DISSERTATION

ANTIMICROBIAL RESISTANCE SURVEILLANCE IN FEEDLOT CATTLE

Submitted by

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ABSTRACT

ANTIMICROBIAL RESISTANCE IN FEEDLOT CATTLE

Objectives: To develop and validate methodological components of a model for surveillance of antimicrobial use and resistance in feedlot cattle.

Methods: A web-based survey of participants knowledgeable and interested in antimicrobial use in beef feedlots was used to solicit responses regarding appropriate metrics for quantifying, analyzing, and reporting antimicrobial exposures. The accuracies of two susceptibility tests commonly recommended for surveillance programs were determined using stochastic latent class analysis. Multivariable logistic and linear regression was used to investigate associations between exposures to antimicrobial drugs and antimicrobial resistance.

Results: When reporting antimicrobial use in the context of antimicrobial resistance, survey participants believed that the Animal Defined Daily Dose metric was the most accurate. The two susceptibility tests investigated had comparable accuracies for the antimicrobial drugs tested. Exposure to parenteral tetracycline in the study feedlots was associated with resistance to tetracycline; however, exposures to all other classes of antimicrobials were not associated with antimicrobial resistance.

Conclusions: Appropriate metrics for reporting and analyzing antimicrobial resistance are necessary to accurately investigate associations between use and resistance, though clarity of what the metric represents may be lost. Testing of susceptibility in surveillance programs is equally valid by way of disk diffusion testing. Multivariable logistic regression was an appropriate and useful method to investigate associations between use and resistance. Parenteral exposures to antimicrobials did not drive antimicrobial resistance at mid-feeding period.

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PREFACE

The three projects presented in this dissertation contributed to a large multi-institution collaborative effort to develop a longitudinal antimicrobial resistance and use surveillance program for the feedlot sector in Canada. The goal of this large-scale effort was to develop and validate a practical model for monitoring antimicrobial susceptibility in populations of feedlot cattle. Lead investigators represented five universities (Colorado State University, University of Calgary, University of Guelph, University of Lethbridge, and University of Saskatchewan), provincial and federal Canadian government (Alberta Agriculture Food, Rural Development Food Safety Division, Agriculture and Agri-Food Canada, and Public Health Agency of Canada), and one private veterinary company (Feedlot Health Management Services) which managed the 4 large, commercial feedlots where the surveillance program was piloted.

In order to implement effective resistance control strategies, surveillance systems must evaluate accurate and reliable data. Prior to collecting this data the methodology related to sampling, shipping, testing, analyzing, and reporting should be validated for efficiency and accuracy. The projects of this dissertation were focused on three specific questions (listed below) about the methodology utilized in this pilot surveillance program.

Research Questions:

1) How should antimicrobial use data be quantified for analysis of antimicrobial resistance and for reporting? (Chapter 2)

2) What is an appropriate testing method for determining susceptibility? (Chapter 3)

3) How should analysis be conducted to investigate associations between exposure to antimicrobial drugs and antimicrobial resistance? (Chapter 4)

Each of these questions was investigated as an independent project. Objectives, methods and materials, results, and discussion for each project are presented separately in Chapters 2-4. Interpretive summaries for each chapter and the final Conclusions (Chapter 5) describe how the project relates back to the aim of the large-scale collaborative effort and the broader implications of the work. Other research investigating the development, dissemination, and persistence of antimicrobial resistance has been conducted globally for decades. A review of the previous work and existing gaps in knowledge related to antimicrobial resistance surveillance in feedlot cattle is presented in Chapter 1.

DEDICATION

For the Benedicts, the Ritsicks, the Roberts, the Ellises, the Munzes, and the Morleys whom have all provided their unconditional support through my training.

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INTRODUCTION

Antimicrobial resistance is an emerging global threat to human and animal health (Levy and Marshall 2004). Awareness of this problem is more widespread due to highly publicized anecdotes about "superbugs" which defy treatment, but the problem itself is nothing new (Newell et al. 2010). Within 2 decades of the discovery of penicillin, researchers were already warning that misuse could lead to selection and propagation of mutant resistant forms of bacteria (Fleming 1929; Levy 2002). One response to these resistant variants in the past has been the application of new and "better" drugs. Different antimicrobials were discovered and synthesized in the latter part of the 20th century on a regular basis. However, no new antimicrobials are currently on the horizon that can adequately compensate for the loss in susceptibilities to existing antimicrobials (The Alliance for the Prudent Use of Antibiotics 2005). A "post-antibiotic" era in which no antimicrobials will be able to combat simple infections is the ultimate fear driving efforts to understand the complexities of antimicrobial resistance (Cohen 1992). It has been suggested that resistances to antimicrobials which develop on the local scale left unmanaged will lead to an untenable global problem and these once powerful will be rendered useless (Levy 2001).

The use of antimicrobials is the hypothesized major driving force for the occurrence of antimicrobial resistance (Figure 1, adapted from Barbosa and Levy 2000). Theoretically, susceptible bacteria in the presence of an antimicrobial are eliminated from heterogeneous populations of bacteria, while the resistant and even marginally susceptible bacteria are left to proliferate. However, other factors such as overuse of

disinfectants and heavy metals in the environment are recognized as having an influence as well (Levy 1998; Levy 2002). Beyond the initial use of antimicrobials, post-therapeutic effects and residues in the environment are also pressures which select for resistant variants of bacteria over susceptible ones (Gibbs et al. 2006; Levy and Marshall 2004).

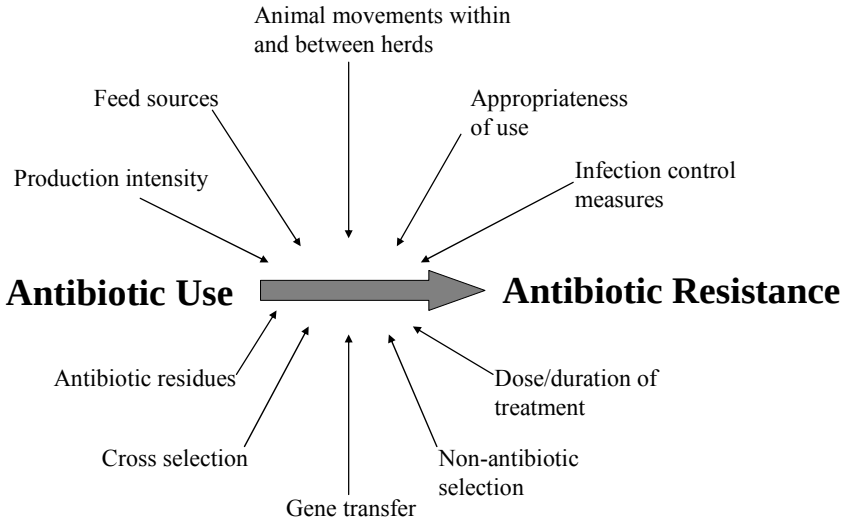

Figure 1: **Relationship between antibiotic use and development of resistance.** Antibiotic use is the main factor in the forward process, i.e. selection of resistance, but other factors can influence that relationship. Factors dependent on management of animals are represented above the horizontal arrow, while factors related to the antibiotic itself and the genetic basis of resistance are represented below the horizontal arrow (adapted from Barbosa and Levy 2000).

Dissemination of antimicrobial resistance through clonal spread as well as by transfer of resistance genes is of greater concern than the initial development (van den Bogaard and Sobberingh 2000). New genetic methods are needed to trace antimicrobial resistance within and between host populations (O'Brien 2002). Though antimicrobial

resistance can spread through many different routes, the transmission from agricultural animals to humans is often scrutinized (Ferber 2000; Shea 2003). A concerning scenario would be that the antimicrobial drugs used in food-producing animals would ultimately lead to preventable health problems in consumers. A direct route between exposures to antimicrobial drugs in food animals to human health problems is unlikely beyond anecdotes of people working or living closely with the animals (Angulo et al. 2004; Fey et al. 2000). However, human and animal microbial ecosystems do overlap in various relationships and efforts to untangle the complexity should also take an ecological approach (Figure 2, adapted from Witte 1998; Bywater 2004; Singer et al. 2006).

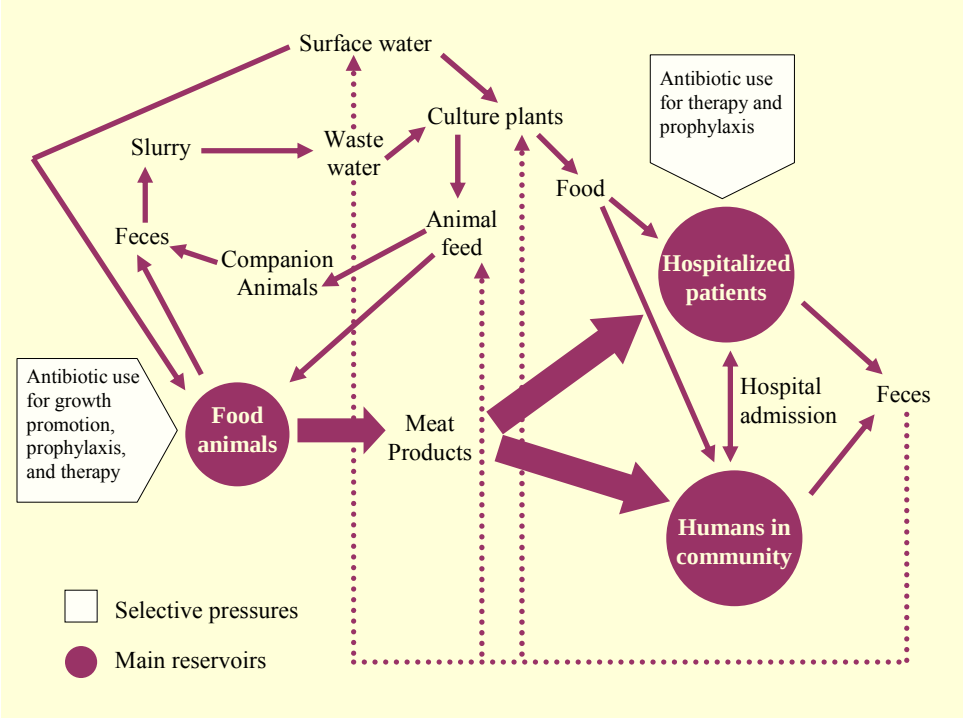

Figure 2: **Network of resistance.** Ecological relationships between antibiotic-resistant bacteria and resistance genes: selective pressures, main reservoirs, and routes of transmission (adapted from Witte 1998).

The concerns about human health related to antimicrobial use are polarized around 2 sets of issues (Barton 1998):

1) Issues that concern proponents of the view that antibiotic use in animals impinges on human health include:

    - The prevalence of antimicrobial resistant bacteria in food-producing animals
    - Evidence that resistant organisms and genes encoding resistance can be passed between animals and people and into the environment
    - The large amount of antimicrobials fed as growth promotants or prophylactic treatments in animals
    - The use in animals of antimicrobials that are used therapeutically in human medicine or which select for cross-resistance to antimicrobials used in human medicine,

2) Arguments for the view that antimicrobial resistance in human pathogens stems from improper use of those drugs in human medicine include:

    - Apart from growth promotants, antimicrobials are used much less in animals than in people
    - Use of antimicrobials in animals has not led to multi resistance problems seen in human medicine
    - The use of antimicrobials as growth promotants is important to the economics and sustainability of intensive livestock production and preventive and therapeutic treatments are essential for animal welfare.

Unfortunately, sound evidence regarding the above issues is sparse and the absence of proof cannot be interpreted as the proof of absence (McGeer 1998). Placing emphasis on the direction of pathogens spreading from food-producing animals to humans may lead investigators to overlook equally important components of the ecology of these pathogens (Barber 2001).

Research is needed in many areas regarding the development, dissemination, and persistence capabilities of antimicrobial resistant organisms and resistance determinants (McDermott et al 2002; McEwen et al. 2008). Food safety concerns have driven investigations into the ability of foodborne bacteria to contaminate all steps in the

production of animal products. In 21 Alberta feedlots, common foodborne bacterial pathogens were rarely detected in carcass and environmental samples (Donkersgoed et al. 2009). Documentation of the transmission of resistant organisms from animals to food products to humans is limited (Piddock 1996). If contamination does occur, data have shown that antimicrobial resistant *E. coli* can enter the food chain regardless of whether or not cattle were administered growth promotants (Alexander et al. 2010). The pathway between the development of resistance in food animals and health threats involves many steps. Likely, the overall probability of transmission through all of these steps is low. However, comprehensive risk assessments are still needed to document these probabilities (Phillips et al. 2004).

The issue of antimicrobial resistance is multi-faceted and cannot be understood with only one approach. However, this overwhelming problem should be attacked one "patch" at a time (Levy 2002). Many calls for surveillance in agricultural populations to monitor antimicrobial resistance have been made (Aarestrup 2005; Anderson 1999; McEwen and Fedorka-Cray 2002; The Alliance for the Prudent Use of Antibiotics 2005). Data from these surveillance programs would theoretically document baseline levels of resistance and would allow earlier response to increasing resistance trends. Responding to low levels of resistance rather than high levels may be crucial since resistance genes are often difficult to eliminate (Austin et al. 1999; Lee et al. 2010; Salyers and Amabile-Cuevas 1997).

SURVEILLANCE

In the past, systems monitoring the usage of antimicrobial drugs may not have been sufficient for specifically documenting and responding to antimicrobial resistance. To comply with drug regulations, these early systems were less focused on antimicrobial resistance than they were on detecting residues in food, allergic reactions, and drug toxicities (Black 1984). However, recent efforts with specific focus on antimicrobial resistance have been conducted as individual cross-sectional studies as well as large scale, ongoing national programs (Aarestrup 2004; Bager 2000; Bronzwaer et al. 2002; Hendriksen et al. 2008; Kaspar 2006). Examples of the organizations monitoring antimicrobial resistance and use on the national scale include:

- ARBAO II Antibiotic resistance in bacteria of animal origin—II (Europe)
- EARSS European Antimicrobial Resistance Surveillance System
- ESAC European Surveillance of Antimicrobial Consumption
- DANMAP Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
- JVARM Japanese Veterinary Resistance Monitoring System
- STRAMA Swedish Strategic Programme Against Antibiotic Resistance
- NARMS National Antimicrobial Resistance Monitoring System (USA)
- CIPARS Canadian Integrated Program for Antimicrobial Resistance Surveillance

Additionally, the Global Advisory on Antibiotic Resistance Data (GAARD) with the Initiative of the Alliance for the Prudent Use of Antibiotics (APUA) has produced comprehensive reports on the state of antimicrobial susceptibility internationally. The efforts of these monitoring systems have provided crucial data for their nations. However, the World Health Organization (WHO) and the World Organization for Animal

Health (OIE) have both made calls for standardization of these programs to allow better comparisons of the global state of antimicrobial resistance and use.

Though harmony is needed among systems, separate surveillance programs with different goals are inevitable. In developing countries, routine and efficient methods for prevention strategies conducted in developed countries may not be practical (Vlieghe et al. 2010). Surveillance in critical care and tertiary care facilities often is more intensive since nosocomial infections have a high probability of involving antimicrobial resistance complications (Ogeer-Gylels et al. 2006). Despite the typical perception that companion animals are not significant reservoirs for antimicrobial resistance, surveillance programs tracking resistance in these populations are also important (DeVincent and Reid-Smith 2006; Guardabassi et al. 2004).

Many surveillance programs are currently in operation, yet optimal methodology for conducting surveillance is unknown. Key features have been suggested for surveillance such as having a statistically valid sampling program, avoiding "copy strains," and using standardized methodology in testing susceptibility (Wallman 2006). Also, due to the need to elucidate associations with resistance, these surveillance programs should document quantities of antimicrobial use (Singer et al. 2006; Bager 2000; Szhotnicki 2004). Beyond the details of program components, overall the greatest current weakness concerning surveillance is simply a lack of adequate data and appropriate response (Williams 2001).

ANTIMICROBIAL RESISTANCE IN CATTLE

The level of antimicrobial resistance in cattle is relatively low according to studies in dairy, cow calf beef, and feedlot herds. Less than 10% of *Pasteurella* spp. and *Mannheimia haemolytica* isolates recovered from healthy calves on 16 dairy herds were able to grow on oxytetracycline-selective media (Catry et al. 2006). Cattle with respiratory infections also had overall low levels of resistance in isolates of respiratory pathogens, except for resistance to sulfamethoxazole in *P. multocida* and *M. haemolytica* and resistance to ampicillin in *M. haemolytica* (Schwarz et al. 2004). The majority of commensal *E. coli* and *Salmonella* spp. recovered from the feces of dairy cows on farms in 21 states had no resistance to a broad range of antimicrobial drugs (Lundin et al. 2008).

Resistances which do commonly exist in these populations are not classified as being of very high importance to human health. Genetic investigations of antimicrobial resistance in healthy lactating dairy cows have found that *E. coli* is an important reservoir for tetracycline and other antimicrobial resistance determinants (Sawant et al. 2007). Investigations of calves and cow-calf pairs found that resistance was rare to antimicrobials classified as being of very high importance to human medicine. The most common resistances in these populations were to tetracycline, sulfamethoxazole, and streptomycin (Gow et al. 2008a). Cow calf farms were at lower risk than feedlots for having *E. coli* isolates that were resistant to tetracycline, sulfamethoxazole, and streptomycin. No resistances to ceftriaxone or ciprofloxacin were observed in the feedlot isolates and less than 1% of isolates were resistant to gentamicin, nalidixic acid, and ceftiofur (Carson et al. 2008b). A separate study also found that resistances to tetracycline and sulfamethoxazole were common in feedlots (Dargatz et al. 2002).

However, most isolates of *Salmonella* recovered from pen floor samples at these 100 feedlots were susceptible to all antimicrobials tested. Despite lacking evidence of direct threats to human health by way of antimicrobial resistance in these populations, the perception is that the emergence of such a problem is possible and should be closely monitored.

Molecular investigations have revealed multiple mechanisms of resistance that are both transferable (plasmids and transposons) as well as permanent (chromosomal changes) (Wilson 1990). Plasmids and transposons have a role in the spread of the resistant genes in *Pasteurella* and *Mannheimia* isolates (Kehrenberg et al. 2001). Plasmids also have been documented to conjugate with commonly between *E. coli* and *Salmonella*. In an outbreak investigation of salmonellosis in calves, plasmids conferring resistance to apramycin and several other antibiotics were transferred by conjugation *in vitro* from *E. coli* to *S. typhimurium* (Hunter et al. 1992). Recently, a novel mechanism (radical-induced mutagenesis) has been documented for the development of resistance to antimicrobials when sublethal levels of different antimicrobials are applied (Kohanski et al. 2010). Unfortunately, traditional testing methodologies which can identify susceptibilities in antimicrobials may not be able to detect novel resistance phenotypes (Tenover 2001). Selective pressures on bacteria can encourage the development of novel resistance genes or can help establish acquired resistance traits (Kehrenberg et al. 2001). However, the genes themselves are not responsible for the greater fitness advantage of antimicrobial resistant *E. coli* in calves. Other factors such as the farm environment and diet exert selective pressures (Khachatryan et al. 2006).

Multiple factors participate in establishing and maintaining antimicrobial resistance. Feed can harbor genetic elements associated with resistance for feedlot cattle by way of contamination with *E. coli* and *Salmonella* or residual determinants from feed components such as wet distillers grain with solubles (Dargatz et al. 2005; Jacob et al. 2010). The environments of intensively managed animals such as feedlot cattle can harbor resistant bacteria and resistance determinants (Alexander et al. 2009; Berge et al. 2010; Gibbs et al. 2006; Holzel et al 2009). Resistance occurrence also varies dependent on certain host factors such as age (Berge et al. 2010; Gow et al. 2008a). Environmental and host factors likely interact with other selective pressures and it is unlikely that any single exposure factor can wholly account for the development and maintenance of resistance (Harada and Asai 2010; Witte 2000).

INDIRECT ASSOCIATIONS BETWEEN ANTIMICROBIAL USE AND RESISTANCE

Within the web of factors which are associated with antimicrobial resistance, antimicrobial use is hypothesized to be a significant component. A classic model for investigating associations between antimicrobial use and resistance indirectly has been to compare resistance in production using conventional practices which include the use of antimicrobials versus production in populations which have specifically excluded the use of antimicrobials. These studies hypothesize that if antimicrobial use is significantly associated with antimicrobial resistance, then differences in resistance will be detected between the production methods. An investigation of *Campylobacter* spp. on swine farms found no difference in the prevalence of this bacterium between antimicrobial-free and conventional production methods, but did find a lower prevalence of antimicrobial resistance in the antimicrobial free farms (Rollo et al. 2010). Though these authors noted that resistances tended to decline as the number of years that a farm was antimicrobial-free increased, they suggested that investigation of other interventions to reduce resistance levels was warranted. Conversely, investigations of antimicrobial susceptibility in organic (i.e., no or severely limited antimicrobial use) and conventional dairy herds have documented that resistances in *Campylobacter* spp. were no different between the production methods (Sato et al. 2004). Interestingly, these authors did find that calves had higher levels of resistance than cows supporting previous statements about other factors contributing to resistance. Additional comparison studies between these dairies revealed that resistance prevalence in *E. coli* isolates were different for 7 antimicrobials, but not significantly different for 10 other antimicrobials (Sato et al.

2005). Controlling for age, conventional dairy farms had significantly higher rates of resistance to ampicillin, streptomycin, kanamycin, gentamicin, chloramphenicol, tetracycline, and sulfamethoxazole. Production practices of swine and dairy operations are different from that of feedlot cattle, so extrapolation of these conclusions to feedlot cattle may be limited.

A recent study compared resistances in pens of feedlot cattle reared using conventional practices with those being fed without antimicrobial exposures (Morley et al. 2011). These authors concluded that conventional feedlot production methods (including parenteral and in-feed use of antimicrobials) do not predictably or uniformly increase the prevalence of a resistance in non-type specific *E. coli* when compared to production methods which restrict exposure to antimicrobial drugs. Additionally, though no tetracyclines were administered in these populations of feedlot cattle, the resistance to tetracycline increased temporally through the feeding period. Similarly, in a separate study, resistance to streptomycin, sulfamethoxazole, and tetracycline increased significantly from arrival to mid-point during the feeding period and persisted until market-readiness (Carson et al. 2008b). Therefore, temporal and transient trends in the prevalence of resistance, which vary between antimicrobial drugs, might account for resistance levels rather than exposure to antimicrobial drugs. Conflicting conclusions from these comparison studies support the need for more direct investigations in the association between antimicrobial use and resistance. Well-designed association studies are needed to shed more light on the lesser understood quantitative aspects of antimicrobial resistance (Phillips 1998).

DIRECT ASSOCIATIONS BETWEEN ANTIMICROBIAL USE AND RESISTANCE

Evidence for and against direct associations between the use of antimicrobial drugs and antimicrobial resistance has been documented. An early study tracking antimicrobial use in feedlot calves and relating it to levels of resistance revealed that therapy with a particular antimicrobial in the week prior to death, increased the level of resistance to *P. haemolytica* to that antimicrobial (Martin et al. 1983). These authors also made observations that resistance to penicillin, tetracyclines, and chloramphenicol occurred more frequently together than expected by chance alone. Injectable oxytetracycline in addition to in-feed chlortetracycline administered to cattle was associated with an increase in the prevalence of resistance in commensal *E. coli* to chloramphenicol and sulfisoxazole, but no other tested antimicrobials (O'Connor et al. 2008). Exposure to chlortetracycline for feedlot cattle was associated with a temporary increase in the recovery of resistant *E. coli* and *Enterococcus* isolates (Platt et al. 2008). Also of note, the ceftiofur-resistant *E. coli* isolates in this study actually declined during the exposure to chlortetracycline. The transient expansion of multiple-resistant variants of *E. coli* was found to be associated in a separate study with the parenteral administration of ceftiofur crystalline-free acid to feedlot steers (Lowrance et al. 2007). Susceptibility returned to baseline levels approximately 2 weeks after completion of the ceftiofur crystalline-free acid administration. Positive associations between in-feed as well as injectable tetracycline were found for resistance to tetracycline, streptomycin, and sulfadiazine among non-type specific *E. coli* in feedlot cattle (Rao et al. 2010). However, these authors concluded that the differences noted were relatively small and of

questionable practical relevance. In cattle receiving antimicrobials for metaphylaxis and treatment in the absence of in-feed macrolides and tetracyclines, no associations were found between antimicrobial use and resistance in recovered isolates of *E. coli* (Checkley et al. 2008). A lack of any associations in *Salmonella* isolates between resistances and the presence of antimicrobials in feed were noted in another study of feedlot cattle (Dargatz et al. 2002). Specific investigation into resistances in *E. coli* isolates recovered from feedlot cattle given subtherapeutic administration of tetracycline in combination with sulfamethazine revealed associations with tetracycline and ampicillin resistances (Alexander et al. 2008). However, these authors acknowledged that additional environmental factors such as diet may be related to these resistances.

The studies described above specifically investigating the association between antimicrobial use and antimicrobial resistances in cattle do provide some evidence that associations exist in these populations. However, as always it is important to keep in mind that association is not causation and further studies are warranted that can account for confounding variables and other biases.

ANTIMICROBIAL USE

Antimicrobial drugs are crucial to the health and management of agricultural populations of animals. Administration of antimicrobials in feedlots is largely for the prevention of liver abscesses and the prevention and treatment of bovine respiratory disease. On all feedlots included in a representative national study, bovine respiratory disease was the most common disease condition and nearly all of the feedlots included injectable antimicrobial drugs (most commonly tilmicosin, florfenicol and tetracyclines) as part of an initial course of treatment for bovine respiratory disease (NAHMS 1999). If the therapeutic regimen used for initial treatment failed to result in a favorable response, 84% of the feedlots changed their choice of antimicrobial. Large feedlots were more likely than small feedlots to administer antimicrobials metaphylactically to groups of cattle to prevent bovine respiratory disease, though overall only 10.4% of cattle placed in feedlots were administered antimicrobials for this reason. Many (83.2%) of the surveyed feedlots also included antimicrobials in feed or water as a health or production management tool. A Canadian study quantified the commonly used antimicrobials by injection (oxytetracycline, penicillin, macrolides, florfenicol, and spectinomycin), in feed (monensin, tylosin, lasolocid, and tetracyclines), and in water (lincomycin-spectinomycin, chlortetraycline, and oxytetracycline) (Carson et al. 2008a). Though usage of antimicrobials is common in North American feedlots, veterinarians weigh multiple factors in the decision to utilize appropriate antimicrobials. A survey of feedlot veterinarians indicated that the effects of moral beliefs on behavioral beliefs were contingent on the condition such as the level of risks associated with treating or not

treating cattle and the effectiveness of antimicrobials in acute illness (McIntosh et al. 2009).

