ANTIMICROBIAL RESISTANCE AND BOVINE RESPIRATORY DISEASE; A PHARMACOKINETIC/PHARMACODYNAMIC APPROACH TO MACROLIDE RESISTANCE

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Bovine respiratory disease (BRD) remains a major disease in beef production systems. The administration of antimicrobials for both the control and treatment of acute BRD is common. According to most published accounts, antimicrobial resistance among BRD pathogens is increasing; therefore, judicious antimicrobial usage is vital for continued efficacy. The introduction of a novel antimicrobial class has not occurred for well over a decade, therefore it is paramount to maximize efficacy of the antimicrobials currently available. The challenge is targeting the perfect scenario: maximizing clinical efficacy while minimizing antimicrobial resistance. The host-pathogen-drug interaction is very complex and despite current sophisticated technology, this interaction is still not well understood for many infectious diseases.

This dissertation work sought to investigate the effects of the administration of a macrolide for both control and treatment of acute BRD on the prevalence of resistance among isolated *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni.* Whole genome sequencing of *M. haemolytica* was utilized to investigate the presence/absence of macrolide resistance genes and their relationship to the observed minimum inhibitory concentration. Cattle were sampled (plasma and pulmonary epithelial lining fluid) after administration of gamithromycin for drug concentration analysis. A non-linear mixed effects approach was used to fit a compartmental model to the resulting sparse pharmacokinetic data so that a complete time concentration curve could be simulated. From these curves, the C_{MAX} and AUC were measured and used to calculate standard PKPD indices using the MIC values of the isolated bacteria.

Clear associations between the use of gamithromycin for control and treatment of BRD and a statistically significantly increased likelihood of macrolide resistance were not found, possibly due to sample size limitations. The calculation of pharmacokinetic-pharmacodynamic

indices found that a longer drug exposure was more closely associated with a successful treatment outcome, but there was not a statistically significant correlation. However, there were few clinical failures in this study giving further credence to the complexity of the *in vivo* system. There are many factors beyond pharmacokinetics/pharmacodynamics and MICs that contribute to the success of a treatment regimen for cattle suffering from BRD.

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Dedication

This dissertation is dedicated to my family, for without them my success would most certainly not be possible. To my wife Sarah for her unending support and tireless effort in helping me to succeed professionally and personally. To my children (Kerrick, Kason, and Kolden) that inspire me to be a better person each and every day. To my parents to whom I owe so much, in particular, my work ethic and desire to achieve. To my wife's parents for which many late night and early mornings would have not been possible.

Chapter 1 - A review of the expected effects of antimicrobials in bovine respiratory disease treatment and control using outcomes from published randomized clinical trials with negative controls

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KEYWORDS

Antimicrobial drugs, Antibiotic susceptibility, Antibiotic resistance, Bovine respiratory disease, Number needed to treat, Randomized clinical trial, Evidence based medicine

Abstract

Bovine respiratory disease (BRD) remains a major disease both from an economic and an animal welfare standpoint in beef production systems. Antimicrobial administration is a mainstay in both the control of and in the therapeutic treatment of acute BRD. However, the pipeline of novel antimicrobial classes has remained dry for well over a decade. Therefore, the judicious use of antimicrobials in both human and animal health remains paramount to ensure efficacy of treatment remains acceptable. In order to judiciously prescribe antimicrobials the clinician needs to have an understanding of reasonable expectations for antimicrobial efficacy in the treatment and control of clinical disease. As such, a systemic review was conducted in the scientific literature including the Freedom of Information Summaries of New Animal Drug Approvals on the FDA website. The objective of this article was to present a cumulative review of the data from published randomized clinical trials utilizing a negative control in the treatment and control of BRD using the number needed to treat as a means to effectively convey this information to the bovine practitioner.

Key Points

- The randomized clinical trial (RCT) is the gold standard for efficacy determination
- RCTs with negative (no treatment) controls are not clouded by a control group treatment effect
- Absolute risk reduction (ARR) is the difference in the probabilities of an event in the control and treatment groups and is estimated as the corresponding difference in the event rates
- Number needed to treat (NNT) is the reciprocal of the ARR
- NNT is more clinically intuitive as it describes the effect in terms of the number of patients a clinician needs to treat to expect a given (typically positive) outcome

Outline

- **Introduction**
- **External validity of clinical trials**
- **Ancillary therapy use in BRD**
- **Antimicrobial use in BRD**
- **Material and Methods**

    - **Search Strategy and Inclusion Criteria**
    - **Article Exclusions**
    - **Data Extraction**
- **Results**
    - **The Effects of Antimicrobials**
    - **Number Needed to Treat**
- **Discussion**

Introduction

Bovine respiratory disease (BRD) is a multi-factorial disease that has been well described by many researchers as a complex or syndrome involving an interaction of stressors, viruses, and bacteria. Despite decades of dedicated research, BRD remains a major disease in all types of beef and dairy production systems with an estimated global economic impact in excess of $3 billion/year.[1] Antimicrobial administration is a mainstay in both the control of disease in populations at high risk of BRD and in the therapeutic treatment of acute clinical disease. However, the pipeline of novel antimicrobial classes for the therapy of BRD has remained dry since the introduction of enrofloxacin (Baytril 100®, Bayer Animal Health, Shawnee Mission, KS) in 1998. Therefore, the judicious use of antimicrobials in both human and animal health remains paramount to ensure efficacy of treatment remains acceptable.

The objective of this chapter is to evaluate the use of antimicrobials for the therapy of bovine respiratory disease through the lens of a cumulative review of published randomized clinical trials investigating the effects of an antimicrobial drug for treatment or control of BRD against a negative control. The number needed to treat NNT will be used to describe these trials.[2] There are numerous ways to express the value of an active treatment over that of its control group, such as odds ratios and risk reduction. Number needed to treat is the reciprocal of the absolute risk reduction which is the difference in the probabilities of an event in the control and treatment groups, and is estimated as the corresponding difference in these event rates. The NNT statistic

has the one major advantage of being more straightforward to readers less versed in thinking of events (clinical outcome) in terms of probabilities. As such, NNT is much easier interpreted by the practicing clinician and speaks in terms of number of treatments needed to make a difference in one patient. The use of NNT, by expressing the effect of the drug in relation to disease recovery of negative controls over the same period, also incorporates the severity of the disease challenge into the estimate of drug effect. Therefore, the use of the NNT value must be carefully relegated to the disease, regimen, animal species, and the specific disease challenge.

Ancillary Therapy Use in BRD

A systematic review was conducted and published in 2012 by Francoz et al. on the use of ancillary drugs in the treatment of BRD.[3] While finding very little reliable and consistent data, they did conclude that there were not enough data at that time to recommend the use of any ancillary therapy alongside antimicrobials in the treatment of BRD. Using the same search criteria as performed in that study we were unable to identify any recent publications of relevance to expanding that conversation, therefore interested readers should consult that publication. Likewise, no clinical trial data addressing the use of more than one antimicrobial at a time vs. a single antimicrobial for the therapy of BRD was found. The focus of this review is therefore limited to the use of antimicrobials alone in the treatment of BRD.

Antimicrobial use in BRD

The prospective, masked, randomized clinical trial conducted in naturally occurring disease is the gold standard for the evaluation of efficacy for disease intervention in both human and veterinary medical clinical research. In regard to naturally occurring BRD studies of antimicrobial efficacy, the literature can be divided into those utilizing a negative control and those comparing the test article to a positive control. When using a positive control treatment, the goal of the experiment

is to prove either superiority or non-inferiority, either of which require quite different study designs. The lack of a significant difference in treatment outcomes in a trial which was not adequately designed to demonstrate non-inferiority cannot necessarily be interpreted as equivalence of the treatments. Analysis and interpretation of trials with positive controls provides many challenges and is beyond the scope of this article. An excellent meta-analysis was recently performed in this area and readers wishing for that scope should refer to the article by O'Connor et al.[4] However, a brief comparison of their approach and objectives to those of this report will be offered in the following paragraph.

O'Connor et al. used a mixed treatment comparison meta-analysis to compare the efficacy of antimicrobial treatments for BRD.[5] In addition to including trials utilizing a negative control, their inclusion criteria and methods of analysis allowed the inclusion of study designs utilizing a positive control as a treatment arm in their analysis. As such the number of article inclusions was much larger than those included in this report. In Figure 1, reproduced with permissions, a visual network of the treatment arms compared in their meta-analysis can be appreciated. Their publication offered comparisons of 60 trials of active drug to negative controls (including FOIs) and 33 comparisons of active to active (one antimicrobial to another) controls. By means of their type of meta-analysis method they were able to rank antimicrobial treatments of BRD by efficacy (Figure 2). Using their rankings, it can be seen that with the published data available in the literature, tulathromycin ranks as the most efficacious treatment for BRD and the older molecules such as the ceftiofur formulations, trimethoprim and oxytetracycline are among the least efficacious antimicrobial treatments for BRD. However, as pointed out by O'Connor et al. one limitation not unique to their meta-analysis, is that the analysis is limited to those data publically available. If more privately held trial data were offered, better estimates might be possible.

In this chapter, we have not attempted to include those publications with a positive control as we are not attempting to compare efficacy between treatments, instead we wish to eludicate the actual effect gained from the use of an antimicrobial in therapy or control of BRD. As such, a simplified interpretation of results is possible when the control group is administered either no treatment or a sham such as a saline injection, since comparisons to the control group are not clouded by an actual treatment effect. It is in this type of research where the true effect of the antimicrobial, in that population of cattle, can be discerned. A certain level of spontaneous recovery is expected, either due to misdiagnosis, a fully competent and successful immune response, or other factors that are unidentifiable. Therefore, comparing treated to untreated controls using the NNT allows an easily interpreted measurement of the antimicrobial effect in a specific clinical situation that can be readily comprehended by veterinary practitioners and their clients alike.

Deciding what data drives decisions

A systematic review of the literature was performed using the online resources PubMed and Web of Knowledge in August of 2014 with similar search terms as outlined previously.[4] Studies were limited to those published in English and originating from North America. Criteria for inclusion in this review were that the publications must have been investigations into the treatment or control of BRD with an antimicrobial in a randomized, blinded, negative control field trial study design where subjective evaluators were masked and the disease was naturally occurring. Trials had to involve the administration of a single antimicrobial, with no ancillary therapy, either for treatment of naturally occurring BRD or in the control of naturally occurring BRD. Studies on the treatment of naturally occurring BRD must have involved animals that were not treated for control of BRD with an antimicrobial prior to or as part of the study. All trials involving positive controls or experimental challenge models were excluded. Additionally,

publications were screened to ensure that they were not duplicative of FOI data, as was the case on many occasions.

Additionally, the Freedom of Information (FOI) New Animal Drug Approvals (NADA) summaries were searched on the FDA website at the same time. Inclusions from the FOI summaries include efficacy trials (ET), dose response studies (DRS), dose selection studies (DSS), single location field studies (FS), and multi-location field studies (MLFS). The only arms of dose selection studies reported here are those which used current labeled dosages in the trial.

Article Exclusions

Notably, articles from Hibbard et al. and Hamm et al., which have been included in other review articles on antimicrobial treatment of BRD, were not included in this analysis as they were both found to be publications of data from their NADA FOI summaries.[6,7] Additionally, in the Hibbard et al. article the third trial was not included in the control analysis because the allocation to each treatment group was not presented. Percentages were given in tables of the trial outcome but did not allow the calculation of confidence intervals. An article from Messersmith et al. was not included because there was no mention of blinding the evaluators and in the article they actually mention tagging animals in accordance with their treatment which would suggest that evaluating investigators were aware of treatment during evaluation.[8]

Data Extraction

Articles were organized by those investigations of BRD treatment efficacy or those reporting on efficacy of treatment for control of BRD. Outcome measures of treatment efficacy papers were treatment failures and mortality, those measurements in the control papers were morbidity. A summary of the individual animal treatment studies included in this report are included in Table 1. All NADA approval studies are listed by pharmaceutical company currently holding rights to

the drug. The published journal articles are listed by the name of the company as it was when the study was supported and published. Table 2 includes the publications providing data for the control/prevention portion of the analysis.

General information from FOI summaries and study reports were gathered including authors, publication source, pharmaceutical sponsorship, treatment regimen, number of animals per treatment group, and treatment outcomes.

The Effect of Antimicrobials in Treating and Controlling BRD

The box plots in Figures 1, 2, and 3 represent a grouping of all the studies in regard to the trial outcomes of those animals receiving antimicrobials versus those receiving no treatment. A median spontaneous recovery rate of 24% was found across all trials in the control group versus a 71% recovery rate of those treated with active ingredient for the therapeutic trials (Figure 1). This indicates an increase of 47% recovery rate with the use of antimicrobials as compared to a negative control. The median mortality rates by treatment group are 1% and 17% in treated and negative controls, respectively (Figure 2). The impact of treatment for control of BRD with an approved antimicrobial in cattle at high risk for developing disease can be seen in Figure 3. Treatment for control of BRD decreased the incidence of morbidity by more than half in these populations of cattle (22% in treated cattle versus 48% incidence in control cattle).

Number Needed to Treat

Using the extracted data, a spread sheet was constructed in Microsoft Excel (2013) with the information in Tables 1 and 2 along with the outcomes of each individual trial. Absolute risk reduction, number needed to treat and their respective 95% confidence intervals were calculated with the use of a spreadsheet calculator developed utilizing the Newcombe-Wilson method without continuity correction.[9] The outputted ARR and confidence intervals were inputted into JMP 11.0.0

(SAS Institute, Cary, NC) for the creation of forest plots. Next, an additional axis for NNT was manually added to each forest plot as described previously by Altman.[10]

Those trials that have confidence intervals crossing the null axis (dashed line at 0 and ∞) display insignificant results. Meaning, that it cannot be stated with 95% confidence that the use of that drug, in that group of cattle, had a positive effect compared to treatment with saline sham injection. It should be pointed out that each of the antimicrobials reported with insignificant confidence intervals in this report were shown to have substantial evidence of clinical efficacy by different statistical tests of significance, as can be found in each of their respective FOIs. Confidence interval estimation for the comparison of two proportions is not without its drawbacks, including the creation of intervals that do not make logical sense, termed aberrations, and an achieved confidence level that can be quite different than the intended 1-α.[9] However, the method used in this paper performs well with large sample sizes and is less affected by unequal sample sizes as compared to many other methods.[11]

As discussed by Altman, trials resulting in ARR lower confidence intervals that are negative present a problem when presenting data as the NNT. The inverse of a very small negative number is another larger negative number which is completely illogical, clinically. As an example, the reciprocal of the ARR 95% lower confidence interval of Ceftiofur sodium, 1.1 mg/kg, intramuscularly daily for 3 days (DSR1) in Figure 4 is -438, which by definition means that -438 animals would need to be treated to have a positive outcome in one case. While this makes little clinical sense, it also presents a problem when graphing these data. The solution is to think of this in terms of that the drug has no effect or an infinite number of animals could be treated without seeing a positive outcome due to the antimicrobial, as can be seen graphically in Figures 4, 5, and 6.

The overwhelming majority of trials show a positive effect on case outcome in the therapy and control of BRD. The median NNT in therapeutic trials involving negative controls is 2. Therefore, for every 2 animals treated for BRD in the overall population of these studies, one case was a treatment success, seen in the last row of Figure 4 (labeled Median). The median NNT for preventing one mortality due to BRD in the trials reviewed is 6; for every 6 animals treated, therapeutically, 1 BRD death is prevented displayed in Figure 5. In Figure 6, for BRD control studies, the median number of animals that need to be treated to prevent one acute case of BRD is 5.

External validity of the included trials

The vast majority of the clinical trial data used in this review were generated in what would be classified as "high risk" calves where significant morbidity is expected due to comingling and accumulated stress, plus the lack of optimal vaccination or nutritional protocols in many cases. Therefore, application of these NNT values to low risk calves, yearling cattle, or cows would be inappropriate other than as a very general indication of potential effects.

Other key determinants of external validity are the study entrance and success/failure criteria. These are summarized for the referenced sources of clinical trial data in Table 3. It is apparent that the case definitions for both study entrance and success/failure vary by study. We would also expect interpretation of the criteria to vary by investigator. In the opinion of the second author, the case definitions for success required to achieve concurrence with the FDA Center for Veterinary Medicine on pivotal clinical trial protocols are likely to result in a lower success rate than would criteria commonly applied in commercial settings. While this may still result in valid testing when applied to all treatments, there is the potential for being overly conservative when estimating the effect of these antimicrobials in a commercial setting.

Discussion

The objective of this article was to explore the effectiveness of antimicrobials in the therapy and control of BRD as compared to no treatment. Extreme caution should be taken in directly comparing the NNT of one antimicrobial to another due to differences in trial design, sample size discrepancies, risk classification differences of BRD between trials, resultant spontaneous recovery rates, and potential differences in case definition and success/failure outcome between trials. However, in the author's opinion since a bulk of the data come from FOI summaries a majority of the variables (population risk status, case and success/failure definitions) are very similar and provide a much better means of comparison than meta-analysis of trials utilizing positive treatment controls.

The presentation of the data in this manner also makes for a succinct way of defining reasonable expectations of efficacy in the treatment and control of BRD in a field setting for cattle at high risk of BRD. It should be pointed out once again that the clinician interpreting these data needs to bear in mind the external validity of these studies when applying it to the type of cattle, environmental setting and typical BRD pathogens encountered in their practice.

Figures and Tables

Author	Citation	Antimicrobial	Date	Pharm Sponsor	Study Length (days)	Tot al N	Mortality Reported
NADA 140-338	[12]	Ceftiofur Sodium	Approved 1988	Zoetis	28	84	Yes
NADA 140-338	[12]	Ceftiofur Sodium	Approved 1988	Zoetis	28	88	Yes
NADA 140-338	[12]	Ceftiofur Sodium	Approved 1988	Zoetis	28	88	Yes
NADA 140-338	[12]	Ceftiofur Sodium	Approved 1988	Zoetis	28	88	Yes
NADA 140-338	[12]	Ceftiofur Sodium	Approved 1988	Zoetis	28	88	Yes
NADA 140-338	[12]	Ceftiofur Sodium	Approved 1988	Zoetis	28	405	Yes
NADA 140-338	[12]	Ceftiofur Sodium	Approved 1988	Zoetis	28	405	Yes
NADA 141-063	[13]	Florfenicol	Approved 1996	Merck	28	50	Yes
NADA 141-063	[13]	Florfenicol	Approved 1996	Merck	15	95	Yes
NADA 141-063	[14]	Florfenicol	Approved 1998	Merck	12	75	Yes
NADA 141-063	[14]	Florfenicol	Approved 1998	Merck	12	75	Yes
NADA 141-063	[14]	Florfenicol	Approved 1998	Merck	11	150	Yes
NADA 141-063	[14]	Florfenicol	Approved 1998	Merck	11	150	Yes
NADA 141-209	[15]	Ceftiofur CFA	Approved 2003	Zoetis	14	108	No
NADA 141-068	[16]	Enrofloxacin	Approved 1996	Bayer	15	24	Yes
NADA 141-068	[16]	Enrofloxacin	Approved 1998	Bayer	15	24	Yes
NADA 141-068	[16]	Enrofloxacin	Approved 1996	Bayer	28	445	Yes
NADA 141-068	[16]	Enrofloxacin	Approved 1998	Bayer	28	145	Yes
NADA 141-068	[16]	Enrofloxacin	Approved 1996	Bayer	28	456	Yes
NADA 141-068	[16]	Enrofloxacin	Approved 1998	Bayer	28	152	Yes

NADA 141-207	[17]	Danofloxacin	Approved 2002	Zoetis	10	238	Yes
NADA 141-244	[18]	Tulathromycin	Approved 2005	Zoetis	14	474	Yes
NADA 141-328	[19]	Gamithromycin	Approved 2011	Merial	10	497	No
NADA 141-328	[19]	Gamithromycin	Approved 2012	Merial	10	242	No
NADA 141-328	[19]	Gamithromycin	Approved 2012	Merial	10	260	No
NADA 141-334	[20]	Tildipirosin	Approved 2012	Merck	14	600	Yes
NADA 141-207	[21]	Danofloxacin	Approved 2011	Zoetis	10	240	No
NADA 141-265	[22]	Florfenicol	Approved 2008	Merck	11	244	No
Kilgore et al.	[23]	Tulathromycin	Published 2005	Pfizer	14	480	Yes
Kilgore et al.	[23]	Tilmicosin	Published 2005	Pfizer	14	480	Yes
Booker et al.	[24]	Florfenicol	Published 1997	Schering-Plough	45	125	Yes

Table 1.1- Outline of publications presented for analysis in the therapy of bovine respiratory disease in cattle from studies in North America. Pharmaceutical sponsors are listed by name as it exists today for each of the New Animal Drug Application (NADA) studies. Where applicable, pharmaceutical companies are listed as they were named when sponsoring the independently published research studies.

Author	Citation	Antimicrobial	Date	Pharma Sponsor	Study Length (days)	Total N
NADA 141-209	[15]	Ceftiofur CFA	Approved 2003	Zoetis	29	1504
NADA 141-244	[18]	Tulathromycin	Approved 2005	Zoetis	14	799
NADA 141-063	[14]	Florfenicol	Approved 1998	Merck	28	399
NADA 141-063	[14]	Florfenicol	Approved 1998	Merck	28	198
NADA 141-143	[25]	Oxytetracycline	Approved 2003	Norbrook	30	1199
NADA 141-143	[25]	Oxytetracycline	Approved 2003	Norbrook	30	1200
NADA 140-929	[26]	Tilmicosin	Approved 1996	Elanco	28	707
NADA 141-328	[19]	Gamithromycin	Approved 2011	Merial	10	159
NADA 141-328	[19]	Gamithromycin	Approved 2011	Merial	10	308
NADA 141-334	[20]	Tildipirosin	Approved 2012	Merck	14	773
Kilgore et al.	[27]	Tulathromycin	Published 2005	Pfizer	14	819
Kilgore et al.	[27]	Tilmicosin	Published 2005	Pfizer	14	817
Galyean et al.	[28]	Tilmicosin	Published 1995	none	28	57
Galyean et al.	[28]	Tilmicosin	Published 1995	none	28	116
Galyean et al.	[28]	Tilmicosin	Published 1995	none	56	121
Vogel et al.	[5]	Tilmicosin	Published 1998	Elanco	28	1096
Johnson et al.	[29]	Ceftiofur CFA	Published 2008	Pfizer	69-83	1045
Hendrick et al.	[30]	Oxytetracycline	Published 2013	Merck	24-49	3784

Table 1.2 - Outline of publications presented for analysis in the control/prevention of bovine respiratory disease in cattle from studies in North America. Pharmaceutical sponsors are listed by name as it exists today for each of the New Animal Drug Application (NADA) studies. Where applicable, pharmaceutical companies are listed as they were named when sponsoring the independently published research studies.

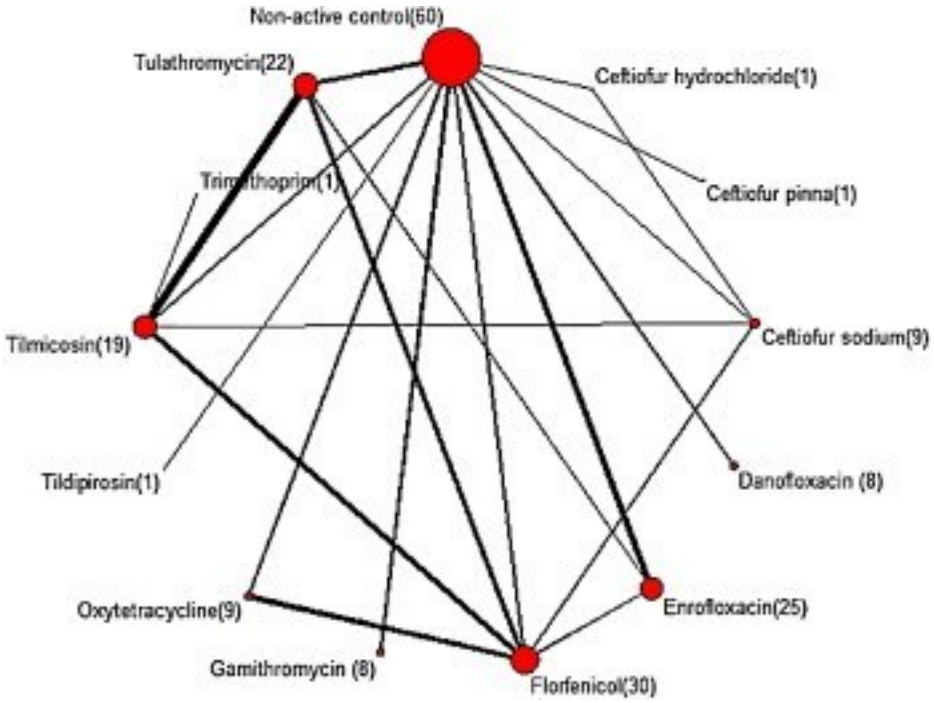

Figure 1.1 The network of treatment arms used in O'Connor et al. mixed treatment comparisons met-analysis. The size of the dot is a relative indicator of the number of arms and the width of the lines is a relative indicator of the number of direct comparisons (number of arms). Reproduced with permission from Elsevier.

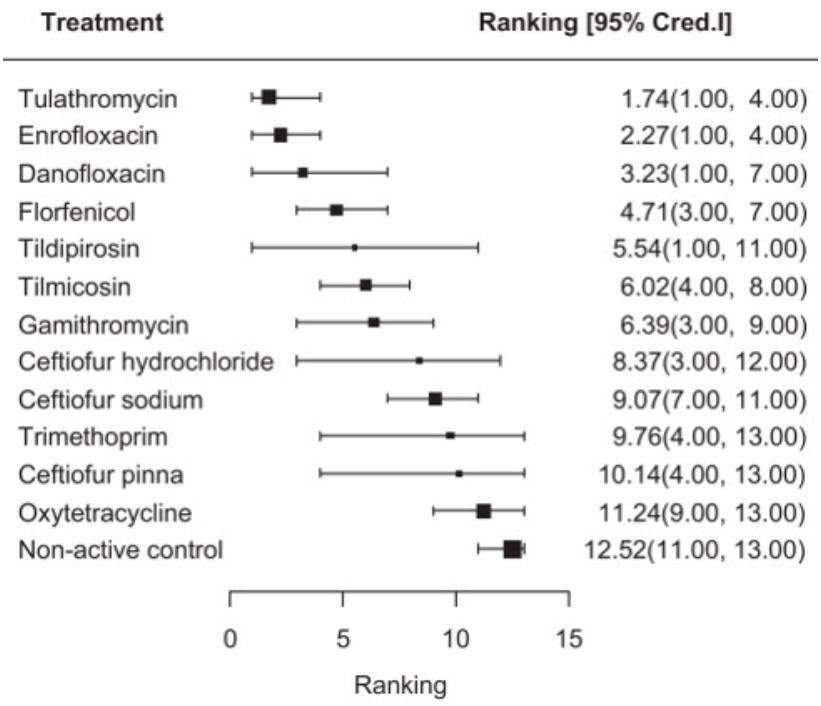

Figure 1.2– Ranking forest plot for treatment arms in O'Connor et al. mixed treatment comparison meta-analysis of antibiotic protocols for BRD (mean rank and 95% credibility interval). Reproduced with permission from Elsevier.

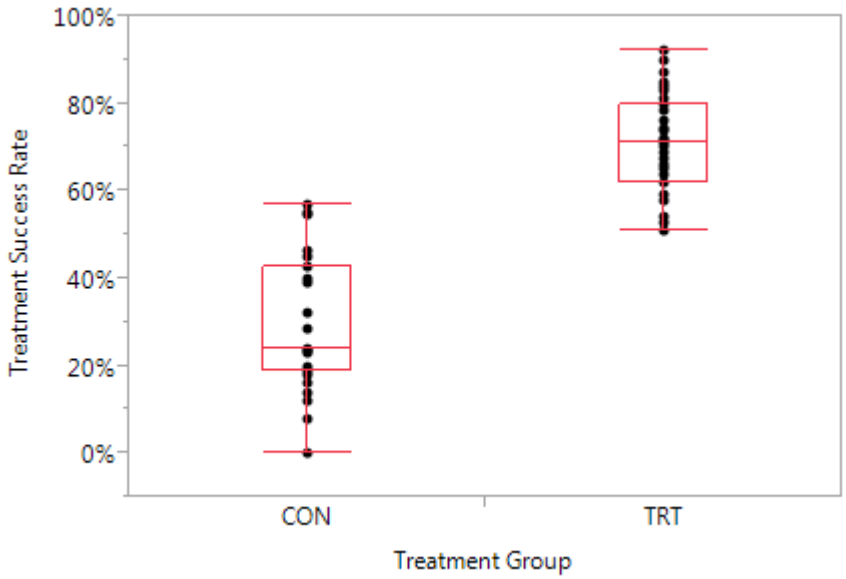

Figure 1.3 - Treatment success rate in all trials included in the therapeutic portion of the analysis. Control (CON) animals received either no treatment or sham-saline injection, treated (TRT) animals received an antimicrobial for the treatment of acute bovine respiratory disease.

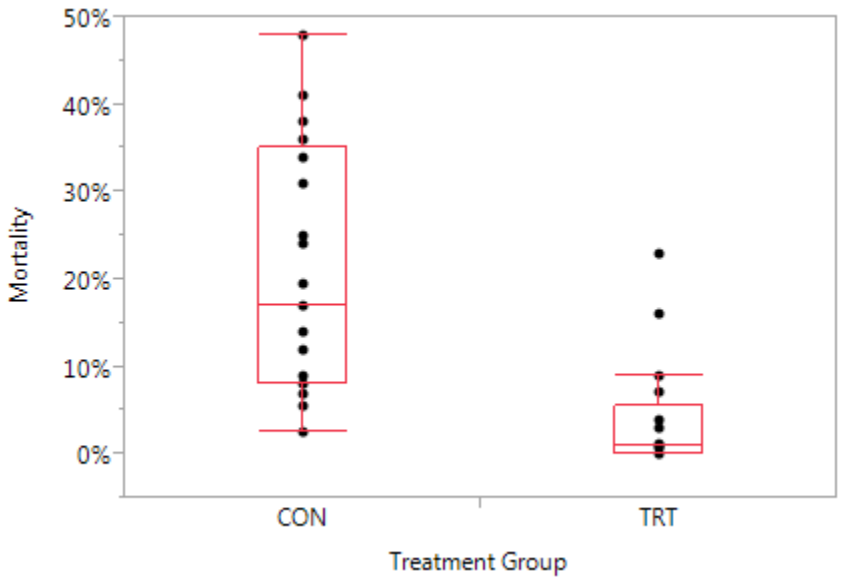

Figure 1.4 - Mortality incidence rates in all trials included in the therapeutic portion of the analysis. Control (CON) animals received either no treatment or sham-saline injection, treated (TRT) animals received an antimicrobial for the treatment of acute bovine respiratory disease.

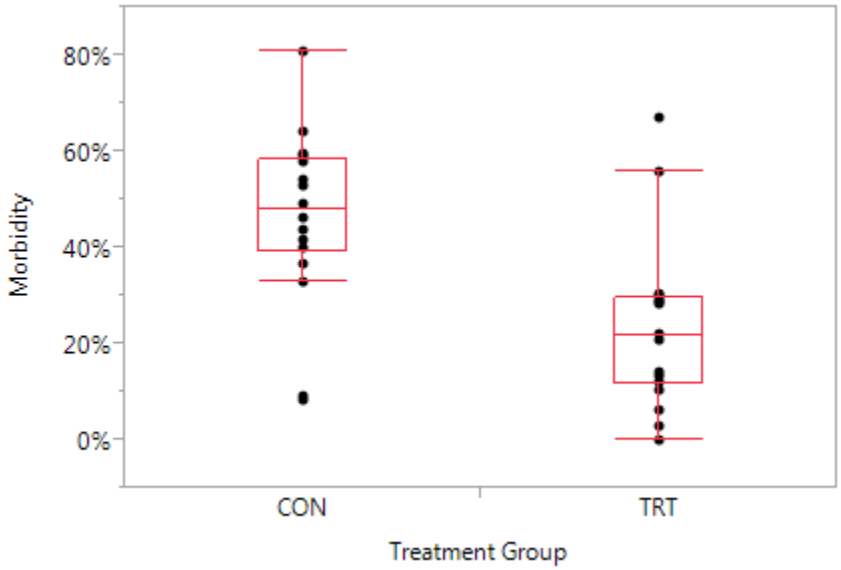

Figure 1.5 - Morbidity incidence rates in all trials included in the mass medication portion of the analysis. Control (CON) animals received either no treatment or sham-saline injection, treated (TRT) animals received an antimicrobial for the prevention/control of bovine respiratory disease.

