, 1993). Integron-associated *sulI* genes have been found among *A. salmonicida* isolates.

The production of the CAT enzymes and beta lactamases are examples of the resistance mechanisms which modify and inactivate the targeted antimicrobial agent. The CAT-I enzyme, conferring chloramphenicol resistance has been found in *P. piscicida*, and the CAT-II enzyme in *V. anguillarum*, *A. salmonicida*, and *E. tarda* (Aoki, 1988). More recently another chloramphenicol resistance gene *catA2*, was identified in *A. salmonicida* (Sorum et al., 2003). Three beta-lactamases, including a carbapenemase were found in seven strains of *A. salmonicida* resistant to amoxicillin (Hayes et al., 1994).

Class 1 integrons are elements that contain genetic determinants of the components of a site-specific recombination system that recognizes and captures mobile gene cassettes. Gene cassettes commonly encode proteins involved in resistance to antibiotics (Fluit and Schmitz, 1999). Integrons have been found among environmental bacteria (Petersen et al., 2000; Rosser and Young, 1999), some motile *Aeromonas* species (Schmidt et al., 2001a), and many isolates of *A. salmonicida* (Sorum et al., 2003; L'Abée-Lund and Sorum, 2001; Schmidt et al., 2001b). Chromosomal mutation is another mechanism of antimicrobial resistance that has been extensively studied in mammalian

bacterial pathogens. Two types of chromosomal mutations are associated with resistance to the quinolones. These mutations have been shown to lead to alterations in the target enzyme DNA gyrase that reduce the accumulation of 4-quinolones (i.e., oxolinic acid) inside the bacterial cell (Lewin et al., 1990). The 4-quinolones, which each share the same core ring structure are antimicrobial agents that target two essential bacterial enzymes, DNA gyrase and DNA topoisomerase IV (Drlica and Zhao, 1997; Courvalin, 1990). The genes *gyrA* and *gyrB* encode for DNA gyrase, essential for uncoiling DNA during replication and transcription. A nucleotide substitution within the *gyrA* polypeptide chain is suspected to alter the binding ability of quinolones to the assembled DNA gyrase (Lewin et al., 1990), as seen in a study with *A. salmonicida* (Oppegaard and Sorum, 1996). Chromosomal resistance due to mutations have also been associated with quinolone resistance in *V. anguillarum* (Aoki et al., 1974), *Pasteurella piscicida*, *E. tarda*, *Y. ruckeri*, and *A. salmonicida* (Tsoumas et al., 1989). *In vitro* experiments, demonstrating low level resistance to oxolinic acid and other 4-quinolones (including fluoroquinolones) in *A. salmonicida*, revealed alterations in outer membrane proteins which may function as porins (Barnes et al., 1990b; Wood et al., 1986). Porins form water-filled channels that regulate the outer membrane permeability of low molecular-mass solutes, including antibiotics (De et al., 2001; Koebnik et al., 2000).

Other structural defenses have been found in studies with *A. salmonicida* (Barnes et al., 1990b; Wood et al., 1986). Outer membrane protein modifications have been associated with the expression of low-level resistance against quinolones and tetracyclines. The presence of the outer membrane protein in *A. salmonicida*, or A-layer has been shown to play a role in virulence (Maurice et al., 1999), and enhanced the

uptake of hydrophobic antibiotics such as chloramphenicol and streptomycin (Garduno et al., 1994). Alternatively, A-layer negative mutants had a reduced susceptibility to these same antimicrobials.

Antimicrobial Susceptibility Testing of Aquatic Bacteria

Introduction

Because similar mechanisms of resistance are found in bacterial pathogens of mammalian and aquatic species, there is increased public concern about antimicrobial resistance from aquatic sources. This concern has spawned efforts to standardize AST methods. In 1998, scientists from multiple countries met at the *Workshop on MIC Methodologies in Aquaculture* (WMA) (Alderman and Smith, 2001) to develop a ‘core’ set of protocols for AST of aquatic microorganisms. These protocols, based primarily on those found in the NCCLS documents (CLSI/NCCLS, 2007b; CLSI/NCCLS, 2003; CLSI/NCCLS, 2000), were created to facilitate interlaboratory data evaluation and begin development of standardized, quality controlled methods for AST. The ‘tentative’ protocols assembled by participants of the workshop were comprised of protocols for disk diffusion, agar dilution, broth microdilution, and broth macrodilution testing. Included were procedures for the following components (as applicable): antimicrobial stock solutions, media preparation, inoculum, diffusion disks, incubation, results interpretation, quality control, and rejection criteria.

Importance of Temperature for Susceptibility

Standardized methods of AST for bacteria isolated from aquatic animals are required because isolates from homeotherms typically require growth temperatures for

testing $\geq 35^{\circ}\text{C}$, whereas most isolates from the aquatic environment including those from fish prefer or require growth at temperatures $\leq 28^{\circ}\text{C}$. AST methods and QC guidelines have been extensively standardized for testing many different organisms and drugs at 35°C , but relatively little work has been done at the lower temperatures. The effect these low temperatures and potentially longer incubation times may have on susceptibility test results has not been addressed extensively in the literature. One could reason that several factors may affect results of AST when conducted at temperatures $< 35^{\circ}\text{C}$. For example, chemicals themselves may be more stable at the lower temperatures compared to temperatures $\geq 35^{\circ}\text{C}$ (Michel and Blanc, 2001). The slower metabolism of the bacteria at the lower temperatures may also affect the results, however lower temperatures may require longer incubation times. This in turn may increase the risk of drug inactivation.

Despite the problems identified previously, AST data using diffusion and dilution protocols at the lower temperatures has repeatedly been proven useful, and does in fact yield consistent reproducible results (Miller et al., 2003; Ho et al., 2000). Therefore, with adequate validation, protocols using low temperatures should be able to be standardized.

As alluded to above temperature may influence the susceptibility of microorganisms to antimicrobials by altering rates of drug transport, intracellular protein binding, and possibly the decreased metabolic rate of the bacterial pathogen. If temperatures are lowered, the decreased temperature favors the passive binding of the drug to intracellular proteins, and the drug may be less likely to attain its target (Michel and Blanc, 2001). Likewise, the lower temperature could alter the drug's affinity for the target. Additionally, if growth at lower temperatures reduces the drug concentration in the microorganism, one would expect a decreased susceptibility compared to isolates

tested at 35°C. In fact, data has shown that AST performed at temperatures below the preferred temperature of the given isolate, produced lower MICs and larger zones of inhibition indicating an increased susceptibility at the lower temperature (Miller et al., 2003). The role temperature may have in altered susceptibility due to factors such as growth kinetics and intracellular pharmacokinetics needs to be investigated.

Current Status of Disk Diffusion Methods

The Kirby-Bauer method (Bauer et al., 1966) is the most commonly used disk diffusion method for determining the susceptibility of aquatic bacteria to antimicrobial agents (Barker and Kehoe, 1995). However, over the years, many different types of disk diffusion methods, including types of media have been used by researchers testing aquatic pathogens by disk diffusion. Dalsgaard (2001) published a comprehensive list of the different types of media used for testing various aquatic pathogens. Dalsgaard (2001) and Barker and Kehoe (1995) both found Mueller-Hinton agar to be the best medium for diffusion testing, based upon its consistent performance with a wide range of aquatic pathogens. It is also a well-known, chemically defined medium that is recommended by the NCCLS for performing disk diffusion testing (CLSI/NCCLS, 1996). Those in attendance at the WMA also recommended this medium (Alderman and Smith, 2001).

In addition to the variation in media used, there has been an even greater disparity in disk diffusion methods used by laboratories (Ottaviani et al., 2001; Guerin-Fauble et al., 1996; Dalsgaard et al., 1994). Such differences include: incubation time, agar content, incubation conditions, media supplements, disk size, disk contents, inoculum preparation, inoculum density, and inoculation technique. The extent of this list of variations can have profound effects on test validity and outcome. Recognizing the

problem, scientists worldwide have acknowledged an urgent need to standardize the disk diffusion method for testing the susceptibility of bacteria isolated from aquatic animals and their environment.

Building on the efforts and recommendations of the WMA, researchers have focused recently to standardize the disk diffusion testing method including the formation of QC standards and parameters used for monitoring performance and reproducibility. To establish NCCLS QC ranges for two organisms, researchers conducted a multi-laboratory collaborative study (Miller et al., 2003). This study established QC ranges for *E. coli* (ATCC[®] 25922; NCIMB 12210) and *A. salmonicida* subsp. *salmonicida* (ATCC[®] 33658; NCIMB 1102) at two temperatures, 22°C and 28°C, against nine antimicrobial compounds commonly used in global aquaculture. These antimicrobials included ampicillin, erythromycin, florfenicol, gentamicin, oxolinic acid, oxytetracycline, ormetoprim-sulfadimethoxine, trimethoprim-sulfamethoxazole, and enrofloxacin (only *A. salmonicida* subsp. *salmonicida*). The standardized methods of disk susceptibility testing used in that study and the resulting QC ranges were recently published in the NCCLS report, M42-R entitled, *Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated from Aquatic Animals* (CLSI/NCCLS, 2006a). The NCCLS-approved methods, including the QC ranges are for testing isolates found in Group 1 (**Table 3**). Organisms in Groups 2-5 may require additional standardized testing methods as bacteria in these groups require supplemented media or different incubation temperatures or times. It is hoped researchers will use the existing methods and QC ranges, as well as future editions of the M42 report to validate their work when conducting disk diffusion testing of aquatic isolates.

Current Status of Dilution Methods

The sizes of the zones of inhibition generated by disk diffusion testing are dependent on the rate of diffusion of the antimicrobial agent through the agar media, thus zone sizes do not correlate directly with the level of *in vivo* drug activity. Broth dilution results on the other hand, have a more direct relationship to the MIC which has greater clinical relevance. Studies with various aquatic pathogens including *A. salmonicida* (Adams et al., 1998) and *Vibrio* spp. (Roque et al., 2001; Ho et al., 2000) have used broth dilution methods to obtain MICs without using universally standardized conditions. Unfortunately, without the use of standardized testing conditions it is difficult to compare data between laboratories or over time. Thus, it is imperative to standardize methods for broth dilution susceptibility testing under optimal growth conditions needed by aquatic isolates such as lowered temperatures.

Unlike disk diffusion studies, which are done in aquatic laboratories using many different types of media, cation-adjusted Mueller-Hinton broth (CAMHB) has been the preferred medium for broth microdilution testing (CLSI/NCCLS, 2000). CAMHB yields consistent results within and between laboratories, and facilitates growth of a wide range of bacterial pathogens.

Again, building upon the recommendations of those at the WMA and using the NCCLS documents describing dilution testing (CLSI/NCCLS, 2007b; CLSI/NCCLS, 2000), researchers at the U.S. FDA are currently developing a method for broth microdilution susceptibility testing, along with QC guidelines for testing organisms at the lower temperatures. A 96-well microtiter plate containing 10 antimicrobial agents (Trek Diagnostic Systems, Inc.; Cleveland, OH) has been developed, and is currently being

Table 3. NCCLS VAST - Aquaculture Working Group recommended grouping for standardizing disk susceptibility tests of various bacteria isolated from fish

Group #	Bacterial spp.	Temperature	Incubation time	Suggested media
Group 1	- <i>Enterobacteriaceae</i> - <i>Aeromonas salmonicida</i> (non-psychrophilic strains) - <i>Aeromonas hydrophila</i> (and other mesophilic Aeromonads) - <i>Pseudomonas</i> spp. - <i>Plesiomonas shigelloides</i> - <i>Shewanella</i> spp. - <i>Vibrio</i> spp. (non-obligate halophilic strains) - <i>Listonella anguillarum</i>	22±2°C and/or 28±2°C	24-28 hours and/or 44-48 hours	—Mueller-Hinton agar
Group 2	- <i>Vibrio</i> spp. (obligate halophilic strains) - <i>Photobacterium damsela</i> subsp. <i>piscicida/damsela</i>	22±2°C and/or 28±2°C 28±2°C	24-28 hours and/or 44-48 hours 44-48 hours	—1.5% NaCl addition where basal media NaCl content is not known; 1.5% final NaCl concentration where basal NaCl content is known ^a —Same as above
Group 3	- <i>Flavobacterium columnare</i> - <i>Flavobacterium branchiophilum</i> - <i>Flavobacterium psychrophilum</i>	28±2°C 15±2°C	24-28 hours and 44-48 hours 44-48 hours and 68-72 hours	—Dilute Mueller-Hinton agar ^b —Dilute Mueller-Hinton agar with 5% serum ^c
Group 4	- <i>Streptococcus iniae</i> - <i>Streptococcus dysgalactiae</i> - <i>Lactococcus garviae</i> - <i>Vagococcus salmoninarum</i> -Other Gram-positive cocci	35°C 35°C	16-18 hours 16-18 hours	—Mueller-Hinton agar with 5% sheep blood —Mueller-Hinton agar
Group 5	-Psychrophilic <i>Aeromonas salmonicida</i> strains - <i>Vibrio salmonicida</i> - <i>Streptococcus difficilis</i> -Gram-positive rods (<i>Renibacterium salmoninarum</i> , <i>Mycobacterium</i> spp., <i>Nocardia</i> spp., <i>Erysipelothrix rhusiopathiae</i> , and <i>Corynebacterium</i> spp.)	15±2°C 15 ± 2°C 28±2°C Multiple variations	44-48 hours 44-48 hours 44-48 hours Multiple variations	—Mueller-Hinton agar —Mueller-Hinton agar with 1.5% NaCl —Mueller-Hinton agar with 5% sheep blood —Multiple variations

^a recommended conditions by Alderman and Smith (2001) and Ottaviani et al. (2001)

^b recommended conditions by Hawke and Thune (1992)

^c recommended conditions by Michel and Blanc (2001)

evaluated using NCCLS recommendations for broth microdilution testing (CLSI/NCCLS, 2007b; CLSI/NCCLS, 2000). Preliminary results show reproducible MICs when testing *Vibrio* spp., *Aeromonas* spp., *Edwardsiella* spp., *Ph. damselae* subsp. *damselae/piscicida*, *Y. ruckeri*, and *Listonella anguillarum*. Once a standardized method and QC parameters are available, diagnosticians will be able to generate data that is comparable both between laboratories and over time. If standard panels, similar to the one described above, become commercially available, researchers will have a valuable new tool to assist in the discrimination between resistant and susceptible bacterial isolates of the aquatic environment.

Future Needs and Challenges

Interpretive Criteria

Interpretive criteria or clinical breakpoints, defining resistant, intermediate, or susceptible phenotypes, are derived from multidisciplinary studies including pharmacokinetics-pharmacodynamics (PK-PD), MIC determinations, and trials involving treatment of diseased animals which may include clinical cases and/or experimentally induced infection. Interpretive criteria are currently available for various warm-blooded target species for several antimicrobial compounds. However, limited work has been conducted on generating interpretive criteria for bacterial pathogens of aquatic species. Thus, there are no aquaculture specific clinical breakpoint concentrations for antimicrobials used in aquatic medicine. Such specific interpretive criteria are urgently needed for all aquatic isolates to enable attending veterinarians to more accurately prescribe an antimicrobial for treatment. Studies attempting to establish clinical breakpoints must include analyses of the relationship between PK-PD parameters in the

target animal species and efficacy in treating infections caused by the specific bacterial pathogen. These analyses should include such PD parameters as length of time for serum or plasma levels to exceed the MIC of the pathogen, peak serum or plasma level to MIC ratio, or 'area-under-the-serum drug concentration' (AUC) to MIC ratio (CLSI/NCCLS, 2001). Studies generating MICs from at least 500 isolates of the bacterial pathogen in question should represent clinically relevant strains that exhibit resistance and susceptibility to the drug in question. Lastly, AST results (MICs and zone sizes) should be correlated with therapeutic outcomes observed in research and in clinical experience (CLSI/NCCLS, 2007a).

Methods of AST for the Other Isolates

A standardized method, as well as QC ranges now exist when conducting disk diffusion susceptibility tests of most aquatic isolates requiring or preferring growth at temperatures <35°C. Testing conditions, including media supplementation, incubation times and temperatures need to be determined when testing the bacterial pathogens in Groups 2-5. Once a method is developed and has been demonstrated to yield consistent results after testing isolates in the 'group' for which the method was standardized, QC parameters should then be determined. To facilitate this process, additional QC organisms may need to be employed. There are over 40 drugs used in global aquaculture (**Table 1**), and currently only 9 of them have QC ranges established for susceptibility testing isolates at temperatures <35°C. It is hoped that this list of antimicrobials will in the not-too-distant future expand to encompass many more drugs of interest to aquaculture.

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Chapter 3: Standardization of a broth microdilution susceptibility testing method to determine minimal inhibitory concentrations of aquatic bacteria

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Abstract

A multiple laboratory study was conducted in accordance with the standards established by the Clinical and Laboratory Standards Institute (CLSI, formerly the NCCLS), for the development of quality control (QC) ranges using dilution antimicrobial susceptibility testing methods for bacterial isolates from aquatic animal species. Quality control ranges were established for *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 when testing at 22°C, 28°C, and 35°C (*E. coli* only) for ten different antimicrobial agents (ampicillin, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, ormetoprim-sulfadimethoxine, oxolinic acid, oxytetracycline, and trimethoprim-sulfamethoxazole). Minimal inhibitory concentration (MIC) QC ranges were determined using dry- and frozen-form 96-well plates and cation-adjusted Mueller-Hinton broth. These QC ranges were accepted by the CLSI -

Subcommittee on Veterinary Antimicrobial Susceptibility Testing. This broth microdilution testing method represents the first standardized method for determination of MICs of bacterial isolates whose preferred growth temperatures are below 35°C. Methods and QC ranges defined in this study will enable aquatic animal disease researchers to reliably compare quantitative susceptibility testing data between laboratories, and will be used to ensure both precision and inter-laboratory harmonization.

Introduction

In the area of anti-infective therapy, researchers and fish disease specialists have made great strides in recent years towards developing standardized methods to determine MICs of bacteria isolated from the aquatic environment. A number of studies have provided valuable data to assist in the determination of the most appropriate growth media, incubation temperatures and times, and antimicrobial agent concentrations for testing various bacterial genera found in the aquatic environment (Coyne et al., 2004a; Michel et al., 2003; Miller et al., 2003; McGinnis et al., 2003; Samuelsen et al., 2003; Rigos et al., 2003b; Alderman and Smith, 2001; Hawke and Thune, 1992; Martinsen et al., 1992; Bandin et al., 1991; Inglis and Richards, 1991; Barnes et al., 1990a). Some of these studies employed dilution methods of antimicrobial susceptibility testing derived from accepted standards, such as those published by the CLSI (CLSI/NCCLS, 2002b; CLSI/NCCLS, 2000). Because there are no QC ranges established for tests conducted at temperatures below 35°C, these studies lacked required internal controls.

Most of these studies recommended using Mueller-Hinton medium for routine susceptibility testing of non-fastidious organisms. Alderman & Smith (2001) published a

‘tentative’ set of protocols wherein they outlined the problems commonly encountered when comparisons were made of data generated by laboratories employing different media and methods. Data generated using these varied protocols differ greatly from laboratory to laboratory, making inter-laboratory correlations of susceptibility results difficult. Thus, there is a pressing need for fish health diagnostic laboratories, veterinarians, and researchers to have standardized antimicrobial susceptibility testing methods available for bacterial isolates of aquatic origin.

Three standardized antimicrobial susceptibility testing methods are recommended by the CLSI for testing bacterial pathogens of mammalian origin (CLSI/NCCLS, 2002b). These are agar disk diffusion, broth dilution, and agar dilution. The E-test[®] (AB Biodisk) is a commercial proprietary system based on a modified agar diffusion method, which is currently not recommended by the CLSI for use as a standardized antimicrobial susceptibility testing method. Prior to the completion of the study reported here, only the agar disk diffusion method was standardized for the testing of aquatic isolates (CLSI/NCCLS, 2006a; Miller et al., 2003). However, agar disk diffusion test results can be less reliable when slower growing organisms are tested. In these cases, fairly large zones of inhibition may indicate susceptibility or may simply represent the effect of delayed growth (Acar and Goldstein, 1996). These factors help justify the need for susceptible, intermediate, and resistant breakpoints for aquaculture drugs and pathogens at the lower temperatures. Disk diffusion tests also yield zones of inhibition which are generally not as useful to the clinician, even when the extrapolation of a MIC value is possible using a linear regression analytical system. Despite its limitations, the agar disk diffusion method is still commonly used in aquatic diagnostic laboratories. In the past

decade, however, several studies have been published where dilution susceptibility testing methods were used on aquatic isolates (Coyne et al., 2004a; Michel et al., 2003; McGinnis et al., 2003; Samuelsen et al., 2003; Rigos et al., 2003b; Torkildsen et al., 2000; Rangdale et al., 1997; Park et al., 1995).

A standardized dilution susceptibility testing method provides two advantages over the disk diffusion test. First, results generated by a dilution testing method may be quantitative (MIC), in addition to qualitative (susceptible, intermediate, and resistant). Quantitative results increase the potential for optimizing a dosing regimen based on the pharmacokinetic and pharmacodynamic parameters that drive clinical efficacy. Secondly, broth dilution methods permit the testing of bacteria whose growth characteristics are less amenable to disk diffusion testing (i.e., slower growing or fastidious organisms). Agar dilution, although considered to be the ‘gold-standard’ for antimicrobial susceptibility testing, can be labor intensive and time-consuming. Therefore, agar dilution tests tend to be performed less frequently than disk diffusion and broth dilution tests. Broth dilution methods offer a preferred choice for quantitatively evaluating slower growing aquatic microorganisms.

To develop a standardized and internationally harmonized dilution susceptibility testing method for aquatic isolates, some members of the CLSI Subcommittee on Veterinary Antimicrobial Susceptibility Testing – Aquaculture Working Group (VAST-AWG) coordinated a multiple laboratory study to standardize a MIC testing method for bacterial isolates that grow at 22°C and 28°C. Temperatures chosen for this study were based upon their routine use in aquatic animal disease diagnostic laboratories worldwide, on recommendations of members of the CLSI Subcommittee on VAST-AWG, on an

effort to coordinate methodologies with international investigators, and to accommodate temperature optimums for aquatic bacteria isolated from both cold- and warm-water species. The methods used in this study were based on the broth microdilution testing methods described in the CLSI standard M31-A2 (CLSI/NCCLS, 2002b). Also incorporated were recommendations of experts in the field of aquatic microbiology, such as incubation temperature and duration, and testing media which were summarized by Alderman and Smith (2001). This method was developed for testing aquatic bacterial isolates which prefer or require temperatures below 35°C, and do not require supplementation of the standard Mueller-Hinton growth medium. Since these aquatic isolates prefer or require these lower temperatures, previously they could not have been tested accurately employing the QC parameters established in the CLSI protocols for testing organisms from mammalian origin whose optimal growth temperatures are $\geq 35^{\circ}\text{C}$ (CLSI/NCCLS, 2002b). The CLSI Subcommittee on VAST - AWG generated a list of aquatic bacterial pathogens which prefer lower temperatures (Table 4), and on which the standardized susceptibility testing method described herein may apply. Commercially-prepared MIC test plates which contained dehydrated antimicrobial agents (dry-form plates) were used in this study, and validated against a commercially-prepared frozen-form plate, which is the CLSI reference method (CLSI/NCCLS, 2001). Ten different antimicrobial agents were chosen to represent major classes of antimicrobial agents, some of which are approved for use in aquaculture in the U.S. and other countries. Some of these antimicrobial agents have been prescribed for ‘extra-label’ use by veterinarians treating non-food commercial and hobby aquarium fish. In addition, some of these antimicrobial agents have been identified in the aquatic environment

Table 4. Broth dilution susceptibility testing conditions for Group 1 organisms, as recommended by the CLSI^a/NCCLS Subcommittee on VAST – AWG.

Group #	Aquatic pathogens	Incubation	Suggested media
Group 1	<i>Enterobacteriaceae</i> <i>Aeromonas salmonicida</i> (non- psychrophilic strains) <i>Aeromonas hydrophila</i> and other mesophilic Aeromonads <i>Pseudomonas</i> spp., <i>Plesiomonas</i> <i>shigelloides</i> , <i>Shewanella</i> spp. <i>Vibrio</i> spp. (non-obligate halophilic strains) <i>Listonella anguillarum</i>	22 °C (24-28 h and/or 44-48 h) & 28 °C (24-28 h)	CAMHB

^a CLSI, Clinical and Laboratory Standards Institute (formerly the NCCLS)

(Capone et al., 1996), and are of growing concern to environmental regulatory agencies (Daughton and Ternes, 1999).

The standardized methods established in this work will assist in the more precise monitoring of resistance in bacteria commonly isolated from the environment, as well as aid aquatic disease specialists in the treatment of bacterial infections in aquatic species.

Materials and Methods

Standardization Study

Participating laboratories. In this study, data was generated in ten participating laboratories. These included the Food and Drug Administration, Center for Veterinary Medicine (FDA-CVM), Office of Research, Laurel, Md.; Fish Health Unit, Department of Primary Industries Water & Environment, Prospect Launceston, Australia; Atlantic Veterinary College, University of Prince Edward Island, Prince Edward Island, Canada;

Institute of Marine Research, Department of Aquaculture, Bergen, Norway; Danish Institute for Fisheries Research, Fish Disease Laboratory, Frederiksberg, Denmark; University of Wisconsin, Wisconsin Veterinary Diagnostic Laboratory, Madison, Wis.; Alparma, Animal Health Division, Chicago Heights, Ill.; University of Patras, Laboratory of Public Health, Rio Patras, Greece; Florida Department of Agriculture and Consumer Services, Division of Animal Industry, Bartow, Fla.; Washington State University, Washington Animal Disease Diagnostic Laboratory, Pullman, Wash.

While the study was initiated with ten participating laboratories, QC ranges presented here are based on data from nine testing laboratories for *Escherichia coli* ATCC 25922 and seven laboratories for *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658. Data from one laboratory was consistently askew with the values observed in the other nine laboratories for both QC strains, and thus the data from this laboratory was eliminated from the entire study. In the case of the *A. salmonicida* subsp. *salmonicida* strain, one laboratory was unable to receive the *A. salmonicida* subsp. *salmonicida* QC strain due to import restrictions, and the other generated data for all antimicrobial agents consistently out of line from the values obtained in the other seven laboratories. As a result, the maximum total QC data points per organism/antimicrobial agent/temperature/incubation time condition were reduced from 300 to 270 for *E. coli*, and to 210 for *A. salmonicida* subsp. *salmonicida*. Previous CLSI methods standardization and QC studies have eliminated data from laboratories based on inconsistent data (Miller et al., 2003; McDermott et al., 2001; Marshall et al., 1996; Jorgensen et al., 1996). In this study the number of data points produced in the nine and seven laboratories for *E. coli* and *A. salmonicida* subsp. *salmonicida* respectively,

satisfied the requirements of the CLSI for the establishment of QC ranges (CLSI/NCCLS, 2002a).

MIC test plates. All plates used in the multiple laboratory trial were dry-form plates manufactured by Trek Diagnostic Systems (Cleveland, OH) (lot #3222) in the standard 96-well format. This custom plate consisted of two-fold dilutions centering on 1 µg/mL, of the following antimicrobial agents: ampicillin, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, ormetoprim-sulfadimethoxine, oxolinic acid, oxytetracycline, and trimethoprim-sulfamethoxazole. **Tables 5-10** include the concentration range tested for each antimicrobial agent. Two wells in each MIC test plate were used as positive controls.

