DISSERTATION

ANTIMICROBIAL USE IN DAIRY CATTLE AND ITS POTENTIAL IMPACT ON ANTIMICROBIAL RESISTANCE IN ENTERIC BACTERIA: SAMPLING, DATA COLLECTION AND ANALYTICAL METHODS

Submitted by

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In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall, 2007

COLORADO STATE UNIVERSITY

October 15, 2007

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY **AURORA VILLARROEL** ENTITLED "*ANTIMICROBIAL USE IN DAIRY CATTLE AND ITS POTENTIAL IMPACT ON ANTIMICROBIAL RESISTANCE IN ENTERIC BACTERIA: SAMPLING, DATA COLLECTION AND ANALYTICAL METHODS*", BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ANTIMICROBIAL USE IN DAIRY CATTLE AND ITS POTENTIAL IMPACT ON ANTIMICROBIAL RESISTANCE IN ENTERIC BACTERIA: SAMPLING, DATA COLLECTION AND ANALYTICAL METHODS

Some researchers have suggested that antimicrobial-use in animals may have an effect on antimicrobial resistance in bacteria isolated from humans. Before this belief can be studied, a possible connection between antimicrobial-use in food-animals and resistance in bacteria isolated from those animals needs to be studied. Some estimates exist on national antimicrobial-use in food-animals. However, trying to relate these estimates to resistance prevalence among bacteria isolated from food-animals across the country may not appropriate. Potential problems that may surface when attempting to study this relationship are explored. Because of these problems, standard methodology to measure antimicrobial-use and resistance needs to be developed. In this study, various methods for data collection on antimicrobial-use and resistance were evaluated, as well as sampling strategies and statistical analysis methods, to study the possible relationship between antimicrobial-use in dairy cattle and resistance among enteric bacteria isolated from those cattle. Potential confounding and interaction factors were evaluated.

Measurement of resistance in enteric bacteria was studied testing one or more isolates per animal, the latter resulting in a better estimate of resistance prevalence. Comparison of handwritten and computerized records for data collection on antimicrobial-use resulted in concluding that, currently, computerized records lack an appropriate field code to record detailed data on antimicrobial-use. As for sampling strategies, random sampling stratified across pens was a better strategy than repeatedly sampling a cohort group of treated and non-treated animals. The main reasons for this conclusion were the lack of attrition of studied animals, effective representation of subpopulations of the dairy, easiness and time-effectiveness of the stratified random sampling strategy. Evaluation of statistical analysis methods for the study at hand showed that crude analysis resulted in erroneous conclusions as to the effect of antimicrobial-use on resistance, likely because it ignores the lack of independence between isolates obtained from the same animals on different occasions. A better option was the use of hierarchical multivariate analysis, which revealed that other factors such as age in calves and production group and parity in cows were strongly associated with the probability of isolation of resistant enteric bacteria. New questions that arose during this study are outlined.

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ACKNOWLEDGEMENTS

The completion of this doctorate degree has been possible thanks to the support of many people throughout the years.

First, I would like to thank Drs. Robert H. BonDurant and V. Michael Lane from the University of California, Davis for planting the seed that got me here. Their mentorship throughout my residency encouraged me to become a better veterinarian, teacher, scientist and person in general. Their commitment and dedication to their students will always stay with me. I hope I can become half as good mentors as they both are.

I would like to thank all my committee members for taking the time in helping me with this program. In special, I would like to thank my co-advisors, Dr. David A. Dargatz and Dr. Mo Salman, for their insight and thoughtful comments during our discussions and the preparation of this dissertation. It was always interesting to meet with Dr. Dargatz and learn of some other way of looking at a problem. From him I have learned to question every possible angle of a problem or situation. From Dr. Salman I take with me his dedication to his students. I still don't know how he manages to mentor so many people and keep on top of things.

I would also like to thank Drs. Brian McCluskey and Claudia Gentry-Weeks, for their help with this program. Their inquisitive review of my work helped me focus and express myself better. Dr. Paul Morley allowed me to use some of the data he had collected prior to the beginning of my program, and this resulted in a key chapter in this dissertation and in the project in general.

I would like to acknowledge the support for this project received from the USDA: Cooperative State Research, Education and Extension Service through the Colorado State University – Center for Economically Important Infectious Animal Diseases, the Animal Population Health Institute and the Integrated Livestock Management.

Last, but not least, I would like to thank my beloved Mike for his invaluable support during this program. You kept me sane and grounded, and your love has taken me where I would have not gotten by myself.

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INTRODUCTION

Science can be defined as the verifiable knowledge derived from the systematic study of phenomena through observation and experimentation.[1] We can ask many types of questions in science.

- Existence questions: what phenomena occur in the world?
- Origin questions: how did the phenomena come to be?
- Process questions: how do things interact and change?
- Applied questions: how can we manipulate the universe to our advantage?

There are scientific specialties that deal with each type of question. Epidemiologists asks process questions about causal relationship and the association of factors with disease.[2]

The ideal methodology to answer scientific questions, the *scientific method,* is a cyclic process of inquiry based on observations, synthesis, hypotheses, and prediction that lead to more observations.[1,3] The first step in the scientific method involves observation and collection of data about the question at hand. These data are then synthesized in an

attempt to recognize a pattern. When a pattern is found, scientists develop one or more hypotheses to explain why this pattern exists. The hypotheses that are generated may vary according to underlying paradigms that the working scientists believe in.[1]

Sir Arthur Eddington graphically explained this issue with the example of an ichthyologist exploring the life in the ocean with a fishing net.[4] In his example the ichthyologist found two patterns: all creatures he fished were longer than two inches and all had gills. Based on his observations, he concluded that *"(1) no sea-creature is less than two inches long and (2) all sea-creatures have gills."*... [I]n short, *"what my net can't catch isn't fish."* These are in fact hypotheses that need to be tested by making more observations to prove that the results are systematic and reproducible.

The conclusions made by the ichthyologist are warranted by his observation, and yet we know that both of his conclusions do not reflect reality. What would happen if he used a fishing net with smaller holes? He would probably change both of his conclusions. How do *we* know that his conclusions don't reflect reality? We know of other scientists who have studied life in the ocean with methods other than a fishing net and have found sea creatures that are smaller than two inches and sea creatures that don't have gills. Sir Eddington uses the fishing net to explain the scientific method; in his own words:

> *In applying this analogy, the catch stands for the body of knowledge which constitutes physical science, and the net for the sensory and intellectual equipment which we use in obtaining it. The casting of the net corresponds to observation; for knowledge which has not been or could not be obtained by observation is not admitted into physical science.*

This dissertation approaches a question for which scientists have long been seeking an answer: does the use of antimicrobials in food-animals have any effect on antimicrobial resistance in bacteria isolated from humans? There are multiple intermediate steps between the use of antimicrobials in food-animals and a possible effect on human disease due to resistant bacteria. Food-animals can receive antimicrobials in order to treat or prevent disease, or to improve production. Before, any product from this animal can be used for human consumption, it has to be tested for quality and processed according to regulated standards. These processes are designed to minimize risk of contamination with pathogens that can cause disease in humans. If foods of animal origin harbor resistant bacteria, resistance determinants could be transferred to other bacteria that colonize man and eventually cause disease.

Before looking into such a complex relationship, it seems reasonable to look first into a possible relationship between antimicrobial-use in food-animals and resistance in bacteria isolated from those animals. However, the methods used to answer this question have to be evaluated first, and they may vary for different food-animal species. This research project was aimed at evaluating the methods to be used to study a possible effect of antimicrobial-use in dairy cattle on resistance in enteric bacteria isolated from these cattle. The study was conducted at a commercial dairy farm in Northern Colorado, and focused on two bacterial species commonly found in dairy cattle: *Escherichia coli* and *Salmonella* spp. A brief review of the current knowledge in antimicrobial resistance is offered as a preface for understanding the scope and design of this study.

CURRENT KNOWLEDGE IN ANTIMICROBIAL RESISTANCE

Bacteria are unicellular organisms that are ubiquitous in the environment. Bacteria can interact with animals (including man) as symbiotic organisms, as commensal organisms or as disease agents.[5] Bacteria usually share their environments with other microorganism, such as protozoa, viruses, fungi, and other multi-cellular organisms. Microorganisms have to compete for nutrients to survive in that environment. Different species have varied methods of out-competing the rest.[5,6] One such method is the synthesis and secretion of antibiotic substances that kill other bacteria or inhibit their growth and multiplication.[5,7,8]

Researchers have discovered that antibiotic compounds or chemically synthesized substances, which are similar to the natural antibiotics, can be used to help overcome disease produced by susceptible microorganisms. Although the host has defense mechanisms to deal with pathogenic organisms, antibiotics can help by shortening the length or severity of the exposure to pathogens.

Antibiotic is the term preferably used to describe those compounds naturally produced by microorganisms, while the term *antimicrobial* describes all substances (naturally produced or chemically synthesized) that can kill or inhibit the growth of microorganisms and at the same time cause little or no damage to the host.[9]

Antimicrobial Mechanisms of Action

Antimicrobials have two primary mechanisms of action:[8,10]

- Inhibition of the synthesis of some structural or functional component of the bacterial cell (*e.g.* cell wall and proteins).

- Competition with substrates of certain metabolic pathways, and therefore blocking production of a substance that may be necessary for bacterial survival (*e.g.* the folic acid pathway).

Based on the knowledge of these mechanisms of action, two or more active ingredients with different but complementary effects can be combined to improve the efficacy of antimicrobial treatment of disease agents.[9] One commonly used combination involves the concurrent use of sulfonamides and trimethoprim. Both of these drugs interfere with the synthesis of folic acid at different points in the metabolic pathway. Therefore, the effect of one drug is enhanced by the addition of the other.

A summary of the mechanisms of action of antimicrobials approved for use in dairy cattle is presented in Table 1.1, along with the antimicrobials that are tested by the National Antimicrobial Resistance Monitoring System (NARMS).[11]

Table 1.1 Mechanisms of action of antimicrobials currently approved for use in dairy cattle (bold) and antimicrobials tested under the National Antimicrobial Resistance Monitoring System (NARMS). Antimicrobials currently approved for use only in dairy calves and heifers under 20 months of age are marked with an (*) and those approved only for local intramammary use are marked with an (†). Antimicrobials tested under NARMS are indicated by the abbreviations used throughout this dissertation.

Mechanism of Action	Target	Antimicrobial class		Antimicrobial	NARMS
Disruption of the integrity of the cell wall	synthesis of cell wall	β - lactam antimicrobials	penicillins	**ampicillin**	*Amp*
				cloxacillin †	
				penicillin G	
			cephalosporins – 1st generation	cephalothin	*Ceph*
				cephapirin †	
			cephalosporins – 2nd generation	cefoxitin	*Cefx*
			cephalosporins – 3rd generation	**ceftiofur**	*Ceft*
				ceftriaxone	*Ceftr*
			potentiated penicillins	**amoxicillin** + clavulanic acid	*Amox*
	permeability	ionophores		**monensin**	
				lasalocid	
Blockage of metabolic pathways	synthesis of folic acid	sulfonamides		sulfamethoxazole	*Sul*
				sulfadimethoxine	
		potentiated sulfonamides		sulfamethazine + trimethoprim	*TMS*

Mechanism of Action	Target	Antimicrobial class	Antimicrobial	NARMS
Inhibition of protein synthesis	DNA	fluoroquinolones	nalidixic acid	*NalA*
			ciprofloxacin	*Cipr*
	ribosome	tetracyclines	**oxytetracycline**	*Tet*
			chlortetracycline	
		macrolides	**erythromycin ***	
			tylosin	
			tilmicosin *	
		lincosamides	**pirlimycin †**	
		aminoglycosides	amikacin	*Amik*
			gentamicin	*Gen*
			kanamycin	*Kan*
			neomycin *	
			spectinomycin *	
			streptomycin †	*Str*
		phenicols	chloramphenicol	*Chlor*
			florfenicol *	

Epidemiology of Antimicrobial Resistance

Microorganisms that secrete antibiotics have the prerequisite that they need to protect themselves from these substances, *i.e.* they need to be *resistant*. Antimicrobial-resistant bacteria have the ability to grow and multiply in the presence of an antimicrobial compound.[5,8,12] Known mechanisms of antimicrobial resistance portrayed by bacteria are:[7,8,10,12,13]

- Reduced bacterial uptake of the antimicrobial.
- Increased excretion of the antimicrobial outside the bacterial cell.
- Modification of the target of antimicrobial action.
- Increased production of the target of the antimicrobial.
- Synthesis of an alternative protein that is not susceptible to the antimicrobial.
- Synthesis of enzymes that can degrade or inactivate the antimicrobial.

Depending on which bacteria and antimicrobial drug are involved and on what amount of antimicrobial reaches and binds its target, some resistance mechanisms will produce low-level resistance and others will produce high-level resistance.[10,12] Low-level and high-level resistance are defined relative to the most common MIC's of an antimicrobial for a given bacterial species.[14-16] Low-level resistance is determined by MIC's slightly above the susceptible level, while high-level resistance has higher defined MIC's.

Resistance can be inherent to a group of bacteria based on their structure and the metabolic pathways available to them, or it can be acquired over time through mutations and adopting determinant genes from other bacteria.[8,10] An example of inherent resistance is that of gram-negative bacteria to the action of penicillins. Penicillins kill bacteria by inhibiting the cross-linkage of peptidoglycan molecules within the cell wall. In gram-positive bacteria, the cell wall is composed mostly of peptidoglycan, and thus they are susceptible to the action of penicillins. Gram-negative bacteria, however, have two inherent means to avoid the action of penicillins: an outer membrane involving the cell wall prevents penicillins from accessing the cell wall; and the cell wall itself has limited peptidoglycan molecules, the target of penicillins.

Acquired resistance arises when new genes are created (*e.g.* by mutation) or genes from other bacteria are adopted (*e.g.* horizontal transfer). The means to the mechanisms of antimicrobial resistance (functional proteins and structural components) are encoded by one or more genes that can be located in two sites: the bacterial genome (*chromosome*) or the free DNA molecules (*plasmids*).[8,10] Genes located on plasmids can be integrated in the bacterial genome, and viceversa.[17]

It has been shown that individual genes can be part of mobile genetic elements that can change position within the DNA or even be transferred between bacteria.[8,10] Mobile genetic elements are difficult to classify in a hierarchical manner because of their very different structure.[17,18] Plasmids, transposons, integrons and phages are among the mobile genetic elements that have been reported to contain genes encoding for antimicrobial resistance.[10,17]

Plasmids are double-stranded circular or linear molecules of DNA that replicate independent from the chromosome.[8,10,19] Reportedly, not all bacteria carry plasmids, but those that do can harbor multiple copies of a given plasmid or even multiple plasmids encoding for different information. Plasmids that carry genes encoding for antimicrobial resistance are called R-factors [8] or R-plasmids.[12]

Transposons contain genes flanked on both ends by repeat sequences (insertion sequences [IS]) that can change positions within a DNA strand.[5,8,12,19] Recognition of these IS allows certain enzymes, called transposases, to cleave the DNA (chromosomal or plasmid) and relocate the transposon to another position in the DNA strand where an IS is found.[12] The insertion process into the new site can disrupt other genes if the IS fall within the sequence of those genes, because the relocation usually implies deletion of adjacent DNA.[12] Many resistance genes are believed to be carried in transposons.

Integrons are other important mobile genetic elements encoding for resistance. They contain a site-specific integrase (*intI* gene), a recombination site (*attI* site), a promoter (*Pc*), and a varying number of gene cassettes[12,20,21] similar to a train with multiple wagons. The integrase determines where the gene cassettes will be inserted into the DNA, which makes this a site-specific process. Gene cassettes are in fact the smallest mobile genetic elements although they can contain up to hundreds of genes.[22] The variability in gene cassettes is immense, as shown by findings of over 2,000 gene cassettes from bacteria isolated from soil in a confined 50 m^2 area.[22] Usually genes that encode for antimicrobial resistance will be adjacent and therefore can be transferred

together, conferring multidrug resistance. Integrons can be found mostly in gram-negative bacteria.[10,21]

Phages, also called bacteriophages, are viruses that infect bacteria.[19] They are composed of a capsid that encloses transferable genetic material and a tail that allows injection of the genetic material into bacterial cells.[19] Bacteria infected by a phage are tricked into replicating the genetic information injected by the phage along with their own.[19] Errors during the replication process (generation of multiple copies of the phage) can include DNA from the host bacteria into the viral capsid; therefore DNA from the host bacteria can now be transferred by the phage to other bacteria.[5] The transfer of genetic material between bacteria via phages is called *transduction,*[8,12,19] which is not considered a common method of transferring antimicrobial resistance genes due to the host-specificity of the phages, where only closely related bacteria are infected. The main mechanism of transferring antimicrobial resistance genes is *conjugation,*[8,12,19,23] where donor and recipient bacteria have direct contact via a "sex pilus" that acts like a bridge. Because conjugation does not require homology between bacteria,[12,19,23] it is a possible mechanism for the transfer of DNA between bacteria from different species. Another possible method for the transfer of antimicrobial resistance genes between bacteria from different species is *transformation*, the acquisition of naked DNA from the bacterial environment.[12,19] Some disadvantages for the transfer of resistance determinants between bacteria by transformation are that bacteria up-taking free DNA have to be "competent" and the free DNA needs to have an area in its sequence that is homologous to that of the bacterial DNA to be inserted into the bacterial DNA. Competent bacteria have a cell wall that allows the up-take of free DNA.

The previous methods describe how bacteria can acquire resistance genes from other bacteria, but new resistance methods can also be acquired by DNA mutation.[6,10,13] In fact, mutation is the most important method of acquisition of antimicrobial resistance for fluoroquinolones.[10,24] Mutations can occur at frequencies of one mutation in 10^6 - 10^{10} cells.[25-27] Some of these mutations are "silent", meaning that they show no effect on the cell,[6] while other mutations can show a wide array of effects, from slightly altered metabolism to cell death. Many mutations, including some that confer antimicrobial resistance, may cause deleterious effects on the bacteria challenging its ability to survive, even in the absence of the antimicrobials.[13,26] Other mutations that confer antimicrobial resistance allow the bacteria to thrive in the presence of the specific antimicrobials, but bacteria may be unable to survive in the absence of those antimicrobials.[13] The situation in which mutant bacteria may have problems surviving in an environment without antimicrobials is referred to as "fitness cost".[28,29] In all these cases, the recently acquired resistance is not very likely to spread to later generations or other bacteria, because resistance would die with the bacteria when environmental conditions return to normal (no antimicrobials). Under certain conditions of stress, some bacteria can express a hyper-mutable phenotype with frequencies of mutation 100 to 1000 fold higher than normal.[6,26] These hyper-mutable types are called *mutators*; they can be transient mutators able to revert to the wild-type phenotype after the stress has disappeared, or they can be stable mutators.[6,26] In theory, these stable mutators could disseminate clonally and spread resistance through the genus or to other bacterial genera and species.

Recently it has been shown for fluoroquinolones that there is a range of concentrations, called the "mutant selection window," that selects for mutant phenotypes *in vitro*.[27,30] The

lower limit for this window is marked by the minimum inhibitory concentration (MIC), the lowest concentration of an antimicrobial capable of inhibiting the growth of an inoculum of 10^4 to 10^5 cells.[27,30] The upper limit of the window is marked by the mutant prevention concentration (MPC), which is the minimum concentration of antimicrobial that inhibits the growth of the least-susceptible single-step mutant.[27,30]

If we assume that the probability of a single-step mutation in a highly mutant strain is at least 1 in 10^5, the probability of a double-step mutant would be 1 in $(10^5)^2$, or 1 in 10^{10}. Therefore the MPC is estimated as the MIC for a 10^{10} inoculum, meaning that growth of bacteria beyond the MPC would require 2 mutations. In this range of fluoroquinolone concentrations, the bacterial inoculum shows a plateau-growth that is maintained by mutant resistant bacteria instead of being completely inhibited.[27,30] These mutants are considered to be "selected" by these antimicrobial concentrations, but not "generated" or "caused" by the presence of fluoroquinolones. A selection process implies the existence of these mutants and their distinction from non-mutants, in this case by killing non-mutants (susceptible to fluoroquinolones). In contrast, a causation process implies that the mutants did not exist before exposure to the antimicrobial. Mutants can in theory mutate further and acquire new resistance genes (by mutation or from other bacteria).

Resistance against Specific Antimicrobial Groups

The following description of resistance mechanisms to specific antimicrobials focuses on antimicrobials approved for use in dairy cattle by the Food and Drug Administration (FDA)[31] and antimicrobials tested by NARMS [11] (Table 1.1).

Antimicrobials Acting on the Bacterial Cell Wall

Two groups of antimicrobials that act on the bacterial cell wall are used in dairy cattle: β-lactam antimicrobials and ionophores. They are described below, briefly.

β-lactam Antimicrobials (penicillins and cephalosporins) [10,12,32-35]

Penicillins and cephalosporins are time-dependent antimicrobials, meaning that they have to maintain their concentration above the MIC for a minimum time to exert their effect. Their mechanism of action is by binding to and inactivating a set of enzymes that act in the cross-linking of peptidoglycan in the cell wall. For this reason, these enzymes are commonly referred to as "penicillin-binding proteins" (PBPs). β-lactam antimicrobials are considered bactericidal drugs during bacterial multiplication, but can also act as bacteriostatic antimicrobials depending on the extent of the damages to the bacterial cell wall. When the cell wall cannot be synthesized correctly, it cannot protect the cell from the outside environment. A second mechanism of action of penicillins against bacteria is blocking the inhibition of autolysins. Autolysins are enzymes that hydrolyze the peptidoglycan of the cell wall.[5] They are active during new cell wall formation but are inhibited by the cell when it is not growing. When penicillins block the inhibition of autolysins, these become active and destroy the cell wall leading to the death of the cell.

Mechanisms of resistance:

- Drug inactivation due to the synthesis of β-lactamases is the most important mechanism of resistance against this class of antimicrobials. β-lactamases are enzymes that inactivate penicillins and some cephalosporins by breaking part of the molecule. There are multiple penicillinases and cephalosporinases. Found both in gram-negative and gram-positive bacteria, the genes encoding for β-lactamases can be located in the chromosome or in plasmids. They are released into the periplasmic space in gram-negative bacteria and outside the cell wall in gram-positive bacteria, where they could in theory protect other bacteria that do not carry the genes to express β-lactamases. Some of these β-lactamases can be induced by the presence of the specific β-lactam antimicrobial, while others are over-expressed when an insult occurs. A special group, called "extended spectrum β-lactamases" (ESBLs), is found mostly in *Enterobacteriaceae* that have originated through point mutations in specific regions of the encoding genes. Bacteria with ESBLs are resistant to a wider spectrum of penicillins and cephalosporins, hence their name. Over 150 ESBLs are recognized to date.

- Target alterations through mutations in PBPs, which reduces their affinity for β-lactam antimicrobials usually confers low-level resistance. Alternative PBPs that have very low affinity for β-lactam antimicrobials can also be generated, conferring high-level resistance against penicillins.

- Reduced uptake due to changes in the cell wall can cause low-level resistance.

Ionophores [36,37]

Monensin and lasalocid are ionophores approved for use in dairy cattle to improve production. Their mechanism of action is on the cell membrane where they interact with cations (like sodium, potassium, calcium, and magnesium), disrupt their gradient, and cause cell death. This effect is not limited only to bacterial cells, but can also affect host cells, which make ionophores toxic at high doses and potentially lethal, especially in horses. They are thus bactericidal drugs with efficacy limited mostly to gram-positive bacteria, because the cell wall of gram-negative bacteria impedes the entrance of ionophores and their access to the cell membrane. Because the different drugs have different affinities for the various cations, the ionophores do not show high cross-resistance. Their use as a feed additive in ruminants forces a change in the ratio of gram-negative and gram-positive bacteria in the rumen, where gram-negative bacteria have a more positive effect than gram-positive bacteria. The ultimate result is an improved feed conversion, both in beef and milk production. Monensin is an antibiotic produced by *Streptomyces cinnamonensis.*

Mechanisms of resistance:

- Efflux systems are thought to be the mechanism of resistance against ionophores antimicrobials but it has not been demonstrated.

It is assumed that either an active efflux of the drug takes place, or alternative pumps to retain the targeted cations are expressed.

Antimicrobials that Inhibit Protein Synthesis

Most antimicrobials used in dairy cattle exert their action inhibiting the synthesis of proteins in the bacterium. Five groups of antimicrobials will be described briefly.

Fluoroquinolones [10,12,35,38]

Fluoroquinolones include enrofloxacin, ciprofloxacin, moxifloxacin and nalidixic acid. They are concentration-dependent antimicrobials, meaning that the higher the concentration of an antimicrobial is present at the infection site, the better it kills susceptible bacteria. Nalidixic acid is the prototype quinolone, although it is rarely used in clinical settings. Fluoroquinolones exert their effect by inhibiting either topoisomerase II (DNA gyrase) or topoisomerase IV. Topoisomerase II disrupts super-coiling of the bacterial DNA rapidly killing the bacteria (bactericidal antimicrobials), and topoisomerase IV separates the DNA daughter strand from the mother strand, impeding further multiplication of the mother strand. Inhibition of topoisomerase II is the preferential mechanism of action in gram-negative bacteria, while inhibition of topoisomerase IV is preferred in gram-positive bacteria, because their DNA gyrase is less susceptible to the action of fluoroquinolones.

Quinolones can act both as bacteriostatic and bactericidal, depending on the concentration at which they are used. High-level of resistance is associated with sequential mutations that implicate both enzymes. Inhibition of the effect on the topoisomerase IV is associated with low-level resistance to fluoroquinolones.

Mechanisms of resistance:

- Target alteration that reduces the drug-binding ability of the enzymes. These target alterations are usually coded by mutations in the quinolone resistance-determining region (QRDR), which is a small section of genes encoding for the DNA gyrase and the topoisomerase IV that determines the presence of resistance to quinolones. Mutations in this area usually confer low-level resistance.

- Decreased uptake is a collateral method of resistance, because to be able to affect the enzymes described above fluoroquinolones need to cross the bacterial membrane. Changes that render the bacterial membrane more resistant to the entry of the drug will result in increased resistance to fluoroquinolones and other antimicrobials that exert their action inside the bacterial cell.

- Increased efflux of the antimicrobial out of the bacterial cell by efflux pumps, decreasing the internal concentration of antimicrobials. This action can confer low-level resistance against fluoroquinolones

Aminoglycosides [10,35]

Gentamicin, amikacin, kanamycin and streptomycin are the aminoglycosides tested by NARMS. Although spectinomycin is not an aminoglycoside, it is included in this group because it is an aminocyclitol that lacks the aminosugars, and therefore is very similar in structure and function. The mechanism of action of these antimicrobials is by binding to

the ribosome and interfering with protein synthesis (bactericidal effect). Aminoglycosides are concentration-dependent antimicrobials.

Mechanism of resistance:

- Drug inactivation by enzymes that modify the antimicrobial is the main mechanism of resistance against aminoglycosides, especially in gram-negative bacteria. More than 50 enzymes have been described, with possibly several genes encoding for similar enzymes. Resistance genes are usually located in plasmids.
- Target modification has been described, but is not considered to be as important as drug inactivation.
- Increased efflux has been described.

Bacteria resistant to specific aminoglycosides often show cross-resistance to other antimicrobials in the group. Because of the mechanism of action, aminoglycosides are sometimes used with other antimicrobials that exert their action on the cell wall of the bacteria, obtaining a synergistic effect.

MLS Antimicrobials [10,35]

The acronym MLS stands for macrolides, lincosamides and streptogramins, which are different chemical compounds showing a similar mechanism of action (binding to the 50S ribosomal subunit to inhibit protein synthesis) and resistance. Macrolides used in

dairy cattle are erythromycin, tylosin and tilmicosin, while pirlimycin is the only lincosamide approved for use in dairy cattle (intramammary). Streptogramins are not approved in dairy cattle.

Mechanisms of resistance:

- Target modification (methylation) of the binding site for these antimicrobials on the rRNA of the bacteria is the most common mechanism of resistance which decreases the affinity for the drugs. Resistance can be constitutive or inducible. Constitutive resistance is equally active against all MLS antimicrobials, while inducible resistance only confers resistance to some of these antimicrobials (depending on their structure).

- Increased efflux by proteins that pump the drugs out of the bacteria, decreasing the concentration of antimicrobials inside the bacteria so that less ribosomal binding occurs. Efflux genes can be located in conjugative elements within the chromosome, making it possible to be transferred to different species.

- Decreased uptake by the outer membrane of bacteria confers intrinsic resistance.

- Drug inactivation by enzymes is another mechanism that confers resistance to structurally related antimicrobials.

It is interesting to note that most isolates tested resistant to MLS antimicrobials in the U.S. are due to the presence of efflux pumps, while isolates from Japan and Europe show

resistance due to target modification. Because MLS antimicrobials have similar mechanisms of resistance, the possibility of cross-resistance exists. Resistance due to efflux pumps has been attributed to at least three different genes, while mutations are responsible for target modifications. Enzymes that inactivate the drugs are encoded by multiple genes of varying types according to the antimicrobial type (>10 genes).

Phenicols [10,35,39]

Chloramphenicol is a broad-spectrum antibiotic secreted by *Streptomyces venezuelae* with bacteriostatic activity. Chloramphenicol is approved for use in humans but not in dairy cattle, while florfenicol -a structural analog- is approved in dairy cattle but not in humans. Their mechanism of action is by binding of the 50S ribosomal subunit, which inhibits protein synthesis. Genes encoding for resistance against both chloramphenicol and florfenicol are mostly found in plasmids, but are hypothesized to be in the chromosome, too.

Mechanisms of resistance:

- Drug inactivation by metabolizing enzymes is the most common mechanism, and it is encoded by several variants of a gene found in plasmids.
- Decreased uptake has been reported.
- Increased efflux has been observed in some gram-negative bacteria.

Tetracyclines [10,35,40]

The two tetracyclines approved for use in dairy cattle, chlortetracycline and oxytetracycline, are natural antibiotics produced by *Streptomyces aureofaciens* and *S. rimosus*, respectively. Tetracyclines exert their action by binding to the 30S ribosomal subunit, and therefore inhibiting protein synthesis (bacteriostatic). The effect of tetracyclines is reversible when resistance mechanisms remove the tetracycline molecules from the ribosomal subunit and allow the ribosome to resume its function of protein synthesis.

Mechanisms of resistance:

- Increased efflux confers resistance to some tetracyclines, but not others. Most of the identified resistance genes for tetracyclines code for efflux proteins.

- Target alteration via ribosomal protection confers resistance by producing a new protein that binds to the ribosomal subunit and interferes with the binding of tetracyclines, without altering protein synthesis.

- Drug inactivation by oxidative enzymes has been found in some bacteria, but is considered to be of lesser importance.

Most of the genes encoding for tetracycline resistance are located on mobile genetic elements, and can be found in commensal bacteria that share the environment with the host and other microorganisms. Tetracycline resistance genes are classified according to their homology. Using a classification system that confers designations of tetracycline

resistance genes according to their homology in amino acid sequences, 32 different genes have been designated. To receive the same designation, any two genes have to share at least 80% of their amino acid sequences. This shows the wide range of genes that have been defined to date. Therefore, phenotypic resistance seen in two bacteria of the same species can be encoded by completely different genes. This implies that not all bacteria resistant to tetracycline have exchanged genetic material or are clones. Due to the structural similarities that exist among the various tetracyclines, it is common to find cross-resistance. It is interesting to note that obligate intracellular pathogens like *Chlamydia* and *Rickettsia* have not yet shown resistance to tetracycline.

Antimicrobials Acting on Metabolic Pathways

The two groups of antimicrobials acting on the metabolic pathway of the synthesis of folic acid are used in dairy cattle. They are described below briefly.

Sulfonamides [12,35,41]

Bacteria cannot utilize preformed folic acid, but have to synthesize their own. The target for sulfonamides is dihydropteroate synthase (DHPS), an enzyme required for the synthesis of dihydrofolic acid. Sulfonamides compete with the para-amino-benzoic acid (PABA) to bind to DHPS, therefore inhibiting the synthesis of folic acid, causing a bacteriostatic effect.

Mechanisms of resistance:

- Target modification seems to be the most observed mechanism of resistance against sulfonamides. Most of the resistance to sulfonamides is attributed to mutations. The new DHPS synthesized by these altered genes usually has less affinity for sulfonamides.

- Over-production of the target enzyme is another mechanism of resistance, as plasmids encoding for new DHPS enzymes are theoretically transferred horizontally between bacteria. These new DHPS select the regular substrate (PABA) over the sulfonamides.

Trimethoprim [10,35]

Trimethoprim's mechanism of action is similar to that of the sulfonamides, by competing with enzymes in the pathway of folic acid synthesis. Trimethoprim competes with dihydrofolic acid to bind to the enzyme dihydrofolate reductase in the last step of folic acid production. Therefore, the action of sulfonamides and trimethoprim is seen as complementary, in that they inhibit the same process at two different points. Because of this, they can be combined to form the so-called "potentiated sulfonamides".

Mechanisms of resistance:

- Target alteration is the main mechanism of resistance against trimethoprim, which appears by acquisition of a gene that encodes for a new dihydofolate reductase

that does not bind to trimethoprim. This mechanism has been shown to produce several variant enzyme types that are resistant to trimethoprim. At least nine genes have been identified that encode for an altered target.

- Over-production of dihydrofolate reductase, so there is enough for both substrates; the original substrate (dihydrofolic acid) and trimethoprim.

Multidrug Resistance (MDR)

From the information detailed above, it can be extrapolated that some microorganisms may be resistant to more than one antimicrobial. Several mechanisms have been reported to explain MDR. One mechanism of MDR is the expression of altered membrane porins that decrease the uptake of various compounds, including some antimicrobials. This mechanism can be associated with a high fitness cost to the bacteria, because some nutrients may also be prevented from entering the cell.[12,13]

Another mechanism of MDR is the expression of efflux pumps, which are proteins that actively excrete substances from inside the cell to the environment.[7,10,12] Some efflux pumps are able to transport only substances that are closely related, while others can transport many different substrates.[7] Some efflux proteins can transport a variety of products, such as antimicrobials, disinfectants, antiseptics, steroids, bile salts, fatty acids and even chemotherapeutic agents like anti-tumor drugs.[7,42] It is believed that most efflux pumps had a preexisting role of transporting compounds other than antimicrobials, and

that over time they adapted to transport these, too. It has been reported that specific events like sporulation or stress influence the expression of efflux pumps.[7] Many efflux pumps, but not all, have a regulatory system that prevents over-expression, because this could lead to the loss of essential molecules (like H^+) and therefore cell death.[7] When these regulatory proteins bind to the substrate of the pumps they regulate, they signal for an increased expression of efflux pumps. The regulation mechanisms are specific for each efflux pump, and many require to be controlled at various levels.

MDR is considered to be acquired by the spread of mobile genetic elements (like integrons, transposons, and plasmids) between bacteria of different species, as opposed to sequential mutations, although multidrug resistance due to the action of unspecific efflux pumps has been reported.[7,10] Multiple genes can be integrated over time on to the mobile genetic elements resulting in MDR. Still, over-expression of efflux pumps causing MDR may arise due to mutations in the regulatory pathways that control the expression of the efflux pumps.[7] A specific region within the chromosomal DNA of many *Enterobacteriaceae* called the *mar* locus or *mar* regulon (to denote *m*ultiple *a*ntimicrobial *r*esistance) contains an operon that regulates the expression of many genes encoding information for the expression of efflux pumps and porins that influence antimicrobial resistance to multiple drugs and disinfectants.[43,44] The wide range of genes that can be regulated by the *mar* locus result in multiple phenotypes of MDR isolates. The *mar* locus can be induced by the presence of certain substrates like salicylate and fluoroquinolones.[43,44]

Finally, MDR due to sequential mutations has also been reported,[8] especially in bacteria that have high mutation frequencies (intrinsic or induced). This is an important mechanism for gram-positive bacteria to acquire MDR because they lack integrons.

An MDR *Salmonella* strain that has received special attention due to its virulence and inherent resistance to multiple antimicrobials is S. Typhimurium Definitive Type 104.[45-49] The resistance pattern seen in this strain is referred to as R-type ACSSuT, describing resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines. Its special feature lies in the fact that resistance is encoded in the chromosome instead of in plasmids, although it has been suggested that some of these genes were once part of a plasmid.[45] It was reportedly first described in human isolates in the UK in 1984,[45] and since then it has been reported both in human and animals around the world.[45,50,51]

Scope and Impact of Antimicrobial Resistance

There are several reasons why the presence of resistance in microorganisms isolated from food-animals may not imply transfer of this resistance to pathogenic bacteria that infect humans.

- Many bacteria are adapted to a specific host, such as *Salmonella typhi* in humans and *Salmonella enterica* serotype Dublin (*S.* Dublin) in cattle.
- The connections between human and animal populations are very complex and differ widely according to species. For example, pets live in close relationship with humans allowing direct contact with multiple people. Dairy cattle on the

other hand are managed in herds and come in contact with people mostly during milking and health care (production and reproduction); therefore, direct contact is restricted in time and extent (only people and health care professionals working on the farm).

- Humans are exposed to food-animals through the food that these animals produce for human consumption; for dairy cattle these products are milk and meat. Food of animal origin entering the legal market has to be inspected under federal supervision and should be appropriately processed and handled to ensure food safety and avoid contamination by food handlers.[52] Therefore, the possible exposure of humans to resistant bacteria via food of animal origin is minimized if safe food handling guidelines are followed.

- Although the use of antimicrobials in animals and people can "select" for resistant bacteria, how the use of antimicrobials is distributed in these populations needs to be taken into account. Some animals may be consuming antimicrobials during part of their production period (*e.g.* medicated milk replacer in calves) or only during a short period for the treatment of a disease, as would happen in people. The use of antimicrobials in food-animals is heavily regulated, mainly to avoid residues in food. No regulation is present for the use of antimicrobials in people, which can lead to unscrupulous use. Unrestricted use of antimicrobials in humans can lead to the spread of resistance among human pathogens. If this route for the spread of resistance is not considered as a "baseline", *any* resistance present in human pathogens can be erroneously attributed to a possible effect from animals.

- The potential 'traffic' of bacteria (resistant or not) cannot be assumed to be unidirectional from animals to humans. The interaction of animals and humans at any level has the potential of transferring bacteria in both directions: from animals to humans *and* from humans to animals.

Most studies conducted on prevalence of resistant bacteria in food-animals by the researchers around the world have focused on enteric bacteria, especially zoonotic agents (transmissible between animals and man).[53-57] The target microorganisms vary according to the type of animal studied. A brief overview follows on the two agents that are the focus of this study and are monitored in cattle by NARMS.

Salmonella spp. [11]

One of the main organisms studied within NARMS is *Salmonella.* Sources of samples for isolation of *Salmonella* from cattle (beef and dairy) have varied since NARMS started in 1996 (Table 1.2). An important distinction should be made between diagnostic samples and non-diagnostic samples (on-farm and slaughter), in that diagnostic samples are collected from cattle with some disease and most probably after antimicrobial treatment. Although on-farm and slaughter samples cannot be assumed to originate only from non-treated cattle, according to federal regulations on slaughter of food-animals treated with antimicrobials, most of these samples should originate from untreated cattle and cattle that were not treated with antimicrobials recently. The population dynamics of microorganisms within the animal host after antimicrobial treatment is ceased are currently unknown. No information could be found on how resistant bacteria compete with susceptible bacteria when the levels of antimicrobial decrease in the tissues.

Due to the variability in source of samples throughout the years, results from different years cannot be directly compared, although some features can be noted. Prevalence of resistance ranged between 35.0% in 1997 and 51.3% in 2003, when a higher proportion of diagnostic samples were tested; prevalence of multidrug resistance varied accordingly (19.4% in 1997 and 34.7% in 2003). In spite of these differences, the most common resistance pattern has consistently been single resistance to tetracyclines.

Only one isolate resistant to amikacin in 1999 and one isolate resistant to ciprofloxacin in 2000 were recovered, both from diagnostic samples. Isolates resistant to nalidixic acid have also been rare throughout the monitoring period, remaining at a prevalence of 2.2% or less in diagnostic samples (Table 1.3) and 0.4% or less in slaughter samples (Table 1.4). In general, an upward trend in prevalence of resistance to β-lactam antimicrobials and chloramphenicol has been observed. Resistance to chloramphenicol is an interesting case because its use in cattle has been prohibited by law since 1994,[58] but its use in companion animals and people remains legal.

Frequency of isolation of the different *Salmonella* serotypes (from all animal species, not only cattle) has shifted during in the past 20 years to the point that two of the most frequently isolated serotypes in 1997 (*S.* Montevideo and *S.* Anatum) were rarely isolated in 2003. This shift in the isolation of different serotypes may indicate that individual *Salmonella* serotypes should be investigated and reported, as opposed to grouped in the genus.

Table 1.2 Number of *Salmonella* isolates obtained from cattle that were tested for antimicrobial resistance by the National Antimicrobial Resistance Monitoring System (NARMS) between 1998 and 2003.

	Year						
	1997	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**
Diagnostic samples (cattle)	183	321	550	450	347	366	354
Diagnostic samples (dairy cattle)	0	0	470	376	0	932	475
Slaughter samples (cattle)	26	284	1610	1388	894	1008	672
On farm samples (unspecified)	859	78	4		0	294	0
beef cattle				706			
dairy cattle				490			
Total	1068	683	2634	3410	1241	2600	1501

The impact of resistance among *Salmonella* isolates in humans has been reported to be an increase in the number of hospitalized patients, deaths and longer treatment courses,[59-62] especially if the strains are multidrug resistant.

Escherichia coli

E. coli is a commensal microorganism that is usually studied as a proxy for *Salmonella* because they are genetically related (approx. 70% common genes).[63] *E. coli* is also easily isolated from feces of humans and other animals. In its inception, NARMS studies were focused more on the isolation of *E. coli* from meat (chicken and beef) than from live animals. The first NARMS report of antimicrobial resistance in *E. coli* isolated from cattle was in 2002.[11] In the last report available at this time from NARMS (2004), it is shown that resistance among *E. coli* isolated from cattle was very similar to those isolated from horses, dogs and chickens, with differences in only a few antimicrobials.[11]

For instance, fluoroquinolone resistance was more prevalent in *E. coli* isolated from cattle than from horses, dogs and chickens, while gentamicin resistance was less prevalent. Resistance to cephalosporins was lowest in dogs, intermediate in cattle and highest in horses. The most common resistance pattern among *E. coli* from all animals was *GenStrSulTet*, representing 9.2% of the isolates. No reports have been found on the specific impact of antimicrobial resistance of non-specific *E. coli*, although it has been proposed that due to its close relationship with other *Enterobacteriaceae* it could represent a risk for the spread of resistance to other bacteria, especially *Salmonella.*[64,65]

Table 1.3 Frequency (%) of *Salmonella* isolates resistant to individual antimicrobials among **diagnostic** samples obtained from cattle between 1997 and 2003. (Source: National Antimicrobial Resistance Monitoring System).[11]

Antimicrobial	1997 n=183	1998 n=321	1999 n=550	2000 n=450	2001 n=347	2002 n=366	2003 n=354
Amikacin	0	0	0	0	0	0	0
Amoxicillin/Clavulanic Acid	1.6	8.1	4.5	20.4	19.6	26.5	35.3
Ampicillin	32.2	42.1	32.9	45.3	39.5	45.6	45.5
Apramycin	0.5	0.9	0.9	3.3	2.6	N/A	N/A
Cefoxitin	N/A	N/A	N/A	19.8	19.3	23.8	29.7
Ceftiofur	0.5	8.1	4.5	18.9	19.6	24.9	35.6
Ceftriaxone	0.5	2.5	0.4	0	0.3	0.8	0.6
Cephalothin	1.6	9	7.5	21.6	21	27.6	35.6
Chloramphenicol	5.5	14.3	16	29.6	30.8	34.4	44.1
Ciprofloxacin	0	0	0	0.2	0	0.5	0
Gentamicin	2.2	4.7	4.2	8.9	8.9	9.8	7.1
Imipenem	N/A	N/A	N/A	N/A	0	N/A	N/A
Kanamycin	29	36.8	26	34.7	26.2	30.1	26
Nalidixic Acid	0	0	0.2	2	2	2.2	1.4
Streptomycin	33.9	43.6	40.7	46.4	47.8	50	47.5
Sulfamethoxazole	30.6	43.9	34.9	43.8	46.4	44	46.9
Tetracycline	36.6	43	37.5	47.8	52.2	50.8	50.8
Ticarcillin**	31.7	39.9	N/A	N/A	N/A	N/A	N/A
Trimethoprim/Sulfamethoxazole	1.6	5.9	5.1	10	8.1	10.7	7.3

** Ticarcillin was replaced by florfenicol in 1999, when no data is available because there are no interpretive criteria for resistance.

Table 1.4 Frequency (%) of *Salmonella* isolates resistant to individual antimicrobials among **slaughter** samples obtained from cattle between 1997 and 2003. (Source: National Antimicrobial Resistance Monitoring System).[11]

Antimicrobial	1997 n=26	1998 n=284	1999 n=1610	2000 n=1388	2001 n=894	2002 n=1008	2003 n=672
Amikacin	0	0	0	0	0	0	0
Amoxicillin/Clavulanic Acid	7.7	2.5	3.9	9.9	11.9	17.7	21
Ampicillin	19.2	9.2	12.5	18.7	18	23.9	28
Apramycin	0	0	0.2	0.2	0.1	N/A	N/A
Cefoxitin	N/A	N/A	N/A	9.1	11.2	15.9	17.7
Ceftiofur	0	2.1	4.2	9.8	11.5	17.4	21
Ceftriaxone	0	0.7	0.1	0.1	0.1	0.2	0.1
Cephalothin	0	2.1	4.7	9.9	11.7	17.7	21.1
Chloramphenicol	11.5	5.6	8.5	15.1	16.6	20.6	25
Ciprofloxacin	0	0	0	0	0	0	0
Gentamicin	0	1.8	1.6	2.1	2.1	2.6	2.7
Imipenem	N/A	N/A	N/A	N/A	0	N/A	N/A
Kanamycin	7.7	9.5	7.1	6.6	6.9	10.1	15.2
Nalidixic Acid	0	0.4	0.1	0.4	0.4	0.4	0.4
Streptomycin	19.2	16.2	15.4	21.3	20.4	25.9	28.6
Sulfamethoxazole	26.9	15.5	15	19.9	19.8	22.3	25
Tetracycline	30.8	24.3	20.9	25.8	26.4	32	36
Ticarcillin**	19.2	8.5	N/A	N/A	N/A	N/A	N/A
Trimethoprim/Sulfamethoxazole	3.8	2.5	2.4	2.2	2.6	2.5	3.3

** Ticarcillin was replaced by florfenicol in 1999, when no data is available because there are no interpretive criteria for resistance.

JUSTIFICATION

Many articles imply that antimicrobials in food-animals are used indiscriminately as feed-additives at sub-therapeutic doses for production enhancement, and that use in this manner is a major factor for the development and transmission of resistant bacteria to humans by food of animal origin.[66-71] This posit conceals several inaccurate generalizations:

- The impression is given that any and all antimicrobials can be used in food-animals for production enhancement at sub-therapeutic doses.

- All food-animals are considered equal, while in fact many differences exist in the management and the intrinsic characteristics of different food-animal species; a simple example are the differences between ruminants and monogastric species.

- All foods of animal origin (meat, fish, dairy products, and eggs) are considered equal in their risk of selection for resistant bacteria and the risk of transmission of these resistant bacteria to the consumer.

- It seems to be suggested that once a food-animal is exposed to antimicrobials, bacteria within the animal will become and remain resistant indefinitely. This may point towards an assumption that food can originate from animals immediately after they have been treated with an antimicrobial, disregarding the need for mandatory withholding periods. Another possible assumption is that there is no

difference between short-term and long-term effects, if any, that antimicrobial-use may have on resistance in food-animals.

The reality of antimicrobial-use in dairy cattle is that strict governmental regulations severely limit their use.[58,72-74] The main purpose of dairy cattle is the production of milk for human consumption. Additionally, dairy cattle represent approximately 7-8% of animals slaughtered for human consumption in the U.S.[75] Because the main product from dairy cattle is milk, the animals of interest as food-producing animals are lactating dairy cattle. Very few antimicrobials are approved for use in lactating dairy cows.[74,76-78] Resistance to non-approved antimicrobials is not as likely in dairy cattle as in food-animal species for which they are approved, although co-selection of resistance and cross-resistance may exist.[8,12] Furthermore, each drug (antimicrobial or other) that is approved for use in dairy cattle has federally enforceable withholding periods before milk and meat can be destined for human consumption. Some drugs that are not approved for use in dairy cattle can be used in an "extra-label" manner under the supervision of a veterinarian, using extended withholding periods. Use of drugs in dairy cattle that may pose risk to public health is forbidden.[58] Therefore, dairy cattle are not exposed to as many antimicrobials as suggested for all food-animals.

Milk residue regulations are enforced at the creamery level through testing of *all* milk shipments.[79] Meat residue regulations are enforced by the Food Safety and Inspection Service of the USDA (USDA-FSIS).[80] Milk and meat from dairy cattle destined for human consumption must originate from animals that have not received antimicrobials

for some time, and thus the prevalence of resistant bacteria may have changed from when individual animals might have been treated.

Most antimicrobials used in dairy cattle are approved for parenteral treatment of disease, not for production enhancement.[81] In fact, monensin is the only antimicrobial currently approved for production enhancement in dairy cattle (lactating and non-lactating).[76] At the time of this writing only six antimicrobials were approved for oral administration in non-lactating dairy cattle (calves, heifers and dry-cows);[76] three of them for production enhancement purposes and all for medicinal use. Potential antimicrobial-use in non-lactating dairy cattle should not represent a risk for the consumer, neither through residues nor by the possible presence of resistant bacteria, because non-lactating dairy cattle do not produce food for human consumption. In the rare event that non-lactating dairy cattle have to be slaughtered because there is no expectation of efficient milk production, carcasses will undergo inspection at the slaughterhouse as established under the Meat Inspection Act.[80] Calves raised at dairies are not used for meat consumption; they are raised to eventually replace lactating cows.

