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Antimicrobial Susceptibility of Fecal *Escherichia coli* Isolates in Dairy Cows Following Systemic Treatment with Ceftiofur or Penicillin

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Wer nicht fragt, bleibt dumm!

Volker Ludwig (* 13. Juni 1937)

TABLE OF CONTENTS

ABBREVIATIONS

ATCC	American Type Culture Collection
AST	Antimicrobial Susceptibility Testing
CCFA	Ceftiofur crystalline-free acid
Cfu	Colony Forming Unit
CIPARS	The Canadian Integrated Program for Antimicrobial Resistance
DANMAP	Danish Programme for surveillance of antimicrobial resistance
DNA	Deoxyribonucleic adic
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ESBL	Extended spectrum beta-lactamase
HGMF	Hydrophobic grid membrane filter
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
PBP	Penicillin-binding protein
QC	Quality control
RNA	Ribonucleic acid
RS	Reduced susceptibility
S	Svedberg unit, physical unit of sedimentation

I INTRODUCTION

The discovery of antimicrobial agents at the beginning of the last century was undoubtedly one of the most important milestones in the history of human and veterinary medicine alike. Soon after the first antibiotic was found by Alexander Fleming, researchers all over the world came up with descriptions of new drug classes. Yet, the awareness of the existence of drug resistant bacteria is as old as the knowledge about the benefits of antimicrobial treatment. For decades after Fleming's discovery, this fact was deemed unimportant as new drugs became readily available any time an older drug was rendered incapable of action. In the last 30 years this attitude has changed due to the rising concerns about emergence and spread of resistance while the discovery of new classes of antimicrobial agents has slowed considerably. The study of resistance in bacteria has gained importance in many places and monitoring programs have been established both in human and veterinary medicine. The EU-wide ban of growth promoters in veterinary medicine was a direct outcome of the research focusing on antimicrobial resistance, as was the formulation of guidelines for prudent use of antimicrobial agents.

The present doctoral thesis reports on the effect of an antimicrobial agent important to the dairy industry (ceftiofur) on a model organism: *Escherichia coli*. This organism has proven to be ideal for screening studies since it is easy to culture and generally present in large numbers in animal feces. It is not only important in its own right because of the potential to serve as a source of resistance determinants for bacterial pathogens, but also mirrors the effect of drugs on other bacterial species such as *Salmonella*.

The objective of this work was to study the effect of ceftiofur on resistance of *E. coli* isolates obtained from dairy cow fecal samples. This was done by applying a screening method to large numbers of isolates in order to enable the investigators to pick up small effects that the drugs might have on the bacterial population and the epidemiology of antimicrobial resistance.

II LITERATURE OVERVIEW

1 Antimicrobial agents

Although many compounds used for treatment of bacterial disease in humans and animals are commonly referred to as antibiotics, the terms "antimicrobial agent" and "antibiotic" are not interchangeable. The term "antibiotic" refers to substances that are produced by microorganisms and that act on other microorganisms by selectively inhibiting their growth and even destroying them; it should not be used to indicate synthetic or semisynthetic compounds, substances of plant or animal origin, and substances active against animal cells (Guardabassi and Courvalin, 2006).

1.1 The discovery of antimicrobial drugs in the 20th century

At the beginning of the 20th century, the German chemist and physician Paul Ehrlich, was systematically testing chemical agents, searching for the "magic bullet" that could be used as a medicine to fight infection. This effort resulted only in a potentially toxic arsenic-based treatment for syphilis (Goldsworthy and McFarlane, 2002).

The discovery of penicillin and its publication in 1929 by the bacteriologist Alexander Fleming in London was a "fortunate occurrence" (after he left unclean culture plates with staphylococci in a corner while on holidays) as he described it in his 1945 Nobel prize lecture (Fleming, 1945). It is commonly considered as the start of antibiotic history, although Fleming himself considered lysozyme as the first antimicrobial agent he discovered, its antimicrobial action becoming clear to him when he accidentally sneezed on a Petri dish (Goldsworthy and McFarlane, 2002).

Interestingly, Fleming used penicillin as an additive to culture media for differentiation of bacteria. Its use as an antibiotic drug for the treatment of infections in humans was established only when Ernst B. Chain and Howard Florey took up the investigation twelve years later. They were awarded the Nobel Prize in Medicine together with Alexander Fleming.

Prior to widespread use of penicillin for disease treatment, another discovery earned a scientist the Nobel Prize in Medicine in 1939. Gerhard Domagk, a German pharmacologist, discovered that a dye used to tint cloth cured streptococcal infections in mice and the active compound sulfanilamide was identified shortly thereafter by Daniel Bovert, a Swiss-born

scientist (Khardori, 2006).

Francis F. Schwentker was the first to report successful treatment of meningococcal meningitis in humans by subcutaneous and intraspinal injection of sulfanilamide (Schwentker *et al*., 1937).

Another researcher contributed greatly to the beginnings of antibiotic history. Selman A. Waksman, a Russian-Jewish immigrant to the United States was investigating microorganisms when he came across a mold adhered to a clump of dirt, which had been taken from the neck of a sick chicken. When Waksman used the mold producing the antibiotic streptomycin against tubercle bacilli, the mold was able to kill the bacteria (Okonko *et al.*, 2008). In 1944, streptomycin was administered to a young woman who had advanced pulmonary tuberculosis and resulting in cure of the disease (Khardori, 2006). Waksman was awarded the Nobel Prize in Medicine in 1952.

Since then a multitude of antimicrobials have been discovered, most of which are produced by actinomycetes and bacteria. Nowadays, most antimicrobial agents are chemically altered. Today, some 5,000 antibiotics are known but only about 1,000 of these have been carefully investigated and about 100 are currently used to treat infections in humans (Okonko *et al*., 2008). The majority of antimicrobial drugs used in animals belong to a small number of major classes with the most important ones being beta-lactams, aminoglycosides, macrolides, lincosamides, chloramphenicol, tetracycline and sulfonamides. Only one class, namely fluoroquinolones, has been added in the last 30 years (Prescott, 2006).

The introduction of antimicrobial drugs in veterinary medicine was similar to that in human medicine although their use as growth promoters has marked one of the major differences. The enhancement of growth rates and improved efficiency of feed were first noted when pigs and poultry were fed waste derived from an antibiotic production plant (Giguère, 2006).

1.2 Antimicrobial mode of action

There are five major mechanisms of action of antimicrobial drugs: inhibition of cell wall synthesis, damage to cell membrane function, inhibition of nucleic acid synthesis or function, inhibition of protein synthesis, and inhibition of folic acid synthesis. A short description of each of these mechanisms is included below to review how antimicrobial drugs interfere with biological processes in bacteria, together with examples of important antimicrobials in veterinary medicine.

1) Inhibition of cell wall synthesis:

Multi-layered glycan and peptide strands form a peptidoglycan layer conferring mechanical protection and a solid surface for cell wall proteins (Guardabassi and Courvalin, 2006). It is considerably thicker in Gram-positive than in Gram-negative organisms where it is surrounded by an outer lipopolysaccharide layer, but it is also more permeable, allowing for larger molecules (such as beta-lactam antimicrobials) to pass (Tipper, 1985).

The cell wall synthesis happens in three consecutive steps, namely the formation of muramyl pentapeptide in the cytoplasmic phase, the translocation of muramyl pentapeptide through the cell membrane (membrane-associated phase) and the cross-linkage of glycan and peptide in the extracytoplamic phase (Guardabassi and Courvalin, 2006).

Interference with cell wall function leads to lysis of the cell resulting in death of the bacterium.

Beta-lactam drugs (e.g. penicillin or cephalosporins) target the enzymes responsible for joining glycan and peptide, the transpeptidases, better known as PBPs (penicillin-binding-proteins) (Tipper, 1985). Glycopeptides (e.g. vancomycin, avoparcin) also inhibit the extracytoplamic phase by making the pentapeptide precursor unavailable to the PBPs by binding to acyl-D-alanyl-D-alanine (Reynolds, 1989).

Bacitracin blocks the membrane-associated phase by forming a complex with the undecaprenyl pyrophosphate lipid carrier (Toscano and Storm, 1982).

2) Damage to cell membrane function

The cell membrane, which forms part of the cell wall of Gram-negative bacteria, acts as a sieve, and allows only small molecules to pass, in sharp contrast to the cell wall of Gram-positive bacteria (Decad and Nikaido, 1976). The polymyxins (e.g. polymyxin B, colistin) increase the permeability of the outer cell membrane of Gram-negative bacteria by binding to the lipopolysaccharides, destroying the barrier to noxious agents. Gram-positive cells are typically intrinsically resistant (Vaara, 2010).

Drugs belonging to the family of aminoglycosides (e.g. gentamicin, amikacin, neomycin, streptomycin) not only act on protein synthesis but also increase membrane

permeability by drug induced disruption of Mg^{2+}-bridges (Mingeot-Leclerq, 1999). The combination of those mechanisms is seen as the reason for bacteriocidal activity of these drugs since all other protein synthesis inhibitors only have bacteriostatic property (Jana and Deb, 2006).

3) Inhibition of nucleic acid synthesis or function

Fluoroquinolones (e.g. enrofloxacin, danofloxacin) target two enzymes that are essential for unzipping during replication and transcription and packing of DNA: topoisomerase II (DNA gyrase; an enzyme important for initiation of DNA replication) and topoisomerase IV (an enzyme acting at the end of replication so that segregation into daughter cells can occur), leading to fragmentation of the chromosome and cell death (Martinez *et al.*, 2006). While older drugs of this family have a greater potency against toposiomerase II, newer ones have a more balanced activity against both (Hooper, 2000).

Rifamycins (e.g. Rifampicin) inhibit protein transcription of DNA into mRNA by blocking the RNA polymerase; it continues to be used in the therapy of mycobacterial diseases (Tupin *et al.*, 2010).

4) Inhibition of protein synthesis

Most antibacterial drugs inhibiting protein synthesis act as inhibitors of translation by binding to specific sites on the bacterial ribosome. Bacterial organisms have 70 S ribosomes that consist of 50 S and 30 S subunits.

Aminoglycosides (e.g. gentamicin, amikacin, neomycin. streptomycin) and tetracyclines (e.g. oxytetracycline) inhibit ribosome function by binding to the smaller 30 S subunit (Chopra and Roberts, 2001). Aminoglycosides have been shown to not only bind to the 30 S subunit of the ribosome and thereby leading to aberrant protein synthesis, but to also inhibit subunit assembly (Mehta, 2002). Tetracyclines inhibit the binding of the aminoacyl-tRNA to the mRNA ribosome complex.

It is also possible that drugs that inhibit protein synthesis bind to the 50 S subunit in chloramphenicol (Xaplanteri *et al.*, 2003) or macrolides (e.g. Erythromycin, Tylosin, Tulathromycin) (Zhanel *et al.*, 2001).

Apart from aminoglycosides, which also affect membrane permeability (see above),

all other classes of drugs acting on the ribosome are bacteriostatic.

5) Inhibition of folic acid synthesis

Prokaryotes are dependent on producing folic acid, a precursor in nucleic acid synthesis.

Sulfonamides (e.g. sulfadimidine) and diaminopyrimidines (e.g. trimethoprim) act on different steps of the bacterial folic acid synthesis and therefore exhibit a synergistic effect when used together. Diaminopyrimidines are dihydrofolate reductase inhibitors while sulfonamides act as competitive inhibitors of 4-aminobenzoic acid. It is important to note that the half-life of trimethoprim is only 30 minutes in sheep, one hour in the adult bovine and goat, three hours in horses and dogs and seven hours in calves: therefore, monotherapy with trimethoprim is of little use in veterinary medicine (Kroker et al., 2002) and the effect of the combined drugs mostly attributable to the sulphonamide component.

6) Other modes of action

Nitroimidazoles (e.g. metronidazole) lead to strand breaks in bacterial and protozoal DNA under anerobic conditions by producing toxic metabolites through a reduction step (Van der Wouden, 2001).

2 Antimicrobial resistance

2.1 History of antimicrobial resistance

"But I would like to sound one note of warning. Penicillin is to all intents and purposes non-poisonous so there is no need to worry about giving an overdose and poisoning the patient. There may be a danger, though, in underdosage. It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."

Sir Alexander Fleming, Nobel Prize Lecture (1945)

The knowledge of resistance is as old as the awareness that certain substances like penicillin

act as antimicrobials (Abraham, 1940). Because the first antimicrobial agents, excluding the synthetic sulfonamide drugs, were all identified or derived from natural products, resistance determinants were already present in the environments from which these agents originated (Bush, 2004). Bacteria collected between 1914 and 1950 (the Murray Collection) were found to be completely sensitive to antimicrobial agents, including sulfonamides that had been introduced in the mid 1930s (Hughes and Datta, 1983).

The great discovery of streptomycin for treating tuberculosis was diminished by the rapid development of resistance by mutation of the target genes (Hawkey, 1998).

In general, soon after the introduction of antimicrobial agents in veterinary medicine, bacteria resistant to antibiotics rapidly emerged while others have not developed resistance in spite of long term exposure (such as the susceptibility of *Streptococcus agalactiae* towards beta-lactam antimicrobials; Aarestrup and Schwarz, 2006). The importance of the spread of antimicrobial-resistant bacteria from food animals to humans became gradually more and more recognized during the 1950s and 1960s. For decades, the strategy to deal with bacteria resistant to the applied drugs was the development of new classes of antimicrobials.

2.2 Antimicrobial resistance in human and veterinary medicine

"Use of antibiotics in food-production animals has resulted in healthier, more productive animals: lower disease incidence and reduced morbidity and mortality in humans and animals: and production of abundant quantities of nutritious, high-quality, and low-cost food for human consumption. In spite of these benefits, there is considerable concern from public health, food safety, and regulatory perspectives about the use of antimicrobials in food-production animals."

