THE IMPACT OF OXYTETRACYCLINE DOSING ON BACTERIAL POPULATIONS AND TRANSFER OF RESISTANCE ELEMENTS *IN VITRO* AND *IN VIVO*

by

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B.G.S., University of Kansas, 1997
D.V.M., Kansas State University, 2002

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine / Pathobiology
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Abstract

The discovery of modern antimicrobials in the early 20th century revolutionized treatment of infectious diseases. Less than 100 years later, antimicrobial resistance has become a global threat to public health. With the rise of antimicrobial resistance, the question that remains to be answered is: Can dosing regimens provide maximal clinical efficacy, yet minimize the development of antimicrobial resistance?

A pharmacokinetic / pharmacodynamic approach was utilized to investigate oxytetracycline regimens that would impart efficacy while minimizing the potential for resistance development due to plasmid transfer. An *in vitro* pharmacodynamic model was used to quantify the response of a *Pasteurella multocida* isolate to two oxytetracycline dosing regimens. The PK/PD index most closely related to efficacy was the Cmax:MIC.

The *in vitro* pharmacodynamic model was then used to investigate the effects of antimicrobial exposure on plasmid transfer. A mixed population of oxytetracycline-susceptible and resistant bacteria was exposed to two dosing regimens and plasmid transfer was quantified. When oxytetracycline concentrations exceeded the MIC of the recipient, development of resistance was suppressed.

The same donor and recipient bacteria were used in an *in situ* swine model to validate the *in vitro* findings. Following surgical implantation of porous membrane straws containing the mixed bacterial population, animal subjects in the treatment groups received one of two oxytetracycline treatments. Oxytetracycline concentrations in the plasma and interstitial fluid were quantified. Plasmid transfer within the implant membranes was quantified and correlated

to pharmacokinetic measures in the animal. Plasmid transfer rates in the implant membranes did not correlate to the investigated pharmacokinetic parameters.

The study methodologies in this dissertation should serve as a foundation for future studies in antimicrobial pharmacokinetic/pharmacodynamic research. The results presented here show that the bacterial response to oxytetracycline can be optimized in a concentration dependent manner and that antimicrobial resistance development through plasmid transfer can be suppressed *in vitro* when oxytetracycline concentrations exceed the MIC of the recipient bacteria. These results suggest that a proper balance between clinical efficacy and minimizing antimicrobial resistance can be achieved for oxytetracycline through appropriate dosing regimens and drug formulations.

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Dedication

I would like to dedicate this work first to God, Whose plan for me continues to unfold before me and to Whom I entrust the future.

I would also like to dedicate this to my family. To my wife, who sacrificed to come here and has supported me graciously during our stay. To my children (Liv and Brock), who have given me a reason to continue during the roughest times. This is as much your accomplishment as it is mine.

CHAPTER 1 - Literature Review

Antimicrobial Pharmacokinetics – Pharmacodynamics

Pharmacokinetics literally translates to "drug movement". It has been defined as the use of mathematical models to quantify the time course of drug absorption and disposition in man and animals[1]. Traditionally, this is represented by the ADME scheme: Absorption, Distribution, Metabolism and Excretion[2]. In lay terms, pharmacokinetics is described as "what the body does to the drug".

Pharmacodynamics is the discipline which studies "how a drug acts on a living organism, including the pharmacologic response and the duration and magnitude of response observed relative to the concentration of the drug at an active site in the organism"[3]. This is often described as "what the drug does to the body". However, antimicrobial pharmacodynamics represents a unique situation in which the intended drug – receptor interaction is not with the host organism, but within the invading microorganism. In a 2004 review article, Drusano describes this as an advantage over traditional pharmacodynamics in that with antimicrobials we can directly measure the ability of the anti-infective agent to dock into its 'receptor' and cause the effect for which it was designed by using the Minimum Inhibitory Concentration (MIC) or Effective Concentration (EC)[4]. While these pharmacodynamic measures are relatively easy to obtain for a bacterial isolate or population (MIC and EC_{90}), it deserves mention that they are not the only pharmacodynamic measures and that other measures of antimicrobial efficacy may be appropriate. However, the MIC is the current accepted standard and will be utilized as the antimicrobial pharmacodynamic measure in the research described herein.

The combination of antimicrobial pharmacokinetics and pharmacodynamics, or PK/PD, is the integration of these two disciplines which describes the bacterial population response to the dynamic changes in drug concentrations in a patient over time. Once the correct PK/PD index value has been determined for the specific drug – pathogen combination, dosing regimens can be optimized based on the MIC of the isolated pathogen and pharmacokinetics of the individual patient. On a larger scale, the index value can be integrated with procedures such as Monte Carlo simulation to set susceptibility breakpoints or assess probability of successful treatment outcome based on population pharmacokinetics[5].

Development of Pharmacokinetic – Pharmacodynamic Indices

The field of antimicrobial pharmacokinetics / pharmacodynamics (PK/PD) developed shortly after the first antibiotics were discovered. In the late 1940's and early 1950's, Dr. Harry Eagle performed a series of experiments that would become the earliest PK/PD studies. His group first demonstrated the concentration-independent nature of penicillin. They noted that there was an optimal concentration of penicillin which maximized the kill rate of bacteria; even a 32,000-fold increase in concentration above the optimal concentration did not accelerate kill rate[6]. In a later study, Dr. Eagle concluded that "the factor that primarily determines its [penicillin's] therapeutic efficacy is the total time for which the drug remains at effective levels at the focus of infection"[7]. The practical application was best summarized: "Such continuous levels could be provided by a continuous drip, or small doses of aqueous sodium penicillin repeated at intervals of approximately 2-4 hours". Two other noteworthy observations from these early experiments were: the persistent bacterial effects on Gram-positive organisms when penicillin concentrations declined below the inhibitory concentration and the observation that an over-extended dosing interval results in re-growth of the organism. Taken together, these

conclusions show that there is an optimal dosing strategy for penicillin based on a minimum time above a concentration at the site of infection to elicit maximal kill. Unknown to him, Dr. Eagle also alluded to what is the basis for this doctoral research, when he stated: "With the latter group of antibiotics [including oxytetracycline] the optimal time-dosage relations remain to be determined".

In 1974, Klastersky[8] *et al.* described the bacterial inhibitory effects of serum and urine from antimicrobial-treated patients. The objective of the study was to correlate individual patient antimicrobial concentrations with clinical outcome. Serum and urine were collected from cancer patients with confirmed bacterial infection. Serum was collected 1 hr post-antimicrobial administration (peak) and 1 hr prior to the following administration (trough). Urine was collected on the second day of treatment over the interval between administrations. For septicemia, respiratory and wound infections, the clinical outcome was correlated to the peak serum inhibitory titer. For urinary tract infections, the outcome was correlated to peak urinary inhibitory concentrations. However, the study results are confounded by the use of multiple antimicrobials in mono- and combination-therapy. The study results are also limited because a breakpoint of 3µg/mL was used to correlate clinical outcome to inhibitory titer regardless of the antimicrobial treatment. Nevertheless, this study was the first to correlate peak concentrations to bacterial effect.

A hybrid parameter, the area under the time-concentration curve (AUC) is the pharmacokinetic measure for total drug exposure. In that regard, it is actually the oldest index although not in a scientific sense. Prior to the pioneering work of Drs. Eagle, Klastersky and others, antimicrobial treatment was administered based on a standard dose. Individual patient and microbe variation were not truly accounted for, but recognized when clinical failures

occurred. The first study to use statistical analysis of antimicrobial pharmacokinetics and bacterial pharmacodynamics to determine an optimal PK/PD index and the magnitude of that index was conducted by Dr. James Thayer on prison inmates in Georgia[9].

The Georgia Prison Study

Dr. Thayer's research (conducted in 1964, published posthumously in 1979) was conducted to determine the pharmacokinetic variables of penicillin associated with cure of gonococcal urethritis. Forty-five prison volunteers were experimentally inoculated with *Neisseria gonorrhoeae.* After developing clinical signs of infection, volunteers were allocated to one of six different penicillin regimens and serial blood samples were collected for determination of penicillin concentration in each subject. Clinical cure was defined as negative urethral and urine cultures at 72 and 96 hours. The researchers examined 46 subject and pharmacokinetic variables in a stepwise discriminant analysis to determine which contributed to the differences between volunteers that were cured and those that were not. In the final analysis, the authors noted the correlation between pharmacokinetic parameters, but the indices including time above the MIC best predicted the probability of correctly classifying subjects as treatment cures or failures.

In vivo Models of Antimicrobial Pharmacodynamics

To determine antimicrobial PK/PD relationships *in vivo,* either clinical or microbiological outcome measures can be utilized. If a fatal disease model is utilized, animals can be administered varying dose regimens to determine which PK/PD index is most predictive of survivorship. For studies with microbiological outcome measures, PK/PD indices are correlated to changes in colony forming units to provide the optimal dosing strategy.

Virtually any animal model can be utilized for antimicrobial PK/PD research, but the neutropenic mouse thigh infection model has emerged as the standard model for this type of research. The murine infection model was first used in the 1960's to improve on existing animal models of *Mycobacterium tuberculosis* infection[10]. The purpose of the original model was to describe a non-fatal model for tuberculosis that could be used to provide pathologic description of disease progression. The authors also mentioned the value in assessing chemotherapy, but commented that "modification will be necessary before the lesion can be used directly as a critical test in chemotherapeutic studies". In the early 1980's, Gerber, Vastola, Brandel and Craig described the procedure for inducing neutropenia, inoculating thigh tissue and quantifying bacterial responses in mice[11]. In that study, the model was used to investigate the development of resistance during therapy *in vivo* without the interaction of a functional immune system. In 1988, Craig's group published another study that would become the foundation for modern day *in vivo* PK/PD research[12]. That paper described the comprehensive methods for using a neutropenic mouse thigh infection model, a dose fractionation design and statistical regression to determine optimal PK/PD indices for 7 different antimicrobial-pathogen combinations. Since this time, the murine thigh infection model has become the standard *in vivo* dose fractionation model for determination of the optimal PK/PD index and magnitude of the index[13, 14]. In a dose fractionation study, one treatment consists of a single administration of the antimicrobial, while the subsequent treatments will receive the same total dose divided into 2 or more administrations. This study design therefore limits the interdependence of the primary PK/PD indices.

However, other murine models (inhalational, systemic) can and have been used to derive the PK/PD relationships for pneumonia[15,16], bioterrorism exposures[17], mycobacterial[18] and

systemic fungal infections[19]. Other disease models including: pyelonephritis, meningitis, osteomyelitis and endocarditis have been reviewed elsewhere[20].

Ideally, the PK/PD relationship for any antimicrobial – pathogen combination is tested in the target animal. As described previously, Dr. Thayer used an induced model of gonococcal urethritis to derive the PK/PD relationship for penicillin. In the present day, disease challenge models in humans are prohibited due to ethical considerations. However, the potential to determine PK/PD relationships does exist under certain circumstances. In his 1991 landmark study, Schentag collected respiratory and plasma samples from patients with nosocomial lower respiratory tract disease to determine the association between pharmacokinetic parameters and clinical outcome for ciprofloxacin[21]. In the final analysis, time above MIC was predictive of bacterial eradication with ciprofloxacin. The methodology does not describe further statistical analysis and whether other parameters were useful for prediction. Subsequent studies have demonstrated that fluoroquinolones act in a concentration-dependent manner and thus, the optimal PK/PD index is either the Cmax:MIC[22] or AUC:MIC[23,24].

Limitations of in vivo animal models

One of the potential disadvantages of the neutropenic models (murine or otherwise) is the lack of a functional immune system. For investigations of a clinical nature this represents the "worst case scenario", the immunocompromised patient. However, for basic PK/PD research this is considered an advantage as it will overestimate the magnitude of the PK/PD index required for a positive outcome[25].

One of the challenges of working with translational animal models in PK/PD research is the effects of interspecies differences. The two potential differences that should be accounted for in an animal model are protein binding and intrinsic clearance. The effects of interspecies

clearance differences were demonstrated recently in a study by Deziel *et al.* in which murine and primate models failed to replicate the pharmacodynamic relationship between levofloxacin and *Bacillus anthracis* seen *in vitro* with humanized drug exposures[26]. The authors hypothesized that the shorter $T_{1/2}$ in animals (2 hrs vs. 7.5 hrs in humans) resulted in drug concentrations below the MIC of the organism for a large portion of the dosing interval. When animal pharmacokinetics were "humanized", bacterial response was correctly predicted. As demonstrated by this example, if the exact values for these factors are known, dose adjustments (quantity or frequency) can be made to approximate desired pharmacokinetics.

The ability to serially quantify both antimicrobial and pathogen in the same animal is desirable; however, for the murine models listed, bacterial quantification requires tissue homogenization, obviously a terminal measurement. A PK/PD methodology that allows repeated sampling of the same host for both drug and bacterial quantification would reduce inter-animal variation and the number of animal subjects necessary for this type of study.

Interdependence of the primary PK/ PD Indices

The primary objectives of antimicrobial PK/PD research are to derive the index and magnitude of that index that is most predictive of efficacy for a given antimicrobial-pathogen combination. Deriving the index in a clinical study can be difficult due to the variation in immune response between individuals. However, the greatest challenge to this objective lies in the interdependence of the three PK/PD indices. This has been described by others[27,28], but merits further illustration here.

Three integrated indices have become widely accepted in antimicrobial PK/PD research. They are the ratio of the antimicrobial maximal plasma concentration to the minimal inhibitory concentration of the pathogen (Cmax:MIC), the area under the plasma time concentration curve

to MIC ratio (AUC:MIC), and the time the plasma antimicrobial concentration remains above the MIC of the target pathogen (T>MIC)[29]. Of the three pharmacokinetic components utilized in PK/PD indices, only the Cmax is truly independent of the others. For intravenous dosing, it is dependent only on the dose given and the volume of distribution.

The area under the time-concentration curve (AUC) has both time and concentration components. This is most easily illustrated using the pharmacokinetic equation for AUC following a one-compartment intravenous bolus (equation 1), where Cp0 is the extrapolated plasma concentration at time zero and T1/2 is the apparent elimination half life for plasma concentrations.

Equation 1: Calculation of AUC

$$AUC = \frac{(Cp0) \times (T1/2)}{0.693}$$

From this equation, it can be seen that AUC is a hybrid parameter with both time and concentration components. It is also obvious that it is a direct relationship where doubling either the peak concentration or the half-life will result in a doubling of the AUC.

Time above MIC is also a hybrid index, but the relationship is even more complex. If the half-life is doubled, then the T>MIC will be twice as long. If the peak concentration is doubled, the T>MIC will increase by one half-life of the antimicrobial.

The effect of changes in the pharmacodynamic component of the indices, the MIC of the pathogen, is quite simply a different permutation of the concentration effects illustrated above. If the pharmacokinetics are constant and the MIC for a pathogen doubles, the Cmax:MIC and AUC:MIC will be one-half of their original value. Given the same scenario, the Time above MIC will be reduced by one half-life; therefore, the effect will depend on the pharmacokinetics of the specific antimicrobial. Special emphasis is given to the effect of increasing or decreasing

the MIC value by a factor of two because antimicrobial concentrations in microwell susceptibility testing are twofold serial dilutions with an accepted error of ± one dilution. This fact is often overlooked in antimicrobial PK/PD research, but it has great impact on the final interpretation of the data.

The most widely used approach to minimize the mathematical interdependence of the PK/PD indices is the dose fractionation study. Dose fractionation studies can be used *in vivo* or *in vitro.* If designed properly, the single administration will result in a greater Cmax:MIC ratio, while the multiple (fractionated) administration regimen will result in a longer T>MIC. Given this design, the AUC values will be identical for the two regimens. Thus, AUC:MIC becomes the default index to be disproven by showing superiority of one dosing regimen. If the single administration has a more favorable outcome, Cmax:MIC will be most predictive of outcome. If the fractionated dose is superior, then the optimal PK/PD index is T>MIC.

A second approach to determine the optimal PK/PD index is to collect individual subject / patient pharmacokinetic data and correlate the antimicrobial concentrations to the bacterial response in the individual. The resulting effect-parameter relationship is modeled; generally using an inhibitory E-max model. This model is a sigmoid shaped model with inflection points at the concentration where bacterial response is first observed and at the maximally effective concentration. The parameter with the highest correlation value is considered the optimal PK/PD parameter. The biggest disadvantage to this approach is that it requires sufficient pharmacokinetic data for each individual subject/patient to determine the exact value of each PK parameter. For experimental challenge models, the pathogen MIC is known but this may prove difficult to determine for clinical cases, especially in cases of polymicrobial infection.

Additionally, a wide range of pharmacokinetic parameters must be achieved to define the optimal magnitude of the parameter.

In vitro Pharmacodynamic Models

In an *in vitro* phamacokinetic model (IVPM), exponential decay of drug concentration is simulated by dilution of the antimicrobial-containing media with fresh media. The two general types of *in vitro* pharmacodynamic models are one-compartment and two-compartment. In a one-compartment model, the antimicrobial is administered directly into the bacterial inoculum. In a two compartment model, the bacteria are retained in their respective compartment by a membrane or filter, while the antimicrobial moves by gradient diffusion into and out of that compartment. Although this is greatly oversimplified, if the antimicrobial is added directly to the compartment containing the bacterial culture, it will be referred to as a one-compartment model. The presence of a retaining membrane will suffice to classify the model as two-compartment. Presented below is a description of the historical evolution of these models and a critique of their respective usefulness.

One compartment models

Early in the history of antimicrobial PK/PD, researchers realized that *in vitro* scenarios of bacterial kill curves had one major limitation, namely the bacteria were exposed to static antimicrobial concentrations. However, in living organisms, the time-concentration profiles of antimicrobials are dynamic. Thus, conclusions based on fixed drug concentrations may not correlate with clinical outcome.

To achieve exponential decay of the antimicrobial in an *in vitro* setting, the first researchers added media to the bacterial culture at selected time points. To simulate absorption,

the added media contained antimicrobial. To simulate the elimination phase, the added media was free of antimicrobial. The resulting time-concentration profile was not a smooth curve, but rather a stepwise rise and decline in antimicrobial concentrations[30, 31]. Thus, the resulting pharmacokinetic simulation only approximated serum antimicrobial concentrations. A smoother curve could be produced by more frequent addition of media; however, this increased the labor requirements of the system.

In 1968, Sanfillipo *et al.*[32] compared bacterial responses to short-acting sulfadiazine and long-acting sulfamethoxypyrazine. Although the authors demonstrated a similar bacterial inhibition for both treatments compared to the control, the published paper is inadequate to support the conclusions. The greatest limitation to this early model was that the pharmacokinetic simulation did not represent the exponential decline of antimicrobial concentrations *in vivo.* One appraisal of this study cited that because intermittent, not continuous, media flow was used the model resulted in a stepwise dilution[33]. Also, the volume of the bacterial reservoir was not kept at a constant volume, so the model did not replicate exponential decay[34]. In addition, the lack of correlation between optical density measurements and viable bacteria counts (colony forming unit counts) and the fact that the materials and methods do not describe the method for confirmation of antimicrobial concentration within the experimental system makes the conclusions difficult to support.

Improving on this design in 1978 and 1980, two groups of investigators employed the use of peristaltic pumps to continually add fresh media to a growing culture of bacteria containing antibiotic while also using pumps to maintain constant volume in the central reservoir. In the experimental setup published by Grasso *et al.* in 1978, IV and parenteral administrations based on human pharmacokinetics of three cephalosporins were simulated to study the effects of

antimicrobial elimination rate on antibacterial activity[35]. The addition of a supplementing flask allowed for the parenteral simulations. In this study, the researchers first compared the dilutional effects at three rates of media flow (30, 60, 120 min half-life simulations) to a static control culture. They concluded that the bacterial populations were not significantly affected by dilution and that results could be compared directly for the three simulated regimens. Based on viable bacterial counts and corresponding drug concentrations, the authors concluded that the potential *in vivo* activity against *Klebsiella* and *E. coli* was superior for cefazolin (vs. cephradine and cephacetrile) due to its slower elimination and resulting increased time above MIC.

Murakawa *et al.* (1980) used a more complex 4 flask model to simulate IV dosing of cephalosporins in humans[36]. In contrast to the conclusions of Grasso *et al.*, these authors stated that a mathematical correction factor was necessary to account for bacterial dilution. The difference between the two studies may have been a result of the initial inoculum. Grasso *et al.* used a starting inoculum of approximately 1×10^6 cells/mL to show that media flow did not affect the bacterial population over time. Murakawa *et al.* used a 1×10^2 cells/mL starting inoculum and compared dilution at 3 flow rates to a static control in deriving their mathematical corrections. However, in their actual IVPM experiments, Murakawa *et al.* used a starting inoculum of approximately 5×10^6 cells/mL. Because the proposed mathematical corrections were only tested at low inoculums, they may not hold true at the bacterial populations used in the actual experiments. For any bacterial culture there will be a physical space limitation to growth, but nutrient availability may be the primary limitation.

Schneider *et al.* (discussed below)[37] showed that media flow had no effect on the growth curves (viable counts and optical density) for *Salmonella typhimurium* with a starting inoculum of 5×10^5 CFU. To the knowledge of this author, there has not been a publication that compares

dilution effects in one-compartment pharmacodynamic models relative to starting inoculum and bacterial species growth rates (doubling time).

