The Pennsylvania State University

The Graduate School

Department of Veterinary Science

DESCRIPTIVE AND MOLECULAR EPIDEMIOLOGY OF ANTIBIOTIC-RESISTANT GRAM-NEGATIVE ENTERIC BACTERIA FROM DAIRY CATTLE

A Thesis in

Pathobiology

by

Ashish Anant Sawant

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2005

The thesis of Ashish Anant Sawant was reviewed and approved* by the following:

Bhushan Jayarao
Associate Professor of Veterinary Science
Thesis Advisor
Chair of Committee

Avery August
Associate Professor of Immunology

Brenda Love
Research Associate

Lorraine Sordillo
Professor and Meadow Brook Chair

Stephen Knabel
Associate Professor of Food Science

Jeffrey Watts
Senior Principal Scientist

Channa Reddy
Distinguished Professor of Veterinary Science
Head of the Department of Veterinary Science

*Signatures are on file in the Graduate School

ABSTRACT

A survey was conducted to study the antibiotic usage on dairy herds (n=113) in Pennsylvania. The findings of the study showed that antibiotics were extensively used on dairy herds for therapeutic and prophylactic purposes. Beta-lactams and tetracyclines were the most widely used antibiotics on dairy herds. Beta-lactams were used for clinical mastitis, dry cow therapy, metritis, and pneumonia; while tetracycline was primarily used in medicated milk replacers. Extralabel use of antibiotics was practiced on many farms. Extralabel use of a third generation cephalosporin, Ceftiofur was reported by 18% of the dairy herds surveyed. Recommended prudent practices of antibiotic usage (record keeping, written treatment plans, following labeled instructions, and veterinarian's advice on antibiotic usage) were not widely practiced. It is felt that current practices related to the antimicrobial usage on farms could contribute to the development of antibiotic resistant bacteria.

Lactating dairy cattle (n=313) on 33 dairy herds were examined for antibiotic resistant gram-negative enteric bacteria. Gram-negative enteric bacteria resistant to ampicillin, oxytetracycline, florfenicol, spectinomycin, and neomycin were isolated from feces of 31, 31, 7, 5, and 1% of lactating cows, respectively. Gram-negative enteric bacteria resistant to ampicillin, florfenicol, neomycin, tetracycline, and spectinomycin accounted for 9, 5, 1, 14, and 10% of the total Gram-negative enteric bacteria. Gram-negative enteric bacteria belonging to 12 species were isolated from dairy cattle, of which *Escherichia coli* (87%) was the most predominant species. Monte Carlo analysis revealed that *E. coli* would require 126 days to undergo a 3 log reduction when held in sterile water at 7^{o}C. It was observed that dairy producers that fed medicated milk replacers to calves were 3.4 fold more likely to have lactating cattle shed

tetracycline-resistant Gram-negative enteric bacteria. However a similar relationship was not observed with calves. Ampicillin- and tetracycline-resistant *E. coli* were the predominant isolates among the gram-negative species isolated and these isolates were genetically diverse. The findings of the study suggest that resistant Gram-negative enteric microflora was widely prevalent on farms.

Tetracycline determinants *tet*(B) and *tet*(A) were detected in 93 and 7% of the isolates, respectively. These *tet* determinants were located on the chromosome, and sequence analysis revealed association of *tetR* and *tetA* with transposon Tn*10*. This is the first report of a chromosomally located *tet* efflux pump associated with Tn*10* in enteric *E. coli* isolated from lactating cattle. The results of this study show that one of the pathways through which tetracycline resistance can be mediated is through transposable element Tn*10* that harbor a *tet*(B) determinant.

Ampicillin resistant enteric *E. coli* (n=94 isolates) from lactating cattle were examined for susceptibility to other antibiotics and genetic determinants that encode for beta-lactam resistance. The majority of the *E. coli* isolates were resistant to tetracycline (88%) followed by chloramphenicol (30%), spectinomycin (35%), ticarcillin (33%), ticarcillin/ clavulanic acid (23%) and ceftiofur (27%). Multidrug resistance (≥3 to 8 antimicrobial agents) was observed in 44 of 94 (47%) isolates, interestingly on most occasions multidrug resistant ceftiofur isolates were also resistant to chloramphenicol. All *E. coli* isolates were susceptible to the 4th generation cephalosporin cefepime. The extended-spectrum beta-lactamase enzymes were not detected in ceftiofur resistant isolates. Ampicillin-resistant isolates contained bla_{TEM}, while ceftiofur-resistant isolates contained cephamycinase (bla_{CMY}). Class I integrons of ≥1 kb were observed in

10 of 75 *E. coli* isolates. Sequence analysis revealed that the integrons encoded for streptomycin and spectinomycin (*aadA*), and/or trimethoprim (*dfr*) resistance.

These findings suggest commensal enteric *E. coli* and other Gram-negative enteric bacteria from lactating cattle can be a significant reservoir of antibiotic-resistant determinants. Widespread prevalence of antibiotic-resistant *E. coli* could also pose a risk to public health as these organisms have the ability to survive for long periods in the environment and could gain access to the food chain.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

This thesis has been by far the most significant scientific accomplishments in my life whereby I have been accompanied and supported by many people. It is my pleasure that I now have the opportunity to express my gratitude for all of them.

The first and foremost person I would like to thank is my advisor Dr. Bhushan Jayarao. Working with him was an honor and experience that I will cherish forever. He may not even realize how much I have learnt from him and grown as a better person and a better student of science. He always encouraged me to be competitive and got the best out of me. His approach towards science and his vision are a permanent part of me that will always inspire and motivate me.

I would like to express my appreciation to the members of my committee-Dr. Avery August, Dr. Brenda Love, Dr. Steve Knabel, Dr. Lorraine Sordillo, and Dr. Jeff Watts whose guidance and many valuable suggestions have very much enriched my work.

I am indebted to Dr. Narasimha Hegde for guiding me through some of my experiments. It was a pleasure to work with my colleagues Jonathan Dohanich, Beth Straley, Antonette Smith, Sara Donaldson, Abhijit Gurjar , and Kristin Jury.

I would like to express my sincere gratitude to Dr. David Wolfgang for his constant encouragement, help with my field work, and always being there whenever I needed assistance. I would like to thank Carol Burns and Louise Byler who helped me extensively during my farm visits. I also express my sincere thanks to Brenda Brooks for her continued support as a graduate student in pathobiology.

I feel the deepest sense of gratitude for my parents Anant Sawant and Sharada Sawant and my mother-in-law Vidhya Pathare, who formed part of my vision and taught me the good things that really matter in life. I save all my reverence for my wife Nutan for her loving support and encouragement that enabled me to complete this work.

Chapter 1

LITERATURE REVIEW

1.1 INTRODUCTION

Antimicrobials are considered as one of the greatest discoveries of the twentieth century that have improved the quality of life in humans. In the pre-antibiotic era, infectious diseases accounted for most mortality and morbidity. Antibiotics have significantly reduced the incidences of pneumonia, tuberculosis and other diseases that were the leading causes of death in the 1900s. The life expectancy in the U.S. has improved from 47.3 to 76.5 years mostly due to control of infectious diseases (Department of Health and Human Services, 1999).

The use of antibiotics in animal agriculture has helped in the production of safer animal products, improving growth rate, and conserving land, feed, and water resources (Animal Health Institute, www.ahi.org). The use of drugs in food animals is fundamental to animal health and well-being as well as to the economics of the industry. The USDA's Economic Research Service report estimated that the hog production industry saved about $45.5 million by using low levels of sub-therapeutic drugs in the year 1999 (Mathews, 2001).

Though antibiotics use has its advantages, the intensive and extensive use of antibiotics has lead to the emergence of antimicrobial-resistance. Bacteria isolated from sick patients in the 1940s had negligible antibiotic-resistance, whereas in today's hospitals we are able to routinely isolate bacteria that are resistant to more than one antibiotic (Hughes and Datta, 1983; Tenover and McGowan, 1996). Resistance is also now commonly observed on farms that use antibiotics (Levy, 1994). More than 100 genes encoding for antibiotic resistance to different antimicrobials, varying in their vectors, linkages, and pathways have emerged and disseminated in bacterial species spread out in various ecosystems throughout the world over the last 60 years including pathogenic strains of bacteria (O'Brien, 1997).

The Swann Committee (1969) from the UK was the first to address the concern of antibiotic-resistance. The committee recommended that antibiotics used in animal feed should not include drugs used in humans, and therapeutic use should be by prescription only. The important setback to livestock production came when the use of avoparcin as a growth promotor in animals was linked to vancomycin-resistant enterococci in food-animals (Wegener, 2003). Outbreaks of multidrug resistant *S.* Typhimurium definitive type 104 were observed in humans and animals (Akkina et al., 1999). The prevalence of fluoroquinolone-resistant *C. jejuni* in the USA increased from 0% in 1990 to 18% in 1999 (Butzler, 2004). Governments and international health agencies are very concerned with the trends in antimicrobial-resistance and are taking steps to review and analyze the issues with a view toward instituting appropriate measures.

The Scientific Steering Committee of the European Commission (1999) recommended prudent use of antibiotics, that antibiotic-resistant organisms be contained, developing new prevention and treatment strategies, and monitoring effects of interventions and evaluating them.

The Australian Joint Expert Technical Advisory Committee on antibiotic-resistance (1999) identified antibiotic regimen, bacterial load, and prevalence of resistant-bacteria as factors that influence emergence and spread of antimicrobial-resistant bacteria. They recommended a risk analysis approach in reviewing use of antibiotic growth promotants, and effective reporting of antibiotic usage and resistance.

The Canadian perspective was reported by the Advisory Committee on Animal Uses of Antimicrobials and Impact on Resistance and Human Health (2002). The important recommendations included better control of therapeutic and extralabel (using approved drug in a manner that is not in accordance with the approved label directions) use of antibiotics, evaluation

of safety and efficacy of antibiotic growth promoters by risk analysis, and effective surveillance of antibiotic resistance.

Scientists at the Joint FAO/OIE/WHO workshop (2004) suggested that risk associated with non-human antimicrobial use and antibiotic resistance be treated as a public health issue. It was decided to develop a list of "critically important" antibiotics used for non-human and animal purposes, to establish a global surveillance network, and to develop a risk management task force for antimicrobial-resistance.

In the United States, the FDA has developed a framework document for managing the potential risks of antimicrobial drugs. The framework places emphasis on classification of antimicrobials into groups based on risk associated with its use to humans, monitoring safety of drug pre and post approval in context to resistance development, collecting data on antimicrobial use in food animals and establishing regulatory thresholds. To combat antimicrobial-resistance the FDA, CDC, and USDA established the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) in 1996 to monitor changes in antimicrobial susceptibilities in foodborne pathogens.

There is a general consensus among various national and international agencies that antimicrobial-resistance is a growing global public health issue. There are critical research gaps that needed to be addressed before developing and implementing "science-based" programs to address antibiotic-resistance in humans and animals. The scope and relevance of antimicrobial-resistance in commensal *E. coli* from healthy lactating cattle for developing such programs was the main driving force for undertaking this work as my doctoral research.

1.2 Antibacterial agents

Antibacterial agents are broadly classified into two groups; antibiotics and biocides. Antibiotics are chemical compounds produced by actinomycetes, fungi, or bacteria that interfere with cell wall formation or processes essential to bacterial growth or survival but do not harm eukaryotic hosts harboring the infecting bacteria (Mascaretti, 2003c). Biocides are chemicals that possess antiseptic, disinfectant, and/or preservative activity. Biocides differ from antibiotic agents in their relative lack of selective toxicity and often lack target specificity, but have broad spectrum of activity (Denyer and Stewart, 1998).

Antiseptics are biocides that destroy or inhibit the growth of microorganisms in or on living tissue and disinfectants have similar properties but are generally are used on inanimate objects or surfaces. The cell wall, cytoplasmic membrane and the cytoplasm are the main target areas for biocides. Antibacterial effects can include metabolic and replicative inhibition (aldehydes); metabolic inhibition (halogen releasing agents, pyrogens); leakage, disruption of energy, transport and respiratory processes (phenols); leakage, respiratory inhibition, and intracellular coagulation (quarternary ammonium compounds, chlorhexidine, and biquanides) (Denyer and Stewart, 1998; McDonnell and Russell, 1999).

Most antibiotics inhibit a specific target in a biosynthetic process and have selective toxicity. By contrast, biocides have multiple, concentration-dependent targets, with subtle effects occurring at low concentrations and more damaging ones at higher concentrations (Russell and Chopra, 1996). Interestingly they do share similarities in some inactivation pathways and effects on bacteria. This is evident by 1) inhibition of enoyl reductase, involved in fatty acid synthesis by both isoniazid, an important antitubercular antibiotic, and the bisphenol triclosan (biocide)

(McMurry et al., 1999); 2) autolysis caused due to phenolic, inorganic, and organic mercury compounds has been observed to be similar to that following bacterial exposure to penicillin (Hugo, 1999); and 3) the filament formation in Gram-negative bacteria caused by both antibiotics (beta-lactams, novobiocin, fluoroquinolones) and biocides (phenoxyethanol, phenylethyl alcohol, chloroacetamide, acridines) (Russell, 2003).

The antibiotic-era started in 1920s, when Alexander Fleming discovered that *Penicillium notatum* fungus was able to inhibit the growth of *Staphylococcus aureus*. Gerhard Domagk in 1935 published his work on a compound named prontosil that was highly effective in vivo and was non-toxic for treating streptococcal infections. This chemical dye was later called sulphonamide. The chemotherapeutic value of penicillin was first studied by Chain and Florey (1940). They were able to produce penicillin extract and demonstrated that it could cure infections in animals. The development of the tetracycline antibiotics was the result of systemic screening of soil specimens collected from many parts of the world for antibiotic-producing microorganisms. The first of these compounds, chlortetracycline, was introduced in 1948 followed by oxytetracycline and tetracycline in 1950 and 1952, respectively.

These remarkable discoveries were the beginning of the antibiotic revolution that changed the course of modern medicine. In the next several decades the quest for better antimicrobial agents with greater stability and broader activity lead to the discovery and development of many antibiotics including streptomycin, aminoglycosides, glycopeptides, cephalosporins, carbapenems, beta-lactamase inhibitors, and monobactam. All were identified from natural products as agents with improved or differentiated properties (Bush, 2004).

1.2.1 Mechanisms of action

Inhibition of cell wall biosynthesis: The bacterial cell wall contains peptidoglycan, a meshwork of strands of peptide and glycan that can be covalently crosslinked. The crosslinkage of adjacent peptide strands to an amide linkage is via the action of a family of transpeptidase enzymes. Beta-lactam antimicrobials (penicillins, cephalosporins, carbapenems, monobactams) bind to transpeptidase and inhibit peptidoglycan formation, thus interfering with cell wall synthesis (Figure **1.2** and **1.3**). These transpeptidases are called penicillin-binding proteins (PBPs). The PBPs are different for Gram-negative, Gram-positive, and anaerobic bacteria. Glycopeptides like vancomycin also inhibit biochemical reactions in the cell wall catalyzed by transpeptidases and carboxypeptidases (Walsh, 2000).

Inhibition of protein synthesis: Several classes of antibiotics are able to interfere with protein synthesis in the ribosome (Figure **1.2**). Bacterial ribosomes contain two subunits, the 50S and 30S subunits. Antibiotics can act by interrupting the timing and specificity of different steps of protein synthesis, or inhibiting decoding and A-site occupation in the ribosome. Tetracyclines bind to the A site and prevent movement of tRNA (Harms et al., 2003). Aminoglycosides bind directly adjacent to the decoding site in the 30S subunit, rendering translation highly inaccurate. Peptide bond formation is another target that is employed by lincosamides, which prevents accurate positioning of tRNA, thereby preventing peptide bond formation, whereas erythromycin and other macrolides bind at the entrance of the polypeptide export tunnel and prematurely block elongation of the polypeptide (Harms et al., 2003; Schlunzen et al., 2001). Aminoglycosides such as spectinomycin prevents conformational changes in the 30S subunit, effectively blocking translocation (Carter et al., 2000).

Inhibition of DNA replication and repair: Fluoroquinolones are synthetic antibiotics that kill bacteria by targeting the enzymes DNA gyrase and topoisomerase (Figure **1.2**). DNA gyrase is responsible for relieving topological stress in translocation and replication complexes by uncoiling the intertwined DNA strands by introducing negative supercoils into DNA (Hawkey, 2003). Quinolones act by binding to the DNA-gyrase enzyme complex. Shortly after binding, the quinolones induce a conformational change in the enzyme. The enzyme breaks the DNA strands for unwinding, but quinolone prevents religation of the broken DNA strands forming a quinolone-gyrase-DNA complex. Accumulation of double strand breaks, triggers the SOS system is triggered leading to bacterial cell death (Drlica and Zhao, 1997).

