Transfer of rifampicin-resistant *Escherichia coli* among feedlot cattle

by

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ABSTRACT:

Transfer and shedding of a rifampicin-resistant strain of *Escherichia coli* (RREC) among cattle was studied in a research feedlot comprised of 30 pens of 11 or 12 yearling steers. On 3 separate occasions, 9, 6 and 6 of the 12 steers in 3 different peripheral pens in the lot were orally inoculated with 10^{11} cells of an unmodified RREC isolate from bovine feces. Fecal swabs were performed on all 360 steers in the feedlot immediately prior to and at approximately 5-week intervals thereafter. Following inoculation, fecal grab samples were collected daily from all 12 pen mates and from the 12 steers in the adjacent pen(s) for 2 weeks, then 3 times per week for up to 4 months. In all 3 trials, the inoculated steers each shed RREC within 24 h of inoculation. All 12 steers in each inoculated pen were positive for RREC within 48 h; all 36 steers shed RREC intermittently throughout the three sampling periods. Transfer to 4 steers in an adjacent pen was confirmed only during the first trial (3 steers shed once each on day 8, day 26 or day 40; the fourth shed on 6 occasions between days 8 and 40). Transfer to non-adjacent pens was not detected during any of the 3 trials. All recovered RREC isolates were compared to the inocula using LMX agar and fatty acid methyl ester (FAME) analysis. Additionally, select recovered isolates were subjected to carbon source utilization tests. The three inocula were further subjected to 16S rRNA sequence analysis, minimum inhibitory antibiotic concentration profiles and pulsed-field gel electrophoresis and were determined to be the same strain. It was observed with the exception of the pen floor, that the resistant strain did not move through the animal feedlot environment, as easily or pervasively as other studies suggested. The RREC did not persist in the feedlot environment beyond the 4-month trial period. Fecal contamination from the pen floor,

animal-to-animal contact and the chute system may have facilitated transfer of the resistant strain between animals. Animal stress may have facilitated the pen-to-pen transfer observed during trial 1, as the inoculation was conducted within 1 week of the steers' arrival in the feedlot.

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Sam

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LIST OF ABREVIATIONS

APHs	Aminoglycoside Phosphotransferases
AST	Antibiotic Sensitivity Testing
CFU	Colony Forming Units
FAME	Fatty Acid Methyl Ester
GI	Gastro-intestinal
MDR	Multi-Drug Resistance
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
PFGE	Pulsed-Field Gel Electrophoriesis
Rm	Rifampicin
Rm^r	Rifampicin Resistant
RREC	Rifampicin Resistant *Escherichia coli*
RREC I	Rifampicin Resistant *Escherichia coli* First Inoculum
RREC II	Rifampicin Resistant *Escherichia coli* Second Inoculum
RREC III	Rifampicin Resistant *Escherichia coli* Third Inoculum
VRE	Vancomycin-Resistant Enterococci

1. INTRODUCTION:

The use of antibiotics in livestock production has been increasingly implicated as a possible cause of the emergence of antibiotic resistant bacteria (Davies, 1996; Khachatourians, 1998; Levy, 1998). Scientists, government policy makers and the public are fearful of the possibility of resistant bacteria being transferred from livestock to humans through direct animal contact, indirect environmental contact or by contaminated food products. The emergence of antibiotic resistance in potential pathogens, such as *Escherichia coli*, *Shigella* spp., *Salmonella* spp. and *Staphylococcus aureus,* is of particular concern because these bacteria may be readily transferred from livestock to humans (Novick, 1981).

Animals are raised for human consumption in North America and most receive antibiotics at some point during their lives (Endtz et al., 1991; Helmuth and Protz, 1997; Witte 1998; Wegener *et al.,* 1999). Antibiotics are used in cattle production for the treatment of disease (therapeutic), prevention of disease (prophylactic) or sometimes to increase the production of meat (i.e., to increase weight gain) or milk (sub-therapeutic). The sub-therapeutic use of antibiotics is viewed as being economical and the widespread adoption of this practice has promoted the intensification of livestock production (Cohen and Tauxe, 1986; Gustafson and Bowen, 1997). Large feedlots have found each dollar invested in the administration of antibiotics to steers on arrival returns three dollars in profit (Gustafson and Bowen, 1997). Broad-spectrum antibiotics that promote growth and feed efficiency have also been shown to control endemic diseases in large groups of animals. Sub-therapeutic concentrations of antibiotics in feed are high enough to have marginal effects on bacterial competition but are low enough to allow bacterial growth

and potentially select for resistant bacteria. Of the 22.7 billion kg of broad spectrum antibiotics produced each year in the United States, it is estimated that 40% are used in the agriculture industry and only 20% are used for therapeutic treatment of animals (Levy 1998). It has been estimated of the 20 million kg of antibiotics imported and produced each year in Canada, half are used in agriculture or aquaculture and only 10% are used for therapeutic treatment of animals (Khachatourians, 1998).

How bacteria spread within the environment is important in understanding the incidence and prevalence of antibiotic resistant bacteria and the means by which antibiotic resistance may be introduced and maintained within microbial populations (Marshall *et al.,* 1990). Further assessment of the risks to human health is imperative. Although there are numerous reports of antibiotic resistant bacteria being isolated from livestock, the majority of this research focuses on poultry and swine where antibiotic use is more prevalent (Center for Veterinary Medicine, 1999). Transmission of antibiotic resistant bacteria has not been extensively studied in cattle and never under commercial feedlot conditions.

To examine transfer of an antibiotic resistant bacterium among cattle, it is necessary to have a bacterial strain that is readily traceable and detectable within the feedlot environment. The strain should also be easily cultured and relatively abundant in the gastro-intestinal (GI) tract of cattle. These characteristics will greatly facilitate tracking of a marked strain introduced into the heavily populated complex microbial community found in the lower GI tract of cattle (Hrubant *et al.,* 1972). Members of the family Enterobacteriaceae and enterococci are abundant in cattle feces (Carroll and Jasper, 1978). Selective and differential media (e.g., Enterobacteriaceae - Eosin

Methylene Blue, MacConkey, LMX; enterococci – Bile Esculin Azide) are readily available for the cultivation of these bacteria (Manafi *et al.,* 1991). Furthermore, the incidence of antibiotic resistant pathogens from these taxa is of growing concern (Cohen and Tauxe, 1986).

A genetically modified strain is undesirable as regulatory restrictions prevent the release of such bacteria into the feedlot environment. Although antibiotic resistant bacterial stains can be generated spontaneously by exposure of a large number of cells to antibiotics in the laboratory, such strains may exhibit reduced competitiveness and often fail to establish within an environment that does not have the selective pressure for the resistance trait (Marshall *et al.,* 1990; Williams *et al.,* 1996). Consequently, screening for an unique antibiotic resistance marker in bacteria directly isolated from cattle may be the approach that is most likely to yield a traceable bacterium capable of establishing within feedlot cattle.

The objectives of this study were to monitor establishment, transfer and persistence of an antibiotic resistant bacterium in feedlot cattle. To address these goals, a lactose fermenting, rifampicin resistant strain of *E. coli* (RREC) from cattle feces using rifampicin gradient plates was isolated. Cattle in a research feedlot were inoculated with this strain and its spread and persistence among animals in the same pen and an adjacent pen were documented. The effect of multiple passages of the RREC through cattle hosts on transfer and persistence was also examined.

2. LITERATURE REVIEW

2.1 Definition and History of Antibiotics:

Antibiotics are natural metabolites that kill or inhibit the growth of competing bacteria. These compounds are produced by a variety of microorganisms and have evolved likely due to inter- and intra-specific competition (Wiener *et al.*, 1998). Their discovery is credited to Sir Alexander Fleming of Great Britain, who noted that a *Penicillium* isolate produced a substance that inhibited streptococcal growth. Unaltered or chemically modified antibiotics are used by mankind to treat bacterial infections in humans and agricultural products. Some bacteria have become antibiotic resistant by adapting to the challenges of the antibiotic environment created by either man or competing microbes.

Penicillin was hailed as a miracle drug, killing bacteria that caused many human diseases including tuberculosis and pneumonia. Between the mid 1940's and 1970's, rapid discovery of new antibiotics made the issue of resistance to older antibiotics of little consequence. We are now on the verge of returning to pre-penicillin days when even seemingly small infections could turn lethal for the lack of effective drugs. Ironically, during a 1945 conference held in New York, Sir Alexander Fleming predicted the demise of the new medical antibiotic breakthrough (Staff Reporter, 1945). At a time when all antibiotics were administered by injection, he predicted that the public would demand a preparation that could be taken by mouth. This in turn would lead to self-medication and wrong source treatment with too small of a dose. Fleming stated that bacteria would develop resistance rather than be eliminated and that the resistant bacteria would be passed on to other individuals.

Several years prior to the introduction of penicillin to clinical practices, Abraham and Chain described a bacterial enzyme that catalyzed hydrolysis of the β-lactam ring thereby inactivating these antibiotics (Gabay, 1994). Plasmids bearing coding regions for antibiotic resistance have been recovered from bacteria stored from the pre-antibiotic era and organisms producing β-lactamases have been retrieved from plant specimens stored during the 17th century (Gabay, 1994; Bergogne-Berezin, 1997). Abraham and Chain predicted that these enzymes would interfere with the potential therapeutic use of penicillin (Gabay, 1994).

2.1.1 Agricultural Use:

During the 1940's, commercial producers of penicillin and vitamin B_{12} began using corn as a fermentation substrate. Fermentation residues were evaluated as poultry and livestock feeds (Fisher, 1994). Favorable effects on livestock performance were noted, including an improvement in growth and feed efficiency as well as a reduction in health problems such as post-weaning diarrhea. It was soon discovered that these beneficial effects were linked to antibiotics in the fermentation residues. This led to the common practice of including sub-therapeutic levels of antibiotics in animal feed (Cheeke, 1999).

Most animals raised for human consumption in Canada and the United States receive antibiotics at some point during their lives (Endtz *et al.,* 1991; Helmuth and Protz, 1997; Witte, 1998). Antibiotics are used in cattle production for the treatment of disease (therapeutic), prevention of disease (prophylactic) or to increase productivity such as weight gain or milk production (sub-therapeutic). The mechanisms of growth

promotion by antibiotics are speculated to be linked to affects on intestinal micro flora. Visek (1978) proposed five hypotheses to explain growth promotion: i) suppression of sub-clinical infections, ii) reduction of microbial toxins, iii) reduction of nutrient destruction by microflora, iv) enhancement of nutrient absorption due to a thinning of intestinal walls and v) increased energy for growth rather than immune system response to gut micro flora. Although antibiotics have been used in feeds since the 1950's, their effectiveness as growth promotants has not diminished with time and there remain large economic incentives for their use (Schell, 1984; Levy, 1992; Gustafson and Bowen; 1997; Dunlop *et al.,* 1998; Khachatourians, 1998).

Bacterial diseases common in cattle are listed in Table 2.1. Therapeutic treatment of these diseases is the most common reason for antibiotic administration. Table 2.2 lists the bacterial targets of antibiotics commonly used for beef and dairy cattle. If label recommendations are followed, this type of chemotherapy shortens the duration of administration and usually reduces the amount of antibiotics used. Consequently, the risk of bacteria developing resistance is minimized.

Broad-spectrum antibiotics that promote growth and feed efficiency have also been shown to control endemic diseases in intensively raised livestock. Prophylactic treatment of an entire herd or flock when only a few animals appear sick is considered both efficient and effective in maintaining herd health. The use of antibiotics to control bovine respiratory disease is a common practice in North America. Operators of large feedlots have found that each dollar invested in the administration of antibiotics to steers on arrival to the feedlot, returns three dollars in profit (Gustafson and Bowen, 1997). Economic advantages have made the use of antibiotics practical and promoted

Table 2.1: Common diseases in beef and dairy cattle treated with antibiotics and antimicrobial agents (McAllister *et al.*, 2001).

Condition	**Causative Bacteria**
Common	
Bovine Respiratory Disease (Pneumonia)	*Pasteurella haemolytica* *Pasteurella multocida* *Haemophilus somnus* *Mycoplasma bovis*
Enteric Disease (Diarrhea)	*Escherichia coli* *Clostridium perfringens* *Salmonella* spp.
Mastitis	*Staphylococcus aureus* *Streptococcus agalactiae* *Klebsiella/ E. coli/ Enterobacter* *Pseudomonas* spp.
Foot Rot	*Actinomyces pyrogenes* *Fusobacterium necrophorum* *Bacteroides* spp.
Metritis (Uterine Infection)	*Actinomyces pyrogenes* *Fusobacterium necrophorum* *Bacteroides* spp.
Ocular (Pink Eye)	*Moraxella bovis*
Less Common	
Lumpy Jaw	*Actinomyces bovis*
Listeriosis	*Listeria* spp.
Anaplasmosis	*Anaplasma marginale*
Tetanus (Black Leg)	*Clostridium* spp.
Wooden Tongue	*Actinobacillus lignieresii*

Table 2.2: Examples of common antibiotics and antimicrobial agents administered to cattle (McAllister *et al.*, 2001).

Antibiotic Family (Source)	Trade Names	Target-action
Aminoglycosides (*Micromonospora* spp., *Streptomyces* spp.)		
Gentamicin	Gentamicin	Primarily Gram negative, Inhibit protein synthesis
Cephalosporins (*Cephalosporium acremonium*)		
Ceftiofur sodium	Excenel	Broad spectrum activity,
Cephapirin	Meticure Sus.	Inhibits cell wall synthesis
Ionophores (*Streptomyces* spp.)		
Monesin	Rumensin	Primarily Gram positive
Lasalocid	Bovatec	Interferes with ion transport
Salinomycin	Posistac	
Macrolides (*Streptomyces* spp.)		
Tilmicosine	Micotil	Primarily Gram positive,
Erythromycin	Erythro-36	Inhibits peptide bond
Tylosin	Tylan	formation
Penicillins (*Penicillium* spp.)		
Penicillin G	Formula 17900	Primarily Gram positive,
	Propen LA, Albacillin	Inhibits cell wall synthesis
Cloxacillin	Orbenin Quick Release,	
	Dry Clox	
Ampicillin	Polyflex sterile	
Tetracyclines (*Streptomyces* spp.)		
Tetracycline HCl		Broad spectrum, inhibits
Chlorotetracycline	Aureomycin	protein synthesis
Oxytetracycline HCl	Oxy LP, Tetraject LP	
	LA 300, Liquamycine	
Others		
Florfenicol	Nuflor	Broad spectrum; inhibits bacterial protein synthesis
Novobiocin	Albadry	Inhibits protein and nucleic acid synthesis
Pilimycin HCl	Pirsue	Primarily Gram positive; inhibits protein synthesis
Trimethoprim/	Trivetrin, Borgal	Broad spectrum inhibiting thymidine synthesis
Sulfadoxine	Sulfamethazine, AS-700	Broad spectrum inhibiting folic acid synthesis

intensification of livestock and poultry production (Cohen and Tauxe, 1986; Gustafson and Bowen, 1997).

Antibiotic feed additives benefit both livestock and consumers. Healthy animals are stronger and grow more efficiently. They incur lower veterinary bills, require less feed and are brought to market earlier. This allows the same amount of meat to be produced by fewer animals, requiring less feed and water and producing less excrement. Thus, it also helps preserve the environment.

Another benefit associated with the use of antibiotics in food-producing animals is a potential decrease in pathogen shedding (Ebner and Mathew, 2000). Animals carrying increased amounts of pathogens at the time of slaughter may present an increased risk for food contamination and human disease. Consequently, it is possible that the use of antibiotics in livestock production dramatically decreases the incidence of zoonotic disease (e.g., *Salmonella*).

2.2 Development of Antibiotic Resistance in Bacteria:

Antibiotic resistance exists in microbes found in numerous environments. Natural environments that have never been exposed to manufactured antibiotics have microorganisms that exhibit antibiotic resistance (Hart, 1998). This may be a result of organisms producing metabolites as a means of competing for limited resources. Resistance has developed in the producing organisms as a defense against their own synthesized antibiotics. The competing microbe may have also evolved antibiotic resistance as protection in response to these competitive attacks.

2.2.1 Naturally Occurring Antibiotic Resistance:

In the soil environment, antibiotics are synthesized by saprophytic organisms such as streptomyces and actinomycetes. These organisms represent 10 – 50% of all culturable bacteria found in soil (Bergogne-Berezin, 1997). A gram of dry soil can contain up to 10^6 culturable colony-forming units (CFU). Plasmids are known to be a major vehicle for the natural transfer of antibiotic resistant genes and are frequent, particularly in actinomycetes like streptomyces. As a result, the soil and natural environment constitute a huge reservoir of antibiotic resistance genes (Wiener *et al.,* 1998).

It has been generally accepted that antibiotic resistance has a fitness cost to the producing bacteria (Lenski, 1997). A resistant bacterium is considered disadvantaged when compared to a susceptible bacterium unless they are in the presence of an antibiotic (Hartley and Richmond, 1975).

There are a number of studies that have described the low prevalence of antibiotic resistant bacteria in populations of humans or animals that have never been exposed to antibiotics (Hartley and Richmond, 1975; Hughes and Datta, 1983; Rolland *et al.,* 1985). The most current example of this examines antibiotic resistant Enterobacteriaceae cultured from the feces of bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*) (Hart, 1998). These animals were not exposed to antibiotics or near areas of agriculture where antibiotic therapy may have been a factor. Of all the coliforms cultured during this study, 14-16% were resistant to tetracycline, 90% were resistant to ampicillin and amoxycillin and 100% were resistant to chloramphenicol.