One compartment models with filters

Bacterial dilution and loss in the early one compartmental pharmacodynamic models were recognized as problematic in the early days of PK/PD. Mathematical corrections had been suggested by Murakawa[38] and later by White *et al.*[39]. Other investigators, however, attempted to circumvent the dilution issue by using filters to retain the bacteria within the central reservoir.

In 1980, Shah used a 3 flask model (central, fresh and waste media) similar to the system originally used by Grasso. However, the waste media was filtered through a porous microglass filter (0.5 micron) prior to exiting the central reservoir. As the author reports, the microglass filter was not 100% effective, but reduced bacterial loss to less than 0.14% per minute[40]. However, antimicrobial quantification was not performed in this study; conclusions were based on expected drug concentrations from the administered dose and pump flow rates. The authors failed to cite the potential effects that the filter may have had on simulated drug concentrations. For one, the filter could directly bind antibiotic and reduce drug concentrations. Second, bacteria may occlude the filter pores and reduce media outflow, thus prolonging half-life in the system. Without measurement of drug concentration either in the central reservoir or on the filter (radio-labeled drug), the effects of direct binding are unknown. Reduction in media outflow may have been explained by an increase in central reservoir volume or by measurement of total waste media. Neither observation is reported in the publication.

A slightly more intricate, but improved, system design was described in 1982 by Schneider *et al.*[41]. The study objective was to measure bacteria responses to three cephalosporins. Target drug concentrations in the system were based on human

pharmacokinetics of the respective antibiotics. Fresh media was pumped into a supplemental reservoir prior to entering the central reservoir to simulate the absorption phase of simulated oral doses. Waste media flow was set equal to fresh media flow to maintain constant volume in the central reservoir. The central reservoir was a water jacketed vessel with a fresh media port at the top and an outflow port at the bottom. Above the outflow port was a Millipore GS/HA filter (0.45 micron) to retain bacteria in the central reservoir and a stir bar to limit bacterial occlusion of the filter. The antibiotic agent concentrations were quantified by agar well diffusion testing and expected concentrations were a near perfect fit to the desired human pharmacokinetic curves. Another important aspect of this study pertains to bacterial quantification. The authors presented the direct comparison of turbidimetric (photometric) measurements to viable cell counts (plating). Although not discussed by the authors, it is quite clear that the photometric measurements are not sensitive to decreases in viable cells. This is most likely due to the fact that the dead bacteria contribute to turbidity measurements.

Two compartment models

Two compartment models began to see widespread use in the early 1980's. Although these models still use dilution to simulate the dynamics of drug loss, they incorporate a selective barrier to prevent a dilution effect on the bacterial population. These models differ from the model of Schneider *et al.* only in that the antimicrobial must diffuse across the retaining membrane. In Schneider's model, the drug is directly administered into the compartment containing the bacterial inoculum (one compartment model). The first report of a two compartment model setup was by Al-Asadi in 1979[42]. He added connection ports to two test tubes and placed a cellulose acetate membrane between them. One tube held the bacterial inoculum, while the antimicrobial (gentamicin) was administered into the other tube. Diffusion

across the membrane resulted in a gradient driven rise in the gentamicin concentrations in the bacterial compartment. Once the desired peak concentration was achieved, media flow into the sealed bacterial compartment resulted in back-diffusion of the gentamicin. The major limitation of this model was the imprecision in the control of drug concentrations compared to other models. Two factors contributed to this imprecision: membrane pores became blocked by bacterial debris and membrane composition (type, pore size, and surface area) affected diffusion across the membrane.

Three years later, Craig *et al.* described an improved two compartment model[43]. In this system, two hemodialyzers were placed in series to increase membrane surface area to approximately 1 m^2. One side of the membrane was designated the bacterial compartment, while the other was designated the drug compartment. On each side was a closed loop that circulated media within the respective compartment. The drug compartment contained an exchange loop through which fresh media was introduced, while at the same rate, drug containing media was removed. In this study, the authors used radio-labeled penicillin to quantify drug concentrations. Using radioactivity, they established that equilibrium across the membranes was rapidly achieved and that drug binding to the membrane was minimal. The authors discussed the limitation of this method of quantification, namely that radioactivity does not necessarily relate to <u>active</u> drug concentration. The authors also suggested that changing the membrane type and/or size would be necessary to alter the absorption characteristics for parenteral dosing simulations.

The model was able to effectively simulate desired drug concentrations. The primary advantage to this model was that the bacterial cells were retained in the system and not diluted with the addition of fresh media. The disadvantages of the system design were: large systems

requiring suitable incubators and very large volumes of media to simulate short half-lives (1-2 hrs), antimicrobial binding must be verified for each membrane/drug combination, directional flow of drug containing media could result in an antimicrobial concentration gradient in the bacterial compartment, the system is not commercialized - thus difficult to replicate, and the potential for biofilm formation on the membrane.

A variation on the two compartment model by Garrison[44] was described in 1990. In this model, the second (peripheral) compartment consisted of a T-tube with polycarbonate membranes on each end that was submerged in the media of the central compartment. In this publication, the figures showed the lag in concentration between the central and peripheral compartments. This lag is a result of the diffusion necessary for drug movement into the peripheral compartment and as such represents a major limitation to all two compartment models.

Capillary models

In 1981, a publication titled "An artificial capillary *in vitro* kinetic model of antibiotic bactericidal activity" by Zinner, Husson and Klastersky had described what was to become the next evolution of PK/PD *in vitro* models[45]. The studies subsequently reviewed will attempt to highlight the capabilities of the capillary diffusion (hollow fiber) infection models with brief discussion of the model limitations.

The first capillary diffusion model used by Zinner *et al.* consisted of a reservoir, connection tubing (with a single port for sampling and drug injection), a circulating pump and a commercially available capillary unit. The capillary unit was a polycarbonate tube with two sampling ports. Inside the tubes were a bundle of polysulfone capillaries. The molecular cutoff for the capillary pores was 10,000 Daltons. The bacterial culture could therefore be inoculated

and retained in the extracapillary space of the polycarbonate tube. As growth media (and antibiotics) flowed through the lumen of the capillaries, the antibiotic and other small molecules would diffuse based on the concentration gradients established in the circulating media and extracapillary space. Decreasing antimicrobial concentration was accomplished by removal and addition of equal volumes of media from the reservoir at intervals of 30 minutes, 1, 2, 3, 4, 5, and 6 hours. This resulted in a stepwise pharmacokinetic curve similar to the earlier methods of Nishida *et al.* and Leitner *et al.* Although it had not been done in this study, the authors recognized that continuous dilution and removal could be performed in the central reservoir to simulate a more realistic pharmacokinetic curve.

In this experiment, the authors compared bolus to infusion dosing of azlocillin. Only one dose regimen of each administration was evaluated. Determinations of antibiotic concentration in the central and bacterial compartments were by agar well diffusion assay.

Capillary models, like previous two-compartment models, represented an improvement on the one compartment models in that it did not dilute the bacterial culture along with the antibiotic. However, the authors only speculated on the ability to manipulate drug concentration in the extracapillary space. Manipulation of antibiotic concentrations in the bacterial compartment was further complicated by the manner in which antibiotic was administered into the system. For bolus dosing, the antibiotic was injected in the circulation loop toward the capillary unit. For infusion dosing, the antibiotic was injected into the circulation loop toward the central reservoir. In addition, the extracapillary space (1.5 mL) in this study was very limited.

In 1985, Blaser *et* al described the first two compartment *in vitro* model using commercially available capillary units[46]. The system consisted of a central reservoir which

contained culture media (type not given) into which antibiotic was infused to simulate human pharmacokinetics of netilmicin and azlocillin. Connected to the central reservoir was a fresh media reservoir, a supplementing reservoir (for infusion of netilmicin due to its longer half-life), an elimination reservoir and a circulating loop in which the serially placed capillary units were connected.

The commercial availability of the capillary units (manufactured by Amicon Corporation) represented a major improvement in the ease of use and consistency of model use between researchers. In this setup, peristaltic pumps circulated the media in the extracapillary space to limit the formation of antibiotic gradients within the individual modules. This system design also represented a potential decrease in the spatial requirements for experiments. However, this presents a disadvantage as well. The small volumes present in the extra capillary space of the modules leads to a larger impact of direct sampling (on the bacterial population). For example, a 1 ml sample from the extracapillary space for bacterial and antibiotic quantification in a 350 mL reservoir represents a 0.3% decrease in the bacterial population. The same 1 mL sample from a 20 mL extracapillary space represents a 5% decrease in the bacterial population. This limitation could be improved by collecting samples from serial modules in a population pharmacokinetic manner, but this assumes homogeneity of the capillary units. This assumption is potentially dangerous due to the serial placement of the units. An antibiotic gradient may be formed in the circulating loop as drug diffuses into the extracapillary space of the consecutive units, possibly resulting in a time delay of maximal antibiotic concentration in the extracapillary space of the downstream serial modules. Variations of this model have been used to determine optimal dosing for therapy of bacterial[47,48] and viral[49] infections.

Limitations of in vitro models

Many of the limitations to these models have been discussed above, but for clarity and completeness will be summarized here. Correlation to *in vivo* results from all *in vitro* models is limited by the lack of immune response *in vitro.* The immune component has been successfully added to an *in vitro* pharmacodynamic model in the form of macrophage monolayers and an *ex vivo* implant containing PMN and inflammatory exudate[50]. However, the lack of a functional immune response will likely overestimate PK/PD index values required for efficacy, and thus may lend a safety factor to the prediction of *in vivo* efficacy.

Although *in vitro* pharmacodynamic models have been used for over 40 years, the methodology for their use has not been standardized, as demonstrated by the number of model types currently being used and the publication of two articles to standardize the terminology in use by investigators[51, 52]. Extrapolation between studies is difficult due to the bacterial species/strains, initial bacterial inoculum, antimicrobial treatment, and outcome measured.

These models, like other models, were not intended to provide the final answer to questions of therapeutic efficacy. They should be considered an instrument capable of providing intermediary information concerning the interaction of an antimicrobial and a pathogen. When available, model outcomes should be validated through comparison to clinical outcome. In cases where induced disease models are ethically prohibited or naturally occurring disease is rare, these models can provide valuable supporting information. However, they should not be viewed as standalone predictors of clinical efficacy.

Conclusion

The literature review herein describes the evolution of antimicrobial pharmacokinetic / pharmacodynamic theory. As our understanding of the interactions between antimicrobial and

pathogen has grown, the study methodologies and models have concurrently become more sophisticated. However, the full potential of these models in simulating the infectious disease process remains to be realized. Significant obstacles remain in the application of PK/PD model outcomes to patient care in the form of questions about the translation of PK/PD indices determined *in vitro* to treat infections *in vivo,* the utility of the AUC:MIC ratio due to the hybrid makeup of the index, and the differences in pathogen MIC values between test systems and actual patient disease. Another key concern is the application of the PK/PD index value determined for a single antimicrobial-pathogen combination across the entire antimicrobial group and across multiple pathogens. Additionally, the use of a PK/PD approach to study antimicrobial - dosing regimen - pathogen combinations of veterinary importance is absent in the scientific literature. To address these concerns, the research presented here describes a combined *in vitro* – *in vivo* approach to study the pharmacodynamics of oxytetracycline in terms of both clinical efficacy and contribution to antimicrobial resistance development.

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CHAPTER 2 - Effects of two oxytetracycline dosing regimens on a *Pasteurella multocida* isolate in an *in vitro* pharmacodynamic model

Introduction

The objective of this research was to utilize an *in vitro* pharmacodynamic model (IVPM) to compare the responses of a *Pasteurella multocida* isolate to two oxytetracycline dosing regimens. Our hypothesis was that the shape of the time – concentration curve is therapeutically important, thus bacterial responses would be different for the two dosing regimens. These results will enable clinicians to optimize oxytetracycline dosing regimens in the treatment of *Pasteurella multocida* infections by matching the PK/PD index to patient pharmacokinetics and minimum inhibitory concentration of the infecting pathogen.

Pasteurella multocida is a Gram-negative facultative anaerobic bacterium. In humans, it is recognized as a primary agent of infection following animal bites, but has been associated with skin, soft tissue, bone and joint infections, as well as, pneumonia, meningitis, endocarditis and septicemia[1]. Although multiple presentations are possible in veterinary species, it is of primary importance as a respiratory pathogen of cattle and swine and as the etiologic agent of fowl cholera.

Oxytetracycline is generally considered to be bacteriostatic, inhibiting protein synthesis by reversibly binding to the 30s ribosomal subunit. The bound oxytetracycline molecule interferes with the binding of the aminoacyl-transfer RNA at the acceptor site on the mRNA-ribosome complex and prevents the addition of amino acids to the growing peptide chain[2,3].

Oxytetracycline is a broad spectrum antimicrobial agent with activity against Gram-positive and Gram-negative, aerobic and anaerobic bacteria. It is also indicated for treatment of atypical infections such as chlamydia, rickettsia, *Mycoplasma* spp., protozoa and spirochetes. Veterinary product label indications are listed in Table 1.

The scientific discipline of pharmacokinetics / pharmacodynamics (PK/PD) ultimately seeks to correlate drug concentrations with a biologic effect. In antimicrobial PK/PD, the desired effect is death or stasis of the infecting pathogen, which is correlated to measured drug concentrations. The IVPM is a unique tool that has been developed to investigate antimicrobial-pathogen interactions in a controlled laboratory setting. This model can be used to elucidate the concentration / time-dependent nature of an antimicrobial using a combination of drug exposure simulations. In this study, the IVPM was used to determine the pharmacodynamic relationship between oxytetracycline and a *Pasteurella multocida* field isolate.

Materials and Methods

Bacterial Growth Media

All liquid growth media, including that used in the central and fresh media reservoirs and for the growth of the stock cultures, was brain- heart infusion (BHI) broth. To prepare the broth, 37 grams of BHI powder[a] were mixed with 2 L of deionized water. The mixture was autoclaved at 250° F / 18 psi for 45 minutes. Prepared media was stored at 4° C until IVPM assembly.

Pasteurella multocida isolate

Multiple *Pasteurella multocida* isolates were screened for antimicrobial susceptibility to oxytetracycline. A swine respiratory disease field isolate with a minimum inhibitory

[a] brain-heart infusion media, Sigma Aldrich Chemical Co, St. Louis, MO

concentration (MIC) of 1 µg/mL was used for this study. The minimum inhibitory concentration was determined by use of an extended-range micro-well dilution system[b]. Testing was conducted in accordance with guidelines established by the CLSI and determination of susceptibility was performed in accordance with CLSI-approved interpretive criteria[4].

The selected isolate was streaked on Trypticase Soy Agar (5% sheep blood) plates[c] and incubated overnight at 37°C in 5% carbon dioxide. Isolated colonies were inoculated into brain-heart infusion broth[d]. The broth culture was incubated on a rotary shaker[e] (180 rpm) for 18 hours at 37°C. Aliquots (1500µL) were stored in 20% glycerin[f] at -70 C.

Pasteurella multocida growth curve correlation to optical density

A growth curve for the bacterial isolate was obtained by inoculating 75 mL of sterile BHI broth with 500 µL (approximately $1.25 * 10^9$ CFU) of the frozen isolate. The inoculated broth was placed on a shaking incubator[g] at 37°C and 180 rpm for the duration of the experiment.

Samples (2 mL) were collected at 0, 2, 4, 8 and 12 hours using sterile technique. The samples were divided after which approximately 1 mL of the sample was placed into a spectrophotometer[h] set to read absorbance at 650 nm. The spectrophotometer was zeroed using blank BHI broth. The remaining 1 mL was used to perform CFU determination as described below.

[b] Sensititer, Trek Diagnostic Systems Inc, Cleveland, Ohio
[c] Tryptic Soy Agar Plates with 5% sheep blood. Remel, Inc. Lenexa, KS
[d] Brain Heart Infusion Broth, Fluka / Sigma Aldrich, Buchs, Switzerland
[e] Innova4000, New Brunswick Scientific, Edison, NJ
[f] Sigma Aldrich, St. Louis, MO
[g] Innova4000, New Brunswick Scientific, Edison, NJ
[h] Spectronic 601, Milton Roy, Rochester, NY

Following incubation at 37°C for 18-24 hours, colonies were counted as described below and the average CFU of the duplicates was plotted against the OD reading for that time point (Figure 1).

Macrodilution Susceptibility Testing

A six hour culture of the *P. multocida* isolate was grown in brain-heart infusion broth on a 180 rpm shaking incubator[i] at 37°C. The OD_{650} was obtained by spectrophotometric[j] reading. The OD reading was converted to a CFU/mL value from the growth curve (equation 6). The bacterial sample was then diluted with blank BHI to obtain a final bacterial inoculant containing $1x\ 10^6$ CFU/mL.

Borosilicate tubes[k] (12 x 75mm) were pre-filled with blank BHI and freshly prepared oxytetracycline stock (10 µg/mL) so that the total volume of each tube was 0.5 mL. Tube dilutions, in duplicate, from 0.5 to 2 µg/mL were prepared in 0.1 µg/mL increments. A 0.5 mL aliquot of the bacterial inoculant was then added to each tube resulting in a starting bacterial concentration of 5×10^5 CFU/mL as per CLSI guidelines[5]. Tubes were loosely covered with paraffin and incubated at 37°C for 20 hours.

The lowest concentration with no visible sign of bacterial growth was determined to be the MIC for each duplicate. The MIC for the isolate was considered to be the average of the two replicate measurements.

[i] Innova4000, New Brunswick Scientific, Edison, NJ
[j] Spectronic 601, Milton Roy, Rochester, NY
[k] Fisherbrand disposable glass tubes, Thermo Fisher Scientific, Pittsburgh, PA

Experimental stock culture

Prior to each experimental run, a single aliquot of the seed culture was thawed at room temperature and inoculated into approximately 500 mL of brain-heart infusion broth[l]. The inoculated broth was incubated on a rotary shaker[m] (180 rpm) for 18 hours at 37^{o}C. Fifteen milliliters of the culture were collected to sub-inoculate each of the four experimental arms in the IVPM setup. Following inoculation of the central reservoirs, a four hour growth period was observed prior to antibiotic treatment.

Pharmacokinetic targets and dosing regimens

The targeted pharmacokinetic drug exposure was an area under the time-concentration curve (AUC) of 40 µg*hr/mL. Two dosing regimens were designed *a priori* using a spreadsheet program[n]. The simulations of the high concentration-short half-life regimen (HC-SHL) and low concentration-long half-life regimen (LC-LHL) were based on the published maximal plasma concentration (C_{max}) and plasma elimination half-life ($T_{1/2}$) values following intravenous and intramuscular dosing, respectively, in swine[6,7]. These values were then manipulated to fit a one-compartment pharmacokinetic model following intravenous bolus dosing and minimize the interdependence of Cmax and Time>MIC with the same AUC for the two regimens. To achieve the desired exposures in the *in vitro* pharmacodynamic model (equation 1), the C_0 and $T_{1/2}$ for the HC-SHL were set at 11.1 µg/mL and 2.5 hrs, respectively. The C_0 and $T_{1/2}$ for the LC-LHL were set for 2.5 µg/mL and 11.1 hrs, respectively.

[l] Brain Heart Infusion Broth, Fluka / Sigma Aldrich, Buchs, Switzerland
[m] Innova4000, New Brunswick Scientific, Edison, NJ
[n] Excel 2003, Microsoft Corp., Redmond, WA

Equation 1: Pump calculations in the IVPM

$$CL = \frac{0.693 \times Volume_{CR}}{T_{1/2}}$$

Where CL is the pump speed in mL/hr, $Volume_{CR}$ is the volume of the central reservoir in mL and T½ is the desired half-life in hours.

In vitro Pharmacokinetic Model

Variations of the model used in this study have been described by others[8,9,10,11]. The model consists of a 500 mL central reservoir (CR)[o], a 4 L fresh media reservoir[p], a 4 L waste collection reservoir[q], reservoir caps[r] and connecting tubing[s] (Figure 2). All components were autoclaved prior to system assembly. For each experimental run, four of the above described systems (two designated oxytetracycline treatments and two associated controls) were assembled. During the experiment, the central reservoir of each system was housed in an incubator[t] at 37 C. The central reservoir media was constantly stirred by use of a stir rod/plate[u] setup.

Fresh media pump speed was based on equation 1, to achieve a specified half-life. Waste media pump speed was set slightly faster, with the exit port set just above the CR fluid line to maintain a constant CR volume. Dual pump heads were used to control the fresh and waste media flow for a given treatment and the associated control arm. To eliminate carryover effects, a single CR port was designated for bacterial inoculation and oxytetracycline dosing. A separate

[o] Bellco Technologies, Vineland, NJ
[p] Thermo Fisher scientific, Pittsburgh, PA
[q] Thermo Fisher scientific, Pittsburgh, PA
[r] Fibercell systems, Fredrick, MD
[s] Masterflex L/S 13 Platinum cured silicon tubing, Cole Parmer, Vernon Hills, IL
[t] NuAire IR Autoflow, NuAire, Inc., Plymouth, MN
[u] IKA Big Squid, Sigma Aldrich, St. Louis, MO

port was designated for bacterial and antimicrobial sampling. Three replicates of each treatment (High Concentration / Low Concentration) and the associated control were conducted.