Test strains and growth conditions. American Type Culture Collection (Manassas, Virginia) reference strains, *E. coli* ATCC 25922; NCIB 12210; DSM 1103 and *A. salmonicida* subsp. *salmonicida* ATCC 33658; NCMB 1102 were incubated at both 22 ± 2°C for 24 to 28 h and 44 to 48 h, and at 28 ± 2°C for 24 to 28 h in cation-adjusted Mueller-Hinton broth (CAMHB).

Quality control. Following guidelines for QC described in the CLSI standard M31-A2 (CLSI/NCCLS, 2002b) *E. coli* ATCC 25922 was incubated at 35°C for 16 to 20 h in CAMHB.

Broth microdilution susceptibility testing. This study was designed in accordance with guidelines described in the CLSI guideline M37-A2 (CLSI/NCCLS, 2002a) for conducting QC studies, and followed procedures outlined in the CLSI standard M31-A2 (CLSI/NCCLS, 2002b). On ten testing days, each laboratory tested *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 in three lots of

CAMHB. CAMHB was prepared by Trek Diagnostic Systems using powders from three lots from three different sources: BD Diagnostic Systems (Sparks, MD) 212322 - lot #1254009; Hardy Diagnostics (Santa Maria, CA) C7521 - lot #2049; and Difco (Sparks, MD) 275710 - lot #2218968. Sterility and pH measurements were taken, and cation supplementation was made based on the certificate of analysis for each lot of powder, and adjusted in accordance with the CLSI standard M7-A6 (CLSI/NCCLS, 2000). Trek Diagnostic Systems distributed all media in liquid form to the 10 participating laboratories.

On each 'testing day', one suspension was prepared for each QC strain in demineralized water. The CLSI guideline M31-A2 states sterile water, Mueller-Hinton broth, or 0.9% saline may be used to prepare inocula of some fastidious pathogens (CLSI/NCCLS, 2002b). Turbidities were measured using one of the following; colorimeter (0.5 McFarland suspension), spectrophotometer (0.08-0.10 at OD₆₂₅), turbidimeter (60-70 NTUs), or the line method (CLSI/NCCLS, 2006a). Suspensions targeted an inoculum density equivalent to approximately 1.0×10^8 colony forming units per mL (CFU/mL). Bacterial suspensions were diluted 1:200 in CAMHB to target an inoculum concentration of approximately 5.0×10^5 CFU/mL. Dry-form MIC test plates were inoculated with 100 μ L per well using either a Trek Autoinoculator[®] apparatus (Trek Diagnostic Systems) or a multichannel pipetter.

Test plates were covered with plastic adhesive seals and incubated within 15 min of inoculation at 22°C and 28°C (*E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658) and 35°C (*E. coli* ATCC 25922). Plates were stacked no more than two plates high to ensure proper humidity and air circulation. Test plates were

read by removing the seal. This permitted the detection of slight growth detectable with the unaided eye. The seals were carefully replaced for those plates which required incubation at 22°C for an additional 20 to 24 h.

Immediately following inoculation, colony counts were performed for each isolate on each test day, from a positive control well from one MIC test plate. To perform the colony counts a 1:1000 dilution was made in demineralized water. A 100 μ L aliquot was used to inoculate a tryptic soy agar plate supplemented with 5% sheep blood. The inoculum was uniformly spread across the surface of the agar using an L-shaped spreader. Colony count plates were incubated at 28°C for 24 to 28 h and the number of CFUs counted.

Definition of minimum inhibitory concentration. MICs were defined as the lowest concentration of antimicrobial agent that prevented visible growth of the microorganism. Following recommendations detailed in the CLSI standard M7-A6 (CLSI/NCCLS, 2000), when a single skipped well occurred the highest MIC was read (i.e., the first well with no growth after the skipped well), and when two skipped wells occurred the test was repeated.

Definition of quality control ranges. In accordance with CLSI guideline M37-A2 (CLSI/NCCLS, 2002a) the organisms were tested using three lots of media tested in at least seven laboratories on ten test days. The percentage of participant MICs that fell within the approved QC ranges for *E. coli* ATCC 25922 exceeded 95% for all antimicrobial agents tested (**Tables 5-7**). In tests on *A. salmonicida* subsp. *salmonicida* ATCC 33658, trailing endpoints were observed by researchers in two laboratories (**Figure 12**), which caused the percentage of participant MICs within the approved QC

ranges to be slightly lower than the targeted 95% for four of the antimicrobial agents (ampicillin, florfenicol, flumequine, and oxolinic acid) at 22°C 44 to 48 h and 28°C 24 to 28 h (**Tables 9 and 10**).

Validation Study

To be in compliance with the CLSI guidelines M37-A2 (CLSI/NCCLS, 2002a) and M23-A2 (CLSI/NCCLS, 2001), for establishment of QC criteria, a single laboratory (FDA-CVM) study was required to show comparability between MIC results generated using the dry-form and frozen-form reference plates.

Test plates consisted of two-fold dilutions of the following antimicrobial agents: ampicillin (0.03-16 µg/mL), enrofloxacin (0.002-1 µg/mL), erythromycin (0.25-128 µg/mL), florfenicol (0.03-16 µg/mL), flumequine (0.008-4 µg/mL), gentamicin (0.06-4 µg/mL), ormetoprim-sulfadimethoxine (0.008/0.15-4/76 µg/mL), oxolinic acid (0.004-2 µg/mL), oxytetracycline (0.015-8 µg/mL), and trimethoprim-sulfamethoxazole (0.015/0.3-1/19 µg/mL). Two wells in each MIC test plate were used as positive controls.

The CLSI recommends that a minimum of 100 isolates should be tested to validate MIC test results using the reference frozen-form plates against those obtained in dry-form plates (CLSI/NCCLS, 2002a). In this study over 100 distinct isolates of *E. coli* and *A. salmonicida* combined, were tested at all temperatures and times for which MIC QC ranges were proposed. *E. coli* isolates were obtained from the FDA-CVM culture collection originating from non-piscine host species. *A. salmonicida* isolates were obtained from various aquatic disease research laboratories in the United States, Canada, and the United Kingdom. Both *E. coli* ATCC 25922 and *A. salmonicida* subsp.

salmonicida ATCC 33658 were used as QC organisms at $22 \pm 2^\circ\text{C}$ and $28 \pm 2^\circ\text{C}$ and only *E. coli* at $35 \pm 2^\circ\text{C}$, using the ranges approved by members of the CLSI Subcommittee on VAST for testing in dry-form plates as a result of the standardization study described above.

Tests were conducted in CAMHB (Trek Diagnostic Systems) using the same procedure employed in the multiple laboratory trial. Bacterial suspensions equivalent to a 0.5 McFarland suspension were prepared and diluted in CAMHB to a standardized inoculum concentration of approximately 5.0×10^5 CFU/mL for the dehydrated plates, and 1.0×10^6 CFU/mL for the frozen plates. Dry-form plates were inoculated with 100 μL per well, and frozen plates with 50 μL per well (making a 1:2 dilution with the thawed antimicrobial agent solution in each well) using a Trek Autoinoculator[®].

Table 5. *Escherichia coli* ATCC 25922 minimum inhibitory concentration QC results at 22°C - 24-28 h with CAMHB^a.

Antimicrobial agent	Testing range ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)			# Data points	% Within QC range
		Inter-laboratory range	Median	CLSI/NCCLS-approved QC range ^b		
Ampicillin	0.06 - 32	2 - 16	4	2 - 16	267	100
Enrofloxacin	0.002 - 1	0.004 - 0.03	0.008	0.004 - 0.015	266	98.1
Florfenicol	0.12 - 64	2 - 16	8	2 - 16	266	100
Flumequine	0.015 - 8	0.12 - 0.5	0.25	0.06 - 0.5	266	100
Gentamicin	0.12 - 8	?0.12 - 2	0.25	0.12 - 0.5	266	95.1
Ormetoprim-sulfadimethoxine ^c	0.008/0.15 - 4/76	0.12/2.4 - 2/38	0.5/9.5	0.12/2.4 - 1/19	267	99.6
Oxolinic acid	0.004 - 2	0.06 - 0.25	0.06	0.03 - 0.25	266	100
Oxytetracycline	0.03 - 16	0.25 - 2	0.5	0.25 - 1	267	99.3
Trimethoprim-sulfamethoxazole ^d	0.03/0.6 - 2/38	?0.03/0.6 - 0.25/4.8	0.06/1.2	0.03/0.6 - 0.12/2.4	267	99.6

^a CAMHB was made from three lots of media, common to all nine laboratories.
^b CLSI, Clinical and Laboratory Standards Institute (formerly the NCCLS)
^c First value indicates concentration of ormetoprim; second value indicates concentration of sulfadimethoxine
^d First value indicates concentration of trimethoprim; second value indicates concentration of sulfamethoxazole

Table 6. *Escherichia coli* ATCC 25922 minimum inhibitory concentration QC results at 22°C - 44-48 h with CAMHB^a.

Antimicrobial agent	Testing range ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$) ¹⁾			# Data points	% Within QC range
		Inter-laboratory range	Median	CLSI/NCCLS-approved QC range ^b		
Ampicillin	0.06 - 32	4 - >32	8	4 - 16	267	99.6
Enrofloxacin	0.002 - 1	0.004 - 0.03	0.008	0.004 - 0.015	265	97.4
Florfenicol	0.12 - 64	4 - 16	8	4 - 16	264	100
Flumequine	0.015 - 8	0.12 - 1	0.25	0.12 - 0.5	264	99.2
Gentamicin	0.12 - 8	0.25 - 4	0.5	0.25 - 1	264	96.2
Ormetoprim-sulfadimethoxine ^c	0.008/0.15 - 4/76	0.12/2.4 - 2/38	0.5/9.5	0.25/4.8 - 2/38	266	95.5
Oxolinic acid	0.004 - 2	0.06 - 0.5	0.12	0.06 - 0.25	264	99.2
Oxytetracycline	0.03 - 16	0.5 - 4	1	0.5 - 2	267	99.3
Trimethoprim-sulfamethoxazole ^d	0.03/0.6 - 2/38	\leq 0.03/0.6 - 0.25/4.8	0.06/1.2	0.03/0.6 - 0.25/4.8	265	100

^a CAMHB was made from three lots of media, common to all nine laboratories.
^b CLSI, Clinical and Laboratory Standards Institute (formerly the NCCLS)
^c First value indicates concentration of ormetoprim; second value indicates concentration of sulfadimethoxine
^d First value indicates concentration of trimethoprim; second value indicates concentration of sulfamethoxazole

Table 7. *Escherichia coli* ATCC 25922 minimum inhibitory concentration QC results at 28°C - 24-28 h with CAMHB^a.

Antimicrobial agent	Testing range ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)			# Data points	% Within QC range
		Inter-laboratory range	Median	CLSI/NCCLS-approved QC range ^b		
Ampicillin	0.06 - 32	2 - 32	4	2 - 16	265	99.6
Enrofloxacin	0.002 - 1	0.008 - 0.03	0.015	0.008 - 0.03	263	100
Florfenicol	0.12 - 64	4 - 16	8	4 - 16	264	100
Flumequine	0.015 - 8	0.12 - 1	0.25	0.12 - 0.5	264	99.6
Gentamicin	0.12 - 8	0.25 - 4	0.5	0.25 - 1	260	97.7
Ormetoprim-sulfadimethoxine ^c	0.008/0.15 - 4/76	0.06/1.2 - 2/38	0.5/9.5	0.12/2.4 - 1/19	265	98.5
Oxolinic acid	0.004 - 2	0.06 - 0.5	0.12	0.06 - 0.25	262	99.6
Oxytetracycline	0.03 - 16	0.5 - 8	1	0.5 - 2	264	99.2
Trimethoprim-sulfamethoxazole ^d	0.03/0.6 - 2/38	\leq 0.03/0.6 - 0.25/4.8	0.06/1.2	0.03/0.6 - 0.25/4.8	263	100

^a CAMHB was made from three lots of media, common to all nine laboratories.
^b CLSI, Clinical and Laboratory Standards Institute (formerly the NCCLS)
^c First value indicates concentration of ormetoprim; second value indicates concentration of sulfadimethoxine
^d First value indicates concentration of trimethoprim; second value indicates concentration of sulfamethoxazole

Table 8. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 minimum inhibitory concentration QC results at 22°C - 24-28 h with CAMHB^a.

Antimicrobial agent	Testing range ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)			# Data points	% Within QC range
		Inter-laboratory range	Median	CLSI/NCCLS-approved QC range ^b		
Ampicillin	0.06 - 32	0.12 - 4	0.25	0.12 - 1	206	97.1
Enrofloxacin	0.002 - 1	0.004 - 0.06	0.015	0.008 - 0.03	207	98.6
Erythromycin	0.03 - 16	4 - 16	8	4 - 16	206	100
Florfenicol	0.12 - 64	0.25 - 4	0.5	0.25 - 1	205	99.0
Flumequine	0.015 - 8	0.015 - 0.25	0.06	0.015 - 0.12	207	98.6
Gentamicin	0.12 - 8	0.25 - 4	0.5	0.25 - 1	207	98.1
Ormetoprim-sulfadimethoxine ^c	0.008/0.15 - 4/76	0.03/0.6 - 0.5/9.5	0.12/2.4	0.06/1.2 - 0.25/4.8	205	97.1
Oxolinic acid	0.004 - 2	0.004 - 0.06	0.015	0.008 - 0.03	205	99.0
Oxytetracycline	0.03 - 16	0.06 - 1	0.12	0.06 - 0.25	207	99.5
Trimethoprim-sulfamethoxazole ^d	0.03/0.6 - 2/38	0.03/0.6 - 0.12/2.4	0.06/1.2	0.03/0.6 - 0.12/2.4	207	100

^a CAMHB was made from three lots of media, common to all seven laboratories.
^b CLSI, Clinical and Laboratory Standards Institute (formerly the NCCLS)
^c First value indicates concentration of ormetoprim; second value indicates concentration of sulfadimethoxine
^d First value indicates concentration of trimethoprim; second value indicates concentration of sulfamethoxazole

Table 9. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 minimum inhibitory concentration QC results at 22°C - 44-48 h with CAMHB^a.

Antimicrobial agent	Testing range ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)			# Data points	% Within QC range
		Inter-laboratory range	Median	CLSI/NCCLS-approved QC range ^b		
Ampicillin	0.06 - 32	0.25 - 8	0.5	0.25 - 1	201	90.5
Enrofloxacin	0.002 - 1	0.008 - 0.06	0.015	0.008 - 0.03	205	99.0
Erythromycin	0.03 - 16	8 - >16	16	4 - 32	205	100
Florfenicol	0.12 - 64	0.25 - 8	1	0.5 - 2	203	93.6
Flumequine	0.015 - 8	0.015 - 0.5	0.06	0.03 - 0.12	202	94.1
Gentamicin	0.12 - 8	0.5 - 4	0.5	0.25 - 2	203	98.0
Ormetoprim-sulfadimethoxine ^c	0.008/0.15 - 4/76	0.06/1.2 - 2/38	0.25/4.8	0.06/1.2 - 0.5/9.5	202	98.0
Oxolinic acid	0.004 - 2	0.008 - 0.12	0.015	0.008 - 0.03	197	90.4
Oxytetracycline	0.03 - 16	0.12 - 1	0.25	0.12 - 1	205	100
Trimethoprim-sulfamethoxazole ^d	0.03/0.6 - 2/38	0.03/0.6 - 0.25/4.8	0.12/2.4	0.03/0.6 - 0.25/4.8	202	100

^a CAMHB was made from three lots of media, common to all seven laboratories.
^b CLSI, Clinical and Laboratory Standards Institute (formerly the NCCLS)
^c First value indicates concentration of ormetoprim; second value indicates concentration of sulfadimethoxine
^d First value indicates concentration of trimethoprim; second value indicates concentration of sulfamethoxazole

Table 10. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 minimum inhibitory concentration QC results at 28°C - 24-28 h with CAMHB^a.

Antimicrobial agent	Testing range (µg mL ⁻¹)	MIC (µg mL ⁻¹)			# Data points	% Within QC range
		Inter-laboratory range	Median	CLSI/NCCLS-approved QC range ^b		
Ampicillin	0.06 - 32	0.12 - 8	0.5	0.12 - 1	205	91.7
Enrofloxacin	0.002 - 1	0.004 - 0.25	0.008	0.004 - 0.03	207	99.5
Erythromycin	0.03 - 16	4 - >16	8	4 - 32	207	100
Florfenicol	0.12 - 64	0.25 - 8	1	0.5 - 2	205	91.7
Flumequine	0.015 - 8	0.03 - >8	0.06	0.015 - 0.12	206	92.7
Gentamicin	0.12 - 8	0.25 - >8	0.5	0.25 - 1	204	98.0
Ormetoprim-sulfadimethoxine ^c	0.008/0.15 - 4/76	0.06/1.2 - 1/19	0.25/4.8	0.06/1.2 - 0.5/9.5	206	97.6
Oxolinic acid	0.004 - 2	0.008 - 0.12	0.015	0.008 - 0.03	206	92.7
Oxytetracycline	0.03 - 16	0.12 - 8	0.25	0.12 - 1	207	99.5
Trimethoprim-sulfamethoxazole ^d	0.03/0.6 - 2/38	0.03/0.6 - 0.5/9.5	0.12/2.4	0.03/0.6 - 0.25/4.8	204	99.5

^a CAMHB was made from three lots of media, common to all seven laboratories.
^b CLSI, Clinical and Laboratory Standards Institute (formerly the NCCLS)
^c First value indicates concentration of ormetoprim; second value indicates concentration of sulfadimethoxine
^d First value indicates concentration of trimethoprim; second value indicates concentration of sulfamethoxazole

Results and Discussion

Standardization Study

An obligatory component of all antimicrobial susceptibility tests is the establishment of QC ranges for a given QC strain for each antimicrobial agent it is tested against. In this standardization study, QC ranges for ten different antimicrobial agents were established for broth microdilution susceptibility testing at 22°C (24 to 28 h and 44 to 48 h) and 28°C (24 to 28 h) for *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658, and at 35°C (16 to 20 h) for *E. coli* ATCC 25922. These QC strains are well characterized and have been approved by the CLSI for use in disk diffusion tests (CLSI/NCCLS, 2006a).

Tables 5-7 and 8-10 summarize the MICs and QC limits for the ten antimicrobial agents tested for *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658, respectively. The CLSI Subcommittee on VAST approved these MIC QC ranges using a modification of the median method described by Gavan et al. (1981) for disk diffusion testing. In many cases there was a single defined median MIC, in which case the QC range was defined as ± 1 dilution from the median MIC (**Figure 8**). There were some cases where an underlying distribution of MICs appeared to be asymmetric (**Figure 9**). In these cases, the QC range was expanded one dilution above or below any shoulder $\geq 66.7\%$ of the peak MIC frequency. There was one instance of an asymmetric distribution (*E. coli* ATCC 25922 – ormetoprim-sulfadimethoxine at 35°C) where a five dilution range was approved (**Figure 10**).

Minimal variability of MIC results was observed with the three lots of CAMHB within and between the laboratories for both QC strains (data not shown). However, trailing endpoints (**Figures 11 and 12**) in tests on both organisms were observed by 8 of 10 laboratories in one of the lots of media for all antimicrobial agents tested.

Colony count data generated by laboratories were between 5.0×10^4 - 1.1×10^6 CFU/mL. While the cell concentrations in some cases were slightly lower or higher than the desired 5.0×10^5 CFU/mL concentration, this did not affect the results, as the MICs were within the approved QC range for *E. coli* ATCC 25922 at 35°C.

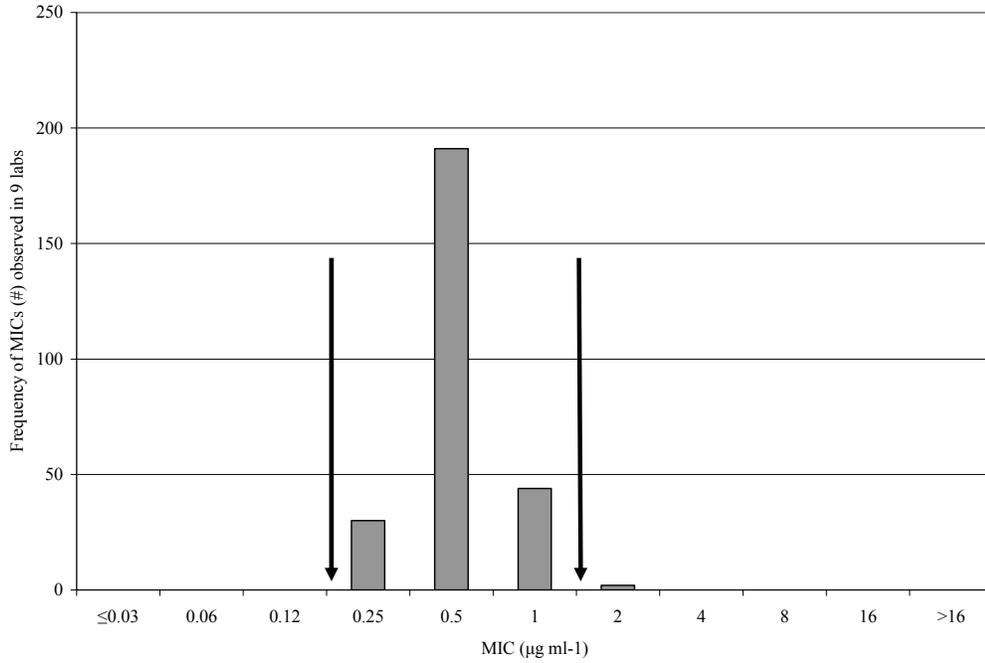


Figure 8. *Escherichia coli* ATCC 25922, oxytetracycline at 22°C 24 to 28 h. An example of a distribution with a single mode resulting in the approved three-dilution QC range 0.25 – 1 µg mL⁻¹.

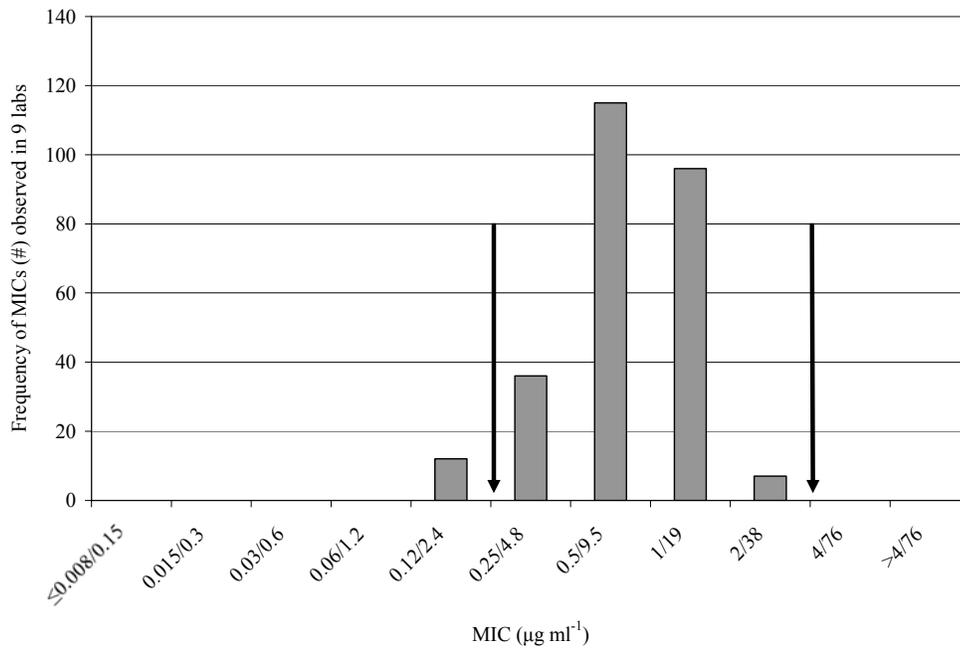


Figure 9. *Escherichia coli* ATCC 25922, ormetoprim-sulfadimethoxine at 22°C 44 to 48 h. An example of a bimodal distribution with the smaller mode (1/19 µg mL⁻¹) representing 83.5% of the larger mode (0.5/9.5 µg ml⁻¹). The approved four-dilution QC range is 0.25/4.8 – 2/38 µg mL⁻¹.

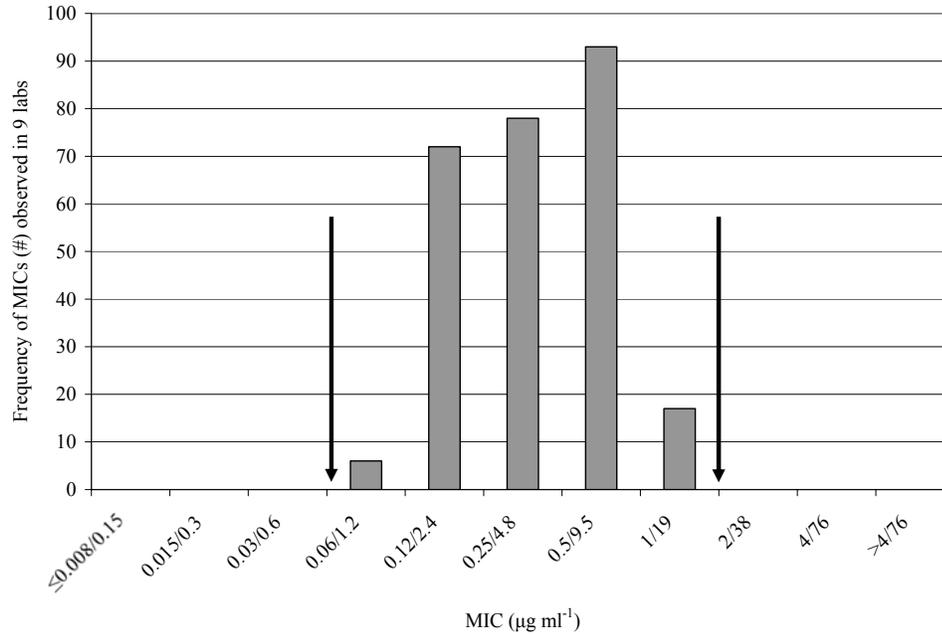


Figure 10. *Escherichia coli* ATCC 25922, ormetoprim-sulfadimethoxine at 35°C 16 to 20 h. An example of a trimodal distribution with the two smaller modes (0.12/2.4 µg mL⁻¹ and 0.25/4.8 µg mL⁻¹) representing 77.4% and 83.9% of the largest mode (0.5/9.5 µg/mL⁻¹), respectively. The approved five-dilution QC range is 0.06/1.2 – 1/19 µg mL⁻¹.