If antimicrobial-use in food-animals is considered to be responsible for resistance in bacteria isolated from humans, by reductive reasoning, it is also responsible for resistance in bacteria isolated from treated animals. Some studies have tried to assess the effect of antimicrobial-use in treated animals with non-uniform results for different antimicrobials even within the same studies.[82-90] A positive association between antimicrobial-use and resistance was found in epidemiologic studies performed in groups of animals.[82,83,88] These studies performed group sampling and reported whether or not antimicrobials were

used on that group. Some clinical trials where antimicrobial resistance was evaluated in individual animals after exposure to antimicrobials have reported a transient positive increase in resistance prevalence[84,86-88] that returned to original levels after discontinuing the treatment.[86,88] Some studies, including some of those that showed a positive relationship (in different groups of animals), failed to show an association between antimicrobial exposure in host animals and resistance among bacteria isolated from those individual animals.[64,83,84,87,88,90,91] A gradual decrease of prevalence of resistance in enteric bacteria (mainly *E. coli*) has been reported as calves increased in age.[64,90,92,93] No information could be found in these studies on whether this decrease in prevalence was in the absence of or in spite of potential therapeutic use of antimicrobials.

Given that no uniform association can be found between antimicrobial-use in cattle and resistance in bacterial isolates, other factors need to be studied that could be confounding the results. The methodology used in the previously described studies is quite different, so that comparisons and extrapolations are difficult. There is a need to develop a uniform methodology that is appropriate to determine the effect, if any, of antimicrobial-use in animals on resistance induced in microorganisms isolated from those animals. Using the analogy of Sir Arthur Eddington [4] that compared a fishing net with the methodology for scientific studies, we need to determine the size and mesh of the fishing net and where and how to fish before we can make conclusions based on our catch. The purpose of this dissertation is to evaluate methods that could be used to assess the effect of antimicrobial-use in food-animals on resistance in microorganisms cultured from those animals.

DISSERTATION OVERVIEW

This section provides an overview of the structure of this dissertation. The broad theme of the dissertation involves the study of methods to evaluate the effect that antimicrobial-use in dairy cattle may have on antimicrobial resistance in enteric bacteria.

Chapter 2 focuses on biosecurity and biocontainment practices that can be implemented on a dairy farm to avoid importing novel bacteria onto the farm and avoid spread of bacteria among the different groups of animals present on the farm. New bacteria may harbor antimicrobial resistant determinants that can be spread through commensal and pathogen populations already present on the dairy. The chapter includes a flowchart that has been designed to be used by dairy farms as a template to easily identify critical control points for the importation of extraneous pathogens. This chapter is formatted according to guidelines for publication in the Journal of the American Veterinary Medical Association.

Chapter 3 is a critical review of available data on antimicrobial-use and resistance in food-animals in the U.S. In addition, false conclusions that can be drawn from comparing data aggregated at different levels are revealed and discussed. This chapter includes proposed stratification of antimicrobial-use data that needs to be collected before an educated conclusion can be made on the potential effect that antimicrobial-use in animals has on resistance among enteric bacteria isolated form those animals. This chapter includes a comparison of data of antimicrobial-use that was collected at a commercial dairy with handwritten and computerized

records. The chapter has been submitted for publication and is formatted according to guidelines for publication in the Journal of the American Veterinary Medical Association.

Chapter 4 evaluates two sampling strategies for bacterial isolates that could be used in the evaluation of the effect that antimicrobial-use in dairy cattle may have on antimicrobial resistance in enteric bacteria. Stratified random sampling among animals in the various pens of a dairy farm is compared to cohort sampling, where the same animals were repeatedly sampled over 1 year. This chapter is formatted for the Journal of the American Veterinary Medical Association.

Chapter 5 compares the use of hierarchical analysis versus crude analysis to evaluate factors associated with antimicrobial resistance in *Salmonella* and *E. coli* isolated from dairy cattle during a longitudinal study performed on a dairy farm. This longitudinal study involves two different cattle populations (adult cows and calves), and the comparison of antimicrobial-treated versus non-treated animals. Additionally, resistance to antimicrobials in samples obtained from environmental samples and feces is compared. This chapter is formatted according to guidelines for publication in the Journal of the American Veterinary Medical Association.

Chapter 6 describes the development of a mathematical model to determine the best sampling strategy to describe the prevalence of antimicrobial resistant non-type specific *Escherichia coli* in dairy farms. This model compares two sampling approaches: single isolate per animal from a larger number of animals versus

multiple isolates per animal from fewer animals. This paper has been published in the American Journal of Veterinary Research,[94] and is formatted according to the guidelines for this publication.

Chapter 7 provides a summary of the conclusions obtained from the studies described in Chapters 2 through 6. Specific recommendations are presented for conducting a national longitudinal study to evaluate the effect of antimicrobial-use on resistance and suggestions for future research that would answer some questions that arose during this project.

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Chapter 2

BIOSECURITY AND BIOCONTAINMENT ON DAIRY FARMS: SUGGESTED OUTLINE FOR A COMPREHENSIVE PLAN

SUMMARY

Many U.S. dairy farms rely on external sources for replacement animals, feedstuffs, transportation, and labor. This translates into an increased risk of disease transmission. Effective biosecurity and biocontainment protocols can be designed for individual dairy farms by the veterinarian working closely with the dairyman. A diagram of the facilities and the possible input and output routes of the dairy farm can help identify critical control points where management practices may increase or decrease the risk of transmission of disease agents. We outline major critical control points that can be found on most dairy farms, and we discuss recommended control and monitoring procedures. A flowchart is provided that can be used as a standard approach for dairy farms in the U.S. An example is presented to demonstrate the use of the flowchart to help design a customized biosecurity and biocontainment program on a dairy farm.

INTRODUCTION

The dairy industry is progressing towards a smaller number of dairy farms with more animals per farm. Over the last 10 years, the number of dairy farms has decreased by 41% (from 180,640 to 97,560), but the total number of milking cows decreased only 4% (from 9.7 mill to 9.1 mill) despite an increase in milk production of over 20 billion pounds.[1] Consequently the average herd size for dairies in the U.S. has almost doubled from 53.9 milking cows in 1991 to 93.4 milking cows in 2001.[1] Western states such as Arizona, New Mexico, Idaho, and California have seen their average herd size more than tripled from 160 cows/dairy to 570 cows/dairy in this same period.[1]

As a result of these changes, it has become rare for dairy farms to be completely self-sufficient, self-contained, production units. Dairy farms are at risk of introducing disease agents, because most rely on external sources for labor, feedstuffs, replacement animals, supplies, and transportation. As per a USDA report, an estimated 45.7% of U.S. dairy farms brought new animals into the herd in 2001.[2] Farms that take animals to shows or temporarily house animals at another farm, and then bring those animals back, are not truly closed herds either, because these animals can come in contact with other animals. Many vehicles can travel among several dairy farms on a daily basis: some to collect milk, calves, or carcasses; others to deliver feedstuffs, pharmaceuticals or semen. Wildlife, rodents, and birds have access to many dairy farms and pose a risk for transmission of disease.[2-6] It is estimated that wild ruminants have physical contact with dairy cows or their feed on 53% of US dairy operations.[2]

Cattle on dairy farms are usually grouped by age or stage of production and are often managed within close proximity to each other. Larger dairy farms tend to focus on milk-producing cows and may contract outside services to manage their calves, heifers, and dry cows. These practices have led to the development of specialized operations, such as calf/heifer rearing facilities and transition management facilities[7] that can specialize and manage animals more efficiently, thus minimizing costs.

Interaction with all these outside resources can increase the possibility of introducing disease agents to the farm. This risk can be minimized by establishing and following a structured biosecurity plan. Biosecurity is the result of management practices designed to avoid introduction of disease agents to the farm.[8-10] Disease agents may include toxins or infectious pathogens, like bacteria, viruses and fungi. In contrast, biocontainment is the result of actions to prevent the spread of disease agents already present on the farm between groups of animals.[8] It is recommended that dairy producers, working closely with their veterinarian, develop a written plan reflecting the health history of the farm and future health goals. A written document makes it easier to objectively execute and revise the plan.

Dairy cattle can be exposed to pathogens through routes such as: oral exposure to contaminated feed or water; inhalation of dust and manure particles; exposure through other natural openings (*e.g.* teat-ends) and wounds; indirect contact via fomites; and exposure through vectors. Calves, heifers, and adult cows differ in their resistance and their exposure to pathogens, even throughout the various production phases. Therefore, some diseases that easily affect calves are not so important in adult cattle, and other

diseases such as mastitis are restricted to lactating cows. Despite these differences, the methods to control the spread of disease agents are based on the same principles: 1) avoid exposure to the agent, 2) ensure better resistance (specific and non-specific) to disease in the animals, and 3) ensure appropriate hygiene and disinfection.

A visual representation of the farm and its dynamics can help identify potential routes of introduction and spread of diseases that need to be considered when developing optimal biosecurity and biocontainment strategies. Here we describe the development of a flowchart that identifies important critical control points to be included in a biosecurity and biocontainment plan for dairy farms. Visual identification of critical control points within each area of the farm can be a very valuable aid in preventing introduction of diseases (intentional or un-intentional) and avoiding the spread of disease agents between areas of the dairy farm. This flowchart can also be used to detail the biosecurity protocol to the personnel that work on the dairy farm. This approach utilizes the same principles as the Hazard Analysis and Critical Control Point (HACCP) system.[11] The HACCP system is mainly a risk assessment approach that focuses on manageable risk factors identified as critical control points.[9] The HACCP system is based on three principles: 1) identifying hazards, 2) defining critical control points and potential mitigation procedures, and 3) designing a monitoring system to evaluate the effectiveness of any control method. If any of the implemented control methods is deemed to be of limited effectiveness, it should be changed. The strength of the HACCP system is its flexibility of implementation through improvement of suboptimal control methods to maximize effectiveness.

BIOSECURITY

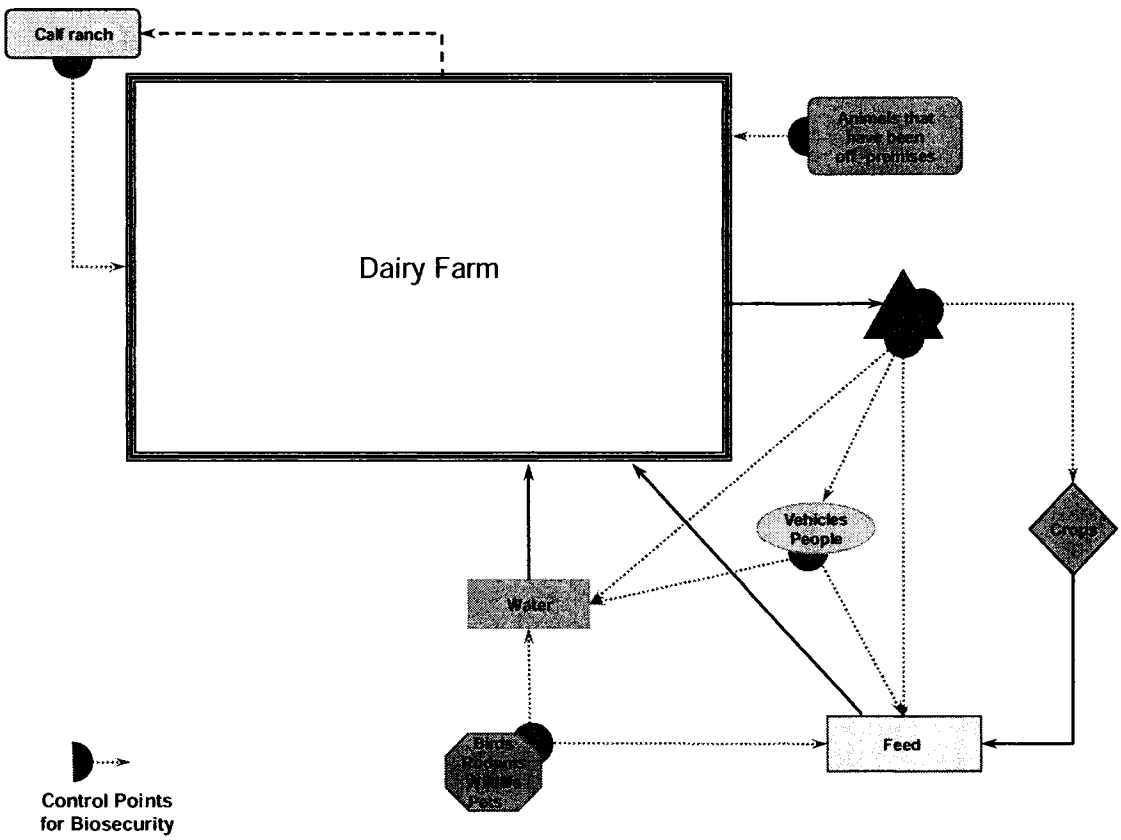
Figure 2.1 shows the subsection of the flowchart of potential routes for introducing disease agents onto a dairy farm. Potential hazards, control methods, and options for monitoring are discussed for each subsection.

Off Premises Cattle

Exposure to animals from other herds has been shown to be a risk factor for introduction of disease agents from other farms.[5,12] Cattle returning from off-premises could bring new pathogens onto the farm. Purchased animals could harbor pathogens that are endemic to the source farm but different from those present on the dairy farm where the animals are going. Incoming animals may be incubating a disease or may be infected with an organism that can recrudesce when the animal is exposed to the stress of transport and a new environment. Control measures to avoid introduction of new disease agents onto a dairy farm may require testing new animals before introduction and/or isolating the animals upon arrival at the farm.

It would be best if every new animal brought onto the dairy farm originated from farms for which complete and accurate health and vaccination records are provided. Before an animal arrives on the farm, its vaccination status and the absence of specific pathogens of interest to the farm should be assessed. The USDA's National Animal Health Monitoring System (NAHMS) Dairy 2002 survey estimated that 48.4% of all dairy farms did not

Figure 2.1 Diagram of potential routes for the introduction of pathogens onto dairy premises. The dairy farm is represented by the black box, and every arrow pointing to the farm is a biosecurity risk. Black solid arrows represent events expected to happen in most dairies, while black dashed arrows represent events that may apply to some dairy farms. Red dashed arrows with a half circle at their start represent hazard and control points for transmission of disease agents.



require any vaccination for incoming animals, and 75.5% did not require any testing of new animals.[13]

Table 2.1 shows the most commonly encountered infectious diseases and pathogens on dairy farms and how they can be prevented from entering a herd (testing or vaccination). Note that vaccination may be an appropriate method to control some diseases in a herd, but vaccination does not imply 100% protection for each animal, much less the herd. In working with populations (dairy herds), vaccination is an important method to develop herd immunity[14] and lower the risk of spreading a disease in the eventual case of exposure to a pathogen. Each producer may choose to focus on a different subset of these pathogens, depending on prevalence of disease in their area and their perceptions of risk and economic impact of disease. Appropriate testing procedures to be used for any of these pathogens will depend on the specific situation at that dairy. For example, different tests and/or strategies could be appropriate when purchasing 50 heifers that have been housed together in the same pen for the past 6 months compared to purchasing 50 milking cows bought at the livestock market and originating from 10 different sources.

It is important to take into account the predictive value of a negative result of a diagnostic test (NPV),[14,15] *i.e.* confidence that a negative test actually reflects a negative status. The NPV depends on the prevalence of the disease in the population, and the sensitivity and specificity of the test. Therefore, a given test will have a higher NPV when used on animals from source herds with a low prevalence of the disease than in source herds with a higher prevalence. Furthermore, when herds import animals from various sources, the

Table 2.1 List of infectious diseases most commonly encountered on dairies and possible control methods to avoid introduction of disease onto a dairy farm.

	Testing	Vaccination
Main	BVD – PI	BVD
	Brucellosis	Brucellosis
	Staphylococcus aureus	IBR
	Mycoplasma spp.	PI-3
	Streptococcus agalactiae	BRSV
	Mycobacterium avium subsp. *paratuberculosis* (MAP)	*Neospora* spp.
	Salmonella spp.	
	Tuberculosis	
Optional	*Leptospira* spp.	*Leptospira* spp.
	BLV	*Salmonella* spp.
	Tritrichomonas foetus	

NPV will not be uniform for all tested animals. Tests with low NPV give a false sense of security when an animal tests negative. This is an important issue in testing for pathogens, such as *Mycobacterium avium* subsp. *paratuberculosis* (MAP), where when lactating cows are introduced to the dairy farm, it is advisable to test them for the presence of contagious intramammary infections caused by *Mycoplasma* spp., *Streptococcus agalactiae* or *Staphylococcus aureus* by culturing their milk.[18] Animals of reproductive age may be tested for the presence of antibodies against *Neospora* spp. and *Leptospira* spp., taking into account that the presence of antibodies only indicates an exposure to the organism, which could be due to natural exposure or vaccination.

Historically, U.S. dairy farms have not routinely isolated or separately housed new arrivals. Of all cattle intended for dairy purposes brought onto dairy farms in the U.S., only 7 – 37% are estimated to be isolated, depending on the type of animal; adult cows being less commonly isolated than calves and heifers.[2,13] It is highly recommended that all purchased animals and all animals that have been off-premises be isolated for a period of time after returning to the operation (*e.g.* show cattle, unsold market animals, animals hospitalized at a veterinary clinic, animals temporarily housed at other farms or cattle facilities, and leased cattle). Any animal that leaves the dairy farm and comes in contact with other animals or premises that hold (currently or at some point) other animals should be housed separately from the rest of the herd upon reentry to the farm for at least 10 days, preferably 3 weeks. This period would allow for incubation and manifestation of clinical signs of highly infectious diseases such as salmonellosis, vesicular stomatitis, foot-and-mouth disease, clinical BVD, and infectious bovine rhinotracheitis (IBR).[19,20]

Therefore, if any of these agents had been imported, the animals would show signs while in quarantine and they would be prevented from spreading the disease agents to the rest of the animals on the farm, as long as strict hygiene practices are maintained in the quarantine facility. These short isolation periods, however, will not allow detection of diseases with long incubation periods, such as Johne's disease and neosporosis.[19] Infections imported via gestating fetuses are best tested for after the calf is born; an example of this is the persistent infection with Bovine Viral Diarrhea virus (BVD-PI).[19,21] The best biosecurity practices would not allow any animal to be released into the general population until all submitted biological samples are found to be negative.

Ideally, the isolation area should be a separate facility as far as possible from the rest of the herd and should be attended by separate personnel with separate and very visibly identified equipment and clothing (coveralls and boots). It would be impractical to hire specific personnel for the isolation facility if it is used only occasionally or in small dairy farms. An alternative would be to assign trained personnel from the farm to attend the isolation facility, but only after their regular tasks are completed. Strict protocols of hygiene and disinfection should be observed by these personnel. Under no circumstances should these personnel have immediate access to the neonatal calves or maternity areas after working in the isolation facility, because these immature animals are more susceptible to infections.[22-25] If maintaining a separate isolation facility is not an option for a particular dairy farm, an alternative would be to keep new or returning animals in a separate group in the most remote location on the farm possible. If the dairy farm has tie

stalls and there is only one building, the new animals can be tethered at the end of one row with as much separation from the other animals as possible.

Replacement heifer-calves sent to a calf ranch are a special case of off-premises cattle. The NAHMS Dairy 2002 survey reported that 10.5% of all replacement heifers are raised away from the dairy farm.[2,13] At these calf-rearing facilities, calves are usually raised in individual hutches until weaning and later commingled in small groups. Calves in these groups may have originated from more than one dairy farm. This situation creates a high risk of transmission of pathogens to the calf ranch when animals from several different farms with potentially different pathogen prevalence are commingled.

The larger concern for dairy producers is the return of these calves (now heifers) to the farm of origin, because their heifers have been commingled with heifers from other farms and may have acquired different microorganisms than those present on their farm. Heifers returning from the calf-ranch constitute a relatively steady flow of animals to be isolated as they arrive on the dairy farm. It may be more efficient to modify an existing heifer-pen for receiving the heifers rather than using the isolation area. Modifications to this pen should prevent fence-line contact with other pens through the use of double or solid fencing, and should provide unshared drinking water. Advantages of using a modified "receiving heifer-pen" over using the isolation area include prevention of possible contact with older animals and immediate adoption of on-farm management systems, such as lock-ups and total mixed ration (TMR). One disadvantage is a possibly easier indirect contact with the resident population. Personnel attending this "receiving

heifer-pen" should observe the same biosecurity protocols as designed for the isolation area.

Currently, some calf-raising facilities who are concerned with the health status of their animals are already requesting certain biosecurity standards in the source herds. These concerned calf-raising facilities will only accept calves that originate from dairy farms of a certain health status or from farms that follow standard procedures to improve biosecurity on their own premises. These requirements will allow the calf-raising facilities to minimize health problems in the calves and deliver a better product back to their clients. Biosecurity standards for both facilities should be discussed and agreed to by both the dairy farm and the calf-raising facility; we suggest including them in the contract negotiation.

To allow monitoring of compliance with isolation practices, records should be kept for all cattle that enter the farm. The foundation of any record system is unique and permanent identification for each animal. The records should contain the identification for each animal, place of origin, vaccination history, tests performed, date of entry to the isolation area, and date released to the general population.

To prevent the spread of reproductive and respiratory diseases such as BVD, leptospirosis, IBR, and bovine respiratory syncytial virus (BRSV) infections, it is especially important to ensure that the general population of the dairy farm has high herd immunity before the arrival of the new animals through proper vaccination protocols,[19] because these diseases spread rapidly among animals when biosecurity is breached.

Vaccination is important to avoid catastrophes at the herd level with certain diseases such as BVD,[19,21] although not all animals may be completely protected. When vaccination is selected as an option to control a disease, vaccination according to manufacturer instructions should be encouraged. Vaccinated animals should be allowed enough time to develop an immune response before being exposed to potentially infectious animals. As a general rule, four weeks should be allowed for adequate protection to be developed after vaccination is completed (one inoculation for live vaccines and two inoculations for inactivated vaccines).[26]

Manure

Excessive application of manure as plant fertilizer may result in potential contamination of groundwater sources.[27-29] *Salmonella* spp.[30,31], *E. coli* [30,32], and MAP [33] have all been isolated from soil and crop cultures over three months after fertilization with manure. Ideally manure should be treated as biological risk material and be composted or removed, but this can be prohibitively expensive when considering disposal costs of manure, opportunity cost of lost nutrients, and increased costs of commercial fertilizers. Economic considerations may limit the implementation of these control methods, because losses are certain, while the consequences of potential losses due to infection transmitted by manure are uncertain.

Methods of using manure include recycling for bedding (see biocontainment), anaerobic digestion as an alternative energy source (methane), or composting for use as commercial plant fertilizer.[34] For most dairy farms, though, the additional investment necessary for

implementing any of these technologies can be cost-prohibitive. Therefore, it would be best to look for other options to dispose of manure. The potential risk for disease introduction could happen when purchasing crops that have been fertilized with manure from other dairies.

Drinking Water

Well water for livestock should be tested regularly for potability.[35] Many pathogens can be spread through contaminated drinking water.[36,37] Two distinct areas need to be accounted for when potential contamination of drinking water is evaluated: water source and water delivery systems.

The NAHMS Dairy 2002 survey reported that 35.1% of all dairy farms used lakes, ponds, or rivers as water sources for their animals.[38] These water sources may carry disease agents from other animal facilities (cattle, swine, poultry, small ruminants, equine), as well as bird droppings, wildlife feces, and human waste. According a USDA report, wild ruminants had access to the water sources used by cattle on an estimated 40 and 60% of the dairy farms.[2]

Biosecurity control methods for drinking water would thus include restricting birds and wildlife from accessing the water source, and minimizing risk of amplification of pathogens through filtering and chemically sterilizing the water. If naturally open water sources such as streams, rivers, ponds or lakes are accessible to the dairy cows or heifers,

an option would be to restrict their access to these water sources and provide them with an alternate source of potable water that cannot be contaminated by wildlife and birds. Monitoring of water quality can be accomplished by regular analysis: chemical, mineral and bacteriological.

Feedstuffs

Feed can be contaminated by spreading manure on the fields where the crops are grown or irrigating the fields with contaminated water. If feedstuffs are cultivated and harvested by the dairy producer, keeping complete records of fertilization practices will allow monitoring crop production. It is advisable to request information on fertilizers used on purchased feedstuffs.

All batches of feedstuffs should be visually inspected for the presence of mold and tested for the presence of toxins. Regular monitoring of feedstuffs by laboratory methods can be prohibitively expensive, and therefore many farmers avoid this practice. An alternative is to store at least one sample from each lot of every feedstuff. In the event of health or production problems, samples can be analyzed for the presence of detrimental bacteria,[39,40] natural and chemical toxins,[41] molds, and fermentation products.[42] Samples should be stored at least until the whole batch of feed has been consumed without incident.

Another possible contamination of feedstuffs is through fecal material from birds, rodents, and wildlife, because most dairy farms do not have tightly closed facilities that would impede access of these animals.[43] Multiple methods are available to help control access of these animals to the dairy farm and feedstuffs. The best method for each dairy farm can be determined taking into account the design of their facilities and the cost of different methods of control.

Vehicles and People

Dairy farms can be considered open facilities in that they have major traffic on and between farms. Many dairy farms share the same milk transport truck from the processor, as well as vehicles that pick up calves, cull animals, or carcasses.[6] Some farms share equipment and service providers (hoof trimmer, veterinarian and artificial insemination technician).[5] According to the USDA, 38% of U.S. dairy farms shared heavy equipment with other livestock operations in 2001.[38]

Methods of control to avoid introduction of unfamiliar pathogens with vehicles or people include restricted access to certain areas of the farm, identified as "Authorized Vehicles Only" areas and disposable footwear covers for visitors. Additional measures to prevent access of unauthorized vehicles to some areas of the farm could include a well identified receiving area at the entrance of the dairy farm as far away from the animals as possible, where all delivery trucks could unload their goods. Similarly, there could be a designated loading area where cull animals or carcasses are loaded onto trucks to be transported

away. This may involve extra handling of goods or animals to and from those areas, but it could be designed so that on-farm vehicles and off-farm vehicles do not cross paths. Visitors should not have access to any area beyond the business office without invitation. Farm biosecurity rules can be clearly indicated with traffic signs forbidding access, similar to biosecurity policies of swine operations.[44] Monitoring practices for the traffic of people and vehicles onto the dairy farm may range from paper records (logs) to sophisticated camera surveillance systems.

Farm workers that have contact with other animals outside the farm can introduce diseases to the farm. Farm workers can also be the source for transmission of diseases such as tuberculosis, salmonellosis, beef measles (*Taenia saginata)*, and *Staphylococcus aureus* to animals.[20,45] Ideally, the best prevention practice would be to hire only workers that test negative for these agents and don't work with cattle outside the farm. For many dairy farmers, though, this may not be an option. Education of workers in basic hygiene procedures and in the areas of disease awareness, prevention, and control will provide an additional layer of biosecurity that cannot be duplicated by any single critical control point. Workers need to have easy access to equipment and facilities necessary to maintain proper hygiene.

BIOCONTAINMENT

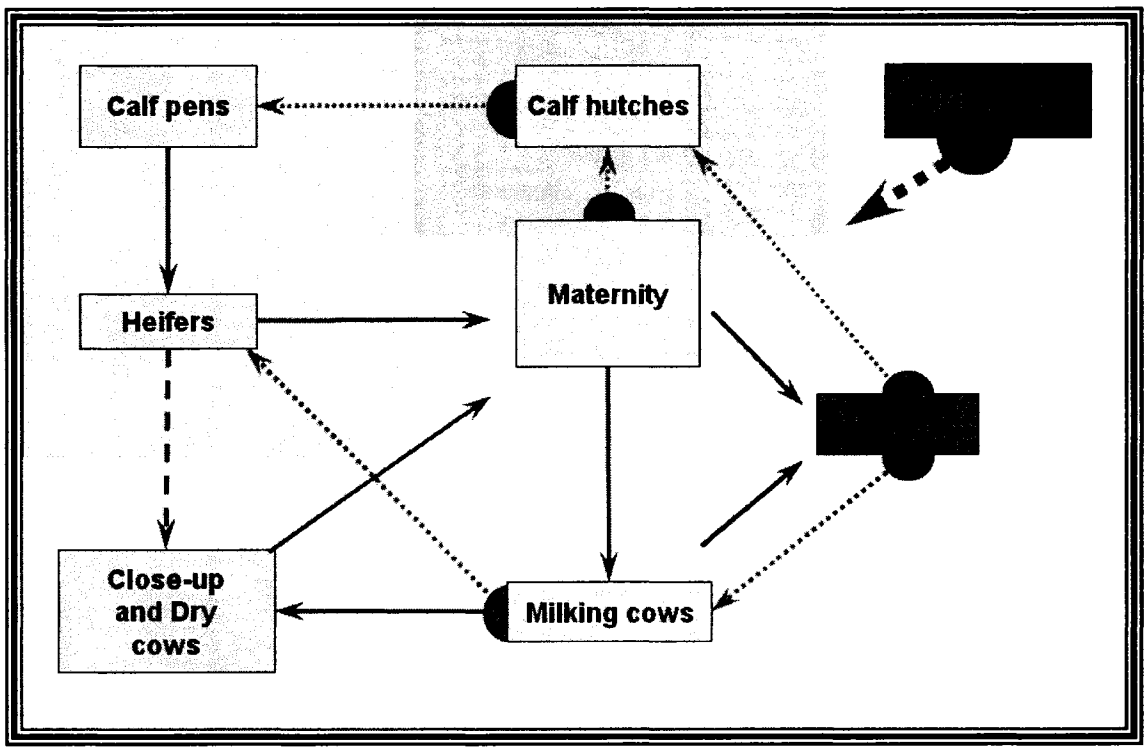
Biocontainment is the result of processes to prevent transmission of pathogenic agents within and between areas of the dairy farm.[8] Figure 2.2 shows the different operational

sections of a dairy farm and critical control points for biocontainment (red dashed arrows with a half circle).

Most animal traffic within a dairy farm is unidirectional: milking cows for example move from the maternity area to the fresh pen, continuing to the high-production pens, and then to lower production pens until cows are either dried or culled. The maternity area houses cows in the peri-parturient period, the time when cows are most susceptible to disease.[24,46] No part of the maternity area should be used to house sick animals. Similarly, cows usually don't go back and forth between different milking groups. Instead they move to lower producing pens as new cows freshen and produce more milk than they do. The only bi-directional traffic for adult cows should be between the general population of milking cows and the hospital pen, where sick cows are housed during treatment and elimination of drug residues. Identifying in the flowchart where a possible bi-directional traffic of animals can exist will help determine potential control methods.

Uni-directional traffic may be better defined in the young stock, because calves and heifers are grouped according to their age. Some dairy farmers may decide to separate a sick heifer that has not kept up with the growth of her group and to house her with younger calves. Older heifers have had the chance to be exposed to disease agents for a longer period of time than younger calves, which can result in either better immunity or chronic infections.[19] Therefore, if by chance older heifers come in contact with younger calves, these heifers may pose a risk for transmission of disease agents to the younger calves. Maybe a better option to avoid potential spread of disease agents to the younger

Figure 2.2 Diagram of potential routes for internal traffic of pathogens on dairy premises. Black full-line arrows represent common practices on U.S. dairy farms, black dashed arrows represent practices in some dairy farms, and red dashed arrows with a half red circle represent critical control points.



calves is to create a "recovery-pen" where these unthrifty heifers can catch up with the rest of their group and then return to it. This would be the equivalent to a hospital-pen for young stock. In smaller dairy farms, where all animals could be tied in the same building, an alternative option could be to either move the unthrifty heifer to one of the ends of the row, or to leave some space between her and the rest of the heifers to at least avoid direct contact.

Biocontainment integrity is best maintained by avoiding direct and indirect contact between groups, especially for high-risk groups. The facilities design for the dairy farm can help prevent the spread of pathogens to more susceptible animals (sick cows, fresh cows and newborns). On most dairy farms there are four distinctive groups of animals: neonates, young stock, milking cows, and dry cows. The dairy farm can be organized such that each group occupies a different area to avoid direct contacts with other groups. In Figure 2.2 each area is identified by a different color: red for neonates/calves, green for young stock, blue for milking cows, and yellow for dry cows.

Indirect contacts can arise through personnel movement, shared transit areas, shared equipment, vehicle traffic, shared feed (such as using feed-refusals from cows for young stock), shared water, or movement of waste from one group to another. On large dairy farms with designated personnel, it may be possible to provide color-coded clothing (e.g. coveralls) and equipment (colored tape) for each area to identify indirect contact. For example, a person with blue coveralls using a shovel with green tape in the calf area (red) is a highly visible breech of biocontainment. In smaller farms, where different groups of

animals may be managed by the same person, the same principle of avoiding direct and indirect contact applies and can be followed by using specific clothing and equipment in high risk areas, such as the hospital-pen and the neonate area, and thorough hand and utensil washing and disinfection between areas. In this case, it is advisable to organize the work in sequence starting with the most susceptible animals and working towards the least susceptible animals. In smaller dairy farms with tie-stalls, an alternative method could be to reorganize the animals in the stalls according to their production and health status, reserving the opposite ends of the rows one for the most susceptible animals (neonates and fresh cows) and the other one for sick animals. The NAHMS Dairy 2002 survey reports that less than half of U.S. dairy farms (42.1%) trained their employees in prevention of introduction and spread of disease.[2,38]

Specific risk points for transmission of pathogens, within the different production areas of a dairy farm, are represented in the flowchart in Figure 2.2 by red dashed arrows, and are detailed below.

Isolation Area

The risk of transmission of pathogens from the isolation area can extend to all other areas of the farm if biocontainment is breeched. The recommendations stated above under the biosecurity section apply to the isolation area as part of the biocontainment protocol. When milk-producing cows are isolated pending results of their milk culture, following strict milking hygiene protocols can help prevent transmission of contagious mastitis pathogens during the isolation period.

Equipment must be routinely cleaned and disinfected to prevent disease transmission within the isolation facility. The design of the isolation area ideally should allow for thorough cleaning and disinfection between periods of use. Therefore, a design for ample capacity should be considered. Records that reflect date of entry of the animals to the isolation facility, date of exit, biological samples submitted, and results of the tests can be monitored to assess compliance with the biocontainment protocols.

Newborn Calves

Calves are usually born in the maternity area and then moved to individual hutches to avoid direct contact with other calves and to prevent transmission of gastrointestinal and respiratory pathogens. Calves are particularly vulnerable while in the maternity area because of the lack of humoral protection at birth.[24,25]

Biocontainment principles include general cleanliness and hygiene of the maternity area to decrease pathogen load. Ideally, the maternity area should be designed to separate a cow in labor from the rest of the cows in the maternity area. This can help provide a less stressful environment for the cow to calve and a cleaner environment for the neonate. Shared maternity areas for multiple cows can result in higher levels of contamination and risk of exposure for newborns. Regardless of the calving facility design, it is recommended that all calves be removed from their dams soon to minimize exposure to fecal pathogens from adult animals.[17,47] Thorough cleaning and disinfection of maternity

pens and proper colostrum management are important control measures to avoid transmission of pathogens from cows and their environment to newborn calves.

Colostrum is the basis of neonatal health and immunity,[48] and the only exogenous source of energy and nutrients for the newborn. Therefore, dairy producers should make sure that all calves consume adequate amounts (1 gallon) of good quality colostrum within 24 hours after birth.[48,49] Optimum immunoglobulin levels in calves can be achieved by proper vaccination of dry cows to ensure transmission of immunoglobulins through colostrum, and controlled feeding of colostrum to the newborn calf to confirm the amount ingested.[50] Special attention should be paid to cleanliness and hygiene of all equipment used to feed and house newborn calves, because contamination of colostrum through inadequate hygiene of equipment counteracts the beneficial properties of colostrum.[49,51]

The NAHMS Dairy 2002 survey showed that 27% of all U.S. dairy farms and 70% of large dairy farms (more than 500 cows) fed pooled colostrum.[2] The feeding of pooled colostrum should be discouraged because of the potential to disseminate pathogens that are transmitted through colostrum such MAP [17,47] and bovine leukemia virus (BLV).[52] Selective feeding of cow-to-offspring colostrum allows future vertical testing and culling for diseases such as neosporosis and MAP infection.[53-55] If colostrum pooling is necessary, it is recommended that only colostrum from cows that have tested negative to MAP, BLV and neosporosis is used. If this is not possible, pasteurizing the colostrum can be considered to destroy the pathogens, with the caveat that pasteurization also destroys more than 25% of immunoglobulins in colostrum.[56,57] There are immunoglobulin

products now on the market that could help supplement the calves when adequate amounts of safe colostrum is not available.[58]

To monitor attention given to newborn calves, random or systematic testing of serum total protein in calves 2-10 days old can be performed.[49] Other possible monitoring practices may include regular bacteriologic count of bacteria in colostrum, specific-gravity testing of colostrum and recording the time of birth, time of colostrum feeding, and source of the colostrum for each calf. Monitoring the incidence of disease in calves within the first 1-2 weeks of life may also be a valuable indicator of colostrum management.

Feeding of Waste Milk to the Calves

Feeding of waste milk to the calves is represented by the dashed arrow from the hospital to the calf hutches in Figure 2.2. Waste milk can be any abnormal milk that cannot be used for human consumption: colostrum, mastitic milk or milk containing drug residues. The NAHMS Dairy 2002 survey reports that 87.2% of all U.S. dairy farms used some waste milk to feed their calves. Transmission of bacteria from antibiotic-treated cows to calves through milk has been reported.[59]

The potential risk of transmission of these bacteria could be avoided by discarding the waste milk and feeding calves either normal milk or milk replacer. If dairy farmers don't want to discard the waste milk, effective pasteurization offers an option to minimize

transmission of highly contagious bacteria such as *Mycoplasma* from cows to calves through milk.[18] The NAHMS Dairy 2002 report estimates that only 1% of the dairy farms fed pasteurized waste milk.[13] Another option could be to feed the pasteurized waste milk to the older calves to spare potential exposure of younger calves with less immunity, and feed younger calves normal milk or milk replacer.

Calf Grouping

Appropriate management of calves moving from hutches to group pens may be a major determinant of the incidence of respiratory disease in the calves after grouping. Coordinated immunization must precede the movement of calves from hutches to group pens. Calves should be vaccinated at least 3 weeks - preferably 4 - before grouping to allow for development of active immunity.[26] When using killed vaccines, the first dose of vaccine should be given at a time to allow 3-4 weeks between administration of the booster dose and time of grouping.

It is recommended that weaning (cessation of milk feeding) occur while the calves are still in the hutches to avoid adding the stress of weaning to the stress of grouping.[60,61] Other stressful events, such as dehorning and removal of supernumerary teats (castration, if males are raised), can be performed 2-3 weeks before or after grouping, also to avoid the additive stress. Leaving time between procedures to prevent additive stress, a logical sequence may be to: vaccinate, dehorn/castrate, revaccinate, wean, and group.

Sick calves housed in individual hutches should not be moved to groups until they are healthy, weaned, and vaccinated. In tie-stall dairy farms, where calves are not grouped, attention can be focused on avoiding contact between sick and healthy calves by leaving space between them. Compliance can be monitored through record-keeping of event dates for vaccination, weaning, dehorning, and animal movement.

Feed-Refusals

The critical control point for feed-refusals is represented by the red dashed arrow from the milking cows to the heifers in Figure 2.2. Current nutritional guidelines for milking dairy cows indicate that cows are not able to produce to their potential unless feed is available at all times.[35] Therefore, milking-cows are "overfed" to insure that they don't go hungry between feedings. A common target for nutritionists is to have 5 to 10% feed-refusals.[1] This left-over feed comes from the most expensive ration on the dairy farm. Additionally, nutrient requirements for growing heifers are similar to those for lactating cows,[35] and therefore economics encourage feeding feed-refusals from high-producing cows to growing heifers. These feed-refusals, however, have been in contact with oro-nasal fluids from milking cows, and therefore may have been exposed to pathogens that inhabit the digestive and respiratory system of adult cows.

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[1] "100 feeding thumb rules revisited". Hoard's Dairyman. September 25, 1995

An option to avoid losses through feed-refusals and not expose the replacement heifers is to feed the refusals to cows that are destined to be culled soon, which could be housed in a separate group. If economics dictate that feed-refusals be fed to growing heifers, it is recommended that the heifers have active immunity against respiratory and enteric diseases through rigorous vaccination. It should be noted, however, that feeding refusals to heifers entails a higher risk of transmission diseases, such as MAP infection or *salmonellosis*, that are not controlled by vaccination alone.

Hospital Pen

Once a sick cow has recovered and eliminated drug residues, it is returned from the hospital-pen to the appropriate production group. The flow of cows from the general population of milking cows to the hospital is represented in Figure 2.2 by a black arrow. The red dashed line with arrow represents the risk for transmission of pathogens when cows are returned from the hospital to the general population.

Many dairy farms have a single milking parlor that is used to milk all cows in the herd, including the fresh cows and the hospital cows. In these farms, the risk for transmission of intramammary infections is higher than in farms with separate milking parlors for sick cows and healthy cows. Good milking procedures and a logical milking order [62-64] of the production groups are necessary to minimize the risk of transmission of contagious intramammary pathogens from infected to healthy animals. The logical milking order is based on milking groups in increasing prevalence of subclinical infection, as determined

by SCC. [62-64] Fresh cows and first lactation heifers should have the lowest prevalence of mastitis, and yet are the most susceptible to infections due to immunosuppression. These groups should be milked first when the rubber liners are the least contaminated; they are followed by high producing cows, lower producing cows, and lastly cows ready to be dried off.

Cows with contagious untreatable infections should be grouped in a separate pen (e.g. *S. aureus* and *Mycoplasma* spp. udder infections).[18] If a pen of chronically infected cows exists, it is recommended that cows in this pen are milked after all other healthy cows have been milked or even with a different milking unit. If only one milking parlor exists to milk all cows on the dairy, cows in the hospital-pen should be the very last group to be milked, but only after thoroughly cleaning and disinfecting the equipment that just milked the chronic infection group. Bulk-tank milk can be monitored through cultures [18,65] to signal new infections with contagious pathogens and therefore a breach in the biocontainment protocols.

Other diseases in addition to intramammary infections may be spread by cows returning from the hospital-pen to the general population. Cows that recover clinically may be returned to the general population while they still have the potential of shedding pathogens, such as *Salmonella* spp.[19] This poses a risk for transmission to other animals. Control of this hazard and monitoring should be customized for the specific disease that caused the animal to be in the hospital-pen. In conjunction with the herd veterinarian, a decision should be made regarding prolonged periods of isolation or even culling of these cows.

Feed and Water

Animals from different production areas (calves, heifers and adult cows) have different prevalent disease agents and different susceptibilities to infection. Therefore, caution should be exercised to avoid shared water, feed, and equipment, as well as direct contact between these groups. According to the USDA Dairy 2002 report, more than half of U.S. dairy farms (58.8%) had at some point used the same equipment for handling manure and animal feed, with 15.2% of them not using any procedure to clean or disinfect it.[2] Vehicles and equipment used for waste management have been identified as a risk factor for contamination of feed when they are also used for feed-handling procedures,[66] such as distributing, loading, mixing, or pushing feed. Sharing equipment for carcass disposal and feed-handling may also be a risk factor. Care should be taken to ensure that vehicles with manure-covered wheels do not contaminate feed bunks or silos during packing or during loading and delivering of feed.

Contamination of water delivery systems can arise due to feces and oral droppings (feed and saliva). Water troughs can be designed to allow easy cleaning, both periodically and *impromptu* as necessary. Metal water troughs that can be tilted to allow fast emptying and located in easily accessible areas will be easier to clean. MAP [33,67] and *Salmonella* spp.[68] can both survive in drinking water for months if proper decontamination is not accomplished.

Monitoring the possibility of cross-contamination can be performed by visual inspection of equipment to ensure it is clean of manure before handling feed. Visual inspection of

water troughs for cleanliness seems to be a simple and yet effective method to monitor possible water contamination. Monitoring of water quality can be accomplished by regular analysis: chemical, mineral, and bacteriological.

Water Recycling

Some dairy farms use recycled flush-water, after separation of solid material, to remove fecal material from alleyways and holding areas. This water runs along all the areas where cows ambulate. Therefore, cows can have direct access to this water (drinking/licking), and feed can become contaminated by splashing into the feedbunk.[69] Reducing pathogen transmission at this point can be achieved by timing the flushes when no animals are present in the areas to be flushed (*e.g.* while milking), feeding after the flush water has been released, and designing the facilities to prevent oral access by any animal to run-off water after flushing. In farms where no flush-water is used, care should be taken not to splash the feedbunk when pressured water is used to clean the areas contaminated with feces. Monitoring of this process can be achieved by observation.

Manure Recycling

Large dairy farms have developed systems that compost manure and reuse it as bedding.[70,71] Manure has an enormous load of bacteria (10^3 - 10^8 bacteria/g in recycled manure)[70] and is the most problematic waste produced on dairy farms.[34] All nutrients contained in manure could be recycled as fertilizer for crops.[29,34] With large dairy farms,

it is increasingly difficult to find adequate crop acreage under cultivation where it can be used. Although bacteria such as *Salmonella, E. coli, Listeria* and MAP have been shown to be promptly undetectable in composted manure,[72,73] incomplete composting could pose a risk for exposure to fecal pathogens from some areas in the dairy farm to others.

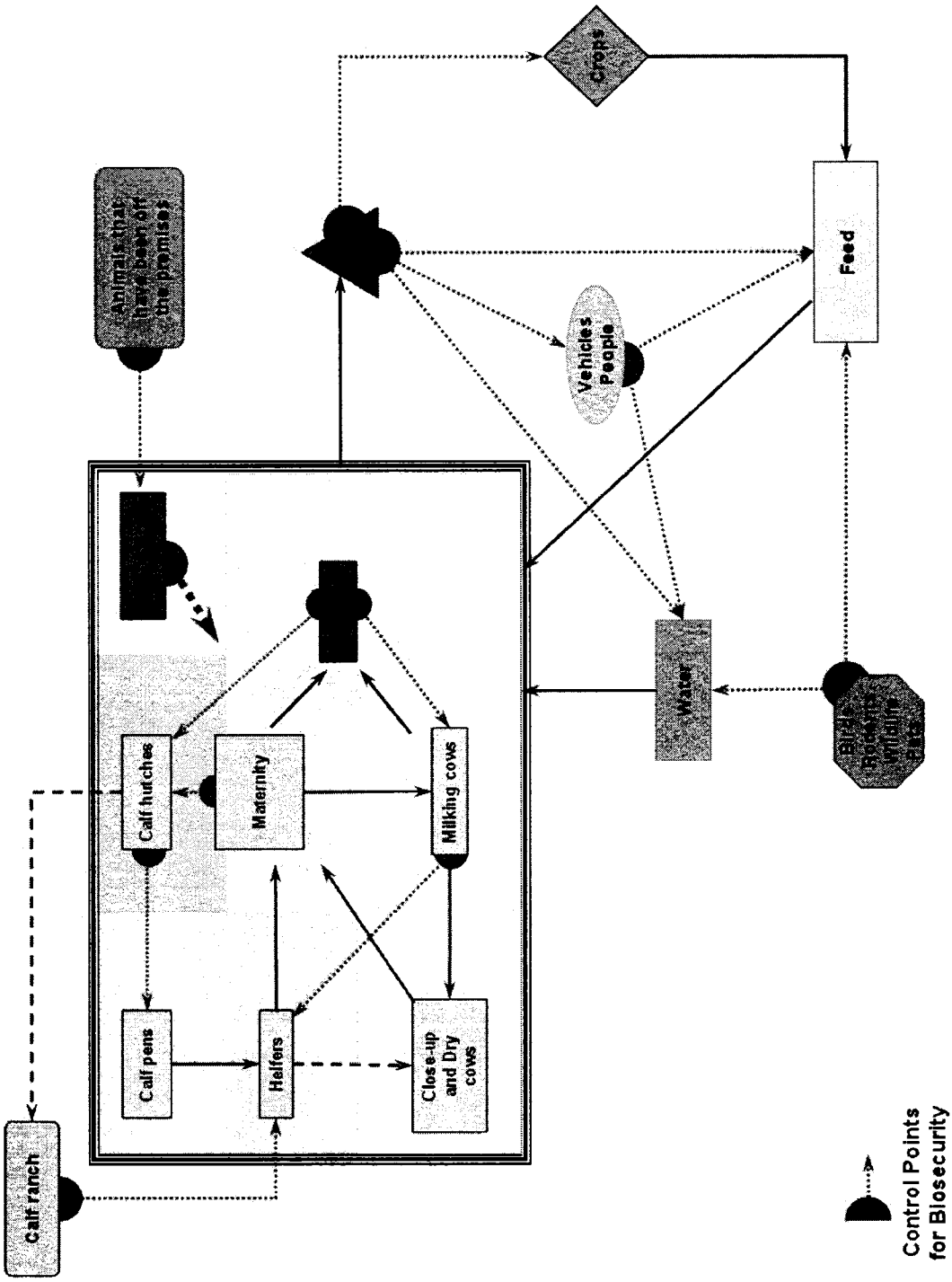
Control measures may include avoiding the use of manure from high-pathogen concentration areas, such as the hospital-pen and the isolation area, and ensuring proper composting procedures. Monitoring practices can include recording the origin of manure used for the different composting batches and quality control procedures for the composting process, such as temperature and microbial activity.[74,75]

The complete diagram for critical control points that need to be considered for effective biosecurity and biocontainment in dairy farms is presented in Figure 2.3. Maintenance of complete and accurate records of disease events, treatments, vaccinations and corrective actions to be implemented to address breaches in biosecurity or biocontainment protocols are important to monitor compliance and effectiveness.

SPECIAL CIRCUMSTANCES

Dairy farms in expansion may import animals from various sources having different exposure histories and that will be commingled. Expanding dairy farms also tend to keep lower producing animals for longer periods to avoid a decrease in herd size due to culling. Animals with low production could be harboring some production-limiting disease agents such as *Salmonella* spp, MAP, or any of the contagious mastitis agents.

Figure 2.3 Comprehensive diagram for biosecurity and biocontainment in dairy premises. Black arrows represent common practices on U.S. dairy farms, while red dashed arrows with a half red circle represent critical control points.



Requirements for managing a rapidly growing herd can result in insufficient time for regular tasks on the farm because human resources are usually overtaxed.[76] In the dairymen's mind, short-term economic survival may take priority over long-term herd health, and thus some farmers may decide to relax biosecurity and biocontainment safeguards in favor of a short-term economic advantage.

By having a defined biosecurity and biocontainment plan that prioritizes diseases of interest, managers can focus on those with the highest perceived risk for the dairy. Some biosecurity measures can still be followed in these circumstances. For example, rather than testing every animal in the group individually, pooled samples of all animals or a representative sample of the animals can be tested. Pooled samples could be appropriate for detection of BVD-PI and contagious mastitis, while a representative sample of animals can be tested for *Leptospira* spp.