The ability of antimicrobials to treat or prevent an indication (efficacy) and the ability to do this well (effectiveness) are major components in the decision to use these drugs. Considering the health risks associated with antimicrobial resistance and the potential association with antimicrobial drug use, evidence of usefulness of these drugs is most definitely necessary. The approval process for new animal drug applications through the Food and Drug Administration (FDA) requires that antimicrobial drugs meet standards of effectiveness and safety. However, further independent field trials in the feedlot sector often follow FDA approvals to further evaluate antimicrobials. Tilmicosin and oxytetracycline in feedlot cattle have been shown to be useful as prophylactic (given prior to an expected infection) antimicrobial drugs for reducing morbidity due to bovine respiratory disease (Donkersgoed 1992; Frank and Duff 2000; Merrill et al. 1994; Schunicht et al. 2000a). Given metaphylatically (at the time of an expected infection) antimicrobials such as florfenicol and tulathromycin are also useful in managing bovine respiratory disease (Booker et al. 2007; Duff and Galyean 2007; Frank et al. 2002). Administration of antimicrobials for treatment of bovine respiratory disease is primarily more effective if disease is recognized early (Cusack et al. 2003). The drugs which have been found to be effective as treatment of undifferentiated fever include tulathromycin, florfenicol, tilmicosin, trimethoprim-sulfadoxine, oxytetracycline, penicillin, and ceftiofur (Batemen et al. 1990; Booker et al. 1997; Guichon et al. 1993; Harland et al. 1991; Jim et al. 1992; Jim et al. 1999; Mechor et al. 1988; Schunicht et al. 2007). Many of these antimicrobials have been compared to one another to assert the comparable or

superior efficacy of one drug to another. The antimicrobial drugs discussed above also improve growth efficiency which is a characteristic in feedlot production that is highly regarded (Gorham et al. 1990; Merrill et al. 1994; Encinias et al. 2006; Schumann et al. 1990; Schunicht et al. 2002b). Cost effectiveness is another crucial characteristic of these antimicrobials in the context of antimicrobial resistance and has also been investigated for these drugs (Perrett et al. 2008; Booker et al. 2006; Schunicht et al. 2002a).

The impact of antimicrobial resistance on bovine respiratory disease is not well established (Watts and Sweeney 2010). As previously described in the antimicrobial resistance section, resistance in feedlot cattle is relatively low. Despite more common resistances to tetracycline in feedlot populations, the efficacy of tetracyclines does not seem to be compromised (Rao et al. 2010). However, a deficiency in information about antimicrobial use complicates antimicrobial research and proper risk assessments are needed to evaluate the potential loss of usefulness of antimicrobial drugs (Fraser et al. 2004; McEwen and Singer 2006). Additionally, since the microbial ecologies of animals and humans are intertwined, any shared loss of usefulness (loss of susceptibility) becomes a concern beyond the feedlot (Witte 1998; Bywater 2004; Singer et al. 2006).

ANTIMICROBIAL USE POLICY

In the United States, the safety of drugs in target species was first regulated by the Federal Food, Drug, and Cosmetic Act in 1938. Among many amendments to this act, ones pivotal in the context of antimicrobial use in feedlot cattle categorized prescription and over-the-counter drugs separately (1951) and provided for the authority of the Food and Drug Administration Center for Veterinary Medicine (FDA-CVM) (1962). More recently, the Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA) began to regulate extra-label use of drugs by veterinarians. A current bill (Preservation of Antibiotics for Medical Treatment Act of 2009 [PAMTA]) is still in the first step of the legislative process and has the objective to preserve the effectiveness of medically important antibiotics used in the treatment of human and animal diseases (Wren 2007). Though not a formal regulation requirement, the Food Animal Residue Avoidance and Depletion Database (FARAD) is a national tool sponsored by the United States Department of Agriculture which aids in avoiding illegal drugs in foods of animal origin. An extensive review of the scientific evidence related to antimicrobial resistance threats to human health due to the use of antimicrobial drugs in animals was conducted by a scientific advisory panel known as "The Facts about Antimicrobials in Animals and the Impact on Resistance" (FAAIR 2002). This collection of researchers has made the following recommendations:

1. Antimicrobial agents should not be used in agriculture in the absence of disease
2. Antimicrobials should be administered to animals only when prescribed by a veterinarian
3. Quantitative data on antimicrobial use in agriculture should be made available to inform public policy

4. The ecology of antimicrobial resistance should be considered by regulatory agencies in assessing human health risk associated with antimicrobial use in agriculture
5. Surveillance programs for antimicrobial resistance should be improved and expanded
6. The ecology of antimicrobial resistance in agriculture should be a research priority

In Canada, regulation of veterinary biologics and medicated feeds is done by the Canadian Food Inspection Agency (CFIA). The Veterinary Drugs Directorate (VDD) is the branch of Health Canada that approves drug products and determines withdrawal times. Currently, extra-label use of drugs by veterinarians is not regulated by any legislation, though Canadian offices of the global FARAD aids in determining withdrawal times for such extra-label drug use. The list of drugs prohibited in food animals in Canada is different from that of the United States (Dowling 2003).

International organizations have also addressed issues of antimicrobial resistance. The World Organization for Animal Health (OIE) has published guidelines for veterinary pharmaceutical industry, veterinary practitioners, dispensing pharmacists, and farmers with the objective "to maintain antibiotic efficacy, to avoid dissemination of resistant bacteria or resistance determinants, and to avoid the exposure of humans to resistance through food" (Anthony et al. 2001). The World Health Organization (WHO) ranks and updates antimicrobials according to their importance in human medicine in efforts to develop risk management strategies (Collignon et al. 2009). These two organizations also have made a joint report with the Food and Agriculture Organization (FAO) on Critically Important Antimicrobials (2007). This meeting was a continuation of another meeting of the three organizations in 2003 after recommendations from the Executive Committee of the Codex Alimentarius Commission were discussed in 2001. Among

many recommendations from the Report of the Joint FAO/WHO/OIE Expert Meeting on Critically Important Antimicrobials in 2007, one relevant to current surveillance efforts in feedlot cattle is:

> 5. Antimicrobial resistance monitoring of foodborne pathogens and commensals (animal, human, food and commodity) should be implemented by all countries considering risk management measures, to enable the detection of hazards and accurately assess the success of selected interventions. Ideally, quantitative standardized minimum inhibitory concentration methods should be applied.

Precautionary bans on growth promotants have been established in Sweden (1986) and the European Union (1997). These bans had roots in recommendations dating back to 1969 with the Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine in the United Kingdom which concluded that "the administration of antibiotics to farm livestock, particularly at sub-therapeutic levels, poses certain hazards to human and animal health; in particular it has led to resistance in enteric bacteria of animal origin." Since these bans, conflicting reports of success and failure as a result of the bans have been reported. The occurrence of antimicrobial resistance in a national population of food animals was ultimately reduced after the government of Denmark banned avoparcin in 1995 and virgniamycin 1998 (Aarestrup et al. 2001). However, a list of adverse consequences such as a deterioration of animal health and an increase in the usage of therapeutic antibiotics in food animals which are of direct importance to human medicine has also been reported (Casewell et al. 2003; Bywater 2005). A separate study in Switzerland reports that the ban on growth promotants in feedstuffs did not result in an increase in therapeutic use of antibiotics in medicated feed (Arnold et al. 2004). Additionally, long-term evaluation of the bans in

swine showed an improvement in productivity (Aarestrup et al. 2010). A report investigating the possibility of a similar ban in the United States has stated that discontinuing use of antimicrobial drugs in swine production would initially decrease feed efficiency, raise feed costs, reduce production, and raise prices to consumers (Matthews 2001).

Prudent and judicious use of antimicrobial drugs has been suggested as a means to reduce consumption and manage resistance in both human and veterinary medicine (Shlaes et al. 1997; Morley et al. 2005). In Germany, the change in prescription patterns of veterinarians in response to prudent use guidelines dramatically reduced antimicrobial drug consumption within 2 years (Ungemach et al. 2006). Antibiotic stewardship and consumption varies across European human hospitals, but studies are currently underway to evaluate the impact of prudent use guidelines including optimal approaches to respiratory infections, cycling antimicrobials in intensive care units, patient education materials, and strategies to improve doctor-patient communication (Bruce et al. 2004; McGowan 2000; Schwartz 1999). The FDA-CVM has recently distributed a draft guidance for the judicious use of medically important antimicrobial drugs in food-producing animals (2010) which puts forth two measures to phase in:

1. Limiting medically important antimicrobial drugs to uses in food-producing animals that are considered necessary for assuring animal health; and
2. Limiting such drugs to uses in food-producing animals that include veterinary oversight or consultation.

In addition to prudent use, improved infection control and hygiene have been suggested to further reduce consumption of antimicrobial drugs (van den Bogaard and Stobberingh 1999). These efforts together may have the ability to "turn the tide of antimicrobial

resistance" (Monnet and Kristinsson 2008). Yet, expectations of reversals in antimicrobial resistance should be accepted with caution since adequate data are lacking to detect these changes (Phillips 2001).

CHALLENGES IN ANTIMICROBIAL RESISTANCE SURVEILLANCE

The burden of antimicrobial resistance has both health and economic impacts and efforts to reduce these are warranted (Holmberg et al. 1987; Howard et al. 2001; Howard and Scott 2005; McGowan 2001). Improved surveillance systems which investigate associations between use and resistance can serve as "information for action" in developing policies which reduce unnecessary prescribing and prolong the usefulness of antibiotics (Livermore 1998). Minimum epidemiological and microbiological requirements for establishing surveillance of antimicrobial resistance in bacteria of animal origin have been defined (Caprioli et al. 2000). However, the intricacies of surveillance components are not well understood. This chapter has described issues surrounding antimicrobial resistance, surveillance efforts currently in place, the prevalence of antimicrobial resistance in feedlot cattle, indirect and direct associations between antimicrobial resistance and use, the necessity of antimicrobial use in feedlot cattle, and the regulatory policies surrounding these issues. Some areas to consider which represent gaps in knowledge about antimicrobial use and resistance are listed below.

*Summary of Gaps (**bold** indicates gaps being further considered in this dissertation):*

- Genetic methods to trace antimicrobial resistance within and between host populations

- Ecological approaches to evaluation of microbial relationships between humans and animals
- **Accurate quantification of antimicrobial use**
- Investigations of the dissemination and persistence of antimicrobial resistance
- Comprehensive risk assessments of antimicrobial resistance
- More antimicrobial resistance surveillance programs; local, national, and international
- **Optimization of methodology and standardization for surveillance programs**
- Susceptibility testing capable of detecting novel resistance
- **Prevalence of resistance in food-producing animals**
- **Studies investigating direct associations between antimicrobial use and resistance**
- Identification of other factors inflating or hiding true associations
- Evaluation of prudent use and other interventions in changing trends in resistance

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CHAPTER 2: Metrics for quantifying antimicrobial use in beef feedlots

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INTERPRETIVE SUMMARY

In the investigation of antimicrobial resistance, antimicrobial use must be accurately quantified since it is hypothesized to be one of the major forces driving the development of resistance. Without accurate representation of exposures to antimicrobials, the association between antimicrobial use and resistance can be falsely inflated or hidden. The selective pressures which encourage the development, dissemination, and persistence of antimicrobial resistance vary by a number of factors including the antimicrobial drug and the degree of exposure in host tissues to heterogeneous populations of bacteria. Yet, the common metrics (sales value and mass of active ingredient) currently used to quantify antimicrobial use do not account for the selective pressures in any manner.

The following project investigates the appropriateness of a series of metrics for quantifying antimicrobial use in beef feedlots. Individuals knowledgeable and interested in this particular topic contributed their perceptions about the related issues in a web-based survey. Participants were prompted to indicate the accuracy of each metric to describe antimicrobial use as well as the clarity of each metric in reference to how easily it is understood by user groups. These responses help to validate portions of the methodologies recommended for analysis and reporting of antimicrobial use information in surveillance of antimicrobial resistance.

ABSTRACT

Objectives: In order to further enlighten discussions regarding the impact of antimicrobial drug use in agriculture, accurate antimicrobial drug use data are needed. The primary objective of this study was to investigate the preferences of stakeholders for reporting antimicrobial drug use data that are collected from beef feedlots.

Materials and Methods: Producers, veterinarians, industry representatives, public health officials, and other knowledgeable beef industry leaders were invited to complete a web-based survey. Participants were asked to provide demographic information and to comment on the most appropriate portrayal of antimicrobial drug use data for different purposes. The survey also explored perceptions and concerns about antimicrobial resistance.

Results: A total of 156 participants in 33 U.S. states, 4 Canadian provinces, and 8 other countries completed the online survey. Preference for methods of presenting antimicrobial drug use data varied and was influenced by participant perceptions regarding clarity and accuracy of the method to represent antimicrobial drug use in large cattle populations. Antimicrobial drug use has most commonly been reported as mass of active compound or sales value; however, participants in this study indicated that these methods were the least appropriate for reporting data to the general public. Compared to 10 years ago, many participants had greater concern about antimicrobial resistance as a health issue for both humans and animals.

Conclusions: To effectively communicate antimicrobial drug use data, evaluation of the target audience is critical to presenting the information clearly and accurately. Metrics that are most accurate need to be carefully and repeatedly explained to the audience.

INTRODUCTION

Antimicrobial drugs (AMDs) are used in the feedlot for prevention of disease (prophylaxis/metaphylaxis), treatment of disease and improvement of production efficiency (Apley 1997; Apley 2008; Barton 2000). Due to concerns about the potential impact on public health and the development of antimicrobial resistance (AMR), antimicrobial drug use (AMU) in cattle and other animals is a controversial subject (Barbosa et al. 2000; Casewell et al. 2003; Phillips et al. 2004; van den Bogaard et al. 2000; Witte 1998).

Obtaining accurate data about AMU is critical to improving our understanding of the controversial public health and AMR issues (Caprioli et al. 2000; McEwen and Singer 2006; Phillips 1998; van den Bogaard et al. 1999; FAAIR Scientific Advisory Panel 2002; Livermore et al. 1998). In order to report accurate usage and investigate potential associations with AMR, we need accurate and practically relevant measures to objectively quantify AMU (Carson et al. 2008; Filius et al. 2005; Singer et al. 2006). Metrics which have been and are still being used are defined (Table 1). Each of these methods of reporting drug usage has both advantages and disadvantages (Chauvin et al. 2001; Jensen et al. 2004; Merlo et al. 1996; Kritsotakis 2006). The goal of this survey was to identify a method for presenting AMU data that is easily understood by user groups and that accurately portrays drug use data in a meaningful and relevant way.

Table 1: **Definitions of antimicrobial drug use (AMU) metrics.**

AMU Metric	Definition
Sales Value	cost of the antimicrobial drug in standard currency
Drug Mass in Kilograms	kg of active ingredient
Number of Animals Treated	count of animals treated with antimicrobial drug
Treatment Rate	the percentage of animals receiving a given treatment in a given population
Animal Defined Daily Dose (ADDD)	number of days of treatment for an animal based on an assumed average maintenance dosage
ADDD per 1000 Animals	standardized exposure rate based upon the Animal Defined Daily Dose relative to a fixed number of animals; used to make standardized comparisons in drug exposure among populations or over time

MATERIALS AND METHODS

Sampling Procedures

The study was conducted as a cross-sectional survey. The targeted study population consisted of owners and operators of beef production facilities, veterinarians, beef industry representatives, and public health officials familiar with AMU in the beef industry. Potential participants were contacted through email listservs managed by relevant professional associations or agencies[a] and by email sent to a list of individuals compiled through recommendations of beef industry and public health leaders. Additionally, participants were encouraged to freely distribute the survey to other knowledgeable and interested colleagues.

For each association or agency that was identified as being interested in this issue, the association president or another administrative leader was contacted by e-mail to determine whether their group was willing to participate. If so, an invitation was posted to the association's listserv. This same invitation was also sent to people specifically identified by stakeholders as being knowledgeable and interested in the topic. Direct access to the web-based survey instrument[b] was provided in the email invitation as a hyperlink. Additionally, this email invitation included a second hyperlink which allowed the invited participant to specifically decline the opportunity to participate (Appendix 2). Approximately 2-3 weeks after the initial invitation, a reminder email was sent through each listserv and to the list of individuals. The web-based survey was available for completion for a 3-month period between June and August 2009. The survey collection instrument was set to only allow one response to be submitted per computer.

Study participation was voluntary and anonymous. Response information and participation was confidential. Prior to initiation of the study, the research protocol was reviewed and approved by the Colorado State University Institutional Review Board.

Survey instrument

The survey contained 22 questions characterizing 3 different general topics: participants' demographics and activities related to the beef industry, opinions on the issues of AMU and AMR, and perceptions about how information regarding AMU is best reported for beef cattle (Appendix 1). Most of the questions required participants to select from a closed series of responses or Likert scale categories. For all questions, response options of "Unknown" and "No Preference" were available. Additionally, open-ended responses were solicited on some questions to allow elaboration if desired by the participant. The questionnaire was pretested by 9 experts that matched the demographics of the intended study population.

Demographics. Individual participants were characterized through questions regarding the number of years of active involvement with the beef industry, the primary state/province and nation of their professional activities, and their highest level of education (high school diploma/GED, degree/diploma from a technical school or community college, bachelor's degree/BS/BA, advanced degree—specify). Additionally, participants were characterized by the primary professional role in which they used AMU information (producer, production consultant, veterinarian, federal government representative, state government representative, university employee, nutritionist, feed salesperson, pharmaceutical industry representative, other—specify) and the top three

sources from which they obtain information about AMDs (feed or drug companies, veterinarians, government extension officers, universities, farm magazines and newsletters, friends/relatives/neighbors, internet/world wide web, peer reviewed journals, beef specialists, other—specify).

Perceptions about AMR and AMU. Regarding AMR as a health issue, participants were asked whether their concerns had changed over the 10 years prior to the study (much greater, somewhat greater, no different, somewhat less, much less) at different organizational scales (locally/individual operations, regionally, nationally, globally). Similarly, participants provided their perceptions on the true risk of health problems as a result of AMR. Participant perceptions regarding the importance of five uses of AMDs in feedlot cattle were also solicited. This was achieved through providing categories that specified the necessity of AMDs for each use (feedlots need AMDs for this specific use, feedlots would be difficult to manage without this specific use, feedlots could be managed without this specific use, feedlots do not need AMDs for this specific use). The five uses of AMDs investigated were prophylaxis/metaphylaxis at arrival, prophylaxis/metaphylaxis after arrival, use in feed or water for treatment of disease, injectable drugs for treatment of disease, and use in feed to prevent liver abscesses.

AMU Metrics. In order to investigate the appropriateness of different methods of quantifying AMU for various purposes, participants were asked to select the first and second most appropriate methods (which are reported cumulatively) of quantifying AMU relative to hypothetical scenarios. Scenarios included a comparison AMU for 2 AMDs in a large cattle population (e.g., feedlot), describing AMU data for investigation of AMR in a scientific paper, and reporting of AMU data to the general public. Participants were

also asked to identify the least appropriate quantification method for reporting AMU to the general public. The scenarios were all structured around hypothetical situations which summarized the use of 2 AMDs according to label instructions for respiratory disease in an 'average' population of feedlot steers shortly after placement. An average steer was considered to weigh approximately 250kg. Quantification methods were presented in tabular form with analogous calculations between the two hypothetical AMDs. The quantification methods investigated in this survey were number of treated animals, total mass of active drug, Animal Defined Daily Dose (ADDD), ADDD per 1000 animals, treatment rate, and sales value (Table 1). Definitions of each method were provided in each relevant section of the survey to ensure that participants were able to appropriately distinguish the different metrics (Appendix 1).

In the context of summarizing AMU for large cattle populations, participants specified the clarity and accuracy for two of the investigated metrics, number of animals treated and ADDD per 1000 animals (clarity categories: very clear, clear, somewhat clear, not clear, unknown; accuracy categories: very accurate, accurate, somewhat accurate, not accurate, unknown). In reference to an ongoing prospective surveillance program, participants were asked to select the best method for summarizing AMU for different organizational scales (local/individual operations, regional, national, global) and if a different definition or measurement was more appropriate for surveillance programs than the ones provided in this survey (unknown, no, yes—specify).

In regards to use of the ADDD method, participants were asked if data should be 1) calculated separately and reported separately for high and low dose exposures of the same drug, 2) calculated separately for high and low dose exposures, summed and

reported as one summary number, or 3) calculated using a common dose regardless of exposure and reported together. An open-response question asked participants to interpret "400 ADDD of tetracycline." The definition of ADDD was available on the same page as this open-response question.

Since AMD dosages can differ for various intended uses (e.g., prophylaxis, metaphylaxis, treatment of clinical disease, or improvement of production efficiency), participants were asked whether it was appropriate to combine these four categories when summarizing AMU data (yes, no, unknown, it depends—specify). A similar question asked if combining AMU data across different classes of AMDs would be appropriate (yes, no, unknown, it depends—specify).

Data analysis

Survey responses were downloaded directly from the web-based collection instrument into a computer spreadsheet and summarized. Odds ratios with associated 95% CIs were calculated for contingency tables and the χ^2 test was performed with statistical software.[c] For the purposes of analysis, some response categories were collapsed to facilitate evaluation of simple associations.

Demographic Classification for Categorical Analysis. The responses for the number of years of active involvement in the beef industry were dichotomized as being < or ≥ the median of the response distribution. Participant locale was categorized into North American (U.S. and Canada), and non-North American. Professional role was categorized as veterinarian, university employee, producer, and other. The preferred

sources of AMD information were categorized as peer reviewed journal, veterinarians, feed or drug companies, and others.

Categorical Analyses of opinions about AMR and AMU Metric Classification. In order to facilitate analyses, Likert scale responses were dichotomized into categories for greater (much greater and somewhat greater) and not greater (no difference, somewhat less, much less, and unknown). Quantification metrics were grouped into three categories: 1) ADDD or ADDD per 1000 animals, 2) number of animals treated or treatment rate, and 3) sales value or total mass of active ingredient. Responses of unknown and no preference about appropriate metrics were excluded from analysis due to low response frequency for these categories. Responses to questions regarding the clarity and accuracy of ADDD per 1000 animals and number of animals treated were dichotomized into clear/accurate (very clear/accurate, clear/accurate) and not clear/accurate (somewhat clear/accurate, and not clear/accurate). A single evaluator (KMB) categorized the open-response question for defining "400 ADDD of tetracycline." Responses which indicated participant understanding of the definition of ADDD were considered correct. Other responses were designated as incorrect if an obvious misunderstanding was described in open-response or as unknown if the participant volunteered their lack of understanding of this metric.

RESULTS

Survey Participants

Twenty associations and agencies were identified as having goals or interests that would be relevant to the issue of AMU in cattle.[a] Administrative leaders from 10 organizations agreed to post the invitation to their listservs and an additional 6 associations or agencies provided a list of specific individuals to contact directly with an invitation to participate. The survey was initiated by 250 individuals and 156 of these participants fully completed the survey. Only responses from completed surveys were summarized. Ninety-eight individuals specifically declined to take the survey using the hyperlink that was included for this purpose (Appendix 2).

Respondents resided in 33 U.S. states, 4 Canadian provinces, and 8 other countries (Belgium, Denmark, Germany, Ireland, Italy, Portugal, South Africa, and United Kingdom). The majority of respondents were from the U.S. (81%; 124/154) and Canada (12%; 19/154). The median number of years of reported involvement in the beef industry was 20 (Q1=10, Q3=34). Veterinarians (51%; 79/156), university professionals (19%; 29/156), and producers (10%; 16/156) were the professional roles most commonly represented by the participants. Other participants reported their professional roles as pharmaceutical industry representatives (8%; 13/156), federal government representatives (5%; 8/156), feed sales representatives (1%; 2/156), state government representatives (0.6%; 1/156), production consultants (0.6%; 1/156), or other (4%; 7/156). As their highest earned degree, 90% (140/156) of participants held advanced degrees (e.g., MS, PhD, DVM), 6% (10/156) had baccalaureate degrees, 1% (2/156) had degrees from a technical school or community college, and 3% (4/156) had high school diplomas/GEDs.

Sources of Information. Seventy-two percent (112/156) of participants used peer-reviewed journals as one of the top three sources of information about AMDs, 60% (93/156) obtained information from veterinarians, 59% (92/156) gained their knowledge from feed or drug companies, and 37% (58/156) referenced universities. The world wide web (29%; 46/156) was used more often as one of the top three sources of AMD information than beef specialists (13%; 20/156), government extension officers (5%; 8/156), and farm magazines or newsletters (5%; 8/156).

Importance of Antimicrobial Drug Resistance

The study attempted to differentiate perceptions about differences in awareness or perceived risk from differences in true risks related to AMR. In general, participants had greater concern about AMR as either a human health issue or an animal health issue than they did 10 years prior to the study (Figure 1). Compared to their attitudes 10 years ago, 61% (95/156) of participants indicated that they had a much greater or somewhat greater concern about AMR as a global health issue for people. Similarly, about half (78/156) of the participants indicated that they had a greater or somewhat greater concern about AMR as a global health issue for animals. Participant concerns about AMR as a global health issue for people were no different than concerns about AMR as a global health issue for animals (P=0.17). When comparing responses regarding AMR as a global health issue for people to other scales, no differences were found at the national level (P=0.77). However, fewer participants had greater concern about AMR as a local (41%; 64/156; P=0.0032) and regional (49%; 76/156; P=0.027) health issue for people than at the global level. No differences were detected between responses about concern

expressed regarding AMR in animals at the global scale when compared to the local (*P*=0.76), regional (*P*=0.88), or national (*P*=0.81) scales.

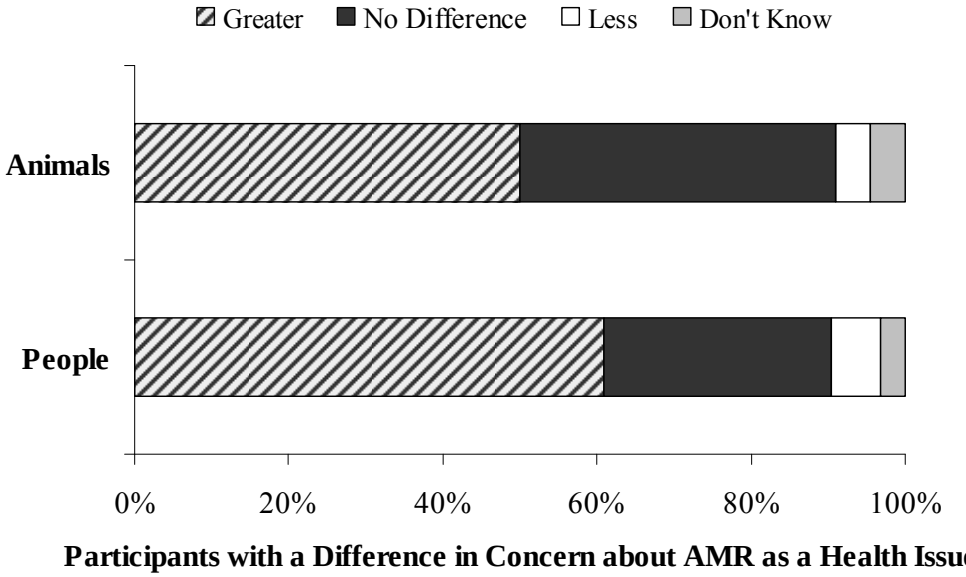

Figure 1: **Change in participants' level of concern about antimicrobial resistance** (AMR) as a global health issue in people and animals during the previous decade (n=156).*Distributions of participant perception of greater or less true risk of health problems due to AMR in people and in animals compared to their perception of true risk 10 years prior the study were no different than the distribution of concern level for Animals presented here (P>0.05).