All enterococci exhibit inherent low-level resistance to aminoglycosides such as gentamicin, kanamycin and streptomycin with minimum inhibitory concentrations (MICs) of 2-16μg/ml (Rice *et al.*, 1995). Levy (1992) argues this low prevalence of resistant bacteria is a result of long-term exposure to antibiotics entering the environment from human or agricultural treatment sources. Others have argued it may reflect a continuing selective pressure from low-level antibiotics naturally occurring in the environment (Hartley and Richmond, 1975; Hart, 1998). This selective pressure is acting upon genotypes that may have arisen by one of two processes, spontaneous mutation or lateral gene transfer.

2.2.2 Spontaneous Mutation:

Evolutionary forces include spontaneous mutation and lateral gene transfer. All living organisms acquire mutations because of normal cellular processes and exposure to mutagens within the environment. The opportunities for spontaneous mutation resulting in a unique genetic trait such as antibiotic resistance, even occurring at low rates (10^{-11} per generation), are far greater for prokaryotes than they are with any other life form (Davies, 1994). Unlike organisms that are more complex, the generation time of a bacterium such as *Escherichia coli* may be as short as 20 minutes. Furthermore, bacteria exist in astronomical abundance (4-6 x 10^{30} cells) on the earth (Whitman *et al.*, 1998). There are more bacteria in 1 ml of rumen fluid in a cow (1 x 10^{10}) than there are people inhabiting the earth (McAllister *et al.*, 2000).

Spontaneous mutation is considered the least significant in the development of antibiotic resistance. By example, the mutation rates for the acquisition of resistance to

streptomycin by Enterobacteriaceae such as *E. coli* or *Salmonella typhimurium* occur at frequencies of 10^{-9} or less per bacterial generation (Davies, 1994). Lateral gene transfer is considered more relevant when assessing the risk of antibiotic resistance transfer between bacteria.

2.2.3 Lateral Gene Transfer:

Lateral gene transfer allows resistance that has evolved many generations ago through spontaneous mutation, to be shared with other bacteria of different species or even genera (Courvalin, 1996). Genetic exchange among bacteria occurs by one of three distinct mechanisms: conjugation, transformation or transduction. These processes are often mediated by a number of mobile genetic elements including plasmids, transposons, bacteriophage and gene cassettes (Recchia and Hall, 1997; Cleaver and Wickstrom, 2000).

2.2.3.1 Conjugation:

Conjugation allows for the transfer of blocks of heterologous DNA between donor and recipient cells. Conjugative transfer can occur between Gram-negative and/or Gram-positive bacteria (Coffey *et al.,* 1998; Khachatourians, 1998). Conjugative transfer of plasmids and transposons has been suggested as the major factor contributing to the rapid rise in antibiotic resistant pathogens (Waters, 1999). This subject has been extensively reviewed (Davies, 1997; Coffey *et al.,* 1998; Hawkey, 1998; Khachatourians, 1998; Davidson, 1999; Mazel and Davies, 1999; Waters, 1999; de la Cruz and Davies, 2000).

2.2.3.2 Transformation:

Free DNA exists in the environment because of bacterial cell lysis. During transformation, (under natural conditions) competent cells take up naked DNA and may incorporate it into their genome. Little is known about the proportion of bacteria of a given community that are naturally transformable.

Few studies have been published dealing with transformation in the field (Graham and Istock, 1978; Bechet *et al.,* 1993; Paul *et al.,* 1993; Barbosa *et al.,* 1999; Haack and Andrews, 2000; Demaneche *et al.,* 2001; Nwosu, 2001). The degree of horizontal gene transfer was studied in bacteria under ideal noncompetitive conditions in the lab and in bacteria living in the natural environmental. Conditions conducive to transformation are far less frequent under *in vivo* conditions than during *in vitro* conditions and are therefore considered a minimal risk in the transfer of antibiotic resistance (Courvalin, 1996; Droge *et al.,* 1999). An example of laboratory versus field conditions was conducted in a fresh water habitat (Williams *et al.,* 1996). Transformation rates under "field" conditions differed significantly from those done under parallel laboratory microcosm conditions. Replicated field studies showed a high degree of variability in transformation frequencies. In the laboratory, treatments were minimized to single parameter influences. In the field, the bacteria are submitted to numerous changing conditions that cannot be replicated in the lab. Under environmental conditions the transformation rate was far less, if it occurred at all, and changed each time the rate of gene transfer was assessed. Environmental experiments are difficult to replicate given all of the confounding factors involved. In the lab, replication is achievable but this does not accurately represent field conditions. The study conducted by Williams and co-workers suggests that lab-based

estimates of transformation may substantially overestimate the extent to which this process occurs in natural environments.

Broad host range plasmids and transposons conferring antibiotic resistance have been successfully introduced into rumen bacteria *in vitro* (Wallace, 1994). Sulfonamides and trimethoprim have effectively been used for many decades in human and animal health treatments. Resistant to both of the antibiotics has spread extensively and rapidly. It is believed that this increased resistance is due mainly to lateral gene transfer of plasmids or transposons (Skold, 2001).

2.2.3.3 Transduction:

DNA transfer occurring through transduction is mediated by bacteriophages through faulty packaging of donor DNA and transfer of these sequences to a recipient cell (Khachatourians, 1998; Droge *et al.,* 1999). Transduction is limited to closely related species because of the high degree of specificity of the absorption step during bacteriophage invasion (Courvalin, 1996). The potential for gene transfer via transduction in nature has been inferred from studies that report on high concentrations of bacteria and phages in terrestrial habitats as well as aquatic habitats (Wommack *et al.,* 1992; Paul *et al.,* 1993).

2.3 Mechanisms of Antibiotic Resistance:

In order for an antibiotic to be effective at restricting growth or killing a bacterial population, it must meet three requirements: a biochemical target, a mechanism for transport or uptake and absence of enzymes that could inactivate or modify the antibiotic

in a manner that renders it ineffective (Khachatourians, 1998). A change in any of these conditions could render a sensitive bacterium resistant to one or several drugs. Bacteria have developed at least nine antibiotic resistance mechanisms (Table 2.3). The following examples of antibiotic resistance mechanisms are described in order to give the reader an appreciation for the diversity of the subject. Mechanisms of antibiotic resistance have been extensively reviewed in the scientific literature (Gutmann *et al.*, 1990; Dever and Dermody, 1991; Davies, 1997; Hawkey, 1998; Khachatourians, 1998; Mazel and Davies, 1999; Olsen, 1999).

2.3.1 Reduced Uptake into the Cell:

Gram-positive bacteria are surrounded by a thick peptidoglycan cell wall. This structure however offers little resistance to the diffusion of small antibiotic molecules that carry a charge such as fluoroquinolones (Nikaido, 1994). In contrast, Gram-negative bacteria have an additional outer membrane that surrounds a thin peptidoglycan layer and acts as a barrier to antibiotics. This outer membrane is more complex than the plasma membrane and includes lipopolysaccharides (carbohydrates that are bonded to lipids). In order for nutrients to diffuse across this barrier, cells such as *E. coli* have developed membrane channels called porins. Porins act as an entry point for some antibiotics like fluoroquinolones (Nikaido, 1994). By reducing the uptake of the drug into the target cell through the modification of the cell membrane and the utilization of efflux pumps, a cell can become resistant (Nikaido, 1994; Hooper, 2001).

Table 2.3: Molecular mechanisms of antibiotic resistance in bacteria (modified from Hayes and Wolf 1990).

Mechanism	**Antibiotic**
Reduced uptake into cell	Chloramphenicol
Active efflux from cell	Tetracycline
Modification of target to eliminate or reduce binding of antibiotic	β-lactams Erythromycin Lincomycin Rifampicin
Inactivation of antibiotic by enzymatic modification:	
Hydrolysis	β-lactams Erythromycin
Derivatization	Aminoglycosides Chloramphenicols Fosfomycin Lincomycin
Sequestration of antibiotic by protein binding	β-lactams Fusidic acid
Metabolism bypass of inhibited reaction	Sulfonamides Trimethroprim
Binding of specific immunity protein to antibiotic	Bleomycin
Overproduction of antibiotic target	Sulfonamides Trimethoprim
Biofilms	Multiple Drug Resistance

2.3.2 Efflux Pumps:

Efflux mechanisms have become broadly recognized as major resistance components to many classes of antibiotics. Some efflux pumps selectively extrude specific antibiotics while others referred to as multi-drug resistance (MDR) pumps, expel a variety of structurally diverse compounds with differing antibacterial modes of action.

Multiple antibiotic resistance in bacteria was at first thought to be caused exclusively by the combination of several resistance genes, each coding for resistance to a single drug. More recently, it has become clear that such phenotypes are often achieved by the activity of drug efflux pumps (Nikaido, 1994). Some of these efflux pumps exhibit an extremely wide specificity covering practically all antibiotics, chemotherapeutic agents, detergents, dyes, and other inhibitors. These efflux pumps work with exceptional efficiency in Gram-negative bacteria through their synergistic interaction with the outer membrane barrier (Nikaido, 1994). Efflux pumps developed in *E. coli, Staphylococcus aureus* and *Streptococcus pneumoniae* have been studied extensively (Levy, 1992; Hinrichs *et al.,* 1994; Aminov *et al.,* 2001; Hooper, 2001).

2.3.3 Modification of Target:

Rifampicin binds to the β-subunit of RNA polymerase, inhibiting DNA transcription. By modifying the targeted enzyme without disrupting its function the bacterium is able to continue with DNA replication and counteract the inhibiting effects of rifampicin. Rifampicin resistance is expressed as an altered target enzyme through numerous insertions, deletions or amino acid substitutions within the target enzyme

(Fukuda and Nagasawa-Fujimori, 1983). Most of the seventeen reported alterations occur within a highly conserved region (residue 507 to 534) of the β-subunit of RNA polymerase (Spratt, 1994; Ingham and Furneaux; 2000).

In some clinical Gram-positive isolates, enzymatic modification of rRNA is the most prevalent mechanism of resistance to macrolides. Erythromycin and other macrolides inhibit bacterial growth by blocking protein synthesis through binding to the 50S subunit of the bacterial ribosomal subunit. RNA methylase adds a methyl group to the 50S subunit, thereby preventing the binding of erythromycin. The methylated subunit however, remains functional and normal protein synthesis continues in the erythromycin-resistant cells (Davies, 1994).

2.3.4 Enzymatic Modification:

Hydrolytic inactivation by β-lactamases is one mechanism of resistance to β-lactam antibiotics. The cleavage of a bond in the β-lactam ring renders antibiotics like penicillin and ampicillin inactive (Davies, 1994). A single base change in the gene for β-lactamase can change the substrate specificity of the enzyme β-lactamase demonstrating how easily resistance to β-lactams can evolve (Davies, 1994).

The aminoglycoside phosphotransferases (APHs) are responsible for the bacterial inactivation of many clinically useful aminoglycoside antibiotics (Thompson *et al.*, 1998; Suter *et al.*, 1997). By transferring a phosphate group from ATP on to an antibiotic such as neomycin, the cell renders the antibiotic ineffective (Thompson *et al.*, 1996). In a similar manner, molecules such as acetyl or adenyl groups may be attached to aminoglycoside antibiotics and result in antibiotic inactivation (Daigle *et al.*, 1999).

2.3.5 Antibiotic Sequestration:

Coumarins such as coumermycin bind to the β-subunit of DNA gyrase. This enzyme is essential for maintaining DNA supercoiling and is critical to DNA replication. Cellular production of a gene product that binds to the coumermycin as it enters the cell, allows DNA replication to continue in the resistant cell (Del Castilla *et al.*, 1991).

2.3.6 Metabolic Bypass:

Synthesis of antibiotic-insensitive bacterial targets is the primary mechanism for resistance against trimethoprim, and sulfonamides (Dever and Demody, 1991). Resistance by *E. coli* to sulfanomides (sulfa drugs) and trimethoprim is acquired by the acquisition of a gene encoding a new enzyme that is insensitive to these antibiotics. These drugs inhibit different reactions in the bacterial metabolic pathway that produce tetrahydrofolic acid, an essential cofactor in the synthesis of nucleic acids (Spratt, 1994). The altered enzymes are fully functional and allow the metabolic pathway to continue producing tetrahydrofolic acid uninhibited.

2.3.7 Biofilms:

Biofilms represent complex communities with functional heterogeneity allowing a protected mode of growth in a challenging environment (Costerton *et al.*, 1999). It has been demonstrated that members of biofilms are less susceptible than their planktonic counterparts to antibiotic therapies (Bardouniotis *et al.*, 2001). Sessile cell barriers prevent bacteriostatic antibiotics from penetrating the full depth of the biofilm thereby

protecting the embedded active cells. Polymeric substances that make up the matrix of the biofilm restrict antibiotic diffusion. A variety of cell metabolic states such as those found in biofilms are best suited to fend off metabolic attacks by an antibiotic (Brown *et al.,* 1988; Costerton *et al.,* 1999; De Kievit *et al.,* 2001; Stewart and Costerton, 2001).

2.4 Factors Contributing to the Development of Resistance:

Resistance poses not only a financial burden to our health system with direct costs estimated at $100 million per year in the United States, but also can result in a traumatic loss of human life (Khachatourians, 1998). At the turn of the century tuberculosis, pneumonia and diarrhea were the leading causes of illness accounting for almost 30% of human deaths (Cohen, 1997). Over the next few decades the frequency of deaths related to these infectious diseases declined because of societal changes, including improved hygiene and sanitation, better housing, food safety and water quality. Advances in antibiotic therapy are also credited for improved human health (Cohen, 1997). For many diseases, antibiotics were key in both the treatment of the infection and in restricting transmission of the disease among the general public.

Antibiotics have been incorporated into human and agricultural health systems. The debate as to which of the two has contributed to the emergence of the antibiotic resistant bacterial "super bug" has been the topic of scientific and non-scientific reviews for many years.

2.4.1 Human Misuse and Overuse of Antibiotics:

As personal incomes have increased, so too have demands for better health care. Easy access to antibiotics and promises of cures to illness have increased the number of prescriptions and inappropriate prescriptions for antibiotics (Schaffner *et al.*, 1983; McCraig and Hughes, 1995; Schwartz *et al.*, 1997). Cohen (1997) suggests that at the same time we are making huge strides in technology with respect to disease cures, a larger complacency towards infectious diseases has developed (Figure 2.1).

The complacency towards infectious diseases has led to the emergence of antibiotic resistance by either influencing antibiotic use and/or increasing the transmission of the infectious agents. Factors such as i) changes in human demographics and behavior, ii) changes in technology and industry, iii) economic development and land use, iv) international commerce and travel, v) bacterial adaptation and change and vi) the breakdown of public health measures have all played a part in the emergence of antibiotic resistant bacteria (Cohen, 1997). For many pathogenic bacteria, combinations of these different factors have resulted in increased frequencies of resistance.

An increase in two income and single parent families has been correlated with increased daycare usage for children (Cohen, 1997). This gives many opportunities for the transmission of resistant bacteria between small children. Changing patterns of immigration needs to also be considered (Cohen, 1997). Today's populations are more interactive and diverse than ever before. We meet and interact with individuals from all over the world every day. This means we increase the likelihood of transmission of antibiotic resistant bacteria beyond our local communities.

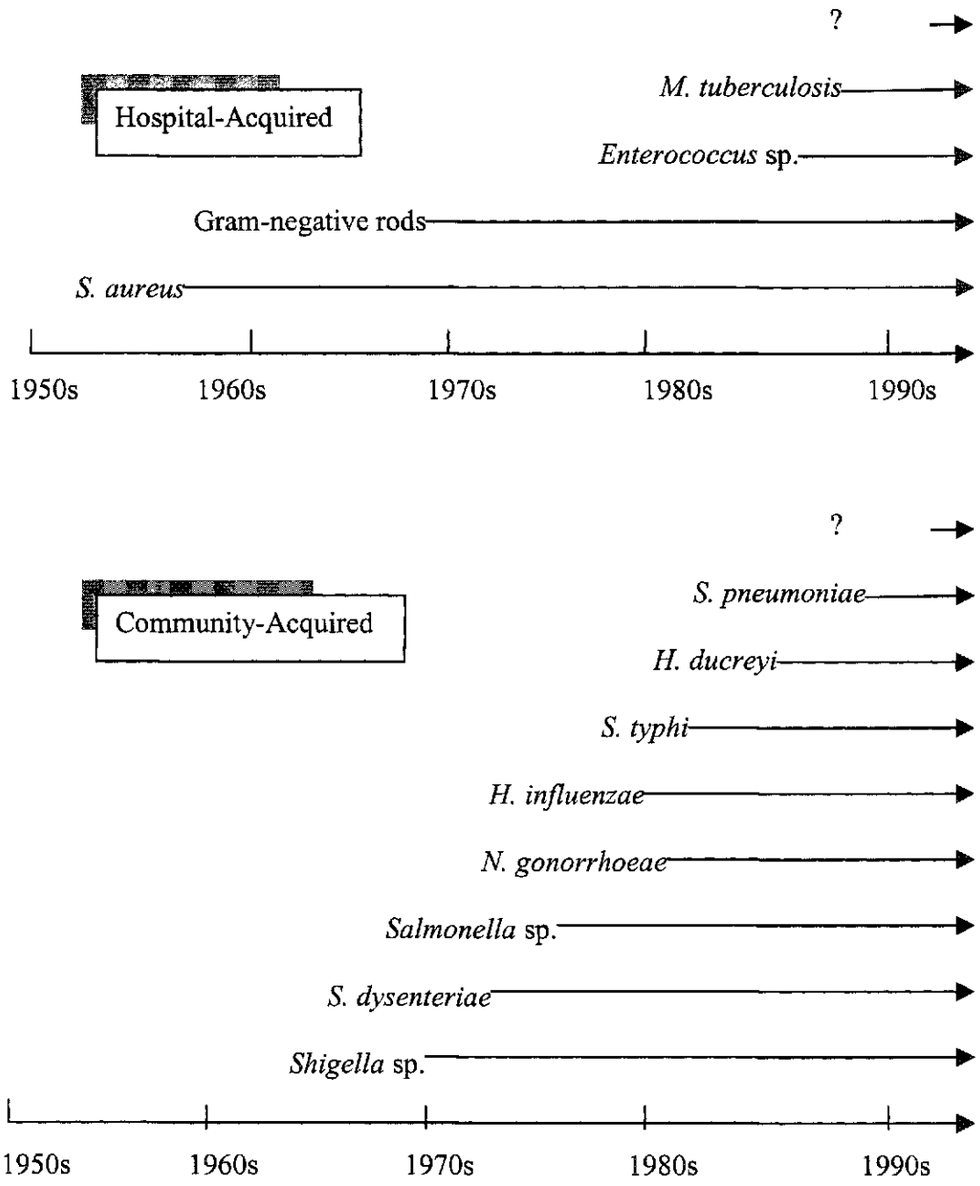

Figure 2.1: Emergence of clinically important antibiotic resistant human pathogens from 1950 to present. Question marks represent those strains of bacteria that we have yet to identify as resistant. (modified from Cohen, 1992).