All incoming animals (even if originating from different sources) can be grouped together in one or more "receiving pens" as explained for replacement heifers and still be separate from the general population. These pens can be located at the edges of the dairy farm. This way, resident animals could be spared from potential direct transmission of pathogens. Additionally, since the incoming cows are already segregated, they can be milked after the resident cows, avoiding potential transmission of contagious mastitis pathogens. If the expanding dairy farm intends to use feed-refusals for the heifers, they should feed the refusals from the resident cows of the herd, not the incoming cows.

Discarding the feed-refusals of the incoming cows for 2-3 weeks is a viable option that should not have a large economic impact on the dairy farm.

PRACTICAL EXAMPLE (DEMONSTRATION)

Let's consider a specific dairy farm whose main concern is *salmonellosis*, both in calves and cows. We visited the farm and noted on the flowchart those hazards that are currently present and need correction. These control points are identified in Figure 2.4 with a red semicircle and arrow. The control points are detailed below. Black arrows denote actual input or output routes present at the dairy.

Control Point 1. Approximately 200 purchased replacement heifers between 22-30 months of age (either before calving or already milking) and occasional breeding-age bulls for service have entered the herd at different points during the past year. All these off-premises animals are commingled with the resident population of cows and heifers without a period of isolation, because there is no specific isolation facility. Therefore, the most critical control point on the dairy is the absence of an isolation area. This situation is denoted by the thick red arrow on the flowchart. The main reason for not having an isolation area is the cost of building a new facility. A temporary pen at the edge of the dairy could be used as an isolation area, making minor changes to avoid contact between incoming and resident animals.

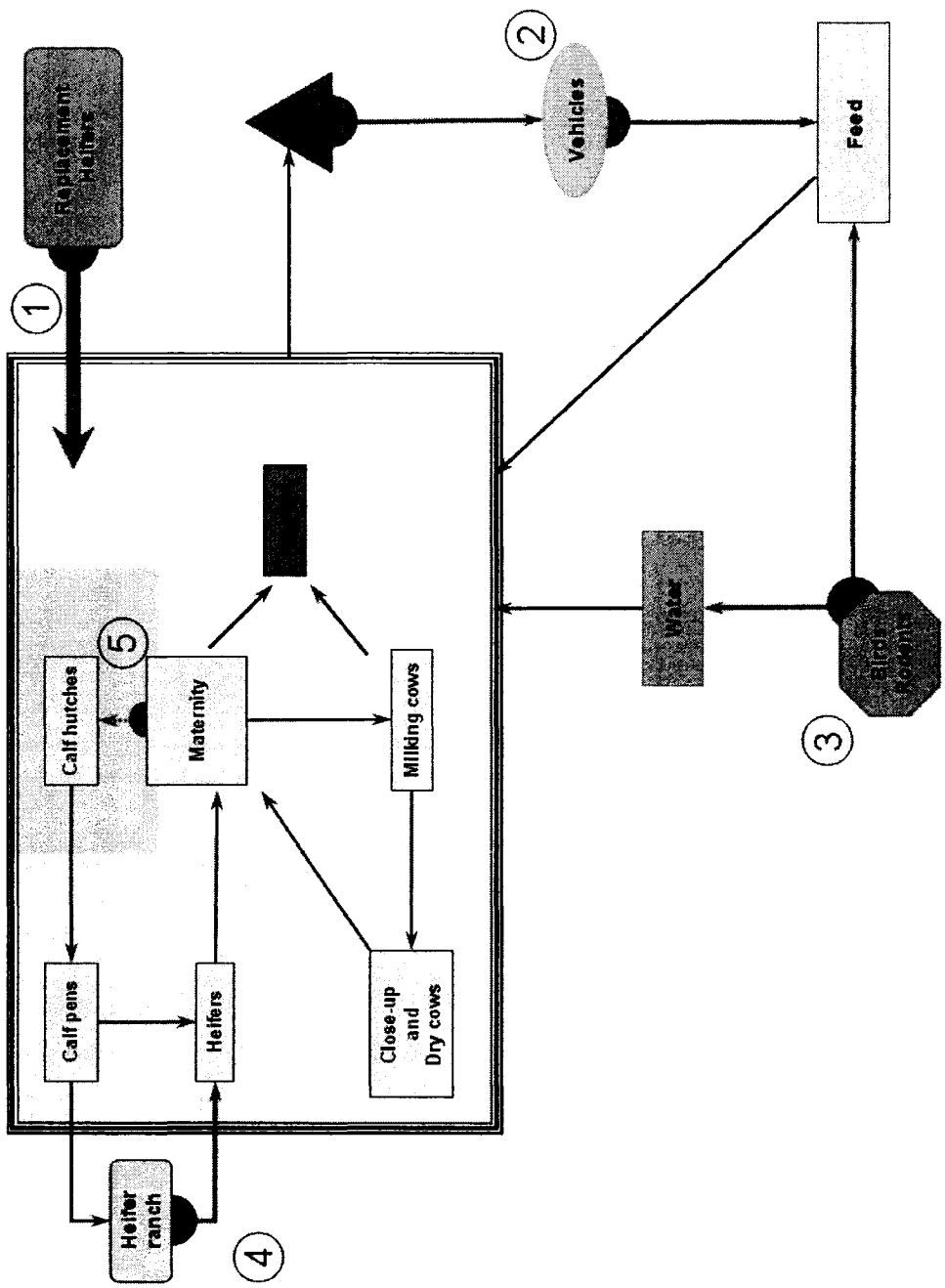
Control Point 2. Vehicles that sweep the feed-alleys have to drive over areas heavily contaminated with feces, mainly transit alleys to-and-from the milking parlor, to access the feedbunk. A recommendation is to sweep the feed-alleys after the transit alleys have been washed and have dried, which diminishes the potential for contamination without major construction or changes in the facilities.

Control Point 3. All waterers and feed sources have open access to birds and rodents, which are difficult to control due to the ubiquitous nature of these pests. Possible control methods to be discussed at this dairy farm include traps, bait, predators, and plastic predator decoys.[2]

Control Point 4. This particular dairy farm sends the heifer-calves to be reared at a heifer ranch when they are 10 months old. When they are seven months pregnant, they return to the farm to a separate pen at one of the corners of the farm. Heifers are moved in and out of the dairy once a month. The separate pen functions as an isolation area for heifers returning from the ranch, which allows the detection of clinical signs of disease in the heifers before they are commingled with the rest of the cows. One potential problem is the size of the receiving-pen, because it is too large for all-in-all-out movement. When new heifers are moved in from the ranch, they are mixed with heifers that are left from the last move. New incoming animals can pose a risk for animals already present in the pen. Because the dairyman considers these heifers his property even when at the heifer ranch and

[2] Lee, C.D. 2005. Got Starlings? Integrated Livestock Management, Colorado State University. Colorado Dairy News. Vol 11, No 3 (May/June).

Figure 2.4 Diagram for biosecurity and biocontainment at an example farm concerned about salmonellosis, both in calves and cows. Black arrows represent actual practices on the farm, while red arrows with a semicircle represent critical control points for salmonellosis.



he argues that they come from the same ranch, he has not enforced any isolation between groups coming in at different times. The recommendation in this instance is to split this pen in two halves so that animals cannot come in contact, and make sure all animals in one half are gone before moving new animals in. This strategy would also allow for cleaning and disinfection of the pen between groups. An additional option may be moving fewer animals every 2-3 weeks instead of once a month. It would be advisable to investigate if there were any infectious disease episodes at the heifer ranch and the health records of the individual incoming heifers, to assess the risk of introducing pathogens onto the dairy farm.

Control Point 5. Calves are born in a common maternity area where 20-30 cows are housed. A problem with this design is that it allows clustering of animals when they are most susceptible to disease (peri-partum and newborn). The advantage of the design is that it allows for specialized personnel to attend these animals. The present design allows contact of newborn calves with multiple cows. It has been suggested to build individual calving pens to prevent contact of the newborn with other cows and to diminish stress to cows in labor. A design is under study to allow fast cleaning of these individual calving pens between two resident cows, thus avoiding indirect contact of newborns with other cows.

Comparison of Figure 2.3 and Figure 2.4 will show that some red dashed arrows from Figure 2.3 are missing in Figure 2.4. These potential hazard points are not present at this

dairy because of control measures that are already in-place. Note that this dairy has no crop production and therefore does not apply manure to them, although the purchased feedstuffs may have been fertilized with manure from one or more of several animal species.

The recommendation for storing a sample of each batch of every feedstuff for eventual testing in the case of an outbreak would be valid at this dairy, too. This dairy farm has a protocol to ensure that no cow housed in the hospital-pen diagnosed with diarrhea returns to the general population until two cultures for *Salmonella* spp. in feces taken two to three days apart are negative. This may be the best method of ensuring no latent shedders return to the general population, short of culling every animal with diarrhea. Therefore, no additional control measures at the hospital-pen were considered.

Feed-refusals are discarded, and waste-milk is pasteurized and regularly cultured to monitor the process of pasteurization, which is why those potential control points are not marked in red. Dedicated personnel is assigned to the calves and never come in contact with the cow area. Random blood samples are taken every week from the calves born during that week to measure total plasma protein levels and to monitor proper colostrum management. Only calves that have not shown any sign of disease within the preceding two weeks are moved from the hutches to the pens (grouping). Finally, the design of waterers and feed-bunks is such that it minimizes direct contamination with manure, plus plenty of access points for personnel permit them to enter and exit pens without stepping on the feed with manure-contaminated boots.

BIOSECURITY AND ANTIMICROBIAL RESISTANCE

Knowledge of the epidemiology of antimicrobial resistance is an important first step to evaluate how biosecurity and biocontainment control measures can impact emergence and spread of antimicrobial resistance on dairy farms. Resistance genes can be transmitted in two ways:

- Vertical transmission happens among the different generations of bacteria.[77,78] Transmission of resistance genes in this manner creates bacterial clones that carry the same genetic information. These genes however can later be lost or altered by mutation or new resistance genes can be acquired.

- Horizontal transmission happens among bacteria that share the environment.[77,78] The genes can be contained in plasmids or free in the environment (from lysed bacterial cells). In this manner, genes can be transferred between bacteria of different species and genera.[78,79]

Antimicrobial resistant bacteria can be imported to dairy farms in any commodity. The most important factors are feedstuffs and live animals, because they represent extensive contact with resident animals. Control methods described in the biosecurity section for prevention of pathogen import can be applied here in order to prevent resistant bacteria. New resistant bacteria that are introduced to the dairy will have to compete for nutrients with resident bacteria (susceptible and resistant). If the new bacteria survive, they can actively transfer resistance genes to other bacteria; and if they die, other bacteria may acquire their DNA from the environment (transformation).[77]

Antimicrobial use in calves and cows can create a selective pressure in favor of resistant bacteria by killing or inhibiting the growth of susceptible bacteria.[77,78] Resistant bacteria may multiply during this time of positive selection and perpetuate the resistance genes (vertical transmission). Resistant bacteria can also transfer genes to the commensal flora that survives the action of the antimicrobial (horizontal transmission). These commensal organisms may already be resistant, given that they have survived. It is important to highlight that resistant bacteria may be less competitive with the rapidly colonizing commensal bacteria once the selection factor (antimicrobial) is absent. This is known as "fitness-cost",[80,81] and may explain why the prevalence of resistance among enteric bacteria in organic dairy farms (no antimicrobial use) is similar to that found in conventional dairy farms.[82-84] If resistant bacteria cannot compete for nutrients with the commensal flora of the animal, they will die and clonal transmission will be prevented. However, even the temporary existence of resistant bacteria may be a risk for propagation of resistance genes, since the release of DNA when bacteria die can result in horizontal transmission.

Milk obtained from cows that are being treated with antimicrobials cannot be used for human consumption during treatment and for a specified amount of time (withholding period) after the last treatment while residues of the antimicrobial may still exist in the milk.[85] The withholding period varies according to the type of antimicrobial used, dose, and route of administration.[11,86] To avoid antimicrobial residues in milk, cows that require treatment are housed separately in large dairies. The separate housing functions as an isolation area for treated cows that prevents contact with healthy cows. Therefore,

cows under selective pressure in favor of resistant bacteria due to the antimicrobial treatment probably do not facilitate transfer of resistance genes to healthy cows (horizontal transmission). On the other hand, housing all sick cows together implies that *all* antimicrobials used in milking cows are used in the same group of animals. This clustering of antimicrobial use within a pen could theoretically create the perfect environment to select multi-resistant bacteria because several antimicrobials may be used in the group at a given time. Additionally, the fact that antimicrobials are constantly being used in this group of cows may favor the clonal spread of resistant organisms (vertical transmission) within single cows or transferred among cows.

By definition, cows in the hospital pen either are sick at the moment or have been sick recently, which makes them more susceptible to acquire other infections from the rest of the animals in the pen. For example, a cow that is housed in the hospital to be treated for pneumonia and shares her environment with another cow that is being treated for mastitis is more likely to acquire mastitis than a cow in the general population. Prevention of disease using biosecurity and biocontainment protocols will decrease the number of animals that are housed in the hospital, and therefore antimicrobial use, which in turn will prevent selective pressure favoring resistant bacteria.

In tie-stall type dairies, sick cows that need antimicrobial treatment are not usually moved from their stall during treatment. Therefore, antimicrobial use is not clustered in a group of animals or in time as happens in larger dairies. If in large dairies this clustering of antimicrobial use was considered a factor for multidrug resistance, no clustering should be protective in tie-stall type dairies. In contrast, sick and healthy cows are now

commingled. It is not known how this commingling can affect transmission of resistance genes. Antimicrobial treatment in sick cows will presumably reduce the number of susceptible bacteria within treated cows. The surviving resistant bacteria could in theory transfer resistance genes to other bacteria within the treated cow or bacteria from healthy cows and the environment. However, if resistance bears a "fitness-cost", it may be difficult for resistant bacteria to compete with the commensal flora for nutrients, and they will die. Once the bacteria have lysed and freed the DNA into the environment, other bacteria can uptake resistance genes by transformation.

An important feature of cows housed in tie-stall conditions is the limited oral exposure to fecal material compared to cows roaming free in a pen. When cows defecate in tie-stalls, some fecal material may splash and contaminate their feed and drinking water, but the exposure to feces in tie-stall is minimal compared to that in a pen of loose cows. Animals in tie-stall housing are usually limited in their direct contact to two neighboring animals, as opposed to multiple animal contacts in a pen. Reducing the number of contacts among animals will prevent the spread of bacteria (resistant or susceptible). Although exposure to enteric bacteria may not be as important for tied animals as it is for penned animals, exposure to respiratory pathogens may be elevated due to enclosed housing. Therefore, implementing management practices that improve air quality in the barn should help in preventing respiratory disease and avoiding a possible spread of resistance genes among airborne pathogens.

It is important to note that most infections requiring antimicrobial treatment in dairy cows are due to mastitis (approx. lactational incidence 15%).[13,87] Given the localized infection

and the anatomy of the udder, direct transmission of pathogens from one cow to another is not likely. Acquisition of contagious mastitis pathogens happens during the milking process, by improper hygiene of the milking machine.[64] Contagious mastitis organisms do not survive well in the environment.[64] In contrast, environmental mastitis pathogens can be acquired when cows lie in contaminated bedding material.[64] Knowledge of the epidemiology of mastitis infections can help in prevention, and therefore reduce the need of antimicrobial use to combat infections. Mastitis is mostly treated with intramammary antimicrobials only, and the potential effect of intramammary antimicrobials on enteric bacteria, if any, is not currently known. Furthermore, similar prevalence of antimicrobial resistance in mastitis-causing bacteria has been reported in organic dairy farms that don't use antimicrobials and conventional dairy farms that regularly treat mastitic cows.[88,89]

Sick calves are usually not separated from healthy calves. When calves are housed in individual hutches, they are considered to be isolated because they don't have direct contact. Indirect contact however, through the personnel and equipment may play an important role in transmission of resistant bacteria. Weaned calves are usually grouped in pens, and when a calf needs treatment it is applied *in situ.* Therefore, resistant bacteria selected by the antimicrobial treatment may spread to healthy calves, and share their resistance genes with susceptible flora. Creating a separate pen for sick calves can help prevent this horizontal spread of resistance to healthy calves.

An important difference between calves and cows is that the most prevalent health problems in calves are diarrhea and pneumonia.[90-92] Both disease conditions are treated using parenteral antimicrobials, and sometimes oral antimicrobials. In addition, many

calves are fed waste milk or milk replacer containing antimicrobials. Oral consumption of antimicrobials is a more rational risk for resistance in enteric bacteria than the risk posed by parenteral or intramammary antimicrobials in cows. However, resistance did not differ between enteric bacteria isolated from calves that were fed milk with or without antimicrobials.[82,93,94] It has been suggested that resistance may depend on the amount of antimicrobial ingested with the milk.[95] Unfortunately, no effective method has been reported to reduce the amount of antimicrobials contained in waste milk, not even pasteurization.[94,96,97] Penicillins in milk can be inactivated by use of β-lactamases,[98] but the health consequences, if any, of the use of β-lactamases is unknown. Although the use of medicated milk replacers can be avoided, it may not be economically viable to discard waste milk. However, preventing disease in lactating cows will minimize the use of antimicrobials and therefore the amount of waste milk produced on the dairy farm.

In summary, implementing an effective biosecurity and biocontainment program will help in preventing the introduction and spread of resistant bacteria on dairy farms. Following effective biocontainment protocols will prevent the spread of resistant bacteria and pathogens. An effective control program will act in two ways:

- preventing the introduction of pathogens that may require the using antimicrobials to treat diseased animals,
- preventing the introduction of bacteria containing new resistance genes that are not present in the resident bacteria.

CONCLUSION

Conceptually, biosecurity and biocontainment are easy to embrace: the goal is to avoid the introduction and spread of disease agents on the dairy farm. The difficulties arise in the practical implementation, i.e. how to determine what specific control methods should be adopted. The solution lies in accurately identify critical control points and prioritizing control methods according to economic considerations (cost-benefit analysis). The flowchart described here is a useful tool to depict the dynamics within a dairy farm, and therefore identify specific practices that represent a risk for the introduction and spread of disease agents. Clear identification of risk practices within a dairy farm will facilitate the transition from the concept to its implementation.

Specifying the disease concerns of the veterinarian and the dairy producer will ensure that a robust biosecurity and biocontainment program is implemented. Involvement of key personnel will provide an additional level to ensure compliance with the program. Disease concerns should be prioritized to focus control methods towards the most important ones. Corrective actions designed to control the main concerns will resolve some of the lower ranked concerns. The biosecurity and biocontainment program should be monitored and regularly reevaluated and updated. Regular reviews of the program will help identify opportunities for the development of new control methods or further refinement of existing control methods to prevent introduction and spread of disease agents on the dairy farm. The use of this flowchart as a template will facilitate evaluating the integrity of an existing program, and identifying critical control points to update or create new protocols.

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Chapter 3

Evaluation Of Current Limitations For The Measurements Of Antimicrobial-Use In Food-Animals In The U.S.

Introduction

Antimicrobial resistance of both human and animal bacterial isolates has become a topic of great interest. Several articles incriminate the use of antimicrobials in food-animals as a cause of antimicrobial resistance in bacteria isolated from humans.[1-6] There have been calls for action to mitigate the emergence and amplification of antimicrobial resistance,[5-8] suggesting that regulatory action should be taken to "*minimize the negative public health impact of the use of antimicrobial agents in food-producing animals*".[7] There is however little information on the effect that antimicrobial-use in food-animals may have on resistance in bacteria isolated from those animals. The existence of this link in food-animals needs to be proven and quantified before antimicrobial-use in food animals can be incriminated as a cause of resistance in bacteria isolated from humans.

Antimicrobials are an important component in the overall strategy necessary to treat and prevent disease, both in humans and animals.[9,10] Therefore, antimicrobial-use can be

categorized as therapeutic (for the treatment of disease) or prophylactic (for disease prevention). Additionally, in food-animals, antimicrobials have proven helpful in enhancing production.[11-13] The increase in production has been attributed to treatment and prevention of disease,[12] or shifts in bacterial populations that improve how nutrients in the diet are used.[11]

An overall improvement in animal health allows for a safer food supply, protecting the consumer.[14] Establishing regulatory actions that limit the use of antimicrobials in food-animals could negatively impact animal health and, therefore, the food supply by limiting the use of effective therapies.[15,16] New regulations that restrict antimicrobial-use beyond current limits would further decrease the limited number of approved chemotherapeutic tools currently available to veterinarians to fight disease.[17,18]

The term "food-animal" describes multiple animal species that represent a range of body mass (from a 1 oz chick to a 2,000 lb bull), digestive systems (monogastric *vs.* ruminant), management systems, productive age, and production purposes. Furthermore, there can be large differences, such as with size or metabolism, within animal species. In chickens for example, body mass can vary from a 1 oz chick to an average weight of 6 lb. in a broiler: a 75 fold weight increase. Metabolism in cattle changes from monogastric calves to ruminant adults. Therefore, antimicrobial-use data summarized across strata (types) of food-animals may result in erroneous conclusions regarding the number of animals exposed or the dose received by each animal.

Control measures to limit the development and spread of antimicrobial resistance should be designed to be efficient. Control measures should have the largest possible impact on preventing resistance while sustaining human and animal health. Policy-makers should rely on sound and appropriate data to identify the most efficient control measures. Using currently available data on antimicrobial-use is an easier and cheaper option than obtaining new data. Although collection of new data requires large investments in resources and delays policy decisions, available data may not be adequate and additional data on antimicrobial-use will likely be necessary as a basis for informed antimicrobial policies.

The purpose of this chapter was to evaluate currently available data on antimicrobial-use in food-animals in the U.S. and identify further data needs for adequate data-driven policy to limit the development and spread of antimicrobial resistance.

AVAILABLE DATA ON ANTIMICROBIAL-USE IN FOOD-ANIMALS IN THE U.S.

Antimicrobial-use in food-animals has not been regularly or systematically monitored in the U.S. Most countries in the world are in this same situation. To the authors' knowledge, Denmark is the only country that has a system in place to collect data on antimicrobial-use in all animal species, because in Denmark it is mandatory to obtain a veterinary prescription for all antimicrobials, except those approved by the E.U. as feed additives.[19]

However, some estimates exist of annual antimicrobial sales and use in the U.S. The most recent estimates of antimicrobial-use in food-animals were reported by the Union of Concerned Scientists (UCS) in 2001[20] and the Animal Health Institute (AHI) in 2005.[1,21] The UCS reported some calculated estimates based on available data on food-animal census, recommended uses and doses, and other estimates as "*educated guesses*".[20] AHI is a trade organization that represents most, but not all, manufacturers of animal health care products in the U.S. The 2005 reports from AHI showed estimates of annual sales figures[21] and annual tonnage of active compound[1] sold by its members between 2002 and 2004, based on sales surveys. Estimates of antimicrobial-use from UCS and AHI are compared in Table 3.1.

Estimates of total antimicrobial-use reported by these two organizations are markedly different. For example, UCS estimated non-therapeutic use of tetracyclines, penicillins, sulfonamides, aminoglycosides, and other antimicrobials in food-animals to exceed AHI estimates of total antimicrobial-use (therapeutic and non-therapeutic uses) for those categories. UCS estimated that approximately 84% of total antimicrobial mass used in livestock were for non-therapeutic purposes.[20] In contrast, AHI reported sales of antimicrobials for therapeutic use at levels of 91%, 92% and 95% of total sales in 2002, 2003 and 2004, respectively.[1] Therefore, sales of non-therapeutic antimicrobials would represent 9%, 8% and 5% for 2002, 2003 and 2004 respectively. These estimates are

[1] Animal Health Institute. June 27, 2005. Press Release. Antibiotic use in animals rises in 2004. Washington, D.C. http://www.ahi.org/Documents/Antibioticuse2004.pdf

Table 3.1 Comparison of estimates of antimicrobial-use (10^6 lb) in animals in the U.S. reported by the Union of Concerned Scientists (UCS) and the Animal Health Institute (AHI).

Antimicrobial class	UCS Year 2001 Non-therapeutic use only	AHI Year 2004 Non-therapeutic and therapeutic use
Ionophores/ arsenicals	6.58	9.44
Tetracyclines	7.12	6.49
Sulfonamides and penicillins	2.37	1.12
Aminoglycosides	0.43	0.35
Fluoroquinolones	0.01	0.05
Other	8.46	4.30
Total	24.97	21.75

similar to U.K. estimates (voluntary report by marketing companies) of approximately 6% sales of non-therapeutic antimicrobials in 2002, 5% in 2003 and 4% in 2004.[22-25] The use of antimicrobials as growth promoters was banned in Denmark in 1999.[19] Therefore U.S. estimates of non-therapeutic antimicrobial-use cannot be compared to Danish data. It is important to emphasize that estimates of antimicrobial-use for non-therapeutic purposes reported by both organizations cannot be directly compared because UCS reported estimated total pounds of antimicrobials consumed while AHI reported non-therapeutic use as sales figures in dollars. Sales figures do not accurately represent the amount of drugs used because the total amount spent on antimicrobials cannot be converted into dosages administered due to widely disparate prices among products with the same active ingredients. It is evident that marked discrepancies exist in the estimates of these two organizations.

AHI reported that approximately 58% of all animal health product sales (not only antimicrobials) were used in companion animals.[21] Reports on animal demographics in the U.S. in 2000-2003 show over 9.1 billion animals, out of which 96.8% (8.8 billion) are chickens and turkeys.[26,27] Among the remaining 300 million animals, 43.9% (130 million) were dogs and cats compared to 34.9% (103 million) ruminants, 20.0% (59 million) pigs. According to these figures, 58% of all animal health care products (not only antimicrobials) were destined to only 1.4% of all animals in the U.S. (130 million dogs and cats/9.1 billion total animals). These estimates cannot be directly compared to those of other countries because they include sales of all types of animal health products, not just antimicrobials. However, it has been estimated that in Denmark in 2003, almost

half of all fluoroquinolones and cephalosporins used in veterinary medicine were in fact used to treat companion animals.[28]

UCS reported an "*educated guess*" of 1 million lb of antimicrobials used in companion animals. For food-animals, the UCS estimated that 24.6 million lb of non-therapeutic antimicrobials were used in cattle, swine and poultry. In addition, "*educated guesses*" of 3 million lb for non-therapeutic use in other livestock species and 2 million lb for therapeutic uses in all livestock species were reported. Therefore, a total of 29.6 million lb of antimicrobials were estimated as total use in approximately 8.87 billion food-animals (9.1 billion animals – 0.13 billion dogs and cats). The resulting annual use according to these estimates is 0.0033 lb per individual food-animal; independent of whether this food-animal is a chicken or a bull.

Assuming the term "companion animal" refers solely to dogs and cats, 1 million lb would have been used in 130 million companion animals. This translates into 0.0077 lb of antimicrobials used per dog or cat. The estimates of antimicrobial-use in all animal species reported by UCS, suggest a 2.5 fold higher relative use of antimicrobials in companion animals than in food animals. In spite of this, the UCS report refers in its title to antimicrobial "abuse" in food-animals. Food-animals have indirect contact with a large part of the human population through consumption of animal products. However, companion animals have direct contact as live animals with a larger part of the human population than food-animals (direct contact only with farm workers). Neglecting the potential effect that antimicrobial-use in companion animals may have on resistance in

humans may result in overlooking another factor that may play a role in the development and spread of resistance in human pathogens.

Government-sponsored estimates of antimicrobial-use in food-animals in the U.S. include a report by the National Academy of Sciences dated in 1999.[29] This report published data on antimicrobial-use only for the poultry industry (reported as $/ ton of feed or $/ lb of live weight). The most recent government-sponsored estimates are species-specific data from the National Animal Health Monitoring System (NAHMS) of the USDA.[30-40] Through NAHMS, the USDA periodically collects and analyzes data on animal health, management, and productivity across the United States via questionnaires of representative operations for each food-animal production commodity. Each NAHMS study is designed based on current issues relevant to the livestock species under study. As such, the objectives of each study vary, as do data collection methods and survey questions. Limited antimicrobial-use data are available through NAHMS prior to 2000. A summary of antimicrobial-use estimates in food-animal species in the U.S. and the most commonly used antimicrobial for each production group is presented in Table 3.2. It is important to note the large variability between species and animal classes.

LINKING ANTIMICROBIAL-USE AND ANTIMICROBIAL RESISTANCE DATA

It can be seen from these data that available information on antimicrobial-use in food-animals is scarce and differs across species. To evaluate a possible relationship between

Table 3.2 Summary of reported antimicrobial-use in food-animal species. Source: NAHMS surveys (2000 – 2005).

Species	Animal class	% operations that used antimicrobials	% animals that received antimicrobials	Most commonly used antimicrobial	% of animals in class treated with this antimicrobial
Cattle	Dairy calves (milk replacer)	55.7	-	Tetracyclines	46.4
	Dairy heifers (ionophores in feed)	44.0	-	-	-
	Dairy heifers (antimicrobials in feed)	17.5	26.9	Tetracyclines	83.9
	Dairy cows (intramammary antimicrobials)	95.0	-	β- lactams	93.7
	Feedlot (injectable antimicrobials at arrival)	56.4	18.8	-	-
	Feedlot (antimicrobials for respiratory disease)	41.7	10.4	-	-
	Feedlot (antimicrobials in feed or water)	83.2	-	Macrolides	42.3
	Feedlot (ionophores in feed)	92.9	95.9	-	-
Sheep	Sheep – adults (therapeutic antimicrobials in feed)	19.6	-	Tetracyclines	21.2
	Sheep – adults (therapeutic antimicrobials in water)	4.0	-	Tetracyclines	3.4
	Sheep – growth (therapeutic antimicrobials in feed)	6.0	-	-	-
	Sheep – growth (therapeutic antimicrobials in water)	0.3	-	-	-
	Sheep – feedlot (antimicrobial in water at entry)	30.0	-	-	-
	Sheep – feedlot (growth promotants)	40.7	-	-	-
	Sheep – feedlot (therapeutic antimicrobials)	90.3	6.0	-	-

Species	Animal class	% operations that used antimicrobials	% animals that received antimicrobials	Most commonly used antimicrobial	% of animals in class treated with this antimicrobial
Poultry	Layer hens	-	-	-	-
	Broilers	-	-	-	-
	Backyard poultry (therapeutic antimicrobials)	10.1	-	-	-
	Gamefowl (therapeutic antimicrobials)	72.3	-	-	-
Swine	Sows (therapeutic antimicrobials)	61.3	-	-	-
	Nursery (therapeutic antimicrobials)	16.0	-	Tetracyclines	48.3
	Weanlings (therapeutic antimicrobials)	11.3	-	-	-
	Growers/finishers (therapeutic antimicrobials)	27.1	-	β- lactams	83.5
	Growers/finishers (growth promotants)	63.7	-	Macrolides	56.3

antimicrobial-use and resistance it is crucial to define and measure the exposure and the outcome as objectively as possible.

Measurement of Antimicrobial-use

Standardized measures are necessary to compare antimicrobial consumption across countries, subpopulations and time. Researchers in human medicine, in attempting to compare data of drug consumption collected from various sources and across different countries, have used different measurement indices.[41-47] The most commonly used measure of drug consumption is the defined daily dose (DDD). The DDD is the average dose used in a day for the main indication of the drug.[41,43,45-47] For the purpose of population studies, it is calculated as the number of standard adult doses consumed per 1,000 inhabitants per day. Based on the DDD, the number of average adults treated with a drug can be estimated from sales and the average maintenance dose for an adult. The use of this measurement in populations calculates *average* drug consumption over time for individuals of a population and does not account for duration of the treatment course, actual dose used or compliance with the treatment regimen.[43,46,47] Using antimicrobials for varying lengths of time may have different effects on isolation of resistant bacteria. Measuring antimicrobial-use in DDD/1,000 individuals/day may not be a useful index because it assumes a steady rate of consumption, and does not account for clustering within time. For example, a measurement of 100 DDD/1,000 inhabitants can represent 100 people consuming one DDD at a single point in time, or one person consuming ½ DDD over 200 days. In these two scenarios, different selective pressures will affect the

bacterial populations colonizing the patients. In the first scenario, a short effect is exerted on multiple bacterial populations. In the second scenario, a long-term effect is exerted on the bacterial population of a single individual. Further, the fractional dose in the second scenario may represent an incomplete dose of an adult or a complete dose (or even overdose) of an infant. DDD does not account for differences in doses between adults and infants and may lead to underestimation of the number of individuals exposed to an antimicrobial. Therefore, because a single DDD can be representative of multiple exposures, and exposure dose and length affect antimicrobial resistance,[48,49] it is not the best index for the purpose of this article.

Another measurement of drug consumption that has been described in the literature is the prescribed daily dose (PDD).[43,45] PDD may be useful in human medicine, however its usefulness in food-animals is questionable due to the lack of a central access point for all prescriptions and over-the-counter sales (no prescription) of some antimicrobials directly to food-animal producers. Other measurements have been described in the literature,[45] but none of them seem to present any clear advantage over the DDD.

The development of DDD for animals (ADD) was proposed by the WHO in 2001, but its implementation was postponed due to "*confusion with respect to what purposes such a unit should and could serve*".[2] In 2004 a Danish study on antimicrobial-use in veterinary medicine was published based on the animal defined daily dose per kg bodyweight

[2] WHO Collaborating Centre for Drug Statistics Methodology. *Defined Daily Dose$_{animal}$ (DDD$_{animal}$) – Status January 2006.* ATCvet Working Group. Oslo, Norway, January 2006.

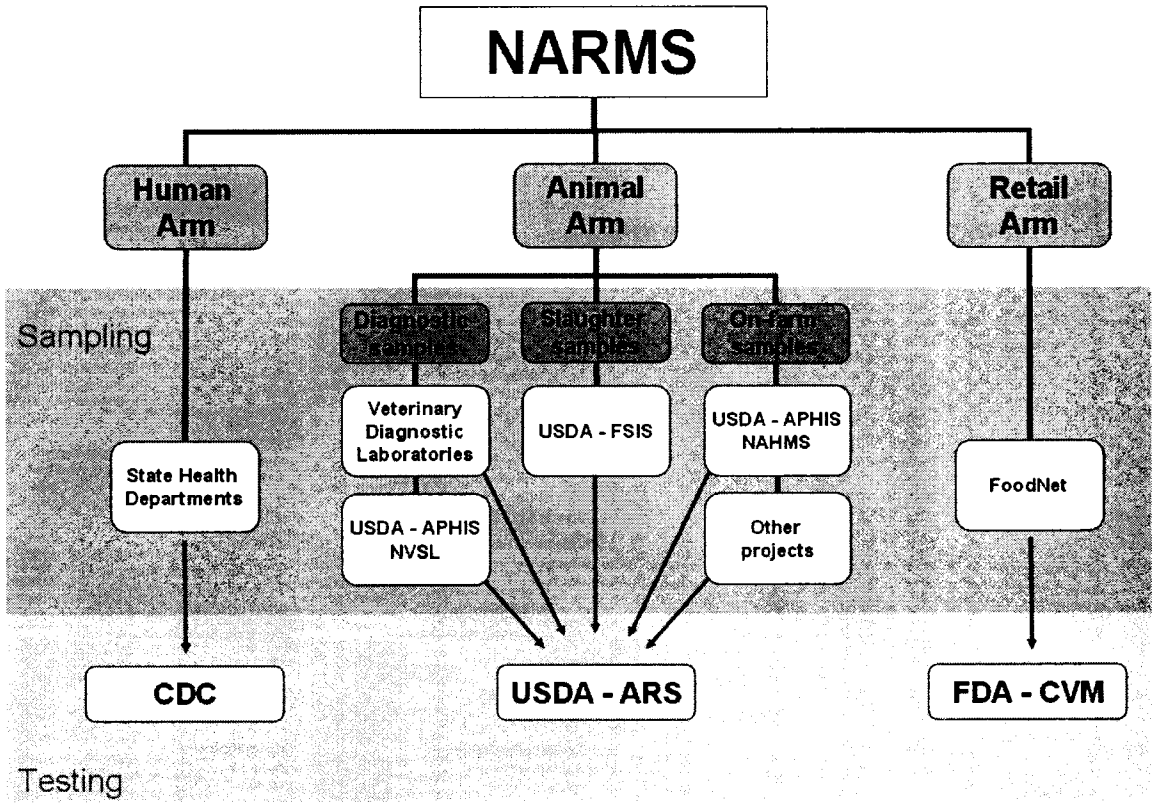
(ADD_{kg}).[47] The ADD is then calculated for a *standard* animal weight within age group. The conclusion of this study was that the number of animals in the target group needs to be taken into account. Since ADD is a group-level measure, even if it were adopted as a standard measure of antimicrobial-use, it would not provide the data granularity necessary to study the relationship between antimicrobial use and resistance in bacteria.

Measurement of Antimicrobial Resistance

In the U.S. antimicrobial resistance among enteric bacteria has been monitored since 1996 by the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS-EB).[50-54] Monitoring of resistance is based on isolates of sentinel and pathogenic bacteria collected from humans and animals across the U.S. NARMS-EB has 3 distinct components: human arm, animal arm and retail meat arm. Four entities participate in this effort (Figure 3.1): the FDA–CVM (Food and Drug Administration–Center for Veterinary Medicine) tests retail meat samples submitted through the FoodNet program; the CDC (Centers for Disease Control and Prevention) tests isolates obtained from human clinical cases; and the USDA–ARS (Agricultural Research Service) tests clinical, non-clinical and slaughter isolates obtained from animals through veterinary diagnostic laboratories, NAHMS, USDA–FSIS (Food Safety and Inspection Service), and other projects.

Animal isolates are submitted to the ARS from samples collected at various sites across the U.S.: (1) clinical *Salmonella* isolates obtained from any animal species at sentinel diagnostic laboratories and other veterinary diagnostic laboratories, (2) non-clinical *E.*

Figure 3.1 Graphical representation of the involvement of the different entities that participate in the National Antimicrobial Resistance Monitoring System (NARMS).



coli, *Salmonella*, *Campylobacter* and *Enterococcus* isolates from food-animals obtained at federally inspected slaughterhouses and processing plants, and (3) non-clinical *E. coli*, *Salmonella*, *Campylobacter* and *Enterococcus* isolates obtained at individual food-animal operations during focused studies on healthy animals. This design should allow representation of sick and healthy animals across the U.S. in the final estimates for antimicrobial resistance. Due to changes in the NARMS-EB program over time, limited numbers of isolates have been tested per year and the tested isolates did not represent domestic animal species proportionally. For example, no *E. coli* isolates were reported for dogs, cats, horses, cattle or swine in 2000 and 2001. For 2002, only two *E. coli* isolates were reported for dogs and cats, and one isolate for swine. Additionally, samples are not evenly distributed across the U.S. Diagnostic samples submitted from eight sentinel sites ranged from 23 to 429 samples per site. This uneven distribution of samples across the U.S. may impact the results on estimating national resistance levels, as some areas of the country may have different resistance patterns than others. Furthermore, merging resistance data from different geographical areas or even different animal groups within species may bias results because the measured outcome is a result of different exposures. For example, according to NARMS-EB in 2003, overall resistance level to tetracyclines among *Salmonella* isolated from swine (n = 680) was 66.8%.[32,33] At least four production groups have been identified in swine (Table 3.2), and each group preferentially uses a different type of antimicrobial.[33] Thus, using currently available data, resistance to tetracyclines (outcome) in swine as a species should be evaluated in relation to the use of tetracyclines in nursery pigs, and β-lactam and macrolide use in grower/finisher pigs (exposure). However, these evaluations cannot be done because

currently available antimicrobial resistance data are not broken down by age or production group within species.

Due to the sources of the isolates (veterinary diagnostic laboratories, slaughterhouses, processing plants and farms) most of them originate from food-animals, therefore under-representing companion animals in the overall measurement of antimicrobial resistance. In fact, only 2.4% (100/4168) of all *Salmonella* isolates tested from animal samples in 2003 were collected from dogs and cats, and all originated from diagnostic samples (i.e. no healthy animal samples).[52] Interpretation of the information resulting from these data is difficult because the degree to which these samples represent the total population is uncertain. Isolates collected from diagnostic samples may have been exposed to antimicrobials. Furthermore, they may have been exposed to different doses and for varying periods of time. Because antimicrobials kill or inhibit growth of susceptible bacteria they cause a shift in the proportion of susceptible and resistant bacteria within an animal during treatment. There is a higher probability of isolating resistant bacteria when a sample is collected during antimicrobial treatment than if the sample is collected once the antimicrobial has cleared from the body or before treatment. To more accurately assess a possible association between antimicrobial-use and resistance, concurrent antimicrobial treatment, drug(s) used, and time from treatment to sample collection need to be reported for diagnostic samples.

In summary, bacterial isolates currently tested by NARMS-EB, although they originate from diseased and healthy animals from varying species across the U.S. are not a representative sample of the US animal population and therefore may show a distorted

picture of resistance levels. The proportion of sampled isolates exposed to antimicrobials in the animal arm is likely much higher than that in the underlying population because of the large number of diagnostic samples. This inaccurate representation of exposure status likely leads to an over-representation of antimicrobial resistance. Standard methodology for reporting of antimicrobial-use (drug, dose, route, duration, etc.) in animals sampled must be developed before the relationship between use and resistance can be appropriately evaluated.

According to NARMS-EB guidelines, resistance is tested in a single isolate obtained from each sample.[50] Although resistance of an isolate to a specific antimicrobial is measured by serial dilutions or diameter of inhibition zone, the final outcome is usually categorized as resistant, intermediate or susceptible.[52,53,55] NARMS-EB reports the percentage of tested isolates that were resistant to specific antimicrobials.[52,53] Data are aggregated within species and within clinical status, and not reported for individual animals. In fact, if a single isolate per sample is tested, it is not possible to determine "levels" (prevalence) of resistance within animals. The isolate chosen to be tested is either resistant or not.

If a "level" of resistance is sought for an individual animal, the best option may be to test several isolates and report the proportion of these that are resistant.[56,57] Reports from NARMS-EB include the number of antimicrobials to which an isolate is resistant, although these data are not reported for individual animals species.[52,53] However, the number of antimicrobials to which an isolate is resistant does not give insight to the level of resistance for specific antimicrobials. Two isolates that are each resistant to three

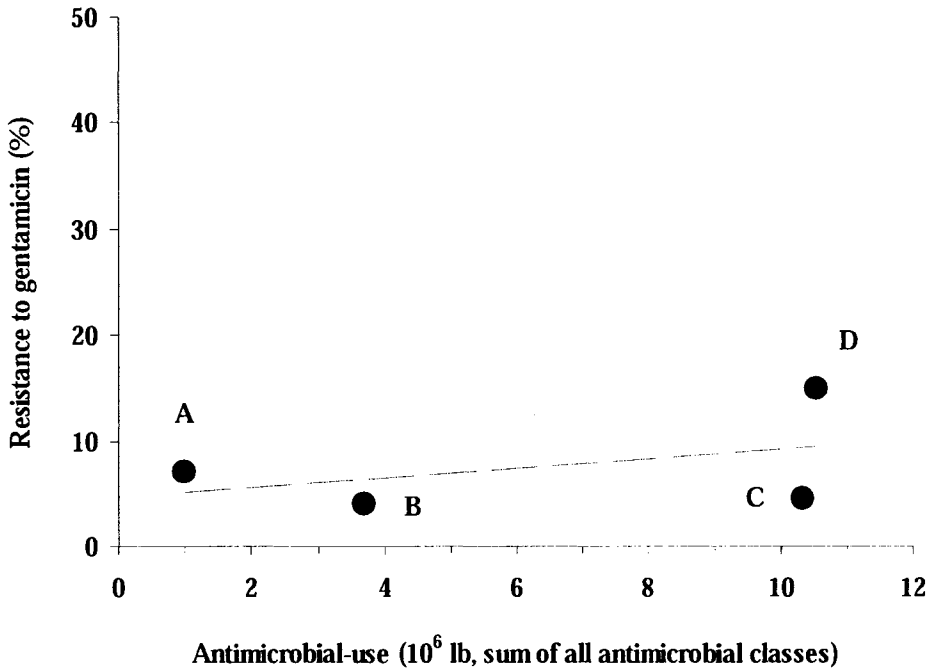
antimicrobials of the sixteen tested under NARMS-EB, can be resistant to different combinations of antimicrobials. Therefore, comparison of resistance levels between isolates should be related to the same antimicrobial. If a "level" of resistance is sought for an individual isolate, the diameter of the inhibition zone or dilution for susceptibility need to be reported.

DATA NEEDS ON ANTIMICROBIAL-USE IN FOOD-ANIMALS IN THE U.S.

The various estimates of antimicrobial-use in food-animals are aggregated within farms, species, regions or for the entire U.S.,[20,21,30-40] and national resistance data are aggregated within species and clinical status.[52,53] Because antimicrobial-use and resistance are aggregated at different levels, the categories are not comparable. Establishing a relationship between these data can result in misleading conclusions, and therefore should not be attempted.

To our knowledge, at the time of this writing, there is no single institution that provides the individual measurements of antimicrobial-use and resistance. Therefore, if currently available data are to be used, data from two different organizations will have to be compared. Figure 3.2 graphically depicts the relationship that results when currently available data on antimicrobial-use (UCS or AHI) and resistance (NARMS-EB) are used. The lowest aggregation level at which both types of data are available is at the animal species level. For the *x* axis data on antimicrobial-use from the UCS (published in 2001) was used.[20] Antimicrobial-use was estimated by UCS only for certain species: companion

Figure 3.2 Relationship between total antimicrobial-use in four animal species and resistance to **gentamicin** in *Salmonella* isolated from those animals. Sources: antimicrobial-use data (x axis) from the Union of Concerned Scientists (2001) and resistance data (y axis) from the National Antimicrobial Resistance Monitoring System (2001).



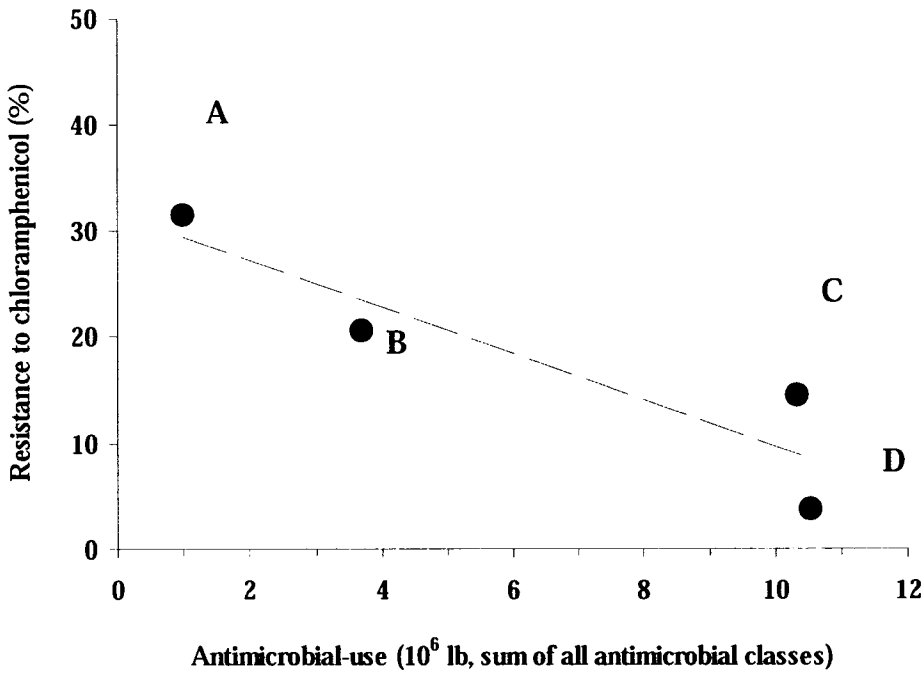
Species: A=companion animals, B=cattle, C=swine and D=poultry

animals, cattle, swine and poultry. For the *y* axis data reported by NARMS-EB on resistance in *Salmonella* isolates (diagnostic and non-diagnostic) during 2001 was used.[53] Due to limitations of available data, resistance levels for the graph were defined as proportion of *Salmonella* isolates that were resistant to a specific antimicrobial among all tested *Salmonella* isolates within each animal species.

Based on this graph, it is tempting to conclude that higher antimicrobial-use resulted in higher prevalence of resistance. However, there are several problems with this rationale. The graph in Figure 3.2 relates only to gentamicin resistance in *Salmonella* isolates submitted to NARMS-EB. It is unknown if the relationship would hold true for enteric bacteria other than *Salmonella* because similar data are unavailable for other bacteria. Furthermore, the *x* axis measures exposure as total antimicrobial-use (any antimicrobial), while the *y* axis measures resistance to a single antimicrobial.

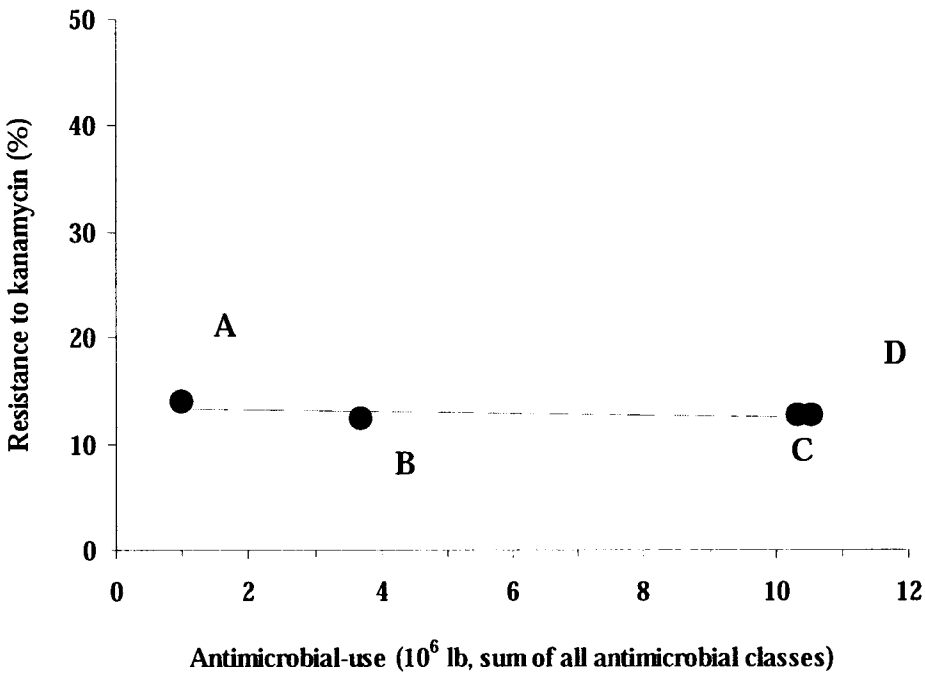
The positive relationship shown in Figure 3.2 does not hold true for other antimicrobials such as chloramphenicol (Figure 3.3) and kanamycin (Figure 3.4). The conclusion drawn based on Figure 3.3 would be exactly the opposite as that of Figure 3.2, i.e. the overall use of larger amounts of antimicrobials would result in lower prevalence of resistance. According to Figure 3.4, resistance to kanamycin in *Salmonella* isolates would be independent of the total amount of antimicrobials used. Therefore, using available data, different conclusions could be reached according to the antimicrobial selected for the measurement of resistance, since total antimicrobial-use remained constant throughout the various graphs.