1.3 Role of antibiotics in animal agriculture

It is difficult to obtain precise estimates on consumption of antimicrobial drugs in the U.S. as there is no national collection or surveillance system for drug usage information. Although accurate data on antibiotic use are not available, estimates indicate 50.6 million pounds of antibiotics are consumed annually and almost half of this use is directed towards agriculture (Harrison and Lederberg, 1998). In animal agriculture, approximately 15.4 million pounds of antibiotics are used each year (Levy, 2002). Almost 90% of all antibiotics used in farm animals and poultry are administered at subtherapeutic concentrations. About 70% of all antibiotics used in subtherapeutic concentrations in animal feeds are given for the purpose of disease prevention (prophylaxis) and the remainder of this amount is administered for growth promotion (CAST, 1981; Hay, 1986; US. Int. Trade Com. 1987).

There are three categories of use; as feed antibiotics, as over-the-counter drugs, and as veterinary prescriptions. The administration of antibiotics for the treatment of existing disease condition is termed therapeutic, while use of antibiotics when the risk of disease is high is considered prophylactic, and administration of antibiotics for enhanced production is termed subtherapeutic (McAllister et al., 2001). Although the duration of antimicrobial use differs between subtherapeutic and prophylactic purposes, the amount of antibiotics used is typically the same, i.e., less than 200 Grams/ton of feed (IOM, 1989).

The National Animal Health Monitoring System (NAHMS) indicated that antibiotics were delivered by injection to 1 to 39% lactating cows on 87.8% of the farming operations. Nearly 50% of dairy producers used antibiotics for dry cow treatment. Antibiotics were also used in the feed and water on feedlot operations of all sizes (USDA/APHIS/VS, 1995). Large operations (> 1000 head) were three times more likely to use antibiotics than small operations. Cattle in large operations were almost twice as likely to receive antibiotics in their feed and water. The tetracycline group of antibiotics was the most frequently used in feed and water on feed-lot operations (Figure **1.1**).

The benefits of using antibiotics for growth promotion were first reported by Stokstad and Jukes (1950) when chickens exposed to small doses of chlortetracycline grew more rapidly than non-exposed chickens. At sub-therapeutic levels, antibiotics are helpful in: (1) improving growth, (2) reducing risk of disease, (3) improving digestion, (4) fattening of domestic animals, and (5) decreasing time and the amount of feed needed to reach slaughter weight (Crawford and Teske, 1983; Droumev, 1983; Frost, 1991; Luetzow, 1997).

Several antimicrobial classes are approved for use in food animals including beta-lactams (e.g., penicillin, ampicillin, and cephalosporin), tetracyclines (e.g., oxytetracycline, tetracycline,

and chlortetracycline), aminoglycosides (e.g., streptomycin, neomycin, and gentamicin), macrolides (e.g., erythromycin), lincosamides (e.g. lincomycin and pirlimycin), and sulfonamides (e.g., sulfamethazine) (Mitchell et al., 1998; Hoeben et al., 1998).

A comprehensive list of drugs that are approved for use in dairy cattle is presented in Appendix A; Table **A-10**. Several antibiotics of the beta-lactam category have been approved for use in dairy cattle. The majority of the beta-lactams are approved for mastitis therapy. Penicillins like amoxicillin, cloxacillin, hetacillin, and penicillin G are available as intramammary preparations. Amoxicillin is also approved for systemic use in treating respiratory diseases and foot rot, whereas penicillin G is used for various conditions including black leg, rhinitis, pneumonia, and metritis. Penicillin G in combination with novobiocin is used for both lactating and dry cow mastitis and in combination with streptomycin for dry cow therapy. Other penicillins that can be used for treating mastitis in dry cows include benzathine cloxacillin and penicillin G. Among the cephalosporins, cephapirin, a 1st generation cephalosporin is approved for treating mastitis in both lactating and dry cows.

Ceftiofur, a new broad-spectrum third-generation cephalosporin antibiotic originally developed for respiratory diseases, which is now approved for treating metritis and foot rot. Ceftiofur is rapidly gaining popularity as an effective drug against bovine and swine respiratory pathogens. It has been found to be effective against bacteria that are resistant to older generation antibiotics (Watts et al., 1994; Salmon et al., 1995). If used according to labeled instructions, no residues of ceftiofur above the safe concentration are observed (Jaglan et al., 1992). The absence of any withdrawal period is an added advantage over other drugs.

The other antibiotics that are approved for mastitis in lactating cattle include pirlimycin, while erythromycin and novobiocin can be used for both lactating and dry cow mastitis therapy.

Antibiotics like florfenicol, spectinomycin, sulfadimethoxine, and tetracyclines are primarily approved for conditions like pneumonia and foot rot. Tetracycline and neomycin are approved for enteritis treatment.

Another important use of tetracyclines in the dairy industry is in medicated milk replacers, for preventing colibacillosis and improving weight gain in calves (Quigley et al, 1997). More than 60% of dairy producers in the United States use antibiotics in milk replacers (Heinrichs et al., 1995). Milk replacers supplemented with chlortetracycline and oxytetracycline are widely used on dairy farms. Their combination with neomycin, decoquinate, and lasalocid are approved for use in medicated milk replacers for dairy calves (USDA/APHIS/VS/CEAH, 1998).

1.4 Antimicrobial resistance

Antimicrobial resistance is defined as "a property of bacteria that confers the capacity to inactivate or exclude antibiotics, or a mechanism that blocks the inhibitory or killing effects of antibiotics, leading to survival despite exposure to antimicrobials" (IOM, 1998).

Bacteria can exhibit resistance in different ways. Some bacteria like *Providencia stuartii* have natural genetic ability to resist antimicrobial agents like tetracyclines, and therefore had an evolutionary advantage at the beginning of the 'antibiotic era' (Mateu and Martin, 2001). In addition, antimicrobial resistance can occur as a result of random genetic mutations in bacteria, leading to variation in susceptibility within any bacterial population. Single-step mutation in the DNA gyrase enzyme makes an *E. coli* resistant to norfloxacin (Hooper et al., 1986). More

commonly, resistance is not due to a chromosomal event, but due to the presence of extrachromosomal DNA (plasmids) acquired from another bacteria.

Different mechanisms that render antibiotics ineffective have evolved in bacteria. One of the most common mechanisms is the production of enzymes that degrade antibiotics. Resistance to aminoglycosides, beta-lactam (penicillins and cephalosporins), and chloramphenicol is by enzyme inactivation (Davies, 1994). Bacteria have also developed the ability to modify their cell surfaces to have reduced affinity for antibiotics (Spratt, 1994). Resistance to fluoroquinolones is chromosomally encoded and involves mutation in the target genes including DNA gyrase and topoisomerase (Chen and Lo, 2003). Active efflux of antibiotics as a resistance mechanism was first described for resistance to tetracycline in *E. coli* by McMurry et al. (1980). Some types of efflux pumps are responsible for multiple antibiotic resistances. These pumps cover a relatively wide spectrum of antibiotics, chemotherapeutic agents, detergents, dyes, and other inhibitors (Nikaido, 1998).

Bacteria can acquire antimicrobial-resistance through different mechanisms. One of the most significant ways of acquiring resistance is by horizontal transmission of genetic material (McDermott et al., 2003). Resistance genes that can transfer resistance to one or several antimicrobial agents at the same time are reported to be carried on mobile integrative and conjugative elements and plasmids (Burrus et al., 2002; Hastings et al., 2004). The broad host range of conjugative plasmids gives them the ability to transfer antimicrobial resistance genes between species or genera (Davies, 1996). The ability of bacteria to exchange antimicrobial resistance genes presents complex scenarios like persistence of resistance without exposure to antibiotics. In the case of apramycin resistance, a conjugative plasmid with high transfer frequency was responsible for maintaining and spreading resistance in genetically diverse

commensal *E. coli* in calves that were never exposed to aminoglycoside antibiotics (Yates, 2004). Mobile elements like transposon Tn21 with a class 1 integron play an important role in horizontal transfer of multidrug resistance in avian *E. coli* (Bass et al., 1999). Gebreyes and Altier (2002) have described conjugative plasmids that encode for multidrug-resistance in pathogens like *Salmonella enterica* serovar Typhimurium isolated from swine.

Environmental factors are also known to play an important role in antibiotic-resistance gene transfer. Salyers and Shoemaker (1996) observed that the transfer of a conjugative transposon carrying antibiotic-resistance genes in *Bacteriodes* spp. is stimulated considerably by exposure to sub-therapeutic concentration of antibiotics. Antibiotics in this case not only selected for the resistant strains but also stimulated transfer of resistance genes (Salyers and Shoemaker, 1996). Selective antibiotic pressure in a farm environment was considered as an impetus for horizontal transfer of a multidrug-resistant plasmid between coliform bacteria of human and bovine origin (Oppegaard et al., 2001).

1.5 Beta-lactam and tetracycline class of antibiotics

In the 1970s, the FDA proposed a ban on the then permitted use of penicillins and tetracyclines for subtherapeutic use. In 1978, Congress directed the Food and Drug Administration (FDA) to hold such actions until additional studies were completed by the National Academy of Sciences. The Academy's review found that the "postulated hazards to human health were neither proven nor disproved." The Academy recommended that additional research be conducted to fill data gaps (FDA-CVM website: www.fda.gov/cvm). These antibiotics have been under scrutiny since then. The national surveys indicate that even after

their use for decades in the dairy industry, to date beta-lactams and tetracyclines are still the most widely used class of antibiotics.

1.5.1 Beta-Lactams

The beta-lactam-based antibiotics inhibit bacterial growth by blocking the final stage in cell wall synthesis that relates to cross-linking of peptidoglycan polymers by transpeptidation (Figure **1.3**). The peptidoglycan polymer is an important constituent of the bacterial cell wall and is composed of alternating residues of N-acetylglucosamine and N-acetylmuramic acid. The N-acetylmuramic acid residues are substituted by peptide chains which are cross-linked to form a mesh-like character and provide structural integrity to the cell wall (Weidel and Pelzer, 1964). The cross-linking is carried out with the help of transpeptidases through a catalytic process (Figure **1.3**). After each catalytic cycle, one free alanine amino acid is released and the transpeptidase enzyme is regenerated which can then participate in another catalytic cycle (Walsh, 2003).

The transpeptidation step of cell-wall biosynthesis is blocked by beta-lactams. Inhibition of transpeptidase activity by penicillins occurs through the formation of covalent acyl enzyme intermediates. The transpeptidase gets depleted by irreversible binding with penicillin, which presents normal crosslinking and eventually results in loss of integrity of the cell wall structure, which leads to cell death (Walsh, 2003; Blumberg and Strominger, 1974).

The penicillins (G and V) were the first beta-lactams to be introduced and were effective against Gram-positive bacteria but had poor activity against Gram-negative bacteria. Within a few years of their introduction, penicillinase producing *Staphylococcus aureus* strains showed resistance to pencillin (Rammelkamp and Maxon, 1942). This propelled search for new forms of beta-lactams that were not inhibited by penicillinase and had a wider spectrum of activity against both Gram-positive and Gram-negative bacteria. By the early 1960s, semi-synthetic penicillins

such as ampicillin and carbenicillin were introduced. These antibiotics were more effective against Gram-negative bacteria than penicillins. In 1965, a plasmid-borne beta-lactamase (TEM-1) was first reported in *E. coli,* which conferred resistance to ampicillin. The enzyme was rapidly disseminated among species and was spread to *P. aeruginosa* by 1969, to *Vibrio cholerae* by 1973, and to *Haemophilus* and *Neisseria* species by 1974 (Matthew, 1979, Rahal et al., 1973). Later on, more plasmid-borne beta-lactamases, notably SHV-1, TEM-2, and OXA-1 were disseminated widely in the bacterial populations. In the early 1970s, chromosomally-encoded *AmpC* beta-lactamases were first reported that could rapidly inactivate ampicillin (Livermore 1987 and 1995; Sanders and Sanders, 1992).

The first generation cephalosporins were developed with the objective of combining the broad-spectrum activity of ampicillin and achieving stability to staphylococcal penicillinase. However, like ampicillin they lacked activity against bacterial species with inducible chromosomally encoded *AmpC* enzymes and against strains that had plasmids encoding TEM and SHV enzymes (Livermore, 1995). The emergence of resistance to ampicillin and first generation cephalosporins fostered the search for newer versions of beta-lactams that were stable to beta-lactamases of Gram-negative species, especially TEM-1. This resulted in the development and introduction of second generation cephalosporins, such as cefoxitime, cefuroxime, and cefamandole. These antibiotics were able to achieve acceptable stability to TEM-1 enzymes; however, they were less effective against chromosomally encoded *AmpC* beta-lactamases (O'Callaghan, 1979).

The third generation of cephalosporins included cefotaxime, ceftriaxone, ceftizoxime, ceftazidime, and cefoperazone, which were more resistant to beta-lactamases including TEM-1, TEM-2, SHV-1, and to chromosomal Class A enzymes of *Klebsiella* spp. Activity against

chromosomally encoded *AmpC*-inducible species was also observed (Livermore, 1995; O'Callaghan, 1979).

1.5.2 Resistance to beta-lactam antibiotics

Resistance to beta-lactams can arise through the following pathways: 1) modification of their penicillin-binding protein targets, 2) beta-lactamase production, and 3) impermeability and efflux. Beta-lactams bind to transpeptidase enzymes to block cell wall synthesis. The transpeptidases, also called penicillin binding proteins (PBPs), and alteration of their structure can reduce their affinity for beta-lactams. Such a mechanism was detected in *Streptococcus pneumoniae* where decreased susceptibility was linked to multiple mutations in the PBP proteins (Pernot et al., 2004). The broad-spectrum beta-lactam resistance in methicillin-resistant *Staphylococcus aureus* strains is attributed to the low affinity of PBP2a transpeptidase for beta-lactams (Lim and Strynadka, 2002). One important mechanism of resistance in Gram-negative bacteria is via beta-lactamases (Livermore, 1995). The beta-lactamase enzymes are classified into four classes (A-D) based on their sequence similarities. Classes A, C, and D comprise evolutionarily distinct groups of serine enzymes, and class B contains the zinc type (Waley, 1992).

Molecular classification of beta-lactamases, their distribution in different species and type of expression is provided in Table **1-2**. Most Gram-negative bacteria except *Salmonella* produce species specific beta-lactamases. The low-level production of beta-lactamase may not be associated with clinically relevant beta-lactam resistance. In most Gram-negative species chromosomally encoded specific beta-lactamases are called class C (*AmpC*) (Medeiros, 1997). Expression of class C beta-lactamases may be inducible or constitutive at a high or low-level, according to the species and the strain (Table **1-2**). The amount of *AmpC* produced in *E. coli* is

not sufficient to exhibit resistance to ampicillin and narrow-spectrum cephalosporins (Normark et al., 1980; Sykes and Matthew, 1979) (Table **1-1**). Resistance to ampicillin and narrow spectrum cephalosporins in *E. coli* is mostly through acquisition of class A plasmid encoded beta-lactamase (Table **1-2**) (Liu et al., 1992; Sanders and Sanders, 1992). Chromosomal *AmpC* beta-lactamases of *Enterobacter* spp., *Citrobacter* spp., *Morganella morganii*, *Providencia* spp., and *Pseudomonas* spp. are induced by ampicillin, and hydrolyze ampicillin and certain cephalosporins (Table **1-1** and **1-2**).

Plasmid mediated TEM-1 enzyme is the most common type of beta-lactamase and is responsible for most of the resistance to ampicllin in *E. coli* isolates. Other enzymes along with TEM-1 that are nowwide spread in enterobacteria include TEM-2, SHV-1 and OXA-1 (Sanders and Sanders, 1992). These beta-lactamases are easily hydrolyzed by broad spectrum cephalosporins. The last 20 years has seen the emergence of extended spectrum beta-lactamases (ESBLs). These enzymes are resistant to newer cephalosporins, monobactams, and penicillins. These beta-lactamases are the result of mutations in molecular class A plasmid encoded enzymes like TEM and SHV (Jacoby and Medeiros, 1991). The rising incidence of ESBL-producing Gram-negative bacteria from hospital acquired infections is a major health problem (Lautenbach et al., 2001).

A new form of plasmid encoded beta-lactamase emerged in the early 1990s. These enzymes exhibit properties similar to *AmpC* chromosomally encoded beta-lactamases. However these beta-lactamases (cephamycinase) exhibit resistance to extended spectrum cephalosporins, cefoxitin, and cephamycin. The enzymes can now be subclassified by their degree of genetic relationship to *AmpC* genes of *Citrobacter freundii* (CMY-2, LAT-1, and BIL-1), *Enterobacter cloacae* (MIR-1), or *Pseudomonas aeruginosa* (MOX-1 and FOX-1) (Bauernfeind et al., 1996a).

The public health importance of this type of resistance was highlighted by the work done by Zhao et al. (2001), who showed that isolates of *E. coli* and *Salmonella* containing bla_{CMY} gene were isolated from food animals as well as retail ground meat. They also showed that bla_{CMY} could be horizontally disseminated via large, broad-host-range plasmids or mobile transposons. Winokur et al. (2001) has provided epidemiological evidence that the CMY-2 *AmpC* beta-lactamase plasmid has been transferred between *E. coli* and *Salmonella* isolates. Dunne et al. (2000) studied *Salmonella* Typhimurium isolates collected by the National Antimicrobial Resistance Monitoring System between 1996 and 1998. They concluded that domestically acquired ceftriaxone resistant *Salmonella* have emerged in the United States and most isolates carried plasmid encoded *AmpC* type resistance. Plasmid encoded *AmpC* beta-lactamases have been frequently isolated from Gram-negative bacteria including *E. coli*, *Salmonella* spp., *P. mirabilis* and *C. freundii* (Bauernfeind et al., 1998). The high intrinsic resistance to penem antibiotics in *Pseudomonas aeruginosa* is attributed to the membrane impermeability and the presence of multidrug efflux system MexAB-OprM (Table **1-1**) (Okamoto et al., 2001).

