Antibiotic resistance in the lower intestinal microbiota of dairy cattle: longitudinal analysis of phenotypic and genotypic resistance

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Dedication

This dissertation is dedicated to Karen, my mother, father, sisters, and the rest of my wonderful and supportive family, in particular my grandfather who's last words to me were to work hard in school.

Abstract

This research focused on methods of measuring antibiotic resistance and analysis of antibiotic resistance data in dairy cattle that were sampled repeatedly over time. Specific objectives included: characterization and longitudinal analysis of phenotypic antibiotic resistance of commensal *Escherichia coli*, development of a statistical model for the analysis of low quantity resistance genes measured by quantitative real-time polymerase chain reaction (qPCR), measurement of antibiotic resistance genes in the lower intestinal bacterial communities of dairy cattle that received a short-term therapeutic dose of antibiotic and untreated cattle, and measurement and longitudinal analysis of the quantities of six antibiotic resistance genes in the lower intestinal bacterial communities of dairy cattle.

Enteric *E. coli* collected from dairy cattle over 1.5 years were tested for phenotypic resistance to 17 antimicrobials. A total of 93 phenotypic patterns were observed among 3,402 isolates tested, with a majority (67%) susceptible to all 17 antimicrobials. The most prevalent resistances were to tetracycline, sulfamethoxazole, and streptomycin. Latent class and latent transition analyses were carried out to group the animals into classes according to their resistance phenotypes and to estimate the probabilities of transitioning into and out of classes over time. Probabilities of transitioning to a pan-susceptible class were high, as were the probabilities of remaining in the pan-susceptible class. Probabilities of transitioning form a pan-susceptible class to a resistant class were very low.

Measurement of antibiotic resistance genes by qPCR presents challenges for genes that are present in very low quantities. A statistical model was developed to analyze qPCR data made up of a significant proportion of observations that fall below the limit of quantification of

a qPCR assay. Computer simulations showed that the statistical model produced less biased estimates of regression parameters than common methods of handling low quantity qPCR data.

qPCR was applied to a cohort of dairy cattle that received a five day course of ceftiofur and matched untreated cattle. Quantities of a gene (bla_{CMY-2}) that confers resistance to ceftiofur were measured and analyzed using the statistical model developed for low quantity genes. Treated animals had significantly higher quantities of bla_{CMY-2} during treatment than untreated animals. By the first day post-treatment, gene quantities had returned to pre-treatment levels.

The quantities of six different antibiotic resistance genes were measured by qPCR in the fecal community bacterial DNA of a cattle population that was sampled repeatedly over 2.5 years. Significantly increasing trends over time were observed for three of the six genes conferring resistance to tetracyclines, macrolides, and cephalosporins. Comparison of phenotypic resistance and qPCR data showed that qPCR performed on community DNA is a more sensitive method of detection that phenotypic testing of cultured isolates.

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Summary

Antibiotic resistance is a well-recognized threat to public health. A long standing question relating to antibiotic resistance that has been a challenge to answer is: to what degree does antibiotic use in food producing animals affect the risk of infection with resistant pathogens in humans? A key to answering this question is an understanding of factors that influence antibiotic resistance in the enteric bacteria of food producing animals. However, there are several issues relating to measurement of antibiotic resistance and analysis of antibiotic resistant data that make it a challenge to interpret resistance data and make comparisons across studies difficult. The research presented in this dissertation explores some of these issues through studies of antibiotic resistance in the lower intestinal bacteria of a population of dairy cattle that was followed over 2.5 years. The specific objectives of the study were to 1) characterize phenotypic antibiotic resistance of commensal *Escherichia coli* isolated from dairy cattle and to identify temporal patterns in resistant *E. coli,* 2) To develop a statistical model for the analysis of low quantity resistance genes measured by quantitative real-time polymerase chain reaction (qPCR), 3) to use qPCR to measure the quantity of an antibiotic resistance gene in the bacterial communities of dairy cattle that received a short-term therapeutic dose of antibiotic and untreated cattle, and 4) to use qPCR to measure the quantities of six antibiotic resistance genes in the lower intestinal bacterial communities of the same dairy cattle population that was studied for objective 1 and to identify temporal trends in resistance gene quantities.

Most studies of antibiotic resistance rely on cultivation-based methods of measuring phenotypic resistance. These methods involve growing particular bacterial species, usually a pathogen or indicator species, in selective media and picking one or more bacterial colonies to

be grown in the presence of antibiotic. These bacterial isolates are often tested against a panel of antibiotics, producing an antibiogram comprised of multiple outcomes for each isolate. It can be difficult to identify patterns in antibiogram data and often the individual antibiotics are analyzed as independent outcomes which can be misleading. In addition, if repeated samples are collected over time it can be difficult to identify temporal patterns in antibiogram data. We applied a latent variable approach to analyze antibiogram data for *E. coli* in dairy cattle. Using latent class and latent transition analysis we showed that cattle could be classified according to the resistance of levels of their *E. coli* isolates and that there was a tendency toward being susceptible to all antimicrobials over time. Animals that were in the susceptible group had high probabilities of remaining in that group over time, while animals that were in a resistant group had high probabilities of transitioning to the susceptible group in the subsequent time point. The probability of transitioning from a susceptible status to a resistant status was very low.

An alternative to measuring antibiotic phenotypic resistance of bacterial isolates is to measure the quantities of resistance genes carried by bacteria. Quantitative real-time polymerase chain reaction (qPCR) is a method of measuring quantities of a gene of interest in a sample. When applied to bacterial DNA that has been extracted in a cultivation independent manner, qPCR can provide estimates of resistance gene quantities in the entire bacterial community of a sample. However, analysis of qPCR data can pose difficulties for rare genes that are present in very low quantities and are often censored. A statistical model was developed to analyze censored qPCR data. Computer simulations were used to compare the performance of the statistical model to common methods of handling censored qPCR data. Of all the methods evaluated, the statistical model yielded the least biased results.

A common study design for investigating the relationship between antibiotic treatment and resistance is to measure levels of resistance between treated and untreated individuals before, during, and after treatment to look for changes in resistance over time and differences between treatment groups. Most of these studies are experimental, and often animals are housed in conditions that do not resemble their normal production environments where they can share bacteria. An observational study design was used to measure antibiotic resistance in dairy cattle that were treated with the antibiotic ceftiofur and untreated animals. Quantities of an antibiotic resistance gene that confers resistance to cefiofur, $bla_{\text{CMY-2}}$, were measured in the fecal community DNA of these cattle by qPCR. Overall quantities of $bla_{\text{CMY-2}}$ were very low in both groups but there was a significant spike in the treatment group during treatment that returned to pre-treatment levels the day after treatment ended.

The previously described study of phenotypic resistance in dairy cattle over time focused on cultivated *E. coli*. This focus on a particular species ignores the potential reservoir of resistance in the wide variety of species that make up the community of bovine intestinal bacteria, many of which cannot be grown in the laboratory. The combination of qPCR performed on community DNA provides a way to quantitatively measure resistance in the entire bacterial community. This approach was used to measure the quantities of six antibiotic resistance genes in fecal samples collected from the same population of dairy cattle that was followed for the study of phenotypic resistance in *E. coli*. The results showed that the reservoir of resistance to antimicrobials represented by these genes is much more widespread than what was reflected in the *E. coli* data. In addition, mixed effects linear regression models revealed significantly increasing trends over time for some of the genes. These trends were not detected in the *E. coli* data set. These results highlight the potential for qPCR performed on community

DNA as a surveillance tool that may be more sensitive for detecting the effects of selection pressures on antibiotic resistance than cultivation-based methods of testing for resistance.

Chapter 1: Introduction

Resistance of microbial pathogens to antibiotics is a serious threat to public health. Increasing rates of antibiotic resistance are well documented and treatment options for some pathogens that were once easily treated with antibiotics have been greatly reduced (9,31,107,108,178,179). The relationship between clinical use of antibiotics in humans and the dissemination of antibiotic resistance is well recognized. The first mass-produced antibiotic, penicillin, became widely available in the mid-1940's. However, penicillin resistance had already been documented a few years earlier in *Escherichia coli*, and reports of penicillin resistant strains of *Staphylococcus aureus* in hospital patients had become common by the late 1940's (3,18). Similar patterns followed with subsequently developed antibiotics; by the end of the 1950's *S. aureus* was widely resistant to tetracycline and macrolides. Methicillin was introduced in 1961 as a solution for *S. aureus* infections that were resistant to penicillin. However, methicillin resistant *S. aureus* (MRSA) strains emerged within a year and have continued to be a serious problem both in hospitals and in the community among individuals who have had no direct link to health care settings (50).

The alarming increase in antibiotic resistance led to calls for prudent use of antibiotics in health care settings along with other infection control measures in an attempt to reduce the incidence of resistant infections. These efforts were met with mixed success; while incidence of hospital acquired MRSA infections have decreased, community acquired MRSA infections have continued to increase (30). The incidences of other types of resistant infections have continued to increase or have remained stable, while new types of resistance have continued to emerge (49). This has led to questions of how antibiotics used for purposes other than treatment of bacterial infections in humans have influenced the emergence and spread of resistance.

Antibiotic use in animal agriculture, particularly for growth promotion has received considerable attention. The first major call for a reduction in the use of antibiotics for growth promotion was issued by the Swann report in 1969 and has been repeated multiple times in the four decades since then (16,73,108,179,186,212). Despite these efforts, antibiotics continue to be used in animal agriculture for purposes other than to treat sick animals. There are multiple reasons for this, including valid disagreement over what constitutes therapeutic use of antibiotics in animal agriculture. One of the reasons the debate over antibiotic use in animal agriculture has not been settled is the fact that the ecology of antibiotic resistance is extremely complicated. Policy makers who decide on how antibiotics should be regulated have to weigh the potential benefits to human health with the potential risks to animal health and potential costs to livestock producers. However, efforts to document a direct causal link between antibiotic use in food producing animals and the risk of human infection with resistant pathogens have yielded conflicting results. It is not clear that eliminating some uses of antibiotics in agriculture will improve human health but it may reduce animal health.

The purpose for the research presented in this dissertation is to explore some of the difficulties surrounding the study of antibiotic resistance in animal agriculture using dairy cattle as a model. The rest of this chapter will provide an overview of antibiotics, antibiotic resistance, agricultural uses of antibiotics with a focus on dairy cattle, and a review of the current state of knowledge of antibiotic resistance in dairy cattle.

Antibiotic modes of action and mechanisms of resistance

Antibiotics are generally defined as low-molecular-weight, biologically active compounds produced by microorganisms that, at sufficiently high concentrations, are harmful to other

microorganisms (61). Antibiotics[1] used in human and veterinary medicine are either directly produced from these naturally occurring compounds or are synthetic derivatives of those compounds. Their function in the environment is not well understood. There has been a long standing theory that antibiotics evolved to play an antagonistic role against other organisms in nature and examples of this type of activity have been documented (109,208). Following that line of reasoning it was thought that mechanisms of antibiotic resistance evolved to counteract the specific antagonistic effects of antibiotics both for bacteria that produced the antibiotic and for targets of those antibiotics (86). However, the concentrations necessary to produce antagonistic antibiotic effects in the environment have rarely been demonstrated (71). At sub-antagonistic concentrations, these small molecules have been shown to have multiple functions including cell to cell signaling and modulation of bacterial metabolism (62,63,109,122,123).

Because the functions of antibiotics vary by concentration, the target, pathway, and mechanisms by which antibiotics function also vary. At therapeutic concentrations, antibiotics function by two primary mechanisms; bacteriostatic antibiotics inhibit bacterial growth and bactericidal antibiotics kill bacteria (182,188). These mechanisms are achieved by five primary modes of action: 1) inhibition of cell wall synthesis or cell wall disruption, 2) inhibition of protein synthesis, 3) alteration of cell membranes, 4) inhibition of nucleic acid synthesis, and 5) antimetabolite activity.

Antibiotic resistance refers to the ability of microorganisms to protect themselves from the antagonistic activities of antibiotics. The definition of antibiotic resistance varies according to context. Resistance can refer to a microorganism's ability to survive or grow in therapeutic concentrations of antibiotic that would normally inhibit or kill susceptible members of the same

[1] The terms antibiotic and antimicrobial will be used interchangeably throughout this document to include the definition provided in the text as well some purely synthetic compounds such as fluoroquinolones and sulfonamides.

strain. Resistance can also refer to microorganisms that may be susceptible to therapeutic concentrations of antibiotic but have reduced susceptibility to lower concentrations. Bacteria can also be phenotypically susceptible but possess resistance genes that are not expressed (12). Resistance can occur either through structural characteristics or metabolic pathways that are inherent to all members of some strains (intrinsic resistance), or through determinants that have evolved to counteract the activities of antimicrobials (acquired resistance). Some examples of intrinsic resistance are reduced susceptibility of gram-negative bacteria to erythromycin which has poor outer membrane permeability, and gram-positive enterococci resistance to most β-lactam antibiotics because they lack the proteins that are the targets of those drugs (131,203).

Acquired resistance refers to the process by which initially susceptible bacteria become resistant to an antibiotic. Acquired resistance can occur through mutations, or through horizontal gene transfer. For example, fluoroquinolones target the type IIA topoisomerases that are required for DNA replication. Point mutations in these genes can lead to resistance to fluoroquinolones, and successive mutations in the same genes can lead to higher levels of resistance (220). Horizontal gene transfer refers to the ability of bacteria to acquire foreign DNA through transformation, transduction, or conjugation. Transformation is the uptake of naked DNA by bacterial cells. Transformation involves integration of DNA into the genome of a recipient cell without the help of integrative enzymes and requires that the foreign DNA have regions of high similarity to regions of the recipient genome (189). Therefore transformation is thought to involve DNA belonging to closely related species. Transduction is the transfer of DNA from one bacterium to another by way of a bacterial virus (bacteriophage) (57). Conjugation is the process by which a bacterial cell shares genetic material located on mobile or mobilizeable

genetic elements such as plasmids or transposons, with another bacterium through direct cell-to-cell contact (74).

The mechanisms by which bacteria resist antibiotics fall into four general categories, antibiotic target modification, reduced uptake, active efflux, and enzymatic inactivation (164,188,220). Target modification can occur by point mutations in the genes encoding the target of the antibiotic such as the bacterial ribosome so that the antibiotic is no longer able to bind to that target. Reduced uptake and active efflux involve enzymes that restrict the entry of antibiotics into the cell or facilitate the removal of antibiotics from the cell before they can reach harmful concentrations. Enzymatic inactivation refers to enzymes produced by bacteria that bind to and inactivate the antibiotics. Most antibiotics are subject to more than one of these mechanisms. A brief description of each of the primary modes of action of antibiotics, examples of antibiotics that work by those mechanisms, and common forms of resistance to those antibiotics are presented in the following sections.

Cell wall synthesis

Antibiotics that inhibit synthesis of the bacterial cell wall include the β-lactam antibiotics, penicillins and cephalosporins. These classes of antibiotics inhibit cell-wall synthesis by binding to and inactivating the enzymes, referred to as penicillin binding proteins (PBP's), that are involved in linking the peptidoglycan chains that form the cell wall structure (35,160,188,211). It has been hypothesized that this class of antibiotics originally evolved from PBP's (219). They are considered bactericidal drugs but they can have bacteriostatic properties under some circumstances. Because they target the cell wall β-lactam antibiotics were traditionally used to treat gram-positive infections. However, newer broad spectrum β-lactams, such as 3rd and 4th generation cephalosporins and carbapenems, are also effective for treatment

of gram-negative infections (190). Six β-lactam antibiotics, four penicillins and two cephalosporins, are approved for use in dairy cattle (Figure 1) (133).

The most common mechanism of resistance to β-lactam antibiotics in gram-negative bacteria is through the drug inactivating group of enzymes known as β-lactamases (67). These enzymes can be narrow spectrum β-lactamases, working on only penicillins and narrow spectrum cephalosporins or they can be extended spectrum β-lactamases (ESBL's) conferring resistance to extended spectrum cephalosporins. These enzymes are encoded by genes that can reside on the genome or plasmids of gram-negative and gram-positive bacteria. In addition to drug inactivation, some bacteria resist β-lactam antibiotics through changes in the active site of PBP's, reducing their affinity for β-lactam antibiotics (67). Another mechanism that confers reduced susceptibility to β-lactam antibiotics is through modification of outer membrane proteins resulting in decreased uptake of β-lactam antibiotics (136).

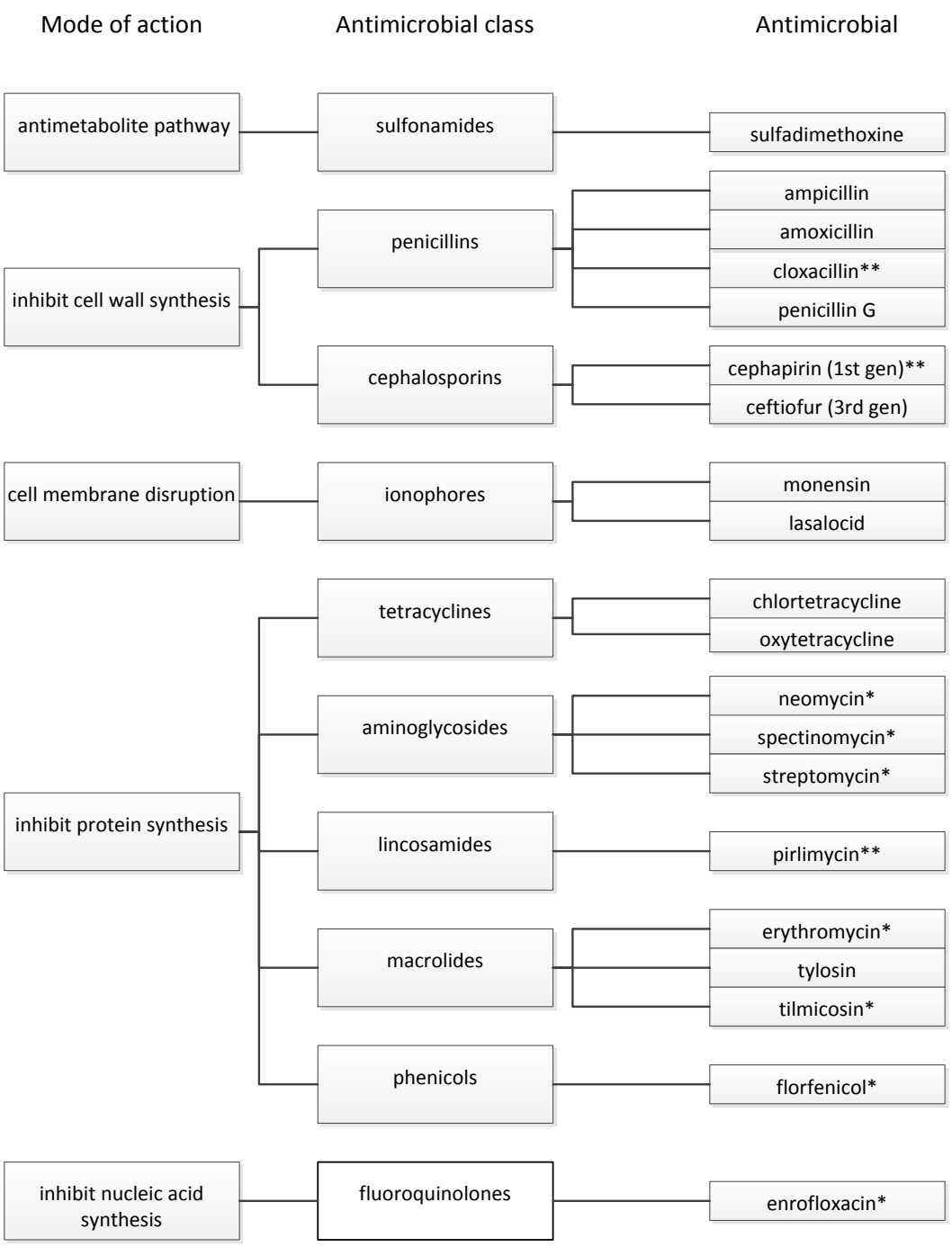

Figure 1. Modes of action, antimicrobial classes, and names of antimicrobials approved for use in U.S. dairy cattle. * indicates antimicrobials that are permitted for use only in calves and heifers less than 20 months of age. ** Indicates antimicrobials that are permitted only for intramammary use.

Cell membrane interference

Ionophores are a class of antimicrobials that interfere with ion transport systems across cellular membranes (44,134,154). Ionophores accumulate in cell membranes and facilitate movement of ions across the membranes, disrupting ion concentration within the cell and causing cell death. Most gram-negative bacteria are intrinsically resistant to ionophores because these compounds are too large to penetrate their outer membranes. Other mechanisms of resistance to ionophores are not well described but are thought to be mediated by efflux pumps. Ionophores are primarily used to improve feed efficiency by acting on gram positive bacteria and reducing negative byproducts of ruminal fermentation. Ionophores act on both prokaryotic and eukaryotic cells and can be toxic in sufficiently high doses. For this reason, ionophores are not used in human medicine.

Protein synthesis

The largest group of antibiotics used in dairy production is bacteriostatic compounds that inhibit protein synthesis. Aminoglycosides bind to the 16S rRNA component of the bacterial ribosome and interfere with translation resulting in incorrectly synthesized proteins (82). These proteins can accumulate in the bacterial membrane and increase its permeability resulting in an additional bactericidal effect. Aminoglycosides are commonly used to treat gram-negative infections and some gram-positive infections. Anaerobic bacteria are intrinsically resistant to aminoglycosides due to reduced permeability of their cellular membranes (119). Additional mechanisms of resistance to aminoglycosides include reduced intake and efflux pumps, modification of the 16S rRNA target site of aminoglycosides, and drug inactivation. Three aminoglycosides, neomycin, spectinomycin, and streptomycin are approved for use in dairy cattle (Figure 1) (133).

Macrolides, lincosamides, and streptogramins are three chemically distinct classes of antibiotics that have similar modes of action (148,151,152). These three classes of antibiotic bind to the 50S ribosomal subunit thereby inhibiting protein synthesis by blocking peptide bond formation. Macrolides are commonly used to treat infections caused by a variety of gram-positive bacteria. A large number of macrolide resistant bacteria have been identified, possessing resistance genes that alter the target site of these drugs, increase efflux, or inactivate the drugs. The binding sites of the three classes of drugs overlap. Therefore resistance mediated by alteration of the binding site leads to cross-resistance among the three classes. Three macrolides (erythromycin, tylosin, tilmicosin) and one lincosamide (pirlimycin) are approved for use in dairy cattle (Figure 1)(133).

Tetracyclines are a bacteriostatic drug class that inhibit drug synthesis by binding to the 30S subunit of the ribosome (55,147,149). Tetracyclines are broad spectrum antibiotics that are active against a wide range of gram-positive and gram-negative bacteria. The first tetracycline drugs were introduced in the 1940's and prior to the 1950's most bacteria tested were susceptible to tetracyclines. Since the 1950's tetracycline resistance has emerged in many commensal and pathogenic bacteria. The primary mechanisms of resistance to tetracycline include efflux pumps and ribosomal protection proteins. Tetracycline inactivating enzymes have also been identified but they are thought to have lesser clinical importance. Over sixty tetracycline resistance genes have been sequenced from a wide variety of gram-negative and gram-positive bacteria and are normally acquired via plasmids or transposons (http://www.antibioresistance.be/tetracycline/menu_tet.html). Two tetracycline drugs, oxytetracycline and chlortetracycline are approved for use in dairy cattle(Figure 1)(133).

Phenicols are a class of bacteriostatic drugs that bind to the bacterial ribosome and inhibit protein synthesis (47). Florfenicol is the only a member of this class that is licensed for use in food producing animals. Florfenicol is not used in human medicine but florfenicol resistant isolates have cross-resistance with chloramphenicol due to a gene that encodes an efflux pump effective against both drugs (127,165,215). Resistance to chloramphenicol can also be mediated by a drug inactivating enzyme that does not deactivate florfenicol.

Inhibition of nucleic acid synthesis

Fluoroquinolones are a class of antibiotic that interfere with DNA replication by inhibiting the type IIA topoisomerase enzymes that are required for relaxation of supercoiled DNA. Resistance to fluoroquinolones occurs through point mutations in the genes encoding those enzymes (219). Successive mutations in the same genes can lead to increased levels of resistance. Efflux pumps that are effective against fluoroquinolones have also been identified (164). One fluoroquinolone, enrofloxacin, was recently approved for use in dairy cattle but only for the treatment of bovine respiratory disease in calves less than 20 months of age (195).

Antimetabolite pathway

Sulfonamides are a class of antibiotic that interfere with metabolic activity by inhibiting an enzyme needed for folate synthesis, a metabolite that is necessary for synthesis of nucleic acids (166). The first antibacterial drug identified in 1932 was a sulfonamide produced from a synthetic dye (174). Although not produced by microorganisms, acquired resistance to sulfonamides is conferred by genes that modify the enzymes that are targets of sulfonamides. Intrinsic resistance also occurs for some bacteria through outer membrane impermeability or the ability to use folate from other sources.

Dissemination and persistence antibiotic resistance

Knowledge of the mechanisms of resistance and the genes that confer those resistance traits are important keys to understanding the problem of antibiotic resistance but are not sufficient to explain how resistance spreads, and persists. Resistance traits can provide bacteria with a competitive advantage in the presence of antibiotic pressure. This advantage can allow a resistant bacterium to displace susceptible bacteria and spread clonally, passing on its resistance traits to successive generations. An example of clonal dissemination is methicillin resistant *Staphylococcus aureus* (MRSA). Infections with MRSA have been dominated by successive waves of small numbers of clones since penicillin went into widespread use in the 1940's (50).

The other way that antibiotic resistance can spread is through the movement of resistance genes from resistant bacteria to susceptible bacteria. As previously discussed, bacteria can acquire novel DNA through horizontal gene transfer and this phenomenon has been shown to occur frequently in nature among widely diverse strains of bacteria (156). This means that the vast diversity of bacteria present in different ecosystems, including the guts of humans and animals, can act as reservoirs of resistance capable of passing resistance genes to susceptible pathogens (66,177). Many of the bacteria harboring resistance traits have not yet been identified or isolated in the laboratory (58,219).

Many resistance genes are also known to be physically linked to other genes, moving together as a single unit during horizontal gene transfer (155). These linked genes can confer resistance to multiple classes of antibiotics. Therefore, use of one antibiotic may select for bacteria that are resistant to multiple antibiotics. Resistance genes can also be linked to other genes that offer bacteria an advantage such as genes that confer resistance to heavy metals (173,184).

The conventional wisdom for decades had been that the maintenance and expression of resistance genes exerts a fitness cost on bacteria and that, in the absence of the selective pressure of antibiotics, these genes would eventually be lost from the population (15). This was backed up by studies showing that reductions in uses of some antibiotics have led to declines in resistant strains (167). However, in other cases removal of antibiotics led to initial reductions in levels of resistance that leveled off, or did not decline at all (70,100,185). This could be because the genes conferring resistance to the antibiotic that has been removed are linked to other genes that confer resistance to antibiotics still in use (2,185). Alternatively, individual resistance genes may confer resistance to multiple antibiotics such as the *erm* family of genes that modify the bacterial ribosome, the target of macrolides, lincosamides and streptogramin antibiotics. In addition to genetic linkage, resistance genes can be maintained in the absence of antibiotic pressure due to secondary mutations that counteract the fitness cost of resistance. In some cases acquisition of resistance genes can actually lead to improved fitness (14). The factors that influence the spread and persistence of antibiotics are complicated. These examples highlight the point that resistant bacteria are unlikely to completely disappear through reduction in antibiotic use.