Figure 3.3 Relationship between total antimicrobial-use in four animal species and **chloramphenicol** resistance among *Salmonella* isolated from those animals. Sources: antimicrobial-use data from the Union of Concerned Scientists (2001) and resistance data from the National Antimicrobial Resistance Monitoring System (2001).



Species: A=companion animals, B=cattle, C=swine and D=poultry

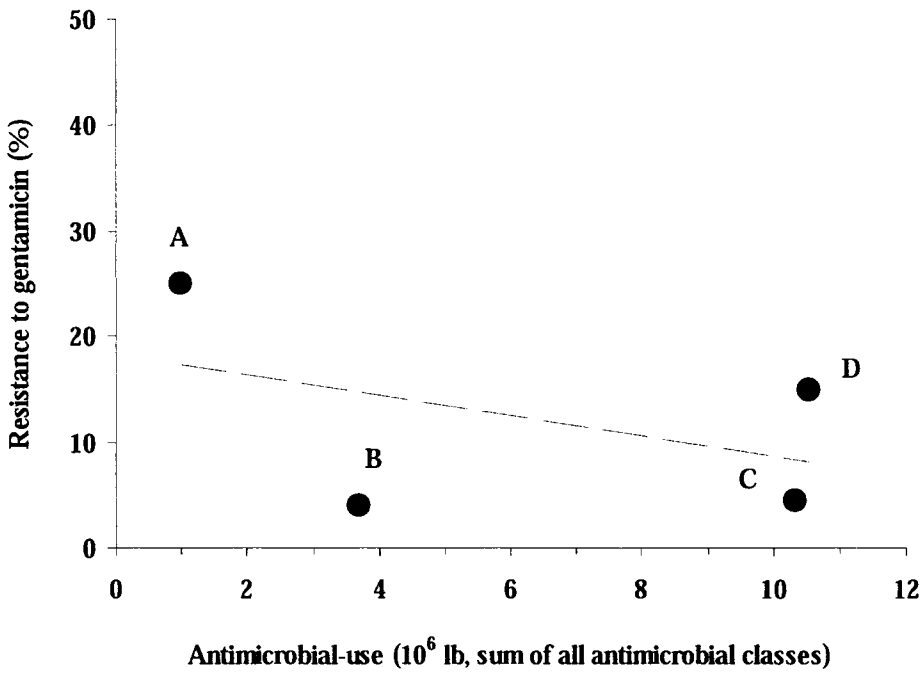
Figure 3.4 Relationship between total antimicrobial-use in four animal species and **kanamycin** resistance among *Salmonella* isolated from those animals. Sources: antimicrobial-use data from the Union of Concerned Scientists (2001) and resistance data from the National Antimicrobial Resistance Monitoring System (2001).



Species: A=companion animals, B=cattle, C=swine and D=poultry

Current understanding of the relationship between antimicrobial-use and resistance at the national level is limited because the lowest stratum at which data are available for both is at the animal species level. Assessment of this relationship using the sparse data currently available requires predicting a trend by comparing data across animal species. A major weakness with this approach is that it introduces a confounding factor, species, in the relationship. Another weakness is that currently there are few data points and thus a significant change in one can affect the overall conclusion drastically. Assume for example that in Figure 3.2 resistance in *Salmonella* isolates obtained from companion animals were higher, while resistance in cattle, swine and poultry remained the same (Figure 3.5). The trend line would change so dramatically that the opposite conclusion as in Figure 3.2 would be drawn. Changing a single point in the graph would result in the conclusion that resistance is lower at higher levels of antimicrobial-use, even though antimicrobial-use in those other species did not change. It is important to emphasize that although antimicrobials such as chloramphenicol have been banned from being used in food-animals for 22 years (1984),[58] bacteria isolated from animals may still be resistant to chloramphenicol. Resistance to an antimicrobial in the absence of use of that specific antimicrobial could be due to cross-resistance to other antimicrobials or to inherent resistance.[9] Thus, resistance may exist in the absence of antimicrobial-use.[59,60] Baseline levels of resistance should be considered when evaluating a possible link between antimicrobial-use and resistance.

Figure 3.5 Hypothetical relationship between total antimicrobial-use in four animal species and **gentamicin** resistance among *Salmonella* isolated from those animals. This graph represents an assumed increase in resistance among *Salmonella* isolated from companion animals compared to Figure 3.2, while resistance in the other species remains as in Figure 3.2.



Species: A=companion animals, B=cattle, C=swine and D=poultry

Another factor that can lead to false conclusions using this aggregate approach is merging resistance data from clinical and non-clinical isolates. For example, NARMS-EB data for 2001[53] show that 71.1% (64/90) of *Salmonella* isolates collected from companion animals originated from clinical samples, compared to only 16.5% (393/2388) in poultry. The bias arises due to potential differences in resistance for clinical and non-clinical isolates. Among *Salmonella* isolates collected from slaughter (non-clinical) samples from poultry, 7.9% in chicken (n = 1307) and 20.9% in turkeys (n = 550) were resistant to gentamicin. Among clinical samples however, 5.1% of chicken and 41.7% in turkey isolates were resistant. Therefore, resistance to gentamicin is higher in clinical samples than in non-clinical samples for *Salmonella* isolated from turkeys, but it is lower in isolates obtained from chickens. The change in resistance between clinical and non-clinical isolates shows an inverse relationship between the two species. In this example, measuring overall antimicrobial resistance at aggregate levels can result in false conclusions. No detailed resistance data are available for non-clinical *Salmonella* isolated from companion animals, and therefore resistance cannot be compared between clinical and non-clinical samples.

Some researchers may assume that clinical isolates are more likely to have been exposed to antimicrobials than non-clinical isolates, because diseased animals are likely treated. However, some diagnostic samples were likely collected before treatment. Thus, isolates from treated and non-treated animals may be lumped in the category of clinical samples. Additionally, non-clinical isolates may originate from animals treated with sub-therapeutic levels of antimicrobials or untreated animals. Classification of isolates by

clinical status does not objectively represent treated and non-treated animals. Therefore, it provides no substantive data to answer the central question. More complexity is added to the issue by the effect of management (husbandry practices) on potential selection of certain types of bacteria, which could then disseminate their inherent resistance genes. An example of this situation is presented by vaccination of adult cattle against specific serotypes of *Salmonella* spp. Vaccination may prevent colonization by certain serotypes but not others. If the selected serotypes had specific resistance genes selection of resistance would be independent of antimicrobial-use.

Formulation of drugs varies according to manufacturer so that the same active ingredient can be mixed with different inactive ingredients that alter the distribution of the active ingredient in the body.[61] For example, injectable formulations containing tetracyclines as the active ingredient have different inactive ingredients that allow for short-term levels of the tetracyclines in blood (24 hours) or long-term levels (up to 8 days).[61] Thus, different formulations of the same antimicrobial may have different effects on resistance, and may need to be studied separately.

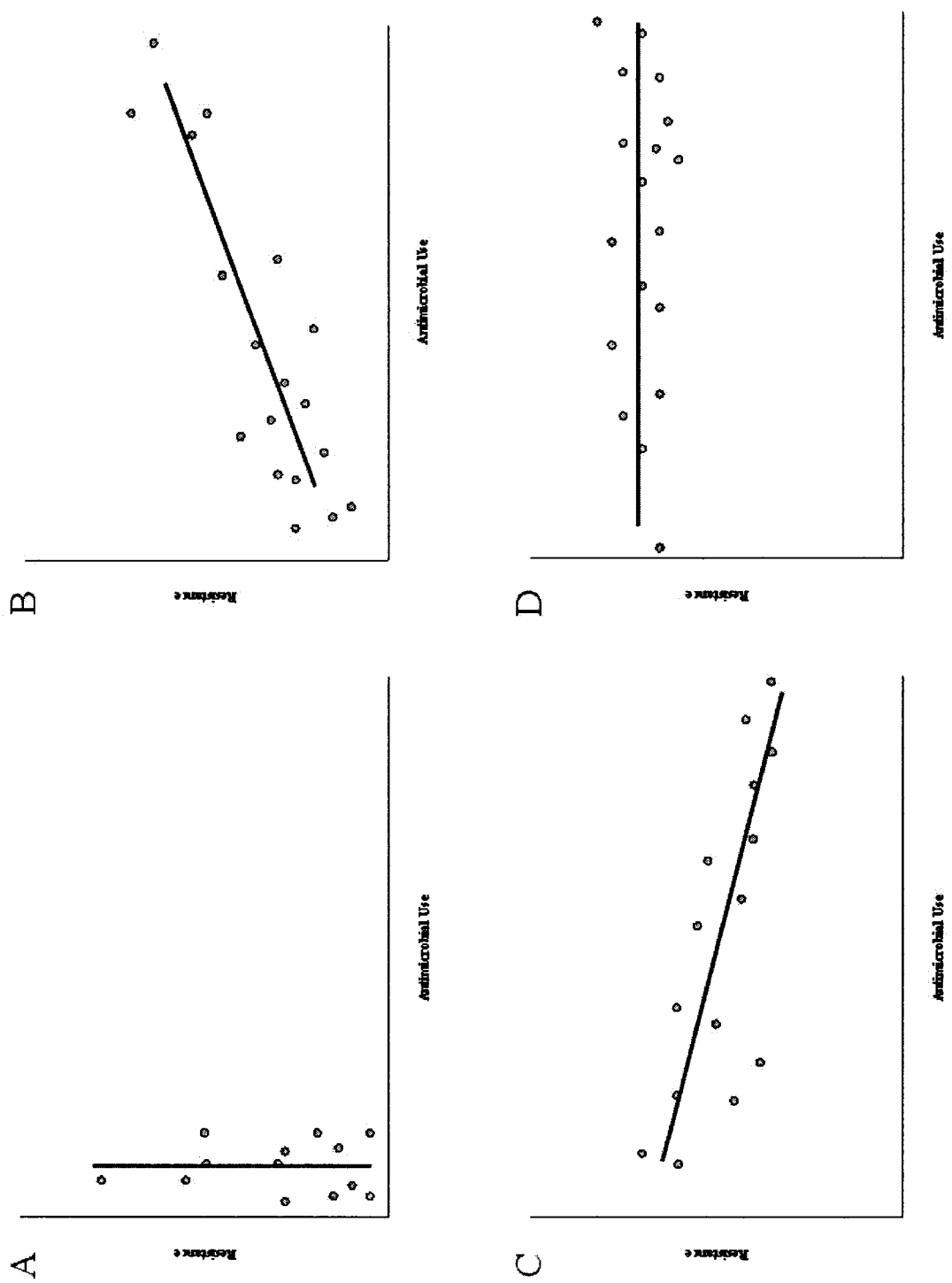
Additionally, when a treatment regimen is not effective in curing a disease, an animal may be exposed to a different regimen: it might be treated with the same antimicrobial for a longer time or it could be treated with a different antimicrobial. Longer treatments and treatment changes could hypothetically affect resistance in bacterial isolates in that animal. Longer exposure to a given antimicrobial may select for the most resistant isolates by killing all susceptible bacteria. Changes in treatments could select for multi-

drug resistant bacterial isolates. However, these hypotheses have not been tested. These potential confounding factors need to be considered during the study design and analysis.

The link between antimicrobial-use in animals and resistance among bacteria isolated from those animals has been evaluated in small groups of animals at the individual animal level. The four graphs in Figure 3.6 show some of the relationships between antimicrobial-use and resistance that have been reported. Scenario A represents a group of animals with minimal antimicrobial-use and varying levels of resistance. This scenario has been described in the literature in pastured beef cattle[62] and organic farms.[60,63,64] In these studies, levels of resistance were defined by the number of antimicrobials to which individual bacterial isolates were resistant.

Scenario B represents a positive linear relationship between antimicrobial-use and resistance. This type of relationship has been reported in studies that evaluated resistance among enteric isolates during antimicrobial treatment and the time immediately following treatment. However, these studies also showed the scenario shown in graph D, where apparently no relationship was observed between antimicrobial-use and resistance. In other words, the same level of resistance was observed for different levels of antimicrobial-use. This situation occurred when resistance was evaluated for antimicrobials other than the ones used for treatment (*e.g.* tetracycline use versus resistance to penicillins).

Figure 3.6 Hypothetical spectrum of relationships between antimicrobial-use in food-animals and resistance in enteric bacteria isolated from those animals.



Scenario C, where higher levels of resistance are observed at lower levels of antimicrobial-use, may be observed when different administration routes are not considered. For example, cephalosporins in cattle can be administered at different doses for systemic injection and intramammary application. The approved dose for systemic administration of cephalosporins in cattle ranges from 0.5 to 3.0 mg/lb body weight every 24 hours for up to 3 days.[61] Young calves (~100 lb) treated with the lowest dose would receive 50 mg of cephalosporins, while approved intramammary doses for cephalosporins are 200 mg/dose (lactating cows) and 300 mg/dose (dry cows). Therefore, intramammary administration of cephalosporins in cattle may result in higher levels of antimicrobial-use than certain parenteral uses. If resistance is evaluated in enteric bacteria, the lower doses of cephalosporins administered to the calves will relate to higher levels of resistance than those observed in cows treated with higher doses of intramammary cephalosporins. This situation has several explanations. First, it has been shown that calves have higher levels of resistant enteric bacteria than adult cows.[60,65,66] And secondly, levels of resistance in enteric bacteria isolated from cows treated with intramammary antimicrobials has been reported to be equivalent to untreated cows. Thus, enteric bacteria (the object of study of NARMS-EB) may not be exposed equally when antimicrobials are administered by differing routes. Therefore, exposure by differing routes should be studied separately.

These issues are an example of degrees of information loss that occur when data are summarized.[67,68] It is possible that evaluating detailed data (individual animals) may result in different conclusions than those reached based on evaluation of aggregate data. This effect can be described by the term "ecological fallacy".[67,69-71] The specific problem

of the ecological fallacy is that conclusions reached in analyses at aggregate levels can differ from conclusions at the individual level. In other words, if a relationship is found between 2 variables studied at the population level, this relationship cannot be assumed to be valid in individuals that form that population. A major reason for this discrepancy is the effect of confounding factors[67,70-72] that may exert their action at lower aggregation levels.

The examples presented above show how data reported at aggregate levels can lead to invalid conclusions, and support the need for more detailed information on antimicrobial-use to evaluate potential effects on resistance. This need for individual-level data has been identified also for the study of antimicrobial resistance in bacterial isolates obtained from humans.[73] Although data on resistance levels for bacterial isolates obtained from animals exist for each state participating in NARMS-EB, there are no parallel data on antimicrobial-use in animals for individual states. Similarly, resistance data are available for specific antimicrobials within an animal species, yet no corresponding data exist on use of specific antimicrobials. Table 3.3 shows a summary of available data on antimicrobial-use in animals (companion and food animals) and resistance within specific strata. Minimum data requirements on antimicrobial-use in order to control possible confounding factors are suggested in Table 3.4.

The best case scenario would be that antimicrobial resistance is an independent state within animals; in other words, there is no relationship between bacteria isolated from different animals. Worst case scenario would be that resistance can be transmitted

Table 3.3 Summary of available data on antimicrobial-use in animals and resistance within specific strata in the U.S.

Data levels	Antimicrobial use	Antimicrobial resistance
National	+	+
Individual states *	-	limited
Animal species	limited	limited
Production group	limited	-
Clinical status	limited	limited
Individual animal	-	limited

* Data for some states, not all.
Shaded area denotes data needed for proper analysis

Table 3.4 Suggested stratification of antimicrobial-use for proper assessment of the effect on antimicrobial resistance.

Animal Class	Purpose for use	Extent of use	Methods of use	Other
Companion animals	Therapeutic	Individual animals	Dose	First choice
Sport animals †	Preventive	Groups	Route	Treatment failure
Food-animals	Production enhancement		Treatment regimen	
Dairy cattle				
Beef cattle				
Small ruminants				
Swine				
Laying hens				
Poultry				
Fiber animals				

† race horses, greyhounds, sled dogs, etc.

between bacteria of different animals similar to a contagious disease. If antimicrobial resistance followed contagious disease dynamics, consideration would have to be given to transmission rates between animals and between bacteria, accounting for possible contagious contacts.[72,74] These factors are inherent to a group of animals but not to the individuals. Therefore, the effect of antimicrobial-use on resistance may be confounded not only by factors that act at the individual level, but also by factors at higher aggregation levels.

DATA COLLECTION ON ANTIMICROBIAL-USE IN DAIRY CATTLE

Food-animal operations of different animal species and production purposes use different management practices, and these practices need to be taken into account when designing a data collection strategy. Milk produced by dairy cattle is under constant scrutiny implemented directly by the creamery for drug residues (especially antimicrobials) following federal regulation.[75] Therefore, dairy farms isolate milking cows that need treatment in the hospital area in order to avoid drug residues in milk (milk obtained from cows in the hospital is not used for human consumption). Given this clustering of antimicrobial-use in the hospital, a simple option for recording antimicrobial-use would be a hospital log. A caveat to this log is that it would record only treatments administered to milking cows, and not to calves, heifers or dry-cows. These animals are not usually segregated from their location to the hospital pen, because drug residues in milk are not an issue with these animals. To solve this problem, treatment logs can be maintained for each of the production areas and record the location of the animal within that area.

Ideally, data recorded in these logs can be transferred to individual animal cards that can readily inform the personnel on how many times an animal has been treated and for how long. These individual animal cards can be maintained with computerized dairy management software such as DairyComp 305 (DC305), DHI-Plus®, PCDART, DairyCHAMP and DairyQuest. Computer programs allow handling large amounts of data, but they need to be designed to allow effective data input and retrieval. Some problems may be encountered when performing queries when the software is not designed to store required data in an appropriate format. An evaluation of the possible problems when commonly used computerized records are used was conducted by comparing data on antimicrobial-use collected from DC305 and handwritten records (individual cow cards).

Comparison of two methods of recording antimicrobial use on the same dairy

A dairy farm in Northern Colorado that used a herd management software (DC305) to maintain computerized records of individual events for each animal was visited to compare handwritten and computerized records. A query was run on DC305 to identify all antimicrobial treatments in 150 female calves between August 2004 and July 2005. Handwritten record cards for these animals were investigated and the information contained in both recording systems was compared. Treatments were recorded on the handwritten cards by each one of three persons administrating the antimicrobials, and then entered onto the software by one of two administrative personnel of the dairy farm.

A total of 201 treatments were identified in the computer records and 202 treatments in the individual cards. Although the total number of treatments is very similar between both types of records, there were 47 calves that had discordant information when comparing data from both records. For 22 calves more treatments were found in the cards compared to the DC305 records, while for the other 25 calves more treatments were recorded in DC305 than those written into the cards. The discrepancies found when more treatments were recorded in DC305 than in the cards was always due to a treatment that was systematically administered to all calves in a group. Group entries are easy to perform in DC305; however, it would take a certain amount of time to record that information on the individual card for each calf. When more treatments were recorded in the individual cards compared to DC305, the most common reasons were prolongation of the initial treatment course or a change to another antimicrobial.

There is no dedicated input record field in DC305 for the recording of antimicrobial treatment. Dairy herd management software has special fields to record some specific incidents such as reproductive events (heats, breedings, pregnancy confirmation) and milk production, but not for treatments or vaccinations. Treatments are recorded in a field called EVENTS, where any other information that has no predetermined field can be entered. The EVENTS field allows only 8 characters to describe an event, which makes it very difficult to record complete information on type of antimicrobial, dose, route and duration of treatment. A separate field is allowed for date of the event, however this can only describe the start of a treatment course. Some of these limitations on field length could be overcome in our project by on-farm records. Administration routes could be

easily determined from label directions of products used (limited by federal regulation). Doses and duration of treatment could be extracted from the SOP's as established by the herd veterinarian, however problems arose when treatment courses were changed or prolonged. For example, a calf with a record of being treated with tetracycline in DC305 on January 1st and with penicillin on January 3rd, 2005 could represent 2 scenarios. The first possibility, and the most common, was that the calf was not improving while being treated with tetracycline, prompting a change in treatment. Therefore tetracycline was discontinued and penicillin was administered as a new treatment regimen. The other option was that the calf showed clinical signs consistent with a second diagnosis and was treated with penicillin for the other signs. For example, it may have had a diagnosis of pneumonia on January 1st and a diagnosis of diarrhea on January 3rd. This treatment regimen is not meant to represent rational therapy, but rather that some antimicrobials are sold OTC and lay people can decide what to use in a calf until it can be seen by a veterinarian.

Written records seem to be easier to maintain and adapt to specific needs than computerized records. It is very easy to add any comment in any available space in a page of written records. Computerized systems usually limit the amount of information that can be entered. Therefore, a national study on antimicrobial-use in dairy cattle should most probably involve written records. There is no specific need to use individual cards for each animal; instead treatment logs can be used in the hospital (milking cows), the calf and the dry-cow areas to record all treatments. An example of a treatment log to effectively record all aspects of antimicrobial-use is presented in Figure 3.7. To use

Figure 3.7 Example of a treatment log to record antimicrobial-use in dairy cattle.

Date	Animal ID	Pen	Diagnosis	Treatment	Dose	Route	Times treated

computerized herd management records for a systematic evaluation of antimicrobial-use in dairy cattle across the U.S., there should be a standard format for data recording. Herd management software can be easily updated to include a field dedicated only to treatments that can then be coded according to the use of any antimicrobials, and even specific types of antimicrobials. This has already been implemented for the use of common codes for the most common culling reasons in dairy cattle. Farmers that use computerized record systems need to be instructed as to what information needs to be recorded and in what format. Farmers that do not use computerized record systems can record the same information in the aforementioned treatment logs. Losses of large amount of information are a concern with either records kept on paper or computer hard-drives. For a national data collection program, periodic collection of data to off-farm sites would reduce potential for significant losses of information.

CONCLUSIONS

In this chapter we provide support to show that detailed data on antimicrobial-use and resistance are necessary to evaluate their potential relationship. With the examples above we show that nationally, antimicrobial-use is unevenly distributed among subpopulations of animal species, purpose, methods and extent of use. However, it is unknown at this point how antimicrobial-use is distributed among these subpopulations. Ignoring the different aggregate levels may result in false conclusions about the effect that antimicrobial-use in food-animals may have on antimicrobial resistance in bacteria isolated from those animals.

To conclude that the effect of using 1 oz of tetracycline in 1,000 chickens is the same as using the same amount in a single cow is nonsensical. The total amount used is the same, and the amount of antimicrobial per body weight is similar. However the number of animals exposed is not the same, and the animals have different digestive systems and management; all of these factors could potentially affect development of resistance. Furthermore, the effect on resistance may differ whether this amount of tetracyclines is used all at once (high dose) vs. over a prolonged period of time at a lower dose. In the end, the same active ingredient - *e.g.* tetracycline - may be used in several animal species, for different purposes, in different doses, routes of administration and varying combinations with inactive ingredients. In summary, measurement of antimicrobial-use at the national level is not representative of use at the individual animal level, and resistance develops at the individual animal level.

The importance and urgency of understanding the relationship between antimicrobial-use and resistance has attracted both human and monetary resources to answer the question. Unfortunately, standard methodology does not exist for measurement of either the exposure factor or the outcome. A standard national methodology should be developed to ensure that study variables are defined in the same way and confounding factors are reported similarly and consistently. Common definitions for study variables will ensure that results from different studies can be compared, and as more studies are published, data from different researchers can be pooled to answer the central question.

Even after many years of research on antimicrobial resistance, the potential impact of antimicrobial-use is unclear. Baseline levels of resistance in bacteria not exposed to antimicrobials need to be established before the effect of antimicrobial-use can be evaluated. However, at the time of this writing, there is no data acquisition system in the U.S. to capture the parameters needed to assess antimicrobial exposure at a level that avoids the potential for erroneous conclusions due to an ecologic fallacy. To clarify the relationship between antimicrobial-use and resistance, data should first be studied at the most detailed level possible. Later, data can be grouped and results of aggregate data analysis can be compared to those of individual data analyses. Only if the data granularity is at the individual animal level, can conclusions be drawn that will enable policy makers to provide proper guidelines to control the development and spread of antimicrobial resistance.

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Chapter 4

Comparison of Random vs. Cohort Sampling Strategies to Evaluate the Effect of Antimicrobial-use in Dairy Cattle on Resistance in Enteric Bacteria

Abstract

Antimicrobial-use in food-animals is considered by some to be a major factor in selection of resistant bacteria in humans. By reductive reasoning, antimicrobial-use in food-animals should be a major factor in selection of resistant bacteria in food-animals. Short-term studies have been performed to test this hypothesis. To study possible long-term consequences of antimicrobial-use on resistance in bacteria, appropriate methodology must be used. The objective of this study was to compare two sampling strategies (random selection and cohort sampling) for collection of bacterial isolates to evaluate the effect of antimicrobial-use in dairy cattle on resistance in enteric bacteria. The ability to represent the exposure factor (antimicrobial-use) and the outcome (resistance in enteric bacteria) were compared. Fecal samples were collected from cows and calves on a commercial dairy farm in Northern Colorado at 8-week intervals for a total of 6 sampling

events for each group. Two subgroups were formed both for cows and calves: (1) a cohort group of 100 animals (50 animals treated with any systemic antimicrobial within 30 days prior to enrollment and 50 untreated animals), and (2) a group of 50 animals randomly selected at each sampling event. Prevalence of resistance in *Salmonella* and *E. coli* isolates from random samples was similar to that of cohort samples in all sampling events in calf samples and five of six sampling events in cow samples. Resistance patterns were also similar for both sampling strategies. Important attrition occurred in the cohort groups (72% in calves and 23% in cows) due to death, culling, or transfer to a heifer ranch. Collection of samples from cohort animals was more laborious than from randomly selected animals, because specific animals had to be located and often fecal material had to be retrieved manually from the rectum as opposed to sampling fecal pats. Under field conditions, random sampling may be preferred to cohort sampling in studies involving antimicrobial treatment because of the dynamic nature of the animal population and the exposure factor (antimicrobial treatment), and ease of sampling.

INTRODUCTION

Antimicrobial resistant bacteria have the ability to grow and multiply in the presence of an antimicrobial compound.[1-3] Infections with antimicrobial resistant bacteria may result in treatments that are less successful and more expensive. As such, antimicrobial resistance, especially in enteric bacteria, has become a major concern in public health.[4-8]

Several articles imply that antimicrobial-use in food-animals is significantly responsible for resistance in bacteria isolated from humans.[7,9-13] By reductive reasoning, antimicrobial-use in food-animals would be responsible for resistance in bacteria isolated from treated animals. Multiple studies have tried to assess the effect of antimicrobial-use in treated animals with different results.[14-19] A positive association seemed to exist between the use of antimicrobials and the detection of resistance to specific antimicrobials, while no association was observed for other antimicrobials within the same studies. These studies used different methodology, and therefore direct comparisons and extrapolations are not possible.

In principle, when antimicrobials are used, resistant bacteria should be more likely to be isolated from samples because susceptible bacteria are inhibited or killed by the antimicrobial.[1-3] Therefore, it seems reasonable to observe an apparent higher prevalence of resistance when it is measured shortly after the use of an antimicrobial. This situation however, may simply represent a selection bias and not an association between the use of antimicrobials and development or spread of resistance among microorganisms. To study the long-term effects, if any, that antimicrobial-use may have on resistance, specific methodologies such as longitudinal studies need to be used.[20] Several sampling strategies may be considered for use in these longitudinal studies. One approach would be the collection of fecal samples from a cohort group[20,21] comprised of treated and non-treated cattle. The cohort groups may be selected according to characteristics such as age, housing, location and production status. For longitudinal studies, though, this may not be the best option, because some animals enrolled in the study as non-treated may eventually

need antimicrobial treatment during the course of the study, and animals may change status during the study. The loss of "control" individuals in this manner may hinder the objective evaluation of the effect of antimicrobial treatment on resistance due to a lack of power.[20,21] Additionally, "treated" animals may not respond to the treatment and die or be culled. The loss of animals from the "treated' group may bias the results because the long term outcome cannot be measured.

Another potential sampling strategy could be random selection [20,21] of cattle throughout the dairy. A possible limitation of this sampling scheme is that animals in a dairy farm are usually grouped according to age (calves) or production (cows). These different groups may have specific characteristics that could be considered confounding factors in the evaluation of the effect of antimicrobial-use on resistance. Several studies have reported a gradual decrease of the prevalence of antimicrobial resistant enteric bacteria (mainly *E. coli*) as age increases.[19,22,23] Newborn calves and peri-parturient cows have been reported to be more susceptible to disease than other cattle,[24,25] which implies a higher probability of antimicrobial treatment. Furthermore, to avoid drug residues in milk treated lactating dairy cows are usually housed in a separate pen (hospital pen) and are therefore clustered. Thus, simple random sampling could result in under-representation of cows treated with antimicrobials due to clustering. A more suitable sampling strategy for studies on dairy farms may be stratified randomization,[20,26] where all pens are sampled and cattle are randomly selected within each pen to ensure representation of all groups in the final sample.[20]

The objective of this study was to compare antimicrobial resistance in enteric bacteria isolated from fecal samples obtained from dairy cattle and calves at a commercial dairy in Northern Colorado using cohort and stratified random sampling strategies. *Salmonella* spp. and generic *Escherichia coli* (*E. coli*) were selected as representative of the *Enterobacteriaceae* family. *Salmonella* was chosen for its importance as a potential zoonotic pathogen. *E. coli* was chosen because it is easily isolated from feces and because it may pose a risk for the spread of resistance genes to *Salmonella*;[23,27] both species have approximately 70% of their genes in common.[28]

MATERIALS AND METHODS

The study was conducted at a commercial dairy in Northern Colorado that milked 1,200 dairy cows and kept records on antimicrobial-use. Adult cows were grouped in 13 pens according to stage of lactation (quantified as "days in milk" or DIM), milk production (high, medium, low, or dry) and reproductive management (artificial insemination or natural-service). Cows needing any kind of treatment (with or without antimicrobials) were housed in a separate hospital pen. All pens that housed milking cows, except the natural-service pen, had typical free-stall housing and a flush system for cleaning alleyways. The natural-service pen was set up as drylot housing.

Only female calves were kept at the farm; males were sold within 2 days of birth. Calves were fed pasteurized non-salable milk (waste milk) obtained from cows that were housed in the maternity and the hospital pens, and therefore waste milk contained antimicrobial

residues. Residues in waste milk were from dry-cow treatments (long-acting intramammary antimicrobials) and current treatments administered in the hospital (intramammary and parenteral). Calves were housed in individual hutches while they were being fed waste milk. Approximately 2-3 weeks after weaning from milk (45 days of age), calves were grouped in super-hutches (6-8 calves per group) for a period of about 1 month. After socially acclimating to this small group of animals, calves were grouped in drylot-type pens of increasing size and animal numbers as age increased (6 to 50 calves per pen). At about 10 months of age, all calves were moved to a custom heifer rearing ranch.

Sampling Strategies

Two study groups, one of cows and one of calves, were used. The populations of sampled cows and calves were each divided into two subgroups:

- A cohort group of 100 animals, which included 50 animals that had been treated with antimicrobials within the 30 days prior to the first sampling event and 50 animals that had not been treated with antimicrobials within 30 days of the first sampling event. Lactating cows treated with intramammary antimicrobials were excluded both from the treated and non-treated groups in this study. Control (non-treated) animals were matched to treated animals according to location and age in calves, and according to lactation number, DIM and location in cows (and therefore, production).

- A group of 50 animals that were randomly selected at each sampling event: 5 cows in each one of the 10 milking pens (lactating cows) and 5-10 calves per pen, according to pen size.

All cows were lactating at the time of enrollment. During the study 22 cows enrolled in the cohort group were dried-off, and subsequently 22 fecal samples were collected from cows in two dry-cow pens. These samples were collected during 5 sampling events, although most (13/22) were collected on sampling event 6. All cows sampled as part of the random group were selected among lactating cows.

Approximately 50 g of feces were collected from each animal, either by manual retrieval from the rectum or by collection of a freshly produced manure pat. Visual confirmation of the animal producing the fecal pat was necessary to correctly identify each animal and later establish its treatment status. Samples were collected on six sampling events at approximately 8-week intervals. Repeated samples were collected from animals enrolled in the cohort group that were still present at the dairy during each sampling event. Animals to be sampled as part of the random group were randomly selected at each sampling event among all animals within a pen. In some instances individual animals contributed samples both to the random and the cohort groups.

The study period extended from September 2004 into July 2005. Samples from calves and cows were collected on alternating months to avoid cross-contamination between cow and calf areas. Actual sampling dates were:

	Calves	Cows
Event 1	Sep-14, 2004	Oct-12, 2004
Event 2	Nov-9, 2004	Dec-6, 2004
Event 3	Jan-4, 2005	Jan-31, 2005
Event 4	Mar-1, 2005	Mar-28, 2005
Event 5	Apr-21, 2005	May-23, 2005
Event 6	Jun-20, 2005	Jul-18, 2005

During the study, more calves had to be enrolled in the cohort group at the third sampling event (6 months), because 37 of the original 100 calves (37%) had been shipped to a heifer ranch and were lost to follow-up.

Bacterial Isolation and Susceptibility Testing

Although susceptibility testing for *Salmonella* and *E. coli* was performed in a similar manner, isolation methods for both bacteria differed.

Salmonella Culture and Isolation

Bacteriologic culture method for Salmonella was performed as described elsewhere.[29] Feces (1 g) were enriched by incubating in 10 mL of Gram Negative (GN) Hajna broth[a] for 18-24 h at 37° C, and tetrathionate broth[a] for 40-48 h at 37° C. After the initial enrichments, aliquots (100 μL) were transferred to 10 mL of Rappaport-Vassiliadis R10

[a] BD-Difco, Sparks, MD

broth[a] which were incubated for 18-24 h at 37° C. Ten (10) μL aliquots of Rappaport-Vassiliadis R10 broth were then streaked onto xylose-lysine-tergitol-4[b] and brilliant green (BG) Sulfa agar[a] for isolation of *Salmonella*. Plates were incubated for 18-24 h at 37° C. Isolated colonies characteristic of *Salmonella* were inoculated into triple sugar iron and lysine iron agar slants for biochemical confirmation (black). One colony was selected from each sample for further characterization. Presumptive *Salmonella* isolates were serogrouped using serogroup-specific antisera[a] and were sent to the National Veterinary Services Laboratories (Ames, IA) for serotyping.

E. coli Culture and Isolation

One hundred (100) μL aliquots of fecal dilutions (1:9 weight/volume, in PBS) were streaked for isolation onto MacConkey agar[a] plates. The plates were incubated for 24 h at 37° C. Isolated colonies characteristic of *E. coli* were inoculated into tryptic soy agar (TSA) slants and incubated with a loose cap for 18-24 h at 37° C. A presumptive identification of *E. coli* was assigned when colonies were 1 to 2 mm in diameter, pink, uniform in color, flat with smooth margins, and had a positive indole reaction (pink) when tested.

Susceptibility Testing

One colony per sample was selected for susceptibility testing. Susceptibility to 16 antimicrobials was tested according to guidelines from the Clinical Laboratories

[b] Hardy Diagnostics, Santa Maria, CA

Standards Institute (CLSI, formerly known as the National Committee for Clinical Laboratory Standards [NCCLS]).[30] Susceptibility was determined by broth-microdilution using a semi-automated system[c] as per manufacturer directions. Minimum inhibitory concentrations (MICs) were established for antimicrobials used both in human and veterinary medicine and were configured in a 96-well custom made panel. Susceptibility results were interpreted by use of the following breakpoints for resistance:

amikacin (64 μg/mL)	amoxicillin/clavulanic acid (32 μg/mL)
ampicillin (32 μg/mL)	cefoxitin (32 μg/mL)
ceftiofur (8 μg/mL)	ceftriaxone (64 μg/mL)
cephalothin (32 μg/mL)	chloramphenicol (32 μg/mL)
ciprofloxacin (4 μg/mL)	gentamicin (16 μg/mL)
kanamycin (64 μg/mL)	nalidixic acid (32 μg/mL)
streptomycin (64 μg/mL)	sulfisoxazole (512 μg/mL)
tetracycline (16 μg/mL)	trimethoprim/sulfamethoxazole (4 μg/mL)

Quality control strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* 29212) were included in susceptibility testing on a weekly basis.

Isolates with an MIC value out of the tested range were assumed to have the MIC value immediately following the tested range. For example, for tetracycline the lowest MIC tested was 4 μg/mL and therefore the lowest MICs reported was ≤ 4 μg/mL. The highest

[c] Sensititre, Trek Diagnostics, Westlake, OH

MIC tested for tetracycline was 32 μg/mL, and so isolates with higher MICs were reported as > 32 μg/mL. For calculation and graphic representation purposes, isolates with an MIC of > 32 μg/mL were assumed to have an MIC of 64 μg/mL (the immediately following MIC value).

Data Analysis

Outcome variables for the study were; 1) isolation, 2) resistance, and 3) multi-drug resistance, and were defined as follows:

- *Isolation*: growth of any colony (susceptible or resistant). Relative frequency of isolation for a group of samples was calculated as the number of samples with positive growth divided by total number of samples within the group.

- *Resistance:* MICs for at least one antimicrobial was above the breakpoint for resistance. Relative frequency of resistance for a group of samples was calculated as the number of samples with an isolate resistant to one or more antimicrobials divided by total number of isolates within the group. Therefore, samples from which no isolates were cultured were not included in this calculation.

- *Multidrug resistance (MDR):* resistance to three or more antimicrobials. Relative frequency of MDR for a group of samples was calculated as the number of samples with an isolate resistant to three or more antimicrobials divided by total

number of isolates within the group. Samples from which no isolates were cultured were not included in this calculation.

Pairwise comparisons were performed between samples obtained from the random and cohort groups, between samples obtained from calves or cows, and between *Salmonella* and *E. coli* isolates. For pairwise comparisons, the study variables were dichotomized as isolation or no isolation, resistance or no resistance (susceptible and intermediate), and MDR or no MDR (resistant to less than 3 antimicrobials). Differences in proportions were analyzed using a standard *Z*–test. Statistical significance was determined at a P-value of 5% or less ($P \leq 0.050$).

Descriptive parameters evaluated for MICs were:

- Range
- MIC_{50}, defined as the MIC that inhibited the growth of 50% of isolates (median).
- MIC_{90}, defined as the MIC that inhibited the growth of 90% of isolates.
- Geometric mean, to describe central tendency of MICs.

Frequency of resistance patterns was also evaluated, highlighting patterns that included resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (ACSSuT).

RESULTS

A total of 1,665 fecal samples were collected: 844 from calves and 821 from cows. At each sampling event, 50 random samples were collected. The number of samples per sampling event in the cohort groups changed over time due to culling or transfer of animals to the heifer ranch. A summary of samples collected from each animal group on each event is presented for calves in Table 4.1 (absolute frequencies) and Table 4.2 (relative frequencies), and for cows in Table 4.3 (absolute frequencies) and Table 4.4 (relative frequencies).

Overall, isolation was significantly ($P < 0.001$) less frequent for *Salmonella* (54/1,665. 3.2%) than for *E. coli* (1,464/1,665, 87.9%). Frequency of isolation was significantly ($P \leq 0.001$) lower for *Salmonella* from calves (16/844, 1.9%), compared to *Salmonella* from cows (38/821, 4.6%), *E. coli* from cows (696/821, 84.8%), and *E. coli* from calves (768/844, 91.0%). Stratum-specific results for isolation are presented in Table 4.5. Overall, *Salmonella* of seven serotypes were isolated (Table 4.6). Four serotypes were isolated from calf samples and five serotypes were isolated from cow samples. Only *S.* Anatum and *S.* Typhimurium serotypes were isolated both from calf and cow samples.

In contrast to isolation, resistance was significantly ($P = 0.009$) higher among *Salmonella* isolates (35/54, 64.8%) than among *E. coli* isolates (694/1,464, 47.4%). Frequencies of resistance differed significantly ($P \leq 0.006$) between subgroups, being highest for *Salmonella* in calves (16/16, 100.0%), lower for *E. coli* in calves (518/768, 67.5%), lower

Table 4.1 **Number** of samples collected from **calves** in a study on antimicrobial resistance in enteric bacteria performed at a commercial dairy farm in Northern Colorado. Distribution of samples among categories of studied factors (rows), and studied outcome variables (columns). Samples with isolation are shaded in gray and added in the total isolates

Sampling group	Sampling event	Sampling location	Total number of samples	*E.coli*				Total isolates	*Salmonella*				Total isolates
				Isolation					Isolation				
				No	Yes				No	Yes			
					Resistance					Resistance			
					No	Yes				No	Yes		
						MDR					MDR		
						No	Yes				No	Yes	
Cohort	1	Individual hutches 6 super-hutches and 5 pens	64	3	9	15	37	61	59	0	0	5	5
	2		99	1	31	24	43	98	94	0	0	5	5
	3		90	3	20	43	24	87	89	0	0	1	1
	4		131	17	39	35	40	114	130	0	0	1	1
	5		115	14	42	40	19	101	115	0	0	0	0
	6		45	6	16	18	5	39	45	0	0	0	0
Random	1	Individual hutches 6 super-hutches and 5 pens	50	2	24	14	10	48	49	0	0	1	1
	2		50	1	20	20	9	49	49	0	0	1	1
	3		50	0	11	24	15	50	50	0	0	0	0
	4		50	9	6	10	16	41	50	0	0	0	0
	5		50	11	13	5	10	39	50	0	0	0	0
	6		50	9	11	18	12	41	48	0	1	1	2

Table 4.2 **Proportion** of samples (%) collected from **calves** in a study on antimicrobial resistance in enteric bacteria performed at a commercial dairy farm in Northern Colorado. Distribution of samples among categories of studied factors (rows), and studied outcome variables (columns).

Sampling group	Sampling event	Sampling location	Total number of samples	*E.coli*				*Salmonella*			
				% Isolation				% Isolation			
				No	Yes			No	Yes		
					% Resistant				% Resistance		
					No	Yes			No	Yes	
						% MDR				% MDR	
						No	Yes			No	Yes
Cohort	1	Individual hutches 6 super-hutches and 5 pens	64	4.7	14.1	23.4	57.8	92.2	92.2	0.0	0.0
	2		99	1.0	31.3	24.2	43.4	94.9	0.0	0.0	5.1
	3		90	3.3	22.2	47.8	26.7	98.9	0.0	0.0	1.1
	4		131	13.0	29.8	26.7	30.5	99.2	0.0	0.0	0.8
	5		115	12.2	36.5	34.8	16.5	100.0	0.0	0.0	0.0
	6		45	13.3	35.6	40.0	11.1	100.0	0.0	0.0	0.0
Random	1	Individual hutches 6 super-hutches and 5 pens	50	4.0	48.0	28.0	20.0	98.0	0.0	0.0	2.0
	2		50	2.0	50.0	30.0	18.0	98.0	0.0	0.0	2.0
	3		50	0.0	28.0	42.0	30.0	100.0	0.0	0.0	0.0
	4		50	18.0	12.0	38.0	32.0	100.0	0.0	0.0	0.0
	5		50	22.0	26.0	24.0	28.0	100.0	0.0	0.0	0.0
	6		50	18.0	22.0	36.0	24.0	96.0	0.0	2.0	2.0

Table 4.3 **Number** of samples collected from **cows** in a study on antimicrobial resistance in enteric bacteria performed at a commercial dairy farm in Northern Colorado. Distribution of samples among categories of studied factors (rows), and studied outcome variables (columns). Samples with isolation are shaded in gray and added in the total isolates

Sampling group	Sampling event	Sampling location	Total number of samples	*E.coli*					Total isolates	*Salmonella*					Total isolates
				Isolation						Isolation					
				No	Yes					No	Yes				
					Resistance						Resistance				
					No	Yes					No	Yes			
						MDR						MDR			
						No	Yes					No	Yes		
Cohort	1	10 milking pens and 2 dry pens	100	3	68	24	5		97	100	0	0	0		0
	2		87	0	77	9	1		87	85	0	0	2		2
	3		89	3	72	14	0		86	89	0	0	0		0
	4		85	32	45	8	0		53	79	0	6	0		6
	5		83	20	29	34	0		63	78	5	0	0		5
	6		77	13	39	25	0		64	75	1	1	0		2
Random	1	10 milking pens	50	6	38	6	0		44	50	0	0	0		0
	2		50	2	42	6	0		48	50	0	0	0		0
	3		50	3	35	11	1		47	48	0	2	0		2
	4		50	23	23	4	0		27	44	1	5	0		6
	5		50	14	23	10	3		36	39	10	0	1		11
	6		50	7	36	7	0		43	46	2	2	0		4

Table 4.4 **Proportion** of samples (%) collected from **cows** in a study on antimicrobial resistance in enteric bacteria performed at a commercial dairy farm in Northern Colorado. Distribution of samples among categories of studied factors (rows), and studied outcome variables (columns).

Sampling group	Sampling event	Sampling location	Total number of samples	*E.coli*				*Salmonella*			
				% Isolation				% Isolation			
				No	Yes			No	Yes		
					% Resistant				% Resistance		
					No	Yes			No	Yes	
						% MDR				% MDR	
						No	Yes			No	Yes
Cohort	1	10 milking pens and 2 dry pens	100	3.0	68.0	24.0	5.0	100.0	0.0	0.0	0.0
	2		87	0.0	88.5	10.3	1.1	97.7	0.0	0.0	2.3
	3		89	3.4	80.9	15.7	0.0	100.0	0.0	0.0	0.0
	4		85	37.6	52.9	9.4	0.0	92.9	0.0	7.1	0.0
	5		83	24.1	34.9	41.0	0.0	94.0	6.0	0.0	0.0
	6		77	16.9	50.6	32.5	0.0	97.4	1.3	1.3	0.0
Random	1	10 milking pens	50	12.0	76.0	12.0	0.0	100.0	0.0	0.0	0.0
	2		50	4.0	84.0	12.0	0.0	100.0	0.0	0.0	0.0
	3		50	6.0	70.0	22.0	2.0	96.0	0.0	4.0	0.0
	4		50	46.0	50.0	4.0	0.0	88.0	2.0	10.0	0.0
	5		50	28.0	46.0	20.0	6.0	78.0	20.0	0.0	2.0
	6		50	14.0	52.0	28.0	6.0	92.0	4.0	4.0	0.0

Table 4.5 Isolation of ***Salmonella*** and ***E. coli*** from **calves** and **cows** at a Colorado dairy. Data are presented as percentage of random or cohort samples (column heading). Data in parenthesis are the actual number of isolates for each category.

	Calves				Cows				
	Salmonella		*E.coli*		*Salmonella*		*E.coli*		
	Random	Cohort	Random	Cohort	Random	Cohort	Random		Cohort
	$n = 300$	$n = 544$	$n = 300$	$n = 544$	$n = 300$	$n = 521$	$n = 300$		$n = 521$
Isolation	1.3 (4)	2.2 (12)	89.3 (268)	91.9 (500)	7.7 (23)	2.9 (15)	81.7 (245)	*	86.6 (451)

* Values of random and cohort samples are significantly different ($P < 0.05$) for that specific category.

Table 4.6 *Salmonella* serotypes isolated from **cows** and **calves** at a commercial dairy in Northern Colorado.

	Calves		Cows	
	Random	Cohort	Random	Cohort
	$n = 4$	$n = 12$	$n = 23$	$n = 15$
9,12 Non-motile		4		
S. Anatum	1		2	1
S. Cubana			8	6
S. Dublin		1		
S. Infantis			12	6
S. Newport				2
S. Typhimurium	3	7	1	

yet for *Salmonella* in cows (19/38, 50.0%), and lowest for *E. coli* in cows (176/696, 25.3%). Stratum-specific results for resistance and MDR are summarized in Table 4.7. A visual summary of the distribution of actual MIC values is presented for *E. coli* isolated from calves (Table 4.8), *E. coli* isolated from cows (Table 4.9), *Salmonella* isolated from calves (Table 4.10) and *Salmonella* isolated from cows (Table 4.11). The MICs were truncated, both for the upper and lower limits. A comparison of MICs (range, MIC_{50}, MIC_{90}, and geometric mean) for *E. coli* isolates from calves and cows is presented in Table 4.12.

Overall (Table 4.7), MDR frequency was highest among *Salmonella* isolated from calves (15/16 or 93.8%), lower for *E. coli* in calves (322/768 or 41.9%), *Salmonella* in cows (3/38 or 7.9%), and lowest for *E. coli* in cows (42/696 or 6.0%). Frequencies of MDR were significantly different ($P \leq 0.001$) among all categories, although not between *Salmonella* and *E. coli* isolates obtained from cows ($P = 0.453$).

A total of 81 resistance patterns were found jointly among *Salmonella* and *E. coli* isolates, from calves (Figure 4.1) and cows (Figure 4.2). Most resistance patterns were observed in *E. coli* (77/81), while seven were observed in *Salmonella* isolates, and only two were observed in both bacterial species. In total, 17 patterns showed the ACSSuT phenotype, four were observed in *Salmonella* and 14 in *E. coli* isolates (one was observed in both species). Approximately 45.9% (17/37) of all *Salmonella* isolates included the ACSSuT phenotype, compared to only 14.8% (103/694) of *E. coli* isolates.

Table 4.7 Summary of resistance and multidrug resistance (MDR) of ***Salmonella*** and ***E. coli*** isolates cultured from **calves** and **cows** at a Colorado dairy. Data are presented as percentage of isolates obtained from random or cohort samples within calves or cows. Data in parenthesis are the actual number of isolates for each category.

	Calves				Cows			
	Salmonella		*E.coli*		*Salmonella*		*E.coli*	
	Random	Cohort	Random	Cohort	Random	Cohort	Random	Cohort
	$n = 4$	$n = 12$	$n = 268$	$n = 500$	$n = 23$	$n = 15$	$n = 245$	$n = 451$
Resistance	100.0 (4)	100.0 (12)	65.3 (175)	68.6 (343)	43.5 (10)	60.0 (9)	22.9 (56)	26.6 (120)
MDR	75.0 (3)	100.0 (12)	38.4 (103)	43.8 (219)	4.3 (1)	13.3 (2)	6.9 (17)	5.5 (25)

Table 4.8 Distribution of minimum inhibitory concentrations (MICs) for ***E. coli*** isolates cultured from **calves** on a commercial dairy farm in Northern Colorado (n = 768). The range of tested dilutions is represented by the boxed values. MICs for resistant isolates are denoted by the dark shading, intermediate MICs by intermediate shading, and susceptible MICs have no shading.