Stephen P. Oliver (2011)

Infections caused by resistant bacteria are associated with higher morbidity and case fatality rate than those caused by susceptible pathogens and the occurrence of genes coding for antimicrobial resistance in a bacterial pathogen can have a pronounced negative impact on humans and animals alike (Helms *et al.,* 2002). For all antimicrobials currently known in veterinary and human medicine, antimicrobial resistance mechanisms have been reported.

In a Danish study, the investigators compared 2-year case fatality rates of patients infected with *Salmonella* Typhimurium with the mortality in the general Danish population. Death rates were 2.3 times higher when compared to the general population in cases where

Salmonella strains were pan-susceptible, 4.8 times higher if the strain was multi-drug resistant and 10.3 times higher if the bacteria showed resistance to quinolones (Helms *et al.*, 2002). Travers and Barza (2002) report a relationship between antimicrobial resistance in *Salmonella* and *Campylobacter* with excess days of hospital stay and diarrhea in patients in the United States and consider the administration of antimicrobials to food animals as the source of resistance. Other authors reported a higher rate of hospitalization during disease outbreaks with antimicrobial-resistant *Salmonella* strains compared to outbreaks with pan- susceptible strains (22 % vs. 8 %) (Varma *et al.*, 2005). Niederman (2001) concludes that excessive length of therapy and reconvalescence, higher death rates and higher cost is attributable to one important mechanism: the likelihood that initial empirical therapy will be inadequate in patients who are infected with resistant organisms. He points out the importance of collecting regular microbiological data to be able to make an educated choice on treatment.

Multi-drug resistant bacteria can be present in a population of animals without leading to increased morbidity or mortality (Kaneene *et al.*, 2010). However, their presence may also significantly change survival or cure rates of animals when infection occurs (Craven *et al.*, 2010; Giguere *et al.*, 2010).

While some authors clearly emphasize the evidence of antimicrobial-resistant bacteria, which lead to infections in humans, and their animal origin as foodborne pathogens (e.g. Swartz, 2002), another view is that "almost every case made for or against antibiotics in animals is complicated by the use of the same antibiotics in humans, which are equally able to give rise to resistance" (Phillips *et al.*, 2004).

Yet, although direct harm to humans is hard to prove in general, the connection between drug-resistant bacteria of food animal origin and illness in humans has been documented in detail in some cases (Spika *et al.*, 1987; Fey *et al.*, 2000) and that selection of resistant bacteria in animals has a potentially deleterious effect on human health. Also, introduction of such an organism of animal origin may be a very rare event in humans, but a phase of amplification in the human population through use of antimicrobials may exacerbate the problem (Turnidge, 2004).

No doubt exists that antimicrobial resistance is a problem in both veterinary and human medicine alike, and this has led to the establishment of monitoring programs on the antimicrobial resistance in bacteria of animal and human origin.

The Danish Integrated Antimicrobial resistance Monitoring and Research Programme (DANMAP) established in 1995 reports yearly on the surveillance of antimicrobial resistance in bacteria from livestock, food, and humans. DANMAP also collects data about bacteria producing extended spectrum beta-lactamase (ESBL), an enzyme that is responsible for bacterial resistance towards a broad range of beta-lactam antimicrobials (including all generations of cephalosporins). Its 2009 report states: "The parallel increase in prevalence in ESBL-producing bacteria in both humans and animals indicates that antimicrobial selection takes place in both reservoirs, and food-derived spread of ESBL-production *E. coli* may be the origin in at least part of the human cases." (DANMAP, 2009).

A similar surveillance program exists in the United States of America: The National Antimicrobial Resistance Monitoring System (NARMS) was built by joining forces of the FDA (United States Food and Drug Administration), the USDA (United States Department of Agriculture), and the CDC (Center of Disease Control) (Mathew *et al.*, 2007).

2.3 Types and dissemination of resistance encoding genes

There are two classes of resistance: intrinsic and acquired.

Intrinsic resistance in bacteria is defined as the "lack of cellular mechanisms required for antibiotic action" (Boerlin and White, 2006). Among many examples is the resistance of most Gram-negative bacteria toward penicillin since the size of the molecules does not allow the antimicrobial to pass the outer membrane.

Acquired resistance: The influence of an antimicrobial chemical substance on bacteria can encourage them to obtain new genes by horizontal gene transfer or can lead to mutation of the bacterial genome encoding for resistance. Horizontal gene transfer and mutation can "act in a synergistic way because horizontal gene transfer introduces new alleles into a population and mutation produces new variations of these alleles" (Blazquez, 2003). Genetic mutations often lead to structural changes in the organism, while horizontal gene transfer usually codes for enzymes that enhance metabolization of antimicrobials.

Unlike intrinsic resistance, acquired resistance does not have to be associated with all strains of a particular bacterial genus or species (Guardabassi and Courvalin, 2006).

2.3.1 Horizontal gene transfer in bacteria

Horizontal gene transfer describes the exchange of DNA between different bacterial cells as

opposed to vertical gene transfer from mother to daughter cell. Prokaryotes are dependent on rapid access to new genetic material in order to adapt to different environments and stressors. One such stressor can be the exposure to antimicrobial drugs.

Microbes typically use one of the following methods for horizontal gene transfer: conjugation, transduction and transformation (Frost *et al.*, 2005; Tenover, 2006).

Conjugation requires cell-to-cell contact and the setting up of a conjugation pore to exchange genetic information in the form of plasmids (Kelly *et al.*, 2009). The process is sometimes initiated by the induction of sex pheromones by the mating pair (Tenover, 2006). This transfer may occur between bacterial strains of the same species, within species of the same genus but also those belonging to different families.

Transduction describes a bacteriophage-mediated process of transfer. It was first described by Zinder and Lederberg (1952) when they were studying genetic exchange in *Salmonella* species. Bacteriophages are viruses capable of infecting bacterial host cells (Sorensen *et al.*, 2005). They typically have limited host range. There are two forms of bacteriophages, depending on their life cycle. Lytic: reproducing themselves while destroying their host; and lysogenic: being able to integrate themselves into the host´s genome (Kelly *et al.*, 2009).

Transformation is defined as the stable uptake, integration and functional expression of extracellular "naked" DNA that can occur under natural bacterial growth conditions (Thomas and Nielsen, 2005).

2.3.2 Mobile genetic elements

The above mentioned mechanisms transfer plasmids and other mobile genetic elements such as transposons and integrons.

The term plasmid was first used by Lederberg and defined as "a generic term for any extrachromosomal hereditary component" (Lederberg, 1952). Plasmids are extra-chromosomal, self-replicating genetic elements that are not essential to survival. The R-factors found in enteric bacteria are quite complex plasmids carrying antibiotic resistance genes, but also encode for transfer functions allowing a rapid transmission within a population of related Gram-negative organisms (Normark and Normark, 2002). Plasmid-mediated resistance is also common in Gram-positive bacteria. For example benzyl-penicillin resistance achieved by the production of beta-lactamase in *Staphylococcus aureus* is mediated by the *bla* gene carried on plasmids (Normark and Normark, 2002).

Transposons (also called "jumping genes") can move from one location on the chromosome to another or from plasmid to plasmid and create phenotypically significant mutations. This is the reason why some identical transposons can be found on many different plasmids of different species and origins. The first transposons were discovered in corn (*Zea mays*) by Barbara McClintock in 1948 for which she was awarded the Nobel Prize in Medicine in 1983 (McClintock, 1950) .

They can also function as conjugative transposons in the sense that they excise from and integrate into DNA like transposons but that they also have a covalently closed circular form for transfer via conjugation in the same way as for plasmids (Salyers *et al.*, 1995).

Insertion sequences (IS) are simple forms of transposons containing only those genes required for transposition (lacking genes encoding for antibiotic resistance or other traits) but that result in mutations due to their translocation (Mahillon and Chandler, 1998).

The integron (gene cassette) system provides another powerful mechanism for the acquisistion of new genes and hence the evolution of bacterial genomes (Hall and Collis, 1995; Recchia and Hall, 1995). Each cassette contains a single gene. Although any typical gene can be part of a cassette, for a long time most genes known to be found in integrons were resistance genes (Hall *et al.*, 1999). Integrons are also frequently included in transposons.

Recently, researchers have described another possibility. The so-called gene transfer agents (GTA) are "virus-like particles that only carry random pieces of genome of the producing cell in a process similar to generalized transduction" (Lang and Beatty, 2007).

2.3.3 Mutation

Compared to gene transfer, the generation of new genetic material by mutation is known to happen much more slowly. On the other hand, Blázquez (2003) showed that under selective pressure (such as in the case of exposure to antimicrobial drugs), mutator strains are favored over strains that are nonmutant. As many as 1% of *E. coli* and *Salmonella* natural isolates are already stable strong-mutators (those with a very high mutation rate) (Matic *et al.*, 1997). Mobile genetic elements such as plasmids are also subjected to genetic alterations by mutations (Normark and Normark, 2002). Although most mutations in bacteria are non-specific and happen "naturally", Kohanski and others (2010) recently demonstrated that the influence of sublethal levels of antibiotic treatment induce mutagenesis due to bacterial production of reactive oxygen species and leading to higher mutation rates in treated

compared to untreated *E. coli.*

Certain bacterial species, such as *Mycobacterium tuberculosis*, have an extremely low capability to exchange DNA with their surroundings, therefore resistance to antimycobacterial drugs is generally the consequence of spontaneous mutations (Normark and Normark, 2002).

2.4 Mechanisms of resistance in bacteria

Antimicrobial resistance mechanisms can be classified into the following categories:

1) The antimicrobial agent can be prevented from reaching its target by reducing its penetration into the bacterial cell:

   Mutations leading to loss, reduction in size, or decreased expression of porins in the bacterial cell membrane are a mechanism of acquired, generally low-level resistance to various antibacterial agents (Guardabassi and Courvalin, 2006). Reduced uptake is a clinically important mechanism of resistance to beta-lactams and fluoroquinolones in Gram-negative bacteria, especially in *Pseudomonas aeruginosa* and Enterobacteriaceae (Guardabassi and Courvalin, 2006). Gram-negative bacteria are coated by an outer membrane which serves as a substantial permeability barrier. This fact explains, at least in part, the enhanced resistance of those bacteria compared with Gram-positive organisms (Vaara and Nurminen, 1999).

   Biofilm formation by some pathogenic bacteria such as *P. aeruginosa* has been discussed as a mechanism for drug resistance by reducing the penetration of antimicrobials into the cell (Normark and Normark, 2002). Other authors argue that the degree of susceptibility is not different from the one expressed by the same bacteria in a non-biofilm (planctonic) mode of growth (Spoering and Lewis, 2001).

2) General or specific efflux pumps may expel antimicrobial agents from the cell:

   Resistance is determined by reduction in the concentration of the drug in the cytoplasm, preventing or limiting access of the drug to its target (Guardabassi and Courvalin, 2006). Drug-specific efflux mechanisms are generally encoded by plasmids and/or other mobile genetic elements that carry additional resistance genes. This explains their association with multidrug resistance (Poole, 2005). They can either be activated by environmental stimuli or by a mutation in a regulatory gene. Efflux pumps were first described for the mechanism of tetracycline resistance (Levy, 2002).

So-called multi-drug efflux systems have been described which provide resistance to a broad range of structurally unrelated antimicrobials and biocides (e.g. triclosan) (Poole, 2002).

3) The antimicrobial agent can be inactivated by modification or degradation, either before or after penetrating the cell:

Enzymatic inactivation is the main mechanism of resistance to beta-lactams, aminoglycosides, and phenicols. The most widespread and clinically important enzymes are the beta-lactamases and the aminoglycoside-modifying enzymes (Guardabassi and Courvalin, 2006).

4) The antimicrobial target may be modified so that the antimicrobial cannot act on it anymore, or the microorganism´s acquisition or activation of an alternate pathway may render the target dispensable:

This mechanism is particularly important for resistance to penicillins (for example methicillin resistance in *Staphylococcus aureus*, target site: peptidoglycan precursors), glycopeptides, and macrolides (target site: ribosome) in Gram-positive bacteria and for resistance to quinolones (target site: DNA gyrase and topoisomerase IV) in both Gram-positive and Gram-negative bacteria (Poole, 2002; Guardabassi and Courvalin, 2006).

A transpeptidase with low affinity for methicillin (PBP2A) partially replaces the normal transpeptidase (PBP2) in methicillin-resistant *S. aureus* (MRSA) containing the *mecA* gene. Changing the structure of the ribosome binding site is usually due to methylation by genetically acquired methylases and less common due to mutation of the target nucleotide sequence.

The modification of target enzyme sequence in quinolones is due to mutation (Guardabassi and Courvalin, 2006).

5) Drug Trapping or Titration:

Bacteria may resist a drug by increasing the production of the drug target or another molecule with affinity for the drug. In both cases, the organism accomplishes a reduction in the proportion of target sited affected by the drug (titration) (Guardabassi and Courvalin, 2006).

2.5 The cost of resistance

A higher mutation rate is associated with loss of fitness as mutators accumulate deleterious mutations more frequently than non-mutators (Chopra *et al.*, 2003). Mutations and alterations of the bacterial genome through horizontal gene transfer harbor the danger of leading to reduction or even loss of viability, so that these cells may be at a disadvantage compared with the parent and may therefore be displaced from the population in the absence of antibiotic selection. Giraud et al. (1999) were able to show that strains experimentally selected for high-level fluoroquinolone resistance had reduced fitness and were not selected *in vivo* in the absence of selective pressure. It is also clear that the replication of plasmids (which frequently carry the genes encoding for resistance) is costly and "if it were always favorable to be resistant, resistance to naturally occurring antibiotics would be ubiquitous" (Wassenaar, 2005). Some mutants, however, do not lose fitness compared to their parents and will clearly be at an advantage in the case of antibiotic use which leads to their selection over non-resistant strains.