New medical technologies such as organ transplants and management for diseases such as AIDS have been particularly important in the emergence of antibiotic resistance (Cohen, 1997). Along with the advances that prolong life, are those that increase hospital stays of individuals that are immune compromised as a result of surgeries and various diseases. These individuals are not only susceptible to infections but also act as reservoirs for resistant bacteria within the hospital or clinical institutions.

Greater populations and extended land use have increased person-to-person contact and the potential transmission of resistant bacteria. Increased populations also lead to stresses on water and sanitation systems (Cohen, 1997; Williams and Heymann, 1998).

More than 50 million unnecessary antibiotic prescriptions are written each year for patients of hospitals according to estimates by the Center for Disease Control and Prevention in the United States (Center for Veterinary Medicine, 1999) (Figure 2.2). Respiratory infections account for more than 75% of antibiotic drug prescriptions annually in the United States (Schwartz *et al.*, 1997). Although some patients suffering from cough or bronchitis may benefit, the majority of the patients receiving antibiotic prescriptions for these illnesses do not (Orr *et al.*, 1993; Gonzales and Sande, 1995). The main reason cited for the inappropriate prescribing during a 1993 study by physicians was related to unrealistic patient expectations and insufficient time for physicians to explain why an antibiotic prescription was inappropriate (Vinson and Lutz, 1993). Educating both the physicians and the public to the implications of antibiotic misuse and overuse is essential to the reduction of emerging antibiotic resistance.

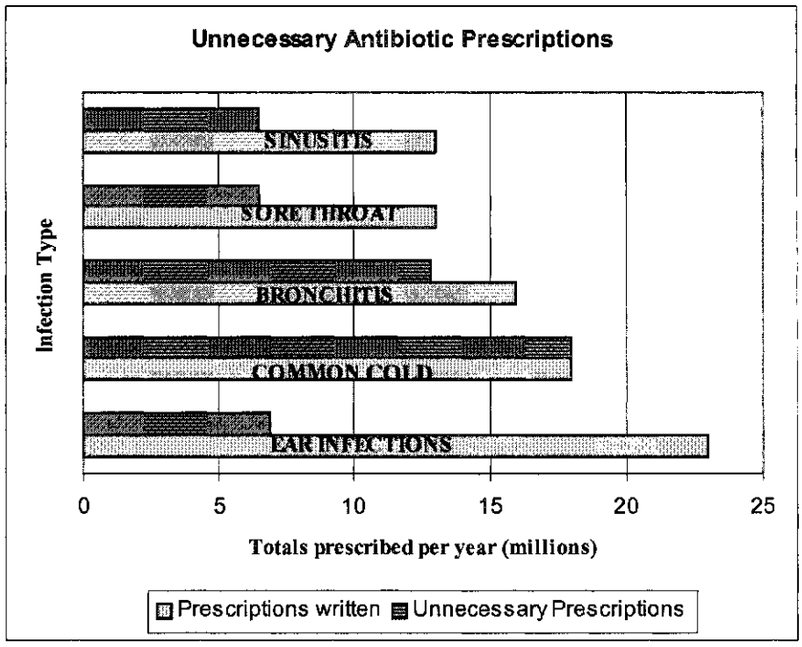

Figure 2.2: Estimated unnecessary antibiotic prescriptions prescribed by clinical physicians per year in the United States compared to the total number of prescriptions written for those conditions (Center for Veterinary Medicine, 1999).

The development of antibiotic resistant populations of bacteria continues every time a patient takes a specific antibiotic for an appropriate bacterial infection. The drug may kill most of the infecting bacteria but some bacteria may survive by mutating or acquiring resistance genes from other resistant bacteria through lateral gene transfer. Patients receiving antibiotics often experience an increase in resistant gut micro-flora (Cohen, 1992; Perchere, 1994; Weinstein, 2001). Antibiotics lead to a shift in the competitive balance resulting in a proliferation of resistant bacteria due to their advantage under the antibiotic selective pressure (Levy, 1992). The surviving cells can multiply quickly as new antibiotic-resistant strains. As a result, the patient's next infection may not respond to the antibiotic therapy used during the previous treatment and this new resistant strain may be transferred to other patients in the community. Hospitals, nursing homes and daycares pose the greatest risks for this profile of resistance transmission (Cohen, 1997).

2.4.2 Role of Animal Production in Antibiotic Resistance:

While numbers have not been established with certainty, it is estimated that of the 22.7 billion kg of antibiotics produced in the United States during 1998, over 40% were used for animal husbandry and crop purposes and that only 20% of those were used for therapeutic treatment of disease in animals (Levy, 1998). The remaining 80% of antibiotics employed in livestock production were used for prophylactic or growth promotion purposes.

Many studies have been cited with respect to the isolation of antibiotic resistant bacteria from animals. The focus has been on poultry and swine where the majority of

antibiotics are used (Tables 2.4 and 2.5). Five conclusions arise from a survey of the literature in these two tables. The studies involving poultry indicate that the use of antibiotics in food-producing animals is generally not associated with an increase in pathogen shedding. Similarly, with the exception of penicillin, antibiotics had no impact on pathogen shedding in swine. However, the scope of these studies is rather narrow, as most studies have focused on *Salmonella* spp. with other potential pathogens essentially being ignored. Finally, although most of the studies indicate that pathogen loads are not increased in the animals as a result of antibiotic use, they suggest that antibiotics may contribute to the prevalence of antibiotic resistance bacteria. This association is made by the authors regardless of having scientific data to support this conclusion. The following are further examples of studies that contribute to the hypothesis that antibiotic use in agriculture leads to antibiotic resistance without sufficient scientific data to support this claim.

Levy (1976b) studied resistant patterns in *E. coli* isolated from the feces of farm personnel after tetracycline was introduced into chicken feed. An increase in tetracycline resistant *E. coli* in the chickens was observed within one week of administration of the tetracycline supplemented feed and was undetectable soon after removal of the antibiotic from the animal feed. Similar increases were observed for tetracycline resistant *E. coli* cultured from the feces of farm personnel working with the chickens within 5 to 6 months after the start of the experiment. However, more tetracycline resistant *E.* coli were simultaneously cultured from people in the community that had never been knowingly exposed to the antibiotic during the study. This suggests that other factors may have been involved in the resistant bacteria colonizing the human gut and minimizes the suggested

Table 2.4: Effect of antibiotics on bacteria during challenge studies involving calves, swine and chickens. Symbols are interpretcd as follows: (~) approximately the same, (<) less than

Animal Species	Antibiotic Tested	Concentration of Antibiotic in Feed	Outcome Species Analyzed	Result (excretion of test animal vs. controls)	Publication
Calves	oxytetracycline	2) 101.01 g/ton	*S. typhimurium*	<(quantity) ~ prevalence and rate of decrease of shedding)	Evangelisti *et al.*, (1975)
Calves	oxytetracycline and neomycin	94.4 g/ton	*S. typhimurium*	< (quantity, prevalence), ~ (rate of decrease of shedding)	Girard *et al.*, (1976)
Pigs	lincomycin	110 mg/kg	*S. typhimurium*	~	DeGreeter *et al.*, (1976)
Pigs	1) ceftiofur sodium oxytetracycline	1) Unknown 100 g/ton	*S. typhimurium*	~	Ebner and Mathews (2000)
	2) apramycin/oxytetracycline	2) 150, 100 g/ton		2) <	
	3) carbadox/oxytetracycline	3) 50, 100 g/ton		3) ~	
Pigs	oxytetracycline	150 g/ton	*S. typhimurium*	~	Evangelisti *et al.*, (1975)
Pigs	oxytetracycline and neomycin	150 g/ton each	*S. typhimurium*	1) <	Girard *et al.*, (1976)
Pigs	1) chlortetracycline	1) 220.5 g/tonne	*S. typhimurium*	1)<	Gutzmann *et al.*, (1976)
	2) chlortetracycline, sulfamethazine, penicillin G	110.2, 110.2, 55.1 g/ton		2)~	
Pigs	efrotomycin	16 mg/kg	*S. typhimurium*	~	Jacks *et al.*, (1988)
Pigs	neomycin, oxytetracycline, nitrofurazone	110, 110-440 g/ton 100 mg/l	*S. typhimurium*	~	Wilcock and Olander (1978)
Pigs	chlortetracycline	110 mg/kg	*S. typhimurium* (rcsistant and sensitive strains)	> (resistant strain), < (sensitive strain)	Williams *et al.*, (1978)
Chickens	virginiamycin	25 g/ton	*S. typhimurium*	~	Abou Youssef *et al.*, (1982)
Chickens	avoparcin	2.5-100 mg/kg	*S. typhimurium, S. pullorum, S. cholerae S. dublin, S. arizonae, E.coli,* streptococci and obligate aerobes, lactobacilli	> (*Salmonella* spp., *E.coli* at 100mg/kg), < (streptococci and obligate aerobes at 100mg/kg), ~ (lactobacilli)	Barrow *et al.*, (1984)
Chickens	nosiheptide	20 g/ton	*S. typhimurium* and *E. coli*	~ (*S. typhimurium* and *E. coli)*	Benazet and Cartier, (1980)
Chickens	1) flavophospholipol	1) 9 mg/kg	*S. enteritidis, Campylobacter jejuni, Clostridium perfringens*	< (*Salmonella*), ~ (*Clostridium* and *Campylobacter*)	Bolder *et al.*, (1999)

Table 2.4: Continued

Animal Species	Antibiotic Tested	Concentration of Antibiotic in Feed	Outcome Species Analyzed	Result (excretion of test animal vs. controls)	Publication
Chickens	oxytetracycline	200 g/ton	*S. typhimurium*	<	Evangelisti *et al.*, (1975)
Chickens	oxytetracycline and neomycin	200 g/ton each	*S. typhimurium*	<	Girard *et al.*, (1976)
Chickens	avoparcin with and without monensin	10 mg/kg, with 100 mg/kg	*S. typhimurium*	~	Gustafson *et al.*, (1982)
Chickens	aviamycin with and without monensin	2.5 and 10 ppm 100 ppm	*Salmonella kedougou*	~	Hinton (1988)
Chickens	1) avoparcin	10 mg/kg	*Salmonella infantis*	1) <	Holmberg, (1984)
	2) monensin	90 mg/kg		2) <	
	3) avoparcin and monensin	10 mg/kg and 90 mg/kg		3) >	
Chickens	chlortetracycline	200 g/ton	*S. enteritidis, S. infantis, S. typhimurium*	<	Jarolmen *et al.*, (1976)
Chickens	enrofloxacin	10 mg/kg	*S. enteritidis*	<	Seo *et al.*, (2000)
Chickens	1) virginiamycin, Bacitracin flavomycin, tylosin	10 and 100 mg/kg	*S. typhimurium*	1) ~ or >	Smith and Tucker (1975a)
	2) nitrovin	10 and 100 mg/kg		2) >	
	3) sulphaquinoloxaline	100 and 500 mg/kg		3) <	
Chickens	1) neomycin, ampicillin spectinomycin, polymixin streptomycin, furazolidone, chloramphenicol, oxytetracycline	100 and 500 mg/kg	*S. typhimurium*	1) < or ~	Smith and Tucker (1975b)
	2) trimethoprim, sulphadiazine	20-100 mg/kg 100-500 mg/kg		2) <	
Chickens	1) lincomycin avoparcin	10 mg/kg 100 mg/kg		1) >	Smith and Tucker (1978)
	2) ampolium, menosin dimetridazole, arsenilic acid, nitro-hydroxyphenylarsonate	125, 100 mg/kg 150, 250 mg/kg 446 mg/kg		2) ~	
Chickens	1) avoparcin	10 mg/kg	*S. typhimurium* (nalidixic-acid resistant and sensitive), *S. heidelberg, S. oranienburg, S. infantis, S. senftenberg*	1) >	Smith and Tucker (1980)
	2) Bacitracin	10 mg/kg		2) ~	
	3) sodium arsenilate	250 mg/kg		3) <	

Table 2.5: Effect of antibiotics on bacteria during observational studies involving poultry, swine and steers. Symbols are interpreted as follows: (~) approximately the same, (<) less than and (>) greater than. Multiple studies in single papers are represented in numerical format (i.e. 1), 2) or 3); modified from Center for Veterinary Medicine, 1999).

Animal Species	**Antibiotic Tested**	**Concentration of Antibiotic in Feed**	**Outcome Species Analyzed**	**Result (excretion of test animal vs. controls)**	**Publication**
Steers	monensin	33 mg/kg	Total anaerobic bacteria	>	Dawson and Boling (1983)
Pigs	1) penicillin 2) streptomycin 3) penicillin and streptomycin combo.	1) 227 mg/100 lbs 2) 250 mg/100lbs 3) 227 and 250 mg/100lbs	Total bacteria, enterobacteriacae	1) > 2) ~ 3) ~	Bridges *et al.*, (1952)
Pigs	1) penicillin 2) streptomycin 3) penicillin and streptomycin combo.	1) 227 mg/100 lbs 2) 250 mg/100lbs 3) 227 and 250 mg/100lbs	Coliform bacteria, *Proteus, Shigella, Staphylococcus*	1) (coliform), ~ (others) 2) ~ (all organisms) 3) >(coliform, *Proteus)* ~ *(Shigellaa, Staphylococcus)*	Bridges *et al.*, (1953)
Pigs	chloratetracycline, oleandomycin, sulphamethazine, procaine penicillin, oxytetracycline	10-100 mg/lb 2 mg/lb 50 mg/lb 25 mg/lb 8 mg/lb	*Bordetella bronchisptica, Hemophilus suis, Pasturella multicoda, Streptococcus equisililis,* and *Mycoplasma* spp.	~ or <	Woods *et al.*, (1972)
	1) penicillin	1) 10 g/ton	Streptococci, lactobacilli and coliforms	1) < (Streptococci), ~ (lactobacilli , coliforms)	Fuller *et al.*, (1960)
	2) Aurofac	2) 3 lb/ton		2) ~	
Chickens	1) monensin sodium 2) avoparcin, notrivon, virginiamycin	100 mg/kg 10-20 mg.kg	*Salmonella* spp.	1) ~ 2) No conclusion could be made.	Hinton *et al.*, (1986)
Chickens	Bacitracin, erythromycin, penicillin, streptomycin or oxytetracycline	50 g/ton	*E. coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas* spp.	~	Mamber and Kaltz (1985)
Turkeys	avoparcin, virginiamycin	20 ppm	*Salmonella* spp.	~	Smith and Green (1980)

link between the farm personnel and feeding of antibiotics to chickens. Current studies show prevalent tetracycline resistance exists naturally in the environment and in gut flora of wild animals that have never been exposed to tetracycline (Hart, 1998). Specific numbers of cultured tetracycline resistant *E. coli* are not presented in this study. As a result, there is insufficient evidence to support the presented conclusion that there is direct evidence for potential transfer of antibiotic resistant bacteria (and in particular tetracycline resistant *E. coli*) between chickens and humans.

Perhaps the greatest health threat is the lateral gene transfer of antibiotic resistant determinants from livestock to people and their subsequent colonization of the intestinal tract. A second study conducted by Levy (1976a) provides potential evidence for transfer of plasmids between bacteria that reside in livestock and those that reside in humans. This study involved cloacal inoculation of chickens with a transformed tetracycline-resistant strain of *E. coli*. Chickens were fed tetracycline as a feed supplement to enhance bacterial colonization of the gut with tetracycline-resistant bacteria. Transfer of the resistance plasmid was detected in fecal micro-flora from the chickens and in fecal samples collected from the handlers of the chickens. Because the strain was unmarked, it is unknown if the plasmid transferred from bacteria in the animals to bacteria in the humans or vice versa. There is the possibility that the resistant *E. coli* were selected for in the human gut as a result of the exposure of handlers to antibiotic dust during animal feeding. This was not taken into consideration during this study (Levy, 1976a).

Water could serve as a reservoir for the transfer of resistant bacteria to humans. This may be accomplished through consumption of contaminated recreation water, untreated drinking water or cultivated crops that have been watered with contaminated

irrigation water (Beuchat, 1999; Venkitanarayanan *et al.*, 1999). Contamination of water sources may be accomplished by downward movement of fecal material through the soil profile into the groundwater or fecal material flowing with surface water runoff from livestock production facilities. Improper handling of contaminated post-harvest produce, may serve as an antibiotic-resistance contamination source (Jawson *et al.,* 1982; Crane *et al.,* 1983; Thornley and Bos, 1985; Moore, 1991; Hooda *et al.*, 2000).

It is generally agreed that the use of antibiotics inevitably results in some degree of bacterial resistance. The question remains, however, if the resistant bacteria in cattle pose a significant health risk to humans. Many of the antibiotics that could potentially lead to antibiotic resistant bacterial pathogens in humans have not been approved for use in North American cattle (McAllister *et al.,* 2001).

2.5 Transfer of Antibiotic Resistant Bacteria:

The uses of antibiotics in clinical medicine and in livestock production represent potential point sources for the development of antibiotic resistant bacteria (Figure 2.3). Bacteria move through the environment by a variety of routes and their presence ultimately has consequences to human health (Khachatourians, 1998) (Table 2.6).