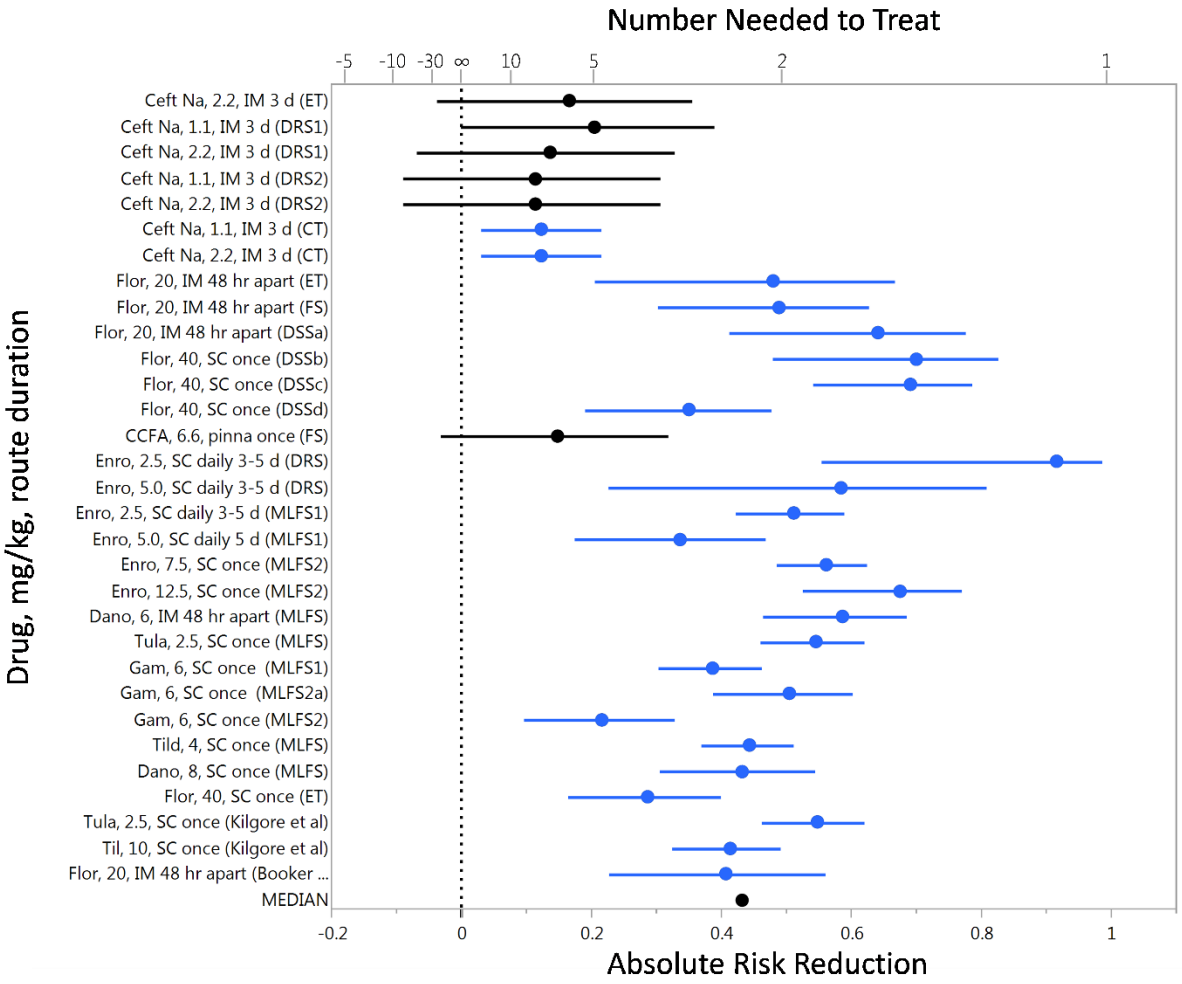

Figure 1.6 - Forest plot of point estimates and 95% confidence intervals of the Absolute Risk Reduction of morbidity (bottom x-axis) and the corresponding Number Needed to Treat (top x-axis) found from analysis of the therapeutic studies. Studies listed on the y-axis correspond in order to those listed in Table 1 and are listed by active ingredient, dose (mg/kg), route of administration and duration of therapy. (ET = efficacy trial, DRS = dose response study, CT = clinical Trial, FS = field study, DSS = dose selection study, MLFS = multi-location field study)

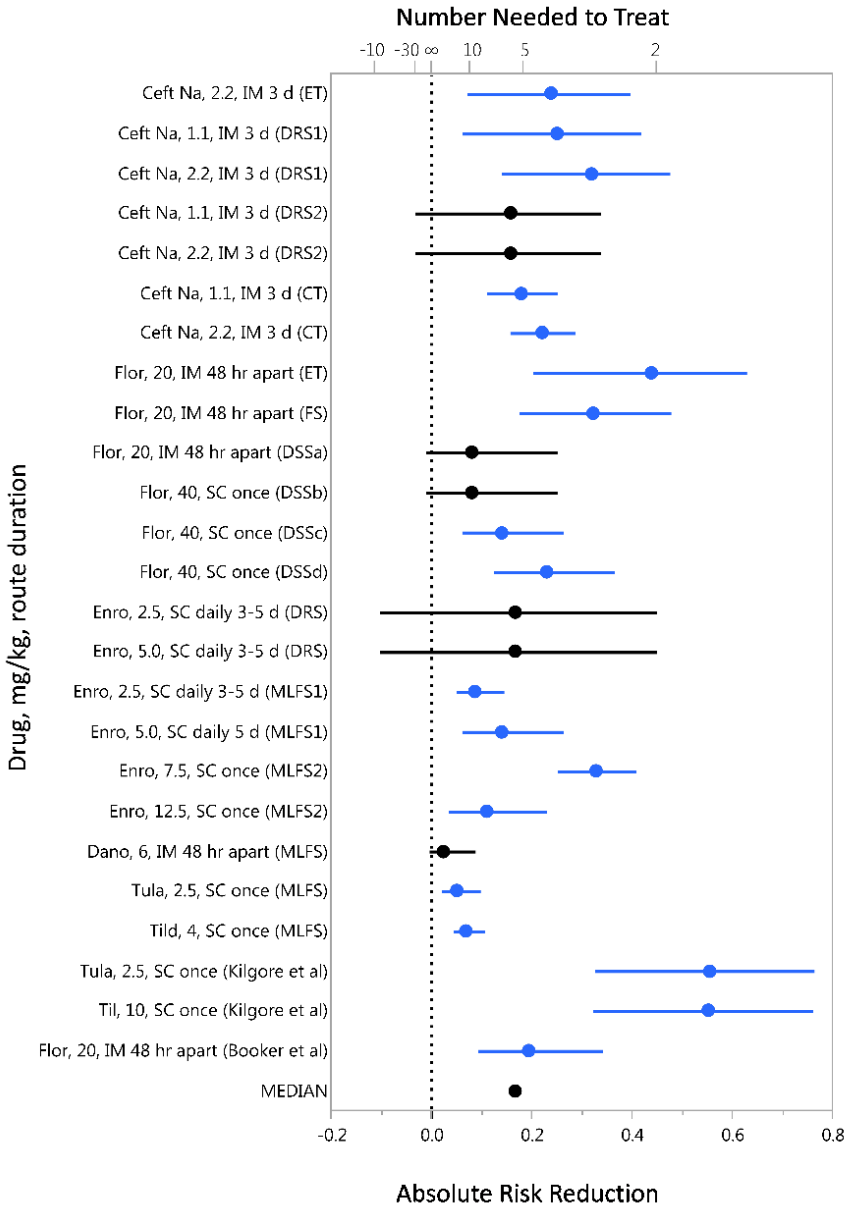

Figure 1.7- Forest plot of point estimates and 95% confidence intervals of the Absolute Risk Reduction of mortality (bottom x-axis) and the corresponding Number Needed to Treat (top x-axis) found from analysis of the therapeutic studies. Studies listed on the y-axis correspond in order to those listed in Table 1 and are listed by active ingredient, dose (mg/kg), route of administration and duration of therapy. (ET = efficacy trial, DRS = dose response study, CT = clinical Trial, FS = field study, DSS = dose selection study, MLFS = multi-location field study)

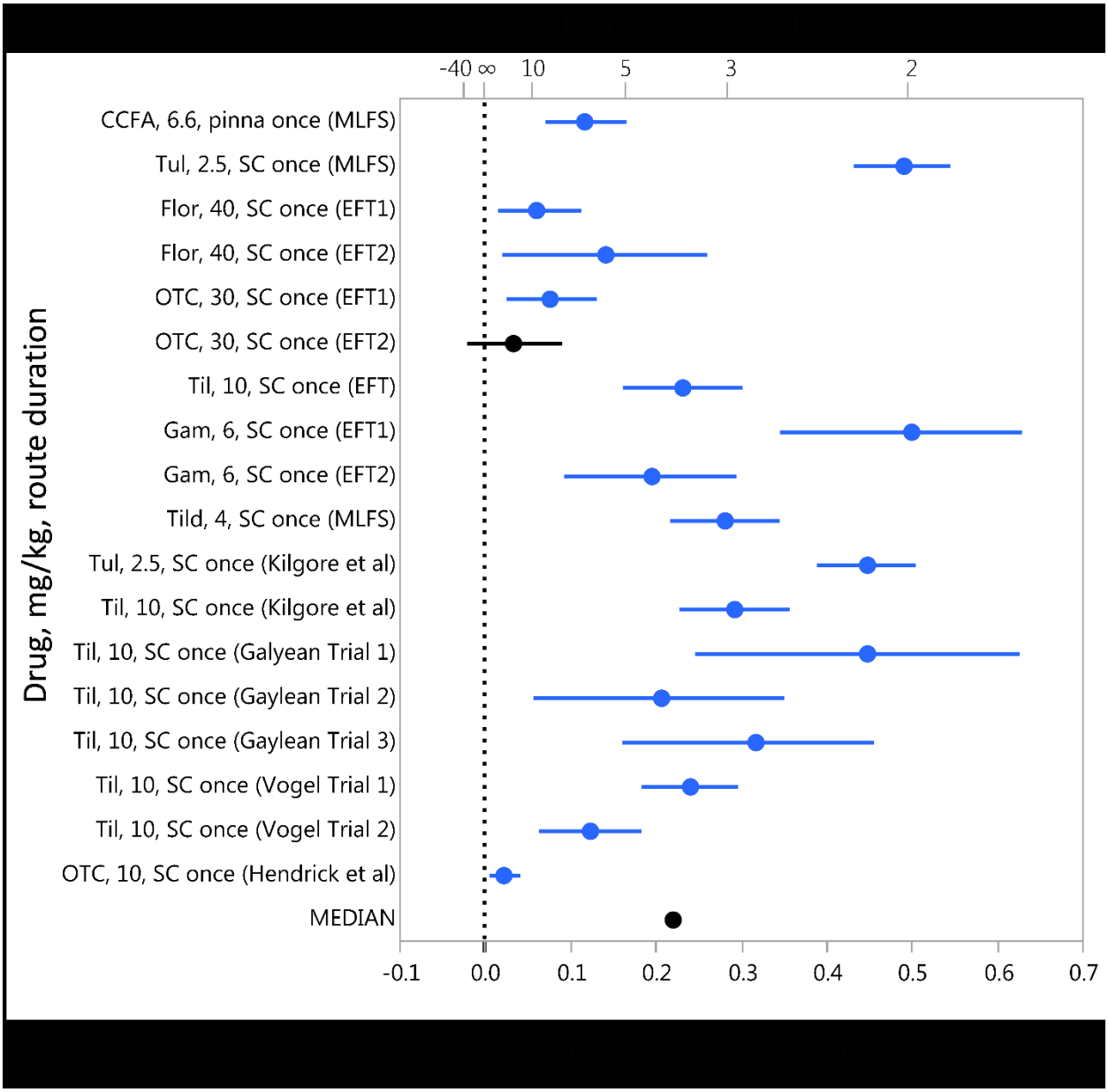

Figure 1.8 - Forest plot of point estimates and 95% confidence intervals of the Absolute Risk Reduction (bottom x-axis) and the corresponding Number Needed to Treat (top x-axis) found from analysis of the control studies. Studies listed on the y-axis correspond in order to those listed in Table 1 and are listed by active ingredient, dose (mg/kg), route of administration and duration of therapy. (ET = efficacy trial, DRS = dose response study, CT = clinical Trial, FS = field study, DSS = dose selection study, MLFS = multi-location field study)

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Chapter 2 - A Literature Review of Antimicrobial Resistance in Pathogens Associated with Bovine Respiratory Disease

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Abstract

The objective of this manuscript was to perform a critical review of the literature as it pertains to the current status of antimicrobial resistance in pathogens associated with bovine respiratory disease (BRD) in beef cattle and to provide a concise yet informative narrative on the most relevant publications available. As such the scientific literature contained in PubMed, AGRICOLA, and CAB were searched in February of 2014 for articles related to susceptibility testing of *Mannheimia haemolytica, Pasteurella multocida* and *Histophilus somni* from cases of BRD. Titles and abstracts were read and 105 articles that were relevant to the subject of BRD antibiotic resistance were attained for further review. After the application of exclusion criterion (publications must have originated from North America, be in English, adhere to standards set forth by the CLSI, and be concerning antimicrobial resistance in BRD in beef cattle), 16 articles remained and are the focus of this publication. Due to the disparate data from the few studies that investigate susceptibility testing of BRD pathogens, a quantitative assessment or meta-analysis was not performed on the studies presented in this review. However, considering diagnostic lab data, there appears to be a clear trend of a decrease in susceptibility of the three major BRD pathogens to the antimicrobials used commonly for treatment and control of BRD. Studies performing sensitivity testing on healthy cattle report much lower resistance, but it remains unclear if this is because of a true lack of resistance mechanisms, or if the isolates do contain quiescent genes for resistance that are only phenotypically expressed following the administration of an antimicrobial for either treatment or control of bovine respiratory disease. Future research to address this question of genotype and phenotypic expression before and after antimicrobial administration will further advance our knowledge in this area.

Introduction

Bovine respiratory disease (BRD) is a multi-factorial disease complex involving an interaction of stressors (weaning, passage through auction markets, commingling, shipping, etc.), viral infections (bovine viral diarrhea virus, bovine respiratory syncytial virus, para-infulenza-3 virus, bovine herpes virus-1), and bacteria (*Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, Mycoplasma bovis*). Global economic impact of BRD is estimated to be >$3 billion/year(Watts and Sweeney, 2010). Antimicrobial administration is a mainstay of both prevention/control of disease and treatment of clinical disease. Table 1 contains a non-exhaustive list of antimicrobial products currently licensed in the United States (U.S.) for the treatment of BRD. Widespread bacterial pathogen resistance to antimicrobials commonly used for BRD is a very real concern shared by producers, practitioners, and the animal health industry. In the U.S. there are no routine surveillance programs to monitor antimicrobial resistance among BRD pathogens, however; independent and industry sponsored research can be found reporting surveillance-type data from different areas of the world.

Observations from the medical field on antimicrobial resistance were first reported by medical practitioners as early as the late 1940s to early 1950s(Glisan et al., 1982). The first documented case of multiple drug resistance transfer by conjugation was reported in 1959 by Akiba and Ochiai in Japan(Watanabe, 1967). Specific to BRD, the first reports of multiple drug resistance in *P. multocida* and *M. haemolytica* (formerly *Pasteurella haemolytica*) were published by Chang and Carter in 1976(Chang and Carter, 1976).

The first publications of research into the mechanisms of antimicrobial resistance are from the early 1960s. Similar publications specific for pathogens of BRD did not begin appearing in the literature until the early 1980s. Resistance genes have been found and described for the common

bacterial pathogens associated with BRD for the tetracyclines,(O'Connor et al., 2010, D'Amours et al., 2011, Kehrenberg et al., 2005, Klima et al., 2011, Singer et al., 1998, Michael et al., 2012b) fluoroquinolones,(Pardon et al., 2013, Michael et al., 2012b) beta-lactams,(Michael et al., 2012b, Klima et al., 2011, Alexander et al., 2013a) macrolides, (Desmolaize et al., 2011a, Desmolaize et al., 2011c, Kadlec et al., 2011, Klima et al., 2011, Michael et al., 2012b) sulfonamides,(Michael et al., 2012b) lincosamides,(Desmolaize et al., 2011c, Kadlec et al., 2011, Michael et al., 2012b) phenicols,(Katsuda et al., 2012, Kehrenberg et al., 2008, Michael et al., 2012b) and aminoglycosides(Michael et al., 2012b, Alexander et al., 2013a).

Lubbers and Hanzlicek described the available literature on antimicrobial resistance by categorizing the information into two categories: 1) authors reporting the percentage of isolates that are susceptible or resistant, or 2) the minimal inhibitory concentration (MIC) distribution, i.e. the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test for either 50% (MIC_{50}) or 90% (MIC_{90}) of isolates tested(Lubbers and Hanzlicek, 2013). Also present in the literature are molecular investigations into the mechanisms of resistance from small pools of field isolates or specific strains known to carry single or multidrug-resistance.

Studies reporting either an MIC distribution or a percentage of susceptible/resistant isolates often, but not always, provide a description of criteria used to determine isolate classification as susceptible, intermediate, or resistant. Comparison of results between publications is difficult, if not impossible, when these criteria are not described adequately. Even when described appropriately, differences in methodology may make comparison between datasets inappropriate.

Standardized antimicrobial susceptibility testing methods and interpretive criteria are described by the Clinical and Laboratory Standards Institute (CLSI) in document VET01-A4(Clinical and Laboratory Standards Institute VET01-A4, July 2013). The most recent listing of CLSI interpretive criteria are contained in the supplemental document VET01-S2(Clinical and Laboratory Standards Institute VET01-S2, July 2013). The Veterinary Antimicrobial Susceptibility Testing (VAST) subcommittee within the CLSI determines veterinary-specific interpretive criteria based on evaluating clinical isolates, wild-type isolate, and pharmacokinetic/pharmacodynamic cutoffs as described in CLSI document VET02-A3(Clinical and Laboratory Standards Institute and VET02-A3, February 2008).

The CLSI, in VET01-A4, defines Susceptible, Intermediate, and Resistant as follows:

> **Susceptible** – a category that implies that an infection due to the strain may be appropriately treated with the dosage regimen of an antimicrobial agent recommended for that type of infection and infection species, unless otherwise indicated;
>
> **Intermediate** – a category that implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of drug can be used; also indicates a "buffer zone" that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations;
>
> **Resistant** – resistant strains are not inhibited by the usually achievable concentration of the agent with normal dosage schedules and/or fall in the range where specific resistance mechanisms are likely (e.g., β-lactamase), and clinical outcome has not been predictable in effectiveness studies.

As outlined in Table 1, eleven antimicrobials have (non-generic) veterinary breakpoints established for the treatment of BRD as described in VET01-A4(Clinical and Laboratory

Standards Institute VET01-A4, July 2013). A CLSI-approved veterinary breakpoint applies to a specific combination of disease, pathogen, animal species, and antimicrobial treatment regimen. When one of these parameters is altered, implied clinical outcome as related to the breakpoint is no longer valid, and therefore, the predictive value of the breakpoint is suspect. Unfortunately, the literature contains a plethora of studies containing information based on non-standardized testing methods or interpretive criteria not validated for the condition being treated.(Apley, 2003) Applying CLSI interpretive criteria to data generated from susceptibility testing which did not adhere to CLSI standards is an example of inappropriate reporting. As such, all studies described herein have either used the criteria described by CLSI or have adequate reasoning for using different criteria.

Literature Review

The scientific literature contained in PubMed, AGRICOLA, and CAB were searched in February of 2014 using the following combinations of terms: (((((((bovine respiratory disease) AND antibiotic resistance) OR antimicrobial resistance) AND *Mannheimia haemolytica*) OR *Pasteurella haemolytica*) OR *Pasteurella multocida*) OR *Histophilus somni*) OR *Haemophilus somnus*). Titles and abstracts were read and 105 articles that were relevant to the subject of BRD antibiotic resistance were attained for further review. Relevance, defined as an abstract stating an objective (or conclusion) related to an obvious attempt to characterize susceptibility or resistance of the three BRD pathogens within a population of cattle. Additionally, if the title was related to BRD and antimicrobial susceptibility or resistance and did not contain an abstract, the article was attained for review.

The first exclusion criteria involved removing publications not originating in North America or not in English. The next exclusion criterion applied was the removal of publications that did not

utilize CLSI (or the former title NCCLS) criteria. The final exclusion criterion was the removal of articles not focused on BRD in beef cattle. Ten articles investigating phenotypic resistance and six articles on genetic components involved in macrolide resistance in BRD bacterial pathogens are included in this review.

Reports of Phenotypic Resistance in North America

Chang and Carter (1976) analyzed isolates of *M. haemolytica* (n=262) and *P. multocida* (n=141) from clinical cases of BRD sent to the Michigan State Laboratory during a three year period from 1971 to 1974(Chang and Carter, 1976). Careful interpretation of these data must be used as the publication predates the establishment of CLSI interpretive criteria. However, this report was the first report of multi-drug resistant BRD bacterial pathogens in the scientific literature. They found that most of the isolates were resistant to at least one of the four antimicrobials tested (dihydrostreptomycin, tetracycline, penicillin, and chloramphenicol). Using susceptibility interpretive criteria described by Kirby and Bauer, they reported resistance to at least a single drug in 79.1% (148/187) and 96.1% (122/127) of *P. multocida* and *M. haemolytica*, respectively. This article is included as a historical reference, but the interpretive criteria differ from current criteria, and the methods pre-date CLSI standards, so comparison to susceptibility distributions using current standards would be inappropriate.

In 1991, Post *et al.* published a report regarding the antimicrobial susceptibility patterns and MICs of *P. multocida* (n=158) and *M. haemolytica* (n=421) isolated from nasal swabs and tissue specimens submitted from cattle with BRD to the Texas Veterinary Medical Diagnostic Laboratory between February and June 1989(Post et al., 1991). The susceptible or resistant findings from this study are only marginally useful for comparison to current findings because of changes in CLSI interpretive criteria and uncertainty in comparing the plates used in this study and

the commercial plates currently used in microwell dilution testing. The *M. haemolytica* MIC_{50} and MIC_{90} values for ceftiofur were at or below 0.125 µg/ml, the lowest dilution tested. For tetracycline, the corresponding values for MIC_{50} and MIC_{90} were 2 and 16 µg/ml, respectively.

Watts *et al.* published a survey of antimicrobial susceptibility findings involving a total of 888 isolates recovered from BRD cases over a four year period from 1988-1992(Watts et al., 1994). The isolates (461 *M. haemolytica,* 318 *P. multocida*, and 109 *H. somni*) were sent from veterinary diagnostic laboratories to an Upjohn (Zoetis) laboratory for MIC determinations. Only isolates of *M. haemolytica*, *P. multocida,* and *H. somni* obtained from lungs of animals that had died from acute BRD were requested. No more than two isolates of each species from each herd (or feedlot) were received from the following states: Pennsylvania, Wyoming, Iowa, Washington, California, Missouri, Nebraska, Oregon, Kansas, Arizona, Texas, South Dakota, Montana, Minnesota, Oklahoma, Colorado, and Utah. The Canadian provinces of Saskatchewan, Alberta, and Quebec were included in year four. Minimum inhibitory concentrations using CLSI approved interpretive criteria were determined for the following antimicrobials: ceftiofur, tilmicosin (breakpoint not yet established at publication), and spectinomycin. The other antimicrobials were evaluated using CLSI interpretive criteria adapted from human medicine, not approved in relation to bovine respiratory disease.

The cumulative results of this 4 year study are reported in Table 2. These MIC distributions represent the earliest published documentation of MIC distributions for major BRD pathogens using CLSI methods and microdilution methods consistent with current methods in diagnostic laboratories. The last two years of the studies utilized commercially available serial dilution microwell dilution plates.

The finding of significant resistance to tilmicosin in *M. haemolytica* and *P. multocida* using the breakpoints in the study led to the Watts *et al.* speculating on reasons for resistance to a new antimicrobial. Although the findings of tilmicosin resistance would have been dramatically decreased with the use of the subsequently approved breakpoints for *M. haemolytica*, their speculation of cross-resistance within the macrolide class of antimicrobials has since been substantiated(Kadlec et al., 2011, Van Donkersgoed et al., 2008, Desmolaize et al., 2011c).

Welsh *et al.* monitored the trends in antimicrobial susceptibility on a total of 842 isolates over the span of 1994-2002 (Welsh et al., 2004). In total, they collected 390 *M. haemolytica*, 292 *P. multocida*, and 160 *H. somni* were isolated at the Oklahoma Animal Disease Diagnostic Laboratory from lungs from six to eighteen month-old beef cattle succumbed to pneumonia. They reported variable susceptibility to tetracycline (range 23-74%) and relatively stable susceptibility to both ceftiofur (96-100%) and enrofloxacin (89-98%) for *M. haemolytica* isolates over that time period. *Pasteurella multocida* susceptibility profiles remained constant for both ceftiofur (96-100%) and enrofloxacin (96-100%) but a decline in florfenicol susceptibility from 100% to 86% was observed from when florfenicol first came to use in 1996 through 2002. As for *H. somni*, they found that susceptibility to commonly used antimicrobials remained consistently high (87-100). A summary of their observed data is reported for each pathogen across all years in Table 3.

In a study on healthy feedlot cattle, Klima *et al.* isolated *M. haemolytica* from deep nasal swabs from a random selection of approximately 10% of animals from 30% of feedlot pens within two feedlots in southern Alberta, Canada during 2007 and 2008(Klima et al., 2014a). Swabs were taken from the same cattle on arrival and within 30 days of feedlot exit. Cattle were subjected to normal commercial practices; BRD cases were treated with either tulathromycin or ceftiofur. During the feeding period, in-feed ionophores (lasalocid or monensin) were administered, chlortetracycline

was fed at levels for liver abscess control, cattle deemed to be at high risk of BRD were administered pulse (1-6 g/hd per day) doses of chlortetracycline in the feed, and tylosin was fed in the diet for liver abscess control.

Susceptibility testing by disk diffusion was performed on 409 *M. haemolytica* isolates collected during the Klima *et al.* study. Antimicrobial resistance was found to consistently be low. All isolates were found to be susceptible to ceftiofur, enrofloxacin, and florfenicol. Resistance was most common for oxytetracycline (n=16) and one isolate was found intermediate to tilmicosin. They reported no obvious trend in resistance among these isolates at entry or exit from either feedlot. Additionally, they found no trend correlating an isolates' expression of resistance and the antimicrobial that was administered to their host. The authors used CLSI Interpretive breakpoints for applications other than bovine respiratory disease for amoxicillin/clavulanic acid, sulfamethoxazole/trimethoprim, ampicillin, gentamicin, and oxytetracycline.

McClary *et al.* identified cattle that had received tilmicosin for treatment of BRD based on records of 16 randomized clinical trials conducted in confined cattle feeding facilities in seven different states from 1996-2004(McClary et al., 2011). The cattle in these studies had no previous history of antimicrobial usage (including metaphylaxis) and met the individual study definition for BRD. Isolates of *M. haemolytica* (n=878) and *P. multocida* (n=359) were collected via deep nasal swab prior to treatment with tilmicosin; calves yielding >1 pathogen were not included in the analysis. The minimum inhibitory concentration to tilmicosin was determined using CLSI standard methods. Only 0.8% of *M. haemolytica* and 6.9% of *P. multocida* pre-treatment isolates were phenotypically resistant to tilmicosin and most (73.7%) of the *M. haemolytica* isolates categorized as either not susceptible or resistant were obtained during two of the 16 clinical trials. Additionally, they found no associations between case outcome and the classification (susceptible or not

susceptible) of the isolate for either pathogen. The authors reported the case outcome data in figures only, with approximate clinical success rates for *M. haemolytica* of 62% for susceptible isolates (n = 688), 47% for intermediate susceptibility isolates (n = 57), and 38% for resistant isolates (n = 6). The P value for a difference in clinical success rate between susceptible isolates as compared to intermediate or resistant isolates was 0.08. The authors acknowledge the lack of power in the study due to the low number of resistant isolates. While the paper did not find a significant difference in clinical outcome between susceptible and resistant isolates, the low power of the paper does not allow the conclusion that there is no difference.

In an attempt to find correlations of ante-mortem treatment regimens with post-mortem susceptibility patterns, Lamm *et al.* searched the Oklahoma Animal Disease Diagnostic Laboratory database for animals that died at the Oklahoma State University Willard Sparks Beef Research Center in 2007(Lamm et al., 2012). Cattle were included in the analysis if they had died due to BRD, had chronicity of the lesions recorded, bacterial organisms were isolated, and susceptibility patterns of the bacterial organisms determined.

The authors (Lamm *et al.*) reported highly variable susceptibility patterns to tilmicosin between *M. haemolytica*, *P. multocida,* and *H. somni* with 88% (15/17), 42% (6/14), and 0% (0/12) of those isolates, respectively, either being intermediate or resistant. When grouping all three pathogens together, only a small portion of the isolates were susceptible to tetracycline (17/42, 40%) but a large number of isolates were susceptible to enrofloxacin (42/43, 98%), ceftiofur (38/42, 90%), and florfenicol (31/43, 72%); the authors indicated that ceftiofur and enrofloxacin were administered to these cattle prior to death. Overall, they reported that their study showed susceptibility patterns of the bacterial organisms isolated from the lungs of feedlot cattle that died

with bronchopneumonia may not always relate to the antimicrobial treatments administered prior to death.

A large investigation was undertaken by Portis *et al.* using isolates from pre- and post-mortem bovine specimens to detect changes of in vitro susceptibility of BRD clinical isolates from 2000 to 2009(Portis et al., 2012). Isolates of *M. haemolytica* (n=2,977), *P. multocida* (n=3,291), and *H. somni* (n=1,844) from 24 diagnostic labs across the U.S. (isolates from 47 states) and Canada (isolates from 6 provinces) were included in the study. All isolates were from diseased or deceased animals without any knowledge of either the age or the previous antimicrobial treatments administered to the animals. All susceptibility testing was carried out in two Pfizer (Zoetis) laboratories using CLSI standardized testing methods and approved interpretive criteria. The testing panel consisted of ceftiofur, enrofloxacin, florfenicol, penicillin, tetracycline, and tilmicosin. Danofloxacin and tulathromycin were added to the panel in 2004. Individual MIC distributions are reported for each drug and year for *M. haemolytica* (n=2,977), *P. multocida* (n=3,291), and *H. somni* (n=1,844) in this report. The reader is referred to the original publication for review of the extensive tables.

Among *M. haemolytica* isolates Portis *et al.* observed no apparent changes in MIC distributions for penicillin and ceftiofur. There was a decline in the percentage of isolates susceptible to danofloxacin over this study period. Declines in susceptibility, upward shifts in MIC distributions, and increases in MIC_{90} were reported for enrofloxacin, tilmicosin and tulathromycin. A marked decrease in isolate susceptibility to tilmicosin from 89.4% in 2000 to 59.5% in 2009 was observed. Additionally, a decrease in florfenicol susceptibility by 10% over the ten year period was observed. Approximately 50% of all *M. haemolytica* isolates demonstrated in vitro susceptibility to tetracycline over the ten year study period.