Methods of testing for antibiotic resistance

Methods of testing for antibiotic resistance fall into two general categories, those that rely on cultivation of bacteria and cultivation-independent methods. Techniques that use cultivation involve growing bacteria from a sample in media and selecting isolates. The isolates can be tested for phenotypic resistance, by attempting to grow them in the presence of antibiotics or they can be tested for the presence of known resistance genes by PCR (216). The variations on this approach can yield results that take on different forms such as minimum inhibitory concentrations (MIC's) or disc diffusion zone sizes. Quasi-quantitative outcomes such as those

are often converted into binary susceptible/resistant data based on predetermined standards. The most frequently used standards are those published by the Clinical Laboratory Standards Institute which determines cut points for MIC's based on the likelihood that an infection with a particular pathogen will respond to treatment with an antibiotic at concentrations normally achieved in therapeutic doses of that antibiotic (56).

The advantages of cultivation techniques are that they are relatively easy to implement, inexpensive, and reproducible. They can also provide answers to questions that have clinical relevance such as whether or not a patient with a bacterial infection is likely to respond to treatment with a particular antibiotic. A major downside is that cultivation of bacteria is highly selective for particular species depending on the type of media being used. Most bacterial species cannot be grown in the laboratory. Therefore the majority of types of bacteria in a sample are overlooked when cultivation methods are used. Also, when a particular species of bacteria is cultivated, multiple strains of the same species can be present. When one or a few isolates per sample are selected for testing, the less predominant strains have a lower probability of being picked. For example, The Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP) has been monitoring trends in antibiotic resistance since 1996. To monitor antibiotic resistance in livestock, DANMAP collects isolates from animals at slaughterhouses (60). Only one isolate per farm is tested and this result represents the resistance status of the whole farm. A recent study compared this type of one isolate/sample testing to a method that calculates the prevalence of resistant isolates/sample to tetracycline and sulfonamide (206). The investigators found that the percentage of resistant isolates per sample was correlated with the binary (resistant/susceptible) resistance status of a sample using the one isolate/sample method. However, 54% of the samples that had >1% resistant isolates to tetracycline and 50% of the samples that had a high level of resistance (>50% positive

isolates) were considered susceptible using the one isolate/sample method. The results were similar for sulfonamide. Thus, the one isolate/sample method typically used by surveillance systems underestimated the level of resistance, illustrating the potential for bias in commonly used methods of surveillance for antibiotic resistance. An additional potential source of bias in cultivation based methods of resistance relates to the common practice of reporting the results as proportion of isolates tested that are resistant which is dependent on the susceptible population (163). Factors that affect the susceptible population of bacteria can affect the proportion of isolates that are resistant, even if the burden of resistance remains unchanged.

Culture independent methods of testing for resistance involve extraction of all DNA in the entire bacterial community of a sample. This community DNA can be tested for the presence of particular resistance genes by end-point PCR, or quantitative real-time PCR (qPCR) can be used to quantify copies of a gene of interest in the sample. This method permits measurement of resistance in the entire bacterial community rather than a particular type of bacteria or a small subset of isolates grown from a sample. In contrast to cultivation based techniques where the unit of study is usually the bacterial isolate, methods based on community DNA extraction carry the implicit assumption that the resistance gene is the unit of study. This assumption has some appeal considering the previously discussed mobility of resistance genes. The disadvantage of the community DNA approach is that there is no way to relate the results to particular species of bacteria. In addition, DNA extraction methods can vary in their ability to yield DNA that is truly representative of the entire bacterial population (120,126). Also, the sequence of the gene of interest must be known. Resistance to a particular antibiotic can be mediated by multiple genes, and it is likely that there are many resistance genes that have not yet been identified (58,102,219). Therefore, measurement of one or a few resistance genes does not necessarily estimate all potential sources of resistance to a particular antibiotic.

Antibiotic uses in livestock production

Antibiotics are used in livestock production in four ways 1) disease treatment, 2) disease control, 3) disease prevention, and 4) growth promotion. Treatment of disease refers to administration of a therapeutic dose of antibiotic to a sick animal. Disease control refers to administration of antibiotics to animals that are not necessarily diseased because disease is present in the herd or flock. This practice is most common in poultry production where antibiotics are administered in the water because treatment of individual birds can be difficult. Disease prevention refers to administration of antibiotics to healthy animals that are at elevated risk of disease. Growth promotion refers to adding low doses of antibiotic to feed to improve feed efficiency, allowing animals to reach market weight more quickly (91,124).

Comprehensive data on antibiotic use in U.S. livestock are not available. A 2008 amendment to the Animal Drug User Fee Act (ADUFA) requires antimicrobial drug sponsors to report to the Food and Drug Administration the quantities of antimicrobial active ingredients that have been sold or distributed for domestic use in food producing animals (194). The law also requires the FDA to provide annual summaries of this information to the public. A summary of the relative amounts of antibiotics produced for use in food producing animals by class is provided in Table 1 (197). The FDA also released a report in 2010 summarizing the quantities of antimicrobials distributed for use in humans (198). Direct comparison of these numbers should be made with caution because they do not take into account the populations of food producing animals compared to humans or differences in body weight and dosage. In addition, care should be taken when interpreting the quantities of particular classes of antimicrobials because of differences in dosages. These data do not reflect the quantities of antibiotics purchased by end-users. Data on particular uses of antimicrobials (e.g. growth promotion versus disease treatment) and uses by animal species were not provided. However, it is clear that the total

weight of antimicrobials distributed for use in food producing animals is substantially higher than the amount produced for human use. Tetracyclines and ionophores make up the largest quantities of antibiotics produced for animal consumption. Higher quantities of penicillins and cephalosporins are produced for use in humans than in animals.

Table 1 Quantity of antimicrobials produced and distributed for domestic use in food producing animals and humans, 2010 (Source: FDA).

Antimicrobial class	Animals kg (%)	Humans kg (%)
Aminoglycosides	200,794 (1.5)	9,381 (0.3)
Cephalosporins	24,588 (0.2)	499,616 (15.1)
Ionophores	3,821,138 (28.9)	NA
Lincosamides	154,653 (1.2)	69,737 (2.1)
Macrolides	553,229 (4.2)	176,278 (5.3)
Penicillins	870,948 (6.6)	1,459,219 (44.0)
Sulfonamides	506,218 (3.8)	471,442 (14.2)
Tetracyclines	5,592,123 (42.2)	131,137 (4.0)
Other	1,517,447(11.0)	497,536 (15)
Total	13,241,138 (100)	3,316,906 (100)

Antibiotic use in dairy production

Antibiotics are used in dairy production primarily for the purposes of disease treatment and disease prevention. Every antibiotic approved for use in lactating dairy cattle has an associated withdrawal period for which a treated cow must be removed from production. Every tanker load of milk that is shipped to a milk plant in the U.S. is tested prior to processing for the presence of specific antibiotics. Shipments that fail testing are discarded and there is a possibility that the producer's license to sell milk could be suspended (202) For this reason, antibiotic uses in lactating cattle are generally limited to disease treatment with preventive uses applied to calves, heifers, and adult cows that are not in milk production. Antibiotics are not generally used explicitly for the purpose of growth promotion in dairy cattle. However, some of the preventive uses may also improve feed efficiency.

Published information on antibiotic usage in dairy production in the U.S. is limited. Information that has been collected has been based on surveys of producers that relied on the quality of record keeping systems which can be highly variable (84,162). For this reason most of the information available describes general patterns of use such as the percent of herds that used particular antibiotics over some time span or the percent of animals that received particular antibiotics. Specific information on frequency, duration, and amount of antibiotics used on dairy operations are generally lacking.

A study of 131 dairy farms in Michigan, Minnesota, New York, and Wisconsin compared antibiotic use and other management practices on conventional and organic dairies (223). Farmers were asked to recall the percent of adult cows, bred heifers, and heifer calves that had received at least one antibiotic treatment in the past 60 days. A majority (85%) of conventional farms reported that 1% to 10% of adult cows had received antibiotic treatment in the prior 60 days, and 57% reported that 1 -10% of heifer calves received antibiotics in the prior 60 days. A majority of conventional farms (83%) reported no treatment of bred heifers during the prior 60 days. Antibiotic usage on organic dairy farms was significantly lower than conventional farms. Antibiotics were used to treat animals on 50% of organic herds, with all but one of these herds reporting that treated animals were either sold or were not allowed to have contact with animals in the organic herd. The most commonly used antibiotics on conventional farms were penicillins (86% of herds), cephalosporins (78%), tetracyclines (27%), and florfenicol (26%). The most common purpose of antibiotic use in adult cattle was for treatment of respiratory illness (97% of herds), lameness (83%) mastitis (80%), and reproductive illness (80%). Approximately half of conventional herds reported feeding milk replacer containing tetracycline or tetracycline combined with neomycin to calves. A small proportion (17%) of conventional dairy producers reported the use of antibiotics (excluding ionophores) in the feed or water of weaned heifers

and calves, and 4% reported continuous use of antibiotics for this purpose. Information on ionophore use not reported.

A survey of 113 dairy farms in Pennsylvania reported that 50% of farms kept written or computerized records on antibiotic usage and only 33 farms kept records that were considered by the authors to be complete (162). Of those 33 farms, the most common use of antibiotics was to treat diarrheal illness in calves (36% of calves), followed by mastitis in lactating cows (14% of cows), foot rot (16% of cows), and metritis (11% of cows). The most commonly used antibiotic for treatment of clinical mastitis was cephapirin followed by other penicillins and cephalosporins. Cephapirin was also the most commonly used drug for dry cow therapy. Tetracyclines and neomycin were the most common antibiotics used to treat diarrheal illness in calves but extra label use of spectinomycin for this purpose was also common. Most farms (70%) reported feeding milk replacer with tetracycline and neomycin to calves for prevention of diarrheal illness. Ten different drugs were used to treat respiratory illness with ceftiofur (48% of farms), ampicillin (45%), and florfenicol (30%) being the most common.

In 2007, the USDA National Animal Health Monitoring System surveyed 2,194 dairy producers in the 16 largest dairy producing states, representing 80% of U.S. dairy herds, to gather information on production and management practices, including antibiotic use (202). The most common diseases for which dairy cattle received antibiotics were mastitis, respiratory illness, diarrheal illness, and reproductive illness (Table 2). The most common antibiotic classes administered to adult cows are listed in Table 3. Patterns of use in unweaned calves and heifers were similar to adult cows with the exception that florfenicol and macrolides were more commonly used for treatment of respiratory illness in calves and heifers than they were for adults.

Common preventive uses of antibiotics on dairy operations included feeding medicated milk replacer to calves to prevent diarrheal illness, adding antibiotic to the feed of newly weaned heifers to prevent respiratory illness, and intramammary administration of antibiotics at dry off to prevent mastitis in adult cows. More than half (58%) of operations reported feeding milk replacer containing tetracycline or tetracycline with neomycin to unweaned calves. Over half of the operations surveyed administered antibiotics in the feed of weaned heifers with 33% feeding ionophores only, and 18% of operations using non-ionophore antibiotics. Ten non-ionophore drugs were used for this purpose with tetracyclines being the most common (30% of operations) followed by sulfamethazine (6%). Most operations (90%) administered dry cow therapy to some cows during a 12 month period and 72% treated all of their adult cows. Eight antibiotics were documented as used for this purpose with penicillins and cephalosporins accounting for 60% and 38% of antibiotics used on cows, respectfully.

In summary, β-lactam antibiotics are the most commonly used antibiotics for treatment of dairy cattle, with florfenicol and macrolides commonly used in younger animals. Tetracyclines, β-lactams, ionophores, and neomycin are the most commonly used antibiotics for disease prevention in calves, heifers, and non-lactating adult cows.

Table 2. Most common diseases requiring antibiotic treatment on U.S. dairy operations and percent of unweaned calves, heifers, and adult cows that received treatment during a 12 month period (Source: USDA National Animal Health Monitoring System).

	Mastitis	Respiratory disease	Diarrheal disease	Reproductive illness	Other
Unweaned Calves	0%	11.4%	17.9%	0%	2.1%
Heifers	0%	5.5%	1.6%	0%	1.4%
Adult cows	16%	2.8%	1.9%	7.4%	7.6%

Table 3. Primary antibiotics used for treatment of disease in adult dairy cows, by percent of cows receiving treatment.

	Mastitis	Respiratory disease	Diarrheal disease	Reproductive illness	Other
Aminoglycoside	0.2%	0.6%	6.4%	0%	0%
β-lactam	19.1%	11.0%	30.3%	19.7%	29.9%
Cephalosporin	53.2%	70.5%	36.0%	27.9%	23.6%
Florfenicol	0%	1.9%	0.4%	0.2%	0%
Lincosamide	19.4%	0%	0%	0%	0%
Macrolide	0.2%	1.1%	1.1%	0.2%	0%
Sulfonamide	1.2%	2.8%	15.6%	1.2%	0%
Tetracycline	2.0%	6.4%	7.0%	2.0%	2.6%
Other	4.7%	5.7%	3.2%	7.6%	43.9%

Surveillance of resistant enteric bacteria in cattle

Several countries have surveillance systems in place to monitor temporal trends in antibiotic resistance. The National Antimicrobial Monitoring System (NARMS) is the U.S. based collaboration between the CDC, FDA, and USDA that monitors resistance in enteric bacteria from human clinical isolates, animal isolates obtained from federally regulated slaughter plants, and retail meats (49). The NARMS system currently tests for resistance to 16 antimicrobials that are classified by the World Health Organization (WHO) and the World Organization for Animal Health (OIE) as critical or important to either human or animal health.

The NARMS system monitors resistance for *Salmonella* spp., *Campylobacter* spp, and *E. coli* O157. However, for samples collected from cattle, only *Salmonella spp*. are isolated and tested. Figure 2 shows the annual prevalence of resistance to four antibiotics in *Salmonella* isolates collected from cattle from 1997 to 2010. The sampling methods used for the animal

testing component of NARMS changed in 2006 from a non-targeted sampling system to a targeted, risk-based system. Therefore the data are not a nationally representative sample and comparisons between data collected before and after 2006 are inappropriate, as are year-to-year comparisons after 2006. Prior to 2006, the data show an increasing trend in resistance to three of the antimicrobials in Figure 2 until 2004. The relative differences among antimicrobials show tetracycline resistance to be consistently the highest, followed by resistance to streptomycin and ampicillin. Resistance to nalidixic acid was consistently below 3% prevalence.

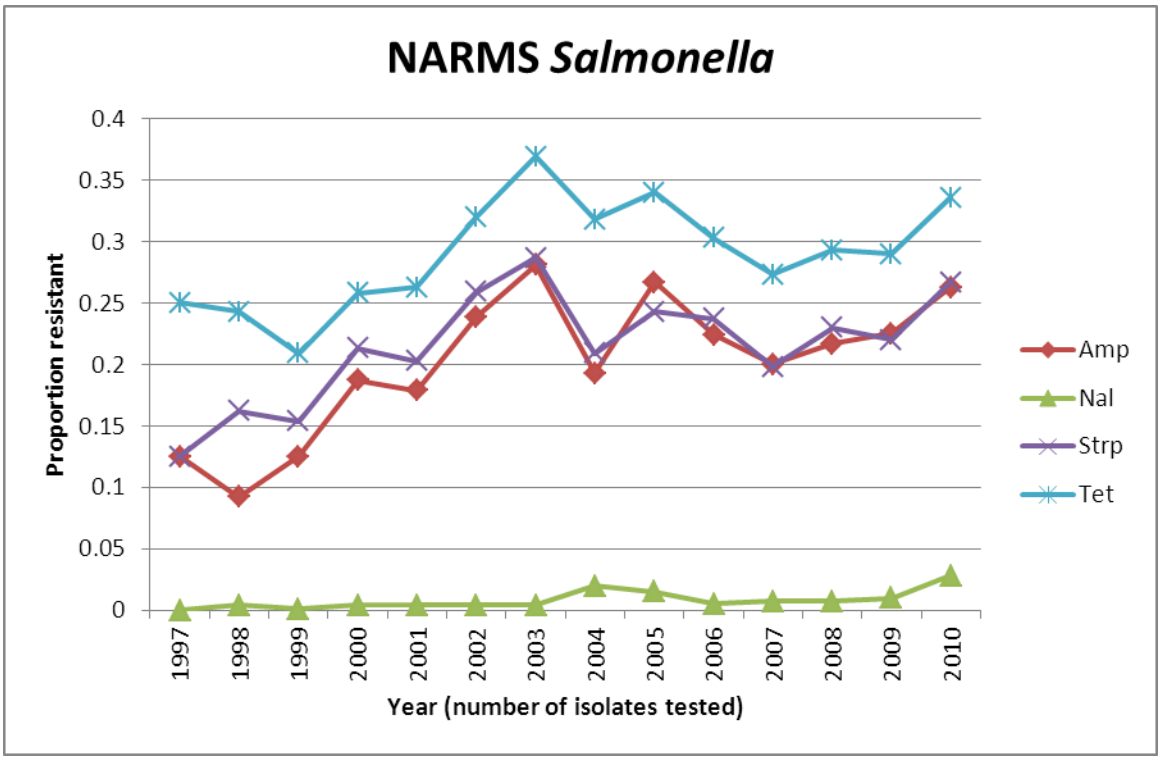

Figure 2. Proportion of *Salmonella* spp. isolates that were resistant to selected antibiotics collected by the National Antimicrobial Resistance Monitoring System, 1997 – 2010. Legend key: amp=ampicillin, nal=nalidixic acid, strp=streptomycin, tet=tetracycline.

The Canadian Integrated Program for Antimicrobial Resistance Surveillance monitors resistance levels in generic *E. coli* from healthy slaughter cattle (143). This system uses a random sampling scheme, weighted by slaughter volume, to obtain a nationally representative

sample. Figure 3 shows levels of resistance from 2003 to 2009 for the same four antimicrobials as Figure 2. Resistance levels in tetracycline, streptomycin, and nalidixic acid are similar for Canadian *E. coli* and U.S. *Salmonella* isolates, but resistance to ampicillin has been lower in *E. coli* than in *Salmonella* isolates.

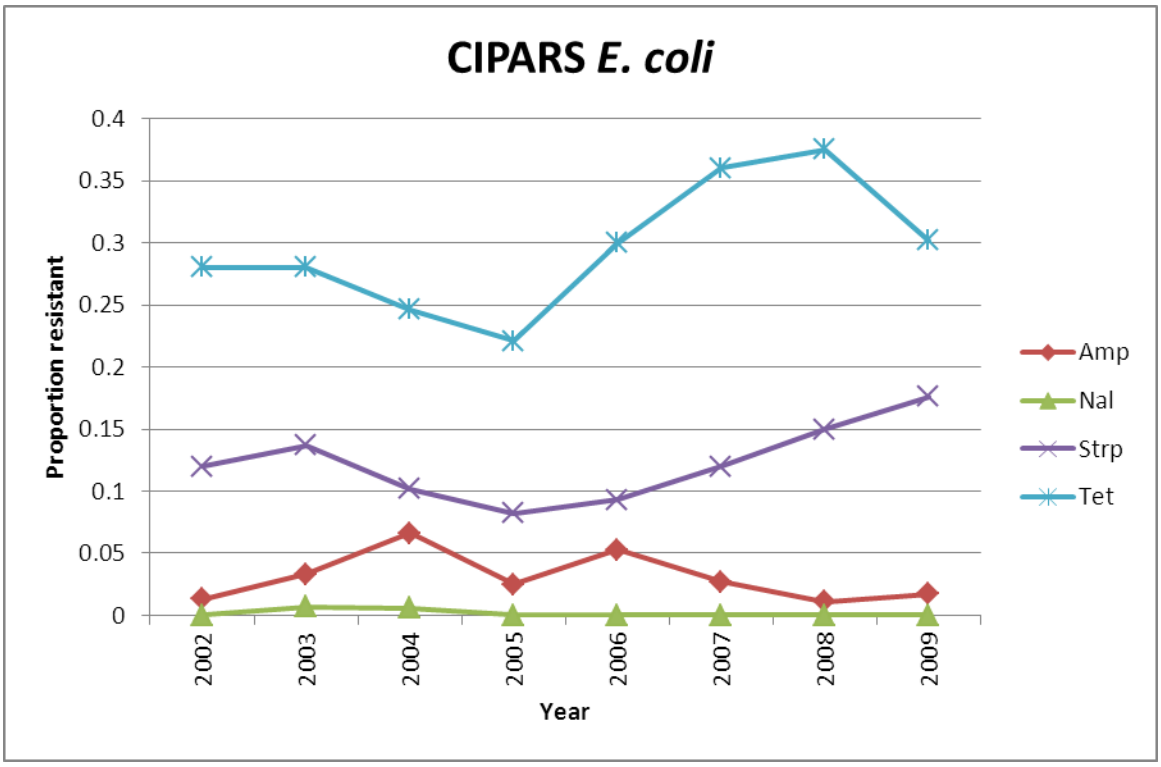

Figure 3. Proportion of *E. coli* isolates collected from healthy slaughter cattle that were resistant to selected antibiotics tested by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2003-2009. Legend key: amp=ampicillin, nal=nalidixic acid, strp=streptomycin, tet=tetracycline.

The Danish Integrated Antimicrobial Monitoring and Research Program (DANMAP) has collected data on resistance in human and slaughter animals, and retail meat in Denmark since 1995 (60). In addition, DANMAP has monitored antimicrobial consumption in food producing animals, through sales data prior to 2001 and through a national veterinary prescription reporting program since 2001. Denmark underwent a voluntary phasing out of all growth promotion uses

of antibiotics that was completed in 1999. The amount of testing, record keeping, and regulation in Denmark has resulted in a more comprehensive surveillance system than the North American systems and provides an opportunity to observe the outcome of the natural experiment of removing growth promotion from animal agriculture.

Figure 4 presents the prevalence of resistance of generic *E. coli* isolated from healthy slaughter cattle to the same four antibiotics presented in figures 2 and 3. The levels of resistance in commensal *E. coli* to tetracycline was lower in Denmark than in Canadian *E. coli* but there was not a noticeable difference among the other three antibiotics between Canadian and Danish *E. coli* isolates. Antibiotic consumption data for cattle in Denmark was provided only from 2005 to 2010. During that time overall consumption of antimicrobials in cattle was stable. However, consumption of penicillins, tetracycline, and phenicols increased over that time. Consumption of macrolides, 3rd and 4th generation cephalosporins decreased over that time, and use of aminoglycosides was stable. Overall, no compelling relationship is evident between antimicrobial use in cattle and trends in resistant commensal *E. coli* in cattle in Denmark.

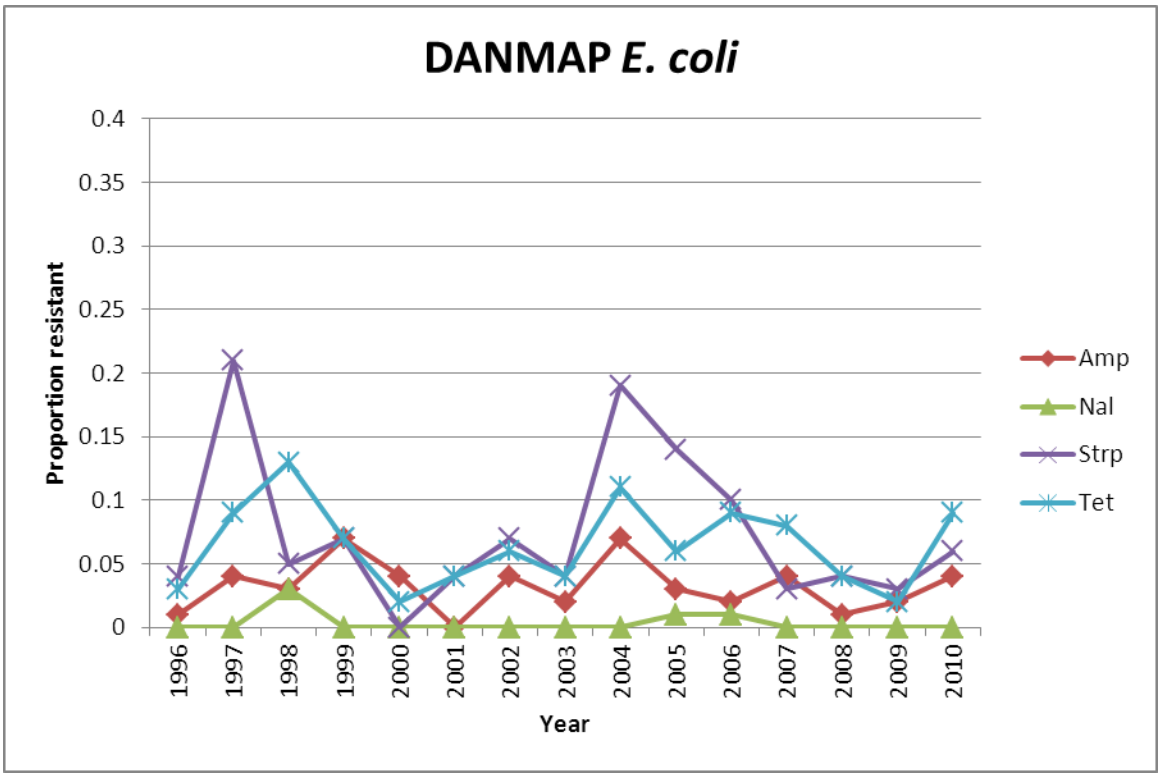

Figure 4. Proportion of commensal *E. coli* isolates from healthy slaughter cattle that were resistant to selected antibiotics collected by the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) 1996 – 2010. Legend key: amp=ampicillin, nal=nalidixic acid, strp=streptomycin, tet=tetracycline.

Studies of antibiotic resistance in enteric bacteria of dairy cattle

There have been several medium to large scale cross-sectional studies of dairy farms to measure the prevalence of resistance to antibiotics in enteric bacteria of dairy cattle. The largest were two studies of 97 dairies that varied in size and management methods in 21 states (28,115). *Escherichia coli (1,389)* and *Salmonella* spp. (294) isolates from healthy adult cows were obtained from 1,450 fecal samples. Both *E. coli* and *Salmonella* spp. were isolated from 6% of the samples. The isolates were tested by broth microdilution and classified as susceptible or resistant to 16 antibiotics according to Clinical Laboratory Standards Institute (CLSI) guidelines(56). Most *E. coli* (77%) and *Salmonella* spp. (83%) isolates were susceptible to all antibiotics. All *E. coli* isolates were susceptible to amikacin, ceftriaxone, and ciprofloxacin, and

all *Salmonella* spp. isolates were susceptible to amikacin, ciprofloxacin, nalidixic acid, and trimethoprim-sulfamethoxazole. Resistance was higher for *E. coli* isolates that came from samples where *Salmonella* was also isolated. The most common resistances were tetracycline, sulfonamide, streptomycin, and cephalothin. The most common phenotypic patterns for *E. coli* were tetracycline only, cephalothin only, and streptomycin-sulfonamide-tetracycline (SSuT). The most common resistance patterns for *Salmonella* spp. were tetracycline only and streptomycin only. The resistance profiles did not vary between *E. coli* that were isolated concurrently with *Salmonella* and *E. coli* that were not isolated with *Salmonella*. Likewise, *E. coli* and *Salmonella* that were isolated from the same sample had similar profiles. The authors concluded that the occurrence of resistance to at least one antibiotic at the farm level is common but overall the levels of resistance in dairy cattle are low.