All ESBL producers, plasmid encoded *AmpC* beta-lactamases, as well as bacteria devoid of outer membrane porins are susceptible to the carbapenems imipenem and meropenem, making carbapenems the last resort against infections caused by Gram-negative bacteria that are resistant to other beta-lactams (Jacoby and Carreras, 1990). Molecular class B zinc-type metallo beta-lactamases are a unique group as they can even hydrolyze carbapenems. Cardoso et al., (1999) isolated metallo beta-lactamase from *P. aeruginosa* associated with nosocomial infections in Portugal. Senda et al. (1996) reported the presence of metallo beta-lactamases in *P. aeruginosa* from 5 hospitals from different geographical locations in Japan. They also observed that these genes were carried on large plasmids.

1.5.3 Tetracyclines

In 1948, the first tetracycline, chlorotetracycline was obtained from fermentation of a soil actinomycete *Streptomyces aureofaciens*. Shortly thereafter oxytetracycline was isolated. Tetracycline was discovered in 1953 whereas methacycline, doxycycline, and minocycline were obtained by semisynthesis in 1965, 1967, and 1972, respectively (Mascaretti, 2003a). At present tetracycline, oxytetracycline, demeclocycline, doxycycline, and minocycline are marketed in the United States for human medicine (Mascaretti, 2003a).

To interact with their targets, tetracyclines traverse the outer membrane of Gram-negative bacteria through the OmpF and OmpC porin channels, as a positively charged cations, probably a magnesium-tetracycline complex (Chopra et al., 1992; Schnappinger and Hillen, 1996). The cationic-metal-ion-antibiotic complex is attracted by the Donnan potential across the outer membrane, leading to its accumulation in the periplasm, where the metal-ion-tetracycline complex dissociates to liberate uncharged tetracycline, a weakly lipophilic molecule able to diffuse through the lipid bilayer regions of the inner (cytoplasmic) membrane (Chopra and Roberts, 2001). Once inside the cytoplasm, tetracycline interacts with the acceptor (A) site for aminoacyl-tRNA. It forms electrostatic interactions, directly or through an Mg^{+2} ion with the phosphodiester links of the 16S rRNA present in the 30S ribosomal subunit. Tetracycline blocks the subsequent rotation of aminoacyl-tRNA into the A site. This results in premature release of polypeptide chain and terminates that cycle without peptide bond formation, thus preventing protein synthesis. This mechanism of action makes tetracycline largely bacteriostatic (Walsh, 2003c).

1.5.4 Resistance to tetracyclines

Resistance to tetracycline may be mediated by one of three different mechanisms: (i) an energy dependent efflux of tetracyclines carried out by transmembrane spanning proteins, which results in reduction of the concentration of tetracyclines in the cytosol; (ii) ribosomal protection, whereby the tetracyclines no longer bind productively to the bacterial ribosome or (iii) chemical modification, requiring oxygen and NADPH and catalysis by enzymes (Mascaretti, 2003b). Presently there are twenty nine different resistance (*tet*) genes and three oxytetracycline resistance (*otr*) genes that have been characterized. Eighteen of the *tet* genes and one *otr* gene code for efflux pumps, and seven of the *tet* genes and one *otr* gene code for the ribosomal protection mechanism (Chopra and Roberts, 2001). The efflux genes are divided into 6 groups (Table **1-3**).

The Gram-negative efflux determinants belong to group 1 (Table **1-3**) and exchange a proton for a tetracycline-cation complex. The majority of the efflux pumps are made up of 2 genes, one coding for an efflux protein and one coding for a repressor protein. The two genes are oriented in opposite directions and share a central regulatory region with overlapping promoters and operators (Hillen and Berens, 1994). In the absence of tetracycline, the repressor protein binds to the two tandemly oriented *tet* operators and blocks transcription of both the repressor and the efflux gene (Hillen and Berens, 1994; Kisker et al., 1995). Induction in the system occurs when a tetracycline-Mg^{+2} complex enters the cell (Figure **1.4**). This complex readily binds to the tet repressor protein which allows *tet*(A) transcription (Orth et al., 2000). The K_d for binding of tetracycline-Mg^{+2} complex with TetR protein is some thousand fold tighter than the binding with the 30S ribosome, so *tet*(A) transcriptional expression initiates even at low levels of drug in the bacterial cell (Orth et al., 2000). The 42-kDa TetA pump protein is overproduced, which is then

inserted into the cytoplasmic membrane, and acts in an antiport mode to pump out tetracycline in exchange of a proton (Yamaguchi et al., 1990) (Figure **1.5**). Export of tetracycline reduces the intracellular drug concentration and protects the ribosomes within the cell.

Spread of tetracycline efflux genes is dictated by the genes or genetic elements they are associated with. The *tet*(B) efflux gene can be transferred between *Actinobacillus* and *Hemophilus* species through conjugative plasmids, whereas the same gene is not mobile in *Treponema* species (Roe et al., 1995; Roberts et al., 1996). The *tet*(E) gene is associated with large plasmids but these plasmids are neither mobile nor conjugative while *tet*(M) is associated with conjugative mobile elements in *Haemophilus ducreyi* (DePaola et al., 1988; Roberts, 1989). Tetracycline resistance determinants have also been detected on transposons. The conjugative transposon Tn916 that harbors the *tet*(M) gene has a wide host range and can transfer the *tet*(M) gene from Gram-positive to Gram-negative bacteria (Bertram et al., 1991). Conjugative transposons carrying tet(Q) encode for ribosomal protection in *Bacteroides* spp. (Shoemaker et al., 1989). One of the most extensively characterized resistances to tetracycline is the Tn*10* transposon based efflux pumps (Hillen and Berens, 1994). The regulation mechanism of the efflux operon (Figure **1.4**) that encodes for the TetA(B) efflux and TetR(B) repressor proteins was elucidated using the Tn*10* transposon (Roberts, 1996). Tn*10* was originally discovered by Nakaya et al. (1960) on a drug resistance factor in *Shigella* species. The factor was referred to as R100 by Sugino and Hirota (1962). The R factor acts as a sex factor in that it brings about transfer of the host chromosome as well as its own transfer by conjugation (Sugino and Hirota, 1962). The translocation ability of Tn*10* was later documented by Kleckner et al. (1975). They observed that Tn*10* carrying tetracycline-resistance is capable of translocation as a unit from one DNA molecule to another. Tn*10* present on a drug resistance plasmid (R-factor) in a *Salmonella*

strain was selected by a P22 bacteriophage during a lytic cycle of growth. This phage was able to insert Tn*10* into a number of different sites on the *Salmonella* chromosome. Insertion of Tn*10* was usually precise, occurring without loss of information on the recipient DNA molecule. Similar experiments showed that the tetracycline-resistance determinant was able to translocate from the plasmid (R100-1) to the chromosome of *Escherichia coli* K-12 (Foster et al., 1975). In the Tn*10* encoded *tet* operon, the repressor protein synthesis occurs before the resistance protein. This type of regulation is believed to be common to all *tet* determinants (Hillen and Berens, 1994).

Multidrug efflux pumps (MAR locus) that can export tetracycline have also been observed in *E. coli* (George and Levy, 1983). The resistance is mediated by mutation in the negative regulator (*MarR*) of the mar operon (Alekshum and Levy, 1997; Levy, 1992). A mutation in the *marR* region over-expresses *marA*, a transcriptional activator of a common group of promoters. Over-expression of MarA increases the expression of the multiple drug efflux pump AcrAB in *E. coli* (Oethinger et al., 2000). *Escherichia coli* exposed to increasing concentrations of tetracycline or chloramphenicol may select for mutations in the *marR* region that enhance intrinsic resistance to a variety of antibiotics including penicillins, cephalosporins, rifampin, nalidixic acid, and quinolones mediated by the AcrAB efflux system (George and Levy, 1983; Levy, 1992).

1.6 Antibiotic resistant bacteria in food animals

The emergence and spread of antibiotic resistance genes is enhanced through the intensive use of antimicrobials in over-crowded populations of production animals. This

phenomenon is known as “selective pressure.” Selective pressure is a general concept that refers to the many factors that create an environmental landscape which allows organisms with novel mutations or newly acquired characteristics to survive and proliferate (Baquero et al., 1998). The classic example of continuous selective pressure in the animal production industry is the use of antibiotics in feed at subtherapeutic concentrations (IOM, 1989).

There is considerable scientific evidence to suggest that the practice of feeding antibiotics can result in selection of antibiotic resistant bacteria in animals (Bager et al., 1997; Klare et al., 1995; Aarestrup et al., 1996). Studies conducted by Smith (1973) found the presence of tetracycline resistant enteric *E. coli* in the late 1950s shortly after tetracycline was introduced in the UK as a feed additive. By the year 1970 when tetracycline was still in use, a substantial increase in the number of tetracycline-resistant *E. coli* was observed. Levy et al. (1976) studied the effect of feeding tetracycline-supplemented feed to chickens. They observed tetracycline resistance in the chicken intestinal flora within one week. Selective pressure selecting for tetracycline resistance in bacteria in chickens was transferred gradually to farm personnel within 5 to 6 months. Recently ampicillin-resistance was detected in *E. coli* from chicks that were exposed to low doses of ampicillin in feed (al-Sam et al., 1993). Antibiotics for treatment incorporated in starter and grower finisher rations of pigs exhibited a significant association with resistance to important antibiotics like ampicillin, spectinomycin, carbadox, and tetracycline in fecal *E. coli* (Dunlop et al., 1998). The influence of selective pressure is well recognized through the work done by Langlois et al. (1983). They observed a significant decrease in tetracycline resistant fecal coliform from 82 to 42% after complete withdrawal of therapeutic and subtherapeutic use of antibiotics on a swine herd for 126 months. Most of this literature has focused the attention of researchers towards the use of antibiotics in animals, especially

antibiotic growth promoters. The Food and Drug Administration acknowledges the likelihood that an antimicrobial drug used to treat an animal may cause antimicrobial-resistance. Recent guidelines issued by FDA (2003) encourages drug sponsors to use a risk assessment process to demonstrate that an antimicrobial drug used to treat food-producing animals will not create a risk of antimicrobial resistant bacteria likely to lead to human health problems.

Resistance to the antimicrobial drugs needed to treat human illnesses is a serious public health threat. Antimicrobial-resistance in foodborne pathogens is therefore an important issue. Foodborne pathogens like *Campylobacter*, *Salmonella*, and pathogenic *E. coli* isolated from beef, pork, and poultry have been found to be resistant to quinolones and tetracycline (Mayrhofer et al., 2004). A study conducted on the zoonotic pathogen *Campylobacter coli* isolated from fattening pigs in France exhibited resistance to tetracycline, erythromycin, or multidrug-resistance (Payot et al., 2004). Multidrug-resistant Salmonella including those resistant to ceftriaxone have been isolated from retail ground meat (White et al., 2001). A shiga-toxin-producing *E. coli* O157:H7 *marR* mutant isolated from cattle feces showed multiple antibiotic resistance to chloramphenicol, tetracycline, nalidixic acid, and ciprofloxacin (Yaron et al., 2003). This can become a major clinical problem as in a case study of a child with diarrhea in Nebraska, whose stool revealed the presence of ceftriaxone-resistant *Salmonella* and cattle were identified as the source of infection (Fey et al., 2000).

To deal with the growing problem of antimicrobial-resistance, the United States National Antimicrobial Resistance Monitoring System (NARMS) was established in the year 1996 for monitoring antimicrobial-resistance in human enteric bacteria including *Campylobacter*, *Salmonella*, *E. coli* O157, and *Shigella*. NARMS maintains a database of antibiotic susceptibility patterns of these pathogens. In the year 2002, a report was published by the NARMS on

susceptibility of 4,689 veterinary origin *E. coli* isolates, of which 1389 were from dairy cattle. In these baseline *E. coli* isolates of veterinary origin, predominant resistance was observed against tetracycline (36%), streptomycin (35%), sulfamethoxazole (26.4%), gentamicin (18.8%), ampicillin (13.7%), and cephalothin (10.4%). Resistance was also observed to important antibiotics like amoxicillin/clavulanic acid (7.1%), cefoxitin (5.5%), and ceftiofur (3.7%). Multidrug-resistance (> 3 to 14 antibiotics) was observed in 31.5% of *E. coli*. The most common resistance pattern included resistance to gentamicin, streptomycin, sulfamethoxazole, and tetracycline and was observed in 5% of the *E. coli* isolates. This type of data is useful in understanding emerging trends in antimicrobial resistance. NARMS data can also be helpful in epidemiological investigations of outbreaks.

In the United States, the Food and Drug Administration has the regulatory responsibility of ensuring that the use of antimicrobial drugs in food-producing animals does not result in adverse health consequences to humans. In 1999, the FDA proposed guidelines with a document entitled, "A proposed framework for evaluating and assuring human safety of the microbial effects of antimicrobial new animal drugs intended for use in food producing animals" (Framework document, FDA, 1999). The document elucidated a 5-pronged strategy for managing risks associated with the use of antibiotic drugs in food-producing animals. Strategies included: 1) categorization of antibiotics based on their importance in human medicine; 2) revision of the pre-approval safety assessments for new animal drug applications to access microbial safety; 3) post-approval monitoring for resistance development; 4) collection of food animal antibiotic use data; and 5) establishment of regulatory thresholds.

Table **1-1**: Distribution, classification and expression of important beta-lactamases*

	Molecular Class			
	A	B	C	D
Chromosomal	*K. oxytoca* Ki (C[a]) *K. pneumoniae*-SHV-1[b] (C) *P. vulgaris* (I) *C. diversus* (I) *Bacteroides* A (C)	*S. maltophilia* L1 (I) *F. odoratum* (C) *Aeromonas* (I)	*E. coli AmpC* (c) *Shigella AmpC* (c) *Enterobacter AmpC* (I) *C. freundii AmpC* (I) *Serratia AmpC* (I) *M. morganii AmpC* (I) *Providentia AmpC* (I) *P. aeruginosa AmpC* (I)	
Plasmid	TEM family[c] (C) SHV family[b,c] (C) HMS-1 (C) *Staphyloccal* penicillinase (I)	IMP-1 and related (C)	Plasmidic *AmpC* (C) (BIL-1, CMY family, FOX-1, LAT-1, MOX-1, MIR-1)	OXA- family[c]

* Livermore (1996).
[a] Mode of production: c, constitutive at insignificant level; C, constitutive at significant level; I, inducible,
[b] SHV-1 is the chromosomal beta-lactamase of *K. pneumoniae*; but may also be encoded by plasmids in other Gram-negative bacilli
[c] Includes extended spectrum derivatives, active against newer generation beta-lactams

Table **1-2**: Natural resistance mechanisms prevalent in different Gram-negative Genera/species of bacteria*

Genus/ Species	**Ampicillin**	**Ampicillin-Sulbactam**	**1st gen Cephalosporin**	**2nd gen Cephalosporin**	**3rd gen Cephalosporin**
Citrobacter	β / #	β	β	β	S
Enterobacter	β / #	β	β	β	S
E. coli	S	S	S	S	S
Morganella	β / #	β	β	β	S
Klebsiella	β	S	S	S	S
Pseudomonas	β / #	β / #	β / #	β / #	#

β = Resistance conferred by chromosomal beta-lactamase
# = Resistance conferred by impermeability/efflux
S = Sensitive

* Table compiled from Livermore (1996).

Table **1-3**: Tetracycline efflux genes*

Efflux groups	**Genes**	**Species**
Group 1	*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(Z), *tet*(I), *tet*(J), and *tet*(30)	*tet*(Z) found in Gram-positive; remainder found in Gram-negative
Group 2	*tet*(K) and *tet*(L)	Gram-positive
Group 3	*otr*(B) and *tcr*3	*Streptomyces* spp.
Group 4	*tet*A(P)	*Clostridium* spp.
Group 5	*tet*(V)	*Mycobacterium smegmatis*
Group 6	unnamed determinant	*Corynebacterium striatum*

* Table compiled from Chopra and Roberts (2001).