Therefore, an organism that has no fitness cost on account of being resistant will behave as any susceptible bacterial clone within a population and will rapidly expand if antibiotic selection pressure is exerted (Normark and Normark, 2002).

3 Implications of resistance in veterinary and human medicine

"Everyone using antimicrobial drugs, including those in agriculture, shares the blame for the rise of resistance and the burden to use these drugs responsibly."

John F. Prescott (2006)

The use of antimicrobials in veterinary medicine and its potential effect on resistance in bacteria has led to the establishment of surveillance programs, scientific investigations, and debates.

Concerns have risen about drug-resistant pathogens originating from an animal related source (foodborne infections, direct contact) as well as bacteria of human origin acquiring resistance genes through gene transfer from these organisms and causing infections that are difficult to treat. Antimicrobial therapy of disease will be less effective if the underlying organism is resistant to the drug used. Antimicrobials that are used frequently in veterinary medicine and that are of the same class of drugs or even identical to the ones used in human medicine, as well as those that are the source of transferred genetic material coding for multi-drug resistance, are in the focus of these concerns.

Following this thought, several questions are important to consider when discussing the topic of antimicrobial resistance in veterinary medicine:

- Who is responsible to monitor antimicrobial resistance in animal and human pathogens?
- Are antimicrobials or classes of antimicrobials used in veterinary and human medicine frequently identical?
- Are bacterial organisms of animal origin a major source of resistance encoding genes in human pathogens?
- What can be done to minimize and optimize antimicrobial usage both in human and veterinary medicine?
- And what are strategies for alternative treatment of bacterial infections and in what way can treatment be reduced by preventing disease in animals?

3.1 Monitoring antimicrobial resistance

The first major review of the effect of antimicrobial drug use on resistance in human and animal pathogens was carried out in the United Kingdom under the chairmanship of M.M. Swann and reported in 1969: "The administration of antibiotics to farm livestock, particularly at sub-therapeutic levels, poses certain hazards to human and animal health" and the authors recommended research into alternative means of growth promotion as well as improved surveillance programs (The Swann Report; Swann, 1969).

Many surveillance programs have been instigated since then. Among the major ones are the National Antimicrobial Resistance Monitoring System (NARMS), which was established in 1996 in the United States, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), and the Danish Integrated Antimicrobial resistance Monitoring and Research Programme (DANMAP). These three include data from livestock, foods and humans. The European Antimicrobial Resistance Surveillance Network (EARS-Net), which became part of the European Centre for Disease Prevention and Control (ECDC) on January 1st 2010, reports data on seven major human pathogens. The European Food Safety Authority (EFSA) publishes an annual European Union report on zoonoses, food-borne outbreaks and antimicrobial resistance.

Surveillance data often are in the form of antibiograms originating from human or veterinary medicine that are used to compare isolates in both populations. One problem with this method is that results are not comparable because of various MIC definitions used to describe the particular subpopulations (Wassenaar and Silley, 2008).

Yet, reports of surveillance programs are indispensable for providing data on the prescribing of antibiotics for physicians and veterinarians alike and are able to define areas where detailed supervision needs to be enacted (as in the example of increased extended spectrum beta-lactamase (ESBL) producing bacteria).

3.2 Overlap of antimicrobials used in veterinary and human medicine

Although individual antimicrobial drugs have been specifically developed for use in veterinary or human medicine only (e.g. ceftiofur in animals and cefotaxime in humans, both belonging to the class of 3rd generation cephalosporin drugs), most major classes of drugs are used in both human and veterinary medicine (Moulin *et al.*, 2008). Classes of drugs solely used in veterinary medicine include feed additives such as ionophores, quinoxalines or

bambermycins (Collignon *et al.*, 2009). When studying antimicrobial consumption in France, Moulin *et al.* (2008) found that four antimicrobial classes were accounting for 80% of sales in veterinary medicine (tetracyclines, sulphonamides/trimethoprim, beta-lactams and aminoglycosides) whereas beta-lactam antimicrobials dominated in human medicine (accounting for more than 50% of the total consumption). As an example of overlapping use, clinicians often treat *Salmonella* infections in humans with cephalosporin or fluoroquinolone antimicrobials and both classes of drugs are frequently administered to animals (Collignon *et al.*, 2009). Emborg and co-authors (2001) conclude that "…it has proven almost impossible to maintain this distinction between antimicrobials used for animals as antimicrobial growth promoters (AGP) and those used as human therapeutics. Because of the increasingly limited therapies for humans – and with new technical possibilities for drug design – pharmaceutical companies have developed many old AGPs into drugs for human use. Some of these drugs (vancomycin, quinopristin/dalfopristin) are considered essential for the treatment of serious life-threatening infections in humans". As an answer to the problem, the World Health Organization (WHO) has developed criteria in order to rank them according to their importance for the treatment of disease in humans (Tollefson *et al.*, 2006). The list was created based on certain criteria and needs to be updated regularly; nevertheless it will be helpful in prioritizing the reduction in usage of certain classes of antimicrobials in veterinary medicine. Categories applied to the antimicrobial classes are "critically important", "highly important" and "important" since all were found to be at least important in human medicine.

The following definitions are taken from Tollefson *et al.* (2006):

Critically important:	Antimicrobial drugs which meet BOTH WHO criteria 1 and 2.
Highly important:	Antimicrobial drugs which meet EITHER WHO criterion 1 or 2.
Important:	Antimicrobial drugs which meet EITHER WHO criterion 3 and/or 4 and/or 5.

WHO Criteria:

1. Antimicrobial drugs used to treat enteric pathogens that cause food-borne disease.
2. Sole therapy or one of few alternatives to treat serious human disease or drug is essential component among many antimicrobials in treatment of human disease.

Serious diseases are defined as those with high morbidity or mortality without

proper treatment regardless of the relationship of animal transmission to humans.

3. Antimicrobials used to treat enteric pathogens in non food-borne disease.

4. No cross-resistance within drug class and absence of linked resistance with other drug classes.

   Absence of resistance linked to other antimicrobials makes antimicrobials more valuable.

5. Difficulty in transmitting resistance elements within or across genera and species of organisms.

   Antimicrobials to which organisms have chromosomal resistance would be more valuable compared to those antimicrobials whose resistance mechanisms are present on plasmids and transposons.

Among the classes ranked "critically important" were aminoglycosides, cephalosporins (3rd and 4th generation), glycopeptides, macrolides, penicillins and quinolones (Collignon *et al.*, 2009), all of which are also used in veterinary medicine.

A classification to define critically important antimicrobial agents in veterinary medicine is currently being prepared by the World Organization for Animal Health (formerly Office International des Épizooties) (Collignon *et al.*, 2009).

3.3 Source of resistance encoding genes in human pathogens

Wassenaar (2005) argues that "most resistance developing in pathogens posing a serious risk to human health, are the result of human application rather than veterinary use of antimicrobials". One bacterium accounting for serious problems with drug resistance in humans, prolonged hospital stay, treatment failure and death is methicillin-resistant Staphylococcus aureus (MRSA). After the first strain of MRSA was isolated in 1961 and only two years after the introduction of methicillin, MRSA spread to many countries within the next decade (Robinson and Enright, 2004). Vancomycin had been the treatment of choice for infections with this pathogen but a recent report describes how a MRSA isolate acquired resistance to vancomycin yet retaining its MRSA phenotype (called VRSA) through conjugative plasmid transfer from *Enterococcus faecalis* while a patient was treated repeatedly with this drug (Weigel *et al.*, 2003). It is one of the examples of a multi-drug-resistant pathogen that, after having evolved and spread in the human population, might pose

a danger to major domestic animal species (Cohn and Middleton, 2010).

In contrast, an example of resistance developing in a foodborne pathogen is *Campylobacter*. Fluorquinolone-resistant *Campylobacter* infections in humans increased after the introduction of ciprofloxacin for human use and enrofloxacin in the poultry industry (Endtz *et al.*, 1991). In countries where fluoroquinolones were not legalized for application in poultry medicine, a rise in resistance towards this class of drugs was not reported (Osterlund *et al.*, 2003). Resistance is conferred by a single mutation in the *gyrA* gene (Wassenaar, 2005).

A study on human and chicken *E. coli* isolates resistant to ciprofloxacin showed that resistant human isolates were distinct from susceptible human isolates but largely indistinguishable from chicken isolates implying a high possibility that they originate from the same source (Johnson *et al.*, 2006).

3.4 Prudent antimicrobial usage

3.4.1 Human medicine

In most countries, patients will receive (systemic) antimicrobial treatment only after seeing a doctor first. Although the physician might come to a different opinion after a physical examination, a survey showed that 48% of patients expected a prescription for antibiotics when feeling sufficiently ill from “a common cold” to seek medical attention (Vanden Eng *et al.*, 2003).

Also, self-medication with antibiotics occurs in many countries where over-the-counter drug sale is legal or minimally supervised (Grigoryan *et al.*, 2006) or when leftovers are available to the individual from a previous course of prescribed treatment.

From another study carried out by Grigoryan et al. (2008), the authors concluded that interventions aiming to prevent self-medication should include the implementation and enforcement of laws for dispensing of antibiotics and expressed a specific concern about the lack of public education; awareness about antibiotic resistance was the lowest in countries with higher prevalence of resistance (Finch, 2004; Grigoryan *et al.*, 2007).

Education of the public and prescribing physicians will become more and more important to reduce unnecessary use of antimicrobial drugs in human medicine especially when it is already facing bacterial strains that have gained multi-drug resistance.

3.4.2 Veterinary medicine

There are several different uses of antimicrobials in veterinary medicine: therapeutic use, prophylaxis, metaphylaxis and use of antimicrobials as growth promoters.

Mercer et al. (1971) described the relationship between feeding cattle and swine antimicrobial agents as growth-promotors and the higher incidence of multi-drug resistant *E. coli* isolates in 1971. In 1986, Sweden was the first country to ban the use of antimicrobial growth promoters in Europe. The growth-promoting use of bacitracin (a polypeptide antimicrobial), spiramycin, tylosin (both macrolide antimicrobials) and virginiamycin (streptogramin antimicrobial) was banned in the EU in 1999 because of the Precautionary Principle (Phillips, 2007). The use of avoparcin had been banned in the EU since 1997 and a EU-wide ban of the use of the last four antimicrobials as growth promoters in animal feed (monensin, salinomycin, avilamycin, flavophospholipol) came into effect on January 1st, 2006 (EC/1831/2003), marking the end of the phase-out process. Reduction of resistance in the pathogens belonging to the genera *Enterococcus* and *Campylobacter* was the target of the new legislation because they are the only major food-borne bacteria susceptible to the selected antimicrobials (Phillips, 2007). Prevalence of resistance towards spiramycin, tylosin and virginiamycin in indicator bacteria such as *Enterococcus faecium* was shown to have decreased rapidly since the ban (Bywater *et al.*, 2005). There was no marked change in resistance of *E. faecium* from pigs (Denmark, The Netherlands, Spain, Sweden) and chickens (France, The Netherlands, Sweden, UK) towards bacitracin, but enterococci had been shown to be intrinsically resistant. This was shown by results from Sweden where it had not been used for many years (Phillips, 2007).

It seems logical that the decreased resistance in indicator bacteria after the ban of growth promoters can only be an advantage for animals and humans alike.

Nevertheless, for some observers a benefit for human health by reducing the number of infections with vancomycin-resistant enterococci after the ban of avoparcin (also a glykopeptid antimicrobial) as a growth-promotor in 1996 remains “not apparent, unless it is argued that the increases in incidence, escalating almost ten years after the ban, might have been still greater” (Phillips, 2007). It was argued that “the diminution of the resistance pool in animal and human fecal commensal enterococci has been at the cost of a deterioration in animal welfare” (Casewell *et al.*, 2003) and led to a temporary rise in the use of antimicrobials for therapeutic reasons as was seen in the Swedish example (Wierup, 2001).

Apart from reducing the amount of antimicrobial agents as growth promoters, the prudent use

of these drugs as therapeutic agents and for metaphylactic reasons plays a key role in trying to minimize consumption in veterinary medicine to a minimum.

Recommendations for clinicians on how to use antimicrobials responsibly, includes the "guidelines for the careful handling of antibacterial veterinary drugs" ("Leitlinien für den sorgfältigen Umgang mit antibakteriell wirksamen Tierarzneimitteln"), that have been developed in Germany by the working committee on veterinary drugs (Arbeitsgruppe Tierarzneimittel) (BTK, 2010). The guidelines that have recently been published in an updated version offer information about antimicrobial usage (such as indication, treatment length, dosage, choice of drug) as well as more specified information on treatment for certain animal species.

It is the responsibility of each veterinarian prescribing drugs to make sure that he or she is aware of the necessary information on how to use antimicrobials judiciously and to reduce their application to a scientifically validated minimum.

3.5 Alternatives to antimicrobial treatment

The "Golden Age" of antimicrobial discovery has long passed. In the last 35 years very few novel antimicrobial drug classes were found (Taylor and Wright, 2008). It has become increasingly more difficult for scientists to come up with new, safe, and chemically stable drugs to make up for those becoming useless due to increasing resistance of pathogens. Analyzing whole genome data of actinomycetes, screening large quantities of novel and potentially useful natural compounds, revisiting "old" substances that were discarded in the last century for several reasons, exploring marine actinomycetes or development of completely synthetic antibiotics have been the focus of researchers in order to supply the market with new antimicrobial agents (Taylor and Wright, 2008).

Yet, in the light of what we now know about transfer and spread of resistance, it is clear that each new class will eventually be rendered incapable of fighting certain pathogens.