A study of 10 dairies in Washington State compared resistance in *E. coli* isolates from farms where multidrug resistant cases of Salmonellosis had occurred in the previous year to control farms, under the hypothesis that farms with multidrug resistant *Salmonella* would have higher levels of resistance in *E. coli* (64). Case farms had significantly higher proportions of resistance to five antimicrobials (ampicillin, chloramphenicol, sulfadiazone, trimethoprim, and gentamicin) than control farms. Case farms also had a significantly higher proportion of multidrug resistant *E. coli* isolates than control farms. The authors speculated that the higher levels of *E. coli* resistance on farms that had reported multidrug Salmonellosis may have higher selective pressure due to antibiotic use. However, antibiotic use was not reported.

A study of 213 healthy lactating cattle from 23 herds in Pennsylvania screened gram-negative bacteria for resistance to ampicillin, tetracycline, spectinomycin, enrofloxacin, and florfenicol (158,161). Thirteen bacterial species were isolated and MIC's for the five antibiotics

were determined for all of the isolates. In addition, the tetracycline resistant isolates were screened for six tetracycline resistance genes. Resistance was most common for tetracycline (42% of animals) and ampicillin (34% of animals). Florfenicol and spectinomycin resistance were less common (8% and 5% of animals respectively). No resistance to enrofloxacin was detected. *Escherichia coli* was the most common species isolated (86%), and was the only species isolated from the plate used to screen for tetracycline resistance. Of those 113 tetracycline resistant *E. coli* isolates, 93% possessed the *tetB* gene and 7% possessed the *tetA* gene. Among the tetracycline resistant *E. coli* isolates, 60 pulsed-field gel electrophoresis (PFGE) subtypes were identified, suggesting that the tetracycline resistant *E. coli* population across all of the herds is diverse and not dominated by a small number of clones. However, within herd diversity was lower, with the number of PFGE subtypes within a herd ranging from one to eight. The authors did not attempt to correlate antibiotic use with resistance but they did state that tetracycline and ampicillin were used by all 23 herds and concluded that lactating cattle can be a significant reservoir of tetracycline and ampicillin resistance.

A study of 30 conventional and organic dairy farms in Wisconsin tested 1,121 *E. coli* isolates from fecal samples of 300 cows and calves for resistance to 17 antimicrobials by broth microdilution (158). The most frequent resistances on both types of farms were, in decreasing order, tetracycline, kanamycin, streptomycin, ampicillin, and sulfamethoxazole. Isolates from conventional dairies were significantly more likely to be resistant to those five antimicrobials and also to gentamicin than isolates from organic dairies. There were no significant differences between the two farm types for eleven other antibiotics. No isolates resistant to ceftriaxone, amikacin, nalidixic acid, or ciprofloxacin were detected in that study. Sixty-nine percent of isolates were susceptible to all 17 antibiotics and there was no difference between farm types in the number of multi-resistant isolates. However, calves on both conventional and organic

dairies had more isolates that were multi-drug resistant than adult cows. Frequency and type of antibiotic use on the conventional dairies was not reported.

A follow up study to the previous one characterized the genetic composition of 678 of the *E. coli* isolates and tested for associations between farm type, resistant/susceptible status and phylogenetic group (209). The authors found no association between farm and phylogenetic group for susceptible isolates but found an overabundance of one phylogroup amongresistant isolates on conventional farms. The fact that this abundance was not observed among susceptible isolates on conventional dairy farms and was not seen among susceptible or resistant isolates on organic dairy farms led the authors to conclude that the distribution of that phylogroup was caused by antibiotic use on conventional farms. This phylogroup was associated with ampicillin resistance and the presence of the β-lactamase gene bla_{TEM} . At least one of the tetracycline resistance genes, tetA, tetB, and tetC were detected in all of the tetracycline resistant isolates with tetB the most common (65%) followed by tetA (28%).

A study of cattle in 18 Ohio dairy herds screened 1,266 *E. coli* isolates for reduced susceptibility to ceftriaxone. The 436 (34%) isolates with reduced susceptibility were tested by PCR for the presence of the Amp C β-lactamase gene bla_{CMY-2}, which confers resistance to ceftriaxone and ceftiofur (192). *Escherichia coli* with reduced susceptibility to ceftriaxone was isolated from 12 of the 18 herds, including 10 of 11 herds that reported ceftiofur use and 2 of 7 herds that reported no ceftiofur use. The bla_{CMY-2} gene was identified in 355 (81%) of the isolates with reduced susceptibility to ceftriaxone. Eighty-one isolates possessing the bla_{CMY-2} gene were tested by broth microdilution for resistance to 16 antimicrobials. All of those isolates were classified as resistant to seven antimicrobials, and 53% were resistant (as opposed to reduced susceptibility) to ceftriaxone. Although *E. coli* with reduced susceptibility to ceftriaxone

had significantly greater odds of coming from farms that reported ceftiofur use, there was not a significant association between ceftiofur use and reduced susceptibility to ceftriaxone at the individual animal level.

Another study examined the relationship between therapeutic doses of ceftiofur and ceftiofur resistance by culturing *E. coli* from dairy cattle that were treated with ceftiofur for *Leptospira* infection and matched untreated cattle (171). The authors collected samples from the cattle before, during, and after treatment. Three *E. coli* isolates per sample were tested against a panel of 16 antimicrobials by broth microdilution. In addition, quantities of *E. coli* in each sample were estimated as log colony forming units (CFU) per gram of sample. The *E. coli* isolates were tested for the presence of the bla_{CMY-2} gene by PCR and community bacterial DNA extracted from each sample was also tested for the presence of the bla_{CMY-2} gene. *Escherichia coli* isolates were also genotyped by repetitive element PCR (Rep-PCR) to assess the genetic diversity of *E. coli* isolates before and after treatment. The authors found significantly higher *E. coli* counts in treated versus untreated animals on the two sampling days during the 5-day course of treatment and two sampling days immediately after treatment. The bla_{CMY-2} gene was detected in 6% of the *E. coli* isolates, all from the treated group, on the last day of treatment and the first two days immediately following the end of treatment. Cluster analysis of the isolates revealed two multidrug resistant clusters made up of only animals from the treated group. The phenotypic and genotypic data suggested that ceftiofur treatment had selected for resistant *E. coli*. However, the PCR data from the community bacterial DNA revealed that the bla_{CMY-2} gene was present in samples from both groups on every day of the study. In addition, the Rep-PCR data showed that the genetic diversity of the *E. coli* isolates was the same after treatment as it was before treatment. Therefore, the authors concluded that the bla_{CMY-2} gene was always present in the gut microbiota of those cattle, that ceftiofur treatment temporarily

decreased the susceptible population of *E. coli* allowing for the detection of resistant *E. coli*. The *E. coli* community after treatment was genetically similar to the *E. coli* community prior to treatment suggesting that resistant *E. coli* did not benefit from any temporary competitive advantage offered by the ceftiofur treatment.

Study investigators of florfenicol and chloramphenicol resistance in healthy dairy cattle of varying ages from four Illinois dairies measured the MIC's of those two antibiotics on 1,987 *E. coli* isolates (168). The isolates were also tested by PCR for the presence of two resistance genes, *flo* and *cmlA*. The MIC's showed a bimodal distribution and all of the 149 (7.5%) isolates that were positive for the *flo* gene had MIC's ≥ 256μg/ml, while all isolates that were negative for the *flo* gene had MIC's that were ≤ 16 μg/ml. Fifteen (0.76%) of the isolates were positive for both the *flo* and *cmlA* genes but no isolates were positive for the *cmlA* gene only. A subset of the *flo* positive isolates were tested to determine if the *flo* gene resided on conjugative plasmids and 37% were able to transfer resistance to florfenicol by conjugation. The authors concluded that florfenicol resistance in commensal *E. coli* of dairy calves is mediated by the *flo* gene which can reside on either conjugative plasmids, non-conjugative plasmids or on the chromosome.

A study of 297 dairy calves, followed over the first six weeks of life on three dairies and three calf ranches in California, tested 5,366 *E. coli* isolates by disk diffusion to twelve antibiotics (21). To account for dependence among the 12 antibiotics due to genetic linkages between resistance genes, the individual test results were analyzed using cluster analysis to classify isolates by their overall phenotypic patterns. The isolates were classified into a total of 14 clusters. A pan-susceptible cluster included 21% of the isolates. The remaining clusters were all characterized by tetracycline resistance and twelve of the clusters were characterized by tetracycline, sulfonamide, and streptomycin resistance (SSuT phenotype). Ten clusters were

characterized by the SSuT phenotype plus resistance to one or more other antibiotics. Eight of the clusters, representing 49% of the isolates were characterized by resistance to six or more antimicrobials.

A follow-up study of those dairy calves in California analyzed the associations between cluster membership and risk factors including farm type, calf age, and prior therapeutic dose of antibiotic (23). Age was a significant factor with the odds of membership in a cluster with a higher level of resistance increasing with age compared to one-day old calves. Calves from ranches, where medicated milk replacer was used had greater odds of being in a higher resistance cluster than calves raised on dairies where hospital or fresh milk with no added antibiotics were used. Calves that had received a therapeutic dose of antibiotic in the five days prior to sample collection also had greater odds of being in a higher level resistance cluster than animals that received no treatment. The association between age and cluster membership was stronger than the associations for farm type or therapeutic treatment. In addition, the association between cluster membership and therapeutic treatment was transitory and not observed 7 days past treatment. The authors noted the emergence and persistence of multiresistant clusters in calves that were not administered antibiotics and speculated that treatment of some individual calves may have been sufficient to maintain those clusters at the farm level.

A series of studies in dairy cattle in Washington State examined the relationship between tetracycline use in milk fed to newborn calves and the prevalence of resistant *E. coli* isolates. First, the research group compared the prevalence of resistance to five antibiotics (ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline) by agar dilution in 18,888 *E. coli* isolates from fecal samples of animals of varying ages (98). Samples were

collected weekly for approximately 40 weeks from fecal pats in pens that separated animals by age. Thus samples could only be linked to age group and not individual animals. The authors found the highest prevalence of resistance to all five antimicrobials among calves less than 3 months of age and higher prevalence to all five antibiotics in heifers 3-6 months of age versus older animals. Nearly 80% of isolates from lactating cattle were susceptible to all five antibiotics. The predominant phenotype among calves less than 3 months of age was the SSuT phenotype (approximately 40% of isolates) followed by SSuT plus either ampicillin or chloramphenicol resistance. The authors also conducted an experiment where they fed milk supplemented with tetracycline to newborn calves and milk without tetracycline to a control group of newborn calves for 6 weeks. They found that calves receiving milk with tetracycline had a significantly higher proportion of pan-susceptible isolates and a significantly lower proportion of SSuT isolates than calves receiving non-medicated milk. They also found that the prevalence of the SSuT phenotype in all calves peaked during the period when they were given milk and then declined as they were weaned at six weeks. The authors concluded that removal of the selective pressure applied by tetracycline did not result in lower levels of resistance. To support this they conducted an in vitro and in vivo competition experiments and found that SSuT isolates out competed pan-susceptible isolates in the absence of antibacterial selective pressure in both experiments. Genetic analysis of a subset of SSuT isolates found that all of the SSuT isolates possessed the *strA*, *sul2*, and *tetB* resistance genes.

A follow up experiment by that same research group compared the ability of SSuT *E. coli* isolates to compete both in vivo and in vitro with null mutant *E. coli* strains that were manipulated to spontaneously lose the genes that convey resistance to those three antimicrobials (100). They found that the null mutant strains were as competitive as strains possessing the resistance genes and concluded that the resistance genes are linked to other

unknown genes that enhance the fitness of resistance isolates in the calf gut. A subsequent study in the same Washington dairy, conducted after the dairy had ceased using the tetracycline dietary supplement, found a decline in SSuT *E. coli* isolates among calves, contrary to the authors' expectations given their previous findings (97). They then conducted an experiment comparing resistance levels in *E. coli* isolates among calves that were fed the tetracycline dietary supplement, a non-medicated dietary supplement, and no supplement. Fecal samples were collected weekly from calves for three months and *E. coli* were isolated and tested for resistance to the same five antimicrobials in the original study plus nalidixic acid. There were no statistical differences among the three groups for resistance to any single antibiotic with the exception that the group receiving no supplement had significantly higher proportion of isolates resistant to chloramphenicol. There was no difference between the group receiving the tetracycline supplement and the group receiving a non-medicated supplement in the prevalence of the SSuT phenotype. However, both groups receiving dietary supplements had significantly higher proportions of the SSuT phenotype than the group that received no supplement. Examination of the growth characteristics of isolates with the SSuT phenotype and non-SSuT isolates in the individual components of the dietary supplement revealed that vitamin D supported a higher density of growth in SSuT isolates. The authors concluded that removal of tetracycline was not what caused the prevalence of the SSuT phenotype to decline on that dairy, but rather another component of that supplement, possibly vitamin D, was selecting for the SSuT phenotype in *E. coli* isolates from calves.

Finally, the authors characterized the genetic element that carries the resistance genes that confer the SSuT phenotype in commensal *E. coli* (96). They found that the *tetB*, *strA*, *strB*, and *sul2* genes to be located in a 14,258bp region and that this arrangement is widely distributed among genetically diverse *E. coli* on 18 farms in Washington, suggesting that the

element conferring the SSuT phenotype is carried on a mobile genetic element and that this element offers a competitive advantage to *E. coli* isolates in the farm environment. The authors were unable to isolate a plasmid carrying those genes and the gene conferring the competitive advantage to these isolates was not identified. However, this series of studies confirmed higher levels of resistance in calves versus adults, characterized a genetic element conveying resistance to many of those isolates and established that isolates carrying that genetic element have a competitive advantage compared to isolates without that genetic element and that this competitive advantage was independent of tetracycline use.

The overall conclusions that can be taken from these studies of dairy cattle are that resistance levels in enteric bacteria, as expressed by proportion of resistant isolates, are generally low for most antibiotics evaluated. The highest levels of resistance among isolates are approximately 20% to 30% with resistance to tetracycline, sulfonamides, and streptomycin being the most common. This is similar to what has been observed in national surveillance programs of slaughter cattle. Low levels of resistance to antibiotics that are rarely or never used on dairy operations such as gentamicin, kanamycin, and chloramphenicol is commonly observed, suggesting that there are selective pressures other than use of those drugs maintaining antibiotic resistance on these farms. Tetracycline resistance in commensal *E. coli* is usually associated with the presence of the *tetB* or *tetA* genes. Calves have higher levels of resistance than older animals and this is not necessarily related to antibiotic use in dairy calves. There is a common phenotypic pattern of SSuT that occurs along with SSuT plus additional resistances and these are more prevalent in calves than adults. Resistance to ceftiofur and ceftriaxone are low relative to other antimicrobials, even though ceftiofur is in widespread use in dairy production. Ceftiofur and ceftriaxone resistance in *E. coli* is strongly associated with the $bla_{\text{CMY-2}}$ gene. Therapeutic doses of antimicrobials have been associated with transitory

increases in resistance. There have been some statistical differences in resistance levels reported among farms including differences between conventional and organic farms. However, efforts to study the relationship between resistance and antibiotic use and resistance have been hampered by a lack of detailed information on antibiotic use on farms.

Although some of the above studies sampled individual animals repeatedly, the repeated measures were for short periods of time or cross-sectional analyses were used. Trend data at the farm level does not exist for dairy farms and is very rare for agricultural antibiotic resistance data in general. Furthermore, attempts that have been made to assess trends in resistance at the farm level have had difficulty, in part because of challenges related to longitudinal analysis of cultivation-based resistance data (4,6). The focus of the research described in the subsequent chapters will therefore deal with issues of longitudinal analysis of antibiotic resistance in dairy cattle. First, phenotypic resistance of commensal *E. coli* isolates will be characterized and a latent variable approach to longitudinal analysis will be applied to the data. Then the focus will turn to quantitative measurement of antibiotic resistance genes in community bacterial DNA. An analytical challenge of using this laboratory method to measure low quantity genes will be addressed. Then this approach will be used to measure resistance genes in the community DNA of cattle that were sampled over a short period of time before and after receiving antibiotic treatment, and in cattle that were sampled repeatedly for more than two years.

Chapter 2: Longitudinal analysis of phenotypic antimicrobial resistance: Case study of *Escherichia coli* in the intestinal tract of dairy cattle.

INTRODUCTION

There is concern that antimicrobial usage in agriculture may select for resistant bacteria, leading to the dissemination of resistance determinants to human or zoonotic pathogens(16). Efforts to monitor antimicrobial resistance often involve the study of indicator species such as *Escherichia coli.* As part of the normal flora of humans and animals, *E. coli* are considered to be well suited to study the selection pressures exerted on a bacterial population and the potential for resistant pathogens to emerge (13,13,204). Studies of phenotypic resistance in *E. coli* isolates collected from fecal samples of healthy cattle have shown resistance levels to be associated with a number of factors. Calves generally have higher levels of resistance than older cattle. This difference is not necessarily related to antimicrobial treatment in calves or use of medicated milk replacer (24,26,99,159). Farm type has also been associated with resistance, with levels generally decreasing in order of calf-ranches, feedlots, dairies, and beef cow-calf operations (26,48,51,76,77,193). Organic dairies have been found to have lower levels of *E. coli* resistant to some antimicrobials (ampicillin, tetracycline, sulfonamides, kanamycin, gentamicin, and chloramphenicol) than conventional dairies but no differences between the two farm types for ten other antimicrobials (159,210).

Therapeutic doses of antimicrobials have been shown to increase levels of phenotypic resistance in *E. coli*, but this effect was transient with resistant *E. coli* quickly returning to pre-treatment levels after treatment was stopped (25,45,112). One study, using multiple phenotypic and genotypic methods, concluded that treatment of dairy cattle with ceftiofur did

not result in an increase of ceftiofur resistant *E. coli,* but rather treatment temporarily decreased the susceptible *E. coli* population, making it easier to detect resistant *E. coli* that were already present (172).

Analysis and interpretation of phenotypic resistance data poses challenges due to the different methods of phenotypic testing available, the variety of forms that the data can take, and the different approaches required to analyze the outcomes (214). In addition, bacterial isolates are often tested against a panel of antimicrobials, producing an antibiogram comprised of multiple outcomes for each bacterial isolate. Often the individual antibiotics are analyzed as independent outcomes which can result in multiple comparison problems and can be inappropriate if resistances to multiple antimicrobials are correlated due to cross-resistances or linkages among different resistance genes. When considered as a whole it can be difficult to identify patterns in the multivariate antibiogram. In addition, if repeated samples are collected over time it can be difficult to identify temporal patterns in antibiogram data.

Several multivariate analytical methods have been applied to antibiogram data. Cluster analysis has been applied to disk diffusion data for twelve antimicrobials tested on *E. coli* isolates obtained from fecal samples of dairy (22). Cluster analysis has also been used to classify *E. coli* isolates from fecal samples of cattle, swine, and humans (6,26). All of these studies used the results of the cluster analyses as dependent variables in separate risk factor analyses involving multivariate GEE models (6,24,26).

Factor analysis is another multivariate method that assumes the observed phenotypic data measure underlying latent variables that follow Gaussian distributions (140). Factor analysis was used to describe the structure of *E. coli* isolates from feedlot cattle fecal samples according to the MIC's of 17 antimicrobials (207). In contrast to cluster analysis, where isolates

are grouped into distinct clusters, factor analysis identifies a number of continuous factors with each of the antibiogram outcomes loading on the factors to varying degrees. The analysis identified six factors that could be interpreted in terms of combinations of antibiotic classes or historical usage patterns. In this case, factor analysis functioned not only as a method to detect structure in the data but also served as a data reduction method, in contrast to the three cluster analysis studies that identified 14 - 16 clusters as the optimal solution for antibiograms made up of 12 to 14 antimicrobials.

Another analytical method for both data reduction and identification of patterns is latent class analysis (LCA) (106). Like factor analysis, LCA assumes an underlying latent variable describes the relationship among a set of observed items. However, LCA is distinguished from factor analysis by the fact that the assumed latent variable is categorical, and the observed variables are categorical. LCA can also be considered a model-based analogue to *k*-means cluster analysis (118). In the case of antibiogram data LCA categorizes each bacterial isolate into a subset of mutually exclusive latent classes based on similarities in phenotypic patterns. One study used a Bayesian approach to latent class analysis to study resistant isolates of *Salmonella* Typhimurium DT104 collected from human clinical samples (65). The purpose of the analysis was to infer the underlying (i.e. latent) genotype that is imperfectly represented by the antibiogram. This study evaluated three competing models and found that a model with six latent classes, one for each variant of a chromosomal region (SGI1) where resistance genes are often located on the DT104 genome provided the best fit to the data.

All of the previously described analysis methods are cross-sectional in nature. However, one of the goals of antimicrobial resistance surveillance programs is to monitor temporal trends. Surveillance systems tend to monitor temporal trends at the population level, usually reporting

data on an annual basis, but do not monitor the progression of resistance within animals on shorter time scales (49,60). On the other hand, experimental and observational studies that take repeated measurements of the same animals over time tend to be short term, often sampling animals before and after antimicrobial treatment on a time scale of days or weeks (24,25,87,88,112,172). These types of studies generally find that any detected changes in resistance levels that are associated with antimicrobial treatment are transient and that resistance levels at the animal level are so variable that identification of longer term trends is very difficult.

An alternative method of analyzing repeated measures data is a longitudinal extension of latent class analysis referred to as latent transition analysis (LTA) or latent hidden Markov models (103,135). As with LCA, latent transition analysis differentiates individuals into discrete and mutually exclusive latent classes (usually referred to as latent statuses to reflect the fact that individuals can change membership over time), based on similarities in phenotypic patterns. In addition, using phenotypic patterns of isolates from the same animal at two or more time points, LTA estimates the probability that individual animals in a particular latent status at an initial time point will remain in that status at a subsequent time point or transition to another status. This may enable the identification of patterns in data that are temporally variable or when no linear trend is evident. Covariates can also be added to an LTA model as predictors of transition probabilities.

The objective of this study was to characterize phenotypic resistance in *E. coli* isolates from dairy cattle that were sampled repeatedly over time. Latent class analysis and latent transition analysis were applied to the data set to identify temporal patterns in resistance. We hypothesized that population level resistance would remain relatively static over time but would

be higher in calves than in older animals, and that isolates from calves would be likely to transition to less resistant phenotypes as the animals aged.

METHODS

Study population and sampling design

From June, 2002 through March, 2004, dairy cattle on four farms in Illinois were sampled. Each farm was visited six times at approximately three month intervals. The four farms varied in size, management practices, and antibiotic use, ranging from a small pasture-based dairy with very little antibiotic use to a fairly large free-stall dairy with frequent antibiotic usage. On the first visit to each farm fecal samples were obtained rectally from randomly selected animals of all ages. On successive visits, fecal samples were collected from the same animals unless they were no longer present in the herd. Calves aged 0-3 months were added to the study on each trip so that the cohort would have animals in the youngest age group on every sampling date.

E. coli isolation and susceptibility testing

From each fecal sample, one gram was suspended in 9ml of Luria-Bertani broth. Ten-fold serial dilutions were prepared and 100 µl of each dilution was spread onto individual MacConkey plates using sterile glass beads. The plates were incubated at 37°C for 18 to 24 hours. For each sample 24 lactose-positive colonies were picked as presumptive *E. coli* colonies and were transferred to MacConkey agar plates. A subset of up to 10 colonies was randomly selected from each of the 24 transferred colonies. Biochemical confirmation of the strains was obtained using the Simmons citrate test and triple sugar iron agar test. The selected isolates were grown in skim milk and stored at -80°C until further testing.

Three confirmed *E. coli* isolates were randomly selected for each sample and were tested for susceptibility to 17 antimicrobials by broth micro-dilution (Table 1). All antimicrobial preparations, laboratory techniques, MIC breakpoints, and control strains were in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines, except for the breakpoint for florfenicol for which there is no CLSI breakpoint defined for *E. coli.* Based on the results of a previous study, an MIC of 16µg/ml was used as the breakpoint for florfenicol (169). Each isolate tested was inoculated to non-selective semi-solid media and grown overnight. Colonies were picked and suspended in 0.85% saline, and were visually adjusted to a turbidity equivalent to the 0.5 McFarland standard. Ten microliters of this suspension was added to 5ml of Mueller-Hinton broth, and this bacterial suspension was then added to the commercial 96-well plates containing the 16 antibiotics (CMV1AGNF, Sensititre Gram-negative NARMS plate, Trek Diagnostic Systems, Westlake, OH) and to the 96 well plates containing florfenicol. Each plate was covered and incubated at 37°C for 18 to 24 hours. The plates were visually inspected to determine the MIC for each isolate, and the MIC was recorded manually. Isolates were classified as susceptible or resistant and with isolates falling in the intermediate range classified as susceptible.

Latent class analysis

Two primary types of parameters are identified by LCA: latent class membership probabilities and item response probabilities conditional on latent class membership (103,135). In this case study, the latent class membership probabilities provide the proportions of *E. coli* isolates falling into each of the latent classes. Item response probabilities provide the basis for interpretation of each latent class. These are the probabilities that an individual *E. coli* isolate in a given latent class will be resistant to each antimicrobial in the analysis. If covariates are included in the

model then logistic regression coefficients predicting latent class membership are estimated and the latent class membership probabilities are calculated directly from the coefficients.

The latent class model is described as follows (103). For a latent class model with n_c classes created from M binary observed variables, the vector $Y_i = (Y_{i1},\ldots,Y_{iM})$ represents the individual *i's* responses to the M observed variables where Y_{iM} are $1,\ldots,r_M$. Let L_i = the latent class membership of individual i, γ equals the latent class membership probabilities and ρ equals item-response probabilities. Let X_i equal the value of the covariate for individual *i*. The likelihood of the model is:

$$P\left(\mathbf{Y}_i = \mathbf{y} \mid X_i = x\right) = \sum_{l=1}^{n_c} \gamma_l(x) \prod_{m=1}^{M} \prod_{k=1}^{r_m} \rho_{mk|l}^{I(y_m=k)}$$

where *I(y=k)* is an indicator function that equals 1 if *y=k* and 0 otherwise. Let β represent the logistic regression coefficients for the covariates *X* that are used to estimate the latent class membership probabilities (γ):

$$\gamma_l(x) = P(L_i = l \mid X_i = x) = \frac{\exp\left(\beta_{0l} + x\beta_{1l}\right)}{\sum_{j=1}^{n_c} \exp\left(\beta_{0j} + x\beta_{1j}\right)} = \frac{\exp\left(\beta_{0l} + x\beta_{1l}\right)}{1 + \sum_{j=1}^{n_c-1} \exp\left(\beta_{0j} + x\beta_{1j}\right)}$$

This model allows for the estimation of the log odds of individual *i* belonging to latent class *l* relative to the baseline class.