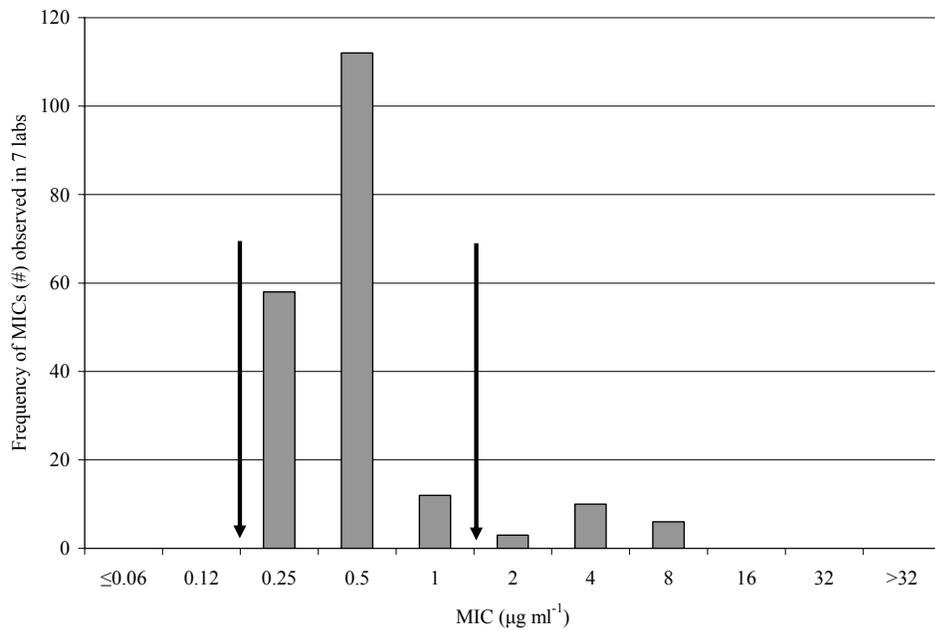


Figure 11. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, ampicillin at 22°C 44 to 48 h. An example of an approved three-dilution QC range (0.25 – 1 µg mL⁻¹) with a single mode, comprised of <95% of the total data points caused by trailing endpoints observed in two of the testing sites.

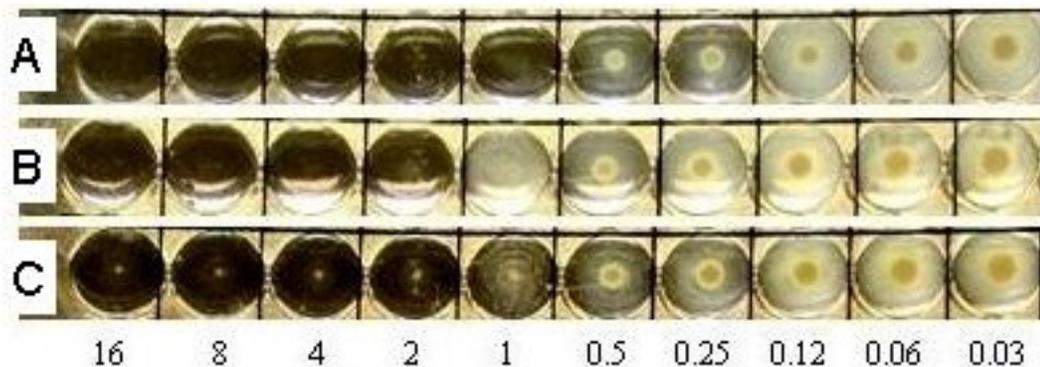


Figure 12. *Escherichia coli* ATCC 25922, oxytetracycline 28°C 24 to 28 h. Using two lots of CAMHB from two different manufacturers, rows A and B show normal growth in wells yielding MICs of 1 µg mL⁻¹ and 2 µg mL⁻¹, respectively. Row C shows small pellets of growth or ‘trailing endpoints’ using the third lot of CAMHB, with an MIC of 2 µg mL⁻¹

Approved CLSI guidelines for testing bacterial isolates at 35°C suggest that equivalent MIC results should be observed from tests using tetracycline in place of oxytetracycline, and trimethoprim-sulfamethoxazole in place of ormetoprim-sulfadimethoxine (CLSI/NCCLS, 2002b). After QC tests were conducted on *E. coli* ATCC 25922 at 35°C in each of the nine laboratories, modifications to those approved QC ranges were necessary. These new modifications were approved by members of the CLSI Subcommittee on VAST, and were included in the CLSI guidance document (CLSI/NCCLS, 2006b). The previously approved CLSI QC range for *E. coli* ATCC 25922 and tetracycline at 35°C was 0.5 – 2 µg/mL (CLSI/NCCLS, 2002b). However, this range did not correlate with the data observed in this study (QC range of 0.5 – 4 µg/mL). The CLSI-approved QC range for both trimethoprim-sulfamethoxazole and ormetoprim-sulfadimethoxine against *E. coli* ATCC 25922 was ≤0.5/9.5 µg/mL (CLSI/NCCLS, 2002b). Since this value was not a true range, it was important to attempt to establish a QC range with an upper and lower limit. QC ranges were established for

trimethoprim-sulfamethoxazole and ormetoprim-sulfadimethoxine using *E. coli* ATCC 25922 at 35°C.

CLSI QC ranges for dilution susceptibility testing are typically three or four dilutions wide (CLSI/NCCLS, 2002b), and this was the finding for the majority of the ranges determined in this work. The affect of temperature on the MICs was clearly demonstrated for both organisms. With an increase in temperature from 22°C to 28°C after 24 to 28 h incubation, a clear increase of approximately one dilution was observed in the approved ranges for both QC strains, indicating an amplified growth rate and/or antimicrobial agent metabolism. Additionally, incubation time affected the MICs for both organisms, where a one dilution increase in the approved QC range at 22°C was observed with the majority of the antimicrobial agents. The increase in MICs with an increased incubation time suggests there may be an amplified antimicrobial agent metabolism and/or degradation during the second 24 h of incubation at 22°C.

Validation Study

MICs were obtained for each isolate from the dry-form and frozen-form plates. MICs were evaluated by comparing the number of log₂ dilution steps from the dry-form plates to the MIC results on the frozen-form plates. Although there were some trends of increasing or decreasing MICs depending upon the organism, antimicrobial agent, and temperature condition, most of the MIC results (>95%) for each condition of organism, antimicrobial agent, temperature, and incubation time were within ±1 log₂ dilution step of one another (**Table 11**). When the percentage agreement within ±1 log₂ dilution fell below 95%, these results were primarily due to trailing endpoints observed in the dry-form plates. Despite using an automated inoculation system, there was a relatively high

frequency of occurrence of skipped wells, which contributed in some instances to a >1 log₂ dilution step difference between the plate-types. Isolates were only retested if two skipped wells were observed in two or more antimicrobial agent-dilution series (CLSI/NCCLS, 2000).

Colony count data for the *E. coli* and *A. salmonicida* isolates consistently yielded inocula concentrations in the 2.0 x 10⁵ - 8.0 x 10⁵ CFU/mL range. Counts slightly out of this range, did not appear to impact the validity of the test and were included.

Table 11. Percentage agreement within 1 log₂ dilution between MIC results on dried- and frozen-form panels.

	N	Temperature / time	AMP	ENRO	ERY	FFN	FLUQ	GEN	PRI	OXO	OXY	SXT
<i>E. coli</i> isolates	74	22°C 24 h	98.6	75.7	100.0	100.0	95.9	95.9	98.7	93.2	98.6	98.2
	74	22°C 48 h	95.9	84.3	93.2	100.0	98.6	87.8	100.0	95.9	98.6	100.0
	69	28°C 24 h	100.0	88.4	100.0	100.0	100.0	92.6	98.6	100.0	97.0	100.0
	112	35°C 16 h	98.2	91.9	99.0	100.0	99.0	87.9	98.2	96.2	78.6	99.0
		AVERAGE		98.2	85.1	98.1	100.0	98.4	91.1	98.9	96.3	93.2
<i>A. salmonicida</i> isolates	40	22°C 24 h	100.0	97.4	97.5	100.0	97.5	100.0	97.5	100.0	94.3	100.0
	40	22°C 48 h	97.5	92.3	97.5	97.6	95.0	87.5	100.0	97.5	97.1	100.0
	40	28°C 24 h	90.2	97.3	100.0	100.0	94.9	97.5	97.5	97.4	97.1	100.0
		AVERAGE		95.9	95.7	98.3	99.2	95.8	95.0	98.3	98.3	96.2

AMP, ampicillin; ENRO, enrofloxacin; ERY, erythromycin; FFN, florfenicol; FLUQ, flumequine; GEN, gentamicin; PRI, ormetoprim-sulfadimethoxine; OXO, oxolinic acid; OXY, oxytetracycline; SXT, trimethoprim-sulfamethoxazole

Conclusions

Based on these standardization and validation studies both *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 are acceptable QC strains for broth microdilution tests in dry- and frozen-form (or in-house prepared) MIC plates.

The methods and QC ranges (**Table 12**) described in this study were presented to members of the CLSI/NCCLS Subcommittee on VAST, and accepted for inclusion in the CLSI guideline M49-A (CLSI/NCCLS, 2006b).

This study represents the first published multiple laboratory study conducted in accordance with approved guidelines to establish MIC QC ranges at lower temperatures. These standardized methods and approved QC ranges should establish a foundation for the establishment of more ranges for other economically important antimicrobial agents,

and will serve as a model for the development of additional standardized testing methods for bacterial pathogens of aquatic animals. The utility of these methods and associated QC ranges should also extend to the development of susceptible, intermediate, and resistant breakpoints for antimicrobial agents used in aquaculture against economically important aquaculture pathogens.

Table 12. Summary of CLSI/NCCLS-approved MIC QC ranges for broth dilution susceptibility testing in CAMHB.

Antimicrobial agent	<i>Escherichia coli</i> ATCC 25922 ($\mu\text{g mL}^{-1}$)				<i>Aeromonas salmonicida</i> subsp. <i>salmonicida</i> ATCC 33658 ($\mu\text{g mL}^{-1}$)		
	22°C 24-28 h	22°C 44-48 h	28°C 24-28 h	35°C 16-20 h	22°C 24-28 h	22°C 44-48 h	28°C 24-28 h
	Enrofloxacin	0.004 - 0.015	0.004 - 0.015	0.008 - 0.03	(0.008 - 0.03)	0.008 - 0.03	0.008 - 0.03
Ampicillin	2 - 16	4 - 16	2 - 16	(2 - 8)	0.12 - 1	0.25 - 1	0.12 - 1
Oxytetracycline	0.25 - 1	0.5 - 2	0.5 - 2	0.5 - 4	0.06 - 0.25	0.12 - 1	0.12 - 1
Erythromycin	----	----	----	----	4 - 16	4 - 32	4 - 32
Florfenicol	2 - 16	4 - 16	4 - 16	(2 - 8)	0.25 - 1	0.5 - 2	0.5 - 2
Flumequine	0.06 - 0.5	0.12 - 0.5	0.12 - 0.5	0.25 - 1	0.015 - 0.12	0.03 - 0.12	0.015 - 0.12
Ormetoprim-sulfadimethoxine	0.12/2.4 - 1/19	0.25/4.8 - 2/38	0.12/2.4 - 1/19	0.06/1.2 - 1/19	0.06/1.2 - 0.25/4.8	0.06/1.2 - 0.5/9.5	0.06/1.2 - 0.5/9.5
Oxolinic acid	0.03 - 0.25	0.06 - 0.25	0.06 - 0.25	0.06 - 0.25	0.008 - 0.03	0.008 - 0.03	0.008 - 0.03
Gentamicin	0.12 - 0.5	0.25 - 1	0.25 - 1	(0.25 - 1)	0.25 - 1	0.25 - 2	0.25 - 1
Trimethoprim-sulfamethoxazole	0.03/0.6 - 0.12/2.4	0.03/0.6 - 0.25/4.8	0.03/0.6 - 0.25/4.8	0.03/0.6 - 0.12/2.4	0.03/0.6 - 0.12/2.4	0.03/0.6 - 0.25/4.8	0.03/0.6 - 0.25/4.8

() indicate QC ranges established previously (CLSI/NCCLS 2002b)

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Chapter 4: Epidemiologic cutoff values for antimicrobial agents against *Aeromonas salmonicida* isolates determined by frequency distributions of minimal inhibitory concentration and diameter of zone of inhibition data

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Abstract

Prior to prescription of antimicrobial therapy, clinicians tasked with the decision to treat or not to treat an animal or population, should be familiar not only with the disease but also with the antimicrobial susceptibility of the bacterial pathogen if present. The accuracy of *in vitro* antimicrobial susceptibility results to predict therapeutic efficacy can be improved through the use of interpretive criteria or clinical breakpoints. These breakpoint values are derived from *in vitro* antimicrobial susceptibility distributions of isolates of the target bacterial species or genus, as well as *in vivo* pharmacokinetic and pharmacodynamic (PK/PD) and clinical efficacy data. This study provided *in vitro* antimicrobial susceptibility distributions for four antimicrobial agents for 217 *Aeromonas salmonicida* isolates from 12 different countries. Minimal inhibitory concentration (MIC) and diameter of the zone of inhibition for oxytetracycline, ormetoprim-sulfadimethoxine, oxolinic acid, and florfenicol were determined for each isolate in accordance with standardized antimicrobial susceptibility testing methods for bacterial

isolates from aquatic animals that have been approved by the Clinical and Laboratory Standards Institute (CLSI). Susceptibility data were tabulated in a scattergram and analyzed by use of error rate bounding. Susceptibility tests for oxytetracycline, ormetoprim-sulfadimethoxine, and oxolinic acid revealed 2 distinct populations of bacteria. Isolates tested against florfenicol clustered into a single population. Use of frequency distributions of susceptibility results to develop epidemiologic cutoff values appears to be applicable to aquatic isolates. Frequency distributions of susceptibility results for *A. salmonicida* revealed clear divisions between isolate susceptibilities. This type of data, considered in conjunction with pharmacokinetic and efficacy data, may be useful for developing clinical breakpoints for use in aquaculture.

Introduction

Veterinarians are expanding their practices to include exotic species, including fish (Francis-Floyd, 2006; Kuehn, 2002). In the United States, only a few antimicrobial agents (including ormetoprim-sulfadimethoxine, oxytetracycline, and florfenicol) are approved for use in fish farmed for food production. Legislation such as the Minor Use and Minor Species Animal Health Act of 2004 is fostering the availability of additional therapeutic agents for use in fish (FDA-CVM, 2006). Before such drugs can be used, it is important for clinicians treating fish to become familiar with aquatic bacterial diseases and the susceptibility of those pathogens to various antimicrobial agents.

Although to our knowledge clinical breakpoints or interpretive criteria (susceptible, intermediate, and resistant) have not been developed for any aquatic pathogens in any aquatic animal species, standardized AST methods for aquatic isolates (Miller et al., 2005; Miller et al., 2003) should improve a clinician's ability to choose an

appropriate antimicrobial agent. Historically, veterinarians and researchers of aquatic diseases have used laboratory-specific clinical breakpoints. These values have had limited application or reliability outside of the regions in which they were generated. These limitations can be attributed to variations among *in vitro* testing procedures, limited diversity of isolates, and unique environmental conditions that may have affected therapeutic efficacy. Efforts to enhance the probability of therapeutic success when relying on AST results are dependent upon interpretive criteria which are as specific as possible for a given bacterial pathogen in a given animal species. The reliability of such interpretive criteria is enhanced when standardized AST methods, such as those published by the CLSI, are used (CLSI/NCCLS, 2006a; CLSI/NCCLS, 2006b). These two guidance documents, M42 and M49, provide standardized test conditions for nonfastidious aquatic bacterial isolates and provide details on methods for quality control and quality assurance.

Frequency distributions of MICs can be used to delineate epidemiologic cutoff values (also known as species-specific microbiological breakpoints), as defined by the European Committee on AST (EUCAST, 2006; Kahlmeter and Brown, 2004). These cutoff values can be used to discriminate wild type (i.e., originally susceptible bacterial populations) from nonwild type (i.e., populations with acquired and mutational resistance mechanisms) isolates. These cutoff values are not to be confused with clinical breakpoints, which are used primarily for predicting clinical outcomes.

The purpose of the study reported here was to develop epidemiologic cutoff values by use of frequency distributions of MICs and diameters of zones of inhibition for 217 typical and atypical (slow growing) isolates of *Aeromonas salmonicida* (causative

agents of furunculosis, goldfish ulcer disease, and carp erythrodermatitis) against 3 FDA-approved antimicrobials and 1 antimicrobial commonly used in some European countries. These distributions may be useful in developing clinical breakpoints when combined with data from pharmacokinetic-pharmacodynamic studies in targeted fish species and, if possible, clinical outcome data from fish with furunculosis or outbreaks of associated disease.

Materials and Methods

Sample Population

Isolates of *A. salmonicida* were obtained from 16 contributors located in various countries (8 in the United States, 2 in Israel, and 1 each in Canada, the United Kingdom, Switzerland, Spain, Norway, and Finland). Contributors were contacted by the authors and requested to provide typical and atypical *A. salmonicida* isolates from a wide geographic region that included clinical and wild-type strains representing a wide range of susceptibilities.

A total of 217 *A. salmonicida* isolates were used for AST, including 112 isolates from the United States representing 20 states, 99 isolates from 11 other countries, and 6 isolates from an unknown origin. Strains were originally isolated from 28 fish species. The year of original isolation for the isolates ranged from 1955 to 2004 (median year of original isolation, 1995).

All isolates were stored in tryptic soy broth with 20% glycerol at -80°C and then cultured on tryptic soy agar supplemented with 5% sheep blood at 22°C for 48 h. After culture, cells were harvested for DNA extraction and AST.

Six isolates, 1 from the National Collections of Industrial, Food and Marine Bacteria (Aberdeen, United Kingdom), and 5 from the American Type Culture Collection (ATCC 33658, ATCC 14174, ATCC 33659, ATCC 27013, ATCC 49393) (Manassas, VA) served as control isolates in the PCR assays. These isolates were not included in the sample population used for AST.

PCR Assay

Genomic DNA was extracted from all bacterial strains by use of a commercially available Generation capture column kit (Gentra Systems, Minneapolis, MN). Extraction was conducted in accordance with the manufacturer's instructions.

The PCR assays were performed in 0.2-mL thin-walled PCR tubes in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). Genomic DNA from *A. salmonicida* subsp *salmonicida* ATCC 33658 was used as a positive control sample for each of the 2 PCR assays (MIY and AP). Nuclease-free water was used as a negative control sample. Template DNA (10 to 100 ng) was added for each reaction, and a 1-kb Ready-Load DNA ladder (Invitrogen, Carlsbad, CA) was used. Products were separated by use of electrophoresis on 1.5% agarose gels and developed with ethidium bromide staining and UV illumination in a Bio-Rad Gel Doc 2000 documentation system (Hercules, CA).

The MIY primer set, which is specific for only typical strains of *A. salmonicida* subsp *salmonicida* (Miyata et al., 1996), was used. The MIY primer set comprises MIY1 (5'–AGCCTCCACGCGCTCACAGC–3') and MIY2 (5'–AAGAGGCCCCATAGTGTGGG–3'). Each reaction (volume, 25 µL) contained 0.6 units of Platinum *Taq* DNA polymerase (Invitrogen); 2.5 µL of 10X PCR buffer

(Invitrogen); 1.5mM MgCl₂ (Invitrogen); 16 pmol of each amplification primer (i.e., MIY1 and MIY2) (Invitrogen); and 0.2mM of each of the 4 deoxynucleotide triphosphates (Applied Biosystems). Reaction mixtures were maintained at 94°C for 2 min, amplified for 35 cycles with denaturation at 94°C for 30 seconds, annealing at 68°C for 90 seconds, and elongation at 68°C for 90 seconds. A final extension was performed at 68°C for 3 min. Expected size of the PCR product was 512 base pairs (bp).

The AP primer set, which is specific for all strains of *A. salmonicida* (Gustafson et al., 1992), was used. The primer set comprised AP1 (5'–GGCTGATCTCTTCATCCTCACCC–3') and AP2 (5'–CAGAGTGAAATCTACCAGCGGTGC–3'). Each reaction (volume, 25 µL) contained 0.25 units of Platinum *Taq* DNA polymerase; 2.5 µL of 10X PCR buffer; 2.5 mM MgCl₂; 8 pmol of each of amplification primer (i.e., AP1 and AP2), and 0.2mM of each of the 4 deoxynucleotide triphosphates. Reaction mixtures were maintained at 94°C for 2 min, amplified for 30 cycles with denaturation at 94°C for 15 seconds, annealing at 57°C for 30 seconds, and elongation at 72°C for 90 seconds. A final extension was performed at 72°C for 3 min. Expected size of the PCR product was 421 bp.

Disk Diffusion Testing

Disk diffusion tests were conducted in accordance with CLSI guidelines (CLSI/NCCLS, 2006a). *Escherichia coli* ATCC 25922 or *A. salmonicida* subsp *salmonicida* ATCC 33658, or both, were used as quality-control isolates. All tests were conducted on Mueller-Hinton agar (Difco, Sparks, MD), with incubation at 22°C for 44 to 48 h. Disks containing florfenicol (30 µg), oxolinic acid (2 µg), oxytetracycline (30 µg), and ormetoprim-sulfadimethoxine (1.25 and 23.75 µg of ormetoprim and

sulfadimethoxine, respectively) were obtained from BD Diagnostic Systems (Sparks, MD). Diameters of the zones of inhibition were measured with a ruler and rounded to the nearest millimeter. Bacterial inocula were standardized and monitored for cell densities in the range of 1×10^8 to 2×10^8 CFU/mL.

MIC Testing

Broth microdilution tests were conducted in accordance with CLSI guidelines (CLSI/NCCLS, 2006b). *E. coli* ATCC 25922 or *A. salmonicida* subsp *salmonicida* ATCC 33658, or both, were used as quality-control organisms. All tests were conducted in 96-well plates produced by Trek Diagnostic Systems (Cleveland, OH); plates were incubated at 22°C for 44 to 48 h. Plates contained dehydrated antimicrobial agent in each well and were formatted in 2 identical series of twelve 2-fold dilutions for florfenicol (32 to 0.015 µg/mL), oxolinic acid (4 to 0.002 µg/mL), and oxytetracycline (32 to 0.015 µg/mL) and eleven 2-fold dilutions for ormetoprim-sulfadimethoxine (8/152 to 0.008/0.15 µg/mL). Two wells were used as positive control wells. A Trek autoinoculator unit (Cleveland, OH) was used to dispense 100 µL of standardized inoculum prepared in cation-adjusted Mueller-Hinton broth (Trek Diagnostic Systems) into each well. Bacterial inocula were standardized and monitored for cell densities of approximately 5×10^5 CFU/mL.

Scattergram Analysis

The MIC and corresponding diameter of the zone of inhibition for each isolate were tabulated to generate a frequency distribution for each antimicrobial agent in the form of a scattergram (Microsoft Excel, Redmond, WA). As recommended by the CLSI

(CLSI/NCCLS, 2002a; CLSI/NCCLS, 2001), an error rate bounding method initially described elsewhere (Metzler and DeHaan, 1974) was modified to calculate discrepancy rates on the basis of MICs and diameters of zones of inhibition for all *A. salmonicida* isolates, typical *A. salmonicida* isolates, and atypical *A. salmonicida* isolates.

Discrepancy rates were calculated for use in selecting epidemiological cutoff values for the diameters of the zones of inhibition. The MIC₅₀ and MIC₉₀ values were also calculated for all isolates, isolates from the United States, and isolates from other countries.

Results

Analysis of PCR results by use of AP (*salmonicida* species-specific for typical or atypical isolates) and MIY (*salmonicida* subspecies-specific for typical isolates) primer sets revealed a pool of isolates consisting of 163 typical and 54 atypical *A. salmonicida* isolates; these results did not include the 6 reference isolates. Of the 163 typical isolates, 110 were from the United States, 49 were from other countries, and 4 were from an unknown origin. Of the 54 atypical isolates, 2 were from the United States, 50 were from other countries, and 2 were from an unknown origin.

Assay of a subset of the population show species-specific AP primers had positive results for only *A. salmonicida* isolates (**Figure 13**). One atypical isolate (*A. salmonicida* subsp *pectinolytica*) had negative results in PCR assays for both primers (data not shown). As expected, all atypical *A. salmonicida* isolates had negative results in the PCR assay for the MIY primer set. Some background banding was observed, but PCR products with intensely positive results made identification by use of PCR assays unambiguous.

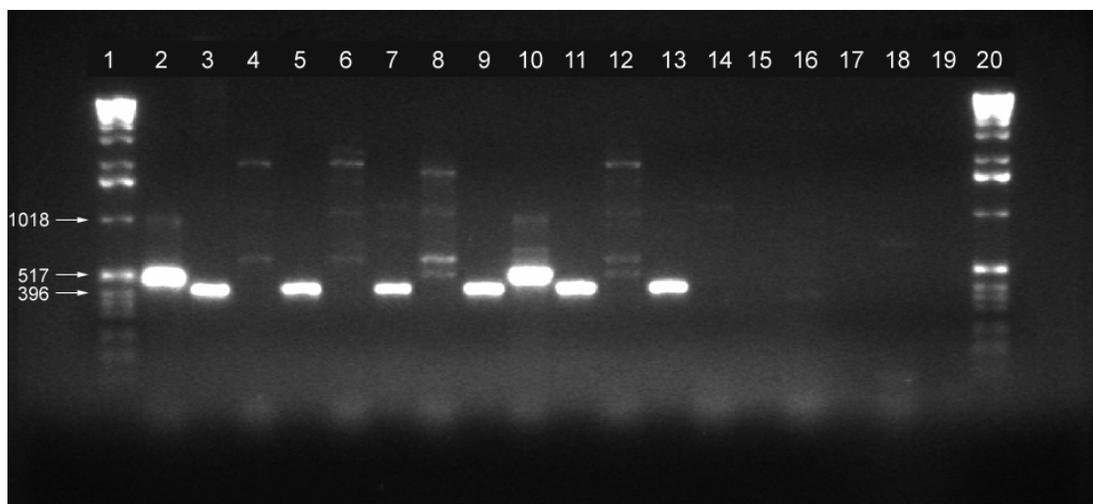


Figure 13—Agarose gel revealing PCR products obtained by use of the *salmonicida* subspecies-specific MIY primer set (512-bp product) for typical isolates only (lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18) and the *salmonicida* species-specific AP primer set (421-bp product) for typical and atypical isolates (lanes 3, 5, 7, 9, 11, 13, 15, 17, 19). Lanes were as follows: 1 and 20, 1-kilobase DNA ladder; 2 and 3, *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658; 4 and 5, *A. salmonicida* subsp. *masoucida* ATCC 27013; 6 and 7, *A. salmonicida* subsp. *achromogenes* ATCC 33659; 8 and 9, *A. salmonicida* subsp. *smithia* ATCC 49393; 10 and 11, Maine91 (typical); 12 and 13, 4059 (atypical); 14 and 15, *A. caviae* ATCC 15468; 16 and 17, *A. veronii* ATCC 9071; and 18 and 19, negative control samples. Values on the left represent molecular size in number of bp. Notice that the MIY primer set did not generate a band at 421 bp in lanes 4, 6, 8, and 12, which is as expected for atypical *A. salmonicida* isolates.

On the basis of evaluation of scattergrams that contained plots of the MICs versus the diameters of the zones of inhibition for oxytetracycline, ormetoprim-sulfadimethoxine, and oxolinic acid, 2 clearly discernible populations of isolates were observed (wildtype [susceptible to antimicrobials; no resistance mechanisms] and non-wildtype [acquired and mutational resistance mechanisms]; **Figure 14A, 14B, 14C**). A wide range of diameters of the zones of inhibition for oxytetracycline (13 to 30 mm) was observed between the 2 populations. Similar separation was evident for ormetoprim-sulfadimethoxine (7 to 19 mm) and oxolinic acid (22 to 34 mm). Distribution of the plotted points for florfenicol revealed a single wildtype population with all isolates having MICs ≤ 2 $\mu\text{g/mL}$ and zone diameters ≥ 34 mm (**Figure 14D**).