Antimicrobial treatment

Oxytetracycline[v] was used for all experimental replicates with dosing corrections for salt and purity. Drug administration to be simulated was an intravenous bolus; therefore, the oxytetracycline powder was dissolved into 15 mL of brain-heart infusion broth immediately prior to direct injection into the central reservoir of the IVPM.

Bacterial quantification

All bacterial samples were plated immediately after collection. Bacteria in the central reservoir of the IVPM were quantified at inoculation, at oxytetracycline administration, and at 4, 8, 12, 24, 36, 48, 60, and 72 hours post-oxytetracycline administration. For each treatment / time point sample, 200 µL of sample was pipetted into 1800 µL of sterile phosphate buffered saline (PBS) containing 0.015% of a non-ionic detergent[w] and vortexed for 1-2 seconds. Two hundred microliters (200 µL) of dilution 1 were withdrawn and added to the second tube containing 1800 µL of PBS/detergent. This vortex and dilution procedure was repeated for 10 total dilutions. Dilutions 3-10 were plated in duplicate; 100 µL of bacterial suspension were pipetted onto blood agar plates[x] and plated using a disposable plating rod[y].

Plated samples were incubated at 37 C for 18-24 hours. Dilution plates with at least 20 but no more than 200 isolated colonies were used for bacterial quantification according to Equation 2.

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[v] Oxytetracycline hydrochloride, Sigma-Aldrich, St. Louis, MO
[w] Tween 80, Sigma Aldrich, St. Louis, MO
[x] Tryptic Soy Agar Plates with 5% sheep blood. Remel, Inc. Lenexa, KS
[y] Lazy L spreader, Sigma Aldrich, St. Louis, MO

Equation 2: Colony Forming Unit determination

$$CFU/mL = Number\ of\ isolated\ colonies * \left(\frac{1}{DilutionFactor} \right) * 10$$

At each time point, all plates that met the above criteria were counted. If more than one dilution per time point was quantifiable, the average CFU / mL was used for that time point. The lower and upper limits of bacterial quantification were $2*10^5$ and $2*10^{13}$, respectively.

Oxytetracycline quantification

Sample storage

Samples taken for bacterial quantification were divided. A 1-2 mL aliquot was immediately frozen at -70° C until analysis.

Sample preparation

All chemical reagents were analytical grade. Briefly, 50 µL of internal standard (doxycycline[z] 2,000 ng/mL) were added to 50 µL of sample. The analyte and internal standard were extracted using 10 µL of concentrated phosphoric acid[aa] followed by the addition of 150 µL of deionized water. The samples were loaded on HLB solid phase cartridges[bb] for the extraction procedure. Following the first elution with 5:95 (methanol:water), the columns were washed with 500 µL of 5:95 (Methanol:Water) and dried under high vacuum (≈ 20 inches Hg) for 10 minutes. Oxytetracycline was recovered from the SPE cartridge using a methanol wash (300 µL). A 150 µL sample was then transferred to a HPLC vial and stored at 4°C in the sample carousel until analysis.

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[z] Doxycycline hyclate, Sigma-Aldrich, St. Louis, MO
[aa] O-Phosphoric acid, Fisher Scientific, Pittsburgh, PA
[bb] HLB 10 mg, Waters Corp., Milford, MA

Oxytetracycline quantification

Quantitiation of oxytetracycline was performed using high performance liquid chromatography and triple quadrupole mass spectrometry (HPLC/MS/MS). Chromatographic separation was achieved using a gradient elution of 100% (0.2% glacial acetic acid[cc] in H_2O) moving to 5% (0.2% glacial acetic acid in H_2O):95% (0.2% glacial acetic acid in acetonitrile[dd]) on a HPLC system[ee] with a C18 analytical column[ff]. Injection volume was 2 µL and flow rate was 0.35 mL/min with a total run time of 5 minutes. Retention times for oxytetracycline and doxycycline (IS) were 2.12 and 2.15 minutes, respectively.

Mass spectrometry utilized an electrospray ionization source[gg]. The instrument[hh] was set to operate in positive ion mode. Transitions were monitored at m/z 461 → 426 for oxytetracycline and m/z 445 → 321 for doxycycline (internal standard). The standard curves were prepared daily and consisted of 7 non-zero points ranging from 20 to 14,000 ng/mL. Runs were accepted if the concentrations were within 15% of the expected value and the fit of the standard curve achieved an R^2 of at least 0.99. Two quality control samples, low (350 ng/mL) and medium (6,000 ng/mL), were run for every 12 samples. Accuracy and coefficient of variation using 2 replicates each of the low and medium quality controls was ±13% and <8%, respectively.

Protein Binding

Protein binding was determined in brain-heart infusion broth by ultrafiltration / centrifugation. Triplicate 200 µL aliquots of the low (20 ng/mL), medium (500 ng/mL) and high

[cc] Glacial Acetic acid, Fisher Scientific, Pittsburgh, PA
[dd] Acetonitrile, Fisher Scientific, Pittsburgh, PA
[ee] Shimadzu LC-20AD, Shimadzu Scientific – North America, Columbia, MO
[ff] Sunfire C18, Waters Corp., Milford, MA
[gg] Turbo-Ionspray Atomospheric Pressure Ionization Source, MDS Analytical Technologies, Concord, ON
[hh] Sciex API 4000, MDS Analytical Technologies, Concord, ON

(14000 ng/mL) calibration solutions from the standard curve were pipetted into centrifugal filtration vials[ii]. The vials were centrifuged at 14,000 x g for 30 minutes. The preparation and quantification procedures were as detailed previously with the following exception: the standard curve for protein binding estimate was fit with a quadratic equation ($r^2 = 0.9998$) consisting of 6 points across the range of concentrations. Accuracy of the standards was within ± 3% of expected concentration. The analytic run consisted of only the standard curve and 9 ultrafiltered samples. Protein binding was calculated using equation 3.

Equation 3: Calculation of protein binding

$$\text{Protein Binding} = 1 - \left[\frac{\text{Concentration in Ultrafiltered Sample}}{\text{Concentration in Known Standard}} \right]$$

For filtered samples at the low concentration, the centrifuged / filtered sample concentrations were above the LOD but below the LLOQ. For these samples, concentration was calculated by dividing the area ratio of the sample by the area ratio of the standard and multiplying by the known concentration of the standard.

Pharmacokinetic analysis

Time – concentration data were analyzed based on a one compartment intravenous bolus model with 1st order elimination. The analysis was completed with a commercially available pharmacokinetic software program[jj]. Concentration at time 0 is calculated by Equation 4. The elimination rate constant for each experimental unit was determined by regression of all points of the time-concentration curve.

[ii] Microcon YM-10m, Millipore Corp., Bedford, MA
[jj] WinNonlin 5.2, Pharsight Corp., Cary, NC

Equation 4: Calculation of C0

$$C_0 = \frac{\text{Dose}}{\text{Volume of Distribution}}$$

Where dose is the amount of drug administered in milligrams and volume of distribution is the calculated volume of the central compartment (reservoir).

Statistical Analysis

In the initial analysis, CFU/mL data were transformed by the natural logarithm prior to analysis using a commercial statistical package[kk]. Data were fit to a repeated measure in time model which can be analyzed as a split-plot model when the Huynh-Feldt condition is satisfied[12]. Statistical significance was set at $p \leq 0.05$.

Results

Pasteurella multocida Growth curve

The data from growth kinetic experiments were best fit to the following exponential equation:

Equation 5: CFU/mL determination from optical density measurement

$$\text{CFU/mL} = 2E+07 * e^{(5.1851)(OD)}$$

Macrodilution Susceptibility Testing

The minimum inhibitory concentration in brain-heart infusion broth for the *Pasteurella multocida* isolate used in this study was between 1.1 and 1.2 µg/mL for the two replicates.

[kk] SAS 9.3.1, SAS Institute, Cary, NC

Protein Binding

The protein binding in brain-heart infusion broth was non-linear (figure 3). Measured values were corrected according to equation 6. For oxytetracycline concentrations greater than 700 ng/mL, protein binding was estimated to be < 15% and corrections were not applied.

Equation 6: Protein binding correction in brain - heart infusion broth

% protein bound = -0.066 * Ln (measured OTC concentration) + 0.587

Pharmacodynamic Determination

Actual pharmacokinetic parameters were less than target values for both dosing regimens (Tables 2 & 3). Peak drug concentration and AUC values were both numerically higher in the HC- SHL treatment, while time above MIC was slightly longer in the LC – LHL treatments. Contrast statements (Table 4) did not show statistical differences between the HC – SHL control and the LC – LHL control, therefore treatments were compared directly (Figure 4). When the bacterial response in the treated experimental arms was subtracted from the associated control arm response, (HC-SHL – HC control vs. LC-LHL – LC control) the comparison of differences approached statistical significance (P=0.056) (Figure 5). However, only the comparison of the HC – SHL to the HC – SHL control reached statistical difference (Figure 6).

Discussion

This study is the first reported use of an *in vitro* pharmacodynamic model to study the interaction of an antimicrobial and pathogen primarily of veterinary importance. Although the limitations of the model have been discussed previously, several considerations merit mention as a guide to future work in this area.

To determine the optimal PK/PD parameter in the present study, a variation on the traditional dose fractionation study was utilized. Both dosing regimens were designed to simulate an AUC of approximately 40 µg*hr/mL. Rather than using a fractionated dose to minimize the interdependence of time above MIC and maximal concentration, antimicrobial exposure simulations of two different half-lives were utilized. One of the disadvantages to this approach in a one-compartment IVPM is the differential rate of bacterial dilution; which may confound interpretation of the results. This effect has been previously shown to be negligible and mathematically correctable. However, these corrections were applied with reference to a non-flowing or static system. Grasso *et al.*[13] and Murakawa *et al.*[14] had only considered the effect of dilution on the bacterial population. White *et al.*[15] considered the effect of media flow on growth kinetics, but only corrected for mathematical overestimation of the bacterial population as the culture approached the stationary growth phase. In our study, the comparison of HC – SHL control and LC – LHL controls demonstrates that the higher media flow (i.e. shorter half-life) resulted in a numerically greater bacterial population. Utilizing a simultaneous control setup for each respective treatment corrects for the possibility of either enhanced or impeded growth due to media flow rates and, thus, is superior to the mathematical corrections proposed.

In this study, a period of bacterial growth was observed prior to antimicrobial treatment in order to simulate the bacterial population of an established infection. However, following this period, the cultures were in the stationary phase of growth, which poses a limitation on the ability to detect bacteriostatic antimicrobial effects. For pharmacodynamic studies involving bacteriostatic agents, it is advisable to initiate treatment during the logarithmic phase of growth.

Because the older tetracycline antibiotics were discovered before the advent of modern PK/PD investigations, there is very little information in the published literature. Most has been extrapolated from the newer tetracyclines (doxycycline, minocycline and tigecycline). A mouse thigh infection model was used by Van Ogtrop *et al.*[16] to demonstrate that both time above MIC and AUC:MIC were correlated with bacterial response to tigecycline, with a slightly higher correlation for time above MIC. In contrast, Bowker et al[17] showed the antibacterial effect of minocycline against *Staphylococcus aureus* was best correlated to AUC/MIC in an *in vitro* pharmacodynamic model simulating human serum concentrations following clinical doses. However, these authors also reported that at doses which only achieved bacterial stasis, once daily dosing was superior to fractionated doses at the same mg/kg total daily dose. Thus, at lower doses, oxytetracycline acts in a concentration-dependent manner. This conclusion is in contrast to the earlier work of Cunha *et al.*[18], who concluded that doxycycline acted in a time-dependent manner at low multiples of the MIC, and in a dose-dependent manner at concentrations 8 -16 x MIC. The differences could be a result of the study methodology (static time-kill for Cunha, *in vitro* pharmacodynamic model for Bowker), antimicrobial (doxycycline for Cunha, minocycline for Bowker) or the pathogen studied (*S. aureus* ATCC 25923 for Cunha, 2 clinical strains and 2 laboratory strains [SMH 32985/SMH32034] for Bowker).

In a recent study using the murine thigh infection model, Crandon *et al.* demonstrated that the free drug AUC:MIC ratio best predicted efficacy against hospital and community acquired strains of *Staphylococcus aureus* for tigecycline[19]. In this study, time> MIC was least predictive of bacterial response for the 7 isolates used. The authors noted that the poor correlation to time was driven by relatively large reductions in the bacterial populations in dosing regimens where the time above the MIC was 0% of the dosing interval. Differences from the

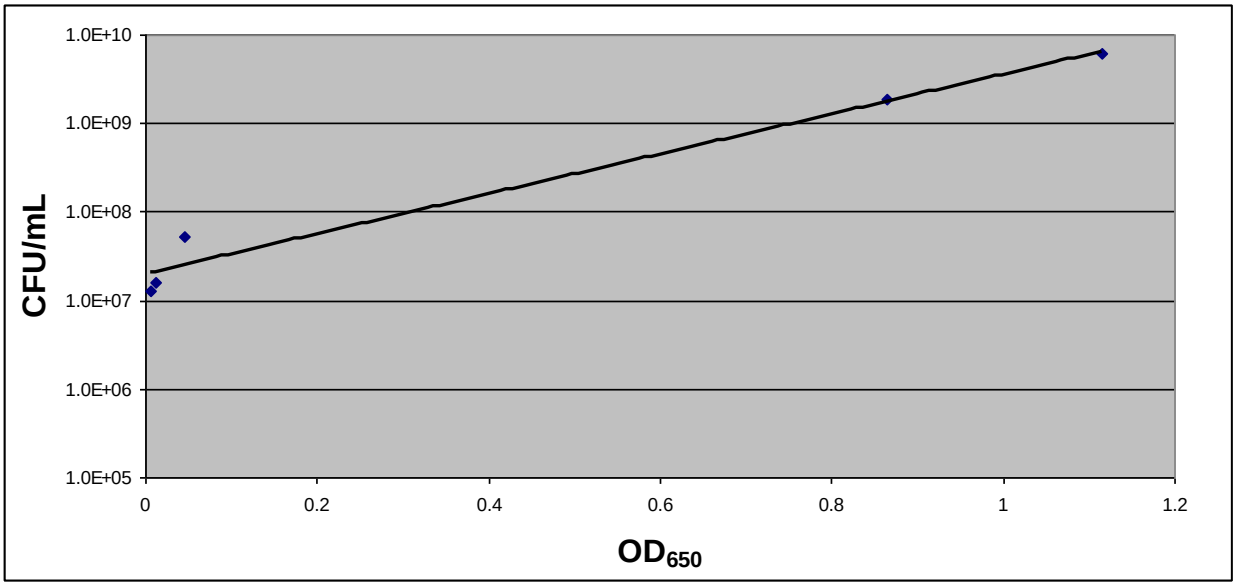
results reported here for oxytetracycline and *Pasteurella multocida* need to be evaluated in light of the different antimicrobials and pathogens studied.

Christianson *et al.* also showed that AUC:MIC was the most predictive index of bacterial response to doxycycline in the murine thigh infection model[20]. The conclusion as stated by the authors of that study was "The 24 hr AUC:MIC is the PK/PD parameter that best predicts the *in vivo* activity of doxycycline against *S. pneumoniae*". This statement emphasizes that PK/PD relationships may be drug-pathogen specific. However, to this author's knowledge, no studies have previously investigated the pharmacodynamics of oxytetracycline and *Pasteurella multocida* under simulated *in vivo* exposures.

The statistical differences between the two dosing regimens reported here suggest that oxytetracycline acts in a concentration dependent manner against *Pasteurella multocida* and that the Cmax:MIC is the PK/PD index most closely associated with efficacy. For the low concentration – long half-life exposures, no statistically significant effect on the bacterial population was seen compared to respective controls, while the high concentration – short half-life regimens resulted in a nearly 2 log reduction in the bacterial population. The comparison of the two oxytetracycline dosing regimens shows that a greater reduction in the bacterial population can be achieved by increasing the maximal drug concentration. These results also demonstrate that the same AUC applied over different lengths of time may result in different bacterial responses. Further research is required to investigate the implications of the bacterial response on clinical outcome of *Pasteurella multocida* infection, to derive the optimal PK/PD index, and the magnitude of that index that optimizes treatment success.

Figures and Tables

Figure 1: *Pasteurella multocida* growth curve



Growth curve was determined in brain-heart infusion broth. Optical density is absorbance read at 650 nm. This growth curve was used to provide proper inoculum size in macrobroth dilution determination of MIC.

Figure 2: *In vitro* pharmacodynamic model setup

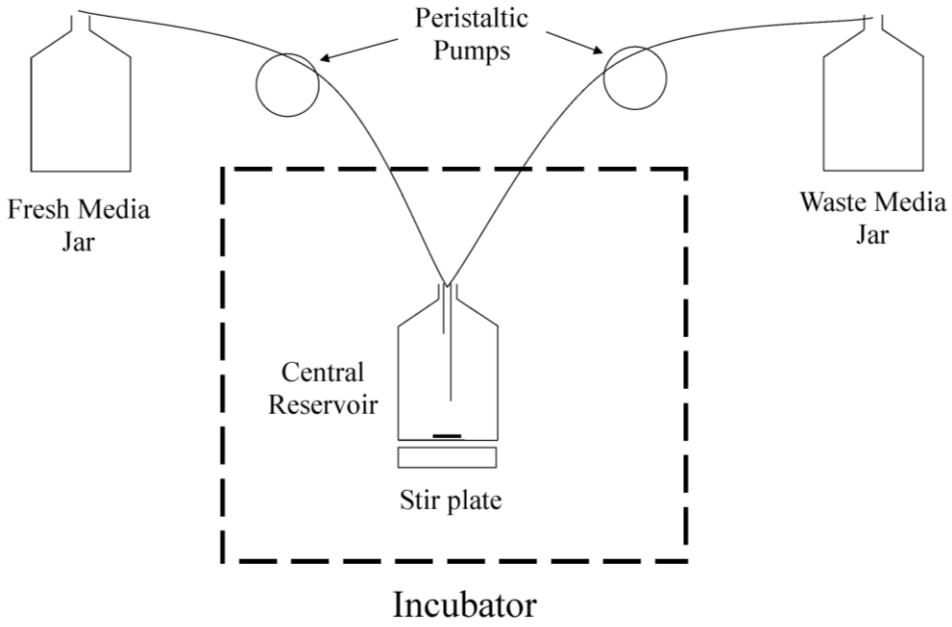


Table 1: Veterinary Label Indications for oxytetracycline

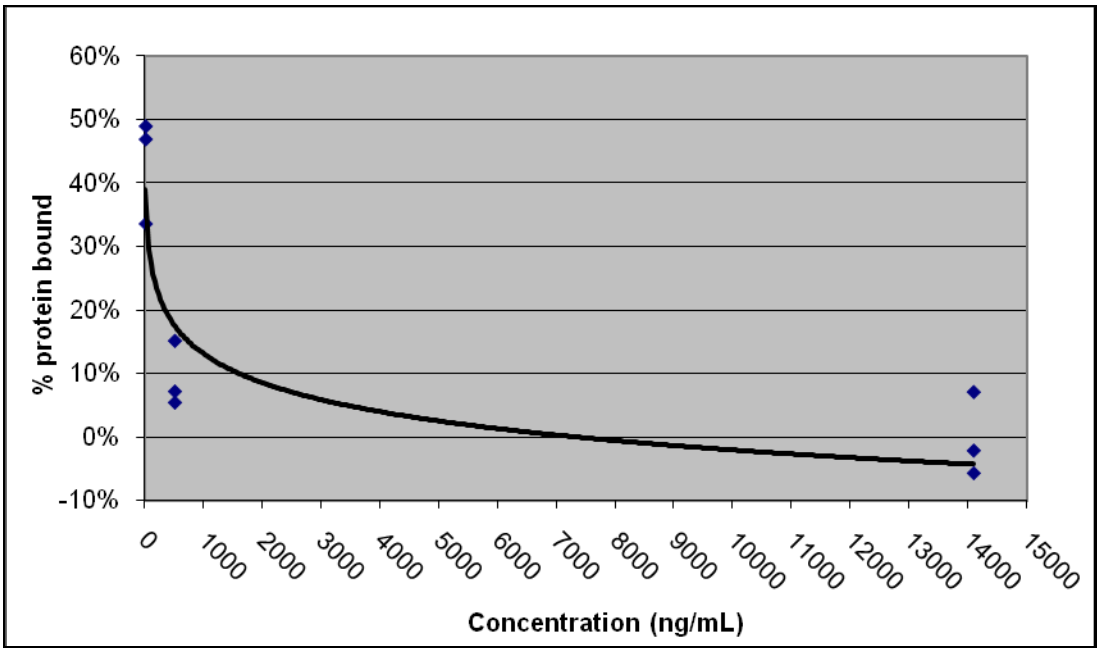
Species / Class	Route of administration	Disease Indication
Finfish*	Immersion	• Fluorescent Marker
Chickens‡	Water additive	• Infectious synovitis caused by *Mycoplasma synoviae* • Chronic respiratory disease caused by *Mycoplasma gallisepticum* and *Escherichia coli* • Fowl cholera caused by *Pasteurella multocida*
Turkeys‡	Water additive	• Hexamitiasis caused by *Hexamita meleagridis* • Infectious synovitis caused by *Mycoplasma synoviae* • Growing turkeys- complicating bacterial organisms associated with bluecomb
Swine‡	Water additive	• Bacterial enteritis caused by *Escherichia coli* and *Salmonella cholerasuis* • Bacterial pneumonia caused by *Pasteurella multocida* • For breeding swine: Leptospirosis caused by *Leptospira pomona*
Calves, Beef cattle and Non-lactating Dairy cattle‡	Water additive	• Bacterial enteritis caused by *Escherichia coli* • Bacterial pneumonia (shipping fever complex) caused by *Pasteurella multocida*
Sheep‡	Water additive	• Bacterial enteritis caused by *Escherichia coli* • Bacterial pneumonia (shipping fever complex) caused by *Pasteurella multocida*

* Oxymarine product label (Alpharma) Compendium of Veterinary Products 10th ed. North American Compendiums. 2007.
‡ Oxy-mycin 10 product label (AgriPharm) Compendium of Veterinary Products 10th ed. North American Compendiums. 2007.