In this same study, isolates of *P. multocida* showed little or no change in MIC distributions, MIC_{50} or MIC_{90} for both penicillin and ceftiofur. Approximately 88% of isolates were susceptible to danofloxacin in 2004 and little change was observed over the five years it was included in the panel. There was an emergence of a few resistant isolates and a small increase in MIC_{90} for enrofloxacin. Tilmicosin displayed a shift towards a higher MIC distribution and the percentage of susceptible isolates decreased to 59.7% by the end of the study. No discernable trends up or down were observed for florfenicol or tetracycline. While a susceptibility of at least 90% was observed to tulathromycin, the MIC_{90} increased 3 fold over the 6 year period in which it was included in the panel.

Trends in isolates of *H. somni* included more than 90% susceptibility to penicillin, a steady increase of MIC_{90} for danofloxacin, and a drop in susceptibility for enrofloxacin from 100% in 2000 to 86% in 2009. Additionally, while the MIC_{50} remained constant, a decrease in florfenicol susceptibility and a shift towards higher MIC distributions was reported. The MIC_{50} of tetracycline increased multiple dilutions and therefore a decrease in tetracycline susceptibility was observed. A shift in MIC distributions for both tilmicosin and tulathromycin was reported.

While commenting on trends across all three bacteria, Portis *et al*, reported that a majority of BRD isolates remained susceptible to danofloxacin, enrofloxacin, and florfenicol, but they did observe a slow increase in resistance to these drugs. All three pathogens remained 100% susceptible to ceftiofur throughout the ten year study period. Although there was substantial variation in levels of susceptibility to tetracycline, MIC distributions did not appear to change over the study period in this report. This report once again demonstrated a cross-resistance effect in the macrolide class of antimicrobials. In 2004, one year prior to the marketing of tulathromycin, a 2-6% resistance rate was noted across the three major BRD bacteria pathogens.

Using isolates from two previous studies(Klima et al., 2011, Alexander et al., 2013b) Alexander *et al.* evaluated tulathromycin resistance in *M. haemolytica* isolated from cattle with a known history of antimicrobial use over a three year period from four commercial feedlots in southern Alberta, Canada(Alexander et al., 2013a). This period started one year after approval of tulathromycin for treatment and prevention of bovine respiratory disease in Canada. Tulathromycin was approved in 2005 in the United States and in 2006 in Canada. As described above, deep nasal swabs were obtained from a random selection of 10% of animals from 30% of feedlot pens of healthy cattle upon arrival and again at ≥60 days on feed (DOF). All *M. haemolytica* isolates (n= 4,548 isolates from 796 of the 5814 cattle sampled) were initially screened for tulathromycin susceptibility by plating onto brain heart infusion plates supplemented with 2 µg/mL tulathromycin. Isolates that grew on these plates (n=5) were isolated from three calves and were subjected to PCR analysis for resistance genes. All five isolates contained *aphA-1* and *tet*(H), conferring resistance to neomycin and oxytetracycline. Two isolates contained $bla_{\text{ROB-1}}$ encoding resistance to ampicillin and penicillin. However, none of the isolates contained the macrolide resistance genes screened for in this study, namely, *erm*(A), *erm*(B) , *erm*(F), *erm*(X), *erm*(42), *msr*(E)-*mph*(E). The three animals that harbored the resistant isolates received metaphylactic doses of tulathromycin and were isolated only at ≥60 DOF. However, in the opinion of those authors, the remarkably low rates of resistance in that study did not support any association between macrolide use and tulathromycin resistance. The current study showed that tulathromycin resistance from this population of feedlot cattle in western Canada was exceptionally low even after this antimicrobial had been used in Canada for a period of four years. In their opinion (Klima et al. and Alexander et al.), there was no evidence that the commercial practices used by the feedlots in this study selected for tulathromycin-resistant *M. haemolytica.*

Isolates from the Kansas State Veterinary Diagnostic Laboratory were used by Lubbers and Hanzlicek in determining the prevalence of multidrug-resistant *M. haemolytica* from BRD cases over the time period of 2009 to 2011(Lubbers and Hanzlicek, 2013). _ENREF_23Isolates (n=389) included in the analysis had to be bovine lung specimen culture positive for *M. haemolytica* from clinical cases (research cases excluded) that had susceptibility test results available. Citing a low rate of resistance to ceftiofur (n=2) the authors reported that no single isolate was resistant to all six drugs (ceftiofur, enrofloxacin, florfenicol, oxytetracycline, spectinomycin, and tilmicosin) analyzed in this study. Enrofloxacin and danofloxacin yielded equivalent results, so only enrofloxacin was included in the analysis. Tilmicosin and tulathromycin yielded the same susceptibility interpretation in 85.5% (153/179) of the isolates; of the remaining 26 isolates, 14 were interpretation discrepancies of intermediate and susceptible, which had no effect on the resistant finding. Seven isolates were resistant to tulathromycin and intermediate or susceptible to tilmicosin; 5 isolates had the opposite relationship, displaying resistance to tilmicosin but susceptible or intermediate to tulathromycin.

The prevalence of multidrug-resistance was alarming and increased over time in the Lubbers and Hanzlicek study. Using resistance to three or more antimicrobials as the definition for multidrug resistance, 42% (23/55), 46% (71/155), and 63% (113/179) of the isolates were classified as multidrug resistant in 2009, 2010, and 2011, respectively. By 2011, 25% of the isolates were resistant to 4 of 6 key antimicrobials (typically all but florfenicol and ceftiofur), and 35% were resistant to 5 of 6 key antimicrobials (all but ceftiofur). In this study, isolates found to be resistant to oxytetracycline were 3.52 times more likely (P=0.04) to be resistant to one or more additional antimicrobials compared to non-oxytetracycline-resistant isolates. Isolates resistant to tilmicosin were 2.64 times more likely (P=0.06) to be resistant to at least one other antimicrobial. There were

no statistically significant coresistance patterns for enrofloxacin, florfenicol, or spectinomycin over the three year period.

Investigating the effects of subtherapeutic vs. therapeutic administration of macrolides on antimicrobial resistance in *M. haemolytica*, Zaheer *et al.* conducted a study on 40 eleven-month old beef steers in Alberta, Canada(Zaheer et al., 2013). All steers originated from the same ranch and had not received antimicrobials during their lifetime prior to inclusion in this study. Steers were housed in individual pens with 10 replicates per treatment (controls no antimicrobials, tilmicosin single subcutaneous injection, tulathromycin single subcutaneous injection, and tylosin phosphate at 11 ppm in the feed). Deep nasopharyngeal swabs were collected on arrival, prior to administration of antimicrobials and then weekly thereafter for the 28 day study period. Their data demonstrated that seven days post injection, *M. haemolytica* was detected in only one steer treated from the tulathromycin treatment group and none of the steers treated with tilmicosin, whereas 60% of all steers were positive for this bacterium upon arrival (day 0). This is suggestive that *M. haemolytica* were not macrolide resistant upon arrival and likely a reflection that these calves had no previous exposure to antimicrobials. In contrast to injectable macrolides, tylosin, in the feed, had no effect on the number of *M. haemolytica* in steers receiving this antimicrobial as compared to cattle that received no antimicrobials. *M. haemolytica* isolated from animals belonging to control and all three macrolide treatment groups throughout the study were found to be susceptible to all tested macrolides, an indication that both therapeutic and subtherapeutic administration did not contribute to macrolide resistance in *M. haemolytica* during the study. Isolates in the present study were also susceptible to all other antimicrobials.

Genetic Components of Bovine Respiratory Disease Resistance

While this review focuses on phenotypic characterization of antimicrobial resistance in bovine respiratory disease isolates, a selected review of genetic components helps in understanding the epidemiology of resistance spread. The understanding of macrolide resistance genetics in *M. haemolytica* and *P. multocida* were greatly advanced in a flurry of publications starting in 2011.

In 2011, Desmolaize *et al*., reported on a novel rRNA methylase gene, *erm*(42), which had diverged from all previously characterized *erm* genes and therefore was previously undetected on PCR assays(Desmolaize et al., 2011b). Genetic analysis suggested acquisition from other members of the Pasteurellaceae and recent gene transfer among *M. haemolytica* and *P. multocida*.

Soon thereafter, Kadlec *et al*., published a whole-genome sequencing evaluation of a 2005 Nebraska *P. multocida* isolate which was resistant to tulathromycin(Kadlec et al., 2011). Prior to this analysis, multiple techniques had failed to determine the mechanism of resistance. Three new resistance genes were identified in this study: the rRNA methylase gene *erm*(42), the macrolide transporter gene *msr*(E), and the macrolide phosphotransferase gene *mph*(E).

Desmolaize *et al*. demonstrated that these three genes are arranged in 3 distinct classes of resistance in *M. haemolytica* and *P. multocida*(Desmolaize et al., 2011c). The *erm*(42) gene alone confers resistance to macrolides, lincosamides, and streptogramin group B antimicrobials. The second class consists of a tandem arrangement of *msr*(E) and *mph*(E). The third class contains all 3 resistance genes and displayed high resistance to all macrolides tested in this study (tulathromycin, gamithromycin, tilmicosin).

Michael *et al*. characterized these genes within an integrative conjugative element (ICE) in an isolate of *P. multocida* that contained 12 antimicrobial resistance genes(Michael et al., 2012c).

This ICE, designated ICE*Pmu1,* contains the resistance genes *aadA*25 (streptomycin/spectinomycin), *str*A and *str*B (streptomycin), *aad*B (gentamicin), *aph*A1 (kanamycin/neomycin), *tet*R-*tet*(H) (tetracycline), *flo*R (chloramphenicol/ florfenicol), *sul*2 (sulfonamides), *erm*(42) (tilmicosin/clindamycin), and *msr*(E)-*mph*(E) (tilmicosin/ tulathromycin). A complete bla_{OXA-2} (penicillins, first and second generation cephalosporins) was also identified but appeared to be nonfunctional in this isolate. The authors pointed to the sequences obtained as evidence suggesting that plasmids, gene cassettes, and insertion sequences have contributed to the development of the two resistance gene regions in this ICE. The presence of these 12 resistance genes in a single ICE demonstrates the potential for transfer of multiple antimicrobial resistance genes in one horizontal gene transfer event.

Michael *et al.* (Michael et al., 2012a) evaluated the same three genes in a recently published investigation of MICs of gamithromycin and tildipirosin using field isolates of *M. haemolytica* (n=29) and *P. multocida* (n=40) collected between 1999 and 2007(Van Donkersgoed et al., 2008). These isolates had previously been shown to carry the genes *erm*(42) and/or *msr*(E)-*mph*(E)(Kadlec et al., 2011). If all three genes were present, the *P. multocida* isolates showed MICs of 16–64 µg/mL for gamithromycin and 16–32 µg/mL for tildipirosin, whereas similar MICs of 32–64 µg/mL for both macrolides were seen for the corresponding *M. haemolytica* isolates.

Ten *P. multocida* isolates that carried only *erm*(42) exhibited low MICs of 2–4 µg/mL for gamithromycin, but had higher MICs of 16–32 µg/mL for tildipirosin(Van Donkersgoed et al., 2008). The single *M. haemolytica* that harbored only *erm*(42) showed MICs of 4 and 32 µg/mL for gamithromycin and tildipirosin, respectively. The two *P. multocida* isolates that carried only the *msr*(E)-*mph*(E) operon exhibited a high MIC of 32 µg/mL for gamithromycin and a low MIC of 2 µg/mL for tildipirosin. The genes *erm*(42) and *msr*(E)-*mph*(E) are part of the resistance gene

regions of the recently identified integrative and conjugative element ICE*Pmu1*, which has been shown to move across genus boundaries and express its resistance genes in different hosts, such as *P. multocida* and *M. haemolytica*. Pronounced increases in the gamithromycin MICs were seen in the presence of *msr*(E)-*mph*(E), whereas distinct increases in the tildipirosin MICs were detected in the presence of *erm*(42). This report, yet again, demonstrates cross-resistance exists in the macrolide class of antimicrobials and is further evidence of BRD bacterial pathogens, due to cross-resistance, exhibiting resistance to antimicrobials prior to them being available for use in the industry.

The most recent evaluation of ICE-mediated antimicrobial resistance in BRD pathogens was published by Klima *et al*. in 2014, and documents the presence of ICE in *M. haemolytica* and *H. somni* isolated from U.S. feedlots(Klima et al., 2014b). Their investigation centered on 42 BRD mortalities in Alberta, Canada, 6 mortalities in Texas, and 20 mortalities in Nebraska. Isolates of *M. haemolytica* (55), *P. multocida* (8), and *H. somni* (10) were collected from lungs and nasal swabs. Forty-five percent (33/73) of all bacterial isolates displayed resistance to 3 or more antimicrobials. Thirty-three percent (18/55) of *M. haemolytica* isolates,37.5% (3/8) of *P. multocida* isolates, and 30% (3/10) of *H. somni* were resistant to more than seven antimicrobial classes, including aminoglycosides, penicillins, fluoroquinolones, lincosamides, macrolides, pleuromutilins, and tetracyclines. All of the multidrug-resistant isolates originated from the Texas and Nebraska feedlots. These isolates varied between 60% and 100% similarity based on PFGE analysis, which the authors point out indicates that resistance was not spread strictly by clonal dissemination.

Eighteen of the *M. haemolytica* isolates, 3 of the *P. multocida* isolates, and 3 of the *H. somni* isolates contained ICE that conferred resistance for up to 7 antimicrobial classes. These ICE were

demonstrated to be transferred by conjugation from *P. multocida* to *Escherichia coli* and from *M. haemolytica* and *H. somni* to *P. multocida*.

Discussion

Antimicrobial resistance among the bacterial pathogens commonly associated with BRD is well documented in the scientific literature and the occurrence of resistant isolates appears to be steadily increasing in reports with the ability to analyze temporal trends. Foreign researchers, although utilizing different industry wide production practices and drug prescription practices, cite the same concerns over increases and patterns in resistance in BRD pathogens, especially in concern to the fluoroquinolone class of antimicrobials(Kaspar, 2006, Katsuda et al., 2013, Pardon et al., 2013, Shin et al., 2005). Japanese researchers have observed resistance rates in fluoroquinolone antimicrobials to have increased up to four-fold from the period of 2006 to 2009(Katsuda et al., 2013).

In the United States, publications from diagnostic laboratory submissions(Lamm et al., 2012, Lubbers and Hanzlicek, 2013, Portis et al., 2012, Watts et al., 1994, Welsh et al., 2004, Chang and Carter, 1976) utilizing specimens from animals that likely died from un-responsive BRD report consistently higher rates of resistance as compared to those that report resistance rates of isolates from pre-treatment(McClary et al., 2011) or healthy cattle(Alexander et al., 2013a, Klima et al., 2011, Zaheer et al., 2013). These contrasts fuel the controversy over whether diagnostic laboratory data are a valid means of monitoring antimicrobial resistance trends due to the biased nature of diagnostic laboratory submissions. Unless the bias towards challenging cases with high morbidity and/or case fatality has dramatically changed, then trends in diagnostic laboratory data suggest that the pathogens involved in non-responsive and high-morbidity BRD challenges are displaying an

increasing incidence of resistance to many of the antimicrobials used in control and therapy of this disease.

Studies which point to limited or non-existent resistance prevalence in small, confined populations exposed to antimicrobials do little to inform the discussion concerning the potential for antimicrobial use to enable spread of resistance through plasmids, integrative conjugative elements (ICE), or clonal spread. Exposure of a population to antimicrobials in the absence of an existing resistant pathogen population relies on de-novo mutations for resistance and then selection for the isolates with these genetic characteristics or phenotypic expression of quiescent genes already present. The fact that many of the isolates discussed in this review contain multiple resistance genes grouped together on some type of transferrable element argues against the concept of de-novo mutation in individual animals or small populations, and instead supports the hypothesis that much of our challenge in resistant BRD pathogens stems from selection for existing multi-drug resistant mobile genetic elements or the resistant pathogens which bear them.

The characterization of these pathogen isolates related to the combination of antimicrobial resistance, virulence, and dominance in colonization remains to be established. It is reasonable to hypothesize that pathogens which contain this "trifecta" would be an enormous challenge within existing beef production systems. The data reviewed in this paper suggest that extensive investigations are needed on where selection pressure is being applied for organisms with resistance phenotypes encoded for by mechanisms such as an ICE. The design of these investigations will be informed by findings of recent and ongoing studies evaluating the epidemiology of BRD pathogens, including investigations of *Mannheimia haemolytica* population distributions within individual animals and across cattle populations.

Conclusion

Due to the disparate data from the few studies that investigate susceptibility testing of BRD pathogens, it is difficult, if not impossible to perform a quantitative assessment or meta-analysis of the studies presented in this review. Even though diagnostic lab data is heavily criticized by some, it is difficult to argue against the appearance of a trend in these data. Following these reports chronologically shows an apparent trend of a decrease in susceptibility of the three major BRD pathogens to most of the antimicrobials commonly used for treatment and control of BRD. It is possible that the cattle in diagnostic studies represent a specific niche in the population that has a preponderance for harboring resistant pathogens and this needs further investigation. However, the fact that there are isolates within the population that phenotypically express high levels of pan-resistance to the antimicrobials used both in treatment and control might suggest that it is unwise to use the same antimicrobials for both treatment and control.

An additional point to consider is the previous lack in technology allowing for the detection of the presence of genetic resistance. Most studies in this review focus on culture and sensitivity which are essentially testing phenotypic expression without any knowledge of the genotype. Perhaps, the "healthy cattle" that display lower levels of resistance have not been exposed to the selection pressure through disease and subsequent treatment, to necessitate the phenotypic expression of resistance genes currently present in their genome as ICE elements or other stand-alone resistance mechanisms? Owing to the relatively recent advances in technology allowing rapid and economically efficient genetic sequencing methods, our research group is currently investigating this in further detail.

This review highlights the continued importance of judicious use of antimicrobials in all sectors veterinary medicine to ensure antimicrobials will remain effective into the future.

Figures and Tables

Trade Name	Generic Drug Name	Product Class	Approval Year	CLSI Approved Breakpoint
LA-200	Oxytetracycline 200 mg/ml	Tetracycline	1980	Yes*
Dual-Pen	Penicillin	β-Lactam	1984	Yes*
Tylan	Tylosin	Macrolide	1985	-
Polyflex	Ampicillin trihydrate	β-Lactam	1985	-
Micotil	Tilmicosin	Macrolide	1992	Yes
Nuflor	Florfenicol	Amphenicol	1996	Yes
Naxcel	Ceftiofur sodium	β-Lactam	1998	Yes
AdSpec	Spectinomycin	Aminocyclitol	1998	Yes
Baytril 100	Enrofloxacin	Fluoroquinolone	1998	Yes
Excede	Ceftiofur crystalline free acid	β-Lactam	2003	Yes
Tetradure	Oxytetracycline 300 mg/ml	Tetracycline	2003	Yes*
Draxxin	Tulathromycin	Macrolide	2005	Yes
Excenel	Ceftiofur hydrochloride	β-Lactam	2008	Yes
Advocin	Danofloxacin	Fluoroquinolone	2002	Yes
Zactran	Gamithromycin	Macrolide	2011	Yes
Zuprevo	Tildipirosin	Macrolide	2012	Yes

Table 2.1– Non-exhaustive list of antimicrobial products licensed in the United States for treatment and/or prevention/control of Bovine Respiratory Disease. *Generic label breakpoints established using field wild-type isolates, in vitro pharmacokinetic, and pharmacodynamic data in the absence of randomized clinical field trials with treatment outcomes

Mannheimia haemolytica susceptibility determined by Kirby-Bauer disc diffusion (%)									
	1994	1995	1996	1997	1998	1999	2000	2001	2002
Ceftiofur	97	98	100	100	98	100	98	96	97
Enrofloxacin	-	-	-	-	-	96	98	89	98
Florfenicol	-	-	100	96	98	97	96	87	90*
Spectinomycin	65	49	71	53	55	63	45	29	51*
Tilmicosin	90	78	93	83	80	74	85	71	79*
Ampicillin	42	64	82	81	63	76	76	57	58
Cephalothin	-	-	-	-	-	-	98	96	97
Erythromycin	90	91	39	84	69	77	67	38	18*
Sulfachloropyridizine	92	-	93	94	88	93	87	90	-
Tetracycline	23	46	74	58	42	63	44	34	54
SMX/TMP	99	90	98	94	95	93	94	94	96

Pasteurella multocida susceptibility determined by Kirby-Bauer disc diffusion (%)									
	1994	1995	1996	1997	1998	1999	2000	2001	2002
Ceftiofur	98	100	100	100	97	100	99	96	100
Enrofloxacin	-	-	-	-	-	96	97	96	100
Florfenicol	-	-	100	100	100	97	86	88	96*
Spectinomycin	34	63	33	63	46	63	31	42	47*
Ampicillin	83	96	100	100	95	76	96	93	98
Cephalothin	-	-	-	-	-	-	96	100	100
Erythromycin	93	89	90	89	79	77	51	41	34*
Sulfachloropyridizine	43	-	65	62	57	93	29	50*	-
Tetracycline	71	58	52	53	56	63	40	44	58*
Tilmicosin	92	81	76	82	84	76	60	58	73*
SMX/TMP	100	88	95	94	95	93	85	76	89*

Histophilus somni susceptibility determined by Kirby-Bauer disc diffusion (%)									
	1994	1995	1996	1997	1998	1999	2000	2001	2002
Ceftiofur	100	100	88	100	100	89	100	97	100
Enrofloxacin	-	-	-	-	-	100	100	100	100
Florfenicol	-	-	100	100	100	100	96	100	100
Spectinomycin	65	70	81	83	74	78	73	86	71
Ampicillin	96	100	94	100	95	89	100	97	100
Cephalothin	-	-	-	-	-	-	100	97	100
Erythromycin	100	100	94	94	95	100	96	97	96
Sulfachloropyridizine	68	-	69	76	78	59	85	86	-

Tetracycline	88	100	94	100	94	100	96	100	100
Tilmicosin	-	88	87	94	87	93	88	97	96
SMX/TMP	96	100	94	100	100	94	100	100	100
*indicates significant decline ($P < 0.05$)									
CLSI approved interpretive criteria related to bovine respiratory disease and this pathogen for these antimicrobials									

Table 2.2 – Summary of susceptibly data of *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni* isolated from lungs of bovine respiratory disease cases submitted to the Oklahoma Animal and Disease Diagnostic Laboratory between 1994 and 2002. (Welsh *et al.*) Note: Tilmicosin has CLSI approved interpretive criteria only for *Mannheimia haemolytica* in BRD

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Chapter 3 - Pharmacokinetics and pharmacodynamics of gamithromycin in pulmonary epithelial lining fluid in naturally occurring bovine respiratory disease in multi-source commingled feedlot cattle

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Abbreviations used:

BRD – bovine respiratory disease

PELF – pulmonary epithelial lining fluid

PK – pharmacokinetics

PD – pharmacodynamics

MM – mass medication

CON – control

NPS – deep nasopharyngeal swab

BAL – bronchoalveolar lavage fluid

Cl – clearance

V – volume of distribution

NCA – noncompartmental analysis

AUC_{24} – area under the curve from time 0 to 24 hours

AUC_{∞} - area under the curve extrapolated to infinity

$AUC_{0\text{-}t}$ – area under the curve from time 0 to the time at which the last sample was collected

C_{MAX} – maximum concentration

Kel – elimination rate constant

MIC – minimum inhibitory concentration

MRT – mean residence time

SC – subcutaneous

KSVDL – Kansas State Veterinary Diagnostic Laboratory

USMARC – United States Meat Animal Research Center

Running Title:

PKPD of gamithromycin in cattle

ABSTRACT

The objectives of this study were to determine if 1) an association exists between individual pharmacokinetic parameters and treatment outcome when feeder cattle were diagnosed with bovine respiratory disease (BRD) and treated with gamithromycin (Zactran®) at the label dose and 2) if there was a stronger association between treatment outcome and gamithromycin concentration in plasma or in the pulmonary epithelial lining fluid (PELF) effect compartment. The study design was a prospective, blinded, randomized clinical trial utilizing three groups of 60 (362-592 lb) steers/bulls randomly allocated within origin to sham injection or gamithromycin mass medication. Cattle were evaluated daily for signs of BRD by a veterinarian blinded to treatment. Animals meeting the BRD case definition were enrolled and allocated to a sample collection scheme consisting of samples for bacterial isolation (bronchoalveolar lavage fluid and nasopharyngeal swabs) and gamithromycin concentration determination (PELF and plasma).

Gamithromycin susceptibility of *M haemolytica* (n=287) and *P multocida* (n=257) were determined using broth microdilution with frozen panels containing gamithromycin at concentrations from 0.03 to 16 µg/mL. A two compartment plasma pharmacokinetic model with an additional compartment for gamithromycin in PELF was developed using rich datasets from published and unpublished studies. The sparse data from our study were then fit to this model using nonlinear mixed effects modeling to estimate individual parameter values. The resulting parameter estimates were used to simulate full time-concentration profiles for each animal in the current study. These profiles were analyzed using non-compartmental methods so that PK/PD indices (AUC_{24}/MIC, AUC_{∞}/MIC, C_{MAX}/MIC) could be calculated for plasma and PELF (also T>MIC) for each individual.

The calculated PK/PD indices were indicative that for both *M haemolytica* and *P multocida* a higher drug exposure in terms of concentration, and duration of exposure relative to the MIC of the target pathogen was favorable to a successful case outcome. A significant association was found between treatment success and PELF $AUC_{0\text{-}24}$/MIC for *P multocida*. The calves in this study demonstrated an increased clearance and volume of distribution in plasma as compared to the healthy calves in two previously published reports. Ultimately, the findings from this study indicate that higher PK/PD indices were predictive of positive treatment outcomes.

KEY WORDS

gamithromycin, antibiotic resistance, pharmacokinetics, pharmacodynamics, bovine respiratory disease, feedlot, cattle

INTRODUCTION

Gamithromycin (ZACTRAN®, Merial Animal Health, Duluth, GA, USA), a macrolide of the azalide subclass, is approved for both treatment of BRD caused by *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni,* and *Mycoplasma bovis* and the control of BRD caused by *M haemolytica* and *P multocida (Merial, 2011)*. Macrolide antibiotics, in general, are bacteriostatic through inhibition of bacterial RNA-dependent protein biosynthesis (Jain & Danziger, 2004). However, gamithromycin can also be bactericidal with minimum bactericidal concentrations only 1 dilution higher than the respective MIC (Huang, Letendre et al., 2010). The tendency for macrolides, especially azalides, to accumulate in inflamed tissue has been described previously (Amsden, 2001). This fact coupled with the known extensive tissue distribution of macrolides, has sparked interest in the veterinary literature concerning the exposure response relationship at the site of action as it pertains to the newer long-acting injectable macrolide formulations (Nowakowski, Inskeep et al., 2004; Womble, Giguere et al., 2006; Venner, Peters et al., 2010; Menge, Rose et al., 2012; Villarino, Brown et al., 2013; Villarino, Lesman et al., 2013; Villarino, Brown et al., 2014).

Recent work examining the concentrations of antibiotics in PELF of healthy animals has been performed to describe the disposition of gamithromycin in beef calves (Giguere, Huang et al., 2011). Giguere et al. found that gamithromycin was rapidly absorbed and reached potentially therapeutic concentrations in PELF within 30 minutes after SC administration. To the authors' knowledge all previous work describing the distribution of these drugs in cattle has been done in healthy subjects and there are no publications describing the PK and PD of a macrolide class antibiotic in the PELF under the conditions of naturally occurring BRD. To that end, the objectives of this study were to: 1) develop a compartmental PK model based upon existing PK data, 2) use

sparse data collected in the present study to estimate parameter values for the animals in this study population, 3) use the model estimated parameters to simulate complete concentration-time profiles of gamithromycin in the central and PELF effect compartments of each animal, 4) determine if a relationship exists between plasma and/or PELF concentrations and treatment outcome, and finally 5) determine the PK/PD indices associated with treatment success in naturally occurring BRD.

MATERIALS AND M ETHODS

Animals and husbandry

One hundred and eighty cattle judged to be at high risk for BRD (overall average body weight of 470 pounds (362-592 lbs)) were sourced from Athens, Tennessee (n=60), Richmond, Kentucky (n=60), and Maryville, Missouri (n=60) as part of another study. Commingled steers and bulls of multiple origins and mixed breeds were acquired at each sale barn and transported to a small research feeding facility in Kansas where they were housed in open air, dirt floor group housing pens for the duration of the trial.

Study design and treatment allocation

The study design was a prospective, blinded, randomized clinical trial with masked subjective evaluators and was approved by the Kansas State University Institutional Animal Care and Use Committee.

Cattle were randomized to one of two treatments prior to arrival (by ear tag ID administered at the sale barn) so that each load was randomly allocated into two pens per source, one for each treatment (6 total pens in the study, 3 for each treatment). At initial processing, cattle assigned to

treatment 1 served as untreated controls (CON) receiving saline at 2 mL/110 pounds subcutaneously in the neck; while cattle randomly allocated to treatment 2 received treatment (MM) for the control of BRD with gamithromycin at the label dose of 6 mg/kg (2 mL/110 lb) subcutaneously in the neck. Additionally, all cattle received a modified live viral respiratory vaccine, clostridia vaccine, growth implant, injectable anthelminthic, duplicate tags for study identification, and were examined to ensure that no clinical signs of BRD were present on arrival. Once in their pens, cattle were fed a ration according to practices typical of the feedlot industry.

Clinical scoring and disease diagnosis

Daily pen observations were performed by a veterinarian masked to treatment allocation. Clinical scoring was by exclusion, i.e. only cattle scoring 1-4, as described in Table 1, were recorded on daily observation forms and brought to the chute for further evaluation. Cattle having a rectal temperature of ≥104.0˚F (≥40.0˚C) and a clinical score of ≥1 were diagnosed with BRD and included in this study. Cattle with a clinical score of ≥1 but not meeting the temperature requirements were returned to their home pen without treatment for further observation. Animals were clinically scored each day but were not eligible for treatment of BRD until the post-control treatment moratorium had elapsed. Control animals were immediately eligible for treatment of BRD and those receiving mass medication were eligible for treatment on study day 8 (7 day moratorium).

Sampling allocation and collection procedures

The day of enrollment (first diagnosis of BRD) was designated as Day 0 for each calf. Cattle meeting inclusion criteria for the study were randomly allocated to a sample collection

scheme consisting of collection of NPS, BAL, and plasma (Table 2). Allocation to sample collection procedures were performed in advance, in blocks of three, to ensure that there was an equal distribution across sampling time points in order to account for the fact that actual number of cases of naturally occurring BRD was unknown beforehand. Samples collected on Day 0 were collected just prior to treatment with gamithromycin. All treatments and procedures at the chute were performed by trained personnel not involved in clinical scoring of cattle. The veterinarian responsible for clinical scoring was not present for treatments and procedures performed at the chute and therefore remained masked throughout the duration of the study period. Deep nasopharyngeal swabs and an aliquot of BAL fluid were immediately sent for bacterial culture while samples of plasma and BAL fluid were processed and frozen at < -70˚C for later analysis by HPLC-MS/MS.

The BAL procedure was used to collect PELF fluid from manually restrained, non-sedate cattle. The BAL tube (Bivonna, BAL-240) was introduced into the trachea via the nasal passage and advanced until wedged into a deep bronchus. Sterile saline (240 mL) was infused in 60 mL aliquots and aspirated immediately after each aliquot. Recovered BAL fluid was collected into a 250 mL centrifuge tube, mixed well, divided evenly among four 50 mL centrifuge tubes, then placed on ice and centrifuged in the feedlot laboratory within 40 minutes of collection. One randomly selected cell pellet was resuspended in liquid Amies media and submitted to the KSVDL for bacterial isolation. The BAL tubes were cleaned by plasma sterilization between collection procedures to prevent cross contamination.