Perhaps the primary concern with regard to the emergence of resistance should focus on those classes of antibiotics that are used in both animal and human medicine including those that are used in livestock production and that cause cross-resistance to antibiotics used by humans. Human clinical treatments include aminoglycosides such as gentamicin and streptomycin to treat serious bacterial infections. Ampicillin, amoxacillin and penicillin G are used to treat a variety of human infections including typhoid fever,

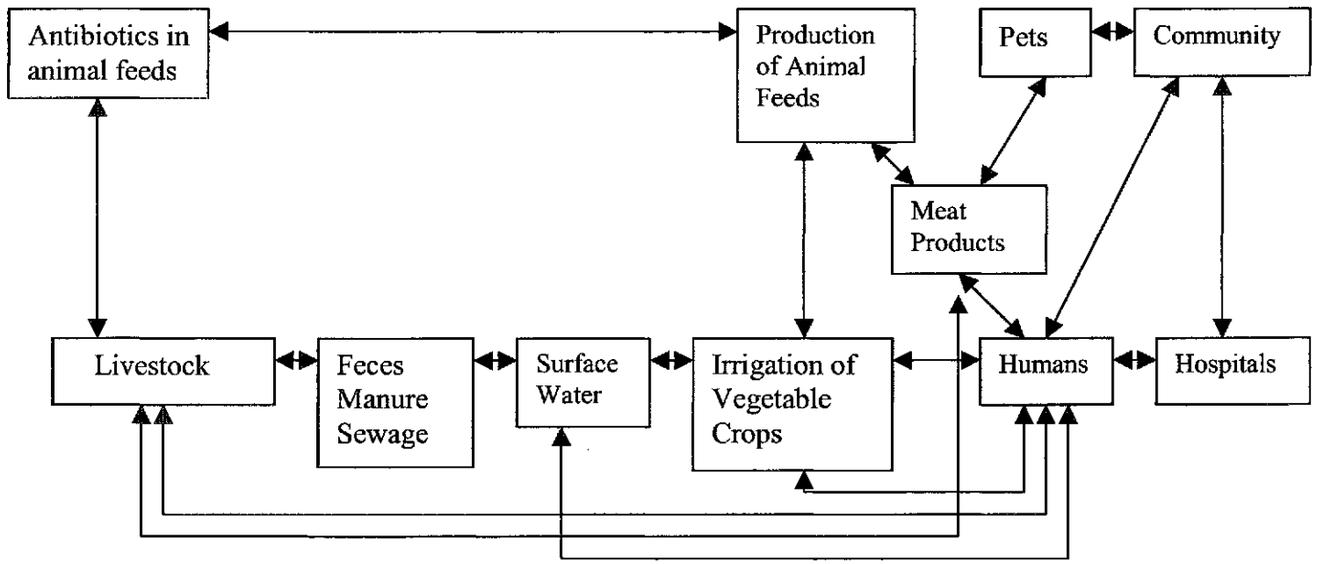

Figure 2.3: The agricultural use of antibiotics in animal feed can result in the selection and transmission of antibiotic resistant bacteria. These bacteria move through the environment by a variety of routes, and their presence ultimately has consequences to human health. (modified from Khachatourians, 1998).

Table 2.6: Transferring bacterial pathogens in livestock and humans including illnesses caused in humans by each bacterial strain (modified from Novick, 1981).

Species	Illness Caused in Humans
Escherichia coli	Intestinal Infection
Enteric disease-producing *E. coli*	Intestinal Infection
Shigella sp.	Intestinal Infection
Salmonella sp.	Food Poisoning
Pseudomonas sp.	Meningitis
Klebsiella-Aerogenes group	Urinary Tract Infections
Yersinia enterocolitica	Food Poisoning
Yersinia psuedotuberculosis	Food Poisoning
Brucella sp.	Brucellosis
Pasteurella multocida	Wound Infections
Listeria	May be a vertebrate pathogen
Erysipelothrix	Eyrsipeloid
Bacillus anthracis	Malig. Pustule Pneumonia
Mycobacteria	Tuberculosis
Leptospira	Weil Disease
Staphylocccus aureus	Cellulitis
Streptococcus agalactiae	Neonatal Meningitis
Clostridium perfringes	Gas Gangrene
Chlamydia psittacii	Orithosis
Mycoplasma sp.	Bronchitis

chlamydia, peptic ulcer disease and Lyme disease. Tetracyclines are also used to treat various human diseases including shigellosis and food poisoning. Sulfonamides and trimethoprim combinations are used to treat urinary infections. These same antibiotics are commonly used in animal husbandry (Table 2.2).

Possible risk to human health comes from the movement of antibiotic-resistance genes from bacteria in food to bacteria that cause human disease such as *Salmonella* spp., *Enterococcus* spp., *E. coli*, *Streptococcus* spp., and *Campylobacter* spp. These bacterial strains killed many people during the pre-antibiotic era and even though they are susceptible to some antibiotics, they have exhibited resistance to multiple antibiotics. The following is a brief summary of antibiotic resistance in these pathogens and their transfer between animals and humans.

2.5.1 Methicillin resistant *Staphylococcus aureus*:

Methicillin is a last resort antibiotic treatment for *S. aureus* infections. *S. aureus* is the number one cause of infection in hospital patients in the United States. It is a common source of infection in individuals recovering from burn and surgical wound (Roman *et al.*, 1997). In Canada, the first methicillin-resistant *S. aureus* (MRSA) was reported in 1981 and since then MRSA account for about 5% of all *S. aureus* infections reported (Health Canada, 1997). Glycopeptides such as vancomycin are the last resort control of MRSA infections in humans. To date, antibiotic use in cattle has not been linked to the development of resistant staphylococci that are of primary concern in human health (McAllister *et al.*, 2000). In May 1993, however, an outbreak of MRSA was identified in western Canada (Roman *et al.*, 1997). Investigation indicated that the strain

had entered the country by a patient who had been hospitalized in Punjab, India. Incidents of illness followed the movement of the index case through Canada and further through those he was in contact with prior to identifying his infection. The freedom with which individuals travel not only locally but globally further emphasizes the risks antibiotic-resistant bacteria pose.

2.5.2 Vancomycin-resistant Enterococci (VRE):

Increasing reliance on vancomycin as a last resort treatment may have led to the emergence of vancomycin-resistant enterococci (VRE). Until 1989, such resistance had not been reported in American hospitals. By 1993, more than 10% of the hospital-acquired enterococci infections reported to the Center for Disease Control were vancomycin resistant (Levy 1998). In some groups of hospital patients, VRE is on the rise and now represents 20% to 40% of all enterococci related infections in United States hospitals (Khachatourians, 1998).

Vancomycin-resistant enterococci are of increasing interest due to their clinical implications. In Europe, the rate of human fecal carriage of VRE isolates in the community is greater than those tested in the United States. Until recently, avoparcin, a glycopeptide that promotes cross-resistance to vancomycin (Stobberingh *et al.,* 1999), was commonly used in the Netherlands. Two separate studies confirmed Pulse Field Gel Electrophoresis (PFGE) patterns of VRE isolated from animals and the human caregivers of those animals were different suggesting the transfer of the resistance bacteria between animals and humans was nonexistent or occurred at very low frequency (van den Braak *et al.,* 1998; Stobberingh *et al.,* 1999). Perhaps it may be hypothesized from these studies that selective pressure for the

human VRE isolate is located within practices in human medicine as opposed to agricultural production practices. Incidences of VRE in the United States support this hypothesis. Although serious outbreaks have occurred in Europe, VRE-associated infections have not increased at the same rate or to the same extent as they have in the United States where glycopeptide antibiotics have not been approved for use in animal husbandry (Wegener *et al.*, 1999; Rice, 2001).

2.5.3 *Campylobacter:*

Reports from the scientific and public health communities, both domestically and internationally, have identified concerns about the relationship between the approval of fluoroquinolones for therapeutic use in animals reared for food and the development of fluoroquinolone resistance in *Campylobacter.* The use of these drugs in livestock production in the Netherlands, the United Kingdom and Spain slightly preceded increases in fluoroquinolone-resistant *Campylobacter* isolates from humans. In the United States, ciprofloxacin-resistant *Campylobacter* were recently isolated from 20% of domestic retail chicken products sampled (Smith *et al.*, 1999). *Campylobacter* spp. isolated during a seven-year period in the Netherlands from poultry products and human stool samples showed patterns of increasing resistance that could be correlated to increased fluoroquinolone use (Endtz *et al.*, 1991). This study concluded that increases were both in human and animal isolates, but failed to identify the origin of the resistant bacteria.

A recent study in Vancouver, Canada revealed a natural resistance to the fluoroquinolone ciprofloxacin in bacterial populations isolated in city-area soil (Waters and Davies, 1997). There was a high degree of variability in the fluoroquinolone target

DNA gyrase. This meant that even without selective pressure these bacteria showed fluoroquinolone resistance suggesting that *Campylobacter* resistance to fluoroquinolones may be innate. The implications of this study may be that blaming the poultry industry for fluoroquinolone resistance is unjustified.

2.5.4 *Escherichia coli*:

E. coli is a normal inhabitant of many animal and human intestinal tracts. Normally *E. coli* serves a useful function in the body by suppressing the growth of harmful bacteria and by synthesizing appreciable amounts of vitamins (Khachatourians, 1998). In recent years, *E. coli* O157:H7 has come to the forefront of public and government concern. *E. coli* O157:H7 is an emerging cause of food borne illness with an estimated 73,000 cases of infection and 611 deaths occurring in the United States each year (Center for Veterinary Medicine, 1999). Most persons recover without antibiotics or other specific treatment in 5-10 day. There is no evidence that antibiotics reduce the duration of the disease, and it is thought that treatment with some antibiotics may cause kidney complications.

Intestinal *E. coli* can be a reservoir for transferable antibiotic resistance plasmids (Khachatourians, 1998). Transfer of nalidixic acid or rifampicin resistant *E. coli* strains between different animals species has been studied under non-selective conditions (Marshall *et al.*, 1990). Primary screens for the resistant *E. coli* strains were constructed by transformation of nalidixic acid resistant or rifampicin resistant *E. coli* with a traceable multi-resistance plasmid. Using antibiotic selection, the study followed the transfer of the resistance plasmids and *E. coli* isolates from of each of a heifer and a bull inoculated

with each strain, through the environment to other animal hosts within the barn environment including humans, pigs, mice, chickens and flies. No antibiotics were given to any of the animals. Approximately 9 x 10^{10} of the combined nalidixic acid and rifampicin resistant *E. coli* cells were orally administered on three occasions over a 4-day period to the heifer and bull. Despite high frequencies of plasmid transfer between different gut bacteria during laboratory experiments, very low frequencies of plasmid transfer occurred in the inoculated bovine gut community and no transfer of the plasmids was detected in the other animal's (flies, mice, chickens or pigs) intestinal micro-flora. Antibiotic resistant *E. coli* were recovered up to 4 months post inoculation but the quantity of transformed *E. coli* cells shed from the cattle decreased quickly following inoculation. The results of this study suggest that transfer of the antibiotic resistant bacteria amongst different animal species occurred under non-selective conditions, the transfer of an antibiotic resistant plasmid was not detected despite gut colonization in the cattle for up to 4 months post inoculation. Competitive exclusion (and thus the necessity of high inoculation for isolate establishment) could be considered as a reason for non-transfer.

2.5.5 *Salmonella*:

In 1997, salmonellosis accounted for 41,901 reported cases of illness in the United States (Center for Veterinary Medicine, 1999). It is estimated that this is a fraction of the 2 to 4 million cases of salmonellosis occurring annually in the United States (Center for Veterinary Medicine, 1999). *Salmonella* spp. are commonly isolated from livestock, especially poultry and swine. Environmental sources of the organism

include water, soil, insects, factory work surfaces, animal feces, raw meats, raw poultry and raw seafood.

Salmonella enterica (MR-DT104) was studied over a 15-year period by Davies and co-workers (1999). Increases in the isolate resistance patterns were noted in both cattle and humans during this period. Isolates susceptible to all drugs tested were more common in humans than in cattle. Although antibiotic-resistant *S. enterica* (MR-DT104) were found in both cattle and humans so too were sensitive strains of *S. enterica* (MR-DT104). Davies and co-workers (1999) suggested that local antibiotic selection pressure may have played a role in the dissemination of the resistant isolates from cattle to humans. The study also noted that early in the global dissemination of MR-DT104, the bacteria were isolated from wildlife that had little or no exposure to antibiotics. Davies and co-workers (1999) suggested that factors other than antibiotic selective pressure are necessary for the spreading of resistant organisms.

2.6 The Politics of Antibiotic Resistance:

During the course of this literature review, many (>280) peer-reviewed articles that addressed aspects of antibiotic resistance were reviewed. Of the 130+ articles cited in this paper, >75% were review articles. Only four articles (Evangelisti *et al.*, 1975; Girard *et al.*, 1976; Dawson and Boling, 1983; Marshall *et al.*, 1990) deal with cattle and of these only one (Marshall *et al.*, 1990) deals with the transfer of resistant strains. Evidence for the risk of antibiotic resistant bacteria directly related to cattle and agricultural use is lacking. Despite this lack of scientific data, numerous political and public inquires have proposed that agricultural use of antibiotics is a major contributor to

antibiotic resistance in the environment. Several governments around the world have responded to real or perceived factors contributing to antibiotic resistance.

2.6.1 The Swann Committee:

In 1965, a large outbreak of *Salmonella* food poisoning occurred in Britain (Fisher, 1994). Although past outbreaks were quickly controlled with the use of antibiotics, patients during this outbreak failed to respond to treatment resulting in six deaths. In 1969, the government appointed Swann committee was established. The committee was made up of microbiologists and physicians charged with finding an explanation for the failure of treatment during the 1969 incident. Sub-therapeutic use of antibiotics in animals for extended periods and hence the selection for resistant bacteria was cited as placing humans at risk of infection by antibiotic-resistant bacteria. The committee recommended banning routine use of antibiotics in animals and banning all use of antibiotics that were concurrently used in human therapy. Use of other antibiotics in feed could continue but would require a veterinarian's prescription. The recommendations were accepted in 1970 in Britain and soon after other European countries adopted the same recommendations. In April 1977, the Federal Drug Administration of the United States lobbied to adopt the Swann Committee recommendations, but failed due to the protests and lobbying from the agricultural community (Marshall, 1980).

2.6.2 Europe's Response to Antibiotic Resistance:

In the United Kingdom, public and government organizations paid little attention to antibiotic resistance during the period between the 1969 report of the Swann Committee and the emergence of MRSA and antibiotic-resistant tuberculosis in humans. With the emergence of MRSA, it was decided that a new committee should be appointed to consider the role of science and technology in addressing antibiotic resistance (Seventh Report to the House of Lords, 1998). The inquiry was described as an alarming experience for committee members convincing them that antibiotic resistance was a major threat to public health.

The Seventh Report to the House of Lords (1998) made recommendations for the prudent use of antibiotics in human medicine and for the use of antibiotics in animals. Antibiotic growth promoters such as virginiamycin should be phased out, preferably by voluntary agreement between the professionals and the industries concerned. It was recommended that a code of practice be introduced to monitor the use fluoroquinolones, reported to be excessively prescribed by veterinarians. Further recommendations included an improved surveillance system to track the emergence of antibiotic resistance and the establishment of a single multidisciplinary Government committee with the mandate of overseeing all aspects of antibiotic use.

Infection control, surveillance, new drug development, vaccines, antiviral drugs and the support of the World Health Organization (WHO) Division of Emerging Diseases were also covered in the House of Lords Report (1998). By the committee members' own admissions, many of the conclusions drawn during the study were assumptions but they continued to put recommendations forth despite the lack of scientific data. In some

cases researchers warned of the complete ban of antibiotics in agriculture could have undesirable consequences such as an increase in potential human pathogens within livestock populations (Hinton *et al.,* 1984).

In the August 9, 1999 issue of Cattle Buyer's Weekly it was mentioned that the EU is moving toward a total ban on the use of antibiotics in animal feed, despite warnings that a ban would make European meat more expensive not only due to increased production costs but also due to import restrictions. Regardless of the potential economic costs, the EU has been steadily moving towards a complete ban on the sub-therapeutic administration of antibiotics. It previously allowed the use of eight antibiotics but now has banned four of them and the remaining four antibiotics will be phased out, starting in 2001. The reasoning behind the ban is that antibiotics are being used to prevent disease, rather than curing it. Authorities believe this constitutes misuse of antibiotics and has advocated that their use in livestock production be discontinued. EU officials admit they lack clear scientific evidence to link the use of antibiotics to the growing antibiotic resistance in humans, but they say that the risk of potential antibiotic resistance to human health outweighs any economic impact of a ban.

2.6.3 The United States' Response to Antibiotic Resistance:

In 1988 the Institute of Medicine (IOM) of the United States reviewed all of the information available on risk to human health associated with the use of antibiotics for treatment of animals used for food production and their inclusion in livestock feed to promote growth. The committee found a considerable amount of indirect evidence implicating both sub-therapeutic and therapeutic use of antibiotics as a potential human

health hazard. The committee strongly recommended further study of the issue (Center for Veterinary Medicine, 1999). Since 1988 the Food and Drug Administration (FDA) Center for Veterinary Medicine (CVM) has approved new antibiotics for use in food-producing animals on a prescription only basis. This prescription only policy is based on the need to assure the proper use of antibiotics through pre-diagnosis and correct treatment of disease to minimize animal suffering and to avoid drug residues in food. Antibiotics for use in animals must meet FDA's standards for safety, efficacy and quality to be approved in the United States (Center for Veterinary Medicine, 1999).