Preliminary, cross-sectional LCA was performed for each of the six time points to determine the appropriate latent class structure prior to undertaking latent transition analysis (103). The latent class structures were determined by fitting models with 2 to 5 latent classes at

each time point. The likelihood ratio statistic and information criteria (AIC, BIC, and adjusted BIC) were used to compare each model (103,135). After determining the appropriate number of classes at each time point, age, coded as a binary variable (1 for calves < 6 months and 0 for non-calves), was added as a covariate to each of the models to determine if being a calf was a significant predictor of latent class membership. All isolates (3 per animal) were used in the cross-sectional analysis with standard errors of estimates adjusted for clustering within animal. The analyses were performed using the SAS procedure PROC LCA v1.2.6 (104).

Latent transition analysis

As with LCA, LTA estimates parameters for latent status membership probabilities and item response probabilities. In addition, LTA estimates latent transition probabilities which describe the propensities for individuals in their respective latent statuses at an initial time point to remain in that status or move into another status in the subsequent time point (103,135). Therefore, latent transition probabilities provide a description of how individuals move into and out of latent statuses over time.

The latent transition model with n_s latent statuses is estimated from M binary observed variables measured at each of T times for a total of MT items. The individual i's responses to variable M at time T are represented by the vector Y_{itm}, and S_{ti} is the individuals latent status membership at time t. As with the latent class model X_i equals the value of covariate X for individual i. The latent transition model can be expressed as:

$$P(\mathbf{Y}_i = \mathbf{Y} \mid X_i = x, G_i = g)$$
$$= \sum_{s_1=1}^{n_s} \cdots \sum_{s_T=1}^{n_s} \delta_{s_1|g}(x)\tau_{s_2|s_1,g}(x) \cdots \tau_{s_T|s_{T-1},g}(x) \prod_{m=1}^{M} \prod_{k=1}^{r_m} \prod_{t=1}^{T} \rho_{mk|s_t,g}^{I(y_m=k)}.$$

where the probability of belonging to latent status *s* at time 1 is given by the parameter *δ* (103). The grouping variable, if used, is represented by G. The *δ*'s are related to the covariates by a standard multinomial logistic model:

$$\delta_{s|g}(x) = P(S_{1i} = s \mid X_i = x, G_i = g) = \frac{\exp\left(\beta_{0s|g} + x\beta_{1s|g}\right)}{1 + \sum_{j=1}^{n_s-1} \exp\left(\beta_{0s|j} + x\beta_{1s|j}\right)}$$

Similarly, the transition probabilities, *τ*, are estimated my a multinomial logistic model estimating the probability that individual *i* transitions to latent status *s* at time *t* given its latent status membership at time *t*-1.

$$\tau_{s_2|s_1,g}(x) = \frac{\exp\left(\beta_{0s_2|s_1,g} + x\beta_{1s_2|s_1,g}\right)}{1 + \sum_{j=1}^{n_s-1} \exp\left(\beta_{0s_2|s_1,j} + x\beta_{1s_2|s_1,j}\right)}.$$

The six time points were evaluated as four separate latent transition analyses, each with two time points that spanned two sampling intervals (i.e. 1-3, 2-4, 3-5, 4-6), or approximately six months. They data were separated into four analyses to speed estimation and improve model identifiability. A six month time interval was used to ensure that all calves less than 6 months of

age had transitioned out of that age group during the time interval. Age was included as a binary covariate (1 for calves < 6 months of age and 0 for animals older than 6 months) both as a predictor of latent status membership at the initial time point and as a predictor of transition probabilities.

The analyses were performed using the SAS procedure PROC LTA v1.2.6, which does not accommodate clustered data (104). Therefore bootstrap methods were used to randomly generate 100 data sets of one isolate per sample from the three isolates per sample in the entire dataset to estimate how within sample variability affects parameter estimates. LTA was performed on the bootstrap data sets to obtain mean values and 95% confidence intervals for both the latent transition probabilities and odds ratios for the age covariates.

RESULTS

Over the six visits to each farm, 1,134 samples were collected from 241 animals: 137 (12%) of the samples were collected from calves less than 6 months of age and 997 (88%) from cows older than 6 months. There were 168 – 203 animals sampled at each time point, with 9% – 16% of samples at each time point collected from calves. The number of animals sampled per farm at any given time point ranged from 31 - 61. Nearly half (49%) of the animals were sampled at all six time points and 91% were sampled at least twice.

From the 1,134 samples, 3,402 *E. coli* colonies were isolated and tested against the panel of 17 antimicrobials (Table 4). The antimicrobial with the greatest frequency of resistance was tetracycline (22% of all isolates), followed by sulfamethoxazole (20%) and streptomycin (16%). The remaining 14 antimicrobials had resistance prevalences of 6% or less. No isolates exhibited resistance to amikacin, ceftriaxone, or ciprofloxacin, and only one isolate was resistant

to nalidixic acid. There was considerable variability among the three colonies isolated from each sample (Table 4).

Two-thirds of the isolates (67%: 32% calf and 71% non-calf) were not resistant (pan-susceptible) to any of the 17 antimicrobials, 15% were resistant to a single antimicrobial and 18% were multi-resistant. All but 36 isolates were resistant to 6 antimicrobials or fewer. The greatest number of resistances observed was thirteen, in three colonies all isolated from the same sample. The most common multiple resistance pattern observed was streptomycin-sulfamethoxazole-tetracycline (SSuT) (Table 5). All but 82 (93%) of the isolates that were not pan-susceptible were resistant to at least one of those three antimicrobials.

Excluding pan-susceptible isolates, the three most common phenotypes among the non-calf isolates were tetracycline only (T = 5.2%), sulfamethoxazole only (Su = 4.3%), and SSuT (3.9%). Among calf isolates, the most common phenotype was streptomycin-sulfamethoxazole-tetracycline-kanamycin (SSuTK = 8.3). The T (6.8%) and Su (6.3%) phenotypes were the second and third most common phenotypes respectively. The SSuT phenotype (4.3%) was only the fifth most common among calf isolates. There was no time trend evident among the T phenotypes in either calves or non-calves. However, there appeared to be a decreasing trend in both the Su and the SSuT phenotypes among both calves and non-calves (Figure 5). Along with SSuTK there were two other relatively common phenotypes among calves that included SSuT plus additional resistances, SSuT-kanamycin-ampicillin (SSuTKA =3.9%), and SSuT-chloramphenicol (SSuTC = 3.9%). Proportions of these three phenotypes among calf isolates were variable over time and were relatively rare in non-calf isolates (Figure 6).

Latent Class Analysis

To limit the number of parameters that would need to be estimated by the latent variable analysis and improve model identifiablity, only antibiogram data for the six antimicrobials with the highest frequencies of resistance were used: tetracycline, streptomycin, sulfamethoxazole, kanamycin, ampicillin, and chloramphenicol. When comparing models with 2 to 5 latent classes, the AIC, BIC, and adjusted BIC unanimously pointed to three classes at time points 1, 4, 5, and 6. There was disagreement among the three information criteria at time points 2 and 3 with the AIC and adjusted BIC pointing toward 4 classes and the BIC pointing toward 3 classes. However, adding a fourth class at these time points resulted in two of the four classes having a very low percentage of isolates. In these cases, the substantive interpretation of the classes was not improved by adding a fourth class. Therefore, for consistency and ease of interpretation, three classes were chosen for all time points.

There was some variation in item response probabilities over time but the three latent classes had the same general interpretation at every time point (Figure 7). The most prevalent class was a pan-susceptible class, made up of isolates that had a very low probability of being resistant to any of the six antimicrobials. The second latent class, which will be referred to as the SSuT class, was made up of isolates with high probabilities of being resistant to streptomycin, sulfamethoxazole, and tetracycline. Isolates in this class had low probabilities of being resistant to the other three antimicrobials. The least prevalent class, here on referred to as SSuT+, was comprised of isolates that, like the SSuT class, had high probabilities of being resistant to tetracycline, sulfamethoxazole, and streptomycin. In contrast to the other two classes, isolates in the SSuT+ class also had relatively high probabilities of being resistant to kanamycin, ampicillin, and chloramphenicol. However, the item response probabilities for those three antimicrobials were usually less than 50%, reflecting the overall low prevalence of resistance to those three antimicrobials in the data set.

The isolates in the pan-susceptible class were the most numerous at every time point (70% - 87% prevalence), followed by the SSuT class (10% - 21% prevalence), and the SSuT+ class (3% - 9% prevalence) (Figure 8). The prevalence of the pan-susceptible class appeared to drop during time point 2 while the SSuT class prevalence increased during that same time point. After time point 2, the prevalences of the pan-susceptible class appeared to increase slightly over time while the SSuT and SSuT+ classes decreased in prevalence.

Including age as a binary covariate improved the fit of the latent class models at every time point (likelihood ratio χ^2 statistic $p < 0.001$ at every time point). Calves less than six months old had significantly higher odds than older animals of being in the SSuT+ class versus the pan-susceptible class at every time point (Table 6). Age as a predictor of membership in the SSuT class was inconsistent. Calves had significantly higher odds than older animals of being in the SSuT class versus the pan-susceptible class at time points 3, 5, and 6. At time point 4, calves had lower odds than older animals in being in the SSuT class versus the pan-susceptible class. There were no significant differences between calves and older animals in SSuT membership at time points 1 and 2.

Latent Transition Analysis

Attempting to model three latent statuses in LTA resulted in identifiability problems for some of the bootstrap data sets. This was due to data sparseness and the inclusion of age as a covariate. In some circumstances there were no calves in one of the latent statuses and odds ratios could not be estimated. Therefore the three latent classes in cross-sectional analysis were collapsed into two latent statuses, a pan-susceptible and resistant status. The result of this approach was that most isolates that belonged to the SSuT class in the cross-sectional analyses were combined with isolates from the SSuT+ class.

Animals that started in the pan-susceptible status had consistently high probabilities (mean = 0.88 – 0.96) of remaining in that status at the subsequent time point and consistently low probabilities (mean = 0.04 – 0.13) of transitioning to the resistant status (Table 7). Animals that started in the resistant status had high probabilities (mean = 0.69 – 0.96) of transitioning to the pan-susceptible class and low probabilities (mean = 0.04 – 0.31) of staying in the resistant status at the subsequent time point. However, there was a trend toward increasing probability of transitioning from the resistant to the pan-susceptible status over time and a corresponding decreasing trend of remaining in the resistant status over time (Table 7).

As with the cross-sectional analyses, age was a significant predictor of latent class status at the initial time point of the four latent transition analyses, with calves having greater odds than older animals of being in the resistant status versus the pan-susceptible status (data not shown). Calves also had higher odds than older animals of transitioning from the resistant to the pan-susceptible status versus remaining in the resistant status in three of the four LTA's (Table 8). Over the final time interval (time periods 4 to 6), calves had lower odds than adults of transitioning from the resistant to the pan-susceptible status. Age was an inconsistent predictor of transitioning from the pan-susceptible class to the resistant class, with calves having lower odds than older animals over time intervals 1 to 3 and 3 to 5, and higher odds over time interval 4 to 6.

DISCUSSION

The levels of resistance observed in this data set are consistent with what has been reported elsewhere for commensal *E. coli* isolated from healthy dairy cattle. A survey of 1,389 *E. coli* isolates from 97 dairy farms across the United States found more than 77% of isolates to be pan-susceptible (115). As with results presented here, no isolates were resistant to amikacin,

ceftriaxone, and ciprofloxacin. Only 2-3 isolates were resistant to nalidixic acid, similar to the one nalidixic acid resistant isolate in our data set. The antimicrobials with the highest individual prevalence of resistance and the most common phenotypic patterns in that study matched our results. However, the overall levels of resistance in that study were lower than what we observed. This difference likely reflects the fact that all animals sampled in that study were older than two years while 12% of the samples in our data set came from calves, which tend to have higher levels of resistance.

Another study of 1,120 *E. coli* isolates from dairy calves and cows, raised on organic and conventional dairies in Wisconsin, reported 69% of the isolates to be pan-susceptible (159). As with our results and the previously mentioned study, no isolates were resistant to amikacin, ciprofloxacin, or ceftriaxone. In addition, no isolates were resistant to nalidixic acid. Only 7% of isolates were resistant to a single antimicrobial but 24% of isolates were resistant to multiple antimicrobials. This may be related to the fact that more than 25% of the isolates came from calves. There were no significant differences between conventional and organic farms in the number of isolates with multiple resistances. However, organic farms did have lower prevalence of resistance to seven individual antimicrobials, including the six antimicrobials used for latent class analysis in this study.

The latent class analyses reported here succeeded in reducing the data set from 40 phenotypic patterns observed among the six antimicrobials analyzed to three classes that had biologically plausible interpretations. Multiple studies have reported the SSuT phenotype to be The most common multiple resistance pattern in *E. coli* isolated from bovine feces, while isolates with the SSuT phenotype plus additional resistances were found to be less common (24,26,76,77,99,115,207). The SSuT phenotype was the most common multiple resistance

phenotype among non-calves in this study, while two other phenotypes, SSuTK and SuT were more common than SSuT among calves in this study.

With latent transition analysis it was possible to identify patterns in latent status membership over time. In this case the most obvious pattern was a tendency toward being in the pan-susceptible status. Animals that started in the pan-susceptible status were likely to remain in that status over time and animals that started in the resistant status were likely to transition to the pan-susceptible status at the subsequent time point. The probability that an animal in the resistant status would remain in that status decreased over time, reflecting a generally increasing proportion of pan-susceptible isolates in the latter two-thirds of the study.

As was expected, age was a significant predictor of both latent class membership and transition probabilities. Calves had greater odds than older animals of being in the SSuT+ class and calves also had greater odds than older animals of transitioning from the resistant status to the pan-susceptible status over three of the four time intervals throughout the study. During the last interval (time 4 to 6) calves had lower odds than adults of transitioning to the pan-susceptible status. This occurred because, in most of the bootstrap samples, all adults that were in the resistant status at time 4 transitioned to the pan-susceptible status at time 6 while only a proportion of the calves made that transition.

In the cross-sectional latent class analyses, three classes were identified as the structure that best fit this particular data set. It is possible that including more antimicrobials in the LCA's would have differentiated the isolates into more than three classes. However, the very low prevalences of resistance to the eleven excluded antimicrobials would have resulted in poorly identified models with only one or a few isolates in some classes. Analysis performed on a data set with fewer pan-susceptible isolates and higher prevalences of resistance to the

antimicrobials would likely differentiate the isolates into a larger number of classes. Although this data set included a large number of isolates, individual animals rather than isolates are the units of study in LTA. Therefore the maximum sample size in the longitudinal analyses was only 241 animals, a lower number than is typically seen in studies utilizing LTA (103,135). Analysis with a larger sample size may be better able to avoid some of the problems with data sparseness that was encountered in this study.

Other studies have used cluster analysis to identify structure in antibiogram data, followed by separate risk factor analysis using cluster membership as a multinomial dependent variable (6,24,26). It would be possible to use latent class analysis to assign individuals to classes based on their posterior (predicted) probabilities of class membership and then to use class membership as a categorical outcome in separate risk factor analysis. However, LTA offers the advantage of providing a framework to model longitudinal data while permitting the inclusion of covariates within the same modeling framework. In a dataset that is temporally variable, as is often the case with antimicrobial resistance data, identification of a trend using traditional methods of longitudinal data analysis can be difficult. Latent transition analysis permits the use of covariates such as antibiotic treatment or age to predict movement among latent statuses. Although longitudinal extensions of factor analysis are available, the output of factor analysis describes the correlation between observed data and underlying continuous variables (103). We found the interpretation of latent class analysis and latent transition analysis, the probabilities of individuals belonging to discrete and mutually exclusive classes to be a more appealing description of underlying genotypes.

Table 4. The number of isolates (n =3,402) that are resistant to each of seventeen antimicrobials and the breakdown of number of isolates per sample (n=1,134) that were resistant to each antimicrobial.

Antimicrobial	Total resistant isolates (%)	Resistant isolates per sample			
		3 of 3	2 of 3	1 of 3	0 of 0
Amikacin (AMI)	0 (0.0)	0	0	0	1,134
Ampicillin (AMP)	166 (4.9)	19	26	57	1,032
Augmentin (AUG)	28 (0.8)	4	4	8	1,118
Ceftriaxone (AXO)	0 (0.0)	0	0	0	1,134
Cephalothin (CEP)	77 (2.3)	7	10	36	1,081
Chloramphenicol (CHL)	112 (3.3)	13	15	43	1,063
Ciprofloxacin (CIP)	0 (0.0)	0	0	0	1,134
Trimethoprim/sulfamethoxazole (COT)	26 (0.8)	3	2	13	1,116
Florfenicol (FLO)	81 (2.4)	10	7	37	1,080
Cefoxitin (FOX)	21 (0.6)	2	4	7	1,121
Gentamicin (GEN)	19 (0.6)	3	0	10	1,121
Kanamycin (KAN)	215 (6.3)	35	27	56	1,016
Naladixic acid (NAL)	1 (0.0)	0	0	1	1,133
Sulfamethoxazole (SMX)	669 (19.7)	101	106	154	771
Streptomycin (STRP)	543 (16.0)	67	90	162	815
Tetracycline (TET)	759 (22.3)	126	102	177	729
Ceftiofur (TIO)	21 (0.6)	3	2	8	1,121

Table 5. The frequencies of 40 phenotypic patterns observed among the 3,402 isolates tested for resistance to six antimicrobials (Amp – ampicillin, Chl – chloramphenicol, Kan – kanamycin, Smx – sulfamthoxazole, Strp – streptomycin, Tet – tetracycline). There were 93 phenotypic patterns observed among all 17 antimicrobials included in the panel.

Phenotypic pattern	Num isolates (%)	Phenotypic pattern	Num isolates (%)
Amp, Chl, Kan, Smx, Strp, Tet	20 (0.59)	Chl, Kan, Smx, Strp, Tet	17 (0.50)
Amp, Chl, Smx, Strp, Tet	19 (0.56)	Chl, Kan, Smx, Tet	10 (0.29)
Amp, Chl, Smx, Tet	5 (0.15)	Chl, Kan, Strp	1 (0.03)
Amp, Chl, Strp	1 (0.03)	Chl, Smx, Strp, Tet	33 (0.97)
Amp, Kan, Smx, Strp, Tet	39 (1.15)	Chl, Smx, Tet	2 (0.06)
Amp, Kan, Smx, Strp	1 (0.03)	Chl, Strp, Tet	1 (0.03)
Amp, Kan, Smx, Tet	6 (0.18)	Chl, Strp	1 (0.03)
Amp, Kan, Strp	2 (0.06)	Chl	2 (0.06)
Amp, Kan, Strp, Tet	14 (0.41)	Kan, Smx, Strp, Tet	62 (1.82)
Amp, Kan, Tet	7 (0.21)	Kan, Smx, Strp	1 (0.03)
Amp, Kan	1 (0.03)	Kan, Smx, Tet	15 (0.44)
Amp, Smx, Strp, Tet	8 (0.24)	Kan, Strp, Tet	6 (0.18)
Amp, Smx, Strp	1 (0.03)	Kan, Strp	3 (0.09)
Amp, Smx, Tet	7 (0.21)	Kan, Tet	3 (0.09)
Amp, Smx	1 (0.03)	Kan	7 (0.21)
Amp, Strp, Tet	12 (0.35)	Smx, Strp, Tet	136 (4.00)
Amp, Strp	1 (0.03)	Smx, Strp	25 (0.73)
Amp, Tet	4 (0.12)	Smx, Tet	109 (3.20)
Amp	15 (0.44)	Smx	153 (4.50)
Other resistance*	57 (1.68)	Strp, Tet	40 (1.18)
Pan-susceptible	2,269 (66.70)	Strp	101 (2.97)
		Tet	184 (5.41)

* These isolates were susceptible to the six antimicrobials included in this table but were resistant to at least one of the other eleven antimicrobials in the panel.

Table 6. Odds ratios and 95% confidence intervals for calves less than 6 months of age versus older animals of being in the SSuT and SSuT+ classes at each time point.

Time point	Pan-susceptible	SSuT: OR (95% CI)	SSuT+: OR (95% CI)
1	Ref	1.9 (0.6 – 6.0)	228.3 (10.4 – 5008.1)
2	Ref	3.1 (0.7 – 13.7)	30.4 (10.0 – 92.0)
3	Ref	6.7 (2.2 – 20.0)	26.7 (7.6 – 93.1)
4	Ref	0.02 (0.0 – 0.18)	123.0 (3.3 – 4633.6)
5	Ref	5.6 (2.6 – 12.1)	34.7 (10.7 – 112.1)
6	Ref	4.1 (1.2 – 14.4)	40.6 (9.9 – 166.3)

Table 7. Probabilities (95% CI) of transitioning from one class to another over approximately 6 six month intervals (two time steps). For each time interval, the diagonal elements are the probabilities that an animal in the pan-susceptible or SSuT+ classes stay in their respective classes. The off-diagonal elements are the probabilities that an animal will transition from on class to another.

		Pan-susceptible	Resistant
Time 1 – 3	Pan-susceptible	0.89 (0.88 – 0.89)	0.13 (0.11 – 0.12)
	Resistant	0.69 (0.64 – 0.74)	0.31 (0.26 – 0.36)
Time 2 – 4	Pan-susceptible	0.88 (0.86 – 0.89)	0.12 (0.11 – 0.14)
	Resistant	0.77 (0.71 – 0.82)	0.23 (0.18 – 0.29)
Time 3 – 5	Pan-susceptible	0.96 (0.95 – 0.97)	0.04 (0.03 – 0.05)
	Resistant	0.84 (0.81 – 0.87)	0.16 (0.13 – 0.19)
Time 4 – 6	Pan-susceptible	0.91 (0.90 – 0.93)	0.09 (0.08 – 0.10)
	Resistant	0.96 (0.95 – 0.96)	0.04 (0.02 – 0.05)

Table 8. Odds ratios (95% CI), calves versus adults (referent group), of transitioning to the pan-susceptible of SSuT+ class over the time interval versus staying in their respective classes.

		Pan-susceptible	SSuT+
Time 1 – 3	Pan-susceptible	Ref	0.50 (0.21 – 0.78)
	SSuT+	1.84 (1.25 – 2.42)	Ref
Time 2 – 4	Pan-susceptible	Ref	1.10 (0.79 – 1.41)
	SSuT+	2.00 (1.08 – 2.94)	Ref
Time 3 – 5	Pan-susceptible	Ref	0.17 (0.08 – 0.25)
	SSuT+	17.8 (7.22 – 28.39)	Ref
Time 4 – 6	Pan-susceptible	Ref	1.36 (1.34 – 1.37)
	SSuT+	0.10 (0.09 – 0.11)	Ref

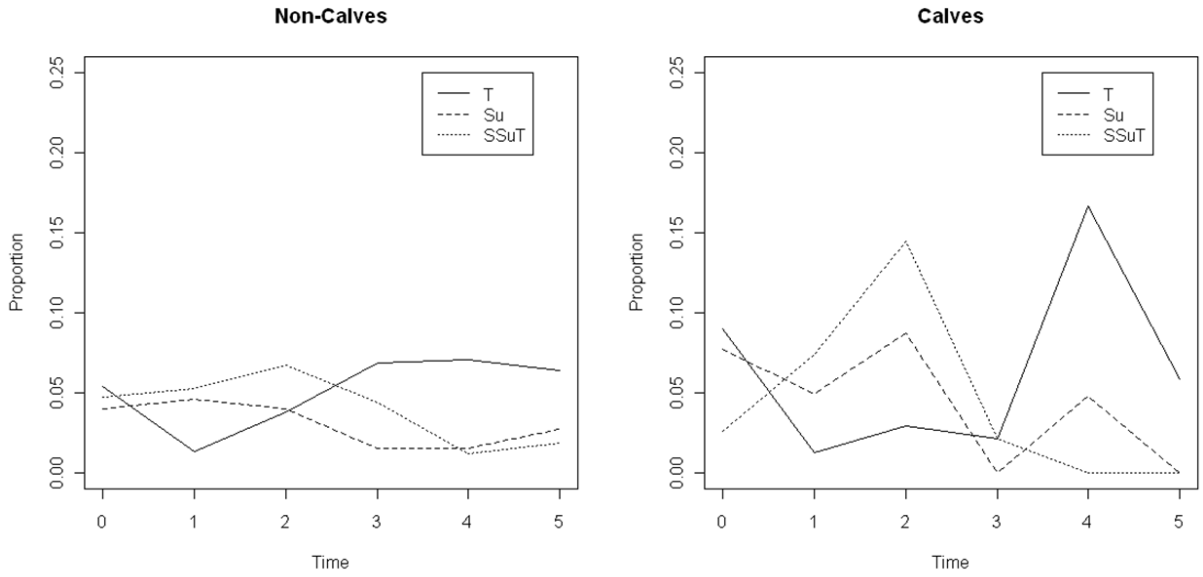

Figure 5. Proportions of resistant isolates over time to three phenotypes, tetracycline only (T), sulfamethoxazole only (Su), and streptomycin-sulfamethoxazole-tetracycline (SSuT) among non-calves (left) and calves (right).

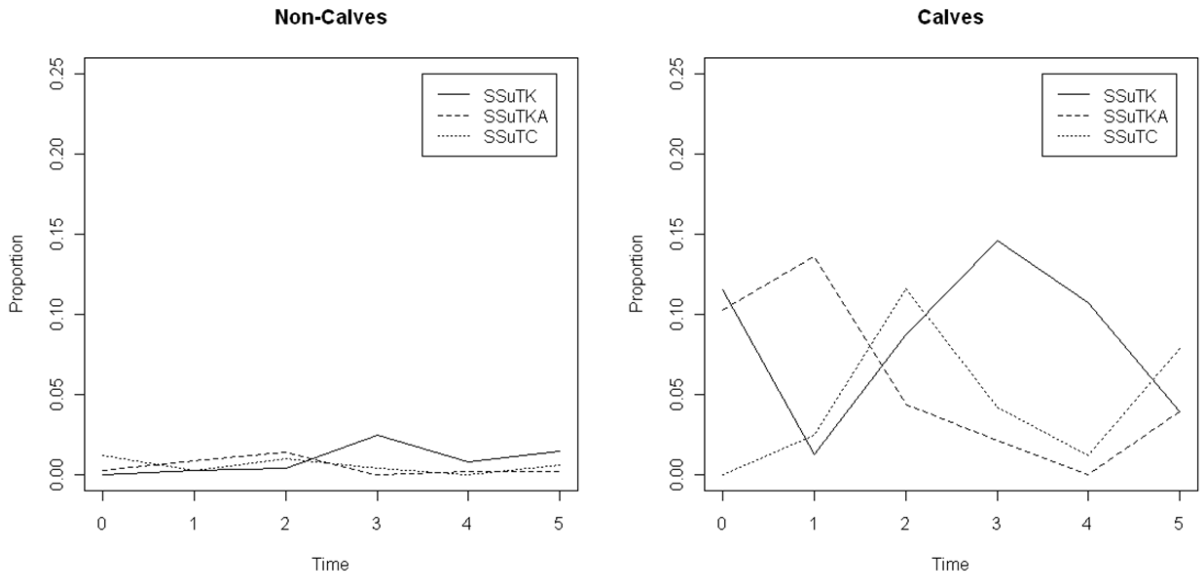

Figure 6. Proportions of resistant isolates over time to three phenotypes, streptomycin-sulfamethoxazole-tetracycline-kanamycin (SSuTK), streptomycin-sulfamethoxazole-tetracycline-kanamycin-ampicillin (SSuTKA), and , streptomycin-sulfamethoxazole-tetracycline-chloramphenicol (SSuTC) among non-calves (left) and calves (right).