Discrepancy rates and error rate bounding were used as recommended by the CLSI (CLSI/NCCLS, 2002a; CLSI/NCCLS, 2001) to determine epidemiological cutoff values (**Tables 13 and 14**). The epidemiological cutoff values were adjusted until the number of false wildtype results on disk diffusion tests (very major discrepancies; type I errors) and false non-wildtype results (major discrepancies; type II errors) were held to a minimum. As specified by the CLSI for collections of clinical isolates (CLSI/NCCLS, 2002a), all rates for major and very major discrepancies were held at < 1.5% and < 3%, respectively. Minor discrepancies (i.e., when 1 test result was classified as intermediate and the other was wildtype or non-wildtype) were also considered in the calculations.

Analysis of MIC₅₀ and MIC₉₀ values calculated for all isolates, isolates from the United States, and isolates from other countries revealed a pattern only for oxolinic acid (**Table 15**). Isolates from the United States had considerably lower MICs for oxolinic acid, compared with the MICs for isolates from other countries. Isolates from the United States had slightly higher MICs for oxytetracycline, compared with the MICs for isolates from other countries (**Table 16**). Isolates from other countries had slightly higher MICs for ormetoprim-sulfadimethoxine, compared with the MICs for isolates from the United States (**Table 17**). No difference was observed with regard to MICs for florfenicol on the basis of geographic origin of the isolates (**Table 18**).

Gross observations of values for MICs and diameters of the zones of inhibition revealed that typical *A. salmonicida* isolates had slightly higher MICs than the atypical isolates for oxytetracycline, ormetoprim-sulfadimethoxine, and florfenicol (data not shown). Slower growth rate, characteristic of atypical *A. salmonicida* isolates, and subsequent increased growth inhibition may help explain this increased susceptibility. In

Table 13. Discrepancy between MICs and diameters of the zones of inhibition for *Aeromonas salmonicida* when tested against various antimicrobials.

<u>Antimicrobial</u>	<u>Isolates</u>	<u>MIC range[‡]</u>	<u>No.</u>	<u>Discrepancy*,</u>		
				<u>Very major</u>	<u>Major</u>	<u>Minor</u>
Oxytetracycline						
	All isolates	$\geq I_{\text{high}} + 2$	217	0	NA	0
		$I_{\text{high}} + 1$ to $I_{\text{low}} - 1$	217	0	0	1 (<0.01)
		$\leq I_{\text{low}} - 2$	217	NA	0	1 (<0.01)
	Typical isolates	$\geq I_{\text{high}} + 2$	163	0	NA	0
		$I_{\text{high}} + 1$ to $I_{\text{low}} - 1$	163	0	0	1 (0.01)
		$\leq I_{\text{low}} - 2$	163	NA	0	1 (0.01)
	Atypical isolates	$\geq I_{\text{high}} + 2$	54	0	NA	0
		$I_{\text{high}} + 1$ to $I_{\text{low}} - 1$	54	0	0	0
		$\leq I_{\text{low}} - 2$	54	NA	0	0
Ormetoprim-sulfadimethoxine						
	All isolates	$\geq I + 2$	217	1 (< 0.01)	NA	3 (0.01)
		$I + 1$ to $I - 1$	217	1 (< 0.01)	0	3 (0.01)
		$\leq I - 2$	217	NA	0	3 (0.01)
	Typical isolates	$\geq I + 2$	163	1 (0.01)	NA	3 (0.02)
		$I + 1$ to $I - 1$	163	1 (0.01)	0	3 (0.02)
		$\leq I - 2$	163	NA	0	4 (0.02)
	Atypical isolates	$\geq I + 2$	54	0	NA	0
		$I + 1$ to $I - 1$	54	0	0	0
		$\leq I - 2$	54	NA	0	0
Oxolinic acid						
	All isolates	$\geq I_{\text{high}} + 2$	217	0	NA	0
		$I_{\text{high}} + 1$ to $I_{\text{low}} - 1$	217	0	0	0
		$\leq I_{\text{low}} - 2$	217	NA	0	0
	Typical isolates	$\geq I_{\text{high}} + 2$	163	0	NA	0
		$I_{\text{high}} + 1$ to $I_{\text{low}} - 1$	163	0	0	0
		$\leq I_{\text{low}} - 2$	163	NA	0	0

Table 13. Discrepancy between MICs and diameters of the zones of inhibition for *Aeromonas salmonicida* when tested against various antimicrobials.

<u>Antimicrobial</u>	<u>Isolates</u>	<u>MIC range</u> ‡	<u>No.</u>	<u>Discrepancy*</u> ,		
				<u>Very major</u>	<u>Major</u>	<u>Minor</u>
Florfenicol	Atypical isolates	$\geq I_{\text{high}} + 2$	54	0	NA	0
		$I_{\text{high}} + 1$ to $I_{\text{low}} - 1$	54	0	0	0
		$\leq I_{\text{low}} - 2$	54	NA	0	0
	All isolates	$\geq \text{NWT} + 1$	217	0	NA	NA
		$\text{NWT} + \text{WT}$	217	0	0	NA
		$\leq \text{WT} - 1$	217	NA	0	NA
	Typical isolates	$\geq \text{NWT} + 1$	163	0	NA	NA
		$\text{NWT} + \text{WT}$	163	0	0	NA
		$\leq \text{WT} - 1$	163	NA	0	NA
	Atypical isolates	$\geq \text{NWT} + 1$	54	0	NA	NA
$\text{NWT} + \text{WT}$		54	0	0	NA	
$\leq \text{WT} - 1$		54	NA	0	NA	

*Very major discrepancies represent the number of false wild-type (WT) results on disk diffusion tests (type I errors), major discrepancies represent the number of false non-wild-type (NWT) results on disk diffusion tests (type II errors), and minor discrepancies represent when 1 test result was classified as intermediate and the other was WT or NWT. †Values reported are number (%). ‡ $I_{\text{high}} + 1$ and $I_{\text{high}} + 2$ represent 1 and 2 dilutions above the highest MIC within the intermediate range, respectively, and $I_{\text{low}} - 1$ and $I_{\text{low}} - 2$ represent 1 and 2 dilutions below the lowest MIC within the intermediate range, respectively. The $I + 1$ and $I + 2$ represent 1 and 2 dilutions above the intermediate MIC value, respectively, and $I - 1$ and $I - 2$ represent 1 and 2 dilutions below the intermediate MIC value, respectively. The NWT represents results for the population of isolates with acquired and mutational resistance mechanisms. The WT represents results for the population of isolates susceptible to antimicrobials (no resistance mechanisms). The MIC range for florfenicol was defined such that $\text{NWT} + 1$, $\text{NWT} + \text{WT}$, and $\text{WT} - 1$ represent 1 dilution above the NWT cutoff value, the NWT cutoff value and WT cutoff value, and 1 dilution below the WT cutoff value, respectively.

NA = Not applicable.

Table 14. Epidemiologic cutoff values for diameters of the zones of inhibition and MICs for all *A. salmonicida* isolates when tested against various antimicrobials.

Antimicrobial	Diameter of zone of inhibition (mm)			MIC (mg/mL)		
	WT	Intermediate range	NWT	WT	Intermediate range	NWT
Oxytetracycline (30 mg)	≥28	24-27	≥23	<1	2-4	≥8
Ormetoprim-Sulfadimethoxine (1.25 and 23.75 mg)*	≥20	17-19	<16	<0.5/9.5	1/19	≥2/38
Oxolinic acid (2 mg)	≥30	26-29	<25	<0.12	0.25-0.5	≥1
Florfenicol (30 mg)	≥31	NA	<30	<4	NA	≥8

*Values reported are for ormetoprim and sulfadimethoxine, respectively.

See **Table 13** for remainder of key.

Table 15. Cumulative percentage of MICs for 217 isolates of *A. salmonicida* (112 isolates obtained from the United States, 99 isolates obtained from other countries, and 6 isolates obtained from an unknown origin) when tested against oxolinic acid.

MIC (mg/mL)	All isolates	United States	Other countries
>4	100	100	100
4	98.6	100	97.1
2	95.9	100	91.2*
1	94.0*	100	87.3
0.5	89.9	100	78.4
0.25	89.9	100	78.4
0.12	89.9	100	78.4
0.06	88.0	98.3	76.5
0.03	82.0†	93.9*,†	68.6†
0.015	9.7	8.7	10.8
0.008	0.0	0.0	0.0
0.004	0.0	0.0	0.0
0.002	0.0	0.0	0.0
≤0.002	0.0	0.0	0.0

*Represents the MIC₉₀. †Represents the MIC₅₀.

contrast, atypical isolates had noticeably higher MICs for oxolinic acid; however, most of these isolates were from countries in which oxolinic acid is approved for use.

Analysis of frequency distributions of susceptibility results for all 4 antimicrobial agents revealed a wider range of zones of inhibition for most MICs than the range of MICs at specific diameters of zones of inhibition. These noticeable variations may be explained by a decreased robustness of disk diffusion tests on slower growing (atypical *A. salmonicida*) and fastidious organisms (Amsterdam, 1996). However, the distinct separation of wildtype and nonwild type isolates on the basis of diameters of the zones of inhibition alone should still provide accurate and useful epidemiological cutoff values for isolates of this pathogen. Susceptibility data revealed that both disk diffusion and broth microdilution testing methods may be used to monitor for the development of antimicrobial resistance in *A. salmonicida* isolates.

On the basis of the epidemiological cutoff values developed in the study, 6 (2.7%) isolates were classified as nonwild type for oxytetracycline, ormetoprim-sulfadimethoxine, and oxolinic acid, 15 (6.7%) isolates were classified as nonwild type for 2 of these antimicrobials, and 56 (25.1%) isolates were classified as nonwild type for only 1 of these antimicrobials.

Discussion

To our knowledge, the study reported here represents the first large-scale study in which standardized AST methods were used to generate frequency distributions of MICs and diameters of the zones of inhibition for a disease-causing bacterium in aquaculture. As recommended by the CLSI for the development of interpretive criteria, more than 100

Table 16. Cumulative percentage of MICs for 217 isolates of *A. salmonicida* (112 isolates obtained from the United States, 99 isolates obtained from other countries, and 6 isolates obtained from an unknown origin) when tested against oxytetracycline.

MIC (mg/mL)	All isolates	United States	Other countries
>32	100	100*	100
32	92.2*	89.6	95.1*
16	81.1	75.7	87.3
8	71.0	67.0	75.5
4	69.1	63.5	75.5
2	69.1	63.5	75.5
1	68.7	63.5	74.5
0.5	68.2	62.6†	74.5
0.25	50.7†	48.7	52.9†
0.12	8.8	10.4	6.9
0.06	0.0	0.0	0.0
0.03	0.0	0.0	0.0
0.015	0.0	0.0	0.0
≤0.015	0.0	0.0	0.0

See Table 15 for remainder of key.

Table 17—Cumulative percentage of MICs for 217 isolates of *A. salmonicida* (112 isolates obtained from the United States, 99 isolates obtained from other countries, and 6 isolates obtained from an unknown origin) when tested against ormetoprim-sulfadimethoxine.

MIC (mg/mL)*	All isolates	United States	Other countries
>8/152	100	100.0	100.0
8/152	94.0	96.5	91.2
4/76	94.0	96.5	91.2
2/38	94.0	96.5	91.2
1/19	92.6	94.8	90.2†
0.5/9.5	90.3†	93.9†	86.3
0.25/4.8	86.6	88.7	84.3
0.12/2.4	74.7‡	76.5‡	72.5‡
0.06/1.2	11.1	6.1	16.7
0.03/0.6	0.5	0.0	1.0
0.015/0.3	0.0	0.0	0.0
0.008/0.015	0.0	0.0	0.0
≤0.008/0.015	0.0	0.0	0.0

*Values represent concentrations for ormetoprim and sulfadimethoxine, respectively. †Represents the MIC₉₀. ‡Represents the MIC₅₀.

Table 18. Cumulative percentage of MICs for 217 isolates of *A. salmonicida* (112 isolates obtained from the United States, 99 isolates obtained from other countries, and 6 isolates obtained from an unknown origin) when tested against florfenicol.

MIC (mg/mL)	All isolates	United States	Other countries
>32	100	100	100
32	100	100	100
16	100	100	100
8	100	100	100
4	100	100	100
2	100	100	100
1	98.2*	98.3*	98.0*
0.5	78.8†	72.2†	86.3†
0.25	23.5	15.7	32.4
0.12	3.7	0.9	6.9
0.06	0.0	0.0	0.0
0.03	0.0	0.0	0.0
0.015	0.0	0.0	0.0
≤0.015	0.0	0.0	0.0

See **Table 15** for remainder of key.

clinical and wildtype isolates relevant to the class of antimicrobial and representing multiple geographic locations were tested.

The study reported here relied on donors providing us with isolates from their own stocks; thus, it did not fully represent a random sample of *A. salmonicida* isolates. Also it is possible some isolates used in this study may have been derived from the same bacterial clone. Clonality was not addressed in this study. Nevertheless, the large number and diversity of isolates in terms of location and species of origin should contribute to credibility of these data.

General recommendations can be made on the basis of the distinct separation (or clustering in the case of florfenicol) of the test population with regard to susceptibility. These epidemiologic cutoff values for isolates of *A. salmonicida* should not be

considered in a clinical context because they are based solely on susceptibility distributions determined *in vitro*. These cutoff values can be used to detect the development of resistance.

Discrepancy between *in vitro* test results of susceptibility and therapeutic effectiveness is a result of the numerous factors that influence the interactions of antimicrobials and bacteria *in vivo*. To have clinical application, these cutoff values must subsequently be correlated (and adjusted when necessary) with serum kinetics of the antimicrobial agent when administered at therapeutic doses and, if possible, clinical outcome data. In the United States, such clinical breakpoints for antimicrobials used in humans have been determined by panels of experts who review large data sets. The data provided here should assist in efforts to determine clinical breakpoints for antimicrobials used in aquatic animal medicine.

In 1 study (Tsoumas et al., 1989), investigators reported the frequency distribution of MICs for 70 isolates of *A. salmonicida* against oxytetracycline and 5 other antimicrobial agents and suggested a susceptible breakpoint of ≥ 1 $\mu\text{g}/\text{mL}$ for oxytetracycline. Data reported here reinforces this recommendation that an oxytetracycline cutoff value of 1 $\mu\text{g}/\text{mL}$ clearly separates the wild type from the nonwild type population (i.e., susceptible from resistant). In another study (Smith and Hiney, 2005), investigators evaluated frequency distributions for MICs and diameters of the zones of inhibition for oxolinic acid against *A. salmonicida* isolates and postulated classifying *A. salmonicida* strains into 3 groups (susceptible, ≤ 0.0625 $\mu\text{g}/\text{mL}$; intermediate, 0.125 to 0.5 $\mu\text{g}/\text{mL}$; and resistant, ≥ 1 $\mu\text{g}/\text{mL}$). Those results are extremely similar to the findings of the study reported here.

When veterinarians are faced with a decision to treat a patient or population, oftentimes the only tools they possess are susceptibility data for the test isolate, recommendations from the supplier of the disk diffusion tests, clinical experience, and information extracted from published reports. Other important considerations are the pharmacokinetic and physiologic differences among species, overall health of the patient or population, and route of administration. Data sets collected by Reimschuessel et al. (2005) summarize the multitude of external factors that can alter the pharmacokinetics of many drugs in piscine patients. Some of these include route of administration, species, temperature, salinity, and disease state. Effects of such variables must also be considered when treating fish.

Clinical aquatic animal medicine is challenging because of a lack of available antimicrobial agents, minimal efficacy data in many cultured fish species, and little information regarding frequency distributions of susceptibility results. The study reported here was an attempt to provide clinicians with some of this much needed data. These data represent a valuable component in the development of interpretive criteria and should be useful as researchers and clinicians move closer to establishing true clinical breakpoints for a major aquatic pathogen, *A. salmonicida*. Additional high-quality *in vivo* pharmacokinetic-pharmacodynamic and efficacy data will be required to allow clinicians and researchers to make comparisons and correlations with *in vitro* data on frequency distributions of susceptibility results reported here.

Chapter 5: Determination of Oxytetracycline Levels in Rainbow Trout Serum on a Biphenyl Column using HPLC

This manuscript is included as published under Miller R.A., R. Reimschuessel, and M. Carson. (2007) Determination of oxytetracycline levels in rainbow trout serum on a biphenyl column using high-performance liquid chromatography. J Chrom B. 852:655-658.

Abstract

We developed a simple and sensitive high performance liquid chromatography method on a biphenyl column to determine oxytetracycline (OTC) levels in rainbow trout serum. The assay used deproteination, filtration, and subsequent separation on a reverse-phase biphenyl column, with UV detection at 355 nm. OTC (7.8-7.9 min) was completely resolved from the structurally similar riboflavin (10.4-10.5 min), a common feed supplement. Estimated limits of detection and quantitation of OTC were 0.01 µg/mL and 0.04 µg/mL, respectively. The average recovery for OTC was 102% with a RSD of 8.34%. Calibration standards were linear from 0.01 µg/mL to 10 µg/mL.

Introduction

Oxytetracycline (OTC) is one of only three antibiotics currently approved by the United States Food and Drug Administration and available for use in aquaculture. It is approved for use against selected indications in salmonids, catfish, and lobsters (FDA-CVM, 2007).

Tetracyclines are known to have low bioavailability and are poorly absorbed when administered with feed (Agwuh and MacGowan, 2006). Elema et al. (1996) showed feed pellets contained many metallic ions that formed complexes with OTC and reduced the amount of drug available for fish to absorb from the intestinal tract. To choose an appropriate therapeutic agent, it is essential to have information about the serum concentration, over time, at the site of infection. Additionally, the minimal inhibitory concentration (MIC) of the pathogen and clinical efficacy data are also important to predict therapeutic efficacy.

Using various methods, researchers have studied the pharmacokinetic profile of OTC in multiple species including shrimp (Chiayvareesajja et al., 2006), hens (Singh et al., 2005), sea turtles (Harms et al., 2004), fish (Chen et al., 2004), goats (Payne et al., 2002), sheep and calves (Craigmill et al., 2000), pigs (Nielsen and Gyrd-Hansen, 1996), and humans (Wojcicki et al., 1985). Some of these methods evaluated drug activity- or microbiological-based methods to estimate OTC concentration in the blood, while others used analytical methods such as HPLC. Generally, HPLC is a more sensitive and precise method, and is especially useful when low concentrations of analyte are present. Very few, if any, studies have provided data resolving OTC from the structurally similar molecule riboflavin (**Figure 15**) in serum or plasma. Riboflavin or vitamin B₂, found in dairy products, eggs, vegetables, organ meats, whole grains, and wheat germ, is recommended as an aquaculture feed supplement (>20 mg/kg feed) to increase energy metabolism and promote physiological health (Cowey and Young Cho, 1993). Ichinose et al. (Ichinose et al., 1985) reported riboflavin concentrations ranging between 0.21 µg/mL and 0.37 µg/mL in serum of black carp, gibel, and eel. Due to their similarities in

structure and excitation range (riboflavin = 325-405 nm; OTC = 310-420 nm) (Garcia et al., 2004), we compared the retention times of riboflavin and OTC on a polymer column. Riboflavin retention time was only 30 s longer than that of OTC (unpublished data). Low levels of riboflavin absorbed from intestinal contents may co-elute with OTC, increasing the area of the OTC peak. In pharmacokinetic studies, exclusively OTC must be quantified in serum or plasma to predict therapeutic efficacy and calculate the pharmacodynamic parameters: time above MIC, C_{max}/MIC and AUC/MIC .

This is the first published method that uses a biphenyl column to quantify OTC in fish serum. This method resolves OTC from surrounding peaks, such as riboflavin.

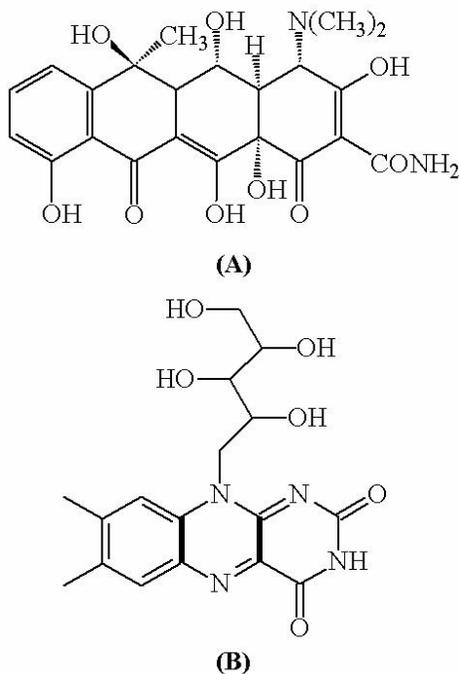


Figure 15. Molecular structures of OTC (A) and riboflavin (B).

Experimental

Chemicals

All chemicals were reagent grade unless otherwise specified.

We obtained OTC from the United States Pharmacopeia (Rockville, MD). We prepared a stock OTC solution, 1.0 mg/mL, by solubilization of 100 mg in 100 mL methanol, and stored it up to 3 months at -20 °C.

We prepared McIlvaine buffer (Oka et al., 1984) by solubilization of 12.9 g anhydrous citric acid (Mallinckrodt Baker Inc., Phillipsburg, NJ) and 10.9 g dibasic sodium phosphate (Fisher Scientific, Fair Lawn, NJ) in 1 L water. We prepared McIlvaine-EDTA buffer by solubilization of 37.2 g EDTA disodium salt dihydrate (Sigma-Aldrich, St. Louis, MO) up to 1 L of McIlvaine buffer with gentle heating. Prior to use we filtered McIlvaine-EDTA buffer through a 0.2 µm nylon filter and stored at 4 °C.

We used methanol (Mallinckrodt Baker Inc.) and acetonitrile (Honeywell Burdick and Jackson, Morristown, NJ), both HPLC grade, without further purification. We obtained oxalic acid dihydrate from Sigma-Aldrich. Water used throughout this study was purified by the Milli-Q plus Ultra-Pure Water System (Millipore Corporation, Bedford, MA).

Equipment

We used an Agilent Series 1100 (Agilent Technologies, Palo Alto, CA) Quaternary Pump, Vacuum Degasser, Autosampler, Thermostatted Column Compartment, and Variable Wavelength Detector for chromatographic separation. We

completed data acquisition and analysis with a PE Nelson 900 Series Interface Controller and TotalChrom™ software (PerkinElmer Life and Analytical Sciences, Wellesley, MA).

Sample Preparation

We collected control serum from 200-600 g rainbow trout obtained from a commercial source. Fish were held in tanks in our laboratory at 14-18 °C, and fed a standard diet which contained 35-45 mg riboflavin/kg feed according to the feed manufacturer (Rangen Inc., Buhl, ID). We euthanatized fish by immersion in a lethal dose of tricaine methanesulfonate (MS-222) (Crescent Research Chemicals, Phoenix, AZ). We stored serum at -80 °C until use. We used the stock 1.0 mg/mL OTC solution diluted in water to concentrations of 10 µg/mL and 3.0 µg/mL, to fortify serum samples at concentrations of 3.0, 0.50, and 0.10 µg/mL.

We vortex mixed serum samples (600 µL) with an equal volume (600 µL) McIlvaine-EDTA buffer, incubated at 30 °C for 30 min, and centrifuged at 12,000 g for 10 min to deproteinate. We removed supernates, and filtered through a Whatman (Florham Park, NJ) Puradisc™ 13 mm PVDF 0.2 µm syringe filter. We loaded the filtered samples into autosampler vials and injected a 100 µL aliquot onto the LC column.

We prepared chromatographic standards by serial dilution of the stock 1.0 mg/mL OTC solution in water to concentrations of 10, 3.0, 1.0, 0.30, 0.10, 0.03, and 0.01 µg/mL. We then diluted the standards 1:1 with McIlvaine-EDTA buffer. We analyzed chromatographic standards in a bracketed sequence (i.e., calibration standards + samples + calibration standards).

Column Liquid Chromatography

Separation was performed on an Allure™ biphenyl column 4.6 x 150 mm, 5 µm particle size, and 60 Å pore diameter (Restek Corporation, Bellefonte, PA). The column was maintained at 40 °C. We used mobile phases which were 0.2 µm nylon-filtered aqueous 0.01 M oxalic acid and acetonitrile. We employed gradient elution with initial conditions of 5% acetonitrile for 1 min, and linear changes to 30% acetonitrile at 10 min, 80% at 12 min, 80% at 13 min, 5% at 15 min, and 5% at 20 min. The flow rate was 1 mL/min.

Validation

We analyzed control and fortified serum samples to determine selectivity, accuracy, and precision. We extracted and analyzed four replicates of control serum and serum fortified at 3.0, 0.50, and 0.10 µg/mL on each of two different days, for a total of eight replicate analyses at each concentration.

We performed an exhaustive extraction procedure to assess accuracy with a real sample. In this experiment we used incurred rainbow trout serum samples obtained from one fish fed an OTC-medicated aquaculture feed (Rangen Inc.). As described above, we extracted and filtered five replicates of the incurred serum sample (200 µL each) with an equal volume of McIlvaine-EDTA buffer (normal extract). We resuspended the remaining pellet after centrifugation in 400 µL of McIlvaine-EDTA buffer, vortex mixed, centrifuged, and filtered as described above (exhaustive extract). We loaded normal and exhaustive extracts into autosampler vials and injected a 100 µL aliquot onto the LC column.

Results and Discussion

Table 19 summarizes the results of the validation study where the mean recovery of OTC was uniformly high (97.1-107%) at all concentrations tested. We did not find OTC in the unfortified rainbow trout serum control samples. Relative standard deviations were acceptably low at all three OTC concentrations tested, indicative of a precise method. Estimated lower limits of detection (LOD) and quantitation (LOQ) of OTC were 0.01 µg/mL and 0.04 µg/mL, respectively. We calculated these values as the average background signal plus 3 X the standard deviation (LOD) or plus 10 X the standard deviation (LOQ) in analyses of rainbow trout control serum. Calculated LOD and LOQ values were lower than those reported in previous OTC pharmacokinetics studies where serum was analyzed on a C8 column (LOD = 0.05 µg/mL) (Doi et al., 1998) and plasma was analyzed on a C18 column (LOQ = 0.1 µg/mL) (Haug and Hals, 2000). In addition, the mean percent recovery for all samples in this study ($102 \pm 8.34\%$) was greater than the same parameter measured previously by Doi et al. ($76.16 \pm 0.14\%$) (Doi et al., 1998) and Haug and Hals ($83.0 \pm 8.9\%$) (Haug and Hals, 2000).

Table 19. Validation using control and OTC fortified rainbow trout serum.