During the first session of the 107th Congress May 9th, 2001, an act cited as "Antibiotic Resistance Prevention Act of 2001" was presented. The act requested funding for top priority action items in response to the public health action plan to combat antimicrobial resistance. Highlights of the report included a national antibiotic resistance surveillance plan that included the monitoring of antibiotic use in the United States. Prevention and control of resistance would be addressed as well as research and product development. Committee co-chairs included the Task Force on Antibiotic Resistance (established 1999), the CDC and FDA. Members included Agencies for Healthcare Research and Quality, Health Resources and Services Administration, Healthcare and Finance Administration, Environmental Protection, Departments of Agriculture, Defense and Veteran's Affairs. In general the committee promoted comprehensive strategies that use multiple interventions for appropriate drug use and infection control. The committee was equally committed to intervention in human and agricultural environments. As a result of their broad-spectrum approach, the

recommendations of the committee seem more conservative when compared to the European response of totally banning antibiotic use in animal husbandry applications.

2.6.4 Canada's Response to Antibiotic Resistance:

The Laboratory Centre for Disease Control (LCDC) in collaboration with the Canadian Infectious Disease Society (CIDS) organized a conference May 1997, entitled "Controlling Antimicrobial Resistance: An Integrated Action Plan for Canadians". Approximately 200 people from health, agriculture, aquaculture, and pharmaceutical industries, the public and media participated in the conference. The objectives of the conference included reporting current factors contributing to antibiotic resistance, creating an action plan to be implemented in public and professional education regarding the issue of resistance, implementation of a national surveillance program and development of a plan for anticipating and responding to obstacles that could prevent the realization of the action plan.

To address some of the industry and consumer concerns regarding antibiotic resistance, a medical research team from the University of Calgary in June 1999 began a two-year study to look for links between the use of antibiotics in cattle and the rise of antibiotic resistant bacteria in people. The Canada Alberta Beef Industry Development Fund has committed $976, 462 for this research. The pork producers also have expressed interest in pursuing similar studies.

The National Information Program on Antibiotics (NIPA) is a coalition of eight health organizations concerned with the issue of appropriate use of antibiotics in Canada. This group focuses on public and physician understanding of the issue of antibiotic

resistance. NIPA has been active since 1996 to raise awareness in Canada about the need for responsible use of antibiotics. During the past five years, NIPA has developed a wide range of educational materials to encourage dialogue between health care professionals and patients, including the coalition's website (www.antibiotics-info.org); the NIPA Antibiotics Tool Kit which contains antibiotics compliance pads, non-prescription pads and information pamphlets; and an "Antibiotics: Use Them Wisely" poster for use in pharmacies and physicians' offices.

January 2001, NIPA took the opportunity to unveil its Canada-wide consumer advertising campaign about the importance of prescribing antibiotics appropriately and using them wisely. The program expected to reach more than 3.5 million Canadians through an extensive print campaign in popular Rogers Media magazines like Chatelaine (English and French), Today's Parent, Healthy Woman, Enfant Quebec and Sante Femme. The campaign is aimed at not only creating greater awareness of the issue but also motivating significant change in Canadians' attitudes toward antibiotics.

2.7 The Take Home Message:

The present review includes references addressing the issue of antibiotic resistance, its development, mechanisms and transmission between animals and animals and humans. Most studies directly challenging animals involved poultry, a few involve swine and even fewer involve cattle. With respect to experimental design, all fall into one of two categories: those that involved the artificial inoculation of animals and those that monitored transmission of resistance under laboratory conditions. The most common bacteria used in these studies were *Salmonella* spp. and the transmission of plasmids

involved laboratory competent strains of *E. coli*. The results of the inoculum studies suggested resistance could be mediated through animal husbandry but were based on laboratory results of plasmid transfer.

Several European countries have banned sub-therapeutic antibiotic use in livestock production. North American restriction of antibiotic use in livestock production is also gaining political popularity despite the lack of evidence that antibiotic resistance is a direct result of antibiotic use in animal husbandry.

The message established by this review is the lack of current relevant studies to assess the degree to which antibiotic use in beef cattle production poses a threat to human health. Further studies monitoring establishment and transmission such as the study currently being conducted by the University of Calgary are imperative in allowing us to make informed and prudent choices regarding antibiotic usage in livestock production systems.

3. MATERIALS AND METHODS:

All animals employed in these studies were cared for under the guideline of the Canadian Council of Animal Care (1993).

3.1 Isolation of Inoculum (RREC I):

Fecal samples (50-100 g) were collected from 10 yearling steers housed at the Lethbridge Research Centre feedlot using plastic sample bags and digital palpation (fecal grab samples). Samples were then transferred to the lab, weighed and 1g sub-samples were combined with 24 ml sterile double distilled water (d_2H_2O) and mixed in a model 400 stomacher (Seward; Mississauga, ON) on high speed for 2 min to create a slurry. Duplicate aliquots (250 µl) were spread on antibiotic gradient plates (0 – 100 µg/ml; Bryson and Szbalski, 1952; Gentile *et al.*, 1992) prepared with MacConkey (Difco, Beckton Dickinson, Sparks, MD) or Bile Esculin Azide (BEA) agar (Sigma Aldrich Canada Ltd., Oakville, ON) and rifampicin (Rm; Sigma), streptomycin (Sigma), gentamicin (Sigma) or nalidixic acid (Sigma). In addition, 100 µl of the fecal slurry from each animal were spread on MacConkey or BEA agar plates to ensure that the antibiotics were the selective criterion rather than the MacConkey or BEA media.

The gradient plates were examined for distinct lactose fermenting (MacConkey plates) or distinct black (BEA plates) colonies that were growing on the plates in the area of high antibiotic concentration following incubation (24 h at 37°C). The level of resistance of these colonies was estimated and recorded. A distinct rifampicin resistant (Rm^r) colony (15 µg Rm/ml) was harvested from a MacConkey + Rm agar gradient plate and streaked for purity on Trypticase Soy agar (TSA; Difco) and incubated (24 h at 37°C). The Rm^r isolate, named Rifampicin Resistant *E. coli* (RREC I), was assessed for

growth on Eosin Methylene Blue agar (EMB; Difco) and tryptophanase activity in peptone water (Holt, 1994). Stocks of RREC I (1 ml) were created in triplicate using brain heart infusion (BHI; BDH Inc., Toronto, ON) broth and 15% glycerol and stored at -80°C for future use.

3.2 Stability of RREC I:

The RREC I cells were grown in 5 ml of TSB (Difco) in a shaking incubator (Model Inova 4080 New Brunswick Scientific Company Inc.; Edison New Jersey) at 39°C and the resulting culture was transferred, using an inoculation loop, into 5 ml of fresh TSB every 12 h for 7 d (>200 generations). Each 12 h culture was enumerated by the spread plate technique. Serial dilutions prepared with 50 mM sodium phosphate buffer (pH 7.0) dilution blanks (9 ml) were spread in triplicate on MacConkey agar and MacConkey agar containing 50 μg Rm/ml (MacConkey + Rm agar) and incubated overnight at 39°C.

3.3 Preliminary Cattle Study (Small Scale):

A preliminary study was performed to assess the ability of RREC I to colonize and persist in cattle. Prior to inoculation with RREC I, 15 yearling steers from the Lethbridge Research Centre feedlot were screened for background lactose fermenting Rm^r CFU to determine if background Rm^r isolates would be of concern for tracking our marked strain. Fecal samples were placed in plastic bags and transported back to the lab for processing within 1 h of collection. Fecal material (10 g wet weight) was combined with 90 ml sterile sodium phosphate buffer and stomached for 2 minutes on high speed to

create a slurry. The resulting suspension was spread in triplicate on MacConkey + Rm, incubated at 39°C overnight and examined for lactose fermenting Rm^r colonies.

Following screening for lactose fermenting Rm^r CFU, fistulated yearling Jersey steers (n = 3) were inoculated with RREC I to determine if the isolate could be established and recovered from inoculated animals. The inoculum for each animal was prepared by growing RREC I cells in 100 ml of TSB in a shaking incubator at 39°C. Cells were harvested by centrifugation and re-suspended in 50 ml sodium phosphate buffer. Animals were inoculated directly into the rumen through rumen cannulae. Rumen samples were collected at 0, 12 and 24 h post inoculation (p.i.) and 100 µl aliquots were plated in triplicate on MacConkey + Rm agar and incubated overnight at 39°C. Fecal grab samples were also collected from each animal every 12 h (0 h – 96 h) and then daily for 2 weeks p.i. Samples were enumerated for the presence of Rm^r lactose fermenting CFU/g feces (wet weight) as previously described.

3.4 Feedlot Cattle Study:

3.4.1 Feedlot Environment and Animal Background:

3.4.1.1 Sample Collection:

Prior to the arrival of experimental animals at the Lethbridge Research Centre feedlot, background environmental samples were collected from feed troughs, fences, soil samples from the pen floor and water bowls. Fences were swabbed (1-1.5 m above the pen floor) in two locations, near the water trough and above the feed bin for each pen using a sterile swab. Duplicate heavy feed particles and dust samples (10-100 g) were collected from each feed bunk using a plastic sample bag. Pen floor samples (10 – 50 g)

were collected using plastic sample bags from two areas, near the water trough and near the feed bunk from all thirty pens to be used during the trial. Duplicate water samples (500 ml) were collected from each of the water troughs (n = 15). Samples were collected from the edge of the bowl after stirring contents to ensure sediment was collected along with the water sample.

Within 24 h of arrival at the Lethbridge Research Centre, yearling steers (n = 360 weighing 350 – 400 kg) were weighed and fecal swabs were collected from each animal. Sterile transport swabs (Starplex Scientific; Etobicoke, ON) were inserted into the rectum (5-7cm) of each animal and immediately placed in modified Amies media prior to being transported to the lab where they were examined for the presence of Rm^r bacteria. All pens (13.54 m x 19.7 m) were open to the environment with windbreaks installed between pens restricting animal-to-animal contact except at water bowls that were located at the fence line and shared between 2 adjacent pens. Feed bunks were located at the roadway face of each pen (Figure 3.1). Animals were bedded using barley straw. Standard pen maintenance and feeding protocols were followed throughout the trial periods.

3.4.1.2 Enumeration and Isolation of Rm^r Cells:

Feed bunk samples (10 g) were combined with 90 ml of sterile sodium phosphate buffer and mixed for 2 min using a stomacher set on high speed to create a slurry. Fence swabs were removed from the transport media and streaked across the agar plates. Pen floor materials were mixed by hand until homogeneous and 10 g was placed into 90 ml of sterile sodium phosphate buffer. Samples were stomached for 2 minutes on high to

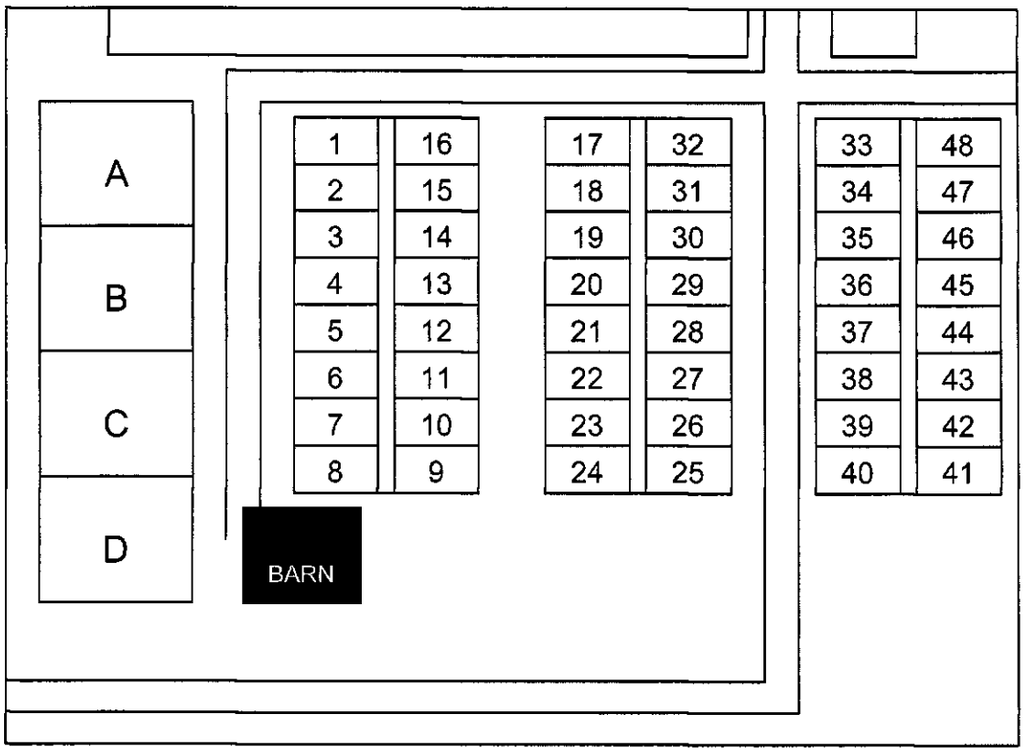

Figure 3.1: Schematic drawing of the research feedlot at the Lethbridge Research Centre. Yearling steers (n = 360) were housed in groups of 12 in pens 17- 46. Animal sampling was conducted in the sheltered barn. Animals involved in the first inoculation resided in pens 24 (inoculated steers and pen mates) and 23 (adjacent pen steers). Animals involved in the second inoculation were housed in pen 17 (adjacent pen 18) and animals involved in the third inoculation were housed in pen 21 (adjacent pen 22).

create a slurry. Water samples were agitated in the collection jars prior to filtration. Samples (100 ml) were filtered through a 0.2 μm membrane filter (Gelman Sciences, Pall Corporation; Ann Arbor, MI) using a vacuum filtration system and filters were plated. The cotton tips were removed from the rectal swabs and placed in 1.5 ml microtubes containing 500 μl sterile sodium phosphate buffer and mixed with a vortex mixer (Model Genie 2 Fisher Vortex; Bohemia, NY).

All materials processed above were plated (100 μl) or streaked on MacConkey + Rm agar plates. Random samples were selected from each environmental group and plated on MacConkey agar to ensure rifampicin was the selective criterion. The plates were incubated for 24 h at 39°C and then examined for lactose fermenting (bright pink) colonies. Fence and rectal swabs were scored qualitatively for growth (positive or negative) while CFU/g or CFU/ml were determined for feed trough and pen floor samples or water bowl samples, respectively.

Cells from isolated lactose fermenting colonies from MacConkey + Rm agar were streaked onto TSA and LMX agar and incubated 24 h at 39°C. Stock cultures were created by suspending cells from isolated colonies on the TSA plates in 1 ml BHI broth containing 15% glycerol. Stock cultures were placed in 1.5 ml cryotubes and stored at -80°C for future use. In addition, cells used to prepare stock cultures were subjected to fatty acid methyl ester (FAME) analysis and compared to the inoculated strain.

3.4.2 Inoculation of Cattle with RREC I:

RREC I cells from a glycerol stock were grown overnight in 10 ml TSB containing 50 μg Rm/ml in a shaking incubator at 39°C. Cells from the resulting culture

(10 μl loop) were used to inoculate 18 (1 L) flasks containing TSB (250 ml). Cultures were incubated at 39°C overnight. The following day, the cultures were enumerated by the spread plate technique. Serial dilutions were plated on both MacConkey agar and MacConkey + Rm agar to determine the number of Rm^r CFU.

Nine of twelve yearling steers in pen 24 were orally inoculated with 5×10^{12} RREC I cells followed by a water wash (500 ml). Animals were subsequently sampled over a four-month period. Prior to feeding, steers from pen 23 were run through the feedlot chute system followed by cattle from pen 24. The order of the steers through the chute was not consistent within pens as it was dependent on their arrival at the chute system. Feces (10 – 50 g) from each animal was grab sampled daily from all steers present in pen 24 and adjacent pen 23. Samples were collected and processed as described for the preliminary cattle study.

Environmental samples were collected and processed from feed bunks and water bowls twice each week as previously described for environmental background samples. Standing manure samples (10 – 50 g) were collected within 2 h of the morning feeding time. Triplicate samples were collected from manure near water bowls, feed troughs and between pen fence line in the inoculated and adjacent pens using plastic collection bags. Manure patties were chosen to reflect approximately a 24 h stand on the feedlot floor (i.e. those showing a dry surface but maintained moisture within the pat). The chute system floor was sampled twice each week (one sampling day and one non-sampling day) throughout the first inoculation sampling period. The manure samples were enumerated for Rm^r lactose fermenting CFU as described for the fecal grab samples.

3.4.3 Inoculation of Feedlot Cattle with RREC II and RREC III:

RREC II was isolated from steer 501 in pen 24 after this animal had continuously shed Rm^r lactose fermenting CFU for a period of 22 days. Following collection, RREC II was streaked for purity on TSA and stored in BHI + glycerol broth at -80°C for future use. RREC II was believed to be a direct descendant of RREC I, based on lactose fermentation, fluorescence on LMX agar and similarity of FAME profiles. RREC II cultures were prepared as described previously for RREC I and six of twelve animals in pen 17 were inoculated with RREC II. Shedding of Rm^r lactose fermenting CFU was monitored for all animals in pen 17 and adjacent pen 18 as described previously for RREC I including sampling the uninoculated pen 18 prior to sampling the inoculated pen 17.

RREC III was isolated in an identical manner as RREC II from steer 421 in pen 17 after this animal continuously shed Rm^r lactose fermenting CFU for a period of 17 days. This isolate was used in a third inoculation of six of twelve animals in pen 21. RREC III cultures, animal inoculations and monitoring of shedding of Rm^r lactose fermenting CFU were performed as described for RREC II and I however both adjacent pens 22 and 20 were monitored for transfer of the RREC III isolate.

3.4.4 Monitoring Transfer to Non-adjacent Pens:

All yearling steers (n = 360) were weighed and fecal swabs were collected from each animal on 3 subsequent dates (5, 11 and 16 weeks post feedlot arrival) following the initial inoculation of RREC I - III. Fecal samples were collected and processed as previously described above.