Despite the majority of participants having greater concern about AMR as a global human health issue, fewer participants believed that the true risk of global human health problems as a result of AMR was greater than 10 years prior to the study (41%; 64/156; *P*=0.006). The percentage of participants with increased perceptions of true risk in animal health due to AMR was not statistically different than that for humans (45%; 70/156*P*=0.33). Compared to the global level, perceptions of true risk of human health problems due to AMR on the local (*P*=0.20), regional (*P*=0.17), and national (*P*=0.64)

scales were not statistically different. Likewise, the true risk of animal health problems as a result of AMR were not statistically different on the local (P=0.74), regional (P=0.51), and national (P=0.77) scales when compared to the global level.

Importance of Antimicrobial Drug Use

Participants indicated a similar spread of opinions on the necessity of AMDs for prophylaxis/metaphylaxis at arrival, prophylaxis/metaphylaxis after arrival, use in feed or water for treatment of disease, and use in feed to prevent liver abscesses (Figure 2). Four to 11% (6/156 – 17/156) of participants indicated that AMDs are not needed for these uses. Overall, about 20% of participants (16% - 22%; 25/156 - 34/156) indicated that AMDs are required for these uses. Whereas about one third of participants indicated that feedlots could be managed without AMDs (31% - 37%; 48/156 - 58/156) or management would be difficult without AMDs (30% - 42%; 47/156 - 65/156) for these uses. In contrast, the majority of participants agreed that injectable AMDs were needed for treatment of diseases of feedlot cattle (87%; 136/156). The remaining 13% (20/156) of participants indicated that management would be difficult without injectable AMDs for treatment of diseases in feedlot cattle.

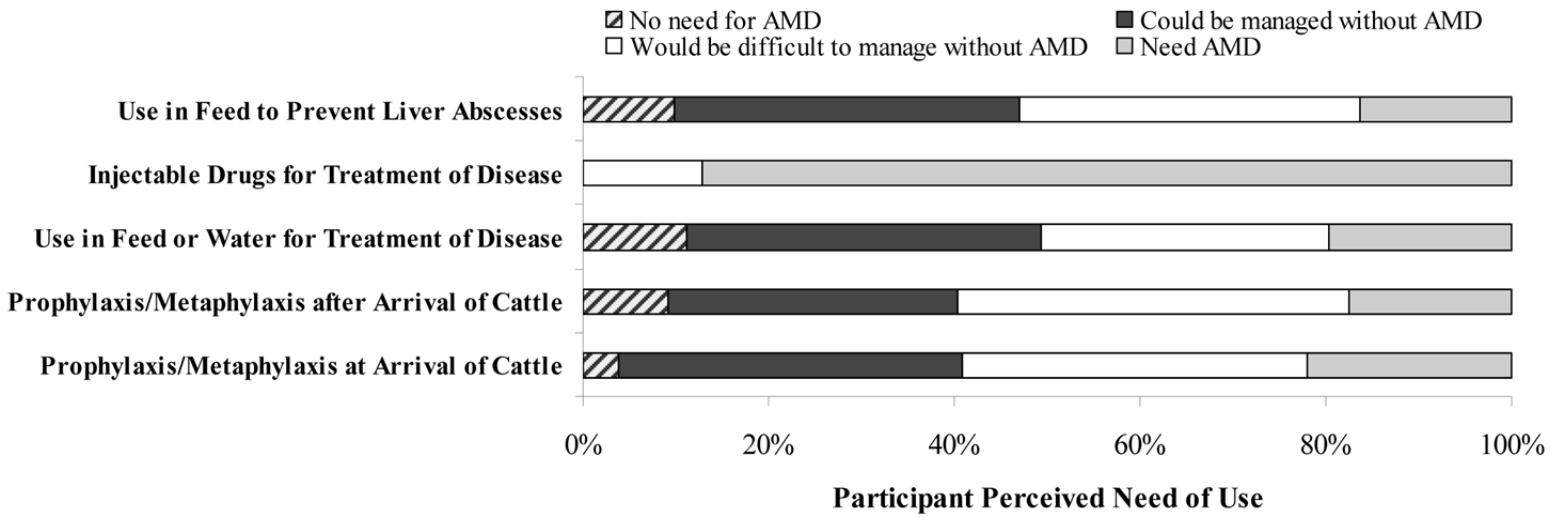

Figure 2: Participants' perceived need for five different uses of antimicrobial drugs (AMDs) in feedlot cattle (n=156).

AMU Metrics

Comparing 2 different classes of AMDs (e.g. macrolides vs. tetracycline). When comparing amounts of two different classes of AMDs, a similar percentage of participants indicated that the total mass of active drug (43%; 67/156) and the ADDD per 1000 animals (41%; 64/156) metrics were most useful (Figure 3). The sales value (11%; 17/156) metric was selected least often as an appropriate metric for comparing 2 different classes of AMDs.

Describing AMU relative to AMR in a scientific paper. When describing AMU data relative to investigating AMR in a scientific paper, ADDD per 1000 animals was the metric selected by the 46% of participants as most useful (72/156) (Figure 3). Sales value (2%; 3/156) and total mass of active drug (31%; 48/156) were selected by even fewer participants as appropriate metrics for this purpose than in the previous scenario.

Reporting AMU data to the public. More than half of the participants selected treatment rate (55%; 86/156) as the method which would allow the clearest interpretation in reporting data regarding AMU to the general public (Figure 3). As with the two previous scenarios, sales value (8%; 12/156) was selected by the smallest percentage of participants as an appropriate metric for reporting AMU data to the public. When asked about the least appropriate metric (as opposed to the most appropriate metric), respondents indicated that sales value (44%; 69/156) and total mass of active drug (31%; 49/156) were the most inappropriate metrics for reporting AMU data to the general public. All other metrics for this question were each selected by less than 8% (12/156) of the participants.

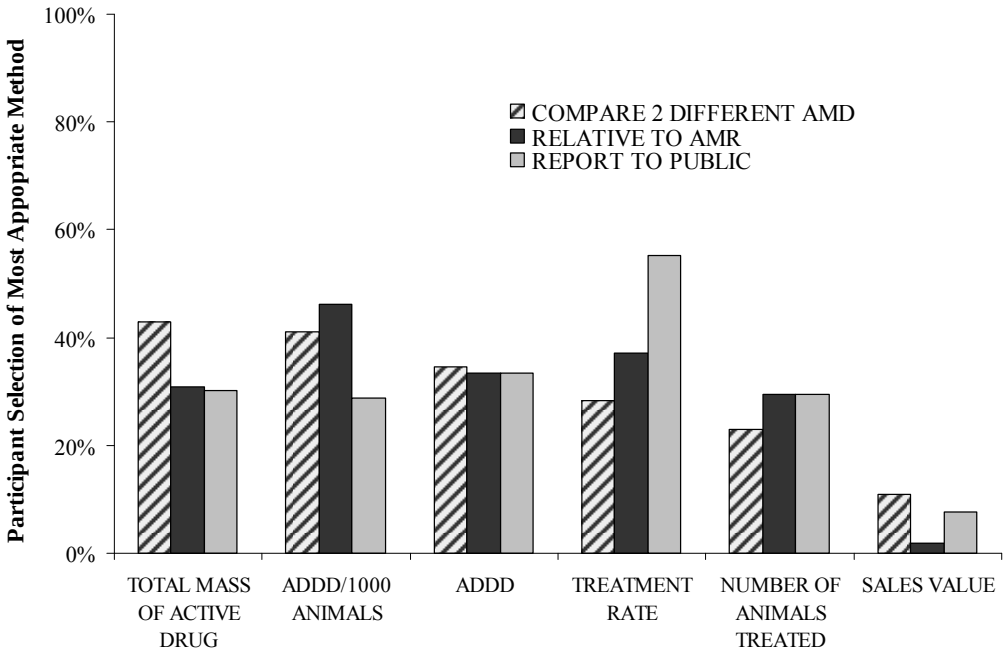

Figure 3: **Participant selection of the top two antimicrobial drug use (AMU) metrics** (cumulatively presented) most appropriate for three separate scenarios; 1) when comparing the amount of two hypothetical drugs in a large cattle population (hatched), 2) when describing AMU data relative to antimicrobial resistance (AMR) in a scientific paper (black), 3) when clearly reporting data regarding AMU to the general public (gray) (n=156).* Categories available for participant selection not displayed in this figure include Unknown, and No Preference.

Summarizing large-scale surveillance. Quantifying AMU as the number of animals treated was judged as the clearest metric for representing AMU when reporting this information for large cattle populations. Quantifying AMU by ADDD per 1000 animals was deemed the most accurate metric for representing AMU for large-scale surveillance of AMU and AMR. The majority (88%; 137/156) of the respondents specified that number of animals treated was clearly understood, but only 36% (56/156) specified that it was accurate. Conversely, only 32% (50/156) of respondents specified that the ADDD per 1000 animals method was clearly understood, while 76% (119/156) specified that it was accurate.

Approximately half of the participants selected ADDD per 1000 animals as the best method of summarizing AMU for prospective surveillance programs at the state/provincial (51%; 79/156), national (53%; 83/156), and global (50%; 78/156) organizational scales. All other metrics were selected by less than 15% (24/156) of participants for these purposes. Specifically, sales value was selected by less than 3% (5/156) of participants as the best method for summarizing AMU. For surveillance programs, most participants (74%; 116/156) were unaware of a more appropriate definition or measurement than the ones investigated in this survey. Participants who indicated that a different definition or measurement was more appropriate than the ones provided in the questionnaire commented on refining specific definitions and stratifying metric summaries according to different confounders.

Utilizing the ADDD metric. Participants indicated that the usage of the ADDD or ADDD per 1000 animals requires specific and clear definitions. Most participants (74%; 116/156) recommended the separate calculation of high and low dose exposures of the same drug and subsequent separate reporting of these amounts. Less than 10% of participants (14/156) held the opposing views of calculating ADDDs in some variant of averaging dosages. In a free-response, 64% (98/153) of participants correctly interpreted "400 ADDD of tetracycline" while 20% (31/153) incorrectly did so and 16% (24/153) indicated upfront that they did not know how to interpret the phrase.

Summarizing Metrics. In the reporting of any AMU metric, most participants believed that separately reporting amounts by the intended use of the AMDs and the class of AMDs is critical to accurately portraying AMU information. Summarizing quantities for the combined uses of prophylaxis, metaphylaxis, treatment of clinical disease, and

improvement of production efficiency was not considered an accurate representation by 67% (105/156) of participants. Only 9% (14/156) considered the combination of these uses in reporting as an appropriate summarization. Another 22% (34/156) of participants indicated that the accuracy of such a summary measure would depend on other factors beyond just these categories such as the purpose of collecting the data, how the data were collected, and the particular AMD or class of AMD. Combining AMU information across different classes of AMDs was also considered inappropriate by 60% (94/156) of participants. Some participants (13%; 21/156) specified that the accuracy of AMU data which combined different classes of AMDs would depend on the reporting situation.

Response Associations

Participants with ≥20 years of beef industry involvement were half as likely (OR=0.5, 95%CI: 0.2-0.9, *P*=0.02) to have increased concern about AMR today as a global human health issue compared to 10 years prior to the study when compared with participants with <20 years of beef industry involvement. These experienced participants were also more likely than the participants with <20 years to believe that AMU in feed or water for treatment of disease was not needed rather than needed in the management of feedlots (OR=11.3, 95%CI: 2.4-., *P*=0.01). When comparing two different classes of AMDs (OR=2.6, 95%CI: 1.2-5.5, *P*=0.01) or describing AMU data relative to AMR (OR=2.6, 95%CI: 1.2-5.9, *P*=0.02), participants with ≥20 years of beef industry involvement were more likely to select a method other than ADDD and ADDD per 1000 animals than participants with <20 years of involvement.

In comparison to North American participants, non-North American participants had different perspectives on the health risks of AMR, the necessity of AMDs in the management of feedlots, and appropriate AMU metrics. Participants from non-North American countries were more likely to believe that the true risk of health problems in people (OR=5.2, 95%CI: 1.4-19.0, P=0.01) and animals (OR=4.0, 95%CI: 1.1-14.7, *P*=0.03) because of AMR as compared to 10 years ago was greater on the global scale. Non-North American participants were more likely than North American participants to indicate that AMDs are not needed rather than needed for the typical uses in North American feedlots of prophylaxis/metaphylaxis at arrival (OR=32.0, 95%CI: 4.0-264.3, *P*<0.001) and in feed for prevention of liver abscesses (OR=5.5, 95%CI: 1.0-28.8, *P*=0.05). When asked about the best metric, non-North American participants were more likely than North American participants to select ADDD or ADDD per 1000 animals as appropriate for the scenarios of comparing two different AMDs (*P*=0.01) and for reporting data regarding AMU to the public (*P*=0.01).

Both professional role and highest degree earned influenced participant responses. Participants with professional roles in universities were more likely than veterinarians to believe that the true risk of AMR as a health issue to people is greater than 10 years prior to the study on the regional (OR=5.6, 95%CI: 2.3-13.9, *P*<0.001), national (OR=3.5, 95%CI: 1.4-8.3, *P*<0.001), and global (OR=3.2, 95%CI: 1.3-7.5, *P*=0.01) scales. When describing AMU data relative to AMR data, producers were more likely than participants from other professional roles to select metrics considered inappropriate by most survey respondents (sales value and total mass of active drug) (*P*=0.03). In a related manner, participants with advanced degrees were more likely than participants with a bachelors

(*P*=0.05) or high school diploma (*P*=0.05) to select ADDD or ADDD per 1000 animals as the most appropriate measure for the same scenario. Additionally, participants with advanced degrees were more likely to indicate that combining information across different AMD classes was not appropriate (*P*=0.02).

DISCUSSION

Results of the present study suggested that there is no single, best method to appropriately represent AMU data related to beef cattle production. To effectively communicate AMU data, evaluation of the target audience is critical to clearly presenting the information. Metrics that are most accurate may need to be carefully and repeatedly explained to the audience. In the past, reports of AMU that focused on sales value or mass of active ingredient metrics as estimates of AMU have not allowed for appropriate investigation of associations between AMU and AMR. Theoretically, differences in the physical characteristics of AMDs, the doses, the dosages, numbers of animals treated, and the reasons for use all modify the effect that AMU has on AMR. Metrics relying on sales value or mass of active ingredient do not account for these differences. Incorporating such selection pressures in AMU metrics is crucial to understanding the development, persistence, and dissemination of AMR within and between populations of animals and humans. Quantification of AMU with metrics which does not account for selection pressures distorts discussion regarding the impact of AMU and cannot be used to investigate AMR.

Data regarding AMU is presented in a variety of formats depending on the purpose of reporting and the intended audiences. Some surveillance programs have quantified specific use of AMDs with a direct focus of investigating the impact of AMU on AMR. This work can be performed on a small scale, such as within a single facility or on a grander scale, such as on a national level (Dunowska et al. 2006; Bager 2000; Bergman 2009; Bunner 2007). With data specifically and accurately gathered, researchers can evaluate associations or lack of associations between AMU and AMR. In

contrast, other reports have summarized various estimates of the quantity of AMDs used to illustrate discrepancies in use between humans and animals or between reasons for AMU (Institute of Medicine 1989; Mellon 2001). These latter reports commonly present AMU information in terms of mass of active ingredient or sales value of the AMDs.

AMU in humans and animals creates a selection pressure that contributes to a local increase in AMR. In theory, bacteria susceptible to the AMD are eliminated and resistant bacteria in the previously heterogeneous bacterial population persist (Levy et al. 2004). However, the probability of occurrence of this phenomenon in association with AMU and the strength of this association is unknown. Additionally, little is understood about the duration of persistence within populations of animals and humans as well as about the likelihood of transmission of resistance between populations (Singer et al. 2007). To better elucidate the existence of human and animal health risks as well as the burden of such risks associated with AMU, a proper quantification and reporting metric is needed (Menendez Gonzalez et al. 2010; Carson et al. 2008).

Choosing an appropriate metric for reporting data regarding AMU is a deceptively complex matter. Challenges in the accuracy and clarity of reporting AMU vary by the AMD of concern and the organizational scale of reporting (e.g., comparisons between farms vs. comparisons between regions or countries). AMDs are provided by pharmaceutical companies in different combinations of ingredients and are administered by different routes and dosing schedules (Ferarro et al. 2001; McEwan et al., 2002; Bywater 2004; Khachatourians 1998; Parveen et al. 2006; Rifenburg et al. 1996). Selection pressures against target bacteria are analogous between the formulations of the same AMD or between similarly structured AMDs (same class of drug) or both (Bywater

2004; Wang et al. 2006). However, even the exact same formulation of a drug (e.g. tetracycline) can apply selection pressures differently. For example, a "High Dose" situation to treat disease could apply a stronger pressure to a population of bacteria and eliminate bacteria with marginal susceptibility to the AMD. Yet, a "Low Dose" of the same AMD to improve production efficiency may lead to the quicker development of AMR since the marginally susceptible and the resistant bacteria would survive and comingle resistance traits (Craig 2001, Funk et al. 2006; Ghosh and LaPara 2007; Guillemot et al.1998; Kohanski et al. 2010).

The web-based format of this study was an easy and quick method to solicit the opinions of a variety of people that are affected by policy decisions regarding AMU in cattle. However, there are limitations that must be considered when interpreting these results. Conducting an extensive survey utilizing probability based sampling strategies was not possible since the total population (sampling frame) of experts in beef cattle AMU and AMR was unknown. With no prior knowledge of the sampling frame, a convenience sample was considered the best method to quickly and easily obtain a reasonably wide distribution of the questionnaire to individuals that were knowledgeable and interested in the topic. Therefore, the representativeness of the sampled individuals to the theoretical target population of experts in beef industry AMU and reporting could not be validated. However, the associations, agencies, and individuals targeted by invitation to the questionnaire were all recognized as important stakeholders.

Though this survey may not have included or represented all experts in the beef industry, stakeholders with advanced degrees were well represented. Likely, holding an advanced degree would aid an individual in evaluating appropriate AMU metrics since

the complexities of the related issues are not directly intuitive. However, the ADDD or ADDD per 1000 animals metrics were not clearly definable by all of the highly educated respondents to this survey. The lack of participants' knowledge about the ADDD metrics may have contributed to nondifferential misclassification when selecting useful metrics. These participants might have been drawn towards or away from selecting ADDD metrics if they were unable to distinguish them from other metrics or if they ignored metrics they did not understand, respectively. Additionally, stakeholders that were willing to participate may not have submitted a complete survey (thus, not included in this report) if they were not comfortable with their grasp on the intricacies of the ADDD metrics. If stakeholder groups which were not well represented in this study were included, different distributions of responses might be expected.

This study investigated a finite number of quantification methods which represent categories of a large number of metrics that have been used. The ones used in the study were chosen to encompass the metrics most commonly used (sales value and total mass of active ingredient) and those which more fully account for selection pressure (ADDD and ADDD per 1000 animals). Since more than one metric may be viewed as appropriate in a specific situation, we solicited responses which allowed for the top two choices. The results have been presented here as a cumulative percentage of the top two choices because the interval perceived by the participant between their two choices can vary. Depending on the purpose of the research, how the data were collected, the organism being investigated for AMR, and the AMDs of interest, other metrics could be appropriate. However, in every case the concepts of clarity and accuracy of reporting should always be highly regarded.

Accuracy and clarity together are hard to come by in quantifying AMU. If we describe AMU to the absolute detail of what it represents, often we lose the simplicity of the representation. Participants indicated that though ADDD metrics lack clarity, they are quite accurate as opposed to the clearly understood metric, number of animals treated, which lacks accuracy. Participants of this study indicated the superior accuracy of the ADDD metric, yet not all participants were able to correctly define an ADDD metric. Therefore, though the details behind this metric may not be wholly understood, participants were still able to recognize that this metric better represents AMU than the other metrics. The absence of a complete understanding of ADDD does not invalidate audience ability to interpret reports.

In designing the questionnaire, AMU in the feedlot setting was the primary focus. AMU and AMR data in North American feedlots has not been available in the past and current efforts to develop an appropriate surveillance system in Canada are underway. Therefore, our research group was specifically interested in the responses of stakeholders in the feedlot industry. Other animal production industries such as swine, poultry, or dairy operations may utilize AMDs differently, but types of use are generally similar. Other animal agribusinesses with scenarios which are not analogous to the feedlot scenarios presented would need further investigation since we did find that the appropriate metric depends on the user audience of the information and the research question. Ideally, a common method or pairing of methods would be used in all animal agribusinesses as well as within the public health sector for more closely comparable estimates.

FOOTNOTES

[a]Associations and agencies that were contacted to solicit their participation included the Academy of Veterinary Consultants, the American Association of Bovine Practitioners, the American College of Veterinary Clinical Pharmacology, the American College of Veterinary Preventive Medicine, the American College of Veterinary Internal Medicine, the American Public Health Association, the Association of State and Territorial Health Officials, the Association for Veterinary Epidemiology and Preventive Medicine, Canadian Cattlemen's Association, the Centers for Disease Control and Prevention, the Council of State and Territorial Epidemiologists, the EPIVET listserv, the United States Food and Drug Administration, Feedlot Health Management Services, the International Conference on the Use of Antimicrobials in Cattle Production, the National Association of State Public Health Veterinarians, the National Cattlemen's Beef Association, the Public Health Agency of Canada, the Texas Cattle Feeders Association, and the United States Department of Agriculture.

[b]SurveyMonkey.com. Portland, Oregon USA.

[c]StataCorp. 2007. *Stata Statistical Software: Release 10*. College Station, TX: StataCorp LP

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CHAPTER 3: Evaluation of resistance classification accuracy by latent class analysis of data from disk diffusion and broth microdilution for *Escherichia coli* and *Mannheimia haemolytica* recovered from feedlot cattle.

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INTERPRETIVE SUMMARY

The resistance status of isolates representing an individual or a population is determined by susceptibility testing. Surveillance programs must accurately identify and monitor levels of resistance in order to effectively respond to susceptibility problems or emerging resistance threats. Yet, the unbiased accuracies of the common susceptibility testing methods (disk diffusion and broth microdilution) are unknown. Therefore, resistance information from these tests might provide false impressions of the true status of resistance. It is unlikely that mitigation strategies that are based on inaccurate information will be as useful as those based on unbiased estimates.

The following project investigates accuracies of disk diffusion and broth microdilution using a novel analysis technique. The ability of each test to correctly identify resistance and non-resistance to different antimicrobial drug and organism combinations is determined without assuming the superiority of one test over the other. The dogmatic approach in developing antimicrobial resistance surveillance programs has been to use broth microdilution susceptibility testing over the disk diffusion method. However, results of this project show that disk diffusion can be an appropriate choice for susceptibility testing when conducting surveillance for antimicrobial resistance.

ABSTRACT

Objective: It is crucial to establish an appropriate understanding of error rates for antimicrobial susceptibility tests when working to establishing reliable estimates of antimicrobial drug resistance. A degree of misclassification is expected with all diagnostic tests and the true state of resistance is ultimately unknown despite rigorous standardization of susceptibility testing methods. Latent class analysis techniques are capable of modeling such uncertainty in classification for diagnostic tests. The objective of this study was to estimate and compare the accuracy of the disk diffusion and broth microdilution methods for surveillance of antimicrobial drug resistance in feedlot cattle.

Methods and Materials: Isolates of *E. coli* and *M. haemolytica* were tested for susceptibility to panels of antimicrobial drugs by standardized methodology for disk diffusion and broth microdilution. Latent class analysis was used to determine the proportions of correctly identified resistant and non-resistant isolates for each antimicrobial susceptibility testing method. The antimicrobial drugs compared in these analyses were ampicillin, ceftiofur, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole.

Results: A total of 2,316 *E. coli* isolates from individual samples, 885 *E. coli* isolates from composite samples, and 783 *M. haemolytica* isolates were tested by both antimicrobial susceptibility tests. Models for all organism and antimicrobial drug combinations indicated that both testing methods correctly classified non-resistance in a

high proportion of isolates. However, the ability of the test to correctly classify true resistance varied among bacteria-drug combinations in the 2 tests.

Conclusions: Non-resistance predicted by either method likely represents the true non-resistance status in these bacterial populations, but errors can be expected more frequently when tests classify isolates as resistant. Additionally, misclassification of resistance to different antimicrobial drugs occurs at different rates in the 2 bacterial species.

INTRODUCTION

As with any diagnostic test, antimicrobial susceptibility testing is subject to errors which lead to misclassification (Figure 1). Procedural failures (sub-standard laboratory or sampling procedures), unpredictable responses due to biological variability in bacterial isolates, or other unrecognized (chance) sources of variability may all lead to diagnostic test errors (Greenwood 1981, 2000). Optimally, we would always choose to employ a highly accurate test that minimizes such errors, but other test characteristics such as cost and availability can also influence our choice in testing methods. For example, a test method with a lower, yet an acceptable level of accuracy might be chosen for surveillance monitoring if the cost of testing allowed for evaluation of a substantially greater number of isolates.