Therefore, alternative approaches for treating bacterial infections in humans and animals are being investigated.

3.5.1 Phage therapy

One such alternative is the rediscovery of phage therapy. The concept of treating infections with lytic bacteriophages is not new and was discovered by Twart and d´Herelle at the

beginning of the 20th century. The introduction of pencillin and other antibiotics shortly thereafter led to decreased interest in phage therapy (Johnson *et al.*, 2008). Phages are abundant in nature and share a common ecology with their hosts (Bruessow, 2005). They are relatively specific to their target, mostly offering a greater specificity than antimicrobial drugs. When present at a specific ratio of phages to their bacterial host, phages can amplify themselves and kill the target host by repeated cycles of replication (Johnson *et al.*, 2008). A multitude of studies can be consulted by the interested reader on this re-emerging approach (Sulakvelidze *et al.*, 2001; Payne and Jansen, 2003; Fischetti, 2005; Higgins *et al.*, 2005; Wagenaar *et al.*, 2005; Gill *et al.*, 2006; Callaway *et al.*, 2008). In summary, phage therapy has shown potential for the control of animal and zoonotic pathogens in cattle, pigs and poultry, however, the studies showed the need for further investigation about candidate phages, efficacy, safety, administration routes and phage resistance (Johnson *et al.*, 2008).

3.5.2 Disease prevention

The growth-promoter ban has made it essentially clear that preventing disease in livestock through prophylactic measures is necessary when reducing the amount of antimicrobials used in animals.

The most extreme and safest way to prevent a bacterial infectious disease is by eradication of the disease-causing organism. Although eradication schemes to control for example bovine tuberculosis and brucellosis have lead to success in many countries, it is hard to reach eradication of a bacterial organism on a country-wide basis as has been achieved for several viral diseases (foot and mouth disease, classical swine fever).

Control of bacterial diseases is easier to achieve on a herd basis. Examples are specific pathogen free (SPF) production of swine under strict biosecurity measures which have been established on a large-scale basis in some countries such as Denmark (Wierup, 2000) and Johne´s disease eradication programs.

Clearly, optimizing hygiene and housing (e.g. ventilation, temperature) conditions in animal production, isolating sick animals from neighboring animals and maintaining closed herds (ideally also applying an “all in, all out” system) in combination with vaccination programs individually tailored to the farm level will have a marked effect on animal health and reduce the need to treat infections (Wierup, 2000; Potter *et al.*, 2008). Concurrently, in human medicine, the need to encourage new vaccine development and to promote the principles of

infection control and effective hygiene within hospitals and among the community should be highlighted (Finch, 2004).

It becomes clear that antimicrobial therapy is – and should always have been – just one way to deal with pathogenic bacteria.

4 Antimicrobial susceptibility testing

When choosing an antimicrobial drug to treat disease in humans and animals, it is fundamental to take several factors into account, including intrinsic or documented acquired resistance of the pathogen. Walker (2006) also includes the nature of the infection, the identity of the pathogen, the pharmacokinetic behavior of the chosen drug in the target animal species, the pharmacodynamic indices of the drug at the site of infection, host characteristics, cost, ease, route and frequency of administration as well as residue avoidance time in food animals. Yet, in individual cases the clinician will have to rely on culture results to determine which bacteria (if any) are to be blamed for the disease and in most cases treatment needs to be administered before having the culture information. Antimicrobial susceptibility testing (AST) has gained utmost importance in recent years due to increasing antimicrobial resistance. To study the influence of antimicrobials on bacterial populations, AST has also been used in numerous screening studies to learn about the status quo (Dunlop *et al.*, 1998; Jordan *et al.*, 2005; Jiang *et al.*, 2006; Tragesser *et al.*, 2006; Frye and Fedorka-Cray, 2007; Edrington *et al.*, 2008; Lundin *et al.*, 2008) or to study evolution of resistance in a target bacterial species over time (Lowrance *et al.*, 2007; Singer *et al.*, 2008; Alali *et al.*, 2009).

Several methods are suitable for AST such as disc diffusion, agar dilution, broth micro- and macrodilution, PDM Epsilometer test (E test) (Schwarz *et al.*, 2010).

Regardless of the test method selected, the routine application of quality control (QC) is an essential component of the AST and allows the laboratory to verify that its personnel, incubation conditions, test media, and antimicrobial agents are performing at an acceptable level (Watts, 2006).

Since the first description of reduced antimicrobial susceptibility of a Staphylococcus species around the growth of a penicillin-producing mould by Alexander Fleming in 1929 (Fleming, 1980) clinicians and researchers have faced the problem of the lack of standards for testing procedures, documentation of results and differences in reported resistance breakpoints (Bywater *et al.*, 2006). In order to solve standardization issues, several institutes in the United

States and Europe have published guidelines for AST procedures including the Clinical and Laboratory Standards Institue (CLSI; formerly called National Committee for Clinical Laboratory Standards NCCLS)), the British Societey for Antimicrobial Chemotherapy (BSAC), the Deutsches Institut für Normung e.V. (DIN) and the Comité de la Société Française de Microbiologie (CA-SFM) (Schwarz *et al.*, 2010).

5 *E. coli* as a model organism for studying *Salmonella*

Escherichia coli, commonly abbreviated as *E. coli*, is one of the most widely studied organisms today. Since its discovery by the German pediatrician Theodor Escherich in 1885, this Gram-negative rod-shaped bacterium has been used in science worldwide as a model for other prokaryotes (Shulman *et al.*, 2007). Because of its wide application in research, it was the first organism to be suggested for whole genome sequencing; the complete genome was published in 1997 (Blattner *et al.*, 1997). Besides its abundance in the environment, *E. coli* is easy to culture and stable laboratory strains have been developed (for example ATCC authenticated microoganisms, (ATCC, 2011). As the name suggests, this bacterium resides in the intestinal tract of warm blooded organisms (caudal small intestine and large intestine, (Selbitz, 2002) and is commonly found in the feces of animals and humans (about 10^4 to 10^7 cfu/g manure in cattle). It is therefore used as an indicator of fecal contamination (Edberg *et al.*, 2000). Lederberg (Lederberg and Tatum, 1946) discovered that the organism is not only able to reproduce asexually but also by sexual methods and can therefore be used for genetic studies. *E. coli* is classified as a so-called open pan-genome organism allowing for many opportunities of genetic exchange (Kelly *et al.*, 2009). Studies of resistance in *E. coli* have lead to a better understanding of the relationship between the degree of antimicrobial drug use and the extent of resistance (Smith, 1975). Furthermore, standardized procedures for in- vitro susceptibility testing of *E. coli* are readily available

While salmonellosis is still the most common reported food-borne bacterial disease in the United States today with 7,000 reported cases in 2008 (CDC, 2010), it is now second to Campylobacteriosis in the European Union with 131,468 confirmed cases in the same year (Lahuerta *et al.*, 2010). These numbers may greatly underestimate the true extent of disease caused by pathogenic *Salmonella* serotypes and numbers have been estimated to be as high as 1.4 million cases per year in the United States (Mead *et al.*, 1999).

Although many cases of salmonellosis are self-limiting and require no treatment, is is critical

to treat invasive human salmonellosis cases as the infection can lead to serious disease or even death, especially in the elder and in children. Concerns have risen that the emergence of antimicrobial resistance in this pathogen may be compromising the chance of recovery in severely ill patients.

Because of the nature of naturally occurring infections with this bacterium (acute and chronic infections, intermittent shedding, seasonal dynamics) as well as demanding sampling and culturing conditions, in-vivo clinical trials are rare. Because of the ease with which *E. coli* can be obtained both from the environment and as a standardized organism, the simplicity of culturing and storing it, the great possibility of gene transfer and interaction within the species itself and with other bacterial species and the evidence of resistance gene transfer between those two species (Winokur *et al.*, 2001), we used this prokaryote as a model to observe effects of antimicrobial drugs on resistance in *Salmonella*.

Moreover, the colon of animals provides a rich opportunity for transfer of resistance between commensals and pathogens that is not available in the environment (Rollins *et al.*, 1974). This lead us to study E. coli from fecal material.

Measurement of the antimicrobial resistance status of whole populations of bacteria using standard techniques is problematic since these methods are adapted to small numbers of isolates in clinical settings. A technique is needed to assess large numbers of isolates in order to measure small effects that may have biological significance. In this study we applied a mass-screening technique to address the impact of ceftiofur use in dairy cattle.

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III PUBLICATION

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Antimicrobial Susceptibility of Fecal *Escherichia coli* Isolates in Dairy Cows Following Systemic Treatment with Ceftiofur or Penicillin

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1 Abstract

The objective of this longitudinal controlled trial was to determine the effect of systemic treatment with ceftiofur on antimicrobial susceptibility of fecal *Escherichia coli* isolates in dairy cows.

Cows with metritis or interdigital necrobacillosis requiring systemic antimicrobial treatment were sequentially assigned to two treatment groups. The first group was treated with ceftiofur hydrochloride and the second with penicillin G procaine. Untreated healthy control cows were selected for sampling on the same schedule as treated cows. Fecal samples were collected on days 0, 2, 7, 14, 21 and 28.

21983 *E. coli* isolates from 42 cows were analyzed for susceptibility to ampicillin, tetracycline and ceftiofur using a hydrophobic grid membrane filter system to assess growth on agar containing selected antimicrobial drugs. Temporal changes in both the concentration of *E. coli* in feces and the susceptibility of *E. coli* to each drug were analyzed. A significant decrease in the concentration of fecal *E. coli* on days 2 and 7 post-treatment (but not thereafter) was detected in animals treated with ceftiofur. The proportion of all isolates (95% confidence interval in parentheses) showing reduced susceptibility at day 0 was 3.0% (2.5, 3.6) for ampicillin, 10.6% (9.7, 11.6) for tetracycline, and 4.8% (4.2, 5.6) for ceftiofur; 1.7% (1.3, 2.1) of isolates were resistant to ceftiofur based on growth at 8 μg/ml. Treatment did not have any significant effect on the proportion of isolates expressing reduced susceptibility to antibiotics with the exception of decreased tetracycline susceptibility in the ceftiofur treated group on day 2. Although we found the potential for selection pressure by documenting the change in *E. coli* concentration following ceftiofur treatment, an increase in ceftiofur resistance was not found.

2 Keywords

Antimicrobial resistance, reduced susceptibility, *E. coli*, ceftiofur, hydrophobic grid membrane, dairy cow

3 Introduction

Treating livestock with antimicrobial drugs regarded as important in human medicine is a contentious issue. Concern arises because of the potential to select resistance in zoonotic pathogens. There are also fears that commensal bacteria exposed to antibiotics might develop

and propagate genetic elements coding for resistance that are subsequently acquired by human pathogens (Angulo *et al.*, 2004; Wassenaar, 2005). One drug of concern is ceftiofur, a third generation cephalosporin. Systemic use of ceftiofur against common diseases of dairy cows is attractive because milk from treated individuals need not be withheld from marketing and withholding periods for meat are short (Tragesser *et al.,* 2006). In human medicine, third generation cephalosporins are valued for treating serious or life threatening infections. Therefore, the use of ceftiofur in dairy cows is seen as a potential threat to the ability to cure a range of life-threatening infections in people (Allen and Poppe, 2002). There are special concerns about zoonotic forms of multi-drug resistant *Salmonella* acquiring resistance to third generation cephalosporins because dairy cattle are one of the reservoirs for these pathogens (Frye and Fedorka-Cray, 2007; Whichard *et al.,* 2007). In response to these issues the United States Food and Drug Administration recently evaluated whether ceftiofur use should be modified to further protect consumers (FDA, 2009).

Escherichia coli often is used as a model for changes in other bacterial species such as *Salmonella* because of similarities in their microbial physiology, frequently identical genetic determinants of resistance and similar mechanisms of genetic dissemination (Lowrance et al., 2007). Several studies showed that the bla_{CMY-2} gene is responsible for cephalosporin resistance in both *Salmonella* and *E. coli* isolates (Donaldson *et al.*, 2006; Daniels *et al.*, 2009). It is also likely that many resistance genes can be exchanged within the family of *Enterobacteriaceae* (Neuwirth *et al.,* 2001). For every *Salmonella* bacterium in the intestine of humans and animals there are thousands of *E. coli*, subject to the same antimicrobial selection and capable of carrying and spreading the same or similar genetic resistance elements (O'Brien, 2002).

Our objective was to scan large numbers of isolates to determine whether systemic ceftiofur or penicillin treatment of dairy cows under field conditions resulted in changes in antimicrobial susceptibility of fecal *E. coli.*

4 Materials and Methods

4.1 Study population and design

We enrolled cows from two New York State dairy herds in a longitudinal controlled trial. Herd sizes were 140 and 300 milking cows. The dominant breed was Holstein-Friesian; all other cows were Ayrshires. A herd survey filled out by the owners showed that antimicrobial

usage practices for these herds were similar to those reported for conventional farms by Zwald et al. (2004). Ceftiofur was used on both farms prior to the study for typical purposes such as treatment of respiratory disease, metritis, retained placenta, and foot problems. Lactating cows with metritis or interdigital necrobacillosis as identified by the herd owner or veterinarian were enrolled sequentially in one of two treatment groups; ceftiofur or penicillin. The treatment for the first animal within each herd was selected at random. Healthy animals served as untreated controls and were paired with antibiotic-treated animals, alternating groups, and matching calving date and parity whenever possible.

Samples were collected from each animal at enrollment (day 0) and subsequently five times in a four-week period. The target sampling days were 2, 7, 14, 21 and 28 days after enrollment. The study was conducted between July 2008 and July 2009.