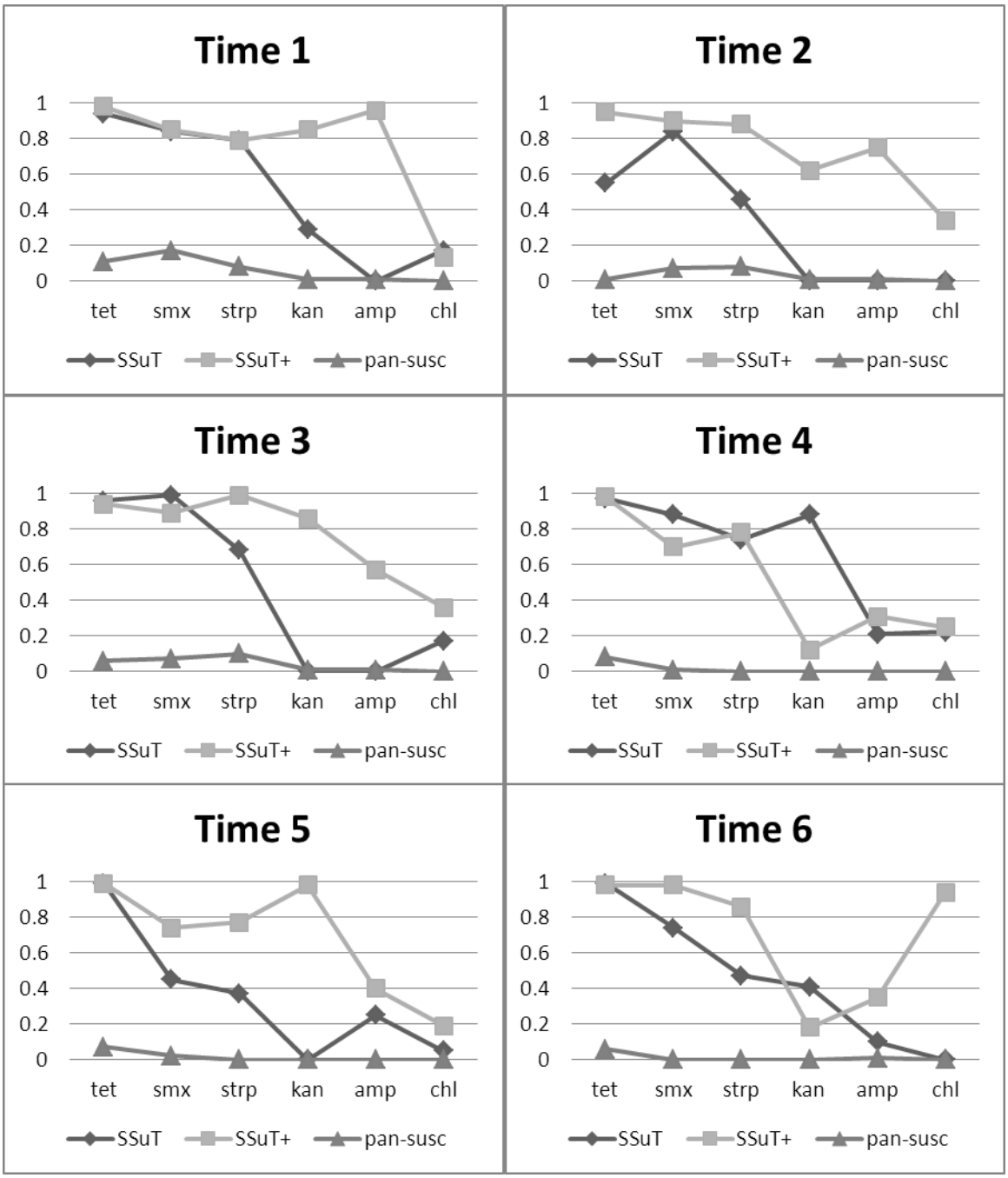

Figure 7. Item response probabilities of resistance to six antimicrobials for isolates in each of three latent classes at each time point.

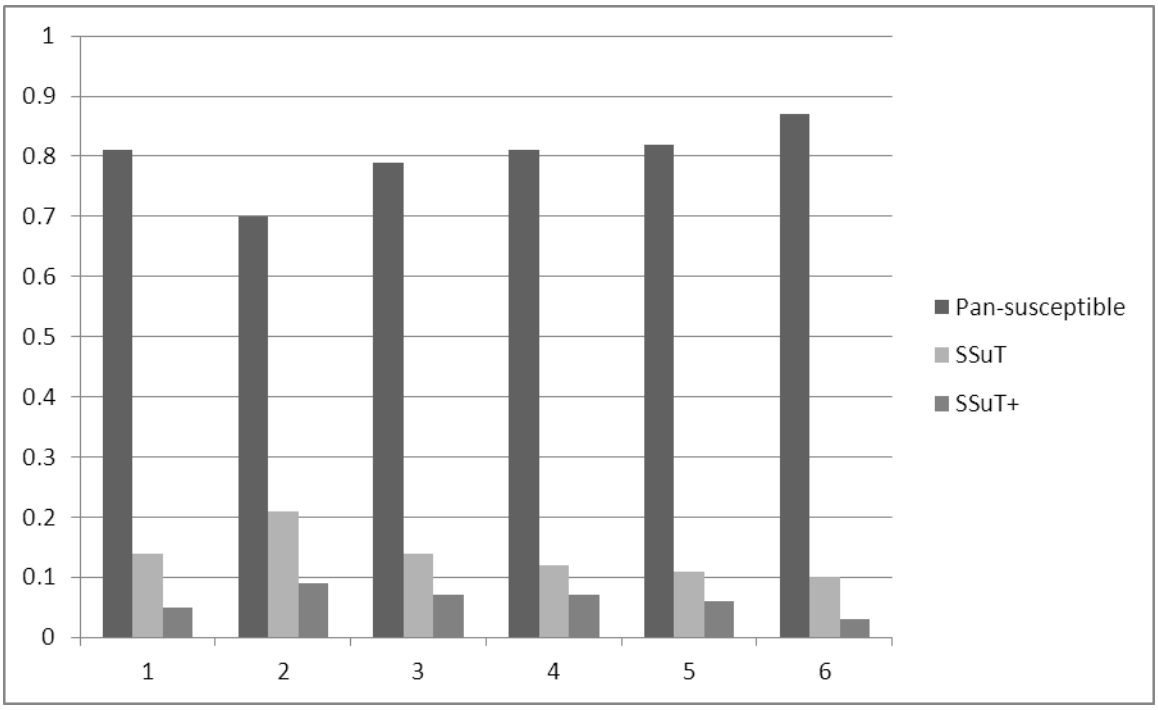

Figure 8. Prevalences of each latent class at each time point.

Chapter 3: Estimation of low quantity genes: a hierarchical model for analyzing censored quantitative real-time PCR data

INTRODUCTION

Quantitative real-time PCR (qPCR) has become an important molecular tool in the life sciences for its ability to both detect and quantify minute amounts of target nucleic acid present in a sample. The performance of a qPCR assay is determined by multiple factors including the assay's limit of detection (LOD), precision, efficiency, and linear dynamic range (43). The LOD is often defined as the lowest analyte concentration that can be detected with reasonable certainty (38,43). The theoretical LOD, the target gene concentration in which at least one copy can be detected with 95% certainty assuming a Poisson distribution, is three gene copies per reaction (43). The practical LOD of a particular assay can be determined through serial dilutions. The precision of the assay is determined by the variation observed among the technical replicates (typically 3) that are run for each sample. The efficiency and linear dynamic range are both determined by the calibration curve constructed from samples of known gene quantity. The efficiency is derived from the slope of the curve and the linear dynamic range is defined by the range of quantities over which the calibration curve is linear. The upper and lower limits of the linear dynamic range define the limits of quantification (LOQ) of the assay. Gene quantities that lie outside of this range cannot be reliably estimated.

Absolute quantification refers to the practice of estimating gene quantities in unknown samples by comparing their cycle thresholds (Cq) to the calibration curve. Absolute quantification is a reliable method for gene concentrations that fall within the linear dynamic range of the calibration curve. Some qPCR applications, such as quantification of genetically

modified organisms in food, require measurement of gene quantities in samples with concentrations near or below the LOQ.

Measurement of genes near the LOQ can present difficulties and is an active area of research (19,20,38,69) (36,37,39,40). For samples with very low quantities of the target gene, it is common to have one or more of the sample's technical replicates produce no signal or produce an estimate that is below the LOQ. A qPCR reaction with no sign of target amplification could indicate that the gene is truly absent from the sample or that the gene of interest was present in the reaction but failed to amplify above the threshold level of fluorescence by the last cycle of the reaction. Alternatively, no signal could result when the gene of interest was present in the sample but in such a low concentration that the probability of including at least one copy of the gene in the small amount of template added to the reaction was very low (42,43,69,139). In these situations, the general recommendation is to increase the number of replicates to improve the probability of detecting the gene of interest. This may improve the likelihood of detection, but will only improve estimates of gene quantity if some of the replicates are above the LOQ. For large studies with many samples, increasing the number of replicates is not always feasible.

When an analytical method is unable to distinguish true negative samples from samples that are positive at a low level, these samples are censored. Common methods for dealing with censored data are to delete the censored observations prior to analysis or to substitute some value for the censored samples, such as a fraction of the detection limit, or zero. All of these methods have been shown to produce varying degrees of bias, depending on the level of censoring present in the data (81,114,199). One qPCR study recommended that substituting

zero for censored observations will produce less biased estimates than deletion of censored observations (69).

Many studies that have investigated the performance of qPCR near the limits of quantification and detection have been focused at the level of individual samples and their replicates (Berdal, 2001, Berdall, 2008, Burns 2004, Burns, 2005, Burns, 2008a, Burns, 2008b, Burns, 2010, Ellison, 2006). However, for some applications, qPCR is used to quantify nucleic acid sequences for the purpose of estimating population level parameters. For example, in epidemiologic studies of antibiotic resistance, qPCR has been used to estimate the association between antibiotic resistance gene quantities in bacterial community DNA and various risk factors (8,27,52-54,138,176,181,221). In these situations it is common to standardize the qPCR measurements either by back-calculating the gene quantity per qPCR reaction to quantity per sample volume or by reporting the gene quantity relative to a reference gene such as 16S rRNA that is used as a proxy for total bacterial quantity (90,175). In both cases the data are often log transformed to normalize the errors prior to statistical analysis. In this situation, substitution of zero will result in a log transformed value that is undefined and some arbitrarily low number on the log scale must be substituted.

The topic of censored observations in analytical methods has been covered in many fields. In general, simple substitution methods or exclusion of censored observations has the potential to bias results and it has been recommended that those methods should never be used (81,93,114,191,199). Alternative approaches have been proposed, such as parametric methods that randomly impute single values for censored observations by maximum likelihood estimation or regression on order statistics (81). However, these methods are generally not adequate for epidemiological studies where the objective is to relate the quantity of a gene to

risk factors, especially when the data come from more complex study designs such as longitudinal or clustered data (114).

Another option to accommodate censored data is to use a Tobit regression approach where it is assumed that the censored and observed data arise from the same underlying normal distribution and the regression parameters are estimated by maximum likelihood (114,191). Another more flexible alternative is to create a hierarchical model that explicitly includes the censoring in the likelihood function and is estimated using MCMC methods (75,93,114). This last option works by iteratively imputing the censored values, using the imputed values and observed data in a regression to obtain parameter estimates, and then using the parameter estimates to randomly impute new values for the censored data. Over many iterations, distributions of the missing values are created. This approach also permits flexibility in the data structure such as multiple technical replicates clustered within sample.

The objective of this study is to develop a hierarchical model for analyzing censored qPCR data. The performance of the model was compared to substitution of fixed values for censored observations using simulated data representing gene quantities in community bacterial DNA. We hypothesized that this model would produce more accurate estimates of regression population parameters than linear regression performed on simulated data where censored values are substituted with a single value.

METHODS

Rationale for model development

Many applications of qPCR measure bacterial genes in a cultivation independent manner that permits estimation of gene quantity in the entire bacterial community of a sample.

This approach requires extraction of community DNA from a sample such as soil or feces to yield a volume of DNA in solution. A small proportion of that DNA solution is then added to each replicate qPCR reaction. Therefore, the output of the qPCR reaction is gene quantity per volume of template added to the reaction. The model developed for this study estimated the unknown total $\log_{10}$ gene quantity per gram of sample from the observed gene quantities in the individual qPCR replicates, some of which were censored.

For this study, it was assumed that that DNA extraction yielded 1000µl of DNA solution for every sample. From that total volume of extracted DNA, it was assumed that 5µl of template DNA was added to each of three replicate qPCR reactions. The three 5µl aliquots of template and the remaining 985µl (X_i) were assumed to follow a Multinomial(n, p_i) distribution, where n equals the unknown quantity of the gene of interest in the entire sample and p_i is a vector of probabilities corresponding to the proportions of sample added to each of the triplicate reactions and the unused sample (i.e. 0.005, 0.005, 0.005, 0.985). Averaging the quantities of the three triplicate reactions reduces the multinomial distribution to two categories and becomes a binomial distribution with the probability parameter equal to the average of the probabilities for the three triplicates: $X \sim Binomial(n, 0.005)$.

Using the Poisson approximation to the binomial distribution, the model directly estimates the $\log_{10}$ gene quantity per sample from the triplicate qPCR reactions, taking the form:

$$
\begin{aligned}
x_{ij} &\sim Poisson(\lambda_j) \\
\lambda_j &= n_j * p_j \\
n_j &= q * 10^{y_j} \\
p_j &\sim Beta(0.005, 0.995) \\
y_j &\sim Normal(\mu, \sigma) \\
\mu &= \beta_0 + \beta_1 * riskfactor
\end{aligned}
$$

where x_{ij} is the observed gene quantity of replicate *i* from sample *j*. The estimated mean, $\lambda_{j,}$ of the replicates for sample *j* approximates the mean of a binomial distribution $n_j * p_j$ where n_j is the unknown gene quantity of the entire volume of extracted DNA. The Poisson approximation to the binomial improves the efficiency of the model because there are fewer parameters to estimate. The unknown quantity (n_j) is the product of a correction factor (q) for DNA extraction efficiency and the unknown $\log_{10}$ gene quantity (y_j) in the entire sample. All DNA extraction protocols yield less than 100% of the DNA present in a sample due incomplete cell lysis and volume losses during the extraction process (121,126). Here the DNA extraction efficiency is assumed to be fixed at 65% but this value can also be assigned a probability distribution of its own. In addition, it is assumed here that the original samples from which the DNA was extracted were all one gram. For samples of varying starting weight, an additional correction factor can be included to adjust for these variations. The probability of including a gene copy in the 5 µl added to a single qPCR reaction (p_j) is assumed to follow a beta distribution centered around 0.005 to allow for extra-binomial variation in the observed data. The unknown $\log_{10}$ gene quantity (y_j) is assumed to follow a normal distribution with a mean estimated by the regression equation β0 + β1**riskfactor*, where the generic risk factor is a binary variable (1 or 0).

To estimate the quantities of censored observations, the censored observations (x_{ij}) are expressed in terms of the true copy number (z_{ij}). For these simulations it was assumed that the LOQ was 10 copies per qPCR reaction. The censored observations are expressed as the observed value if it is greater than or equal to 10, or ranging from between 0 to 9 if censored:

$$x_{ij} = \begin{cases} z_{ij} & if \ z_{ij} \geq 10 \\ 0-9 \ if & z_{ij} < 10 \end{cases}$$

For uncensored data, the likelihood is:

$$p(x \mid \lambda) = \prod_{i=1}^{n} P\left(x_{ij} \mid \lambda\right)$$

where P is the Poisson probability density function (pdf). For a censored observation, the likelihood is:

$$\Pr\left(x < 10\right) = \Pr\left(z_{ij} \leq 10\right) = \int_{0}^{10} P(z_{ij} \mid \lambda) = \Phi\left(z_{ij} \mid \lambda\right)$$

where Φ is the cumulative density function (cdf) of the Poisson distribution.(75) The likelihood for all of the data is

$$p(x_{ij} \mid \lambda) = \begin{cases} P(x_{ij} \mid \lambda) & if\ x_{ij} > 10 \\ \Phi(10 \mid \lambda)\ if\ 0 \leq x_{ij} \leq 10 \end{cases}$$

In other words, initial values for the censored observations are randomly assigned a probability and corresponding value between 0 and 9 according to the cdf of the Poisson distribution, and the imputed and observed data together are used to estimate the parameters of the model. The estimated parameters are then used to iteratively impute new values for the censored observations and then re-estimate the model parameters. A censored observation with a large estimated λ is more likely to be assigned a value closer to 9 on the following iteration than a censored observation with a very small estimated λ. After a number of iterations, posterior distributions of the parameters, and censored observations are obtained.

Simulation of data

The performance of the model was assessed by testing it using simulated qPCR data. Datasets of 500 samples were generated representing $\log_{10}$ gene quantity per gram of sample.

Each simulated dataset was generated assuming a normal distribution using three different mean values to test the model on datasets with three different degrees of censoring (Table 1). All datasets had a standard deviation of 1.5, and an indicator variable was used to randomly assign a binary risk factor to approximately 50% of the samples in each dataset resulting in gene copy numbers that were on average 0.5 $\log_{10}$ copies higher in the group with the risk factor than the group without the risk factor. Because the simulations were repeated using three different mean values on the $\log_{10}$ scale, the risk factor effect of 0.5 $\log_{10}$ copies ranged from approximately 21 gene copies to 2100 copies per sample.

The simulated samples of $\log_{10}$ gene quantity per gram were transformed to gene quantity per gram and multiplied by 0.65 for assumed losses due to extraction efficiency to yield gene quantity per 1000 µl of DNA extract. To obtain the gene quantity added to each of the triplicate qPCR wells per sample, three 5 µl random samples were drawn from the simulated 1000 µl of DNA extract using a Dirichlet-multinomial distribution, a multivariate generalization of the beta-binomial distribution. The copy number per 1000 µl was used as the size parameter (n) and the four probability parameters each followed a beta distribution centered around 0.005, 0.005, 0.005, and 0.985 respectively. The probability parameters were allowed to follow beta distributions to produce sample triplicates that are more variable than what would be produced if the triplicates followed a binomial distribution with a fixed value for p_i. Based on our experience with real qPCR data, this extra-binomial variation yielded simulated sample triplicates that more closely resembled reality than simulations assuming a binomial variance. This process produced three simulated observations per sample of gene quantity per 5 µl of DNA resulting in a dataset comprised of 1500 total observations for 500 samples. An LOQ of 10 copies, the lowest quantity standard typically used in our qPCR assays, was used as the censoring limit. All simulated observations with values less than 10 were set to missing so they

could be estimated by the model. The simulated data were generated using R version 2.12.2 (144).

The model was run using WinBUGS (version 1.4), called from R with the R2Winbugs package, on 500 simulated datasets for each parameter combination using non-informative normal priors for β0 and β1 and a non-informative gamma prior for the inverse of σ (116,183). The model was run for 5000 iterations with a burn-in of 1000 iterations. Preliminary runs were made using three MCMC chains each with different initial parameter values to confirm adequate mixing among the chains. Afterward, one chain was run per dataset to speed the simulation.

The posterior means of β0, β1, and σ for each iteration were stored. For every parameter the bias, mean square error (MSE), and proportion of 95% credible intervals that included the true values of their respective parameters were calculated (41),

$$\text{bias} = \overline{\hat{\beta}} - \beta$$

$$\text{MSE} = \frac{1}{N-1}\sum_{i=1}^{N}\left(\hat{\beta}_i - \overline{\hat{\beta}}\right)^2 + \left(\overline{\hat{\beta}} - \beta\right)^2$$

For comparison with the hierarchical model, simulations were also performed on 500 datasets generated in the same way as described above but censored observations were replaced with 0, or one-half of the LOQ. Triplicate observations were back-calculated to $\log_{10}$ gene quantity and averaged. For situations where all triplicates were below the LOQ, substitution of 0 resulted in an average gene quantity that was undefined when transformed to $\log_{10}$ copies per gram of sample. The minimum observable $\log_{10}$ average possible is 2.01, when one of the three reactions has a copy number of 1 and the other two are 0. For samples with three censored triplicates, a value 1.00 $\log_{10}$ or 1 gene per gram (average per reaction = 0.00325) was

substituted for the log transformed sample average to approximate a sample where the gene of interest in not detected in any of the replicates. The average $\log_{10}$ gene quantity of each sample was used as the dependent variable in a linear regression with the generic risk factor as the independent variable. The model was run using 500 simulated datasets to generate distributions of each parameter. For each of these three scenarios the bias and MSE were calculated for β0, β1, and percent coverage of 95% confidence intervals were calculated for β0 and β1.

RESULTS

For each simulated dataset, the model estimated posterior densities for each sample replicate that was below 10 gene copies, using information present in uncensored observations. For observations that were part of a sample with three out of three censored observations, the mass of the posterior densities were centered over 0 with posterior means that were less than 1 gene copy (Figure 9). For observations that were part of a sample with two of three censored observations, the densities were more evenly dispersed, with posterior means of approximately 6 gene copies. For observations that came from samples with only one of three censored observations, the densities were centered toward the maximum of the range below the censoring limit, with posterior means of approximately 8 gene copies.

To illustrate how the model estimated the $\log_{10}$ gene copy per gram of sample, the posterior means and 95% confidence intervals were plotted against the true values for one simulated dataset with parameters β0 = 3, β1 = 0.5, σ = 1.5, and a sample size of 50 (Figure 10). For samples with 0, 1, or 2 censored observations, the 95% CI's are very narrow. For samples with 3 censored observations the 95% CI's are very wide but still include the true values for all but the most extreme values of simulated data. In this example, samples with 3 censored

observations had standard deviations approximately 0.8 $\log_{10}$ larger than samples with 0, 1, or 2 censored observations. The difference in precision of estimates among samples with 0, 1, or 2 censored triplicates was small. For example, the difference in the standard deviation between a sample with no censored observations and a sample with a similar true $\log_{10}$ gene quantity and two censored triplicates was approximately 0.02 $\log_{10}$.

The regression parameter estimates for the intercept, β0, represented the average $\log_{10}$ gene quantity per gram for samples that were not associated with the binary risk factor, with true values in each scenario ranging from 1.0 – 3.0. The estimates for β1 represented the average increase in $\log_{10}$ gene quantity per gram for samples that were associated with the risk factor, with true values of 0.5 $\log_{10}$ in every scenario. For all three scenarios, the hierarchical model overestimated β0 by 0.15 – 0.20 $\log_{10}$ gene copies per gram (Table 10). The degree of overestimation increased as the amount of censoring increased. The model underestimated the effect of the risk factor, β1, by -0.03 to -0.05 $\log_{10}$ gene copies per gram with the amount of underestimation decreasing as the degree of censoring increased. The model performed well in its estimation of β1 in all three scenarios; the 95% credible intervals for β1 included the true value in 93% - 95% of the iterations. However, the 95% credible intervals for β0 provided inadequate coverage of that parameter, including the true value in only 72% to 85% of iterations.

Substitution of a value approximating 0 copies resulted in β0 values that were underestimated by a range of -0.15 to -0.30. The estimates of β1 were also underestimated by -0.02 to -0.11 $\log_{10}$ gene copies. Coverage of the 95% confidence intervals of β1 were good (95% - 96%) except for scenario three when the coverage dropped to 87%. As with the hierarchical model, 95% CI coverage for β0 was inadequate for all three scenarios (21% - 63%). Substitution

of one half the LOQ resulted in β0 that were over estimated by 0.69 to 2.23 $\log_{10}$ gene copies and β1 estimates that were underestimated by -0,24 to -0.45. Very few of the 95% confidence intervals covered the true values of β0 and β1 when censored values were replaced by one-half of the LOQ.

For scenarios 1 and 2, the hierarchical model performed equally well as substitution of a value approximating 0 in estimation of β1 (Table 9). Both approaches produced similar biases, MSE's and 95% CI coverage. For scenario 3, the bias of the substitution of 0 approach increased and the 95% CI coverage dropped below acceptable levels. This was likely due to the fact that the proportion of observations that were censored were so high that there was not enough information in the scenario 3 datasets to adequately estimate β1. Both the hierarchical model and the substitution of 0 method produced inadequate estimates of β0 in all three scenarios. However, the hierarchical model performed slightly better than the substitution of zero method for scenarios 1 and 2 while the reverse was true for scenario 3. Substitution of one-half the LOQ performed the worst in all three scenarios.

DISCUSSION

The performance of the hierarchical model was approximately the same across all three scenarios; the estimates of the regression coefficient for the risk factor were close to the true value and the regression intercepts were overestimated in every scenario. Substitution of 0 also produced accurate estimates of the risk factor coefficient for scenarios 1 and 2, but in scenario 3 when the level of censoring was extremely high, substitution of 0 was no longer able to adequately estimate the risk factor coefficient. As with the hierarchical model, substitution of 0 inadequately estimated the intercept but, in contrast to the hierarchical model, the intercept was underestimated. Substitution of one-half the LOQ performed poorly in all three scenarios.

For most studies that investigate the relationship between gene quantity and a risk factor, the regression coefficient for the risk factor is the parameter that is of primary interest. Therefore, it is encouraging that, of the three approaches, the hierarchical model provided the most accurate estimates of the risk factor effect overall.

It was observed that the model underestimated the true simulated values when gene quantity was greater than 4 $\log_{10}$ (Figure 10). However, the 95% CI's of the posterior estimates continued to include the true value for large quantity samples except those greater than approximately 6 $\log_{10}$. This decline in performance for high quantity samples is most likely due to the law of small numbers which provides the basis for the use of the Poisson distribution to approximate a binomial distribution(105). The Poisson approximation to the binomial is generally described as appropriate when n is very large and p is very small and when $n*p < 10$. This condition is true when the $\log_{10}$ gene quantity is less than approximately 3.5 (i.e. $10^{3.5} * 0.65 * 0.005 = 10.2$). For the data sets used in these simulations the percentage of large quantity samples was small and not likely to affect the regression parameter estimates. The 95% CI's of estimated $\log_{10}$ gene quantity failed to include the truth only for a small percentage of samples with greater than approximately 6 $\log_{10}$ copies.

In our experience, it is common to measure some antibiotic resistance genes by qPCR at levels greater than 6 $\log_{10}$ per gram. With these high quantity genes, the amount of censoring is very low if not completely absent and special analytical methods are not necessary. However for other genes, the amount of censoring can be very high, similar to the simulated data in this study. For those low quantity genes, samples with greater than 6 $\log_{10}$ gene copies are relatively rare. In this situation, using the Poisson approximation to the binomial should be appropriate.

The general consensus for using qPCR to measure low quantity genes is that the number of replicates should be increased to improve the precision of estimates. For large studies this is not always feasible. Additionally, in epidemiologic studies involving qPCR, the main objective is often to measure an association between gene quantity and a risk factor in a population. In this case, precise estimation of gene quantity in individual samples is secondary to estimation of population parameters. Under the assumptions stated in the methods, this model provides a more accurate estimation of population parameters for moderate to highly censored qPCR data then do substitution of values close to 0 or one-half of the LOQ. The use of a hierarchical model and MCMC methods has the added advantage of being able to accommodate additional levels to account for more complicated study designs that are common in epidemiology such as longitudinal studies with repeated measures within individuals or multilevel data such as animals clustered within herds.