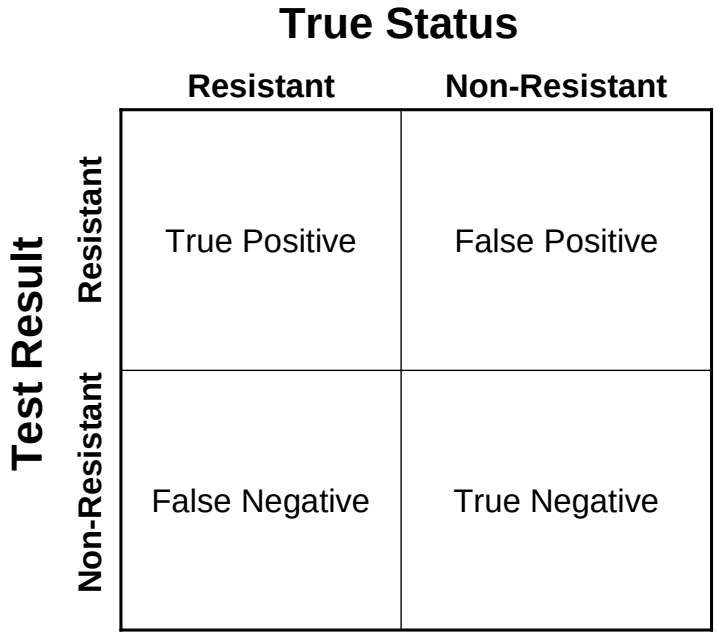

Figure 1: **Misclassification in diagnostic tests.**

Disk diffusion and broth microdilution are antimicrobial susceptibility testing techniques which both provide estimates of phenotypic susceptibility to antimicrobial drugs based on bacterial growth in the presence of varying concentrations of the antimicrobial drug. Internationally recognized and standardized methods for conducting these tests have been established by the Clinical and Laboratory Standards Institute (2008), as have interpretive criteria. These breakpoints for susceptibility classification (based upon MIC for broth microdilution and zone diameter for disk diffusion) are also used in epidemiological surveillance, although some have suggested that different, lower breakpoints may be better suited for early detection of developing resistance in different ecological settings (Bywater et al. 2006; Simjee et al. 2007).

The potential for errors in antimicrobial susceptibility testing is widely recognized. In fact, when considering testing by broth microdilution and disk diffusion, results suspected as incorrect by disk diffusion are often considered to be confirmed or refuted by comparison to broth microdilution, an assumed "gold standard" test (Citron et al. 2005; Murray et al. 1982; Sautter and Denys 1987; Hubert et al. 1998; Woolfrey et al. 1983; Shyrock et al.1996; Metzler and DeHann 1974; Klement 2005). The inherent assumption when we consider a test to be a "gold standard" is that the test is perfectly accurate and that true classification status (e.g. resistance and non-resistance in susceptibility testing) is always correctly identified by the test. However, all diagnostic tests, even highly regarded tests such as broth microdilution, are vulnerable to classification errors (Enoe 2000). For susceptibility testing, cross-classification of two fallible tests such as disk diffusion and broth microdilution (Figure 1) without a reliable understanding of their error rates provides an imperfect understanding of the true

resistance and true non-resistance status of a population. This type of reference-based test evaluation will always yield results that are biased to the extent that the reference test ("gold-standard") does not correctly identify the true classification status of individuals, and the new test can never appear better than the reference test that is being used for cross-classification.

Obtaining accurate antimicrobial susceptibility information for bacterial isolates is critical for testing of isolates in clinical settings and also for surveillance programs evaluating antimicrobial resistance in both commensal and pathogenic bacteria (Aarestrup 2004; APUA 2005; Levy and Marshall 2004; McEwen and Fedorka-Cray 2002; Wassenaar and Silley 2008; Williams 2001). Identifying important population trends of reduced susceptibility to available antimicrobial drugs in different populations and over time is necessary to facilitate an appropriate understanding of the complexities of antimicrobial resistance (Greenwood 2000; Vieira et al. 2008; Phillips 1998). While it is possible to reduce the potential for errors through test selection and rigorous standardization of protocols, it is impossible to eliminate all errors. In order to correctly interpret results generated for either clinical or surveillance purposes, the potential for errors in detecting resistant and susceptible isolates must be understood. Further, even with imperfect susceptibility testing methods the prevalence of true resistance can be accurately established and monitored if the potential for erroneous classification is understood and accounted for (Caprioli et al. 2000; Varaldo 2002).

In contrast to reference-based diagnostic test evaluation, newer methods of test evaluation have been identified which allow estimation of the true, unbiased parameters related to test accuracy (namely sensitivity and specificity) and prevalence of the

condition in question without presuming to know the true classification of individuals. Because these methods attempt to model classification probabilities that are hidden from direct observation, they are sometimes called latent class analysis. The objective of this study was to estimate and compare the accuracy of the disk diffusion and broth microdilution methods for surveillance of antimicrobial resistance in feedlot cattle.

MATERIALS AND METHODS

Study Population— All animal handling and sampling procedures were approved prior to the initiation of the study by the Animal Care Committee of Feedlot Health Management Services (FHMS) and the Institutional Animal Care and Use Committee of Colorado State University. Cattle sampled in this study were managed under typical conditions of feedlot production in North America. Bacterial isolates evaluated in this study were collected as part of an ongoing project for the purpose of surveillance of antimicrobial resistance in feedlot cattle. However, the isolates used in this study were purposefully selected from the entire sample set for the purpose of evaluating test sensitivity and specificity. As such, results were not intended to provide estimates of resistance prevalence for the cattle enrolled in the study or for other populations of feedlot cattle.

Cattle were enrolled from September 17, 2007 to January 16, 2010, and isolates included in this dataset were a convenience sample of the isolates that had been evaluated for antimicrobial susceptibility using both disk diffusion and broth microdilution. To meet an important assumption of the analysis method, this set of isolates was stratified into 2 sample sets that were likely to have different resistance prevalences. The structure of the collected data allowed easy classification of isolates into a population recovered from animals at arrival to the feedlot and a second population that were recovered from animals that had been managed in the feedlot environment for at least 60 days. The resistance prevalences of these 2 sample sets were expected to be different since the latter isolates from established animals were more likely to have been exposed to antimicrobial drugs than the isolates from animals newly arriving at the feedlot. The resistance

prevalences of these isolates were not assumed to be representative of the resistance prevalence of the population sampled for the surveillance project. It is assumed that test accuracy (sensitivity and specificity) was constant across different populations and was not biased by the sampling scheme.

Sampling Procedures— Pens of cattle were usually filled over several days as cattle arrived at the feedlot on different trucks. Once occupancy of a pen of animals had been finalized and cattle had been allocated to the study, pen-level composite fecal samples were collected at three time points: at arrival, at > 60 days on feed (DOF) and at exit (≤ 30 days prior to slaughter). In brief, at each sampling time a new plastic spoon was used to combine feces from 20 fresh pen-floor pats into a new fecal cup (minimum 10 grams feces) for each composite sample. The sample was mixed thoroughly, and approximately 4 grams of feces from each fecal cup were then transferred into a vial containing modified Cary Blair transport media (Enteric Transport Medium, 15 ml, Cat#F01W, Dalynn Biologicals Inc., Calgary, Alberta) (Alexander et al. 2009).

Individual animals enrolled in the trial were sampled twice over the course of the study: during initial processing shortly after arrival to the feedlot and when cattle were re-handled as part of standard feedlot protocols. Each individual animal had two samples collected each time they were sampled: a nasopharyngeal swab sample and a fecal sample collected per rectum. The nasopharyngeal sample was collected in the deep pharynx using a commercially available double guarded swab (# J273, Jorgensen Laboratories, Inc, Loveland, CO, USA). A Cary Blair media tube (BBL CultureSwab™, CA90001-038, VWR International, Mississauga, Ontario) was used to transport the

nasopharyngeal swab. Individual fecal samples were collected per rectum using a new plastic palpation sleeve. A minimum of 4 grams of feces from the rectum of each animal was transferred into a vial containing modified Cary Blair transport media (Enteric Transport Medium,15 ml, Dalynn Biologicals Inc.) (Alexander et al. 2009).

Sample Transport and Data Storage— All samples were labeled with the date and the pen number (and the animal ID for individual animal samples), refrigerated in a chilled cooler and transported to the microbiology laboratory (Agriculture and Agri-Food Canada Lethbridge Research Station, Lethbridge, Alberta) for further processing by overnight courier.

Laboratory Procedures—Nasopharyngeal swabs were processed immediately after overnight delivery to the microbiology laboratory. Fecal samples were stored in a 4ºC cooler while processing of nasal swabs was completed.

Nasopharyngeal Swabs—In a Risk Level II containment laboratory, nasal swabs were aseptically removed from their transport vial and the tips were vortexed at high speed for 30 seconds and then allowed to settle undisturbed for at least 10 minutes.

One hundred microliters of this suspension were spread onto blood agar containing 15μg/mL bacitracin (BAC plates) and incubated overnight at 37ºC. BAC plates were also inoculated with *M. haemolytica* ATCC strain 33396 and *M. glucosida* ATCC strain 38457 as positive controls. Colonies with morphology typical of *M. haemolytica* were selected for further analysis, using the BAC plate with *M. haemolytica*

33396 as a reference (round, medium sized, 'wet', white-grey colored colonies with some degree of hemolysis evident). Three to 5 colonies were streaked onto BAC plates and incubated at 37ºC overnight. Isolated colonies were rechecked to confirm purity and verify that the morphology was similar to the reference plate. Isolates were then tested for oxidase (Oxoid) and a catalase reactions (using 3% hydrogen peroxide). Isolates which were oxidase and catalase positive were prepared for PCR. Positive (*M. haemolytica* and *M. glucosidal*) and negative controls were also prepared for each of the tests. Isolates that were identified as presumed-*M. haemolytica* were stored in 20% glycerol stocks at -80ºC until further phenotypic characterization. Phenotypic tests were performed using Rosco diagnostic tablets (Diatabs ®) and compared against phenotypic profiles (Angen et al. 2002). Phenotypic tests performed included alpha-fucosidase, beta-galactosidase, beta-glucosidase, beta-xylosidase, D-xylose, esculin hydrolysis, indole, L-arabinose, maltose, mannitol, ornithine decarboxylase, sorbitol, trehalose, and urease. A multiplex PCR assay was used to confirm the presumed-*M. haemolytica* isolates as positive (Alexander et al. 2008). All *M. haemolytica* isolates confirmed by PCR were tested for susceptibility.

Composite and Individual fecal samples—Individual fecal samples corresponding to animals from which *M. haemolytica* was recovered and all composite fecal samples were processed by mixing the Cary-Blair transport medium to create a uniform slurry (Alexander et al. 2009). A sterile cotton swab was used to streak for isolation on MacConkey Agar (MAC) and plates were incubated for 24 hours at 37ºC. Isolates that fermented lactose and had appropriate morphology were subcultured on lysogeny broth

(LB) plate, incubated at 37ºC overnight and then tested for indole reaction. A presumptive identification of non-type specific *E. coli* was based on colony morphology, lactose fermentation and positive indole reaction. Up to 3 lactose-fermenting (pink) colonies per individual fecal sample and five colonies per composite manure sample were selected and archived for susceptibility testing by freezing at -80°C in 30% glycerol.

Antimicrobial Susceptibility Testing—Each isolate included in this analysis was tested for susceptibility to panels of antimicrobial drugs by disk diffusion (BioMIC) and broth microdilution (Sensititre). Both procedures were conducted according to protocols of the Clinical Laboratory Standards Institute (CLSI 2008). The antimicrobial drugs included on the panels used in this study were designed independently for surveillance purposes, and as such were not identical. The antimicrobial drugs included on both the disk diffusion and broth microdilution susceptibility panels were ampicillin, ceftiofur, sulfisoxazole, streptomycin, tetracycline, and trimethoprim-sulfamethoxazole. Only isolates which had results for these antimicrobial drugs from both testing methods were included in these analyses. Disk diffusion information was recorded as zone diameter and broth microdilution information was recorded as the MIC. Quality control strains used were *Escherichia coli* ATCC 25922, *Streptococcus pneumoniae* ATCC 49619, and *Staphylococcus aureus* ATCC 29213.

Interpretive criteria—Susceptible, intermediate, and resistant (SIR) designations of *M. haemolytica* were determined using CLSI guidelines for ampicillin (2002) and

oxytetracycline (2006). Interpretive criteria for *E. coli* were also obtained from CLSI guidelines (2005), except for streptomycin, which were based on those used by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) and the National Antimicrobial Resistance Monitoring System (NARMS, CDC 2003).

***Data Analysis*—**Data were evaluated graphically and by calculating descriptive statistics. For analysis purpose, isolate susceptibility was dichotomized as resistant and non-resistant (which included both intermediate and susceptible classifications). Resistance classification obtained from both testing methods was used to cross-classify isolates (i.e. resistant by both methods, non-resistant by both methods, and both discordant classifications) (Figure 1). Using counts of these cross-classifications, stochastic latent class analysis modeling (Markov-Chain Monte Carlo simulation using a Gibbs sampler)[a] was used to estimate the proportion of isolates correctly classified for resistance (sensitivity) and the proportion of isolates correctly classified for non-resistance (specificity) by each testing method, as previously described (Lunn et al. 2000; Branscum et al. 2005). Data were stratified into 2 sample sets based on sample collection timing (samples obtained at feedlot arrival, and those obtained from animals that had been managed in the feedlot environment for at least 60 days). These models assumed that the two testing methods were conditionally dependent and the code was adapted from previously published information (http://www.epi.ucdavis.edu/diagnostictests/AB2deptests2popns.html; code available upon request). Convergence of each model was assessed by running six simultaneous chains with widely different starting values while monitoring history (time series) plots,

autocorrelation plots, and Brooks-Gelman-Rubin plots (Gelman and Rubin 1992). For the final model of each organism (*E. coli* from individual samples, *E. coli* from composite samples, and *M. haemolytica*) and antimicrobial drug combination, a single chain was run with the weakly informative prior probability distributions. An initial burn-in of 5,000 iterations was discarded and the next 50,000 iterations were used to generate posterior probability distributions of the test sensitivities and specificities (Enoe et al. 2000; Johnson and Gastwirth 2000). The median estimates and their 95% probability intervals were determined from these posterior distributions regarding the proportion of correctly classified isolates for resistance (sensitivity), and for non-resistance (specificity). Although there is not a formal method for testing whether point estimates for 2 tests were statistically different, an approximate method was used as a somewhat objective method for evaluating these comparisons of interest. Specifically, probability intervals which overlapped with the point estimate (median) of the other susceptibility test were used as an indication that the antimicrobial susceptibility tests were not different from one another in their ability to correctly classify true resistance or true non-resistance. Conversely, probability intervals which did not encompass the point estimate (median) of the other test were considered to indicate that there was a statistically detectable difference between the tests (assumed probability of Type I error <0.05). Positive and negative predictive values were calculated across a range of true resistance prevalence values and then plotted to allow further assessment of information. The predictive values of resistance (predictive value of a positive test) and the predictive values of non-resistance (predictive value of a negative test) were estimated using standard formulas (Thrusfield 2005). In general, the predictive value of resistance is the

proportion of true positives divided by the sum of true positives and false positives, and the predictive value of non-resistance is the proportion of true negatives divided by the sum of true negatives and false negatives (Figure 1). These predictive values vary dramatically with true resistance and therefore must be considered across a range of relevant prevalences.

Prior probability estimates—Beta distributions of probabilities were created with software[b] to represent the possible values for the prior antimicrobial resistance prevalences of sample set 1 and sample set 2 (Table 1). For the disk diffusion (test 1) susceptibility test, prior probability beta distributions (priors) for sensitivity and specificity were also established. Due to the assumed dependence between the susceptibility tests, the priors for the sensitivity and specificity of the broth microdilution test (test 2) were conditional on the disk diffusion priors. The choice of test order for conducting the analysis with broth microdilution conditionally dependent on disk diffusion was arbitrary. All priors were weakly informative with effective sample sizes (a + b; Table 1) under n=10. Priors for resistance prevalences of *E. coli* isolates in sample set 1 and sample set 2 were taken from a previous study (Rao et al. 2009). For *M. haemolytica* isolates, relevant priors were only available for the sample set with higher prevalence of resistance (sample set 2). Therefore, the priors for resistance prevalences in sample set 2 were taken from a previous study and the estimates for sample set 1 were extrapolated as 5% lower than these estimates (Watts et al. 1994). Since no sources have published estimates of the sensitivity and specificity of disk diffusion or of broth microdilution susceptibility tests, the authors arrived at a consensus opinion for

reasonable estimates and a lower limit with 95% confidence. The disk diffusion sensitivity and specificity priors were assumed to be equivalent and the conditional sensitivity and specificity priors of the broth microdilution test were assumed to be only slightly higher, but also equivalent. These prior estimates were the same for all antimicrobial drugs in the analysis.

Table 1: **Prior probability distributions for prevalence of antimicrobial resistance and for sensitivities and specificities of susceptibility tests.** Parameters for the beta distributions were truncated to 1 significant digit for presentation.

			Beta Distribution Parameters (a,b)	Mode	95% Probability Interval
Resistance Prevalence Sample set 1	***E. coli***				
		Ampicillin	(1.3,6.0)	6.5%	1.2%-50.9%
		Ceftiofur	(1.0,7.0)	0.4%	0.4%-41.3%
		Streptomycin	(2.2,4.0)	27.8%	6.2%-72.8%
		Sulfisoxazole	(2.2,2.1)	52.9%	11.3%-90.5%
		Tetracycline	(2.9,3.0)	48.7%	13.8%-85.0%
		Trimethoprim-sulfamethoxazole	(1.1,4.5)	2.8%	0.8%-57.3%
	M. haemolytica				
		Ampicillin	(1.8,2.4)	37.5%	6.7%-85.7%
		Tetracycline	(1.7,2.0)	40.1%	6.6%-88.9%
Resistance Prevalence Sample set 2	***E. coli***				
		Ampicillin	(1.2,6.0)	3.5%	0.1%-48.7%
		Ceftiofur	(1.0,7.0)	0.7%	0.4%-41.6%
		Streptomycin	(2.2,4.0)	29.3%	6.8%-73.4%
		Sulfisoxazole	(1.8,1.9)	47.1%	7.9%-90.7%
		Tetracycline	(3.1,2.6)	56.4%	16.8%-88.7%
		Trimethoprim-sulfamethoxazole	(1.0,2.2)	0.8%	1.2%-80.9%
	M. haemolytica				
		Ampicillin	(1.8,2.2)	39.5%	6.7%-87.8%
		Tetracycline	(1.6,1.9)	43.0%	6.5%-90.5%
Disk Diffusion		Sensitivity	(2.0,1.1)	67.9%	14.8%-98.2%
		Specificity	(2.0,1.1)	67.9%	14.8%-98.2%
Broth Microdilution		Conditional Sensitivity	(4.8,1.2)	83.3%	43.1%-99.0%
		Conditional Specificity	(4.8,1.2)	83.3%	43.1%-99.0%

Sensitivity Analysis—The influence of prevalence and sensitivity/specificity priors was assessed by running the model with highly informative priors based on the same mode as the weakly informative priors that were used (Georgiadis et al. 2003). Additionally, widely varying distributions were created to assess the limits of model convergence. Without varying the original priors, starting values for the models were assessed by widely varying prevalence as well as sensitivities/specificities separately. The outcomes of the disk diffusion and the broth microdilution antimicrobial susceptibility tests were assumed to be dependent. Correlation between tests was monitored by the calculated correlation value for each model. Additionally, a sensitivity analysis of the dependent models was run by assuming complete independence between tests in separate models (Georgiadis et al. 2003).

RESULTS

Isolates—A total of 2,316 *E. coli* isolates from individual animal samples were evaluated for susceptibility by both tests, as were 885 *E. coli* isolates from composite samples, and 783 *M. haemolytica* isolates. Isolates in sample set 1 had widely different apparent prevalences of resistance to tetracycline, sulfisoxazole, and streptomycin than in sample set 2 (Table 2). However, the apparent prevalences of resistance to ampicillin, ceftiofur, and trimethoprim-sulfamethoxazole were not as disparate between the 2 sample sets (Table 2). Between 20% and 30% of *E. coli* isolates were resistant to a single antimicrobial drug, while <20% of *M. haemolytica* isolates were resistant to a single antimicrobial drug (Table 3).

Table 2: **Apparent prevalence of antimicrobial resistance (AMR) in isolates of each sample set.**

Organism and Antimicrobial Drug (n Sample set 1 : n Sample set 2)	Disk Diffusion		Broth Microdilution	
	Sample set 1 AMR Prevalence	Sample set 2 AMR Prevalence	Sample set 1 AMR Prevalence	Sample set 2 AMR Prevalence
***E. coli* Individual**				
Ampicillin (1130 : 1149)	2.0%	4.3%	3.2%	5.5%
Ceftiofur (1137 : 1158)	0%	0.1%	0.5%	0.3%
Streptomycin (1156 : 1146)	3.9%	20.9%	6.6%	26.8%
Sulfisoxazole (1158 : 1158)	5.2%	27.6%	6.2%	28.7%
Tetracycline (1132 : 1106)	16.4%	78.5%	17.5%	76.9%
Trimethoprim-sulfamethoxazole (1150 : 1158)	0%	1.1%	0.2%	1.1%
***E. coli* Composite**				
Ampicillin (394 : 484)	5.8%	5.0%	5.8%	6.2%
Ceftiofur (395 : 490)	0.3%	0%	0.3%	0.8%
Streptomycin (390 : 482)	20.8%	19.9%	24.1%	26.8%
Sulfisoxazole (381 : 473)	18.4%	20.3%	17.8%	23.0%
Tetracycline (363 : 451)	55.9%	78.7%	51.8%	71.4%
Trimethoprim-sulfamethoxazole (395 : 490)	1.8%	1.2%	3.8%	1.8%
M. haemolytica				
Ampicillin (433 : 350)	0.9%	0.6%	0.7%	0.9%
Tetracycline (433 : 350)	3.7%	6.9%	4.8%	10.3%

Table 3: **Number of resistances detected in isolates by disk diffusion (panel of 12 antimicrobial drugs) and broth microdilution (panel of 15 antimicrobial drugs).**

	E. coli **Individual (n=2316)**		***E. coli*** **Composite (n=885)**		***M. haemolytica*** **(n=783)**	
Resistance Number*	**Disk Diffusion**	**Broth Microdilution**	**Disk Diffusion**	**Broth Microdilution**	**Disk Diffusion**	**Broth Microdilution**
0	1145(49.4%)	1227(53.0%)	282(31.9%)	294(33.2%)	622(79.4%)	738(94.3%)
1	609(26.3%)	591(25.5%)	287(32.4%)	333(37.6%)	146(18.6%)	43(5.5%)
2	229(9.9%)	264(11.4%)	131(14.8%)	145(16.4%)	13(1.7%)	2(0.3%)
3	223(9.6%)	142(6.1%)	127(14.4%)	62(7.0%)	1(0.1%)	0(0%)
4	89(3.8%)	74(3.2%)	45(5.1%)	42(4.7%)	1(0.1%)	0(0%)
5	16(0.7%)	18(0.8%)	11(1.2%)	7(0.8%)	0(0%)	0(0%)
6	4(0.2%)	0(0%)	2(0.2%)	1(0.1%)	0(0%)	0(0%)
7	1(0.04%)	0(0%)	0(0%)	1(0.1%)	0(0%)	0(0%)

* Number of antimicrobial drugs that isolates were resistant to

Test Accuracy—Models for all combinations of organisms and antimicrobial drugs indicated that both antimicrobial susceptibility testing methods were very good at correctly classifying non-resistance of isolates (specificity; Table 4). However, the classification of true resistance (sensitivity) varied. As indicated by extremely wide probability intervals for classification of true resistance, ceftiofur was not modeled well in these analyses.

Table 4: **Proportions of true resistance and true non-resistance status.**

Organism and Antimicrobial Drug (n)	Disk Diffusion True Resistance (PI*)	Broth Microdilution True Resistance (PI*)	Disk Diffusion True Non-Resistance (PI*)	Broth Microdilution True Non-Resistance (PI*)
***E. coli* Individual**				
Ampicillin (2279)	84.3% (59.7-98.9)	94.7% (77.1-99.1)	99.9% (99.5-99.9)	99.0% (98.3-99.8)
Ceftiofur (2295)	36.7% (4.6-94.1)	79.5% (45.8-96.5)	99.9% (99.8-99.9)	99.6% (99.3-99.9)
Streptomycin (2302)	70.5% (51.0-79.8)	87.2% (64.6-93.8)	99.7% (98.8-99.9)	97.9% (96.4-99.2)
Sulfisoxazole (2316)	94.5% (85.2-98.8)	95.9% (88.0-98.5)	99.7% (98.9-99.9)	98.7% (97.7-99.5)
Tetracycline (2238)	94.9% (89.8-96.9)	92.6% (87.9-94.7)	98.0% (94.9-99.4)	96.3% (93.6-97.7)
Trimeth-sulfa (2308)	81.7% (48.7-98.7)	81.4% (58.7-95.9)	99.9% (99.6-1)	99.8% (99.5-99.9)
***E. coli* Composite**				
Ampicillin (878)	87.5% (64.9-99.2)	92.0% (76.8-98.6)	99.5% (97.8-99.9)	99.0% (97.5-99.9)
Ceftiofur (885)	43.5% (6.1-95.2)	79.4% (46.1-96.4)	99.8% (99.4-99.9)	99.5% (98.8-99.9)
Streptomycin (872)	80.3% (58.7-98.5)	93.2% (73.5-98.7)	98.8% (95.6-99.9)	95.5% (90.6-99.5)
Sulfisoxazole (854)	91.2% (76.9-99.2)	95.6% (83.4-99.2)	99.1% (97.0-99.9)	98.6% (96.3-99.8)
Tetracycline (814)	97.1% (92.6-99.5)	89.2% (84.9-93.2)	94.7% (76.9-99.7)	95.8% (82.8-99.3)
Trimeth-sulfa (885)	63.3% (31.5-96.1)	89.4% (62.4-98.3)	99.9% (99.1-99.9)	99.2% (98.1-99.9)
M. haemolytica				
Ampicillin (783)	79.2% (34.5-98.7)	83.8% (54.0-97.3)	99.8% (99.1-99.9)	99.8% (99.2-99.9)
Tetracycline (783)	65.6% (40.3-90.4)	88.1% (60.3-97.4)	99.7% (98.2-99.9)	99.1% (97.0-99.9)

*PI = 95% Probability Intervals. Trimeth-sulfa = trimethoprim-sulfamethoxazole.

For *E. coli* isolates cultured from individual animal samples, disk diffusion was superior to broth microdilution in correctly classifying non-resistance for 5 of the 6 shared antimicrobial drugs tested (ampicillin, ceftiofur, streptomycin, sulfisoxazole, and tetracycline; Table 4). Ability to correctly classify true non-resistance to trimethoprim-sulfamethoxazole was not detectably different between the two susceptibility tests. Disk diffusion was superior to broth microdilution in terms of correctly classifying tetracycline resistance. Conversely, broth microdilution was superior to disk diffusion in correctly classifying ceftiofur and streptomycin resistances. However, the probability intervals for detection of true resistance for both ceftiofur and streptomycin were relatively wide. No difference was detected between the two susceptibility tests for correctly classifying resistance to ampicillin, sulfisoxazole, and trimethoprim-sulfamethoxazole.