4.2 Treatment protocol

Treatment for cows in the ceftiofur group consisted of a subcutaneous injection of 1 gram of ceftiofur hydrochloride suspension *(*Excenel RTU, Pharmacia & Upjohn Co, Division of Pfizer Inc, New York, NY) once per day for a total of four days. Cows enrolled in the penicillin group received 9 million IU of a penicillin G procaine suspension (PenOnePro, Vet One, MWI, Meridian, ID) intramuscularly once per day for a total of four days.

4.3 Sample collection and processing

Fecal samples were collected before treatment by the herd owner or veterinarian (day 0) and on days 2, 7, 14, 21 and 28 after enrollment by the veterinarian. Approximately 50 g of feces were collected directly from the rectum of each cow with a clean plastic sleeve. Samples were placed immediately into a sterile sample container and transported refrigerated to the laboratory to be processed on the day of collection.

Those administering treatments and collecting samples were not blinded to treatment group. Study personnel carrying out laboratory procedures were unaware of the assignment of animals.

Fifteen g of fecal material from each sample were placed in a sterile Whirl-Pak filter bag (Nasco, Fort Atkinson, WI) and mixed with 40ml of a sterile 0.9% saline solution. The sample was then mixed in a stomacher for 2 minutes. The filtrate was placed in a second sterile Whirl-Pak filter bag and processed for another 2 minutes. Five 1 ml replicates of the specimen were aseptically transferred from the filter pocket into 5 ml freezer vials prepared with 1 ml of

glycerol (Fisher Scientific, Fair Lawn, NJ) and tryptose soy broth (Becton, Dickinson and Company, Franklin Lakes, NJ) (1:1 vol/vol) and placed in a -80°C freezer for storage.

4.4 Production of hydrophobic grid membrane filter master grids

HGMF master grids were used to enumerate and replicate bacteria using a modification of a method described previously (Dunlop *et al.,* 1998; Jordan *et al.,* 2005). A master grid containing no more than 200 colonies was chosen to limit the probability of any one colony being derived from more than one bacterium (Jordan *et al.,* 2005).

To generate a master grid, each frozen sample was thawed in a 30 °C water bath. Five-fold serial dilutions were made in 0.1 % peptone water containing 1% Tween 80 (Fisher Scientific, Fair Lawn, NJ). Approximately 1.5 ml of each diluted sample was filtered through a sterile HGMF grid (ISO-GRID®, QA Life Sciences, CA) using an SF-1 Spreadfilter (Filtaflex, Almonte, Ontario, Canada).

HGMF grids were then aseptically transferred onto MacConkey agar (MAC) (Northeast Laboratory Services, Waterville, ME) and incubated at 37°C for 18-24 h to produce a master grid (see Figure 1).

If the dilution process failed to produce a grid as described, another replicate of the sample was chosen, thawed and more extended dilutions carried out until the condition was met. In cases where the diluted sample yielded very low numbers of colonies, either the sample was enriched in GN broth (Hajna) or a dilution was incubated in the waterbath to increase the number of bacteria. Five-fold serial dilutions were then produced from the enriched sample.

4.5 Colony replication

Bacterial growth on the master grid was copied onto HGMF grids on a series of nine different agars using an RP-1 HGMF replicator (Filtaflex, Almonte, Ontario, Canada). In order of replication, these agars were: Mueller-Hinton (MH) containing ceftiofur at three different concentrations - 4, 8 and 16 µg/ml (Northeast Laboratory Services, Waterville, ME); chromogenic agar (BBL Chromagar Orientation, Becton, Dickinson and Company, Franklin Lakes, NJ), MH agar containing either ampicillin (16 µg/ml), tetracycline (8 µg/ml), sulfamethoxazole (256 or 512 µg/ml), or chloramphenicol (16 µg/ml) (Northeast Laboratory Services, Waterville, ME); and MH plates without an added antimicrobial agent (Northeast Laboratory Services, Waterville, ME) to ensure that the inoculum was transferred to all agar plates. All plates were incubated at 37°C for 18-24 hours. The chromogenic media served as a

control for *E. coli* (purple appearance).

Breakpoints were adopted from the Clinical and Laboratory Standards Institute (CLSI, 2008). The presence of the bla_{CMY-2} gene, which has been linked to ceftiofur resistance in *Salmonella* isolates, is strongly associated with a minimal inhibitory concentration of 8 µg/ml (Alcaine *et al.*, 2005).

The concentration of sulfamethoxazole was switched from 256 µg/ml to 512 µg/ml after the beginning of the study; information for this antimicrobial is therefore incomplete and was excluded.

4.6 Quality control

Identity of *E. coli* was confirmed for a subset of typical purple-colored isolates on chromogenic agar either in the Animal Health Diagnostic Center (AHDC), Cornell University College of Veterinary Medicine or by the Sulfide Indole Motility (SIM) tube test. To ensure quality of the filter system, two *E. coli* isolates were chosen as positive and negative controls. These were cultured, processed and replicated as described for replicate samples from study animals. The isolate serving as negative control (ATCC 25922) was confirmed to be susceptible to all study antimicrobials using the Sensititre Automated Microbiology System´s A80 Panel (TREK Diagnostic Systems Inc., Cleveland, OH) in the AHDC. The positive control, originating from a previous study, was characterized as multi-drug resistant by the same method and as having the bla_{CMY-2} gene by PCR. For quality control of the plates and growth conditions, both isolates were streaked onto the side of the antimicrobial plates, outside the grid zone, for at least two sample days per cow.

4.7 Imaging and analysis of colony growth

Digital pictures of HGMF filters on agar were taken with a camera (Canon Power Shot SX110 IS) using remote picture software to enhance quality (PSRemote Version 1.6.4, Breeze Systems Limited, Bagshot, Surrey, UK). Customized software for image analysis (HGMFRES, NSW Department of Primary Industries, Wollongbar, NSW, Australia) was then used to detect bacterial growth and identify the grid cell address (row and column position) of each colony as described previously. (Jordan *et al.*, 2005). A third software (HGMF Image Dr, NSW Department of Primary Industries, Wollongbar, NSW, Australia) was used to exclude all colonies on the chromogenic agar not showing the typical *E. coli* color reaction.

4.8 Statistical analysis

Data were analyzed using SAS ver. 9.1 (SAS Institute Inc., Cary, NC, USA). The distribution of parity and days in milk among treatment groups was analyzed using Kruskal-Wallis tests. The pairwise associations of reduced susceptibility to tetracycline, ampicillin and ceftiofur were analyzed at the isolate-level using chi-squared tests and at the cow-level using Spearman´s rank correlation. Estimation of *E. coli* concentrations in fecal samples was achieved by applying the following formula:

$$cfu \cong n \times (5^{dil}) \times 2 \times \frac{55}{15} \times \frac{1}{1.5}$$

where *n* is the count of isolates on the control grids, *dil* is the number of five-fold dilutions to reach the solution providing approximately 50-150 isolates per grid, and the other values represent volumes transferred in the various steps of the procedure. In the case of enriched samples dilution number was set to 0. Repeated measures analysis of variance (ANOVA; PROC MIXED) was used to analyze the treatment group effect on the $\log_{10}$ concentration over time. Cow was treated as a subject effect and a first-order autoregressive covariance structure was specified. Sample day (0, 2, 7, 14, 21 and 28) was included in the model as a fixed effect. The treatment by day interaction term was tested to determine whether changes in concentration over time depended on treatment group. Pairwise differences between treatment-day combinations were tested using Bonferroni-corrected *p*-values. Assessment of the normality of model residuals and plots of residual values against predicted values were used to evaluate model assumptions and were considered acceptable for this model.

Treatment group effects on reduced antimicrobial susceptibility were analyzed by first calculating the proportion of isolates with growth on the applicable antimicrobial plate among those grid cells with growth on both on the MH and chromogenic agar control plates. This was done for each cow-sample day combination. For each combination of antimicrobial and drug-concentration, the change from the pre-treatment measurement in proportion reduced susceptibility was calculated within cow for each post-treatment day. The data were analyzed initially using repeated measure ANOVA as described above, but model assumptions were not met. Therefore Kruskal-Wallis tests were used to compare treatment groups on each sample day. The effect was also evaluated by calculating the maximum change from baseline

for each drug for each cow. The effect of treatment group, days in milk at enrollment, lactation group (1, 2 or ≥3), and breed on maximum change were evaluated using Kruskal Wallis tests.

5 Results

Of 44 animals enrolled, 2 were excluded because of sale or treatment changes (Table 1). The animals retained in the study included 32 Holstein-Friesians and 10 Ayrshires. All except two cows were enrolled within 14 days after calving. None of the cows received systemic antimicrobial treatment directly prior to enrollment, but one animal started in the study one week after dry treatment. One cow received ceftiofur for six days, another cow was treated with oxytetracycline starting 8 days after treatment with ceftiofur was completed. These were not excluded from the analysis.

A total of 240 individual fecal samples and 21983 *E. coli* isolates were included in the statistical analysis. The distribution of isolates between the treatment groups was 7,237, 8,222 and 6,524 for ceftiofur, control and penicillin, respectively, and there was approximately even distribution of isolates across sample days. There were no significant differences in days in milk (DIM) or lactation number between treatment groups ($p>0.15$). The median and mean true sample intervals are displayed in Table 2. The median number of isolates from each sample was 82 and ranged from 21 to 238. Quality control measures for laboratory outcomes were all consistent with the expected results except that a concentration problem was detected with some chloramphenicol plates which lead us to exclude those data from the analysis.

5.1 Effect of antimicrobial treatment on *E. coli* concentration

Information about dilution was missing for eight samples and calculations were therefore based on 232 samples. A significant effect of ceftiofur treatment on *E. coli* concentration in the feces as estimated by dilution number was detected. The repeated measure ANOVA treatment by day interaction was significant ($p < 0.0001$). Based on pairwise comparisons, this effect was due to a significant decrease in concentration for samples collected on days 2 and 7 from cows in the ceftiofur group, but we also noted a moderate increase in estimated cfu on day 7 in the penicillin group (Figure 2). Because of arbitrary assignment of a dilution of 0 to 15 samples requiring enrichment, this analysis was also checked using a non-parametric rank-based method. This confirmed significant differences among treatment groups on day 2 and 7. Ten of these 15 were from ceftiofur-treated cows on days 2, 7 and 14.

5.2 Prevalence of isolates with RS to antimicrobials

The percentages (95% confidence intervals in parentheses) of RS isolates at day of enrollment were 3.0% (2.5, 3.6) for ampicillin, 10.6% (9.7, 11.6) for tetracycline and 4.8% (4.2, 5.6) for 4 µg/ml ceftiofur (cef 4); 1.7% (1.3, 2.1) showed ceftiofur resistance with growth at 8 µg/ml ceftiofur (cef8) and 0.7% (0.4, 1.0) for 16 µg/ml ceftiofur (cef16). Growth on two or more antimicrobial plates was found for 1.5% (1.1, 1.9) of isolates. Differences in percentage of growth between herds at enrollment were significant for tetracycline, cef4 and cef8 as listed in Table 3. For all sample dates combined, of the 21983 *E. coli* isolates, 1.8% (1.6, 1.9), 12.6% (12.2, 13.1), 4.0% (3.7, 4.2), 0.9% (0.8, 1.0) and 0.3% (0.2, 0.4) exhibited growth on ampicillin, tetracycline, cef4, cef8 and cef16 respectively. Growth on two or more plates was found for 1.8% (1.6, 1.9) of isolates and the percentage of isolates growing on at least one of the antimicrobial agar plates was 16.9% (16.4, 17.4).

Isolates showing RS or resistance to ceftiofur were significantly more likely to show RS to ampicillin or tetracycline ($p<0.0001$). Isolates growing on cef16 were significantly more likely to also grow on cef8 or cef4 (relative risk (RR) 125.5 and 19.8 respectively) and the same was true for isolates growing on cef8 to also grow on cef4 (RR 17.6). Isolates growing on tetracycline plates were significantly more likely to also grow on ampicillin (RR 3.3) and the reverse situation had an even more pronounced effect (RR 4.6). RS or resistant ceftiofur isolates were more likely to show RS to ampicillin (RR 2.6), whereas there was a weaker association with tetracycline RS (RR 1.4). All RR had a p-value <0.0001 in isolate-level analyses.

5.3 Effect of antimicrobial treatment on susceptibility of *E. coli*

The Kruskal-Wallis test for individual treatment days was significant only for the change in tetracycline susceptibility on day 2 (p-value =0.01) with the ceftiofur treated group having the largest increase from baseline, penicillin treated cows intermediate and the control group the lowest. The change in the proportion isolates growing on cef16 followed a similar pattern, though less significant (p-value = 0.1) because of increases from day 0 to day 2 in three cows in the ceftiofur group. The proportion growth at a concentration of 16 µg/ml ceftiofur for these three cows increased from 2 to 8%, 0 to 14%, and 0 to 6%, respectively, and were 0% on all sampling days thereafter. There were no significant differences in maximum change from baseline among treatment groups ($p \geq 0.15$). Additionally, neither DIM at enrollment (0-7, with >7 set equal to 7), lactation group (1, 2, 3 or greater), nor herd had a significant effect

on the maximum change from baseline (≥ 0.19).

No significant changes were found in the susceptibility of isolates from untreated control cows that were enrolled paired with treated cows and sampled on the same schedule.

5.4 Individual cow and herd effect

Cow-level average percentages of RS and resistance for each antimicrobial drug are displayed in Figure 3. The proportion of isolates showing RS was highly variable among cows with a few cows yielding a high percentage of RS isolates across multiple sampling days. In cow-level analyses, parity, DIM and breed did not have statistically significant effects on maximum change in susceptibility of *E. coli* isolates ($p>0.05$).

6 Discussion

The reduction in the concentration of *E. coli* in fecal samples during systemic treatment with ceftiofur found in day 2 and day 7 samples is consistent with previous reports (Jiang *et al.*, 2006; Singer *et al.*, 2008; Daniels *et al.*, 2009) and signifies a very large selection pressure induced by the presence of the drug.