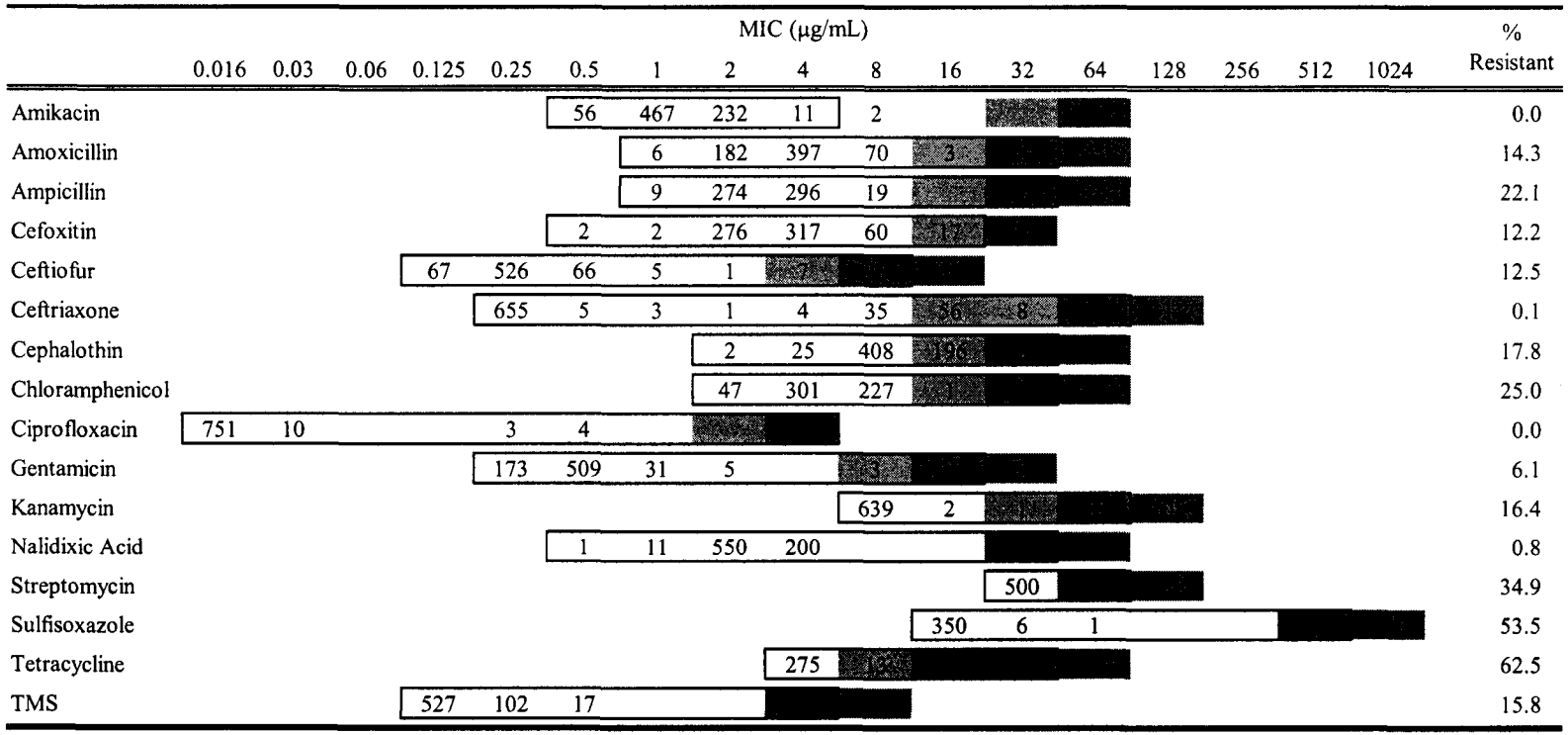


Table 4.9 Distribution of minimum inhibitory concentrations (MICs) for *E. coli* isolates cultured from **cows** on a commercial dairy farm in Northern Colorado (n = 696). The range of tested dilutions is represented by the boxed values. MICs for resistant isolates are denoted by the dark shading, intermediate MICs by intermediate shading, and susceptible MICs have no shading.

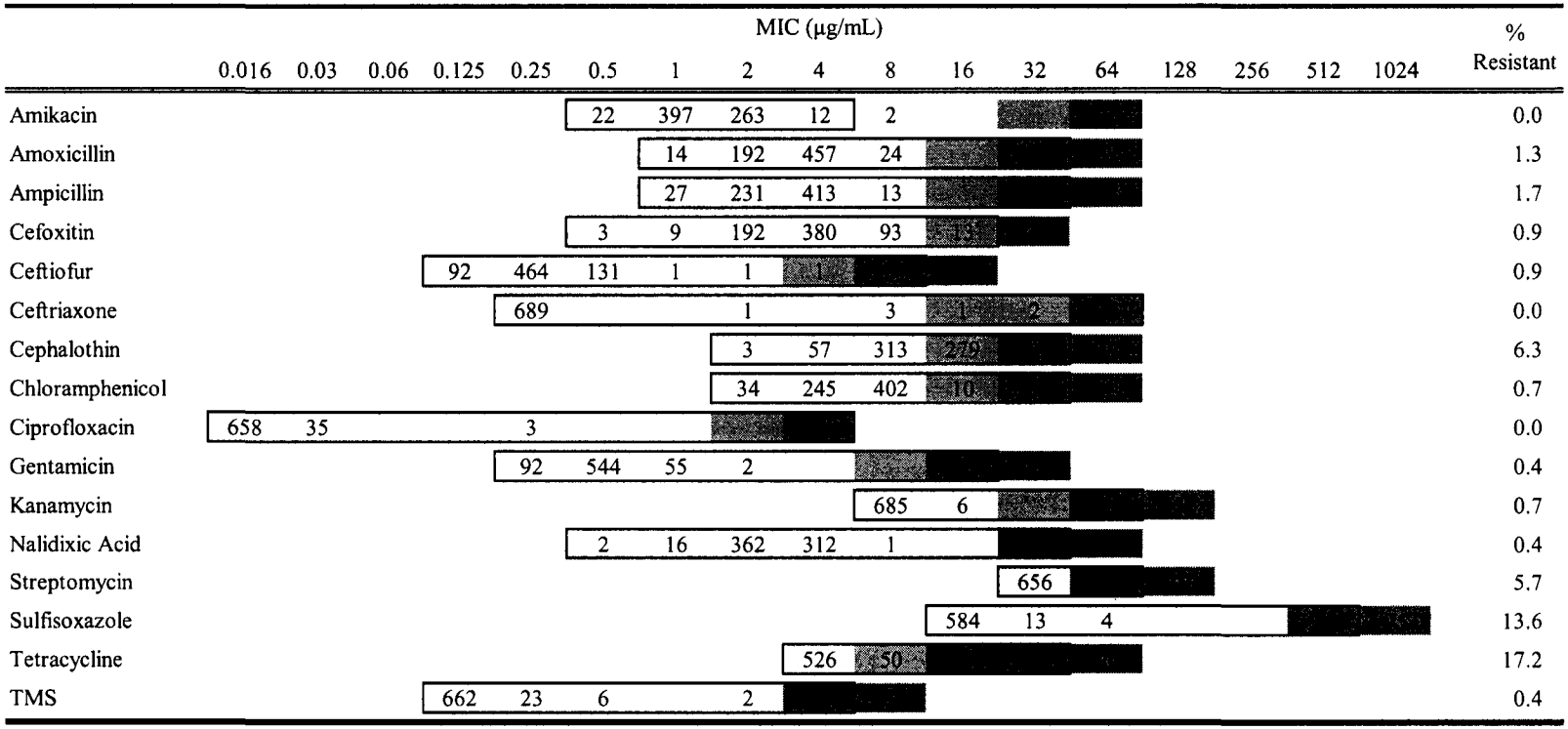


Table 4.10 Distribution of minimum inhibitory concentrations (MICs) for ***Salmonella*** isolates cultured from **calves** on a commercial dairy farm in Northern Colorado (n = 16). The range of tested dilutions is represented by the boxed values. MICs for resistant isolates are denoted by the dark shading, intermediate MICs by intermediate shading, and susceptible MICs have no shading.

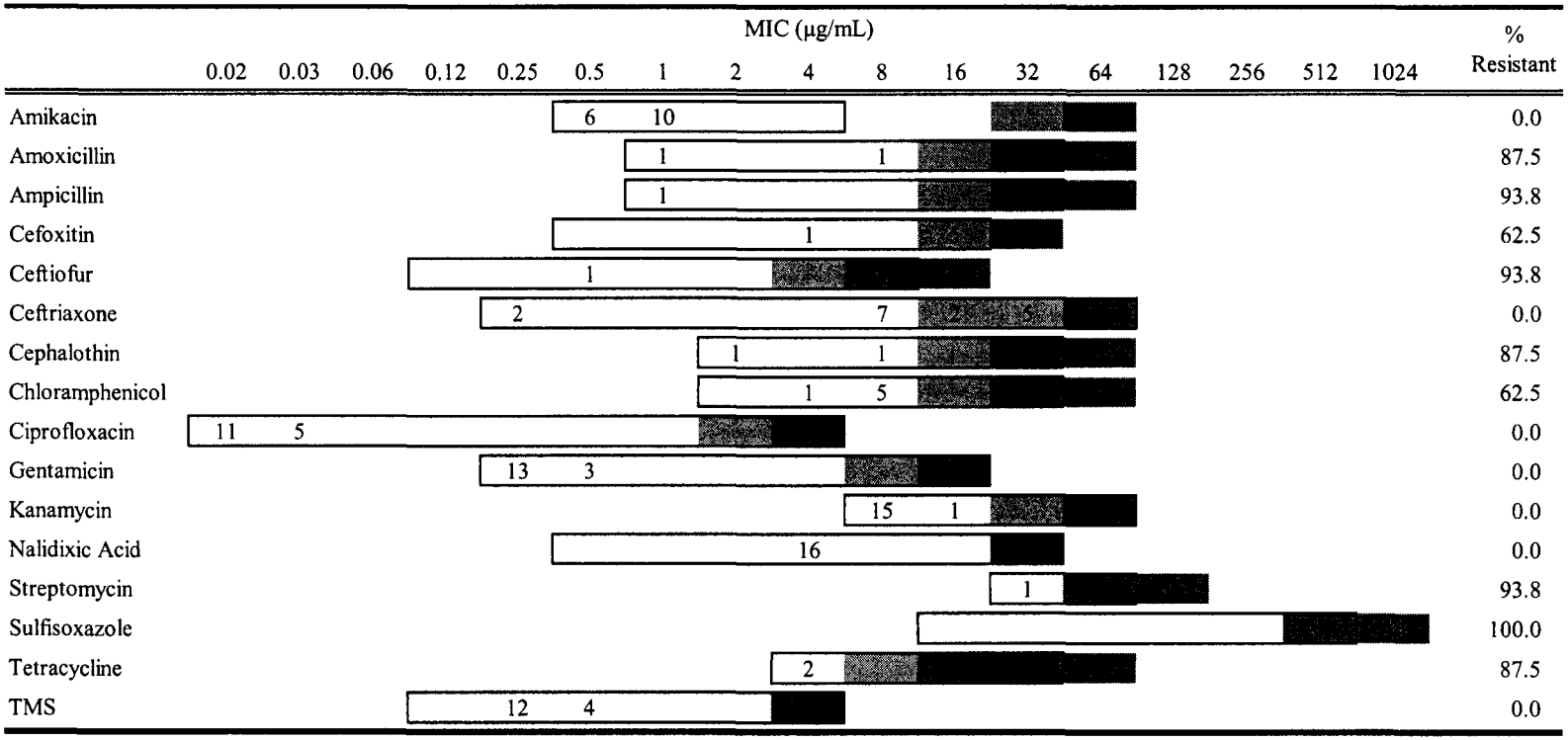


Table 4.11 Distribution of minimum inhibitory concentrations (MICs) for ***Salmonella*** isolates cultured from **cows** on a commercial dairy farm in Northern Colorado (n = 38). The range of tested dilutions is represented by the boxed values. MICs for resistant isolates are denoted by the dark shading, intermediate MICs by intermediate shading, and susceptible MICs have no shading.

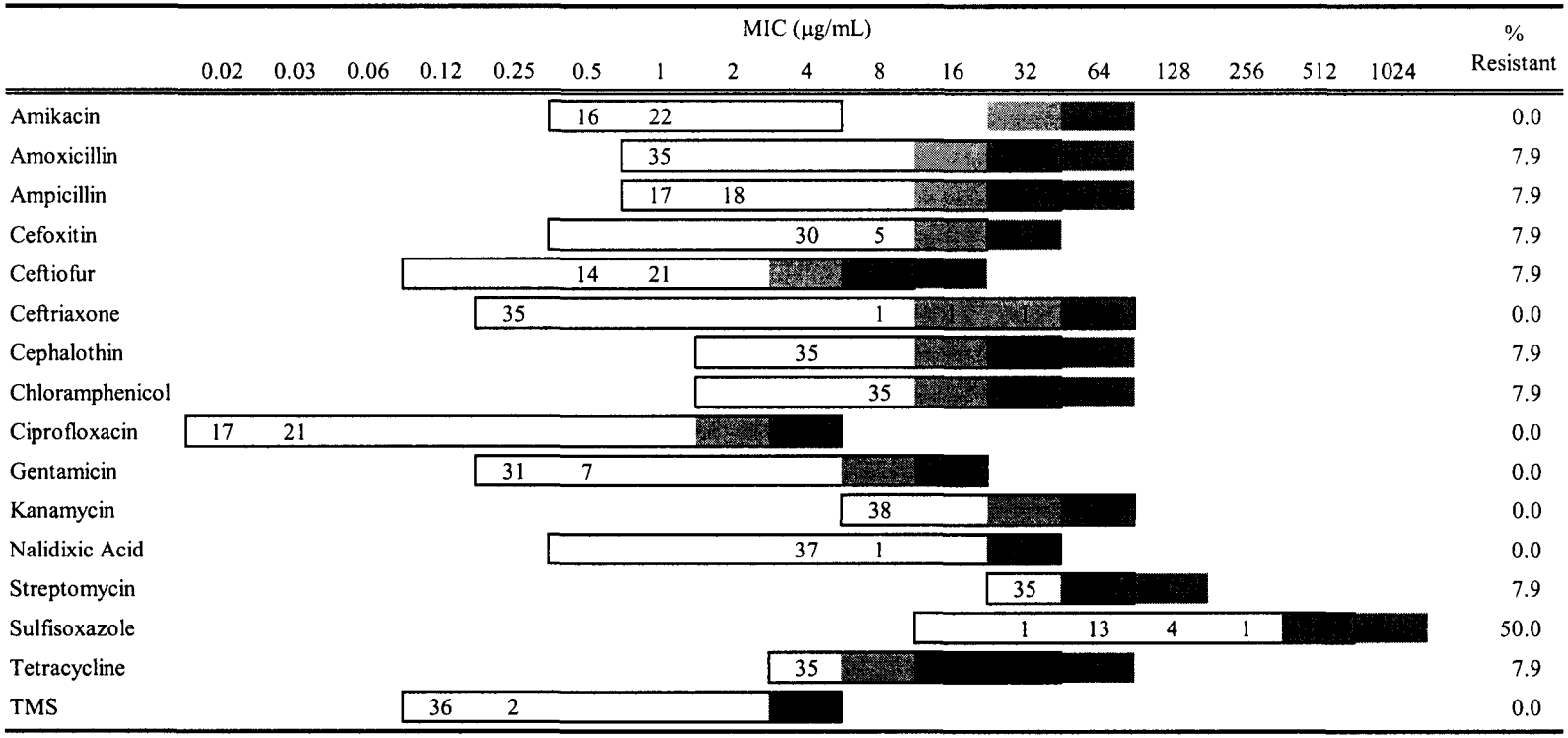


Table 4.12 Descriptive summary of antimicrobial susceptibility parameters for *E. coli* isolates cultured from fecal samples obtained from **calves** and **cows** at a commercial dairy in Northern Colorado.

	Resistance breakpoint ≥	Calves (n = 768)					Cows (n = 696)				
		MIC				%	MIC				%
		Range	MIC_{50}	MIC_{90}	G. mean	Resistant	Range	MIC_{50}	MIC_{90}	G. mean	Resistant
Amikacin	64	0.5 – 8	1	2	1.2	0	0.5 – 8	1	2	1.31	0
Amox / Clavulanic	32	1 – 64	4	32	5.03	14.3	1 – 64	4	4	3.39	1.3
Ampicillin	32	1 – 64	4	64	5.77	22.1	1 – 64	4	4	3.2	1.7
Cefoxitin	32	0.5 – 32	4	32	4.34	12.2	0.5 – 32	4	8	3.69	0.9
Ceftiofur	8	0.125 – 16	0.25	8	0.42	12.5	0.125 – 16	0.25	0.5	0.03	0.9
Ceftriaxone	64	0.25 – 128	0.25	8	0.43	0.1	0.25 – 32	0.25	0.25	0.03	0
Cephalothin	32	2 – 64	8	64	13.21	17.8	2 – 64	8	16	10.93	6.3
Chloramphenicol	32	2 – 64	8	64	9.37	25	2 – 64	8	8	6	0.7
Ciprofloxacin	4	0.016 – 0.5	0.016	0.016	0.01	0	0.016 – 0.25	0.016	0.016	0.02	0
Gentamicin	16	0.25 – 32	0.5	1	0.57	6.1	0.25 – 32	0.5	0.5	0.49	0.4
Kanamycin	64	8 – 128	8	128	12.45	16.4	8 – 128	8	8	8.19	0.7
Nalidixic acid	32	0.5 – 64	2	4	2.43	0.8	0.5 – 64	2	4	2.72	0.4
Streptomycin	64	32 – 128	32	128	48.29	34.9	32 – 128	32	32	48.29	5.7
Sulfisoxazole	512	16 – 1024	1024	1024	149.09	53.5	16 – 1024	16	1024	149.09	13.6
Tetracycline	16	4 – 64	32	64	20.53	62.5	4 – 64	4	64	6.52	17.2
TMS	4	0.125 – 8	0.125	8	0.03	15.8	0.125 – 8	0.125	0.125	0.01	0.4

G. mean = geometric mean

Figure 4.1 Frequency of the most common resistance patterns found among ***Salmonella*** (dark bars) and ***E. coli*** isolates (light bars) cultured in samples collected from **calves**. Patterns coded A through M are ACSSuT phenotype plus any other possible combination of resistance to other antimicrobials. For a legend of pattern codes, refer to Appendix A.

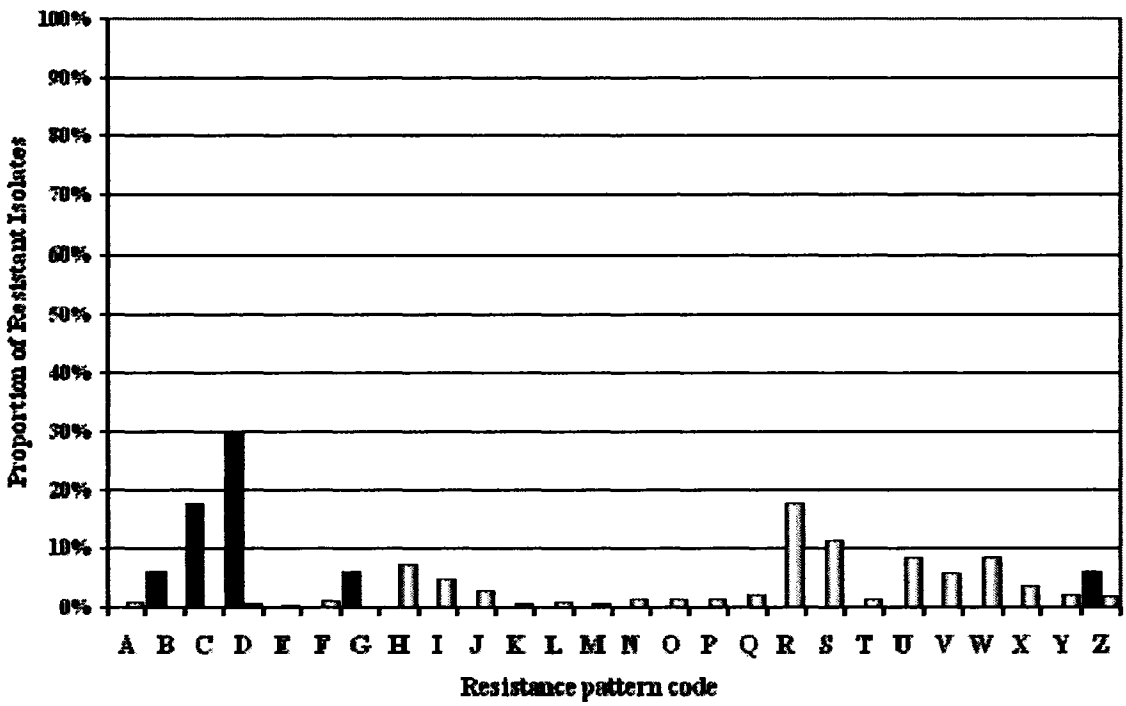
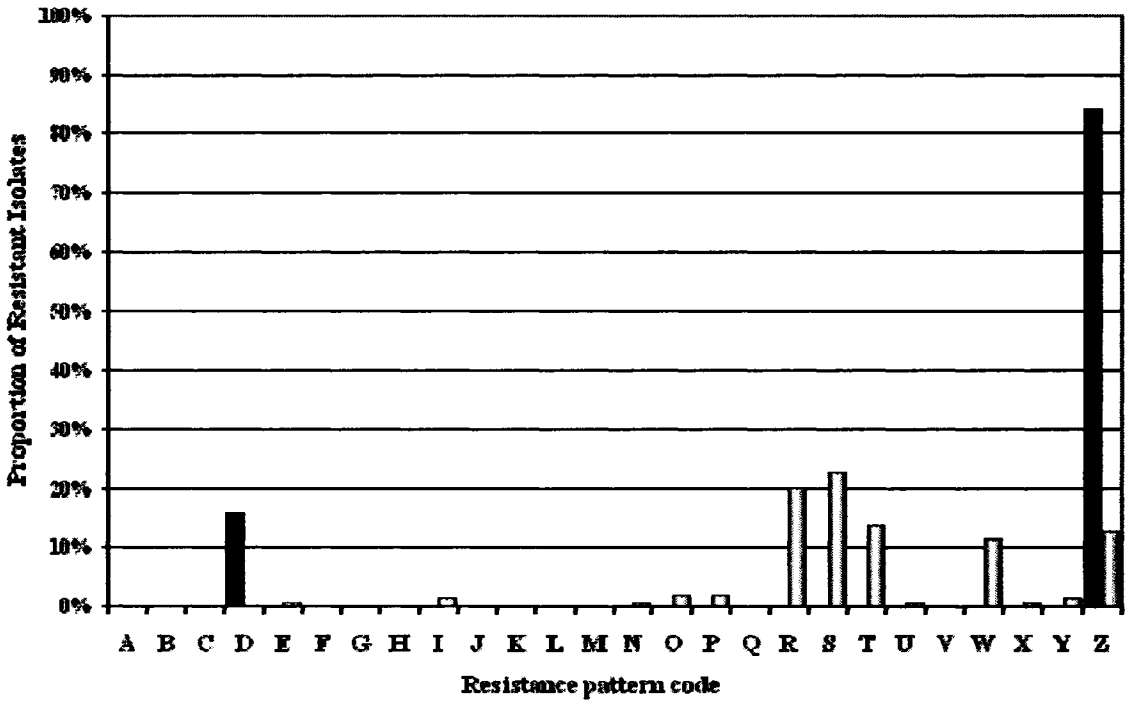


Figure 4.2 Frequency of the most common resistance patterns found among ***Salmonella*** (dark bars) and ***E. coli*** isolates (light bars) cultured in samples collected from **cows**. Patterns coded A through M are ACSSuT phenotype plus any other possible combination of resistance to other antimicrobials. For a legend of pattern codes, refer to Appendix A.



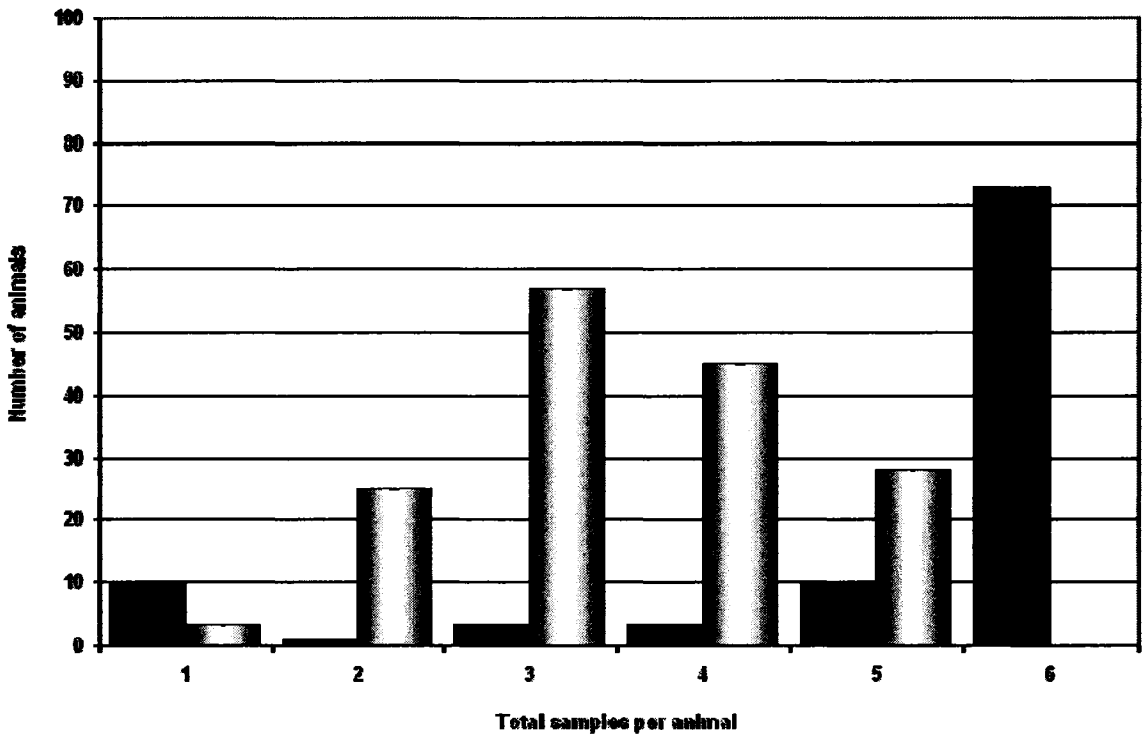
None of the *Salmonella* isolates were resistant to amikacin, ciprofloxacin, gentamicin, kanamycin, nalidixic acid or trimethoprim-sulfamethoxazole (TMS). The highest prevalence of resistance among *Salmonella* isolates was to sulfisoxazole, both in calves (16/16 or 100.0%) and cows (19/38 or 50.0%). In contrast, the only antimicrobials for which no resistance was found in *E. coli* isolates were amikacin and ciprofloxacin. The highest prevalence of resistance among *E. coli* isolates was to tetracycline, both in calves (480/768 or 62.5%) and cows (120/696 or 17.2%). The ACSSuT phenotype (see Appendix A) was found in 62.5% (10/16) of *Salmonella* isolates cultured from calves, 7.9% (3/38) of *Salmonella* isolates from cows, 12.2% (94/768) of *E. coli* isolates from calves, and 0.004% (3/696) of *E. coli* isolates from cows.

Due to the marked differences observed between *Salmonella* and *E. coli* isolated from calves and cows, results will be presented separately for each group.

Calves

In total, 844 fecal samples were collected from calves: 300 from randomly selected animals and 544 from cohort animals. Samples collected from the cohort group were composed of a similar number of treated (n = 247) and non-treated (n = 297) calves. Total number of samples per calf in the cohort group (repeated samples) ranged from one to five, with a median of three samples per calf (Figure 4.3).

Figure 4.3 Distribution of the total number of samples collected per animal in **calves** (light bars) and in **cows** (dark bars) during six sampling events performed at 8-week intervals in enrolled cohorts at a commercial dairy in Northern Colorado.



Salmonella from Calves

Only 16 *Salmonella* isolates were cultured from the 844 calf samples (1.9%). None of the sampled calves had *Salmonella* isolated on more than one sampling event. Almost all (15/16) of the *Salmonella* isolates cultured from calves were MDR, actually resistant to seven or more antimicrobials and all 15 included the phenotype ACSSuT. The single isolate that was different was resistant only to sulfisoxazole (*S.* Anatum). Comparison of resistance frequencies for *Salmonella* in the random and cohort groups showed no difference ($P = 0.267$) either for the whole study (Table 4.5) or individual sampling events (Table 4.2). Although eight different resistance patterns were found, four were exhibited by one single isolate each. Isolates within a *Salmonella* serotype had similar resistance patterns.

Due to the small number of *Salmonella* isolates recovered, no meaningful comparisons could be made between resistance patterns from random and cohort samples. Only one *Salmonella* isolate was found in any of the pens that housed grouped calves. All other isolates were found in calves housed in individual hutches, most of them (13/16 or 81.3%) from calves that were 90 days old or less at the time of sampling. *Salmonella* isolates tended to be recovered more frequently during the first half of the study (September through January) than the second half (March through June). See Table 4.1 for a summary of absolute frequencies of isolation, resistance and MDR by sampling event, and Table 4.2 for a summary of relative frequencies.

E. coli from Calves

E. coli was isolated from 91.0% of the fecal samples collected from calves (768/844). No differences between random and cohort samples were observed in the proportion of *E. coli* positive cultures (P = 0.130, Table 4.5), resistant isolates (P = 0.198) or MDR isolates (P = 0.087, Table 4.7). There were 70 different resistance patterns found in *E. coli* isolated from calves compared to only eight patterns observed in *Salmonella* isolates. Forty of these patterns were found only in one or two isolates. Only two of the patterns observed in *E. coli* isolates were also observed in *Salmonella* isolates (Figure 4.1, patterns D and Z, see Appendix A).

Resistant and MDR *E. coli* isolates were found in all locations where calves were housed. Prevalence of resistance was highest among *E. coli* isolates cultured from calves that were housed either in individual hutches (218/309 or 70.6%) or in super-hutches (24/32 or 75.0%). The lowest prevalence of resistance among *E. coli* isolates was observed in the oldest calves, where 49.6% (62/125) were resistant to at least one antimicrobial. Prevalence of MDR showed a similar decrease as age increased, ranging from 54.4% (168/309) in calves housed in individual hutches to 21.6% (27/125) in the oldest calves. Within sampling group, the frequency of isolation, resistance and MDR of *E. coli* did not vary across sampling events (Table 4.1 and Table 4.2). Of the 768 *E. coli* isolates cultured from calves, none were resistant to amikacin or ciprofloxacin; only one isolate was resistant to ceftriaxone; five were resistant to nalidixic acid; and 14 were resistant to gentamicin. Table 4.13 shows the descriptive parameters for MICs of *E. coli* isolates obtained from cohort calves and randomly selected calves. Range of MICs was identical

Table 4.13 Summary of descriptive MIC parameters for *E. coli* isolates obtained from **calves** enrolled in a cohort group or from randomly selected calves in a study conducted on a commercial dairy farm in Northern Colorado.

	Resistance breakpoint ≥	Random (n = 268)				Cohort (n = 500)			
		MIC Range	MIC_{50}	MIC_{90}	G. mean	MIC Range	MIC_{50}	MIC_{90}	G. mean
Amikacin	64	0.5 – 8	1	2	1.23	0.5 – 8	1	2	1.19
Amox / Clavulanic	32	1 – 64	4	32	4.62	1 – 64	4	32	5.26
Ampicillin	32	1 – 64	4	64	5.1	1 – 64	4	64	6.16
Cefoxitin	32	0.5 – 32	4	16	4.19	0.5 – 32	4	32	4.42
Ceftiofur	8	0.125 – 16	0.25	4	0.37	0.125 – 16	0.25	8	0.45
Ceftriaxone	64	0.25 – 128	0.25	8	0.38	0.25 – 32	0.25	8	0.46
Cephalothin	32	2 – 64	8	64	12.51	2 – 64	8	64	13.6
Chloramphenicol	32	2 – 64	8	64	9.06	2 – 64	8	64	9.54
Ciprofloxacin	4	0.016 – 0.5	0.016	0.016	0.02	0.016 – 0.5	0.016	0.016	0.02
Gentamicin	16	0.25 – 32	0.5	1	0.54	0.25 – 32	0.5	1	0.58
Kanamycin	64	8 – 128	8	128	11.08	8 – 128	8	128	13.25
Nalidixic acid	32	0.5 – 64	2	4	2.43	1 – 64	2	4	2.43
Streptomycin	64	32 – 128	32	128	47.53	32 – 128	32	128	48.71
Sulfisoxazole	512	16 – 1024	32	1024	124.73	16 – 1024	1024	1024	164.05
Tetracycline	16	4 – 64	32	64	19.68	4 – 64	32	64	21
TMS	4	0.125 – 8	0.125	8	0.23	0.125 – 32	0.125	8	0.29

G. mean = geometric mean

for both sampling strategies for all antimicrobials except ceftriaxone, nalidixic acid and TMS. For these three antimicrobials, the difference was due to a single isolate showing the discrepant MIC limit. The geometric mean, MIC_{50} and MIC_{90} were similar for the cohort and random groups for each antimicrobial.

Attrition in the Cohort Group

During the course of the study, all 51 calves initially enrolled as non-treated animals in the cohort group were lost to follow-up due to transfer to the heifer ranch (n = 50) or death (n = 1). Furthermore, 19/51 (37.3%) calves were treated with an antimicrobial and therefore could no longer be considered control calves. The proportion of calves that had been treated between sampling events varied during the study (Figure 4.4). The median time between enrollment and loss to follow-up or treatment was 176 days (Figure 4.5), which represented a heavy attrition for the cohort group. Therefore, more calves had to be enrolled during the cohort study. On sampling event 4, an additional 36 control and 23 treated calves were enrolled. Of the 36 new control calves, 3 (8.3%) were treated with an antimicrobial during the course of the study.

Cows

In total, 821 fecal samples were collected from cows: 300 from randomly selected cows and 521 samples from cohort cows. Cohort samples were composed of a similar number of treated (n = 246) and non-treated cows (n = 275). The total number of samples per cow

Figure 4.4 Proportion of cohort (light bars) and randomly selected (dark bars) **cows** that were treated with an antimicrobial between sampling events (8 weeks) in a study conducted on a commercial dairy farm in Northern Colorado.

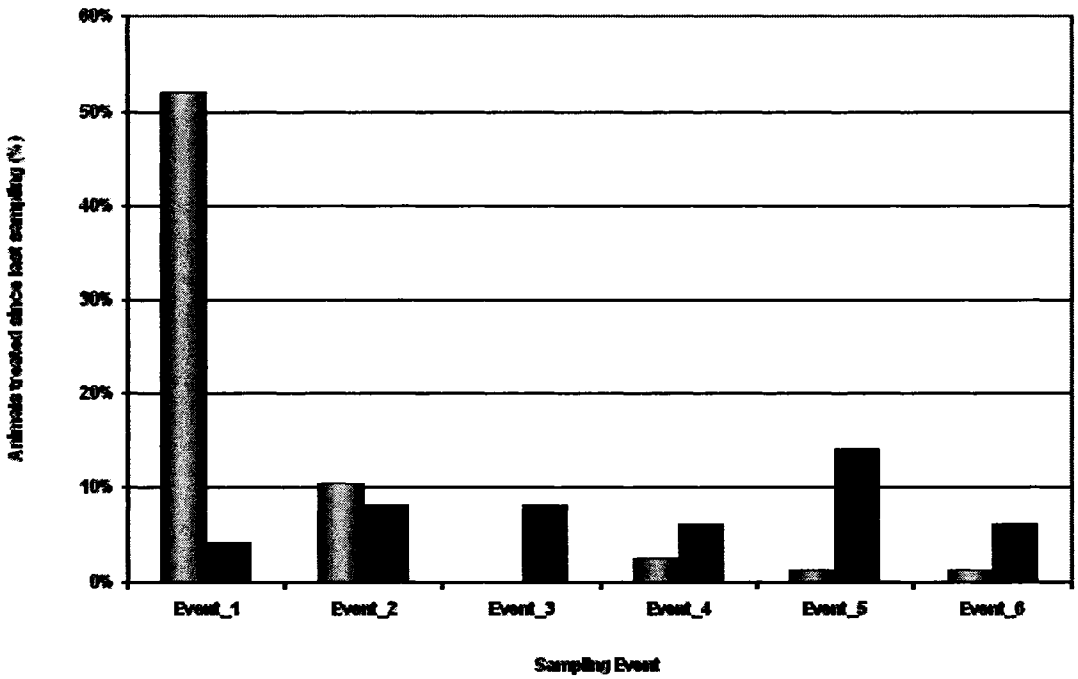
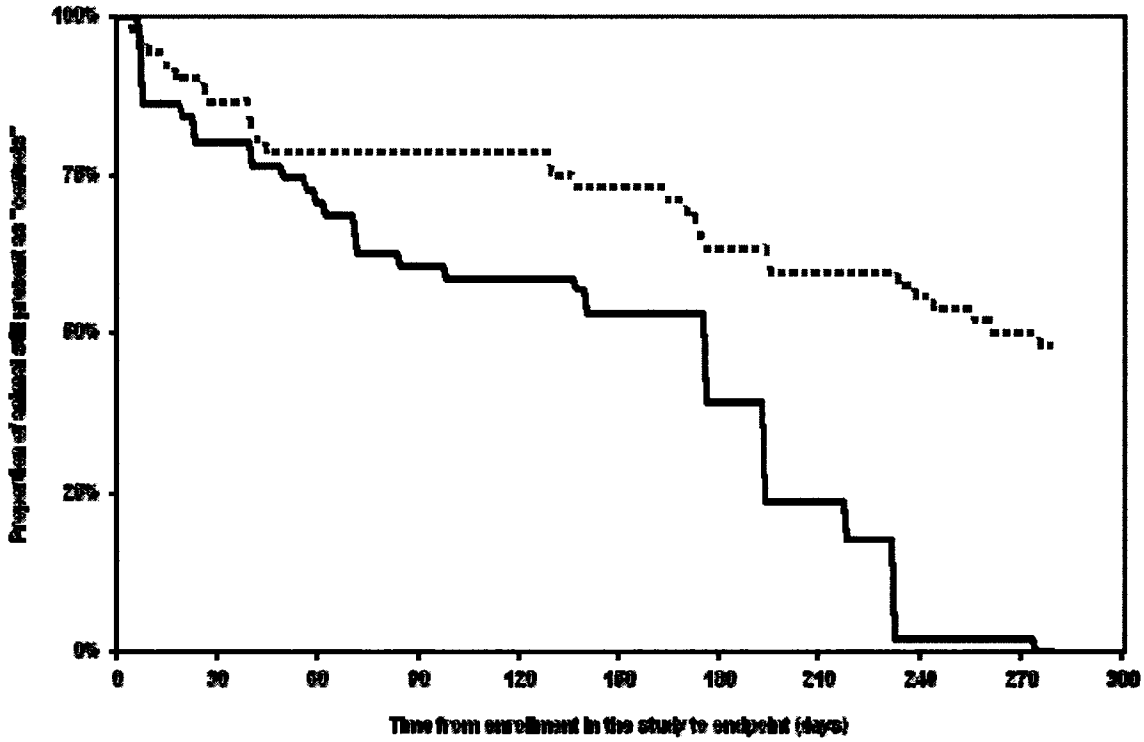


Figure 4.5 Survival of **calves** (solid line) and **cows** (dashed line) enrolled as non-treated cohorts ("controls") during a study of antimicrobial-use and antimicrobial resistance at a commercial dairy in Northern Colorado. Endpoints were defined as treatment with antimicrobials (any route of administration), culling, death or transfer to the heifer ranch.



in the cohort group (repeated samples) ranged from one to six, with a median of six samples per cow (Figure 4.3).

Salmonella from Cows

Overall, *Salmonella* was isolated from 4.6% (38/821) of cow fecal samples, which was significantly higher ($P = 0.001$) than the frequency of isolation in calves (1.9%, 16/844, see Table 4.5). Random samples had a more frequent isolation of *Salmonella* (7.7%, 23/300) than samples from the cohort group (2.9%, 15/521, $P = 0.001$). Most cows from which *Salmonella* was recovered had only one positive sample during the entire study period. Only two control cows (non-treated cohorts) had *Salmonella* isolated twice from their feces: one cow on sampling events 4 and 6 (16 weeks apart) and another cow on sampling events 4 and 5 (8 weeks apart). Both had different serotypes recovered at each sampling event. These *Salmonella* isolates were either susceptible to all tested antimicrobials or resistant only to sulfisoxazole.

Almost half (18/38) of all *Salmonella* isolates cultured from cows were *S.* Infantis (Table 4.6), and all of them were susceptible to all antimicrobials tested. The next most frequent serotype was *S.* Cubana (14/38), which was resistant to only sulfisoxazole ($n = 13$) or susceptible to all tested antimicrobials ($n = 1$). The remaining *Salmonella* isolates were serotypes Anatum ($n = 3$) all resistant only to sulfisoxazole and MDR isolates of serotypes Newport ($n = 2$) and Typhimurium ($n = 1$). All three MDR isolates showed the same resistance pattern; ACSSuT-AxCefxCeftCeph (Figure 4.2, pattern D). Therefore,

among the 19 *Salmonella* isolates cultured from cows, only three resistance patterns were observed.

Salmonella was isolated from each milking pen at least once (including fresh pen and hospital) but never in dry cow pens. Resistant *Salmonella* isolates were found mostly in samples from cows in the mid to high producing pens, plus in one fresh cow and two cows housed in the hospital. The three MDR *Salmonella* isolates were cultured from three cows located in three different pens and sampled during two different sampling events (sampling event 2 and 5). No differences were observed between results from the random and the cohort groups at most sampling events (Table 4.3 and Table 4.4). However, there was a difference during sampling event 5 (March), when more *Salmonella* isolates were recovered from the random group (11/50) than from the cohort group (5/83, $P = 0.007$). This variation was enough to account for the difference observed between the random and the cohort groups when evaluating all sampling events together (23/300 or 7.7% in random samples vs. 15/521 or 2.9% in cohort samples, $P = 0.001$). In contrast with the results observed in calves, *Salmonella* was isolated from cows more frequently during the second half of the study (March through July) than during the first half (October through January).

E. coli from Cows

E. coli was isolated from 84.7% (695/821) of the cow fecal samples, which was significantly less ($P < 0.001$) than isolation from calf samples 91.0% (768/844). In contrast with results found for *Salmonella*, there was a lower ($P = 0.045$) frequency of

isolation of *E. coli* in the random group (81.7%, 245/300) than in the cohort group (86.6%, 421/521), although no difference was observed in prevalence of resistant (P = 0.156) or MDR isolates (P = 0.286, Table 4.7). Frequency of MDR among *E. coli* isolates cultured from cow feces (6.0%, 42/696) was similar to that of *Salmonella* isolates 10.5%, 4/38, P = 0.221).

There were 27 different resistance patterns found among the 696 *E. coli* isolates obtained from cows; 19 of the 27 patterns were observed in only one (12 patterns) or two isolates (7 patterns) (Figure 4.2). Only one of the patterns observed in *E. coli* was also observed in *Salmonella* isolates: single resistance to sulfisoxazole (pattern Z in Figure 4.2). Of the 695 *E. coli* isolates cultured from cows, none were resistant to amikacin, ceftriaxone or ciprofloxacin, and less than 10 isolates were resistant to each of the following antimicrobials: amoxicillin-clavulanic acid, cefoxitin, ceftiofur, chloramphenicol, gentamicin, kanamycin nalidixic acid and TMS. Table 4.14 shows the descriptive parameters for MICs of *E. coli* isolates obtained from cohort and random cows. As observed in calf samples, MIC ranges for the samples from the cohort group and for randomly selected cows were similar for all antimicrobials. Any differences were due to one or two isolates showing the discrepant MIC limit. The geometric mean, MIC_{50} and MIC_{90} were similar for cohort and random groups.

Table 4.14 Summary of descriptive MIC parameters for *E. coli* isolates obtained from **cows** enrolled in a cohort group or from randomly selected calves in a study conducted on a commercial dairy farm in Northern Colorado.

	Resistance breakpoint ≥	Random (n = 245)				Cohort (n = 451)			
		MIC Range	MIC_{50}	MIC_{90}	G. mean	MIC Range	MIC_{50}	MIC_{90}	G. mean
Amikacin	64	0.5 – 8	1	2	1.32	0.5 – 8	1	2	1.3
Amox / Clavulanic	32	1 – 64	4	4	3.55	1 – 64	4	4	3.3
Ampicillin	32	1 – 64	4	4	3.26	1 – 64	4	4	3.17
Cefoxitin	32	0.5 – 32	4	8	3.67	0.5 – 32	4	8	3.69
Ceftiofur	8	0.125 – 16	0.25	0.5	0.27	0.125 – 16	0.25	0.5	0.27
Ceftriaxone	64	0.25 – 16	0.25	0.25	0.27	0.25 – 32	0.25	0.25	0.26
Cephalothin	32	2 – 64	16	16	11.43	4 – 64	8	16	10.66
Chloramphenicol	32	2 – 64	8	8	6.03	2 – 64	8	8	5.98
Ciprofloxacin	4	0.016 – 0.25	0.016	0.016	0.02	0.016 – 0.25	0.016	0.016	0.02
Gentamicin	16	0.25 – 32	0.5	0.5	0.49	0.25 – 16	0.5	0.5	0.49
Kanamycin	64	8 – 128	8	8	8.16	8 – 128	8	8	8.21
Nalidixic acid	32	1 – 64	2	4	2.6	0.5 – 64	2	4	2.79
Streptomycin	64	32 – 128	32	32	34.06	32 – 128	32	32	33.77
Sulfisoxazole	512	16 – 1024	16	1024	25.59	16 – 1024	16	1024	30.75
Tetracycline	16	4 – 64	4	64	6.49	Apr-64	4	64	6.54
TMS	4	0.125 – 8	0.125	0.125	0.13	0.125 - 8	0.125	0.125	0.13

G. mean = geometric mean

Resistant and MDR *E. coli* isolates were found at some point in all locations where cows were housed, including the dry pens. Prevalence of resistance and MDR varied among pens (data not shown). Frequency of isolation, resistance and MDR of *E. coli* recovered by cohort or random sampling did not vary across sampling events (Table 4.3 and Table 4.4), except during sampling event 1, when significantly ($P = 0.031$) more resistant *E. coli* isolates were isolated in the cohort group (97/100 or 97%) than in the random group (44/50 or 88.0%).

Attrition in the Cohort Group

In total 22% (22/100) cohort cows were lost during the study due to death ($n = 3$) or culling ($n = 19$); 10 control cows and 12 treated cows. Among control cows, 51.9% (27/52) were eventually treated with antimicrobials during the study, by either parenteral ($n = 6$), intramammary ($n = 8$) or topical administration (treatment of foot abscess, $n = 13$). Median time that control cows remained untreated throughout the duration of the study was 262 days (range 1 – 280, Figure 4.5). Overall, 38/100 (38%) of cohort cows were lost. The percentage of cows treated between sampling events varied during the study (Figure 4.4).

DISCUSSION

Data from this study allowed comparison of results from various groups: calves vs. cows, and random vs. cohort sampling, and for different bacteria: *Salmonella* vs. *E. coli.*

Additionally, results could be compared among six sampling events and among various locations.

Calves vs. Cows

In general, isolates obtained from calf samples had a higher prevalence of resistance and MDR than cow isolates (both *Salmonella* and *E. coli*). Other studies have also found this difference in resistance between isolates from calves and cows.[31,32] Both, the prevalence of resistance and MDR in calves decreased as age increased, consistent with findings of other studies.[19,22,23,33] Calves were fed waste milk that contained antimicrobial residues. Oral exposure to antimicrobials may represent a risk for the selection of resistant enteric bacteria, especially if it is long-term exposure; calves in this study were fed antimicrobial-containing milk for approximately 45 days. Several studies, however, have found no difference in resistance between enteric bacteria isolated from calves that were fed milk with or without antimicrobials.[19,23,31] The fact that resistance prevalence decreased with calf age also discredits the theory of exposure to antimicrobials through milk as a source of resistance. Alternatively, it has been suggested that resistance may depend on the amount of antimicrobial ingested with the milk.[18] Levels of antimicrobials in waste milk were not measured during this study, and therefore this theory could not be confirmed. However, waste milk was mostly from cows treated with cephalosporins (77%) or penicillins (13%), while the highest prevalence of resistance among *E. coli* isolates cultured from calves were sulfisoxazole (53.5%) and tetracycline (62.5%, Table 4.8). Although waste milk was pasteurized on this farm, pasteurization has not proven effective in reducing the amount of antimicrobials in waste milk.[19,34,35]

Another possible explanation for the higher resistance prevalence among calf isolates as compared to cow isolates is that waste milk fed to calves may have carried resistant bacteria. Because the use of antimicrobials kills or inhibits susceptible bacteria,[1,3] it can be hypothesized that, if high levels of antimicrobials exist in waste milk, most bacteria contained in waste milk are resistant. Therefore, feeding calves with waste milk containing resistant bacteria could be equivalent to direct oral inoculation of resistant bacteria. Waste milk however was pasteurized. A marked reduction in counts of viable bacteria in waste milk by pasteurization[36] would probably invalidate the theory of direct inoculation with viable resistant bacteria as a cause of high resistance frequencies in calves. However, it is possible that resistance genes could be released during inactivation of resistant bacteria and can then be acquired by susceptible bacteria via transformation.

A further explanation for the differences in prevalence of isolation and resistance among enteric bacteria from calves and cows may be the existence of distinct environmental and microbiological niches in calves and cows. During the first few weeks of age, calves are monogastric animals that consume only milk.[37] The rumen does not develop until calves start to consume solid feed at a few weeks of age.[37] Milk escapes ruminal fermentation, while solid feed does not. Therefore, as calves get older, the natural rumen flora may impose a more hostile environment for enteric bacteria to thrive in the rumen and spread through the digestive tract, possibly explaining the decrease in prevalence of resistance as calf age increased.