Species / Class	Route of administration	Disease Indication
Beef cattle, Beef calves, Non-lactating dairy cattle and Dairy calves[§]	Intravenous Intramuscular Subcutaneous	• Bacterial pneumonia and shipping fever caused by *Pasteurella* sp. and *Hemophilus* sp. • Infectious bovine keratoconjunctivitis caused by *Moraxella bovis* • Bacterial enteritis caused by *Escherichia coli* • Necrotic pododermatitis caused by *Fusobacterium necrophorum* • Calf diphtheria caused by *Fusobacterium necrophorum* • Wooden tongue caused by *Actinobacillus lignieresii* • Leptospirosis caused by *Leptospira pomona* • Wound infections and acute metritis caused by infection of oxytetracycline-susceptible strains of staphylococcus and streptococcus
Swine[§]	Intramuscular	• Bacterial enteritis caused by *Escherichia coli* • Bacterial pneumonia caused by *Pasteurella multocida* • Leptospirosis caused by *Leptospira Pomona* • In sows, as an aid in the control of enteritis in suckling pigs caused by *Escherichia coli*
Cattle◊	Medicated Feed	• Bacterial enteritis caused by *Escherichia coli* • Bacterial pneumonia caused by *Pasteurella multocida*
Catfish◊	Medicated Feed	• Control of bacterial hemorrhagic septicemia • Control of pseudomonas disease

[§] Oxytetracycline 200 injection product label (Aspen) Compendium of Veterinary Products 10th ed. North American Compendiums. 2007.
[§] Oxytetracycline 200 injection product label (Aspen) Compendium of Veterinary Products 10th ed. North American Compendiums. 2007.
◊ Terramycin Type A Medicated Article (Phibro animal health) NADA # 008-804. www.accessdata.fda.gov
◊ Terramycin Type A Medicated Article (Phibro animal health) NADA # 008-804. www.accessdata.fda.gov

Figure 3: Protein binding in brain – heart infusion broth



Determination of protein binding is detailed in the text. Plot of oxytetracycline concentration vs. percent protein binding yielded a logarithmic function. Three replicates at three concentrations were ultrafiltered to produce the plotted values.

Table 2: Targeted pharmacokinetics in the *in vitro* Pharmacodynamic Model

Parameter	HC-SHL	LC-LHL
Cmax	11.08 µg/mL	2.5 µg/mL
$T_{1/2}$	2.5 hr	11.08 hr
T>MIC	8.75 hr	13.25 hr
AUC	39.88 hr*µg/mL	39.88 hr*µg/mL

Table 3: Achieved pharmacokinetics in the *in vitro* Pharmacodynamic Model

Dosing Regimen	*f*AUC : MIC	*f*Cmax : MIC	*f*Time > MIC
HC	37.70	11.32	8.08
HC	35.20	8.62	8.8
HC	32.90	8.19	8.45
HC Average ± SD	35.27 ± 2.40	9.38 ± 1.70	8.44 ± 0.36
LC	30.02	1.88	10.08
LC	29.27	1.78	9.48
LC	31.67	2.00	10.98
LC Average ± SD	30.32 ± 1.23	1.89 ± 0.11	10.18 ± 0.75

Table 4: Statistical comparisons of the bacterial responses for the following *in vitro* pharmacodynamic model dosing regimens

Contrast Statement	P- value
HC-SHL control v. LC-LHL control	0.0803
HC-SHL v. LC-LHL	<0.0001
HC-SHL - HC-SHL control v. LC-LHL - LC-LHL control	<0.0001
HC-SHL v. HC-SHL control	0.0016
LC-LHL v. LC-LHL control	0.162

Figure 4: Mean *Pasteurella multocida* (± standard error) concentrations in Low Concentration-Long half-life control and High Concentration-Short half-life control (p=0.227)

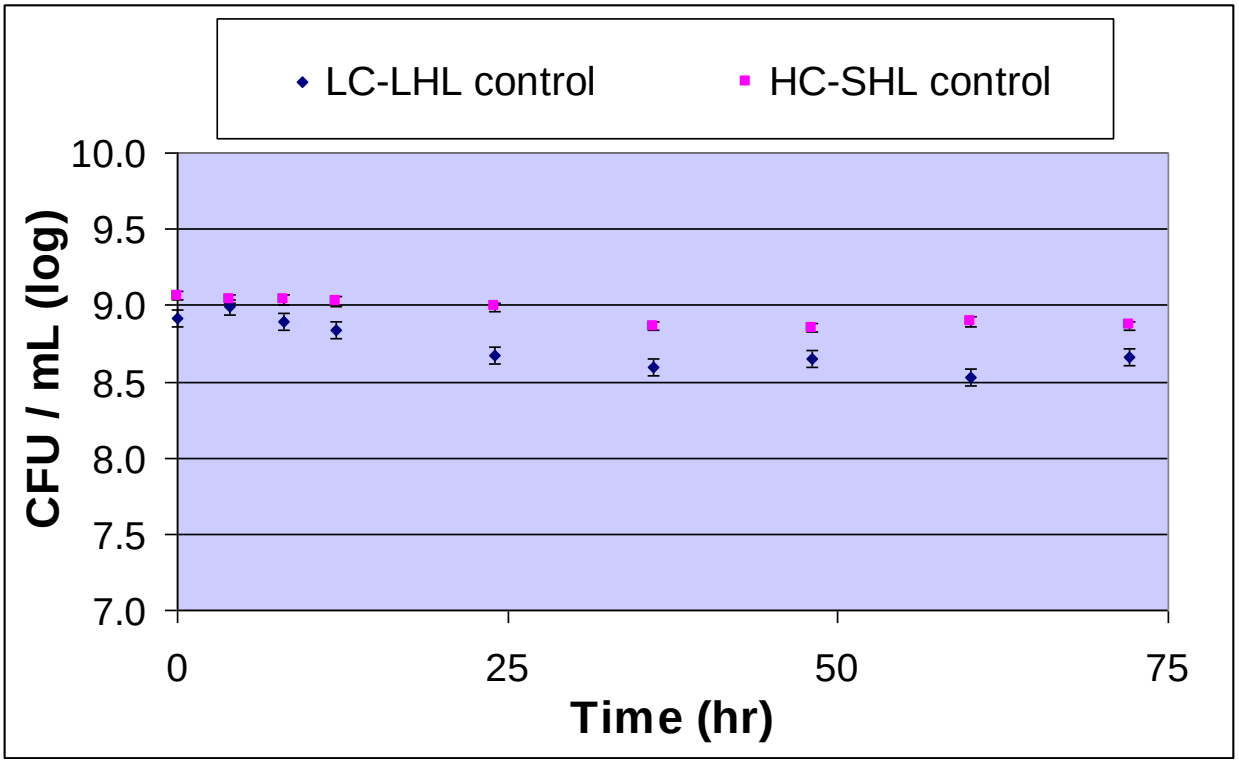


Figure 5: Mean *Pasteurella multocida* (± standard error) concentrations expressed as the differences in High Concentration-Short half-life minus High Concentration-Short half-life control and Low Concentration-Long half-life minus Low Concentration-Long half-life control (p=0.056)

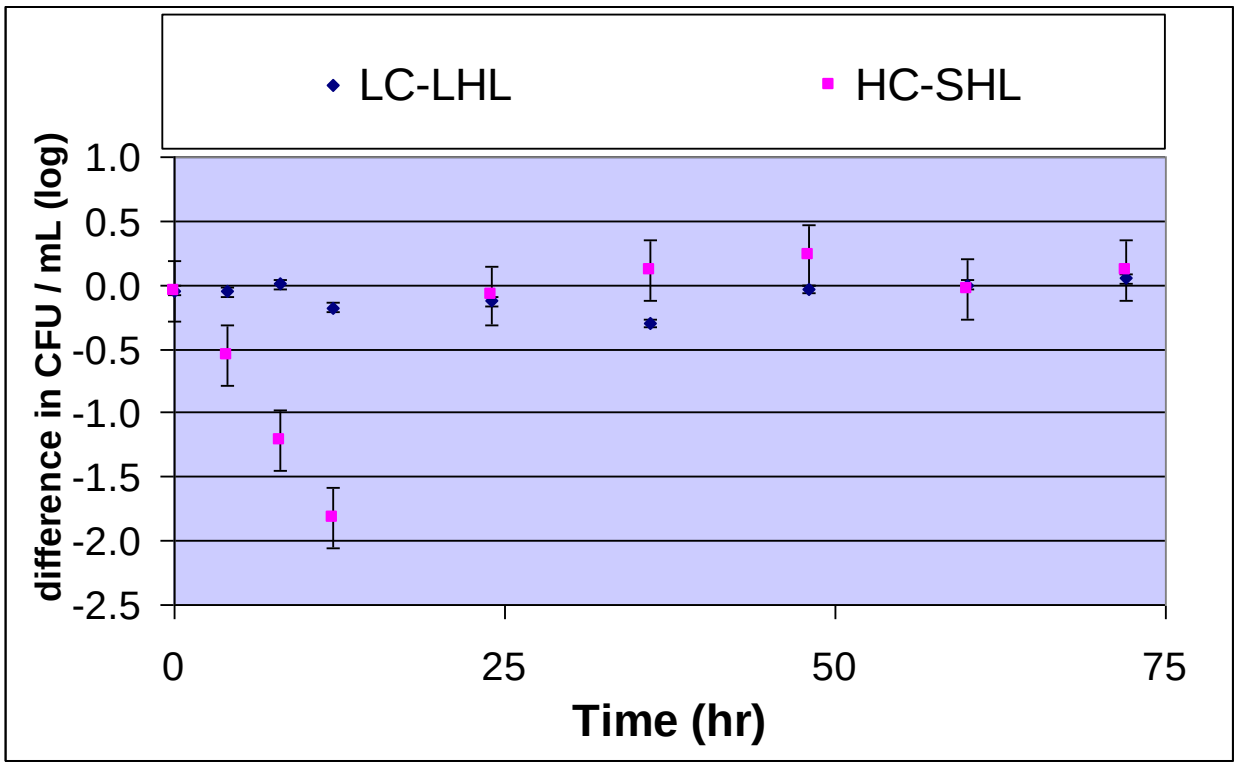
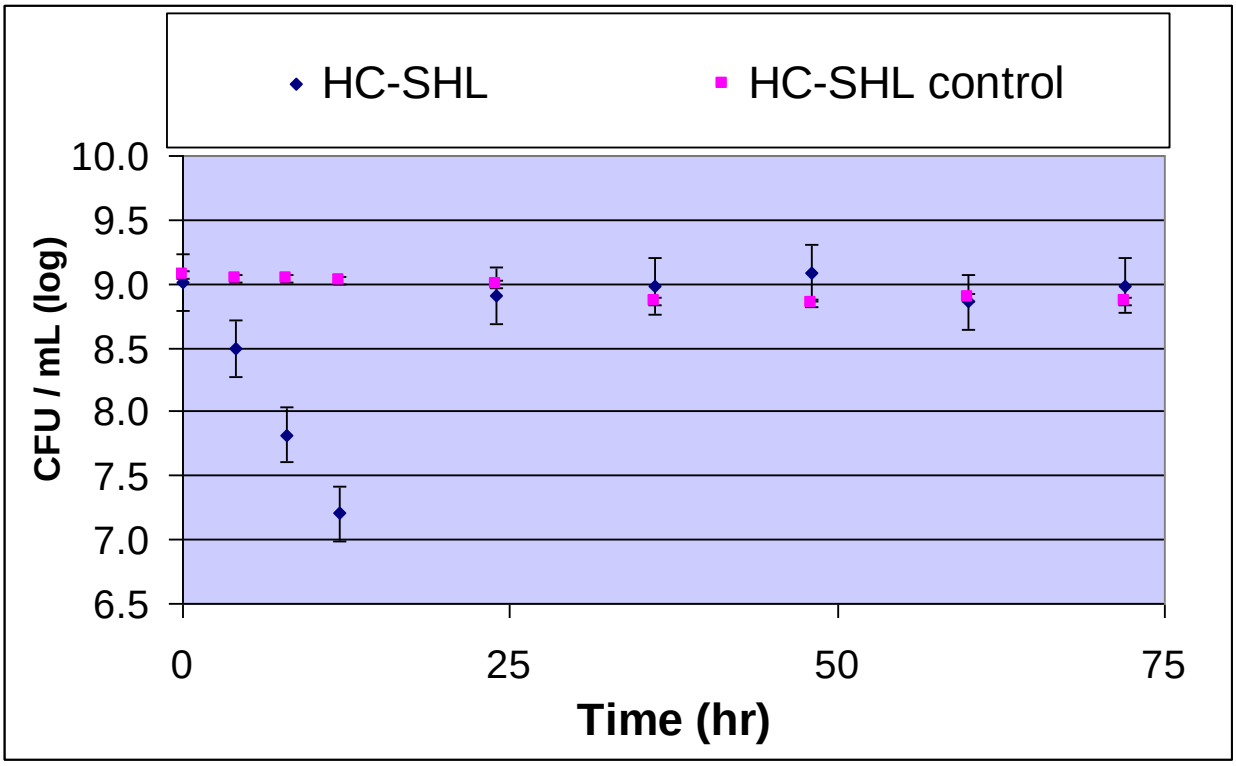


Figure 6: Mean *Pasteurella multocida* (± standard error) concentrations for High Concentration-Short Half Life and High Concentration- Short Half Life control (p=0.0016)



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CHAPTER 3 - Effects of two oxytetracycline dosing regimens on horizontal transfer of antimicrobial resistance plasmids in an *in vitro* pharmacodynamic model

Special acknowledgement goes to Greg Peterson, collaborator on the microbiological aspects of Chapters 3 & 4.

Introduction

The remarkable adaptive abilities of bacteria have lead to the phenomenon of antimicrobial resistance. Just 30 years ago, we were "closing the book on infectious diseases"[1]. Today, we face a global pandemic of re-emerging infectious diseases brought around, in part, by antimicrobial resistance. Three key factors have been recognized in the emergence of bacterial resistance: mutation, bacterial genetic exchange, and selective pressure in health care and community settings[2]. Of these factors, the primary focus has been investigating methods to decrease the selective pressure within health care and the community by focusing on prudent drug use[3,4,5]. The importance of prudent antimicrobial use has also been recognized in veterinary medicine as evidenced by the American Veterinary Medical Association's policy statement on judicious therapeutic use of antimicrobials[6]. For veterinarians, the prevention of antimicrobial resistance has important implications in our obligations to both public health and the successful treatment of our patients. This is particularly true in production animal medicine where these goals are often depicted as contradictory in the public debate.

The roles of mutation and dosing strategies to minimize resistance development have been studied extensively, giving rise to theories such as the much-debated Mutant Prevention Concentration[7,8,9,10]. There is also a plethora of literature in regard to the mechanisms of bacterial gene exchange[11]; however, relatively little has been published regarding the influence of drug exposure on the rates of conjugative transfer[12].

The issue of antimicrobial resistance has resulted in a paradigm shift with regard to investigation of antimicrobial therapeutics. Since the late 1990's, researchers have sought dosing regimens that impart not only clinical efficacy, but also minimize the development of antimicrobial resistance. Consistent with these goals, the objective of this research was to describe the relationship between drug exposure and rate of plasmid transfer in an *in vitro* pharmacodynamic model. Our hypothesis was that oxytetracycline exposure has an impact on the rate of plasmid transfer between bacterial species. Results of this study provide information for the development of oxytetracycline dosing regimens that minimize the horizontal transfer of antimicrobial resistance genes.

Materials and Methods

Bacterial Species/Strains

Salmonella enterica subspecies *enterica* serovar Typhimurium 5678, containing a plasmid approximately 100kB in size with resistance markers for chloramphenicol, streptomycin, sulfisoxazole, trimethoprim-sulfamethoxazole, tetracycline and beta-lactams, was used as the resistant plasmid donor strain. Both donor and the plasmid recipient bacteria, *E. coli* C600N, had been utilized previously for plasmid transfer experiments[13]. Aliquots of the stock bacteria were stored at -80º C.

Determination of Minimum Inhibitory Concentration (MIC)

The minimal inhibitory concentration for each of the study bacteria was determined by modification of CLSI recommended procedures[14]. Ten microliters of a 0.5 McFarland bacterial suspension were pipetted into 11 mL of Luria-Bertani (LB) broth[A]. Aliquots of the bacterial suspension (100 µL) were added to the wells of a 96 well plate[B]. One hundred microliters of oxytetracycline solution had been previously added to the wells, so that the final concentrations tested were: 0, 1, 5, 25, 50, 75, 100, 125, 150, 175, 200, 250, 500, 750, and 1000 ng/mL. Testing was done in duplicate for the donor and recipient strains.

The plates were placed in an incubated spectrophotometer[C] set to read absorbance at 600 nm with readings taken hourly. The inhibitory concentration was determined to be the lowest concentration at which optical density readings were reduced.

Experimental Stock Culture

Single frozen aliquots of the *Salmonella* and *E. coli* were thawed at room temperature. The bacteria were streaked for isolation on LB agar plates[D]. A single colony of each bacteria was transferred to separate flasks containing 10 mL of sterile brain-heart infusion broth[E] and incubated overnight at 37º C on a rotary shaker[F]. The entire 10 mL of the overnight cultures were transferred into separate flasks containing 90 mL of sterile brain-heart infusion broth. Both bacterial cultures were grown to logarithmic phase growth ($OD_{A600} = 0.6$)[G]. Once the desired

[A] BD, Franklin Lakes, NJ
[B] Corning Costar 96 well plates, Sigma Aldrich, St. Louis, MO
[C] SpectraMax 190, Molecular Devices, Sunnyvale, CA
[D] BD, Franklin Lakes, NJ
[E] Sigma Aldrich, St. Louis, MO
[F] Gallenkamp orbital incubator, Sanyo-Gallenkamp, Loughborough, Liecestershire, UK
[G] Spectronic 20D+, Thermo Scientific Corp., Waltham, MA

optical densities were reached, the *Salmonella* and *E. coli* were transferred to syringes[H] in a 1:5 volumetric ratio. Five mL of the bacterial mixture was inoculated into each central reservoir of the *in vitro* pharmacodynamic model systems. A one hour growth interval was observed in the systems prior to oxytetracycline administration.

In vitro Pharmacokinetic Model (IVPM)

The IVPM has been described in detail in Chapter 2. All bacterial growth media, system setup, calculations and components were the same as previously described. Pump speed was set accordingly to achieve the desired half-lives outlined in Table 5. The two dosing regimens were modifications of intravenous[15] and intramuscular[16] dosing of oxytetracycline in swine. The intravenous dose was designed so that initial peak concentrations were above 1000 ng/mL and time within the 150-1000 ng/mL range would be minimized. The intramuscular simulation was designed so that drug concentrations would remain within the 150-1000 ng/mL range for a comparatively longer period of time. Each of the three (3) replicate runs included: High Concentration Short Half-Life, High Concentration Short Half-Life control, Low Concentration Long Half-Life, and Low Concentration Long Half-Life control.

Antimicrobial Treatment

Analytical grade oxytetracycline as the hydrochloride salt was diluted in fresh brain-heart infusion broth prior to dosing in the IVPM. Corrections for salt and purity were made to achieve oxytetracycline concentrations outlined in Table 5.

[H] Monoject 12 mL regular luer syringe, Kendall, Mansfield, MA

Bacterial Quantification & Determination of plasmid transfer

Samples were collected from the central reservoir of the IVPM at 12, 24 and 36 hours after oxytetracycline administration. The 1-1.5 mL sample was centrifuged at 5,000 x g for 10 minutes. The supernatant was pipetted into cryovials and stored at -70º C for oxytetracycline quantification as described below. The bacterial pellet was resuspended with 122.5 µL of phosphate buffered saline (PBS) by brief vortexing. Twenty-two and one-half microliters (22.5 µL) of the suspension was serially diluted in 202.5 µL of sterile PBS to the -8th dilution. Transconjugant bacteria were quantified by duplicate plating of 50 µL of the -1, -4, -6 and -8 dilutions on Hektoen enteric agar containing 50 µg/mL of ampicillin and 12 µg/mL nalidixic acid. *Salmonella* (the donor) were quantified by plating 50 µL of the -4, -6 and -8 dilutions on Hektoen enteric agar containing 50 µg/mL of ampicillin. *E. coli* (the recipient) were quantified by plating 50 µL of the -4, -6 and -8 dilutions on Hektoen enteric agar containing 12 µg/mL nalidixic acid. Colony-Forming unit counts were determined as by Equation 1.