Deep nasopharyngeal swabs were collected from both nares by a veterinarian trained in the procedure. Briefly, a double guarded sterile uterine swab was introduced through the nares into the nasal cavity and guided to the point where resistance was met in the area of the nasopharyngeal

tonsilar tissue. At this point the double guarded swab was retracted slightly, the interior sleeve portion containing the swab was pushed through the exterior guard, and the swab advanced from the sleeve and rotated to ensure a sufficient sample of the mucous and tonsilar secretions from the pharyngeal tissues. The swab was retracted into the guarded sleeve to prevent contamination while exiting the nares. The swabs were placed in liquid Amies media and transported on ice to the KSVDL for bacterial culture and isolation.

Blood was collected into 10 mL sodium heparin vacutainer tubes via jugular venipuncture and tubes were centrifuged in the feedlot laboratory at 500 X g for 15 minutes. Plasma was pipetted into duplicate cryovials and stored along with other cryovial samples of BAL fluid (duplicates of PELF fluid, resuspended cell pellets, and urea analysis samples from both PELF and plasma) at < -70˚C until analysis.

Treatment administration and case outcome determination

Cattle diagnosed with BRD were randomly assigned to a sample collection scheme, treated with gamithromycin according to label directions (6 mg/kg) SC in the neck, and returned to the home pen. Case outcome was determined until Day 9, post treatment. A treatment failure was defined as the calf meeting study inclusion criteria as previously described, if the calf was a clinical score 3 regardless of rectal temperature, or if a calf died from BRD. Those cattle not categorized as a treatment failure by Day 9 were therefore considered treatment successes. Therefore, comparisons within this manuscript are between cattle deemed a treatment success versus those that were deemed a treatment failure.

Gamithromycin concentration analysis

Concentrations of gamithromycin in PELF and plasma were determined by Merial personnel masked to treatment. Samples were analyzed by reversed-phase HPLC with detection via MS/MS transitions by methods previously described (Giguere, Huang et al., 2011). The limit of detection and limit of quantitation were 5 ng/mL and 10 ng/mL, respectively. The concentration of gamithromycin in PELF was estimated using the ratio of urea in BAL fluid to that as measured in serum as described previously (Rennard, Basset et al., 1986).

Bacterial isolation and MIC determination

Nasopharyngeal swabs and PELF samples were plated directly onto trypticase soy + 5% blood, chocolate, and MacConkey agar plates and incubated in 5% CO_2 at 37˚C for 18-24 hours. Up to 12 colonies displaying growth characteristics typical of *M haemolytica* and *P multocida* were isolated in pure culture. Identity was confirmed using MALDI-TOF® (Bruker Daltonics, Billerica, MA, USA) and frozen for later susceptibility testing at the USMARC in Clay Center, Nebraska.

Gamithromycin susceptibility of *M haemolytica* and *P multocida* isolates were performed at USMARC by broth microdilution with frozen panels from TREK Diagnostic Systems® (Thermo-Fisher Scientific Inc., Waltham, MA, USA) containing gamithromycin at concentrations ranging from 0.03 to 16 µg/mL. Bacterial suspensions were prepared and susceptibility plates inoculated as per CLSI guidelines (Clinical and Laboratory Standards Institute & VET01-A4, July 2013). In brief, isolates were cultured on chocolate agar and incubated with increased CO_2 at 37˚C for 18-20 hours. Bacterial suspensions equivalent to 0.5 McFarland standard were made by suspending 3 to 5 isolated colonies from each plate, into 5 mL of demineralized water. Mueller-

Hinton broth tubes were then inoculated with 140 µl of the resulting bacterial suspensions. A 12 channel pipette was used to dispense 50 µl of this suspension into each of 12 wells in the panel, such that each panel could be used to evaluate the susceptibility of 8 strains. Plates were sealed and incubated at 37˚C for 18-20 hours at which time the plates were visually inspected and MIC values determined by noting the lowest concentration of antibiotic that completely inhibited growth. Determination of susceptible, intermediate or resistant were based off of clinical breakpoints established for gamithromycin by CLSI (Clinical and Laboratory Standards Institute & VET01-A4, July 2013).

Pharmacokinetic modeling and pharmacodynamics

A user defined, two compartment plus PELF compartment was built in Phoenix NLME® (Certara L.P., Cary, NC, USA). The model schematic can be seen in Figure 1. The differential equation describing this model is as follows:

$$\frac{dA_1}{dt} = -(A_1 * k_{10}) + (A_a * k_a) - (A_1 * k_{12} - A_2 * k_{21}) - (A_1 * k_{13} - A_{pelf} * k_{31})$$

$$\frac{dA_2}{dt} = (A_1 * k_{12} - A_2 * k_{21})$$

$$\frac{dA_{pelf}}{dt} = (A_1 * k_{13} - A_{pelf} * k_{31})$$

where A_1 is the amount in central compartment, k_{10} is the elimination rate constant from the central compartment, A_a is the amount at the site of the SC injection, K_a is the absorption rate constant from the site of the injection, k_{12} is the rate constant for the central to the peripheral compartment, A_2 is the amount in the peripheral compartment, k_{21} is the rate constant for the peripheral to the central compartment, k_{13} is the rate constant for the central to the PELF compartment, A_{pelf} is the

amount in the PELF compartment, and k_{31} is the rate constant for the PELF to the central compartment.

Data collected from published (Huang, Letendre et al., 2010; Giguere, Huang et al., 2011) and unpublished (personal communication with coauthor RKT) PK trials of gamithromycin in cattle were used to generate initial estimates to develop the model. These data consisted of samples of plasma and PELF concentrations after administration of the label dosage of gamithromycin in healthy beef calves. When specific data were not available in the manuscript, data was extracted using an online tool, WebPlotDigitizer (http://arohatgi.info/WebPlotDigitizer/).

Next, the sparse data from this trial were integrated into the model using a non-linear mixed effect approach, without the inclusion of covariates, to predict parameter values for each animal in our study. Parameter values were calculated for each individual animal within this study by use of the typical value and the individual ETA (e.g. V = tvV * exp(nV)). Individual ETA values for each parameter of the PK model can be found online in supplemental materials. One hundred simulations were conducted predicting complete plasma and PELF time concentration curves for each animal in this current study. Average simulated data were analyzed by NCA in order to compare the results of this study to those in the literature and to calculate PK/PD indices for plasma (AUC_{24}/MIC, AUC_{∞}/MIC, C_{MAX}/MIC) and PELF (AUC_{24}/MIC, AUC_{∞}/MIC, C_{MAX}/MIC, T>MIC). Clearance and V were calculated from extrapolated graphical data contained in each manuscript as follows and for the calves in this study; Cl = Dose/AUC_{inf} and V = Cl/Kel.

The PK/PD indices were calculated from AUC_{24}, AUC_{∞}, and C_{MAX} of the simulated plasma and PELF time-concentration profiles of each individual animal and the MIC of the sample collected from that animal. T>MIC was calculated using predicted PELF data only because plasma concentrations did not reach levels above the MICs in this study. The MIC used for the calculation

was from the isolate with the highest MIC collected from that calf at time 0 only (i.e., prior to therapeutic treatment) but could be from either BAL or NPS. Time above MIC was not calculated for plasma as the plasma concentrations did not reach that of the lowest MIC dilution tested.

Statistical analysis

A generalized linear mixed model fit by maximum likelihood regression was built in STATA (Stata/SE 12.1 for Windows, StataCorp LP, College Station, TX, USA) to compare morbidity between MM and CON using pen as a random clustering effect. Initial covariates included in the model, but ultimately excluded due to lack of significance, were trailer compartment and state of animal origination.

Statistical comparisons of PK and PD parameters between treatment outcome (success and failure) were performed with the Kruskal-Wallis test for nonparametric data using SAS® software (Version 9.3, SAS Institute Inc., Cary, NC, USA). The level of significance was set at $P \leq 0.05$ using a two-tailed test. Both compartmental and NCA PK parameters were compared statistically since this report focuses on both types of modeling.

RESULTS

Morbidity outcome

Descriptive morbidity, mortality, and treatment failure data can be found in Table 3. Treatment for control of BRD with gamithromycin resulted in a numerically lower morbidity but a numerically higher relapse rate (therapeutic failure), however, these differences were not statistically significant ($P = 0.25$). Total morbidity throughout the 28 days of the trial was much lower than anticipated and the power of the study may therefore have been insufficient to detect

significant differences in morbidity between sham injected cattle and those treated for control of BRD with gamithromycin.

PK model development

Model development was guided by goodness of fit plots within the modeling software. The results of the final model can be seen in Figure 2. There is an excellent fit of predicted data to actual data in plasma and PELF at lower concentrations. The model slightly under-predicts PELF at higher concentrations but this is likely due to difficulty in accurately predicting the Ka due to the paucity of data in this area of the curve.

Pharmacokinetics of study animals

The estimated typical value of the compartmental model parameters for the population (regardless of treatment outcome) are summarized in Table 4. Note that the volume parameters (V and V_{PELF}) are not weight normalized because weight was not a significant covariate in the nonlinear mixed effects model.

The simulated full time concentration curves for the treatment successes and failures can be found in Figure 3. The Cl, V, rate constants, C_{MAX}, AUC_{0-t}, AUC_{24}, $AUC_{0-\infty}$, and MRT calculated from these curves were compared between treatment successes and treatment failures and no statistical differences were observed. C_{MAX} was, however, numerically higher in the treatment success group. Variability was high and this may or may not be a true difference (plasma P=0.12, PELF P=0.22).

MIC and pharmacodynamics

The MIC distribution for the clinical (BAL and NPS) isolates of *M haemolytica* (n=287) and *P multocida* (n=257) from this trial can be seen in Figure 4. Isolates of *M haemolytica* in the susceptible category represent 60% of the isolate population and those in the resistant category encompass 36% as compared to 31% and 68% for *P multocida*, respectively. The isolates tested were collected over all time points (0, 12, 24, and 120 hours post treatment) from both CON and MM cattle and therefore represent isolates not yet exposed to therapeutic drug as well as isolates exposed to gamithromycin in the later sample collections. Additionally, nine of the 26 cattle diagnosed with BRD and subsequently sampled were from the treatment group receiving mass medication with gamithromycin on arrival and would have therefore, had previous exposure to gamithromycin.

The bivariate histogram in Figure 5 shows the MIC distribution for both *M haemolytica* and *P multocida* at time 0 by treatment outcome. This graphic suggests that the sample size was not equivalent across outcome groups, but the MIC comparisons within outcome group are quite similar. As such, there were just 22 cattle in the success group and 4 in the failure group with the plots representing a single isolate per calf for those yielding an isolate (4 calves did not yield an isolate at time 0). Due to small sample size and confounding of arrival treatment (CON and MM) within therapeutic treatment outcome it was not possible to statistically compare the MIC by outcome. However, it can be visually appreciated that there is a symmetry in each group with the treatment success group having 10 susceptible isolates and 8 resistant isolates. The treatment failures represent cattle yielding 2 susceptible and 2 resistant isolates, prior to treatment.

The results of the PK/PD index calculations can be found in Table 5 and represent a comparison between treatment successes and failures by pathogen. Although the standard error in

the failure group is relatively large and likely reflective of the small sample size, comparing the means between outcomes indicate that in all cases, more active ingredient is present and for a longer duration in the treatment success group. Statistical differences between treatment successes and failures were noted in AUC_{24}/MIC (P = 0.04) for *P multocida* in PELF. Additionally, an association (P = 0.10) was observed with higher AUC_{24}/MIC, AUC_{∞}/MIC, and C_{MAX}/MIC for treatment successes as compared to failures in plasma, and AUC_{24}/MIC (P = 0.07) in PELF for *M haemolytica.* Half of the failures (2/4) and 41% (7/17) of the cattle in the success group were from the MM group that received gamithromycin on arrival to the feedyard. Those cattle from the MM group accounted for 80% (8/10) of the isolates displaying an MIC of ≥16 μg/mL (at time 0). The isolate yielding the highest MIC, as cultured prior to treatment for BRD, is reported by treatment group (CON, MM) in Table 6.

Comparison of the PK parameters resulting from this study compared to those of previously published studies can be found in Table 7. The calves in this study demonstrated an increased Cl and V in plasma as compared to the healthy calves in the two previous reports. The MRT of both plasma and PELF are quite similar to those reported previously by Giguere et al. Both the C_{MAX} and $AUC_{0-\infty}$ reported herein, are quite different, especially in plasma, from the previously published reports of gamithromycin PK in healthy cattle (Huang, Letendre et al. 2010; Giguere, Huang et al. 2011).

DISCUSSION

Trials involving sparsely sampled data, such as this one, represent difficulties in parameter estimation. Mixed effects models help to overcome these challenges by partitioning sources of variability in hierarchical statistical models, there-by allowing a reduction in the variance of the

estimated population parameters. These models also have the advantage of allowing quality analysis from fewer samples, thereby sparing expense and the stress from additional animal handling. However, quality prior information on the parameters is a requirement to inform these models (Dodds, Hooker et al., 2005; Riviere, 2011; Mould & Upton, 2012). It was fortunate to have had access to rich data to externally validate the model. This allowed a comparison of the simulated data in this study to the results of two previously published reports to confirm the models' accuracy.

Using only two samples of PELF per animal, the mixed effect model utilized in this study allowed for the estimation of the gamithromycin PK/PD values achieved in cattle diagnosed with BRD. The resulting simulated individual animal profiles were used to run a NCA in order to compare our model output to previous publications results. The PK parameter results obtained from that analysis are comparable to the values obtained previously (Huang, Letendre et al., 2010; Giguere, Huang et al., 2011) as shown in Table 7. Considering the differences in the physiological status of the study participants under investigation (healthy cattle in previous publications versus cattle diagnosed with BRD in this report) and the fact that this model under-predicts PELF, the resulting comparable PK parameters from this study demonstrates that our two compartment plus PELF effect compartment model was acceptable. However, the lower drug exposure found in the morbid animals in this study as compared to those utilizing healthy animals is noteworthy.

Table 4 displays the estimated typical values of the compartmental model parameters. It should be noted that the V_{PELF} is much larger than its actual physical volume in cattle because the role of this parameter in the model is a virtual compartment in which relatively low concentrations were observed for the administered dose.

Gamithromycin has been reported to be a low protein binding drug (26%) in the serum of healthy animals (Huang, Letendre et al., 2010). Protein binding was not an objective of this study and therefore was not evaluated. Variation in protein binding is expected to have minimal effect for drugs that display low protein binding. However, it is possible that alterations in the protein binding of the morbid animals in this study did have some effect on the PK differences we observed. Future studies focusing on determining the differences between healthy and diseased animals needs to be considered to determine if this was simply an effect of the modeling/sampling strategy utilized in this study or a difference truly exists.

Much discussion is available in the literature surrounding the selection of the proper pharmacodynamic index to determine the optimal dosing of the macrolide class of antimicrobials. Contemporary thought on the newer 'longer acting' injectable macrolides in veterinary medicine is that the most important index is AUC/MIC. However, intense debate remains whether this should be measured and reported for plasma (Toutain, 2009; Papich, 2014), at the site of infection (Amsden, 2001; Evans, 2005) or both (Rodvold, George et al., 2011). Although not statistically significant, the marginally significant association between plasma PKPD indices and treatment outcome (P=0.10) would seem to substantiate the claims of using plasma drug concentrations. However, we did observe a significant association between PELF AUC_{24}/MIC and treatment outcome (P=0.04) with *P multocida* suggesting that both plasma and PELF are correlated with treatment outcome. This finding is not surprising given the fact that the drug in the PELF is derived from and thus correlated with the drug in the plasma. However, completely ignoring the PELF compartment and confining interpretation to plasma alone could be misleading, especially when considering drugs with very extensive tissue distribution such as gamithromycin and other macrolide class antibiotics.

Prior to the initiation of the study gamithromycin did not have clinical breakpoints determined by the CLSI. However, since the conclusion of the live phase of this study gamithromycin breakpoints have been reported for *M haemolytica, P multocida,* and *H somni* at ≤4.0, 8.0, ≥16.0 µg/mL for susceptible, intermediate, and resistant, respectively. The biphasic population of bacteria cultured from cattle displaying signs of BRD in this report fit those breakpoints. Conversely, in this study, there seemed to be little association between the *in vitro* determined MIC and treatment outcome, especially for *P multocida* (Table 7). Several authors have noted differences between the MIC determined *in vitro* and the MIC determined in the more physiologically relevant matrix, serum (Evans, 2005; Mitchell, McKellar et al., 2012; Mitchell, Goh et al., 2013). It remains a possibility that the lack of association, in this study, between reported MIC and treatment outcome could be due to a similar phenomenon.

Several cattle yielded multiple isolates of *M haemolytica* and *P multocida* from either NPS or BAL, selection of isolate MIC was performed by choosing the isolate with the highest MIC at time 0 (Table 6). Therefore, PD indices reported in this study are likely to represent worst case scenarios as the results of this trial are also confounded by on arrival treatment allocation. Giguere et al. reported MIC_{90} values of 0.5 and 1.0 µg/mL for *M haemolytica* and *P multocida* in 2011 (Giguere, Huang et al., 2011). Using those MICs would have certainly resulted in much different PD indices.

Reported herein were the AUC/MIC ratios for both PELF and plasma, as well as the other standard PKPD indices (C_{MAX}/MIC, T>MIC). Although minimal statistical differences were observed, our findings indicate that for both *M haemolytica* and *P multocida,* a longer drug exposure was more closely related to a successful treatment outcome. While some small differences in exposure were observed, it is unlikely that these differences substantially contributed

to the difference in clinical outcome. However, it is important to keep in mind that due to small sample size, there was a large amount of variability in the data which could contribute to the lack of statistical significance.

Additionally, given the small numerical difference between success and failure PD indices, it seems that there are likely many factors beyond PK, PD, and MICs that contribute to the success of a treatment regimen. For example, the immunological status of the animal and the environmental conditions that the animal is subjected to undoubtedly also play a role in disease outcome. It has been shown that some macrolides have anti-inflammatory and immunomodulatory effects in addition to antimicrobial activity. Tulathromycin, a semi-synthetic macrolide of the subclass triamilide, has been extensively researched in this area (Fischer, Beatty et al., 2011; Er & Yazar, 2012; Fischer, Beatty et al., 2013; Fischer, Duquette et al., 2014; Duquette, Fischer et al., 2015). Azithromycin, a macrolide of the same subclass as gamithromycin, has recently been shown to exert anti-inflammatory properties on lung epithelial cells in humans (Kitsiouli, Antoniou et al., 2015). While data specific to gamithromycin is currently lacking in this area, it is possible that anti-inflammatory activity similar to that of azithromycin and tulathromycin could have facilitated a "self-cure" in this study. This should be further considered, especially considering the high treatment success rate observed in this study given the isolation of many resistant organisms as shown in Table 6.

There appears to be an over-representation of resistant *P multocida* isolates in the MM treatment group. This is likely explained by the fact that gamithromycin was utilized for both mass medication and treatment in this study, a practice that is not common in the field. The reasoning for the lack of resistant *M haemolytica* in the MM group is not clear and deserves further attention in future studies.

Another layer of complexity must also be appreciated; there remains a possibility that bacteria other than *M haemolytica* and *P multocida* are responsible wholly, or in part, for the treatment failures. While *H somni* was isolated (data not shown), it was present very infrequently. It is possible that another resistant pathogen is contributing to the clinical signs associated with the BRD cases in this study.

This report is, to the authors' knowledge, the first of its kind in the veterinary literature, to perform such a large PK/PD examining drug concentrations in PELF within a group of cattle experiencing naturally occurring BRD. Although challenged by sample size limitations of ultimately diseased animals, a compartmental PK model was developed to which the sparse clinical data from this trial were successfully fit. Therefore, complete concentration-time profiles were simulated for the central and PELF effect compartment for each animal in order to determine PK/PD indices for *M haemolytica* and *P multocida* unique to each animal in this study. The findings from this study indicate that PK variability in cattle diagnosed with BRD seems at least as important as the MIC of *M haemolytica* or *P multocida*. Additionally, further consideration should be paid to other possible bacterial pathogens in association to BRD.

ACKNOWLEDGEMENTS

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Figures and Tables

Score	Description
0	No abnormal signs
1	Slower than pen mates but still perks up when approached; does not appear weak; actively follows movements with raised head
2	Stands with head lowered; perks up when approached but returns to depressed stance; moves slowly and falls to back of group; may display signs of weakness such as incoordination
3	Obviously very weak; difficulty in moving with group; raises head only when approached closely
4	Moribund, unable to rise

Table 3.1 - Description of clinical scoring criteria used for daily clinical observations of feeder cattle to assist in diagnosis of bovine respiratory disease. Clinical scoring was performed by a veterinarian masked to study treatment allocation.

N	0 hr	12 hr	24 hr	120 hr
8	BAL/Plasma/NPS	Plasma	Plasma	BAL/ Plasma/NPS
9	Plasma/NPS	BAL/Plasma/NPS	Plasma	BAL/ Plasma/NPS
9	Plasma/NPS	Plasma	BAL/ Plasma/NPS	BAL/ Plasma/NPS

Table 3.2 - Sampling scheme for bronchoalveolar lavage (BAL), deep nasopharyngeal swabs (NPS), and plasma in cattle diagnosed with bovine respiratory disease (BRD). The top row is hours after treatment, 0 hr is just prior to treatment with gamithromycin. The number of cattle enrolled in each collection scheme is represented in the first column (labeled N).

	Control (%)	Mass Medicated (%)	Total (%)
Morbidity from BRD	17/90 (18.9)	9/90 (10.0)	26/180 (14.4)
Mortality from BRD	0	0	0
BRD Treatment Failure	2/17 (11.8)	2/9 (22.2)	4/26 (15.4)

Table 3.3 - Summary comparison of morbidity, mortality, and treatment failure rates of bovine respiratory disease among feedlot cattle allocated to either sham injection or mass medication with gamithromycin at 6 mg/kg. Treatment for BRD was also with gamithromycin at the time of BRD diagnosis by a veterinarian. The numerical differences were not statistically significant. (% = percentage of subjects)

	Units	Estimates
V	L	183
k10	1/hr	1.84
Ka	1/hr	8.83
k12	1/hr	7.09
k21	1/hr	0.26
k13	1/hr	0.98
k31	1/hr	0.02
V_{PELF}	L	201

Table 3.4 –Population pharmacokinetic parameter estimates following administration of gamithromycin administered at an average dose of 6 mg/kg subcutaneously for the treatment of acute bovine respiratory disease.

		M haemolytica		*P multocida*	
	Parameter	Success (±SE)	Failure (±SE)	Success (±SE)	Failure (±SE)
Plasma	AUC_{24}/MIC	1.32 (±0.1) ‡	0.67 (±0.59) ‡	1.24 (±0.41)	0.72 (±0.42)
	AUC_{∞}/MIC	3.49 (±0.4) ‡	1.78 (±1.55) ‡	3.21 (±1.07)	1.91 (±1.12)
	C_{MAX}/MIC	0.09 (±0.01) ‡	0.04 (±0.037) ‡	0.08 (±0.03)	0.04 (±0.03)
PELF	AUC_{24}/MIC	31.5 (±4.4)	24.6 (±22.4)	31.0 (±9.8)*	22.4 (±11.7)*
	AUC_{∞}/MIC	205 (±29)	164 (±150)	209 (±67)	148 (±77)
	C_{MAX}/MIC	1.86 (±0.26)	1.45 (±1.32)	1.83 (±0.58) †	1.32 (±0.69) †
	T>MIC	77 (±10)	44 (±44)	56 (±19)	44 (±25)

Table 3.5 – Pharmacokinetic/pharamcodynamic indices for cattle treated with gamithromycin for acute bovine respiratory disease (mean (±SE)). Parameter comparisons are by pathogen (cultured at time 0) and treatment outcome within either plasma or the effect compartment, PELF. Values are calculated as free unbound drug using 26% protein binding from a previous study (Huang, 2010). (* P = 0.04; † P = 0.07; ‡ P = 0.10)

CON				MM		
ID	*M haemolytica*	*P multocida*		ID	*M haemolytica*	*P multocida*
102	-	-		150	1.0	-
104	≥16.0	-		*195	-	≥16.0
106	-	≥16.0		204	1.0	≥16.0
116	-	0.5		206	1.0	≥16.0
118	-	0.5		209	-	≥16.0
126	-	0.5		*212	-	≥16.0
174	1.0	-		213	-	≥16.0
175	1.0	0.5		218	-	≥16.0
*222	1.0	1.0				
224	-	-				
225	1.0	1.0				
229	1.0	-				
*236	-	1.0				
240	-	-				
241	1.0	-				
243	1.0	1.0				
245	-	-				

*Treatment failure

- = culture yielded no isolate

Table 3.6 – Minimum inhibitory concentration (µg/mL) comparison of time 0 isolates from both PELF and NPS isolates by treatment group. The single isolate with the highest MIC is reported for each calf (four calves yielded no isolates at time 0)

	Huang et al.	Giguere et al.		Current study	
	Plasma	Plasma	PELF	Plasma	PELF
No. animals	32	30	30	26	26
C_{MAX} (µg/mL)	0.27	0.43	4.61	0.13 (±0.003)	3.04 (±0.09)
$AUC_{0-\infty}$ (µg*h/mL)	8.28	7.95	348	5.4 (±0.13)	340 (±12)
Cl (mL/hr/kg)	654*	830*	-	1140 (±27)	-
V (L/kg)	56.9*	94.2*	-	97.4 (±2.4)	-
MRT (hr)	41.3*	43.1	71.1	52.8 (±0.18)	79.0 (±0.29)

Table 3.7 – Comparison of the current study models simulated output of pharmacokinetic parameters to previously published work by noncompartmental analysis. The label dose of 6 mg/kg subcutaneously was administered in each study. Values of C_{MAX} and $AUC_{0-\infty}$ are reported as total drug since neither publication corrected for protein binding. (± SE where available; *Calculated from extrapolation of graphical data in manuscript

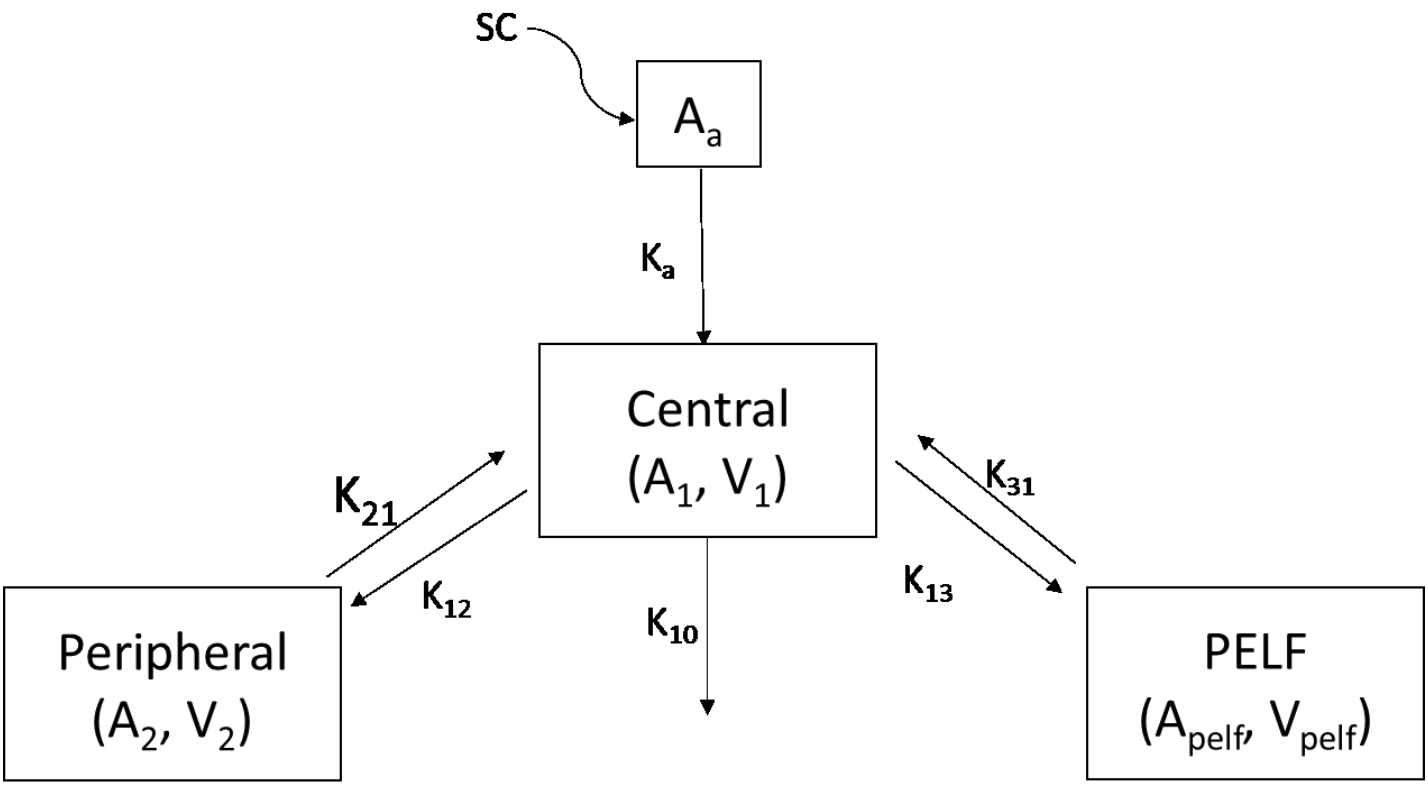
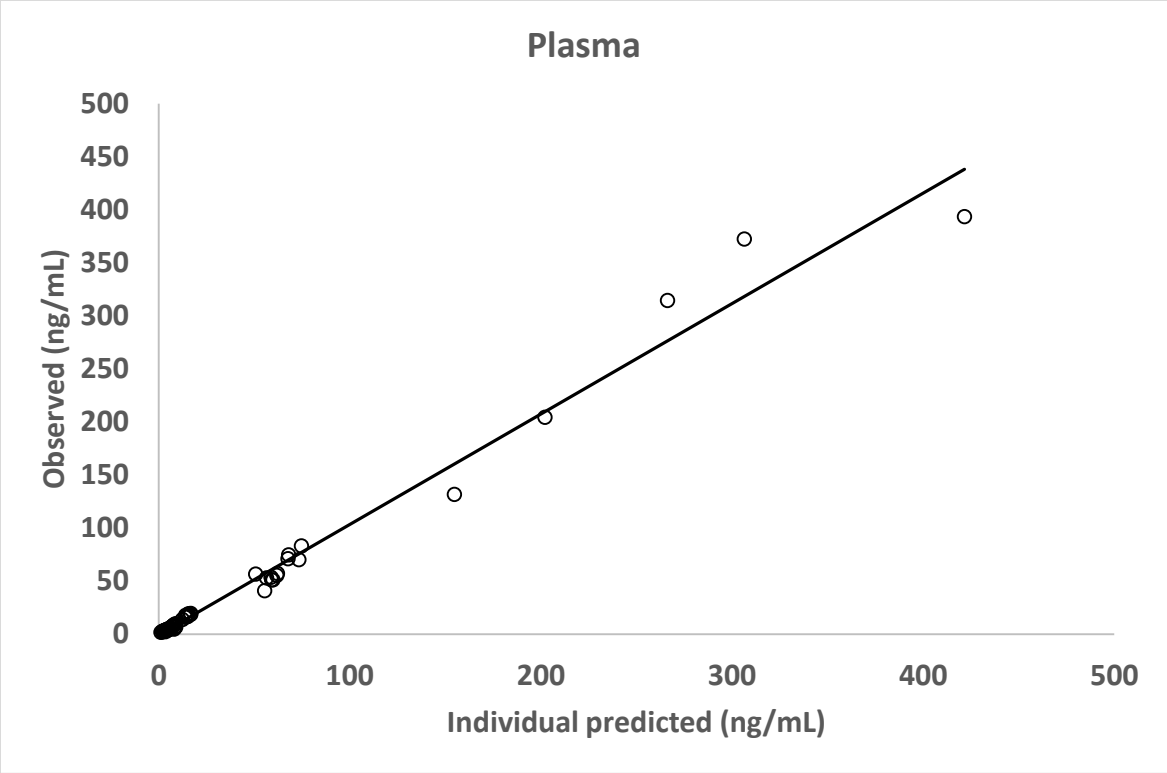


Figure 3.1 – Schematic representing the final pharmacokinetic model for the two compartment plus PELF effect compartment for concentration of gamithromycin in feedlot cattle diagnosed with bovine respiratory disease.