Table 9. Parameter values used to generate simulated data of $\log_{10}$ gene copies per gram of sample, the mean percentage (range) of qPCR reactions in each data set that fell below the limit of quantification, and the mean number of samples that have 0,1,2, or 3 replicates censored.

Scenario	True Parameters	Mean (range) percent of censored observation	Mean number of censored observations per sample (0, 1, 2, 3)
1	$\beta0=3$, $\beta1=0.5$, $\sigma=1.5$	57% (51% - 62%)	189, 27, 26, 256
2	$\beta0=2$, $\beta1=0.5$, $\sigma=1.5$	79% (75% - 86%)	84, 18, 20, 378
3	$\beta0=1$, $\beta1=0.5$, $\sigma=1.5$	93% (89% - 96%)	27, 8, 10, 455

Table 10. Bias, mean square error, and percent coverage of 95% confidence intervals of regression parameters estimated using the hierarchical model described in the text, and by linear regression with censored observations (<10 copies/reaction) replaced by 0.00325 (5 copies per sample). The number of simulations was 500 for each scenario.

Scenario 1	**Bias**	**MSE**	**95% CI coverage**
Model			
β0	0.15	0.03	72%
β1	-0.05	0.02	95%
Substitute 0.00325*			
β0	-0.23	0.06	45%
β1	-0.06	0.02	95%
Substitute 5			
β0	0.69	0.47	0%
β1	-0.24	0.06	16%
Scenario 2			
Model			
β0	0.19	0.06	79%
β1	-0.03	0.03	94%
Substitute 0.00325*			
β0	-0.30	0.10	21%
β1	0.02	0.02	96%
Substitute 5			
β0	1.37	1.87	0%
β1	-0.37	0.14	0%
Scenario 3			
Model			
β0	0.20	0.21	85%

β1	-0.03	0.06	93%
Substitute 0.00325*			
β0	-0.15	0.03	63%
β1	-0.11	0.03	87%
Substitute 5			
β0	2.23	4.99	0%
β1	-0.45	0.20	0%

* The log of zero is undefined. To approximate an average gene quantity of 0 per sample, a value was substituted that, under the assumption of 65% extraction efficiency, back calculates to 1 gene copy per gram or 0 $\log_{10}$ copies.

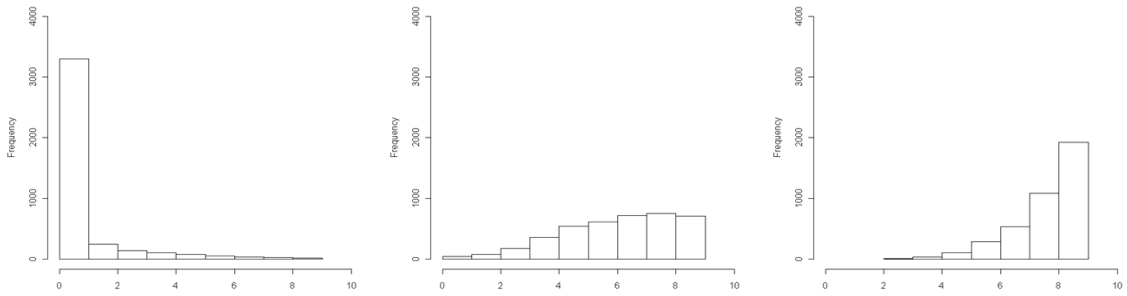

Figure 9. Posterior densities of three censored observations coming from samples with (from left to right) three, two, or one out of three observations that were censored. The true sample averages from left to right were 0, 8.3, and 14.3 gene copies.

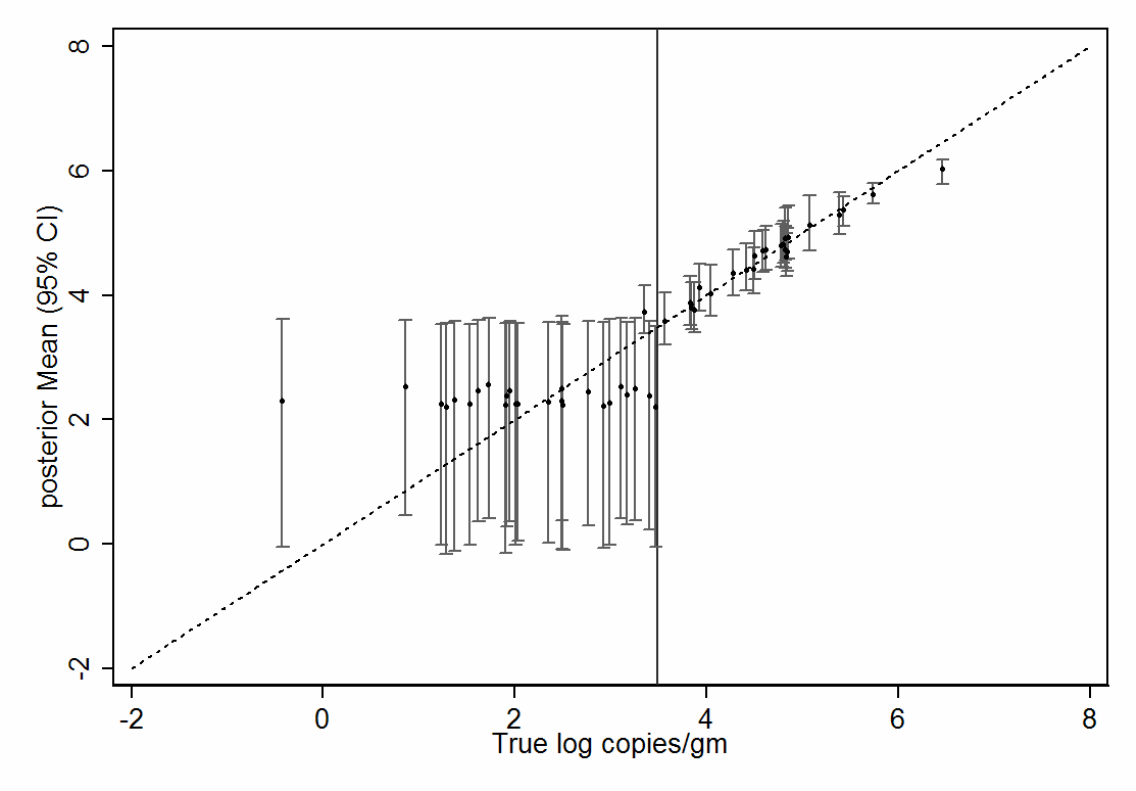

Figure 10. Posterior means and 95% credible intervals of log gene quantity versus the true value for a simulated data set (n=50) generated from a normal distribution with the parameters β0=3, β1=0.5, σ=1.5. The vertical line shows the censoring limit (mean of 10 copies per sample = 3.488 $\log_{10}$ copies per gram).

APPENDIX

```
######################
# R code for simulations    #
######################
library (R2WinBUGS)

# generate 500 simulated data sets of 500 samples each
n.fake<-500
n=500

# place holders for outputs
b0.mean<-rep(NA, n.fake)
b1.mean<-rep(NA, n.fake)
sigma.mean<-rep(NA, n.fake)
b0.sd<-rep(NA, n.fake)
b1.sd<-rep(NA, n.fake)
sigma.sd<-rep(NA, n.fake)
b0.err<-rep(NA, n.fake)
b0.se<-rep(NA, n.fake)
b1.err<-rep(NA, n.fake)
b1.se<-rep(NA, n.fake)
sigma.err<-rep(NA, n.fake)
sigma.se<-rep(NA, n.fake)
b0.2.5<-rep(NA, n.fake)
b0.97.5<-rep(NA, n.fake)
b1.2.5<-rep(NA, n.fake)
b1.97.5<-rep(NA, n.fake)
sigma.2.5<-rep(NA, n.fake)
sigma.97.5<-rep(NA, n.fake)
mn_pcntcen<-rep(NA, n.fake)
y.mean<-rep(NA, n)

# Loop for each of the 500 simulations
for (s in 1:n.fake){
        tx=rbinom(n,1,0.5)
        m=3+0.5*tx
        y1=rnorm(n,m,1.5)       # generates log simple quantity per gram
        q=0.65
        y2=q*10^y1              # transform quantity from the log scale
        y3=round(y2)
        y3[y3>99999999]<-99999999 # round quantity and cap at high value

# The following code takes the vectors of gene quantity per sample and the risk factor into a
# matrix of gene quantity  per 5, 5, 5, and 985 microliters of DNA extract, a sample ID variable, a
# risk factor variable (tx).

        dir=function(p){ g=rgamma(length(p),shape=p,scale=1); g/sum(g)}
```

```
y4=matrix(rep(0,n*3),n,3)
for(i in 1:n){y4[i,1:3]=rmultinom(1,y3[i],dir(c(5,5,5,985)))[1:3]}
data<-cbind(y4, tx)
data<-as.data.frame(data)
data<-reshape(data, direction="long", varying=(1:3), v.names="well")
w<-data$well
t<-data$tx
sample<-data$id
z <- w
z[z<10] <- NA
w[w<10] <- 9
data<-data.frame(sample, w, z, tx)
sortdata <- data[order(sample) , ]
sample<-sortdata$sample
w<-sortdata$w
z<-sortdata$z
t<-sortdata$t
J<-1500
I<-500


# specify the data, initial values, and model to call WinBUGS from R

sim.data <- list(I=I, J=J, z=z, w=w, sample=sample, tx=tx)
sim.inits <- function (){
        list (tau=1, b0=3, b1=1)
        }
sim.parameters <- c("sigma", "b0", "b1", "y", "z")


sim.fake <- bugs(sim.data, sim.inits, sim.parameters, "sim_model.bug",
        n.chains=1, n.iter=5000, n.burnin=1000, n.thin=1, n.sims=4000,
        bugs.directory="c:/Program Files/WinBUGS14/",
        working.directory="Insert path of WinBUGS working directory ",
        clearWD=FALSE, DIC=FALSE, debug=FALSE)

# Store outputs from each simulation
attach.bugs(sim.fake)
b0.mean[s]<-mean(b0)
b1.mean[s]<-mean(b1)
sigma.mean[s]<-mean(sigma)
b0.err[s]<-b0.mean[s]-3
b0.se[s]<-b0.err[s]^2
b1.err[s]<-b1.mean[s]-0.5
b1.se[s]<-b1.err[s]^2
sigma.err[s]<-sigma.mean[s]-1.5
sigma.se[s]<-sigma.err[s]^2
b0.sd[s]<-sd(b0)
```

```
b1.sd[s]<-sd(b1)
sigma.sd[s]<-sd(sigma)
b0.2.5[s]<-quantile(b0, .025)
b0.97.5[s]<-quantile(b0, .975)
b1.2.5[s]<-quantile(b1, .025)
b1.97.5[s]<-quantile(b1, .975)
sigma.2.5[s]<-quantile(sigma, .025)
sigma.97.5[s]<-quantile(sigma,.975)
}

#################
# WinBUGS code    #
#################
model{
for(j in 1:J){
        upperlimit[j]<-10000000+(-10000000+9)*equals(w[j], 9)
        z[j] ~ dpois(lambda[sample[j]]) I( , upperlimit[j])
}
for(i in 1:I){
        lambda[i] <- q[i]*pow(10,y[i])*p[i]
        p[i] ~ dbeta(5,995)
        q[i] <- 0.65
        y[i] ~ dnorm(mu[i],tau)
        mu[i] <- b0+b1*tx[i]

}

 tau ~ dgamma(0.001, 0.001)
 sigma <- 1/sqrt(tau)
 b0 ~ dnorm(0,0.001)
 b1 ~ dnorm(0,0.001)
}
```

Chapter 4: Quantitative measurement of *bla*$_{CMY-2}$ in a longitudinal observational study of dairy cattle treated with ceftiofur

INTRODUCTION

There is concern that a relationship exists between antibiotic use in livestock production and the emergence, spread, and persistence of antibiotic resistance in pathogens or commensal bacteria that can potentially transfer resistance genes to pathogens (16). Most of the criticism of antibiotic use in agriculture has been directed toward use of antibiotics for growth promotion or disease prevention. However, it is also important to understand the impact that therapeutic doses of antibiotics for treatment of disease have on resistance because disease treatment typically involves higher doses of antibiotic over shorter time spans than those used for growth promotion or disease prevention. In addition, disease treatment often involves antibiotics that are critical to both human and animal health.

Third and fourth-generation cephalosporins are a class of antimicrobials that have been identified as critical to both human and animal health (72). Ceftiofur is the only-third generation cephalosporin that is licensed in the U.S. for use in livestock. In cattle, ceftiofur is currently labeled for the treatment of bovine pneumonia, interdigital necrobacillosis, acute metritis (in cows 0 to 14 days postpartum), and mastitis (1). All administrations of ceftiofur are by prescription of a licensed veterinarian only. Bacterial isolates that are resistant to ceftiofur can be cross-resistant to ceftriaxone, a third-generation cephalosporin that has a variety of applications in human medicine and is the primary choice of treatment of invasive salmonellosis in children (85).

Among the numerous genes that have been identified as encoding proteins that convey resistance to cephalosporins, *bla*$_{CMY-2}$, a β-lactamase encoding gene, is commonly associated with resistant *Salmonella* isolates in North America (11,142,217,222). Plasmid transfer of *bla*$_{CMY-2}$ between *Salmonella* and *Escherichia coli* has been demonstrated and *bla*$_{CMY-2}$ has been identified in an *E. coli* 0157:H7 isolate obtained from a human (213,218).

Surveillance data has shown an increase in the frequency of cephalosporin resistance among *Salmonella* isolates obtained from both humans and animals (68,78,145,213). In the summer of 2009 an outbreak of cephalosporin resistant *Salmonella* in western states led to two USDA recalls of ground beef (200,201). Concern over this increase and evidence of cross-resistance among cephalosporins led to an FDA ban on extra label use of cephalosporins in food-producing animals set to take effect in November, 2008. However, the FDA revoked the ban prior to enactment to allow consideration of information that was received during the comment period (196).

Despite evidence of an association between ceftiofur use and cephalosporin resistance in some bacteria, significant gaps remain in our understanding of this relationship. A topic that has not been adequately addressed is the degree and duration of amplification of the resistance pool following ceftiofur use (110,111). One reason for this relates to the methods of susceptibility testing that are most commonly used. Until recently, studies of ceftiofur resistance in agricultural samples have relied primarily on cultivation-based methods of testing for resistance (59,80,94,113,171,192). A major downside of cultivation-based methods of susceptibility testing is that they are highly selective for particular species of bacteria and depend on the type of media being used. Most bacterial species cannot be grown in the laboratory. Therefore the majority of types of bacteria in a sample are overlooked when

cultivation methods are used. In part because of this, most studies of antibiotic resistance have focused on pathogens that can be cultured and ignore the potential reservoir of resistance that resides among the commensal bacterial population. Given the ability of bla_{CMY-2} to be shared among different bacterial species, it is necessary to account for this reservoir when measuring resistance (218).

One method of studying the non-cultivable reservoir of bacteria is to use culture-independent extraction of all bacterial DNA from a sample. One study used this method combined with end-point PCR to detect the presence of bla_{CMY-2} in the community DNA of fecal samples of dairy cattle that were treated with ceftiofur (171). The use of community DNA and PCR provided information regarding the presence of bla_{CMY-2} prior to treatment that was not evident in test results for phenotypic resistance to ceftiofur of cultured *E. coli* isolates.

To accurately assess the degree and duration of resistance gene amplification following antibiotic treatment, a quantitative method of measuring resistance in the entire bacterial population of a sample is necessary. Quantitative real-time PCR (qPCR) combined with community DNA extraction can be used to measure the antibiotic resistance gene load in the entire bacterial community of a sample. The downside of this method is that these measurements cannot be related to any particular species of bacteria.

A recent study used community DNA extraction and qPCR to measure bla_{CMY-2} quantities in fecal samples from feedlot cattle that received 3 different treatment regimens of ceftiofur and were housed together with untreated cattle (8). This study found that overall group means of bla_{CMY-2} quantity were higher in treated cattle than in untreated cattle. The differences between treated and control animals over time varied by treatment regime. The group that received the lowest single dose of ceftiofur (4.4 mg/kg) had significantly higher quantities of bla_{CMY-2}

compared to the control group on two sampling days following treatment, on the fourth and seventh day post-treatment. The group that received a single dose of 6.6 mg/kg was not significantly different than the control group on any day of the study. The group receiving three doses of 6.6 mg/kg spaced over 14 days saw a significant difference versus the control group in bla_{CMY-2} quantities the day following the 2nd dose but a difference was not observed again until 5 days and 8 days following the final dose. On the last sampling day, 14 days after the final dose, there was no difference between the treated and untreated animals. Therefore, the durations of elevated bla_{CMY-2} quantities for two of the three treatment regimens were approximately one week following the cessation of treatment.

Previous studies of cephalosporin resistance have found that although the correlation between cephalosporin resistance and the presence of bla_{CMY-2} is often very high, the prevalence of cephalosporin resistance in cultured isolates from food animals is generally low (11,59,68,171,192). Two studies in the previously mentioned population of treated and untreated feedlot cattle found higher prevalences of cephalosporin resistant isolates and higher quantities of bla_{CMY-2} than has been reported elsewhere but these seem to be the exception (8,113). The majority of evidence suggests that bla_{CMY-2} may not be present in large quantities in fecal samples of food animals and this can present a challenge for measurement of bla_{CMY-2} by qPCR. In qPCR assays, samples are often run in triplicate as a way to assess intra-assay variation. For samples with low concentrations of the gene of interest, it is common to have one, two, or all three of the triplicates show no evidence of the target gene. A qPCR reaction with no sign of amplification could indicate that the gene is truly absent from the sample or that the gene of interest was present in the reaction but failed to amplify due to poor PCR reaction conditions such as the presence of inhibitors. Alternatively, a negative result could indicate that the gene of interest was present in the sample but in such a low concentration that the probability of

including at least one copy of the gene in the small amount of community DNA added to the qPCR reaction was very low (43,69,139). Additionally, qPCR assays have limits of quantification (LOQ) that are determined by the linear ranges of the standard curves used to estimate gene quantity. There may be evidence for some observations below the LOQ (e.g. melt curve analysis and sequencing) that the gene of interest is present in a sample but in a low quantity that cannot be reliably estimated. Estimates of gene quantities outside of the linear range as well as observations with no detected signal are censored. Therefore, sample triplicates of $bla_{\text{CMY-2}}$ quantities measured by qPCR could be comprised of combinations of observations that are non-censored, censored but with evidence that $bla_{\text{CMY-2}}$ is present, and censored observations with no evidence of $bla_{\text{CMY-2}}$.

Deletion of censored observations is not an acceptable option because resulting statistical estimates will most likely be biased upward. Additionally, observations that fall below the limit of quantification, including observations with no signal detection and observations with evidence that the gene of interest is present, contain information that can be useful for trying to detect early emerging resistance or when assessing the effects of antibiotic treatment on the abundance of rare resistance genes.

The objectives of the current study are to use community DNA extraction and qPCR to quantify $bla_{\text{CMY-2}}$ in the feces of matched ceftiofur treated and untreated dairy cattle and to analyze the data using a hierarchical model previously developed to analyze low quantity qPCR data. We hypothesized that short term therapeutic treatment may result in a temporary increase in $bla_{\text{CMY-2}}$ quantity during and shortly after treatment but that average $bla_{\text{CMY-2}}$ quantity will not differ between treated and untreated animals as the time post-treatment increases.

METHODS

Study Design

An observational study of dairy cattle was conducted on an Illinois farm as previously described (171). Briefly, five cows were treated for infertility due to *Leptospira borgpetersenii* serovar Hardjo-bovis. Following the veterinarian's recommendation, these five cattle were treated with ceftiofur according to label instructions (2.2mg/kg, intramuscularly, once daily for 5 days). A cohort of 5 untreated cows in the same milking string was randomly selected. These cows were individually matched to the treated cows based on lactation number.

All five treated cows started therapy at the same time. Treated and untreated animals were sampled prior to (days 0 and 1), during (days 3 and 5), and after (days 6 to 12, 15, and 26) ceftiofur therapy. On day 1, sample collection occurred approximately 1 hour before the first ceftiofur injection was administered. Fecal samples were collected from the rectum of each cow, stored in sterile Whirl-pak bags, and transported on ice until processing (within 4 h of collection).

DNA extraction from feces

DNA was extracted from each fecal sample using the QIAamp DNA stool mini kit and a modified protocol as previously described (137). Briefly, the following modifications were made to the manufacturer's recommended protocol: 0.1 g of sample was used instead of 0.2 g; all centrifugation steps were increased by 30 s; samples were incubated at 95°C instead of 70° during the lysis step (no. 3); the volume of proteinase K was increased to 20 µl; the incubation step (no. 12) was changed from 70°C for 10 min to 55°C for 30 min; and the two wash steps (no. 15 and 16) were repeated. These modifications were found to produce a cleaner DNA preparation from fecal samples without a loss of sensitivity.

Standard preparation

For absolute quantification of *bla*$_{CMY-2}$ in fecal samples, plasmid standards were created by cloning a single copy of the *bla*$_{CMY-2}$ amplicon into the pDrive cloning vector (PCR cloning kit, Qiagen). Plasmid DNA containing the cloned gene was purified using a QIAprep Spin Miniprep Kit. The purified plasmid was checked by PCR using SP6 and T7 primers to assure the gene was present as a single copy. The purified plasmid was quantified by A_{260} and the copy number per µl was calculated based on the size of the plasmid plus the insert. Aliquots of the standards, diluted in nuclease free water to concentrations of 1 x 10^{10} copies per 5µl, were stored at -20°C. For each qPCR experiment a new aliquot was thawed and 10-fold serial dilutions were made in a 1:25 mixture of BSA and nuclease free water down to 1 x 10^{1} copies per 5 µl. Addition of BSA to the dilutions was found to improve the sensitivity of the assay. A range of 1 x 10^{5} copies per 5µl to 1 x 10^{1} copies per 5 µl was used for the qPCR experiments.

Real-time PCR reaction and quantification

We designed, optimized and validated with nucleotide sequencing the primers 5' CAG CAT CTC CCA GCC TAA TC -3', and 5' GAA GCC CGT ACA CGT TTC TC -3'. Primers were designed with Primer3 software(153) using the sequences of the plasmid-borne *bla*$_{CMY-2}$ gene in *E. coli*. QPCR reactions were performed on the unknowns on a Strategene Mx3000p system using Brilliant® II SYBR® Green qPCR mix according to the following recipe: 12.5µl SYBR Green master mix, 0.5µl (300nM) of each primer, 1 µl of BSA, 0.375 µl of ROX reference dye (1:500 dilution in nuclease free water), 5.125 µl of nuclease free water, and 5 µl of plasmid standard or unknown community DNA for a total volume of 25 µl per QPCR reaction. The master mix for the plasmid standards followed the same recipe as the unknowns with the exception that water was substituted for the BSA, which had been previously added to the standards during the serial

dilution step. The reaction conditions for the amplification were 95°C for 10 min and 40 cycles of 94°C for 30 sec 60°C for 1 min and 72°C for 30 sec each. Fluorescence was measured at the end of the extension phase of each cycle. Following the last cycle a continuous dissociation curve for each sample was created by measuring fluorescence at short temperature intervals ranging from 60°C to 95°C. Standards and no template controls were included with all of the assays and all standards, unknowns, and controls were run in triplicate.

Quantification of bla_{CMY-2} in unknown samples

The raw data of qPCR assays consist of a cycle threshold (Cq), the cycle at which the level of fluorescence in a sample rises to a level that is significantly higher than background levels. Individual thresholds for each run were calculated by a background-based algorithm for multiple run projects (MxPro v.4.1 default method) so that samples from different assays could be directly compared. The dissociation curves for all samples were examined to confirm that only one peak at the correct melt temperature was present, providing evidence that no non-specific amplification occurred. Gel electrophoresis and sequencing were performed on a subset of PCR products to confirm that only bla_{CMY-2} was amplified in samples with the correct melt temperature. The log-scale amplification plots were inspected to confirm that all samples with an amplified product were in the exponential phase of amplification when the threshold was crossed. Any spurious results due to non-specific amplification or well evaporation were excluded prior to threshold calculations.

A single consolidated standard curve was created by ordinary least squares regression and the quantities of bla_{CMY-2} in the unknowns were estimated from the standard curve. MxPro (v 1.4) software was used to calculate thresholds, Cq's, the consolidated standard curve, and bla_{CMY-2} quantities in the unknowns.

Statistical analysis

The estimates of gene quantity per 5 µl of sample DNA extract, measured in triplicate, were entered into a previously described model designed to analyze censored qPCR data that was adapted for repeated measurements (32). The model estimates the mean $\log_{10}$ gene quantity in the 0.1g fecal sample from the observed triplicate gene quantities. The model assumes that the three 5 µl aliquots of DNA extract used in the qPCR reactions and the remaining 185 µl of unused DNA solution are assumed to follow a multinomial $X_i \sim Multinomial(n, p_i)$ distribution, where n equals the unknown quantity of $bla_{CMY\text{-}2}$ gene copies in the entire sample and p_i is a vector of probabilities corresponding to the proportions of sample added to each of the triplicate reactions and the proportion of unused sample (i.e. 0.025, 0.025, 0.025, 0.925). Averaging the quantities of the three triplicate reactions reduces the multinomial distribution to two categories which becomes a binomial distribution with the probability parameter equal to the average of the probabilities for the three triplicates (i.e. $X \sim Binomial(n, 0.025)$). The unknown $\log_{10}$ gene quantity per 0.1g of sample (y_j) that is estimated by the model is assumed to follow a Normal distribution with a mean (µ) estimated by the regression equation:

$$y_i = \beta_{0j} + \beta_{1j} * day + \beta_2 * treatment + \varepsilon_{ij}$$

Where β_{0j} is a random intercept for animal, β_{1j} is a random slope for time, and β_2 is a time-varying indicator for treatment (75,125).

The model parameters were estimated by MCMC methods using WinBUGS v1.4 (116). A censoring limit of 10 copies per reaction was used, corresponding to the limit of quantification as determined by dilution assays. For each iteration of the simulation, values for the censored observations are randomly assigned a value between 0 and 10 gene copies. For some censored

observations, there was evidence from melt curves and sequencing of the PCR product that the gene of interest was present in the sample in some unknown quantity below the censoring limit of 10 quantities. These observations were randomly assigned values between 1 and 10 copies to reflect the fact that they were positive but censored. The probability that a censored observation was assigned a value closer to the censoring limit of 10 copies increased with the number of uncensored observations in the set of triplicate observations for each sample. For example, a censored observation that was part of a triplicate set that included two uncensored observations had a higher probability of being assigned a value near 10 copies than a censored observation that was part of a set of three censored triplicates. The imputed and observed data are both used to estimate the parameters of the model. Non-informative normal priors were given to all of the coefficients and non-informative gamma priors were used for the overall precision (inverse of the variance), the precision of the sample means, and the precision of the animal random effects. The model was run with 3 chains, each with different initial values, for 5000 iterations with a burn-in of 1000 iterations.

RESULTS

A total of 390 qPCR reactions representing 130 fecal samples were run in six assays. A threshold of 0.15 was used for the consolidated standard curve which had a calculated efficiency of 1.01, R^2 of 0.990, and y-intercept of 35.78. The efficiencies of the six individual standard curves had a mean of 0.9905 (95% CI 0.9902 – 0.9908). Among the 390 triplicate reactions that were performed, 46 (11%) were excluded prior to threshold calculations because their dissociation curves showed evidence of primer dimer formation or some other non-specific product. No single sample had all three of its triplicates excluded for this reason.