The frequency of isolates with reduced susceptibility found in our study population was equal to or less than the percentage of resistant *E. coli* isolated from dairy herds using conventional production methods in the United States previously reported by DeFrancesco *et al.* (2004), Sato *et al.* (2005) and Lundin *et al.* (2008). However, the observation that certain cows shed much higher levels of reduced susceptible *E. coli* isolates warrants further investigation to see if this is a permanent or transient biological effect.

We did not find a significant change in ceftiofur susceptibility among *E. coli* isolates during or after treatment with ceftiofur. This may be due to several factors. First, the emergence and spread of resistance under selection pressure might be such a rare event that even our study design, which targeted a large number of isolates per sample and used repeated sampling as well as control animals, was unable to detect it. This would be consistent with Tragesser *et al.* (2006) finding a herd-level but not an individual-cow effect in a study investigating ceftiofur use and isolation of *E. coli* with RS to ceftriaxone. Second, the number of ceftiofur-resistant *E. coli* in the population before treatment might determine the amount of change observed after treatment. Lowrance *et al.* (2007) reported a transient increase in the population of ceftiofur-resistant isolates in a cohort study of feedlot cattle treated with ceftiofur crystalline-

free acid. Systemic treatment with ceftiofur may allow *E. coli* isolates already in possession of resistance genes to temporarily dominate the intestinal flora while proportionally killing larger numbers of susceptible strains. Although the proportion of *E. coli* with RS to ceftiofur did not increase significantly in our study during or after treatment, the percentage of isolates with resistance to ceftiofur (16µg/ml) tended to be higher in day 2 samples when the *E. coli* concentration was greatly reduced.

An advantage of this study was the ability to repeatedly test a relatively large sample of the gut *E. coli* population for traits of resistance and reduced susceptibility following antimicrobial therapy. Although our study design allowed for good estimates of the proportion resistant isolates in each sample, we recognize that having a sample size of about 14 cows per group and reliance on nonparametric statistical methods may have limited the study power for detecting differences among treatments.

7 Conclusions

In this study, most *E. coli* isolates were susceptible to all antimicrobials tested; however, there was wide cow-to-cow variation of antimicrobial susceptibility within the *E. coli* population. Ceftiofur treatment significantly reduced the concentration of fecal *E. coli* for several days after treatment, but the proportion of isolates susceptible to ampicillin, tetracycline or ceftiofur was largely unaffected except for a change in tetracycline resistance noted in the ceftiofur-treated group on day 2. The large number of isolates evaluated provided a high probability of detecting major effects of ceftiofur treatment on fecal *E. coli* antibiotic susceptibility in the treated cows. The population dynamics of intestinal microbial flora is complex and unmeasured factors may account for differences among studies evaluating antimicrobial drug treatment effects on the emergence of resistance.

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10 Tables and Figures

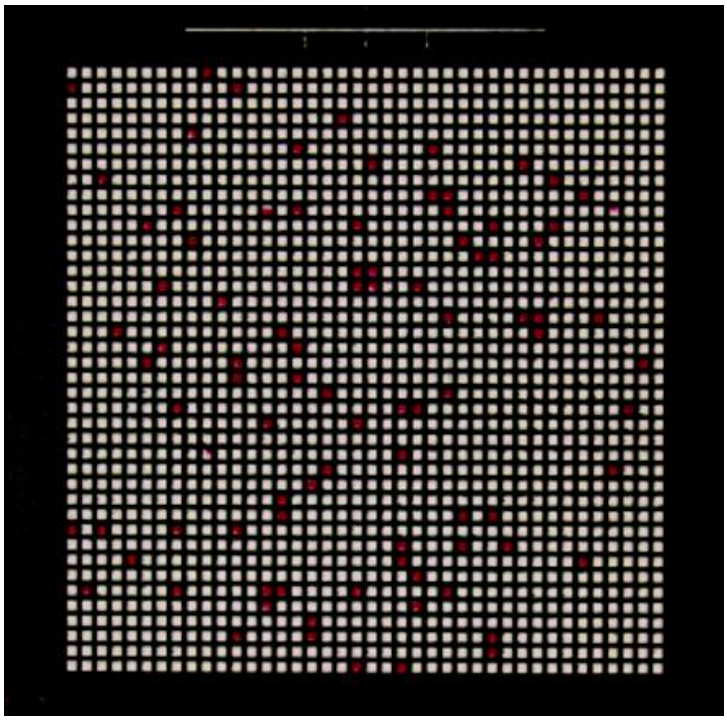

Figure 1. Typical master grid on MacConkey agar: *Escherichia coli* growth within grid cells on the hydrophobic grid membrane filter (red appearance).

Table 1. Descriptive statistics of the 42 cows included in the analysis by herd.

	Herd A	*Herd B*
Total number of cows	27	15
Total number of *E. coli* isolates	11435	6702
Number of animals treated for metritis	17	9
Number of animals treated for interdigital necrobacillosis	0	1
Median lactation number (min-max)	2 (1-5)	1 (1-5)
Median days in milk (min-max)	2 (0-5)	8 (0-282)
Number of animals enrolled in ceftiofur group	9	5
Number of animals enrolled in penicillin group	8	5
Number of animals enrolled in control group	10	5

Table 2. Comparison between the target number of days between sampling events and the actual number of days between sampling given as median, minimum, maximum and mean.

Sampling event	*Target sample interval (days)*	*Actual sample interval (days)*	
		Median (min - max)	*Mean*
Day 0	0	0 (0-0)	0
Day 2	2	2 (1-5)	2.3
Day 7	5	5 (3-7)	4.8
Day 14	7	7 (5-8)	7.0
Day 21	7	7 (3-7)	6.8
Day 28	7	7 (4-8)	6.8

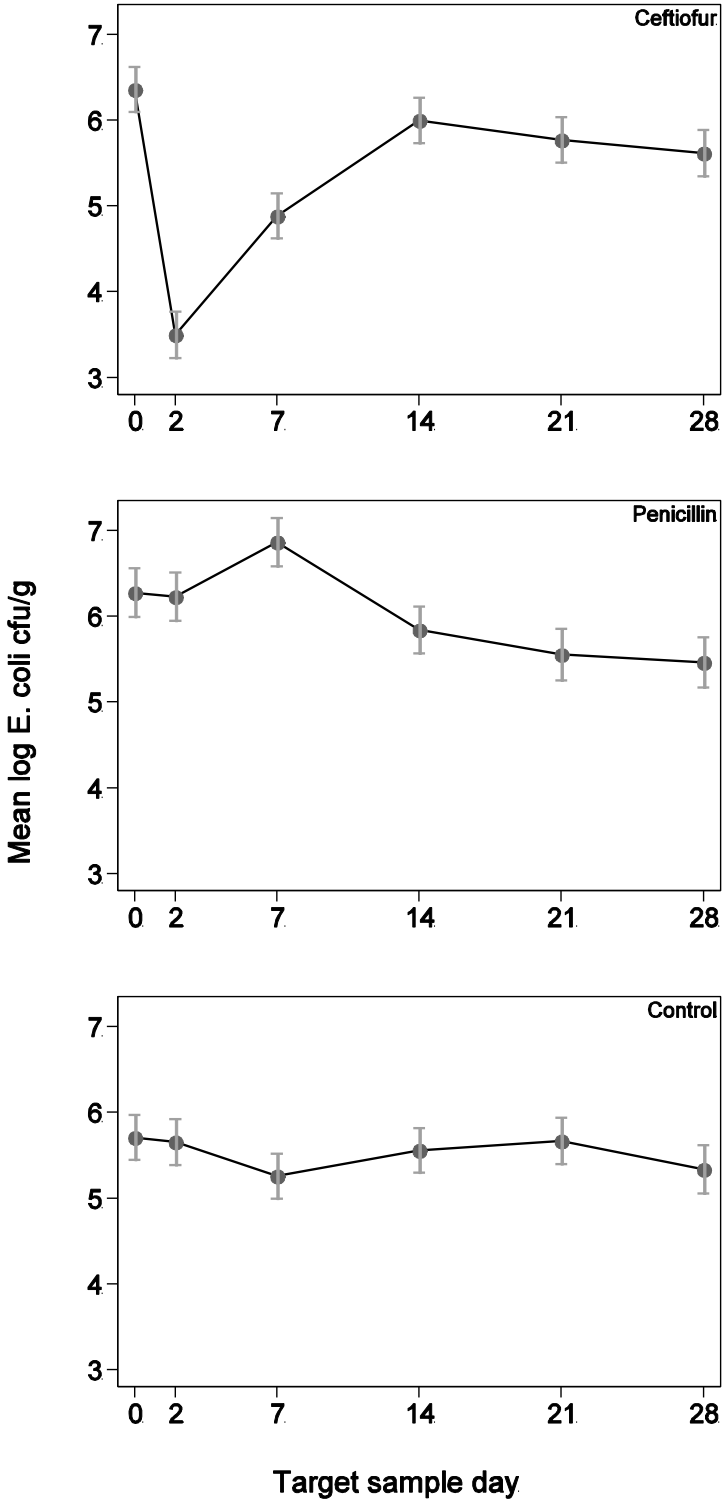

Figure 2. Effect of treating dairy cows with ceftiofur as compared to treatment with penicillin or no treatment (control) on the mean concentration of fecal *Escherichia coli* concentration (cfu/g) over time. Data obtained as predictions from linear mixed models with error bars representing approximate 95% confidence interval for the mean effects.

Table 3. Percentage of fecal *Escherichia coli* isolates at day of enrollment with reduced susceptibility to ampicillin (Amp), tetracycline (Tet) or ceftiofur (Cef). P-values are calculated for differences between farm A and B. P-values test for the hypothesis that percentage of reduced susceptible isolates from Farm A ≠ Farm B for each antimicrobial.

	Percentage of isolates			
	Farm A	*Farm B*	*Combined*	*P-value*
Amp 16µg/ml	3.1	2.8	3.0	0.65
Tet 8 µg/ml	12.5	6.7	10.6	<0.0001
Cef 4 µg/ml	6.5	1.4	4.8	<0.0001
Cef 8 µg/ml	2.3	0.3	1.7	<0.0001
Cef 16 µg/ml	0.9	0.2	0.7	0.019

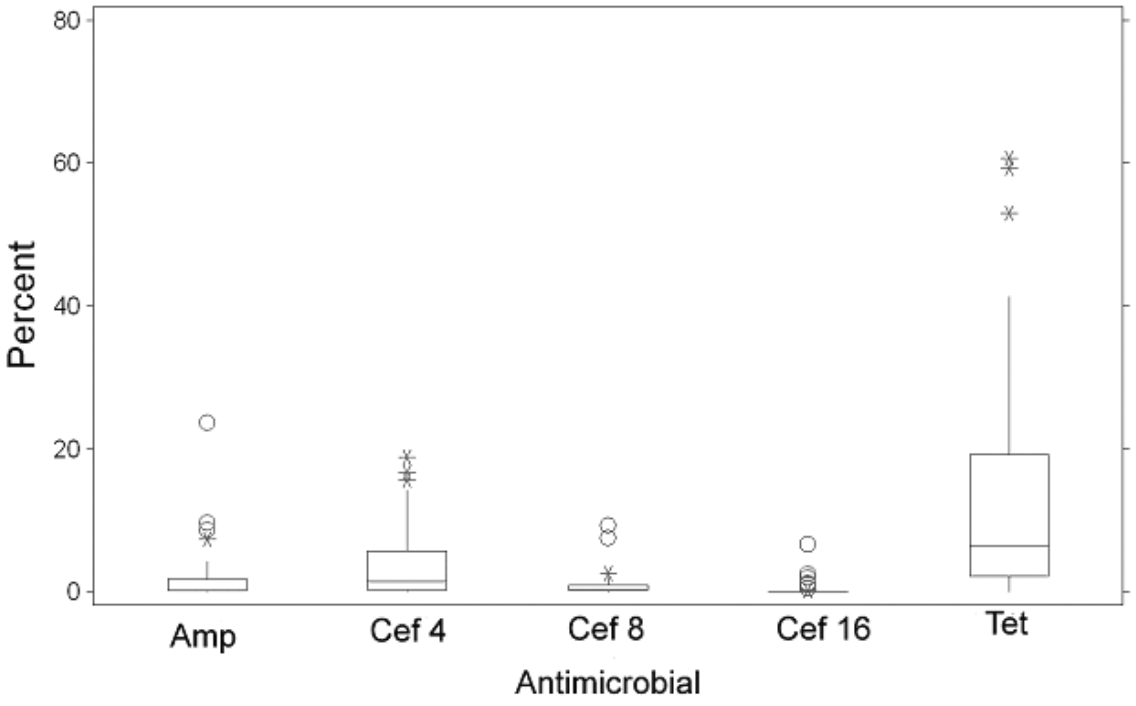

Figure 3. Box and whiskers plots of the cow-level percentages of *Escherichia coli* isolates growing on plates containing antimicrobial agents (n=42 cows). Amp=ampicillin (16 µg/ml), Cef4 = ceftiofur (4 µg/ml), Cef8 = ceftiofur (8 µg/ml), Cef16 = ceftiofur (16 µg/ml) and Tet = tetracycline (8 µg/ml).

IV DISCUSSION

Several effects of antimicrobial drug use on a given strain of bacterial population are possible. A population of bacteria that is fully susceptible to a bactericidal drug will react to the application of the drug with a significant reduction in the total number of bacteria, although in some cases a minimal subpopulation may remain. This effect can be achieved by careful choice of drug, identification of the bacterial population targeted, susceptibility testing prior to treatment and sufficient dosage, adequate route of administration and length of therapy.