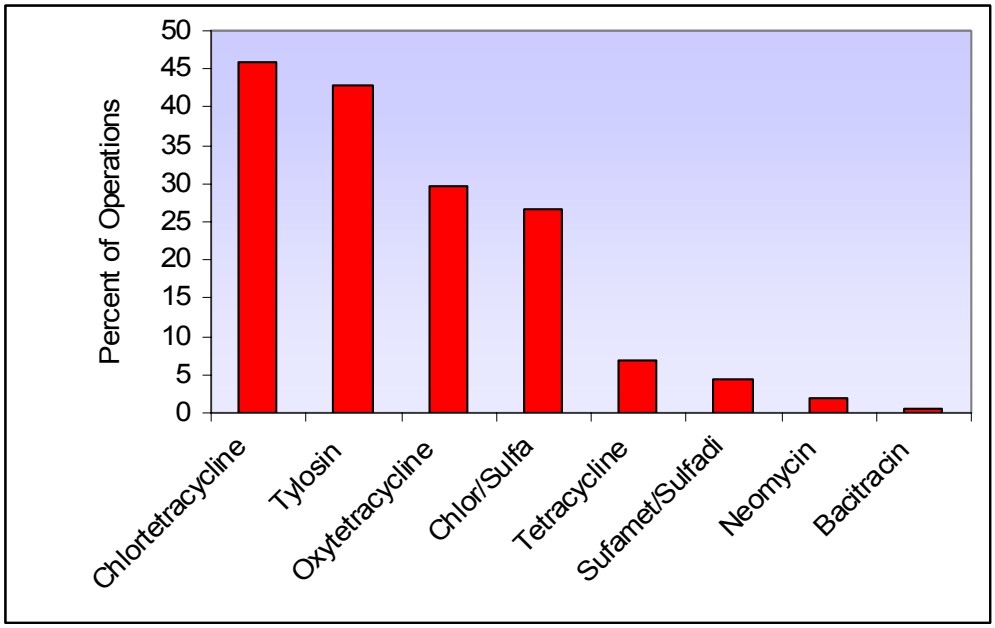


Figure **1.1**: Antibiotic additives used in feed and water on 1000-plus head feedlot operations in the U.S. in the year 1994 (USDA/APHIS Veterinary Services, 1995)

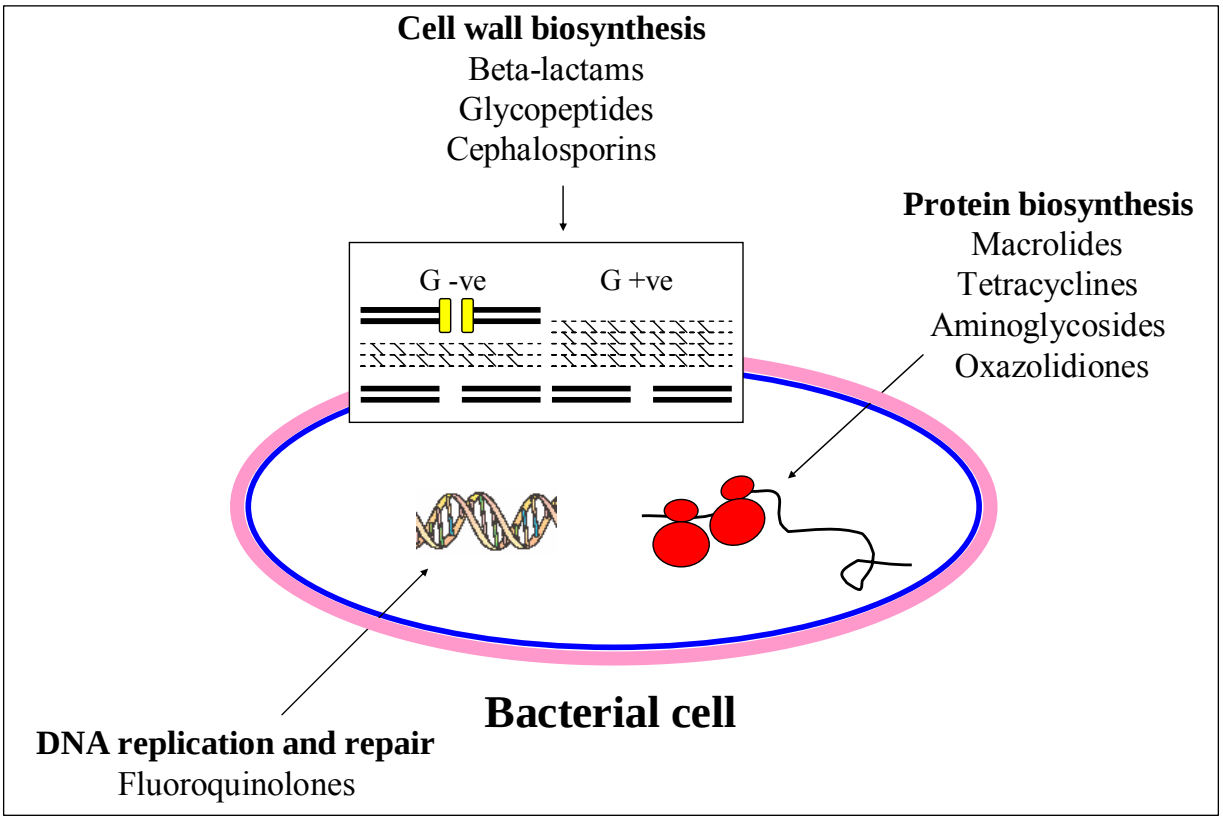


Figure **1.2**: Targets for antibacterial drugs

Figure adapted and based on information by Walsh C (ed.). 2003. Antibiotics: actions, origins, resistance. ASM press. Washington, D.C.

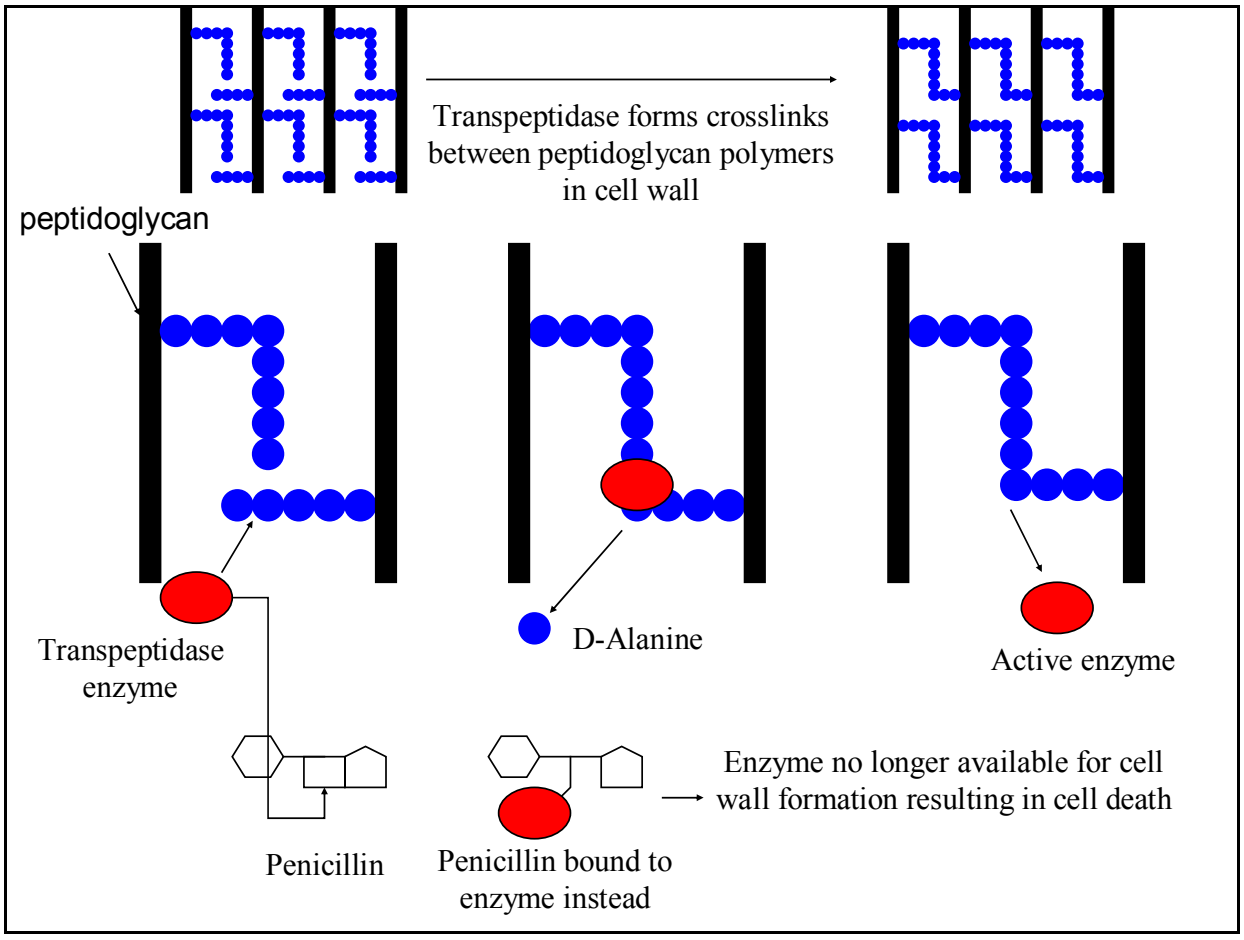


Figure **1.3**: Mechanism of beta-lactam cell wall inhibition

Adapted from a figure available on line at
www.shodor.org/master/biomed/pharmaco/penicillin/penapp.html.

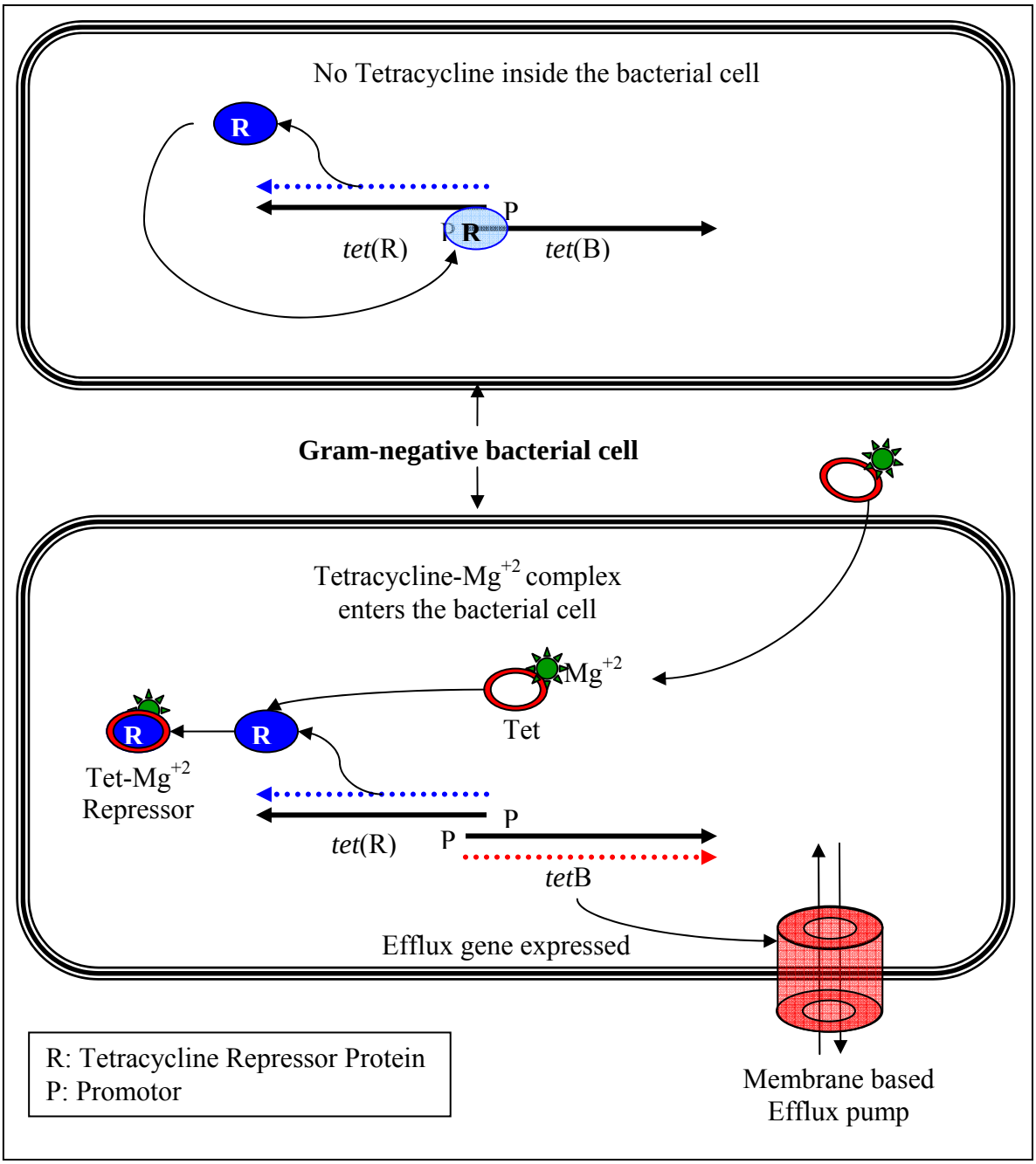


Figure **1.4**: Efflux-mediated resistance in Gram-negative bacteria

Figure adapted and based on information by Lawley et al. (2000) and Chopra and Roberts (2001).

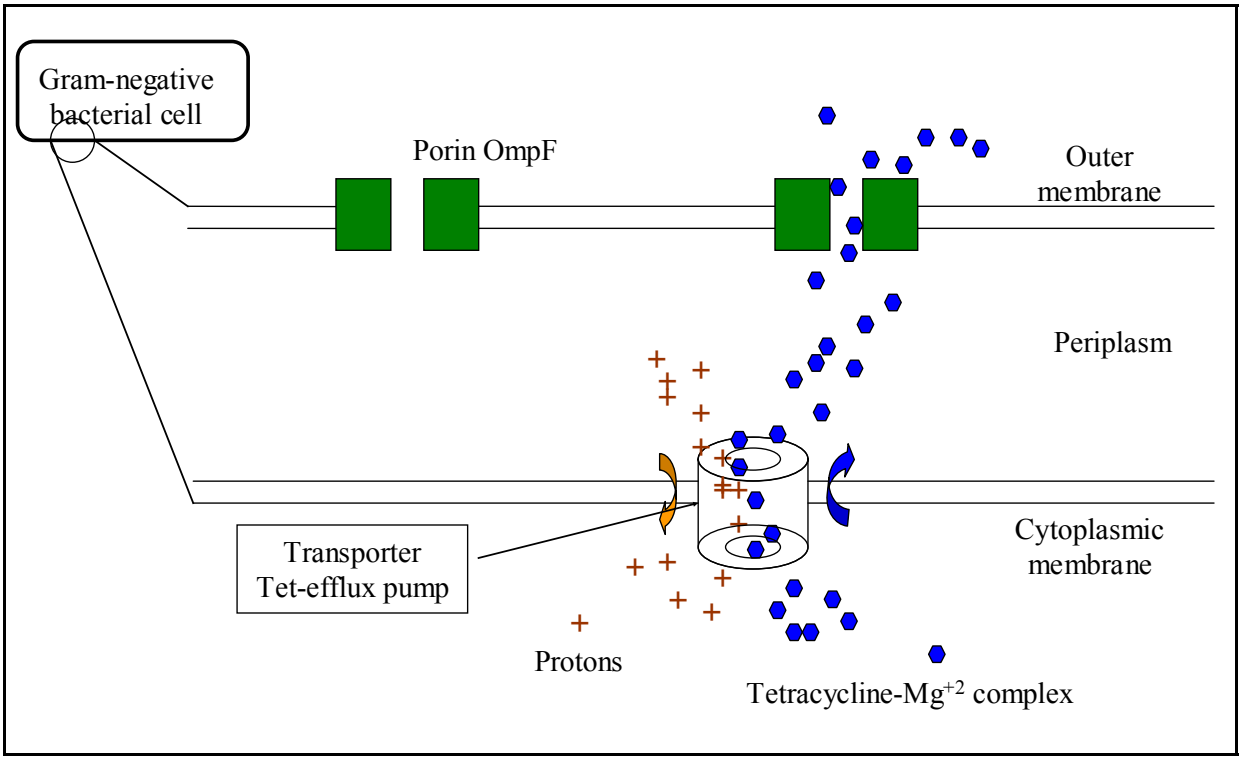


Figure **1.5**: Tetracycline drug: H^+ antiport system

Figure adapted and based on information by Nikaido (1996) and Thanassi et al. (1995).

1.7 REFERENCES

Aarestrup, F. M., P. Ahrens, M. Madsen, L. V. Pallesen, R. L. Poulsen, and H. Westh. 1996. Glycopeptide susceptibility among Danish Enterococcus faecium and Enterococcus faecalis isolates of animal and human origin and PCR identification of Genes within the *VanA* cluster. Antimicrob. Agents Chemother. 40:1938-1940.

Advisory Committee on Animal Uses of Antimicrobials and Impact on Resistance and Human Health. 2002. Uses of Antimicrobials in Food Animals in Canada: Impact on Resistance and Human Health. Available on line at www.hcsc.gc.ca/vetdrugsmedsvet/amr_final_report_june27_12_e.html. Accessed on 9th February, 2005.

Akkina, J. E., A. T. Hogue, F. J. Angulo, R. Johnson, K. E. Petersen, P. K. Saini, P. J. Fedorka-Cray, and W. D. Schlosser. 1999. Epidemiologic aspects, control, and importance of multiple-drug resistant Salmonella Typhimurium DT104 in the United States. J. Am. Vet. Med. Assoc. 214:790-798.