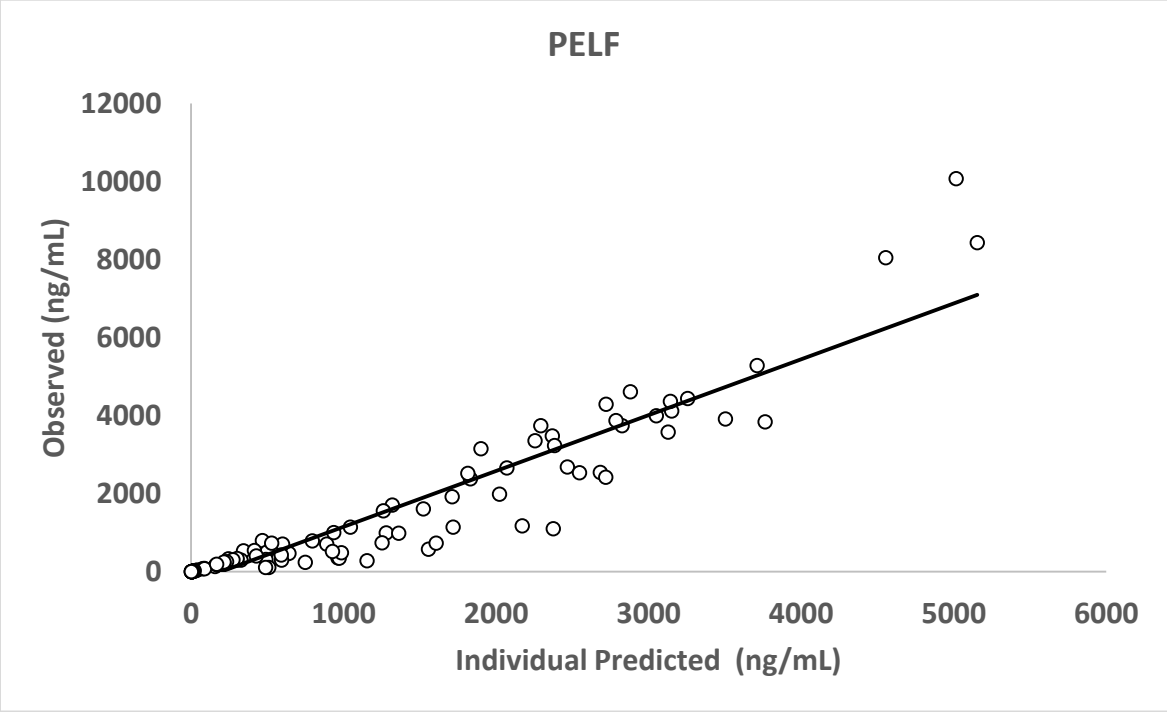


Figure 3.2 – Goodness of fit plots for model predicted data versus actual observed data from two previously published studies (Huang, Letendre et al. 2010; Giguere, Huang et al. 2011) and data obtained from personal communication with one of the co-authors (RKT).

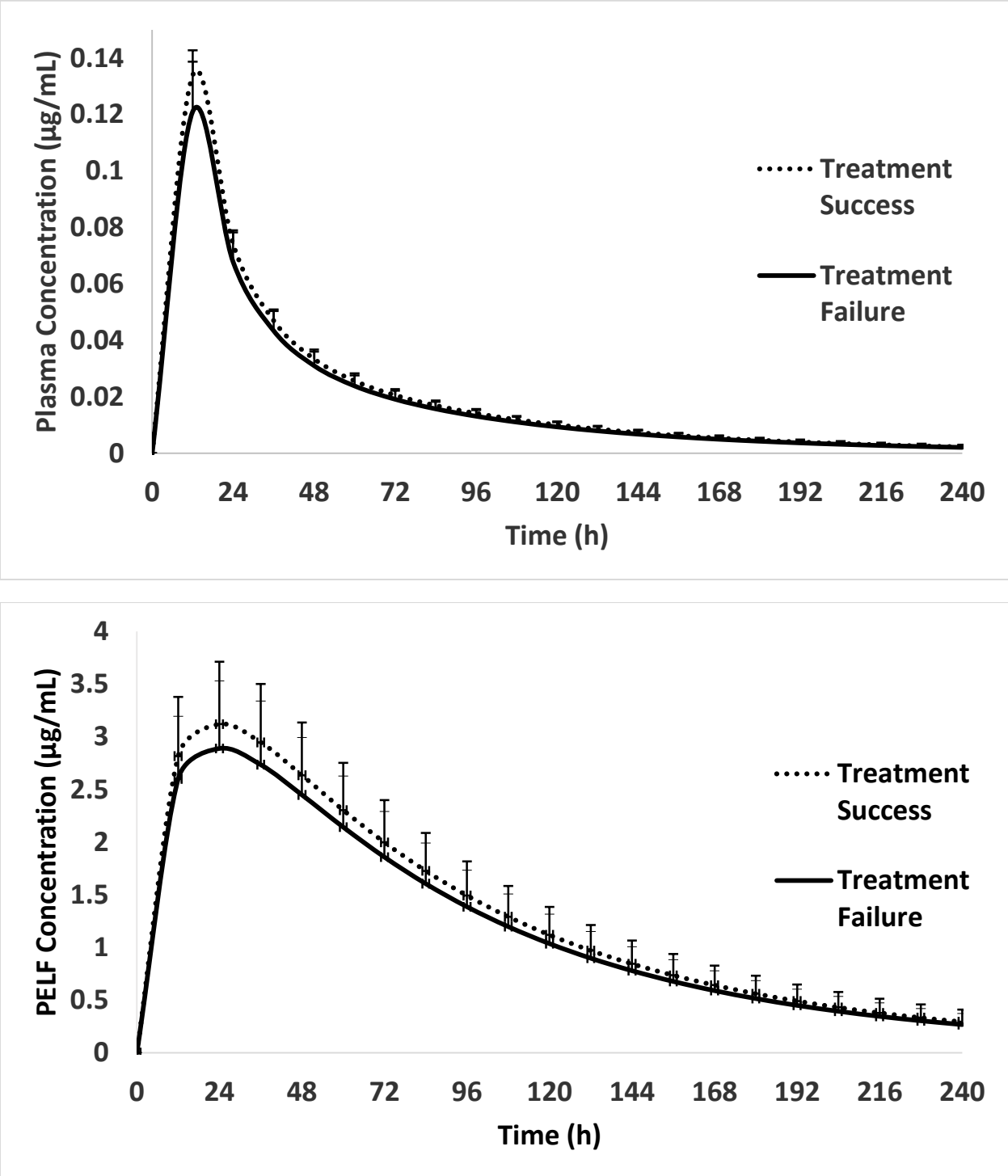


Figure 3.3 – Full simulated time concentration curves for plasma and PELF. Curve comparisons are by treatment outcome, error bars represent standard error. Statistical comparison yielded no significant differences between outcomes in maximum concentration (plasma P=0.12, PELF P=0.22).

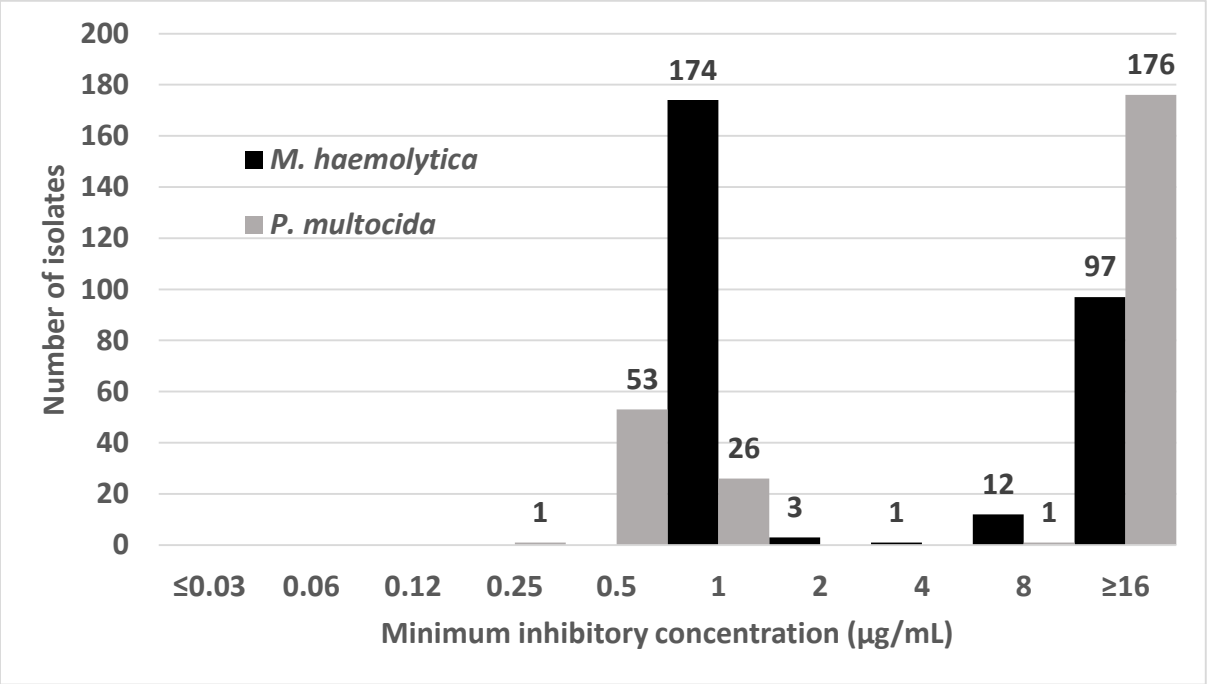


Figure 3.4 – Frequency distribution of clinical isolates of *Mannheimia haemolytica* (n=287) and *Pasteurella multocida* (n=257) MIC to gamithromycin cultured from bronchoalveolar and nasopharyngeal samples from cattle diagnosed with bovine respiratory disease. Samples were collected at time 0, 12 hrs, 24 hrs, and 120 hrs post treatment. Numbers above bars represent isolate number at that respective MIC dilution.

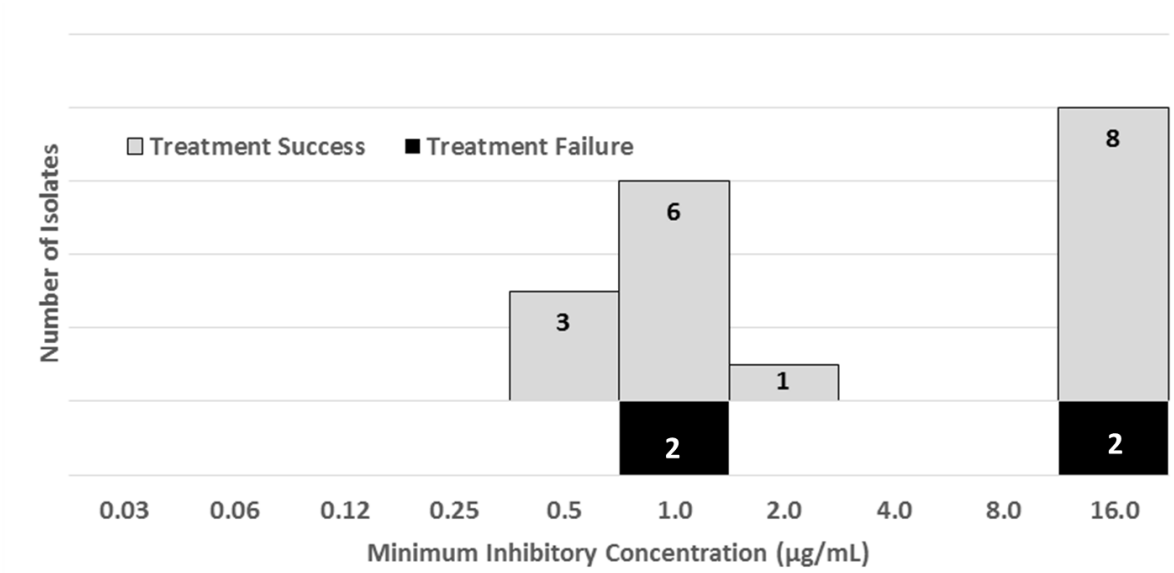


Figure 3.5 - Bivariate histogram of MIC distribution of *Mannheimia haemolytica* and *Pasteurella multocida* cultured from bronchoalveolar and nasopharyngeal samples just prior to treatment (time 0) for bovine respiratory disease in 26 head of cattle (4 calves did not yield an isolate at time 0).

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Chapter 4 - Comparison of minimum inhibitory concentration and macrolide resistance genes in genetically typed, clinical field isolates of *Mannheimia haemolytica*

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Running title: Comparison of MIC and WGS for *M. haemolytica* clinical isolates

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Abstract

The objective of this study was to compare the results of gamithromycin MIC testing, as determined by broth microdilution, with genomic analysis for the determination of macrolide resistance genes, in genetically similar clinical strains of *Mannheimia haemolytica* isolated from feeder cattle experiencing clinical symptoms of bovine respiratory disease (BRD). Feedlot cattle were monitored for signs of BRD for 28 days in a randomized clinical trial. As many as 12 isolates of *M. haemolytica* from each culture sample were subjected to MIC determination and genomic analysis for the determination of the macrolide resistance genes *erm*(42), *msr*(E), and *mph*(E). Genotype served as the gold-standard comparator test for susceptibility categorization in a two by two table. Twenty-six cattle were diagnosed with BRD and contributed 276 clinical strains of *M. haemolytica* for gamithromycin MIC testing. A very high sensitivity (100%), specificity (97.4%), and accuracy (98.2%) was observed in the susceptibility categorization of gamithromycin sensitivity for the *M. haemolytica* that were isolated in this study. Additionally, the positive (resistant) predictive value and negative (susceptible) predictive values of the test for this population were both quite high at 94% and 100%, respectively. In this study, WGS was utilized to compare the results of resistance genotype to the MIC categorization, or phenotype, as determined by broth microdilution technique. The broth microdilution technique proved to be a highly accurate and precise test for gamithromycin susceptibility of *M. haemolytica* in this population of cattle.

Key words:

bovine respiratory disease, feedlot cattle, antimicrobial resistance, gamithromycin

Introduction

Antimicrobial susceptibility testing is currently the test of choice when a clinician is faced with choosing an antimicrobial regimen to treat difficult to cure bacterial infections. Additionally, much attention is paid in the literature and media to antimicrobial resistance trends as measured by these testing procedures in both human and animal populations. One of the more common antimicrobial susceptibility testing methods is MIC testing by a broth microdilution. The MIC is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in a broth (or agar) dilution susceptibility test.[2] In veterinary medicine, the testing methods and interpretive criteria have been standardized by the CLSI and are described in VET01-A4.[2]

A topic of recent interest is the ability of these testing methods to demonstrate repeatable and accurate results using genetically similar clinical isolates from the same or different animals. In other words, if there was a clonal expansion of a given bacteria in a population of animals, would the results of such testing be equal across all isolates? There are few publications in the veterinary literature investigating the repeatability or accuracy of MIC testing as compared to the genotyping of specific antimicrobial resistance elements. One recently published study investigated the identification of antimicrobial resistance genes contained in *Escherichia coli* with the results of MIC testing and found a high correlation between the two methods.[20]

Considerable research has focused on antimicrobial resistance within the arena of bovine respiratory disease (BRD). Bovine respiratory disease is often cited as the number one area of economic loss to the beef industry, with estimates of annual global losses of up to $3 billion US dollars being reported previously.[21] The disease is the result of a complex interaction of stressors, viruses, and bacterial pathogens. The bacteria most often implicated in the pathogenesis of BRD are *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni.* The epidemiology

and antimicrobial resistance of these pathogens has been extensively studied yet many questions remain regarding host-pathogen interactions.

Recently, a research group utilized serotyping along with PFGE for the characterization of *M. haemolytica* isolated from the nasopharynx of cattle[13]. Although repeatability of MIC testing was not an objective of the study, they did report finding that isolates sharing the same serotype and resistance phenotype tended to cluster together in dendrogram analysis. Even though indirect and of lower power, this report does lend some evidence to the repeatability of MIC testing.

To the authors' knowledge no other reports on the accuracy of MIC, as compared to WGS in genetically similar BRD isolates exists within the veterinary literature. Owing to the paucity of this type of data, the objective of this study was to compare the results of gamithromycin MIC testing, as determined by broth microdilution, with genomic analysis for the determination of macrolide resistance genes, in genetically similar clinical strains of *M. haemolytica* isolated from either nasopharyngeal swabs (NPS) or bronchoalveolar lavage fluid (BAL) of feeder cattle experiencing clinical symptoms of bovine respiratory disease (BRD).

Materials and methods

Animals and husbandry

One hundred and eighty cattle perceived to be at a high risk (lightweight (165-269 kgs), commingled, and long transportation) for developing BRD were sourced from Athens, Tennessee (n=60), Richmond, Kentucky (n=60), and Maryville, Missouri (n=60) as part of another study.[3] All sources contained commingled steers and bulls of multiple origins and mixed breeds. This study was approved by the Kansas State University Institutional Animal Care and Use Committee.

Treatment allocation

Cattle were randomized to either treatment for control of BRD, also known as mass medication (MM), with gamithromycin (6 mg/kg subcutaneously in the neck) or a sham injection of saline (CON) administered upon arrival to the feedlot in Manhattan, KS. Allocation was randomized within source (two pens per source (MM and CON) for a total of six pens in the study).

Clinical scoring and disease diagnosis

Daily pen observations were performed by a veterinarian masked to treatment allocation and case outcome utilizing a previously published clinical scoring system.[3] Diagnosis of BRD was determined based on cattle having a rectal temperature of ≥40.0˚C (≥104.0˚F) and a clinical score of ≥1 (general signs of depression and weakness). Cattle assigned a clinical score of ≥1 but not meeting the rectal temperature requirements were returned to their home pen without treatment and further observation.

Sampling allocation and collection procedures

The day of BRD diagnosis served as Day 0 for each calf and included either a bilateral NPS collection, BAL collection, or both as determined by a randomized sample collection scheme utilized to meet the objectives of another study.[3] Samples collected on Day 0 were collected just prior to treatment with gamithromycin and at 12 or 24 hours post administration. All cattle had both NPS and BAL collected at 120 hours post-administration of gamithromycin. The veterinarian responsible for clinical scoring was not present for treatments and procedures performed at the chute and therefore remained masked throughout the duration of the study period. Nasopharyngeal swabs and BAL fluid were immediately sent for bacterial culture.

The BAL procedure was performed on non-sedated, manually restrained cattle. The BAL tube[a] was introduced into the trachea via the nasal passage and advanced blindly until wedged into

a deep bronchus. Sterile saline (240 mL) was infused in 60 mL aliquots and aspirated immediately after each aliquot, starting with the second aliquot. Recovered BAL fluid was divided evenly among four 50 mL centrifuge tubes, then placed on ice and centrifuged in the feedlot laboratory. Following centrifugation, one randomly selected cell pellet was resuspended in liquid Amies media and submitted for bacterial isolation. The BAL tubes were cleaned and sterilized by gas plasma sterilization before the next collection procedure to prevent cross contamination.

Nasopharyngeal swabs were collected from both nares by a veterinarian trained in the procedure utilizing a previously described method.[3] After collection, each swab was placed in individual liquid Amies media and transported on ice for bacterial culture and isolation.

M. haemolytica isolation and MIC determination

Nasopharyngeal swabs and BAL samples were plated directly onto trypticase soy + 5% blood, chocolate, and MacConkey agar plates and incubated in 5% CO_2 at 37˚C for 18-24 hours. Up to 12 colonies displaying growth characteristics typical of *M. haemolytica* were further isolated in pure culture from each calf. Confirmation of isolate identity was performed with MALDI-TOF[b] and frozen for subsequent susceptibility testing.

Gamithromycin susceptibility testing of *M. haemolytica* isolates was determined by personnel masked to treatment group by a broth microdilution technique utilizing frozen panels[c]. The custom plates contained gamithromycin at concentrations ranging from 0.03 to 16 µg/mL. Suspensions of *M. haemolytica* were prepared and susceptibility plates inoculated as per VET01-A4, section 12.1.[2] Briefly, isolates were cultured on chocolate agar and incubated with increased CO_2 at 37˚C for 18-20 hours. Suspensions of *M. haemolytica* equivalent to 0.5 McFarland standard were made by suspending 3 to 5 isolated colonies from each plate, into 5 mL of demineralized water. Mueller-Hinton broth tubes (11 mL) were then inoculated with 140 µl of the resulting *M.*

haemolytica suspensions. A 12 channel pipette was used to dispense 50 µl of this suspension into each of 12 wells in the plate, such that the susceptibility of 8 strains could be evaluated per plate. Plates were sealed with seal strips and incubated at 37˚C for 18-20 hours at which time the plates were visually inspected and MIC values determined by noting the lowest concentration of antibiotic that completely inhibited growth. Interpretation of susceptible, intermediate or resistant was determined using clinical breakpoints established by CLSI. Gamithromycin breakpoints have been reported for *M. haemolytica, Pasteurella multocida,* and *Histophilus somni* at ≤4.0, 8.0, ≥16.0 µg/mL for susceptible, intermediate, and resistant, respectively (RK Tessman, Merial, personal communication). *Staphylococcus aureus* ATCC 29213 was used for quality control in this study.

***M. haemolytica* genomic sequencing, bioinformatic, and phylogenetic analyses**

A manuscript describing the sequencing and analyses of the isolates described in this study, as well as an additional 800 isolates is currently in production. Briefly, single colony isolates of *M. haemolytica* were grown overnight in 1 ml of Brain Heart Infusion (BHI) broth with no shaking in 96 deep well blocks. DNA extractions were performed with DNA kits according to the manufacturer's instructions[d]. The DNA samples were quantified with a fluoromoter[e]. DNA libraries were constructed with original A Indices kits and sequenced on a DNA sequencer[f]. A minimum of 10X genome coverage was obtained for each isolate sequenced. Each library was mapped to an available closed circular *M. haemolytica* genome available in GenBank (CP004752),[10] using the Bowtie 2 mapping algorithm for SNP identification. Bootstrapped Neighbor-Joining phylogenetic trees were constructed from concatenated SNP genotypes of each isolate using an F84 substitution model in PHYLIP (version 3.69).[6] The trees were viewed with Dendroscope (version 3.2.10).[11] Additionally, the raw sequence reads were mapped to previously described integrative and conjugative elements (ICEs) identified in the genomes of two *M.*

haemolytica isolates (GenBank # CP004752 and CP005383.1), and one *P. multocida* (GenBank # NC_016808).[4] This was performed to identify antibiotic resistance genes within the mapped libraries that were homologous to those harbored on the reference ICEs, including the macrolide resistance genes *erm*(42), *msr*(E), and *mph*(E).

Comparing gamithromycin MICs to the presence or absence of macrolide resistance genes erm(42), msr(E)-mph(E)

In order to compare the susceptibility determination (susceptible/intermediate or resistant), genotype, as defined as the presence or absence of macrolide resistance genes *erm*(42) and/or *msr*(E)-*mph*(E), was considered the gold standard. It was assumed that the presence of the *erm*(42) and/or *msr*(E)-*mph*(E) should result in the expression of phenotypic resistance. As such, the phenotype as determined by MIC testing was compared to the presence or absence of *erm*(42) and/or *msr*(E)-*mph*(E) for all *M. haemolytica* isolates.

A two by two table was constructed as shown in Table 1 to calculate MIC test sensitivity, specificity, and positive and negative predictive values as compared to the gold standard (genotype). Sensitivity was calculated as True Positive / (True Positive + False Negative). Specificity was calculated as True Negative / (False Positive + True Negative). Positive (resistant) predictive value was calculated as True Positive / (True Positive + False Positive). Negative (susceptible) predictive value was calculated as False Negative / (False Negative + True Negative). The accuracy was calculated as (True Positive + True Negative) / Total of Isolates. The sensitivity in this study was defined as the proportion of the isolates that were truly resistant by genotyping that were correctly identified as resistant by MIC determination. The specificity was defined as the proportion of truly susceptible isolates that were correctly identified as susceptible. The positive predictive value, for resistance, was defined as the probability that an isolate was truly resistant if

the MIC testing determined it resistant. The negative predictive value, for susceptibility, was defined as the probability that an isolate was truly susceptible if the MIC testing determined it susceptible. Further, for the purposes of this publication, a minor error was defined as an intermediate result from MIC testing in an isolate with or without genotypic resistance. A major error was defined as a MIC determination of resistant without a genotype for resistance, and a very major error was defined as an MIC determination of susceptible with a genotype for resistance.

Analysis

All samples were entered into a laboratory information management system (LIMS) for sample accountability and to ensure biologic samples were not inadvertently lost to follow-up. Output was immediately either exported or transcribed into digital spreadsheets for storage and interpretation. Due to the nature of the data, limited sample size, and objective of this publication, statistics were limited to descriptive summary and interpretation only.

Results

Twenty-six cattle were diagnosed as having BRD during the course of the study. Fourteen of the 26 cattle (53.8%) diagnosed with BRD yielded a positive *M. haemolytica* (presence of at least one isolate) culture from either the nasopharynx or the lungs at some point in the sampling period. Eleven of the 14 cattle were from the CON group and the remaining three cattle were from the MM group. The range of isolates from both sample sources (24 maximum possible isolates) per calf per time point was from 0 – 24 isolates with a median of 6 isolates/calf.

In total, 287 isolates of *M. haemolytica* were collected from the NPS and BAL samples throughout the study. The MIC distribution for all of the isolates collected in this study can be found in Figure 1. As many as 12 colonies were selected per culture per sample source (NPS or BAL), therefore, Figure 1 represents MIC determination of several isolates per calf.

Among the cattle from which *M. haemolytica* was cultured, 10 of 14 yielded isolates by NPS and/or BAL samples prior to treatment (time 0 hr) with gamithromycin; three at 12 hours, zero at 24 hours, and nine cattle gave positive culture results at 120 hours post treatment. The relative frequency of isolation of *M. haemolytica*, and the number of cattle sampled at each time point, are displayed for each sample source, and genetic subtype in Table 2.

The 287 isolates were subjected to WGS, however, 11 isolates were removed from further analysis due to difficulty late in the sequencing process. Based on SNP profiles identified from WGS, the remaining isolates fell into one of two distinct phylogenetic clades. Each clade was further divided into subtypes based on clustering patterns and strong bootstrap support (Figure 2). Isolates placed within subtypes were accepted as highly related by their SNP profiles.

Minimum inhibitory concentration by genetic subtype can be found in Table 3. Five of the clonal populations had MIC determination spread across the susceptible, intermediate, or resistant categories (1f pre-treatment, and 1i, 2b, 2c, and 2d post-treatment). Comparison of the phenotype to the genotype was performed for all of the 276 *M. haemolytica* isolates. There were 16 discrepancies (16 individual isolates from 5 cattle) between phenotypic MIC determination and genotype and each were from isolates collected post-treatment. The animal identification number, sample source (BAL or NPS), treatment group, *M. haemolytica* subtype, MIC determination, and status of the *msr*(E)-*mph*(E) for each of the isolates that had a mismatch of phenotype and genotype may be found in Table 4. Interestingly, each of the isolates which displayed resistance that were not from the 2b subtype (a single isolate from each of subtype 1f, 1i, 2c, and 2d) reverted to susceptible (MIC=1 μg/mL, data not shown) on subsequent MIC testing as part of another experiment. The repeated testing (isolates frozen between testing from late Fall of 2013 to early spring of 2015) for that experiment was targeted at the isolates with an MIC of 16 μg/mL.

However, given that the initial call was resistant, no lab errors could be identified, the retesting was biased and un-blinded, and not all isolates could feasibly be retested, they remained to be interpreted as resistant for this publication.

The *erm*(42) gene was not found in any of the *M. haemolytica* isolated in this study. The isolates in 1f and 1i were predominately susceptible with an MIC value of 1.0, the one isolate from each of these two subtypes that resulted in an MIC of ≥16.0 appears to be either misclassified (due to the absence of the *msr*(E)-*mph*(E) genes) by MIC testing or displayed phenotypic resistance through another mechanism. Isolates of subtype 2b (n=88) resulted in 86 resistant (≥16.0 μg/mL) and two susceptible (1.0 μg/mL) MIC determinations. The calf that yielded the two susceptible isolates (post-treatment) also had 17 other 2b subtype isolates collected post-treatment that were phenotypically resistant. Interestingly, of those 19 2b isolates, three of them did not contain the *msr*(E)-*mph*(E) genes; two of those three 2b isolates lacking resistance genes resulted in the susceptible determinations described above, and the third isolate not containing the resistance genes had an MIC of ≥16.0 μg/mL. All other isolates of the 2b subtype that were called resistant had the *msr*(E)-*mph*(E) genes, and were expressing it phenotypically.

Isolates of the subtype 2c (n=13) were predominately susceptible, with MICs of 1.0 (10 isolates), 2.0 (1 isolate), and 4.0 μg/mL (1 isolate). The one 2c isolate that resulted in an MIC of ≥16.0 μg/mL also did not contain the macrolide resistance genes. The isolates in subtype 2d (n=18) were scattered across each of the categories, with six isolates in the susceptible (1.0 μg/mL), eleven isolates in the intermediate zone (8.0 μg/mL), and one isolate determined to be resistant (≥16.0 μg/mL). The subtype 2d were the only isolates returning a result of intermediate. Once again, all of the isolates from the subtype 2d lacked the macrolide resistance genes evaluated in this study.

The two by two table populated with the *M. haemolytica* isolate results from this study can be found in Table 5. Additionally in Table 5 are the calculated sensitivity, specificity, accuracy, positive predictive value, negative predictive value, minor error, major error, and very major errors rates.

Discussion

We report a positive *M. haemolytica* culture rate (53.8%) similar to previously published study.[19] In that report they found 65% of samples from nasal swabs, transtracheal washes, or both sampling sources to contain at least one *M. haemolytica* isolate across sampling time points in a study of newly received beef bulls at French fattening operations.

The data reported herein found little variability in MIC categorization as determined by a broth microdilution technique when compared to genotype. Of the 276 *M. haemolytica* isolates in this report, only 16 isolates (5.8%) appear to be misclassified by comparing the phenotype to the genotype. That can be further broken down into a minor error rate of 4.0%, a major error rate of 1.8%, and no very major errors. Further, our assumption was that bacteria with the genotype for resistance would result in the expression of phenotypic resistance and in this investigation it was found to be true in every instance. All 85 isolates found to contain the macrolide resistance genes *msr*(E)-*mph*(E) had gamithromycin MIC determinations of ≥16 µg/mL.

All of the minor errors arose from a single subtype isolated from one calf. These were in subtype 2d from the post-treatment samples and as can be seen in Table 2, are spread across all three categories (S, I, and R). Without the use of molecular techniques it would be difficult to determine if there was indeed a correct or incorrect categorization in this subgrouping. In a recent study, researchers reported six isolates of *M. haemolytica* that contained the *erm*(42) but not the *msr*(E)-*mph*(E) genes to have an MIC range of 4 (n=1) to 8 (n=5) µg/mL.[17] However, with the

techniques used in this study it was determined that none of the 2d isolates contained the *erm*(42) or *msr*(E)-*mph*(E) genes.