3.4.5 Collection of Samples from the Chute System:

Triplicate fecal samples (10 – 50 g) were collected from the chute area using plastic collection bags for 6 weeks following the inoculation of animals in pen 24 on two separate occasions each week. One collection per week occurred 4-6 h after the steers were run through the chute system for grab sampling. The second collection per week occurred on a non-sampling day approximately 30 hours after the steers were run through the chute system. All samples were processed as previously described for pen floor samples.

3.4.6 Sampling Following Transport to Slaughter:

With the exception of 5 animals held back for post mortem sampling, steers (n = 355) were transported 200 km for slaughter in semi-trailers 3 months p.i. of RREC III. Five inoculated steers chosen from pen 21 (461, 465, 469) and pen 17 (495, 422) based on shedding patterns > 12 d p.i., were housed together at the front of the trailer for the transport period. Prior to transport the steers were fecal grab sampled and the feces was processed as previously described. Following arrival at the meat packing plant, animals were removed from the trailer unit and feces (10 – 50 g) were collected from the trailer floor using plastic collection bags. Samples were then transported on ice to the lab and processed as previously described for grab samples within 3 h of collection.

3.4.7 Post-mortem Tissue and Digesta Samples:

Steers (5) inoculated with RREC III (460, 464, 476, 468 and 470) were slaughtered and intestinal tissue (50-100 g) and digesta (10-50 g) samples were collected from 6 regions of the gastrointestinal tract (Figure 3.2) using plastic collection bags. This experiment was conducted to determine if the animals' gastrointestinal tracts were still colonized by the RREC III cells despite the lack of shedding events for > 30 d. All 5 steers had shedding episodes for Rm^r lactose fermenting CFU during the trial period. Four of the steers were directly inoculated and shed for >20 p.i., (460, 464, 476 and 468) while the fifth animal was an uninoculated pen mate and shed on 6 occasions (470).

Tissue samples were weighed into 10 g sub-samples, combined with 100 ml of sodium phosphate buffer and gently agitated to remove digesta. The samples were washed a second time, placed in 90 ml fresh phosphate buffer and homogenized in a blender (Model Osterizer, Sunbeam; Ontario, Canada) for 1 min at high speed. The homogenate (100 µl) was plated in triplicate on MacConkey + Rm agar and incubated overnight at 39°C.

Digesta samples (10 g wet weight) were combined with 90 ml sodium phosphate buffer and stomached for 2 minutes to create a slurry. Samples were then processed, plated, incubated and assessed as previously described for fecal samples. One random sample from each tissue and digesta region was plated on MacConkey agar to ensure rifampicin was the selective criterion.

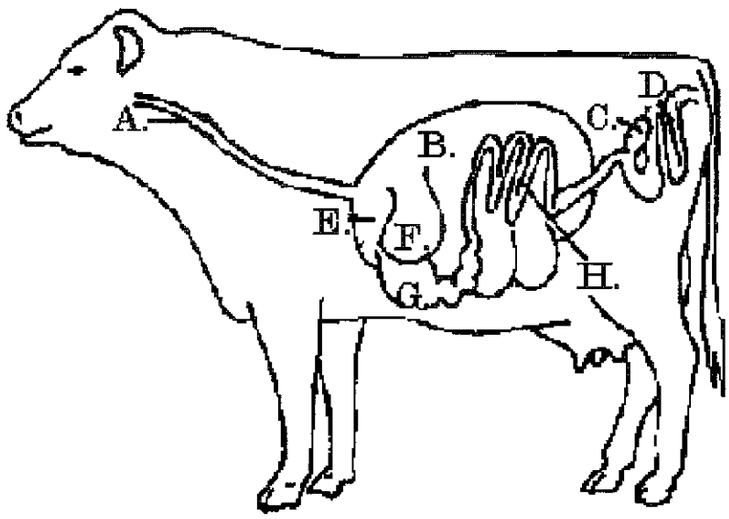

Figure 3.2: The gastrointestinal tract of cattle including A) esophagus, B) rumen, C) cecum, D) large intestine, E) reticulum, F) omasum, G) abomasum and H) small intestine. Tissue and digesta samples were removed and processed from six areas of the large intestinal tract (D) including the cecum (C), colon, rectum and the small intestinal tract (H) including the duodenum, jejunum and ileum to determine if the RREC isolates continued to colonize the gastrointestinal tract.

3.5 Identity of the Rm^r Lactose Fermenting Isolates:

During the course of the three inoculations, 522 Rm^r lactose fermenting isolates were collected. Fatty acid methyl ester, Biolog and MIC tests were conducted on all or a selected subset of the isolates to determine if the Rm^r bacteria were direct descendents of RREC I. RREC inocula were compared further by 16S rDNA sequence analysis and pulsed-field gel electrophoresis. Three *E. coli* isolates were used as reference strains in this study. *Escherichia coli* A291 (Lethbridge Research Centre culture collection), *E. coli* O157:H7 4420 (Lethbridge Research Centre culture collection) strains were isolated from cattle and characterized previously by Jay Yanke (Curator, Lethbridge Research Centre Culture Collection) and *E. coli* K12 DH5α obtained from Gibco Invitrogen Corp. (Burlington, ON).

3.5.1 Fatty Acid Methyl Ester (FAME) Analysis:

Lactose fermenting colonies originating from all three inoculations were harvested from MacConkey + Rm agar plates, streaked onto TSA and incubated at 39°C overnight. Cells were harvested from the TSA and the FAMEs were obtained by saponification, methylation, and extraction, as described previously (Eder, 1995; Diogo *et al.,* 1999) and as recommended by the MIDI standard protocol (MIDI; Newark, DL). The separation of FAMEs was achieved with a Hewlett-Packard (Palo Alto, CA) model 5890 gas chromatograph equipped with a flame ionization detector and controlled by the Sherlock Single Tower Library Generation software (version 1.06; MIDI, Newark, DE). Numerical analysis and mean profiles based on the fatty acid patterns of the strains were

generated. Euclidean distances of the fatty acids generated by the MIDI software package were compared to the initial and subsequent inocula for each positive isolate cultured.

3.5.2 Biolog™ (Carbon Utilization Patterns):

Rifampicin resistant lactose fermenting isolates were assessed (n = 152/ 478) and compared for carbon utilization patterns using the Biolog™ system (Hayward, CA). Biolog™ GN2 (Gram negative) panels incorporate 95 different standardized carbon sources and 1 control (water). Plates were inoculated with 50 µl saline cell suspension (67% transmission) and incubated 37°C for 24 h prior to being read on Biolog™ Microstation. Isolate profiles were compared to GN database within the Biolog™ system (version 4.01B) and identification was proposed based on carbon utilization patterns.

3.5.3 Minimum Inhibitory Antibiotic Concentration (MIC) Assessment:

Biolog™ AST (antibiotic sensitivity testing) panels were used to test and compare bacterial sensitivities using 2-fold serial dilutions to varying concentrations of antibiotic compounds. The test yielded a minimum inhibitory concentration (MIC) value as well as breakpoint interpretations for those antibiotics where standards have been set. Cells were grown up and streaked for purity on TSA at 37°C overnight. Single colonies were selected and sub-cultured again on TSA at 37°C overnight. Following incubation cell suspensions (0.5 McFarland standard) were created in a saline solution (8 ml). An aliquot (100µl) of the cell suspension was transferred to Mueller-Hinton broth and the panels

were inoculated. The AST panels were then incubated for 18-24 h and the results read by the Biolog Microstation™ Reader and interpreted with the Micronaut™ Merlin program that automatically cross-referenced the specific plate schematic and reported the MICs and the breakpoint interpretation for each inoculum.

3.5.4 16S rDNA Sequence Analysis:

Inoculum isolates RREC I-III were subjected to 16S rDNA sequence analysis using a modified protocol from Brumbaugh and co-workers (1988). Cultures were incubated at 39°C in a shaking incubator (200 rpm) overnight in TSB. Each culture (2 ml) was centrifuged at 14,000 rpm for 1 min, the supernatant discarded and the cells were re-suspended in 200 µl of a 1 mM EDTA solution. The cell suspension was heated at 100°C for 5 minutes and stored at -20°C overnight to assist in cell lysis. Cell lysates were thawed on ice, centrifuged (14,000 rpm for 1 min) and the cell pellet discarded. The resulting DNA solutions were used as templates for the amplification of 16S rDNA with FP1 and 1525R primers. Polymerase chain reactions (PCR) were performed with QIAGEN Hotstar Taq™ DNA polymerase and Q solution (Mississauga, ON) according to the manufacturer's recommended protocol. Reaction mixtures were preheated at 95°C for 15 min and subsequently subjected to 45 PCR cycles (94°C for 45 s, 48°C for 30 s 72°C for 2 min). Template size and purity were examined by agarose gel electrophoresis (Sambrook and Russell, 2001).

Nucleotide sequence analysis was performed on the 16S rDNA PCR products prepared from lysed RREC I – III cells by the cycle sequencing method as previously described (Lane, 1991). Sequencing reactions were primed with 519R (GWA TTA CCG

CGG CKG CTG), FP1 (AGA GTT YGA TYC TGG CT), 1100R (AGG GTT GCG CTC GTT G), 338F (ACT CCT ACG GGA GGC AG), 1492R (TAC GGY TAC CTT GTT ACG ACT) and 926F (AAA CTY AAA KGA ATT GAC GG). Labeled reactions were resolved on a LI-COR Model 4000 automated DNA sequencing system (Lincoln, NE). DNA sequence data were assembled with Sequencher version 4.1 (Gene Codes, Ann Arbor, MI), aligned with Clustal W version 1.8 software (Thompson et al., 1994) and compared with sequences in the GenBank database (Benson et al., 2000) by using the BLAST algorithm (Altschul et al., 1990).

The 16S rDNA sequences for RREC I – III will be deposited in the GenBank database.

3.5. 5 Pulsed-field Gel Electrophoresis (PFGE):

Pulsed-field gel electrophoresis was performed on the strains from each of the inoculations and compared to 3 controls (*E. coli* A290, O157:H7 4420 and K12 DH5α) according to a modified protocol previously described by Liu and Sanderson (1995). Cultures were grown (15-18 h) on LB agar plates incubated at 37°C overnight. Cell suspensions were created in 15 ml Falcon tubes by adding approximately 10^9 cells to 0.5 ml cell suspension solution (10 mM Tris-HCl [pH 7.2], 20 mM NaCl, 100mM EDTA) and then pre-heated in a water bath at 70°C and mixed with molten 1.4% agarose held at 70°C. The mixture was then immediately drawn into a tuberculin syringe with the needle adapter removed. When the agarose rod had hardened after 20 minutes at room temperature, it was sliced into 1 mm thick discs that were placed into lysing buffer solution (10 mM Tris-HCl [pH 7.2], 50 mM NaCl, 100mM EDTA, 0.2% sodium dodecyl

sulfate, 0.5% *N*-laurylsarcosine) and gently shaken at 70°C for 2 h. The discs were then drained and washed 2 times (15 min each) in wash solution (20 mM Tris-HCl, [pH 8.0] 50 mM EDTA) at room temperature and gently agitated. Discs were drained and gently agitated with 3 ml of the proteinase K solution (100 mM EDTA, [pH 8.0], 0.2% SDS, 1% N-laurylsarcosine sodium salt, 1.0 mg proteinase K /ml buffer solution) at 42°C for 72 h. The discs were then washed in wash solution and treated with 3 ml PMSF solution (1mM phenyl-methylsulfonyl fluoride in wash solution) and incubated with agitation at room temperature for 2 h. Samples were then gently agitated with d_2H_2O at 37°C for 10 min and drained. Plugs were immersed in 40 µl enzyme buffer solution (2X concentration) and incubated 10 minutes at room temperature. The buffer solution was then discarded and 40 µl/plug buffer solution (1X concentration) 0.006 µL/µl total liquid volume of *I-CeuI* was added to the plugs and incubated for 2 h at 37°C. A Bio-Rad CHEF-DRII apparatus (Bio-Rad; Mississauga, ON) was used to resolve genomic DNA fragments by PFGE. Fragment separation was achieved following the I-*CeuI* digestion with an initial switch time of 18 s, final switch time of 20 s at 5.4 volts and a run time of 10 h using 0.7% agarose gel. Gels were stained with ethidium bromide and photographed for permanent record (Sambrook and Russell, 2001).

4. RESULTS:

4.1 Isolation of RREC I:

Naturally occurring antibiotic resistant enterococci or enterobacteriaceae were screened on antibiotic gradient plates made with BEA agar or MacConkey agar, respectively. Nalidixic acid or streptomycin gradient plates prepared with BEA agar consistently yielded numerous colonies across each gradient plate. In contrast, no colonies grew on BEA agar with gentamicin or rifampicin. Streptomycin gradient plates prepared with MacConkey agar were completely covered with bright pink colonies (lactose fermenting). No lactose fermenting colonies were observed on the gentamicin or nalidixic acid gradient plates made with MacConkey agar. A single isolated colony was observed on a MacConkey + Rm gradient plate. The colony grew at an estimated Rm concentration of 15 μg/ml. The colony was isolated, streaked for purity, given the strain designation RREC I and stored for future use.

4.2 Characterization of RREC I:

Preliminary biochemical tests were used to determine the identity of RREC I. The isolate produced colonies that fluoresced on LMX agar, were metallic on EMB agar and produced tryptophanase when grown in peptone broth. FAME and Biolog™ profiles identified RREC I as a member of the genus *Escherichia.*

The stability of Rm^r for RREC I was tested by multiple passages in TSB. All cells were Rm^r after greater than 192 generations in TSB. There were no differences observed between numbers of colonies cultured on MacConkey + Rm or MacConkey agar.

Approximately 1.5 x 10^9 colonies were enumerated each plating and this number remained consistent throughout the experiment.

4.3 Small Scale Cattle Trial:

Lactose fermenting RREC isolates were not observed either in ruminal or fecal samples from the Jersey yearling steers prior to inoculation with the RREC I strain. Lactose fermenting RREC were cultured for up to 12 h p.i. in the rumen and were detected in the feces after 24 h and persisted for at least 5 days post inoculation (data not shown).

4.4 Feedlot Cattle Study:

4.4.1 Background Rm^r Bacteria:

Lactose fermenting rifampicin resistant colonies were not cultured from any environmental background samples (n = 210) conducted prior to the arrival of the yearling steers. Yearling steers (n = 360) were also negative for Rm^r isolates. Samples from the feedlot and steers produced abundant lactose fermenting colonies when plated on MacConkey agar.

4.4.2 Identification of Rm^r Lactose Fermenting Isolates Recovered from Feedlot Cattle:

A total of 522 Rm^r isolates were collected during the three cattle inoculations of which 501 were lactose fermenting. The MIDI system did not provide a definitive identification to the species level for RREC I, or any of isolates recovered from the

feedlot cattle. However, when the FAME profiles of 501 recovered Rm^r lactose fermenting isolates were compared to RREC I-III, 478/501 isolates clustered with RREC I – III into 1 major branch differing by < 5 – 10 Euclidean units. Only 23/501 isolates did not cluster with RREC I- III. Significant differences were also observed between the FAME profiles of the 3 inocula and 3 control strains, *E. coli* A291 and *E. coli* O157:H7 4420.

Carbon utilization patterns for a sub-set 152/478 of the Rm^r lactose fermenting isolates, including RREC II and III, re-isolated during the feedlot trial and RREC I were determined with the Biolog™ system. All three inocula (RREC I - III) and the 152 Rm^r lactose fermenting isolates had identical carbon utilization patterns. Four carbon sources (Tween-40, D-glucosaminic acid, D-arabitol and D-saccharic acid) were used by all of the Rm^r isolates and inocula, but not by typical *E. coli* according to the Biolog™ database. In addition, 11 carbon sources (p-hydroxyphenylacetic acid, bromosuccinic acid, α-keto butric acid, α-keto glutaric acid, alaninamide, D-alanine, gentiobiose, propionic acid, D-serine, glucuronic acid and mono-methyl succinate) used by the control *E. coli* A291 strain were not used by the Rm^r isolates from this study (Figure 4.1).

The number of Rm^r lactose fermenting isolates collected from various sources during the course of the feedlot trial and analyzed for their similarity to RREC by FAME and Biolog™ analysis is presented in Table 4.1. Data collected from FAME and biochemical profiles and Biolog™ were used to group isolates with the inocula strains. Transfer beyond pens 24, 23, 17, 21 and 22 was not observed.

The 3 inocula, *E. coli* A291 and *E. coli* O157:H7 4420 were subjected to the Biolog™ AST panels to determine levels of antibiotic resistance. MICs were determined

	1	2	3	4	5	6	7	8	9	10	11	12
A			sextrin		Tween 40	Tween 80	N-acetyl-D-glactosamine	N-acetyl-D-glucosamine		L-arabinose	D-arabitol	cellobiose
B		D-fructose	L-fucose	D-galactose		α-D-glucose		α-lactose	α-D-lactose lactulose	maltose	D-mannitol	D-mannose
C	D-melibiose	β-methyl D-glucoside	psicose	D-raffinose	L-rhamnose	D-sorbitol	sucrose	D-trehalose	turanose		methyl pyruvate	
D	acetic acid			formic acid	D-glactonic acid lactone	D-galacturonic acid	D-gluconic acid	D-glucosaminic acid	D-glucuronic acid			
E						D,L-lactic acid				D-saccharic acid		
F			glucuronamide			L-alanine	Lalanyl-glycine	L-asparagine	L-aspartic acid		glycyl-L-aspartic acid	glycyl-L-glutamic acid
G									L-serine			
H		inosine	uridine	thymidine					glycerol	D,L-α-glycerol phosphate	glucose-1-phosphate	

Figure 4.1: Carbon utilization pattern of Rm^r lactose fermenting isolates collected during the feedlot trial. The table is modified from information provided by the manufacturer (Biolog^{TM}).