Alekshum, M. N., and S. B. Levy. 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulation. Antimicrob. Agents Chemother. 41:2067-2075.

Al-Sam, S., A. H. Linton, P. M. Bennett, and M. Hinton. 1993. Effects of low concentrations of ampicillin in feed on the intestinal *Escherichia coli* of chicks. J. Appl. Bacteriol. 75:108-112.

Bager, F., M. Madsen, J. Christensen, and F. M. Aarestrup. 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant Enterococcus faecium on Danish poultry and pig farms. Prev. Vet. Med. 31:95-112.

Baquero, F., M. C. Negri, M. I. Morosini, and J. Blazquez. 1998. Antibiotic-selective environments. Clin. Infect. Dis. 27:S5-S11.

Bass, L., C. A. Liebert, M. D. Lee, A. O. Summers, D. G. White, S. G. Thayer, and J. J. Maurer. 1999. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian Escherichia coli. Antimicrob. Agents Chemother. 43:2925-2929.

Bauernfeind, A., I. Stemplinger., R. Jungwirth., R. Wilhelm, and Y. Chong. 1996a. Comparative characterization of the cephamycinase blaCMY-1 gene and its relationship with other beta-lactamase genes. Antimicrob. Agents Chemother. 40:1926-1930.

Bauernfeind, A., Y. Chong., and K. Lee. 1998. Plasmid-encoded *AmpC* beta-lactamases: how far have we gone 10 years after the discovery? Yonsei. Med. J. 39:520-525.

Bauernfeind, A., Y. Chong., and S. Schweighart. 1989. Extended broad spectrum beta-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. Infection. 17:316-321.

Bertram, J., M. Stratz, and P. Durre. 1991. Natural transfer of conjugative transposon Tn916 Gram-positive and Gram-negative bacteria. J. Bacteriol. 173:443-448.

Blumberg, P. M., and J. L. Strominger. 1974. Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. Bacteriol. Rev. 38:291-335.

Burrus, V., G. Pavlovic, B. Decaris, and G. Guedon. 2002. Conjugative transposons: the tip of the iceberg. Mol. Microbiol. 46:601-610.

Bush, K. 2004. Why it is important to continue antibacterial drug discovery. ASM News. 70:282-287.

Butzler, J. P. 2004. Campylobacter, from obscurity to celebrity. Clin. Microbiol. Infect. 10:868-876.

Cardoso, O., J. C. Sousa, R. Leitão, and L. Peixe. 1999. Carbapenem hydrolysing ß-lactamase from clinical isolates of *Pseudomonas aeruginosa* in Portugal. J. Antimicrob. Chemother. 4:135.

Carter, A. P., W. M. Clemons, D. E. Brodersen, R. J. Morgan-Warren, B. T. Wimberly, and V. Ramakrishnan. 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature. 407:340-348.

Chain, E., H. W. Florey, A. D. Gardner, N. G. Heatley, M. A. Jennings, J. Orr-Ewing, and A. G. Sanders. 1940. Penicillin as a chemotherapeutic agent. Lancet 2:226-228.

Chen, F. J., and H. J. Lo. 2003. Molecular mechanisms of fluoroquinolone resistance. J. Microbiol. Immunol. Infect. 36:1-9.

Chopra, I., and M. Roberts. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 65:232-260.

Chopra, I., P. M. Hawkey, and M. Hinton. 1992. Tetracyclines, molecular and clinical aspects. J. Antimicrob. Chemother. 29:245-277.

Council for Agricultural Science and Technology. 1981. Antibiotics in Animal Feeds. Report No. 88. Council for Agricultural Sciences and Technology, Ames, Iowa.

Crawford, L. M., and R. H. Teske. 1983. Growth promotants approved in the U.S. Vet. Research Commun. 7:83-84.

Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science. 264:375-382.

Davies, J. 1996. Origins and evolution of antibiotic resistance. Microbiologia. 12:9–16.

Denyer, S. P., and G. S. A. B. Stewart. 1998. Mechanism of action of disinfectants. Int. Biodet. Biodeg. 41: 261-268.

Department of Health and Human Services. 1999. Accountability report, A century of progress and a look into the future. Available on line at http://www.hhs.gov/of/reports/account/acct99/execsum/century.html. Accessed on 8th February, 2005.

Domagk, G. J. 1935. Ein Beitrag zur Chemotherapie der bakteriellen infektionen. Dtsch. med. Wochenschr. 61:250-253.

Drlica, K., and X. Zhao. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol. Mol. Biol. Rev. 61:377-392.

Droumev, D. 1983. Review of antimicrobial growth promoting agents available. Vet. Res. Commun. 7:85-89.

Dunlop, R. H., S. A. McEwen, A. H. Meek, R. C. Clarke, W. D. Black, and R. M. Friendship. 1998. Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada. Prev. Vet. Med. 34:283-305.

Dunne, E. F., P. D. Fey, P. Kludt, R. Reporter, F. Mostashari, P. Shillam, J. Wicklund, C. Miller, B. Holland, K. Stamey, T. J. Barrett, J. K. Rasheed, F. C. Tenover, E. M. Ribot, and F. J. Angulo. 2000. Emergence of domestically acquired ceftriaxone-resistant Salmonella infections associated with *AmpC* beta-lactamase. J. A. M. A. 284:3151-3156.

European Commission. 1999. Opinion of the Scientific Steering Committee on Antimicrobial Resistance. European Commission Directorate General XXIV, Consumer Policy and Consumer Health Protection. Brussels, Belgium.

Fey, P. D., T. J. Safranek, M. E. Rupp, E. F. Dunne, E. Ribot, P. C. Iwen, P. A. Bradford, F. J. Angulo, and S. H. Hinrichs. 2000. Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. N. Engl. J. Med. 342:1242-1249.

Food and Drug Administration. 2003. FDA press release-FDA issues guidance on evaluating the safety of antimicrobial new animal drugs to help prevent creating new resistant bacteria. Available on line at www.fda.gov/cvm. Accessed 25th Jan, 2005.

Foster, T. J., T. G. Howe, and K. M. Richmond. 1975. Translocation of the tetracycline resistance determinant from R100-1 to the *Escherichia coli* K-12 chromosome. J. Bacteriol. 124:1153-1158.

Frost, A. J. 1991. Antibiotics and animal production. p. 181-194. *In* J. B. Woolcock JB (ed.), World Animal Science Microbiology of Animals and Animal Products, Elsevier, New York.

Gebreyes, W. A., and C. Altier. 2002. Molecular characterization of multidrug-resistant Salmonella enterica subsp. enterica serovar Typhimurium isolates from swine. J. Clin. Microbiol. 40:2813-2822.

George, A. M., and S. B. Levy. 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: Involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. 155:531-540.

Harms, J. M., H. Bartels, F. Schlunzen, and A. Yonath. 2003. Antibiotics acting on the translational machinery. J. Cell. Sci. 116:1391-1393.

Harrison, P. F., and J. Eds. Lederberg. 1998. Antimicrobial resistance: issues and opinions. Workshop report. National Academy Press. Washington, DC.

Hastings, P. J., S. M. Rosenberg, and A. Slack. 2004. Antibiotic-induced lateral transfer of antibiotic resistance. Trends Microbiol. 12:401-404.

Hawkey, P. M. 2003. Mechanisms of quinolone action and microbial response. J. Antimicrob. Chemother. 51 Suppl 1:29-35.

Heinrichs, A. J., S. J. Wells, and W. C. Losinger. 1995. A study on the use of milk replacers for dairy calves in the United States. J. Dairy Sci. 78:2831-2837.

Hillen, W., and C. Berens. 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annu. Rev. Microbiol. 48:345-369.

Hooper, D. C., J. S. Wolfson, K. S. Souza, C. Tung, G. L. McHugh, and M. N. Swartz. 1986. Genetic and biochemical characterization of norfloxacin resistance in *Escherichia coli*. Antimicrob. Agents Chemother. 29:639-644.

Hughes, V. M., and N. Datta. 1983. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. Nature. 302:725-726.

Hugo, W. B. 1999. Disinfection mechanisms. p. 258-283. *In* A. D. Russell, W. B. Hugo, G. A. J. Ayliffe (ed.), Principles and Practice of Disinfection, Preservation and Sterilization, 3rd ed. Blackwell Science, Oxford.

Institute of Medicine. 1989. Human health risks with subtherapeutic use of penicillin or tetracyclines in animal feed. Committee on human health risk assessment on using subtherapeutic antibiotics in animal feeds. National Academy Press, Washington D. C.

Institute of Medicine. 1998. Antimicrobial Resistance: Issues and Options. P. F. Harrison, and J. Lederberg (eds.), National Academy Press, Washington D.C.

Jacoby, G. A., and A. A. Medeiros. 1991. More extended-spectrum β-lactamases. Antimicrob. Agents Chemother. 35:1697-1704.

Jacoby, G. A., and I. Carreras. 1990. Activities of beta-lactam antibiotics against *Escherichia coli* strains producing extended-spectrum beta-lactamases. Antimicrob. Agents Chemother. 34:858-862.

Jaglan, P. S., F. S. Yein, R. E. Hornish, B. L. Cox, T. S. Arnold, R. D. Roof, and T. J. Gilbertson. 1992. Depletion of intramuscularly injected ceftiofur from the milk of dairy cattle. J. Dairy. Sci. 75:1870-1876.

Joint Expert Technical Advisory Committee on Antimicrobial Resistance (JETACAR). 1999. The use of antibiotics in food-producing animals: antibiotic-resistant bacteria in animals and humans. Canberra, Commonwealth of Australia.

Kisker, C., W. Hinrichs, K. Tovar, W. Hillen, and W. Saenger. 1995. The complex formed between *Tet* repressor and tetracycline-Mg2+ reveals mechanism of antibiotic resistance. J. Mol. Biol. 247:260-280.

Klare, I., H. Heier, H. Claus, R. Reissbrodt, and W. Witte. 1995. *vanA*-mediated high-level glycopeptide resistance in Enterococcus faecium from animal husbandry. FEMS Microbiol. Lett. 125:165-171.

Kleckner, N., R. K. Chan, B. K. Tye, and D. Botstein. 1975. Mutagenesis by insertion of a drug-resistance element carrying an inverted repetition. J. Mol. Biol. 97:561-575.

Langlois, B. E., G. L. Cromwell, T. S. Stahly, K. A. Dawson, and V. W. Hays. 1983. Antibiotic resistance of fecal coliforms after long-term withdrawal of therapeutic and subtherapeutic antibiotic use in a swine herd. Appl. Environ. Microbiol. 46:1433-1434.

Lautenbach, E., J. B. Patel, W. B. Bilker, P. H. Edelstein, and N. O. Fishman. 2001. Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*: risk factors for infection and impact of resistance on outcomes. Clin. Infect. Dis. 32:1162-1171.

Lawley, T.D., V. Burland, and D. E. Taylor. 2000. Analysis of the complete nucleotide sequence of the tetracycline-resistance transposon Tn10. Plasmid. 43:235-239.

Levy, S. B. 1994. The Antibiotic Paradox: How Miracle Drugs Are Destroying the Miracle. Plenum Press, New York.

Levy, S. B. 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. 36:695-703.

Levy, S. B. 2002. The 2000 Garrod lecture. Factors impacting on the problem of antibiotic resistance. J. Antimicrob. Chemother. 49:25-30.

Levy, S. B., G. B. FitzGerald, and A. B. Macone. 1976. Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. N. Engl. J. Med. 295:583-588.

Lim, D., and N. C. Strynadka. 2002. Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*. Nat. Struct. Biol. 9:870-876.

Liu, P. Y., D. Gur, L. M. Hall, and D. M. Livermore. 1992. Survey of the prevalence of beta-lactamases amongst 1000 Gram-negative bacilli isolated consecutively at the Royal London Hospital. J. Antimicrob. Chemother. 30:429-447.

Livermore, D. M. 1987. Clinical significance of beta-lactamase induction and stable derepression in Gram-negative rods. Eur. J. Clin. Microbiol. 6:439-445.

Livermore, D. M. 1995. β-lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. 8:557-584.

Luetzow, M. 1997. Approval procedures for anti-microbial growth promoters: aspects of risk assessment and risk management. *In* The Medical Impact of the Use of antimicrobials in Food Animals. World Health Organization, Geneva.

Mascaretti, O.A. 2003a. Chapter 17. Inhibitors of the 30S ribosomal subunit: aminoglycosides and tetracyclines p. 239-240. *In* Bacteria versus antibacterial agents: An integrated approach. American Society for Micobiology, Washington, D. C.

Mascaretti, O.A. 2003b. Chapter 17. Inhibitors of the 30S ribosomal subunit: aminoglycosides and tetracyclines p. 242-243. *In* Bacteria versus antibacterial agents: An integrated approach. American Society for Micobiology, Washington, D. C.

Mascaretti, O. A. 2003c. Chapter 6. Antibiotics and synthetic antibacterial agents. p. 97. *In* Bacteria versus antibacterial agents: An integrated approach. American Society for Micobiology, Washington, D. C.

Mateu, E., and M. Martin. 2001. Why is anti-microbial resistance a veterinary problem as well? J. Vet. Med. B. Infect. Dis. Vet. Public Health. 48:569-581.

Mathews, K. H. 2001. Antimicrobial Drug Use and Veterinary Costs in U.S. Livestock Production. Agriculture Information Bulletin 766. Available online at www.ers.usda.gov. Accessed on 8, February 2005.

Matthew, M. 1979. Plasmid mediated b-lactamases of Gram-negative bacteria: distribution and properties. J. Antimicrob. Chemother. 5:349–358.

Mayrhofer, S., P. Paulsen, F. J. Smulders, and F. Hilbert. 2004. Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. Int. J. Food Microbiol. 97:23-29.

McAllister, T. A., L. J. Yanke., G. D. Inglis., and M. E. Olson. 2001. Is antibiotic use in dairy cattle causing antibiotic resistance. Adv. dairy technol. 13: 229-247.

McDermott, P.F., R. D. Walker, and D. G. White. 2003. Antimicrobials: modes of action and mechanisms of resistance. Int. J. Toxicol. 22:135-143.

McDonnell, A. D., and A. D. Russell. 1999. Antiseptics and disinfectants: activity, action and resistance. Clin. Microbiol. Rev. 12:147–179.

McMurry, L. M., P. F. McDermott, and S. B. Levy. 1999. Genetic evidence that *InhA* of *Mycobacterium smegmatis* is a target for triclosan. Antimicrob. Agents Chemother. 43: 711–713.

McMurry, L., R. E. Jr. Petrucci, and S. B. Levy. 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. 77:3974-3977.

Medeiros, A. A. 1997. Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. Clin. Infect. Dis. 24:S19-S45.

Mitchell, J. M., M. W. Griffiths, S. A. McEwen, W. B. McNab, and A. J. Yee. 1998. Antimicrobial drug residues in milk and meat: causes, concerns, prevalence, regulations, tests, and test performance. J. Food Prot. 61:742-756.

Nakaya, R., A. Nakamura, and Y. Murata. 1960. Resistance transfer agents in *Shigella*. Biochem. Biophys. Res. Commun. 3:654-659.

National Antimicrobial Resistance Monitoring System-Animal Arm. 2004. 1997-2003 NARMS report. U. S. Department of health and human services, CDC, Atlanta, Georgia.

Nikaido, H. 1998. Multiple antibiotic resistance and efflux. Curr. Opin. Microbiol. 1:516-523.

Nikaido, H. 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. 178:5853-5859.

Normark, S., T. Grundstrom, and S. Bergstrom. 1980. Susceptibility to penicillins and cephalosporins in beta-lactamase producing strains of *E. coli* and relative amount of beta-lactamase produced from these strains. Scand. J. Infect. Dis. Suppl. 25:23-29.

O'Brien, T. F. 1997. The global epidemic nature of antimicrobial resistance and the need to monitor and manage it locally. Clin. Infect. Dis. 24:S2-S8.

O'Callaghan, C. H. 1979. Description and classification of the newer cephalosporins and their relationships with the established compounds. J. Antimicrob. Chemother. 5:635-671.

Oethinger, M., W. V. Kern, A. S. Jellen-Ritter, L. M. McMurry, and S. B. Levy. 2000. Ineffectiveness of topoisomerase mutations in mediating clinically significant fluroquinolone resistance in *Escherichia coli* in the absence of the *AcrAB* efflux pump. Antimicrob. Agents Chemother. 44:10-13.

Okamoto, K., N. Gotoh, and T. Nishino. 2001. Pseudomonas aeruginosa reveals high intrinsic resistance to penem antibiotics: penem resistance mechanisms and their interplay. Antimicrob. Agents. Chemother. 45:1964-1971.

Oppegaard, H., T. M. Steinum, and Y. Wasteson. 2001. Horizontal transfer of a multi-drug resistance plasmid between coliform bacteria of human and bovine origin in a farm environment. Appl. Environ. Microbiol. 67:3732-3734.

Orth, P., D. Schnappinger, W. Hillen, W. Saenger, and W. Hinrichs. 2000. Structural basis of gene regulation by the tetracycline inducible *Tet* repressor-operator system. Nat. Struct. Biol. 7:215-219.