Correct classification of resistance and non-resistance in isolates of *E. coli* from composite samples was similar to that of *E. coli* isolates from individual animal samples. No difference was found between the 2 tests in correctly classifying non-resistance for all of the 6 antimicrobial drugs tested (ampicillin, ceftiofur, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole; Table 4). In correctly classifying resistance, no difference was found between the tests in 4 of the 6 antimicrobial drugs (ampicillin, streptomycin, sulfisoxazole, and trimethoprim-sulfamethoxazole). Analogous to that of *E. coli* isolates from individual samples, disk diffusion was superior to broth microdilution in correctly classifying tetracycline resistance for *E. coli* isolates from composite samples.

Correct classification of true resistance and true non-resistance were not detectably different between the antimicrobial susceptibility tests for isolates of *M.*

haemolytica in the 2 antimicrobial drugs tested. Other shared antimicrobial drugs in the panels of disk diffusion and broth microdilution could not be modeled due to the absence of detected resistance in one or both of the sample sets.

Predictive Value for Resistance and Non-Resistance—Depending on the antimicrobial drug being evaluated and the expected prevalence of resistance, these results suggest that there would be substantial differences in the confidence that users could have regarding how well these susceptibility tests were correctly classifying resistance and non-resistance (Figure 2). The disk diffusion assay had better ability to predict true resistance for ampicillin, streptomycin, and sulfisoxazole than did broth microdilution when resistance prevalence in *E. coli* isolates was low (Figure 2); a similar difference was found regarding the predictive value for resistance when evaluating tetracycline susceptibility in *M. haemolytica* isolates. Conversely, the ability to have high confidence in test results indicating non-resistance to these 4 antimicrobial drug and organism combinations was substantially better for broth microdilution than disk diffusion when resistance prevalence was high (Figure 2). For example, these estimates suggest that in a population of *E. coli* isolates where the true prevalence of resistance to streptomycin resistance was 5%, a disk diffusion test indicating resistance would accurately predict true resistance status 92.5% of the time and a broth microdilution test would only correctly predict true resistance status 68.6% of the time. However, when the true prevalence of resistance to streptomycin was 70%, a disk diffusion test indicating non-resistance would correctly predict true non-resistance status 59.2%, while a broth

microdilution test indicating non-resistance would accurately predict non-resistance 76.6% of the time (Figure 2).

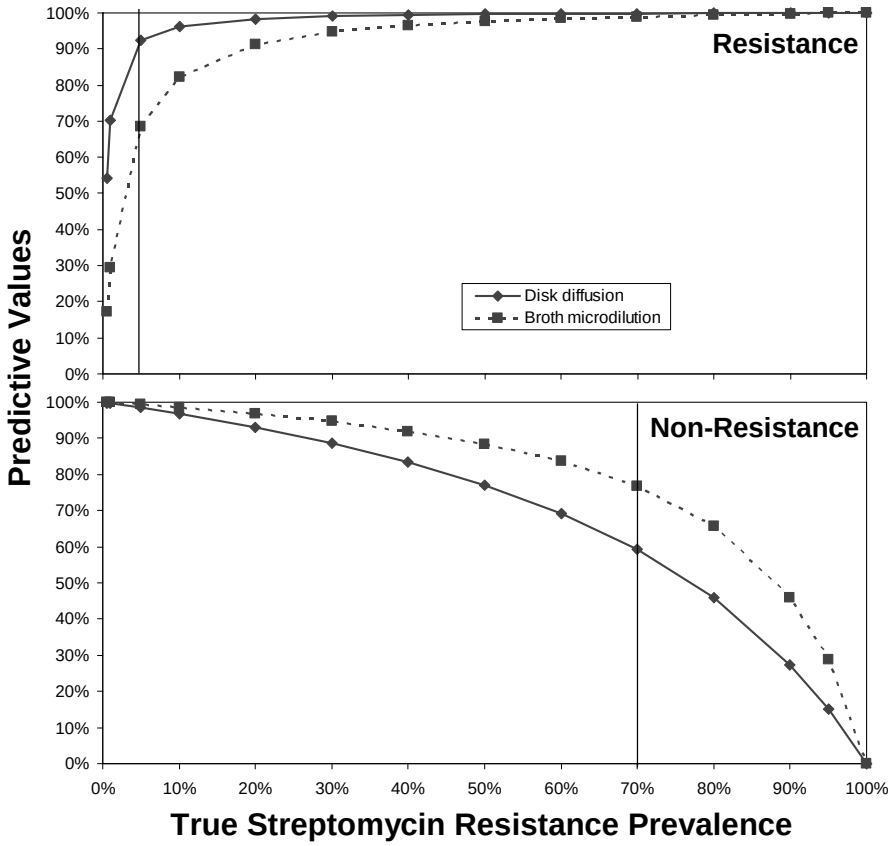

Figure 2: **Predictive values for resistance and non-resistance to streptomycin by disk diffusion and broth microdilution.** The vertical lines represent the true prevalence of streptomycin resistance at arbitrary low and high levels to illustrate large differences in predictive values between the 2 antimicrobial susceptibility tests.

In contrast, the susceptibility tests for other antimicrobial drugs had predictive abilities that were more comparable across a wide range of true prevalence values (ceftiofur, tetracycline, and trimethoprim-sulfamethoxazole in *E. coli* isolates and

ampicillin in *M. haemolytica* isolates; Figure 3). Though the predictive abilities of the two tests were not identical, there are not large, practical differences for these antimicrobial drugs regarding the confidence that a user could have in positive or negative tests results (resistant or non-resistant, respectively) for these two assays. For example, the difference between the predictive abilities of disk diffusion (71.4%) and broth microdilution (56.8%) when the true prevalence of resistance to tetracycline was 5% is much smaller than this difference for streptomycin (Figures 2 and 3). Similarly, there is only a small difference in the predictive ability for non-resistant test results when the true prevalence of resistance to tetracycline was 70% (89.2% for disk diffusion vs. 84.8% for broth microdilution).

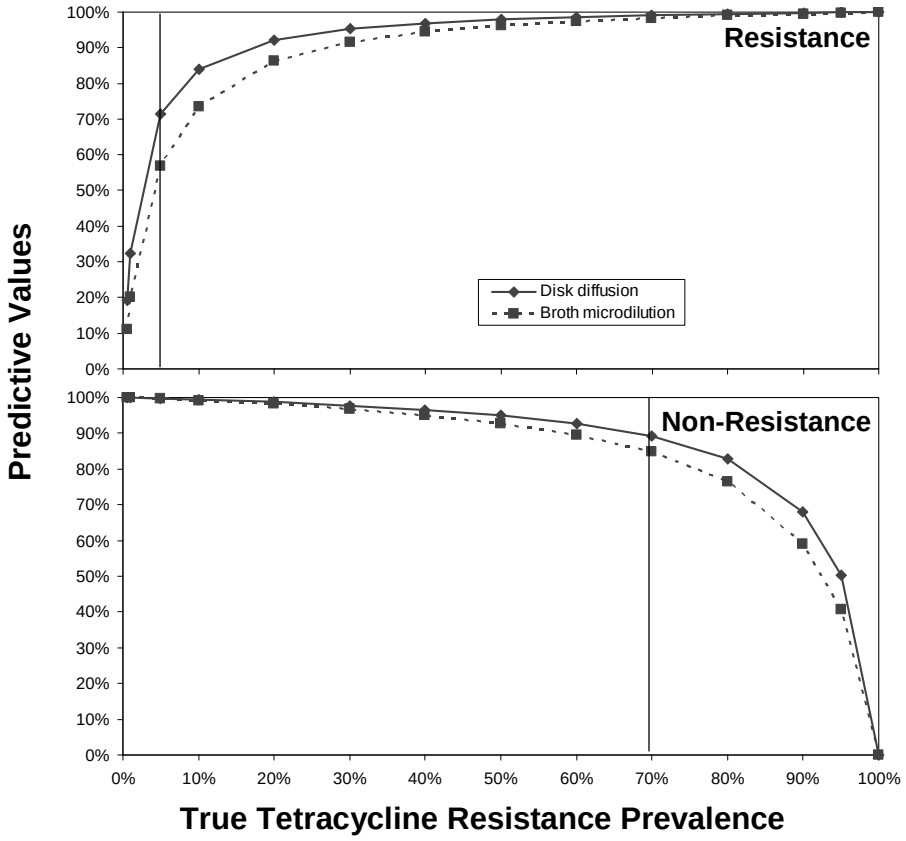

Figure 3: **Predictive values for resistance and non-resistance to tetracycline by disk diffusion and broth microdilution.** The vertical lines represent the true prevalence of tetracycline resistance at arbitrary low and high levels to illustrate small differences in predictive values between the 2 antimicrobial susceptibility tests.

Model Convergence and Sensitivity Analysis — Evidence for model convergence was provided for each model by the overlapping history (time series) plots for 6 simultaneous chains, a relatively immediate drop to zero in autocorrelation plots, and overlapping lines with values of ~1 in Brooks-Gelman-Rubin plots (Gelman and Rubin 1992). Widely varied prior probability distributions and starting values had little influence on results obtained from all of the models. Correlation values obtained from the latent class models for the 2 tests were low (<0.3) for all models suggesting that there was a low or small amount of conditional dependence between the sensitivities and specificities of the tests.

Additionally, this conclusion was supported by the fact that parameter estimates obtained from models which assumed conditional independence for the classification of true resistance and true non-resistance between the two susceptibility tests were generally similar to the estimates obtained from models which included parameters for conditional dependence (results not shown).

DISCUSSION

Results of this study indicate that the ability of disk diffusion and broth microdilution susceptibility tests to correctly classify true resistance varies by the antimicrobial drug and organism of interest. In contrast, both testing methods had consistently strong ability to correctly classify true non-resistance for all antimicrobial drugs tested and in both *E. coli* and *M. haemolytica*. Therefore, non-resistance detected by either method likely represents the true non-resistance status in these populations, but more errors in the correct classification of true resistance can be expected. Additionally, misclassification of resistance to different antimicrobial drugs occurs at different rates in the 2 organisms.

Hui and Walter used a maximum-likelihood procedure to develop deterministic estimates of error rates and true prevalences in 2 populations assuming conditional independence between the tests (1980). More recently, stochastic methods have allowed incorporation of prior knowledge (Bayesian methods) using Monte Carlo simulation to estimate posterior probabilities regarding test accuracy and the true prevalence of conditions in populations (Enoe et al. 2000). Additionally, the methodology has been extended to account for tests which do not meet the traditional assumption of conditional independence (Branscum 2005). Therefore, theoretically unbiased estimates of the accuracies for conditionally dependent tests can be obtained.

The ability of a test to correctly identify resistance or non-resistance dictates our level of trust in describing the true picture of resistance. Clinically, an accurate understanding of true resistance and true non-resistance is important for appropriately selecting antimicrobial drugs to control or prevent disease while also adhering to

principles of prudent use. Both broth microdilution and disk diffusion susceptibility tests have limitations in their methodology for adequately representing *in vivo* factors which contribute to therapeutic failures, such as stage of infection or physiologic barriers. Information obtained from broth microdilution is often considered as more clinically relevant than that obtained from disk diffusion because the MIC information can aid therapeutic decision making regarding drug selection, dosing, and route of administration. However, this study has shown that misclassification of resistance (and non-resistance) in many of the tested antimicrobial drugs was just as likely in broth microdilution testing as it was in disk diffusion. In the case of tetracycline, the broth microdilution test was actually shown to be more prone to errors in resistance classification than disk diffusion. A limited number of antimicrobial drugs were analyzed in this study, so similar results in untested antimicrobial drugs may also be found.

On a broader scale, surveillance for antimicrobial resistance on a regional, national or even international level requires that large numbers of isolates be evaluated to help ensure representativeness of results. Therefore, the cost per test becomes a much larger concern when compared to susceptibility testing for clinical purposes. High accuracy in surveillance is necessary to confidently and efficiently respond to potential antimicrobial resistance threats to health on a population level (Livermore et al. 1998). Given that results for different drugs were largely comparable for disk diffusion and broth microdilution, we conclude that there are several advantages which make disk diffusion susceptibility testing a more practical choice for use in large surveillance programs compared with broth microdilution. The equipment and supplies necessary for

testing by disk diffusion is lower in cost than for testing by broth microdilution. Additionally, disk diffusion testing is more flexible in terms of the ability to easily and cheaply switch antimicrobial susceptibility discs in the antimicrobial drug panel as opposed to producing or ordering customized 96-well plates in broth microdilution testing. The epidemiologic thresholds of emerging resistances are often much lower than the clinical breakpoints between resistance and non-resistance (Greiner and Gardner 2000a; Greiner and Gardner 2000b; Bywater et al. 2006; Simjee et al. 2008). Since lower prevalences of resistance need to be detected, large numbers of isolates must be tested for surveillance purposes and costs can become even more prohibitive. A limitation of this study was that clinical breakpoints were used to designate resistance and non-resistance, so it is possible that the tests would perform differently at other breakpoints or epidemiologic thresholds. However, the breakpoints utilized here were the ones readily available and might practically be used in the development of a surveillance program.

Beyond the considerations of test accuracy, the predictive values calculated in this study illustrate that the decision of an appropriate test for a certain population could also be directed by the expected level of resistance prevalence. In general, at very low prevalences, the predictive value for resistance will always be low while the predictive value for non-resistance is high (Thrusfield 2005). Conversely, at very high prevalences, the predictive value for resistance will always be high while the predictive value for non-resistance is low. This principle applies generally to all tests and has been previously noted as well in the performance of disk diffusion susceptibility testing (Lamy et al. 2004). The susceptibility tests in this study had comparable predictive abilities across a range of resistance prevalence to some antimicrobial drugs indicating that either test

would perform well. However, the tests would perform differently dependent on the prevalence of resistance to antimicrobial drugs such as streptomycin and sulfisoxazole in *E. coli* isolates.

Since disk diffusion and broth microdilution are based on similar biological mechanisms which measure concentration relationships between the organism and the antimicrobial drug, the two tests were assumed to be dependent. In highly dependent tests, the proportions of detecting true resistance or true non-resistance by each test are expected to be the same and correlation values would be high (i.e. >0.7). Correlation values in this study were low and analogous models assuming independence of test accuracies produced similar posterior probability distributions to those of the dependence models. However, we do not believe that this finding universally exempts us from the necessity of assuming dependence between disk diffusion and broth microdilution since both tests were highly accurate (Georgiadis et al. 2003). The dependence models used in this study would be recommended to account for any amount of correlation, no matter how small (Dendukuri and Joseph 2001).

One of the assumptions of latent class analysis for 2 tests in 2 populations is that the 2 populations have different prevalences (Branscum 2005). This was a limitation in our study for ampicillin, ceftiofur, and trimethoprim-sulfamethoxazole resistance since the prevalences of these antimicrobial drugs in sample set 1 were similar to their counterparts in sample set 2. Even tetracycline resistance in *M. haemolytica* isolates and sulfisoxazole resistance in the *E. coli* isolates from composite samples was suspect for this reason as well. Therefore, the estimates for these antimicrobial drugs should not be

considered as reliable as the other antimicrobial drugs with smaller probability intervals (Toft 2005).

The collection of isolates used for this study was a non-representative, convenience sample. Also, multiple isolates were from the same individual or from individuals within the same pen and clustering of resistances would be expected in such a population. Therefore, the posterior probability distributions for prevalence produced by the models were not reported. It was not an objective of this study to describe true resistance prevalence in the 2 sample sets nor was it necessary for the evaluation of the test accuracy by this latent class analysis method. Every isolate was tested by both systems and the effective comparison of the tests was at the isolate level. Theoretically, the proportions of correctly classified true resistance and true non-resistance are constant across populations of different resistance prevalences. Therefore, the estimates produced in this study would be relevant in all populations of feedlot cattle for the antimicrobial drug and organisms presented.

The prior probability distributions (priors) used in these models were potentially biased due to design differences between this study and the studies which documented resistance prevalence in similar populations. However, the priors were all structured to be weakly informative to the Bayesian analyses and were not found to influence the models heavily. The prevalence estimates for antimicrobial drug resistance in *E. coli* isolates for arrival and exit populations were highly relevant since the population of feedlot cattle from Alberta were likely very similar to that of our study (Rao et al. 2009). The previous study only evaluated resistances by way of composite samples, so the use of these same priors for the individual *E. coli* isolates in our models might be different if

more appropriate priors were available. The *M. haemolytica* prevalence priors were even more biased than the individual *E. coli* priors. No other studies have been conducted which estimate the level of resistance in representative populations of feedlot cattle for *M. haemolytica* isolates using analogous susceptibility testing approaches. Therefore, the best estimates were obtained from a study of diagnostic laboratory lung samples for different respiratory pathogens (Watts et al. 1994). The resistance detected in lung isolates may differ from that of the nasopharyngeal swab isolates used in our study. The biased priors as well as the low level of resistance detected in *M. haemolytica* most likely account for the wide probability intervals for the proportions of correctly classified resistance.

FOOTNOTES

[a]WinBUGS 1.4, 1996-2003, Imperial College and Medical Research Council, UK: http://www.mrc-bsu.cam.ac.uk/bugs/

[b]BetaBuster 1.0, free software available at http://www.epi.ucdavis.edu/diagnostictests/home.html

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CHAPTER 4: Associations between parenteral antimicrobial use and antimicrobial resistance in *Escherichia coli* sampled from individual feedlot cattle

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INTERPRETIVE SUMMARY

The veterinary and public health sectors are both interested in the prevalence of antimicrobial resistance in food producing animals. The former has interests in the efficacy of antimicrobial drugs in managing these animals as well as the concerns of the latter. The public health sector hypothesizes that the exposures to antimicrobials in food producing animals detrimentally affects the susceptibility to antimicrobials in humans. Tracing the probabilities of persistence and dissemination of resistance from food-producing animals to humans is beyond the scope of this project. However, this project does evaluate one of the first steps in this transmission possibility; the development of resistance. Not only is the prevalence of antimicrobial resistance important, but also the associations or lack of associations between resistance and exposures to antimicrobial drugs.

This project directly evaluated these associations in feedlot cattle by tracking all exposures to parenteral antimicrobial drugs between time points when samples were collected, cultured, and tested for susceptibility to panels of antimicrobial drugs. Due to the intensive management of feedlot cattle in groups or pens, the antimicrobial pressures applied to an individual in one of these pens might logically apply pressure to other individuals in that pen. Therefore, exposures were tracked for individuals as well as calculated on a pen-level basis to evaluate an ecological exposure to antimicrobials in this environment. Accurately evaluating the complex relationships between antimicrobial use and resistance has always been challenging with the limited techniques that are available to analyze this type of data. Two different analytical approaches were used in this project

to explore options for surveillance programs. One of these two approaches was found to be appropriate and useful, while the other lacked interpretable results.

ABSTRACT

Objectives: The objectives of this study were to 1) estimate the prevalence of antimicrobial resistance in the study population and 2) to investigate the associations between exposures to parenteral antimicrobial drugs and antimicrobial resistance in fecal non-type specific *E. coli* (NTSEC) recovered from individual feedlot cattle.

Materials and Methods: Two-stage random sampling was used to identify cattle for enrollment at 4 western Canadian feedlots. A fecal sample was collected per rectum from each individual at arrival and at a second sampling point around mid-feeding period when cattle were rehandled as part of standard production practices. From samples collected at this second time point, a total of 2,133 NTSEC isolates were tested for susceptibility to antimicrobial drugs by disk diffusion. Parenteral exposures to antimicrobial drugs were recorded for each individual enrolled in the study as well as for other animals in the same pen. The least square means estimates and 95% confidence intervals for the prevalence of resistance at each time point were modeled using Poisson regression. Multivariable logistic regression modeling was used to investigate associations between antimicrobial resistance and exposure to antimicrobial drugs. Regression models were adjusted for clustering of observations among individuals and pens.

Results: The most common resistances identified in arrival samples were sulfisoxazole (7.5%; 95%CI: 6.1-9.2), streptomycin (7.7%; 95%CI: 6.3-9.5) and tetracycline (20.0%; 95%CI: 17.7-22.6). At the second sampling point, resistance prevalence was 25.6%

(95%CI: 23.5-28.0) for sulfisoxazole, 25.0% (95%CI: 22.8-27.3) for streptomycin, and 72.7% (95%CI: 70.5-75.1) for tetracycline. Regression modeling identified an association between exposures to tetracyclines with antimicrobial resistance to tetracycline at the second time point. Exposures to other classes of drug were not associated with increased resistance.

Conclusions: Parenteral exposures to antimicrobial drugs in feedlot cattle did not drive resistance at mid-feeding period.

INTRODUCTION

Though the development mechanisms and factors associated with antimicrobial resistance are complex and multifactorial, exposure to antimicrobial drugs is considered a large contributor to the overall burden of resistance (Witte 2000; Levy and Marshall 2004). Specifically, exposures to antimicrobial drugs in agricultural populations and the associations with resistance dissemination ultimately to human populations are of particular concern (Phillips et al. 2004; Barton 1998; McGeer 1998; Angulo et al. 2004). However, associations between exposure to antimicrobial drugs and resistance need to be characterized accurately before risk assessments of identified associations can be pursued (McDermott et al. 2002). Surveillance studies of antimicrobial resistance in populations of swine, poultry, and cattle have described varying levels of resistance, differences in exposure to antimicrobial drugs and management practices, and associations as well as lack of associations between resistance and exposures to antimicrobial drugs (Thibodeau et al. 2008; Akwar et al. 2008; Checkley et al. 2008). Additionally, temporal trends have been found that show certain antimicrobials may only be associated in a transient fashion (Platt et al. 2008; Lowrance et al. 2007). Continuous surveillance studies are needed to monitor changes in resistance prevalence and potential associations with exposures to antimicrobial drugs for the collective health of humans and animals (Aarestrup 2005).

Challenges in conducting antimicrobial resistance surveillance studies are numerous (Caprioli 2000). Quantifying exposures to antimicrobial drugs in a manner that accurately represents selection pressures is deceptively complex (Singer et al. 2006). Outcome measures of susceptibility testing can be presented in continuous or categorical representations, but the differences in detection abilities between these measures is

unknown. Further, analytic techniques for modeling these measures have inherent shortcomings. While exploring the possible analytical approaches to these data, the objectives of this study were to estimate the resistance prevalence and to investigate the associations between exposures to parenteral antimicrobial drugs and resistance in fecal non-type specific E. coli (NTSEC) recovered from individual feedlot cattle.

MATERIALS AND METHODS

Overview—All individual and pen-level exposures to parenteral antimicrobial drugs were summarized between 2 time points when fecal samples were collected from individual feedlot cattle. These samples were cultured for isolates of non-type specific *E. coli* (NTSEC), which were tested for susceptibility to panels of antimicrobial drugs. Resistance to each tested antimicrobial was evaluated separately for associations with exposures to different classes of antimicrobials in regression models.

Study Population— All animal handling and sampling procedures were approved prior to the initiation of the study by the Animal Care Committee of Feedlot Health Management Services (FHMS) and the Institutional Animal Care and Use Committee of Colorado State University.

Cattle (n=5,913) enrolled in this study were managed at 4 western Canadian feedlots in south central Alberta under production conditions typical of those used at large commercial cattle feedlots throughout western Canada and the U.S. Feedlots had one-time capacities between 15,000 and 20,000 animals, with pens capable of housing 50-350 animals. All feedlots had modern cattle handling facilities. Commercial feedlots were purposively selected for participation based on willingness to participate, and their ability to collect data about exposures to antimicrobial drugs for individual animals.

Candidate animals utilized in the study were procured through the auction market system across western Canada. Various cattle types were fed at these feedlots over the 3 years including cattle of various entry weights, age classes (calves and yearlings), frame sizes, sources (e.g., ranch-direct cattle and back-grounded cattle), and genders (bulls,

steers and heifers). Cattle entering these feedlots typically weighed 500-900 pounds, were managed in the feedlot for 120-250 days, and were slaughtered when body weights reached 1,250-1,400 pounds.

All animals enrolled in the study were subject to standardized animal health management and feedlot production procedures as per the protocols developed by health and production consultants (FHMS). In brief, each animal received a unique identification ear tag, a growth implant, vaccines to selected bacteria and viruses, and topical avermectin anthelmintic for internal and external parasite control. In animals determined to have a higher risk of developing respiratory disease, a parenteral antimicrobial drug was administered as part of the prevention strategies for bovine respiratory disease (Table 1). Water and standard feedlot diets were offered *ad libitum* throughout the feeding period; rations were formulated to meet or exceed the National Research Council nutritional requirements for feedlot cattle.

***Sampling Procedures*—**Animals were allocated to the study from September 17, 2007 to January 16, 2010. A 2-stage random sampling plan was used to determine which pens and animals within those pens were selected for enrollment. During the enrollment period, 30% of all new pens of cattle were randomly selected for inclusion in the study using a pen randomization table. Within each selected pen, 10% of all animals in that pen were then randomly enrolled in the study at initial processing using an individual animal randomization table.

Individual animals enrolled in the trial were sampled twice over the course of the study: during initial processing shortly after arrival to the feedlot and in the middle of the

feeding period when cattle were re-handled as part of standard feedlot protocols (e.g. for replacement of hormonal implants). At each sampling date, all animals had a nasopharyngeal swab sample and a fecal sample collected per rectum. The nasopharyngeal sample was collected in the deep pharynx using a commercially available double guarded swab (# J273, Jorgensen Laboratories, Inc, Loveland, CO, USA). A Cary Blair media tube (BBL CultureSwab™, CA90001-038, VWR International, Mississauga, Ontario) was used to transport the nasopharyngeal swab. Individual fecal samples were collected per rectum using a new plastic palpation sleeve. A minimum of 4 grams of feces from the rectum of each animal was transferred into a vial containing modified Cary Blair transport media (Enteric Transport Medium, 15 ml, Dalynn Biologicals Inc.) (Alexander et al. 2009). All individual animal samples were labeled with the date and a unique study number linked to that animal. Samples were refrigerated in a chilled cooler and transported to the microbiology lab (Agriculture and Agri-Food Canada Lethbridge Research Station, Lethbridge, Alberta) for further processing by overnight courier. Each sample collected over the course of the trial was assigned a unique identification number to ensure blinding of the laboratory staff and uniform labeling of samples.

Laboratory Procedures— To optimize study efficiency regarding the relationship between resistance in NTSEC and *M. haemolytica* for a separate project, only fecal samples recovered from animals that also had *M. haemolytica* isolates recovered from their nasopharyngeal swabs were cultured for NTSEC. Manure samples were processed immediately after overnight delivery to the microbiology laboratory. The feces were mixed with the Cary-Blair transport medium to create a uniform slurry (Alexander et al.

2009). A sterile cotton swab was immersed completely into the sample and then streaked onto a MacConkey Agar (MAC) plate. Plates were incubated overnight at 37ºC. After 24 hours of incubation, the MAC plates were removed from the incubator and examined for lactose-fermenting (pink) colonies with morphology typical of NTSEC. Using aseptic technique, up to 3 colonies were selected and streaked onto fresh Lysogeny Broth (LB) plates and incubated at 37ºC overnight. Using a single colony from each LB plate, an indole test was performed and isolates determined as presumed-NTSEC (lactose-fermenting, indole-positive) were stored in 30% glycerol stocks at -80ºC until further characterization.