Among the remaining 354 reactions, 233 (60%) had no Cq and showed no sign of any amplification. The remaining 122 reactions had dissociation curves that showed a single product with the correct melting temperature within a range of 85.6°C to 86.2°C (Figure 11). The *bla*$_{CMY-2}$ copy number per qPCR reaction ranged from 41 copies down to 1 copy. Five reactions had copy numbers estimated by the standard curve to be less than 1. Reactions with gene quantity estimated to be less than 10 or with no Cq (84% of the observations) were considered censored and their values were set to missing so that they could be imputed by the model. There was one sample from the untreated group on the first day of the study (Day 0) where the quantities of all three triplicates were at least 10-fold higher than all other samples. Including this outlier in the analysis had a large influence on the results; therefore the results presented here exclude the triplicates from that one sample.

Of the 129 fecal samples analyzed, 79 (61%) had at least one of its triplicates test positive for *bla*$_{CMY-2}$. At least 2 cattle from each treatment group were positive for *bla*$_{CMY-2}$ on every sampling day of the study except for days 8 and 26 when only one positive sample was detected in the untreated group. Every animal in the study tested positive for *bla*$_{CMY-2}$ at least one time but no animals were positive on every day of the study. Five of ten samples from treated animals and four of 10 samples from untreated animals were positive on the two days prior to treatment.

The crude *bla*$_{CMY-2}$ quantities estimated by the standard curve increased in both the ceftiofur treated and untreated groups during the treatment period and then returned to near pre-treatment levels on the first day post-treatment (Figure 12). Gene quantities were variable in both groups for the remainder of the post-treatment phase of study. Gene quantities during

the post-treatment period were similar to pre-treatment levels in both groups with the exception of a one day spike in bla_{CMY-2} quantity on day 11 in the treatment group.

The overall trajectories in bla_{CMY-2} quantities over time were essentially flat in both groups; when included in the model the random coefficients for time were slightly negative with 95% credible intervals that all included zero (data not shown). Therefore, the random slopes for time were removed from the final model, which included random intercepts for animal and a covariate for treatment (Table 11). Treatment with ceftiofur was significantly associated with bla_{CMY-2} quantities during treatment (Figure 13). The animal level random intercepts were very similar, and there were no apparent differences between the two groups in baseline bla_{CMY-2} levels. The Median of the posterior intraclass correlation coefficient for the animal random intercept (ICC = $\sigma^2_{animal}/ \sigma^2_{animal} + \sigma^2_{y}$) was 0.23, indicating that between animal variation accounted for 23% of the total variance in the data.

DISCUSSION

The previous study of this cohort by Singer et al. (2008) presented several pieces of evidence to support the conclusion that bla_{CMY-2} was present in both treatment groups throughout the course of the study (171). The authors concluded that the higher frequency of resistant *E. coli* that was observed in the treated group was likely due to a temporary reduction of a much larger ceftiofur susceptible *E. coli* population that permitted the detection of resistant *E. coli*. The authors also showed that the genetic diversity of the *E. coli* population at the end of the study was the same as it was prior to treatment suggesting that resistant *E. coli* did not exploit the temporary competitive advantage offered by the ceftiofur treatment. The results of our analysis showed a statistically significant spike in the quantity of bla_{CMY-2} during the treatment period, indicating that not only was it temporarily easier to detect bla_{CMY-2} in cultured

E. coli isolates from treated animals but the quantity of the gene may have temporarily increased in the bacterial population as well.

An interesting difference between our results and the results from the study of bla_{CMY-2} quantities in feedlot cattle is the higher quantities of bla_{CMY-2} that were observed in all treated and untreated groups in that study (8). The lowest group mean that was reported in their data was nearly 2 $\log_{10}$ higher than the highest group mean that we observed. The data presented here included a high proportion of censored observation and these results are consistent with what was observed in this cattle population when tested for bla_{CMY-2} by end-point PCR of community DNA (171). It is unknown to what degree reactions below the LOQ affected the results of the feedlot study. In addition, the quantities reported here were estimated assuming 65% DNA extraction efficiency which may be overly optimistic (130).

Another possible explanation for the difference between the two studies may relate to study design. Our study was an observational study with five treated animals mixing freely among a herd of approximately 150 milking cows. The feedlot cattle study was an experimental design of 61 animals with approximately half receiving treatment where 50% of 60 cattle were treated with ceftiofur. The higher density of treated animals in the experimental study may have resulted in more shedding of resistant bacteria that could spread to other cattle in the study population (79). However, it may also be the case that bla_{CMY-2} quantities are generally higher in feedlot cattle than in dairy cattle due to differences in their respective environments and management methods. The prevalence of phenotypic resistance in *E. coli* from that population of feedlot cattle were also higher than what was observed in the dairy cattle in this study (113).

It is unlikely that the lower *bla*$_{CMY-2}$ quantities in this study were due to the presence of PCR inhibitors in our samples. Both this study and the feedlot cattle study extracted DNA using the same commercial kit. Our extraction method was modified to further reduce the number of inhibitors present in the extracted DNA. These modifications reduce the yield of DNA during extraction but DNA was still present in sufficiently large concentrations (as measured by A_{260}, data not shown) that sensitivity should not have been reduced (137). Also, the qPCR efficiency and y-intercept of a standard curve created from standards mixed with fecal DNA did not provide any evidence of a loss in efficiency or sensitivity (data not shown).

The hierarchical model that was used to analyze these data offers some advantages over other methods that have been proposed. The R package NADA: Nondetects and Data Analysis offers multiple methods of producing summary statistics for censored data, along with an ANOVA model for censored data estimated by MLE (81,144). However, this package does not offer an ANOVA model for repeated measures. The method proposed by Jacqmin-Gadda et. al. (2000) does accommodate censored data and repeated measurements (93). However, the method proposed here is more flexible, allowing for nested random effects (e.g. triplicate measurements nested within sample nested within animal), and adjustment for samples that are censored but positive for the gene of interest. Performing qPCR on many replicates of the same sample may be preferred method for the precise measurement of low concentration target genes. However, for large studies, running many replicates is not always feasible. Simple substitution of a value such as a fraction of the censoring limit has repeatedly been shown to produce biased results in a wide variety of laboratory assays. Discarding the censored data is also an unacceptable option. In the case of this data set, the majority of observations would have to be removed and there is information contained in these observations that can be useful

for studying the relationship between ceftiofur use and *bla*$_{CMY-2}$ quantities. The method used in this study provides an alternative way of analyzing low quantity qPCR data.

This study demonstrated that treatment of diseased dairy cattle with a therapeutic dose of ceftiofur resulted in a transient increase in the *bla*$_{CMY-2}$ gene in the lower intestinal bacterial population of those cattle. Gene quantities returned to pre-treatment levels immediately after treatment ended suggesting that treatment did not have a lasting effect on the bacterial population. This conclusion is in agreement with a previous study of these cattle that focused on ceftiofur resistance of cultured *E. coli* (170). We also observed a slight increase in *bla*$_{CMY-2}$ quantity in untreated cattle which may be indicative of transmission of resistant bacteria among these cattle. Resistant *E. coli* were not isolated from untreated animals in the previous study suggesting that this cultivation independent approach focusing on community DNA may be a more sensitive method for measuring low levels of antibiotic resistance than one that focuses on particular species of bacteria.

Table 11. Posterior means and 95% credible intervals for treatment and intercept in dairy cattle treated with a 5-day course of ceftiofur and untreated dairy cattle

Variable	mean	95% CI
Intercept	2.33	2.22 – 2.44
Treatment	0.39	0.18 – 0.61

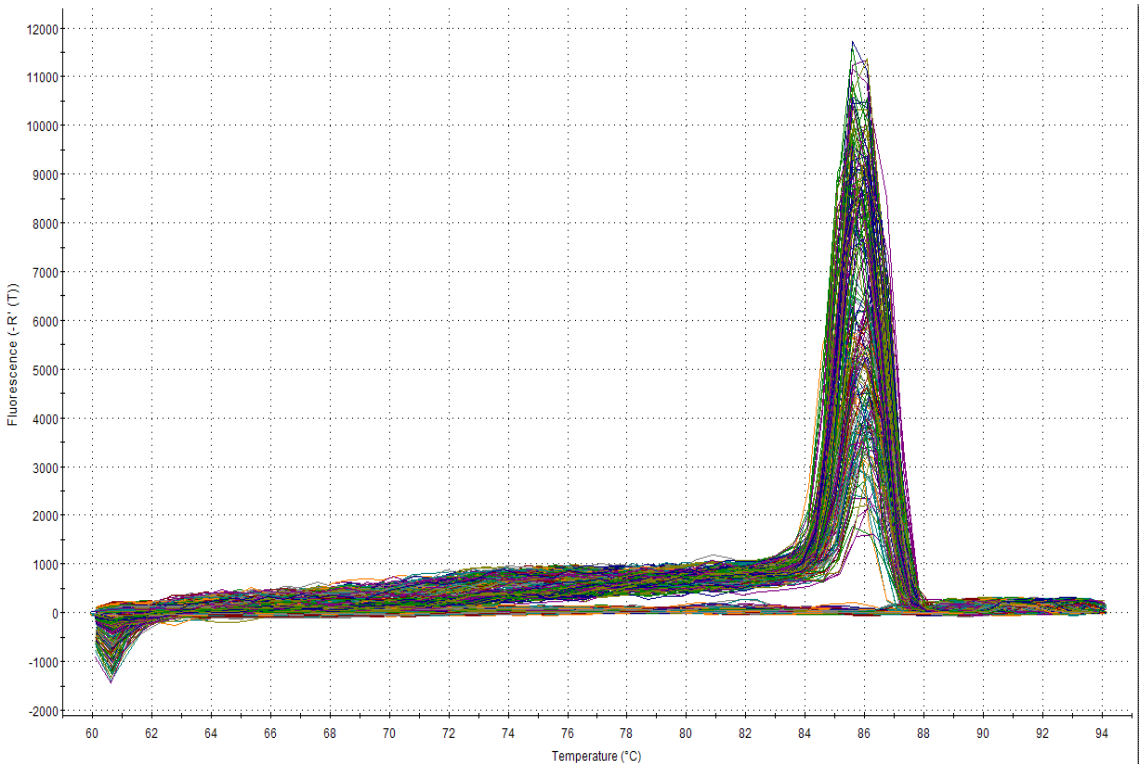

Figure 11. Dissociation curves of all standards and unknown samples included in the analysis.

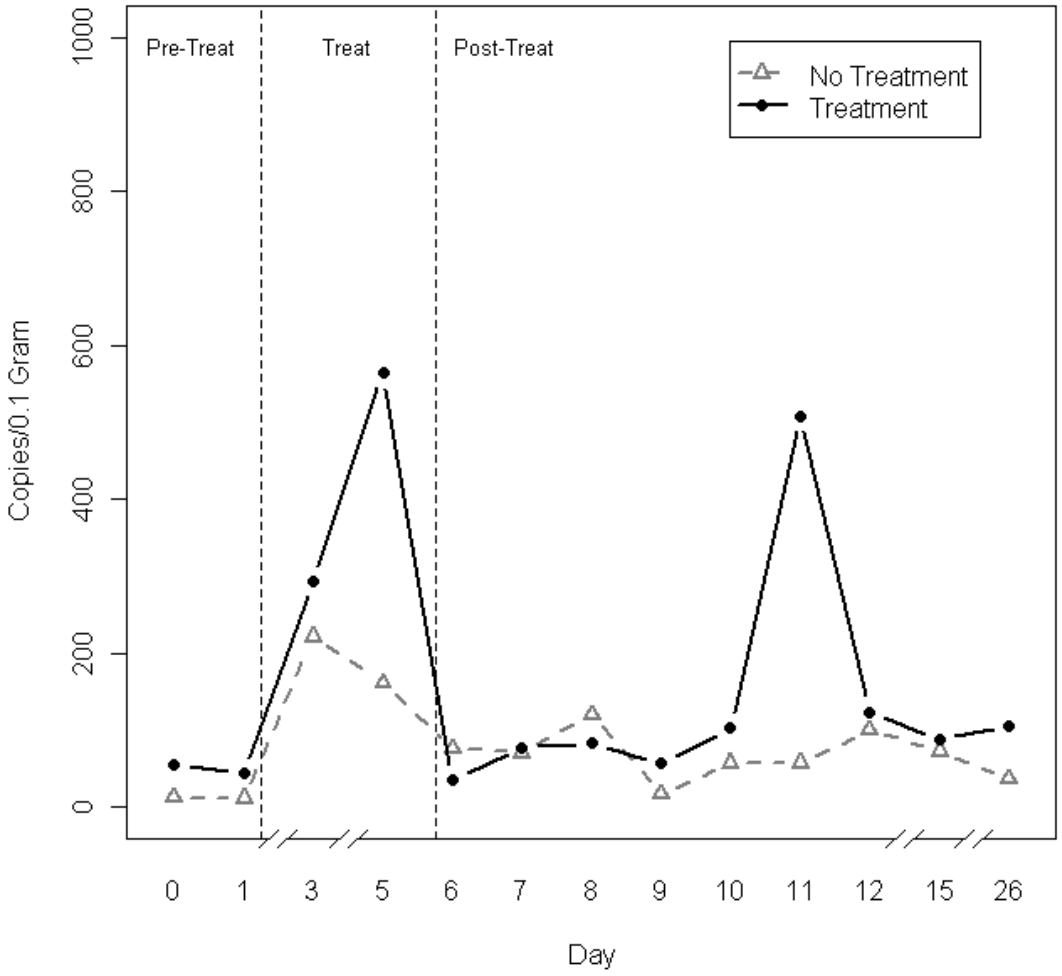

Figure 12. Mean number of copies of *bla*$_{CMY-2}$ per 0.1 gram of sample as estimated by the standard curve in cows treated with ceftiofur (solid line) on days 1 – 5 and untreated cows (dashed line) by day of sample collection.

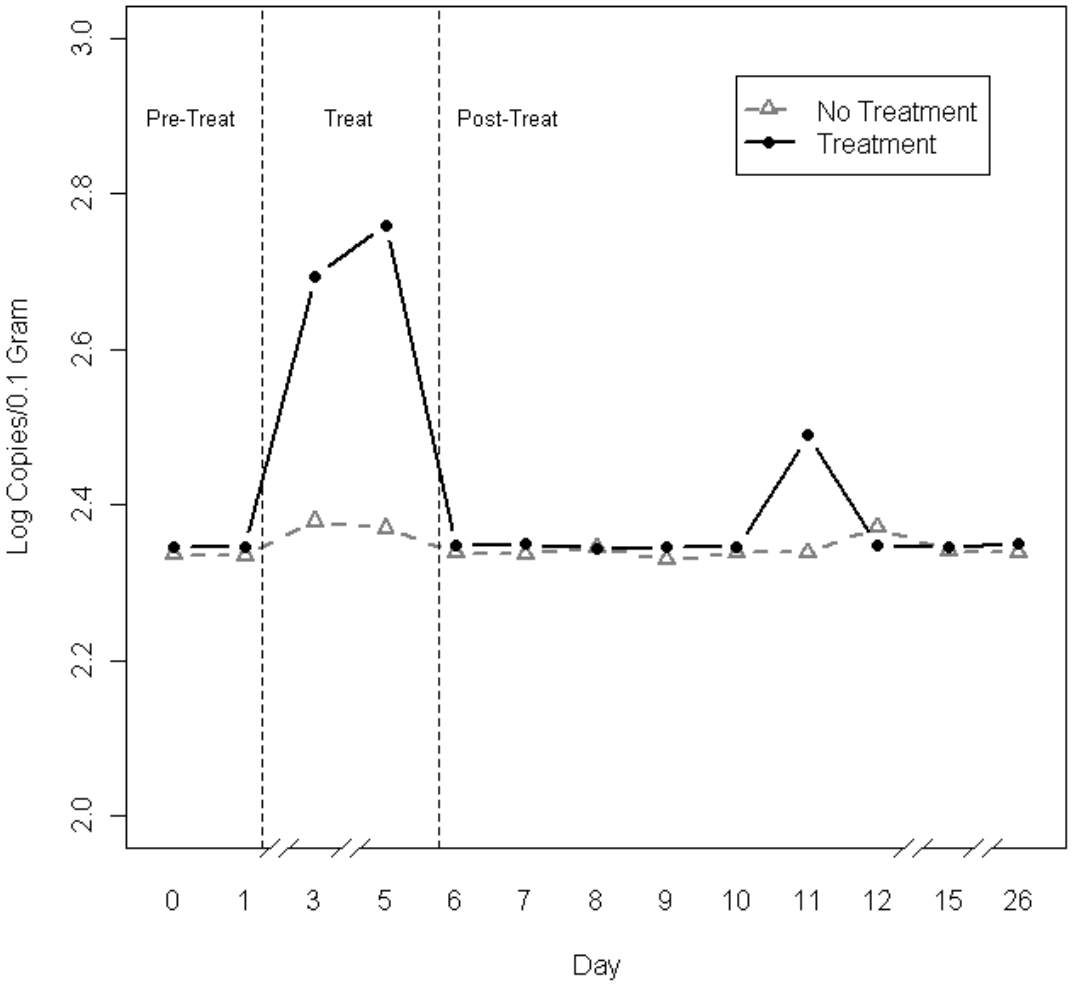

Figure 13. Fitted group means of $\log_{10}$ *bla*$_{CMY-2}$ quantity per 0.1 gram of sample in cows treated with ceftiofur (solid line) on days 1 – 5 and untreated cows (dashed line) by day of sample collection.

APPENDIX

WinBUGS code for hierarchical model. The data, and initial values were imported from R using the R2WinBUGS package

```
model{                                          # observation level (sample triplicate)
for (i in 1:O){
        ulo[i]<-10000+(-10000+10)*equals(wo[i], 9)
        #upper limit of censored observations set to 9 copies

        llo[i]<-1 + (-1)*equals(pos[i], 0)
        # lower limit of censored observation is 1 if observation is censored but positive or 0 if
        # no signal was detected

        o[i] ~ dpois(lambda[sampleo[i]]) I(llo[i], ulo[i])

        }


for (j in 1:J){                                 # sample level
        lambda[j]<-q[j]*pow(10, y[j])*prob[j]
        prob[j] ~ dbeta(5, 195)
        q[j] <- 0.65                    # correction factor for DNA extraction efficiency
        mu[j]<- a[animal[j]] + b1*treatment[j]
        }

for ( i in 1:10){                               # random intercepts for animal
        a[i] ~ dnorm(mu.a, tau.a)
        }
        b1 ~ dnorm(0, 0.001)
        mu.a ~ dnorm(0, 0.001)
        tau.a <- pow(sigma.a, -2)
        tau.y <- pow(sigma.y, -2)
        sigma.a ~ dunif(0, 100)
        sigma.y ~ dunif(0, 100)
        ICC <- sigma.a/(sigma.a + sigma.y)
        }
```

Chapter 5: Longitudinal analysis of antibiotic resistance gene quantities in dairy cattle

INTRODUCTION

With the concern over the emergence and spread of antimicrobial resistant pathogens, well-designed studies need to be able to relate antibiotic use to antibiotic resistance. The majority of attempts to investigate antibiotic resistance have primarily relied on cultivation of bacterial isolates to measure resistance. However, most bacteria in a sample are non-cultivable (89,146). In addition media used to grow the bacteria can be biased toward particular strains (187). Consequently, studies that rely on cultivation of particular species ignore the potential reservoir of resistance that resides among the entire bacterial population of a sample.

An alternative to cultivation based methods is the use of molecular methods on community bacterial DNA that has been extracted in a cultivation independent manner (137). The quantities of known resistance genes in the bacterial community can then be measured by real-time polymerase chain reaction (qPCR). This method implicitly assumes that the resistance genes and not bacterial isolates are the units of interest in a study of antimicrobial resistance. This approach can be appealing, given the ability of resistance genes to be shared among widely diverse bacteria (157). In addition, the quantitative measure provided by qPCR may be more suitable to detecting the effects of selection pressures. The downside to this approach is that the genes measured cannot be related to any particular species of bacteria.

Several studies have used the combination of community bacterial DNA and qPCR to measure resistance genes in agricultural samples. Most of these have focused on samples from waste holding systems, soil, or water in an attempt to monitor the fate of resistance genes in

the agricultural environment (29,52,54,83,101,117,176,181,221). Some of these studies found elevated resistance gene quantities in ground water that was down gradient from manure storage systems (52,101,117). Others found elevated levels of resistance genes in manure storage systems that were correlated with antibiotic residues (176).

Additional studies have combined community DNA extraction with qPCR to measure resistance gene levels in the feces of livestock, before and after animals were administered therapeutic doses of antimicrobials. One study administered a 7-day course of ampicillin to swine and found elevated levels of β-lactamase resistance genes in the bla_{TEM} group in the community DNA of feces from those animals (27). The animals in that study were sampled four times up to the day after treatment ceased. Another study administered three different doses of ceftiofur to feedlot cattle and found elevated levels of the β-lactamase resistance gene bla_{CMY-2} among treated animals on several days as far out as day 21 of the study (8). The animals in that study were followed for 28 days. Two studies, one in swine and the other in feedlot cattle, evaluated the effect of feeding antimicrobial growth promoters (tylosin, monensin, and virginiamycin) on quantities of macrolide resistance genes in the *erm* group and the tetracycline resistance gene *tetM* and found no differences between animals fed growth promoters and animals that did not receive antibiotics in feed(92,95). Animals in those two studies were followed for up to 15 weeks in the swine study and approximately 20 weeks in the cattle study.

The previously mentioned studies used experimental designs in which animals in different treatment groups were housed separately rather than allowed to mingle and potentially share bacteria as they would under most natural situations. One observational study followed cattle that were administered a five day course of ceftiofur along with untreated commingled cattle. This study found that cattle in the ceftiofur treatment group had elevated

quantities of the *bla*$_{CMY-2}$ gene during treatment but that *bla*$_{CMY-2}$ quantities dropped back to pretreatment levels on the first day after treatment ceased (34).

The follow up periods for the previously mentioned studies were relatively short. In general, longer-term longitudinal studies of antibiotic resistance in agricultural samples are rare. One series of studies measured phenotypic *E. coli* resistance in the feces of swine and humans over three years (4,6). These studies found differences in resistances among swine, humans working in swine production, and humans working at a slaughter plant. These studies also found associations between phenotypic resistance and antibiotic use. The authors noted that there was considerable seasonal variability in *E. coli* resistance. However, no seasonal or overall trends could be identified. Another study analyzed phenotypic *E. coli* in the feces of dairy cattle over 1.5 years using a latent transition analysis approach (33). The authors found that animals classified as pan-susceptible had high probabilities of staying in that class, and animals with *E. coli* isolates that were in the resistant class had high probabilities of transitioning to the pan-susceptible class on the subsequent time point.

There have been no studies that have monitored antibiotic resistance gene quantities in livestock under natural production conditions over an extended period of time. The objectives of this study were to use community DNA extraction and qPCR to measure the quantities of six antibiotic resistance genes in the feces of dairy cattle collected over 2.5 years. We hypothesized that gene quantities would be stable over time and that herds with a higher level of antibiotic use would have higher gene quantities than a herd with little antibiotic use.

METHODS

Study design

From September, 2001 through March, 2004, dairy cattle on four farms in Illinois were sampled. Each farm was visited nine times at approximately three month intervals. The four farms varied in size and management practices, including antibiotic usage. Two of the farms had approximately 200 milking cows each and a third farm had approximately 100 milking cows. Those three farms routinely used antibiotics both preventively, in medicated milk replacer for calves and for medicated dry-cow therapy, as well as for treatment of illness. The fourth farm was a small pasture-based dairy with approximately 30 milking cows that rarely used antibiotics, for treatment of illness only. On the first visit to each farm fecal samples were obtained rectally from randomly selected animals of all ages. Ages of the animals ranged from less than 1 month to 57 months. On successive visits, fecal samples were collected from the same animals unless they were no longer present in the herd. Calves aged 0-3 months were added to the study on each trip so that the cohort would have animals in the youngest age group on every sampling date. Samples were stored as 1 gram aliquots at -80° C until further processing.

DNA extraction

Community DNA was extracted from each thawed sample using a previously published protocol(141,205). The protocol, which was developed for fresh fecal samples, was modified by eliminating the initial centrifugation steps to minimize DNA loss due to cell lysis caused by freezing and thawing of the sample. Briefly, 1 gram of feces was suspended in 2.1 ml enzyme mix (40mg.ml lysozyme and 425 units/ml of mutanolysin in TE) and incubated at 37° C for 45 minutes. Seven ml of a solution of Guanidine thiocyanate (600g/L), EDTA (0.1 M), and Sarkosyl (1%) was added, and after briefly mixing, the samples were put on ice for 10 minutes. Ammonium acetate (3.5 ml, 7.5 M) was added, mixed, and placed on ice for an additional 10 minutes. DNA was extracted from the samples twice with 7ml of 24:1 chloroform/isoamyl

alcohol, centrifuging at 4000 g for 10 minutes to separate phases for each extraction. The aqueous layer was transferred to a new tube after each extraction ultimately yielding 9 ml of DNA solution. The 9 ml of DNA solution was combined with 4.9 ml of ice-cold isopropyl alcohol, mixed, and incubated at room temperature for 10 minutes to allow precipitation. The precipitate was collected by centrifugation, and the pellet was washed twice with 4ml of 75% ethanol and allowed to air dry approximately 30 minutes. One ml of buffer AE (10mM tris-HCL, 0.5 mM EDTA, pH 8.0) was added and the DNA was resuspended overnight at 4° C. RNAse (DNase-free, 1.5 µl of 10mg/ml solution) was added and samples were incubated at 37° for 90 minutes. The DNA concentration was determined by biophotometer. This protocol was found during validation to produce yields of DNA from gram-negative bacteria that were comparable to a commercial stool kit (QIAamp DNA Stool Mini Kit, Qiagen) and yields of DNA from gram-positive bacteria that were 10-fold higher than the commercial kit (data not shown). Thus this protocol extracted DNA that was more representative of the bacterial community than the commercial kit(205).

Quantitative real-time PCR (qPCR)

Gene selection

Six resistance genes representing four antibiotic classes commonly used in animal agriculture were selected. Two were macrolide resistance genes, one encoding a ribosomal protection protein (*mefA*) and the other encoding an efflux pump (*ermB*). Both genes have been identified in a wide range of Gram-positive and Gram-negative hosts (152). Two were tetracycline resistance genes, one encoding a ribosomal protection protein (*tetM*) and the other encoding an efflux pump (*tetA*). One tetracycline gene (*tetM*) has been identified in a broad range of Gram-positive and Gram-negative hosts while the other is generally associated with Gram-negative

hosts (55,150). A florfenicol resistance gene (*flo*) encodes non-enzymatic resistance to florfenicol and has been associated with Gram-negative hosts (17). The sixth gene was an Amp-C β-lactamase resistance gene, bla_{CMY-2}, that confers resistance to cephalosporins and has been found in Gram-negative hosts (217). All six genes have been associated with mobile genetic elements allowing them to move among bacteria. The *flo*, *tetA*, and bla_{CMY-2} genes can be co-located on the same plasmids, allowing them to move together among bacteria (46).