There are several possible reasons for the discrepancy observed in the 2d isolates and the other (1f, 1i, 2b, 2c) discrepant results in this study. First, it is possible that another mechanism of resistance, such as a *de novo* mutation, was present in some of the isolates in this subtype which was undetected by our techniques. In a recent report, one *M. haemolytica* (strain Mh14717) and three *P. multocida* (strains Pm14424, Pm14421, Pm14426) that were confirmed negative for the *erm*(42) and *msr*(E)-*mph*(E) displayed high macrolide resistance.[16] Those isolates were from the field and collected from cattle within Europe from 2010 – 2013. The methodology used in that investigation utilized DNA extraction and Multiplex PCR to confirm the absence of previously reported macrolide resistance genes, and subsequent genome sequencing to attempt to determine the mechanism of resistance. In all strains they reported that the observed resistance was conferred by 23S rRNA mutations and speculated that it was from *de novo* mutations followed by selective pressure (administration of macrolide antimicrobial). The isolates in the present study that were resistant and intermediate to gamithromycin but did not contain the erm(42) and *msr*(E)-*mph*(E) genes were investigated for mutations (*post hoc*) in 23S rRNA and no such mutations were found. It is possible that there are other factors or resistant elements not accounted for in this study that are responsible for the observed increase in MIC within these strains.

Another possible explanation is regarding the fact that the MIC determination and DNA sequencing were performed independently at different points in time. Although both diagnostics utilized the same bacterial stock, it is possible that either a mixed culture of two different subtypes of *M. haemolytica* was present in the stock or the genotype of the strain drifted with culture passage resulting in the discrepancies observed.

A final hypothesis for the discrepancy between phenotype and genotype could be due to inherent variability in MIC determination. Even under ideal conditions MIC testing is reported to be subject to variability from sources such as inoculum, consistency of medium composition, incubation temperature and atmosphere, reading of the test, among others.[7,15] Although a high degree of repeatability was observed in this current study, these limitations could help explain the variability that was observed, especially considering the sensitivity in MIC determinations that macrolides can display with even slight changes in pH and media composition.[5,8,14,15]

Overall a very high sensitivity (100%), specificity (97.4%), and accuracy (98.2%) was observed in the MIC determination of gamithromycin susceptibility for the *M. haemolytica* that were isolated in this study. Additionally, the positive predictive value and negative predictive values were both high at 94% and 100%, respectively. Positive and negative predictive values are dependent upon the prevalence of resistance in the dataset and can be limited when resistance is not present in the isolates tested.[12] Resistant strains were well represented in this study; therefore, these values can be considered as reliable indicators of the probability of the MIC determination of susceptibility to gamithromycin by broth microdilution technique being truly correct for *M. haemolytica* isolates in populations of cattle that are similar to those in this study.

Although no similar publications performing an accuracy assessment of MIC categorization of *M. haemolytica* using resistance genotype as a gold standard were found in a search of the literature, previous researchers have compared the accuracy of susceptibility testing by disk diffusion methods for both *P. multocida* and *M. haemolytica* using agar dilution testing methods as the gold standard.[1] Considering the differences in MIC testing methodologies, comparing those results to these in the current study would be inappropriate. Further, their testing did not include an antimicrobial representing the macrolide class. However, it was their opinion

that the gold standard of choice would be to utilize molecular techniques to confirm the presence or absence of resistance mechanisms.

In a study similar to the one reported here, a high correlation between resistance genotype and resistance phenotype was observed.[20] A macrolide similar to gamithromycin, azithromycin, was included in their testing panel and they reported a 100% sensitivity and specificity. However, they observed very low resistance rates, with only one of 76 strains of *E. coli* containing resistance genes to azithromycin. Although the organism in their study was *E. coli* and the antimicrobial panel was more extensive, we too report a similarly high correlation between antimicrobial resistance genotype and phenotypic expression in *M. haemolytica* for the macrolide gamithromycin. Other groups have reported similar high concordance between genotype and phenotype in human[9,18] and porcine[22] isolates using WGS.

Conclusions

In this study, WGS was utilized to determine the presence or absence of the macrolide resistance genes *msr*(E)-*mph*(E). The resulting genotype was used as a gold-standard to compare the results of gamithromycin susceptibility testing by a broth microdilution technique. The MIC testing utilized in this study proved to be a highly accurate test for *M. haemolytica* sensitivity to gamithromycin in this population of cattle. This demonstrates that WGS may be effectively used to determine the presence or absence of the resistant genotype, and could serve as a reasonable alternative to phenotypic testing procedures.

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Sources and Manufacturers

[a]Portex Bivonna, Smiths Medical, St. Paul, MN, USA

[b]Bruker Daltonics, Billerica, MA, USA

[c]TREK Diagnostic Systems, Thermo-Fisher Scientific Inc., Waltham, MA, USA

[d]Mo Bio Ultra Clean, Carlsbad, CA, USA

[e]Promega Quantus, Madison, WI, USA

[f]Illumina MiSeq DNA Sequencer, San Diego, CA, USA

Declaration of conflicting interests

R.K.T. is an employee of Merial. Other authors: none to declare.

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Figures and Tables

Table 4.1 – Two by two layout of genotype and phenotype comparison used to calculate the sensitivity, specificity, and positive and negative predictive values of the minimum inhibitory concentration determinations for *M. haemolytica* isolates in this study. Genotype was determined using WGS and phenotype was determined via broth microdilution.*

		Genotype	
		R	I/S
Phenotype	R	True Positive	False Positive
	I/S	False Negative	True Negative

*R = Resistant; I/S = Intermediate/Susceptible

Table 4.2 – Number of cattle for which at least one *M. haemolytica* was isolated, by time point following diagnosis of bovine respiratory disease and treatment at time zero with gamithromycin. Isolates are reported by genetic subtype (left column) and as a total of all M. haemolytica isolates. The percentage (%) is equal to the number of cattle culture positive/the number of cattle sampled at each time point (N).*

	Nasopharyngeal swab				Bronchoalveolar lavage fluid			
Subtype	0 hr	12 hr	24 hr	120 hr	0 hr	12 hr	24 hr	120 hr
1c	1 (4%)	-	-	-	-	-	-	-
1e	1 (4%)	-	-	-	-	-	-	-
1f	1 (4%)	-	-	2 (8%)	-	-	-	1 (4%)
1i	1 (4%)	1 (11%)	-	1 (4%)	-	1 (11%)	-	-
2b	3 (12%)	-	-	4 (15%)	2 (25%)	-	-	4 (15%)
2c	2 (8%)	1 (11%)	-	1 (4%)	-	1 (11%)	-	-
2d	2 (8%)	1 (11%)	-	1 (4%)	-	-	-	1 (4%)
Total	11* (42%)	3 (33%)	0	9 (35%)	2 (25%)	2 (22%)	0	6 (23%)
N	26	9	9	26	8	9	9	26

* Ten cattle contributed eleven isolates at this time point (i.e. one calf contributed both a 1c and a 2b and is therefore represented twice in this total)

Table 4.3 – MIC distribution for each genetic subtype of *M. haemolytica* as determined for isolates collected pre-treatment with gamithromycin (Pre-Tx) or isolates collected at 12 and 120 hours after treatment with gamithromycin (Post-Tx). The subtype in the left hand column represents the clade and subtype for each group of isolates. *

		Minimum Inhibitory Concentration (μg/mL)									
Subtype	Time Point	≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	≥16
1c	Pre-Tx	-	-	-	-	-	1	-	-	-	-
	Post-Tx	-	-	-	-	-	-	-	-	-	-
1e	Pre-Tx	-	-	-	-	-	2	2	-	-	-
	Post-Tx	-	-	-	-	-	-	-	-	-	-
1f	Pre-Tx	-	-	-	-	-	11	-	-	-	1
	Post-Tx	-	-	-	-	-	19	-	-	-	-
1i	Pre-Tx	-	-	-	-	-	12	-	-	-	-
	Post-Tx	-	-	-	-	-	19	-	-	-	1
2a	Pre-Tx	-	-	-	-	-	1	-	-	-	-
	Post-Tx	-	-	-	-	-	-	-	-	-	-
2b	Pre-Tx	-	-	-	-	-	42	-	-	-	-
	Post-Tx	-	-	-	-	-	2	-	-	-	86
2c	Pre-Tx	-	-	-	-	-	24	-	-	-	-
	Post-Tx	-	-	-	-	-	10	1	1	-	1
2d	Pre-Tx	-	-	-	-	-	22	-	-	-	-
	Post-Tx	-	-	-	-	-	6	-	-	11	1

*Pre-Tx = Pre-treatment; Post-Tx = Post-treatment

Table 4.4 – Sample source, treatment group, *M. haemolytica* subtype, MIC determination and status of resistance genes for each of the isolates whose genotype did not match their phenotype. (No isolates in this study contained the erm(42) gene).*

Animal ID	Sample Source	Treatment	Subtype	MIC	*msr(E)-mph(E)*
104	NPS	CON	1f	≥16	N
175	NPS	CON	1i	≥16	N
222	NPS	CON	2b	≥16	N
206	NPS	MM	2c	≥16	N
243	BAL	CON	2d	≥16	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	BAL	CON	2d	8	N
	NPS	CON	2d	8	N

*NPS = nasopharyngeal swab, BAL = bronchoalveolar lavage fluid; CON = control treatment group; MM = mass medication with gamithromycin

Table 4.5 – Two by two table of genotype versus phenotype test outcome for the *M. haemolytica* isolates in this study. A minor error was defined as an intermediate result from MIC testing in an isolate with or without genotypic resistance. A major error was defined as a MIC determination of resistant without a genotype for resistance, and a very major error was defined as an MIC determination of susceptible with a genotype for resistance. Calculated values are shown as percentages.*

		Genotype	
		R	I/S
Phenotype	R	85	5
	I/S	0	186

Sensitivity	100%
Specificity	97.4%
Accuracy	98.2%
Positive Predictive Value	94%
Negative Predictive Value	100%
Minor error	4.0%
Major error	1.8%
Very major error	0%

*R = Resistant; I/S = Intermediate/Susceptible

Figure 4.1 – MIC to gamithromycin of *M. haemolytica* (n=276) from cattle treated for BRD. Isolates represent multiple isolates per calf, from multiple time points (0, 12, and 120 hours post administration of gamithromycin) collected from both nasopharyngeal swabs and bronchoalveolar lavage fluid.

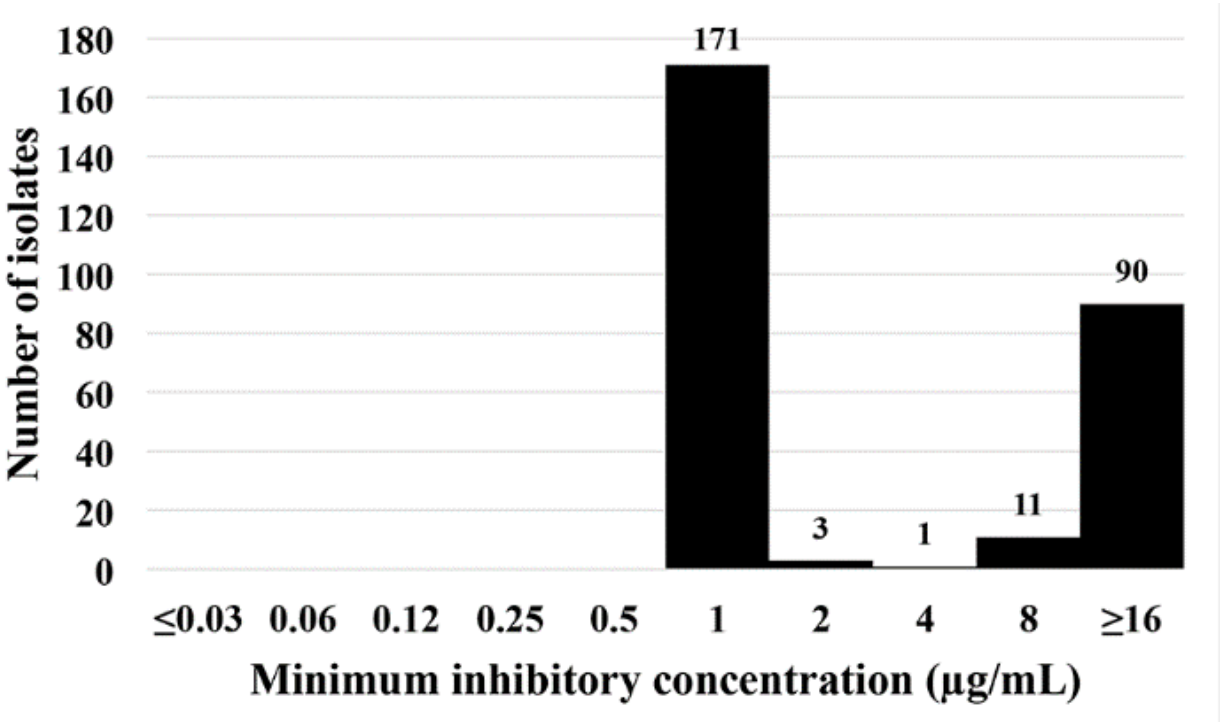
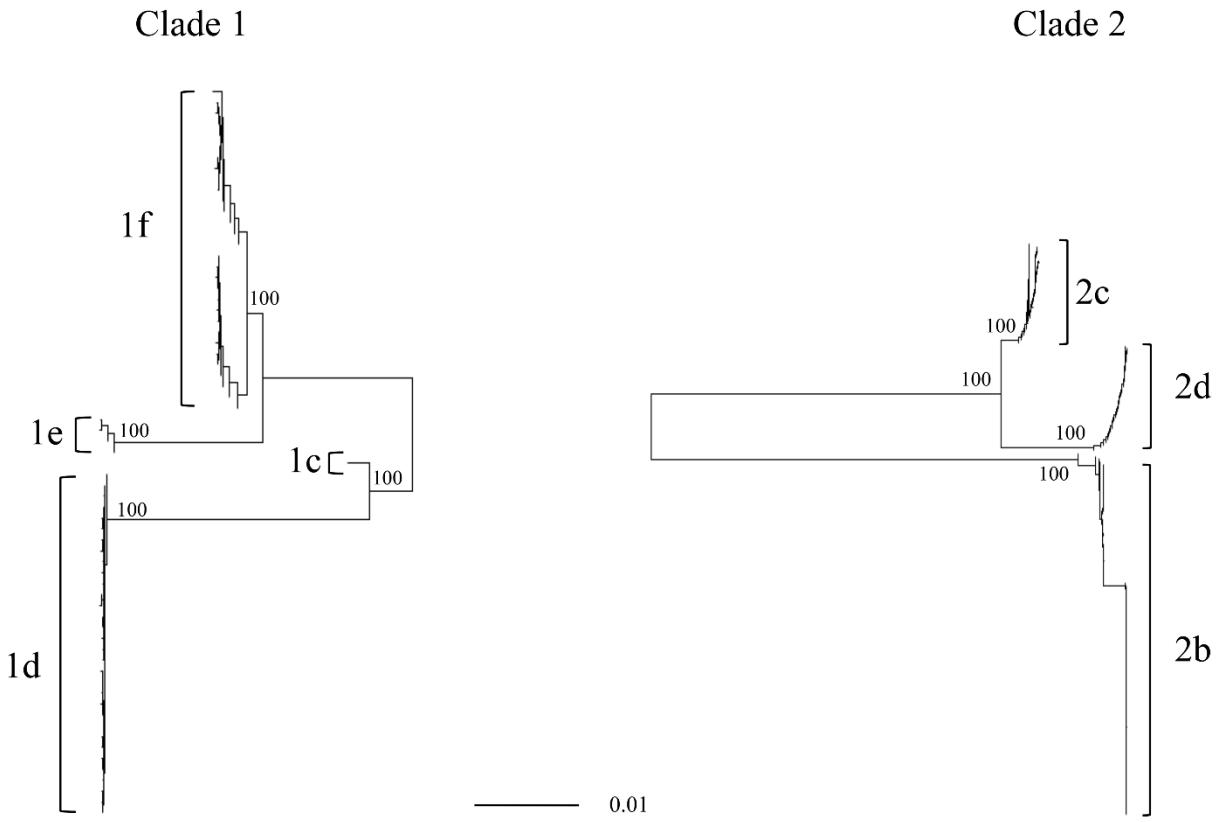


Figure 4.2- Neighbor-Joining trees of *M. haemolytica* clades 1 and 2 based on genome-wide concatenated SNPs from 276 isolates. Subtypes within the clades are denoted by lower case letters. Numbers beside internal nodes of the tree represent bootstrap percentage values from 100 pseudo-alignments. The scale bar represents substitutions per site within trees. The genetic distance between clades 1 and 2 exceeds the genetic distance within the two clades and is not shown.



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Chapter 5 - Characterization of macrolide resistance in bovine respiratory disease clinical field isolates following mass medication and/or treatment with gamithromycin

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Running title: Gamithromycin resistance in BRD field isolates

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Abstract

The first objective of this work was to describe gamithromycin susceptibility of *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni* isolated from cattle diagnosed with bovine respiratory disease (BRD), post-treatment and/or mass medication with this antimicrobial. The second objective was to characterize the macrolide resistance genes present in genetically subtyped *M. haemolytica* isolated from cattle that had either been treated on arrival for control of BRD (MM) or sham saline injection (CON). *M. haemolytica* (n=276), *P. multocida* (n=253) and *H. somni* (n=78) were isolated from feedlot cattle diagnosed with BRD. Determination of susceptibility to gamithromycin was performed by broth microdilution. Whole genome sequencing was utilized to determine the presence or absence of macrolide resistance genes in *M. haemolytica* isolates. Generalized linear mixed models were built for data analysis. Resistance was overrepresented by the 2b subtype of *M. haemolytica.* There was not a significant difference between MM and CON groups in regards to the likelihood of culturing a resistant or intermediate isolate of *M. haemolytica* or *P. multocida*. The likelihood of culturing a resistant or intermediate isolate of *M. haemolytica* differed significantly by state of origin and further investigation in this area is warranted. A single *M. haemolytica* genetic subtype correlated with nearly all of the observed resistance. Additional studies to elucidate the relationships between phenotypic and genotypic resistance and clinical response to antimicrobials are necessary to inform judicious use of antimicrobials in the context of relieving animal disease and suffering.

Key words:

antimicrobial resistance, bovine respiratory disease, Mannheimia haemolytica, Pasteurella multocida, Histophilus somni

Introduction

Antimicrobial administration has been the mainstay for both the treatment and control of BRD for several decades. Considering the ability of bacteria to adapt and survive in changing environmental conditions, it comes as no surprise that the bacterial pathogens associated with BRD are now commonly found to be resistant to these antimicrobials.[6]

Resistance to antimicrobials is either intrinsic or acquired. Intrinsic resistance is the inherent ability of a bacterium to resist the action of a particular antimicrobial agent or class of agents through characteristics that prevent the mode of action of the agent. For example; an anaerobic bacterium would be intrinsically resistant to the aminoglycosides because the bacterium would lack the oxidative metabolism necessary to transport the drug inside the cell, thus preventing the drug binding to the 30S ribosomal subunit and killing the bacteria. Acquired resistance comes from either a genomic mutation or through the acquisition of resistance genes via horizontal transfer (transduction, conjugation, or transformation).[32]

Conjugation requires direct contact between the donor and recipient cell in order for transfer of DNA to occur. Typically the DNA is brought into the cell as a single strand and then converted to double-stranded DNA by the recipient cell. Within the last several years, the presence of integrative conjugative elements (ICE) have been recognized in bacteria of the *Pasteurellacae* family.[12,22,25] These elements are mobile genetic elements that mediate their own excision from the host chromosome, form a circular intermediate and encode their own machinery to transfer themselves by conjugation, and are then able to integrate and replicate as a part of the host chromosome.[32]

The first report on macrolide resistance at the molecular level among BRD pathogens was published in 2011.[9] This was an investigation of field isolates of *M. haemolytica* and *P. multocida*

that were reported as highly resistant to lincosamides and moderately resistant to macrolides, a resistance phenotype commonly termed Type I MLS_B. DNA sequence analysis revealed that none of the resistant isolates had mutations in their ribosomal protein or rRNA components (consistent with Type I MLS_B phenotype), but all isolates were monomethylated on the 23S rRNA nucleotide A2058 which indicated that they had an *erm* gene. Continued genetic analysis suggested that this novel *erm*(42) gene had been acquired from other members of the *Pasteurellaceae* family and was a recent gene transfer to *M. haemolytica* and *P. multocida.* Further, they hypothesized that due to *erm*(42) bearing such little resemblance to members of the *erm* gene family (the closest being a 39% identity in the core sequence) its divergence must have been an ancient evolutionary event predating the existence of MLS_B antimicrobials.

In a nearly simultaneously published report, researchers reported on three macrolide resistance genes found in a strain of *P. multocida* (strain 36950) isolated from a calf in a Nebraska feedlot in 2005.[19] This particular strain had shown resistance to most antimicrobials approved for treatment of BRD and prior to this investigation, the mechanism of resistance had not been determined. Performing whole genome sequencing (WGS) and a series of cloning experiments they discovered three macrolide resistance genes, (the first of which was described previously[9]) *erm*(42), *msr*(E), and *mph*(E). The *erm*(42) gene codes for an rRNA methylase gene, the *msr*(E) gene codes for an ABC transporter protein, and the *mph*(E) gene codes for a macrolide phosphotransferase protein.[19,25] The *msr*(E) and *mph*(E) genes are organized in an operon-like structure and separated only by a non-coding spacer sequence of 55 base pairs.

Working further with the same strain of *P. multocida* (strain 36950) isolated from a Nebraska feedlot in 2005, it was found that the macrolide resistance genes were actually a small part of an ICE.[25] Through WGS, these researchers described two resistance gene regions, termed

153 ICE*Pmu*1 and ICE*Pmu*2, which contained a total of 12 resistance genes. These two regions, among
154 others, contained tetracycline resistance genes that had previously been reported to be present
155 almost exclusively on plasmids; florfenicol and aminoglycoside resistance genes that have rarely
156 been seen in BRD pathogens; and resistance genes (macrolide, gentamicin, spectinomycin,
157 streptomycin, and β-lactam) novel to *P. multocida*. These findings led the authors to conclude that
158 *P. multocida* is able to acquire resistance from other Gram-negative bacteria, incorporate it into its
159 own DNA, and use these genes to phenotypically express resistance.

160 A recent study investigated the effects of these three genes on the MICs of gamithromycin
161 and tildipirosin.[24] In both *P. multocida* and *M. haemolytica* they reported a pronounced increase
162 in MICs to gamithromycin for those isolates containing *msr*(E)-*mph*(E) and a marked increase in
163 MICs to tildipirosin for *erm*(42) containing isolates. The subset of isolates that harbored all three
164 genes displayed increased MICs to both of the newer macrolides in their study. Similar work found
165 the same to be true; greatly elevated MICs for tildipirosin and tilmicosin in the presence of *erm*(42)
166 and greatly elevated MICs for tilmicosin, tulathromycin and gamithromycin in the presence of
167 *msr*(E)-*mph*(E).[28] These two studies remain the only publications to investigate the molecular
168 effects of the macrolide resistance genes on BRD bacterial pathogen sensitivity to gamithromycin.

169 Therefore, the objectives of this work were; first, to describe gamithromycin susceptibility
170 of *M. haemolytica, P. multocida,* and *H. somni* isolated from cattle diagnosed with bovine
171 respiratory disease (BRD), post-treatment and/or mass medication with this antimicrobial, and
172 second, to characterize the macrolide resistance genes present in genetically subtyped *M.*
173 *haemolytica* isolated from cattle that had either been treated on arrival for control of BRD (MM)
174 or sham saline injection (CON).

Materials and methods

Animals and treatment allocation

As described previously, 180 cattle perceived to be at a high risk for developing BRD (165-269 kgs) were sourced from three states within the United States as part of another study.[7] The cattle were randomly allocated to receive either treatment for control of BRD (MM) with gamithromycin (6 mg/kg subcutaneously in the neck) or a sham injection of saline (CON) administered upon arrival to a research feedlot near Manhattan, Kansas. The treatments were assigned to cattle within state of source, yielding a total of six pens in the study. This study was approved by the Kansas State University Institutional Animal Care and Use Committee.

Clinical scoring and disease diagnosis

Cattle were observed for symptoms of BRD once daily in their pen for 28 days by a veterinarian masked to treatment allocation. The diagnosis of BRD was determined based on a calf having a rectal temperature of ≥40.0˚C (≥104.0˚F) and a clinical score of ≥1 (general signs of depression and weakness).[7]

Sampling allocation and collection procedures

The collection procedures of diagnostic samples from cases of BRD in this study have been described previously.[7] Nasopharyngeal swabs (NPS) were collected, bi-laterally and pooled, immediately prior to treatment (0 hour) from all cattle diagnosed with BRD. Additionally, cattle were allocated to receive a bronchoalveolar lavage at either 0, 12, or 24 hours post treatment with gamithromycin. Cattle sampled by BAL at 12 and 24 hours also were simultaneously sampled by NPS. All cattle diagnosed with BRD were sampled by means of both BAL and NPS 120 hours after treatment administration.

197 **Bacterial isolation and MIC determination**

198 After collection, NPS and BAL samples were transported on ice where they were plated
199 directly onto trypticase soy + 5% blood, chocolate, and MacConkey agar plates. The plates were
200 incubated in 5% CO_2 at 37˚C for 18-24 hours. Up to 12 colonies of *M. haemolytica* and up to 6
201 colonies of both *P. multocida* and *H. somni* displaying growth characteristics typical of each were
202 further isolated in pure culture from each calf. Isolate identity was confirmed with MALDI-TOF[a]
203 and frozen for subsequent susceptibility testing.

204 Susceptibility testing to gamithromycin was performed by personnel masked to treatment
205 group by a broth microdilution technique as previously described.[8] *Staphylococcus aureus* ATCC
206 29213 was used for quality control in this study. Determination of susceptible (≤4 μg/mL),
207 intermediate (8 μg/mL) or resistant (≥16 μg/mL) was based off of clinical breakpoints established
208 for gamithromycin by CLSI (RK Tessman, Merial, personal communication).

209 ***M. haemolytica* genomic sequencing, bioinformatic, and phylogenetic analyses**

210 The details of these methods have been previously reported.[8] Briefly, extracted DNA was
211 quantified and Illumina Nextera XT DNA libraries were constructed and sequenced (minimum
212 10X genome coverage) on an DNA sequencer[f]. Resulting libraries sequences were individually
213 mapped to an available closed circular *M. haemolytica* genome available in GenBank
214 (CP004752).[18] Bootstrapped Neighbor-Joining phylogenetic trees were constructed from SNP
215 genotypes that were identified from the mapped sequences. Additionally, antimicrobial resistance
216 genes were identified by mapping to previously described ICEs identified in two *M. haemolytica*
217 isolates (GenBank # CP004752 and CP005383.1),[18] and one *P. multocida* (GenBank #
218 NC_016808)[13] isolate.

Analysis

Intermediate and resistant isolates were lumped together to create a binomial outcome variable (either susceptible or intermediate/resistant) for purposes of analysis. Data were inputted into a statistical software[c] and generalized linear mixed models, fit by maximum-likelihood regression, were built to determine if there was an association between on-arrival treatment and an MIC determination of susceptible or intermediate/resistance. Treatment group, time, and state of origin were modeled as fixed effects and random variables were included to account for clustering of isolates within cattle and cattle within individual pens for the analysis of *M. haemolytica* data. Models including state of origin would not converge for *P. multocida* data. Neither models would converge when a treatment by sampling time point interaction was included. All attempts to model *H. somni* data were unsuccessful due to a low isolation count. A corresponding *P*-value of ≤ 0.05 was considered significant.

Results

As previously reported, 26 cattle met the clinical criteria for BRD during the course of the study.[7] There were only four first-treatment failures, two each from the MM and CON groups. The number of days on study at the time of BRD diagnosis ranged from 4 – 21 days, with a mean and median of 14 days. Of the four failures, two yielded a *M. haemolytica* isolate over the sampling period and each harbored *M. haemolytica* strains containing resistant genes at the final sampling.

There were 287 isolates of *M. haemolytica* collected throughout this study including isolates cultured from samples of both NPS and BAL. Genetic subtype data could not be retrieved for eleven isolates, leaving the total for analysis at 276 isolates of *M. haemolytica.* There were 253 isolates of *P. multocida* and 78 isolates of *H. somni* included in this descriptive analysis. The

241 breakdown of the number of cattle yielding at least one strain of each bacteria are displayed by
242 time point and sampling source in Table 1.

243 As reported previously, the 276 isolates of *M. haemolytica* subjected to WGS fell into one
244 of two distinct phylogenetic clades and each clade was further divided into subtypes based on
245 clustering patterns and strong bootstrap support (Figure 1).[8]

246 The MIC distributions of all three species of bacteria are presented in Table 2. The MIC
247 distributions are segregated by on-arrival treatment group assignment and further by whether they
248 were isolated pre- or post-treatment with gamithromycin. In this table, *M. haemolytica* MIC
249 distribution is presented irrespective of genetic subtype.

250 The cattle that were MM on arrival yielded only a single isolate (2c subtype) displaying
251 resistance to gamithromycin which was from a calf originating from Missouri which was collected
252 120 hours after treatment for BRD. Additionally, the MM cattle had only a single isolate in the
253 2b subtype. This isolate was cultured prior to treatment and categorized as susceptible with an
254 MIC of 1 μg/mL, and was negative for the both the *erm*(42) gene and the *msr*(E)-*mph*(E) operon.

255 Although there are numerical differences in the counts of resistant and intermediate *M.*
256 *haemolytica* between the MM and CON treatment groups, there was no effect of treatment (MM
257 or CON) observed on the likelihood of isolating a resistant or intermediate *M. haemolytica*
258 (P=0.41). There was, however, an observed effect of sampling time point on the likelihood of
259 isolating intermediate or resistant *M. haemolytica* (P<0.001). Samples obtained from either NPS
260 or BAL at 120 hours after treatment administration were more likely to contain resistant or
261 intermediate isolates as compared to those obtained at time 0 (OR=1,580, 95% CI 173-14,412;
262 P<0.001). However, samples obtained at 12 hours post treatment administration were not

significantly different than those obtained prior to treatment (OR=11, 95% CI 0.61-201; *P*=0.10) in regards to the likelihood of culturing resistant or intermediate isolates of *M. haemolytica.*

There was a significant effect of state of origin (P<0.001) on the likelihood of culturing a resistant or intermediate isolate of M. haemolytica. Calves originating from Tennessee were more likely (OR=14, 95% CI 0.56-374; P=0.11) to yield a resistant or intermediate *M. haemolytica* isolate as compared to calves from Missouri, albeit not a significant difference. Calves originating from Kentucky were significantly more likely (OR=446, 95% CI 47-4,232; P<0.001) to yield a resistant or intermediate isolate as compared to calves from Missouri.

No statistical differences were found between treatment groups in regards to the likelihood of isolating an intermediate or resistant *P. multocida* (*P*=0.91) and there was no observed effect of sampling time point (*P*=0.50). Models in which state of origin was included did not converge for P. multocida. Due to the low isolation counts, all attempts at models for *H. somni* were unsuccessful.

The majority of the isolates displaying resistance following treatment for BRD with gamithromycin came from three CON cattle (Table 3). Eighty-six of the 89 (96.6%) post-treatment isolates in the CON group categorized as resistant came from the 2b subtype and all of these resistant 2b isolates came from the same pen of cattle, originating from Kentucky. The three isolates not from the 2b group which displayed phenotypic resistance in CON cattle were isolated from cattle originating from the states of Missouri (1f), Tennessee (1i), and Kentucky (2d), respectively. Just a single isolate, *M. haemolytica* subtype 2c, displayed resistance in MM cattle.

Table 4 displays each *M. haemolytica* genetic subtype by MIC and the presence or absence of the macrolide resistance genes within each subtype. All isolates collected in this study were negative for the presence of the *erm*(42) gene. The only isolates in which the *msr*(E)-*mph*(E)

operon were detected were in the 2b subtype; all of the cattle yielding these isolates originated from Kentucky and the isolates displayed phenotypic resistance of MIC ≥16 µg/mL. All 2b isolates collected prior to treatment had an MIC of 1 µg/mL and were negative for the *msr*(E)-*mph*(E) operon. Eighty-five of 88 (96.6%) of the 2b subtype isolates collected after treatment with gamithromycin contained the *msr*(E)-*mph*(E) operon.