Antimicrobial Susceptibility Testing—Each NTSEC isolate included in this analysis was tested for susceptibility to a standardized panel of antimicrobial drugs by disk diffusion. The antimicrobial drugs included in this panel were ampicillin, amoxicillin-clavulanate, ceftazidime, ceftiofur, enrofloxacin, sulfisoxazole, florfenicol, neomycin, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline. Ceftazidime-clavulanate was also included in the panel for a separate project investigating occurrence of extended spectrum beta lactamases. Due to interruptions in the availability of antimicrobial discs for ceftazidime-clavulanate, not all isolates were tested for susceptibility to this antimicrobial drug. The antimicrobial testing procedures were conducted according to protocols of the Clinical Laboratory Standards Institute (CLSI 2008). Quality control strains used were *Escherichia coli* ATCC 25922, *Streptococcus pneumoniae* ATCC 49619, and *Staphylococcus aureus* ATCC 29213.

Interpretive criteria— Susceptible, intermediate, and resistant (SIR) designations of *M. haemolytica* were determined using CLSI guidelines for ampicillin (2002) and oxytetracycline (2006). Interpretive criteria for *E. coli* were also obtained from CLSI guidelines (2005), except for streptomycin, which were based on those used by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) and the National Antimicrobial Resistance Monitoring System (NARMS, CDC 2003).

Antimicrobial Use Data—Individual animal exposure data regarding antimicrobial drugs were recorded at each feedlot over the course of the study using a chute-side computer system (*i*FHMS, Okotoks, Alberta). Exposures were also recorded for animals not enrolled in the study, but housed in the same pen as study animals. These data included the product used, the dose, and the date of administration. All study data were subsequently compiled, collated in a computer spreadsheet and verified. In-feed antimicrobials, ionophores, and coccidiostats were not included in this analysis.

Data Analysis—To facilitate further analysis involving logistic regression, isolate susceptibility was dichotomized as resistant and non-resistant (which included both intermediate and susceptible classifications). Dosage information for exposures to antimicrobial drugs was converted into an Animal Defined Daily Dose (ADD). The ADD metric represents the number of days of treatment for an animal based on an assumed average maintenance dosage. Dosage conversion to ADD was based on the expected length of drug effect as indicated by approved dosages (Table 1). Aggregate (pen-level) exposures to antimicrobial drugs accounted for the number of animals

receiving the drug in a pen as well as the total number of animals in the pen. This density of exposure in a pen represented the ecological pressure beyond individual exposure to antimicrobial drugs. All exposures to antimicrobial drugs between arrival and the second sampling point were summarized for individuals by class of antimicrobial drug and by exposure context (individual or pen-level).

Table 1: **Parenteral antimicrobial drugs used in study population.** ADD = Animal Defined Daily Dose; BW = body weight; BRD = bovine respiratory disease.

Antimicrobial Drug and Dosage	Class	Primary Reason for Use	ADD
Ceftiofur sodium 1 mg/kg BW	Beta-lactam	BRD Treatment	1
Ceftiofur crystalline free acid 6.6 mg/kg BW	Beta-lactam	BRD Treatment	3
Ceftiofur hydrochloride 1.1 mg/kg BW	Beta-lactam	BRD Treatment	1
Enrofloxacin 7.7 mg/kg BW	Quinolone	Relapse BRD Treatment	3
Florfenicol 40 mg/kg BW	Phenicol	BRD Treatment	3
Florfenicol 40 mg/kg BW and Flunixin meglumine 2.2 mg/kg BW	Phenicol	BRD Treatment	3
Oxytetracycline			
10 mg/kg BW	Tetracycline	BRD Prevention/Treatment	1
20 mg/kg BW	Tetracycline	BRD Prevention/Treatment	2
30 mg/kg BW	Tetracycline	BRD Prevention/Treatment	3
Tilmicosin 10 mg/kg BW	Macrolide	BRD Prevention/Treatment	3
Trimethoprim and sulfadoxine 16 mg/kg BW	Sulfonamide	BRD Treatment	1
Tulathromycin 2.5 mg/kg BW	Macrolide	BRD Prevention/Treatment	3
Tylosin tartrate 29 mg	Macrolide	Implant Site Abscess Prevention	1/275

Descriptive Analysis— Data were evaluated graphically and by calculating descriptive statistics (Tables 2-4). The least square means estimates and 95% confidence intervals for the prevalence of resistance at arrival and >60DOF were modeled using Poisson regression (Figure 1). Regression analysis using generalized estimating equation methods was used to correct prevalence estimates for lack of independence associated with multiple isolates recovered from the same individual and with multiple isolates recovered from individuals within the same pen. Compound symmetry (exchangeable) correlation structures nested unique sets of isolates from individuals within each unique group (pen) of cattle. Sampling point (arrival or >60DOF) was used as the predictor variable of interest for these analyses, and separate models were developed to estimate resistance prevalence for 11 of the 12 antimicrobial drugs evaluated. It was not possible to estimate the prevalence of isolates resistant to ceftazidime-clavulanate, as interpretive criteria for this drug are not available.

For presentation, individual parenteral exposures to antimicrobial drugs were further summarized into 3 sub-periods between the arrival and the >60DOF sampling points. Period 1 included exposures to a class of antimicrobial drugs within the 3 days prior to the >60DOF sampling point. Period 2 included exposures to a class of antimicrobial drugs between 4 and 14 days prior to the >60DOF sampling point. Period 3 included exposures to a class of antimicrobial drugs beyond 14 days prior to the >60DOF sampling point. If exposures exceeding a single day were given just prior to a period cutoff, the amount of ADDs attributed to each period were summarized separately and included only in their respective categories (Table 5). Pen-level parenteral exposures

were presented as the average ADD exposure per individual and the average ADD exposure in the risk period based on an average risk period of 101.5 days (Table 6).

Table 2: **Number of antimicrobials drugs to which non-type specific *E. coli* isolates were resistant** from individual feedlot cattle sampled at arrival (n=1663) and > 60 days on feed (>60DOF) (n=2133). Estimates were not adjusted for lack of independence between isolates sampled from the same individuals and between individuals from the same pens of cattle.

Resistance Number	Arrival Percent Resistance (Frequency)	>60DOF Percent Resistance (Frequency)
Pan-Susceptible	76.5% (1272)	25.4% (541)
1	12.9% (215)	37.9% (808)
2	3.9% (65)	16.7% (357)
3	4.8% (80)	12.8% (273)
4	1.1% (19)	5.1% (108)
5	0.5% (8)	1.8% (38)
6	0.2% (3)	0.2% (5)
7	0.0% (0)	0.1% (1)
8	0.0% (0)	0.1% (2)
9	0.1% (1)	0.0% (0)

***Table 3*: Resistance patterns of non-type specific *E. coli* isolates at arrival (n=1663) and >60 days on feed (>60DOF) (n=2133).** Other phenotypes included different patterns which represented <0.5% of isolates each. Estimates were not adjusted for lack of independence between isolates sampled from the same individuals and between individuals from the same pens of cattle. RES = resistant.

Sample Time	Frequency	Percent of Isolates from Group	Resistance Number	Ampicillin	Amoxicillin-Clavulanate	Ceftazidime	Ceftiofur	Enrofloxacin	Sulfisoxazole	Florfenicol	Neomycin	Streptomycin	Trimethoprim-Sulfamethoxazole	Tetracycline
ARRIVAL	1272	76.5%	0	.	.	.	.	.	.	.	.	.	.	.
	172	10.3%	1	.	.	.	.	.	.	.	.	.	.	RES
	53	3.2%	3	.	.	.	.	.	RES	.	.	RES	.	RES
	23	1.4%	2	.	.	.	.	.	RES	.	.	.	.	RES
	18	1.1%	2	.	.	.	.	.	.	.	.	RES	.	RES
	17	1.0%	1	RES	.	.	.	.	.	.	.	.	.	.
	15	0.9%	3	RES	.	.	.	.	.	.	.	RES	.	RES
	15	0.9%	4	.	.	.	.	.	RES	RES	.	RES	.	RES
	12	0.7%	2	RES	.	.	.	.	.	.	.	.	.	RES
	9	0.5%	1	.	.	.	.	.	RES	.	.	.	.	.
	57	3.4%			Other Phenotypes			Other Phenotypes			Other Phenotypes			
>60 DOF	785	36.8%	1	.	.	.	.	.	.	.	.	.	.	RES
	541	25.4%	0	.	.	.	.	.	.	.	.	.	.	.
	189	8.9%	3	.	.	.	.	.	RES	.	.	RES	.	RES
	167	7.8%	2	.	.	.	.	.	RES	.	.	.	.	RES
	133	6.2%	2	.	.	.	.	.	.	.	.	RES	.	RES
	79	3.7%	4	.	.	.	.	.	RES	RES	.	RES	.	RES
	40	1.9%	2	RES	.	.	.	.	.	.	.	.	.	RES
	34	1.6%	3	RES	.	.	.	.	.	.	.	RES	.	RES
	17	0.8%	3	.	.	.	.	.	RES	RES	.	.	.	RES
	15	0.7%	5	.	.	.	.	.	RES	RES	.	RES	RES	RES
	11	0.5%	4	RES	.	.	.	.	RES	.	.	RES	.	RES
	11	0.5%	4	.	.	.	.	.	RES	.	RES	RES	.	RES
	111	5.2%			Other Phenotypes			Other Phenotypes			Other Phenotypes			

Table 4: **Zone diameters for non-type resistant *E. coli* isolates (n=2133) at > 60 days on feed.** The minimum zone diameter possible is 6mm (the diameter of the antimicrobial susceptibility disk) which indicates that no zone of growth inhibition as occurred up to the disk margin. Breakpoints indicate CLSI susceptible (blue) and resistant (red) breakpoints. Ceftazidime-clavulanate has no established breakpoints.

Zone Diameter	Ampicillin	Amoxicillin-Clavulanate	Ceftazidime	Ceftiofur	Ceftazidime-Clavulanate	Enrofloxacin	Sulfisoxazole	Florfenicol	Neomycin	Streptomycin	Trimethoprim-Sulfamethoxazole	Tetracycline
6	5.5% (117)	0.1% (2)	0.1% (2)	0.0% (0)	0.2% (2)	0.0% (0)	25.8% (551)	6.3% (135)	0.1% (2)	8.5% (182)	2.3% (49)	59.1% (1261)
7	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.1% (2)	0.0% (0)	0.2% (4)	0.0% (0)	1.5% (31)
8	0.0% (0)	0.0% (0)	0.1% (1)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.3% (7)	0.0% (0)	1.9% (40)	0.0% (0)	4.5% (96)
9	0.1% (1)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.1% (1)	0.1% (3)	0.1% (2)	2.9% (61)	0.0% (0)	2.6% (56)
10	0.1% (3)	0.1% (3)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.1% (1)	0.1% (3)	0.3% (6)	7.3% (155)	0.0% (0)	3.5% (75)
11	0.1% (3)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.4% (8)	4.2% (89)	0.0% (0)	1.8% (38)
12	0.1% (1)	0.1% (2)	0.1% (1)	0.0% (0)	0.0% (0)	0.0% (0)	0.1% (2)	0.1% (1)	0.6% (12)	3.2% (69)	0.1% (1)	3.5% (74)
13	0.1% (3)	0.1% (3)	0.0% (0)	0.1% (1)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.6% (12)	1.6% (33)	0.0% (0)	2.2% (47)
14	0.4% (8)	0.1% (1)	0.0% (0)	0.1% (1)	0.1% (1)	0.0% (0)	0.1% (1)	0.0% (0)	0.1% (3)	1.3% (28)	0.1% (3)	3.6% (76)
15	1.4% (30)	0.1% (3)	0.1% (1)	0.1% (1)	0.0% (0)	0.0% (0)	0.1% (3)	0.0% (0)	0.6% (12)	6.4% (137)	0.1% (3)	3.4% (72)
16	2.3% (49)	0.3% (6)	0.1% (1)	0.0% (0)	0.0% (0)	0.0% (0)	0.1% (1)	0.1% (1)	1.0% (21)	12.1% (258)	0.3% (6)	3.1% (66)
17	3.7% (78)	1.3% (28)	0.0% (0)	0.1% (2)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	1.6% (33)	15.8% (336)	0.4% (8)	1.2% (26)
18	8.1% (172)	2.8% (59)	0.1% (1)	0.1% (2)	0.0% (0)	0.0% (0)	0.2% (5)	0.0% (0)	9.9% (210)	22.3% (476)	1.0% (22)	0.3% (7)
19	10.2% (218)	4.0% (86)	0.0% (0)	0.0% (0)	0.1% (1)	0.1% (1)	0.7% (15)	0.1% (1)	16.6% (353)	7.3% (156)	1.1% (24)	0.2% (5)
20	15.3% (327)	6.6% (141)	0.1% (3)	0.3% (6)	0.0% (0)	0.1% (3)	1.4% (30)	0.6% (12)	29.9% (637)	3.2% (69)	2.0% (43)	0.6% (13)
21	18.9% (404)	15.1% (322)	0.1% (3)	1.4% (30)	0.2% (3)	0.4% (8)	3.9% (83)	0.9% (20)	27.7% (590)	1.3% (27)	3.6% (77)	1.4% (29)
22	13.3% (283)	16.3% (348)	0.2% (4)	2.5% (54)	0.1% (1)	0.2% (5)	3.9% (83)	2.3% (48)	7.1% (152)	0.3% (7)	3.4% (73)	2.1% (45)
23	12.2% (261)	26.0% (555)	1.2% (25)	5.7% (122)	0.7% (9)	0.5% (10)	10.6% (227)	6.7% (142)	2.5% (54)	0.2% (5)	6.3% (134)	3.6% (76)
24	4.1% (87)	13.3% (284)	2.3% (48)	7.6% (162)	1.2% (16)	0.6% (12)	10.3% (220)	8.9% (189)	0.3% (6)	0.0% (0)	4.4% (93)	1.2% (25)
25	21% (44)	7.7% (165)	3.9% (83)	12.2% (261)	2.8% (38)	1.6% (33)	12.9% (275)	16.6% (353)	0.3% (7)	0.1% (1)	6.0% (127)	0.5% (11)
26	1.5% (31)	3.8% (81)	9.2% (197)	27.3% (582)	5.6% (76)	3.7% (78)	14.8% (315)	24.9% (532)	0.4% (9)		9.9% (210)	0.2% (4)
27	0.2% (4)	1.2% (25)	10.2% (217)	15.9% (339)	6.9% (93)	4.3% (92)	6.1% (129)	14.9% (317)	0.1% (3)		10.3% (219)	
28	0.2% (4)	0. 6% (12)	15.4% (329)	13.1% (280)	13.8% (187)	7.0% (149)	4.6% (99)	9.5% (203)	0.1% (1)		13.8% (294)	
29	0.2% (5)	0.2% (4)	24.2% (517)	8.8% (187)	26.4% (358)	14.2% (302)	2.3% (50)	5.2% (111)			17.3% (369)	
30		0.1% (1)	15.1% (323)	2.8% (60)	20.5% (278)	15.4% (329)	0.8% (16)	1.5% (32)			9.6% (205)	
31		0.1% (1)	10.1% (215)	1.2% (26)	13.0% (176)	16.5% (352)	0.8% (16)	0.8% (18)			4.7% (101)	
32		0.1% (1)	4.8% (103)	0.7% (15)	6.2% (84)	15.6% (333)	0.2% (5)	0.1% (2)			2.8% (60)	
33			1.9% (40)	0.1% (1)	1.9% (26)	9.8% (209)	0.2% (5)	0.1% (1)			0.5% (10)	
34			0.8% (16)	0.1% (1)	0.4% (6)	5.4% (116)					0.1% (2)	
35			0.1% (2)		0.1% (1)	2.8% (59)						
36			0.0% (0)			1.6% (35)						
37			0.05% (1)			0.3% (6)						
38						0.1% (1)						
Total	100% (2133)	100% (2133)	100% (2133)	100% (2133)	63.6% (1356)	100% (2133)	100% (2133)	100% (2133)	100% (2133)	100% (2133)	100% (2133)	100% (2133)

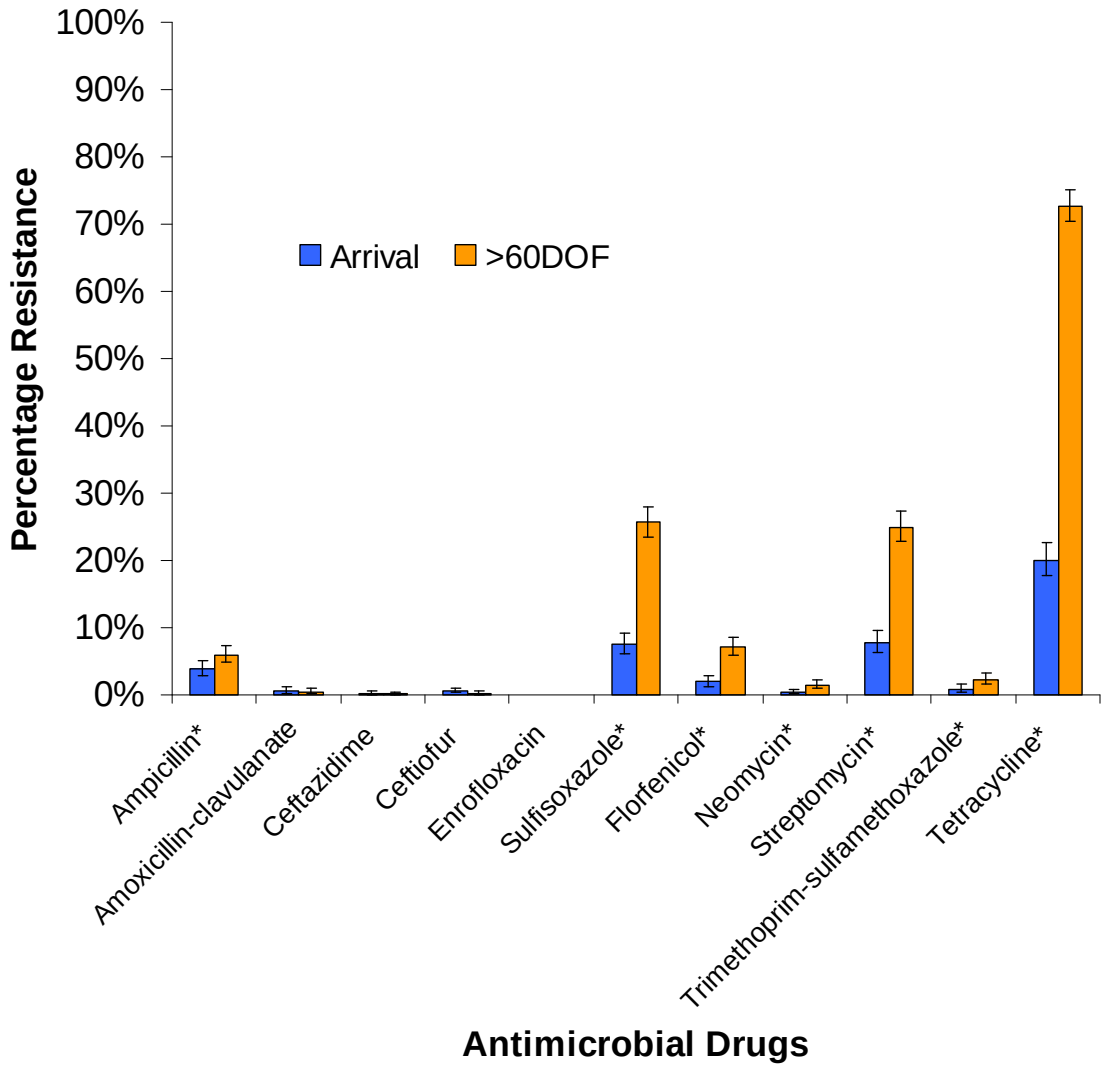

Figure 1: **Prevalence of resistance.** Adjusted for individual and pen effects, least square means estimates of the prevalence of resistance to various antimicrobial drugs among non-type specific *E. coli* isolates obtained from individual fecal samples at arrival (n=1663) and at >60 days on feed (>60DOF) (n=2133). Error bars represent 95% confidence intervals *Least square mean prevalences of resistance varied significantly ($p<0.05$) between arrival and >60DOF adjusted resistance prevalences.

Table 5: **Parenteral exposure to antimicrobials in sampled individuals (n=1048).** The total amount of antimicrobials administered for each period and for the total risk period is quantified as Animal Defined Daily Dose (ADD). DOF = Days on feed.

	Days prior to >60DOF sampling point			Total Risk Period
	0-3	**4-14**	**>14**	
	n (pct)	*n (pct)*	*n (pct)*	*n (pct)*
Class of Antimicrobial	*Sum ADD*	*Sum ADD*	*Sum ADD*	*Sum ADD*
Beta lactam	0 (0.0%)	2 (0.2%)	14 (1.3%)	16 (1.5%)
	0.0	6.0	28.0	34.0
Macrolide	0 (0.0%)	0 (0.0%)	192 (18.3%)	192 (18.3%)
	0.0	0.0	522.1	522.1
Phenicol	0 (0.0%)	1 (0.1%)	14 (1.3%)	15 (1.4%)
	0.0	3.0	42.0	45.0
Quinolone	0 (0.0%)	0 (0.0%)	4 (0.4%)	4 (0.4%)
	0.0	0.0	12.0	12.0
Sulfonamide	0 (0.0%)	0 (0.0%)	3 (0.3%)	3 (0.3%)
	0.0	0.0	9.0	9.0
Tetracycline	0 (0.0%)	0 (0.0%)	271 (25.9%)	271 (25.9%)
	0.0	0.0	778.0	778.0

Table 6: **Exposure to parenteral antimicrobials in pens.** Sampled individuals (n=1048) were housed in pens (n =241) which included a total of 48,216 animals each averaging 101.5 risk days. ADD = Animal Defined Daily Dose.

Class of Antimicrobial	**Pens Exposed (pct)**	**Average in Exposed Pens**	
		ADD per Animal	**ADD Per Animal - day**
Beta lactam	155 (64.3%)	0.0561	0.0006
Macrolide	133 (55.2%)	0.7301	0.0072
Phenicol	66 (27.4%)	0.0712	0.0007
Quinolone	77 (32.0%)	0.0429	0.0004
Sulfonamide	117 (48.5%)	0.0342	0.0003
Tetracycline	158 (65.6%)	1.1209	0.0110

Multivariable Regression Analysis—Separate logistic multivariable models for each antimicrobial drug were built using backward elimination and commercially available software[a]. The associations between exposure to antimicrobial drugs and resistance were examined through 10 logistic models and 12 linear models. In order to analytically control for the potential lack of independence associated with grouped housing, group exposures, etc., unique sets of isolates from individuals were nested within each unique group (pen) of cattle using generalized estimating equation methods. A compound symmetry (exchangeable) correlation structure accounted for the lack of independence with repeated measures from individuals and pens.

The predictor variables for the multivariable logistic were the totals of the antimicrobial drug exposure summaries. Predictor variables (12 total) for parenteral exposure to antimicrobial drugs were summarized by drug classes (beta lactams, macrolides, phenicols, quinolones, tetracyclines, and sulfonamides) for each exposure context (individual or aggregate). Exposures to in-feed antimicrobial drugs, ionophores and coccidiostats were not included as potential predictors of antimicrobial resistance.

The outcome for logistic models was the phenotypic resistance or non-resistance of each NTSEC isolate for each antimicrobial drug tested. Variables were initially screened in univariable models to determine which ones would be included in multivariable model building using a critical alpha for inclusion of 0.25. Backward selection was then used to identify final multivariable models using a critical alpha of 0.05. Final models were assessed for confounding by adding previously eliminated variables one at a time and evaluating the change in the estimates of the remaining model variables. If the estimate of a single remaining variable changed by greater than 20%, or

if estimates of multiple remaining variables changed by greater than 15%, the confounding variable was forced into the model. First order interactions of all main effects were forced into the model based on a critical alpha of 0.05. Any variables displaying characteristics of instability (extreme estimates or confidence intervals) were removed from the final model. Odds ratios, 95% confidence intervals (95%CI), and the associated *P*-values were reported for logistic regression.

RESULTS

A total of 3,796 NTSEC isolates originating from 1,855 individuals in 278 pens were included in this analysis. Of these, 1,663 (43.8%) isolates from 807 individuals in 205 pens were recovered from samples collected at arrival and 2,133 (56.2%) isolates from 1048 individuals in 241 pens were recovered from samples collected at the second time point, >60DOF.

Antimicrobial Resistance

Most arrival isolates (76.5%, 1272/1663) were pan-susceptible to all antimicrobial drugs tested (Table 2). Only 12.9% (215/1663) of arrival isolates were resistant to a single antimicrobial drug, most of which were single resistances to tetracycline (10.3%, 172/1663) (Table 3). The most common multiple resistance (≥ 2 antimicrobial drugs) in arrival isolates were combinations of sulfisoxazole, streptomycin, and tetracycline. Fewer (25.4%, 541/2133) isolates collected at >60DOF were pan-susceptible to the panel of antimicrobial drugs tested (Table 2). A greater proportion (37.9% (808/2133) of >60DOF isolates were resistant to a single antimicrobial drug, which was also most commonly resistance to tetracycline (36.8%; 785/2133) (Table 3). The top 4 resistance patterns in arrival and >60DOF isolates were the same combinations of sulfisoxazole, streptomycin, and tetracycline. Accounting for a lack of independence between isolates from the same individual and individuals from the same pen, the adjusted resistance prevalence in arrival isolates for sulfisoxazole was 7.5% (95%CI: 6.1-9.2), for streptomycin was 7.7% (95%CI: 6.3-9.5) and for tetracycline was 20.0% (95%CI: 17.7-22.6) (Figure 1). At the >60DOF sampling point, the adjusted resistance prevalence was

25.6% (95%CI: 23.5-28.0) for sulfisoxazole, 25.0% (95%CI: 22.8-27.3) for streptomycin, and 72.7% (95%CI: 70.5-75.1) for tetracycline. All other antimicrobial drugs tested had resistance prevalences <10% at both sampling points.

Antimicrobial Drug Use

In this population, individual parenteral treatment with beta lactams, phenicols, quinolones, and sulfonamides was uncommon (Tables 5 and 6). Tetracyclines and macrolides were the most commonly used classes of drug. Aggregate exposures to parenteral drugs occurred in many of the pens, but amounted to very few ADD per animal relative to the individual exposures. Almost all exposures to parenteral antimicrobial drugs occurred shortly after arrival to the feedlot (i.e. preceded >60DOF sampling by many days in period 3).