Sample	No.	Mean OTC concentration (µg/mL) ± SD	Mean recovery (%) ± % RSD ^a
Control serum - 0 µg/mL	8	-----	-----
Fortified serum - 0.10 µg/mL OTC	8	0.11 ± 0.01	107 ± 12.3
Fortified serum - 0.50 µg/mL OTC	8	0.51 ± 0.02	101 ± 3.06
Fortified serum - 3.0 µg/mL OTC	8	2.9 ± 0.06	97.1 ± 2.13

^a Mean recovery ± % RSD in all samples = 102 ± 8.34

We found excellent separation and recovery of OTC in fortified and incurred rainbow trout serum samples following simple deproteination and filtration (**Figure 16**).

From preliminary optimization trials, we found that riboflavin elutes at 10.4 min. Thus, in **Figure 16B-D** presumed riboflavin was found to separate in rainbow trout serum samples at 10.4 min, and estimated concentrations were consistent with those reported previously by Ichinose et al (1985). These data suggest riboflavin can be present in fish serum at relatively high concentrations, and needs to be resolved from OTC when using HPLC methods.

We generated standard curves for OTC by linear (weighted $1/x^2$) regression of peak areas against their respective concentrations. We studied linearity by separate analysis in quadruplicate of the calibration curves, created using the standards (10 $\mu\text{g/mL}$ – 0.01 $\mu\text{g/mL}$). Regression analyses for each line resulted in R^2 values exceeding 0.9991.

Stable retention times were observed throughout the validation (**Figure 16**). We were able to accurately determine OTC concentrations without interference from riboflavin (**Figure 16B-D**) or other serum components.

The mean normal extract concentration of five incurred serum replicates was $0.45 \pm 0.01 \mu\text{g/mL}$, while the mean exhaustive extract concentration was $0.02 \pm 0.01 \mu\text{g/mL}$, a value less than the LOQ of the method (**Figure 17**). The normal extract of the incurred serum was 95.5% of the total extract (normal + exhaustive extracts), consistent with the near quantitative recoveries found in the fortified experiments.

Conclusion

The method we describe here uses simple extraction and chromatographic procedures that accurately quantify OTC concentrations in rainbow trout serum. We believe its selectivity for OTC makes it a highly valuable method for pharmacokinetics and pharmacodynamics studies which require accurate OTC concentrations.

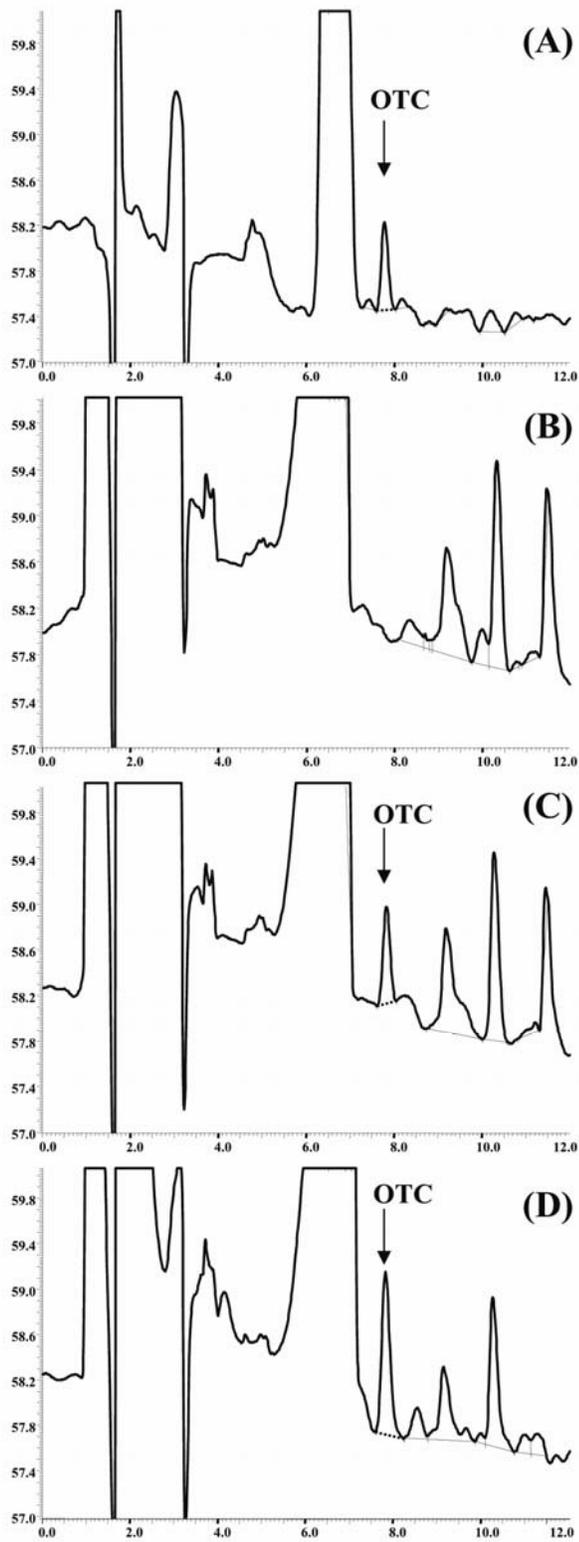


Figure 16. Chromatograms of the (A) 0.10 µg/mL calibration standard, (B) rainbow trout control serum, (C) 0.10 µg/mL fortified rainbow trout serum, (D) incurred rainbow trout serum (0.18 µg/mL).

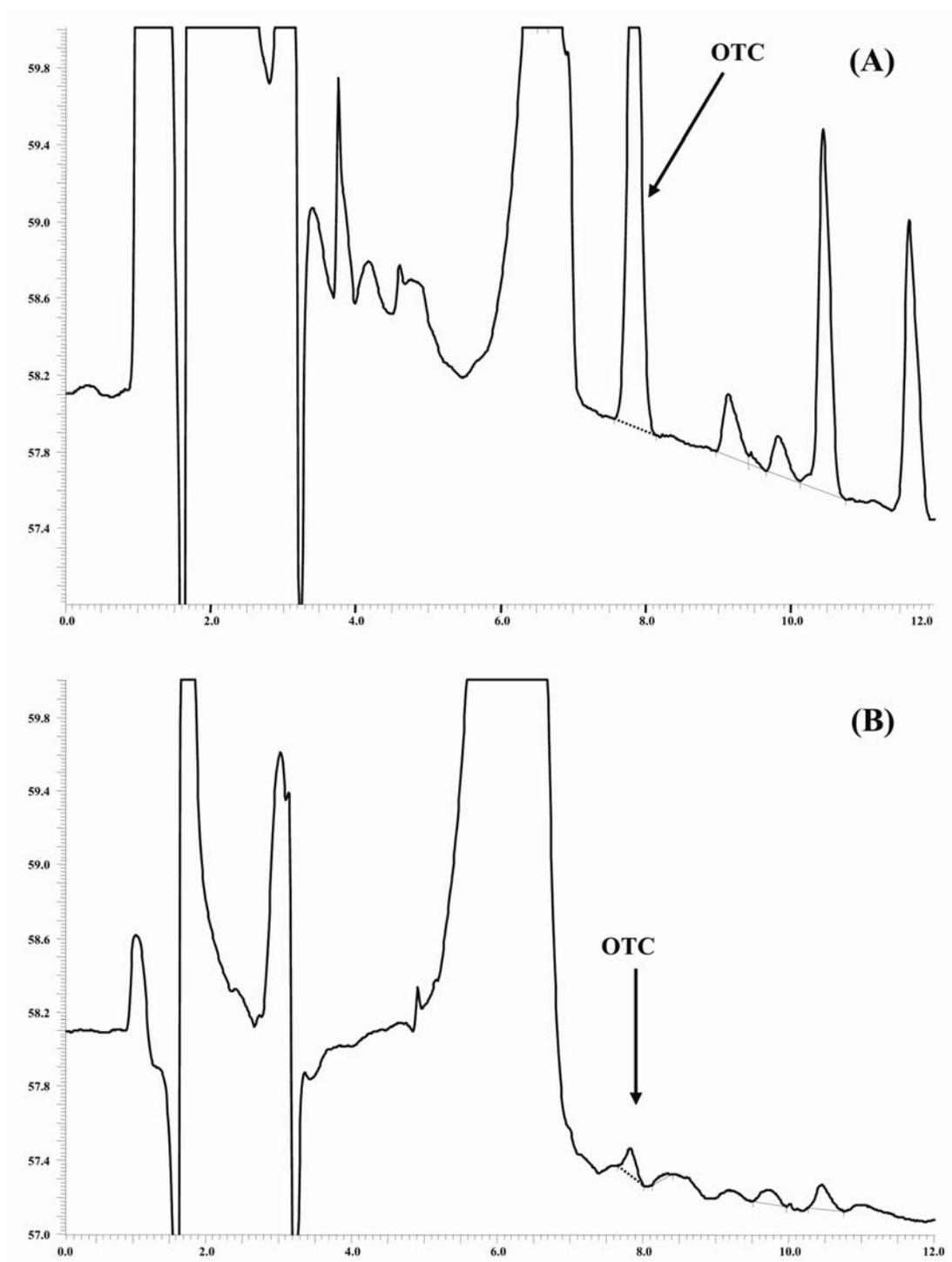


Figure 17. Chromatograms of incurred rainbow trout serum (A) and an exhaustive extraction of the centrifuged serum components (B).

Acknowledgements

The authors would like to thank Cristina Nochetto for her assistance with the chromatography, and Charles Gieseke and Christie Cheely for maintaining the animals used in this work. This study was made possible due to financial support from the Oak Ridge Associated Universities through a grant sponsored by the Food and Drug Administration.

Chapter 6: Pharmacokinetics of Two Oxytetracycline Feed Formulations in Rainbow Trout Using an Individualized Dosing Regimen

Abstract

Different drug formulations can affect pharmacokinetics and therefore may affect therapeutic efficacy. Active serum antimicrobial agent concentrations can be correlated with the *in vitro* derived minimal inhibitory concentration (MIC) of a pathogen to help predict therapeutic efficacy. We used a microbiological method to determine active oxytetracycline (OTC) pharmacokinetic profiles in serum from rainbow trout using two commonly used OTC-medicated feed formulations, quaternary salt (q-salt) and dihydrate salt. In separate experiments 72 rainbow trout held at 12-13 °C received 74.7 ± 1.5 mg OTC q-salt/kg body weight (b.w.) or 73.4 ± 2.0 mg OTC dihydrate salt/kg b.w. via oral gavage on 10 consecutive days. We euthanatized nine fish and collected serum on days 1, 3, 6, 8, 10, 12, 15, and 22 after dosing began. No significant difference ($p < 0.05$) was found in serum OTC concentrations of fish fed the two formulations until late in the elimination phase when serum OTC concentrations neared the limits of detection (LOD) after 15 days. Maximum serum concentrations, C_{\max} , were the same for both formulations ($1.19 \mu\text{g/mL}$) after 10 days. Pharmacokinetic properties including area under the concentration-time curve at steady-state over 24 h (AUC_{ss}), absorptive half-life ($T_{1/2\alpha}$), and elimination half-life ($T_{1/2\beta}$) were also very similar. This study revealed virtually identical pharmacokinetic properties for OTC in fish fed an OTC q-salt and dihydrate salt feed formulation.

Introduction

OTC is one of only three antibiotics currently approved by the U.S. Food and Drug Administration (FDA) and is available for use in aquaculture. It is approved for use against selected indications in salmonids, catfish, and lobsters (FDA-CVM, 2007).

In June 2006, FDA's Center for Veterinary Medicine (CVM) accepted an amended product chemistry package for Phibro Animal Health's new animal drug application which changed the Terramycin[®] OTC formulation from the mono-alkyl (C8-C18) trimethylammonium or q-salt, to the dihydrate salt. A recent study in shrimp hemolymph by Reed et al. (2006) showed the C_{max} and time to achieve the maximum (T_{max}) were not significantly different for either OTC formulation. However, they did show the q-salt form was more stable in water, and suggested it may remain in tact in the environment longer than the dihydrate salt form. They concluded the q-salt form, by persisting in the environment, may facilitate the development of resistant microorganisms. This is particularly important since tetracyclines are not metabolized *in vivo*, but are excreted in tact primarily in the urine (50-80% of a given dose) (Gerding et al., 1996).

The goal of this study was to, for the first time in a finfish species, elucidate whether there is a significant difference in the attainable microbiologically active serum OTC concentrations after administration of different OTC feed formulations (OTC q-salt and OTC dihydrate) administered by oral gavage. This study also provided pharmacokinetic data for OTC in healthy rainbow trout which will be separately correlated (**Chapter 7**) with data from experimentally infected rainbow trout.

Materials and Methods

Animals

We conducted two separate studies using the same experimental design for two OTC feed formulations (q-salt and dihydrate salt). We obtained 72 rainbow trout (*Oncorhynchus mykiss*) from Casta Line Trout Farm (Goshen, VA). We placed three fish in separate compartments using a polycarbonate box within 60 L aquaria. These 24 aquaria had a flow-through water supply maintained at 12-13 °C. We allowed fish to acclimate to their tanks for 5 d, and did not offer feed during this time. Three days into the acclimation period, we weighed each fish to calculate the amount of feed needed to target a dose of 75 mg OTC/kg b.w. The FDA-approved dosing range is 55 to 83 mg OTC/kg b.w. The mean weights of the fish in the OTC q-salt and dihydrate salt studies were 172 ± 44 g (median=182 g) and 130 ± 25 g (median=131 g), respectively.

OTC-medicated and Control Feed

We purchased the two OTC-medicated (8.8 g OTC/kg feed) trout production feeds from Rangen Inc. (Buhl, ID). Crude protein, fat, fiber, ash, and phosphorus levels in the two feeds were identical according to the manufacturer. Eurofins Scientific Inc. (Des Moines, IA) used a microbiological assay to determine the potency of the feed made with OTC q-salt (8130 mg/kg). Covance Laboratories Inc. (Madison, WI) used a high performance liquid chromatography method to determine the potency of the feed made with OTC dihydrate salt (8140 mg/kg). Feed analyses were completed within 3 months of use. We stored homogenized feeds away from light to minimize photodegradation of OTC. Mean doses in the OTC q-salt and dihydrate salt studies were 74.7 ± 1.5 mg

OTC/kg of fish, and 73.4 ± 2.0 mg OTC/kg, respectively. Based upon total feed ingested, administered doses were equivalent to 0.9% b.w. per day.

Study Design

After acclimation, we anesthetized each fish for <1 minute in a 400 mg/L solution of tricaine methanesulfonate (Crescent Research Chemicals; Phoenix, AZ). Dry homogenized feed samples were packed into plastic 3 mL luer-lock syringes with the tip excised and flamed to round the edges and minimize irritation during gavage. We calculated doses based on the body weight of each fish. All doses were within the FDA-approved dose of OTC in salmonids (55-83 mg/kg). We administered the doses by gavage daily for 10 consecutive days. No fish were observed to regurgitate any feed. We euthanized nine fish (three tanks) and obtained blood samples from the caudal vein at 1, 3, 6, 8, 10, 12, 15, and 22 d after beginning dosing. After the blood was allowed to clot at room temperature for 1-3 h, we stored the samples at 4 °C for 24-48 h. We collected serum from centrifuged blood samples (3,000 g for 15 min at 5 °C) and stored all serum at -80 °C until use. After dosing ceased, we fed homogenized non-medicated trout production feed (Rangen Inc.) at 0.9 % b.w. by oral gavage on days 10, 11, 16, and 17 to all fish remaining.

Serum Analysis by Microbiological Assay

We used methods modified from those described by Chen et al. (2004) and Strasdine and McBride (1979) to measure OTC in fish serum. We prepared *Bacillus cereus* ATCC 11778 spore suspensions equivalent to a 3.0 McFarland (9×10^8 CFU/mL) measured on a VITEK colorimeter (Biomérieux Inc.; Durham, NC). We added a 12 mL

aliquot of the spore suspension to 250 mL of Antibiotic Medium #8 agar (BD Diagnostic Systems; Sparks, MD) that had been cooled to 45.5 °C. After pouring the agar into a 150 mm X 150 mm petri plate (Nalge Nunc International; Rochester, NY), we allowed it to harden for 1.5 h. We removed agar wells with a cork borer (diameter = 4.70 mm) connected to a vacuum (**Figure 18**) and placed plates at 4 °C for 30 min, then prepared fortified OTC calibration standards.

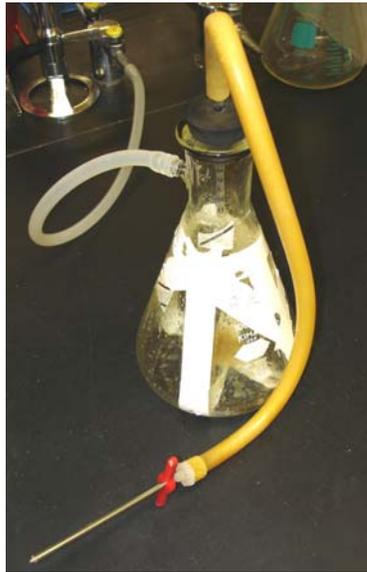


Figure 18. Agar cutter apparatus.

Control serum was collected previously from rainbow trout fed non-medicated trout production aquaculture feed (Rangen Inc.), and was used in the preparation of fortified OTC calibration standards. We prepared fortified serum standards (8, 4, 2, 1, 0.5, and 0 µg/mL) using a stock 1.00 mg/mL OTC methanolic solution (United States Pharmacopeia; Rockville, MD). The 0.5 µg/mL fortification was the lowest detectable fortified concentration. We added 45-50 µL per well. Fortified standards were added as a series of six concentrations in three microassay plates for each of the OTC q-salt and

dihydrate salt serum analyses. Mean zone diameters for each fortification level were used in regression calculations to determine serum OTC concentrations in the ‘unknown’ samples at each time point.

We thawed ‘unknown’ serum samples at room temperature, vortex mixed and added 45-50 μL to one well per sample. We incubated all test plates at 4 $^{\circ}\text{C}$ for 1 h then moved them to a 28 $^{\circ}\text{C}$ incubator for 20-21 h. We measured zone of inhibition diameters using digital calipers to the nearest one-hundredth of a mm.

We incorporated the zone diameters into the corresponding regression equation (based on the regression lines in **Figures 19 and 20**), to solve for the serum OTC concentration.

q-salt calculations: **Serum OTC concentration** = $\log_{10}^{0.064(\text{zone diameter})-1.129}$

dihydrate calculations: **Serum OTC concentration** = $\log_{10}^{0.070(\text{zone diameter})-1.353}$

We performed pharmacokinetic calculations using MS Excel Add-ins available for download online (Usansky et al., 2007).

Results

Fortified Standards

Regression analyses performed on zone diameter measurements of fortified standards were linear for both OTC q-salt (**Figure 19**) and OTC dihydrate (**Figure 20**) serum concentrations. The precision of the method was calculated to be $\pm 0.08 \mu\text{g/mL}$ (OTC q-salt analysis) and $\pm 0.05 \mu\text{g/mL}$ (OTC dihydrate analysis) by inserting the standard deviation of the zone diameter measurements into the above equations. The LOD of the average zone diameter of a well after 20-21 h of incubation was 4.29 mm plus two 2 mm radii (4

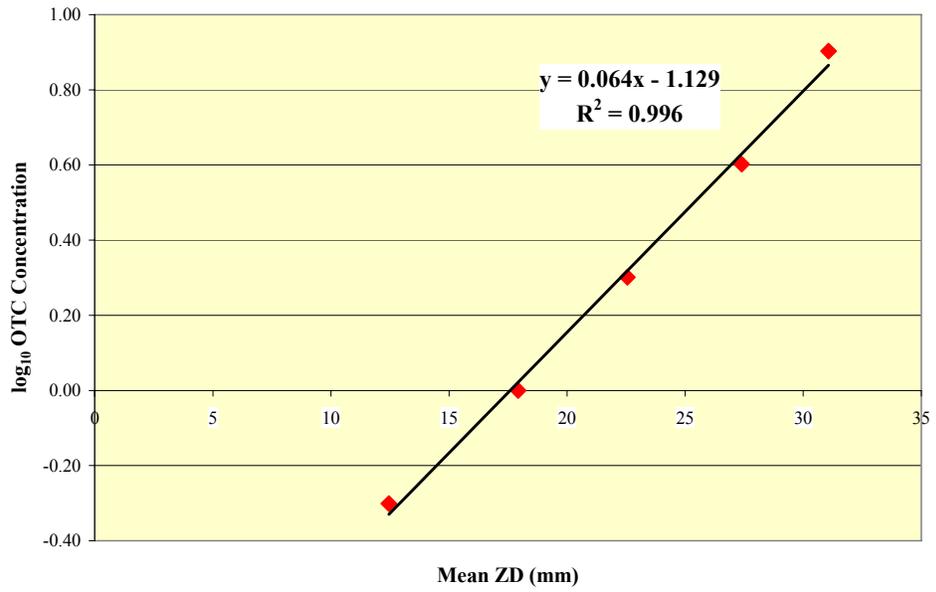


Figure 19. Regression analysis of OTC fortified serum standards used in analysis of serum from fish fed OTC q-salt feed.

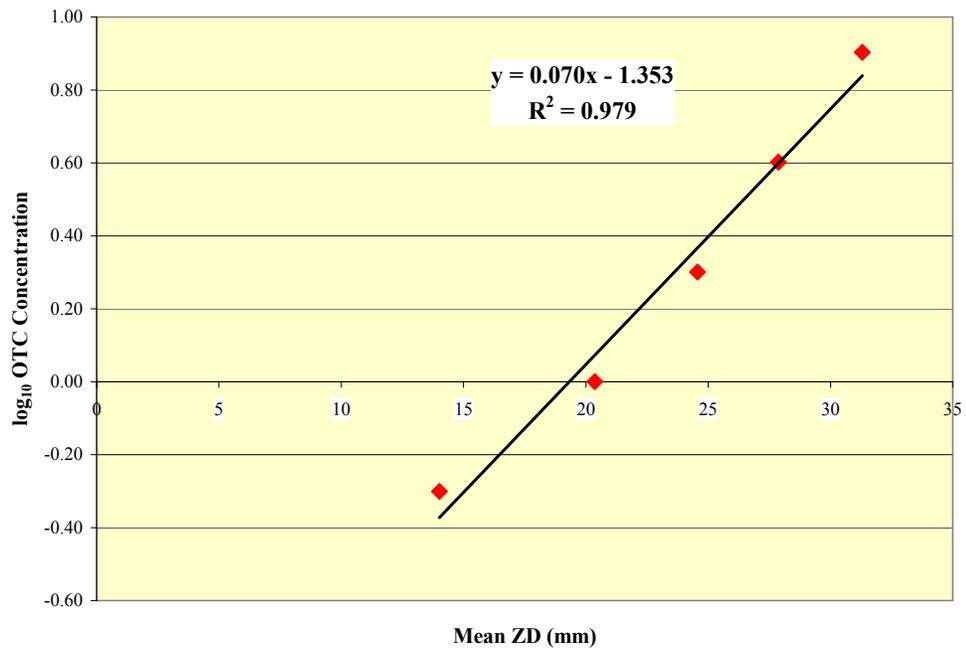


Figure 20. Regression analysis of OTC fortified serum standards used in analysis of serum from fish fed OTC dihydrate salt feed.

mm). This diameter is the smallest zone of growth inhibition surrounding the well that can be measured using this method. The LODs for the OTC q-salt and dihydrate analyses were 0.25 µg/mL and 0.17 µg/mL, respectively.

Incurred Serum OTC Concentrations

Significant differences in serum OTC concentrations between the two medicated feed formulations were found on days 15 and 22 (**Table 20; Figures 21 and 22**).

However, serum OTC concentrations were not detected in 5/9 q-salt samples and 1/8 dihydrate salt samples at 15 d, and no OTC was detected at 22 d in either treatment group. For samples where no OTC was detected, we assigned values equal to the LOD. Assignment of the LOD to these values was responsible for the significant difference found at 15 d and 22 d in the q-salt and dihydrate salt groups. We observed very low standard deviations throughout both studies likely due to the individualized dose calculations where all fish received approximately the same dose every 24 h per their b.w.

A white precipitate formed around the wells of serum samples in which no OTC was found (**Figure 23**). It is not clear why this precipitate was present only in samples where OTC was not detected.

Table 20. Serum OTC Concentrations in Healthy Rainbow Trout

OTC feed formulation	Days after Dose #1	Animals (n)	Mean OTC []	Standard Deviation
q-salt	1	7	0.25	0.00
dihydrate	1	9	0.31	0.07
q-salt	3	9	0.66	0.09
dihydrate	3	9	0.74	0.10
q-salt	6	9	0.79	0.19
dihydrate	6	9	0.85	0.19
q-salt	8	9	1.07	0.15
dihydrate	8	9	1.08	0.21
q-salt	10	9	1.19	0.34
dihydrate	10	9	1.19	0.27
q-salt	12	9	0.75	0.27
dihydrate	12	9	0.62	0.14
q-salt	15	9	0.31 ^a	0.07
dihydrate	15	8	0.21 ^a	0.06
q-salt	22	9	0.25 ^b	0.00
dihydrate	22	9	0.17 ^b	0.00

^a Significantly different (p<0.05)

^b Significantly different (p<0.05)

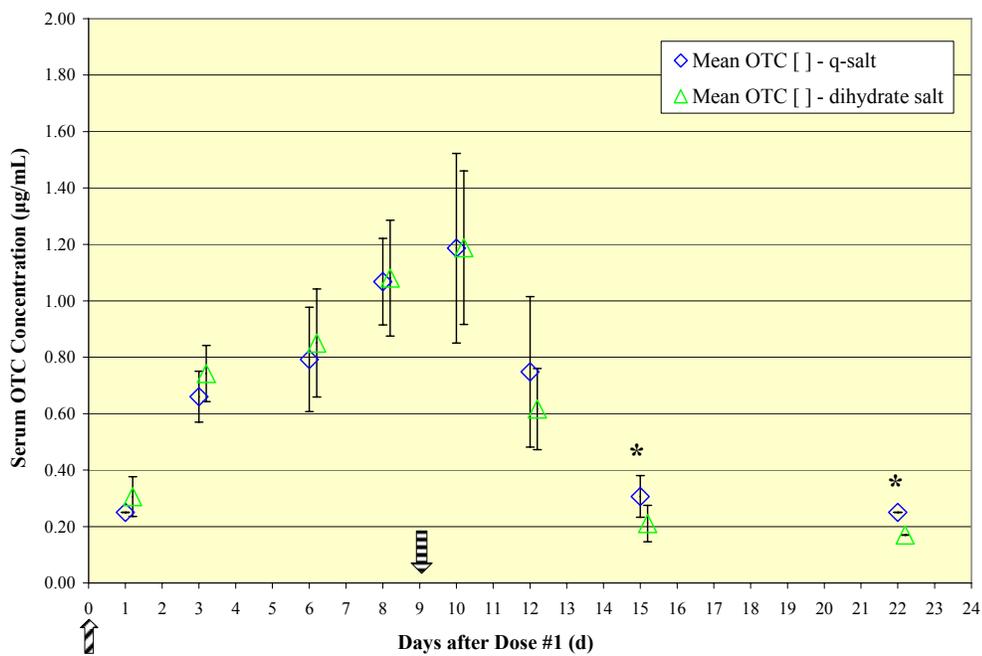


Figure 21. Mean serum OTC concentrations in rainbow trout after oral administration for 10 consecutive days. Bars represent \pm standard deviation. * indicates mean values are significantly different. \uparrow - 1st dose given; \downarrow - 10th dose given.