Discussion

Isolation of specific bacterial BRD pathogens from NPS prior to treatment, expressed as a percentage of cattle from which at least one isolate was recovered were 38% for *M. haemolytica*, and 65% for *P. multocida*. These percentages are similar to a previous publication, which reported 45% and 57% isolation of *M. haemolytica* and *P. multocida* from NPS samples taken prior to treatment.[29] In 1991, researchers used NPS and BAL samples to compare the flora of the upper and lower respiratory tract of feedlot cattle undergoing naturally occurring BRD.[2] They reported isolation of 32%, 47%, and 7% from the NPS and 12%, 43%, and 5% from the BAL fluid for *M. haemolytica, P. multocida,* and *H. somni*, respectively. In a nearly identical study published the following year, they reported isolation of 15%, 70%, and 10% from the NPS (estimated from graphical data) and 14%, 68%, and 12% from the BAL fluid for *M. haemolytica, P. multocida,* and *H. somni*, respectively.[1] The findings from those two studies are similar in regards to the isolation of both *M. haemolytica* and *P. multocida* in the study reported here (Table 1). The isolation percentage reported herein for *H. somni,* from both NPS and BAL is slightly higher than those found previously.[1,2] The differences in *H. somni* isolation percentages could be due to changes in culture techniques or represent a truly different prevalence of that bacteria species between the two studies.

In the study reported here, the observed effect on resistance phenotype, after administering a macrolide antimicrobial for the control of BRD on arrival versus controls was not as expected. Comparing the resistance counts in *M. haemolytica* isolates pre- and post-treatment between MM and CON treatment groups; there appeared to be a protective effect of MM with gamithromycin in regards to the lower total number of resistant isolates in MM cattle. However, when accounting for clustering of cattle within pen there was not a statistical difference between treatment groups (P = 0.41). In contrast, there was a numerically higher number of strains displaying resistance in both *P. multocida* and *H. somni* in the cattle that received MM on arrival to the feedlot. However, there was not a statistical difference between treatment groups (P = 0.91) for *P. multocida.* Statistical models for *H. somni* would not converge due to limited sample size.

There was a significant difference by state of origin in the likelihood of obtaining a resistant or intermediate isolate of *M. haemolytica.* Cattle originating from Kentucky were significantly more likely to yield a resistant or intermediate isolate as compared to cattle sourced from either Missouri or Tennessee. Although not statistically significant, cattle from Tennessee tended to be at an increased likelihood of yielding resistant or intermediate isolates as well. Reasons for this geographical trend are not clear but could include different management practices or antimicrobial usage patterns prior to the cattle being marketed and subsequently included in this study.

Comparing only the MIC distributions of the isolates collected post-treatment between the MM and CON cattle (Table 2); treatment for BRD appeared to increase the prevalence of phenotypic resistance in *M. haemolytica* isolated from CON cattle, and increase the prevalence of phenotypic resistance in *P. multocida* and *H. somni* isolated from MM cattle. It should be mentioned that the post-treatment samples were collected at three different time points and lumped together for the results and discussion. Additionally, while all 26 cattle had NPS pre-treatment,

only nine BAL samples were collected pre-treatment and 26 post-treatment. These factors could possibly confound the results in this study since there were more opportunities for isolation of resistant (or susceptible) bacteria across these three post-treatment time points. However, it is interesting to note that the observed increase of phenotypic resistance in *P. multocida* and *H. somni* did not result in numerous overt clinical failures. This is further evidence of a possible disconnect between *in vitro* testing results and treatment outcome, especially as it pertains to the macrolide class of antimicrobials, as has been previously reported.[3,20,23]

A single *M. haemolytica* subtype (2b) represented all genotypic macrolide resistance and 95.6% (86/90) of phenotypic resistance. Nearly ninety-nine percent (89/90) of the phenotypic resistance in *M. haemolytica* came from CON cattle. Just one single strain, of the 2c subtype, phenotypically expressed resistance among the isolates from the MM cattle and this strain did not harbor macrolide resistance genes. This is suggestive of a possible protective effect of gamithromycin administration for the control of BRD, in terms of the very low prevalence of resistance for *M. haemolytica* observed in the MM group. There is no clear explanation as to the over-representation of *M. haemolytica* resistance observed in the CON cattle. Perhaps there is a synergism between gamithromycin and the immune system (or other factors) when administered as a MM on arrival (i.e. to a non-clinical animal) that is not present when a calf becomes morbid with symptoms of BRD.

Macrolides are known to possess anti-inflammatory and immunomodulatory effects in addition to their antimicrobial activity.[4,20] While no such data are available on gamithromycin, another veterinary macrolide has been extensively studied in this area.[11,14-17] Additionally, azithromycin, a macrolide of the same subclass as gamithromycin, has been shown to exert anti-inflammatory properties on lung epithelial cells in humans.[21] Researchers have noted that the

administration of azithromycin to healthy human volunteers resulted in acute stimulation of neutrophil degranulation and phagocytosis-associated oxidative burst together with increases in serum interleukin-1β which the authors stated may contribute towards its antimicrobial activity.[5]

However, if credibility is given to these immunomodulatory activities as to gamithromycin's effect on *M. haemolytica*, it appeared to have no effect in regards to reducing the burden of resistance due within *P. multocida* and *H. somni* when administered to asymptomatic cattle on arrival to the feedlot in this study. In fact, a greater proportion of the isolates collected from MM cattle were resistant to gamithromycin both pre- and post-treatment than those in the CON group. The *P. multocida* and *H. somni* resistant isolates originated from 12 animals, of which just two were ultimately deemed first-treatment failures and 10 were successes.

A further intriguing observation from this study is the inability to find *M. haemolytica* isolates containing macrolide resistance genes in any samples cultured prior to treatment for BRD (Table 4), especially considering that nine of the 26 cattle had previously received a macrolide class antimicrobial as a MM for control of BRD. One conventional theory is that bacteria may contain the genetic machinery for resistance but not express it phenotypically until after selection pressure is applied (antimicrobial administration), allowing the proliferation of the resistant bacterial strains. However, we observed no macrolide resistance genes in pre-treatment isolates (from both MM and CON cattle). Yet in isolates of *M. haemolytica* that were collected after the antimicrobial was administered for treatment of BRD, we observed the presence of the *msr*(E)-*mph*(E) operon in 85 of 88 (96.6%) of the 2b subtype. It remains unclear as to why just one particular subtype contained the macrolide resistance genes and all of the others did not.

This finding could support multiple hypotheses. It is possible that a small population of *M. haemolytica* strains were present containing the macrolide resistance genotype that were not

successfully cultured because of being below a minimum level of detection out competed by more metabolically fit bacteria, or that the strains harboring the resistance genes were present in refugia, within biofilms or as persister cells, or present in an area of the respiratory tract not sampled. Additionally, it is possible that there were no *M.* haemolytica strains present containing the resistance genes prior to treatment and they were quickly acquired by horizontal transfer from other bacterial cells such as *P. multocida*, as described by Michael *et al.* in 2011.[26]

As discussed in the introduction, a previous report has investigated the effects of *erm*(42) and *msr*(E)-*mph*(E) on the MIC range of gamithromycin and tildipirosin[24]. They reported that *erm*(42), when present in *M. haemolytica* alone increased the MIC from 0.5 to 4 µg/mL and the combination of *erm*(42) and *msr*(E)-*mph*(E) resulted in an MIC increase from 0.5 to as high as 32-64 µg/mL for gamithromycin. They found similar distinct increases in MICs to tildipirosin when isolates contained the macrolide resistance gene *erm*(42). Other researchers have also observed dramatic increases in MICs for isolates harboring the macrolide resistance genes *erm*(42) and *msr*(E)-*mph*(E) for erythromycin, tilmicosin, and tulathromycin.[9,19,22] _ENREF_7 While MIC data for these other macrolides were not determined in the study reported here, a similar increase in MICs to each of those antimicrobials for the isolates containing the macrolide resistance genes would be expected. Further, given prior evidence of macrolide cross-resistance[10,19,30,31] and the fact that resistance to tulathromycin was observed in BRD isolates prior to it being licensed for use[27] also substantiate an expectation of macrolide cross-resistance in the isolates from this study.

Recent studies reported finding *erm*(42) and *msr*(E)-*mph*(E), being carried in *M. haemolytica* isolates independent from one another, very infrequently.[24] In this study no isolates contained the *erm*(42) gene, however, the *msr*(E)-*mph*(E) genes were found frequently in the 2b subtype of *M. haemolytica* isolates. Although the maximum antimicrobial test concentration was

16 µg/mL for MIC testing of gamithromycin, this study demonstrated that the presence of *msr*(E)-*mph*(E) alone can increase the MIC of *M. haemolytica* to gamithromycin from 1 to ≥16 µg/mL for the 2b subtype.

As reported previously, the culture of a resistant pathogen did not seem to associate with an increase chance of treatment failure for the cattle in this study.[7] Additionally, a nearly 50% reduction in morbidity was observed subsequent to administration of gamithromycin for control of BRD along with a 77.8% first treatment response following treatment for BRD with gamithromycin.[7] Other researchers have similarly observed a poor correlation between phenotypic macrolide resistance and BRD treatment outcome following administration of a macrolide class antimicrobial.[23]

Conclusions

The objectives of this publication were to observe and describe the effect of gamithromycin administration for both the control and treatment of BRD on the resistance patterns of *M. haemolytica, P. multocida,* and *H. somni*. The results indicate a very complex, and perhaps bacterial species dependent, interaction is taking place between the host, the bacterial pathogen, and the antimicrobial. Further, this interaction appears to be confounded by the state from which the cattle originate. The geographical trend observed in this study deserves further attention to determine what management practices might cause such a difference.

The *M. haemolytica* subtype 2b was the only strain to display genotypic resistance to gamithromycin in this study. Despite the findings of genotypic resistance, a high likelihood of first-treatment success was observed following administration of gamithromycin for the treatment of BRD and overt clinical failure did not seem to be associated with the macrolide resistance genes identified.

Future studies aimed at further investigating the prevalence and incidence of resistance and its correlation with *M. haemolytica* subtype could lead to the development of a subtype specific vaccine if a suitable epitope was discovered. If an efficacious vaccine could be developed against a strain of *M. haemolytica* that is predisposed for antimicrobial resistance it would be a major advancement in the area of BRD prevention and therapy.

Additional studies to elucidate the relationships between phenotypic and genotypic resistance and clinical response to antimicrobials are necessary to inform judicious use of antimicrobials in the context of relieving animal disease and suffering.

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Sources and Manufacturers

[a]Bruker Daltonics, Billerica, MA, USA

[b]Illumina MiSeq DNA Sequencer, San Diego, CA, USA

[c]STATA/SE 12.1 for Windows; StataCorp LP, College Station, TX, USA

Declaration of conflicting interests

R.K.T. is an employee of Merial. Other authors: none to declare.

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Figures and Tables

Table 5.1 – Number of cattle for which at least one strain of the respective bacteria was isolated (% of cattle sampled), by time point following diagnosis of bovine respiratory disease. The bottom row represents the number of cattle sampled at each time point for each sampling source (nasopharyngeal swab or bronchoalveolar lavage fluid).

	Nasopharyngeal swab				**Bronchoalveolar lavage fluid**			
	0 hr	12 hr	24 hr	120 hr	0 hr	12 hr	24 hr	120 hr
Mannheimia haemolytica	10 (38%)	3 (33%)	0 (0%)	9 (35%)	2 (25%)	2 (22%)	0 (0%)	6 (23%)
Pasteurella multocida	17 (65%)	5 (56%)	4 (44%)	8 (31%)	4 (50%)	6 (67%)	1 (11%)	6 (23%)
Histophilus somni	8 (31%)	0 (0%)	1 (11%)	1 (4%)	2 (25%)	0 (0%)	1 (11%)	2 (8%)
Cattle sampled (n)	26	9	9	26	8	9	9	26

Table 5.2 - MIC to gamithromycin of M. haemolytica (n = 276), P. multocida (n = 253), and H. somni (n = 78) isolated from cattle (n=26) diagnosed with bovine respiratory disease prior to treatment (Pre-Tx) and a summation of isolates collected at 12, 24, and 120 hours following treatment (Post-Tx) with gamithromycin. Distribution is further divided by treatment group, either those receiving mass medication (n = 9 cattle) or control (n = 17 cattle). Isolates were cultured from either deep nasopharyngeal swabs or from fluid recovered from bronchoalveolar lavage and represent several isolates per calf.*

			Minimum Inhibitory Concentration (µg/mL)									
			≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	≥16
Control (n = 17)	*Mannheimia haemolytica*	Pre-Tx	-	-	-	-	-	89	2	-	-	1
		Post-Tx	-	-	-	-	-	46	-	-	11	88
	Pasteurella multocida	Pre-Tx	-	-	-	1	33	17	-	-	-	1
		Post-Tx	-	-	-	-	19	8	-	-	-	2
	Histophilus somni	Pre-Tx	-	3	6	37	1	1	-	-	-	1
		Post-Tx	-	-	-	-	-	-	-	-	-	-
Mass Medication (n = 9)	*Mannheimia haemolytica*	Pre-Tx	-	-	-	-	-	26	-	-	-	-
		Post-Tx	-	-	-	-	-	10	1	1	-	1
	Pasteurella multocida	Pre-Tx	-	-	-	-	-	-	-	-	1	49
		Post-Tx	-	-	-	-	1	-	-	-	-	121
	Histophilus somni	Pre-Tx	-	-	-	-	-	-	-	-	-	6
		Post-Tx	-	-	-	-	-	-	-	-	-	23

Table 5.3 –MIC to gamithromycin of *M. haemolytica* isolates (n=276) from either the control group (CON) or those cattle that were mass medicated (MM) with gamithromycin on arrival. Isolates are grouped by subtype and collection time point; (Pre-Tx = collection just before treatment with gamithromycin for BRD, Post-Tx = collection at either 12, or 120 hours following gamithromycin treatment for BRD). No *M. haemolytica* isolates were obtained at 24 hours following treatment.*

			Minimum Inhibitory Concentration (µg/mL)									
Treatment	Subtype	Time Point	0.03	0.06	0.12	0.25	0.5	1	2	4	8	≥16
CON	1e	Pre-Tx	-	-	-	-	-	2	2	-	-	-
		Post-Tx	-	-	-	-	-	-	-	-	-	-
	1f	Pre-Tx	-	-	-	-	-	11	-	-	-	1
		Post-Tx	-	-	-	-	-	19	-	-	-	-
	1i	Pre-Tx	-	-	-	-	-	12	-	-	-	-
		Post-Tx	-	-	-	-	-	19	-	-	-	1
	2b	Pre-Tx	-	-	-	-	-	42	-	-	-	-
		Post-Tx	-	-	-	-	-	2	-	-	-	86
	2d	Pre-Tx	-	-	-	-	-	22	-	-	-	-
		Post-Tx	-	-	-	-	-	6	-	-	11	1
MM	1c	Pre-Tx	-	-	-	-	-	1	-	-	-	-
		Post-Tx	-	-	-	-	-	-	-	-	-	-
	2b	Pre-Tx	-	-	-	-	-	1	-	-	-	-
		Post-Tx	-	-	-	-	-	-	-	-	-	-
	2c	Pre-Tx	-	-	-	-	-	24	-	-	-	-
		Post-Tx	-	-	-	-	-	10	1	1	-	1

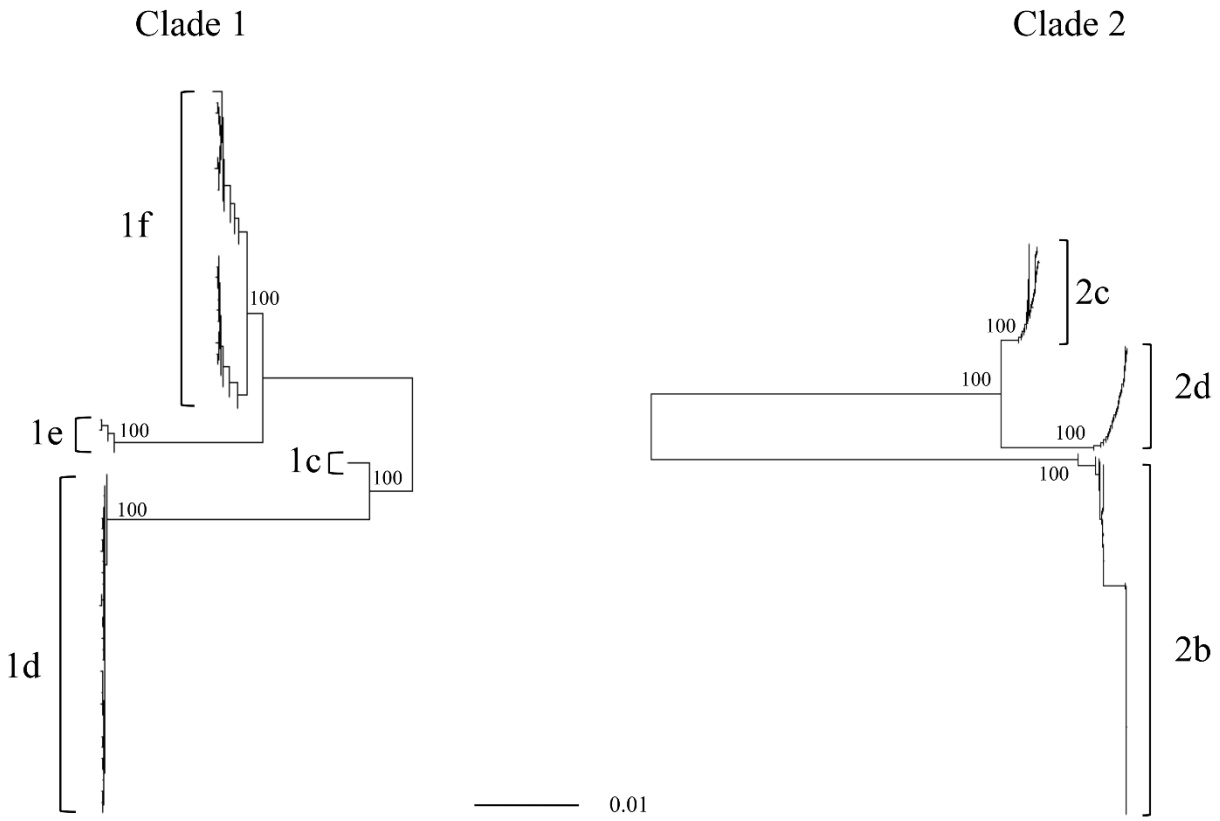
*Pre-Tx = Pre-treatment; Post-Tx = Post-treatment; CON = control treatment group; MM = mass medication with gamithromycin

Table 5.4 – MIC of *M. haemolytica* isolates (n=276) collected either prior to treatment for BRD with gamithromycin (Pre-Tx) or at 12, or 120 hr post treatment (Post-Tx). Isolates of the subtype 2b were the only isolate subtype that displayed msr(E)-mph(E) at any point during collection. No M. haemolytica isolates were obtained at 24 hours following treatment.*

		Minimum Inhibitory Concentration (µg/mL)										*Gene Presence*	
Time Point	Subtype	0.03	0.06	0.12	0.25	0.5	1	2	4	8	≥16	*erm*(42)	*msr*(E)-*mph*(E)
Pre-Tx	1c	-	-	-	-	-	1	-	-	-	-	N	N
	1e	-	-	-	-	-	2	2	-	-	-	N	N
	1f	-	-	-	-	-	11	-	-	-	1	N	N
	1i	-	-	-	-	-	12	-	-	-	-	N	N
	2b	-	-	-	-	-	43	-	-	-	-	N	N
	2c	-	-	-	-	-	24	-	-	-	-	N	N
	2d	-	-	-	-	-	22	-	-	-	-	N	N
Post-Tx	1f	-	-	-	-	-	19	-	-	-	-	N	N
	1i	-	-	-	-	-	19	-	-	-	1	N	N
	2b	-	-	-	-	-	2	-	-	-	1	N	N
		-	-	-	-	-	-	-	-	-	85	N	Y
	2c	-	-	-	-	-	10	1	1	-	1	N	N
	2d	-	-	-	-	-	6	-	-	11	1	N	N

*Pre-Tx = Pre-treatment; Post-Tx = Post-treatment; N = No; Y = Yes

Figure 5.1 – Neighbor-Joining trees of M. haemolytica clades 1 and 2 based on genome wide concatenated SNPs. Subtypes within the clades are denoted by lower case letters. Numbers beside internal nodes of the tree represent bootstrap percentage values from 100 pseudo alignments. The scale bar represents substitutions per site within trees. The genetic distance between clades 1 and 2 exceeds the genetic distance within the clades and is not shown.



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Chapter 6 - *In vitro* characterization of gamithromycin antibacterial activity against both susceptible and resistant *M. haemolytica*

ABSTRACT

Bacterial pathogen sensitivity to antimicrobials is most often assessed by determining the minimum concentration of a given antimicrobial that will visibly inhibit growth *in vitro* after 18-24 hours of incubation, termed the minimum inhibitory concentration (MIC). However, this assay fails to capture the interaction of the antimicrobial and the pathogen over the course of time which is an important aspect in the host-pathogen-drug interaction. Time-concentration killing assays are another type of *in vitro* experiment that can be used to describe a drug's antibacterial effects over time. Given that, the objectives of this study were to: (1) perform *in vitro* time-kill curve studies with gamithromycin on genetically subtyped clinical isolates of *M. haemolytica* that have been previously identified as either resistant or susceptible, (2) determine the concentrations at which gamithromycin demonstrates activity at the two levels of antibacterial action (bacteriostasis and bactericidal) for future PK-PD modeling and integration of gamithromycin. Gamithromycin exhibited both bactericidal and bacteriostatic activity against *M. haemolytica* depending on the concentration of drug. Further, antibacterial activity was observed against highly resistant *M. haemolytica* isolates but this activity was at concentrations above those likely unattainable *in vivo*.

INTRODUCTION

The determination of pathogen susceptibility to an antimicrobial is conventionally done through minimal inhibitory concentration (MIC) testing. The MIC is the minimum concentration

of a given antimicrobial that will visibly inhibit growth *in vitro* after 18-24 hours of incubation, which may be viewed as a pharmacodynamic estimate[1-3]. After an MIC is determined, the value can be compared to breakpoint values established by the Clinical and Laboratory Standards Institute Veterinary Antimicrobial Susceptibility Testing Subcommittee (CLSI/VAST) for the categorization of susceptible, intermediate, or resistant.

The main advantage of the MIC determination procedure is that it is relatively easy to determine experimentally and an organism that is cultured can be rapidly defined as either susceptible, intermediate, or resistant, given that CLSI criteria have been established for the specific pathogen / antimicrobial / disease / host species / dose regimen combination. The main disadvantage of the MIC is that it is determined from a single static measurement at the end of the experiment and it therefore, inherently ignores the interaction of the drug and pathogen over the course of time[3]. A more complete approach involves an investigation of the pharmacodynamic nature of an antimicrobial through *in vitro* exposure of exponentially growing bacteria to a range of static drug concentrations with bacterial counts being monitored over the time course of the experiment, also called a time-concentration kill (TCK) curve experiment. The quantification of growth at numerous time points across the dilution range of drug over the 24 hour incubation period permits the determination of antimicrobial and bacterial interaction dynamics. These experiments can be used to determine if an antimicrobial is bacteriostatic or bactericidal and the speed at which the bacteria are killed. Additionally, TCK curve experiments allow for the development of a pharmacodynamic model that when linked with a pharmacokinetic and statistical model can be used to select optimal antimicrobial dosing strategies which may then be tested in clinical trials[4].

A major food animal disease which could benefit from these types of investigations is bovine respiratory disease (BRD), a disease of significant concern for both the beef and dairy

industry in regards to animal welfare and economic impacts. The three most commonly cited bacterial pathogens involved in BRD are *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni*[5]. Antimicrobial resistance appears to be increasing in populations of these pathogens within some populations of cattle[6], and the need for advanced understanding of optimal dosing regimens to achieve bacteriological cure and minimize selection for resistant target pathogens is paramount. The use of PK-PD integration and modeling in the selection of dosing regimens for the treatment of BRD have been previously performed for danofloxacin[7], marbofloxacin[8], florfenicol[9,10], and amoxicillin[11]. Time-kill curve experiments, performed solely as investigations into antimicrobial potency and kill rate, have been previously reported for several antimicrobials against the pathogens of BRD[12-15]. However, in all of the aforementioned publications, the bacterial isolates were categorized as susceptible to the antimicrobial used in that experiment which may not always be the case in the field.

An example of an antimicrobial class where the classically cited pharmacodynamic properties may not be universally agreed upon is the macrolides. Antimicrobials of this class inhibit RNA-dependent protein synthesis through a reversible binding to the 23S ribosomal RNA in the 50S subunit of the bacterial ribosome[16]. Generally speaking, antimicrobials that interfere with protein synthesis are considered bacteriostatic. However, macrolides have been shown to be both bactericidal and bacteriostatic depending on the concentration of the drug and the particular bacterial species exposed[16].

Gamithromycin is a semi-synthetic, 15 member-ring macrolide of the azalide subclass that is licensed in the United States for the treatment and control of bovine respiratory disease (BRD)[17]. It has demonstrated efficacy as an antimicrobial for both the prevention and treatment of BRD[18-20]. The antibacterial activity of gamithromycin against BRD pathogens has been investigated[12]. Gamithromycin demonstrated bactericidal activity (3 log kill within 24 hours) against both *M.*

haemolytica and *P. multocida* at MIC values as high as 4 µg/mL and bacteriostatic activity against *H. somni* at the MIC_{90} concentration of 0.5 µg/mL. Given this previous work, the objectives of this experiment were to: (1) perform *in vitro* time-kill curve studies with gamithromycin on genetically subtyped clinical isolates of *M. haemolytica* that have been previously identified as either resistant or susceptible, (2) determine the concentrations at which gamithromycin demonstrates activity at the two levels of antibacterial action (bacteriostasis and bactericidal) for future PK-PD modeling and integration of gamithromycin.

MATERIAL AND METHODS

Animals, BRD diagnosis, sample collection, and treatment administration

This study was approved by the Kansas State University Institutional Animal Care and Use Committee. As part of another study, 180 head of feeder cattle (165-269 kg) were sourced from three separate auction markets within the United States and brought to a research feedlot in Manhattan, KS[21]. The cattle were randomly allocated to treatments as described in that manuscript, placed in pens according to treatment, and observed for 28 days for symptoms consistent with BRD. The diagnosis of BRD was performed by a veterinarian masked to treatment allocation with the use of an objective scoring system and rectal temperature criteria. At the time of BRD diagnosis cattle were treated with gamithromycin according to label directions (6 mg/kg, single subcutaneous injection). Samples were collected for aerobic bacterial culture from both deep nasopharyngeal swabs and bronchoalveolar fluid (BAL) at several possible time points around the time of treatment, and then again 120 hours after treatment. Additionally, samples were harvested at the same time points for the determination of gamithromycin concentrations in both plasma and pulmonary epithelial lining fluid (PELF).

Bacterial isolation, MIC determination, and *M. haemolytica* phylogenetic analysis

Nasopharyngeal swab and PELF samples from cattle morbid with BRD were hand delivered to the laboratory where they were plated directly onto trypticase soy + 5% blood,

chocolate, and MacConkey agar plates. The plates were incubated in 5% CO_2 at 37˚C for 18 to 24 hours and as many as 12 individual colonies displaying growth characteristics typical of *M. haemolytica* were further isolated in pure culture from each calf. The identity of each isolate was confirmed with MALDI Biotyper® (Bruker Daltonics, Billerica, MA, USA) and frozen for later susceptibility testing.

The gamithromycin MIC was determined for each isolate by personnel masked to treatment group based on a broth microdilution technique utilizing *Staphylococcus aureus* ATCC 29213 for quality control, as previously described[21]. Determination of susceptible (≤4 μg/mL), intermediate (8 μg/mL) or resistant (≥16 μg/mL) was based on clinical breakpoints established for gamithromycin by CLSI (personal communication with co-author RKT).

Briefly, as previously reported, DNA was extracted, quantified, and Illumina Nextera XT DNA libraries were constructed and sequenced (minimum 10X genome coverage). The library sequences were individually mapped to an available closed circular *M. haemolytica* genome publicly available in GenBank (CP004752)[22,23]. Bootstrapped Neighbor-Joining phylogenetic trees were constructed from SNP genotypes to compare genetic relatedness of individual isolates of *M. haemolytica*.

Selection of isolates

In total, 276 isolates of *M. haemolytica* were obtained over the course of the clinical portion of the study. Following the determination of an MIC for each isolate, six susceptible isolates (MIC = 1.0 μg/mL) and five resistant isolates (MIC ≥16.0 μg/mL) were selected for the in vitro TCK curve experiments. The five resistant isolates were discovered to have an actual MIC of 128 μg/mL (*vide infra)* and will be discussed as such from this point forward. Isolates were selected from a genetically diverse population to evaluate if there were detectable differences in the antimicrobial activity between strains.

***In vitro* time-concentration kill curve experiments**

The *M. haemolytica* strains used in this study were part of a collection of strains described previously[23,24]. Strains were revived from banked samples on Chocolate agar (Hardy Diagnostics, Santa Maria, CA, USA) at 37°C with 5% CO2, for 18-20 h. Individual colonies were selected and struck for isolation onto Mueller-Hinton II (MHII) agar (Becton, Dickinson and Co, Sparks, MD USA) and incubated at 37°C for 18-20 h. The resulting cultures were used as the inoculum for the TCK assays described below.

Six isolates in the MIC = 1 μg/mL range and 5 isolates in the 128 μg/mL MIC range were selected for TCK analysis. Isolates were selected with the goal of capturing the genetic diversity of *M. haemolytica* present in the strain collection. Accordingly, susceptible isolates included representatives of subtypes 1c, 1e, 1f, 1i, and two isolates of the 2b subtype[24]. All resistant isolates originated from a single subtype (2b); care was taken to select isolates from different animals and different study time points.

Two different stock concentrations of gamithromycin were prepared for the TCK assays, one at 5120 μg/mL, which was used for the low range assays (for isolates with MIC = 1 μg/mL), and the other at 10,240 μg/mL, which was used for high range assays (for isolates with MIC 128 μg/ml). For each stock solution the appropriate amount of gamithromycin powder was dissolved in 0.1M sodium phosphate buffer, pH 6.0. Stock solutions were distributed into 1 mL aliquots which were stored at -80°C, and used within one month of preparation. For low range assays, dilutions of gamithromycin ranged from 0.25 to 16 μg/mL (equivalent to 0.25 – 16 x MIC) in a 3 mL assay volume. For high range assays dilutions ranged from 4 - 256 μg/mL (equivalent to .03 – 2 x MIC, for MIC = 128 μg/mL).

For each strain to be assayed, bacterial cultures were prepared by transferring 3 to 5 colonies from a fresh MHII agar plate to 5 mL sterile ddH2O, until the turbidity was equivalent to

a 0.5 McFarland standard. From this suspension, 350 µL was transferred to 5 mL of Mueller-Hinton II (MHII) broth (Becton, Dickinson and Co, Sparks, MD USA) and then 150 µL of the resulting suspension was used to inoculate 30 mL of MHII broth that had been pre-warmed to 37°C. The 30 mL cultures were incubated without shaking, at 37°C for 2h, and the resulting cultures were primed to enter log-phase growth, with starting concentrations of approximately 1 – 3 x 10^5 CFU/mL. Time-concentration kill assays were performed using 48 well, deep-well blocks, with 2.9 mL of each 30 ml culture placed in each of 8 wells. This design allowed two strains to be tested against 8 concentrations of antibiotic, in biological triplicate, in each 48 well block.