Logistic Regression

Ecological (group-level) parenteral exposures were not associated with resistance in NTSEC isolates of the tested animals (Table 7). For every additional dose (3 ADD) of tetracycline given parenterally to an individual, the odds of resistance to tetracycline increased by 3.51 (95% CI: 1.37-8.99). For every additional dose (3 ADD) of beta lactam given parenterally to an individual, the odds of resistance to streptomycin was lower (Odds Ratio: 0.17, 95%CI: 0.01-2.96). Parenteral exposure to macrolides, phenicols, quinolones, and sulfonamides were not associated with differences in the prevalence of antimicrobial resistance. Complex relationships between exposures to antimicrobial drugs and resistance were identified. Exposures to tetracyclines on the pen-

level confounded the association between individual exposures to tetracyclines and tetracycline resistance. Additionally, the individual exposure to sulfonamides confounded the protective association between individual exposure to beta lactams and streptomycin resistance.

Table 7: **Final multivariable logistic models of associations between parenteral drugs and antimicrobial resistance.** The odds ratios and 95% confidence intervals are presented in terms of 3 ADD or 1 treatment of parenteral antimicrobials assuming a 3 day treatment period. *Italics indicate confounders that were controlled in the analyses.*

Outcome Variable	Predictor Variable	Odds Ratio	95% Confidence Interval		*P*-value
Tetracycline	Individual Tetracycline	3.51	1.37	8.99	0.01
	Pen-level Tetracycline				0.11
Streptomycin	Individual Beta lactam	0.17	0.01	2.96	0.02
	Individual Sulfonamide				0.19

DISCUSSION

Results of this study indicate that parenteral exposures to antimicrobial drugs in feedlot cattle did not drive resistance at mid-feeding period. Individual and ecological exposures to these drugs were not associated with increased resistance. The one exception to this was the individual parenteral exposure to tetracyclines which was positively associated with resistance to tetracycline in *E. coli* at mid-feeding period. Tetracyclines were one of the most common antimicrobial drugs used in this population which is not necessarily the case in other feedlots. Other studies have found both transient and temporal trends with tetracycline resistance in feedlot cattle in the absence of exposure to antimicrobials (Morley et al. 2011). Additionally, confounding factors unmeasured in this study such as environmental and management pressures likely contribute to this relationship (Berge et al. 2010; McDermott et al. 2002; Barbosa and Levy 2000). Even if causality was supported, this finding may lack practical significance in terms of important resistance among pathogenic bacteria since tetracyclines continue to be efficacious in feedlot populations for prevention and treatment of bovine respiratory disease, prevention of histophilosis, and liver abscess control.

Using logistic regression to analyze antimicrobial resistance data is heavily dependent on interpretive criteria used to classify bacteria as resistant or susceptible, and thus models may change if more liberal or conservative criteria are suggested. In surveillance of antimicrobial resistance, epidemiologic cutoffs are often much lower than clinical interpretive criteria, so a regression technique capable of detecting a more refined change in resistance might be preferred (Bywater et al. 2006, Simjee et al. 2008). With this capability, detection of changing trends in resistance prevalence could be detected

earlier. Regression of a continuous outcome rather than a categorized one would theoretically provide more detailed information (Wagner et al. 2003). However, the assumptions of linear regression could not all be strictly followed in these analyses. The outcomes modeled in this study had distributions of zone diameters ranging from closely normal (enrofloxacin) to bimodal (any antimicrobial drugs with resistance), or even a lack of an identifiable distribution (tetracycline). Linear regression of the zone diameters would be inappropriate since the distributions are non-Gaussian. Additionally, a 1mm difference in zone diameter at the top of the scale (35mm vs. 36mm) and a 1 mm difference at the bottom of the scale (7mm vs. 8mm) are not equivalent. This is to say that though zone diameter measurements are continuous, they are not interval data. A log-transformation of the zone diameter did not remedy the relationship between each zone diameter. Finally, a non-parametric rank analysis provided conservative levels of significance as well as a reliable estimate indicating the direction of association. However, the parameter estimates derived from analysis of rank-transformed data are not interpretable in a biologically relevant manner as there is not a uniform difference in susceptibility associated with a 1 unit increase in rank. Linear regression may be a more precise analysis technique than logistic regression, but it did not practically make sense in this data set to estimate the magnitude of associations.

Not all isolates in this study were pan-susceptible at the arrival sampling point. Thus, the resistance status at arrival should ideally be controlled as a potential confounding variable for the association between resistance at the second time point and exposure to antimicrobial drugs. Isolates collected at the 2 sampling points in this study were not necessarily from the same individuals. Initially, a subset analysis of only

isolates with matching resistance data from the 2 sampling points was used to investigate the arrival resistance status as a covariate. However, this subset included less than 20% of the full dataset. Another possible approach to including this covariate information for the full collection of isolates at the second time point was attempted by categorizing the arrival resistance status into 3 levels (resistant at arrival, non-resistant at arrival, and resistance status unknown at arrival). Yet, little information was gained in this dataset from this approach and no variables accounting for resistance at arrival were included in the final models.

The lack of independence between isolates from the same individuals and individuals from the same pen was expected to account for some of the variability in the associations investigated in this study (Wagner et al. 2003b). Nested effects for individuals and pens in this analysis allowed for adjustment to account for this lack of independence between individuals and pens. The authors also expected the baseline resistance status of each isolate as well as the extent of the association with exposures to antimicrobial drugs to vary, so analysis with random intercepts and random slopes would be intuitive. Additional variability due to the sampled individuals and pens would have been accounted for with this methodology and models would have produced estimates theoretically closer to the true estimates. The random coefficient models were not possible with this particular data set because the models would not converge to produce estimates of association. However, similar analyses to ours in the future should investigate this possibility in other data sets in attempt to further characterize different sources of variability.

Selection criteria for isolates and feedlots may have biased the conclusions of this study. In an effort to test isolates efficiently within this surveillance program, fecal samples were only cultured for NTSEC if *M. haemolytica* could be cultured from the corresponding nasopharyngeal swab from the same individual. We do not anticipate that there is a difference in resistance prevalence in NTSEC isolates between those cultured from individuals positive for *M. haemolytica* and those negative for *M. haemolytica*. However, this assumption should be investigated to support or refute a similar isolate selection scheme for future surveillance programs. The 4 feedlots sampled in this study were purposefully selected since they had existing data collection instruments (iFHMS). Likely, these feedlots were progressive in more than just their data collection abilities which would contribute to a selection bias. However, for the purpose of developing a surveillance program in feedlot cattle, the design and implementation of this study was valid for evaluating possible sampling, laboratory, and analysis procedures.

FOOTNOTES

[a] SAS version 9.2, SAS Institute Inc, Cary, NC

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CHAPTER 5: Conclusions

The collection of the 3 projects presented as part of this dissertation contributes to a large, pilot surveillance program in Canada for the feedlot sector. Surveillance of antimicrobial resistance is evolving as we learn more about the prevalence of resistances to antimicrobial drugs and the many factors involved in the development, dissemination, and persistence of resistance. Due to the large scale nature of these programs, methodologies are necessary which are accurate, yet practical to implement. Sampling and testing strategies must be representative, but not cost-prohibitive. Investigation into the magnitude of associations between risk factors and resistance to antimicrobial drugs will help to identify mitigation targets for reducing the burden of antimicrobial resistance. The specific gaps in knowledge addressed, conclusions, and limitations of each project are summarized below.

Project 1: Metrics for quantifying antimicrobial use in beef feedlots

The primary objective of this project was to investigate the preferences of stakeholders for reporting antimicrobial drug use data that are collected from beef feedlots. Specifically, the goal of this survey was to identify a method for presenting antimicrobial use data that is easily understood by user groups and that accurately portrays drug use data in a meaningful and relevant way. Therefore, the current knowledge gaps addressed by this project were:

- **Accurate quantification of antimicrobials**
- **Optimization of methodology and standardization for surveillance programs**

Project 1 Inference:

- Choosing an appropriate metric for reporting data regarding antimicrobial use is deceptively complex.
- Evaluation of the target audience is critical to clearly presenting and effectively communicating information regarding antimicrobial use.
- Metrics that are most accurate may need to be carefully and repeatedly explained to the audience. Accuracy and clarity together are difficult to encompass in quantifying antimicrobial use data.
- Quantification of antimicrobial use with metrics which do not account for selection pressures distorts discussion regarding the impact of antimicrobial use and cannot be used to investigate antimicrobial resistance.

- Ideally for antimicrobial resistance surveillance programs, a common quantification metric or pairing of metrics would be used in all animal agribusinesses as well as within the public health sector for more closely comparable estimates
- The Animal Defined Daily Dose (ADDD) or a related metric in the form of a rate (ADDD per 1000 animals) is recommended for quantifying antimicrobial use in antimicrobial resistance surveillance programs.

Project 1 Limitations:

- Surveyed individuals were a convenience sample and the representativeness of the sampled individuals to the theoretical target population of experts on antimicrobial use and reporting in beef industry could not be validated.
- If stakeholder groups which were not well represented in this study were included, different distributions of responses might be expected.
- Other metrics could be appropriate, depending on the purpose of the research, how the data were collected, the organism being investigated for resistance to antimicrobial drugs, and the antimicrobial drugs of interest.

Project 2: Evaluation of resistance classification accuracy by latent class analysis of data from disk diffusion and broth microdilution for Escherichia coli and Mannheimia haemolytica recovered from feedlot cattle.

The objective of this project was to compare estimates of the sensitivity and specificity of disk diffusion and broth microdilution when used for identification of antimicrobial resistance in non-type-specific *E. coli* and *M. haemolytica* recovered from feedlot cattle. These parameters can be used to better estimate the true prevalence of resistance for the combinations of organisms and antimicrobial drugs investigated. Additionally, similarities or differences in accuracy between the 2 tests can aid investigators in selecting the more practical or superior susceptibility test for surveillance programs. Therefore, the current knowledge gap addressed by this project was:

- **Optimization of methodology and standardization for surveillance programs**

Project 2 Inference:

- The ability of disk diffusion and broth microdilution susceptibility tests in correctly classifying true resistance varies by the antimicrobial drug and organism of interest.
- Non-resistance detected by both methods likely represents the true non-resistance status in these populations, but more errors in the correct classification of true resistance can be expected.
- Misclassification of resistance to different antimicrobial drugs occurs at different rates in the 2 organisms tested.

- The predictive values calculated suggest that the expected level of resistance prevalence may dictate a more or less appropriate test for some antimicrobial drugs.
- Susceptibility testing by disk diffusion is recommended for surveillance programs over broth microdilution since it provides comparable accuracy and is more cost-effective.
- Latent class analysis (no gold standard) techniques are useful for investigating accuracy of susceptibility tests for surveillance programs.
- Reference-based analysis (assuming broth microdilution as the gold standard) tended to overestimate the proportion of correct resistance classification and underestimate the proportion of correct non-resistance classification for disk diffusion (results not shown).

Project 2 Limitations:

- Clinical breakpoints were used to designate resistance and non-resistance, so it is possible that the tests would perform differently at other breakpoints or epidemiologic thresholds.
- The few antimicrobial drugs evaluated in this project were selected due to their overlap between the two test panels and do not represent all drugs or drug classes.
- The difference in the prevalence of resistance between the 2 sample sets necessary for the analysis technique was small for some of the organism and drug combinations. Therefore, the estimates for these antimicrobial drugs should not be considered reliable.

- The prior probability distributions (priors) used in these models were biased due to design differences between this study and the studies which documented resistance prevalence in similar populations. However, the priors were all weakly informative and did not influence the models heavily.

Project 3: Associations between parenteral antimicrobial use and antimicrobial resistance in Escherichia coli sampled from individual feedlot cattle

The objectives of this study were to estimate antimicrobial resistance prevalence and to investigate the associations between exposures to parenteral antimicrobial drugs and antimicrobial resistance in fecal non-type specific *E. coli* recovered from individual feedlot cattle. These estimates and associations can be used to document the prevalence of antimicrobial resistance in feedlot cattle and the impact that antimicrobial drugs used has on developing antimicrobial resistance. Therefore, the current knowledge gaps addressed by this project were:

- **Prevalence of resistance in food-producing animals**
- **Studies investigating direct associations between antimicrobial use and resistance**
- **Optimization of methodology and standardization for surveillance programs**

Project 3 Inference:

- Antimicrobial resistance prevalences were relatively low in this population of feedlot cattle, with the exception of tetracycline resistance at the second time point.
- When controlling for lack of independence:
    - Individual parenteral exposures to tetracyclines were associated with an increase in resistance to tetracycline at mid-feeding period.
    - Parenteral exposure to macrolides, phenicols, quinolones, and sulfonamides were not associated with antimicrobial resistance.

    - Parenteral exposures on the pen-level were not associated with antimicrobial resistance.
- Multivariable logistic regression is an appropriate and useful approach for evaluating associations between resistance and exposures to antimicrobial drugs in surveillance.
- Protective associations and confounding were identified and indicate complex relationships between antimicrobial use and antimicrobial resistance.

Project 3 Limitations:

- Logistic regression analyses were based on clinical interpretive criteria. Resistance estimates and associations with exposure may differ based on other interpretive criteria.
- In feed antimicrobials were administered in this population, but were not summarized and included in the association models.
- Random slopes and random intercepts could not be included in this analysis due to lack of model convergence in this dataset.
- Only non-type specific *E. coli* isolates sampled from individuals that were positive for *M. haemolytica* were cultured and tested for susceptibility.
- The resistance status of individuals at arrival could not be included as a covariate since few arrival isolates could be matched to isolates from the second sampling point (i.e. individuals sampled and tested at arrival were not the same individuals sampled and tested at the second time point).
- The 4 feedlots sampled in this study were purposefully selected which may contribute to a selection bias.

APPENDIX 1: Survey Instrument

1. Survey Goal

To identify the method for presenting antimicrobial use data that:

a) is easily understood by user groups, and

b) accurately portrays drug use data in a meaningful and relevant way.

This survey is designed to be completed by the owners or operators of beef operations, veterinarians, beef industry association representatives, or public health officials familiar with antimicrobial use in the beef industry.

The survey contains 4 short sections and will take about 15-20 minutes to complete. Questions are in reference to your perception of how antimicrobial use data is presented. Please answer all questions. Contact Kathy Benedict at kbened@colostate.edu if you have any questions or concerns.

All responses will be kept strictly confidential.

2. Demographics

The first section will characterize your unique perspective within the beef industry.

1. As of June 1, 2009, how many YEARS (in whole numbers) have you actively been involved in/with the beef industry?

2. Where is your business located (current, primary location)?

State/Province:

Country:

3. Which of the following BEST characterizes the professional role in which you primarily use antimicrobial use information?

- ◯ PRODUCER
- ◯ PRODUCTION CONSULTANT
- ◯ VETERINARIAN
- ◯ FEDERAL GOVERNMENT
- ◯ STATE GOVERNMENT
- ◯ UNIVERSITY
- ◯ NUTRITIONIST
- ◯ FEED SALESPERSON
- ◯ PHARMACEUTICAL INDUSTRY
- ◯ Other (Please specify)

4. What is your highest level of education?

- ◯ HIGH SCHOOL DIPLOMA / GED
- ◯ DEGREE/DIPLOMA FROM A TECHNICAL SCHOOL OR COMMUNITY COLLEGE
- ◯ BACHELORS / BS / BA
- ◯ ADVANCED DEGREE [Please list any advanced degrees, (eg. MS, PhD, MPH, DVM, MD, AABP, ACVIM) that you have earned.]

5. By selecting one choice in each column, identify your *TOP 3* sources of information about antimicrobials. Please specify the alternate source you use if you choose the "Other" category.

	1st Most Common Source Used	2nd Most Common Source Used	3rd Most Common Source Used
FEED OR DRUG COMPANIES	◯	◯	◯
VETERINARIANS	◯	◯	◯
GOVERNMENT EXTENSION OFFICERS	◯	◯	◯
UNIVERSITIES	◯	◯	◯
FARM MAGAZINES AND NEWSLETTERS	◯	◯	◯
FRIENDS, RELATIVES, OR NEIGHBORS	◯	◯	◯
INTERNET/WORLD WIDE WEB	◯	◯	◯
PEER REVIEWED JOURNALS	◯	◯	◯
BEEF SPECIALISTS	◯	◯	◯
OTHER	◯	◯	◯

Please specify

3. Antimicrobial Use Scenarios

1. How important are antimicrobial drugs for the following feedlot uses?

	Need antimicrobial drugs	Would be difficult to manage without antimicrobial drugs	Could be managed without antimicrobial drugs	No need for antimicrobial drugs
Prophylaxis/Metaphylaxis at Arrival	◯	◯	◯	◯
Prophylaxis/Metaphylaxis after Arrival	◯	◯	◯	◯
Use in feed or water for Treatment of Disease	◯	◯	◯	◯
Injectable drugs for Treatment of Disease	◯	◯	◯	◯
Use in Feed to Prevent Liver Abscesses	◯	◯	◯	◯

Use the following table for clarification on use methods.

	DEFINITIONS
Number Treated	number of cattle treated with antimicrobial drug
ADDD (treatment head-days)	Animal Defined Daily Dose; Number of head-days that cattle were being treated with drugs based upon the assumed average maintenance dose of a drug used for its primary indication in an average sized animal (e.g., 250 kgs)
ADDD/1000 head	Standardized exposure rate based upon the Animal Defined Daily Dose relative to a fixed number of animals; used to make standardized comparisons in drug exposure among populations or over time
Drug Mass (kg)	kilograms of active ingredient
Treatment Rate	the percentage of cattle receiving a given treatment in a given population

For Questions 2-5, refer to the table on the right.

2. By selecting one choice in each column, identify the *TOP 2* methods that would be most useful when *COMPARING THE AMOUNT* of Drug X used to the amount of Drug Y used in a large cattle population.

Assumption: Each drug is a different type (e.g. tetracycline, florfenicol).

	Most Useful	2nd Most Useful
NUMBER TREATED	◯	◯
TOTAL MASS OF ACTIVE DRUG	◯	◯
ADDD	◯	◯
ADDD/1000 HEAD	◯	◯
TREATMENT RATE	◯	◯
SALES VALUE	◯	◯
DON'T KNOW	◯	◯
NO PREFERENCE	◯	◯

The following table summarizes the use of 2 antimicrobial drugs according to label instructions for respiratory disease in feedlot cattle. Th information was collected from 100 feedlots on the treatment of 2,000,000 head of cattle. Each method of presentation represents the same amount of drug used within the same column.

Assumption: Feedlot steer ~ 250 kg (550 lb).

	Drug X $10 per 10mg (10mg/Kg once for 3 days)	Drug Y $5 per 20mg (20mg/Kg once for 2 days)
Number Treated	200,000	200,000
Total Mass of Active Drug	1,500,000 Kg	2,000,000 Kg
ADDD (treatment head-days)	600,000	400,000
ADDD/1000 head	600	400
Treatment Rate	10%	10%
Sales Value	$15,000,000,000	$500,000,000

*ADDD = Animal Defined Daily Dose

3. By selecting one choice in each column, identify the *TOP 2* methods that would be most useful when describing antimicrobial use data *RELATIVE TO ANTIMICROBIAL RESISTANCE* in a professional/scientific paper.

	Most Useful	2nd Most Useful
NUMBER TREATED	◯	◯
TOTAL MASS OF ACTIVE DRUG	◯	◯
ADDD	◯	◯
ADDD/1000 HEAD	◯	◯
TREATMENT RATE	◯	◯
SALES VALUE	◯	◯
DON'T KNOW	◯	◯
NO PREFERENCE	◯	◯

The following table summarizes the use of 2 antimicrobial drugs according to label instructions for respiratory disease in feedlot cattle. Th information was collected from 100 feedlots on the treatment of 2,000,000 head of cattle. Each method of presentation represents the same amount of drug used within the same column.

Assumption: Feedlot steer ~ 250 kg (550 lb).

	Drug X $10 per 10mg (10mg/Kg once for 3 days)	Drug Y $5 per 20mg (20mg/Kg once for 2 days)
Number Treated	200,000	200,000
Total Mass of Active Drug	1,500,000 Kg	2,000,000 Kg
ADDD (treatment head-days)	600,000	400,000
ADDD/1000 head	600	400
Treatment Rate	10%	10%
Sales Value	$15,000,000,000	$500,000,000

*ADDD = Animal Defined Daily Dose

4. By selecting one choice in each column, identify the *TOP 2* methods you think would lead to *THE CLEAREST INTERPRETATION* in reporting data regarding antimicrobial use to the *GENERAL PUBLIC*.

	Most Clear	2nd Most Clear
NUMBER TREATED	◯	◯
TOTAL MASS OF ACTIVE DRUG	◯	◯
ADDD	◯	◯
ADDD/1000 HEAD	◯	◯
TREATMENT RATE	◯	◯
SALES VALUE	◯	◯
DON'T KNOW	◯	◯
NO PREFERENCE	◯	◯

The following table summarizes the use of 2 antimicrobial drugs according to label instructions for respiratory disease in feedlot cattle. Th information was collected from 100 feedlots on the treatment of 2,000,000 head of cattle. Each method of presentation represents the same amount of drug used within the same column.

Assumption: Feedlot steer ~ 250 kg (550 lb).

	Drug X $10 per 10mg (10mg/Kg once for 3 days)	Drug Y $5 per 20mg (20mg/Kg once for 2 days)
Number Treated	200,000	200,000
Total Mass of Active Drug	1,500,000 Kg	2,000,000 Kg
ADDD (treatment head-days)	600,000	400,000
ADDD/1000 head	600	400
Treatment Rate	10%	10%
Sales Value	$15,000,000,000	$500,000,000

*ADDD = Animal Defined Daily Dose

5. Which method do you think is *LEAST APPROPRIATE* in reporting data regarding antimicrobial use to the *GENERAL PUBLIC*?

- ◯ NUMBER TREATED
- ◯ TOTAL MASS OF ACTIVE DRUG
- ◯ ADDD
- ◯ ADDD/1000 HEAD
- ◯ TREATMENT RATE
- ◯ SALES VALUE
- ◯ DON'T KNOW
- ◯ NO PREFERENCE

The following table summarizes the use of 2 antimicrobial drugs according to label instructions for respiratory disease in feedlot cattle. Th information was collected from 100 feedlots on the treatment of 2,000,000 head of cattle. Each method of presentation represents the same amount of drug used within the same column.

Assumption: Feedlot steer ~ 250 kg (550 lb).

	Drug X $10 per 10mg (10mg/Kg once for 3 days)	Drug Y $5 per 20mg (20mg/Kg once for 2 days)
Number Treated	200,000	200,000
Total Mass of Active Drug	1,500,000 Kg	2,000,000 Kg
ADDD (treatment head-days)	600,000	400,000
ADDD/1000 head	600	400
Treatment Rate	10%	10%
Sales Value	$15,000,000,000	$500,000,000

*ADDD = Animal Defined Daily Dose

6. In your own words, please interpret "400 ADDD of tetracycline." You may respond, "Don't Know."

7. In the use of the ADDD method, should data be recorded separately for *HIGH and LOW* dose uses of the same drug?

For example, cattle in the United States can be treated with chlorotetracycline in feed at:

- **a *HIGH* dose for treatment of respiratory disease (350mg per head per day), or**
- **a *LOW* dose for control of liver abscesses, to increase weight gain, and to improve feed efficiency (70mg per head per day).**

◯ CALCULATE ADDDs SEPARATELY FOR HIGH DOSE AND LOW DOSE EXPOSURES AND REPORT SEPARATELY

◯ CALCULATE ADDDs SEPARATELY FOR HIGH DOSE AND LOW DOSE EXPOSURES, SUM, AND REPORT ONE SUMMARY NUMBER

◯ CALCULATE ADDDs USING A COMMON DOSE (e.g. 350mg/head/day) REGARDLESS OF EXPOSURE, AND REPORT TOGETHER

◯ DON'T KNOW

◯ NO PREFERENCE

4. Antimicrobial Use Perceptions

You have now finished with the first 2 sections and have 2 short sections remaining. The next section will investigate your general perceptior of antimicrobial use data. Please use your professional perspective only for the following questions.

Use the following table for clarification on use methods.

	DEFINITIONS
Number Treated	number of cattle treated with antimicrobial drug
ADDD (treatment head-days)	Animal Defined Daily Dose; Number of head-days that cattle were being treated with drugs based upon the assumed average maintenance dose of a drug used for its primary indication in an average sized animal (e.g., 250 kgs)
ADDD/1000 head	Standardized exposure rate based upon the Animal Defined Daily Dose relative to a fixed number of animals; used to make standardized comparisons in drug exposure among populations or over time
Drug Mass (kg)	kilograms of active ingredient
Treatment Rate	the percentage of cattle receiving a given treatment in a given population

1. For ongoing prospective surveillance programs, select the best method of summarizing antimicrobial use in each row for each of these organizational levels/scales.

	Number Treated	Total Mass of Active Drug	ADDD	ADDD/1000	Treatment Rate	Sales Value	Don't Know	No Preference
Individual Operation	◯	◯	◯	◯	◯	◯	◯	◯
State/Province	◯	◯	◯	◯	◯	◯	◯	◯
National	◯	◯	◯	◯	◯	◯	◯	◯
Global	◯	◯	◯	◯	◯	◯	◯	◯

Please provide additional comments explaining your choices below.

2. In reference to question 1, is a different definition or measurement more appropriate for surveillance programs than the ones given above?

- ◯ DON'T KNOW
- ◯ NO
- ◯ YES (Please specify)

3. In general, antimicrobial drugs are used in feedlot cattle for prophylaxis, metaphylaxis, treatment of clinical disease, or to enhance production efficiency. Dosages used for these different circumstances can differ, and yet we could summarize all these uses together.

If we combine these four categories listed above when summarizing antimicrobial use data, would you consider the summary an accurate portrayal?

- ◯ YES
- ◯ NO
- ◯ DON'T KNOW
- ◯ IT DEPENDS (Please specify)

4. Should antimicrobial use statistics combine information across different types (classes) of antimicrobial drugs (e.g. tetracycline, florfenicol)?

- ◯ YES
- ◯ NO
- ◯ DON'T KNOW
- ◯ IT DEPENDS (Please specify)

5. Please specify whether the following methods of summarizing antimicrobial drug use are *EASILY UNDERSTOOD* for large cattle populations.

	Very Clear	Clear	Somewhat Clear	Not Clear	Don't Know
Number Treated	◯	◯	◯	◯	◯
ADDD/1000 head	◯	◯	◯	◯	◯

	DEFINITIONS
Number Treated	number of cattle treated with antimicrobial drug
ADDD/1000 head	Standardized exposure rate based upon the Animal Defined Daily Dose relative to a fixed number of animals; used to make standardized comparisons in drug exposure among populations or over time

6. Please specify whether the following methods *ACCURATELY* summarize antimicrobial drug use for large cattle populations.