Different diets may exert varying pressure on bacterial proliferation. It has been shown that higher percentage of grain in the diet, which results in lower pH in the digestive tract,

is associated with higher counts of *E. coli*.[38] This theory is consistent with a decrease in resistance prevalence as calf age increased, because after weaning the percentage of grain in the diet is decreased over time.[37]

Apart from the different diets used in calves and cows, the design of the facilities may also help in developing these niches for resistant bacteria. Adult lactating cows were housed in pens with concrete flooring and freestalls in all milking pens except the natural service pen. Concrete floors help in daily cleaning, which at this dairy was done with a flushing system using recycled water. The flushing system may have helped in the distribution of the different strains of bacteria, as flush water was common for all freestall pens. Calves that were housed in hutches were kept on concrete bedded with straw that was cleaned only after the calf moved out. Older calves were kept on drylot-type housing that was cleaned once or twice per year. Therefore, calves of all ages were exposed to unclean environments that could have helped resistant bacteria to survive by providing ample nutrients and reducing the competition with commensal bacteria.

Finally, it is possible that the biosecurity control measures implemented on the dairy were effective in avoiding cross-contamination between cow and calf areas. The calf area on this dairy was distinctly separated from the cow areas. Dedicated personnel were assigned to the calf area, and access was restricted to exclude personnel who worked with adult cows. Heavy machinery used to clean the cow areas was also used in the calf area, but only after thorough cleaning.

Salmonella vs. *E. coli*

Frequency of *Salmonella* isolation in this study was consistent with that obtained in other studies performed in dairy cattle in the absence of clinical salmonellosis,[39-42] as well as a report from the National Animal Health Monitoring System (NAHMS).[43] Significantly fewer *Salmonella* isolates were recovered compared to *E. coli* isolates, both in calves (1.9% vs. 91.0%) and in cows (4.6% vs. 84.8%). However, more *Salmonella* than *E. coli* isolates were resistant to at least one antimicrobial (100.0% vs. 67.5% in calves, and 50.0% vs. 25.3% in cows). The differences in isolation and resistance frequencies observed among *Salmonella* and *E. coli* isolates from calves and cows were suggestive of four independent bacterial populations within the same dairy (see Table 4.5 and Table 4.7). Possible explanations for this may be the effectiveness of biosecurity control measures and that transmission of resistance genes may not happen as easily and rapidly *in vivo* as it has been suggested based on *in vitro* studies.[1,3,7,13,44] Both theories may be reinforced by the absence of an evident similarity between *Salmonella* serotypes isolated from calves and cows (Table 4.6).

Diverse resistance patterns were observed among *Salmonella* and *E. coli* isolates obtained from calves (Figure 4.1) and cows (Figure 4.2). The presence of various combinations of resistance against the different cephalosporins that were tested (Appendix A) suggests the existence of extended spectrum beta-lactamases (ESBLs) in these isolates.[45,46] Of the 81 different resistant patterns, 17 showed the ACSSuT phenotype; four in *Salmonella* and 14 in *E. coli* isolates (one in each). This phenotype, which has been associated with *S.* Typhimurium definitive type 104,[47,48] is important due to its supposed clonal spread.[49]

Because no molecular or genotypic analyses were performed, it can only be speculated on the possible genetic relationship of the different isolates. Finding so many different resistance patterns suggests active genetic modification exists (mutations or acquisition of new genes from other bacteria). The difference in the number of resistance patterns observed for *Salmonella* ($n = 7$) and *E. coli* ($n = 77$) could be due to the limited number of *Salmonella* isolates (54 total, 37 resistant) that were recovered compared to *E. coli* (1,464 total, 694 resistant).

Cohort vs. Random Sampling

Random samples yielded similar results in frequencies of bacterial isolation, resistance, and MDR compared to samples from the cohort group (see Table 4.5 and Table 4.7). Comparison of MICs between random and cohort groups in calves and cows also yielded similar results (see Table 4.13 and Table 4.14). Using the geometric mean to compare MICs highlighted possible differences, while comparing MIC range, MIC_{50} and MIC_{90} showed almost no difference at all. The geometric mean was influenced by the total number of samples, which may explain why isolates from the cohort group seem to have slightly larger geometric means; there were almost twice as many cohort samples as random samples.

Cows that needed antimicrobial treatment were all housed in the hospital pen. The stratified random sampling strategy ensured that all pens were represented in the random groups. Animals within the cohort group were enrolled according to treatment status at the beginning of the study. Due to the design of the study, calves were enrolled within the first month of age while cows were enrolled mostly within the first 100 DIM. The

probability for animals in the cohort group of being treated with an antimicrobial decreased during the study. Thus, treated animals were more consistently represented in the random group (see Figure 4.4) than in the cohort group.

The number of samples for the random groups (cows and calves) was easily maintained at each sampling event during the study ($n = 50$). In contrast, in the cohort groups, many of the animals enrolled as "controls" were lost during the study due to treatment, culling, or transfer to the heifer ranch (100% in calves and 38% in cows). In dairies that raise the calves on the premises, attrition should be much lower. If there are any "blanket treatments" (systematic treatments for all animals in a group), attrition would also be 100%. Examples of such blanket treatments are dry-off intramammary applications[50,51] and parenteral tetracycline administration at dry-off for control of *Leptospira* infection.[52] When selecting a cohort sampling scheme to evaluate any long-term effects of antimicrobial-use on resistance, attrition needs to be accounted for in determining the sample size.

Collection of samples from cohort animals represented a challenge on several occasions, because some animals were not readily found in their pens. Searching for these animals drastically increased time spent on the farm and interrupted some of the normal farm activities. Once found, the animal had to be restrained and feces had to be collected manually from the rectum. In contrast, random sampling was performed in an efficient manner. The only requisite to obtain a random sample was that the animal needed to be correctly identified. Cattle promptly defecate when they are moved. Therefore, the simple act of walking through the pen encouraged many cows to defecate and produce a sample.

Accurately identifying the cow that produced the sample was the only challenge to collecting samples from cows in the random group. Although this method was used with many of the cohort animals too, it was not always possible to see the cows enrolled in the study defecate.

Sampling event

Isolation of *Salmonella* (Table 4.1 and Table 4.2) from calf samples was more frequent during the first half of the study (September through January), while in cows (Table 4.3 and Table 4.4) it was more frequent during the second half of the study (March through July). These differences are difficult to explain by climate alone, because of the wide range in temperatures between these two periods in Colorado.

Results for *E. coli* isolation were similar in calves and cows, with higher frequencies of isolation during the first half of the study than during the second half. Prevalence of resistance among *E. coli* isolated from cows was highest during warmer months (sampling events 1, 5 and 6) than during colder months (sampling events 2, 3 and 4). Prevalence of resistance among calf *E. coli* isolates was similar on all sampling events. Prevalence of MDR in *E. coli* isolated from cohort calves decreased almost in a linear fashion from sampling event 1 through 6 (Table 4.2). A steady decrease of resistance among isolates from the cohort group could be explained by the initial exposure of the treated group to parenteral antimicrobials at enrollment, and the reduction of this kind of exposure over time. However, as mentioned above, all calves were fed waste milk that contained antimicrobials and therefore exposed orally. It is possible that parenteral and oral exposure to antimicrobials affect enteric bacteria in different ways. Although oral

exposure could be expected to have a greater effect than parenteral exposure, the results of this study do not support this idea. If parenteral antimicrobial use is a factor for resistance, an explanation for the similar resistance prevalence throughout the different sampling events may be that treated calves were consistently represented throughout the study. Another possible explanation could be a long-term die-off of resistant bacteria due to competition with commensal flora and a possible fitness cost in the absence of the selecting agent.[53,54] Presence of MDR among *E. coli* isolated from cows was rare. Overall, these results suggest that sampling at different times of the year may have an impact on isolation and prevalence of resistance.

Sampling Location

Using a stratified random sampling strategy ensured that all pens were represented in the random groups. Cohort sampling however failed to always represent all pens; some pens were over-represented while others may have not been represented at all. This situation was especially important when sampling calves. Given that on this dairy all calves were transferred to a heifer ranch at 10 months of age, it was advantageous to enroll cohort calves within the first month of age to ensure collection of repeated samples. Although this issue was most important in calf sampling, it also had an effect on cow sampling. The most susceptible time for disease during the lactation in dairy cows is during the peri-parturient period.[24,55-57] Therefore, when enrolling dairy cows that have been treated with an antimicrobial, it should be expected that most would be fresh cows. In this study 37/48 (77.1%) enrolled treated cows were in their first 100 days of lactation (range 2-560 DIM, median 63 DIM). This problem of over-representation of some subgroups of animals is

further perpetuated because cows are usually grouped according to production, and production is directly correlated with stage of lactation (DIM).[58] As the study progressed, most of the cows tended to be housed in the low-production pens.

CONCLUSIONS

- Random sampling may be a better strategy to evaluate potential consequences of antimicrobial-use on resistance among enteric bacteria from dairy cattle than cohort sampling, especially for long-term studies. There are several reasons:
    - Random sampling yielded similar results of frequencies of isolation, resistance and MDR to cohort sampling.
    - Random sampling was more efficient than cohort sampling. Less time was needed to collect samples from random animals, and farm routines were not altered to accommodate sampling.
    - Random sampling had no problems of attrition, and showed a similar percentage of treated animals on the different sampling events.
    - The cohort groups had marked attrition, and under-represented treated animals as the study progressed.
- Random sampling is not appropriate when monitoring of resistance in individual animals is necessary.
- Resistance patterns and serotypes differed between *Salmonella* isolates from cows and calves. *E. coli* resistance patterns also differed between isolates from calves and cows, suggesting four independent bacterial populations.

- *Salmonella* isolation was more prevalent among cows than among calves.
- *Salmonella* and *E. coli* isolated from calves had a higher prevalence of resistance and MDR that those isolated from cows.
- Resistance among calf isolates decreased as calf age increased.
- Overall, *E. coli* was isolated from a higher proportion of samples than *Salmonella*, but *Salmonella* isolates had a higher prevalence of resistance.
- Isolation, resistance and MDR frequencies were not uniform throughout the year. Therefore short-term studies may result in faulty conclusions.

Appendix A

Correspondence of resistance patterns to codes used in Figure 4.1 and Figure 4.2.

Pattern code	Pattern
A	ACSSuT
B	ACSSuT - AxCeft
C	ACSSuT - AxCeftCeph
D	ACSSuT - AxCefxCeftCeph
E	ACSSuT - AxCefxCeph
F	ACSSuT - AxCeph
G	ACSSuT - CeftCeph
H	ACSSuT - TMS - AxCefxCeftCeph
I	ACSSuT - TMS - AxCefxCeftCeph
J	ACSSuT - TMS - AxCefxCeftCeph
K	ACSSuT - TMS - AxCefxCeftCeph - NalA
L	ACSSuT - TMS - Gen - AxCefxCeftCeph
M	ACSSuT - TMS - Gen - AxCefxCeftCeph - NalA
N	ASSuT
O	AST
P	SuT - Ceph
Q	ASuT
R	SuT
S	T
T	Ceph
U	CSSuT
V	KCSuT
W	SSuT
X	KSSuT
Y	ST
Z	Su

A = ampicillin, C = chloramphenicol, S = streptomycin, Su = sulfisoxazole, T = tetracycline, Ax = amoxicillin-clavulanic acid, Cefx = cefoxitin, Ceft = ceftiofur, Ceph = cephalothin, Gen = gentamicin, NalA = nalidixic acid, K = kanamycin and TMS = trimethoprim-sulfamethoxazole.

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Chapter 5

EVALUATION OF FACTORS ASSOCIATED WITH RESISTANCE IN *SALMONELLA* AND *E. COLI* ISOLATED DURING A LONGITUDINAL STUDY IN DAIRY CATTLE

ABSTRACT

Antimicrobial-use in food-animals has been suggested as a major source of resistance among human pathogens. For this to happen, antimicrobial-use should have an effect on resistance in bacteria isolated from treated animals, however this has not been well established. In dairy cattle, various factors other than antimicrobial-use have previously been shown to be associated with resistance among enteric bacteria isolated from those cattle. The objective of this study was to compare two analytical methods to evaluate factors associated with isolation, resistance, and multi-drug resistance among *Salmonella* and *E. coli* isolates: hierarchical multivariate analysis versus crude analysis. The study was conducted on a commercial farm in Northern Colorado. In total 2,065 samples were collected from calves and cows; 1,665 from feces and 400 from feed, drinking water and flush water. *Salmonella* and *E. coli* isolated from calves and cows showed four different resistance signatures. In general, *Salmonella* isolated from feed/drinking water samples

had similar frequencies of isolation, resistance, and MDR compared to fecal samples. *E. coli* isolates were more frequently isolated from feces than from feed/water samples. Flush water samples had more frequent *Salmonella* isolation than fecal samples, but the frequency of *E. coli* isolation was similar. Results of hierarchical multivariate analyses showed that antimicrobial treatment was a risk factor for recovery of resistant or MDR *E. coli* from calves or cows, but not for *Salmonella* due to the low number of isolates that lead to low statistical power. Conclusions on the association between antimicrobial use in animals and resistance in enteric bacteria isolated from those animals based on results of crude analyses differed often from those based on hierarchical multivariate analysis. Factors found to be strongly associated with resistance both in *Salmonella* and *E. coli* were calf age, lactation number, production group, and sampling event. It is concluded that multiple interacting factors affect the probability of recovery of resistant enteric bacteria.

INTRODUCTION

Antimicrobial resistance, especially in enteric bacteria, has become a major issue in public health.[1-5] Resistant bacteria can cause infections for which there may be fewer treatment options, higher treatment costs, and potential health risks, including death.[6-9] Many references suggest indiscriminate use of antimicrobials in food-animals as feed-additives at sub-therapeutic doses for production enhancement.[5,10-14] Additionally, these references suggest that use in this manner is a major factor for the development and transmission of resistant bacteria to humans by foods of animal origin. The conclusions

from such references are often based on generalizations that do not take into account differences among food-animals. For example, not all antimicrobials approved for use in food-animals are approved in all species or production groups.[15] Dosages vary among different approved uses, and, furthermore, different combinations of antimicrobial and food-animal species (or production group) may have different consequences on resistance, if any.

If antimicrobial-use in food-animals is considered to be responsible for resistance in bacteria isolated from humans, by extension, it is responsible for resistance in bacteria isolated from treated animals. Some studies have tried to assess the effect of antimicrobial-use in treated cattle with non-uniform results for different antimicrobials even within the same studies.[16-24] A positive association between antimicrobial-use in food-animals (pigs and poultry) and resistance among enteric bacteria isolated from those animals was reported in epidemiologic observational studies.[16,17,22] These studies performed group sampling and reported whether or not antimicrobials were used on that group, not in individual animals. Several other studies failed to show an association between antimicrobial exposure in animals and resistance in bacteria isolated from those animals.[17,20-22,24-26] Other studies, have shown a transient increase in resistance to the specific antimicrobial used to treat the animal, but no change in resistance to other antimicrobials.[20,22-24] Prevalence of resistance among isolates form the target bacterial species returned to pre-treatment levels after discontinuing the treatment.[22,23]

Given no consistent association between antimicrobial-use in cattle and resistance in bacterial isolates, other factors should be studied. Some recent studies suggest factors

other than antimicrobial-use that may be associated with resistance found in bacterial isolates.[27,28] For example, some studies have found a significantly higher prevalence of resistance among enteric bacteria isolated from calves compared to cows.[28,29] In fact, a gradual decrease of prevalence of resistance in enteric bacteria (mainly *E. coli*) has been reported as calves increased in age.[21,25,27,30] No information could be found on whether the decrease in prevalence in these studies was in the absence of or in spite of therapeutic use of antimicrobials. Calves raised on calf ranches had higher resistance prevalence than calves raised at the dairy. [27] Prevalence of resistance also varies among farms.[27,31] A higher prevalence of resistance in enteric bacteria was present in larger herds,[32] housed cattle (compared to grazing),[26] and when sampling during warmer months of the year.[32]

Studies of the complex ecology of antimicrobial resistance among enteric bacteria in cattle have included the evaluation of the environment where cattle live. Sources of environmental samples in studies conducted on dairy farms have included drinking water, feed, feed-refusals, bulk-tank milk, milk filter, manure lagoon, bird droppings, bedding, equipment, and pen-floor samples.[32-34] Most of these sources represent a potential for oral exposure to resistant bacteria.

Most studies have collected samples on multiple dairy farms. The inherent differences that exist between personnel, equipment, and management on dairy farms can complicate the study of factors that affect antimicrobial resistance in enteric bacteria. Studying these factors at a single dairy farm can avoid confounding factors due to the underlying and often unmeasured differences between farms. The objective of this study was to evaluate

two methods of statistical analysis to study a possible association between antimicrobial-use in dairy cattle and resistance in *Salmonella* and *E. coli* isolated from those cattle.

MATERIALS AND METHODS

Sample population

This study was conducted at a commercial dairy in northern Colorado milking approximately 1,200 cows and that kept records on antimicrobial-use. Adult cows were grouped in 13 pens according to stage of lactation (quantified as "days in milk" or DIM), milk production (high, medium, low, or dry) and reproduction (insemination or natural-service). Cows needing any kind of treatment (with or without antimicrobials) were housed in a separate hospital pen. All pens that housed milking cows, except the natural-service pen, had typical free-stall housing and a flush system for cleaning alleyways. The natural-service pen was set up as dry-lot housing.

Calves were housed in individual hutches and fed pasteurized non-salable milk (waste milk) obtained from cows that were housed in the maternity and the hospital pens. Therefore, waste milk contained antimicrobial residues from dry-cow treatments (long-acting intramammary antimicrobials) and current treatments administered in the hospital (intramammary and parenteral). Calves were housed in individual hutches while they were being fed waste milk. Approximately 2-3 weeks after weaning, calves were grouped in "super-hutches" (6-8 calves per group) for a period of about 1 month. After socially

acclimating to this small group of animals, calves were grouped in dry-lot pens of increasing size and animal numbers as age increased (6 to 50 calves per pen). At about 10 months of age, all calves were moved to a custom heifer rearing ranch.

Fecal samples

Sampling strategies for fecal samples were described in Chapter 4. All fecal samples collected for that study were used in this study (random and cohort samples).

Environmental samples

Environmental samples were collected from possible sources of oral exposure to enteric bacteria. On each sampling event, a total of ten samples was collected from each of the following sources: feed, drinking water, and flush water in cow areas; colostrum, hospital milk, drinking water, and feed in calf areas. One feed sample and one drinking water sample were collected from each of the ten lactating-cow pens. Samples collected from the calf area included one feed sample and one drinking water sample for each of the pens holding older calves and two of the super-hutches. Five individual hutches were randomly selected, from which one grain sample and one drinking water sample were collected. Additionally, ten waste milk samples were collected at feeding time, before the calf had access to the milk. Colostrum samples were collected from individual fresh cows after the first milking.

Feed samples were collected from 4-6 different parts of the feed alley and mixed in a Ziploc® bag.[1] Drinking water samples were collected in sterile 50 ml conical tubes[2] by first stirring the water in the water trough to allow for collection of sediments.

Bacterial isolation and susceptibility testing

Fecal samples did not require pre-culture processing and were cultured directly as described below. All solid feed samples were processed prior to culturing by thoroughly mixing the Ziploc® bag and delivering 2 g into a WhirlPak® bag and adding 1x buffered peptone to cover the solids. The WhirlPak® bag was incubated for 24 h at 37° C, after which the contents were cultured as described below. Milk and colostrum samples were processed by adding 25 ml of 1x buffered peptone to 5 ml of the original sample and incubating for 24 h at 37° C. Drinking water and flush water samples were processed by adding 5 ml of 10x buffered peptone to 30 ml of the original sample and incubating for 24 h at 37° C.

Salmonella Culture and Isolation

Feces (1 g) were enriched by incubating in 10 ml of GN Hajna[3] for 18-24 h at 37° C, and tetrathionate broth[c] for 40-48 h at 37° C. After the initial enrichments, aliquots (100 µl) were transferred to 10 ml of Rappaport-Vassiliadis R10 broth[c] which were incubated for

[1] WhirlPak® bags, NASCO, Ft. Atkinson, WI
[2] Greiner Bio-One, Monroe, NC
[3] BD-Difco, Sparks, MD

18-24 h at 37° C. Ten (10) µl aliquots of Rappaport-Vassiliadis R10 broth were then streaked onto xylose-lysine-tergitol-4[4] and BG Sulfa[c] agar for isolation of *Salmonella.* Plates were incubated for 18-24 h at 37° C. Isolated colonies characteristic of *Salmonella* were inoculated into triple sugar iron and lysine iron agar slants for biochemical confirmation (black). A single colony was selected from each sample for further characterization. Presumptive *Salmonella* isolates were serogrouped using serogroup-specific antisera[c] and were sent to the National Veterinary Services Laboratories (Ames, IA) for serotyping.

E. coli Culture and Isolation

One hundred (100) µl aliquots of fecal dilutions (1:9 weight/volume, in PBS) were streaked for isolation onto MacConkey agar[c] plates. The plates were incubated for 24 h at 37° C. Isolated colonies characteristic of *E. coli* were inoculated into tryptic soy agar (TSA) slants and incubated with a loose cap for 18-24 h at 37° C. A presumptive identification of *E. coli* was assigned when colonies were 1 to 2 mm in diameter, pink, uniform in color, flat with smooth margins, and had a positive indole reaction (pink) when tested.

Susceptibility Testing

One colony per sample was selected for susceptibility testing. Susceptibility to 16 antimicrobials was tested according to guidelines from the Clinical Laboratories

[4] Hardy Diagnostics, Santa Maria, CA

Standards Institute (CLSI, formerly know as the National Committee for Clinical Laboratory Standards [NCCLS]).[35] Susceptibility was tested by broth-microdilution using a semi-automated system[5] as per manufacturer directions. The minimum inhibitory concentrations (MICs) were determined for antimicrobials that are commonly used in human and veterinary medicine. MICs were evaluated by use of a 96-well custom made panel. Susceptibility results were interpreted using the following breakpoints for resistance:

amikacin (64 μg/ml)	amoxicillin/clavulanic acid (32 μg/ml)
ampicillin (32 μg/ml)	cefoxitin (32 μg/ml)
ceftiofur (8 μg/ml)	ceftriaxone (64 μg/ml)
cephalothin (32 μg/ml)	chloramphenicol (32 μg/ml)
ciprofloxacin (4 μg/ml)	gentamicin (16 μg/ml)
kanamycin (64 μg/ml)	nalidixic acid (32 μg/ml)
streptomycin (64 μg/ml)	sulfisoxazole (512 μg/ml)
tetracycline (16 μg/ml)	trimethoprim/sulfamethoxazole (4 μg/ml)

Quality control strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* 29212) were included in susceptibility testing on a weekly basis.

[5] Sensititre, Trek Diagnostics, Westlake, OH

Data analysis

MICs

Results of susceptibility testing were reported as MICs. Isolates with an MIC value out of the tested range were assumed to have the MIC value at or outside the tested range. For example, for tetracycline the lowest MIC tested was 4 μg/ml and therefore the lowest MICs reported were ≤ 4 μg/ml. The highest MIC tested for tetracycline was 32 μg/ml, and so isolates with higher MICs were reported as > 32 μg/ml. For calculation and graphic representation purposes, isolates with an MIC of > 32 μg/ml were assumed to have an MIC of 64 μg/ml (the next sequential dilution).

Descriptive parameters evaluated for MICs were:

- Range
- MIC_{50}, defined as the MIC that inhibited the growth of 50% of isolates (median).
- MIC_{90}, defined as the MIC that inhibited the growth of 90% of isolates.
- Geometric mean, to describe central tendency of MICs.

Actual MIC values were not used as outcomes for the multivariate analyses because no endpoints were available for MICs. Additionally, few antimicrobials showed a bimodal distribution of MICs (susceptible and resistant MIC modes), meaning that most isolates

that were resistant had the same MIC value. Therefore, susceptible isolates would be represented by multiple MIC values and resistant isolates only by one value.

Outcome variables

The outcome variables used in multivariate analyses were dichotomized for statistical analysis (yes or no), and were defined as follows:

- *Isolation*: growth of any colony (susceptible or resistant). Relative frequency of isolation for a group of samples was calculated as the number of samples with positive growth divided by total number of samples within the group.

- *Resistance:* MICs for at least one antimicrobial were above the breakpoint for resistance. Relative frequency of resistance for a group of samples was calculated as the number of samples with an isolate resistant to one or more antimicrobials divided by total number of isolates within the group.

- *Multidrug resistance (MDR):* resistance to three or more antimicrobials. Relative frequency of MDR for a group of samples was calculated as the number of samples with an isolate resistant to three or more antimicrobials divided by total number of isolates within the group. Samples from which no isolates were cultured were not included in this calculation.

Explanatory variables

Study factors or explanatory variables to be included in the multivariate analyses were recorded at each sampling event. The following explanatory variables were selected:

- age (calves),
- lactation number (cows),
- milk production (cows),
- location of the sampled animal on the dairy (pen),
- treatment with antimicrobials (yes/no),
- type of antimicrobial used,
- number of days from last antimicrobial treatment to collection of samples,
- and sampling event.

Records on antimicrobial treatment for all animals were maintained at this dairy in handwritten and computerized records. Location and age were correlated for calves, because age and housing in individual hutches or group pens is done according to age. Therefore, although both variables were tested, only the most significant of the two was included in the model.

Adult dairy cows were grouped according to milk production, treatment, and lactation number. Milk production in dairy cows is correlated with stage of lactation (quantified as "days in milk" or DIM). Therefore, location of cows during the study was correlated to

milk production, DIM, and lactation number. To allow for best representation of the different pens, the chosen explanatory variables were lactation number and milk production, which was categorized as high (2 pens), medium (4 pens), low (2 pens), fresh cows (2 pens) and hospital (1 pen).

Statistical analyses

Results in cows and calves were analyzed separately due to the inherent differences found in previous studies. Comparisons were performed between environmental and fecal samples for results on isolation, resistance and MDR of *Salmonella* and *E. coli*. Differences in proportions were analyzed using a standard *Z*–test.[36,37] Statistical significance was determined at a p-value of 5% or less ($P \leq 0.05$).

Univariate (crude) analysis of the association of antimicrobial treatment was performed for each outcome variable, and the results compared to those obtained from a multivariate analysis that accounts for other possible explanatory variables. Multivariate analysis was performed using hierarchical modeling (multilevel analysis), because several isolates were tested in individual animals (repeated samples at different sampling events).[37]

Results obtained from repeated samples collected from the same animal over time could not be considered to be independent, but clustered within animals. In the same way that cows are clustered within a pen or a farm, bacterial isolates are clustered within a cow. In other words, it is plausible that isolates collected from the same animal are more alike than isolates collected from different animals. Although bacteria grow in clones, many

different clones co-exist. Therefore, selecting one isolate from a cow on different sampling events may be equivalent to selecting one cow from the same pen. On each sampling event a different cow or clone can be randomly selected.

Multilevel analysis allows analysis of unbalanced data.[38] In other words, not all animals need to have the same number of samples to be included in the analysis; rather any animal with at least one sample can be included. Additionally, multilevel analysis allows modeling of residuals within a cluster,[38] for example cows housed in the same pen.

A two level multivariate analysis was performed. The aggregate level (level 2) was determined as the individual animals (cows and calves), while isolates were set as the lowest level unit (level 1). Location of the animals at the time of sampling was not used as a higher aggregation level but rather as a factor because all animals changed locations during the study. Therefore, animals could not be assigned to just one group. Analyses were performed using MLwiN (Version 2.02, Centre for Multilevel Modelling, University of Bristol, UK).

The predicted outcome variable was identified as y_{ij}; and the probability of a positive outcome was noted as π_i. Due to the binary nature of the data, outcomes were coded as either 1 (positive outcome of interest) or 0 (absence of positive outcome). For example, when evaluating resistance to any antimicrobial in *E. coli* isolates cultured from calves, the predicted outcome (y_{ij}) was assigned a value of 1 if the isolate obtained from the i^{th}

isolate cultured from the j^{th} calf was resistant to at least one antimicrobial, or 0 if it was susceptible to all antimicrobials.

$$\pi_i = P(y_{ij} = 1) \quad (1)$$

The *logit* link function was used to transform these predicted probabilities of a positive outcome to ensure constraint between 0 and 1 (binary outcome). The equation for the general model was:

$$\text{logit}(\pi_{ij}) = \log\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right) = \beta_{0j} + \beta_1 x_{ij} + \beta_2 x_{ij} \quad (2)$$

where:

- $\frac{\pi_{ij}}{1-\pi_{ij}}$ is interpreted as the odds that the binary outcome is equal to 1
- β_{0j} is the random intercept for the model and has 2 terms: $\beta_{0j} = \beta_0 + u_{0j}$
  - a fixed term represented by β_0
  - and the random effect u_{0j} that is animal-specific. It is assumed that u_{0j} follows a normal distribution: $N(0, \sigma^2_{u0})$.
- β_1 and β_2 are the estimates for the different explanatory variables (x_{ij}).

A random intercept model allows the intercept to randomly vary across level 2 units (animals). Quasi-likelihood estimation methods are used by MLwiN instead of maximum likelihood due to the binary nature data. Wald tests were used to determine statistical significance of the estimates (β's) at a level of significance of 5%. The odds ratios (OR) were calculated as e^{β}. Because most explanatory variables had several categories, a variable was kept in the model when at least one of the categories had a significant association ($P \leq 0.05$) with the studied outcome.

RESULTS

A total of 2,065 samples were collected: 1,665 fecal samples (844 in calves and 821 in cows) and 400 environmental samples (220 in calves and 180 in cows). Fecal samples are described in detail in Chapter 4, as well as the number of samples per animal included in the study. Presented below are the comparison of results from environmental and fecal samples, and the evaluation of factors associated with resistance.

Environmental vs. Fecal Samples

Results are presented separately for calves and cows. A summary of the frequencies of isolation, resistance and MDR is presented in Table 5.1 for calf areas and in Table 5.2 for cow areas. Overall, prevalence of resistance among fecal and environmental samples decreased in the following order:

Table 5.1 Frequency of isolation, resistance and multi-drug resistance (MDR) for ***Salmonella*** and ***E. coli*** isolates cultured from fecal and environmental samples (feed/water) collected in the **calf area** at a commercial dairy in Northern Colorado. Data are presented as percentage of random or cohort samples (column heading). Data in parenthesis are the actual number of isolates for each category.

	Salmonella		*E.coli*		
	Fecal	Feed / water	Fecal		Feed / water
	n = 844	n = 220	n = 844		n = 220
Isolation	1.9 (16)	0.9 (2)	91.0 (768)	*	55.0 (121)
Resistance	100.0 (16)	100.0 (2)	67.4 (518)	*	55.4 (67)
MDR	93.8 (15)	100.0 (2)	42.1 (323)	†	34.7 (42)

* Values between fecal samples and feed / water samples are statistically different ($P \leq 0.05$).
† $P = 0.077$

Table 5.2 Frequency of isolation, resistance and multi-drug resistance (MDR) for ***Salmonella*** and ***E. coli*** isolates cultured from fecal and environmental samples (feed/water and flush water) collected in the **cow areas** at a commercial dairy in Northern Colorado. Data are presented as percentage of random or cohort samples (column heading). Data in parenthesis are the actual number of isolates for each category.

	Cows					
	Salmonella			*E.coli*		
	Fecal	Feed / water	Flush water	Fecal	Feed / water	Flush water
	$n = 821$	$n = 120$	$n = 60$	$n = 821$	$n = 120$	$n = 60$
Isolation	4.6 [a]	7.5 [a]	81.7 [b]	84.8 [a]	34.2 [b]	78.3 [a]
	(38)	(9)	(49)	(696)	(41)	(47)
Resistance	50.0	44.4	54.0	25.3	22.0	23.4
	(19)	(4)	(27)	(176)	(9)	(11)
MDR	7.9 [a,b]	22.2 [a]	0.0 [b]	6.0	4.9	10.6
	(3)	(2)	(0)	(42)	(2)	(5)

[a,b] Values with a different superscript are significantly different ($P \leq 0.05$).

- The highest resistance prevalence was for *Salmonella* isolated from calves.
- The second highest resistance prevalence was in *E. coli* isolated from calves.
- The third highest resistance prevalence was in *Salmonella* isolated from cows.
- The lowest resistance prevalence was seen in *E. coli* isolated from cows.

Summary of MIC parameters are presented in Table 5.3 for calves and in Table 5.4 for cows. Discordant MIC limits are highlighted. Resistance patterns of *E. coli* isolates obtained from fecal samples were similar to those obtained in feed/water samples, although the frequencies for each pattern varied. Figure 5.1 shows the distribution of the most common patterns among *E. coli* isolates from calf feces and calf feed/water samples, while Figure 5.2 shows that for cows.

No statistical analyses were performed to evaluate possible differences between feed/water samples and fecal samples obtained from individual pens, because only 2 feed/water samples were available for each pen and each sampling event. Scarcity of data contributed to limited statistical power.

Calf areas

Only two *Salmonella* isolates were cultured from 220 environmental samples (0.9%) in the calf areas (Table 5.1); one from a feed sample, and one from a drinking water sample. Both were isolated from different pens on different sampling events, were different serotypes and showed different resistance patterns (data not shown). *Salmonella* isolation

Table 5.3 Summary of descriptive MIC parameters for *E. coli* isolates obtained from fecal and environmental samples from **calves** on a commercial dairy farm in Northern Colorado. All MIC_{50} and MIC_{90} values are alike, except for those highlighted. (G Mean stands for geometric mean).

	Fecals $n = 844$			Feed/water $n = 220$		
	MIC_{50}	MIC_{90}	G Mean	MIC_{50}	MIC_{90}	G Mean
Amikacin	1	2	1.20	1	2	1.29
Amox/clavulanic	4	32	5.20	4	32	5.42
Ampicillin	4	64	6.05	4	64	6.47
Cefoxitin	4	32	4.48	4	32	5.12
Cefttiofur	0.25	8	0.44	0.25	8	0.49
Ceftriaxone	0.25	8	0.45	0.25	8	0.50
Cephalothin	8	64	13.51	8	64	14.02
Chloramphenicol	8	64	9.42	8	64	10.90
Ciprofloxacin	0.02	0.015	0.02	0.02	0.015	0.02
Gentamicin	0.50	1	0.56	0.50	16	0.82
Kanamicin	8	128	12.36	8	128	12.65
Nalidixic Acid	2	4	2.45	2	4	2.59
Streptomycin	32	128	48.72	32	128	49.17
Sulfasoxazole	1024	1024	152.37	16	1024	94.48
Tetracyline	64	64	21.21	8	64	15.11
TMS	0.12	8	0.28	0.12	8	0.33

Table 5.4 Summary of descriptive MIC parameters for *E. coli* isolates obtained from fecal and environmental samples from **cows** on a commercial dairy farm in Northern Colorado. All MIC_{50} and MIC_{90} values are alike, except for those highlighted. (G Mean stands for geometric mean).

	Fecals $n = 821$			Feed/water $n = 120$			Flush water $n = 60$		
	MIC_{50}	MIC_{90}	G Mean	MIC_{50}	MIC_{90}	G Mean	MIC_{50}	MIC_{90}	G Mean
Amikacin	1	2	1.31	1	2	1.31	1	2	1.45
Amox/clavulanic	4	4	3.39	4	4	3.47	4	4	3.45
Ampicillin	4	4	3.20	4	4	3.17	4	4	3.45
Cefoxitin	4	8	3.69	4	8	4.14	4	8	3.83
Cefttiofur	0.25	0.5	0.27	0.25	0.5	0.24	0.25	0.5	0.28
Ceftriaxone	0.25	0.25	0.26	0.25	0.25	0.25	0.25	0.25	0.25
Cephalothin	8	16	10.93	16	16	10.82	16	16	11.91
Chloramphenicol	8	8	6.00	8	8	6.46	8	8	5.78
Ciprofloxacin	0.015	0.015	0.02	0.015	0.015	0.02	0.015	0.015	0.02
Gentamicin	0.5	0.5	0.49	0.5	0.5	0.47	0.5	1	0.50
Kanamicin	8	8	8.19	8	8	8.14	8	8	8.24
Nalidixic Acid	2	4	2.72	4	4	3.06	4	4	3.02
Streptomycin	32	32	33.87	32	32	33.16	32	64	36.01
Sulfasoxazole	16	1024	28.82	16	64	23.24	16	1024	35.48
Tetracyline	4	64	6.52	4	64	6.82	4	64	6.05
TMS	0.12	0.12	0.13	0.12	0.12	0.12	0.12	0.12	0.13

Figure 5.1 Relative frequency of resistance patterns found in *E. coli* isolates recovered from 844 fecal samples (clear bars) and 220 feed/water samples (dark bars) in the **calf** areas of a dairy farm in Northern Colorado. See Annex A for information on resistance patterns.

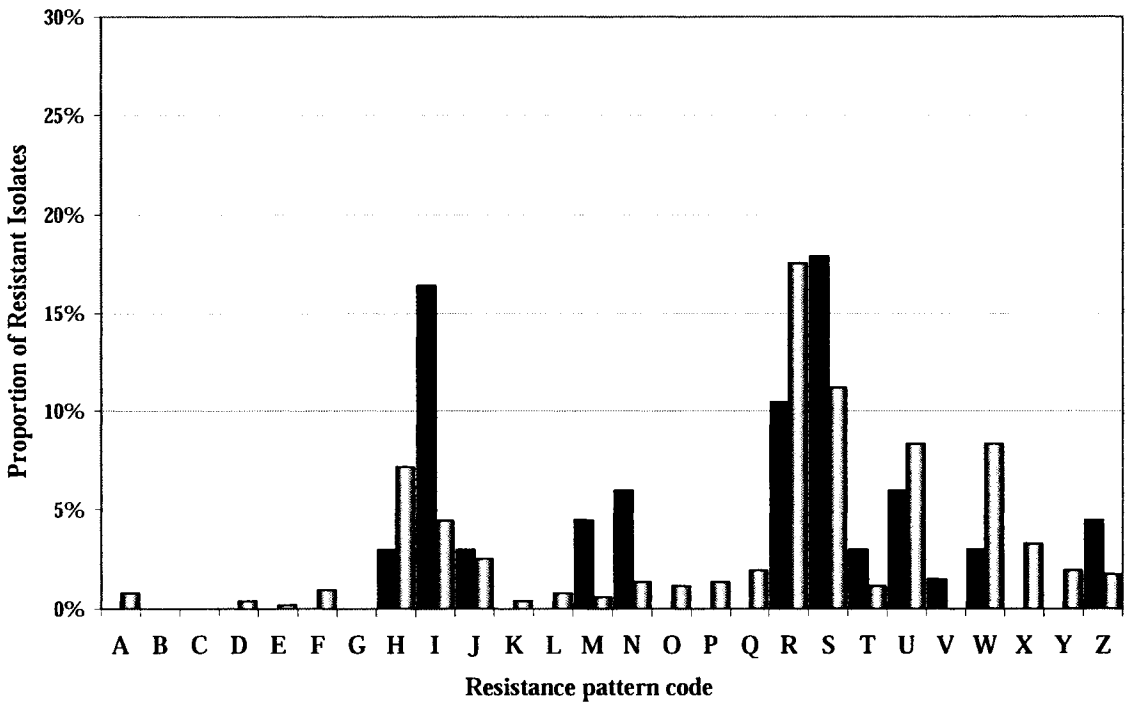
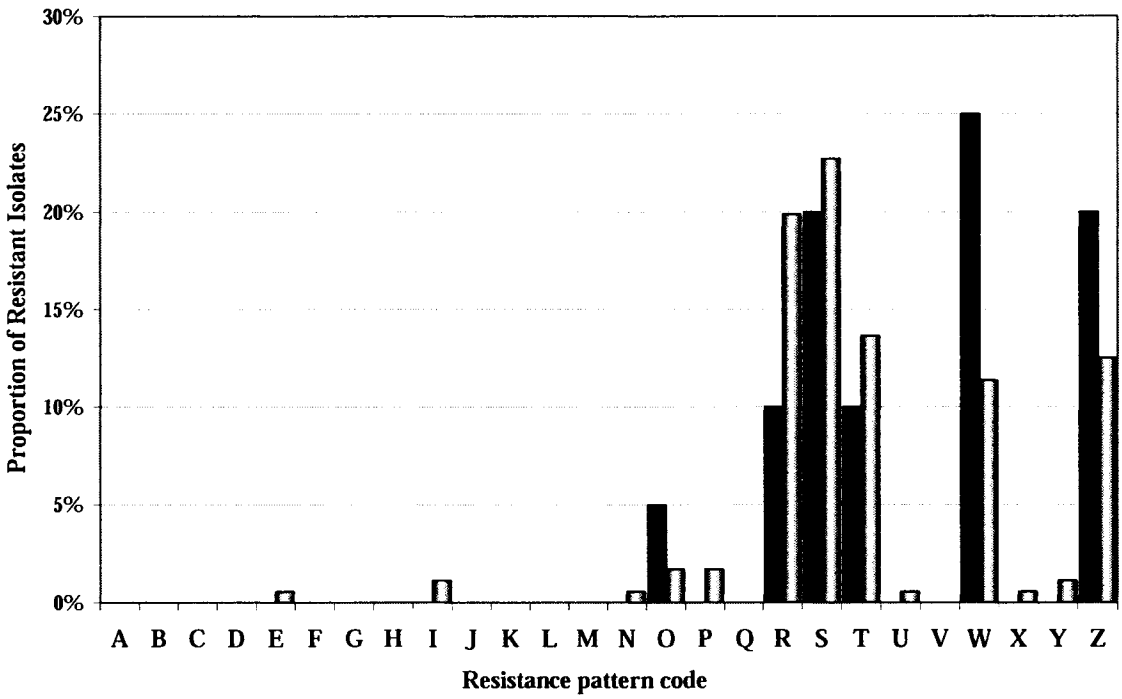


Figure 5.2 Relative frequency of resistance patterns found in *E. coli* isolates recovered from 821 fecal samples (clear bars) and 160 feed/water samples (dark bars) in the **cow** areas of a dairy farm in Northern Colorado. See Annex A for information on resistance patterns.



was also of low frequency among calf fecal samples, where 16 isolates of 844 samples (1.9%) were obtained. Although overall the prevalence of *Salmonella* was low, all isolates were resistant to at least one antimicrobial and most were MDR; 15/16 (93.8%) of fecal *Salmonella* isolates from calves were MDR, and 2/2 (100.0%) of the *Salmonella* isolated from feed/water were MDR. The two *Salmonella* serotypes obtained from feed/water samples were Infantis and Typhimurium (Table 5.5). *S.* Infantis was not isolated from any calf fecal samples, although it was isolated from cow fecal samples. *S.* Typhimurium was the most frequent serotype isolated from calf fecal samples.

E. coli isolation from fecal samples (768/844 or 91.0%) was significantly ($P < 0.001$) higher than from feed/water samples (121/220 or 55.0%). Significantly ($P = 0.006$) more *E. coli* isolates obtained from fecal samples were resistant (518/768 or 67.4%) than those obtained from feed/water (67/220 or 55.4%). Difference in frequency of MDR, although not statistically significant ($P = 0.077$), followed a similar tendency: *E. coli* isolates from fecal samples showed higher MDR frequency (323/768 or 42.1%) than isolates from feed/water samples (42/220 or 34.7%).

Cow areas

Of the 180 environmental samples collected from cow pens, 60 samples were from the recycled flush water used to clean cow traffic areas. Isolation and resistance results for *E. coli* obtained from these flush water samples more closely resembled isolates obtained from fecal samples than from feed/water samples (Table 5.2). Significantly more ($P <$

Table 5.5 Comparison of *Salmonella* serotypes cultured from fecal samples collected from calves and cows and environmental from their areas at a commercial dairy in Northern Colorado.

	Calves		Cows		
	Fecal	Feed / Water	Fecal	Feed / Water	Flush water
	n = 16	*n* = 2	*n* = 38	*n* = 9	*n* = 49
9,12 Non-motile	4				
S. Agona				1	
S. Anatum	1		3	1	9
S. Cubana			14	1	20
S. Dublin	1				
S. Infantis		1	18	3	10
S. Newport			2	2	
S. Senftenberg					10
S. Typhimurium	10	1	1		
S. T. Copenhagen				1	

0.001) *Salmonella* isolates were cultured from flush water samples (49/60 or 81.7%) compared to fecal (38/821 or 4.6%) and feed/water samples (9/120 or 7.5%).

Although most flush water samples collected throughout the study had *Salmonella* isolations, none of the ten samples collected on event 2 were positive. No apparent explanation exists for this discrepancy; several other samples collected and processed on the same day (feed/water or feces) had *Salmonella* isolations, and the management of the farm did not differ from other sampling events.

No differences were observed for resistance frequencies. For MDR, the only significant ($P < 0.001$) difference observed was a higher prevalence among *Salmonella* isolated from feed/water samples (2/9 or 22.2%) compared to fecal samples (3/38 or 7.9%) and flush water samples (0/49). *Salmonella* serotypes recovered from environmental samples from cow areas and fecal samples were alike (Table 5.5).

Similar isolation frequencies were observed for *E. coli* from flush water samples (47/60 or 78.3%) and fecal samples (696/821 or 84.8%), both of which were significantly ($P < 0.001$) larger than frequency of isolation from feed/water samples (41/120 or 34.2%). No differences were observed for frequencies of resistance or MDR in any of the samples.

Factors Associated with Resistance in Salmonella and E. coli Isolates

The possible association of parenteral antimicrobial treatment with isolation, resistance and MDR was evaluated separately in calves and cows. The majority of treated animals

were treated only once during the study. Multiple treatments were recorded on 22/844 calves (2.6%) and 12/821 cows (1.5%). Most of these animals were re-treated only once; 20/22 calves (90.9%) and 10/12 cows (83.3%). Thus, not enough data were available to evaluate whether re-treatment had any association with recovery of resistant isolates.

Calves

Salmonella

Because all *Salmonella* isolates recovered from calves (n = 16) were resistant to at least one antimicrobial and 15/16 (93.4%) were MDR, results of analyses were almost identical for all three outcomes (isolation, resistance and MDR). Therefore, only the results of the analysis for isolation of resistant *Salmonella* are presented (Table 5.6).

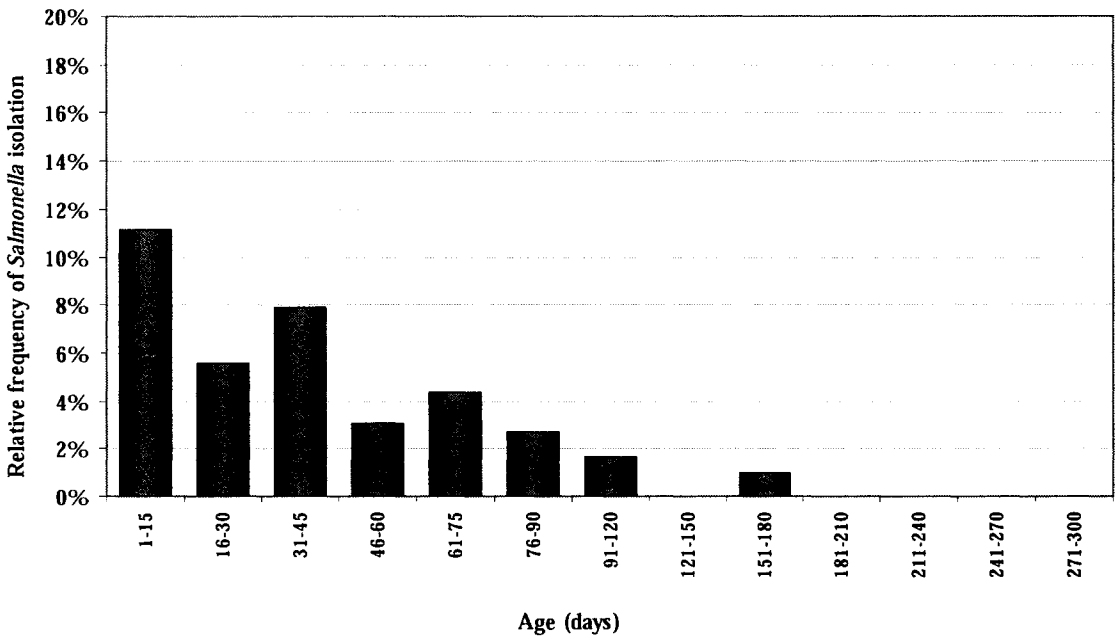
Due to the infrequent isolation of *Salmonella* from calves, most results were non-significant (95% CI for the OR included 1.00). In general, antimicrobial treatment in calves did not show a significant association with isolation of *Salmonella* from calves. The strongest association with *Salmonella* isolation for any of the explanatory variables was for calf location. Calves housed in individual hutches were 26.58 times more likely (95% CI, 8.69 – 81.29) to have *Salmonella* cultured from their feces than calves housed in groups (pens and super-hutches). In fact, isolation steadily decreased as calf age increased (Figure 5.3). No significant association was observed between *Salmonella* isolation and *E. coli* isolation or resistance among *E. coli* isolates.

Table 5.6 Evaluation of factors associated with resistance of *Salmonella* isolates obtained from 844 fecal samples from dairy **calves**. Significant OR ($P \leq 0.05$) are shown in bold. Missing OR are due to non-convergence.

			Resistant *Salmonella* (n = 16)	
	Factor	**Category**	OR	95% CI
Crude analysis	**Treated at any time**		1.73	0.65 - 4.65
	Treated within 8 weeks of sample		2.61	0.92 - 7.40
Multivariate analysis	**Antimicrobial treatment** (forced in the model)	* **Not treated**		
		Tx 1 week ago		
		Tx 2 weeks ago	2.19	0.70 - 6.83
		Tx 3 week ago	**2.51**	**1.07 - 5.92**
		Tx 4 weeks ago	1.56	0.49 - 4.95
		Tx 2 months ago	2.10	0.68 - 6.48
		Tx longer	**4.23**	**1.54 - 11.62**
	Housing	**Individual hutches**	**26.58**	**8.69 - 81.29**
		* **Pens (groups)**		
	Sampling occasion	* **Event 1**		
		Event 2	0.55	0.29 - 1.06
		Event 3	**0.09**	**0.02 - 0.33**
		Event 4	**0.14**	**0.05 - 0.43**
		Event 5		
		Event 6	0.66	0.27 - 1.60

* Reference category

Figure 5.3 Frequency of recovery of *Salmonella* isolates (susceptible or resistant) from 844 fecal samples obtained from **calves**, by calf age.



E. coli

The strongest association with isolation of any *E. coli* isolate was with age (Table 5.7). In contrast with *Salmonella*, it was less likely to isolate *E. coli* from younger calves than from calves older than 6 months (reference category). However, calves less than 60 days of age were 3.39 (95% CI, 2.58 – 4.46) times more likely to have a resistant *E. coli* than the calves 6-month old or older. This association was even stronger for MDR, where calves less than 60 days old were 9.66 (95% CI, 7.01 – 13.30) times more likely to have recovery of an MDR *E. coli* isolate than the reference category (calves > 180 days). Isolation of MDR *E. coli* decreased almost linearly as calf age increased (Figure 5.4).