Equation 1: Colony forming unit calculation for transconjugant, donor and recipient bacteria

$$CFU / mL = \text{Number of colonies} * 20 * \frac{1}{\text{Dilution Factor}}$$

Equation 2: Calculation of Transconjugant (TC) ratio

$$TC \text{ ratio} = \frac{\text{Transconjugant (CFU/mL)}}{\text{Donor Bacteria (CFU/mL)}}$$

Oxytetracycline quantification

Samples were prepared by the solid phase extraction method, and analyzed by a LC/MS/MS method described in Chapter 2 for oxytetracycline in brain-heart infusion broth. The standard curve consisted of 6 non-zero points ranging from 20 ng/mL (LLOQ) to 14,000 ng/mL. The run was accepted if the concentrations of the standards were within 15% of the expected concentration and the fit of the curve was at least 0.99. Two low (350 ng/mL) and two medium (6000 ng/mL) quality controls were run; one low QC was more than 20% different from the expected value. The accuracy and coefficient of variation of the remaining QC samples were ± 19% and ± 17%, respectively.

Corrections for protein binding

Procedures and analytical methods for determination of protein binding in brain-heart infusion broth are described in Chapter 2. Protein binding in brain-heart infusion broth was non-linear between 20 and 14,000 ng/mL. For concentrations > 700 ng/mL, protein binding was estimated at less than 15% and was not corrected. For the Low concentration dosing regimen, one concentration measurement was above 700 ng/mL (751), but for consistency, all data for this regimen were corrected for protein binding according to equation 4 (Chapter 2). For the High concentration dosing regimens, the 12 hour samples were above 700 ng/mL and the 36 hour samples were below the LLOQ of the assay, so no corrections were applied. The 24 hour sample for all high concentration replicates was corrected for protein binding.

Statistical Analysis

Statistical analyses were performed using a commercial software package[I]. Transconjugant (TC) ratios were logarithmically (base 10) transformed prior to statistical analysis. The lower limit for the calculation of the transconjugant ratio is detailed in equations 3-5. The TC ratios were analyzed by timepoint using one-way ANOVA with treatment as the independent variable. Statistical significance was set *a priori* at the p=0.05 level.

Equation 3: Upper limit of quantification for donor

$$\text{ULOQ} = 200\,(\text{max. colonies counted}) * 20\,(\text{volume plated}) * 10\text{^}8\,(\text{highest dilution plated}) = 4 \text{ x } 10\text{^}10\,\text{CFU/mL}$$

Equation 4: Lower limit of quantification for transconjugants

$$\text{LLOQ} = 1\,(\text{colony}) * 20\,(\text{adjustment for volume plated}) = 20\,\text{CFU/mL}$$

Equation 5: Lower Limit for TC ratio

$$\text{Lowest limit for the TC ratio} = \frac{\text{TC LLOQ}}{\text{Donor ULOQ}} = \frac{20}{4 \text{ x } 10\text{^}10} = 5 \text{ x } 10\text{^}\text{-}10$$

Results

During determination of the MIC values, growth reductions were generally seen following 8-12 hours of incubation. The oxytetracycline MIC for the *E. coli* (recipient bacteria) was 125 ng/mL. The MIC for the *Salmonella* (donor bacteria) was 60,000 ng/mL.

Four of the time points in the treated replicates had transconjugant ratios of zero (no transconjugant colonies). Three of these time points were at 12 hours (2 LC-LHL and 1 HC-SHL), while the fourth time point was a LC-LHL replicate at 36 hours (Table 5). The mean transconjugant ratios for the HC-SHL control and LC-LHL controls were not significantly

[I] SAS 9.3.1, SAS Institute, Cary, NC

different at any of the three timepoints. At 12 hours, the transconjugant ratios for the High Concentration and Low Concentration treatments were statistically less than the respective controls (p=0.01 for both contrasts). At the 24 and 36 hour timepoints, the High Concentration treatment was not significantly different from its respective control (p=0.24 and 0.98, respectively), while the comparison of the Low Concentration treatment to its control approached significance at both timepoints (p=0.057 and 0.062, respectively). The transfer rate comparison of the High concentration to Low concentration treatment was not significantly different at 12, 24 or 36 hours (p= 0.65, 0.70, and 0.13), respectively. Results are presented graphically in figures 10, 11, 12.

Discussion

There is a growing body of *in vitro* evidence suggesting that the development of antimicrobial resistance within a population of bacteria can be suppressed by extrapolating the pharmacokinetic/pharmacodynamic indices associated with clinical efficacy[17,18,19]. The primary focus of these studies have been on the outgrowth of resistant bacteria: either originating from genetic mutation or pre-existing within the population. In contrast, the study reported here focuses on the development of antimicrobial resistance due to emergence of resistant organisms following the acquisition of a horizontally transferred plasmid. The importance of this mechanism of resistance development in bacterial populations has been discussed previously[20,21,22].

The *in vitro* pharmacodynamic model is an ideal laboratory tool to study the most basic interactions between antimicrobials and pathogens. A limitation to the one-compartment IVPM utilized here is the dilution effect on the bacteria caused by inflow of fresh media to the central reservoir. Because the transconjugant ratios of the HC-SHL and LC-LHL controls were not

significantly different at any time during the experiments, the effect of dilution rate was considered negligible. Another limitation of the IVPM under the described conditions is the favorable advantage given to the bacterial population. The type of growth media, the constant inflow of nutrients and removal of waste products, inoculum size, timing of treatment and temperature at which the experiments are conducted, and lack of a functional immune system give every conceivable advantage to the bacterial pathogen. For the present study, conjugative events may also be favored (compared to filter mating studies) given the absolute number of donor and recipient bacteria present in the culture system, and the constant stirring present in the IVPM. Results of the present study should be interpreted as the "best case" scenario for plasmid transfer given the *in vitro* conditions of the experiment.

The transfer rates in the present study are in agreement with the conjugative rates found by Showsh and Andrews[23]. Using two *Bacillus* strains, the authors reported filter mating transfer rates ranging from 1×10^{-1} to $<1 \times 10^{-8}$, when the donor was pre-grown in the presence of tetracycline (10 μg/mL). These authors also demonstrated that at low tetracycline concentrations (during mating) the conjugative frequency was enhanced. Although transfer rates noted in the previous work were achievable in our research, the conclusions are quite different with regard to the effects of drug exposure on plasmid transfer. The conclusions from Showsh and Andrews suggest that tetracycline exposure during either the pre-growth or mating period increases conjugation frequency, while in the present study oxytetracycline exposure suppressed transfer rates. The previous authors hypothesized that the enhanced conjugation was a direct effect on the donor strain, not antibiosis of the recipients.

In a paper by Torres *et al.*[24], conjugal transfer rates were also shown to be enhanced in the presence of tetracycline for *E. faecalis.* Filter matings were performed at static

concentrations (10 µg/mL) of tetracycline, as compared to the dynamic pharmacokinetics in liquid culture described here. Due to the static drug exposures in the Showsh *et al.* and Torres *et al.* and the dynamic drug exposures in the present study it is difficult to make direct comparisons of the results. In fact, results in Appendix A of this dissertation would suggest that the static exposures in the studies by Showsh *et al.* and Torres *et al.* were not true static exposures due to oxytetracycline degradation. Failure to account for the actual drug exposure limits the conclusions that can be drawn from these studies. To the author's knowledge, there are no studies investigating the effects of oxytetracycline on conjugative plasmid transfer with simulated *in vivo* antimicrobial exposures.

Previous static concentration experiments[25] with the donor and recipient bacteria used here had shown that plasmid transfer was bimodal, with higher rates of transfer at concentrations less than the growth inhibitory concentration (125 ng/mL) and concentrations between 150-1000 ng/mL. The results presented here show that conjugation was suppressed when antimicrobial concentrations exceeded the inhibitory concentration of the recipient strain. In contrast to previous work, at no time points did oxytetracycline exposure enhance the conjugation rates. The discrepancy may be a result of differences in either the laboratory conditions (filter vs. liquid culture) or the drug exposure profiles (static vs. dynamic).

The transconjugant ratio was calculated according to the method of Hirt *et al.*[26] using Equation 8 above. Because the transconjugant ratio is a hybrid of two individual measures, both require examination to make inferences about the ratio. One limitation of this study was that the plating procedures were not normalized for sample volume. However, the *Salmonella* population in the control replicates was numerically greater than in the treated replicates. The potential impact on the transconjugant ratio due to changes in the *Salmonella* population alone is

approximately 0.5 log decrease for the oxytetracycline exposed populations. The decrease in the transconjugant ratio seen here was driven by relative fewer transconjugants formed in the treated populations, not by a comparative increase in the number of donor organisms.

The results presented here show suppression of conjugative transfer in the oxytetracycline treated systems at 12 hours when compared to the respective controls. This difference is not present at 24 and 36 hours because the transconjugant ratios in the controls have decreased to the level of the treated systems. Two observations merit discussion: the peak rate of plasmid transfer occured early in the time course of the study and the development of transconjugant bacteria was suppressed by exposure to oxytetracycline. Both observations can be related to effects of bacterial growth. If conjugation is a function of bacterial growth, then a change in bacterial growth due to either the inherent growth properties of the bacterial population (stationary / death phase) or the induction of bacterial stasis due to the presence of an antimicrobial, would suppress horizontal gene transfer as was seen in the present study for the treated bacterial populations. Although not statistically significant, the suppression of the transconjugant ratio in the low concentration regimens was numerically present at all time points. This suppression was not statistically different at 24 hours nor numerically present at 36 hours in the high concentration regimen, when corresponding oxytetracycline concentrations were below the MIC of the recipient bacteria (Figures 10-12).

It is inappropriate to use low power estimates to infer differences where statistical significance was not obtained. However, numerical differences within relatively low powered studies are legitimately used as an incentive for further studies with greater power. Using the difference in means and standard deviations in the low concentration regimens at 24 and 36 hours, 3 replicates of each treatment gives a study power of approximately 0.57 or a probability

of > 0.4 for a false negative result. These findings provide preliminary insight into the relationship between drug exposure and the development of antimicrobial resistance due to horizontal gene transfer. Future studies are needed to investigate other antimicrobial-pathogen combinations and to validate these findings *in vivo.*

Figures and Tables

Table 5: Transconjugant ratio (log_{10}) and oxytetracycline concentration by timepoint.

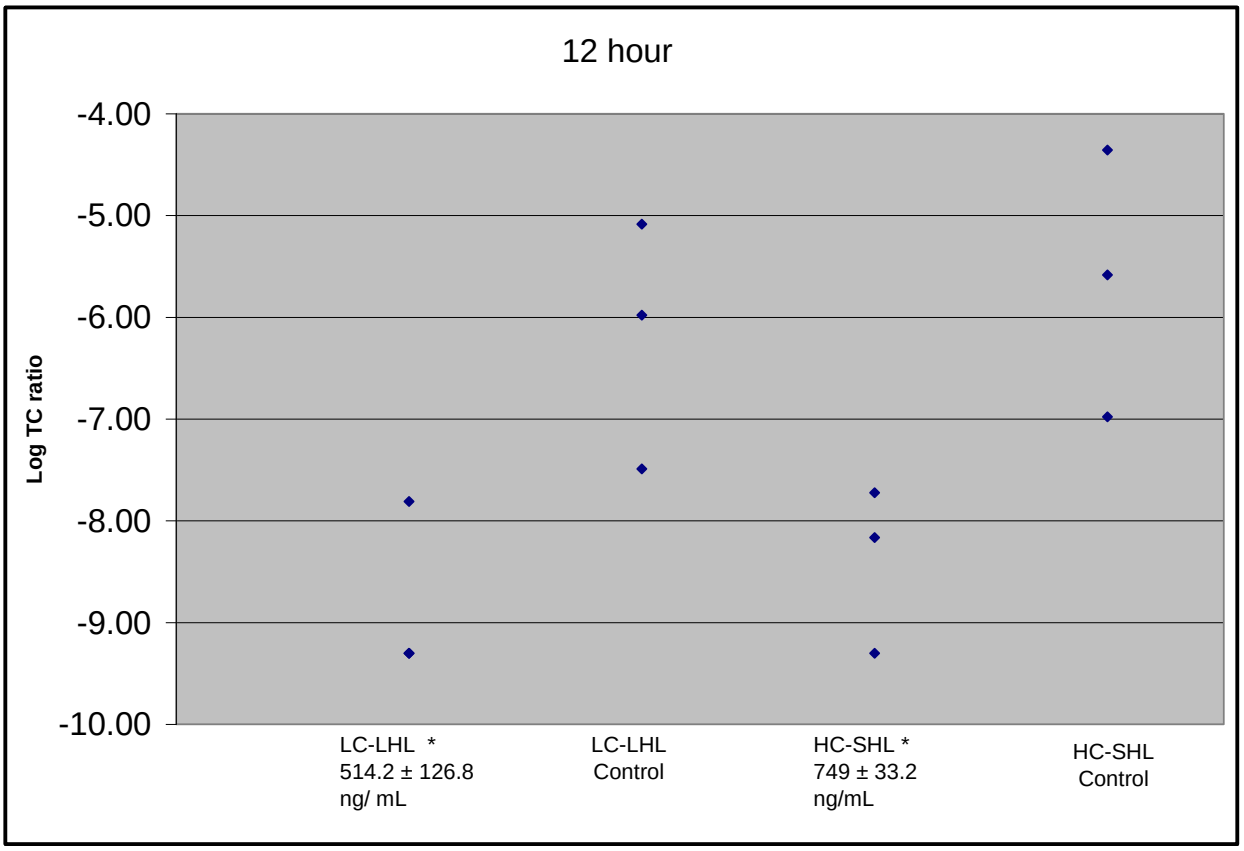
Time	Treatment	Log TC ratio ± SD	free Oxytetracycline concentration ± SD (ng/mL)
12 hour	LC – LHL[J]	-8.80 ± 0.86[a]	514.16 ± 126.77
	LC – LHL control	-6.19 ± 1.21[b]	--
	HC – SHL[K]	-8.40 ± 0.81[a]	636.56 ± 30.34
	HC – SHL control	-5.64 ± 1.31[b]	--
24 hour	LC - LHL	-7.83 ± 0.64 [a]	383.04 ± 86.05
	LC – LHL control	-6.90 ± 0.58 [a]	--
	HC - SHL	-8.00 ± 0.55 [a]	62.83 ± 16.76
	HC – SHL control	-7.46 ± 0.03 [a]	--
36 hour	LC - LHL[L]	-8.66 ± 0.65 [a]	222.51 ± 41.13
	LC – LHL control	-7.74 ± 0.60 [a]	--
	HC - SHL	-7.95 ± 0.34 [a]	Below LLOQ
	HC – SHL control	-7.96 ± 0.43 [a]	--

Transconjugant ratios with different superscripts at each time point differed significantly (p=0.05).

**-- no oxytetracycline treatment.

[J] Ratio set equal to -9.3 (LLOQ) in two of three time points
[K] Ratio set equal to -9.3 (LLOQ) in one of three time points
[L] Ratio set equal to -9.3 (LLOQ) in one of three time points

Figure 7: Transconjugant ratio (log_{10}) by treatment at the 12 hour time point



Replicates with * are significantly different from respective controls

** For the LC-LHL treatment, two replicates had no transconjugant colonies and were set at the lowest possible TC ratio (-9.3)

** For the HC-SHL treatment, one replicate had no transconjugant colonies and was set at the lowest possible TC ratio (-9.3)

Figure 8: Transconjugant ratio (log_{10}) by treatment at the 24 hour time point

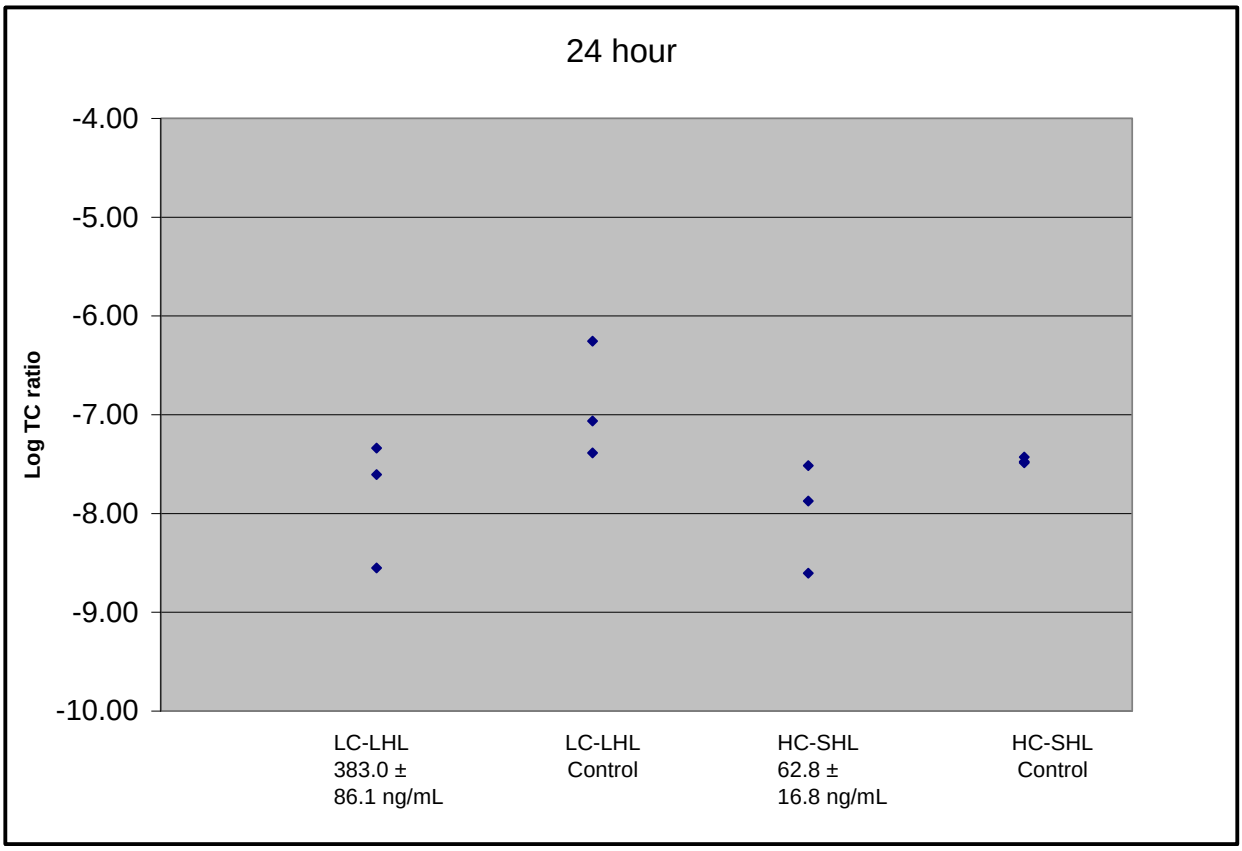
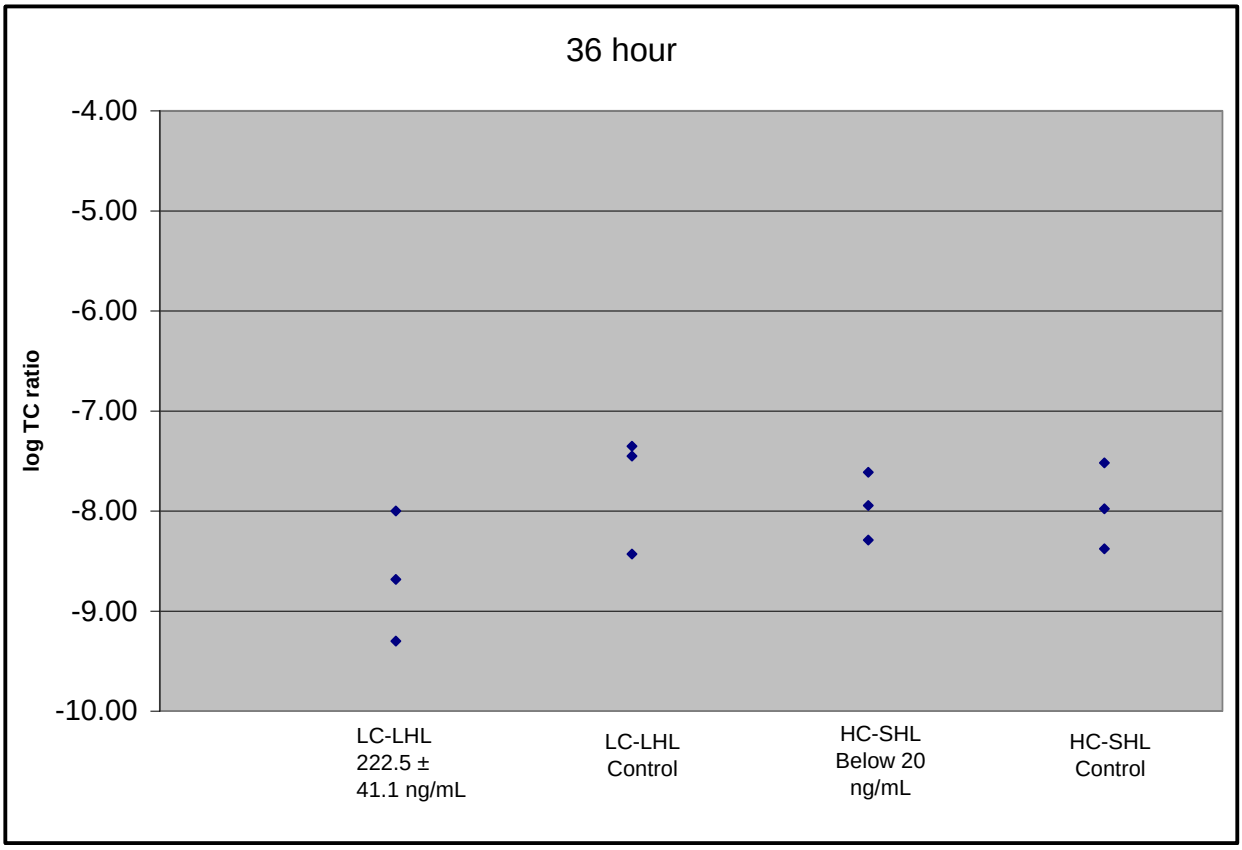


Figure 9: Transconjugant ratio (log_{10}) by treatment at the 36 hour time point



** For the LC-LHL treatment, one replicate had no transconjugant colonies and was set at the lowest possible TC ratio (-9.3)

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CHAPTER 4 - Effects of two oxytetracycline dosing regimens on horizontal transfer of antimicrobial resistance plasmids *in situ*.