In circumstances where bacteria that are intrinsically resistant or resistance to the particular drug in the population has been uniformly established previously, bacterial population dynamics will most likely not be altered after exposure to an antimicrobial agent for which resistance is ubiquitous in the population.

When a drug is used and a mixed population of target bacteria containing resistant and susceptible strains is present, exposure of this population may lead to the disappearance of the susceptible strains, leaving those, that are in possession of resistance-encoding genes towards the drug, without changing the total number of those bacteria. If this situation is present, resistant bacteria will be found more frequently during the course of treatment, yet the drug did not have an effect on the resistant strain itself and the total number of bacteria will be greatly reduced during and possibly for some time after treatment is completed.

Another possibility when antimicrobial selection pressure acts on a mixed population of bacteria is a net gain in total number of resistant bacteria during the reduction of susceptible strains. This may be due to spread of resistance encoding genes to susceptible bacteria under the influence of the antimicrobial or to the enhancement of growth of resistant strains. The total size of the population may or may not be reduced depending on the rise in number of resistant bacteria.

Even when the guidelines for prudent antimicrobial use are heeded, and a population of uniformly susceptible bacteria is opposed to the drug, the effect of selection pressure can lead to the emergence of resistance. Bacteria can gain resistance either through mutation or more likely through horizontal gene transfer from different bacteria that share the same environment and are able to exchange genetic material with the target species. Treatment will therefore potentially result in induction of resistance or more likely the spread of resistance

not only to the target bacteria but to other bacterial populations as well.

All these different scenarios of antimicrobial drugs acting on bacterial populations are theoretically possible and in a real-life situation where different bacteria and different strains are abundant, we will most likely find a mixture of all of the above.

The results of our study indicated that in the case of exposure of fecal *E. coli* isolates to ceftiofur, the most prominent measurable effect was the reduction of total size of the population, making it more likely to find resistant bacteria. We were not able to document the effects of induction of resistance and net gain in resistant population with the study design that was applied. However, this does not mean that they did not occur at levels undetectable by the study methods.

Studies similar to the one we carried out have found similar effects of ceftiofur use on the fecal *E. coli* population while others reported a net increase in resistant isolates or found a combination of both effects (Jiang et al., 2006; Tragesser et al., 2008; Lowrance et al., 2007; Singer et al., 2008; Alali et al., 2009). It is important to mention that protocols varied greatly between the different investigations. Singer *et al.* (2008) studied the effect of ceftiofur treatment in dairy cattle and found a significant drop in total fecal *E. coli* bacterial counts in ceftiofur treated animals (n=5). The authors reported that the ceftiofur-resistant bacterial population did not increase in quantity when treating cows with ceftiofur but that "levels stayed low and were overtaken by a returning susceptible population". While the effect of significant reduction of *E. coli* concentration in feces was also described by Lowrance *et al.* (2007) studying feedlot cattle, the authors stated that administration of ceftiofur crystalline-free acid (CCFA, a long-acting formulation of ceftiofur used as a "one-shot therapy") leads to a "detectable selection pressure within the gastrointestinal tract that favored transient expansion of ceftiofur resistance" in the *E. coli* isolates that were tested. In this study as well as in the previously mentioned study by Singer *et al*. (2008), susceptibility testing was only performed on a subset of three colonies per fecal sample. Tragesser *et al.* (2006) compared *E. coli* fecal isolates with reduced susceptibility to ceftriaxone in herds for which ceftiofur use was reported with herds where ceftiofur was not used and found a significant correlation of ceftiofur use and the existence of cows on a farm from which ceftriaxone-resistant *E. coli* were isolated. This herd-level association was not confirmed at the individual cow level, where "…no association was found between receiving ceftiofur in the previous six months and the isolation of *E. coli* with reduced ceftriaxone susceptibility."

Another study investigated the effect of three different doses of ceftiofur (in the form of CCFA) given to beef feedlot cattle on the quantity of a gene encoding for ceftiofur resistance (*blaCMY-2*) compared to a standardized reference gene (*16SrRNA*) among total community DNA extracted from fecal samples (Alali *et al.*, 2009). The authors concluded that "the administration of CCFA in feedlot cattle may provide selection pressure favoring higher levels of *blaCMY-2* carriage, but this may lead to concurrent reductions in the total bacterial population (as reflected by lowered *16srRNA*) during the treatment period."

The effect of ceftiofur treatment on fecal bacteria has also been investigated in dairy calves. Jiang *et al.* (2006) showed that total bacterial counts in feces were reduced following the first dose of antibiotic treatment and that immediately after treatment the means of ceftriaxone-resistant bacterial counts increased by 1.05, 0.87, or 0.58 log CFU/g for three different ceftriaxone concentrations used (16, 64, or 128 μg/ml) which the authors concluded "…revealed that the treatment of calves with ceftiofur can temporarily change the bacterial flora in the calf´s intestine to favor the growth of ceftriaxone-resistant fecal bacteria."

In conclusion it seems that while most authors that investigated the effect of ceftiofur on the fecal bacterial population documented a reduction in total bacterial *E. coli* counts, the effect of a (transient) net increase in resistant bacteria was not found in all studies.

One of the main differences in our study compared to the investigations described above was that we used a treatment protocol that is often applied in dairy practice with a four-day treatment period. This differed from other studies in that treatment was one day shorter (Singer *et al.,* 2008) or that no long-acting preparation of ceftiofur (CCFA) was used (Lowrance *et al.,* 2007; Alali *et al.,* 2009).

In interpreting the results of this investigation, it is important to recognize that ceftiofur was generally used on both farms prior to this study and that each cow was sampled over a period of four weeks. It could be expected that results are possibly different when introducing a new drug into a population or even when studying fecal populations for a longer period of time.

Cows with clinical metritis and interdigital necrobacillosis were enrolled because these are common indications for ceftiofur use (Chenault *et al.,* 2004) and the study was conducted in a real-life situation in commercial dairy herds. Penicillin is a frequently used alternative to ceftiofur when treating those two diseases and was therefore chosen for the second treatment group. We did not expect penicillin to influence the *E. coli* population significantly because

penicillin acts on the bacterial cell wall synthesis of Gram-positive organisms. But by using a treatment control group we were able to eliminate the possibility that disease was a significant factor on the recovery of resistant fecal *E. coli* isolates. This was important since the third group, the non-treated controls, consisted of healthy cows only.

In our study, the amount of drug was the same for all animals regardless of their actual body weight to be consistent with usual practices on the participating farms. By not measuring the actual weight of each individual cow, one can argue that some animals might have received a higher dose than others. All animals enrolled in the study received a dose in the range indicated on the label instructions (Excenel RTU, ceftiofur hydrochloride, 0,5-1mg/kg, 1-2 ml/100 lbs body weight [1-2ml/45 kg] for 3-5 consecutive days, (Pfizer, 2008). Because we applied the same treatment protocol to all cows being sequentially enrolled we believe that we did not produce a bias and if dose-dependent effects occurred they would have been evenly distributed among both treatment groups.

When estimating *E. coli* cfu/g in fresh fecal matter, we did not consider the possible differences in dry matter which might have differed between individuals and between sampling days. The water content of dairy cow feces usually lies within a range of 79 to 83 % (Shalit *et al.*, 1991) and is typically 81,2 % in fresh samples (Wang *et al.*, 1996), but the moisture content can vary as much as 70 to 85 % (Murphy, 1992). Although we did not account for this possible variation, the results of the estimated cfu/g were of a much higher magnitude (maximum reduction of colony size by the factor 10^{-3} on day 2 of ceftiofur treatment) than can be explained by the relatively small potential difference in water content.

Although our design setup may have had the above mentioned flaws, we believe that there is value in the study results. To our knowledge it is the first investigation on the effect of ceftiofur treatment compared to two control groups on large numbers of *E. coli* isolates by using the HGMF method. We confirmed the findings of other studies documenting the strong effect of reducing total fecal *E. coli* population. When looking at the percentage of resistant isolates in a sample, we did not detect an increase in those isolates as a proportion of total *E. coli* detected. The population dynamics are altered by many factors, making it impossible to guarantee equal sampling conditions over time. Nevertheless, when looking at the results of our study we must conclude that if ceftiofur use leads to the spread and emergence of resistance in fecal *E. coli* isolates in dairy cows under field conditions, this must be a rare event which we were not able to document.

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V SUMMARY

The existence of resistance mechanisms of bacteria against the action of antimicrobial drugs is a well documented fact and has complicated treatment of bacterial disease since the beginning of antimicrobial therapy in the last century.

Drug resistance is a problem of veterinary and human medicine alike, especially when considering infections with food-borne pathogens such as *Salmonella.* Fecal *E. coli* isolates were used as a model to measure the effect of treating dairy cows systemically with ceftiofur on antimicrobial resistance of this important pathogen. Ceftiofur, a third-generation cephalosporin, is an attractive treatment for certain diseases in dairy cattle such as metritis and interdigital necrobacillosis since withholding times for meat are short and milk does not need to be withheld from marketing. Third-generation cephalosporins are also used in human medicine, for example in the treatment of systemic non-typhoidal salmonellosis in children. Therefore, the emergence and spread of resistance towards this class of antimicrobials has been a focus of concern.

Effects of ceftiofur treatment on a group of cattle were compared to a penicillin treated group and a healthy control group not receiving treatment. Reduction of susceptibility of *E. coli* was tested for ampicillin, tetracycline and three different concentrations of ceftiofur. A significant decrease in the concentration of fecal *E. coli* on days 2 and 7 post-treatment was detected in animals treated with ceftiofur. Treatment did not have a significant effect on the proportion of isolates expressing reduced susceptibility to ceftiofur. The result of the reduction of total *E. coli* count in the samples during and after ceftiofur use is consistent with results reported by other authors. The large number of isolates analyzed provided a high possibility of detecting major effects of ceftiofur treatment on *E. coli* susceptibility. However we did not observe a net increase in reduced susceptible *E. coli* isolates with the exception of decreased tetracycline susceptibility in the ceftiofur treated group on day 2.

The results of this work lead to the conclusion that the emergence and spread of resistance-encoding genes in a bacterial population under the influence of ceftiofur are rare and that the effect could not be measured even though we were able to draw information on a large number of isolates analyzed compared to other investigations previously published on this topic.

The results are applicable to commercial dairy herds with typical antimicrobial treatment practices comparable to those used in the US, including prior use of ceftiofur for treating individual sick animals.

VI ZUSAMMENFASSUNG

Das Wissen über das Vorhandensein von bakteriellen Resistenzmechanismen ist so alt wie die Entdeckung der Wirksamkeit antimikrobieller Substanzen. Diese Resistenzen führen seit der Einführung ihres Gebrauches zur Therapie menschlicher und tierischer bakterieller Erkrankungen im letzten Jahrhundert immer öfter zu einer Reduktion des Therapieerfolges.

Antibiotikaresistenz ist ein Problem der Veterinär- und Humanmedizin gleichermaßen, besonders wenn es um Infektionen mit durch Nahrungsmittel übertragene Erreger wie beispielsweise Salmonellen geht. Der Effekt, den die systemische Behandlung von Milchkühen mit Ceftiofur auf die Resistenzlage dieses wichtigen Erregers haben könnte, wurde modellhaft anhand fäkaler *E. coli*-Bakterien untersucht. Ceftiofur gehört zur Klasse der dritten Generation der Cephalosporine und ist ein häufig verwendetes Medikament für bestimmte bakterielle Erkrankungen von Milchkühen, wie z.B. Metritis und interdigitale Nekrobazillose, da die Wartezeit auf Fleisch kurz ist und die Milch trotz antibiotischer Behandlung der Kuh abgeliefert werden kann. Cephalosporine der dritten Generation werden auch in der Humanmedizin angewandt, zum Beispiel für die Behandlung systemischer Infektionen mit nicht-typhoiden Salmonellen beim Kind. Aufgrund dieser Tatsache gibt die Enstehung und Verbreitung von Resistenzen gegenüber dieser Klasse von Antibiotika Anlass zur Sorge.

Gemessen wurde der Effekt der systemischen Behandlung von kranken Tieren mit Ceftiofur im Vergleich zur Behandlung einer Gruppe erkrankter Kühe mit Penicillin und einer unbehandelten, gesunden Kontrollgruppe auf die reduzierte Wirkung von Ampicillin, Tetrazyklin und drei verschiedenen Dosierungen von Ceftiofur gegenüber fäkalen *E. coli*-Bakterien. Ein signifikanter Abfall in der *E. coli*-Konzentration an den Probentagen 2 und 7 nach Behandlungsbeginn konnte festgestellt werden. Die Behandlung hatte keine statistisch signifikanten Auswirkungen auf den Anteil der Isolate mit reduzierter Wirkung gegenüber Ceftiofur. Das Ergebnis der verringerten Menge an fäkalen *E. coli*-Bakterien während und nach der Gabe von Ceftiofur stimmt mit Ergebnissen anderer Autoren überein. Die Wahrscheinlichkeit, mit der bedeutende Effekte der Ceftiofurgabe auf die Wirksamkeit der getesteten Antibiotika zu messen sind, wurde durch die große Menge an Isolaten, die zur Analyse herangezogen werden konnte, erhöht. Abgesehen von einer transienten Zunahme der *E. coli*-Bakterien am Tag 2, die eine Resistenz gegenüber Tetrazyklin aufwiesen, konnte keine

Erhöhung des Anteils an Bakterien mit erhöhter Resistenz nachgewiesen werden.

Die Ergebnisse dieser Arbeit lassen den Schluss zu, dass die Enstehung eines Resistenzgenes und dessen Verbreitung in der Bakterienpopulation unter dem Einfluss von Ceftiofur mit hoher Wahrscheinlichkeit seltene Ereignisse sind, so dass es auch durch Untersuchungen wie diese, der eine im Vergleich zu anderen wissenschaftlichen Studien ungleich größere Menge an untersuchten Isolaten zu Grunde liegt, nur schwer nachweisbar ist.