Table 4.1: Source of isolates collected throughout the feedlot study. All lactose fermenting Rm^r colonies were subjected to FAME analysis and a select sub-group was tested with the Biolog™ system. Isolates are grouped according to source and inoculation period. The numerator in the Biolog™ column represents the number of isolates similar to RREC and the denominator represents the number of isolates tested by the system.

Source	# Rm^r Isolates Retrieved from Samples	# Similar to RREC according to		Total Collected Isolates per Group
		FAME	Biolog™	
Inoculation I				
Pen 24				171
Animal Samples	148	148	33/33	
Floor Samples	23	23	6/6	
Pen 23				25
Animal Samples	19	19	Not tested	
Floor Samples	6	6	Not tested	
Inoculation II				
Pen 17				159
Animal Samples	127	127	68/68	
Floor Samples	32	32	7/7	
Inoculation III				
Pen 21				102
Animal Samples	102	102	37/37	
Floor Samples	n/a			
Chute System				21
	21	21	1/1	
Non-Adjacent Pens				23
Animal Samples	23	0	0/17	
Total:	501	478	152	501

for 24 different antibiotics (Table 4.2). All three inocula showed identical MIC patterns for all 24 antibiotics tested. Differences between inocula and the *E. coli* controls were observed for 5 antibiotics. The RREC inocula were resistant to cefalothin, cefoxitin and ceftiofur and in contrast the *E. coli* controls were sensitive to these antibiotics. The 3 RREC inocula were substantially more resistant to rifampicin than the *E. coli* controls (1600 μg/ml vs 4 μg/ml). Finally, intermediate resistance was observed for the 2 control strains to tilmicosin whereas RREC I - III were determined to be resistant.

The 16S rDNA sequences from the inocula (RREC I - III) were aligned with Clustal W and found to be nearly identical (>99.7%; Figure 4.2). A consensus sequence for the inocula 16S rDNA was compared with entries in the GenBank database and found to be > 99.5% homologous (1312-1309/1316 nt) with entries for *E. coli, Shigella* spp. and *Klebsiella* spp.

The enzyme *I-Ceu*I was used to cleave genomic DNA from RREC I - III and *E. coli* A291, *E. coli* K12 DHα5 and *E. coli* O157:H7 4420. Banding patterns generated by PFGE showed 7 cleavage sites for all 6 strains with differences in band size observed for the controls. The profiles for the inocula were identical to each other but differed significantly from the DH5α control strains by the presence of a band 150kb larger (Figure 4.3).

4.4.3 RREC I Inoculation:

Yearling steers (n = 9/12) from pen 24 were inoculated with approximately $5x10^{12}$ RREC I cells. Rifampicin resistant colonies that appeared similar to RREC I were recovered from all inoculated animals and 2 of the uninoculated animals 24 h p.i. (Table

Table 4.2: Sensitivity of RREC I – III, *E. coli* A291 and *E. coli* O157:H7 (4420) to 24 antibiotics. The strains were determined to be sensitive (S), resistant (R) or intermediate (I) using the Biolog™ AST panels incorporating 2-fold serial dilutions to varying concentrations of each of the listed antibiotic compounds. Differences between inocula strains and the *E. coli* controls were observed for 5 antibiotics (✕).

Antibiotic	RREC I	RREC II	RREC III	*E. coli* A291 and *E. coli* O157 4420	Differences Between RREC and Controls
Amikacin	S	S	S	S	
Amoxicillin/Clavulanic acid	S	S	S	S	
Ampicillin	S	S	S	S	
Cefalothin	R	R	R	S	✕
Cefazolin	S	S	S	S	
Cefoxitin	I	I	I	S	✕
Ceftiofur	R	R	R	S	✕
Chloramphenicol	S	S	S	S	
Clindamicin	R	R	R	R	
Co-trimoxazole	S	S	S	S	
Enrofloxacin	S	S	S	S	
Erythromicin	R	R	R	R	
Gentamicin	S	S	S	S	
Oxacillin	R	R	R	R	
Penicillin G	R	R	R	R	
Penicillin/Novobiocin	R	R	R	R	
Pirlimycin	R	R	R	R	
Rifampicin	R>1600	R>1600	R>1600	R at 4	✕
Spectinomycin	S	S	S	S	
Sulfadimethoxin	R	R	R	R	
Tetracycline	S	S	S	S	
Tiamulin	R	R	R	R	
Ticarcillin	S	S	S	S	
Tilmicosin	R	R	R	I	✕

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InocI          AACGCTGGCGGCAGCCTAACACATGCAAGTCGAACGGTAACAGAAAGAAGCTTGCTTCTT 60
InocII         ----------CACGCCTAACACATSCAAGTCGAACGGTAACAGAA-GAAGCTTGCTTCTT 49
InocIII        ------------------------------------------------------------

InocI          TGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG-ATAA 119
InocII         -GCTGACGAGTGGCGGACGG-TGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAA 107
InocIII        -----ACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAA 55
                    ********** ********************************** ****

InocI          CTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGAC-TTCGG 178
InocII         CTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGAC-TTCGG 166
InocIII        CTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGG 115
               ************************************************** *****

InocI          GCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTTGTTGGTGGGGTAACGGCTCACC 238
InocII         GCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTTGTTGGTGGGGTAACGGCTCACC 226
InocIII        GCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTTGTTGGTGGGGTAACGGCTCACC 175
               ************************************************************

InocI          AAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGG 298
InocII         AAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGG 286
InocIII        AAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGG 235
               ************************************************************

InocI          TCCAGACTC-TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC 357
InocII         TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC 346
InocIII        TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC 295
               ********* ************************************************

InocI          AGCCATGCCGCGTGTATGAAGAAGGC-TTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAA 416
InocII         AGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAA 406
InocIII        AGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAA 355
               ************************** ********************************

InocI          GGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACT 476
InocII         GGGAGTAAAGTTAATACCTTTRCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACT 466
InocIII        GGGAGTAAAGTTAATACCTTTRCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACT 415
               ********************* ************************************

InocI          CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA 536
InocII         CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA 526
InocIII        CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA 475
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InocI          AAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACT 596
InocII         AAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACT 586
InocIII        AAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACT 535
               ************************************************************

InocI          GCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT 656
InocII         GCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT 646
InocIII        GCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT 595
               ************************************************************

InocI          GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTG 716
InocII         GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTG 706
InocIII        GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTG 655
               ************************************************************

InocI          ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG 776
InocII         ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG 766
InocIII        ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG 715
               ************************************************************

InocI          TAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTA 836
InocII         TAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTA 826
InocIII        TAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTA 775
               ************************************************************

InocI          AGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAAATTGACGGGGGCC 896
InocII         AGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA-TTGACGGGGGCC 885
InocIII        AGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA-TTGACGGGGGCC 834
               ********************************************** ************

InocI          CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCT 956
InocII         CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCT 945
InocIII        CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCT 894
               ************************************************************

InocI          TGACATCCACAGAACTTTCCAGAGATGGATTGGTGC-TTCGGGAACTGTGAGACAGGTGC 1015
InocII         TGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGC 1005
InocIII        TGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGC 954
               ********************************** ***********************

InocI          TGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAA 1075
InocII         TGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAA 1065
InocIII        TGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAA 1014
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InocI          CCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAA 1135
InocII         CCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAA 1125
InocIII        CCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAA 1074
               ************************************************************

InocI          CTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACG 1195
InocII         CTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACG 1185
InocIII        CTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACG 1134
               ************************************************************

InocI          TGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGT 1255
InocII         TGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGT 1245
InocIII        TGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGT 1194
               ************************************************************

InocI          GCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCC--ATGAAGTCGGAATCGCTAGTA 1313
InocII         GCGTCGTAGTCCGGATTGGAGTCTGCAACTCGATCTCTCATGAAGTCGGAATCGCTAGTA 1305
InocIII        GCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCC--ATGAAGTCGGAATCGCTAGTA 1252
               ********************************   *  ********************

InocI          ATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCC-TTGTACACACCGCCCGTCA 1372
InocII         ATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCCTTGTACACACCGCCCGTCA 1365
InocIII        ATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCC-TTGTACACACCGCCCGTCA 1311
               ************************************** ******************

InocI          CACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCA 1432
InocII         CACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCA 1425
InocIII        CACCA---------------------------------------------------------- 1316
               *****