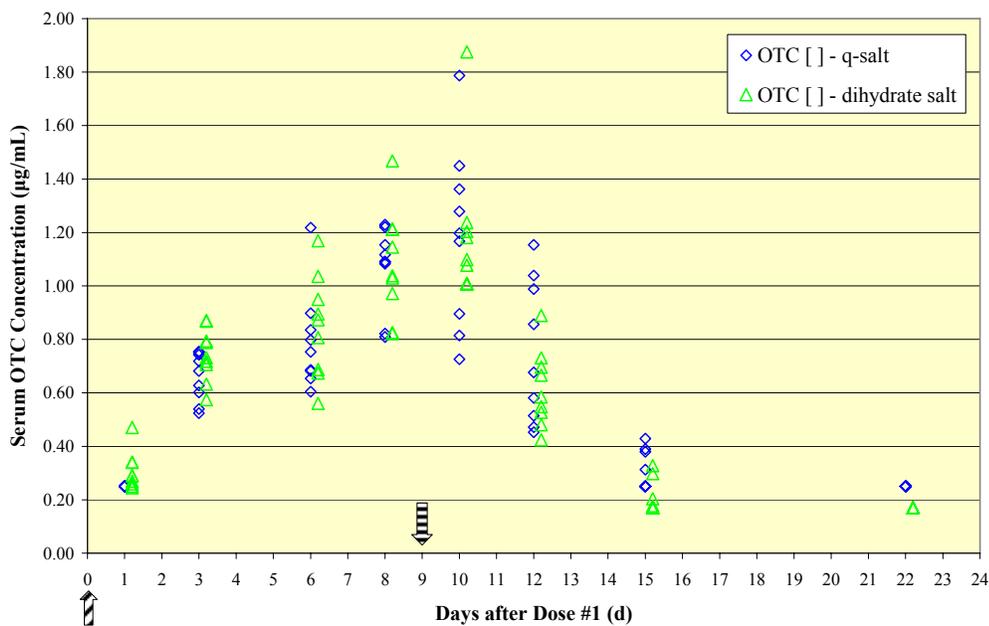


Figure 22. Serum OTC concentrations in 72 rainbow trout - all data. \uparrow - 1st dose given; \downarrow - 10th dose given.

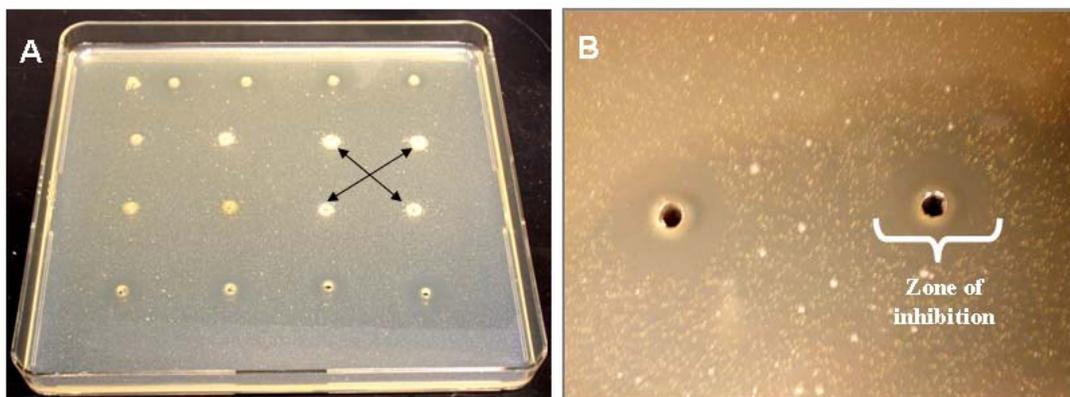


Figure 23. Microassay plate (A) with wells surrounded by a white precipitate (arrows); a diameter of zone of *Bacillus cereus* growth inhibition (B).

Pharmacokinetics Calculations

First order absorption from the gut into the central compartment (blood) was observed. For the purpose of making a comparison in diseased animals (**Chapter 7**), we calculated a $T_{1/2\alpha} (\ln 2/k_a)$ in the dihydrate and q-salt study equal to 5.2 d and 4.4 d, respectively, without using the method of residuals (**Figures 24 and 25**). Similarly, without extrapolation the elimination half-life ($T_{1/2\beta} = \ln 2/k_e$) of the dihydrate salt was 4.5 d and the q-salt was 5.5 d. Limited sampling during the elimination phase made it difficult to choose an appropriate pharmacokinetic model.

An AUC_{ss} was determined for each curve using mean serum OTC concentrations at 8 and 10 d calculated using the trapezoidal rule.

$$AUC_{ss} = ((C_{8d} + C_{10d})/2)(t_{10d} - t_{8d})/2$$

The AUC_{ss} for q-salt was 27.1 $\mu\text{g}\cdot\text{h}/\text{mL}$ and the AUC_{ss} for dihydrate salt was 27.2 $\mu\text{g}\cdot\text{h}/\text{mL}$, almost identical.

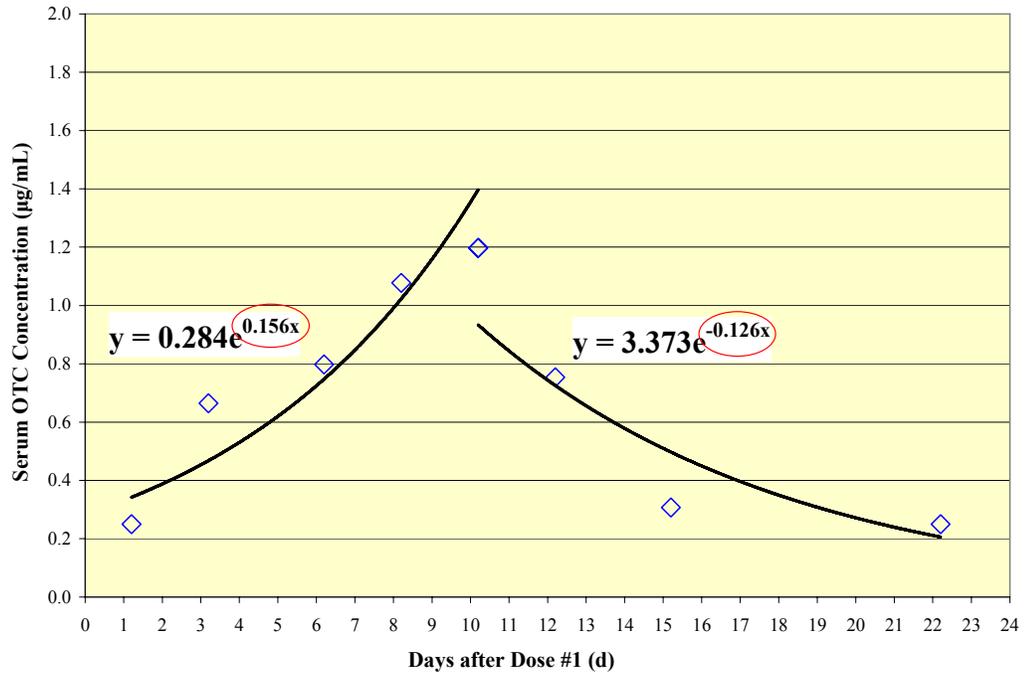


Figure 24. Two compartment pharmacokinetic model for OTC (q-salt formulation). Values circled in red are the k_a (at left) and k_e (at right).

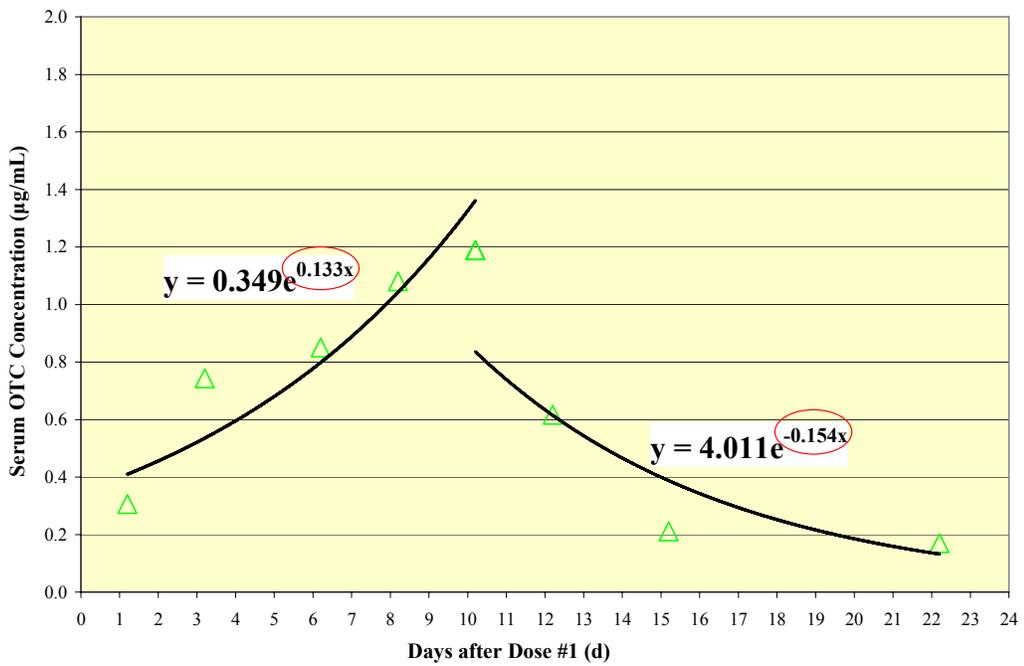


Figure 25. Two compartment pharmacokinetic model for OTC (dihydrate salt formulation). Values circled in red are the k_a (at left) and k_e (at right).

Discussion and Conclusions

This work represents the first published pharmacokinetics study in fish incorporating the following:

- 1) An FDA-approved dosing regimen administered by oral gavage
- 2) Each dose calculated for individual fish based on b.w.
- 3) No repeated bleedings of experimental fish aimed to reduce bias and variability from blood loss

We chose to use a high dose (73-75 mg OTC/kg b.w.), still within the FDA-approved range of 55-83 mg OTC/kg b.w., in an attempt to determine the maximum achievable serum OTC concentrations in healthy rainbow trout under prime experimental conditions. These supra-optimal conditions were meant to mimic what typically occurs in an aquaculture situation, the strongest healthiest fish eat the most, thus receiving treatment, while the out-competed and/or unhealthy fish succumb to disease. Additionally, achievable (or best case scenario) serum OTC concentrations during the dosing regimen in healthy fish can be used to help make clinical decisions based on *in vitro* susceptibility data obtained in the laboratory.

The effects of daily anesthesia and handling stress during this study were not determined. However, Chen et al. (2004) completed a similar study where OTC-medicated feed was broadcasted to summer flounder held at a slightly higher temperature (17 °C). When compared to the present study, following a 10 d dosing regimen Chen et al. found very similar concentrations in summer flounder at 0.04 d ($1.25 \pm 0.30 \mu\text{g/mL}$) and 1 d ($1.19 \pm 0.36 \mu\text{g/mL}$). This suggests the potential stress from daily anesthesia and handling may not markedly effect serum OTC concentrations.

Neither C_{\max} or T_{\max} were significantly different for the two forms of OTC, agreeing with data presented by Reed et al. (2006) in white shrimp. Contrary to the results of Reed et al., we found $AUC_{0-\infty}$ values for the two forms of OTC were not significantly different (q-salt = 175.9 $\mu\text{g}\cdot\text{h}/\text{mL}$, dihydrate salt = 187.0 $\mu\text{g}\cdot\text{h}/\text{mL}$). These findings, as well as the virtually identical AUC_{ss} values, indicate there is no marked difference in the total amount of active OTC absorbed in rainbow trout fed these two formulations.

The microbiological method employed in this study proved to be reproducible and yielded very similar results to those found using high performance liquid chromatography (data not shown).

Ultimately, only when drug effect can be directly predicted or assessed from drug concentrations, does pharmacokinetic analysis become applicable and meaningful. Thus, additional pharmacokinetics studies of OTC in fish exposed to a bacterial pathogen (**Chapter 7**) are needed to provide insight into the correlation of mass transfer of OTC embedded in pharmacokinetic models with drug effect.

Chapter 7: Efficacy and Pharmacodynamics Studies of Oxytetracycline in Rainbow Trout against *Aeromonas salmonicida*

Abstract

Pharmacokinetic/pharmacodynamic (PK/PD) indices quantify the relationship between pharmacokinetic parameters (i.e., area under the concentration-time curve, AUC) and microbiological parameters (i.e., minimal inhibitory concentrations, MICs). These indices can be used to determine both appropriate dosage regimens and index magnitudes required for efficacy and prevention of the emergence of antimicrobial resistance. The goal of our study was to determine the relevant PK/PD index (AUC/MIC) for oxytetracycline (OTC) using a furunculosis disease model in rainbow trout. We began by confirming the efficacy of OTC-medicated feed in rainbow trout against an *Aeromonas salmonicida* (0.25 µg/mL) immersion challenge disease model. We continued with a pharmacokinetics study using a total of 72 dermally abraded, *A. salmonicida*-challenged rainbow trout which were terminally bled 1, 3, 4, 6, and 10 d after medicated dosing began. There was a clear direct correlation between plasma total protein (TP) and fish health. The OTC AUC at steady-state over 24 h (AUC_{ss}) was 20.1 µg·h/mL during a 10 d dosing regimen of 74.7 ± 2.3 mg OTC/kg body weight (b.w.). The estimated AUC_{ss} divided by the current susceptible MIC epidemiologic cutoff value (1 µg/mL) for all *A. salmonicida* isolates (AUC/MIC index) is 20.1. This estimate is well above the reported PK/PD target for tetracyclines in a neutropenic murine-thigh model (≥5). Based on this estimate and AUC_{ss} values calculated previously in healthy

uninfected rainbow trout, the 1 µg/mL epidemiologic cutoff value represents an accurate breakpoint value for OTC against isolates of *A. salmonicida*.

Introduction

Many researchers have studied the pharmacokinetic profile of OTC in multiple aquatic species including shrimp (Uno et al., 2006; Chiayvareesajja et al., 2006; Reed et al., 2006), sea turtles (Harms et al., 2004), and fish (Rigos et al., 2004; Wang et al., 2004; Coyne et al., 2004b; Haug and Hals, 2000; Doi et al., 1998). There is, however, a void in the literature of data showing a drug effect in fish that was directly predicted or assessed from systemic OTC concentrations.

OTC (Terramycin[®], Phibro Animal Health) is approved by the United States Food and Drug Administration (FDA) as an oral antibacterial to treat many bacterial infections in animals and humans, but in aquaculture it is approved to treat furunculosis (*Aeromonas salmonicida*) and pseudomoniasis in salmonids, and bacterial hemorrhagic septicemia and pseudomoniasis in catfish (FDA-CVM, 2007). We chose to develop a furunculosis disease model in the rainbow trout because it is one of the most costly aquaculture diseases worldwide.

Furunculosis is a septicemic disease principally in salmonids and was first described in 1894 (Bernoth, 1997). Furunculosis is enzootic to many hatcheries but can be prevented through good husbandry practices and treatment. Signs of chronic infections are raised red fluid-filled lesions (furuncles) and necrosis of internal organs. Additional gross signs include intestinal swelling and erythema around the base of the fins. The disease is transmitted horizontally, but not vertically, and, as is the case with many fish diseases, severity increases with poor environmental conditions and associated

stress (Pickering, 1997). Recently an FDA-approved commercial vaccine (Furogen[®], AquaHealth Ltd.) has been shown to be highly effective in salmonids (Faisal and Gunn, 2007). Control of the disease by improved water quality and administration of antimicrobial medicated feeds have also been shown to be effective.

As the aquaculture industry expands, questions arise concerning the consequences of antimicrobial use. Because drugs like OTC are incorporated into the feed that is dispersed into the water, they are essentially dosing the environment, resulting in selective pressures in the exposed ecosystem (World Health Organization, 1999). Researchers have documented the emergence of antimicrobial resistance in the environment following use of antimicrobial agents in aquaculture (Huys et al., 2001; Guardabassi et al., 2000a; Sathiyamurthy et al., 1997; Husevag and Lunestad, 1995; Husevag et al., 1991). Thus, haphazard antimicrobial use in aquaculture should be avoided. Prudent use guidelines (National Aquaculture Association Board of Directors, 2003; AVMA Executive Board, 2002) discourage using antimicrobials for chemoprophylaxis. In addition, veterinarians need accurate test results to prescribe drugs appropriately, as well as guidelines for interpreting those from antimicrobial susceptibility tests. The M31-A3 Approved Guideline published by the Clinical and Laboratory Standards Institute states, *“It is important to consider that the judicious use of antimicrobials in the veterinary setting is directly related to the interpretive criteria associated with AST in that a given set of interpretive criteria only applies to that specific antimicrobial and disease combination. It is also important to note that interpretive criteria...apply only if the laboratory has conducted susceptibility testing according to the specific methods found in the documents.”* (CLSI/NCCLS, 2007b)

Currently, no such interpretive criteria (i.e., clinical breakpoints) are available for any fish pathogen in any fish species for any antimicrobial agent. To determine clinical breakpoints for a given antimicrobial agent against an aquatic pathogen at least the following are required; 1) susceptibility frequency distributions for a large number of isolates resulting in epidemiologic cutoff values (also called micro-epi cutoffs), 2) target animal PK/PD studies, and 3) correlations of susceptibility test results with clinical outcome. The latter two requirements are addressed in the present study to correlate with and complement the susceptibility frequency distribution data presented in **Figure 14A**.

The goal of this work was to confirm the efficacy of OTC for an *A. salmonicida* strain with a low MIC, determine its pharmacokinetics in fish exposed to the pathogen, and attempt to correlate the mass transfer of OTC embedded in the pharmacokinetic model with drug effect. We determined the AUC_{ss} for OTC and proposed a PK/PD index target that may predict therapeutic efficacy and prevent resistance from emerging.

Materials and Methods

Animals

For the Efficacy and Parallel study we obtained 144 rainbow trout (*Oncorhynchus mykiss*) from Casta Line Trout Farm (Goshen, VA) where no furunculosis cases have been reported. We placed three fish in separate compartments using a polycarbonate box within 60 L aquaria. The 24 tanks had a flow-through water supply maintained at 12-13 °C (**Figure 26**). We allowed fish to acclimate to their tanks for 3 d, and did not offer feed during this time.



Figure 26. Three rainbow trout per 60 L tank equipped with a polycarbonate box.

Study Design

In the Efficacy study we randomly assigned tanks to two treatment groups (Non-medicated and Medicated) using a randomized block design (**Figure 27**). Fish were observed at least twice daily throughout the study.

In the Parallel study we randomly assigned tanks to two treatment groups (Non-medicated and Medicated) and a Pre-dosing sampling group (**Figure 28**). We also randomly assigned tanks within each treatment group (Pre-dosing, Non-medicated, and Medicated) to a designated sampling day, where all fish in that tank were sacrificed and sampled as described below. The Pre-dosing treatment group was comprised of 12 fish total, euthanized one and two days (2 tanks/day) after challenge. In the Non-medicated feed group unexpected mortalities increased the total number of fish sampled on each pre-determined sampling day (1, 3, 4, 6 d), thus no fish were available to sample on day 10. The Medicated group was comprised of six fish, euthanized and sampled 1, 3, 4, 6, and 10 d after dosing began.

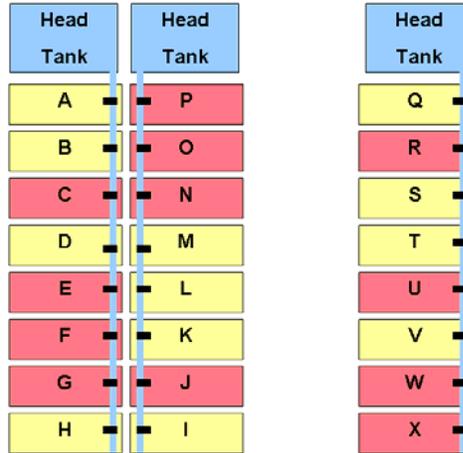


Figure 27. Efficacy study tank assignments - random tank assignment for Non-medicated (light yellow) and Medicated (dark pink) treatment groups.

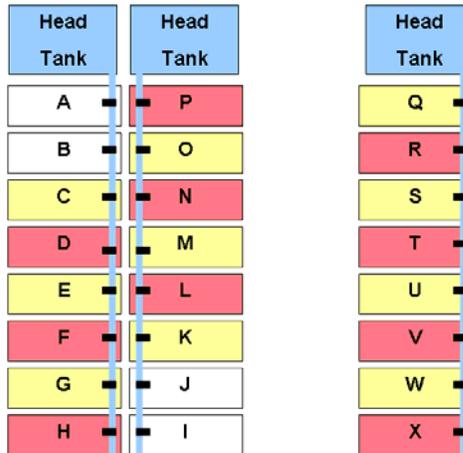


Figure 28. Parallel study tank assignments - random tank assignment for Non-medicated (light yellow), Medicated (dark pink), and Pre-dosing (white) experimental groups.

OTC-medicated and Control Feed

We purchased OTC-medicated (8.8 g OTC/kg feed) trout production feed manufactured using the newly approved dihydrate salt OTC formulation (Rangen Inc., Buhl, ID). Control trout production feed was obtained from the same manufacturer. According to the manufacturer identical crude protein, fat, fiber, ash, and phosphorus levels were present in both feeds. Covance Laboratories Inc. (Madison, WI) used a high performance liquid chromatography method to determine the potency of the OTC dihydrate salt feed (8140 mg/kg) and control feed (<2 mg/kg). Feed analyses were completed within 3 months of use, and the results were used in dose calculations. We homogenized the feeds in separate blenders, and stored them away from light to minimize photodegradation of OTC.

A. salmonicida Challenge Strain

A. salmonicida subsp. *salmonicida* Maine91 (CVM 33722) was originally isolated in 1991 from salmon in Maine and later found to be virulent in rainbow trout by Bowser et al. (1994). It was confirmed to be a ‘typical’ strain previously using PCR primers specific for *A. salmonicida* subsp. *salmonicida* (Miller and Reimschuessel, 2006). To confirm the virulence of this strain we sequentially infected six 150-300 g rainbow trout. We prepared a bacterial suspension equivalent to a 0.5 McFarland ($1-2 \times 10^8$ colony forming units/mL) and injected 0.5 mL intraperitoneally. Within 2-3 days, the fish succumbed to infection and the isolate was aseptically retrieved in near pure culture from the spleen on tryptic soy agar (TSA) with 5% sheep blood after 44-48 h incubation at 22 °C. Once in pure culture, the procedure was repeated in another fish. This was repeated

in a total of six rainbow trout, and the final isolate (CVM 35493) was frozen at -80 °C in tryptic soy broth with 30% glycerol.

Challenge Procedure

Following a 3 d acclimation period, we placed each fish under anesthesia in a 400 mg/L tricaine methanesulfonate solution (MS-222) (Crescent Research Chemicals; Phoenix, AZ) and measured its weight. We used each fish's weight to calculate the amount of medicated feed needed to target a dose within the FDA-approved range of 55 to 83 mg OTC/kg b.w. In the Efficacy study the mean fish weight in the Non-medicated group was 153 ± 27 g (median=152 g) and in the Medicated group it was 151 ± 25 g (median=150 g). In the Parallel study the mean fish weight in the Non-medicated group was 149 ± 24 g (median=147 g), in the Medicated group it was 137 ± 30 g (median=127 g), and in the Pre-dose sampling group it was 154 ± 21 g (median=156 g).

To facilitate infection by the *A. salmonicida* challenge strain we applied a dermal abrasion using a Dremel[®] tool under anesthesia (**Figure 29**) (Gieseke et al., 2006). We removed scales and epidermis from a 1 x 2 cm area on the epaxial musculature caudal to the dorsal fin. The scales and mucus were gently wiped free from the abraded area. Darwish et al. (Darwish et al., 2002) also cited dermal abrasion as necessary to induce disease reproducibly (**Figure 30**). Following dermal abrasion, each fish was returned to their respective tank compartment to recover.

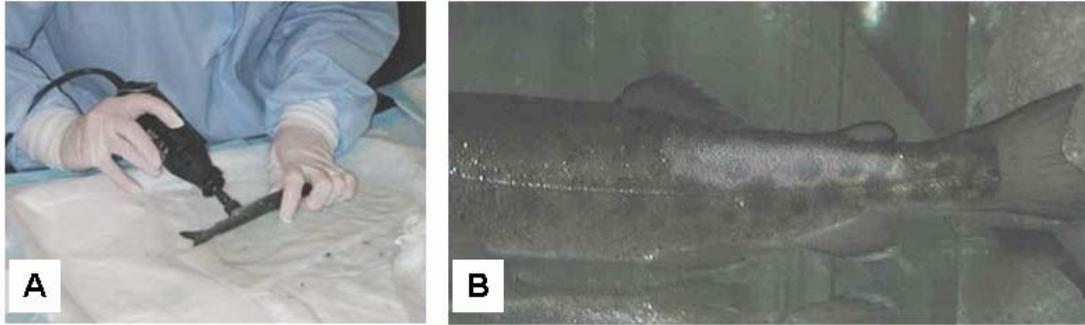


Figure 29. Abrasion method using a Dremel® tool (A) to produce a 1 x 2 cm abraded area (B).

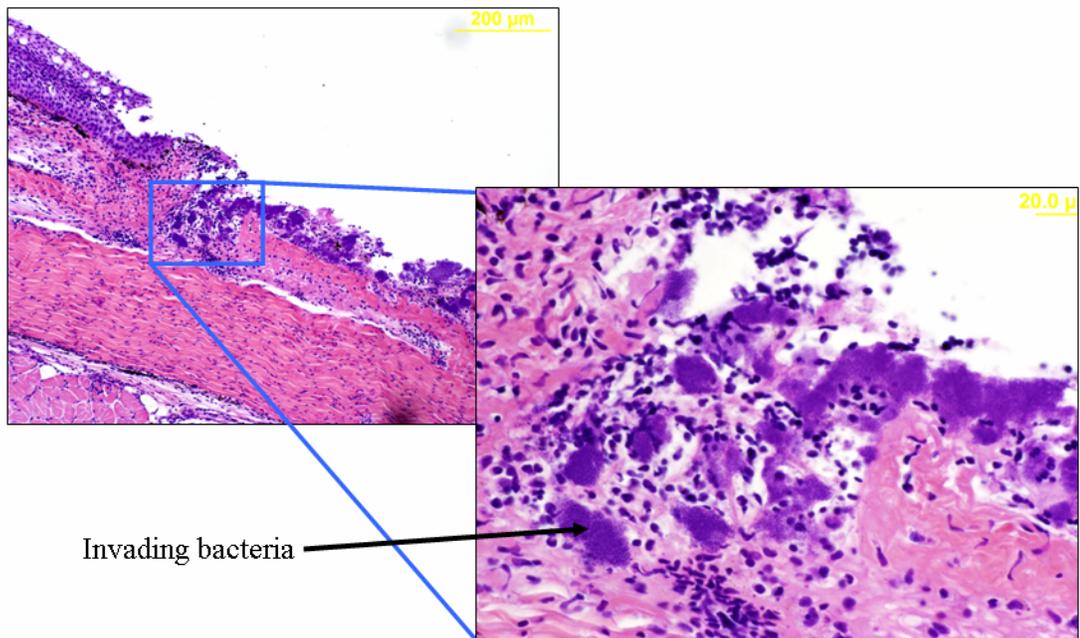


Figure 30. *A. salmonicida* invading the abraded dermis 2 d after immersion challenge.