Palumbi, S. R. 2001. Second Joint FAO/OIE/WHO workshop. 2004. Expert workshop on non-human antimicrobial usage and antimicrobial resistance: management options. Oslo, Norway. Humans as the World's Greatest Evolutionary Force. Science. 293:1786-1790.

Payot, S., S. Dridi, M. Laroche, M. Federighi, and C. Magras. 2004. Prevalence and antimicrobial resistance of Campylobacter coli isolated from fattening pigs in France. Vet. Microbiol. 101:91-99.

Quigley, J. D. 3rd., J. J. Drewry, L. M. Murray, and S. J. Ivey. 1997. Body weight gain, feed efficiency, and fecal scores of dairy calves in response to galactosyl-lactose or antibiotics in milk replacers. J. Dairy. Sci. 80:1751-1754.

Rahal, K., G. B. Gerbaud, and Y. A. Chabbert. 1973. Characterization d'un facteur de re´sistance transferable chez *Vibrio cholerae* biotype *el tor*. Ann. Inst. Pasteur. 124B:245–247.

Rammelkamp, C. H., and T. Maxon. 1942. Resistance of *Staphylococcus aureus* to the action of penicillin, Proc. Soc. Exp. Biol. Med. 51:386-389.

Roberts, M. C. 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. 19:1-24.

Roberts, M. C. 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. 19:1-24.

Roberts, M. C. 1989. Plasmid-mediated *Tet M* in Haemophilus ducreyi. Antimicrob. Agents Chemother. 33:1611-1613.

Russell, A. D. 2003. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. Lancet Infect. Dis. 3:794-803.

Russell, A. D., and I. Chopra. 1996. Understanding antibacterial action and resistance, 2nd ed. Chichester, Ellis Horwood, UK.

Salmon, S. A., J. L. Watts, C. A. Case, L. J. Hoffman, H. C. Wegener, and R. J. Jr. Yancey. 1995. Comparison of MICs of ceftiofur and other antimicrobial agents against bacterial pathogens of swine from the United States, Canada, and Denmark. J. Clin. Microbiol. 33:2435-2444.

Salyers, A. A., and N. B. Shoemaker. 1996. Resistance gene transfer in anaerobes: new insights, new problems. Clin. Infect. Dis. 23 Suppl. 1:S36-S43.

Sanders, C. C., and W. E. Sanders. 1992. Beta-Lactam resistance in Gram-negative bacteria: global trends and clinical impact. Clin. Infect. Dis. 15:824-839.

Schlunzen, F., R. Zarivach, J. Harms, A. Bashan, A. Tocilj, R. Albrecht, A. Yonath, and F. Franceschi. 2001. Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. Nature. 413:814 -821.

Schnappinger, D., and W. Hillen. 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. Arch. Microbiol. 165:359-369.

Senda, K., Y. Arakawa, K. Nakashima, H. Ito, S. Ichiyama, K. Shimokata, N. Kato, and M. Ohta. 1996. Multifocal outbreaks of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum beta-lactams, including carbapenems. Antimicrob. Agents Chemother. 40:349-353.

Shoemaker, N. B., R. D. Barber, and A. A. Salyers. 1989. Cloning and characterization of a Bacteroides conjugal tetracycline-erythromycin resistance element by using a shuttle cosmid vector. J. Bacteriol. 17:1294-1302.

Spratt, B. G. 1994. Resistance to antibiotics mediated by target alterations. Science. 264:388-393.

Stokstad, E. L. R., and T. H. Jukes. 1950. Further observations on the animal protein factor. Proceedings of the Society of Experimental Biology and Medicine. 73:523-528.

Sugino, Y., and Y. Hirota. 1962. Conjugal fertility associated with resistance factor R in *Escherichia coli*. J. Bacteriol. 84:902-910.

Swann, M., K. Blaxter., J. Murdoch, J. Parsons, and E. White. 1969. p. 1-83 *In* Report of the joint committee on the use of antibiotics in animal husbandry and veterinary medicine. Her Majesty's Stationary Office. London.

Sykes, R. B., and Matthew. 1979. Detection, assay and immunology of β-lactamases, p. 17-49. *In* J. M. T. Hamilton-Miller and J. T. Smith (ed.), Beta-lactamases, Academic Press, Ltd., London.

Tenover, F. C., and J. E. Jr. McGowan. 1996. Reasons for the emergence of antibiotic resistance. Am. J. Med. Sci. 31:9-16.

Thanassi, D. G., G. S. Suh, and H. Nikaido. 1995. Role of outer membrane barrier in efflux-mediated tetracycline resistance of Escherichia coli. J. Bacteriol. 177:998-1007.

U.S. International Trade Commission. 1987. Synthetic Organic Chemicals: United States Production and Sales, 1950-1986. U.S. Government Printing Office, Washington, D. C.
USDA/APHIS/Veterinary Services. 1995. Feedlot Health Management Report: Part II: Cattle on Feed Evaluation. N172.0195. Centers for Epidemiology and Animal Health, Fort Collins.

Waley, S. G. 1992. β-Lactamase:mechanism of action, p.198-228. *In* M. I. Page (ed.), The Chemistry of β-lactams. A. and P. Blackie, London.

Walsh, C. 2000. Molecular mechanisms that confer antibacterial drug resistance. Nature. 406:775-781.

Walsh, C. 2003. Antibiotics that act on cell wall synthesis. p. 36-45. *In* C. Walsh (ed.), Antibiotics: actions, origins, resistance, ASM press, Washington, D. C.

Watts, J. L., R. J. Jr. Yancey, S. A. Salmon, and C. A. Case. 1994. A 4-year survey of antimicrobial susceptibility trends for isolates from cattle with bovine respiratory disease in North America. J. Clin. Microbiol. 32:725-731.

Wegener, H. C. 2003. Antibiotics in animal feed and their role in resistance development. Curr. Opin. Microbiol. 6:439-445.

Weidel, W., and H. Pelzer. 1964. Bagshaped macromolecules- a new outlook on bacterial cell walls. Adv. Enzymol. Relat. Areas Mol. biol. 26:193-232.

White, D. G., S. Zhao, R. Sudler, S. Ayers, S. Friedman, S. Chen, P. F. McDermott, S. McDermott, D. D. Wagner, and J. Meng. 2001. The isolation of antibiotic-resistant salmonella from retail ground meats. N. Engl. J. Med. 345:1147-1154.

Winokur, P. L., D. L. Vonstein, L. J. Hoffman, E. K. Uhlenhopp, and G. V. Doern. 2001. Evidence for transfer of CMY-2 *AmpC* beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. 45:2716-2722.

Yamaguchi, A., N. Ono, T. Akasaka, T. Noumi, and T. Sawai. 1990. Metal-tetracycline/H+ antiporter of *Escherichia coli* encoded by a transposon,Tn10. The role of the conserved dipeptide, Ser65-Asp66, in tetracycline transport. J. Biol. Chem. 265:15525-15530.

Yaron, S., D. G. White, and K. R. Matthews. 2003. Characterization of an *Escherichia coli* O157:H7 *marR* mutant. Int. J. Food Microbiol. 85:281-291.

Yates, C. M., M. C. Pearce, M. E. Woolhouse, and S. G. Amyes. 2004. High frequency transfer and horizontal spread of apramycin resistance in calf faecal *Escherichia coli*. J. Antimicrob. Chemother. 54:534-537.

Zhao, S., D. G. White, B. Ge, S. Ayers, S. Friedman, L. English, D. Wagner, S. Gaines, and J. Meng. 2001. Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing Escherichia coli isolates. Appl. Environ. Microbiol. 67:1558-1564.

Chapter 2

ASSESSMENT OF ANTIBIOTIC USAGE IN DAIRY HERDS IN PENNSYLVANIA

2.1 ABSTRACT

A survey (July 2001- June 2002) was conducted on 113 dairy herds, from 13 counties in Pennsylvania, for use of antimicrobial agents. Fifty-six of 113 (50%) dairy farms surveyed maintained written or computerized antibiotic treatment records on the farm. Only 21% of dairy producers had written plans for treating sick animals. On most occasions (93%) antibiotics were administered by the owner/manager or designated herdsman, but only 32% of farms sought a veterinarian's advice before administering antibiotics. However, no more than 24% of the dairy producers followed the instructions for antibiotic usage and completed the course of antibiotic treatment. The majority of dairy producers used antibiotics in an extralabel manner, with guidelines from a veterinarian; separated and visibly marked treated cows; and milked treated cows last with a separate milking unit. About 3% of dairy producers surveyed had antibiotic residue violations in the past 6 months.

An in-depth analysis of the records of 33 of the 113 dairy farms was done to determine the incidence of disease conditions for which antibiotics were administered. Pneumonia and enteritis were recorded on 88 and 100% of the farms, respectively. Nearly 79, 100, and 100% of farms had lactating cattle with metritis, foot rot, and clinical mastitis, respectively.

Use of antibiotics was the largest in calves with enteritis (36%), followed by pneumonia in calves (25%) and foot rot in cattle (16%). The most widely observed health problems in dry cows on farms were clinical mastitis (27%) and pneumonia (24%). A small percent of dry cows were treated with antibiotics for clinical mastitis (8%) and pneumonia (10%).

Twenty four antibiotics including beta-lactams (penicillins and cephalosporins), spectinomycin, and florfenicol were widely used for therapeutic purposes, while oxytetracycline and neomycin were used in milk replacers for prophylaxis. Feeding medicated milk replacers to

calves for prevention of calf scours was widely practiced (70%). Beta-lactam antibiotics were mostly used for dry cow therapy, clinical mastitis, and on some farms for pneumonia and metritis. Ceftiofur, a third generation cephalosporin, was not only used for the prescribed conditions, but also as an extralabel drug on 18% of farms for treating mastitis in lactating cattle. Current practices related to antimicrobial usage on farms could contribute to the development of antibiotic resistant bacteria. The results of this study suggest antibiotics are extensively used on dairy herds for both therapeutic and prophylactic purposes. Beta-lactams and tetracyclines were the most widely used antibiotics. Farm management practices associated with antibiotic use varied considerably. Extralabel use of antibiotics was practiced on many farms. It is felt that a comprehensive study to determine the prevalence and distribution of antibiotic resistant bacteria on dairy herds would be valuable in understanding the relationship between antibiotic use and development of antibiotic resistance bacteria.

2.2 INTRODUCTION

Antibiotics are used in livestock production as therapeutics, growth promoters, and prophylactics. Therapeutic use of antibiotics is often required to manage clinically apparent diseases. The therapeutic regimen is dictated by label instructions from the manufacturer or in accordance with extralabel instructions from a veterinarian. Antibiotics are used as growth promoters in livestock via administration at low doses for extended periods of time. As prophylactics, antibiotics are used at low doses for a period of 2 weeks, to prevent disease. Although the duration of antimicrobial use differs for growth promotion and prophylaxis, the

dosage for both is typically less than 200 gm/ton of feed, and is considered subtherapeutic (IOM, 1989).

The earliest evidence of growth-promoting effects of antibiotics became apparent when chickens exposed to small doses of chlortetracycline grew more rapidly than non-exposed chickens (Stokstad and Jukes, 1950). At subtherapeutic levels, antibiotics are helpful in: (1) improving growth, (2) reducing risk of disease, (3) improving digestion, (4) improving weight gain, and (5) decreasing time and amount of feed needed to reach slaughter weight (Crawford and Teske, 1983; Droumev, 1983; Frost, 1991; Luetzow, 1997). Almost 90% of all antibiotics used on farm animals and poultry are administered in subtherapeutic concentrations. About 70% of all antibiotics used in subtherapeutic concentrations in animal feeds are given for the purpose of disease prevention (prophylaxis), while 30% are used for growth promotion (CAST, 1981; Hays, 1986; US. Int. Trade Com., 1987).

A study conducted by Zwald et al. (2004) on antibiotic usage on conventional dairy farms in Michigan, Minnesota, New York, and Wisconsin reported that use of newer antibiotics such as ceftiofur was a common practice on farms. They also commonly observed use of dry cow therapy on these farms and approximately half of farms fed medicated milk replacers to calves. Some of the farms also reported using antibiotics that were prohibited for use in dairy cattle.

A study conducted in Kenya's prolific milk producing districts showed that small dairy producers produced milk with beta-lactam residues exceeding the established maximum residue levels (Shitandi and Sternesjo, 2004). Use of antibiotics in Sweden and Norway for mastitis treatment has been influenced by policies and recommendations (Grave et al. 1999). In these countries the preference for beta-lactams, including procaine, benzyl penicillin and combinations with dihydrostreptomycin, was based on the withdrawal period. Dairy producers in Sweden use

long-acting drug treatment for subclinical mastitis and dry cow therapy, whereas the same formulations are not accepted in Norway. These examples reveal that antibiotic usage varies from country to country, within a country, and between farms, depending on policies and desired results.

The extensive use of antibiotics has caused bacteria to adapt defenses against antibiotics (Levy et al., 1987). This has resulted in the evolution, spread, and persistence of antimicrobial resistance in bacterial populations in animal agriculture. The emergence and spread of antibiotic resistance genes is enhanced through the intensive use of antimicrobials in over-crowded populations of production animals. This phenomenon is known as "selective pressure." Selective pressure is a general concept that refers to the many factors that create an environmental landscape which allows organisms with novel mutations or newly acquired characteristics to survive and proliferate (Baquero et al., 1998). The classic example of continuous selective pressure in the animal production industry is the use of antibiotics in feed at subtherapeutic concentrations (IOM, 1989).

Usage of antibiotics is known to leave residues in farm products (Levy et al, 1987; Tenover et al., 1996; Corpet, 1996). Several antibiotics enter the aquatic and terrestrial ecosystems through the discharge of effluents from farms (Bates et al., 1994). When applied to the land, farm wastes containing bioactive veterinary drug residues and antimicrobial resistant bacteria are susceptible to runoff into bodies of water and can potentially create reservoirs in the environment for antibiotic resistant bacteria (Chee-Sanford et al., 2001; Austin, 1985).

The present study investigates how antibiotics are used on dairy herds and identifies management practices that could contribute to development of antibiotic resistance.

2.3 MATERIAL AND METHODS

2.3.1 Dairy Herds

A total of 248 dairy farms from 36 counties in Pennsylvania were solicited to participate in the antibiotic usage survey. The 248 dairy farms had participated earlier in a study on foodborne pathogens in bulk tank milk (Jayarao et al., 2002). The dairy herds that participated in that study were referred by 19 county extension educators, and three milk cooperatives. The same 248 herds were contacted again and 173 of the dairy producers indicated their interest in participating in an antibiotic usage survey. Dairy producers were selected for the study who: 1) would allow, if available, reviewing of farm records related to antibiotic usage; 2) were currently members of National Dairy Herd Improvement Association, and 3) granted permission to contact their veterinarian to verify antibiotic use if needed. The 126 dairy producers that met the study criteria were requested to take part in the survey. A total of 113 dairy producers (n=13 counties) participated in the survey. The dairy herds were categorized based on their herd size of <100 lactating cows (n= 42 herds, 3-4 herds/county), between 100-199 lactating cows (n=45 herds, 3-5 herds/county) and > 200 lactating cows (n=26 herds, 2 herds/county). Thirty three of the 113 dairy herds maintained complete records on individual animal health and antibiotic usage. Records from these herds were analyzed to determine the type of antibiotics that were used and the purpose for which they were administered.

2.3.2 Survey Questionnaire

A survey instrument (questionnaire) on antibiotic usage was developed. The questionnaire was modified to improve its usefulness for collecting information on antibiotic usage. The questionnaire survey was administered to the dairy producer or manager of each

farm. It took 45-60 minutes to complete the survey (Appendix A; Tables **A-2** to **A-4**). The first part of the survey was administered to all 113 dairy producers. The second part of the survey was administered to the 33 dairy producers who had records on herd health and antibiotic use (Appendix A; Table **A-5** to **A-9**). The survey was conducted from July 2001 through June 2002.

2.3.3 Data Analysis

Answers to the questionnaire were transferred to Microsoft Excel and grouped by the type of response (e.g., "yes" or "no") obtained. The response for each question was subjected to one-way ANOVA to determine if there was any significant difference in their response. A *P* value of < 0.05 was considered as a significant difference in the type of response for a given question. All statistical analyses were performed using JMP software version 4.0 (SAS Institute, Cary, NC).

2.4 RESULTS

2.4.1 Farm management practices associated with antibiotic usage on 113 farms

One hundred and thirteen farm managers/producers were administered a survey to ascertain why, how, and when antibiotics were used on their dairy herds (Table **2-1**).