Hierarchical multivariate analysis of the association of antimicrobial treatment with *E. coli* isolation could not be performed because the algorithm failed to converge. Non-convergence was due to presence of few isolates in some of the categories. Association of antimicrobial treatment with resistance or MDR in *E. coli* isolates varied according to time elapsed between treatment and sample collection (Table 5.7). This association was not linear. Calves treated within 1 week of sample collection were 4.56 (95% CI, 1.57 – 13.24) more likely to have a resistant *E. coli* isolate recovered than non-treated calves. Calves treated 1 to 2 weeks before sample collection showed a higher risk (OR = 8.24 [95% CI, 2.90 – 23.41]). Association of antimicrobial treatment with recovery of an MDR *E. coli* isolate seemed to follow a negative exponential relationship. Calves treated within one week before sample collection were at highest risk (OR = 4.67 [95% CI, 2.00 – 10.89]), followed by calves treated between 1 and 2 weeks prior to sample collection (OR = 3.05 [95% CI, 1.68 – 5.52]) and calves treated between 2 and 3 weeks prior to

Figure 5.4 Frequency of recovery of multi-drug resistant (MDR) *E. coli* isolates from 844 fecal samples obtained from **calves**, by calf age.

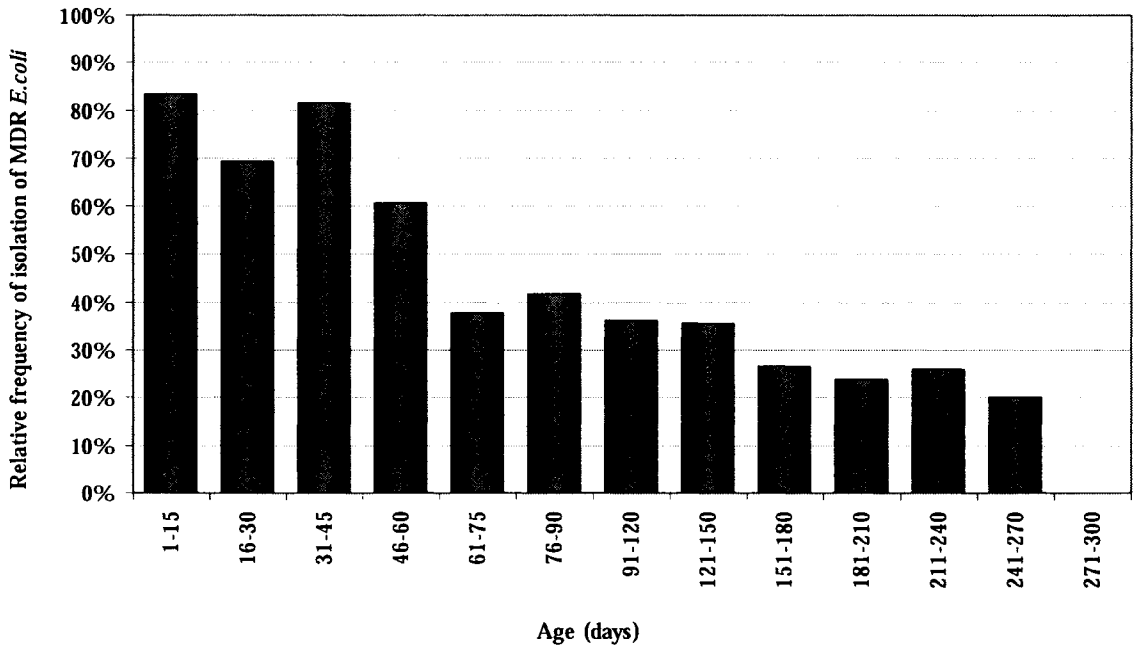


Table 5.7 Evaluation of factors associated with isolation, resistance and multidrug resistance (MDR) of *E. coli* isolates obtained from 844 fecal samples from dairy **calves**. Significant OR ($P \leq 0.05$) are shown in bold. Missing OR are due to non-convergence.

			Any *E.coli* (n = 768)		Resistant *E.coli* (n = 518)		MDR *E.coli* (n = 323)	
	Factor	Category	OR	95% CI	OR	95% CI	OR	95% CI
Crude analysis	Treated at any time		1.14	1.11 - 1.18	1.15	1.03 - 1.28	0.98	0.81 - 1.18
	Treated within 8 weeks of sample		1.11	1.08 - 1.14	1.11	0.97 - 1.28	1.23	1.00 - 1.53
Multivariate analysis	Antimicrobial treatment (forced in the model)	* Not treated						
		Tx 1 week ago			4.56	1.57 - 13.24	4.67	2.00 - 10.89
		Tx 2 weeks ago			8.24	2.90 - 23.41	3.05	1.68 - 5.52
		Tx 3 week ago			3.68	1.70 - 7.94	1.84	1.04 - 3.24
		Tx 4 weeks ago			1.83	1.06 - 3.15	1.62	0.96 - 2.74
		Tx 2 months ago			0.71	0.54 - 0.94	0.80	0.59 - 1.10
		Tx longer			1.77	1.43 - 2.18	0.84	0.66 - 1.07
	Housing	Individual hutches						
		* Pens (groups)						
	Sampling occasion	* Event 1						
		Event 2	2.63	1.12 - 7.92			1.16	0.86 - 1.56
		Event 3	1.33	0.62 - 3.06			2.59	1.90 - 3.54
		Event 4	0.14	0.08 - 0.27			2.08	1.55 - 2.79
		Event 5	0.16	0.09 - 0.17			1.61	1.17 - 2.22
		Event 6	0.14	0.08 - 0.21			1.40	0.99 - 1.97
	Age	0 - 60 days	0.13	0.06 - 0.28	3.39	2.58 - 4.46	9.66	7.01 - 13.30
		61-120 days	0.12	0.07 - 0.22	1.45	1.18 - 1.77	1.96	1.53 - 2.50
		121-180 days	0.27	0.17 - 0.41	1.42	1.16 - 1.73	1.43	1.13 - 1.79
		* >180 days						

* Reference category

sampling (OR = 1.84 [95% CI, 1.04 – 3.24]). No significant association was observed beyond 3 weeks of lag time between treatment and sample collection.

Crude analysis (without possible confounding factors) of the association of antimicrobial treatment with isolation, resistance and MDR of *E. coli* isolates is presented in the top two rows of Table 5.7 for comparison with results of hierarchical multivariate analysis. Using crude analyses, antimicrobial treatment showed a weak association in all instances (OR ~ 1.1), while using hierarchical multivariate analyses varying strength of association could be observed (OR range 0.71 – 8.24).

Risk of *E. coli* isolation varied by sampling event, as did the risk for MDR (Table 5.7). This variation followed different patterns for *E. coli* isolation than for MDR. Using sampling event 1 as the reference category, *E. coli* was more likely isolated during sampling event 2 (OR = 2.63 [95% CI, 1.12 – 7.92]) and sampling event 3 (OR = 1.33 [95% CI, 0.62 – 3.06]), and much less likely during the following occasions (OR ~ 0.14). Recovery of MDR *E. coli* however was significantly more likely during sampling event 3 (OR = 2.59 [95% CI, 1.90 – 3.54]), sampling event 4 (OR = 2.08 [95% CI, 1.55 – 2.79]) and sampling event 5 (OR = 1.61 [95% CI, 1.17 – 2.22]) than on sampling event 1 (reference category).

Cows

Salmonella

Similar to calves, few *Salmonella* isolates were recovered from cows (38/821 or 4.6%). Half (19/38) were resistant to at least one antimicrobial, although only 3 (15.8%) of these resistant *Salmonella* isolates were recovered from treated cows. Thus, evaluation of a possible association between antimicrobial treatment and resistance or MDR among *Salmonella* isolates was not possible.

Evaluation of a possible association between antimicrobial treatment and *Salmonella* isolation (susceptible and resistant isolates, see Table 5.8) showed that cows treated with any parenteral antimicrobial were 0.41 (95% CI, 0.26 – 0.66) times as likely to have *Salmonella* isolated from their feces as non-treated cows. In this case, crude analysis showed similar results to hierarchical multivariate analysis.

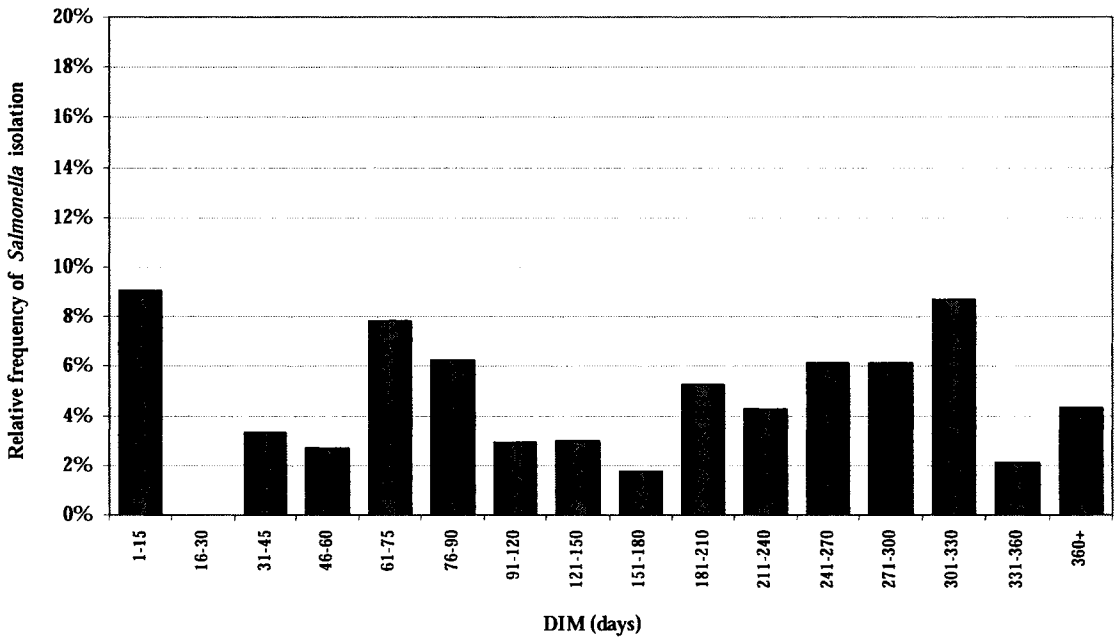
Multivariate analysis showed that the strongest risk factor for isolation of *Salmonella* was production group. Risk of *Salmonella* isolation was higher in hospital cows (OR = 15.23 [95%CI, 4.98 – 46.57]) and fresh cows (OR = 7.29 [2.34 – 22.67]) compared to low production cows (Table 5.8). Cows in their first lactation were also more likely to have *Salmonella* isolated from their feces than adult cows (OR = 2.36 [95% CI, 1.63 – 3.42]). Recovery of *Salmonella* isolates, both susceptible and resistant, was non-uniform across DIM (Figure 5.5). Similar to findings in calves, there was no significant association between the recovery of any *Salmonella* isolate and the isolation of *E. coli*.

Table 5.8 Evaluation of factors associated with isolation of ***Salmonella*** from 821 fecal samples collected from dairy **cows**. Significant OR ($P \leq 0.05$) are shown in bold. Missing OR are due to non-convergence.

			Any *Salmonella* (n = 38)	
	Factor	**Category**	**OR**	**95% CI**
Crude analysis	**Treated at any time**		**0.43**	**0.19 - 0.96**
	Treated within 8 weeks of sample		0.27	0.04 - 1.93
Multivariate analysis	**Antimicrobial treatment** (forced in the model)	* **Not treated**		
		Tx 1 week ago		
		Tx 2 weeks ago		
		Tx 3 week ago		
		Tx 4 weeks ago		
		Tx 2 months ago	0.84	0.26 - 2.68
		Tx longer	**0.41**	**0.26 - 0.66**
	Lactation number	**1st Lact**	**2.36**	**1.63 - 3.42**
		* **2 + Lact**		
	Production	* **Low**		
		Medium	**5.33**	**1.90 - 14.94**
		High	**5.52**	**1.92 - 15.85**
		Fresh cows	**7.29**	**2.34 - 22.67**
		Hospital cows	**15.23**	**4.98 - 46.57**

* Reference category

Figure 5.5 Frequency of recovery of *Salmonella* isolates (susceptible or resistant) from 821 fecal samples obtained from **cows**, by lactation stage (days in milk, DIM).



E. coli

Isolation of *E. coli* was associated with treatment with an antimicrobial within 1 week of sample collection (OR = 5.25 [95%CI, 1.77 – 15.60]), or treatment more than 2 months prior to sample collection (OR = 9.73 [95% CI, 6.42 – 14.73], see Table 5.9). Convergence was not achieved for all categories (time from treatment to sample collection). Crude analysis however, showed a weak association of antimicrobial treatment with *E. coli* isolation (OR = 1.24 [95% CI, 1.19 – 1.31]).

The only other factor associated with *E. coli* isolation among cows was production group. Medium-producing cows were at higher risk of having *E. coli* isolated from their feces compared to low producing cows (OR = 2.51 [95% CI, 1.80 – 3.51]). No other production groups showed a significant association.

The risk of recovery of resistant or MDR *E. coli* in cows was associated with antimicrobial treatment at varying strengths of association. Overall the highest risk for recovery of resistant *E. coli* was in cows treated with a parenteral antimicrobial within four weeks of sample collection, and specifically 2-3 weeks prior to sampling (OR = 10.40 [95% CI, 4.23 – 25.56]). Risk for the recovery of MDR *E. coli* was also highest if sampling occurred within the four weeks after parenteral treatment, with similar OR for each week within this period (Table 5.9).

First lactation cows were 1.68 (95% CI, 1.17 – 2.42) times more likely than adult cows to have MDR *E. coli* isolated from their feces. Other factors associated with risk of

Table 5.9 Evaluation of factors associated with isolation, resistance and multidrug resistance (MDR) of ***E. coli*** isolates obtained from 821 fecal samples from dairy **cows**. Significant OR ($P \leq 0.05$) are shown in bold. Missing OR are due to non-convergence.

			Any *E.coli* (n = 695)		Resistant *E.coli* (n = 176)		MDR *E.coli* (n = 42)	
	Factor	Category	OR	95% CI	OR	95% CI	OR	95% CI
Crude analysis	Treated at any time		1.24	1.19 - 1.31	1.74	1.34 - 2.25	3.42	1.85 - 6.33
	Treated within 8 weeks of sample		1.19	1.14 - 1.24	2.13	1.56 - 2.89	5.53	3.08 - 9.92
Multivariate analysis	Antimicrobial treatment (forced in the model)	* Not treated						
		Tx 1 week ago	5.25	1.77 - 15.60	4.33	2.58 - 7.26	6.86	3.66 - 12.85
		Tx 2 weeks ago			3.12	1.13 - 8.58	5.42	1.49 - 19.81
		Tx 3 week ago			10.40	4.23 - 25.56	6.62	2.43 - 18.01
		Tx 4 weeks ago			5.47	2.36 - 12.69	4.54	1.68 - 12.28
		Tx 2 months ago			2.16	1.37 - 3.41	3.16	1.37 - 7.26
		Tx longer	9.73	6.42 - 14.73	1.68	1.35 - 2.09	2.64	1.70 - 4.10
	Lactation number	1st Lact					1.68	1.17 - 2.42
		* 2 + Lact						
	Production	* Low						
		Medium	2.51	1.80 - 3.51	0.74	0.57 - 0.95	0.65	0.36 - 1.18
		High	0.69	0.43 - 1.11	1.18	0.80 - 1.74	2.81	1.47 - 5.34
		Fresh cows	1.34	0.94 - 1.91	0.92	0.69 - 1.23	0.83	0.45 - 1.53
		Hospital cows	1.34	0.88 - 2.03	1.72	1.25 - 2.38	4.80	2.82 - 8.17
	Sampling occasion	* Event 1						
		Event 2			0.54	0.38 - 0.78	0.39	0.19 - 0.81
		Event 3			0.92	0.66 - 1.29	0.60	0.32 - 1.12
		Event 4			0.34	0.23 - 0.52		
		Event 5			2.27	1.66 - 3.10	1.74	1.03 - 2.96
		Event 6			2.01	1.46 - 2.77	1.34	0.76 - 2.38

* Reference category

resistance among *E. coli* isolates obtained from cows were production group and sampling event. Cows housed in the hospital at the time of sample collection were more likely to have resistant (OR = 1.72 [95% CI, 1.25 – 2.38]) or MDR (OR = 4.80 [95% CI, 2.82 – 8.17]) *E. coli* isolated from their feces compared to low-producing cows. Sampling event showed varying strength of association with recovery of resistant *E. coli* (Table 5.9).

Resistance to Select Antimicrobials

Because resistance among isolated bacteria was tested for multiple antimicrobials, using non-specific results of resistance compared to the potential exposure (treatment) may bias the results. For example, when evaluating a possible association between tetracycline treatment in calves and resistance in *E. coli*, a bias may occur if *E. coli* isolates are designated as resistant if they show resistance to any antimicrobial and not just tetracycline. As described above, an isolate was designated as resistant if it was resistant to *any* antimicrobial. A possible association between treatment with select antimicrobials and resistance of *E. coli* to those antimicrobials was further studied. The antimicrobials selected for these analyses were tetracyclines and beta-lactams because of their common use in dairy cattle. Additionally, in calves, the association between treatment with florfenicol and resistance of *E. coli* to chloramphenicol was studied. As before, treatment was always forced into the model. Results of the analyses for calves are presented in Table 5.10, and for cows in Table 5.11.

Table 5.10 Evaluation of factors associated with resistance to **select antimicrobials** in *E. coli* isolates obtained from 844 fecal samples from dairy **calves**. Significant OR ($P \leq 0.05$) are shown in bold. Missing OR are due to non-convergence.

			E.coli Resistant to tetracyclines (n = 480)		*E.coli* Resistant to beta-lactams (n = 195)		*E.coli* Resistant to chloramphenicol (n = 192)	
	Factor	Category	OR	95% CI	OR	95% CI	OR	95% CI
Crude analysis	Treated at any time		**0.71**	**0.53 - 0.95**	1.25	0.92 - 1.70	0.9	0.52 - 1.54
	Treated within 8 weeks of sample		0.81	0.59 - 1.12	**2.02**	**1.44 - 2.84**	1.49	0.83 - 2.65
Multivariate analysis	*Antimicrobial treatment* (forced in the model)	* Not treated						
		Tx 1 week ago	2.70	0.86 - 8.47	2.04	0.97 - 4.29		
		Tx 2 weeks ago			**2.72**	**1.27 - 5.82**		
		Tx 3 week ago			1.06	0.58 - 1.93	0.75	0.22 - 2.62
		Tx 4 weeks ago	**0.48**	**0.33 - 0.70**	0.61	0.25 - 1.46	0.53	0.16 - 1.73
		Tx 2 months ago	**0.34**	**0.20 - 0.57**	**0.23**	**0.10 - 0.54**	**2.30**	**1.30 - 4.07**
		Tx longer			0.72	0.51 - 1.02	**0.41**	**0.22 - 0.78**
	Housing	Individual hutches			**6.14**	**5.00 - 7.55**	**3.34**	**2.76 - 4.04**
		* Pens (groups)						
	Sampling occasion	* Event 1						
		Event 2	1.20	0.90 - 1.60	**0.53**	**0.39 - 0.71**	1.21	0.89 - 1.65
		Event 3	**2.46**	**1.78 - 3.40**	**0.52**	**0.37 - 0.74**	**1.39**	**1.01 - 1.93**
		Event 4	**2.41**	**1.76 - 3.30**	**1.51**	**1.11 - 2.05**	**2.82**	**2.07 - 3.83**
		Event 5	**2.00**	**1.42 - 2.80**	0.83	0.59 - 1.15	**1.61**	**1.16 - 2.24**
		Event 6	**1.59**	**1.11 - 2.27**	0.88	0.60 - 1.29	1.33	0.90 - 1.96
	Age	0 - 60 days	**7.64**	**5.38 - 10.84**				
		61-120 days	**2.97**	**2.30 - 3.85**				
		121-180 days	**2.09**	**1.67 - 2.61**				
		* >180 days						

* Reference category

Table 5.11 Evaluation of factors associated with resistance to select antimicrobials in *E. coli* isolates obtained from 821 fecal samples from dairy **cows**. Significant OR ($P \leq 0.05$) are shown in bold. Missing OR are due to non-convergence.

			E.coli Resistant to tetracyclines (n = 120)		*E.coli* Resistant to beta-lactams (n = 50)	
	Factor	Category	OR	95% CI	OR	95% CI
Crude analysis	Treated at any time		1.75	0.90 - 3.41	1.36	0.78 - 2.36
	Treated within 8 weeks of sample		1.89	0.71 - 5.03	**3.34**	**1.80 - 6.19**
Multivariate analysis	Antimicrobial treatment (forced in the model)	* Not treated				
		Tx 1 week ago				
		Tx 2 weeks ago				
		Tx 3 week ago				
		Tx 4 weeks ago				
		Tx 2 months ago	2.17	0.62 - 7.58	0.66	0.23 - 1.92
		Tx longer	1.75	0.92 - 3.32	2.07	0.93 - 4.64
	Lactation number	1st Lact	**0.57**	**0.45 - 0.71**		
		* 2 + Lact				
	Production	* Low				
		Medium				
		High				
		Fresh cows				
		Hospital cows				
	Sampling occasion	* Event 1				
		Event 2			1.70	0.96 - 2.99
		Event 3			**1.81**	**1.03 - 3.17**
		Event 4			1.69	0.96 - 2.98
		Event 5			**1.85**	**1.06 - 3.22**
		Event 6			1.12	0.60 - 2.07

* Reference category

Calves

Crude analysis of the association between treatment with tetracyclines at any time during the study and tetracycline resistance of *E. coli* isolates showed a negative association (OR = 0.71 [95% CI, 0.53 – 0.95], see Table 5.10). Multivariate analysis however, showed a positive association –although non-significant– if calves were treated with tetracyclines within one week before sampling (OR = 2.70 [95% CI, 0.86 – 8.47]). A negative association was found between treatment with tetracyclines four weeks or longer prior to the sampling event and tetracycline resistance in *E. coli* isolated from those calves. As before, calf age was a significant risk factor in this analysis. Calves less than 60 days old were 7.64 (95% CI, 5.38 – 10.84) times more likely to have recovery of a resistant *E. coli* isolate than calves 6 months of age or older. Risk of resistance among *E. coli* isolates varied by sampling event.

Similar results were observed for beta-lactam antimicrobials. A positive association between treatment of calves with parenteral beta-lactams (penicillins and cephalosporins) within 2 weeks prior to sampling and resistance to beta-lactams in *E. coli* isolates (Table 5.10). The factor most strongly associated with beta-lactam resistance among *E. coli* isolates from calves was housing in individual hutches (OR = 6.14 [95% CI, 5.00 – 7.55]). Sampling events showed varying strength of association.

Risk of recovery of an *E. coli* isolate resistant to chloramphenicol was more likely among calves housed in individual hutches (OR = 3.34 [95% CI, 2.76 – 4.04]). As observed for other antimicrobials, different sampling events had varying strength of association. Since

chloramphenicol is not approved for use in food-animals, treatment with florfenicol was studied as a risk factor for chloramphenicol resistance in *E. coli* isolates recovered from calves. A positive association was observed in calves treated 1-2 months prior to sample collection (OR = 2.30 [95% CI, 1.30 – 4.07]), while a negative association was observed in calves treated more than 2 months prior to sampling (OR = 0.41 [95% CI, 0.22 – 0.78]).

Cows

No significant association was found for parenteral treatment with tetracyclines or beta-lactam antimicrobials in cows and recovery of *E. coli* isolates resistant to these antimicrobials (Table 5.11). The only study variable for which a significant association could be found with resistance to tetracyclines was lactation number; first lactation cows were 0.57 (95% CI, 0.45 – 0.71) as likely as adult cows to have a resistant *E. coli* isolate recovered from their feces. Risk of resistance to beta-lactams varied across different sampling events.

DISCUSSION

Environmental vs. Fecal Samples

Overall, the frequency of isolation for *Salmonella* in this study was low and resembled frequencies reported in other studies for feed and drinking water,[39-41] flush water,[42] and feces.[41,43-45] Similar frequencies for *Salmonella* isolation and resistance were observed for

fecal samples and feed/water samples, both for calves and cows. Two *Salmonella* MDR isolates were recovered from feed/water samples from cow areas, which represented a significantly higher percentage (22.2%) than the three MDR isolates recovered from feces (7.9%). The biological significance of this result is unclear, due to the limited number of isolates that were recovered. Because so few *Salmonella* isolates were recovered from feed/water samples (n = 2 from calf areas, and n = 9 from cow areas), comparison of isolated serotypes with other samples was not possible.

A much higher proportion of flush water samples from the cow areas yielded *Salmonella* isolates (82.7%), compared to fecal samples (4.6%). This could be because flush water was recycled and stored in two big tanks that allowed continuous exposure of bacteria to nutrients for growth and multiplication. *Salmonella* isolates recovered from flush water samples were of similar serotypes to those isolated from cow feces (Table 5.5). One exception was serotype Senftenberg, which was isolated from flush water samples but not from feces. It is possible that some cows from which no fecal samples were collected were shedding *S.* Senftenberg, and therefore contributed to its presence in the flush water. It is also possible that *S.* Senftenberg originated in wildlife that had access to the manure lagoon, where the flush water was collected to be recycled. Another possibility is that *S.* Senftenberg had been present on the dairy at some point and was perpetuated in the recycled flush water. Although the flush water was released while the cows were absent from the pen (during milking at the parlor) and scraped to minimize residues, cows may still have had access to it when returning to the pen. Further, this study was performed over a year and therefore, if *Salmonella* species are as persistent in the dairy farm environment as previously reported,[33,46,47] the same serotypes should be recovered from

feces and flush water that cleans feces. The most likely explanation for these results may be the shedding by cows that were not selected for sampling of feces.

Frequency of isolation, resistance and MDR for *E. coli* isolates was higher in calf feces than in feed/water samples collected from calf areas. The highest frequency of MDR *E. coli* was among calves housed in individual hutches. It is possible that the reduced space available in individual hutches and the fact that these hutches were only cleaned between calves exposed the calves to higher bacterial loads. Resistant and MDR *E. coli* may have a fitness advantage in this environment, where calves can recycle enteric bacteria through grooming. Additionally, calves housed in hutches are fed a milk-based diet, which may confer an advantage to resistant and MDR *E.coli* to survive.

Frequencies of isolation, resistance and MDR for *E.coli* among feed/water samples from calf areas were higher than those from cow areas (Table 5.1 and Table 5.2, respectively). Fecal contamination of feed/water samples in the calf areas may explain this. Feed and water buckets in the individual hutches were placed inside the hutch to avoid rain and snow, allowing easy contamination with feces. Calves housed in pens can contaminate feed by stepping into the feedbunk while competing for feed. Feedbunk design in cow pens prevented them from contaminating the feed with feces.

Due to the lower frequency of isolation of *Salmonella* and *E. coli* from feed/water samples compared to fecal samples, testing feed/water samples as a proxy for fecal samples would not be efficient. Furthermore, resistance patterns of feed/water vs. fecal *Salmonella* isolates could not be compared due to small sample size. Another reason that feed/water samples are poor substitute for fecal samples is that resistance patterns for *E.*

coli isolates from fecal samples showed higher variability than feed/water samples. No other studies were found that evaluated isolation of *E. coli* in feed and water samples to compare results.

Factors Associated with Resistance

Because of the low frequency of isolation of *Salmonella*, analyses for the association of parenteral antimicrobial treatment with isolation, resistance and MDR of *Salmonella* isolates were limited. If this relationship is to be studied further, an approximate frequency of isolation of *Salmonella* of 3-10% in non-clinical cattle[41,43,44] needs to be taken into account for considerations of sample size calculations for the studies.

Crude analysis of the association of antimicrobial treatment with recovery of resistant or MDR isolates differed from the results of the more appropriate hierarchical multivariate analysis. Therefore, when the only factor studied in relation to resistance or MDR is antimicrobial treatment, results may be biased. During a longitudinal study, several samples may be collected from the same animal. Performing a simple crude analysis implies an assumption of independence between all isolates recovered from an animal over time and that no other factors are part of this potential association. A hierarchical multivariate design allows proper consideration of these issues.

For the limited number of *Salmonella* isolates recovered during this study, hierarchical multivariate analyses showed that antimicrobial treatment may have been associated with *Salmonella* isolation, but much less than other factors. For calves, housing in individual

hutches was the most important risk factor. *Salmonella* isolation from calves consistently decreased as age increased, as observed in other studies.[21,25,27,30] Although most calves housed in individual hutches were less than 60 days old, some older calves, up to 90 days old, were housed in hutches and had a positive *Salmonella* isolation. It is likely that these older calves were kept in the hutches longer because they were sick, and this could explain why being housed in individual hutches was more strongly associated with *Salmonella* isolation than calf age. For cows, production group was the most important risk factor. As reported in other studies,[32,33,41] sick cows (hospital) and fresh cows had higher risk of *Salmonella* isolation. No direct association was found between *Salmonella* isolation and DIM, probably due to varying DIM for cows housed in the hospital pen. All these animals (young calves, sick cows and fresh cows) are the most immunologically challenged on the farm.[48-50] This may explain the higher prevalence of *Salmonella* in these animals compared to the rest of the herd. The association between *Salmonella* isolation and the hospital pen may be spurious due to transfer of sick cows from other pens to the hospital, *i.e. Salmonella* infection may have originated in a pen other than the hospital.

The association between antimicrobial treatment and isolation of *Salmonella* was not uniform for calves and cows. A positive association was observed in calves, although it was not always significant. Low statistical power due to a reduced number of recovered isolates may have prevented the results from being significant. However, other studies have shown a lack of association between antimicrobial treatment and *Salmonella* isolation in calves.[27,41,51] In contrast, cows treated with a parenteral antimicrobial were less likely (OR 0.41 [95% CI, 0.26 – 0.66]) than untreated cows to have a positive

Salmonella isolation. These results agree with those from another study,[43] although a second study showed the opposite effect.[51] In the present study, no convergence (solution) could be achieved by the statistical software for the categories of treatment less than two months prior to sampling. The lack of convergence could have biased the results towards the null hypothesis of no association. No biologically plausible explanation can be offered as to why treatment with an antimicrobial longer than two months prior to sampling would be associated with a higher probability of recovery of resistant bacteria compared to treatment closer to sampling. This result could be an artifact due to the large interval included in the category of "treated two months or more prior to sampling" (8 months) as compared to the other categories. A larger interval allowed for more animals to be included in that category and a better chance to show a significant difference.

Sampling event was also a significant risk factor for isolation of *Salmonella* in calves. There was no apparent link to environmental temperature as previously reported.[44,52] One explanation could be the susceptibility to infection in calves of different ages. As the study progressed, calves increased in age and therefore their susceptibility to infection decreased and could have lowered the risk of *Salmonella* isolation as seen in Table 5.6. However, multivariate analysis showed a higher risk with treatment at 2 or more months prior to sampling, which contradicts this theory. Another possible explanation is that some other factors not evaluated in this study have an effect on the ecology of *Salmonella* in dairy calves.

Recovery of resistant and MDR *E. coli* was more significantly associated with previous antimicrobial treatment than observed for *Salmonella*. It is important to note however,

that other factors were significantly associated with resistance of *E. coli* isolates. Calf age was an important risk factor for resistance and MDR in *E. coli.* The risk followed a gradient, being larger for the youngest calves and decreasing as calf age increased. For cows, housing in the hospital pen had the highest risk of recovery of resistant and MDR *E. coli.* Other production groups varied in their risk, although the scarcity of the data may have compromised the significance of these results. First lactation cows were at higher risk of having MDR *E. coli* isolated from their feces. It is important to emphasize that milk and meat obtained from cows housed in the hospital (treated with drugs that require withholding period) are prohibited from being used for food for human consumption,[53-56] and therefore exposure to antimicrobial residues or resistant bacteria to humans through food is prevented.

Previous studies that have tried to assess the effect of antimicrobial treatment in animals on resistance of enteric bacteria found discrepant results for individual antimicrobials.[16-21] These discrepant results could be due to measuring resistance to antimicrobials to which the animal was not exposed. For example, if treatment with tetracyclines is evaluated as a potential risk factor for resistance, and resistance is measured to 16 antimicrobials, illogical conclusions may occur. Nonetheless, some antimicrobials may show cross-resistance[57] because they use the same resistance mechanisms or because resistance genes are transmitted together in gene cassettes, for example.

The non-uniform results observed in this study for the association of antimicrobial treatment with resistance or MDR could be due to, among other reasons, measuring the exposure and the outcome at different levels. To evaluate if this bias may have existed,

the possible association of treatment with select antimicrobials and resistance of *E. coli* to those antimicrobials was further studied. The studied exposure is treatment with tetracyclines, while the measured outcome is resistance to any combination of the 16 antimicrobials tested, whether these combinations included resistance to tetracyclines or not.

A more focused evaluation of the association of treatment and resistance would be if both the exposure and the outcome are measured to only one antimicrobial. This evaluation was performed in this study for tetracyclines, beta-lactams and florfenicol. For all three antimicrobials, treatment within 2 weeks prior to sampling showed a positive association with resistance in *E. coli* to that specific antimicrobial. These results would support the idea of temporary selection of resistant bacteria when antimicrobials are used. After treatment has ceased, susceptible bacteria can grow and multiply to out-compete the resistant bacteria that thrived while antimicrobials where being used. This can explain reported similarities in the frequency of resistant enteric bacteria in commercial and organic dairy farms.[32,43,44,58]

One major factor that could have contributed to these non-uniform results could be the use of a single isolate from each sample for susceptibility testing. Many studies test a single isolate from a large number of animals to characterize antimicrobial resistance in populations. Collecting samples from large numbers of animals enhances the ability to detect differences between animals and between groups. However, testing a single isolate fails to address the variability among isolates from the same animal. Previous studies

have suggested that more than one isolate per sample are needed to best represent the bacterial flora within an animal.[31,59,60]

Many articles imply that antimicrobial-use in food-animals is responsible for resistance in bacteria.[5,10-14] A review of the criteria that need to be considered to evaluate if a factor is a cause of a certain outcome [61,62] instead of just associated with that outcome shows that strength of association, consistency of the association, and time-relationship between exposure and outcome are important criteria that need to be taken into consideration. Results throughout the analyses showed a non-uniform direction of association between antimicrobial treatment and recovery of resistant *Salmonella* or *E. coli*. The lack of consistency in the direction of the association does not favor the classification of antimicrobial treatment as a causative factor of resistance in bacteria, although it is associated (see below).

Note that when sampling feces from an animal for culture, we are dealing with probabilities of isolation of enteric bacteria, and probabilities that these bacteria are resistant. Millions of bacteria are to be found in only 1 g of feces,[63] but usually only 1 isolate is tested among all those that grow on a culture.[2,64,65] Susceptible bacteria, by definition, don't grow or multiply in the presence of antimicrobials.[66-68] Therefore, the use of antimicrobials should reduce the amount of susceptible isolates within the sites or tissues in an animal where the antimicrobial reaches necessary MICs. A decrease in the number of susceptible bacteria will shift the population towards a higher proportion (not amount) of resistant bacteria. For example, if we assume an animal has 100 isolates of which 10 are resistant and 90 are susceptible, we have 10% (10/100) resistant isolates. If

treatment of this animal with an antimicrobial reduces the susceptible population from 90 to 30, and there are still 10 resistant isolates, we have 25% resistant isolates (10/40).

Thus, the probability of recovering a resistant isolate is larger after treatment than before treatment at those sites or tissues where the antimicrobial reaches effective MICs. In consequence, antimicrobial-use may not be related to the presence of resistant isolates, but rather to the probability of isolating resistant isolates. An example based on the analogy presented by Sir Arthur Eddington to explain the scientific method[69] may help further clarify this point. If we cast a net into a lake (sampling), we will obtain a sex ratio common to that species of fish (*e.g.* female to male ratio of 50:50). If the net is cast a second time after a toxic effluent contaminates the lake and affects the sex ratio in favor of males (*e.g.* 20:80) there are two possible interpretations: (1) that the toxic effluent *caused* female fish (susceptible isolates) to transform into males (resistant isolates), or (2) that female fish have diminished in numbers leaving primarily male fish behind.

The time-relationship between a factor and the outcome is probably the most important determinant of whether that factor can be considered a cause of the outcome or not. To establish a causal relationship between a factor and the outcome, it has to be proven that the outcome did not exist before the factor was present.[36,61] In the study of a possible effect of antimicrobial-use (potential exposure factor) in animals on antimicrobial resistance in bacteria (outcome), this time-relationship is difficult to prove. Several reports show that resistant bacteria can be found in isolates banked before any antimicrobials were commercialized,[68,70,71] and in wildlife species that have no explicit exposure to antimicrobials.[72,73] A study that attributed fluoroquinolone resistance in

human strains of *Campylobacter* spp. to the use of fluoroquinolones in food-animals documented that resistance in humans was first detected several years prior to the approval of these antimicrobials in food-animals, violating the time-relationship criterion of causality.[74]

To test differences due to the exposure to a factor, the same individuals need to be tested before and after exposure. For example, to evaluate if a new diet has any effect on milk production in a herd we would need to compare milk production in the same animals before and after the new diet. We will not be able to fairly evaluate the effect of the diet by comparing milk produced by one cow (or group of cows) before the new diet was implemented to that produced by a different cow (or group of cows) when the new diet is in place. The inherent variability in milk production among cows could bias the results. A similar situation occurs when evaluating a possible effect of antimicrobial-use on resistance in enteric bacteria. Susceptibility should be tested in the same isolates before and after exposure to antimicrobials. This cannot be accomplished in field conditions, where the isolate is collected from the cow and transported to the lab. The next time that a sample is collected from that cow, a different isolate will be tested because the original one was eliminated from the cow and sent to the lab. Although removing this isolate from the pool should not affect the distributions of resident bacteria, the fact is that particular isolate cannot be sampled anymore. Another isolate, maybe even a clone is sampled next time, although the probability that it has undergone some kind of genetic change exists (mutation or exchange of genes with another bacterium).

In laboratory conditions, bacterial cultures are grown in the presence or absence of antimicrobials and conclusions on the effect of an antimicrobial are drawn based on the isolates that grow on the culture plates. When an isolate grows in a media that contains antimicrobials it *does not* imply that the isolate transformed, but rather that an isolate that had resistance determinants survived in that medium. Although studies under laboratory conditions are the best proxies available for now, conclusions cannot be indiscriminately extrapolated to field conditions because of the action of the surrounding environment on bacteria: presence of specific nutrients, environmental conditions (*i.e.,* oxygen, host defenses) and competition with symbiotic bacteria.

Due to the inconsistencies in the association between antimicrobial treatment in dairy cattle and antimicrobial resistance in enteric bacteria isolated from these cattle, and the impossibility of proving the absence of resistance before antimicrobials were used, we cannot conclude that antimicrobial-use is a *cause* of resistance. This does not imply "no *association*" between frequencies of isolation of resistant bacteria and antimicrobial-use. An association exists due to shifts in populations of susceptible and resistant bacteria when antimicrobials are used; susceptible bacteria tend to succumb while resistant bacteria tend to multiply, which translate into a larger proportion of resistant bacteria in the overall population and therefore, higher probabilities of isolation. Additionally, it has been shown that, under laboratory conditions, bacterial cultures may experience high mutation rates in the presence of fluoroquinolones.[75,76] The importance of this feature in field conditions has not been evaluated.

Further research needs to study additional factors that can help explain the ecology of antimicrobial resistance of enteric bacteria in dairy cattle, including a possible effect of the daily interaction with man. Dairy farms are open systems that require daily traffic of vehicles and personnel that have access to other environments where they can be exposed to resistant bacteria that can be introduced to the dairy. It is possible that man could act as a reservoir of bacteria with resistance determinants or simply as a 'fomite' to transfer resistant bacteria from one place to another.

CONCLUSIONS

- The effect of antimicrobial treatment on resistance is best evaluated by studying exposure of animals and resistance of enteric bacteria to a single antimicrobial.
- Antimicrobial treatment with select antimicrobials was associated with a *higher probability* of isolating *E. coli* resistant to those antimicrobials in calves or cows treated less than 2 weeks before sampling.
- Factors such as calf age, production group in cows, lactation number and sampling event significantly affected the risk of isolation, resistance and MDR among *E. coli* and *Salmonella* isolates.
- Due to scarce data, no conclusions can be drawn on the effect of antimicrobial treatment on resistance of *Salmonella* isolates. If this evaluation is to be pursued, the naturally low prevalence of *Salmonella* isolation in healthy dairy cattle (3-10%) needs to be accounted for during sample size calculation.

- In general, enteric bacteria isolates recovered from feed and water samples may not be representative of the isolates recovered from feces.
- Isolates of enteric bacteria from flush water samples closely resembled fecal isolates. To evaluate antimicrobial-use as a risk factor for resistance, exposure to antimicrobials in individual animals needs to be established. Thus, flush water samples would not be a good substitute for fecal samples in this kind of study.

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Chapter 6

USE OF A SIMULATION MODEL TO EVALUATE SAMPLING STRATEGIES FOR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN NON–TYPE-SPECIFIC *ESCHERICHIA COLI* ISOLATED FROM DAIRY COWS

ABSTRACT

Objective - To evaluate various sampling strategies potentially used to measure prevalence of antimicrobial susceptibility in cattle.

Sample Population - 500 isolates of non–type-specific *Escherichia coli* (NTSEC) isolated from the feces of 50 cows from two dairy farms (25 cows/farm and 10 isolates/cow).

Procedure - Diameters of inhibition zones for 12 antimicrobials were analyzed to estimate variation among isolates, cows and farms; and then used as sampling distributions for a stochastic simulation model to evaluate four sampling strategies. These theoretic sampling strategies used a total of 100 isolates in four allocations (1 isolate from 100 cows, two isolates from 50 cows, three isolates from 33 cows, or four isolates from 25 cows).

Results - Analysis of variance composition revealed that 74.2% of variation was attributable to isolates, 18.5% to cows and 7.3% to farms. Analysis of results of simulations suggested that when most of the variance was attributable to differences among isolates within a cow, culturing 1 isolate from each of 100 cows underestimated overall prevalence, compared with results for culturing more isolates per cow from fewer cows. When variance was not primarily attributable to differences among isolates, all four sampling strategies yielded similar results.

Conclusions and Clinical Relevance - It is not always possible to predict the hierarchical level at which clustering will have its greatest impact on observed susceptibility distributions. Results suggested that sampling strategies that use testing of three or four isolates/cow from a representative sample of all animals better characterize herd prevalence of antimicrobial resistance impacted by clustering.

Abbreviations

NTSEC	Non–type-specific *Escherichia coli*
CLSI	Clinical and Laboratory Standards Institute

INTRODUCTION

Antimicrobial resistance has been the subject of intense study by microbiologists, veterinarians, and physicians throughout the world.[1,2] The importance of antimicrobial resistance is in the increased cost and difficulty of treatment and the reduction of options for treatment in patients that are infected with antimicrobial-resistant pathogens, with a consequent increase in risk of transmission of the disease and deterioration of health of infected patients (including death). Many experts have suggested that evolving antimicrobial resistance and zoonotic transmission of resistant bacteria in food is a direct result of antimicrobial use in animal agriculture that is less than optimal. This view is especially prevalent regarding antimicrobial use for animals raised in intensive confinement operations, such as large dairy farms and cattle feedlots. Resistant bacteria may be found in some raw foods of animal origin, but proper processing and safe food handling procedures can prevent cross-contamination, and thorough cooking destroys known pathogens.[3]

Because of the aforementioned concerns, research and surveillance related to antimicrobial susceptibility in bacteria recovered from food-producing animals have greatly intensified worldwide during the past decade.[2,4-11] These monitoring systems have typically focused on zoonotic agents that are considered important in food-borne illness of humans. Although NTSEC is a highly variable commensal organism of many animal species and is not considered a primary pathogen, it is closely related to *Salmonella enterica* and is easily isolated from feces of humans and other animals. Many of these

research and surveillance efforts have used sampling strategies that characterized susceptibility of NTSEC or other bacteria in only one isolate from a large number of animals as a method for characterizing antimicrobial susceptibility in herds or larger populations.[2,4-6,11] Although collecting samples from larger numbers of animals enhances the ability to characterize between-animal and between-group variability in susceptibility, it ignores the potential for variability among isolates that can be cultured from the same animal.

For example, it has been estimated that ruminant intestinal contents or feces can contain up to 10^{12} viable bacterial cells/g from as many as 200 species of bacteria.[12-14] By use of the common strategy of characterizing a single isolate for each animal, it is clearly not possible to investigate diversity in susceptibility that may be present among isolates within specific animals, nor is it possible to investigate how this variability impacts the overall risks related to evolution or spread of antimicrobial resistance. The same way that a farm represents a population or cluster of animals that requires collection of samples from several animals to accurately represent the farm, a cow or any other animal represents a population or cluster of resident bacteria, and it seems logical that collecting a sample of only a single bacterium would not accurately represent the entire cluster.

Furthermore, collecting samples from a large number of animals may be logistically and economically less desirable, especially when monitoring animals longitudinally over time. It is highly desirable to optimize sampling strategies to allow characterization of the true diversity in antimicrobial susceptibility among microbial populations at various levels of animal population hierarchies as well as to maximize logistic efficiency.

It has been suggested[15-19] that evaluating a single isolate for each animal may not be an appropriate sampling strategy because of potential clustering of antimicrobial-resistant bacteria within specific animals or groups of animals. Investigators of these studies suggested that > 1 isolate/sample is needed to best represent the population of bacterial flora. However, we are not aware of any published reports of formal investigations into the specific number of isolates that should be tested per animal. The objective of the study reported here was to evaluate various sampling strategies that could be used to investigate antimicrobial susceptibility in dairy cattle.

MATERIALS AND METHODS

Sample Population

Samples collected for a parallel study were used as the field data for this study. Twenty-five lactating cows were selected as a convenience sample from the highest production groups on each of two large dairy farms in northern Colorado. The farms milked 400 and 900 cows, respectively. Feces were manually collected directly from the rectum of each cow by use of a new plastic sleeve; approximately 25 g of feces were placed in sterile 50-mL conical tubes.[1] Fecal samples were placed on ice and transported to a microbiology laboratory for processing; samples were processed within two hours after collection.

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[1] Corning® 50mL PP Centrifuge Tubes, Conical Bottom with Flat Top Cap (Cat. No. 430829), Corning Life Sciences, Corning, NY.

Experimental Procedure

Isolates of NTSEC recovered from feces of cows on two dairy farms were characterized with regard to antimicrobial susceptibility. These data were analyzed to evaluate the magnitude of variation at three levels of population hierarchy (among isolates, cows, and herds). Distributions of diameters of inhibition zones were then used as sampling distributions for a stochastic simulation model designed to evaluate various sampling strategies that could be used when investigating antimicrobial resistance in dairy herds.

Culture and Antimicrobial Susceptibility Testing

Fecal samples were processed for culture and isolation of NTSEC within one hour of arrival to the laboratory. Briefly, a sterile swab was used to inoculate a small amount of feces onto MacConkey agar plates,[2] which was then streaked for isolation. Plates were incubated in aerobic conditions for 12 to 18 hours at 37°C and then examined to identify growth of NTSEC. A presumptive identification of NTSEC was assigned when colonies were 1 to 2 mm in diameter, dark pink, uniform in color, flat with smooth margins, and had a positive indole reaction when tested. A total of 10 isolates from each fecal culture were arbitrarily selected for further characterization.

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[2] BD BBL MacConkey agar (Cat. No. 221270), Becton Dickinson, Franklin Lakes, NJ.

Antimicrobial susceptibility testing was conducted by use of the agar disc diffusion method in accordance with guidelines published[20] by the CLSI (formerly known as the National Committee for Clinical Laboratory Standards). The selected isolates were streaked onto Muller-Hinton agar plates[3] and incubated in aerobic conditions for 12 to 18 hours at 37°C. Isolates were suspended in saline (0.9% NaCl) solution so that the turbidity was equivalent to that of a 0.5 McFarland standard and streaked again onto 150-mm Muller-Hinton agar plates. Discs containing standardized concentrations of 12 antimicrobial drugs (ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, enrofloxacin, florfenicol, gentamicin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim-sulfamethoxazole) were applied to the plates by use of an automated dispenser; plates were then incubated in aerobic conditions for 12 to 18 hours at 35°C. Diameter of the inhibition zones was measured and recorded by use of a computerized image capture and analysis system.[4] Susceptibility of isolates to antimicrobial drugs was categorized (susceptible, intermediate, or resistant) by use of inhibition zone measurements in accordance with interpretive methods and breakpoints established by CLSI guidelines.[20] When no veterinary breakpoint recommendations were available, breakpoints were extrapolated from available CLSI recommendations of interpretive criteria for humans.

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[3] BD BBL Hinton II agar (Cat. No. 221800), Becton Dickinson, Franklin Lakes, NJ.

[4] BIOMIC, Giles Scientific, New York, NY.

Data Analysis

Statistical software[5] was used to perform all statistical analyses, including descriptive statistics. A nested ANOVA was used to analyze field data to determine the composition of the variance for three (isolate, cow, and herd) and two (isolate and cow by combining data from both dairy farms) levels of clustering, as described elsewhere.[21] This analysis of the composition of the variance estimated the amount that each level of clustering contributed to the total variation of diameters of the inhibition zones. Only main effects were considered in this model; potential interactions of main effects were not analyzed.

Stochastic Simulation Modeling

Stochastic modeling was used to simulate distributions of antimicrobial susceptibilities that could be obtained when sampling microbial populations by use of four sampling strategies. For the purpose of the study reported here, a theoretic herd of 1,000 dairy cows was the assumed reference population from which 100 isolates were randomly selected and tested. The four sampling strategies included testing one isolate from each of 100 cows, two isolates from each of 50 cows, three isolates from each of 33 cows, or four isolates from each of 25 cows.

A spreadsheet computer program[6] with a third-party stochastic modeling software[7] add-in was used to develop the model. Empiric data obtained from the field portion of this study

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[5] MINITAB release 13.1, Minitab Inc, State College, Pa.

[6] Microsoft Excel, Microsoft Corp, Redmond, Wash.

[7] @Risk, Palisade Corp, Newfield, NY.

were used to identify a most probable distribution for inhibition zones for the sampling distributions in the simulation modeling. Susceptibility data for isolates cultured from all cows on both farms were combined for this purpose. A lognormal distribution was determined to most closely fit the observed distributions of inhibition zones, and the empiric means and SD values were used to define the sampling distributions for the model.