Introduction

The objective of this research was to compare the effects of two different oxytetracycline dosing regimens on the rate of horizontal gene transmission between *Salmonella enterica* and *E. coli* in an *in situ* swine model using bacterial cultures contained within semi-permeable membranes. The research hypothesis was that plasmid transfer can be suppressed by proper oxytetracycline dosing. These results serve to increase our understanding of antimicrobial resistance development through horizontal gene transfer, the role of antibiotic exposure on the suppression / enhancement of these transfer rates *in vivo*, and the application of a novel swine model for *in situ* evaluation of the effect of antimicrobials on bacterial gene transfer rates.

Study Overview

Nine pigs were anesthetized for surgical implantation of jugular catheters, interstitial fluid probes and implant membranes containing a mixed bacterial population. Following a brief recovery period, animals were either administered IV or IM oxytetracycline or served as untreated controls based on prior group assignment. Plasma and interstitial fluid samples were collected over a 12 hour period, at which time the animals were euthanized. The implant membranes were then dissected from the subcutaneous space with quantification of the bacterial contents. Oxytetracycline concentrations were quantified using a LC/MS/MS method and correlated to bacterial transconjugant ratios.

Materials and Methods

Study Animals

Nine (9) crossbred pigs were sourced from a commercial swine operation in north central Kansas. All subjects were determined to be healthy by physical examination upon arrival. Prior to initiation of the study, an 8 day acclimation period was observed. Animals were fed a non-medicated commercial swine ration and monitored daily for clinical signs of disease. The study protocol was approved by the Institutional Animal Care and Use Committee at Kansas State University.

Randomization

The study was a randomized complete block design. Each block contained one of each of the following 3 treatments: intravenous oxytetracycline, intramuscular oxytetracycline, or untreated control. Animals were assigned to blocks by use of random numbers, and then randomized within blocks a second time to determine surgical order.

Bacterial strains

Salmonella enterica subspecies *enterica* serovar Typhimurium 5678, containing a plasmid approximately 100kB in size with resistance markers for chloramphenicol, streptomycin, sulfisoxazole, trimethoprim-sulfamethoxazole, tetracycline and beta-lactams, was used as the resistant plasmid donor strain. Both donor and the recipient bacteria, *E. coli* C600N, had been utilized previously for plasmid transfer experiments[1]. Minimum inhibitory oxytetracycline concentrations for these strains had been previously determined to be 125 ng/mL for the *E. coli* isolate and 60,000 ng/mL for the *Salmonella* isolate by the methods described in Chapter 3.

Bacterial implant membranes

Stock cultures were started from single frozen aliquots of both the *Salmonella* and *E. coli* isolates by thawing at room temperature. The bacteria were streaked for isolation on LB agar plates[A]. A single colony of each bacteria was transferred to separate flasks containing 10 mL of sterile brain-heart infusion broth[B] and incubated overnight at 37º C on a rotary shaker[C]. The entire 10 mL of the overnight cultures were transferred into separate flasks containing 90 mL of sterile brain-heart infusion broth. Both bacterial cultures were grown to logarithmic phase growth ($OD_{A600} = 0.6$)[D]. The logarithmic cultures were mixed in a 1:5 (donor: recipient ratio) which was used to fill each full length implant membrane[E] (approximately 250 µL of bacterial suspension). The ends of the membrane were heat sealed. A side-by-side double seal was performed at the midpoint of the membrane and the membrane was cut between the inner seals. Each exposed end was additionally sealed with liquid bandage drops and dipped in ethanol to reduce membrane surface contamination. Implant membranes were randomly assigned to study subjects prior to surgery and stored in PBS overnight at 4º C.

Anesthesia

Prior to anesthetic induction, each animal received atropine[F] (0.02 mg/kg intramuscularly) followed by anesthetic induction with an intramuscular injection of an acepromazine[G] (0.5 mg/kg), xylazine[H] (2 mg/kg), and ketamine[I] (20 mg/kg) combination.

[A] BD, Franklin Lakes, NJ
[B] Sigma Aldrich, St. Louis, MO
[C] Gallenkamp orbital incubator, Sanyo-Gallenkamp, Loughborough, Liecestershire, UK
[D] Spectronic 20D+, Thermo Scientific Corp., Waltham, MA
[E] CellMax implant membrane, 500 KDa, Spectrum Laboratories, Rancho Domingo, CA
[F] Atropine (generic) 15 mg/mL, NeogenVet, Lexington, KY
[G] Acepromazine (generic) 10 mg/mL, Vedco, Inc., St. Joseph, MO
[H] Anased 100 mg/mL, Akom, Inc., Decatur, IL
[I] VetaKet 100 mg/mL, IVX Animal Health, St. Joseph, MO

Following induction, an orotracheal tube[J] was placed and anesthesia was maintained using isoflurane[K] vaporized in 100% oxygen. Inhalant was administered to maintain surgical anesthesia (range 2 to 5 %). Each subject was placed in left lateral recumbency and the surgical area was prepared by clipping the hair and performing alternating cycles of iodine[L] and alcohol[M].

Surgical catheterization and placement of implant membranes and ultrafiltration probes

Using a #10 scalpel[N], a 10-cm curvilinear skin incision was made beginning at a point 4 cm cranial and 4 cm lateral to the manubrium and ending 4-cm caudal to the ramus of the mandible. The incision was continued through the cutaneous coli muscle and parallel to the ventral margin of the sternocephalicus muscle. This muscle was elevated and retracted laterally to expose the external jugular vein. The jugular vein and surrounding tissues were lavaged with lidocaine[O] solution to minimize muscular spasm and optimize venous access.

A 14-gauge hypodermic needle[P] was used to puncture the jugular vein. After removal of the needle, a 20-cm long, 1-mm diameter silicone rubber tube[Q] was advanced approximately 8 cm into the jugular vein. The phlebotomy site was closed and the catheter secured to the jugular vein by imbrication of the adjacent tissues with braided suture[R]. A Carmalt forceps was used to create a tunnel from the dorsal midline approximately 10-cm caudal to the base of the ear and exiting deep to the sternocephalicus muscle adjacent to the jugular catheter. The silicone rubber tubing was grasped with the Carmalt forceps and pulled through the tissue tunnel. A luer lock

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[J] 8mm orotracheal tube, Tyco Healthcare, Pleasanton, CA
[K] IsoFlo, Abbott Animal health, North Chicago, IL
[L] Povidone Iodine 7.5%, Triad Disposables, Inc., Brookfield, WI
[M] Isopropyl alcohol 70%, Barton Solvents, Wichita, KS
[N] #10 scalpel, Bard-Parker (BD), Franklin Lakes, NJ
[O] Lidocaine HCl 2%, Hospira, Inc., Lake Forest, IL
[P] 14 x 50 mm, Kendall Monoject, Mansfield, MA
[Q] Silastic tubing, Dow Corning, Midland, MI
[R] 2-0 Vicryl, Ethicon, Somerville, NJ

adapter[S] was inserted into the tubing to facilitate attachment of an injection port[T]. The catheter assembly was thoroughly flushed with heparinized saline (4 USP units heparin[U] per mL saline[V] solution) to ensure patency. The cutaneous coli muscle was closed with braided suture[W] in a simple continuous pattern and the skin was closed with monofilament suture[X] in a continuous Ford interlocking pattern. Monofilament suture[Y] was also used to secure the silicone rubber tubing at the exit portal by placing a finger trap suture onto the dorsal cervical skin.

During the same anesthetic event, the implant membranes containing the mixed bacterial populations were placed subcutaneously over the right dorsolateral thorax using a subcutaneous tunneling needle[Z]. To implant each membrane, two stab incisions approximately 15 cm apart were made using a #22 scapel blade[AA]. The needle was inserted into the caudal incision, tunneled through the subcutaneous tissue and exited from the cranial incision. Any tissue occluding the tunneling needle lumen was removed with hemostats. The implant membrane was placed into the lumen of the tunneling needle in retrograde fashion. The implant membrane was held in place at the cranial incision with forceps while the tunneling needle was withdrawn from the caudal incision. The skin incisions were closed with a single simple interrupted suture[BB]. The implantation procedure was repeated until three total membranes were placed in the control animals and nine membranes were placed in each of the oxytetracycline treated subjects.

[S] Female luer lock,
[T] Surflo Luer lock injection port, Terumo Corp., Somerset, NJ
[U] Heparin 1000 USP/mL, APP Pharmaceuticals, Schaumburg, IL
[V] 0.9% NaCl solution, Baxter Healthcare Corp., Deerfield, IL
[W] 0 Vicryl, Ethicon, Somerville, NJ
[X] #2 Braunamid, Aesculap Inc., Center Valley, PA
[Y] #2 Braunamid, Aesculap Inc., Center Valley, PA
[Z] 4.8 (OD) x 3.2mm (ID) skin tunneling needles, Harvard Apparatus, Holliston, MA
[AA] Miltex Inc., York, PA
[BB] 0 vicryl, Ethicon, Somerville, NJ

Two ultrafiltration probes[CC] were placed subcutaneously in each study animal in the dorsal lumbo-sacral area. The use of the ultrafiltration probes has been described in detail elsewhere[2]. A two incision method was used as per manufacturer's instructions. The vacuum vial holders were anchored to the study animals by direct suturing with monofilament suture[DD].

An aural catheter was placed for subjects in the intravenous treatment group prior to discontinuation of anesthesia. Following sterile preparation, the auricular vein was distended by occlusion and a catheter[EE] was placed. Following placement, the catheter was capped, flushed, and locked with heparinized saline solution. The aural catheter was then secured to the ear with surgical glue and elastic tape.

Upon completion of the surgical procedures, the isoflurane was discontinued while the animal remained on 100% oxygen for a brief period. The animal was removed from the semi-circle breathing system and returned to its holding stall. The animal was monitored until removal of the endotracheal tube, and a 4 hr recovery period was observed prior to treatment administration.

Oxytetracycline administration

The drug formulation used for this study contained 200 mg oxytetracycline base per mL as the dihydrate form in 2-pyrrolidone and povidone[FF]. Oxytetracycline was administered following the 4 hour post-surgery recovery period. Subjects in the intravenous treatment group received 8 mg/kg of oxytetracycline via the aural catheter. Following drug administration, the catheter was flushed with heparinized saline and removed immediately.

[CC] UF-3-12 Ultrafiltration probe, BASi, West Lafayette, IN
[DD] 0 Braunamid, Aesculap Inc., Center Valley, PA
[EE] 22 ga x 25 mm catheter, Nipro Corp., Osaka, Japan
[FF] Liquamycin LA-200, Pfizer Animal Health, New York, NY

For intramuscular injection, 12 mg/kg of oxytetracycline was administered in the semimembranosus / semitendinosus area using an 18G. x 25 mm needle. Treatment was administered in the hindlimb contralateral to the one used for pre-anesthetic solution injection.

Doses were chosen based on pharmacokinetics from a preliminary study in animals of similar age/production status.

Plasma Sampling

Blood samples were collected by syringe from the jugular catheter immediately prior to oxytetracycline administration and at 0.25, 0.5, 1, 1.5, 2, 4, 6 and 12 hours after oxytetracycline administration. The samples were placed in a lithium heparin tube[GG] and placed on ice. For further processing, the samples were centrifuged at 2350 x g for 5 minutes. The plasma was extracted from the sample using a transfer pipette[HH] and placed in a labeled cryovial[II]. Processed samples were stored at -70 C until analysis.

Interstitial Fluid (ISF) Sampling

Interstitial fluid collection has been described previously[3]. Interstitial fluid samples were collected from the ultrafiltration probes at 2 hour intervals. At each timepoint, the collected ISF from the two probes in each animal was pooled. Interstitial fluid collected during the pre-treatment recovery period for all subjects and from the control animals during the entire study was pooled and used for blank matrix during drug analysis. Samples collected from treated animals were transferred to cryovials using a calibrated pipette[JJ]. To estimate sample volume, collected fluid was pipetted in 50 µL increments with rounding to the nearest 25 µL.

[GG] 3 mL Vacuette LiHep, Greiner bio-one, Monroe, NC
[HH] Fisherbrand transfer pipettes, Fisher Scientific, Pittsburgh, PA
[II] 2 mL cryogenic vial, Fisher Scientific, Pittsburgh, PA
[JJ] EDP2, Rainin Instrument Co., Oakland, CA

Oxytetracycline quantification in plasma

The procedures / conditions for sample preparation and analysis have been detailed in Chapter 2. Blank swine plasma was used to construct the standard curve, which consisted of 6 non-zero points. The standards were fit to a quadratic curve across the range of concentrations (20 ng/mL [LLOQ] to 14,000 ng/mL). The run was accepted if the concentrations were within 15% of the expected concentration and the fit of the standard curve was at least 0.999. Accuracy and coefficient of variation for the quality control samples were ± 3.6% and <5%, respectively.

Oxytetracycline quantification in interstitial fluid

The procedures / conditions for sample preparation and analysis are as above with the exception of the matrix used. Interstitial fluid collected from the 3 control animals served as the matrix for the standard curve. The standard curve, constructed from 7 non-zero points, was quadratic across the range of concentrations (20 ng/mL (LLOQ) to 14,000 ng/mL). The run was accepted if the standard concentrations were within ± 15% of the expected concentration and the fit of the standard curve was at least 0.999. Accuracy and coefficient of variation for the quality control samples were ± 6.0% and <3%, respectively.

Oxytetracycline concentration in implant membranes

Following removal of the implant membranes, the nine straws were divided into 3 pools. The contents of each pool were placed into a centrifuge vial and spun at 5000 x g for 10 minutes. The supernatant was collected for oxytetracycline quantification and the pellet was collected for quantification of *Salmonella, E. coli,* and transconjugant populations. Thus, 3 replicates of bacterial and oxytetracycline quantification were performed per treated animal subject. The contents of the 3 implant membranes in the control subjects were not pooled and were used for bacterial quantification only. Implant membrane and interstitial fluid samples were analyzed

concurrently, utilizing the same standards constructed from blank porcine interstitial fluid. The accuracy and coefficient of variation for the quality control samples were ± 6.0% and <3%, respectively.

Protein binding

Protein binding was determined in porcine plasma by ultrafiltration / centrifugation. Triplicate aliquots (200 uL) of the low (20 ng/mL), medium (500 ng/mL) and high (14000 ng/mL) calibration solutions from the standard curve were pipetted into centrifugal filtration vials[KK]. The vials were centrifuged at 14,000 x g for 30 minutes. The preparation and quantification procedures were as detailed in Chapter 2.

The standard curve for protein binding in porcine plasma was fit with a quadratic equation consisting of 7 points across the range of concentrations. Accuracy was within ± 8.2% of expected concentrations for plasma. Protein binding was calculated by dividing the concentration of the filtered / centrifuged sample by the concentration in the unfiltered standard. The percent free drug was averaged for the 9 samples (3 replicates at 3 concentrations).

Protein binding was also determined in brain-heart infusion broth by ultrafiltration / centrifugation. Triplicate aliquots (200 uL) of the low (20 ng/mL), medium (500 ng/mL) and high (14000 ng/mL) calibration solutions from the standard curve were pipetted into centrifugal filtration vials[LL]. The vials were centrifuged at 14,000 x g for 30 minutes. The standard curve for protein binding estimate was fit with a quadratic equation consisting of 6 points across the range of concentrations. Accuracy was within ± 3% of expected concentration. Protein binding was calculated using equation 4 (Chapter 2).

[KK] Microcon YM-10, Millipore Corp., Bedford, MA
[LL] Microcon YM-10m, Millipore Corp., Bedford, MA

Bacterial quantification & determination of plasmid transfer

The procedures for bacterial quantification and calculation of transconjugation rates were as reported in Chapter 3. Briefly, contents of the *in situ* membranes were centrifuged at 5,000 x g for 10 minutes. The supernatant was collected and the pellet resuspended. An aliquot of the bacterial suspension was serially diluted in 10 fold increments. Plating the serial dilutions on selective media allowed for quantification of the donor and transconjugant populations. Data are reported as the transconjugant ratio (Equation 7, Chapter 3).

Study termination

Based on the *in vitro* experiments detailed in Chapter 3, the plasmid transfer rates were highest at the 12 hour time point. Implant membranes were removed after the subjects were euthanized by barbiturate[MM] overdose following the 12 hour plasma and interstitial fluid time points. The implant membranes were carefully dissected from their subcutaneous sites and placed in tubes of PBS for bacterial quantification (all subjects) and oxytetracycline analysis (treated subjects only).

Pharmacokinetic Analysis

Oxytetracycline concentrations in the plasma were corrected for protein binding. Free drug concentrations (plasma and ISF) were analyzed by noncompartmental analysis using commercial pharmacokinetic software. For the interstitial fluid samples, concentrations were analyzed as representing the midpoint of the two hour collection period.

[MM] Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI

Statistical Analysis

All statistical analyses were performed using a commercial statistical package[NN]. Using the generalized linear model procedure, analysis of variance was performed with treatment (IV, IM or control) as the independent variable and transconjugant ratio (logarithmically $_{(base\ 10)}$ transformed) as the dependent variable. The subject that received subcutaneous oxytetracycline was included in the intramuscular treatment group.

For the linear regression analysis, the transconjugant ratios (logarithmically $_{(base\ 10)}$ transformed) were regressed against 5 pharmacokinetic parameters; the unbound concentrations of oxytetracycline in the implant membranes, plasma observed maximal concentration (C_{max}), ISF observed C_{max}, area under the plasma concentration time curve ($AUC_{observed}$), and ISF $AUC_{observed}$. The null hypothesis to be rejected was that there is no correlation (regression slope = 0) between the transconjugant ratio and drug exposure parameters.

Results

Protein binding of oxytetracycline in swine plasma was linear across the concentration range of the standard curve. A mean free drug proportion of 42% across the concentration range of the standard curve was determined. This is similar to other published estimates of oxytetracycline plasma protein binding in swine[4]. Estimates of protein binding in the implant membranes were between 13-19%, therefore all concentrations were adjusted to free drug based on equation 4 (Chapter 2).

Oxytetracycline administration was perivascular in one subject in the intravenous treatment group. Pharmacokinetics from this animal confirmed this and the subject was analyzed

[NN] SAS 9.3.1, SAS Institute, Cary, NC

using an extravascular input model. See Table 6 for individual animal pharmacokinetics in plasma and ISF.

Free drug concentrations within the implant membranes were well correlated to oxytetracycline concentrations in the interstitial fluid at the last sampling interval for the parenteral administrations (R^2= 0.86, Figure 13). The correlation was negatively affected by inclusion of the IV treated animals due to higher concentrations in the implant membranes compared to ISF. (Figure 14).

There was no statistical difference in the transconjugant ratios between treatment groups in the analysis of variance (p=0.747). The linear regression analysis showed that none of the pharmacokinetic parameters in either plasma or ISF were significantly correlated to transconjugant ratio (Figures 16-19). All calculated measures of drug exposure in this model were poorly correlated to plasmid transfer within the implant membranes at 12 hours, as evidenced by p-values greater than 0.797 for all regression analyses.

Discussion

There is a growing body of research describing the correlation of results from *in vitro* pharmacodynamic models to *in vivo* studies. In a study by Aviles *et al.*[5], fungal response in the IVPM was well correlated (R^2 =0.995) to the response seen in an immunocompetent murine sepsis model when free drug concentrations were used. In a study by Den Hollander *et al.*[6], *in vitro* and *in vivo* dose fractionation studies were performed to determine the optimal PK/PD index for azithromycin. However, the authors noted some discrepancy in the bacterial responses between the two models used. In the IVPM, the single dose resulted in a significantly greater CFU reduction at 6 hrs than the fractionated doses. In the murine sepsis/peritonitis model, bacterial response was similar for both dosing regimens. However, survival was not well

correlated to bacterial counts *in vivo.* When the bacterial response *in vitro* and survival rates *in vivo* were used to determine the optimal PK/PD index for azithromycin, C_{max} was shown to be most predictive of antimicrobial efficacy in both models. The authors contributed the lack of correlation in bacterial responses to differences in growth rates between the two models.