Fifty percent of the farms surveyed kept written or computerized records of antibiotic treatment, including medicated feed use that could be verified. Of these farms, only 33 producers maintained complete records of antibiotic usage. A significant difference was observed among the dairy producers who had written plans (21%) and those dairy producers who did not have written plans (79%) for treating sick animals. About 32% of dairy producers always sought a veterinarian's advice before administering antibiotics (Table **2-1**). Other than the veterinarian,

antibiotics were primarily administered by either the owner/manager or herdsman (93%). Only 24% of the dairy producers followed instructions on antibiotic usage and completed the course of treatment presented for a given condition. The majority of the dairy producers (79%) stated that extralabel medication was practiced on the farm following the orders or written guidelines from a veterinarian (Table **2-1**). Statistically significant percent of dairy producers physically separated treated cows (85%), marked them visibly (87%), and milked them last (84%) in separate milking units (86%). A large percent of dairy producers also visibly marked dry cows treated with antibiotics (73%; Table **2-1**). Ninety seven percent of farms had no antibiotic residue violation for 6 months prior to the survey. A significant percent of dairy producers (58%) routinely screened cows for antibiotics after freshening with an antibiotic residue detection test (Table **2-1**).

2.4.2 Antibiotics approved for use in dairy cattle

Antibiotics approved for use in dairy cattle are listed in Appendix A. The antibiotics approved for use in dairy cattle belong to 10 different classes. Beta-lactam antibiotics comprise the largest class of antibiotics used on these farms. Penicillin G and cloxacillin are approved for use for both mastitis in lactating cows and dry cow therapy. Penicillin G is also allowed for treatment of conditions like black leg, rhinitis, pneumonia, and metritis in dairy cattle (Appendix A). Penicillin G is also used in combination with other antibiotics for mastitis treatment. Combination with novobiocin can be used for both lactating and dry cows, whereas combination with dihydrostreptomycin is only permitted for use in dry cows. Amoxicillin can be used for local treatment of mastitis as well as systemic treatment of respiratory and foot rot conditions, whereas ampicillin can be used only for the treatment of respiratory conditions (Appendix A). Hetacillin, a type of ampicillin is available as an intramammary infusion for treating acute,

chronic, or sub-clinical bovine mastitis (Appendix A). The first generation cephalosporin cephapirin has been approved for prevention and treatment of mastitis (Appendix A). Ceftiofur is a broad spectrum injectable cephalosporin, developed solely for veterinary therapeutic use. First approved by the FDA in 1988 for treating respiratory diseases in cattle, is can also be used for treating metritis and foot rot.

Chlortetracycline and oxytetracycline can be used for treatment of non-milking dairy cattle and for prophylaxis in calves. Chlortetracycline is allowed for improving feed efficiency and weight gain in calves. It can also be used to treat enteritis and respiratory infections in non-lactating cattle. Oxytetracycline, in combination with neomycin, is used in medicated dairy calf milk replacers for prevention of bacterial diarrhea (Appendix A).

Erythromycin, a macrolide class of antibiotic, can be used for treating mastitis in lactating cows and dry cows. Florfenicol, a member of the thiophenicol group of antibiotics, is available as an injectable drug and has been labeled for use in cattle for treatment of bacterial pneumonia and foot rot conditions (Appendix A). Novobiocin is used for treating mastitis in lactating cattle as well as for dry cow therapy. Pirlimycin, a lincosamide antibiotic, is approved for treatment of mastitis in lactating cows only (Appendix A). Use of spectinomycin and sulfadimethoxine has been allowed for treatment of respiratory diseases in cattle. Since the withdrawal period for spectinomycin is not established in pre-ruminating and lactating dairy cattle, this medication cannot be used in dairy cattle 20 months of age or older. Currently sulfadimethoxine is the only sulfonamide that is labelled for treating lactating cattle with respiratory disease and foot rot. Extralabel use of sulfadimethoxine in lactating cattle is prohibited (Appendix A).

2.4.3 Documented disease or health conditions in dairy herds (n=33) that required use of antibiotics

The treatment in animals and prevalence of disease conditions in different age groups on 33 of 113 farms was determined (Table **2-2**). The percent of farms that recorded clinical cases of pneumonia was the highest for calves (88%), followed by lactating cattle (33%), dry cows (24%), and heifers (15%). The total number of animals treated for pneumonia was highest for calves (25%). Although the number of farms reporting cases of pneumonia was greater for lactating cattle than dry cows, the number of animals treated with antibiotics for pneumonia was higher in dry cows (10%) than lactating cattle (3%; Table **2-2**). Clinical cases of metritis that were treated with antibiotics were recorded on more farms (79%) in lactating cattle than in heifers (27%) and cows in early stages of their dry period (21%). Lactating cattle treated with antibiotics for metritis comprised 11% of the total lactating cattle population under this study.

Foot rot cases were detected in lactating cattle on all of the 33 farms, with 16% of the infected lactating cattle treated with antibiotics (Table **2-2**). The incidence of foot rot was also observed in animals of different age groups, but on comparatively fewer farms. Similarly, enteritis in calves was commonly observed on all the farms surveyed, as compared to other age groups. With 36% of the calf population treated with antibiotics for enteritis, it becomes the single most prevalent disease among the recorded diseases on the farms, as well as among various age groups. Another disease that was manifested largely in lactating cattle and reported on all farms was clinical mastitis. Fourteen percent of lactating cattle were treated with antibiotics for mastitis compared to 8% of cows in the early or late phase of their dry period and 5% of heifers (Table **2-2**).

2.4.4 Antibiotics used to treat health conditions in dairy herds (n=33)

Farm records were also studied to collect information on the type of antibiotics used. A total of 24 antibiotics were used for therapeutic and prophylactic purposes. The 24 antibiotics belonged to 13 antibiotic classes (Table **2-3**). The beta-lactam group was the largest single group with 7 antibiotics in this class used on study farms.

A variety of drugs were used for prevention and treatment of mastitis. For dry cow therapy, cephapirin was the drug of choice (52%) followed by novobiocin (27%) and penicillin G procaine (24%). Other antibiotics used were cloxacillin and penicillin G procaine and novobiocin combination. Cephapirin was also a preferred drug for treating mastitis in lactating cattle and was used on 49% of the farms (Table **2-3**). Penicillin G procaine (18%) and erythromycin (9%) were also used for treating mastitis in lactating cattle on farms. Antibiotics like amoxicillin, hetacillin, and pirlimycin were also used (Appendix A; Table **A-8** and Table **2-3**). The most significant observation was the extralabel use of ceftiofur (18%) for treating mastitis in lactating cattle. This antibiotic is not approved for treating mastitis.

On 30% of the farms, spectinomycin was the drug of choice for treating enteritis. This antibiotic has been approved for treatment of pneumonia in dairy cattle less than 20 months of age. For treatment of respiratory diseases ten different antibiotics were used. The most commonly used antibiotics for treatment of pneumonia were ampicillin (45%, only in calves), ceftiofur (48%), florfenicol (30%), and spectinomycin (42%; Table **2-3**). All of these antibiotics have been approved for systemic use. Sulfadimethoxine was also used on 27% of the farms to treat animals with pneumonia. Other antibiotics used were amoxicillin, tetracyclines, danofloxacin, and tilmicosin. Danofloxacin, a broad spectrum floroquinolone, is approved for use for treating respiratory diseases in chickens, cattle, and swine, but is not indicated for use in

dairy cattle. Tilmicosin has been approved for use in calves (< 20 months old) for treatment of respiratory diseases. Ceftiofur was a drug of choice for treatment of metritis on 5 of the 33 farms.

The combination of oxytetracycline and neomycin was widely used on farms (23 of 33; 70%) for prophylaxis in calves to prevent bacterial diarrhea (Appendix A; Table **A-8** and Table **2-3**). Some farms also used chlortetracycline, which has been approved for improving weight gain and feed efficiency. Extralabel prophylactic use of bacitracin-zinc and carbadox was also observed in some dairy herds.

A total of nine antibiotics were used to treat more than one condition/disease on the farms surveyed. Antibiotics that were widely used for treatment of different conditions on farms were ceftiofur (79%), spectinomycin (67%), sulfadimethoxine (49%), and ampicillin (46%).

2.5 DISCUSSION

The risk of transferring antibiotic resistant bacteria to humans via the food chain has been identified as a major public health threat and a priority issue by several expert committees, including the Institute of Medicine, the American Society for Microbiology, and the U.S. Office of Technology Assessment (ASM, 1994; US OTA, 1995; ASM, 1997; IOM, 1998).

The survey in this study included questions that were helpful in gaining insight into farm management practices associated with antibiotic usage. Fifty percent of dairy producers maintained records of antibiotic treatments conducted, including medicated feeds. Similar surveys conducted with dairy producers from Michigan, Minnesota, New York, and Wisconsin showed that 71.7%, 58.6%, and 36.4% of conventional dairy producers kept antibiotic treatment records for lactating, non-lactating cows, and calves/heifers, respectively (Zwald et al., 2004).

Kaneene and Ahl (1987) surveyed dairy producers in Michigan who indicated that insufficient record keeping and poor knowledge about drug withdrawal periods among producers are important factors leading to drug residues in milk.

A significant number of dairy producers failed to administer the entire course of antibiotic therapy. This lapse could lead to improper usage of antibiotics and potentially result in imprudent use of antibiotics on farms. Only 32% of farmers always sought veterinary advice before administering antibiotics. The tendency to rely on personal experience for antibiotic use, dosage and withdrawal period was also common in dairy producers surveyed by Zwald et al. (2004).

One of the important aspects of prudent and proper use of antibiotics is to take advice from the veterinarian before the use of any extralabel antibiotics. The majority of the dairy producers sought written guidelines from the veterinarian before using antibiotics extralabelly. The Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA) allows veterinarians to prescribe extralabel uses of certain approved animal drugs and approved human drugs for animals under certain conditions. The key constraints of AMDUCA are that any extralabel use must be by or on the order of a veterinarian and must not result in violative residues in food-producing animals (http://www.fda.gov/cvm/default.html, accessed September 2004). Taking a veterinarian's advice can avoid extralabel use of antibiotics that are prohibited in food animals. These drugs include enrofloxacin, chloramphenicol, and sulfonamides, other than sulfadimethoxine, along with a voluntary ban on the use of aminoglycosides.

A majority of the farms in our survey (87%) marked their animals treated with antibiotics. This practice has been shown to be effective in preventing drug residues in milk (Talley, 1999). Routine testing of treated cows with a residue detection kit is helpful in reducing

the risk of residue occurrence in milk (McEwen et al., 1991). The regular testing of milk and proper management of treated animals could be an important factor that allowed 97% of dairy producers in our study to produce milk free of antibiotic residues.

The findings of our study revealed that practices such as consulting the veterinarian before using extralabel antibiotics, separating and milking the treated animals last, and routine testing of freshened cows (one that's just given birth to a calf) for antibiotic residues are helpful in using the appropriate antibiotics and preventing antibiotic residues in milk. Absence of antibiotic treatment records, lack of written plans for treating sick animals, not consulting the veterinarian for treating sick animals, and failure to complete antimicrobial treatment course are factors that can lead to the selection of antibiotic resistant bacteria.

Detailed study of the records from 33 farms indicated that prevalence of disease and health conditions was different for each age group (Table **2-2**). Ortman and Svensson (2004) recorded disease and treatments of heifers calves (n=3081) from 112 Swedish dairy herds from birth to first calving. Of the infectious diseases recorded, 26.4% were treated with antimicrobial drugs. The most common diseases observed from birth to 210 days were respiratory diseases and diarrhea. Adult animals (> 420 days of age) were most frequently treated for foot rot and mastitis. The results of our study are similar to these findings. The data indicates that on these farms typical use of antibiotics is relatively high in calves and lactating cattle.

The National Animal Health Monitoring System (NAHMS, 1997) conducted a nationwide survey on antibiotic use on farms that covered 79% of U.S. milk cows. The survey provided broad estimates on the use of antibiotics and the method of use. However, detailed information on the type of antibiotics used and the reasons why they are used was limited. The

present study emphasized identifying the reasons for antibiotic use and farm management practices associated with it.

The majority of the dairy producers surveyed indicated that mastitis was the most commonly treated condition on their farm. A similar observation was made by Mitchell et al. (1998) who reported that mastitis was the most common condition where antibiotics were used. Two of the cornerstone practices of prevention and control of mastitis are teat dipping and dry cow treatment (Pankey, 1989; Berry and Hillerton, 2002). Dry cow therapy (intramammary treatment with antibiotics after the last milking of the cow before the dry period) treating was practiced on most of the farms surveyed. Application of dry cow therapy at the end of lactation is known to significantly reduce the incidence of new intramammary infections both during the dry period and at calving (Oliver and Sordillo, 1988; Berry and Hillerton, 2002). Therefore, dry cow therapy is now considered an integral part of the total management system recommended for controlling intramammary infections.

Foot rot was the second most common condition treated in lactating cattle on the farms surveyed. Common bacterial agents that cause foot rot include *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* (Berg and Loan, 1975). Foot rot is also considered as the most commonly occurring pathological condition in lactating dairy animals (Landais et al., 1989). Foot rot has been previously cited as a frequent health problem by dairy producers surveyed in Canada (Spicer et al., 1994).

Metritis is known to reduce the reproductive efficiency of cows. Cows with dystocia (difficult parturition), retained placenta, twins or still-births, and various metabolic disorders are more likely to develop metritis than are other cows (Lewis, 1997). Analysis of farm records revealed that nearly 79% of the farms used antibiotics to treat metritis. *Arcanobacterium*

pyogenes, either alone or in combination with anaerobes like *Fusobacterium necrophorum* and *Bacteroides* spp. are the most common bacteria associated with metritis (Farin et al., 1989; Griffin et al., 1974; Ruder et al., 1981). Use of systemic or intra-uterine antimicrobial therapy is typical for treating metritis (Smith et al., 1998). Metritis was also observed in heifers on some farms. This could be due to complications associated with first time pregnancy.

In our study, a significant number of dairy producers reported that they used antibiotics to treat calves with enteritis and pneumonia. Calf loss from diarrhea is an important segment of total loss in the United States cattle industry. Specifically, it was reported in the late 1970s (Hunt, 1985) that enteric pathogens kill up to 25% of calves each year, resulting in more than $250 million in losses. The two enteropathogens most commonly encountered in the investigation of field outbreaks of calf diarrhea are enterotoxigenic *Escherichia coli* (ETEC) and rotavirus (Acres and Radostits, 1976; Acres et al., 1977). When colibacillosis or salmonellosis is confirmed or is the suspected cause of diarrhea in calves, use of appropriate antimicrobial therapy plays an important role in restoring and maintaining health of calves (Roussel Jr. and Brumbaugh, 1991).

Calf pneumonia is a disease of calves from 2 to 6 months of age. The morbidity associated with dairy calf pneumonia can vary greatly as this disease can occur both endemically and as outbreaks of respiratory disease (Ames, 1997). Waltner-Toews et al. (1986) reported 15% of Ontario Holstein dairy calves were treated for pneumonia before weaning, based on diagnosis by the dairy producers. Virtala et al. (1996) found the crude risk for pneumonia was 11% when diagnosed by caretaker and 25.6% when diagnosed by a veterinarian. The incidence rates reported in the literature clearly indicate that treatment of calf pneumonia is essential to prevent heavy economic losses. *Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni*

are commonly associated with calf pneumonia (Ames, 1997). Antibiotics including ampicillin, ceftiofur, florfenicol, and spectinomycin are approved for treatment of pneumonia.

Different antimicrobial classes are approved for use in food animals and they include beta-lactams (e.g., penicillin, ampicillin, and cephalosporin), tetracyclines (e.g., oxytetracycline, tetracycline, and chlortetracycline), aminoglycosides (e.g., streptomycin, neomycin, and gentamicin), macrolides (e.g., erythromycin), lincosamides (e.g. lincomycin and pirlimycin), and sulfonamides (e.g., sulfamethazine and others) (Mitchell et al., 1998; Hoeben et al., 1998). A variety of antibiotics were used on the farms that kept complete records (Table **2-3**). For treatment of mastitis, various commercially available preparations were used. The majority of the antibiotics belonged to the beta-lactam class of antibiotics. Cephapirin has been proven effective in treating *S. aureus* udder infections (Owens et al., 1991), and was a drug of choice for treating mastitis in lactating cattle as well as for dry cow therapy on the farms. Dry cow therapy with cloxacillin has proven effective against mastitis pathogens such as *S. agalactiae*, *S. uberis*, *S. dysgalactiae,* and *S. aureus*, as well as lowering the number of new infections during the dry period (Sol and Melenhorst, 1990). Novobiocin alone, and in combination with penicillin G, was used for dry cow therapy. This combination is commonly used to treat cows at drying off to protect against new intramammary infections (Sanchez and Watts, 1999).

Amoxicillin and penicillin G are effective antibiotics against mastitis pathogens (Jousimies-Somer et al., 1996). Pirlimycin therapy has proven to be effective in eliminating intramammary infections caused by environmental streptococci and *S. aureus* in lactating dairy cattle (Gillespie et al., 2002). Interestingly, extralabel use of ceftiofur was observed in 6 of 33 farms (18%) for treatment of mastitis. Ceftiofur is approved for use in lactating cattle for the treatment of pneumonia, metritis and foot rot. Occasional use of ceftiofur in an extralabel manner

for intramammary treatment of mastitis was not uncommon (Smith et al., 2004). Studying the efficacy of ceftiofur use to treat mastitis is currently an important area of research (Oliver et al., 2004; Smith et al., 2004). Ceftiofur is the only third generation cephalosporin that is being studied for both intramammary and parentral mastitis therapy. The advantage of using this drug is that it does not have a withholding period for milk or meat, following the label dose of up to 2.2 mg/kg daily for 5 days (Erskine et al. 2002).