InocI          CTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGG----------------- 1469
InocII         CTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAACCT 1479
InocIII        ----------------------------------------------------
```

Figure 4.2: CLUSTAL W (1.8) multiple sequence pairwise alignments. Sequence type explicitly set to DNA. The sequence format is Pearson. Alignments are set for InocI (RREC I inoculum, 1469 bp), InocII (RREC II inoculum, 1479 bp) and InocIII (RREC III inoculum, 1316 bp). Sequence scores are (1:2) 98, (1:3) 99 and (2:3) 99 (Thompson et al., 1994).

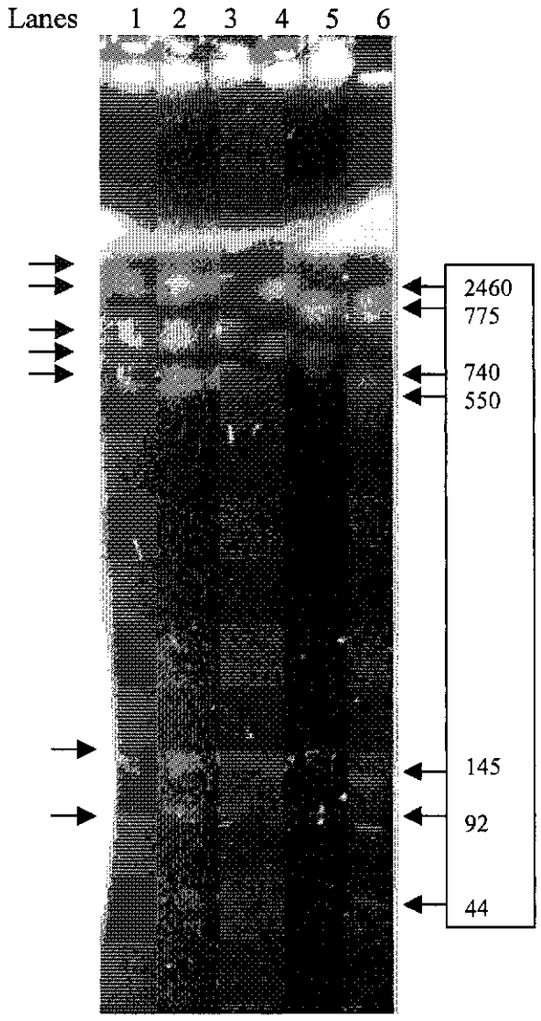

Figure 4.3: Separation by PFGE of fragments from I-*Ceu*I digestion of genomic DNA from RREC I – III (lanes 1 - 3), *E. coli* A291 (lane 4), *E. coli* O157:H7 4420 (lane 5) and *E. coli* K12 DHα5 (lane 6). Arrows (left) mark resolved fragments of RREC I – III and (right) *E. coli* K12 DHα5. Size of fragments (kb) were determined by comparison with previously described *S. typhimurium* I-*Ceu*I digestion of genomic DNA (Liu and Sanderson, 1995).

4.3). All inoculated animals showed continuous shedding events for a minimum of 9 days p.i. (506) and maximum 33 days p.i. (501). Sporadic shedding was detected in all inoculated animals for at least 22 days p.i. (502) and a maximum of 61 days p.i. (501 and 504). Uninoculated animals in pen 24 had sporadic shedding events on 2 (413 and 503) and 4 (416) occasions. Transfer to pen 23 animals (n = 7/12) was detected and RREC I CFU were recovered on several occasions (Table 4.4). No shedding events were detected in either pen after 61 days p.i.

4.4.4 RREC II Inoculation:

Shedding patterns in steers inoculated with RREC II from steer 501, showed similar trends to those observed with RREC I (Table 4.5). All inoculated animals shed within 24 h p.i. as did 2 uninoculated animals (411 and 417). Constant shedding occurred for a minimum of 9 days p.i. (412) and continued for a maximum of 27 days p.i.(422). Sporadic shedding occurred over a 62 d period for animal 422. All uninoculated animals began shedding RREC II sporadically within 72 h p.i. and continued to shed RREC II for up to 38 days. Animal 411 showed the most consistent shedding pattern (9/10 samples over a 15 d period). Transfer of RREC II to the adjacent pen 18 was not observed.

The RREC I and II were not cultured from either feed or water samples during the trial period. However, the strains were recovered from pen floor samples in proportion to animals shedding the isolate in that pen (Figure 4.4 and 4.5). The RREC isolates were also recovered from the chute system throughout sampling period.

Table 4.3: Shedding of RREC I CFU by animals in pen 24 during the time course of the first inoculation. Animal ear tag numbers in **bold** and italicized print identify inoculated animals. Shedding patterns were recorded as positive (+) if RREC CFU were detected by plating a 10^{-2} dilution on MacConkey + Rm agar. Non-shedding events are recorded as blank.

Time/Cattle #	413	416	***496***	***498***	***499***	***500***	***501***	***502***	503	***504***	***505***	***506***
24h	+	+	+	+	+	+	+	+		+	+	+
48h		+	+	+	+	+	+	+		+	+	+
72h		+	+	+	+	+	+	+		+	+	+
96 h			+	+	+	+	+	+		+	+	+
8 Days			+	+	+	+	+	+		+	+	+
9 Days		+	+	+	+	+	+	+	+	+	+	+
10 Days			+	+	+	+	+	+		+	+	
11 Days			+	+	+		+	+	+	+	+	+
12 Days			+	+		+	+	+		+	+	
15 Days			+		+		+			+	+	
18 Days	+		+	+	+		+			+		
22 Days			+		+		+	+		+		
24 Days			+				+					
26 Days			+				+					+
29 Days							+					+
31 Days							+					
33 Days			+			+	+					
36 Days			+							+		
38 Days			+									
40 Days			+							+	+	
43 Days							+					
45 Days				+			+					
47 Days							+					
50 Days			+				+					
52 Days					+							
54 Days												
57 Days												
59 Days							+					
61 Days							+			+		
71 Days												
78 Days												
85 Days												

Table 4.4: Shedding of RREC I CFU in pen 23 following the first inoculation of 9 yearling steers in the adjacent pen 24. All animals were uninoculated and are identified by ear tag numbers. Shedding patterns were recorded as positive (+) if RREC I CFU were detected by plating a 10^{-2} dilution on MacConkey + Rm agar. Non-shedding events are recorded as blank.

Time/Cattle #	483	484	485	486	487	488	489	490	491	492	493	494
72h												
8 Days			+	+							+	
15 Days												
18 Days												
22 Days												
24 Days												
26 Days			+									
29 Days												
31 Days			+									
33 Days		+	+									
36 Days			+									
38 Days			+									
40 Days	+	+	+					+				
43 Days			+									
45 Days	+						+					
47 Days												
50 Days												
52 Days				+								
54 Days												
57 Days												
59 Days												
61 Days												
71 Days												
78 Days												
85 Days												

Table 4.5: Shedding of RREC II CFU by animals in pen 17 during the time course of the second inoculation. Animal ear tag numbers in **bold** and italicized print identify inoculated animals. Shedding patterns were recorded as positive (+) if RREC CFU were detected by plating a 10^{-2} dilution on MacConkey + Rm agar. Non-shedding events are recorded as blank.

Time/Cattle #	411	***412***	414	***415***	417	418	419	***420***	***421***	***422***	***495***	497
24h	+	+		+	+			+	+	+	+	
48h	+	+	+	+	+	+		+	+	+	+	+
72h	+	+		+	+	+	+	+	+	+	+	+
6Days	+	+	+	+	+	+		+	+	+	+	
7Days		+		+	+	+	+	+	+	+	+	+
8 Days	+	+	+	+			+	+	+	+	+	+
9 Days	+	+		+		+	+	+	+	+	+	+
10 Days	+		+	+				+	+	+	+	
13 Days	+	+		+				+	+	+	+	
15 Days	+			+	+			+	+	+	+	
17 Days		+		+				+	+	+	+	
20 Days				+			+			+	+	
22 Days										+	+	
24 Days				+				+		+		
27 Days										+		
29 Days												
31 Days												
36 Days				+						+		
38 Days			+	+						+		
41 Days												
43 Days								+		+		
45 Days										+		
48 Days												
50 Days											+	
53 Days												
56 Days												
57 Days												
59 Days												*n/a
62 Days										+		
69 Days												
76 Days												

* Data not available (n/a).

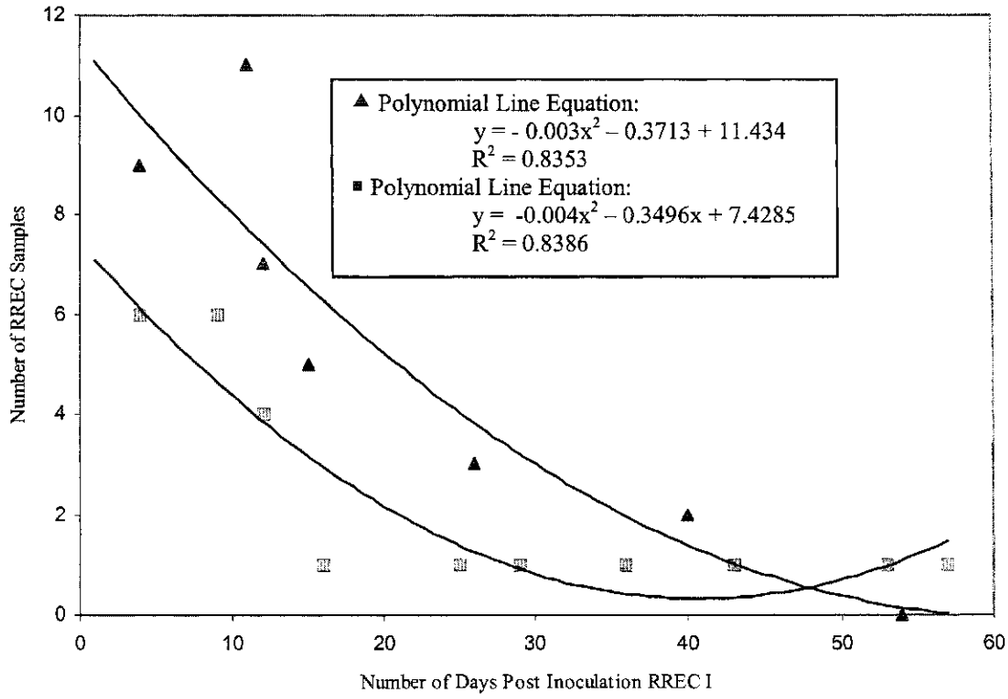

Figure 4.4: Relationship between the number of RREC positive samples (▲) from the floor of pen 24 and the number of cattle shedding RREC positive samples (■) during a 60 d period after steers (n = 9/12) were inoculated with RREC I. The trend lines are indicated for each data set shown.

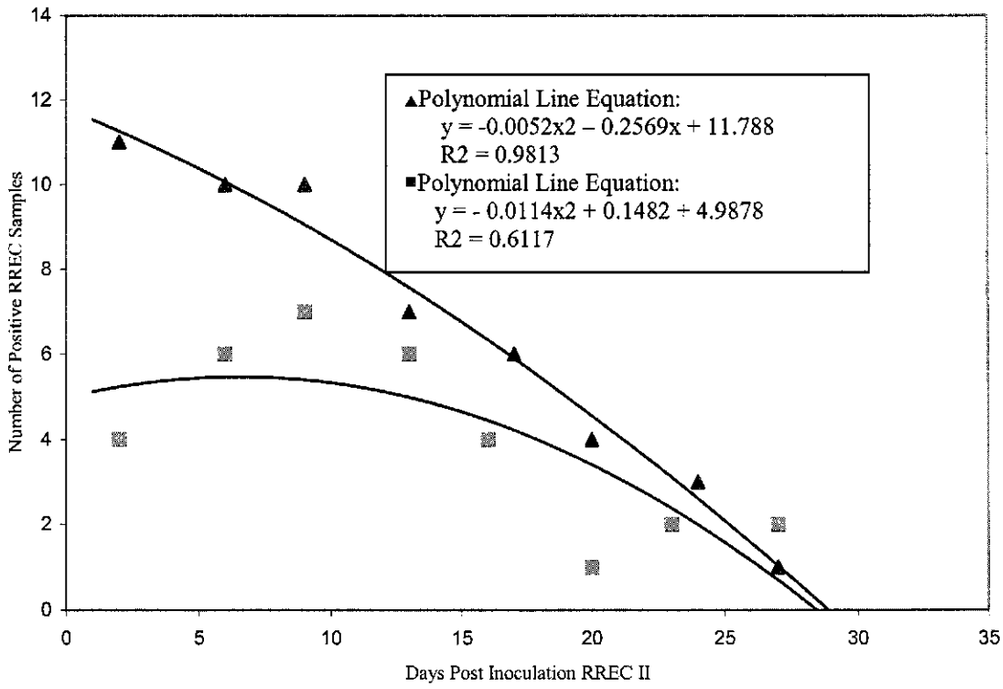

Figure 4.5: Relationship between the number of RREC positive samples (▲) from the floor of pen 17 and the number of cattle shedding RREC positive samples (■) during a 35 d period after steers (n = 6/12) were inoculated with RREC II. The trend lines are indicated for each data set shown.

4.4.5 RREC III Inoculation:

Yearling steers (n = 6/12) from pen 21 were inoculated with RREC III isolated from animal 421 as previously described. Shedding patterns were similar to those observed during the 1st and 2nd inoculations (Table 4.6). All inoculated and uninoculated animals shed within 24 h post-inoculation. The duration of continuous shedding was longer than observed during the previous 2 trials. Constant shedding in inoculated animals occurred for a minimum of 4 days p.i. (469) and for a maximum 30 days p.i. (467). Sporadic shedding occurred over a 54 d period animal 464. All uninoculated animals shed sporadically within 72 h of p.i.

4.4.6 Transport and Post-Mortem Samples:

Rifampicin resistant lactose fermenting isolates were not recovered from either the compartment floor of the transport trailer or the post-mortem tissue and digesta samples collected from 5 yearling steers immediately following slaughter. Sample dilutions spread on MacConkey agar yielded numerous lactose fermenting colonies.

Table 4.6: Shedding of RREC III CFU by animals in pen 21 during the time course of the third inoculation. Animal ear tag numbers in **bold** and italicized print identify inoculated animals. Shedding patterns were recorded as positive (+) if RREC III CFU were detected by plating a 10^{-2} dilution on MacConkey + Rm agar. Non-shedding events are recorded as blank.

Time/Cattle #	459	***460***	***461***	462	463	***464***	465	466	***467***	***468***	***469***	470
24h	+	+	+	+	+	+	+	+	+	+	+	+
48h		+	+	+	+	+	+	+	+	+	+	+
72h		+	+			+	+		+	+	+	+
96 h		+	+	+		+			+	+	+	+
8 Days		+	+			+			+	+		
9 Days		+	+			+	+		+	+		
10 Days		+	+			+	+		+	+	+	
11 Days		+	+			+			+	+	+	+
12 Days		+	+			+			+	+	+	
15 Days		+	+			+		+	+	+		
17 Days		+	+			+			+	+		
20 Days		+	+			+			+	+		
23 Days		+	+						+			+
24 Days		+	+			+			+	+		
26 Days								+	+			+
29 Days						+	+		+	+		+
30 Days						+			+	+		
32 Days		+				+				+		
36 Days		+	+			+				+		
38 Days			+									
40 Days		+	+							+		
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50 Days									+			
52 Days						+						
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57 Days												
59 Days												
61 Days												

5. DISCUSSION:

In this study we tested the hypothesis that transfer of antibiotic resistant bacteria would occur between cattle within and between pens. Although previous studies have shown that *Salmonella* spp. readily move throughout a livestock environment (Holmberg, 1984b; Molbak *et al.,* 1999), or MRSA through a clinical environment (Levy 1992; Roman *et al.,* 1997), transfer of RREC in our study was restricted primarily to the pen into which it was introduced. Furthermore, we found transfer of the strain may not be as promiscuous as previous studies and reviews have suggested (Barrow *et al.,* 1984; Fisher, 1994; Bergogne-Berezin 1997; Gustafson and Bowen, 1997; Helmuth and Protz, 1997; Khachatourians, 1998; Davies *et al.,* 1999; Klare *et al.,* 1999; Wegener *et al.,* 1999). Within 24 h p.i., all inoculated animals (inoculations 1 - 3) shed the RREC inoculum strain. Within 48 h p.i., uninoculated animals within the inoculated pens (24, 17 and 21) also shed the strain. The RREC inocula were shed more prolifically and persisted longer in inoculated animals than uninoculated animals during all three trials. The magnitude and duration of colonization by RREC I - III were similar to those obtained in other cattle studies involving *E. coli* O157:H7 (Brown *et al.,* 1997; Cray *et al.,* 1998). During these *E.coli* O157 studies, persistence of the inoculated strain was reported in cattle given approximately the same dose (~10^{12} CFU) as used in our trials. We found no differences in colonization or transfer between animals regardless of any preconditioning of the inoculum (i.e., RREC II and III were isolated from a shedding animal in trial I and II, respectively). We expected RREC II and III to establish more readily in the GI tract of the inoculated steers. It has been observed in other studies that laboratory culturing reduces the resilience of bacteria cultures (Williams *et al.,* 1996). RREC II was believed

to be a direct descendent of the previous RREC I inoculum strain and therefore had re-colonized in the cattle following culture in the laboratory. Laboratory culturing was limited between isolation and re-inoculation with the RREC II. The RREC III inoculum was cultured under identical conditions but this time from an RREC II inoculated animal. Similarly, increased resilience was not observed and shedding patterns nearly mimicked the first and second inoculation experiments. Warmer climate conditions (data not shown) may have contributed to the slightly longer shedding pattern of animals during the 3rd inoculation experiment due to persistence of the strain in feces on the pen floor.

Animals arrived at the experimental facility one week prior to the first inoculation with RREC I. Transportation to a new environment along with new animals relationships may have contributed to heightened animal stress. Hormones associated with increased stress can change microenvironments within the animal gut (Blecha, 1988; Hinton *et al.*, 1994; Grandin, 1997). Increased prevalence of antibiotic resistant strains of *E. coli* has been recorded in calves following transport (Hinton and Linton, 1983; Hinton *et al.*, 1984). This change was associated with the displacement of sensitive strains that may have been compromised due to subtle changes in the animal digestive tract environment. Several studies have been conducted regarding stress and immunity of animals (Blecha, 1988; Corrier *et al.*, 1990; Hinton *et al.*, 1994). Differences in bacterial competitive exclusion in compromised animals may have allowed RREC I to better colonize the guts of animals in pens 23 and 24. Differences in stress response by individual animals may explain differences observed for shedding events of each animal (Moberg, 1987; Cornick *et al.*, 2000). Transfer between pens was only seen during the first inoculation (e.g.,

between inoculated animals in pen 24 and uninoculated animals in pen 23). Differences in animal health or stress may have contributed to transfer between these two pens.

The RREC inocula did not persist in the GI tract of the cattle beyond the 4 month trial period. Transport has been associated with increased antibiotic resistant bacterial shedding in calves (Hinton *et al.,* 1994). Following transport, we did not observe shedding of the RREC strain and in fact found no traces of the inocula in either post-mortem GI tissue or digesta samples. These results suggest that the RREC isolates were cleared from the digestive tract rather than simply not found as a result of intermittent shedding.

The RREC origin of recovered Rm^r lactose fermenting isolates was grouped by FAME and carbon source analysis (Table 4.1). The results of these tests suggest that 95% isolates tested were descendents of the original RREC I isolate. The RREC I, II and III were observed to be identical using FAME, Biolog^{TM}, MIC, ribotyping (16S rDNA) and PFGE and differed from the three *E. coli* control strains (*E. coli* A291, *E. coli* O157:H7 4420 and *E. coli* K12 DHα5). Fatty acid methyl ester (FAME) derivatives have been examined as a means of characterizing single species derivatives (Livesley *et al.,* 1993). Despite some variations, the isolated strains were found to have very stable fatty acid profiles. Results can vary depending on the strain in question and the exact growth conditions (Brown *et al.,* 1997).

The endonuclease I-*Ceu*I, encoded by a class I mobile intron inserted in the gene for 23S rRNA in *Chlamydomonas eugamatos,* cleaves a specific 19-bp sequence in the *rrn* gene and results in large DNA fragments that can be mapped with PFGE. This sequence is present only in seven genes for rRNA in *S. typhimurium* and *E. coli* (Liu and

Sanderson, 1995; Liu *et al.*, 1999). Because of conservation of I-*Ceu*I sites in genes for rRNA and conservation of the number and locations of the *rrn* gene, I-*Ceu*I provides an excellent tool for the rapid examination of the chromosomes of related species of bacteria. The I-*Ceu*I digestion of genomic DNA resulted in 7 bands for all six digestions indicating all strains were closely related. RREC I, II and III resulted in indistinguishable PFGE patterns suggesting the 3 inocula isolates were representative of the same strain (Tenover *et al.*, 1995). Each succeeding inoculum was isolated from an animal shedding the previous RREC inoculum (e.g. RREC II came from a steer inoculated with RREC I) beyond the normal transition period of a ruminant (>5 d; Brown *et al.*, 1997; Cheeke, 1999). For this reason, it was determined that the RREC inocula had persisted in the steers' digestive system.

During the design phase of this experiment it was determined that an innate antibiotic resistant bacterium should be chosen for uniqueness and ease of selection throughout the trial period. Isolates grown on BEA agar produced slower growing and smaller colonies requiring more incubation time (24 h) than the lactose fermenting RREC isolate chosen from MacConkey agar. The RREC I isolate was recovered from an animal that had never been exposed to rifampicin using an antibiotic gradient plate. Rifampicin resistance is coded chromosomally and consequently more likely to be maintained in the absence of antibiotic selective pressure (Ingham and Furneaux; 2000). Rifampicin binds to the β-subunit of RNA polymerase, inhibiting DNA transcription. By modifying the target enzyme without disrupting its function the bacterium is able to continue with DNA transcription and counteract the inhibiting effects of rifampicin. Rifampicin resistance is expressed as an altered target enzyme through numerous insertions, deletions or amino

acid substitutions within the target enzyme (Fukuda and Nagasawa-Fujimori, 1983). Most of the seventeen reported alterations occur within a highly conserved region (507 to 534) of the β-subunit of RNA polymerase (Spratt, 1994; Ingham and Furneaux; 2000). Throughout this experiment, the cattle were not fed antibiotics and thus the colonization and persistence of RREC cells occurred in the absence of obvious selection. The RREC I isolate remained resistant to Rm > 192 generations during the laboratory experiment without the addition of Rm to the culture media.

E. coli is among the most prevalent microorganisms in cattle feces making up ~3% of the micro-flora population (Hrubant *et al.,* 1972; Carroll and Jasper, 1978; Perotti *et al.,* 2001). Selective media such as MacConkey + Rm and LMX (Manafi *et al.,* 1991) allowed reasonable confidence in selecting a RREC isolate. MacConkey agar controls were incorporated into each experiment involving the re-isolation of RREC I through III cells, to ensure that rifampicin plates were selective due to the inclusion of the antibiotic and not because of medium composition.

Environmental samples were cultured throughout the first and second inoculation experiments in an attempt to determine the mode of isolate transfer between inoculated and uninoculated animals. RREC I cells (or CFU) were not recovered from water bowls or feed bunks. Yearling steers shed inoculated strains within 24 h and as did most uninoculated animals. The short duration to infection and the lack of RREC CFU found in environmental samples supporting fecal-oral transmission rather than fecal-feed-oral or fecal-water-oral mode of transmission. Uninoculated cattle were most likely infected with a large number of transient RREC I cells excreted in feces by the inoculated cattle or through animal-to-animal contact (Clinton *et al.,* 1981). The number of isolates

recovered from pen floor samples was indicative of the number of animals shedding RREC in that pen (Figure 4.4 and 4.5). Positive samples were cultured from feces lying at the fence line between two pens suggesting that the transfer between animals in pens 24 and 23 may have occurred due to fecal contamination. Inoculated pen animals were put through the chute system prior to animals from adjacent pens during each sampling event. The RREC isolates were recovered from the chute system floor on non-sampling days suggesting that the chute system may also be a source of bacteria transfer between animals. During regular handling and processing of cattle in a commercial feedlot system, the chute is a common point to which all cattle are exposed. Cleaning of the chute system periodically during and immediately following use, may reduce transfer not only of antibiotic resistant bacteria but also disease causing bacteria among cattle in the feedlot.

During a study conducted by Marshall and co-workers (1990), *E. coli* isolates of bovine or porcine gut origin spread readily into human and other animal host species including chickens, flies and mice that were in direct contact with each other. During this study it was also observed that minimizing contact between animal hosts (separation into pens) minimized bacterial exchange and in fact transfer was only observed between animals with direct contact. Marshall and coworkers (1990) hypothesized that colonization may have persisted because of re-ingestion from environmental sources despite low environmental density of the test organism. They also suggested that factors other than environmental density, such as bacterial competitive exclusion, animal feed differences or health, enhanced the intraspecies transfer.

Transfer of antibiotic resistant *Salmonella* spp. (Holmberg *et al.*, 1984a; Davies *et al.*, 1999; Molbak *et al.*, 1999) and MRSA (Roman *et al.*, 1997) has been observed in human clinical studies. Although great care is taken with respect to sterility and aseptic technique, it is inevitable that transfer of these pathogenic bacteria occurs between patients. Patients in the clinical environment are likely to be immune compromised and thus more susceptible to colonization of a novel strain in their systems. In addition, antibiotic selective pressure exists within the institution as physicians regularly prescribe antibiotics for hospitalized patients (Lipsitch *et al.*, 2000). Although sterility did not exist in the feedlot environment, animal-to-animal contact was minimized between pens and antibiotics were not used during the trial period. This may explain the reduced transfer observed in this experiment.

Characterization of RREC II and RREC III confirmed that colonization of cattle lower GI tract occurred during this experiment on 3 separate occasions with 3 separate sets of animals in the absence of antibiotic selection. Transfer between animals also occurred within the inoculated pens (pen 24, 17 and 21). Transfer between pens did not occur beyond the first inoculation despite our attempts to increase the persistence of RREC I by passing it through an animal host. Animals did not shed the RREC strains beyond a 62 d period. Post-mortem experiments did not observe colonization in the gastrointestinal tract 30 days after the last shedding event of any animal in the inoculated pen.

Innate antibiotic resistance exists in cattle populations regardless of antibiotic use. The RREC I inoculum was isolated from an animal that had never received rifampicin. Antibiotics including rifampicin were never administered to any animal

involved in this experiment. On 17 occasions during this experiment, lactose fermenting Rm^r isolates that were determined not to be descendents of the RREC inocula were cultured.

We are well aware that antibiotic use patterns are important in the development of bacterial resistance. Antibiotic dosage and time of administration would be expected to have an effect on the frequency that resistant strains are selected in a particular setting (Lipsitch and Levin, 1997). This study supports the hypothesis that risk can be decreased for the transfer of antibiotic resistant bacterial strains under non-selective conditions. In the absence of selective pressure, colonization of the digestive tract of the feedlot cattle may be a short duration process and rampant movement of an antibiotic resistant bacteria throughout the feedlot environment is not the norm. Further study is needed to determine the effects of antibiotic treatment and stress on the transfer of antibiotic resistant bacteria in feedlot cattle.

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