Blaser, *et al.*[7] also demonstrated good correlation between the IVPM and a tissue cage model in guinea pigs for a multitude of antimicrobial-pathogen combinations. Greko, *et al.*, were able to correlate danofloxacin efficacy to AUC:MIC ratio in a tissue cage model in calves[8]. This agrees with other PK/PD literature for fluoroquinolones[9,10]. However, in this model the bacterial inoculum and antimicrobial were both administered directly into the tissue cage.

The results of the present *in situ* study are compared to previous results by the authors utilizing an *in vitro* pharmacodynamic model (IVPM)[11] incorporating the same bacterial isolates and antimicrobial. In the previous study, the rate of horizontal gene transfer between these two bacterial strains was suppressed when antimicrobial concentrations exceeded the MIC of the recipient bacteria. In the present study, the analysis of variance demonstrated no statistical difference in the transconjugant ratios between treated and control animals. In the regression analysis, the transconjugant ratio in the implant membranes at 12 hours was not correlated to any of the measures of drug exposure examined. Additionally, the y-intercept for the regression analyses of the treated animals was approximately -7.3. This is equivalent to the average log transconjugant ratio of the bacteria in the control subjects. In comparison, the previous *in vitro* study found log transconjugant ratios at 12 hours were -5.6 and -6.2 for the untreated controls. This discrepancy may be partially explained by the sample timing; the period from inoculation to treatment administration was slightly different in the two studies. The 12 hour time point *in situ* is the time from treatment administration. In the *in vitro* model, the bacterial populations were in

logarithmic growth prior to inoculation into the model, followed by a one hour growth period. In the *in situ* model, bacterial populations were prepared and placed in the implant membranes, then stored at 4º C overnight. The membranes were removed from refrigeration as the individual animal underwent the surgical procedure, followed by a 4 hour recovery period. Thus, the age of the bacterial populations between the two experiments were also different and may partly explain the results reported here.

Inherent *in vitro – in situ* differences may in part explain the results in plasmid transfer ratios. In the *in vitro* experiment, conditions (media, temperature and lack of immune response) are ideally suited to the bacteria. However, conditions are quite different *in vivo.* Although the *in situ* implant membrane should limit the influence of immunity through size exclusion of host factors, the nutrient influx from interstitial fluid into the bacterial broth may create a less optimal growth environment for plasmid transfer as compared to the constant influx of growth media in the *in vitro* model. This may be especially true for laboratory strains that may have lost virulence factors enabling infection in an animal host. If resistance transfer is limited to baseline levels *in situ* for these reasons, further suppression may not be evident in the present study design.

The membranes themselves may also be a limiting factor to transconjugation. The volume of the membrane limits the absolute number of bacteria that can be placed in close proximity for a contact related event (conjugation), and may therefore be a limiting factor for plasmid transfer.

Another potential explanation for the lack of correlation is difference in drug exposure. Oxytetracycline concentrations in the implant membranes were measured at only one time point (12 hour); however, all concentrations within the membrane exceeded the inhibitory

concentration of the recipient bacteria. Based on previous results, these concentrations should be sufficient to suppress plasmid transfer. Additionally, because the correlation between ISF oxytetracycline concentrations and implant membrane concentrations was good for parenteral treatment, the assumption, at least for this group of study subjects, is that implant membrane exposure would be similar to ISF concentrations.

Although previous experiments *in vitro* did not show diffusion to be a limiting factor (Appendix B), the poorer correlation for IV treated subjects may be a result of diffusional lag across the implant membrane. The peak plasma concentrations reported for the IV treated animals were similar to the *in vitro* concentrations of oxytetracycline in the study reported in the appendix; however, the concentration gradient in the present study would have been reduced more rapidly due to *in vivo* elimination compared to *in vitro* degradation in the study in the appendix. The reduced gradient *in vivo* could result in lower oxytetracycline concentrations in the implant membranes. Perhaps the correlation between drug exposure and horizontal gene transfer would be improved by using different measures of drug exposure. In the present study, only the traditional PK/PD measures were correlated to the transconjugant ratio.

A significant limitation to this study is the terminal sampling of the implant membranes. The 12 hour time point was chosen as the optimal sampling time based on *in vitro* work reported previously. If the time of maximal plasmid transfer occurs at a time other than 12 hours due to discussed differences *in situ,* then effects of drug exposure may not be evident with the current study design. Although actual transconjugant ratios *in vitro* and *in situ* were slightly different, it is important to note that at no time during either experiment were transconjugant ratios enhanced in the oxytetracycline exposed bacterial populations compared to respective controls.

The results of the present study provide an *in vitro – in situ* correlation for the effects of oxytetracycline exposure on transfer of antimicrobial resistance plasmids. Future studies are needed to address the limitations of the *in situ* model and to validate the preliminary findings of the present study.

Figures and Tables

Table 6: Pharmacokinetic parameters for individual animal subjects

Animal ID	Treatment	Plasma		ISF	
		*f*Cmax µg/mL	*f*AUC hr*µg/mL	*f*Cmax µg/mL	*f*AUC hr*µg/mL
31	Intramuscular	0.99	8.91	0.96	8.80
38	Intramuscular	0.75	5.36	0.79	6.64
33	Intramuscular	0.72	5.90	0.77	6.17
Mean ± SD	Intramuscular	0.82 ± 0.15	6.72 ± 1.91	0.84 ± 0.10	7.20 ± 1.40
37	Intravenous	3.32	13.19	1.81	13.75
32	Intravenous	5.17	13.72	2.57	16.56
Mean ± SD	Intravenous	4.25 ± 1.31	13.46 ± 0.37	2.19 ± 0.54	15.16 ± 1.99
35	Intravenous**	0.41	4.15	0.41	2.73

** Pig #35 – Administration was perivascular due to loss of aural catheter. Animal was modeled using a non-compartmental extravascular input model.

Figure 10: Correlation of Interstitial Fluid and Implant membrane oxytetracycline concentrations in IM/SC treated animals

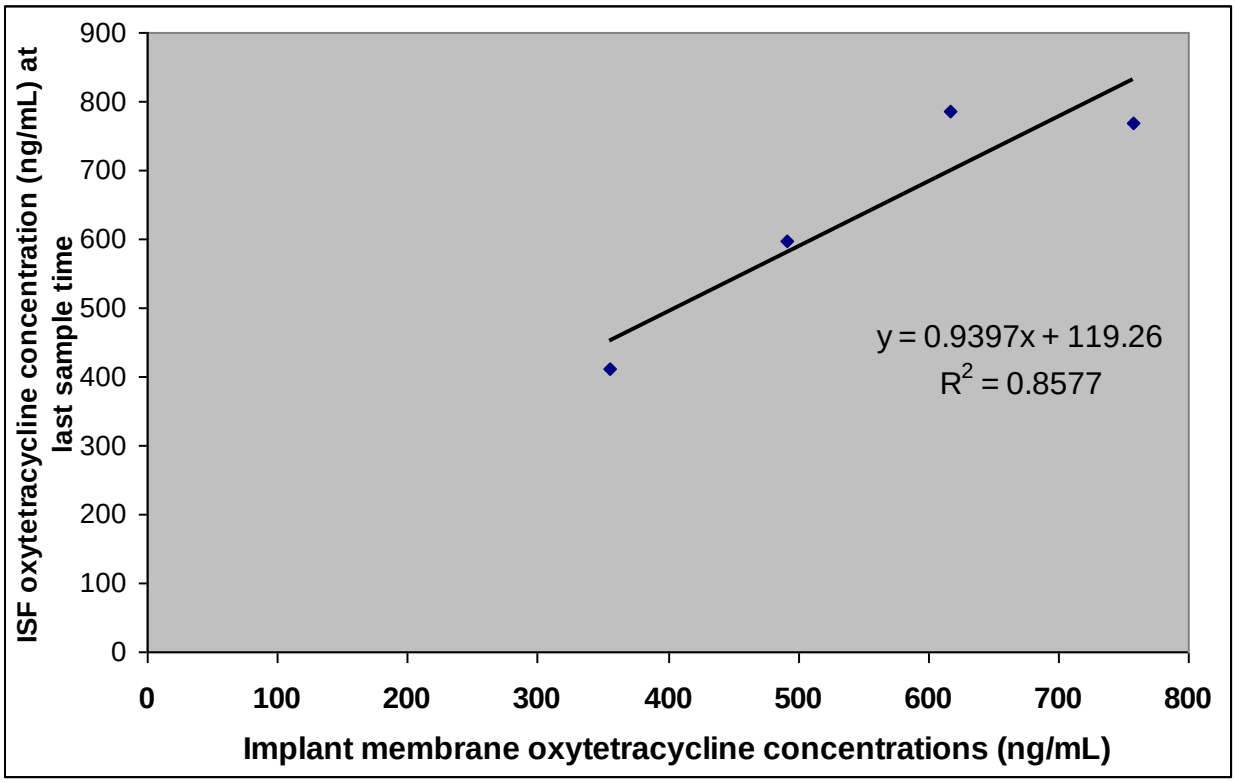


Figure 11: Correlation of Interstitial Fluid and Implant membrane oxytetracycline concentrations in all treated animals

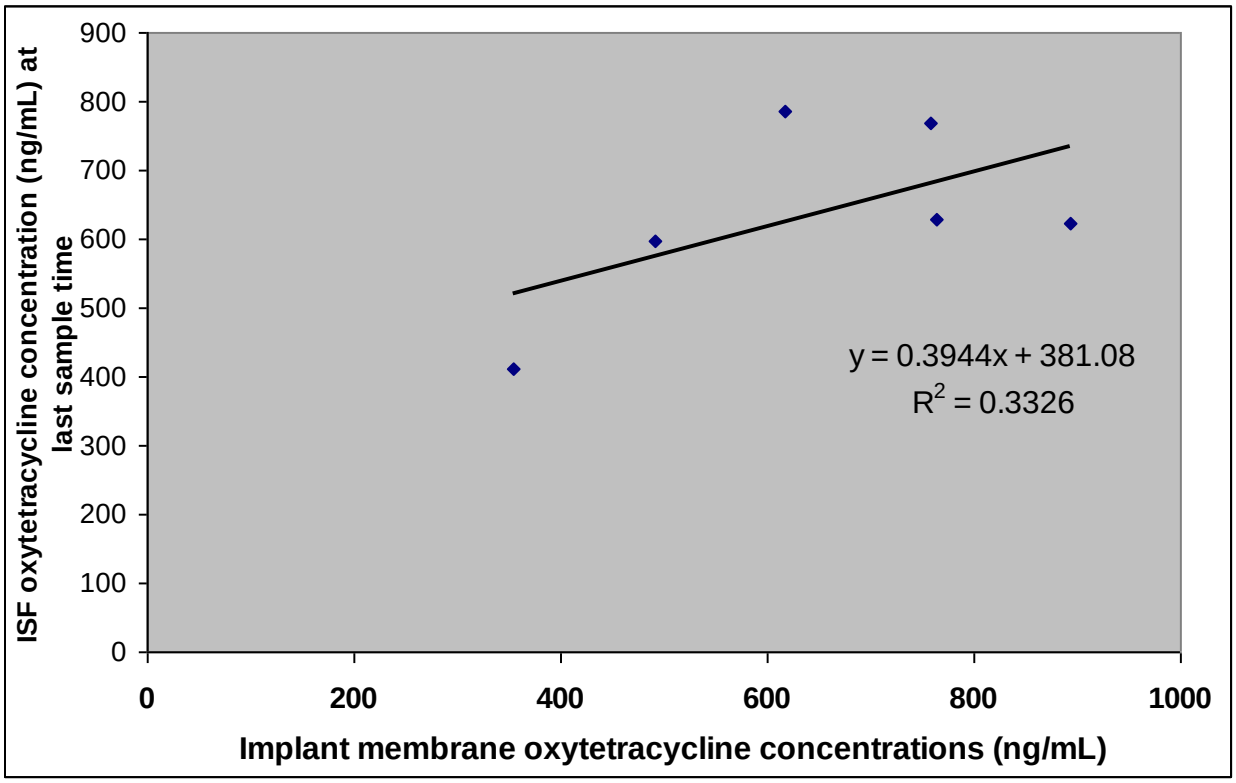
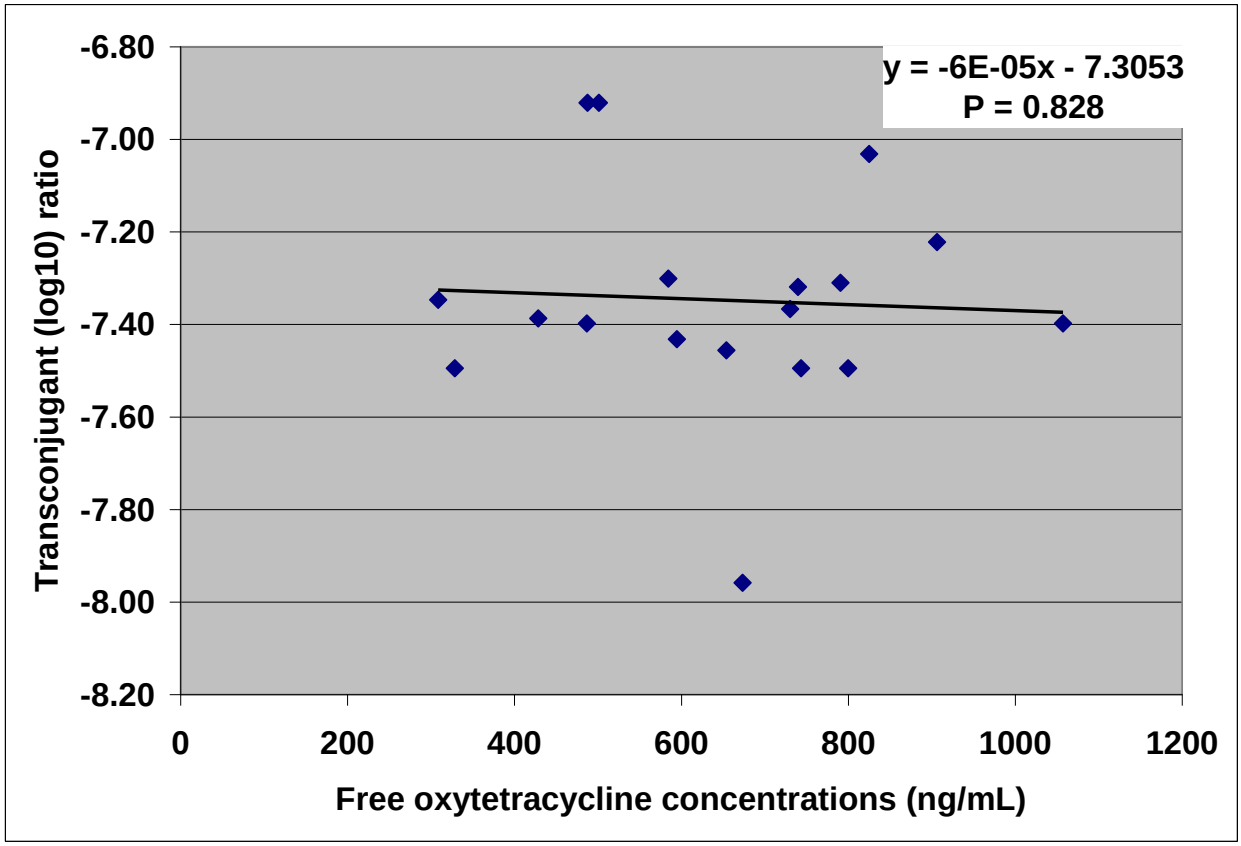
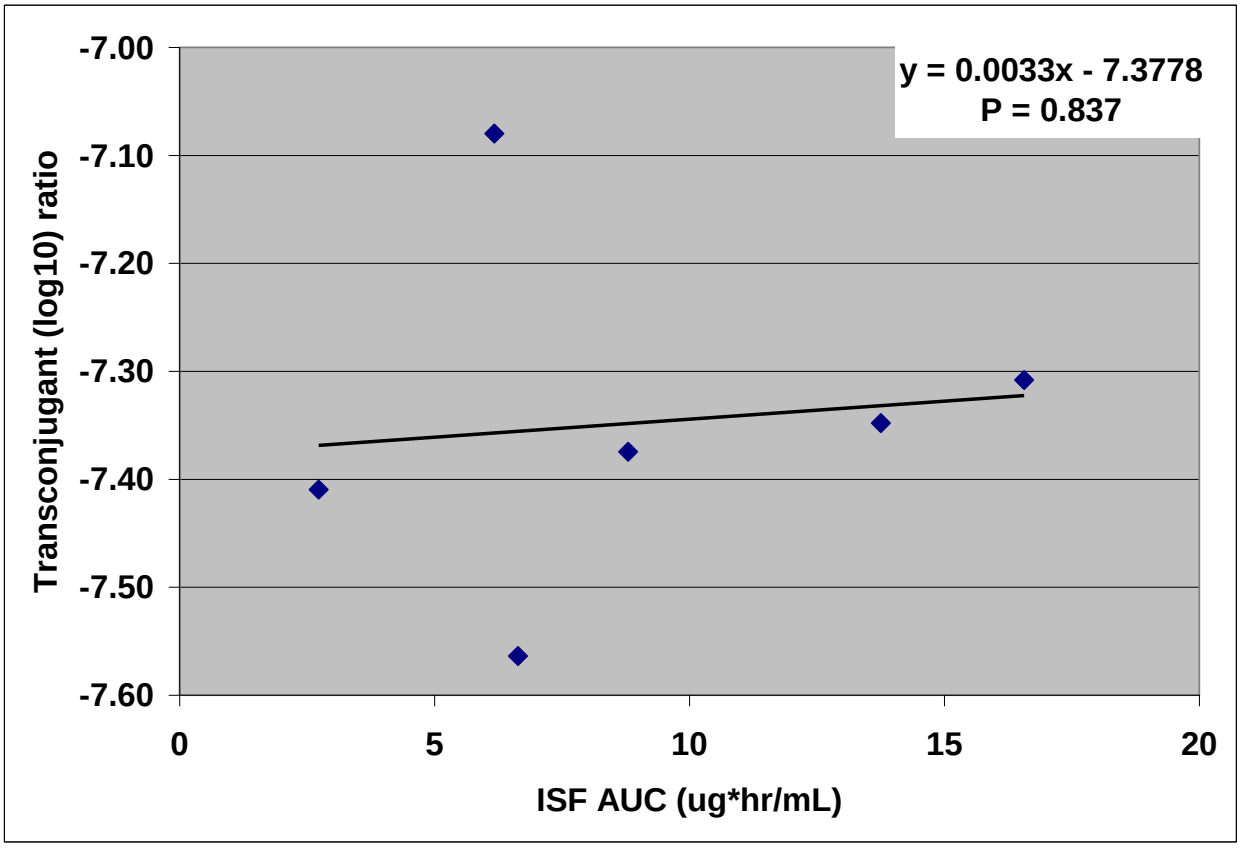


Figure 12: Correlation of free oxytetracycline in implant membranes to Transconjugant ratio (log_{10})



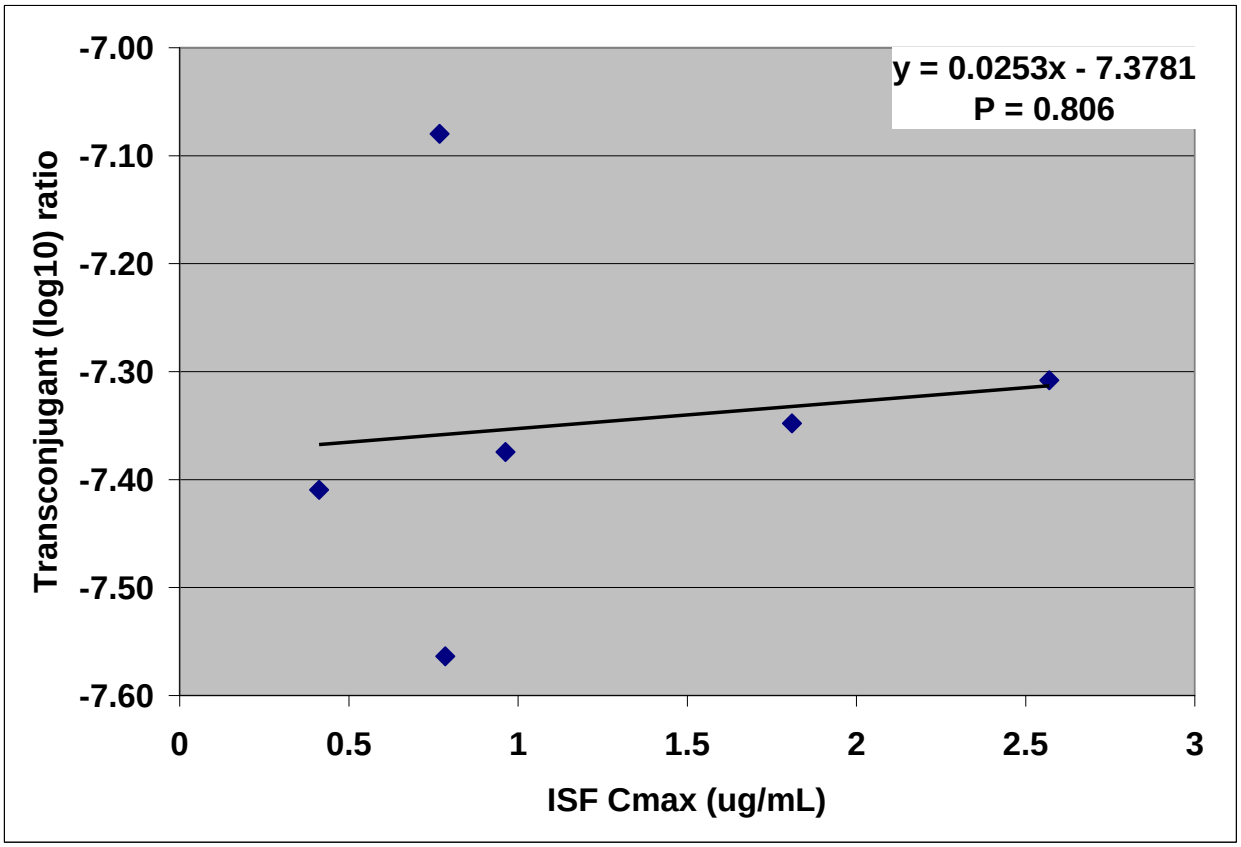
P value tests: H_o: slope = 0 [no correlation between independent and dependent variables]