Die Resultate der Studie gelten für solche Milchviehherden, in denen der Antibiotikaeinsatz dem typischen Gebrauch in den USA gleicht und in denen Ceftiofur routinemäßig für die Behandlung von Einzeltiererkrankungen eingesetzt wird.

VII ANNEX

1 Unabridged materials and methods section

Study population and design- We enrolled cows from two New York State dairy herds in a longitudinal controlled trial. Herd sizes were 140 and 300 milking cows. The dominant breed was Holstein-Friesian; all other cows were Ayrshires. A herd survey filled out by the owners showed that antimicrobial usage practices for these herds were similar to those reported for conventional farms by Zwald *et al.* (2004). Ceftiofur was used on both farms prior to the study for typical purposes such as treatment of respiratory disease, metritis, placental retention, and foot problems. Lactating cows with metritis or interdigital necrobacillosis as identified by the herd owner or veterinarian were enrolled sequentially in one of two treatment groups; ceftiofur or penicillin. The treatment for the first animal within each herd was selected at random. Healthy animals served as untreated controls and were paired with antibiotic-treated animals, alternating groups, and matching calving date and parity whenever possible.

Samples were collected from each animal at enrollment (day 0) and subsequently five times in a four-week period. The target sampling days were 2, 7, 14, 21 and 28 days after enrollment. The study was conducted between July, 2008 and July, 2009.

Treatment protocol- Treatment for cows in the ceftiofur group consisted of a subcutaneous injection of 1 gram of ceftiofur hydrochloride suspension *(*Excenel RTU, Pharmacia & Upjohn Co, Division of Pfizer Inc, New York, NY) once per day for a total of four days. Cows enrolled in the penicillin group received 9 million IU of a penicillin G procaine suspension (PenOnePro, Vet One, MWI, Meridian, ID) intramuscularly once per day for a total of four days.

Sample collection and processing- Fecal samples were collected before treatment by the herd owner or veterinarian (day 0) and on days 2, 7, 14, 21 and 28 after enrollment by the veterinarian. Approximately 50 g of feces were collected directly from the rectum of each cow with a clean plastic sleeve. Samples were placed immediately into a sterile sample container and transported refrigerated to the laboratory to be processed on the day of collection.

Those administering treatments and collecting samples were not blinded to treatment group. Study personnel carrying out laboratory procedures were unaware of the assignment of animals.

Fifteen g of fecal material from each sample were placed in a sterile Whirl-Pak filter bag (Nasco, Fort Atkinson, WI) and mixed with 40ml of a sterile 0.9% saline solution. The sample was then mixed in a stomacher for 2 minutes. The filtrate was placed in a second sterile Whirl-Pak filter bag and processed for another 2 minutes. Five 1 ml replicates of the specimen were aseptically transferred from the filter pocket into 5 ml freezer vials prepared with 1 ml of glycerol (Fisher Scientific, Fair Lawn, NJ) and tryptose soy broth (Becton, Dickinson and Company, Franklin Lakes, NJ) (1:1 vol/vol) and placed in a -80°C freezer for storage.

Production of hydrophobic grid membrane filter (HGMF) master grids- HGMF master grids were used to enumerate and replicate bacteria using a modification of a method described previously (Dunlop *et al.*, 1998; Jordan *et al.*, 2005). A master grid containing no more than 200 colonies was chosen to limit the probability of any one colony being derived from more than one bacterium (Jordan *et al.*, 2005).

To generate a master grid, each frozen sample was thawed in a 30 °C water bath. Five-fold serial dilutions were made in 0.1 % peptone water containing 1% Tween 80 (Fisher Scientific, Fair Lawn, NJ). Approximately 1.5 ml of each diluted sample was filtered through a sterile HGMF grid (ISO-GRID®, QA Life Sciences, CA) using an SF-1 Spreadfilter (Filtaflex, Almonte, Ontario, Canada).

HGMF grids were then aseptically transferred onto MacConkey agar (MAC) (Northeast Laboratory Services, Waterville, ME) and incubated at 37°C for 18-24 h to produce a master grid.

If the dilution process failed to produce a grid as described, another replicate of the sample was chosen, thawed and more extended dilutions carried out until the condition was met. In cases where the diluted sample yielded very low numbers of colonies, either the sample was enriched in GN broth (Hajna) or a dilution was incubated in the waterbath to increase the number of bacteria. Five-fold serial dilutions were then produced from the enriched sample.

Colony replication-Bacterial growth on the master grid was copied onto HGMF grids on a series of nine different agars using an RP-1 HGMF replicator (Filtaflex, Almonte, Ontario, Canada). In order of replication, these agars were: Mueller-Hinton (MH) containing ceftiofur

at three different concentrations - 4, 8 and 16 µg/ml (Northeast Laboratory Services, Waterville, ME); chromogenic agar (BBL Chromagar Orientation, Becton, Dickinson and Company, Franklin Lakes, NJ), MH agar containing either ampicillin (16 µg/ml), tetracycline (8 µg/ml), sulfamethoxazole (256 or 512 µg/ml), or chloramphenicol (16 µg/ml) (Northeast Laboratory Services, Waterville, ME); and MH plates without an added antimicrobial agent (Northeast Laboratory Services, Waterville, ME) to ensure that the inoculum was transferred to all agar plates. All plates were incubated at 37°C for 18-24 hours. The chromogenic media served as a control for *E. coli* (purple appearance).

Breakpoints were adopted from the Clinical and Laboratory Standards Institute (CLSI, 2008). The presence of the bla_{CMY-2} gene, which has been linked to ceftiofur resistance in *Salmonella* isolates, is strongly associated with a minimal inhibitory concentration of 8 µg/ml (Alcaine *et al.*, 2005).

The concentration of sulfamethoxazole was switched from 256 µg/ml to 512 µg/ml after the beginning of the study; information for this antimicrobial is therefore incomplete and was excluded.

Quality control-Identity of *E. coli* was confirmed for a subset of typical purple-colored isolates on chromogenic agar either in the Animal Health Diagnostic Center (AHDC), Cornell University College of Veterinary Medicine or by the Sulfide Indole Motility (SIM) tube test. To ensure quality of the filter system, two *E. coli* isolates were chosen as positive and negative controls. These were cultured, processed and replicated as described for replicate samples from study animals. The isolate serving as negative control (ATCC 25922) was confirmed to be susceptible to all study antimicrobials using the Sensititre Automated Microbiology System´s A80 Panel (TREK Diagnostic Systems Inc., Cleveland, OH) in the AHDC. The positive control, originating from a previous study, was characterized as multi-drug resistant by the same method and as having the bla_{CMY-2} gene by PCR. For quality control of the plates and growth conditions, both isolates were streaked onto the side of the antimicrobial plates, outside the grid zone, for at least two sample days per cow.

Imaging and analysis of colony growth-Digital pictures of HGMF filters on agar were taken with a camera (Canon Power Shot SX110 IS) using remote picture software to enhance quality (PSRemote Version 1.6.4, Breeze Systems Limited, Bagshot, Surrey, UK). Customized software for image analysis (HGMFRES, NSW Department of Primary Industries, Wollongbar, NSW, Australia) was then used to detect bacterial growth and identify

the grid cell address (row and column position) of each colony as described previously (Jordan *et al.*, 2005). A third software (HGMF Image Dr, NSW Department of Primary Industries, Wollongbar, NSW, Australia) was used to exclude all colonies on the chromogenic agar not showing the typical *E. coli* color reaction.

Statistical analysis- Data were analyzed using SAS ver. 9.1 (SAS Institute Inc., Cary, NC, USA). The distribution of parity and days in milk among treatment groups was analyzed using Kruskal-Wallis tests. The pairwise associations of reduced susceptibility to tetracycline, ampicillin and ceftiofur were analyzed at the isolate-level using chi-squared tests and at the cow-level using Spearman´s rank correlation. Estimation of *E. coli* concentrations in fecal samples was achieved by applying the following formula:

$$cfu \cong n \times (5^{dil}) \times 2 \times \frac{55}{15} \times \frac{1}{1.5}$$

where *n* is the count of isolates on the control grids, *dil* is the number of five-fold dilutions to reach the solution providing approximately 50-150 isolates per grid, and the other values represent volumes transferred in the various steps of the procedure. In the case of enriched samples dilution number was set to 0. Repeated measures analysis of variance (ANOVA; PROC MIXED) was used to analyze the treatment group effect on the $\log_{10}$ concentration over time. Cow was treated as a subject effect and a first-order autoregressive covariance structure was specified. Sample day (0, 2, 7, 14, 21 and 28) was included in the model as a fixed effect. The treatment by day interaction term was tested to determine whether changes in concentration over time depended on treatment group. Pairwise differences between treatment-day combinations were tested using Bonferroni-corrected *p*-values. Assessment of the normality of model residuals and plots of residual values against predicted values were used to evaluate model assumptions and were considered acceptable for this model.

Treatment group effects on reduced antimicrobial susceptibility were analyzed by first calculating the proportion of isolates with growth on the applicable antimicrobial plate among those grid cells with growth on both on the MH and chromogenic agar control plates. This was done for each cow-sample day combination. For each combination of antimicrobial and drug-concentration, the change from the pre-treatment measurement in proportion reduced

susceptibility was calculated within cow for each post-treatment day. The data were analyzed initially using repeated measure ANOVA as described above, but model assumptions were not met. Therefore Kruskal-Wallis tests were used to compare treatment groups on each sample day. The effect was also evaluated by calculating the maximum change from baseline for each drug for each cow. The effect of treatment group, days in milk at enrollment, lactation group (1, 2 or ≥3), and breed on maximum change were evaluated using Kruskal - Wallis tests.

1.1 References

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2 Software analysis window

Figure: HGMFRES software, NSW Department of Primary Industries, Wollongbar, NSW, Australia. Original digital picture of the grid to be analyzed on (left), analyzed grid with colonies being highlighted in red (right).

3 HGMF on spreadfilter device

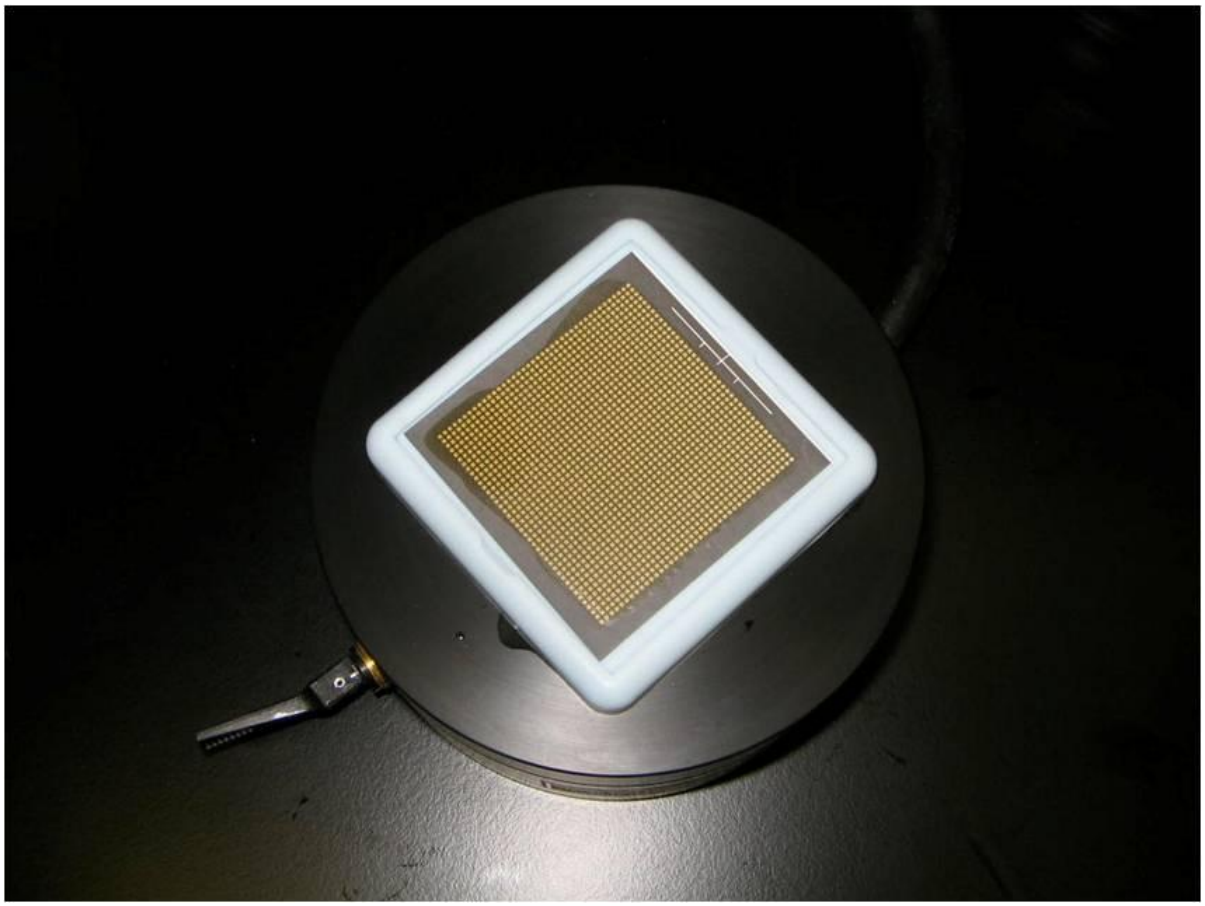

Figure: HGMF being placed on the SF-1 Spreadfilter (Filtaflex, Almonte, Ontario, Canada). The inoculum is filtered through the membrane with the help of vacuum.

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