Fifteen 1.8 L brain heart infusion (BHI) broth cultures of *A. salmonicida* CVM 35493 were grown in 2 L Erlenmeyer flasks incubated at 22 °C for 24 h. We pooled these cultures in a 10 L plastic carboy, and took a sub-sample to determine the colony forming units (CFUs)/mL of broth. We performed 10-fold serial dilutions of

the inoculum in 96-well microtiter plates using 20 μL transfer volumes and 180 μL sterile saline in each well. Four 20 μL drops were placed on TSA with 5% sheep blood, allowed to absorb, and incubated at 22 $^{\circ}\text{C}$ for 44-48 h.

$$\begin{aligned}\text{Cell density of inoculum in Efficacy study} &= \text{Avg colony count/drop} \times 50 \text{ (to get to} \\ &\text{per mL)} \times \text{dilution factor (10}^0 \text{ to 10}^{-6}\text{)} \\ &= 5.75 \text{ colonies/drop} \times 50 \times 10^{-6} \\ &= 2.88 \times 10^8 \text{ CFU/mL of broth culture}\end{aligned}$$

$$\text{Cell density of inoculum in Parallel study} = 1.38 \times 10^8 \text{ CFU/mL of broth culture}$$

We allowed fish to recover from anesthesia for 1.5-2 h prior to challenge. Tank water levels were lowered to 15 cm (~27 L), flow stopped, and 1 L total of undiluted *A. salmonicida* CVM 35493 BHI broth culture was added evenly to the three compartments in each of the 24 tanks. In the Efficacy study, fish were exposed to the 1 to 28 dilution of the *A. salmonicida* CVM 35493 broth culture [(2.88 $\times 10^8$ CFU/mL/28) = **1.03 $\times 10^7$ CFU/mL tank water**]. In the Parallel study, fish were exposed to **4.91 $\times 10^6$ CFU/mL tank water** [(1.38 $\times 10^8$ CFU/mL/28)]. Exposure lasted for 2 h then water flow was restored to all tanks at 0.75 L/min.

Dosing by Oral Gavage

Two days after bacterial challenge, in both Efficacy and Parallel studies we anesthetized all fish individually with MS-222 as before, and fed orally by gavage one of the two trout production feeds (Non-medicated and OTC-medicated). In the Efficacy study 36 fish (12 tanks) received control feed (Non-medicated), and another 36 fish received OTC-medicated feed. In the Parallel study 30 fish (10 tanks) received control

feed (Non-medicated), 30 fish received OTC-medicated feed, and another 12 fish (4 tanks) did not receive any feed and were sacrificed for sampling one day before and on the day dosing began (Pre-dosing group). We packed dry homogenized feed samples into dedicated plastic 3 mL luer-lock syringes with the tip excised and flamed to minimize irritation during insertion and removal (**Figure 31**). We administered doses by gavage daily for 10 consecutive days using separate anesthesia baths to prevent OTC and/or *A. salmonicida* cross-contamination. No fish were observed to regurgitate measurable amounts of medicated feed. Based upon total feed ingested, administered doses were equivalent to 0.9% b.w. per day. We used mass equivalents for fish fed the control feed (Non-medicated group).

In the Efficacy study, the mean dose in the Medicated group was 74.5 ± 1.5 mg OTC/kg of fish. In the Parallel study, the mean dose in the Medicated group was 74.7 ± 2.3 mg OTC/kg of fish. Immediately following dosing each fish was returned to their respective tank compartment to recover.

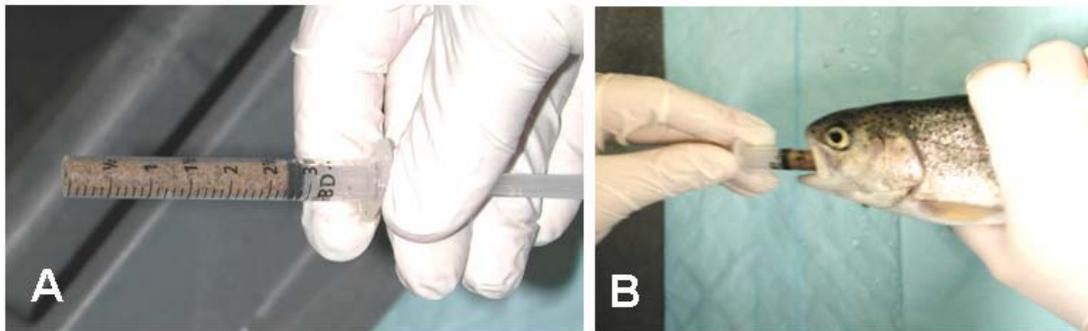


Figure 31. Excised 3 mL luer-lock syringe (A) used in oral gavage dosing (B).

Sampling Procedure

We obtained both plasma and serum from all moribund and surviving fish by exsanguination after euthanization with a lethal dose of MS-222. Plasma was also

collected from five freshly dead fish during the Efficacy study. We filled heparinized microhematocrit tubes and centrifuged them at 10,000 RPM for 5 min. We measured packed cell volumes (PCV, %) or hematocrit using a micro-capillary reader (IEC; Needham Heights, MA), and total protein (TP, g/dL) using a clinical refractometer (Schuco; Williston Park, NY). Preliminary studies suggested the product of PCV and TP is an accurate predictor of the osmoregulatory status of the fish. We allowed the remaining blood volume to clot at 4 °C overnight, centrifuged at 4,000 g for 15 min at 4 °C, and collected the serum which we stored at -80 °C.

We aseptically removed spleens from dead, moribund, and surviving fish, placed each spleen in 200 μ L 0.85% sterile saline, and weighed it to the nearest ten-thousandth of a gram. We homogenized each spleen in a microcentrifuge tube using a sterilized tissue homogenizer with a stainless steel shaft and Teflon pestle (Scientific Laboratory Supplies Inc.; Millville, NH) (**Figure 32**). We performed 10-fold serial dilutions of the vortex mixed spleen homogenate in a 96-well microtiter plate using 20 μ L transfer volumes and 180 μ L sterile saline in each well. We placed four 20 μ L drops from each dilution step onto a TSA with 5% sheep blood plate, allowed them to absorb, and incubated the plate at 22 °C for 44-48 h. We multiplied the average colony counts/drop by the dilution factor and then divided by the spleen weight to calculate the CFU/g of spleen. We randomly selected five colonies



Figure 32. Homogenizing spleen tissue for determination of CFU/g of spleen.

to streak for isolation and have their susceptibility to OTC determined by broth microdilution.

Broth Microdilution Testing

We determined OTC MICs after 44-48 h incubation at 22 °C using the standardized broth microdilution antimicrobial susceptibility testing method described by Miller et al. (Miller et al., 2005) (**Chapter 3**) and found in the CLSI Approved Guideline M49-A (CLSI/NCCLS, 2006b). We included quality control organisms *Escherichia coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 in all tests.

Serum Analysis

We used a modified microbiological method similar to the one described by Chen et al. (2004) and Strasline and McBride (1979) for determination of OTC in fish serum. We prepared a *Bacillus cereus* ATCC 11778 spore suspension in sterile deionized water equivalent to a 3.0 McFarland (9×10^8 CFU/mL) measured on a VITEK colorimeter (Biomérieux Inc.; Durham, NC). We added a 12 mL aliquot of the spore suspension to 250 mL of Antibiotic Medium #8 agar (BD Diagnostic Systems; Sparks, MD) that had been cooled to 45.5 °C. After pouring the agar into a 150 mm x 150 mm petri plate (Nalge Nunc International; Rochester, NY), we allowed it to harden for 1.5 h. We removed agar wells with a cork borer (diameter = 4.70 mm) connected to a vacuum and placed plates at 4 °C for 30 min.

We collected serum from rainbow trout fed non-medicated trout production feed (Rangen Inc.), and used it to prepare fortified OTC calibration standards. We prepared serum standards (8, 4, 2, 1, 0.5, and 0 µg/mL) using a stock 1.00 mg/mL OTC methanolic

solution (United States Pharmacopeia; Rockville, MD). The 0.5 µg/mL fortification was the lowest detectable fortified concentration. After removing the microassay plates from 4 °C, we added 45-50 µL of fortified solutions to each well. We included the series of six fortified standards on three microassay plates. We used mean zone diameters at each fortification level in regression calculations. We calculated a level of detection (LOD) based on the average zone diameter of a well after 20-21 h of incubation (4.29 mm) plus two 2 mm radii (4 mm). We determined this diameter to be the smallest zone of growth inhibition surrounding the well capable of accurate measurement using this method. Using the regression equation provided below we calculated a LOD of 0.18 µg/mL.

We thawed serum samples at room temperature, vortex mixed and added 45-50 µL to one well per sample. We incubated all test plates at 4 °C for 1 h then moved them to a 28 °C incubator for 20-21 h. We measured diameters of zone of inhibition using digital calipers to the nearest one-hundredth of a mm.

We incorporated the zones of inhibition into the corresponding regression equation (based on the regression line in **Figure 33**), to solve for the serum OTC concentration.

Serum OTC calculations: $OTC [] = \log_{10}^{0.070(\text{zone diameter})-1.326}$

We performed pharmacokinetic calculations using MS Excel Add-ins available for download online (Usansky et al., 2007).

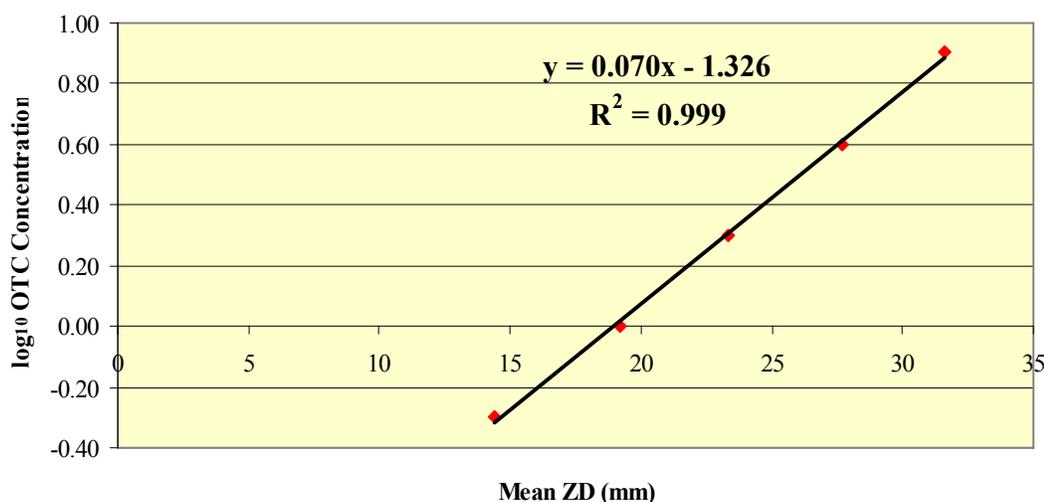


Figure 33. Regression analysis of OTC fortified serum standards used in extrapolation of serum OTC concentrations from the Efficacy and Parallel studies.

Results and Discussion

Efficacy Study

Treatment with OTC-medicated feed drastically decreased the percent mortality when compared to the Non-medicated group (**Figure 34**). Percent mortality in the Non-medicated fish was 88.9%, whereas in Medicated fish it was only 2.8%. All mortalities occurred between 4 and 9 d after dosing began, and all fish had substantial concentrations of *A. salmonicida* in their spleens (**Figure 35**). Clinical signs presented as erratic swimming, tachypnea (respiratory distress), darkening of skin, hemorrhages at the base of the fins. Internally, the presence of ascites and hemorrhage of the abdominal wall and viscera were noted.

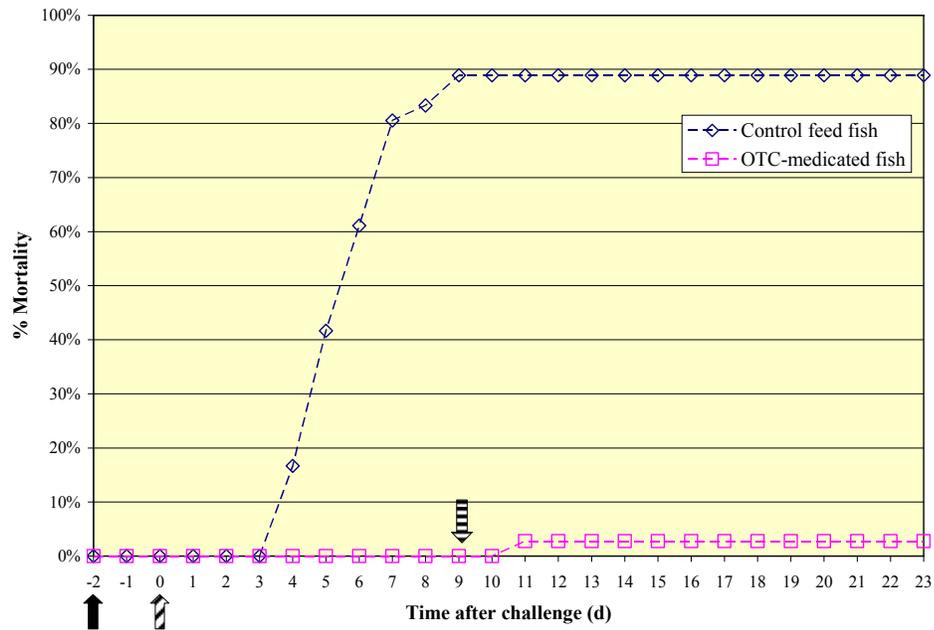


Figure 34. Cumulative percent mortality showing the efficacy of OTC in rainbow trout against an experimental *A. salmonicida* infection. ↑ - day of challenge; ↗ - 1st dose given; ↘ - 10th dose given.

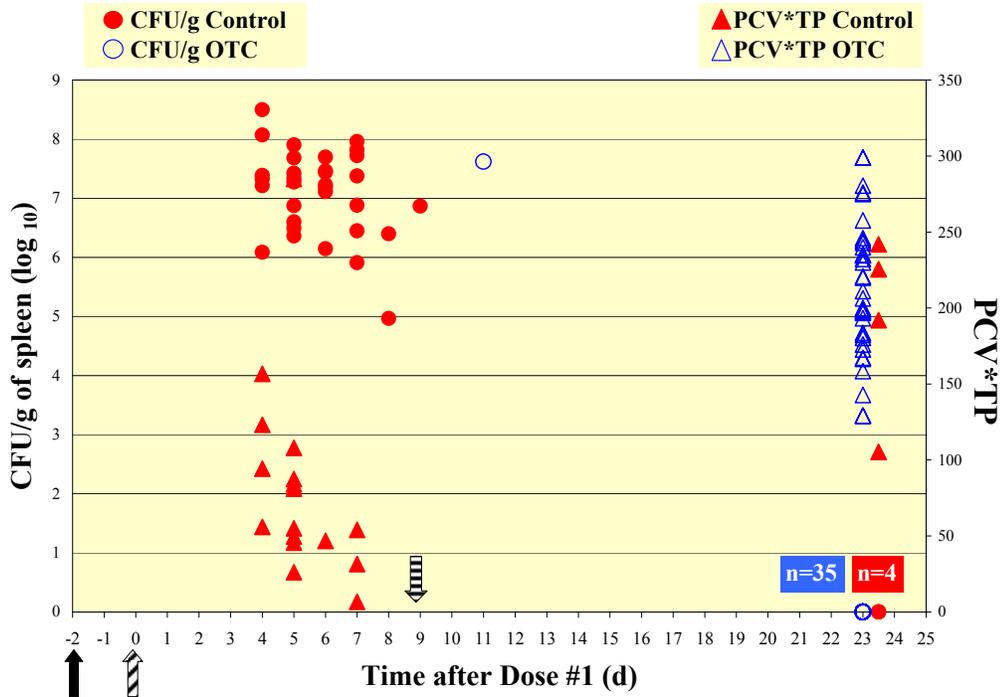


Figure 35. Persistence of *A. salmonicida* CVM 35493 in rainbow trout, each represents one spleen (left axis). Packed cell volume (PCV) x total protein (TP) values in experimentally infected rainbow trout (right axis). ↑ - day of challenge; ↗ - 1st dose given; ↘ - 10th dose given.

Infectivity on the order of 10^6 - 10^8 CFU/g was found in the spleen in virtually all samples. Histologically, focal to multi-focal necrosis in the spleen was common in dead fish and those showing signs of clinical disease (**Figure 36**). The one Medicated fish found dead 11 days after dosing began also had a high concentration of *A. salmonicida* detected in the spleen. Broth microdilution susceptibility tests of the 156 *A. salmonicida* isolates taken from spleens of Non-medicated fish revealed no change in OTC MIC (0.06 – 0.25 $\mu\text{g/mL}$) from the parent

challenge strain CVM 35493 (0.25 $\mu\text{g/mL}$). However, all five *A. salmonicida* isolates randomly taken from the spleen of the Medicated fish found dead were highly resistant to OTC with MICs >32 $\mu\text{g/mL}$. Clonality was not addressed with these isolates.

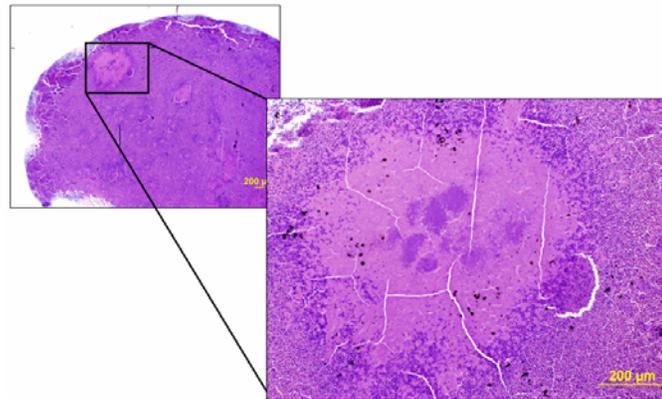


Figure 36. Focal necrosis and *A. salmonicida* microcolonies in rainbow trout spleen, 8 d after immersion challenge.

Direct correlations between PCV*TP values in moribund and freshly dead fish and *A. salmonicida* concentrations/g of spleen were easily recognized (**Figure 35**). Low PCV*TP values (<150) in plasma were consistently found in moribund and freshly dead fish, whereas surviving fish in both treatment groups had higher PCV*TP values (>150). Additionally, in all fish where low PCV*TP values were recorded high concentrations of *A. salmonicida* were found. These low PCV*TP values are due to an inability to maintain ionic homeostasis which is critical to the survival of all fish species. Loss of

Na⁺-K⁺-ATPase, a membrane-bound enzyme that actively transports Na⁺ out of the cells and K⁺ into the cells is likely responsible for the osmoregulatory dysfunction seen in this study. Deane and Woo (2005) also showed osmoregulatory function to be impaired in the kidney at an early stage of infection (vibriosis) in sea bream.

Serum OTC concentrations in all surviving Non-medicated fish sacrificed 14 d after dosing ended were below the LOD (0.18 µg/mL), and four out of 35 surviving Medicated fish had low (0.19-0.25 µg/mL) but detectable levels of OTC (**Figure 37**).

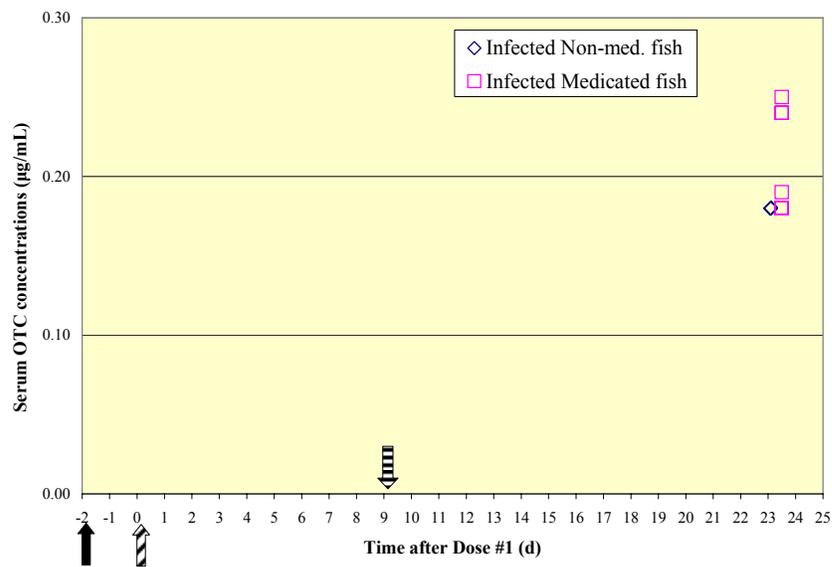


Figure 37. Serum OTC concentrations in surviving fish 14 days after dosing. ↑ - day of challenge; ↑ - 1st dose given; ↓ - 10th dose given.

Parallel Study

A. salmonicida was not found in spleens from any Medicated fish (**Figure 38**). *A. salmonicida* was detected in spleens of Non-medicated fish 1 d (1/6 fish), 3 d (3/7 fish), 4 d (7/9 fish), and 6 d (6/8 fish) after dosing began. Generally higher concentrations of bacteria/g of spleen were detected in dead and moribund fish than in sacrificed fish. Less bacteria/g of spleen were found in Non-medicated fish in the Parallel study than in the Efficacy study. A possible explanation for this difference is a 1 log lower challenge dose was used in the Parallel study.

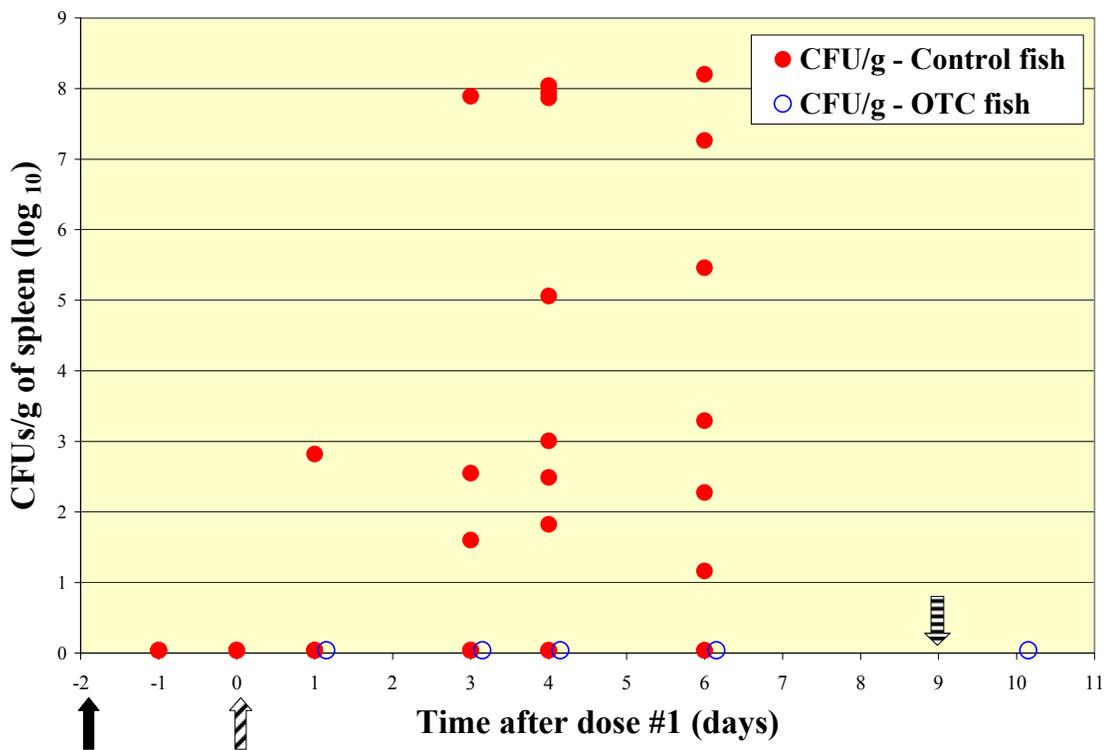


Figure 38. *A. salmonicida* CVM 35493 persistence in experimentally challenged rainbow trout. ↑ - day of challenge; ↗ - 1st dose given; ↓ - 10th dose given.

Calculations of serum OTC concentrations revealed first order absorption from the gut into the central compartment (blood). We calculated an absorptive half-life ($T_{1/2\alpha} = \ln 2/k_a$) equal to 5.1 d (**Figures 39 and 40**), similar to the half-lives calculated for OTC q-salt (4.4 d) and dihydrate salt (5.2 d) in healthy unabrased rainbow trout (**Chapter 6**). Wang et al. (2001) reported an OTC $T_{1/2\alpha}$ after oral administration of 2.3 h.

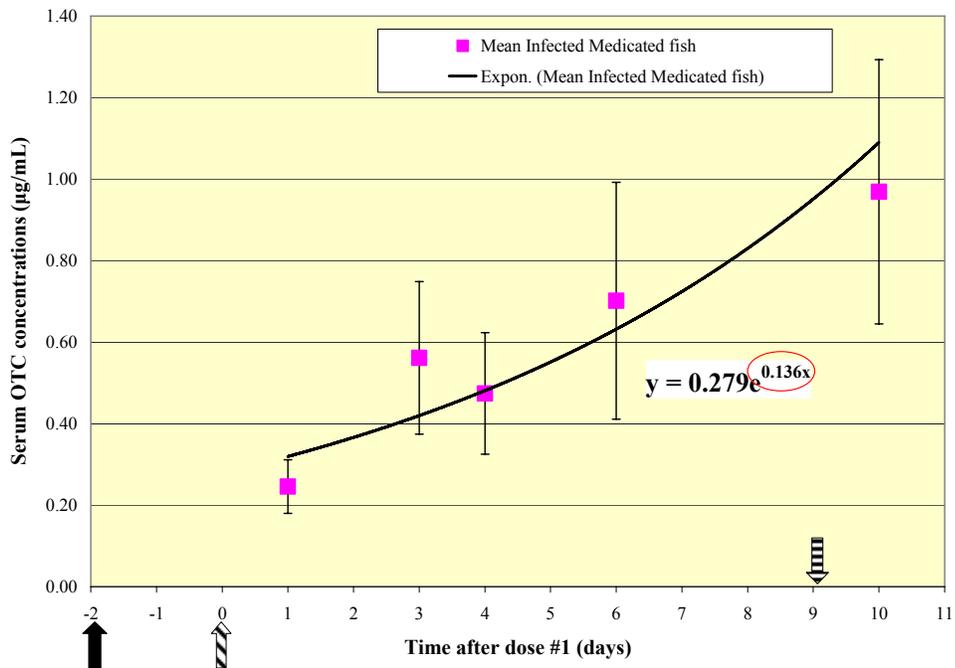


Figure 39. Mean serum OTC concentrations with a fitted exponential curve. Circled value indicates the absorption rate constant, k_a .