Figure 13: Correlation of Interstitial fluid AUC to Transconjugant ratio (log_{10})



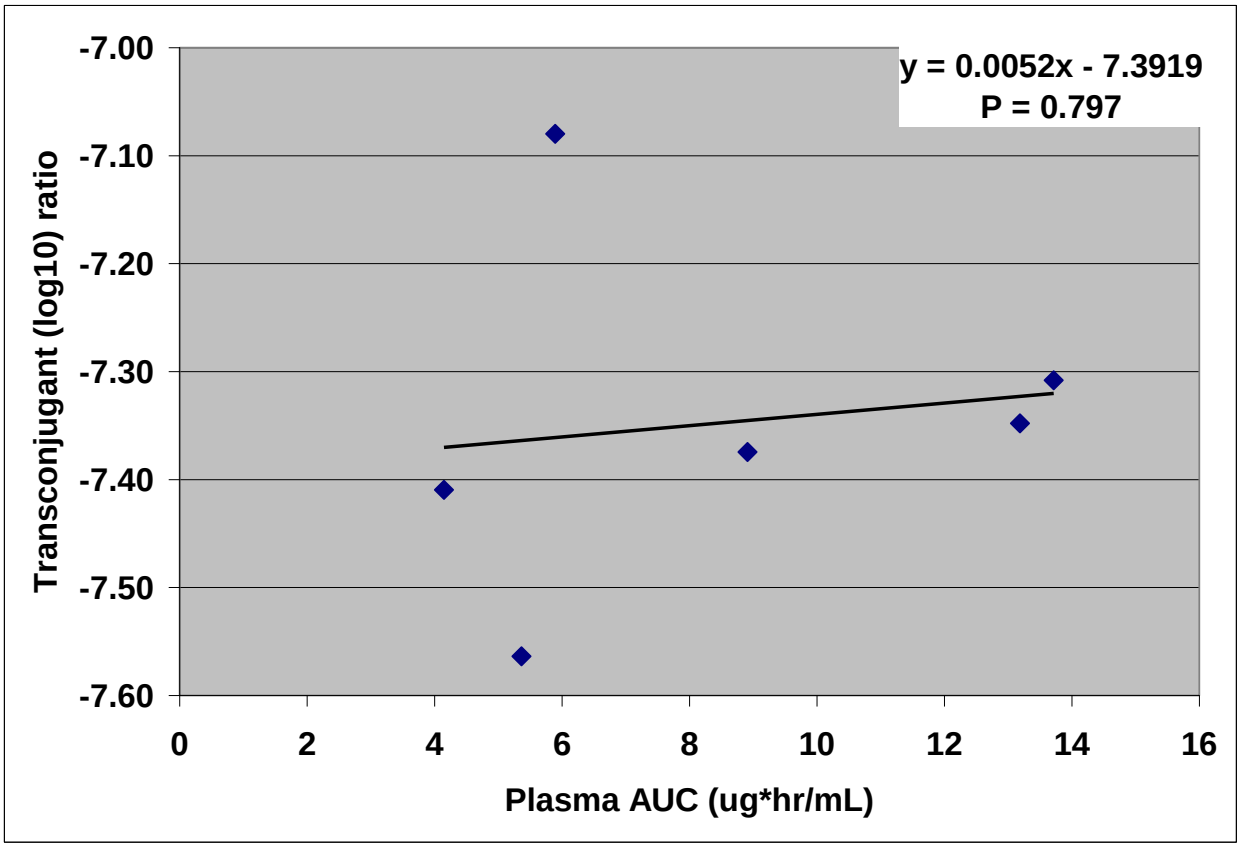
P value tests: H_o: slope = 0 [no correlation between independent and dependent variables]

Figure 14: Correlation of Interstitial fluid Cmax to Transconjugant ratio (log10)



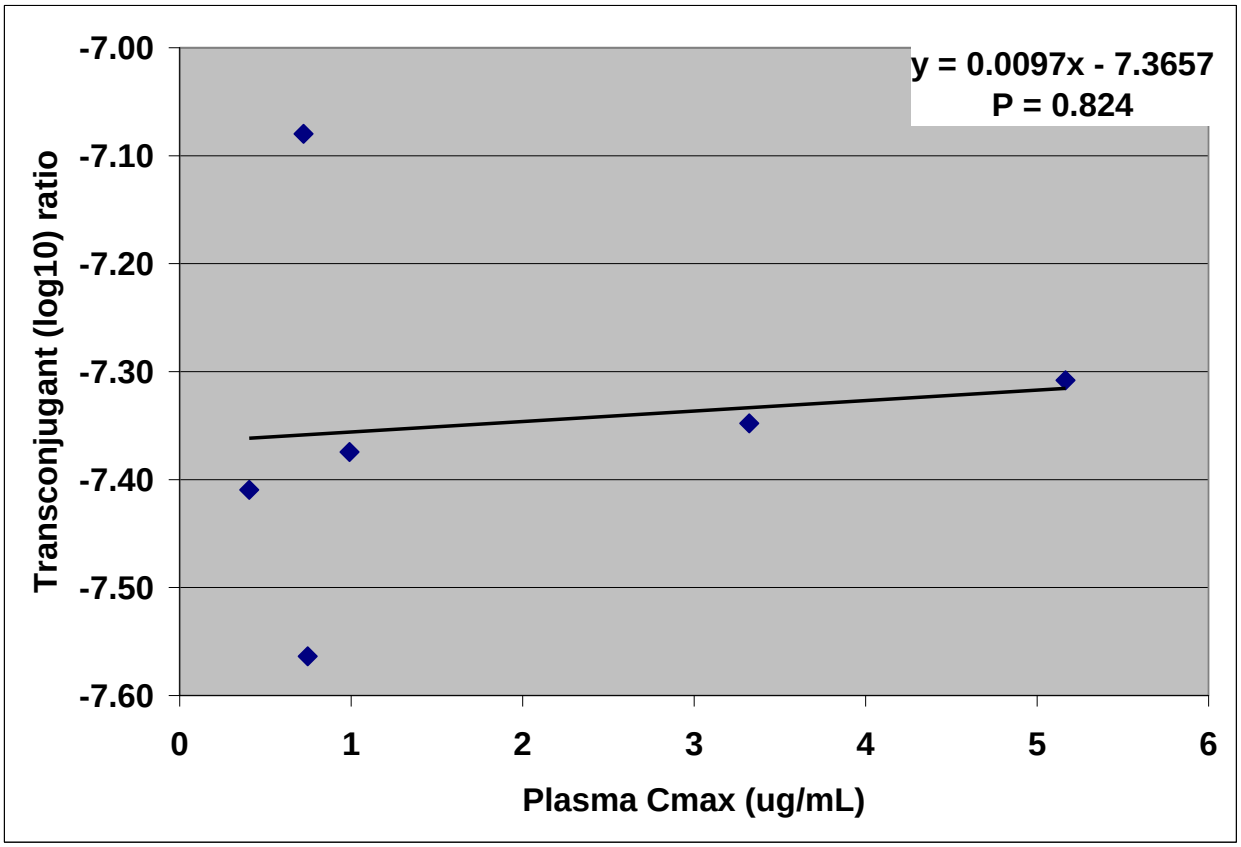
P value tests: H_o: slope = 0 [no correlation between independent and dependent variables]

Figure 15: Correlation of Plasma AUC to Transconjugant ratio (log10)



P value tests: H_o: slope = 0 [no correlation between independent and dependent variables]

Figure 16: Correlation of Plasma Cmax to Transconjugant ratio (log10)



P value tests: H_o: slope = 0 [no correlation between independent and dependent variables]

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Appendix A - Considerations for future studies

The primary focus of this research was the development and utilization of an *in vitro* pharmacodynamic model. During the model development stage, many lessons were learned concerning the nuances of *in vitro* pharmacodynamic models. Included here is a brief discussion of the encountered problems to serve as a guide for future work in this field of study.

Contamination of the *in vitro* Pharmacodynamic Model

Our initial experimental runs in the two compartment model suffered from contamination of the central and fresh media reservoirs. Four simple procedural changes reduced the contamination rates significantly. The central reservoir was autoclaved with the media in place. This decreases the number of media transfers to only the transfer of media into the fresh reservoir. Autoclaving the fresh media in place was considered but not implemented because the fresh media reservoirs were not compatible with extended autoclave times. Due to the large volumes of media being sterilized in each jar (2 L), autoclave time was increased from the recommended 15 minutes to 1 hour. System assembly was originally done in an open-faced biosafety hood, but was moved to a larger biosafety cabinet; decreasing the potential for contamination of the system due to exposure to the personnel assembling the setup. The larger size cabinet also allowed all components to be placed inside prior to assembly, thus preventing the breaks in sterile technique associated with the smaller biosafety hood. The final change that effected contamination reduction was implementing the use of aluminum foil “caps” on any exposed port prior to autoclaving. These caps could then be individually removed during assembly.

The transition to the one-compartment model also had an impact on the contamination of the assembled system. The one-compartment model consists of fewer components and thus results in fewer potential instances for contamination. Although no contaminant bacteria were noted during the plate counting, it is possible that low levels of contamination would be overcome by competitive exclusion due to the large inoculum of *P. multocida* (Chapter 2) and *E. coli* and *Salmonella* (Chapter 3).

Drug Loss

One of the primary limitations to the interpretation of data in Chapter 2 was the failure to achieve the desired pharmacokinetics in the *in vitro* pharmacodynamic model. The achieved targets were less than desired in all replicates. The following discussion addresses each of the contributing factors to undesired loss of oxytetracycline individually.

Drug Binding

Prior to utilizing the model described here, a capillary (two-compartment) *in vitro* pharmacodynamic model had been utilized by the author. The core components of the model were as for the one-compartment model with the addition of a hollow fiber (HF) capillary module[A]. The bacterial culture was inoculated into the extracapillary space of the HF module. Media from the central reservoir was circulated through the HF module by a peristaltic pump specifically designed for use with this module. Pharmacokinetic analysis of data obtained from this model setup showed an absorption phase, which was problematic due to the fact that the dosing simulation was for an intravenous bolus. We hypothesized that either the drug was not being circulated from the central reservoir rapidly enough or that oxytetracycline was binding to

[A] Medium 20 kD cartridge, Fibercell Systems, Fredrick, Maryland.

the polysulfone fibers and not diffusing into the extracapillary space. The circulating pump was set at its highest setting, therefore we attempted to circumvent the issue of circulation/diffusion by using higher concentrations of oxytetracycline in the central reservoir. After several attempts without successfully achieving the target pharmacokinetics, the more simple one-compartment model described here was adopted.

In the one-compartment model, potential issues with oxytetracycline binding continued. However, because of the simplicity of the model only two components contacted the central reservoir media, and only the glass reservoir itself and the stir rod were investigated. The experimental investigation into sources of drug loss is described below.

Drug Degradation

Another consideration for the failure to reach desired pharmacokinetic targets in the *in vitro* model is degradation. Three potential sources of degradation: enzymatic (media enzymes or bacterial enzymes), temperature, and ultraviolet radiation were considered.

A similar loss with respect to oxytetracycline was described by Bergan.[1] Under the conditions of the study (Mueller-Hinton broth (MHB) at 37° C), the estimated half-life of degradation was approximately 3 hrs. In 2008, Loftin *et al.*[2] studied the degradation rates of oxytetracycline and other antibiotics under various conditions of temperature, ionic strength and pH. Although the authors failed to state the starting concentration of oxytetracycline used, the estimated half-life of degradation at 35° C, pH = 7 was 19 hours (effects from changes in osmolality were insignificant).

In an experiment to test the stability of oxytetracycline in brain-heart infusion broth at 37° C, we found that oxytetracycline degrades with an approximate half-life of 23.8 hours[3]. This is quite similar to the findings of Loftin *et al.* described above and stability estimates (26 hrs

under similar conditions) reported in the drug monograph[4]. Differences in growth media and analytic methods could potentially explain the contrasting results of Bergan *et al.* In our experiment, brain-heart infusion broth (BHI) was used; Loftin *et al.* used an aqueous solution of sodium monohydrogen phosphate while Bergan used Mueller-Hinton broth. No enzymes would be expected in the aqueous solution, while there could be enzymes in the MHB that were not present in the BHI, resulting in a faster degradation. In both the present study and the Loftin study, an LC/MS method was used, while Bergan used a microbiological assay for quantifying drug concentration. Since the assay requires further incubation and oxytetracycline is heat-labile, then the extended time at higher temperature could result in falsely decreased concentrations and a shorter half-life. The implication of these findings is that oxytetracycline concentrations *in vitro* are NOT static under the conditions required for bacterial growth. This must be recognized when making comparisons to antimicrobials that are temperature/pH stable.

System Costs

In vitro pharmacokinetic model research can be very expensive to conduct. In addition to the basic equipment required (incubator, pumps, reservoirs, and tubing), there are significant disposable costs associated with each experimental run. Because the hollow fiber modules are by far the most expensive disposable item, transitioning to or starting with a one-compartment model will reduce operating expenses. There are two potential means of reducing system costs: reducing system size and reducing the number of control runs. If the size of the central reservoir is reduced, fresh media volumes will also be reduced for an identical half-life simulation. This was not implemented in our system as we had previously purchased pumps and reservoir caps sized for the two compartment pharmacodynamic model.

Reducing the number of control arms should be given serious consideration. If a traditional dose fractionation experiment is being performed with identical half-lives for both treatments, then one control arm is easily eliminated as the remaining control will suffice for both treatments. However, not utilizing at least one concurrent control arm may lead to questions regarding the inter-run variability in the bacterial populations.

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Appendix B - Implant membrane oxytetracycline diffusion

Experimental objective / hypothesis

A preliminary experiment was performed to assess issues relating to use of implant membranes within the *in vitro* pharmacodynamic model. If the implant membranes could be utilized within the IVPM, an additional variable could be eliminated from the *in vitro – in vivo* correlation. The research hypothesis was that oxytetracycline concentrations within the implant membranes were not limited by diffusion and/or drug binding.

Materials and Methods

Implant membranes were cut to approximately 75 mm segments. Thirty–six segments were filled with sterile BHI and heat sealed as previously described in Chapter 3. Porous cages were fabricated from 10 x 75 mm plastic test tubes. Three implant membrane segments were placed in each plastic cage and stored in sterile PBS at 4° C overnight.

Three reservoirs containing 500 mL of sterile brain–heart infusion broth were placed in the incubator. Four plastic cages containing the implant membrane segments were placed in each reservoir. Oxytetracycline base (1mg/mL) was added to each reservoir resulting in a starting concentration of 8.8 µg/mL. At 2, 4, 8, and 12 hours after the oxytetracycline was added, one cage was removed from each of the replicate reservoirs. The ends of the implant membrane were cut and the media collected in a 2 mL cryovial. All samples were stored at -80° C until analysis.

Samples were prepared by the solid phase extraction method, and analyzed by LC/MS/MS method described in Chapter 2 for oxytetracycline in brain-heart infusion broth. The

standard curve consisted of 6 non-zero points ranging from 20 ng/mL to 14000 ng/mL. The run was accepted if the concentrations of the standards were within 15% of the expected concentration and the fit of the curve was at least 0.99. Two low (350 ng/mL) and two medium (6000 ng/mL) quality controls were run; one low QC was < 20% of the expected value and was excluded. The accuracy and coefficient of variation of the remaining QC samples were ± 19% and ± 17%, respectively.

The results were analyzed statistically by timepoint using one-way ANOVA to test the hypothesis that implant membrane concentration was equal to media concentration at each time point.

Results and Discussion

One time point in the implant membranes (2 hr) was not analyzed due to insufficient sample volume. Although the other time points contained sufficient volume for analysis, the number of implant membranes needed to provide this volume was problematic given the size of the central reservoir used in the present study.

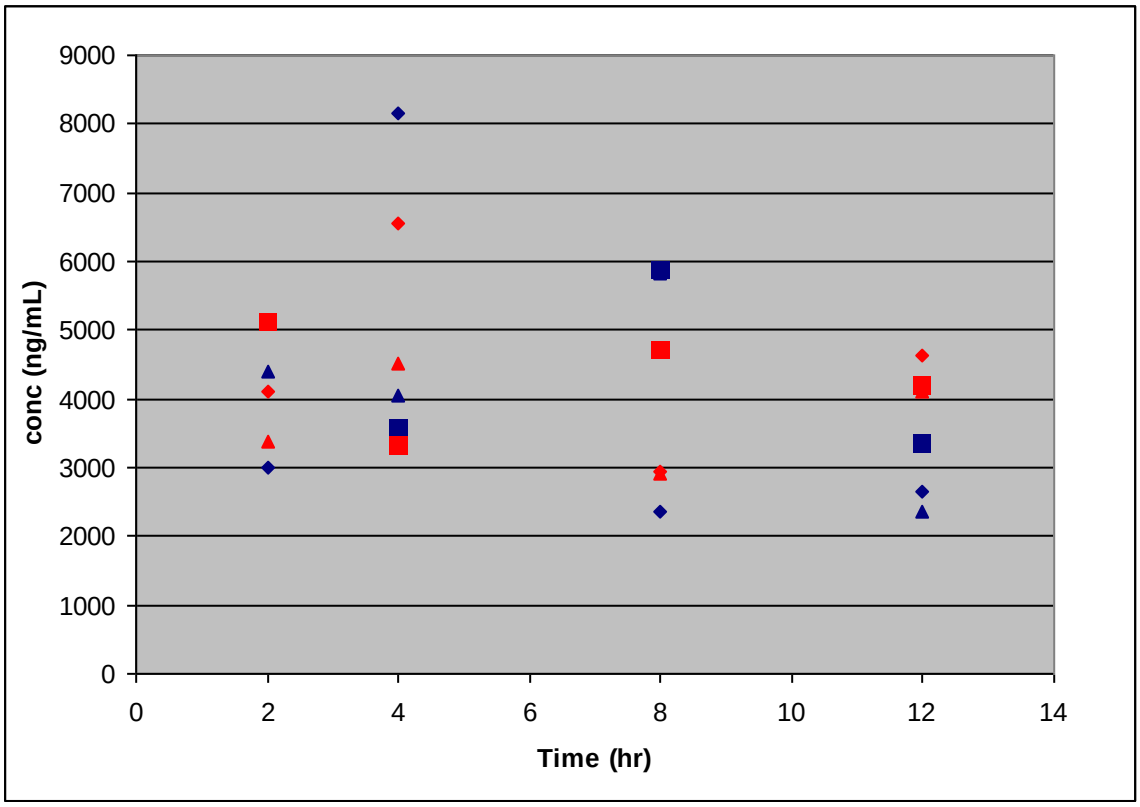
The comparison of media to implant membrane concentration is presented in Figure 16. Oxytetracycline concentrations were not statistically different between the media and implant membranes at 2, 4, and 8 hrs. Concentrations were significantly less in the implant membranes at 12 hours ($p=0.01$). If the disparity in concentrations in media and implant membranes were a diffusion related phenomenon, it would be expected that differences would be seen across time points as the average concentration was approximately 4000 ng/mL for the duration of the experiment. One diffusion related explanation for the discrepancy at 12 hours is that the implant membranes would float near the surface of the broth as the experiment progressed. If the membranes were not fully immersed in the media, surface area of the membrane would be

reduced and diffusion limited. This would be more pronounced at latter time points because the multiple cages present initially would have to be placed on end to fit within the IVPM, however as cages were removed remaining cages tended to float sideways.

Ultimately, the use of the implant membranes within the IVPM was abandoned due to the inability to maintain a sterile IVPM during repeated extraction of implant membranes from the central reservoir at multiple time points.

The use of a sepsis model *in vitro* (Chapter 3) and *in situ* model (Chapter 4) represents a variable that cannot be accounted for in this research. Because the effects of drug exposure on plasmid transfer were different in the two studies, this represents on area of improvement in future studies. The lack of agreement between these models should be investigated further before either model is pronounced superior.

Figure 17: Implant membrane and media concentrations in the *in vitro* pharmacodynamic model at 2, 4, 8 and 12 hours



** Same symbols represents paired (membrane – media) samples from the same replicate

** Implant membrane concentrations are blue

** Media concentrations are red

** One implant membrane sample at 2 hours was not analyzed due to insufficient sample volume