Standard preparation

For absolute quantification of *mefA*, *tetM*, *ermB*, *tetA*, *flo*, and bla_{CMY-2} in fecal samples, plasmid standards were created for each gene by cloning a single copy of each gene amplicon into the pDrive cloning vector (PCR cloning kit, Qiagen). Plasmid DNA containing the cloned gene was purified using a QIAprep Spin Miniprep Kit. The purified plasmid was checked by PCR using SP6 and T7 primers to assure the gene was present as a single copy. The purified plasmid was quantified by A_{260} and the copy number per µl was calculated based on the size of the plasmid plus the insert. Aliquots of the standards, diluted in nuclease free water to concentrations of 1 x 10^{10} copies per 5µl, were stored at -20°C. For each qPCR experiment a new aliquot was thawed and 10-fold serial dilutions were made in a 1:25 mixture of BSA and nuclease free water down to 1 x 10^1 copies per 5 µl. Addition of BSA to the dilutions was found to improve the sensitivity of the assay. A range of 1 x 10^7 copies per 5µl to 1 x 10^1 copies per 5 µl was used for the qPCR experiments.

Real-time PCR reaction and quantification

qPCR reactions were performed on the unknowns on a Stratagene Mx3000p system using Brilliant® II SYBR® Green qPCR mix according to the following recipe: 12.5µl SYBR Green master mix, 0.5µl (300nM) of each primer, 1 µl of BSA, 0.375 µl of ROX reference dye (1:500 dilution in

nuclease free water), 5.125 µl of nuclease free water, and 5 µl of plasmid standard or unknown community DNA for a total volume of 25 µl per QPCR reaction. The master mix for the plasmid standards followed the same recipe as the unknowns with the exception that water was substituted for the BSA, which had been previously added to the standards during the serial dilution step. The reaction conditions for the amplification were 95°C for 10 min and 40 cycles of 94°C for 30 sec 60°C for 1 min and 72°C for 30 sec each. Fluorescence was measured at the end of the extension phase of each cycle. Following the last cycle a continuous dissociation curve for each sample was created by measuring fluorescence at short temperature intervals ranging from 60°C to 95°C. Standards and no template controls were included with all of the assays and all standards, unknowns, and controls were run in triplicate.

For each gene, a single consolidated standard curve was created from the standards of all runs by ordinary least squares regression. The quantities of each gene in the unknowns were estimated from the standard curves. MxPro (v 1.4) software was used to calculate thresholds, C_t's, the consolidated standard curves, and gene quantities in the unknowns.

Statistical analysis

For three of the genes, *mefA*, *tetM*, and *ermB*, the gene quantities per reaction fell within the limits of quantification of the qPCR assays for nearly every observation. The triplicate observations of those three genes were averaged and then back calculated to $\log_{10}$ gene quantity per gram of sample to normalize the data. The log gene quantities were entered into three mixed effects linear regression models with age as a binary variable (1 for animals less than 6 months of age and 0 for older animals), three indicator variables for farms 1, 2, and 3. Farm 4, the smallest farm with the least antibiotic use was used as the referent category. Seasonality of gene quantities was assessed by including three indicator variables for spring

(March – May), summer (June – August), and fall (September – November), with winter (December – February) as the referent category. Sampling trip as an ordinal variable (0 – 8) was included to estimate a linear trend in gene quantities over time, adjusting for season. A random intercept for animal and a random slope to account for repeated measurements within animal were included. The analyses were carried out in Stata Statistical Software (180) using the xtmixed command.

For the *tetA*, *flo*, and bla_{CMY-2} genes, a substantial number of reactions fell below the limit of quantification (LOQ) of 10 copies per reaction and were censored. Quantities for those three genes were analyzed using a hierarchical linear model approach that was previously developed to analyze censored qPCR data (32). Age, farm, season and sampling trip were entered into these models in the same way as mentioned previously with a random intercept for animal correlated with a random slope for time (75). The analyses were carried out in WinBUGS v1.4 called from R v2.12.2 using the R2Winbugs package (144,183).

RESULTS

Over the 2.5 years of the study, 455 fecal samples collected from 81 animals were analyzed. The number of samples analyzed per herd was 107 to 128. Forty-eight (10.5%) of the samples were collected from animals less than 6 months of age. The median number of samples analyzed per animal was 7 (range 1 – 9) and 90% of the animals were sample at least twice. There were 21 to 70 samples analyzed from each time point. The six genes were analyzed by qPCR in triplicate resulting in 1,365 observations for each gene.

The *mefA* and *tetM* genes were detected in 100% of the reactions and *ermB* was detected in all but 20 (1.5%) reactions (Table 12). The other three genes had reactions with no detection in 59% to 75% of the reactions. All genes except *mefA* had some reactions (2% - 18%)

where the gene of interest was detected but the quantity estimated was below the LOQ of 10 gene copies per reaction (Table 12).

The mean log quantity of genes per gram of sample over time was consistently highest for *mefA* followed by *tetM* then *ermB* (Figure 14). The other three gene quantities were 1 – 3 orders of magnitude lower; the *flo* and bla_{CMY-2} genes alternated as the lowest quantity gene at various time points. Age was significantly associated with gene quantity for *tetM*, *ermB, tetA*, *flo*, and bla_{CMY-2} quantities, with animals less than 6 months of age having on average 0.70 – 2.34 more log gene copies than older animals. There was no difference between younger and older animals in *mefA* quantities.

There was a significantly increasing trend in *mefA*, *tetA*, and bla_{CMY-2} log quantities over time (Tables 13 and 14). The *tetM*, *ermB*, and *flo* genes had positive but non-statistically significant associations with time. There was a seasonal association with gene quantities for four of the genes. Samples that were collected during the autumn months had significantly higher quantities of *tetM*, *tetA*, *flo*, and bla_{CMY-2} quantities than samples that were collected during the winter months. In addition, samples that were collected during the summer months had significantly higher quantities of *tetM* and *tetA* genes than samples collected during the winter months.

There was considerable variability in gene quantities among the four farms. Farms 1, 2, and 3 were significantly different than Farm 4 for at least one gene but no farm was significantly different than Farm 4 for all six genes. For both of the macrolide/lincosamide resistance genes (*mefA* and *ermB*), Farm 1 had significantly higher gene quantities than Farm 4 (Table 13). However, Farms 2 and 3 had significantly lower *mefA* quantities than Farm 4 and there were no significant differences between Farms 2, 3 and 4 in *ermB* quantities. For the two tetracycline

resistance genes (*tetA* and *tetM*), there were no significant differences between Farm 4 and the other farms in *tetA* quantities, but Farms 1 and 2 both had significantly (lower) quantities of *tetM* than Farm 4 (Tables 13 and 14). For the *flo* gene, Farms 1 and 2 had significantly higher gene quantities than Farm 4 but there was no significant difference between Farms 3 and 4. For the bla_{CMY-2}, Farm 2 had significantly higher gene quantity than Farm 4 but there were no differences between Farm 4 and the other two farms.

Of the 455 samples analyzed by qPCR, 357 (78%) were also analyzed for phenotypic resistance in a previous study(33). From each of those 357 samples three *E. coli* colonies were isolated (1,071 total isolates) and tested by broth microdilution against a panel of 17 antimicrobials. If at least one of the three isolates tested per sample was resistant to one of the antimicrobials, that sample was categorized as resistant to that antimicrobial. The dichotomous susceptible/resistant status of each sample for ceftiofur, florfenicol, and tetracycline was compared to the gene quantities of the bla_{CMY-2} gene, the *flo* gene, and both the *tetA* and *tetM* genes. There were no macrolides or lincosamides included in the panel, therefore agreement with *mefA* and *ermB* quantities was not evaluated.

Of the 357 samples tested for phenotypic resistance to ceftiofur, 6 (1.7%) were classified as resistant (Table 15). The bla_{CMY-2} gene was detected in 85 (24%) samples including 4 of the 6 that were resistant to ceftiofur. There was 76% agreement between phenotypic ceftiofur resistance and detection of the bla_{CMY-2} gene. Samples that were resistant to ceftiofur had significantly higher log quantities of the bla_{CMY-2} gene than samples that were susceptible (Table 15). There were 19 samples (5%) that were classified as resistant to florfenicol. The *flo* gene was detected in 99 samples (28%) including 11 of the 19 that were resistant to florfenicol. The percent agreement between phenotypic florfenicol resistance and detection of the *flo* gene

was 73%. Samples that were resistant to florfenicol had significantly higher quantities of the *flo* gene than samples that were susceptible to florfenicol. There were 122 samples (34%) that were resistant to tetracycline. The *tetA* gene was detected in 160 of the samples (45%) including 77 of the 122 that were resistant to tetracycline. The percent agreement between phenotypic tetracycline resistance and detection of the *tetA* gene was 64%. Samples that were resistant to tetracycline had significantly higher log quantities of *tetA* than samples that were susceptible to tetracycline. The *tetM* gene was detected in all samples and the percent agreement between phenotypic tetracycline resistance and *tetM* detection was 34%. There was not a significant difference in log *tetM* quantity between samples that were resistant to tetracycline and samples that were susceptible.

DISCUSSION

This is the first study to measure antibiotic resistance gene quantities in a livestock population over an extended period of time. Our results suggest that there was a seasonal trend for four of the genes (*tetM*, *tetA*, *flo* bla_{CMY-2}), with quantities lower in the winter than in the autumn. In addition, there was an overall increasing trend for three of the genes (*mefA*, *tetA*, bla_{CMY-2}). This is in contrast to a three year study of phenotypic *E. coli* resistance that did not detect a seasonal trend(5,7). The authors of that study were unable to conduct a time series analysis to look for an overall trend in resistance, citing the need for a continuous measure of resistance. The increasing trends were contrary to our hypothesis which was based on phenotypic resistance of *E. coli* in the same population where a decreasing trend in tetracycline resistance was observed over time and no trends in ceftiofur or florfenicol resistance were observed(33).

A study that measured six tetracycline resistance gene quantities (including *tetM*) in the wastewater lagoons of cattle feedlots over six sampling periods from June to November found

that *tet* gene levels were higher in the autumn months than in the summer months(138). The authors speculated that higher autumn levels might be related to seasonal preventive antibiotic uses such as adding tetracycline in the feed of newly weaned calves as often occurs in the autumn. Three of the four genes (*tetA*, *flo*, bla_{CMY-2}) that were associated with higher quantities during the autumn months have been observed to be co-located on plasmids in *E. coli* isolated from cattle (46,129). It is possible that the higher quantities of those genes could be reflective of increased prevalence of Enterobacteriaceae that possess those plasmids through horizontal gene transfer or clonal shifts.

There were some general patterns among the farms revealed by the regression models. Farm 1 had the highest quantities of macrolide/lincosamide resistance genes. Farms 1 and 2 had the highest quantities of the florfenicol resistance gene. Farm 2 had the highest quantities of the ceftiofur resistance gene. Farm 4 had higher *tetM* gene quantities than the other three farms but there were no other differences among the farms in either tetracycline resistance genes. Farm 3 did not stand out from the others in quantities of any of the genes. Available antibiotic use data for treatment of disease on these farms was very limited. However, from the data that was obtained, Farm 3 had the highest overall use of antibiotics for treatment and the highest documented macrolide, tetracycline and ceftiofur use. Farm 2 was the only farm that documented florfenicol use during this period. While this information is likely incomplete, it appears that antibiotic resistance gene levels on these farms did not track with therapeutic use of antibiotics on the farms.

The qPCR results for four of the genes (*flo*, bla_{CMY-2}, *tetA*, *tetM*) that were compared to phenotypic resistance in *E. coli* were generally in poor agreement with the culture data. The poor agreement was mainly attributable to qPCR positive samples that were culture negative.

One possible reason for this is that some of the genes were located in bacteria other than *E. coli*; all four of the genes evaluated have been observed in bacterial species other than *E. coli* (55,128,150,217). The discordant results could also be due to false-negative results in the phenotypic data, or the gene could be present in low prevalence strains of *E. coli* that had low probabilities of being isolated. Detection based on qPCR would be expected to be more sensitive than a cultivation-based approach where only three isolates per sample were tested for resistance. However, there were also phenotypically resistant samples that were possibly false-negative by qPCR.

A previous study compared end-point PCR performed on community DNA for the *flo* and bla_{CMY-2} genes to phenotypic resistance in cultured *E. coli* to florfenicol and ceftriaxone, a 3rd generation cephalosporin used in human medicine that is similar to ceftiofur (137). That study found higher agreement between *flo* positive and florfenicol resistant samples than we observed. This difference was mainly due to the higher proportion of samples that were *flo* positive in our study (0.27 versus 0.15). This difference may be reflective of a true difference in the populations studied. Alternatively, the difference could be due to the DNA extraction methods used in the two studies or our qPCR assay may be more sensitive for detection of the *flo* gene than the end-point PCR assay used in the previous study. The previous study used nested PCR for detection of the bla_{CMY-2} gene and detected that gene in all samples that were resistant to ceftriaxone. In our study qPCR failed to detect bla_{CMY-2} in two samples that were resistant to ceftiofur.

The use of qPCR added an additional piece of information for comparison with phenotypic data that was not provided by end-point PCR in that culture positive samples had higher quantities of their respective genes than culture negative samples, with the exception of

tetM. This supports the notion that qPCR may be a more sensitive method for detecting the effects of selection pressures on resistance than cultivation based methods. For example, an analysis of the 357 samples used for this comparison was still able to detect significantly higher bla_{CMY-2} quantities in samples from Farm 2 compared to Farm 4 (data not shown). No significant differences in ceftiofur resistance among the farms were observed because Farm 2 and Farm 4 each had only one resistant ceftiofur isolate. The fact that there was no difference in *tetM* quantities between tetracycline resistant and tetracycline susceptible isolates highlights the fact that appropriate gene selection is important. Genes with very broad host ranges that are ubiquitous in the population being studied may not be particularly useful for evaluating selection pressures.

The combination of qPCR with community DNA may be preferable to cultivation-based methods for evaluating the effect of selection pressures on bacterial populations. For genes that are widely dispersed and present in very high quantities, such as *mefA*, *tetM* and *ermB*, the quantitative measure provided by qPCR may be better able to identify differences among populations. For emerging resistances mediated by relatively rare genes such as bla_{CMY-2}, qPCR appears to be more sensitive than cultivation-based methods. In addition, the quantitative nature of qPCR makes it more suitable than cultivation based methods for monitoring trends in resistance over time.

There are some research questions, namely those addressing whether or not pathogens are likely to respond to treatment, where culture based methods may be preferable to qPCR using community DNA. Surveillance systems that rely on phenotypic resistance data monitor trends in resistant pathogens (49,60). However, these systems also monitor trends in resistant commensal bacteria such as *E. coli* under the assumption that this type of data provides a

measure of the potential for resistance genes to be shared with pathogens. Therefore the resistance gene is the unit of interest in this case and not resistant bacterial isolates. The combination of qPCR and community DNA is a more appropriate method for that objective and would complement the cultivation-based methods used by surveillance systems.

Table 12. The mean (range) log gene quantity per gram of sample for six genes, the number of observations where the gene of interest was not detected, and the number of observations where the gene of interest was detected but below the LOQ (n = 1,367).

	Mean (range) log quantity per gram of sample	Number of observations with no gene detection	Number of observations with gene detection but below LOQ
mefA	9.25 (5.13 – 10.56)	0	0
tetM	6.67* (2.73 – 9.68)	0	23
ermB	5.78* (2.05 – 9.79)	20	71
tetA	2.81* (0 – 8.97)	807	47
Flo	1.28* (0 – 8.76)	851	247
$bla_{\text{CMY-2}}$	1.09* (0 – 8.36)	1023	70

* Means of gene quantities were approximated by substituting 0 for observations below the LOQ. For samples with all three triplicate qPCR reactions below the LOQ, 0 was substituted for the log gene quantity per gram.

Table 13. Results of mixed effects linear regression models for log quantities of *mefA*, *tetM*, and *ermB* resistance genes. A random intercept and random slope were included to account for repeated measurements within the same animal.

	mefA	*ermB*	*tetM*
Intercept	8.96 (8.72 – 9.20)	5.10 (4.75 – 5.46)	6.85 (6.51 – 7.19)
Age	0.16 (-0.03 – 0.35)	2.03 (1.74 – 2.32)*	0.70 (0.42 – 0.97)*
Farm 1	0.21 (0.02 – 0.39)*	0.90 (0.60 – 1.21)*	-0.76 (-1.01 – -0.51)*
Farm 2	-0.21 (-0.39 – -0.03)*	0.15 (-0.16 – 0.46)	-0.97 (-1.21 – -0.72)*
Farm 3	-0.28 (-0.47 – -0.10)*	-0.09 (-0.39 – 0.22)	-0.21 (-0.46 – 0.04)
Spring	-0.02 (-0.18 – 0.14)	0.14 (-0.11 – 0.39)	0.30 (0.06 – 0.55)*
Summer	-0.07 (-0.22 – 0.08)	0.02 (-0.20 – 0.23)	0.25 (0.04 – 0.47)*
Fall	0.03 (-0.12 – 0.17)	0.06 (-0.15 – 0.28)	0.31 (0.10 – 0.52)*
Time	0.08 (0.05 – 0.12)*	0.04 (-0.01 – 0.09)	0.03 (-0.03 – 0.08)

* Indicates significant associations at the $p < 0.05$ value

Table 14. Results of mixed effects linear regression models for log quantities of *bla*$_{CMY-2}$, *flo*, and *tetA* resistance genes. A random intercept and random slope were included to account for repeated measurements within the same animal.

	tetA	*flo*	*bla*$_{CMY-2}$
Intercept	1.30 (0.38 – 2.22)	1.75 (1.09 – 2.35)	0.51 (-0.35 – 1.49)
Age	2.34 (1.69 – 3.03)*	1.34 (0.91 – 1.79)*	1.74 (1.08 – 2.43)*
Farm 1	0.27 (-0.53 – 1.09)	1.32 (0.80 – 1.88)*	0.20 (-0.55 – 0.94)
Farm 2	-0.33 (-1.18 – 0.51)	0.69 (0.15 – 1.26)*	1.70 (1.03 – 2.40)*
Farm 3	0.04 (-0.78 – 0.88)	0.24 (-0.31 – 0.79)	0.09 (-0.62 – 0.83)
Spring	0.58 (-0.06 – 1.26)	0.14 (-0.30 – 0.58)	-0.57 (-1.43 – 0.26)
Summer	1.40 (0.85 – 1.96)*	0.22 (-0.15 – 0.60)	0.10 (-0.49 – 0.72)
Fall	1.44 (0.90 – 2.01)*	0.63 (0.27 – 0.99)*	1.08 (0.51 – 1.67)*
Time	0.15 (0.04 – 0.24)*	0.07 (-0.01 – 0.13)	0.13 (0.02 – 0.23)*

* Indicates 95% Bayesian credible intervals that do not include 0

Table 15. Comparison of culturing versus gene-specific qPCR analysis in the detection and quantification of specific resistance genes (n=357).

Culture	qPCR		% Agreement	Mean log quantity (95% CI)*	t statistic (p)
	bla_{CMY-2} +	bla_{CMY-2} -			
Ceftiofur +	4	2	76.8%	4.58 (0.78 – 8.38)	-3.74 (0.0002)
Ceftiofur -	81	270		1.17 (0.94 – 1.40)	
	flo +	*flo* -			
Florfenicol +	11	8	73.0%	3.56 (1.99 – 5.12)	-4.47 (<0.0001)
Florfenicol -	88	250		1.24 (1.01 – 1.47)	
	tetA +	*tetA* -			
Tetracycline +	77	45	64.1%	3.74 (3.21 – 4.28)	-6.24 (<0.001)
Tetracycline -	83	152		1.86 (1.53 – 2.18)	
	tetM +	*tetM* -			
Tetracycline +	122	0	34.2%	6.78 (6.59 – 6.97)	0.32 (0.75)
Tetracycline -	235	0		6.82 (6.70 – 6.94)	

* Calculations of mean log quantity were performed by substituting 0 for qPCR reactions below the Limit of Quantification (LOQ) of 10 gene copies per reaction.

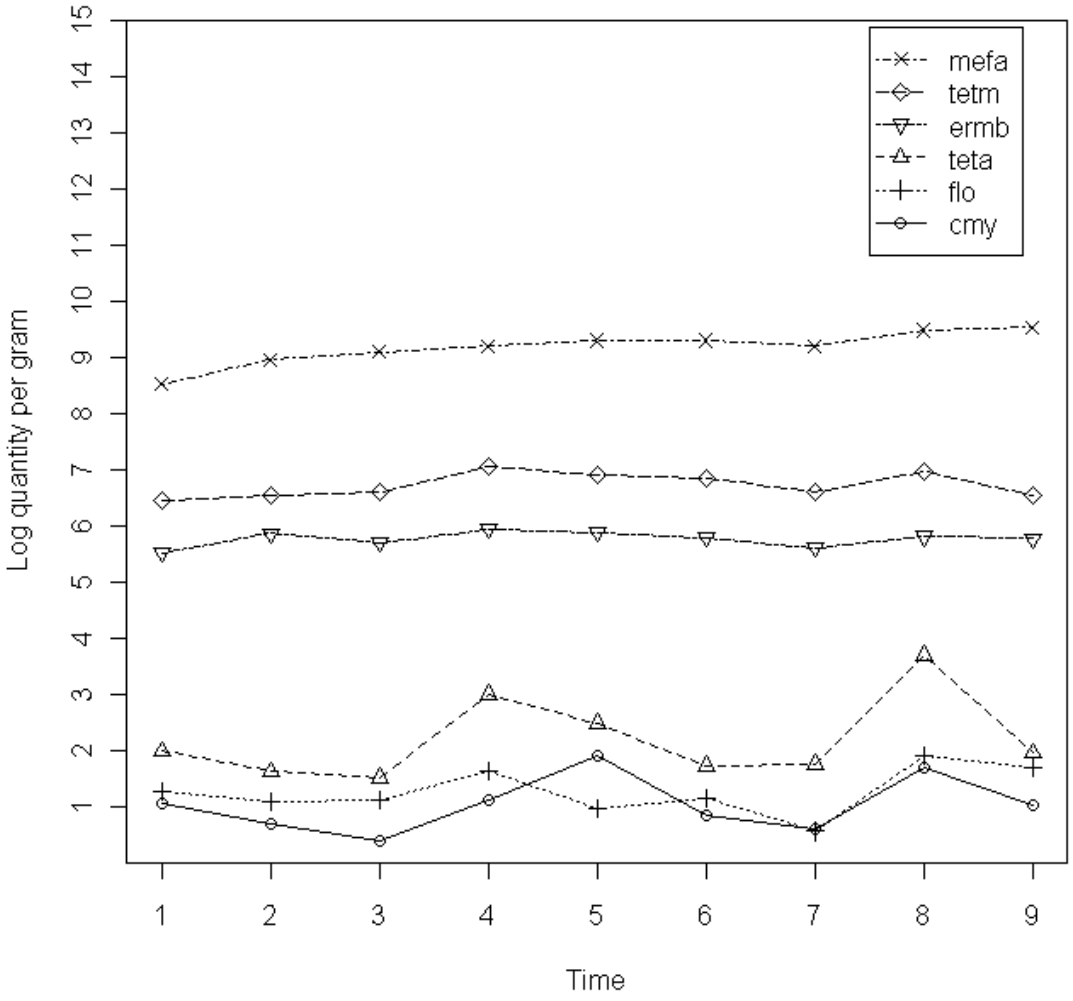

Figure 14. Approximate mean log quantities per gram of six antimicrobial resistance genes in fecal samples collected from dairy cattle over 2.5 years at 9 sampling points each separated by approximately 3 months. Mean quantities were approximated by substituting 0 for observations that fell below the LOQ.

Conclusion

The work in this dissertation has focused on antibiotic resistance in lower intestinal bacteria of dairy cattle and methods of testing for resistance. The overall purpose of the research was to characterize antibiotic resistance over time in a population of dairy cattle. Methodological challenges arose relating to analysis of phenotypic and genotypic resistance data that had not been addressed in the literature. Thus the results of this work have added to the knowledge base of antibiotic resistance in food producing animals and to the methods available for analysis of resistance data.

Most studies of antibiotic resistance in dairy cattle have been cross-sectional and were unable to estimate trends in resistance over time. Surveillance programs that collect data on resistance in food producing animals do monitor trends over time. However, these systems collect data from slaughter plants and report results at a national level. Thus these systems are unable to provide information on trends in resistance at the farm level. Previous attempts to monitor farm-level trends in non-dairy livestock production systems were unsuccessful due to the binary susceptible/resistant data that result from phenotypic testing methods. A latent variable approach to longitudinal analysis was applied to phenotypic resistance data of *E. coli* to estimate the probabilities that animals would transition into and out of classes defined by resistance status over time. The results showed a strong tendency for animals that shed *E. coli* that are pan-susceptible to continue to shed pan-susceptible *E. coli* over time and for animals that shed resistant *E. coli* to transition to the pan-susceptible group at the next time point. Due to the high proportion of isolates that were susceptible to all antimicrobials tested, the latent transition analysis was unable identify more than two groups, a pan-susceptible group and a resistant group. However, this approach may be able to identify more descriptive classes if

applied to populations of animals with higher levels of resistance such as calves only, or bacterial strains that tend to have higher levels of resistance than commensal *E. coli*.

Studies that focus on phenotypic resistance of cultivated bacterial strains fail to take into account the potentially vast reservoir of resistance that can exist among non-cultivated strains in a bacterial population. A community DNA plus qPCR approach can provide estimates of resistance gene quantities and give an indication of the burden of resistance in the entire bacterial community of a sample. However, this approach presents difficulties for rare genes that are present in very low quantities. Methods of analyzing low quantity genes have not been adequately addressed in the literature. A statistical model was developed to estimate gene quantities from data that include observations below the limit of quantification (LOQ) of qPCR assays. Through computer simulations it was found that this approach produces less biased estimates of regression parameters that substitution of a fixed value for observations below the LOQ that has been suggested in the qPCR literature.

Studies that measure antibiotic resistance in animals before, during, and after antibiotic treatment for the most part have detected a transient increase in resistance. One study using multiple methods concluded that treatment of dairy cattle with ceftiofur did not select for resistance *E. coli* but rather temporarily reduced the susceptible population of *E. coli* so that resistant strains that were already present could be detected. The resistant strains were no longer detectable after treatment ceased and there was no evidence that the genetic diversity of the *E. coli* population was different after treatment than before treatment. By using qPCR to measure $bla_{\text{CMY-2}}$ quantities in the community DNA of those same samples, it was found that quantities of this gene actually increased during treatment. This suggests that ceftiofur treatment may have selected for resistant bacteria contrary to the conclusions of the previous

study. However, gene quantities dropped after treatment ended supporting previous studies that have shown a transient increase in resistance caused by antibiotic treatment.

Measurement by qPCR of six antibiotic resistance genes in the community DNA of cattle followed for 2.5 years revealed increasing trends in gene quantities over time for three of the genes. A seasonal trend was also observed for four of the genes with higher levels observed during the autumn months than compared to the winter. These trends were not observed in phenotypic resistance data from the same population. Furthermore, qPCR detected resistance to tetracycline, florfenicol, and ceftiofur in a higher proportion of samples than did phenotypic methods of testing, supporting the idea that qPCR is a more sensitive method than phenotypic testing for measuring the burden of resistance in a bacterial population. Also, the quantitative nature of qPCR data may be better suited for assessing the effect of selection pressures on resistance.

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