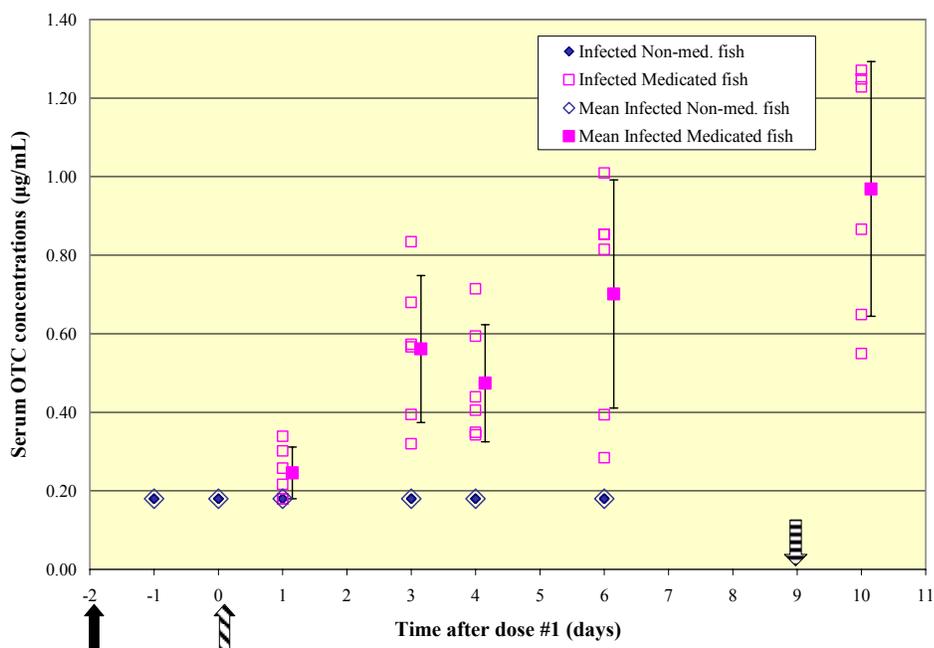


Figure 40. Serum OTC concentrations in rainbow trout experimentally challenged with *A. salmonicida* CVM 35493.

An AUC_{ss} estimated over 24 hours was calculated using mean serum OTC concentrations at 6 and 10 d and the trapezoidal rule.

$$AUC_{ss} = ((C_{6d} + C_{10d}) / 2) (t_{10d} - t_{6d}) / 4$$

The AUC_{ss} in experimentally infected rainbow trout was 20.1. Interestingly, this value and the AUC_{0-10d} are lower but comparable to the AUC_{ss} and AUC_{0-10d} values calculated previously in uninfected rainbow trout fed OTC medicated feed under the same conditions (**Table 21**). This indicates that slightly less OTC makes it into fish that have been subjected to the dermal abrasion and *A. salmonicida* challenge procedures. Whether one or both of these stressors contribute to this decreased absorption, remains to be seen.

Table 21. Summary of AUC values in infected and uninfected rainbow trout held under identical conditions.

Treatment Group	Mean dose \pm SD ($\mu\text{g/mL}$)	AUC_{ss}	AUC_{0-10d}	AUC_{0-∞}
Infected OTC dihydrate salt	74.7 \pm 2.3	20.1	143.0	n/a
Uninfected OTC q-salt *	74.7 \pm 1.5	27.1	175.9	309.7
Uninfected OTC dihydrate salt *	73.4 \pm 2.0	27.2	187.0	293.3

* data from pharmacokinetic study (Chapter 6)

Slightly decreased AUC values in dermally abraded and challenged fish may also be due to an increased residence time of the OTC-medicated food in the gastrointestinal (GI) tract. We found most GI tracts of fish sampled to be full of feed indicating a decrease in gastric emptying possibly due to bacterial challenge and/or stress. Bioavailability was not determined in this study due to difficulties in intravenous administrations in fish, thus the percentage of OTC absorbed from the GI tract could not be calculated. If in a similar study, a more potent OTC-medicated feed were used, and less ingested feed was required to reach comparable therapeutic serum concentrations, presumably, the fish's ability to digest, pass, and absorb the feed should result in higher achievable serum OTC concentrations. If all of this proves true, other than the obvious benefit to the fish, fewer doses and possibly less total OTC administered may prove to be economical for farmers and better for the environment.

Alternatively, the slightly decreased AUC values in the abraded and infected fish (**Table 21**) may be explained by hemodilution. Loss of osmoregulatory function leading to an increased plasma volume with a decreased TP, may help explain the lower AUC values (**Figure 41**). Additional studies aimed at comparing PCV and TP values in dermally abraded and unabraded rainbow trout fed OTC-medicated feed with and without bacterial challenge are needed to prove an affect of bacterial challenge and/or dermal abrasion on hemodilution of OTC in serum. TP values in serum should also be

determined since Hrubec and Smith (1999) found significantly higher TP concentrations in rainbow trout and catfish serum than in plasma.

The plot of mean PCV*TP values in Non-medicated and Medicated fish shows a very similar trend until 6 d after dosing began (**Figure 41**). No significant difference in mean PCV*TP values were observed 1, 3, or 4 d after dosing began. A gradual decline in the health of the Non-medicated fish began with four mortalities (1 at 3 d, 2 at 4 d, 1 at 6 d) and one moribund fish at 4 d. A significant decrease in mean PCV*TP values ($p<0.05$) from 1 to 3 d after dosing began indicated a decline in osmoregulatory function in Non-medicated fish. A critical period during this study was between 4 and 6 d after dosing began when mean TP concentrations only (not PCV values) in Non-medicated fish declined significantly ($p<0.05$), while TP concentrations in Medicated fish increased markedly (not significant). Also at 6 d mean TP values were significantly higher in the Medicated fish ($p<0.05$) confirming a positive effect was afforded by administration of the OTC-medicated feed. TP concentrations in Medicated fish at 10 d were significantly lower than those from fish sacrificed 1 d after bacterial challenge and before dosing, however they appeared to be in the late stages of recovery to normal osmoregulatory function.

Also with the gradual decrease in mean PCV*TP values in Non-medicated fish came a gradual increase in the levels of *A. salmonicida* recovered in the spleen starting 1 d after dosing began (**Figure 42**). Similar to what was observed in the Efficacy study the major decrease in PCV*TP values after challenge was probably due to sepsis in the animal, liver malfunction, and proteinemia, resulting in an inability to regulate ion transport. However, one can postulate that the initial decrease in PCV*TP from one to

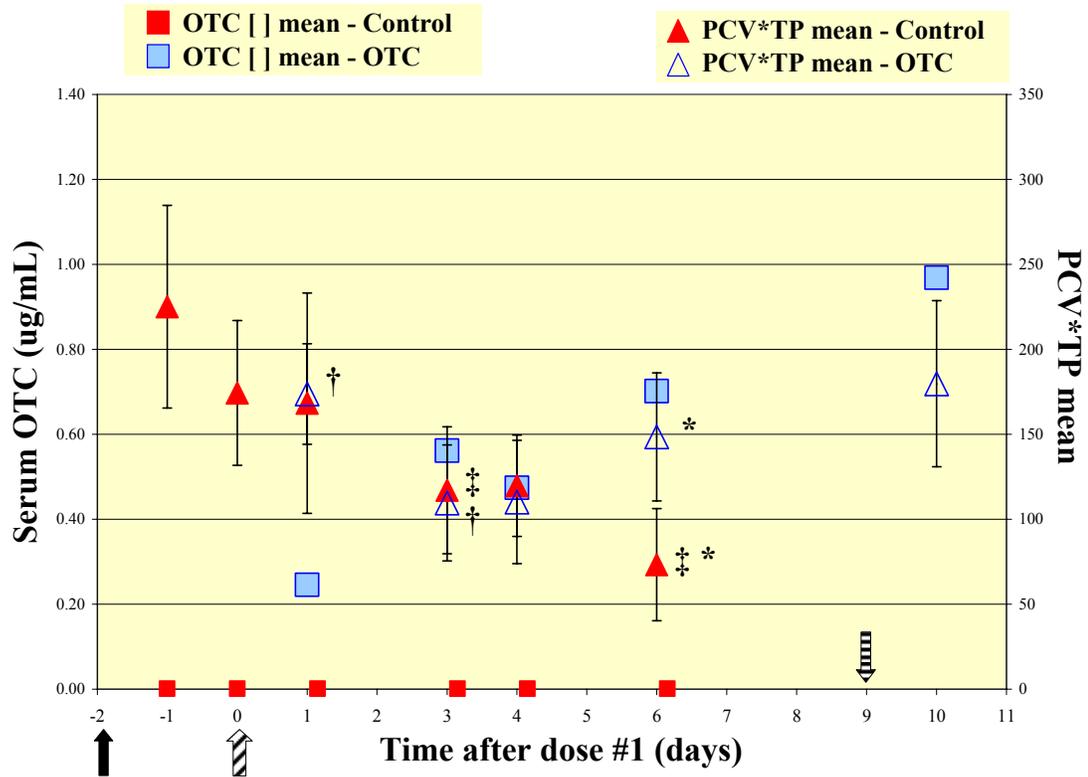


Figure 41. Direct relationship between mean PCV*TP and serum OTC concentration in rainbow trout. Left axis – Mean serum OTC concentrations after challenge; Right axis – Mean packed cell volume (PCV, %) x total protein (TP, g/dL) after challenge. † - OTC-medicated fish mean 1 d and 3 d values are significantly different, ‡ - Control fish mean 4 d and 6 d values are significantly different, * - OTC-medicated fish and Control fish mean values are significantly different; ↑ - day of challenge; ↗ - 1st dose given; ↘ - 10th dose given.

two days after challenge may be due to the dermal abrasion and/or stress. Medicated fish PCV*TP values declined throughout the study until mean serum OTC concentrations approached 0.5 µg/mL (Figure 41). The decline in PCV*TP values cannot be explained with the sepsis assumption however, since no *A. salmonicida* were detected in the spleen in Medicated fish. Many authors have described anemic states in cases of bacterial infection of salmonids. Waagbø et al. (1988) reported hypoproteinemia and a reduction in red blood cells, hematocrit and hemoglobin in Atlantic salmon suffering from Hitra

disease. Barham et al. (1980) also reported hypoproteinemia and a reduction of hemoglobin and hematocrit in rainbow trout infected with *Aeromonas* spp. and

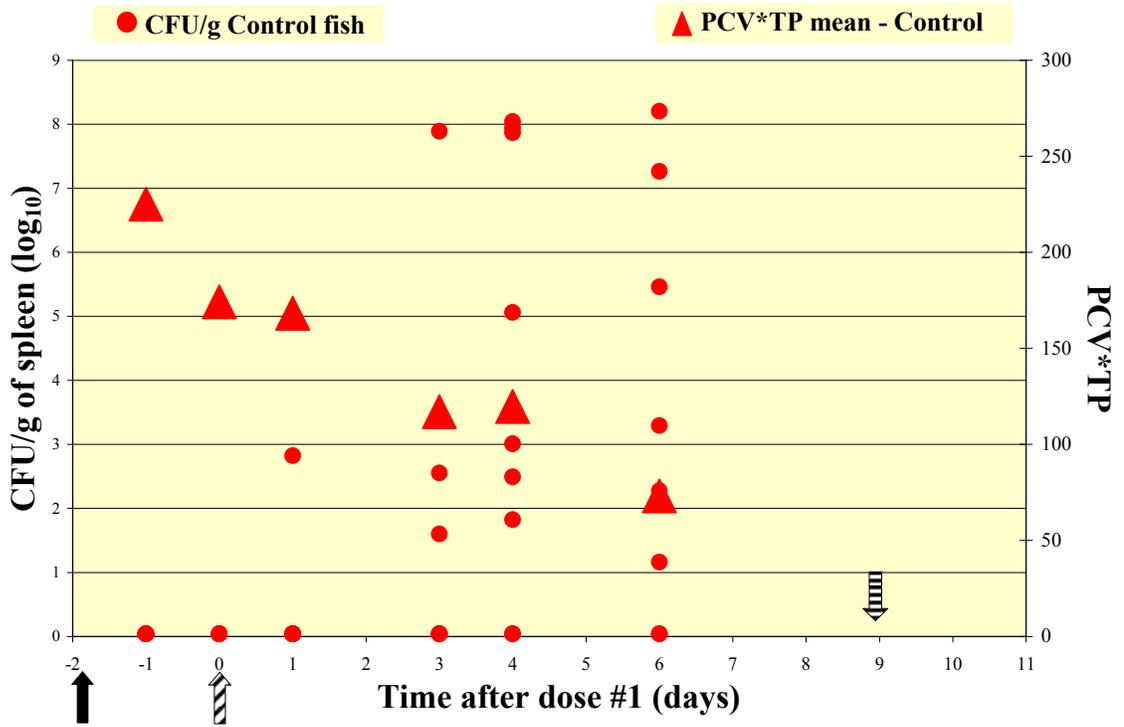


Figure 42. Inverse relationship of PCV*TP and the persistence of *A. salmonicida*.

Streptococcus spp. A decrease in TP in the serum of brown trout and Atlantic salmon with ulcerative dermal necrosis and a single fungal infection *Saprolegnia ferax* was reported by Mulcahy (1971). Řehulka (2003) reported hypoproteinemia to correspond with a histological finding in kidney and acute hepatitis, which are mainly characterized by the loss of albumin and a decrease in its synthesis. Albumin contributes to maintaining colloidal osmotic pressure in the blood flow. Řehulka showed hypoalbuminemia can lead to swelling and ascites (Řehulka, 2003), which was also noted in some fish challenged with *A. salmonicida* in the present study.

Conclusions

Results from this study can be used to determine a pharmacokinetic/pharmacodynamic (PK/PD) index which is a quantitative relationship between a pharmacokinetic parameter (i.e., AUC) and a microbiological parameter (i.e., MIC). Examples of PK/PD indices include AUC/MIC, C_{\max} /MIC, and time above MIC ($T_{> \text{MIC}}$). These indices can be used to determine appropriate dosage regimens and index magnitudes required for efficacy and prevention of the emergence of antimicrobial resistance (Mouton et al., 2005).

Aminoglycosides and fluoroquinolones exhibit concentration-dependent killing and are best represented with the PK/PD indices AUC/MIC and C_{\max} /MIC. Although tetracyclines exhibit time-dependent killing *in vitro*, AUC/MIC has been the major PK/PD index correlating with therapeutic efficacy (Craig, 1998). This may be due to the much longer *in vivo* post-antibiotic effect of these drugs when compared to β -lactams and macrolides (Craig and Gudmundsson, 1996). In PK/PD studies use of AUC_{ss} is preferred over AUC_{0- ∞} (Mouton et al., 2005) when determining indices. However, calculation of these indices using steady-state concentrations obtained during a 24 h period from a single fish is very difficult. Handling, anesthetizing, and bleeding a fish two or more times within a 24 h period would cause considerable stress, vascular injury and skewing of results due to the reduction in circulating blood volume. A more realistic approach is to calculate these indices from blood samples taken once daily or once every two to three days.

The present Parallel study used this approach in an experimental challenge model of *A. salmonicida* (MIC=0.25 $\mu\text{g}/\text{mL}$) in rainbow trout. Based on the epidemiologic

cutoff values for OTC against isolates of *A. salmonicida* (**Figure 14A**) (Miller and Reimschuessel, 2006), the *A. salmonicida* challenge strain fits into the wild-type classification and should be considered susceptible to OTC. We calculated an AUC_{ss} equal to 20.1 $\mu\text{g}\cdot\text{h}/\text{mL}$. If we divide this value by the MIC of the challenge strain the AUC_{ss}/MIC is 80.4. This value is far greater than the PK/PD targets for tetracycline against gram-negative infections reported by Burgess et al. (2007) ($AUC_{ss}/\text{MIC}\geq 25$) using a Monte Carlo simulation, and Stein and Craig ($AUC_{ss}/\text{MIC}\geq 5$) using a murine thigh-infection model (Stein and Craig, 2006). Similarly, Passarell et al. (2005) associated an AUC/MIC of >30 with a $>90\%$ chance of success in a human intra-abdominal infection model.

In summary, if we divide the AUC_{ss} values from **Table 21** (20.1, 27.1, and 27.2) and the current susceptible *A. salmonicida* breakpoint of 1 $\mu\text{g}/\text{mL}$ (**Figure 14A**), the AUC_{ss}/MIC values are still considerably greater than those reported by Stein and Craig and are near those proposed by Burgess et al. (2007) and Passarell et al. (2005). Thus, as in studies of OTC in mammals, studies of OTC in fish share a clear relationship between AUC and efficacy. Increasing the *A. salmonicida* susceptible breakpoint from 1 $\mu\text{g}/\text{mL}$ to 2 or 4 $\mu\text{g}/\text{mL}$ should be avoided unless favorable clinical outcomes are reported against isolates with these higher MICs. Further, due to an inability to detect *A. salmonicida* isolates from the spleen of Medicated fish during the Parallel study, a link between the AUC_{ss}/MIC target (≥ 5) and the prevention of emergent resistant *A. salmonicida* isolates can be identified.

Additional studies investigating the clinical efficacy of OTC in rainbow trout against an infection using a higher MIC (1-4 $\mu\text{g}/\text{mL}$) *A. salmonicida* isolate will provide

crucial information related to the accuracy of the current epidemiologic cutoff values.

Pharmacokinetics studies using a higher potency OTC-medicated feed will also provide more data on the potential serum OTC concentration over the course of an infection.

Summary

Aquatic animal veterinarians are faced with many of the same clinical decisions as clinicians and veterinarians specializing in other disciplines. One such decision is whether or not to prescribe antibiotic treatment for a patient/population. Clinicians and veterinarians most often make rapid judgment calls based on minimal empirical evidence. Fish farmers and pet fish owners, just like poultry farmers and owners of companion animals, want instant answers. Treating with an appropriate antibiotic can save a farmer's investment as well as a beloved family member. However, indiscriminate antibiotic use can put the animal, environment, and even the owner at risk of acquiring an infection caused by bacteria that have become resistant to the originally prescribed antibiotic. The decision to treat should involve multiple factors including:

1. Antimicrobial susceptibility test (AST) results of the pathogen isolated in the disease outbreak
2. Published or unpublished susceptibility distribution data for a given pathogen population
3. Known pharmacokinetic properties of the antibiotic in the target animal
4. Relevant clinical experience and reports of clinical cases

Antimicrobial susceptibility tests need to be conducted using standardized methods and quality control strains. This allows for meaningful comparisons within and between laboratories. Most human and terrestrial animal bacterial pathogens grow best *in vitro* at temperatures at or above 35 °C. It is at these temperatures that antimicrobial susceptibility testing (AST) methods have been standardized (CLSI/NCCLS, 2007b; CLSI/NCCLS, 2003; CLSI/NCCLS, 2000). Conversely, aquatic animal bacterial

pathogens prefer or require *in vitro* growth temperatures well below 35 °C, and thus require different incubation conditions for AST. We standardized methods for AST of non-fastidious aquatic isolates by disk diffusion (Miller et al., 2003) and broth microdilution (Miller et al., 2005). Each method is equipped with two quality control (QC) strains which allow researchers and technicians worldwide, to reproducibly conduct AST of bacterial isolates that prefer or require lower *in vitro* growth temperatures. When used correctly these standardized methods provide a means for scientists to confidently monitor AST data over time, and will also foster more meaningful scientific communication. Other aquatic animal bacterial pathogens that need standardized AST methods are members of *Vibrionaceae*, *Flavobacteriaceae*, gram-positive cocci, and psychrophilic strains (i.e., *Vibrio salmonicida*).

Using standardized AST methods and associating test results with clinical outcome can help veterinarians decide what treatment to prescribe. However, associating AST results with clinical outcome is not always possible, especially in fish medicine where mortalities can be difficult to assess due to bird predation, cannibalism, and sinking carcasses. Interpretive criteria or clinical breakpoints are useful when deciding which drug to use. These breakpoints are zone diameters and minimal inhibitory concentrations (MIC) for a given antimicrobial agent and disease causing agent. They delineate whether an isolate is susceptible, intermediate, or resistant *in vitro*, and ultimately are used to predict how the target animal may respond to treatment using the standard dosing regimen. Clinical breakpoints exist for many human and terrestrial animal pathogens, but none have been determined for any bacterial pathogen in any aquatic animal.

In this work we began the process to establish the first clinical breakpoints for an antimicrobial agent approved for use in aquaculture against an aquatic bacterial pathogen. First we used newly standardized AST methods for aquaculture to determine the susceptible and resistant MIC and zone diameter cutoffs (called epidemiologic cutoff values) for oxytetracycline, ormetoprim-sulfadimethoxine, florfenicol, and oxolinic acid against 217 *Aeromonas salmonicida* isolates. These epidemiologic cutoff values can be used to monitor for the development of resistance to these drugs in *A. salmonicida* isolates. To have clinical application, these MIC cutoff values, based on a large population of isolates, must be correlated with pharmacokinetic data in a target animal species and clinical outcome data where the MIC of the infecting *A. salmonicida* isolate is known. In human and veterinary medicine, the relationship between pharmacokinetic data and MICs of pathogenic isolates, is used to develop pharmacokinetic/pharmacodynamic (PK/PD) indices. The most commonly used indices are $T > MIC$, C_{max}/MIC , and AUC_{ss}/MIC . PK/PD target values for each of these indices can be used to predict therapeutic efficacy, minimize the emergence of resistant organisms, and help establish clinical breakpoints.

In our work, pharmacokinetic studies of OTC in rainbow trout were used to monitor serum concentrations in both healthy rainbow trout and those challenged with a presumably susceptible strain of *A. salmonicida* (MIC = 0.25 µg/mL). The PK/PD index, AUC_{ss}/MIC , is generally accepted as the best predictor of therapeutic success for tetracyclines against gram-negative infections (Burgess et al., 2007; Stein and Craig, 2006; Passarell et al., 2005). When either of the AUC_{ss}/MIC values calculated in healthy and challenged trout were divided by the current susceptible MIC epidemiologic cutoff

value for *A. salmonicida* (1 µg/mL) the products were well above the AUC_{ss}/MIC target value of ≥ 5 reported by Stein and Craig (2006) in a murine thigh-infection model, and very close to ≥ 25 (Burgess et al., 2007) and ≥ 30 (Passarell et al., 2005) reported recently. Based on the findings presented here, the 1 µg/mL susceptible MIC epidemiologic cutoff value for *A. salmonicida* isolates appears to be accurate. This work also showed the currently approved dosing regimen for OTC in salmonids is effective against experimental *A. salmonicida* infections caused by *A. salmonicida* isolates with MICs ≤ 0.25 µg/mL. However, additional studies are needed before an OTC susceptible clinical breakpoint can be established for *A. salmonicida*. Separate efficacy studies using the same OTC dosing regimen against challenge strains with MICs of 1, 2 and/or 4 µg/mL along with pharmacokinetic data (AUC_{ss}) will need to be conducted to determine susceptible and resistant clinical breakpoints.

This work will help aquatic animal disease researchers in the future develop clinical breakpoints for OTC and other antimicrobial agents against economically important aquatic animal pathogens. These studies show PK/PD parameters historically only applied in studies on humans and terrestrial animals can be applied in fish as well. The studies described here may serve as a template for future efforts in this field. A neutropenic mouse thigh-infection model developed by Dr. William Craig (1998) shares some similarities with the fish spleen-infection model used in this work. Both fish spleen and mouse thigh can be used to monitor bacteria levels over time in response to treatment. These types of studies cannot be used to assess efficacy, but can help assess *in vivo* kill kinetics. It is doubtful that an artificially immunocompromised fish would survive for long in the microbial milieu of a fish tank, but does warrant an investigation.

Examinations are also needed into the applicability of similar fish tissue-infection models of other gram-negative septicemias including enteric septicemia of catfish caused by *Edwardsiella ictaluri*.

Future clinical response studies with OTC and other antimicrobial agents should include a procedure to detect the presence of resistant isolates in the entire tissue homogenate. This was not performed in the present work. This would require using a spread-plate technique of each homogenate dilution onto agar media with and without the antimicrobial agent. The benefit of testing the entire tissue homogenate is it would drastically increase the chance of detecting bacteria with a decreased susceptibility, as compared to the current study which only tested the susceptibility of five randomly selected *A. salmonicida* colonies.

Pharmacokinetics studies conducted in laboratory settings where multiple blood samples are taken from a single fish over time can yield data that misrepresent concentrations in normal animals not subjected to such extreme stress. Despite a fish's hematopoietic ability, repeated sampling of 1-2 mL of blood for analysis may reduce the circulating volume in which a drug can enter, thus making meaningful correlations difficult. Also repeated bleeding at or near the same site will probably only result in the withdrawal of fluid from the hematoma. Thus pharmacokinetic studies conducted in a field setting with large numbers of animals or in a laboratory setting where euthanization is used will likely provide the most reliable predictors of achievable serum drug concentrations. In the present work, limited variability was observed in serum OTC concentrations in healthy and infected fish that were euthanized at each time point after

dosing began. Similar weights, environmental conditions and doses of OTC per gram body weight all contributed to this consistency.

The Food and Drug Administration (FDA) approved OTC for use in aquaculture in the 1970's. It is not clear what studies were completed to optimize the approved 10 day dosing regimen for OTC in salmonids. Researchers have reported maximum serum OTC concentrations in healthy fish after a single oral dose (Rigos et al., 2003a; Haug and Hals, 2000; Bjorklund and Bylund, 1990; Iversen et al., 1989) similar to those reported in the present work which used the FDA-approved 10 day dosing regimen. Additional studies are needed to investigate the need of the full 10 day dosing regimen in salmonids. These investigations may reveal a shortened dosing period, more infrequent dosing, and/or a change in the potency of OTC-medicated feed will yield similar results in terms of efficacy and OTC pharmacokinetics. These results may also present a more economical and environmentally friendly alternative to OTC dosing in aquaculture. This presentation is contingent upon the total amount of antimicrobial agent administered using this modified regimen being less than that using the approved 10 day dosing regimen. Only then would there be a presumed benefit to the environment and/or fish farmer.

Release of xenobiotics and pollutants from industry (including aquaculture) into the environment should be minimized or avoided altogether. With a predicted 50% increase in global population by 2050 (The United Nations Population Division, 2001), will come an increased demand on the food supply, in particular high protein sources like fish. Preventative measures taken now to minimize the exposure of fish, shellfish, and

the aquatic environment to xenobiotics like antimicrobial agents can help protect and preserve this important resource.

The American Veterinary Medical Association (AVMA, 2002) and National Aquaculture Association (2003) have made strides to curb unnecessary antimicrobial use in aquaculture by publishing guidelines for judicious and prudent antimicrobial use for food-fish veterinarians. More efforts like these which raise awareness about antimicrobial use in aquaculture and place more responsibility on the food-fish veterinarian are crucial. Recently, the FDA amended the new animal drug regulations to implement the veterinary feed directive (VFD) drugs section of the Animal Drug Availability Act of 1996 (ADAA). A VFD drug is intended for use in animal feeds, and such use of the VFD drug is permitted only under the professional supervision of a licensed veterinarian.

In the healthcare environment, recommendations for preventing and reducing antimicrobial resistance in hospitals stress the importance of improving antimicrobial use, referred to as antimicrobial stewardship. Antimicrobial stewardship programs have served as wake-up calls to both clinicians and health care administrators. Studies and surveys suggest as much as half of all antimicrobial use is inappropriate. If veterinarians (including aquatic animal veterinarians) implement such a program would they come to the same conclusion? The probability is good, and this might begin a movement toward reducing needless antimicrobial use in veterinary medicine.

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