	Very Accurate	Accurate	Somewhat Accurate	Not Accurate	Don't Know
Number Treated	◯	◯	◯	◯	◯
ADDD/1000 head	◯	◯	◯	◯	◯

	DEFINITIONS
Number Treated	number of cattle treated with antimicrobial drug
ADDD/1000 head	Standardized exposure rate based upon the Animal Defined Daily Dose relative to a fixed number of animals; used to make standardized comparisons in drug exposure among populations or over time

5. Antimicrobial Resistance

The final section contains questions on your perception of antimicrobial resistance.

1. Compared to 10 years ago, how different is your level of *CONCERN* about antimicrobial resistance as a health issue *TO PEOPLE* at each of these organizational scales?

	Much Greater	Somewhat Greater	No Difference	Somewhat Less	Much Less	Don't Know
LOCALLY (INDIVIDUAL OPERATIONS)	◯	◯	◯	◯	◯	◯
REGIONALLY	◯	◯	◯	◯	◯	◯
NATIONALLY	◯	◯	◯	◯	◯	◯
GLOBALLY	◯	◯	◯	◯	◯	◯

2. Compared to 10 years ago, how different is your level of *CONCERN* about antimicrobial resistance as a health issue *TO ANIMALS* at each of these organizational scales?

	Much Greater	Somewhat Greater	No Difference	Somewhat Less	Much Less	Don't Know
LOCALLY (INDIVIDUAL OPERATIONS)	◯	◯	◯	◯	◯	◯
REGIONALLY	◯	◯	◯	◯	◯	◯
NATIONALLY	◯	◯	◯	◯	◯	◯
GLOBALLY	◯	◯	◯	◯	◯	◯

3. Compared to the past 10 years, do you believe there is a difference in the *TRUE RISK* of health problems *IN PEOPLE* because of antimicrobial resistance at each of these organizational levels?

	Much Greater	Somewhat Greater	No Difference	Somewhat Less	Much Less	Don't Know
LOCALLY (INDIVIDUAL OPERATIONS)	◯	◯	◯	◯	◯	◯
REGIONALLY	◯	◯	◯	◯	◯	◯
NATIONALLY	◯	◯	◯	◯	◯	◯
GLOBALLY	◯	◯	◯	◯	◯	◯

4. Compared to the past 10 years, do you believe there is a difference in the *TRUE RISK* of health problems *IN ANIMALS* because of antimicrobial resistance at each of these levels?

	Much Greater	Somewhat Greater	No Difference	Somewhat Less	Much Less	Don't Know
LOCALLY (INDIVIDUAL OPERATIONS)	◯	◯	◯	◯	◯	◯
REGIONALLY	◯	◯	◯	◯	◯	◯
NATIONALLY	◯	◯	◯	◯	◯	◯
GLOBALLY	◯	◯	◯	◯	◯	◯

6. Conclusion

1. Please provide any other comments you have regarding the presentation of antimicrobial use data for this survey.

This concludes the survey. Thanks very much for your participation. We plan to use the data to characterize the preferred method(s) of presenting antimicrobial use data within the beef industry.

Contact Kathy Benedict at kbened@colostate.edu if you have any questions or concerns

APPENDIX 2: Survey Instrument – Decline

1.

1. Please select the choice below if you DO NOT wish to participate in this survey.

◯ DECLINE TO PARTICIPATE

APPENDIX 3: Nasopharyngeal Swab Sampling Protocol

TITLE: Nasopharyngeal Swab Sampling	**SOP NO.: 001**
PROJECT MANAGER: Chelsea Flaig	**DVM: Dr. Calvin Booker**
EFFECTIVE DATE: April 19, 2007	**STUDY: FHMS-445**

BACKGROUND

The following procedure applies when using the Jorgenson J273 swab for nasopharyngeal sampling.

COLLECTION EQUIPMENT AND SUPPLIES

- **i.** Study label binder with pre printed labels
- **ii.** Nasopharyngeal swabs.
- **iii.** Transport media tubes.
- **iv.** Blood transport carrying boxes
- **v.** Gloves – all sizes.
- **vi.** Eye protection for the collector

PROCEDURES

1. Store all unused swabs and transport media tubes at room temperature.
2. Each swab package consists of one single white nasopharyngeal swab, one clear inner swab sheath, one clear thick outer guard sheath, and two spare white end caps (which will not be used in this procedure).
3. Restrain the animal in the chute and secure the head tightly with the head gate. If the nasal cavity becomes bloody it is advised that you take the second sample through the opposite nostril.
4. Sampling can be performed using a single individual to both restrain the animal's head and collect the sample. Additional individuals may be utilized if needed.
5. Gauge the approximate length of insertion of the swab by holding the swab against the animal's head from the tip of the nostril to where the white capped end reaches the back of the jaw, below the ear. This distance will be approximately 10 to 14 inches, depending on the size of the animal. Remove the swab apparatus from the plastic package and grasp it with the free hand as close to the distance on the swab that was just measured.

6. Flatten the twist tie allowing it to move freely into the guard sheath. Firmly push the inner swab sheath through the white cap on the guard sheath until it breaks the seal on the end of the cap. Pull the swab sheath back into the guard sheath before insertion.

7. The individual should then face in the same direction as the animal. Using the arm and hand closest to the animal, secure the head by bending the head and neck toward the sample collector. This hand will also be used to guide the swab into the nostril upon insertion. Using your free hand, collect the sample as described below.

8. With the free hand, insert the swab end into the nasal cavity of the nostril to be sampled. Direct the swab ventrally (down) and medially (center of head), maintaining this direction with the hand holding the nostril. The swab should be inserted to the level of the pharynx (approximately 10 to 14 inches as measured above). The swab will move freely and smoothly with only moderate pressure.

9. Caution must be used not to accidentally insert the swab into the false nostril of the animal and obtain the sample from there. The false nostril, which lies medial (central) and dorsal (up) on either side of the septum, is a shallow compartment (6 to 8 inches deep) that could easily be mistaken for the correct collection site. Directing the swab down (rather than up) as it is inserted into the nostril will help to avoid insertion into the false nostril. If the swab only enters a very short distance and will not easily push in further, you are likely in the false nostril. Withdraw the swab and guide it downwards.

10. When the swab reaches the level of the pharynx, you will feel it bump up against the back of the pharynx. Do not force the swab any further. At this point, you should be close to the distance previously measured out on the sheath. Pull the whole swab back approximately one inch. Hold the swab at that level with the hand that is guiding the swab at the nostril. With the other hand, push the swab and inner sheath out of the end of the guard sheath cap. Push the white swab further and rotate the swab with your fingers to collect the nasopharyngeal sample.

11. Pull the swab back into the inner sheath. Pull the inner sheath back into the guard sheath. Remove the whole apparatus from the nasal cavity. Make sure that the swab is held in such a way that it will not slide out of the end of the guard sheath.

12. Remove the inner sheath and the swab from the guard sheath, ensuring the swab remains inside the inner sheath.

13. Insert the swab directly into the transport media tube by pushing it through the inner sheath. Cut off the swab (with the bandage scissors) at a level that will contain the whole length of the swab within the transport media tube.

14. Label the transport media tube with the appropriate label.

15. Place the tube in the transport box and then into the cooler provided.

16. Contact FHMS that the samples are ready for pick up.

APPENDIX 4: Composite Fecal Sample Collection Protocol

TITLE: Pen Floor Composite Fecal Sample Collection	SOP NO.: 002
PROJECT MANAGER: Chelsea Flaig	DVM: Dr. Calvin Booker
EFFECTIVE DATE: September 7, 2007	STUDY: FHMS-445

BACKGROUND

This procedure describes the pen floor fecal sample collection for surveillance of large groups of animals.

Caution should be taken with regards to the reactions and movements of the cattle to ensure worker safety when working in the pens.

You will be notified by the Research Project Manager (RPM) when to sample from the pens. Two people should attend for safety and assistance purposes.

EQUIPMENT

Note: No ice is required in the coolers for collection, only for shipping purposes.

1. Fecal cups (for sampling into)
2. Spoons
3. Apron for each collector
4. Labels - pre made
5. Fecal transport vials
6. Cooler of appropriate size
7. Appropriate sized gloves for the collectors
8. Paper towels
9. Garbage bags - white kitchen
10. An assortment of pens and markers
11. Date stamps

PROCEDURE

1. One composite sample of fecal material will be collected from each pen. Each composite will contain 20 different fecal patties. Try to collect from fresh patties.

2. Load up the apron with all equipment needed, plus some extra in case of mishap. Also take a white kitchen trash bag for disposal of dirty gloves and spoons (You can tie this to your apron).

3. Walk through the pen collecting samples as you go. Twenty different samples need to be collected per cup, by scooping one level spoonful from each sample patty from the pen floor with the spoon. If a fecal patty is somewhat old, scrape the top crust off to get to a fresher sample. Avoid collection of any bedding material. A new spoon must be used for each pen.

4. The sampling pattern for each pen is as follows: start at the gate and head towards the back of the pen collecting samples as you go; then turn and walk along the back of the pen, still collecting samples. Once at the far corner, turn and walk diagonally back to the gate, collecting the remainder of the samples.

5. Once a cup is filled, stir the mixture for 1 minute. Transfer approximately 10 grams into a fecal transport tube and then label the tube. Store the tube in the cooler. Proceed to the next pen.

APPENDIX 5: Rectal Fecal Sample Collection Protocol

TITLE: Allocation Rectal Fecal Sample Collection	SOP NO.: 003
PROJECT MANAGER: Chelsea Flaig	DVM: Dr. Calvin Booker
EFFECTIVE DATE: April 19, 2007	STUDY: FHMS-445

BACKGROUND

This procedure describes the duties of the sample collector for allocation fecal rectal sample collection.

1. **COLLECTION SUPPLIES AND EQUIPMENT:**

   a. Study Label Binder - pre printed labels provided by FHMS

   b. A good selection of blue ink pens, markers, and date stamp

   c. Fecal sample containers

   d. OB Sleeves

   e. Plastic disposable gloves - all sizes

   f. Cooler(s)

   g. Frozen ice packs

   h. Paper towels

   i. Garbage bags

PROCEDURES

1. **COLLECTOR**

   a. Animals will be run into the chute one at a time.

   b. Ear tags will be identified.

   c. Collector to put on a clean OB sleeve on the hand used for collection for every animal.

   d. No sleeve lube can be used.

   e. A glove should be worn on the non-collection hand. This hand can be used to lift the tail, or touch inanimate objects. Change it when it becomes excessively

contaminated. Do not touch the fecal cup with this hand; otherwise there is a chance of cross contamination between samples.

f. **Minimum** quantity per fecal sample is 10 grams.

g. Put the fecal sample into the fecal cup. Do not touch the inside of the fecal cup at any time, other than to deposit or wipe the sample into it and only then with the hand that was used to collect the sample.

h. Hand the fecal cup to the Data Recorder who can wipe off the fecal cup, place the lid on it, and attach the label.

i. When sample collection is complete for the day, place in the fridge (if possible) until picked up or delivered to FHMS.

j. Notify FHMS that the samples are ready to be picked up.

APPENDIX 6: Sample Labeling and Transport Protocol

004 FHMS-445 Sample Submission

* Samples are to be shipped every Sunday evening. The samples will go to the lab in Lethbridge to be processed.

* Samples need to be kept cool at all times. The microbes are temperature sensitive and overgrowth will occur if the samples get warm. Please leave the samples in the fridge, or in a cooler with ice packs, until you are ready to record and ship them.

1) Get the samples out of the walk-in fridge in the Field Services Building. They will either be in a cooler or a box. The cooler/box should say FHMS-445 on the top. If not, look inside, there should be fecal cups and nasal tubes.
2) There are two types of individual animal case #s, arrival and exit.
    a. Arrival: F05**0**04066; the 0 behind the 2 digit feedlot ID indicates an arrival sample.
    b. Exit: F05**1**00106; the 1 behind the 2 digit feedlot ID indicates an exit sample.
3) Record the fecal sample case #s. eg) F05004066. Place them into a Ziploc bag. Keep Arrival and Exit samples separate. Write the type of sample on the bags with a marker (Arrival, Exit).
4) Match the nasal sample case #s to the fecal samples. eg) N05004066. Place them into a Ziploc bag (separate from the fecals). Keep the Arrival and Exit samples separate. Write the type of sample on the bags with a marker (Arrival, Exit).
    a. For each fecal sample there should be a nasal sample, if there is one but not the other please make a note of this on the Case #s and Sample Submission forms.
5) If there are composite samples eg) C050157, record and bag them separate from the other fecal samples. Some feedlots bag the composite samples separately from the other samples when they are collected. Write composite and the type of sample on the bags (Arrival, >60 DOF, Exit).
    a. There are 3 types of composite samples:
        i. Arrival: C05**0**157; the 0 behind the 2 digit feedlot ID indicates an arrival sample. The label should might also say Arrival.
        ii. >60 DOF: C05**1**025; the 1 behind the 2 digit feedlot ID indicates a >60 DOF sample. The label should also say >60 DOF.
        iii. Exit: C05**2**007; the 2 behind the 2 digit feedlot ID indicates an exit sample. The label should also say Exit.
    b. Record all information from the composite label on a separate piece of paper. This does not go in the spreadsheet of case #s but is needed for sample tracking. Information that will be on the label: Case # and Date. Info that may be on the label: Pen #, Lot #, Sort Group, type of sample, etc. Please record all information available and leave it on RPM's desk.
6) Place ice packs above and below the samples in a clean cooler (not the one from the feedlot). The coolers are in a cardboard box for shipping. Use the appropriate sized box depending on the amount of samples.

7) Open the excel spreadsheet called Case Numbers for Samples Submitted 'Date of shipment'; if there is no spreadsheet please make a new one.
    a. Record all case #s in the spreadsheet. Save the sheet and print 2 copies – 1 will go with the shipment, the other will be filed (place on RPM's desk).
    b. Place 1 copy on top of the cooler in the box.
8) Open the Sample Submission Form for the date of the shipment. If there isn't one please make a new one.
    a. Update the shipment date, shipped by, # of samples, and how many of each type of sample (Arrival, Exit). Save and print 2 copies - 1 will go with the shipment, the other will be filed (place on RPM's desk).
    b. Place 1 copy on top of the cooler in the box. Tape the box closed.
9) Fill out the pre-printed waybill. Add the date, No. of Pieces, Wt. The service is Station to Station, Declared value of 0, packaging is a Box. Sign the waybill.
    a. Affix the waybill to the top of the box.
    b. Leave the top copy of the waybill on RPM's desk along with the copies of the case #s and sample submission form.
10) The labels for the box are in the same folder. Ensure that there are labels on the box (From, To, Southbound).
11) Take the box to Greyhound in High River. The Greyhound is in the Grocery Kart store (601 1st St W) by the Beef and Brew Restaurant. Do not take the box to the Greyhound in Okotoks; the restaurant is not usually open when samples need to be shipped and the bus does not always stop to pick up packages.
    a. The bus leaves for Lethbridge at 9:30 pm. The samples need to be there at 9:00 pm at the absolute latest.
    b. At Greyhound, place the box on the scale; tell the attendant the package is going to Lethbridge and that it is charged to the account on the waybill.

APPENDIX 7: *E. coli* Isolation Protocol

Isolation of *Escherichia coli* from bovine feces and manure

sample processing, isolation, identification & storage procedures

OBJECTIVE:

This protocol provides a method for isolating viable lactose-fermenting *Escherichia coli* from bovine fecal and manure samples.

MATERIALS REQUIRED:

- ❑ Sterile, individually wrapped cotton-tip swabs
- ❑ MacConkey agar plates
- ❑ Sterile disposable spreaders
- ❑ 37°C incubator
- ❑ Sterile, disposable 1μL inoculating loops
- ❑ LB agar plates
- ❑ Commercial Kovac's reagent (PML Microbiologicals)
- ❑ Filter paper (9 cm diameter)
- ❑ Sterile Petri dish lid
- ❑ LB broth
- ❑ Sterile 2.0mL screw-cap microtubes containing 1mL of BHI + 20% glycerol

EXECUTION:

Sample Processing

Fecal and/or manure samples (contained in Enteric Transport Vials with Cary-Blair medium) will be delivered to the Lethbridge Research Centre via an overnight delivery service (i.e. Fed-Ex or Greyhound).

Upon receipt, manure samples will be processed immediately. Fecal samples will be stored in a 4°C cooler until processing of the nasal swabs is completed. Only fecal samples from animals which tested positive by PCR for *Mannheimia haemolytica* will be processed.

1. Using the attached 'spoon-lid', mix the feces to create a uniform slurry with the Cary-Blair transport medium (if filled to the fill-line, represents approximately a ¼ dilution).
2. Completely immerse a sterile cotton swab into the sample, and streak then this onto a MacConkey Agar plate (as represented by step 1 on Fig. 1)
3. Using a clean, sterile swab further streak across the surface of the plate for single colonies (Steps 2-3 of Fig. 1).
4. Incubate the plates overnight at 37°C.

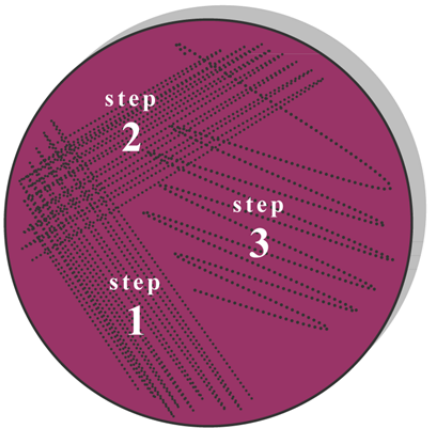

Fig. 1. Schematic of swab-streaking method for fecal slurries plated onto MacConkey agar.

Colony Selection & Isolation

After 24 hours of incubation, remove the MAC plates from the incubator and examine for lactose-fermenting (pink) colonies with morphology typical of *E. coli*.

Using proper aseptic technique, streak single colonies onto ½ of a fresh LB plate and incubate at 37°C, overnight. Streak a maximum of **three isolates per fecal (animal) sample** and **five isolates per composite (manure) sample**

Presumptive Identification

Using a single colony from LB agar, perform the indole test on each isolate by streaking the colony onto a filter paper flooded with 1-1.5mL of commercial Kovac's reagent. A positive reaction is indicated by an immediate color change to pink-red. Discard any isolates that are indole negative.

A positive and negative control should be prepared for the test

Storage

Isolates that have been determined as **presumed**-*E. coli* (lactose fermenting, indole positive) will be stored in 20% glycerol stocks at -80°C until sufficient time for further characterization.

Using a sterile 1 µL inoculating loop, suspend 3-6 (depending on the size) single colonies of the isolate from the LB plate into a single 2.0mL screw-cap tube with 1.0 mL of Brain Heart Infusion broth + 20% glycerol. Gently mix the suspension by flicking the tube. Freeze overnight at -20°C and transfer to -80°C the following day.

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APPENDIX 8: *M. haemolytica* Isolation Protocol

Isolation of *Mannheimia haemolytica* from bovine nasal swabs

sample processing, isolation, identification & storage procedures

OBJECTIVE:

This protocol provides a method for isolating and identifying viable *Mannheimia haemolytica* from bovine upper-respiratory (nasal) samples.

MATERIALS REQUIRED:

- ❑ 2 mL microcentrifuge snap-cap tubes containing 0.7 mL of sterile Brain Heart Infusion (BHI) Broth
- ❑ Metal scissors
- ❑ Blood Agar Plates modified with 15 µg/mL bacitracin (BAC)
- ❑ 37°C incubator
- ❑ *M. haemolytica* ATCC 33396 - reference strain – fresh culture
- ❑ *M. glucosida* – reference strain – fresh culture
- ❑ Sterile disposable inoculating loops
- ❑ Commercial oxidase test strips (Oxoid)
- ❑ 3% hydrogen peroxide
- ❑ PCR primer mix – Lkt1, Lkt2, 16S, HP
- ❑ Qiagen Multiplex MasterMix (Qiagen)
- ❑ Thermocycler
- ❑ Sterile screw-cap 2.0 mL tubes containing 1.0 mL of Brain Heart Infusion broth with 20% glycerol
- ❑ -80°C freezer with inventory boxes
- ❑ Biochemical tablets (Diatabs®) for phenotypic characterization (Rosco Diagnostics)
- ❑ Suitable wells or tubes for conducting the tablet phenotypic characterizations

EXECUTION:

Sample Processing

Nasal swabs contained in Cary-Blair transport media will be delivered to the Lethbridge Research Centre in a cooled container via an overnight delivery service (i.e. Fed-Ex or Greyhound).

Upon receipt, nasal swabs will be immediately processed in a Risk Level II containment laboratory.

1. Label 2.0 mL snap-cap microcentrifuge tubes containing 0.7 mL of Brain-Heart Infusion (BHI) broth with sample designations corresponding to the nasal swabs.
2. Remove the swab from the transport vial with sterile forceps, and aseptically cut the tips of the nasal swabs directly into the corresponding tube, using scissors immersed in 100% ethanol and flamed between each use
3. Vortex each tube at high speed for 30 seconds. Allow any aerosols to settle, by leaving the suspensions capped and undisturbed for at least 10 minutes. Spread plate 100 µL of the suspension directly onto a single Blood Agar Plate containing 15 µg/mL of the selective agent, bacitracin (BAC plates).
4. Incubate at 37°C overnight.

Also, incubate BAC plates inoculated with *M. haemolytica* ATCC strain 33396 and *M. glucosida* ATCC strain 38457 as positive controls.

Colony Selection & Isolation

After 24 hours of incubation, remove the BAC plates from the incubator and examine for colonies with morphology typical of *Mannheimia haemolytica*. Use the BAC plate with *M. haemolytica* 33396 as a reference. Suspect colonies are round, medium sized, 'wet' (but not mucoid), white-grey in color and should have some degree of haemolysis evident directly under the colony.

Using proper aseptic technique, streak single colonies onto ½ of a fresh BAC plate and incubate at 37°C, overnight. Where possible, **three** MH-typical colonies should be isolated. In instances where more than one suspect colony morphology is present, multiple isolates may be selected from a single plate, to a maximum of **five isolates per sample**.

Presumptive Identification

Check the isolated colonies to confirm purity and verify that the morphology is similar to what is expected for *Mannheimia haemolytica* (again, using a reference plate to compare with).

"Typical" colonies should be selected and tested:

1. **Oxidase Test**
   Using a disposable loop, smear a loopful of a single colony directly onto a commercial Oxidase test strip (Oxoid). Observe the strip for a positive reaction (dark blue/purple color) **beginning within 5 seconds**. A "weak"

color-change or a change after **30** seconds should be considered negative. Eliminate any negative isolates from further testing.

> Use caution to only pick up the colony and none of the blood-agar media, as red blood cells contain cytochrome oxidase which may give rise to false-positive results.

2. **Catalase Test**
   Smear a single colony of the isolate directly onto a sterile glass slide using a disposable loop. Apply a single droplet of 3% hydrogen peroxide directly onto the smear and observe for an immediate reaction producing bubbles. Eliminate any negative isolates from further characterization.

3. **PCR**
   Prepare isolates that are catalase and oxidase positive for PCR by lysing a 1 µL loopful of culture suspended in 40 µL of TE buffer (pH 7.0) by heating to 98°C for 5 minutes in a shaking (≥500 RPM) thermomixer.

   Immediately prior to performing the PCR reaction pellet the cell debris by centrifugation at maximum speed for 5 minutes. Use the supernatant as DNA template (2 µL/25 µL PCR reaction)

   Prepare a working PCR mastermix with Qiagen Multiplex Mastermix, as per the manufacturer's instructions (adjust concentrations for 25 µL reactions).

   **Note: the multiplex primers should be pooled together prior to creating the mastermix, as per the manufacturer's recommended concentrations. The required primers are listed in Table 1 below*

   Aliquot 23 µL of the mastermix into individual tubes or wells, and add 2 µL of the DNA template. Run the PCR reaction in a thermocycler with a heated lid with the following settings:

15 min.	95°C	
30 sec	94°C	40 ×
30 sec	60°C	
30 sec	72°C	
10 min.	72°C	
Forever	4°C	

   Load 20 µL of the PCR products along with a suitable marker into a 1.8% agarose gel containing 0.5 µg/mL of ethidium bromide. Run the gel at 90-110V for ¾-1¼ hours. Visualize and photograph the gel with a UV transilluminator. All samples should be positive for the 16S product

(304bp). Samples which are positive for 16S (304 bp), Lkt (206 bp) **and** HP (90 bp), but **not** Lkt-2 should be considered *M. haemolytica*.

A positive (*M. haemolytica* and *M. glucosida*) and negative control should be prepared for each of the tests.

Table 1. Multiplex primers used for the identification of *M. haemolytica*

Primer Set	Sequence (5′ to 3′)	Amplicon
Lkt	(F) GCAGGAGGTGATTATTAAAGTGG	206 bp
	(R) CAGCAGTTATTGTCATACCTGAAC	
Lkt-2	(F) CTCTCTTTAGAAAAGCTGGAAAAC	170 bp
	(R) TTTTGCCAAGTGGTGTATTGC	
HP	(F) CGAGCAAGCACAATTACATTATGG	90 bp
	(R) CACCGTCAAATTCCTGTGGATAAC	
16S	(F) GCTAACTCCGTGCCAGCAG	304 bp
	(R) CGTGGACTACCAGGGTATCTAATC	

Storage

Isolates that have been identified as presumed-*Mannheimia haemolytica* will be stored in 20% glycerol stocks at -80°C until sufficient time for further phenotypic characterization.

Suspend a 1µL-loopful of culture from the previously subcultured colonies into 1 mL of BHI broth with 20% glycerol in a 2.0 mL screw-cap microcentrifuge tube. Gently mix the suspension by flicking the tube. Freeze overnight at -20°C and transfer to -80°C the following day.

Definitive Identification

A subset of the archived isolates should be verified as *M. haemolytica* by traditional phenotypic characterization. Revive the cultures by streaking a loopful of thawed glycerol stock directly onto a BAC plate.

Phenotypic tests should be preformed using Rosco diagnostic tablets (Diatabs ®), as per the manufacturers instructions and compared against the phenotypic profile described by Angen et al., 2002. Phenotypic tests to be preformed include: alpha-fucosidase, beta-galactosidase, beta-glucosidase, beta-xylosidase, D-xylose, esculin hydrolysis, indole, L-arabinose, maltose, mannitol, ornithine decarboxylase, sorbitol, trehlaose, and urease.

REFERENCES:

Angen, O., Ahrens, P., Bisgaard, M. 2002. *Mannheimia* (*Pasteurella*) *haemolytica*-like strains isolated from diseased animals in Denmark. Vet Microbiol. **84**: 104-113

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