Assays were conducted using a multichannel pipette and were initiated by the addition of 100 µl of the appropriate stock concentration of antibiotic (0 to 480 µg/mL for a working concentration range of 0 to 16 µg/mL (low range assay); or 0 to 7680 µg/mL for a working concentration range of 0 to 256 µg/mL (high range assay). Samples were mixed by repeated pipetting (7 - 9 times) and tips were changed between biological replicates. Nine time points were evaluated for each assay, including 0, 0.5, 1, 1.5, 2, 3, 4, 8 and 24 h, and the sample block was incubated at 37°C with shaking for the duration of the assay. At each time point, 100 µL was removed from each well and subjected to serial decimal dilutions. Viable cell counts were determined using the "track dilution" plating method[25], which entailed spotting 15 µL of each appropriate dilution to a MHII agar plate. Inoculated plates were then tilted at a 45° angle, allowing the sample to migrate in a track to the opposite side of the plate. With a 15 µl plating volume, the lower limit of detection (LOD) of this plating method is $\log_{10}$ 1.83 or approximately 67 CFU/mL.

All plates were held at room temperature from time point 0 to 8, after which they were inverted and incubated at 37°C for 14 – 16 h. Throughout the assay, the plated dilutions targeted a viable cell count of 20 to 200 CFU per track. *Staphylococcus aureus* ATCC 29213 served as a quality control strain for concentrations of gamithromycin in the low range assay and for the high

range assay, antibiotic solutions were diluted 1:10 so that values fell within the assayable range. Viable cell counts were recorded and CFU/ml concentrations calculated. All assays were performed in biological triplicate and the geometric mean and standard deviation at each time point were calculated and plotted.

Determination of antibacterial activity

The antibacterial effect of gamithromycin was quantified for two levels of growth inhibition for each isolate of *M. haemolytica;* bacteriostatic and bactericidal activity. The values for bacteriostatic and bactericidal activity were defined as the values which produced no change from the initial bacterial count and values which provided a 3-$\log_{10}$ reduction in initial inoculum count after 24 h incubation, respectively. The LOD of 67 CFU/mL precluded the ability to detect the concentration at which bacterial eradication (4-$\log_{10}$ reduction) was attained.

RESULTS

BRD morbidity

As previously reported, 26 of the 180 (14.4%) calves were diagnosed with BRD during the 28 day study period.[21] Nine calves were from the treatment group administered gamithromycin on arrival for control of BRD and 17 were from the control group. Four calves were classified as treatment failures, two from each treatment group.

MIC verification

The MIC of each isolate was verified, in comparison to the previously determined MIC, using CML1FZAC plates prior to the TCK assay. The MIC of the resistant isolates, previously characterized with MIC of $\geq$ 16 μg/mL[21], were further defined as having an MIC = 128 μg/ml by broth microdilution, using a dilution series of gamithromycin ranging from 4 – 256 μg/ml.

***In vitro* time-concentration kill curves**

The results of the *in vitro* TCK curves for the isolates with an MIC of 1 (n=6) and 128 µg/mL (n=5) are illustrated in Figures 1 and 2, respectively. In each assay the bacterial growth was as expected in control samples, with an increase in count of ~3- $\log_{10}$ CFU/mL over the incubation period of 24 h.

The isolates with an MIC = 1 µg/mL displayed little growth inhibition at 0.25 and 0.5 multiples of MIC for the first 4 hours, however these concentrations resulted in 3-$\log_{10}$ reductions in counts by 24h of incubation. Concentrations at the MIC (1 µg/mL) resulted in little bacterial growth being observed at early time points up to 4h of incubation for five of the six strains. The other strain (33204) had 3-$\log_{10}$ increases in CFU/mL up to 4 hours. However, all six strains were 3-$\log_{10}$ reduced by either 8 (strains 32600, 33204, 32770, 33058, 32917) or 24 hours (strain 33058). At the drug concentrations of 8 and 16 × MIC, colony counts were below the LOD after short exposure (3 h) to gamithromycin.

In vitro antibacterial activity of gamithromycin against resistant strains of *M. haemolytica* (MIC = 128 µg/mL) was more limited. There was an increase in bacterial count at 0.03 – 0.12 × MIC for all isolates. Isolate 32998 displayed bacteriostasis at 0.25 and 0.5 × MIC. Isolate 32711 was held static at 0.25 x MIC and approached a 3-$\log_{10}$ decrease in CFU/mL (2.86) at the 0.5 x MIC concentration. Paradoxically, isolate 33014 was static at 0.25 x MIC but not inhibitory at 0.5 x MIC. Gamithromycin was bactericidal for all isolates at 2 x MIC within 3 – 8 hours of incubation. Bactericidal activity was obtained at 1 × MIC for strains 32711, 33014, and 32998 at 4, 8, and 24 hours of incubation, respectively.

Antibacterial activity

Bactericidal activity was exhibited at 0.25 – 16 multiples of the MIC over the 24 hour incubation period for all six MIC = 1 µg/mL isolates of *M. haemolytica*. In resistant strains (MIC = 128 µg/mL), bactericidal activity was obtained for three of five isolates at 1 x MIC and for all

five isolates at 2 x MIC. Data from TCK assays demonstrated that isolates at a lower MIC (1 μg/mL) were killed in an apparent time dependent manner. The antibacterial activity of gamithromycin against MIC 128 μg/mL was quite limited in this study due to the concentrations tested, however killing appeared to be concentration dependent.

DISCUSSION

Antimicrobials of the macrolide class have been described as either bacteriostatic or bactericidal depending on the concentration and the species of bacteria[16]. Macrolide class antimicrobials were historically grouped with other time-dependent drugs, implying that the PD index of %T>MIC is the parameter best describing their efficacy; however, due to post-antibiotic effects, macrolides might be better evaluated with use of the AUC/MIC ratio[26].

In 2014, Tessman and Bade reported observing a 3-log decrease in CFU/mL of *M. haemolytica* with an MIC of 1 μg/mL at 1 x MIC by 8 hours post gamithromycin exposure with an *in vitro* TCK assay similar to the current study[12]. Additionally, they found that all of the *M. haemolytica* isolates (n = 5, MIC = 1 μg/mL) they tested resulted in a 3-log decrease at the 4 μg/mL concentration by 4 hours (their first quantification time point). The current study found similar results, with five of the six isolates in this study having 3-log10 CFU/mL reductions by 8 hours and all six isolates by 24 hours of incubation. The findings from the current study also demonstrated a 3-log reduction for all six MIC = 1 μg/mL isolates at 4 x MIC by 8 hours incubation. Further, five of the six *M. haemolytica* isolates resulted in a 3-log decrease at the 4 μg/mL concentration by 3 hours and all six were reduced by 3-log CFU/mL at 4 hours.

In the *M. haemolytica* isolates with an MIC = 1 μg/mL growth inhibition was observed at concentrations lower than was expected. In all six strains a 3-$\log_{10}$ decrease in CFU/mL was observed by 24 hours indicating that gamithromycin was bactericidal in a time-dependent manner.

It is possible that residual gamithromycin from the wells was transferred during the dilution procedures leading up to colony forming unit quantification. As another possible explanation for these findings, differences between the MIC and TCK assays should be noted. While the media composition and temperature of incubation were the same, the inoculum size, media fluid volume, and incubation conditions (static incubation for MIC and shaking incubation for TCK) differ between the two tests. Additionally, MIC values were determined at 18-20 hours and the final quantification point for the TCK assays were performed at 24 hours of incubation. Finally, the MIC test outcome is determined by a visual inspection for cell death or growth as compared to the TCK where viable cell counts were determined using the track dilution plating method.

The microbiological assays in this study were limited to *in vitro* assays for both the MIC and TCK analyses. Previous publications have noted differences between broth and serum MIC determinations, especially as it pertains to macrolide antimicrobials[14,15,27-31]. In these studies the MIC of macrolides in heat inactivated bovine serum have been decreased as compared to those determined in broth. Likewise, studies comparing the killing kinetics of macrolides between *in vitro* and *ex vivo* assays have found that the kinetics differ in the different media with improved killing *ex vivo*[31]. It is possible that if *ex vivo* studies would have been performed the killing kinetics of gamithromycin would have differed as well.

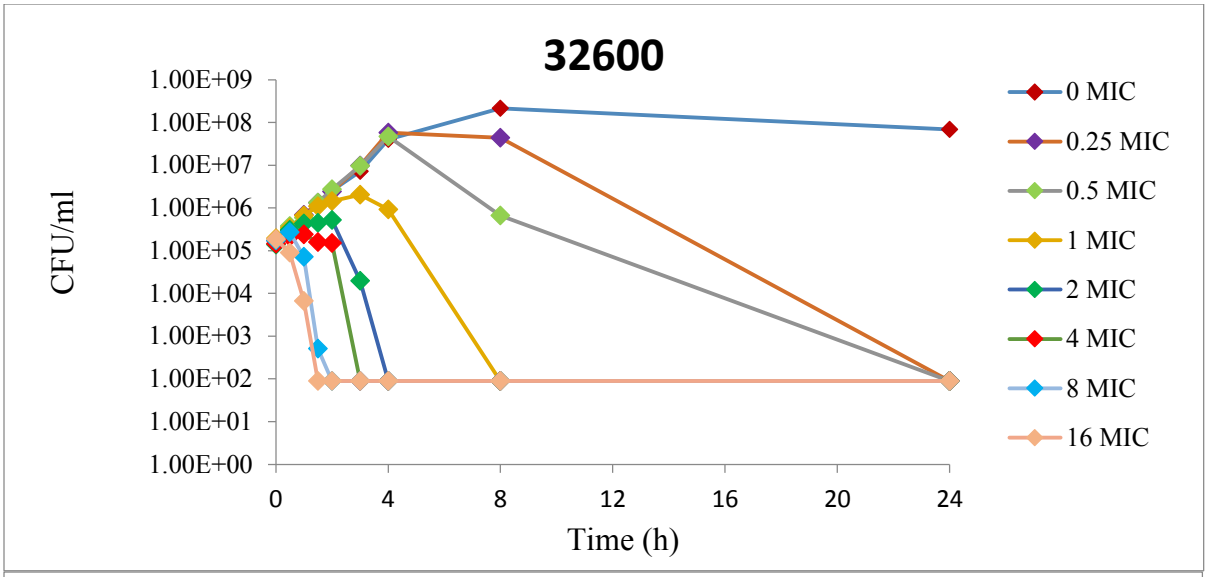
These data are further limited by the relative large gaps in data points for both the time and drug concentrations for the TCK curves. There are large changes in bacteria CFU/mL from the 8 to 24 hour time points, most notably for the susceptible strains. The absence of data to inform the killing over that 16 hour time span limits the usefulness of the final data point. Additionally, the LOD is too high to quantify the concentration where bacterial eradication might be achieved. Finally, the higher dilutions (e.g. 64, 128, and 256 μg/mL) represent large changes in drug concentrations making it difficult to determine where the actual MIC is located. Extending the

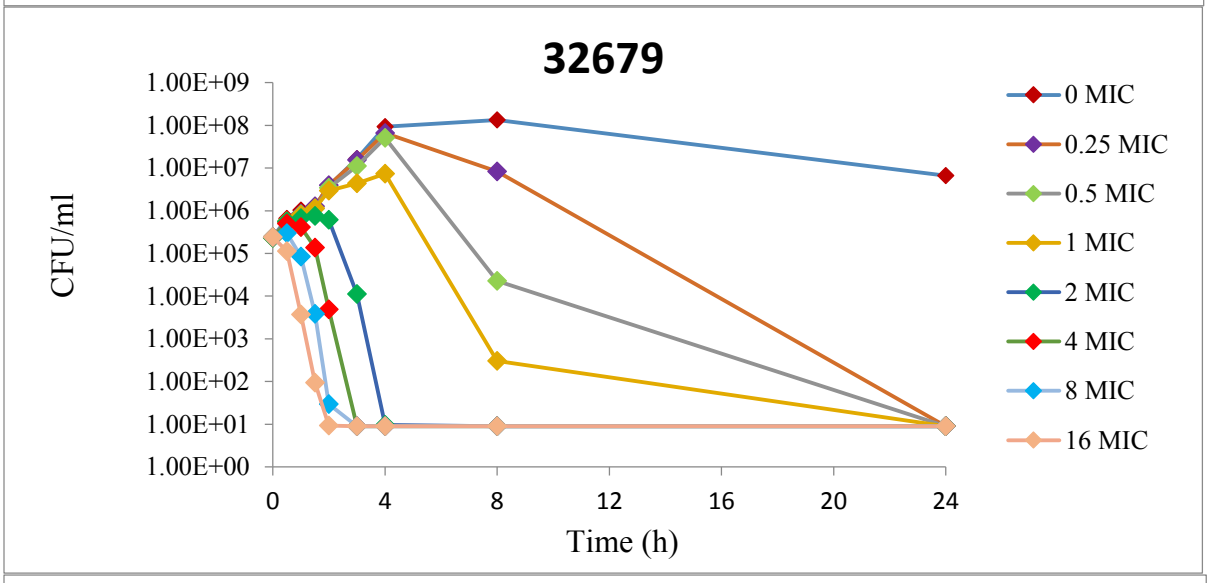
doubling dilution approach in the determination of MIC, as suggested by Aliabadi and Lees (2001 and 2002),[32,33] might be one possible approach to more accurately describe the MIC of the more highly resistant strains used in this study. These factors described above present limitations to a direct integration into a PK-PD model.

A disconnect between *in vitro* MIC determination and BRD clinical case outcome for the macrolide antimicrobials has been previously reported[21,34] which could possibly be explained by the differences between the artificial media and the physiological matrix where the infection resides. Activity of gamithromycin against highly resistant isolates (128 μg/mL) presented in this study was limited to only those concentrations at or 2 multiples above the MIC. Given the results of previous pharmacokinetic data, these concentrations are quite likely physiologically unattainable. However, gamithromycin has been demonstrated to be a clinically effective antimicrobial at its labeled dosage of 6 mg/kg for both the treatment and control of BRD[18,19,35]. However, this efficacy, in relation to low and high MIC isolates was not determined in the clinical trials.

These data included herein demonstrate that gamithromycin can be bactericidal or bacteriostatic against *M. haemolytica* depending on the concentration of drug. Additionally, while antibacterial activity was observed against highly resistant *M. haemolytica* isolates (MIC = 128 μg/mL) it was at concentrations so high (1 – 2 x MIC) as to be physiologically unattainable. However, the significance of these findings in light of all of the *in vitro, in vivo,* and *ex vivo* experimental discrepancies has yet to be determined for the macrolide class of antimicrobials. As such, a word of caution must be advised when these type of data are used to make dosage predictions for clinical use in the treatment of BRD.

FIGURES


32600
CFU/ml
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
4
8
12
16
20
24
Time (h)
0 MIC
0.25 MIC
0.5 MIC
1 MIC
2 MIC
4 MIC
8 MIC
16 MIC


32679
CFU/ml
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1.00E+06
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1.00E+04
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1.00E+02
1.00E+01
1.00E+00
0
4
8
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16
20
24
Time (h)
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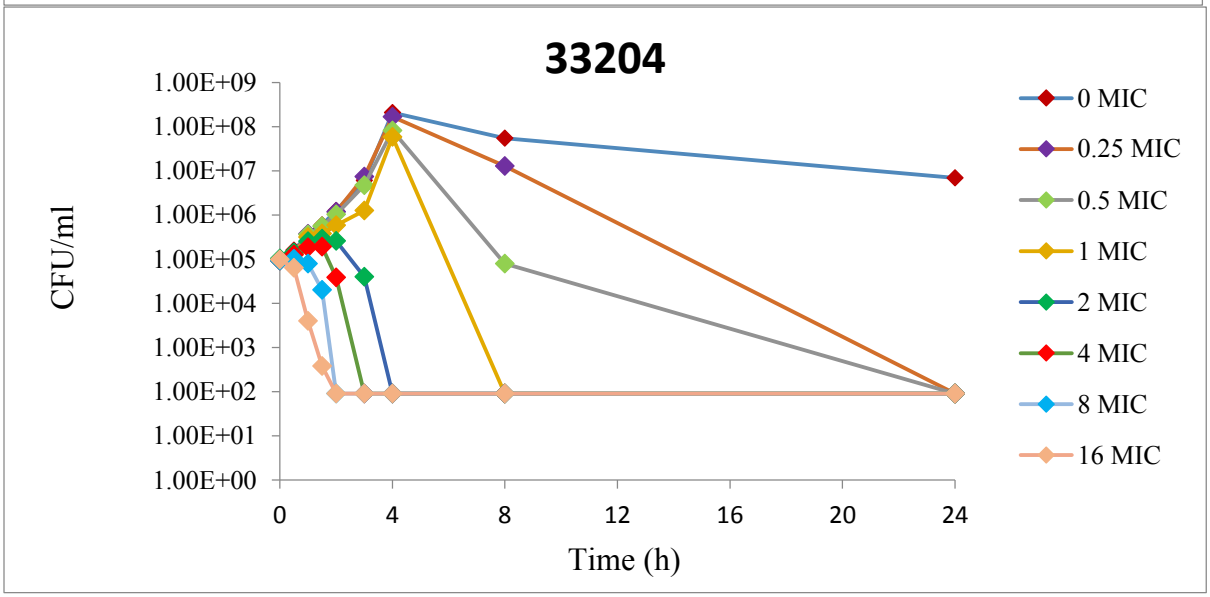
0.25 MIC
0.5 MIC
1 MIC
2 MIC
4 MIC
8 MIC
16 MIC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CFU/ml
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1.00E+07
1.00E+06
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1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
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24
Time (h)
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0.25 MIC
0.5 MIC
1 MIC
2 MIC
4 MIC
8 MIC
16 MIC

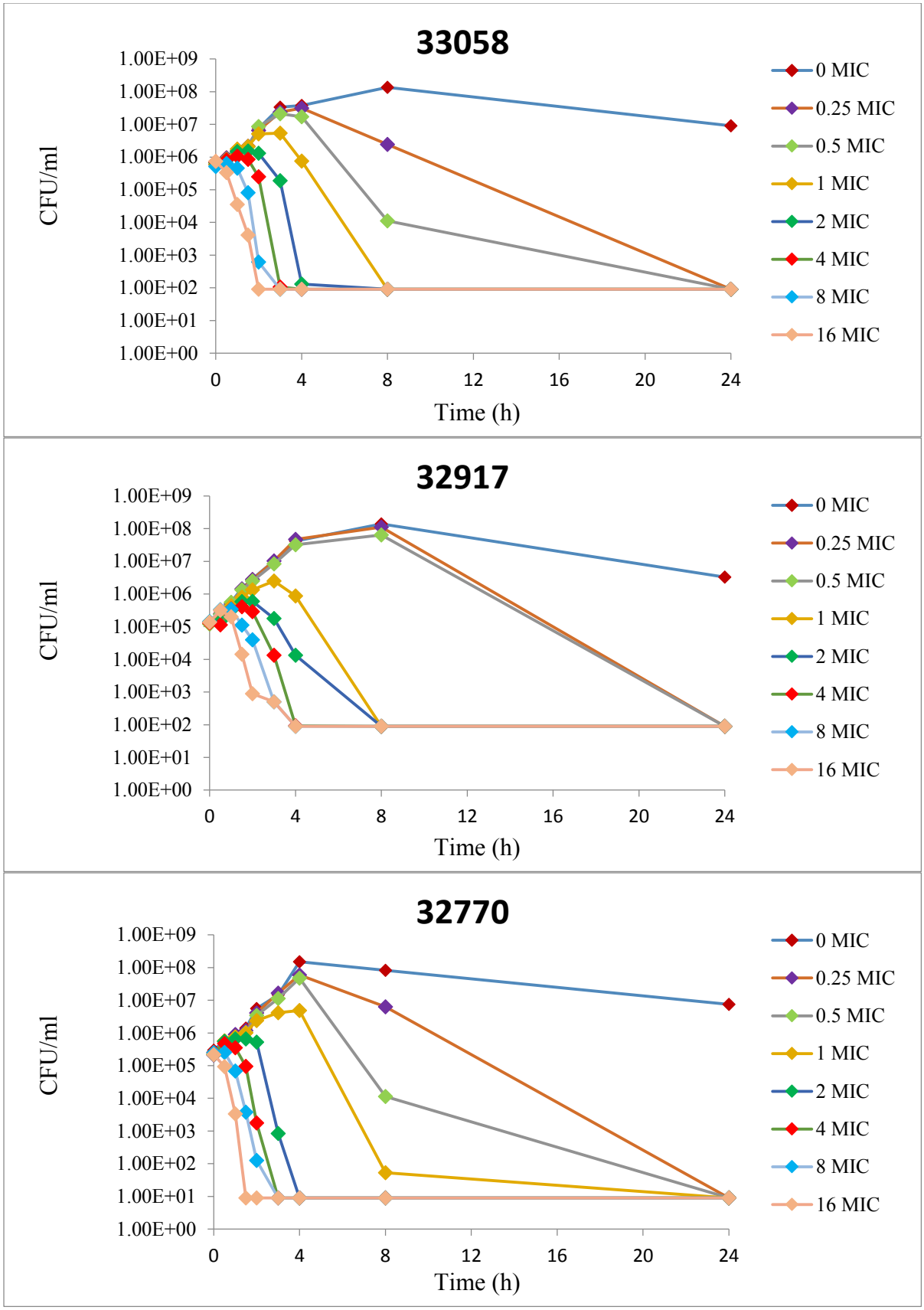
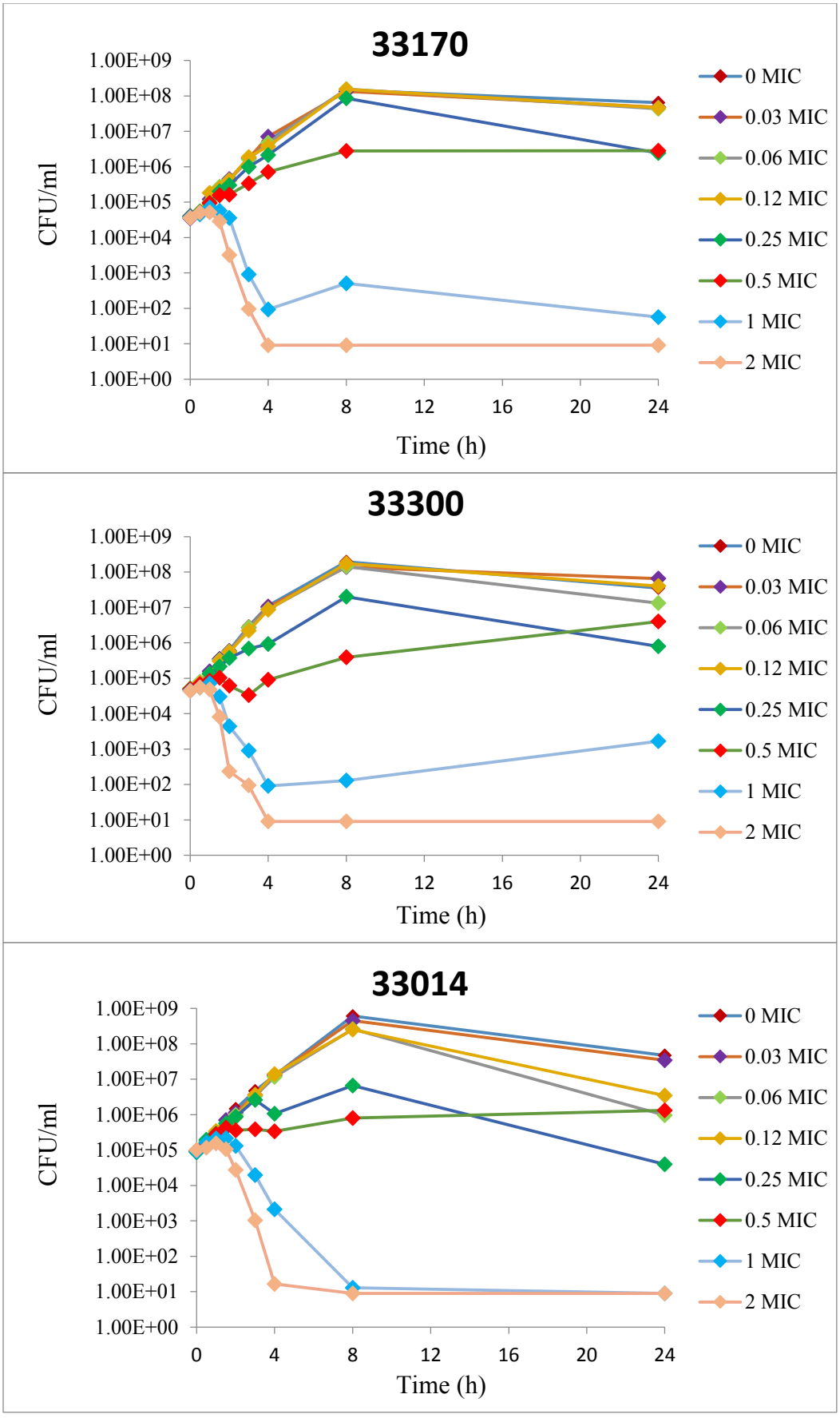


Figure 2 – *In vitro* inhibition of growth of six different strains of *M. haemolytica* (MIC = 1 µg/mL) over 24 hour exposure to 8 MIC multiples of gamithromycin. SEM bars not included for clarity of graphics. (Lower limit of detection 67 CFU/mL)


33170
CFU/ml
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1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
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1.00E+02
1.00E+01
1.00E+00
0
4
8
12
16
20
24
Time (h)
0 MIC
0.03 MIC
0.06 MIC
0.12 MIC
0.25 MIC
0.5 MIC
1 MIC
2 MIC
33300
CFU/ml
Time (h)
33014
CFU/ml
Time (h)

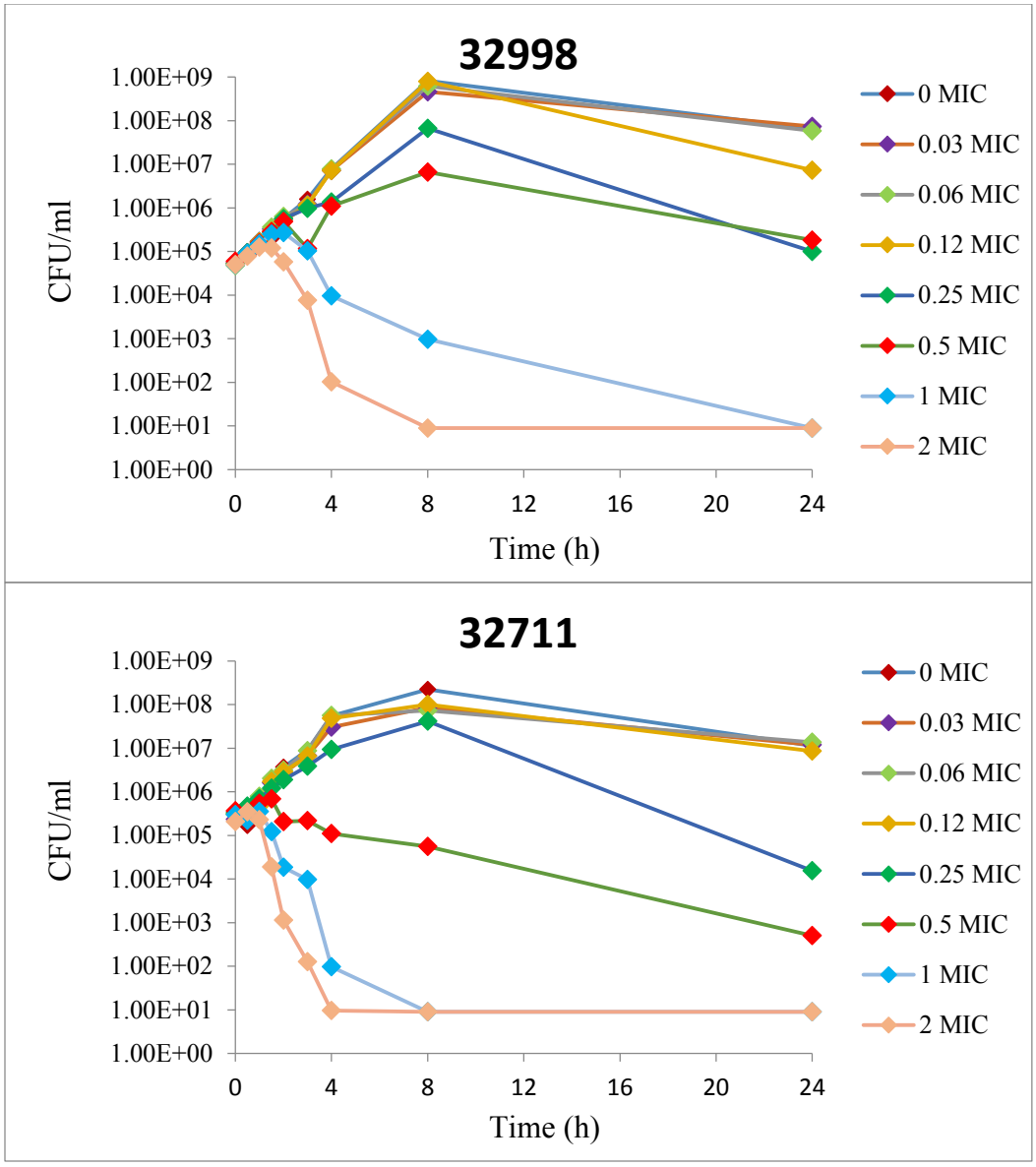

Figure 3 – *In vitro* inhibition of growth of six different strains of *M. haemolytica* (MIC = 128 µg/mL) over 24 hour exposure to 8 MIC multiples of gamithromycin. SEM bars not included for clarity of graphics. (Lower limit of detection 67 CFU/mL)

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Chapter 7 - Consideration for future studies

Our work has built upon techniques utilized for many years in the human medical research field and applied them to naturally occurring BRD. The use of naturally occurring BRD was a more clinically relevant approach yet much more difficult to perform due to not having control over the sample size. The lack of power potentially precluded our research from reaching more definitive answers but led to many new hypothesis which can be built upon in the future.

Considering the ability of an Integrative Conjugative Element (ICE), conferring resistance to multiple antimicrobials, to travel amongst members of the *Pasteurellaceae* family (and to many other bacteria), a similar approach to characterizing the resistance genes for both *P. multocida* and *H. somni* would be of great value. Additionally, repeating the same PK-PD approaches utilized for *M. haemolytica* for both *P. multocida* and *H. somni* would add a tremendous amount of knowledge to this area.

Of great personal interest to me are the macrolide antimicrobial compounds. The apparent disconnect between the results of *in vitro* testing methods (MIC, TCK, etc.) and clinical outcome data are particularly intriguing. All of the work contained herein was performed purely *in vitro.* However, *ex vivo* methods have been shown by some research teams to more closely resemble the observed treatment outcome (i.e. *ex vivo* analysis often result in lower MIC values than *in vitro* methods for macrolide compounds). Finally, the anti-inflammatory and immunostimulatory abilities of macrolides are particularly fascinating to me and as I learn more about the work that has been done in this area it raises the question of how much of a role those properties are contributing to clinical outcome as well.

Conclusions

Currently, by some accounts, we, as a group of human and veterinary health care professionals, are in crisis mode in regards to the increasing appearance of antimicrobial resistance and the lack in the discovery of novel classes of antimicrobial compounds. At no point before have clinicians been faced with a more difficult decision when deciding whether to treat with an antimicrobial or allow an animal to attempt a self-cure. Numerous organizations have released consensus statements and/or prescription guidelines in an attempt to persuade the general practitioner to judiciously use antimicrobials. It will take the work of many different types of scientists working in conjunction with the clinicians practicing in the field to truly make a difference in fine tuning the treatment regimens that can both successfully treat an infectious disease and mitigate the risk of antimicrobial resistance.

The ability with which we were able to integrate so many researchers with different areas of expertise in this work certainly helped to make this project a success. Future work should likewise pull together different research teams for collaborative synergism and novel approaches to the same ultimate end goal.