The model initially simulated one value for each cow, which was assumed to be the mean diameter for the inhibition zones for isolates cultured from that cow when evaluated for susceptibility against a specific drug. This value was estimated by simulating a lognormal distribution with the mean equal to that of the empiric data and the SD adjusted on the basis of the proportion of the total variance that is attributable to differences among cows. The following equation was used:

$$ZD_c = \text{lognormal}\ (\bar{x},\ \sqrt{\sigma^2 \cdot p_c})$$

where ZD_c represents the mean diameter of inhibition zones for isolates obtained from a specific cow, $\bar{x}$ is the empiric mean diameter of inhibition zones relative to a specific drug, σ^2 is the empiric variance of diameters of inhibition zones as estimated from field data, and p_c is the assumed proportion of the variance that is attributable to differences among cows. This simulated mean diameter of the inhibition zones was then used to simulate diameters of inhibition zones for the remainder of the isolates obtained from a specific cow by use of the following equation:

$$ZD_i = \text{lognormal}\ (ZD_c, \sqrt{\sigma^2 \cdot p_i})$$

where ZD_i represents the simulated diameter of the inhibition zone for a specific isolate, and p_i is the assumed proportion of the variance that is attributable to differences among isolates within a cow.

The model simulated all values for the diameters of inhibition zones for the testing of one isolate from each of the 100 cows. These values were used as isolate No. 1 for all other strategies. Then, the model simulated new values for the diameters of inhibition zones for isolate No. 2 when two isolates from each of 50 cows were tested. When testing three isolates from each of 33 cows, values simulated before were used as isolates Nos. 1 and 2, and new values were simulated for isolate No. 3. This mimicked actual laboratory conditions in which testing of multiple isolates would be performed by testing new isolates and combining it with data already obtained from testing fewer isolates.

Once the values for the inhibition zones for all isolates in the various sampling strategies were simulated, susceptibility to antimicrobial drugs was categorized on the basis of measurements of inhibition zones obtained by use of CLSI interpretive guidelines (resistant or not resistant [susceptible and intermediate combined]).[20] The simulated herd prevalence of resistance to the various antimicrobial drugs among the simulated isolates was calculated for each sampling strategy by dividing the number of isolates resistant to each specific drug by the total number of isolates (ie, 100 total isolates).

For the purpose of the study reported here, simulations were conducted for resistance to tetracycline and cephalothin. These antimicrobials were chosen because they had the highest resistance prevalence in the field data (7.1% and 3.0%, respectively). For each of the four sampling strategies, simulations for each antimicrobial were performed by use of three scenarios for the between-cow variance component (assuming the value for p_c was 85%, 50%, and 15% of the total variance). These scenarios were chosen to study the effect of variance among cows on the outcome of number of isolates needed to estimate herd prevalence. Five thousand iterations were performed for each simulation. Differences in mean herd prevalence were evaluated among sampling strategies within each scenario by use of a standard Z test for difference in proportions with significance set at values of $P < 0.05$.

RESULTS

Field Data

A total of 500 NTSEC isolates were cultured, and 495 were evaluated for antimicrobial susceptibility (data on 5 isolates were not available after isolation and archiving). Most of the 495 isolates were susceptible to all or most of the antimicrobial drugs tested. Among the 495 isolates evaluated, 441 (89.1%) were susceptible to all 12 drugs, 41 (8.3%) were resistant to one drug, 10 (2.0%) were resistant to two drugs, two (0.4%) were resistant to three drugs, and one (0.2%) was resistant to four drugs (Table 6.1). For cephalothin and tetracycline, it was notable that most of the isolates were classified as having intermediate susceptibility. None of the isolates were resistant to chloramphenicol, enrofloxacin, florfenicol, or gentamicin.

Table 6.1 Results of antimicrobial susceptibility testing of 495 NTSEC isolates and the proportional variance in diameter of inhibition zones attributable to various hierarchic levels of the population structure (ie, clustering).

	Diameter of inhibition zone (mm)		Proportion of variance in diameter of inhibition zones attributable to level of the population structure (%)			Susceptibility of isolates, by CLSI categorization (%)		
Antimicrobial	Mean	SEM	Farm	Cow	Isolate	Susceptible	Intermediate	Resistant
Ampicillin	17.7	0.08	0	15.9	84.1	51.7	47.3	1
Cefoxitin	22.7	0.09	5.5	50.4	44.1	98	0	2
Ceftiofur	25.8	0.08	12.4	33.7	53.9	99.6	0.2	0.2
Cephalothin	15.7	0.07	4.2	17.9	77.9	3	94	3
Chloramphenicol	24.1	0.08	6.7	11.3	81.9	99.4	0.6	0
Enrofloxacin	28.6	0.08	3.6	15.7	80.7	100	0	0
Florfenicol	24.4	0.09	0	18.1	81.9	99.6	0.4	0
Gentamicin	21	0.07	19.4	13.7	67	99.8	0.2	0
Streptomycin	17.7	0.07	10.5	1.2	88.3	93.5	6.1	0.4
Sulfamethoxazole	20.7	0.15	9.6	13.6	76.9	93.1	4.7	2.2
Tetracycline	17.7	0.13	4.2	8.9	86.9	11.3	81.6	7.1
Trimethoprim-sulfamethoxazole	27	0.1	8.9	8.2	82.9	99.6	0	0.4

Analysis of the composition of the variance for the diameter of the inhibition zone revealed that overall antimicrobial susceptibility varied most among isolates (75.5% of all variance in susceptibility) and less among cows (17.4% of variance) or between farms (7.1% of variance). However, there were exceptions to this generality, and for some antimicrobials (eg, ceftiofur, gentamicin and streptomycin), the proportion of the variance attributable to the farm was > 10% (Table 6.1). Additionally, for cefoxitin and ceftiofur, the variance among cows was greater than the variance among isolates. When data for both farms were combined, the composition of the variance that resulted was 78.2% among isolates and 21.8% among cows.

Model Simulations

Comparison of the four sampling strategies revealed that when most of the variance of the diameter of inhibition zone for tetracycline was assumed to be attributable to differences among isolates within a cow (p_c = 15% and p_i = 85%), obtaining one isolate from each of 100 cows greatly underestimated herd prevalence of antimicrobial resistance (Figure 6.1; Table 6.2). Mean herd prevalence estimated by use of this sampling strategy was 1.3%, which was significantly ($P < 0.001$) less than the estimates of ≥ 3.8% when > 1 isolate/cow were tested. Maximum estimated herd prevalence by use of one sample from each of 100 cows (5.5%) was significantly ($P < 0.001$) less than estimates for the other three sampling strategies (>12.9%). Similarly, evaluating two isolates from each of 50 cows also underestimated the mean and maximum simulated herd prevalence, but to a lesser degree. This sampling strategy estimated a mean herd

Figure 6.1 Frequency distributions for results of simulated herd prevalence of antimicrobial-resistant NTSEC against tetracycline for four sampling strategies (1 isolate from each of 100 cows [solid line], two isolates from each of 50 cows [solid line with white circles], three isolates from each of 33 cows [dashed-and-dotted line], and four isolates from 25 cows [solid line with black squares]) when assuming 85% of the variance in diameters of inhibition zones was attributable to differences among isolates within a cow (n = 5,000 iterations for each sampling strategy).

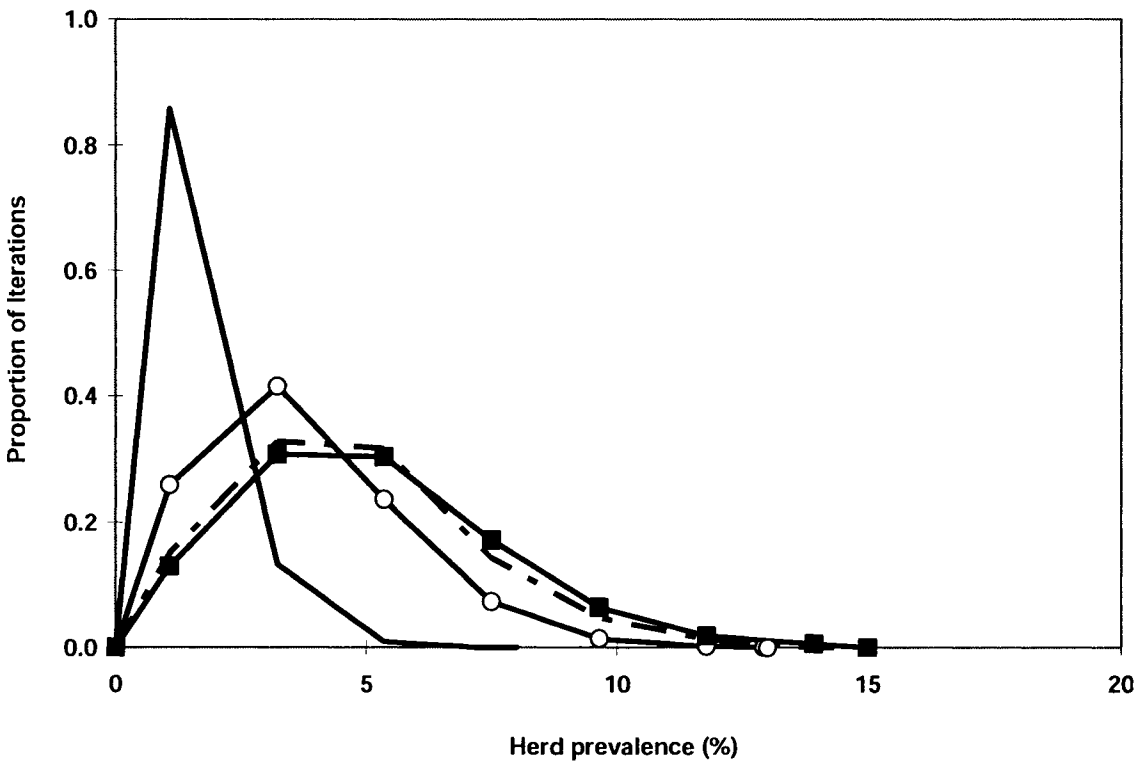


Table 6.2 Mean ± SEM values for the percentage of resistance obtained by use of simulation models (n = 5,000 iterations) for tetracycline and cephalothin among NTSEC isolates selected by use of four sampling strategies, on the basis of the assumed proportional variance in diameters of inhibition zones among isolates within a cow.

Sampling strategy	*n*	Tetracycline			Cephalothin		
		15%	50%	85%	15%	50%	85%
1 isolate from each of 100 cows	100	6.1 ± 0.02	3.9 ± 0.02*	1.3 ± 0.01*	8.2 ± 0.03	7.1 ± 0.03	4.9 ± 0.10†
2 isolates from each of 50 cows	100	6.2 ± 0.03	5.4 ± 0.03	3.8 ± 0.02*	7.6 ± 0.03	7.2 ± 0.03	6.0 ± 0.14
3 isolates from each of 33 cows	99	6.3 ± 0.03	5.8 ± 0.03	4.7 ± 0.02	7.4 ± 0.04	7.2 ± 0.03	6.4 ± 0.17
4 isolates from each of 25 cows	100	6.3 ± 0.04	6.0 ± 0.03	5.1 ± 0.02	7.3 ± 0.04	7.3 ± 0.03	6.6 ± 0.20

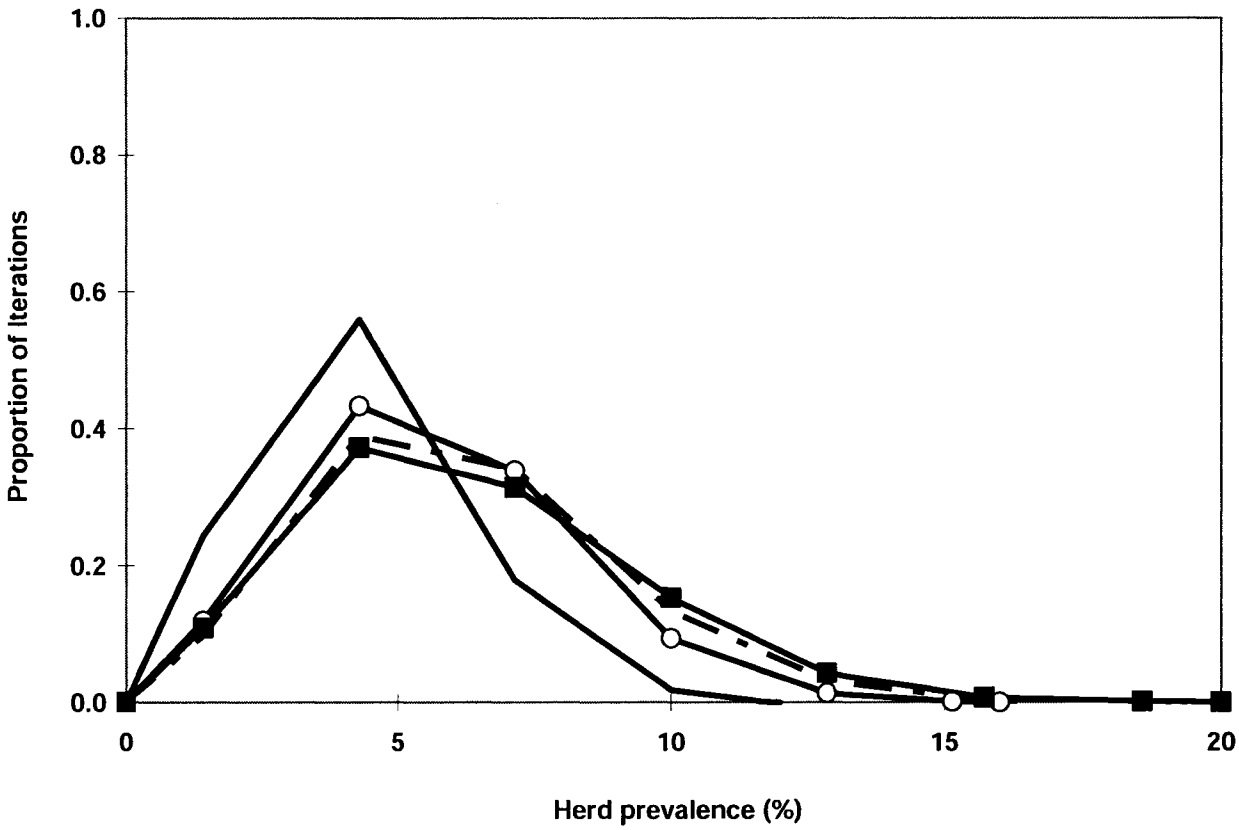
,†Within a column within an antimicrobial, indicates that the value differs significantly ($P < 0.001$; †$P = 0.009$) from the other values in the column.

prevalence of 3.8% of NTSEC isolates resistant to tetracycline, which was significantly ($P <$ 0.015) less than the estimate from the sampling strategies of 3 or 4 isolates/cow (4.7% and 5.1%, respectively). Maximum herd prevalence was estimated to be 12.9% when sampling two isolates/cow, compared with 13.8% and 13.9% when sampling 3 or 4 isolates/cow, respectively. There was not a significant ($P = 0.189$) difference between mean herd prevalence of estimated antimicrobial resistance when evaluating three isolates from 33 cows or four isolates from 25 cows.

When the amount of variability in susceptibility to tetracycline attributable to differences among cows was the same as the variability attributable to differences among isolates within a cow (p_c = 50% and p_i = 50%), the distributions of estimated herd prevalence were similar (Figure 6.2 and Table 6.2). Testing one isolate/cow under this assumption also significantly ($P < 0.001$) underestimated the mean herd prevalence of antimicrobial resistance (3.9%), compared with estimated mean herd prevalence when testing > 1 isolate/cow (≥ 5.4%). Maximum estimated prevalence with one isolate/cow (11.7%) was significantly ($P < 0.001$) less than the prevalence estimated by use of multiple isolates (≥ 15.1%).

When we assumed that p_c = 85% and p_i = 15%, the distribution of herd prevalence of antimicrobial resistance was very similar for all four sampling strategies and there were no significant differences. Cephalothin had a smaller variance and lower prevalence of resistance, compared to tetracycline. Thus, there was a difference in herd prevalence only when 85% of the variance was assumed to be attributable to differences among isolates within a cow (p_c = 15% and p_i = 85%). Again, testing one isolate/cow significantly ($P = 0.009$) underestimated the mean herd prevalence, compared with results when testing more isolates from fewer cows. However,

Figure 6.2 Frequency distributions for results of simulated herd prevalence of antimicrobial-resistant NTSEC against tetracycline for four sampling strategies when assuming 50% of the variance in diameters of inhibition zones was attributable to differences among isolates within a cow (n = 5,000 iterations for each sampling strategy). *See* Figure 6.1 for key.



for this assumption (p_c = 15% and p_i = 85%), testing two isolates from each of 50 cows yielded similar results to testing more isolates from fewer cows. There was no difference in the estimation of the mean herd prevalence among the four sampling strategies for scenarios of higher variation attributable to differences among cows (p_c = 85% and p_i = 15%). Maximum herd prevalence of antimicrobial resistance against cephalothin differed significantly ($P < 0.001$) among all sampling strategies, except when p_c = 50% and p_i = 50%. Minimum herd prevalences were very similar for all simulations.

Discussion

The objective of the study reported here was to evaluate various sampling strategies that could be used to investigate the prevalence of antimicrobial susceptibility in dairy cattle to determine the optimal sampling strategy by use of field data and stochastic modeling. Combining data from both dairy farms to identify source distributions for the simulation models increased the amount of information available for use in the modeling study, but it also diluted some of the variability.

Our results from the field data (overall prevalence of antimicrobial resistance and composition of the variance) are very similar to those from other studies[4,19,22] performed in cattle and pigs. This confirms the high variability in resistance patterns among antimicrobials, despite the fact that herd prevalence of antimicrobial resistance is consistent among these studies for most antimicrobials. Other studies[8,9,23-26] have revealed a higher prevalence of antimicrobial-resistant *Escherichia coli* in dairy cattle. Analysis of the field data revealed several resistance patterns among the cows. Antimicrobials for which at least one resistant isolate was found were those that have been widely used in the dairy industry, namely ampicillin, cephalothin, tetracycline,

streptomycin, sulfamethoxazole, and trimethoprim-sulfamethoxazole. Some of the newer antimicrobials (florfenicol and ceftiofur) used at dairy farms did not have resistance. Enrofloxacin and chloramphenicol, two antimicrobials not approved for use in dairy cattle, did not have any resistant isolates on either farm.

Results for the simulation model varied by antimicrobial compound and distribution of the variance among isolates and among cows. Sampling one isolate from each of 100 cows yielded results similar to those for sampling more isolates from fewer cows only when most of the variation was attributable to differences among cows. This was true both for tetracycline and cephalothin, and agrees with suggestions from other studies.[15-19] When the variance was equally distributed among cows and isolates (p_c = 50% and p_i = 50%), the model revealed that sampling one isolate from each of 100 cows yielded similar herd prevalence of antimicrobial resistance for cephalothin but not for tetracycline. We hypothesized that the smaller overall variance of the diameters of inhibition zones for cephalothin, compared with overall variance of the diameters of inhibition zones for tetracycline, was responsible for this effect. Larger variances cause a wider spread of prevalence estimates, therefore making potential differences more evident than would be possible with smaller variances. Both antimicrobials have the same cutoff value for resistance (diameter of inhibition zone, 14 mm), yet the variance for tetracycline (8.6 mm) was four times larger than that of cephalothin (2.2 mm). Prevalence of antimicrobial resistance from the field data was larger for tetracycline than for cephalothin.

For the purpose of the model described here, we assumed that the diameter of inhibition zones for tetracycline and cephalothin followed a lognormal distribution with mean and SD equal to those obtained from the field study. When data collected from other farms does not follow this

distribution, then this model would not be applicable. The model would need to be evaluated with various distributions to determine whether it will still yield similar results. We used data from only two farms, and the results of this model may vary when data from other dairy farms is used. However, the similarities between tetracycline and cephalothin for each scenario (p_i = 85%, 50%, or 15% of the variance attributable to differences among isolates within cows) are interesting. Use of data from two farms and combining it as if all the cows were from the same farm may have underestimated or overestimated the variances, because there were some disparities in the data obtained from the two dairy farms. One farm had a significantly ($P <$ 0.001) higher prevalence of resistance for cephalothin (25.2%), compared with results for the other farm (2.4%), and also had a significantly ($P < 0.001$) higher prevalence of intermediate susceptibility to ampicillin (20.8%), compared with results for the other farm (10.2%).

An alternative approach to estimate prevalence of antimicrobial resistance has been described in another study[13] in which investigators used spiral plates to count the number of isolates that grew at various antimicrobial concentrations. This approach implies a higher cost, because it uses multiple plates for each sample (1 plate for each concentration of each antimicrobial) and a spiral plating instrument. Spiral plating may be a more precise method of estimating the prevalence of antimicrobial resistance, but when spiral plating is not an available option, clinicians and researchers need to be able to establish an optimal sampling strategy to estimate herd prevalence of antimicrobial resistance.

In addition to providing a better estimate of the prevalence of antimicrobial resistance, testing more isolates from fewer animals should also be a less expensive sampling strategy. Overall the cost of susceptibility testing will not change, because the total sample size is the same. However,

costs for sample collection and initial culture will be minimized by collecting samples from fewer animals and then selecting multiple isolates from one culture. Obtaining samples from fewer animals will enable investigators to spend less time on each farm and therefore allow for the collection of samples from more farms in large-scale surveillance programs. Consequently, this will allow a broader representation of farms.

Ideally, sampling strategies should be customized for the antimicrobial to be tested and the population characteristics in terms of prevalence of resistance. To accomplish this, it would be necessary to collect data for this specific population during the period before the study to evaluate the composition of the variance and distribution. Because this would be cost prohibitive for most studies and it appears to be impractical, we conclude that it is best to establish a sampling strategy that will enable testing of 3 or 4 isolates/cow. The worst-case scenario would be when the variance is primarily attributable to differences among cows, which will have the same results as testing one isolate/cow. However, when the variance is primarily attributable to differences among isolates within a cow, testing multiple isolates from each cow will result in a better estimate of herd prevalence of antimicrobial resistance.

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Chapter 7

Conclusions And Recommendations For Future Study

It is a public health initiative to mitigate the emergence and amplification of antimicrobial resistance both in animals and people. It has been suggested that antimicrobial-use in food-animals is a major factor in the development of antimicrobial resistance that can cause health impacts in humans. However, prior to establishing an association between antimicrobial-use in food-animals and resistance in bacteria isolated from humans, a possible association with resistance in bacteria isolated from those animals needs to be demonstrated. The objective of this dissertation was to evaluate methods that can be used to study a possible relationship between antimicrobial-use in food-animals and the presence and spread of resistant bacteria in those animals. The complexity of this relationship is discussed, as well as the presence of interaction and confounding factors that must be identified to clarify true relationships.

Bacteria susceptible to an antimicrobial will die or cease multiplication in the presence of that specific antimicrobial. Antimicrobial-use may result in a skewed distribution of susceptible and resistant bacteria. Susceptible bacteria will die or not multiply while resistant bacteria will persist and potentially grow. This situation results in a higher

proportion of resistant bacteria, even if the same total number of resistant bacteria remains. For example, assume that initially there are 100 isolates and 10 of these (10%) are resistant. If antimicrobial-use results in the death of 80 of the 90 susceptible isolates, the final population includes 10 susceptible and 10 resistant isolates (10/20 = 50% resistance). Although this is a very simplistic example, it illustrates the mathematical change in prevalence of resistance that may occur during antimicrobial therapy. Resistant isolates are now more probable to be selected from a culture, simply because they represent a higher percentage of the total population.

To evaluate a possible relationship between a factor and an outcome, various steps need to be completed:

- The outcome variable needs to be defined and measured appropriately.
- The exposure variable needs to be defined and measured appropriately.
- Appropriate study designs need to be used.
- The existence of other explanatory variables needs to be evaluated and, if present, measured.
- Appropriate analysis methods need to be defined to evaluate the association between the exposure and the outcome variables.

In this study, the exposure is specified as antimicrobial-use in dairy cattle and the outcome is resistance in enteric bacteria isolated from those animals. Antimicrobial-use and resistance are not defined in a consistent manner throughout the literature and therefore need to be defined to allow data collection and measurement. This lack of a

refined definition may in part explain why, even after many years of research on antimicrobial resistance, it is still unclear what effect, if any, antimicrobial-use has on resistance in bacteria.

CONCLUSIONS

Definition and Measurement of Antimicrobial Resistance

Antimicrobial resistance is defined as the ability of organisms to grow and multiply in the presence of an antimicrobial. Resistance is not defined as growth in the presence vs. the absolute absence of an antimicrobial, but as growth in the presence of an antimicrobial above a specified concentration (breakpoint). The Clinical and Laboratory Standards Institute (CLSI, formerly known as the National Committee for Clinical Laboratory Standards or NCCLS) has developed guidelines for the detection of resistance in individual isolates of various bacterial species. These guidelines specify appropriate culture methods, resistance determination methods, breakpoints for resistance, and methods to interpret the results of established tests. These guidelines provide researchers with the necessary information to determine if a specific bacterial isolate is resistant to a given antimicrobial. However, no information is provided on how to measure resistance in a bacterial population.

Studies on antimicrobial resistance test a single isolate from each animal as representative of the bacterial population of that bacterial species within an animal. However, millions of bacteria can be found in only 1 g of feces. In the quest to evaluate whether

antimicrobial-use in food-animals is associated with resistance among enteric bacteria isolated from those animals, the information delivered by testing a single isolate from those animals may be an inaccurate reflection of the prevalence of resistance within the whole bacterial population. Testing a single isolate to represent the entire enteric flora is similar to sampling one cow per dairy to represent all animals in the dairy with regard to milk production.

Data Collection on Antimicrobial Resistance

Research related to antimicrobial susceptibility in enteric bacteria at the herd level usually represents successive sampling steps from the original population. In other words, a few animals are *sampled* from the whole herd and a *sample* of feces is taken from those animals. In the laboratory, a *sample* of the collected feces is cultured and a *sample* isolate is selected from the final culture to characterize susceptibility for the whole population of enteric bacteria in that animal. This process ignores the potential for variability among isolates that can be cultured from the same animal. The same way that a farm represents a population or cluster of animals, an animal represents a population or cluster of bacteria. Collection of samples from various animals is required to accurately represent the cow population within a farm, and therefore it seems logical that a single bacterium would not accurately represent the bacterial population within an animal. Some studies have suggested that more than one isolate per animal is needed due to the variability between isolates. However, none of these studies have determined how many isolates should be tested.

As reported in Chapter 6, multiple isolates were collected from the same cows to measure variability in resistance and determine how many isolates per animal would be required to estimate herd prevalence of antimicrobial resistance. High variability in zone diameters for resistance was found among these multiple isolates, mostly due to differences among isolates within a cow. A mathematical model was developed using these field data to compare estimates in herd prevalence of antimicrobial resistance when testing one or more isolates per cow. Results from this model suggested that testing antimicrobial susceptibility for 3 or 4 isolates per cow better characterized herd prevalence of antimicrobial resistance than testing a single isolate per cow. According to this model, it was also concluded that a minimum of 100 isolates were needed per group of animals to determine the accurate probabilities of detection of resistant isolates. Therefore, when a group of cattle is to be tested, the best estimation of herd prevalence of resistance would be attained by testing 3 isolates from 33 cows, or 4 isolates from 25 cows.

Definition and Measurement of Antimicrobial-Use

Currently, there are no established guidelines to measure antimicrobial-use, so researchers quantify antimicrobial-use in various ways. Some researchers quantified antimicrobial-use as total pounds of active ingredient *sold* in the U.S., while others have quantified it as total pounds of active ingredient *estimated to have been consumed* by food-animals in the U.S. Although these two measurements may be considered equivalent, they conceal some differences. For example, using an estimate of antimicrobials sold for veterinary use in the U.S. as an estimate for antimicrobial-use in food-animals assumes that all veterinary antimicrobials are used in food-animals.

However, some antimicrobials sold for veterinary use are only approved for use in pets or horses and are prohibited from being used in food-animals. Other antimicrobials may be used in various animal species other than food-animals, and therefore, estimating total use at the national level would over-estimate antimicrobial-use in food-animals.

Another method of quantifying antimicrobial-use is reporting total sales figures. The major problem with this approach, however, is that drugs with the same active ingredient may have different costs, depending on manufacturer and inactive ingredients. Many veterinary products used for pets and sport-animals have a higher price than products used for food-animals, and thus the distributions of sales figures may not correlate accurately to actual amount of product consumed. At this point, little is known about the actual exposure of any animal species to antimicrobials in the U.S.

In the U.S. there is no mandate to collect data on antimicrobial-use for any animal species. Furthermore, because federal laws permit the sale of certain antimicrobials without a prescription, evaluating prescriptions would not provide accurate quantification. These two facts make it impossible to estimate the amount of antimicrobials that is actually used in the food-animal industry in the U.S. today. Furthermore, the term "food-animal" includes multiple animal species such as cattle, small ruminants, pigs, poultry and fish. Each of these species has unique anatomy, physiology, management, nutrition, production cycle and economic considerations. These characteristics may influence the prevalence of resistance among enteric bacteria in distinctive ways that we cannot determine with the currently available data.

Currently, only a few estimates of national antimicrobial-use in food-animals exist in the U.S. These estimates show major differences, as shown in Chapter 3. In spite of the sparse data available on antimicrobial-use, it is evident that it is unevenly distributed among different antimicrobials, animal species, production purposes, intent (therapeutic vs. non-therapeutic use), and extent of use. Still, the actual distribution among these subpopulations is unknown, especially for antimicrobials that are approved for use in multiple animal species.

Measurements of antimicrobial-use in different studies cannot be compared due to the lack of data at the individual animal level or the lack of an agreed upon standard measurement. Some standard measurements have been suggested based on the human literature. However, these measurements cannot be used to evaluate a potential association between antimicrobial-use and antimicrobial resistance. The proposed standard measurements reflect *average* antimicrobial-use in a group of animals. The large variation in body weights between young stock and adult cattle accentuates the difference between actual and *average* antimicrobial-use. Therefore, average measurements could fail to detect an association at the individual animal level. For example, suppose a high dose of a given antimicrobial were associated with a higher prevalence of resistance in the animals where it is used. If only a few animals in a group had received a high dose and none of the other animals were treated, using an *average* measurement across the group would assume that *all* animals in the group were treated with an *average* dose. This assumption could result in two flawed conclusions. First, it could fail to show the supposed association with the high dose. Additionally, if the analysis were to show an association, it would suggest that an *average* dose was associated with resistance, rather

than a high dose in certain animals. These flawed conclusions could be accentuated if the various animals have different body weights (young stock *vs.* adult animals).

Data Collection on Antimicrobial-Use

As detailed above, trying to establish a relationship between data collected at different aggregate levels may result in false conclusions. Data can always be grouped later and results of aggregate data analysis can be compared to those of individual data analysis. If, for example, the aggregate analyses replicate the results of individual analyses, we will be able to determine that there may not be interaction or confounding factors at a specific level. However, failing to analyze the data at the individual animal level will prevent identifying other possible explanatory factors.

Trying to establish a relationship between total antimicrobial-use in the U.S. and resistance of individual bacterial isolates would be comparable to trying to establish a relationship between the national education budget (exposure) and high school student grades (outcome):

- The education budget is distributed across different grade levels, rather than high school alone. Similarly, antimicrobials are used in different animal species, and different production purposes.

- Multiple factors affect academic performance, and these factors vary from grade school, middle school, and high school students. Similarly, multiple factors have

been shown to be associated with varying resistance levels among the different animal species.

- Investments into a high school program may not have the same effect on students early in the investment period compared to years later, at the end of or after the investment period. Similarly, the association of the exposure and the outcome may vary according to the amount of time elapsed since exposure. Antimicrobial-use provides a temporary environment in which resistant bacteria have a survival advantage. However, this advantage may be lost after the antimicrobials are withdrawn.

Because of these problems associated with aggregate data, to test the hypothesis of an association between antimicrobial-use and resistance, it is necessary that antimicrobial-use data be recorded in as much detail as possible at the individual animal level. In dairy cattle, treatments are administered primarily by the producer, and therefore the record system is dependant on producer compliance. Data collection methods need to record detailed data in a uniform, efficient, and uncomplicated manner. Individual animal data on antimicrobial-use would overcome the aforementioned problems of standard measurements.

In our study, which was focused only on dairy cattle, two data collection methods for antimicrobial-use were compared at a single commercial dairy farm in Northern Colorado. The single most important characteristic required in a data collection method used by dairy personnel is time-efficiency; data need to be collected without interfering with their daily activities. During our study, we learned that detailed data collection on

antimicrobial-use in dairy cattle is feasible without disruption of the normal farm activities. However, comparison of handwritten and computerized records showed that commonly used dairy management software is not currently an option to gather uniform high quality data on antimicrobial-use in the U.S. At this time, no uniform and specific record field is available in the many software packages on the market. Designated record fields would need to be created by participating software manufacturers to record treatment. Handwritten treatment logs for each area of the dairy farm may be the best option available at this point until appropriate software or record fields can be developed.

Study Design

Previous studies have attempted to assess the effect of antimicrobial-use in animals on resistance in enteric bacteria isolated from those animals. These studies lack consistency in the methodology used, making comparisons between these studies and extrapolations difficult.

Most published studies on antimicrobial resistance are cross-sectional studies, where data are collected at one point in time. However, antimicrobial resistance is a dynamic situation where a one time measurement does not accurately reflect the dynamic dimension. Different resistance levels can be found when sampling 1 or 10 days after antimicrobial treatment. Therefore, to evaluate the effect of antimicrobial-use on resistance, it is necessary to perform longitudinal studies. These studies will also allow evaluating possible long-term effects, if any, that antimicrobial-use in animals may exert on resistance in bacteria.

In our study two sampling strategies for the collection of feces from dairy cattle were evaluated in their ability to produce similar results in frequencies of recovery of susceptible, resistant, and multi-drug resistant (MDR) isolates of *Salmonella* and *E. coli*. A longitudinal cohort sampling strategy was devised comparing a group of animals treated prior to the first sampling event and a non-treated control group. The normal dynamics of a dairy resulted in large attrition of the cohort groups during the study (culling, transfer to a calf ranch, or treatment). This attrition of sampled animals resulted in lower power of statistical analyses towards the end of the study. Additionally, animals within the cohort group tended to be clustered in specific pens throughout the study, so some pens were under-represented. The other sampling strategy that was evaluated was stratified random sampling, where fecal samples were collected from randomly selected animals within the different pens. This design represented the naturally existing strata of a dairy farm because animals are grouped according to age and production, allowing the uniform representation of each pen throughout the study. Stratified random sampling resulted in a consistent representation of animals that had been recently treated with an antimicrobial. Additionally, random sampling was more efficient than cohort sampling: less time was needed to collect samples from random animals, and farm routines were not altered to accommodate sampling. Due to these advantages, and the fact that similar frequencies of isolation, resistance, and MDR isolates were obtained for both sampling methods, it was concluded that stratified random sampling is a better option for conducting a large scale study across the U.S.

Smaller scale longitudinal cohort studies can be conducted to evaluate any short-term effects of antimicrobial-use over time. For this type of study, individual animals would

need to be sampled repeatedly over time, and therefore random sampling would not be appropriate and a longitudinal cohort study would be required. These studies should start by measuring a baseline of resistance. In other words, resistance prevalence prior to any antimicrobial exposure should be determined. Then, resistance prevalence can be evaluated regularly (*e.g.* daily) as antimicrobial treatment is implemented. Measurement of resistance should be continued after the antimicrobials are withdrawn to determine if prevalence of resistance returns to baseline levels from its expected increase due to selection of resistant bacteria. Based on data from this study and previous studies, a period of two weeks after cessation of antimicrobial-use should be sufficient to return to baseline levels. If baseline levels are not regained, acquisition of new resistance would be suggested.

In this study antimicrobial susceptibility was tested for 16 antimicrobials for two bacterial species (*Salmonella* and *E. coli*) isolated from calves and cows. This resulted in 64 possible outcomes to be studied. The large number of outcomes complicated the statistical analysis of the data. We conclude that more focused studies should be performed, studying resistance and antimicrobial-use of only one or two antimicrobials. Additionally, the naturally low prevalence of certain bacteria such as *Salmonella* spp. in healthy dairy cattle (3-10%) needs to be accounted for during sample size calculation and study design. Therefore, if resistance in *Salmonella* is the objective of the study, a much larger sample size would need to be projected. In our study, this low prevalence resulted in low power in many statistical analyses, and the inability to draw many conclusions.

Evaluation of Interaction and Confounding Factors

It has been shown with *in vitro* studies that resistance genes can be transmitted horizontally between bacteria. Therefore resistance could be conceptualized as a contagious disease that can be transmitted between bacteria within a specific animal and bacteria in different animals. If antimicrobial resistance follows contagious disease dynamics, transmission rates between animals and between bacteria need to be considered (accounting for possible contagious contacts) when studying the epidemiology of resistance. Therefore, the effect of antimicrobial-use on resistance may be confounded not only by factors that act at the individual level, but also by factors at higher aggregation levels (contagious contacts between individuals of different populations).

Population factors: biosecurity and biocontainment

Control of introduction and spread of resistance determinants on the dairy farm can be accomplished by establishing effective biosecurity and biocontainment measures. The most important feature of a good biosecurity and biocontainment program is to accurately identify and prioritize critical control points. A graphical representation of the animal flow within a dairy farm, and in and out of the farm, will help identify practices that represent a risk for the introduction and spread of bacteria with new resistance determinants. Biosecurity and biocontainment protocols should be monitored and regularly reevaluated and updated. Regular reviews of the program will help identify opportunities for the development of new control methods or further refinement of

existing control methods to prevent introduction and spread of disease agents on the dairy farm.

Effective biosecurity and biocontainment protocols on the dairy may have prevented the spread of *Salmonella* and *E. coli* from cows to calves, as evidenced by the different serotypes and resistance patterns. Additionally, the results of this project suggest that transmission of resistance determinants between bacteria may not occur as easily and rapidly *in vivo* as it has been suggested based on *in vitro* studies.

Individual factors

Frequencies of isolation and resistance differed between *Salmonella* and *E. coli* obtained from calves and cows, showing four different signatures. In general, bacteria isolated from calves had a higher prevalence of resistance than those isolated from cows, and *Salmonella* isolates had a higher prevalence of resistance than *E. coli* isolates. These findings support the conclusion that calves and cows should be sampled as two distinct subpopulations of animals, and that conclusions in one subpopulation cannot be readily extrapolated to the other. Individual factors that influence these differences in calves and cows need to be elucidated. Factors such as calf age, production group (cows), lactation number (cows), and sampling event showed a significant effect on the risk of isolation, resistance and MDR among *E. coli* and *Salmonella* isolates during this study. Other factors that could have impacted the outcome but were not measured during this study include differences in nutrition, genetic makeup or immunosuppression due to subclinical chronic disease conditions.

In the calf area, *Salmonella* was isolated only from calves that were housed in hutches and not in grouped pens, even though the initial grouping is considered to be the most stressful time for young calves. *Salmonella* isolation only in hutched calves may point towards an effect of calf immunity/resistance to infections and/or personnel handling practices, because calves don't have direct contact with each other in the hutches but there is significantly more contact with personnel than in older calves. We did not identify each hutch with a specific code or number. Therefore we could not determine if all resistant isolates were cultured from the same hutches over time, or whether calves with resistant isolates were housed close to each other. Further studies may consider identifying individual hutches, to determine if a resistance is more likely in bacteria isolated from calves housed in hutches that had been occupied by other calves from which resistant isolates were obtained.

Environmental factors

Time at which samples were collected (sampling event) was an important factor associated with differences in antimicrobial resistance among isolates. The effect of sampling event cannot be readily explained by differences in ambient temperature, because more resistant isolates were recovered in calves during the first half of the study (Sep – Jan), while in cows more resistant isolates were recovered during the second half of the study (Mar – Jun). These results may be indicative of changes in other environmental factors between sampling events, such as wind and humidity. Whatever the explanation is for these results, it can be concluded that short-term and long-term studies may result in different conclusions if these factors are not identified.

In general, enteric bacterial isolates recovered from environmental samples (feed and drinking water) were not representative of isolates recovered from feces. The main reason for this discrepancy may be the timing of sample collection; environmental samples were collected the same day that fecal samples were collected. It is well established that material ingested by ruminants requires multiple days of transit time through the gastro-intestinal tract. Therefore, it is possible that if environmental samples were collected 2-4 days prior to fecal sample collection, the results may have been different. Further studies may consider comparing serial samples obtained from the environment and the animals to assess if any relationship exists.

Other factors

The potential 'traffic' of bacteria (resistant or not) cannot be assumed to be unidirectional from animals to humans. The interaction of animals and humans at any level has the potential of transferring bacteria in both directions: from animals to humans *and* from humans to animals. Dairy cattle come in contact with humans almost on a daily basis during milking, feeding, and handling for medical treatments. The presence of the bacteria with identical resistance patterns in humans and animals, when measured in a cross-sectional study, can only suggest a common exposure. However, the direction of this relationship cannot be established unless longitudinal studies are performed that begin with the absence of resistance in either humans or animals.

The external validity of findings in this study may be limited because it was performed at a single dairy to eliminate the possible effect of confounding factors across dairies (different personnel and management practices).

Statistical Analysis Methods

The analysis of data collected during a study needs to take into account the design of the study to correctly evaluate the relationship of the studied factors and outcomes. In longitudinal studies, where an animal is sampled multiple times, the isolates collected from this animal cannot be considered independent. This lack of independence must be included in the analysis. The crude analysis of the association of antimicrobial-use in dairy cattle with isolation of susceptible or resistant *Salmonella* and *E. coli* from fecal samples, set up as a simple 2x2 table, assumes that all isolates recovered from the same animal over time are independent of each other and that no other factors are part of this potential association. The appropriate statistical method for the data collected during our study was a hierarchical multivariate analysis. Hierarchical analysis accounted for non-independence of samples, while multivariate analysis allowed evaluation of potential confounding and interaction factors.

In our study, the results of the crude analysis of a possible association between antimicrobial treatment and resistance were compared to results of hierarchical multivariate analysis. Results of the crude analysis did not agree with those of hierarchical multivariate analysis. The assumption of independence among isolates collected from the same animal (fallacy in crude analysis) biased the results of the analyses away from the null hypothesis (no association between antimicrobial-use and isolation of resistant isolates). Therefore, assuming independence among isolates could result in erroneously concluding that antimicrobial-use in animals was associated with resistance in bacteria isolated from those animals. Hierarchical multivariate analyses also

showed that other factors such as age and sampling event in calves, and production group in cows were more strongly associated with the probability of isolation of *Salmonella* (susceptible, resistant or MDR) than antimicrobial treatment. These results may be biased due to recovery of few *Salmonella* isolates, showing lack of power to determine certain relationships. In spite of this potential problem due to low isolation, there was an extremely strong association of isolation of *Salmonella* with other variables such as location and age in calves.

The outcome variables of this project were dichotomized as either resistant or not (combining susceptible and intermediate isolates) due to the difficulty of analyzing and interpreting the meaning of serial dilution data (MIC) that had been truncated at different dilutions for the different antimicrobials. Truncation methods differed between the upper and lower limits of MIC, complicating statistical analyses. Further studies may consider using non-truncated MIC values or using the same truncation method on the upper and lower limits to allow better statistical analysis.

An association between antimicrobial-use and resistance was found inconsistently throughout the study. In general, a stronger association was observed when antimicrobial-use and resistance were evaluated for the same antimicrobials. In other words, use of tetracyclines for example was associated with resistance to tetracyclines, while it was not associated with resistance to other antimicrobials.

The Special Situation of Dairy Cattle

Antimicrobial-use in dairy cattle is heavily regulated by the government, probably because dairy cattle's main product (milk) is harvested on a daily basis. Few antimicrobials are approved for use in lactating dairy cows, while some more are approved for use in non-lactating dairy cattle (replacement heifers and dry cows). Therefore, dairy cattle are not exposed to as many antimicrobials as suggested for food-animals in general. Furthermore, each drug (antimicrobial or other) that is approved for use in dairy cattle has federally enforceable withholding periods before milk or meat can be designated for human consumption. Given the results of this study and others, where resistance levels returned to baseline levels within two weeks post-treatment, and the regulatory withholding periods, potential exposure of humans to resistant bacteria from treated cows should be less likely than direct exposure to antimicrobials, or direct contact with treated animals (mostly pets). Milk and meat from dairy cattle destined for human consumption originates from animals that have not received antimicrobials for some time, and the prevalence of resistant bacteria should have diminished from the time the animal was being treated. However, this hypothesis has not been tested.

Food for human consumption in the U.S. currently originates internationally and nationally. Livestock and produce are subject to different regulations according to their country of origin. Therefore, meat or milk available for human consumption in the U.S. may be from cattle exposed to antimicrobials not approved for use in food-animals in the U.S. The study of a possible association between antimicrobial-use in dairy cattle and

resistance in enteric bacteria isolated from humans, needs to be expanded to consider international sources.

RECOMMENDATIONS

Based on the results of this project, following is a set of recommendations for the development of a larger longitudinal study to evaluate the possible associations between antimicrobial-use in dairy cattle and resistance in enteric bacteria:

- Study calves and cows separately at each dairy.
- Test at least 100 isolates per herd or group by stratified random sampling.
- Test three or four isolates per animal for antimicrobial susceptibility. This entails testing 25 to 33 animals per herd or group.
- Establish a method to maintain accurate and detailed records on antimicrobial-use at the individual animal level.
- Record time elapsed since last treatment with an antimicrobial.
- To evaluate a possible association between antimicrobial-use in food-animals and resistance of enteric bacteria isolated from those animals a longitudinal study is preferable. Collect samples daily, starting before antimicrobial treatment and continuing for at least two weeks after treatment is discontinued.

- Collect information on management factors that can influence immunity, stress and direct contact with sick/stressed animals or personnel.

- Identify hutches with a number or code to be able to trace back a resistant isolate to a specific hutch and not just to the hutches in general.

- Study the resistance to a specific antimicrobial in relationship to the use of that same antimicrobial or one of the same family.

- To use MIC data based on serial dilutions, avoid truncation and measure all possible dilutions to exactly determine the MIC value for each isolate.

FUTURE DIRECTIONS FOR STUDY

In science, as some questions are answered, others arise. Most questions cannot be answered completely due to inadequate design, inherent variability and economic constraints on sample size. It has been suggested that in the exception you discover the rule. The answers obtained during this project have led to other questions that need to be explored to elucidate a possible long-term relationship between antimicrobial-use in dairy cattle and resistance in enteric bacteria isolated from dairy cattle.

- Consistent with findings of several other studies, the frequency of resistant isolates, both for *Salmonella* and *E. coli*, was highest in the youngest calves and decreased as the age of the calf increased. A hypothesis related to antimicrobial-use that could explain this situation would be that when newborns feed on colostrum they are actually ingesting residues of antimicrobials used for the prevention and treatment of intramammary infections during the dry period of cows. With the growing concerns on the perceived "extensive" use of antimicrobials in food-animals, new products have emerged to prevent intramammary infections during the dry period that do not contain antimicrobials. Comparison of the frequency of resistant isolates cultured in neonatal calves born from cows treated with or without intramammary antimicrobials at dry-off could help answer this question. Additional factors that could be studied in this relationship could be certain environmental factors, by separating calves from these two groups in different areas with no direct contact between them.

- This project focused on evaluating the effect of antimicrobials that had been administered by parenteral injection. There are other routes of administration for antimicrobials that could show different results in frequency of isolation of resistant enteric bacteria. It is not likely that intramammary treatments will show a significantly larger association with isolation of resistant enteric bacteria than parenteral antimicrobials, but oral antimicrobials may be different due to their direct contact with the digestive system. Another possible concern may be topical antimicrobials that are used on superficial infections (mainly on feet) because infections are commonly painful and/or itchy and the animal may tend to lick it. Therefore, although topical antimicrobials may not be easily absorbed through the skin, they may enter the digestive system when the animal licks the wound.

- A further step in the evaluation of antimicrobial-use in dairy cattle on resistance in enteric bacteria could address differences in doses by comparing administration of antimicrobials for preventive purposes with therapeutic administration. Preventive antimicrobials are usually administered orally to a group of animals in much lower concentrations compared to therapeutic injectable antimicrobials administered to those animals that are showing clinical signs of disease. A national study in Denmark has shown an increase of antimicrobial-use for therapeutic purposes after banning the use of antimicrobials for preventive purposes. The emerging question in this situation would be if sick animals treated to help fight an infection have a higher probability of recovery of resistant isolates compared to healthy animals that are administered an antimicrobial for the

prevention of disease. Different colonization probabilities in sick and healthy animals could explain this process.

- The previous point can be studied further by evaluating possible changes in the prevalence of antimicrobial resistant bacteria during the necessary withdrawal time that needs to be implemented when food-animals are treated with antimicrobials. Milk and meat from dairy cattle treated with antimicrobials cannot be used for human consumption until the specified withdrawal time has passed. When antimicrobials are administered to an animal, there is usually no "adaptation" period, i.e. the full dose is given from the initiation of the treatment regimen instead of an increasing dose over time (there may be some exceptions). However, when the treatment regimen is finished and no more antimicrobials are administered, antimicrobial levels in tissues will decrease gradually as they are metabolized and excreted. It would be of interest to see if and how the populations of resistant enteric bacteria change over during the withdrawal time.

- The mathematical model that evaluated sampling strategies for the number of isolates per animal to be collected was based on diameters of inhibition zones. Although this is still a valid and approved technique to characterize antimicrobial susceptibility, over the past few years testing via broth micro-dilution has become more widespread due to the potential for automation. Due to the inherent differences in the nature of the results between the two testing techniques (zone diameters are measured in millimeters and micro-dilution MICs are measured in serial dilutions), it would be of interest to replicate the model for data collected

with the broth micro-dilution method. Both models (zone diameter and micro-dilution) need to be validated, since mathematical models are only as valid as their assumptions and the external validity of the data.

- One of the most overlooked issues, in my opinion, in the relationship of antimicrobial resistance in bacteria isolated from food-animals and people is the direction of the relationship. Most published studies imply there is only one direction: from animal to man. Yet, the existence of 'beef measles" (*Taenia saginata*) in beef carcasses, proves fecal-oral contamination from human to animal. Therefore, the personnel on a dairy should always be considered as a potential source of resistance determinants.

- Because most veterinary products sold in the U.S. are used in companion animals, and given their close relationship with people, the study of use of antimicrobials in companion animals should be evaluated as a selection point for resistant isolates that can influence resistance in human isolates.