Several antibiotic formulations are available and have been approved for treatment of mastitis. This indicates the importance of this disease within the dairy industry. The majority of antibiotics approved for mastitis therapy are beta-lactams for both lactation and dry cow therapy. Use of ceftiofur as an extralabel antibiotic was the most relevant observation. The data from this survey shows extensive use of beta-lactam antibiotics on dairy farms and dairy cattle.

Different antibiotic classes currently labeled in the United States for the treatment of enteritis in calves include amoxicillin, chlortetracycline, neomycin, oxytetracycline, streptomycin, sulfachloropyridazine, sulfamethazine, and tetracycline (Constable, 2004). Interestingly, among the above approved antibiotics, only chlortetracycline, tetracycline, and neomycin were used on some farms. The drug of choice for treatment of enteritis among dairy producers was spectinomycin. Spectinomycin is approved for treating enteritis in swine (McOrist et al., 2000) and was used as extralabel in dairy animals.

A total of 10 antibiotics were used for treatment of pneumonia. The antibiotics that were preferred on most farms were ampicillin, ceftiofur, florfenicol, and spectinomycin. Ceftiofur was the drug of choice on most farms. Since ceftiofur is a relatively new drug, most of the available literature deals with studies regarding the efficacy of ceftiofur for the treatment of bovine respiratory disease (Yancey et al., 1987; Hibbard et al., 2002; Hornish and Kotarski, 2002). Very

few reports on the amount of actual ceftiofur usage are available. Zwald et al. (2004) reported that 80% of conventional dairy farms located in Michigan, Minnesota, New York, and Wisconsin used ceftiofur for treating respiratory diseases. Ampicillin (22%) and florfenicol (7%) were also used on these farms for the same diseases. Antibiotics like spectinomycin, sulfadimethoxine, and tetracyclines are commonly used for the treatment of pneumonia in cattle (Burrows and Ewing, 1989; Prescott and Baggot, 1988). Tilmicosin was used on one farm and is a relatively new macrolide drug approved for treatment of bovine respiratory disease in the United States since 1991.

The most commonly used drug for foot rot therapy was sulfadimethoxine. Sulphonamides are not directly effective against most obligate anaerobes, but may affect aerobic organisms that create the microenvironment in which *Fusobacterium* thrive during a foot rot condition according to the United States Pharmacopiea (website http://www.usp.org, accessed March 2005). Ceftiofur is also used for treatment of acute foot rot conditions and has been evaluated and recommended for such foot rot treatment (Kausche and Robb, 2003; Morck et al., 1998). Ceftiofur has been proven to be effective for the treatment of acute post-partum metritis in dairy cows (Chenault et al., 2004) and was the drug of choice for metritis treatment on some farms.

Medicated milk replacers containing oxytetracycline and neomycin were used on the majority of farms surveyed. Use of medicated milk replacers has been reported to reduce the severity of diarrhea and the number of days of diarrhea in calves (Quigley et al., 1997). Chlortetracycline and oxytetracycline in combination with neomycin, decoquinate, and lasalocid are the only approved antimicrobials agents that can be used in medicated milk replacers (USDA/APHIS/VS/CEAH, 1998). According to the National Dairy Herd Evaluation Project around 60% of US dairy producers use milk replacers for feeding neonatal calves. Medicated

milk replacers were used by 59.6% of dairy producers for calves from birth to 3 weeks of age, while the use increased to 70.7% for older calves from 3 weeks to weaning age (Heinrichs et al., 1995). The extensive use of medicated milk replacers in calves was also observed on the farms we surveyed. The majority of the dairy producers (70%) indicated that they fed medicated milk replacers, with tetracycline and neomycin, to calves as prophylaxis against enteritis.

Another antibiotic that was used for prophylaxis in dairy animals was carbadox, which is actually indicated for use in swine feed for growth promotion, improved feed efficiency, increased weight gain, as well as to control swine dysentery, and bacterial swine enteritis (FDA-CVM, 2004). Similarly bacitracin-zinc, a coccidiostat indicated for use in poultry (CFR, 2004), was used in cattle. It was also observed that 20 of 23 (86.9%) farms used the recommended route of administration and only 7 of 20 (35%) farms completed the treatment for mastitis, pneumonia, and metritis.

This use of antibiotics in animal agriculture, including extralabel use and especially the use of antibiotics for growth promotion, has been controversial because of the potential evolution of antibiotic resistance bacteria and their subsequent transfer from animals to humans. Such transfer could have severe public health implications as it might lead to treatment failures (Kelly et al., 2004). Selective pressure exerted by the use of antibiotics as growth promoters in food animals may develop reservoirs of transferable antibiotic resistance in various ecosystems (Witte, 2000). Many antibiotics used in animal agriculture are poorly absorbed in the animal gut. It is estimated that 25% to as much as 75% of the antibiotics administered to feedlot animals could be excreted unaltered in feces (Elmund et al., 1971; Feinman and Matheson, 1978) and can persist in soil after land application (Donoho, 1984, Gavalchin and Katz, 1994). Multiple classes of antimicrobial compounds were detected in swine waste storage lagoons and surface and

groundwater proximal to swine and poultry farms, indicating that animal manure and waste serve as sources of antimicrobial residues in the environment (Campagnolo et al., 2002).

The findings of this study demonstrate that different types of antibiotics were used on farms, including extensive extralabel use of antibiotics. Mastitis was the single most important disease on dairy farms and antibiotics were used extensively to treat it. A major emphasis was given to use of antibiotics in dry cow therapy to prevent mastitis in the freshening cows. Different classes of antibiotics were used for treatment of pneumonia. Treatment of mastitis and foot rot was common in lactating cattle, whereas enteritis and respiratory disease were the primary ones treated in calves. Beta-lactam was the largest and most widely used class of antibiotics. Ceftiofur was a drug of choice on many farms and its usage also included extralabel use. Another class of antibiotics extensively used was oxytetracycline and neomycin, for prophylaxis in calves. It is felt that management practices on these farms do not address proper antibiotic usage. It is felt that a comprehensive study to determine the prevalence and distribution of antibiotic resistant bacteria on dairy herds would be valuable to understand the relationship between antibiotic use and development of antibiotic resistant in bacteria.

Table **2-1**: Response to questionnaire survey on use of antibiotics on 113 dairy herds

	Herd Characteristics	**Herd size**			
		< 100	**100-199**	**>200**	**Total**
	No. of dairy herds surveyed	42	45	26	113
	No. of cows in milk (average)	64	137	228	75
	Avg. milk prod./cow/milking	34 lbs.	35 lbs.	36 lbs.	33 lbs
	Survey question	**% Response**			**Mean**
1	Does the farm maintain written records for antibiotic treatments including medicated feeds				
	Yes	52.4	42.2	57.7	50
	No	47.6	57.8	42.3	50
2	Does the farm have written plans for treating sick animals with antibiotics?				
	Yes	31	13	19	21*
	No	69	87	81	79
3	Is the veterinarian's advice sought before administering antibiotics?				
	Always	21	46	57	32
	Most of the times	57	50	32	38
	Sometimes	22	4	11	9
4	Other then the veterinarian, who is allowed to administer antibiotics to animals?				
	Owner/Manager/Herdsman	83	94	96	93*
	Family	8	3	0	4
	Milker/Farm worker	9	3	4	3
5	Following administration of an antibiotic, is the course of treatment completed?				
	Always	14	36	20	24*
	Sometimes	80	62	71	71
	Never	6	2	9	5
6	Is extralabel usage of medication done only based on the orders or written guidelines from a veterinarian?				
	Yes	71.4	86.7	76.9	79*
	No	28.6	13.3	23.1	21

Table **2-1**: Continued

	Survey question	**Herd Size**			
		<100	**100-199**	**>200**	**Total**
		% Response			**Mean**
7	Are treated cows always visibly marked as "treated"?				
	Yes	92.9	80	88.5	87*
	No	7.1	20	11.5	13
8	Are treated cows physically separated from other milking cows?				
	Yes	83.3	88.9	80.8	85*
	No	16.7	11.1	19.2	15
9	Are treated cows milked last?				
	Yes	85.7	82.2	84.6	84*
	No	14.3	17.8	15.4	16
10	Are treated cows milked with a separate milking unit?				
	Yes	95.2	73.3	92.3	86*
	No	4.8	26.7	7.7	14
11	Are dry cows treated with antibiotics visibly marked as being dry cow treated?				
	Yes	71.4	73.3	76.9	73*
	No	28.6	26.7	23.1	27
12	Were there any antibiotic residues violations in past 6 months?				
	Yes	2.4	2.2	3.8	3*
	No	97.6	97.8	96.2	97
13	Are cows routinely screened after freshening for antibiotics with an antibiotic residue detection test?				
	Yes	52.4	57.8	65.4	58*
	No	47.6	42.2	34.6	42
* Difference between answers with each question is significant ($p \leq 0.05$)					

Table **2-2**: Prevalence of clinical diseases and health conditions in animals of different age groups and clinical cases treated with antibiotics in each age group on 33 dairy farms

Dairy cattle	**Total number of animals on 33 farms**	**Farms with recorded clinical cases N (%)**	**Range of clinical cases/farm/yr**	**Animals treated with antibiotics N (%)**
	Pneumonia			
Calves	390	29 (88)	0-14	99 (25)
Heifers	667	5 (15)	0-2	7 (1)
Lactating Cattle	2783	11 (33)	0-11	71 (3)
Dry cow	361	8 (24)	0-7	37 (10)
	Metritis			
Heifers	667	9 (27)	0-6	26 (4)
Lactating Cattle	2783	26 (79)	0-14	294 (11)
Dry cows (early)	361	7 (21)	0-9	27 (7)
	Foot rot			
Calves	390	3 (9)	0-3	6 (2)
Heifers	667	4 (12)	0-4	10 (1)
Lactating Cattle	2783	33 (100)	2-21	459 (16)
Dry cows	361	7 (21)	0-5	17 (5)
	Enteritis			
Calves	390	33 (100)	3-30	141 (36)
Heifers	667	6 (18)	0-7	19 (3)
Lactating Cattle	2783	9 (27)	0-15	43 (2)
Dry cows	361	4 (12)	0-4	11 (3)
	Clinical mastitis			
Heifers	667	10 (30)	0-6	34 (5)
Lactating Cattle	2783	33 (100)	3-30	389 (14)
Dry cows (early and late)	361	9 (27)	0-7	30 (8)

Table **2-3**: Antibiotics used for different documented disease or health conditions on 33 dairy herds farms

Antimicrobial agent	**Therapeutic use**									**Prophylactic use**	
	Dry Cow Treatment	Mastitis	Enteritis	Pneumonia	Foot rot	Metritis	Other	Total farms*	%	Total farms	%
Cephapirin benzathine	17							17	52		
Cloxacillin benzathine	5							5	15		
Novobiocin	9							9	27		
Novobiocin & Penicillin G Procaine	4							4	12		
Penicillin G Procaine	8	6					1[e]	15*	46		
Pirlimycin		4						4	12		
Amoxicillin		4		5	1		1[c]	9*	27		
Cefapirin sodium		16						16	49		
Ceftiofur		6[a]		16	3	5	1[a]	26*	79		
Erythromycin		3					1[c]	3*	9.1		
Hetacillin		1						1	3		
Ampicillin				15[b]			2[d]	15*	46		
Bacitracin-zinc										1[a]	3
Bambermycin										1	3
Carbadox										1[a]	3
Oxytet-neomycin										23	70
Chlortetracycline			1	2				3*	9.1	1	3
Danofloxacin				1				1	3		
Florfenicol				10				10	30		
Lincomycin			1[a]					1	3		
Neomycin			2					2	6.1		
Spectinomycin			10[a]	14				22*	67		
Sulfadimethoxine				8	9			16*	49		
Tetracycline			1	2				3*	9.1		
Tilmicosin				1				1	3		

a: Extralabel use; b: Used in calves; c: Pink eye; d: Navel ill; e: Wounds

* Farms used same antibiotic to treat different clinical conditions.

2.6 REFERENCES

Acres, S. D., and O. M. Radostits. 1976. The efficacy of a modified live reo-like virus vaccine and an *E. coli* bacterin for prevention of acute undifferentiated neonatal diarrhea of beef calves. Can. Vet. J. 17:197-212.

Acres, S. D., J. R. Saunders, and O. M. Radostits. 1977. Acute undifferentiated neonatal diarrhea of beef calves: the prevalence of enterotoxigenic *E. coli*, reo-like (rota) virus and other enteropathogens in cow-calf herds. Can. Vet. J. 18:113-121.

American Society of Microbiology. 1994. Report of the ASM Task Force on Antibiotic Resistance. Washington, D.C.

American Society of Microbiology. 1997. New and Reemerging Infectious Diseases: A Global Crisis and Immediate Threat to the Nation's Health, The Role of Research. Washington, D.C.

Ames, T. R. 1997. Dairy calf pneumonia. The disease and its impact. Vet. Clin. North. Am. Food Anim. Pract. 13:379-391.

Austin, B. 1985. Antibiotic pollution from fish farms: effects on aquatic microflora. Microbiol. Sci. 2:113-117.

Baquero, F., M. C. Negri, M. I. Morosini, and J. Blazquez. 1998. Antibiotic-selective environments. Clin. Infect. Dis. 27 Suppl 1:S5-S11.

Bates, J., J. Z. Jordens, and D. T. Griffiths. 1994. Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. J. Antimicrob. Chemother. 34:507-514.

Berg, J.N., and R. W. Loan. 1975. Fusobacterium necrophorum and Bacteroides melaninogenicus as etiologic agents of foot rot in cattle. Am. J. Vet. Res. 36:1115-1122.

Berry, E. A., J. E. Hillerton. 2002. The effect of selective dry cow treatment on new intramammary infections. J. Dairy Sci. 85:112-121.

Burrows, G. E., and P. Ewing. 1989. In vitro assessment of the efficacy of erythromycin in combination with oxytetracycline or spectinomycin against Pasteurella haemolytica. J. Vet. Diagn. Invest. 1:299-304.

Campagnolo, E.R., K. R. Johnson, A. Karpati, C.S. Rubin, D. W. Kolpin, M. T. Meyer, J. E. Esteban, R. W. Currier, K. Smith, K. M. Thu, and M. McGeehin. 2002. Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. Sci. Total Environ. 299(1-3):89-95.

Chee-Sanford, J. C., R. I. Aminov, I. J. Krapac, N. Garrigues-Jeanjean, and R. I. Mackie. 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. Appl. Environ. Microbiol. 67:1494-1502.

Chenault, J. R., J. F. McAllister, S. T. Jr. Chester, K. J. Dame, F. M. Kausche, and E. J. Robb. 2004. Efficacy of ceftiofur hydrochloride sterile suspension administered parenterally for the treatment of acute postpartum metritis in dairy cows. J. Am. Vet. Med. Assoc. 224:1634-1639.

Code of Federal Regulations. 2004. Title 21-part 558-New animal drugs for use in animal feeds. Available online: http://www.gpoaccess.gov/cfr/index.html. Accessed 28th September 2004.

Constable, P. D. 2004. Antimicrobial use in the treatment of calf diarrhea. J. Vet. Intern. Med. 18:8-17.

Corpet, D. E. 1996. Microbiological hazards for humans of microbial growth promoter use in animal production. Rev. Med. Vet. 147:851-862.

Council for Agricultural Science and Technology. 1981. Antibiotics in Animal Feeds. Report No. 88. Council for Agricultural Sciences and Technology, Ames, Iowa.

Crawford, L. M., and R.H. Teske. 1983. Growth promotants approved in the U.S. Vet. Research Commun. 7:83-84.

Donoho, A. L. 1984. Biochemical studies on the fate of monensin in animals and in the environment. J. Anim. Sci. 58:1528-1539.

Droumev, D. 1983. Review of antimicrobial growth promoting agents available. Vet. Res. Commun. 7:85-89.

Elmund, G. K., S. M. Morrison, D. W. Grant, and M. P. Nevins. 1971. Role of excreted chlortetracycline in modifying the decomposition process in feedlot waste. Bull. Environ. Contam. Toxicol. 6:129-135.

Erskine, R. J., P. C. Bartlett, J. L. VanLente, and C. R. Phipps. 2002. Efficacy of systemic ceftiofur as a therapy for severe clinical mastitis in dairy cattle. J. Dairy Sci. 85:2571-2575.

Farin, P. W., L. Ball, J. D. Olson, R. G. Mortimer, R. L. Jones, W. S. Adney, and A. E. McChesney. 1989. Effect of *Actinomyces pyogenes* and Gram-negative anaerobic bacteria on the development of bovine pyometra. Theriogenology. 31:979.

Feinman, S. E., and J. C. Matheson. 1978. Draft environmental impact statement: subtherapeutic antibacterial agents in animal feeds. p. 372. *In* Food and Drug Administration Department of Health, Education and Welfare Report, Food and Drug Administration, Washington, D. C.

Food and Drug Administration-Center of Veterinary Medicine. 2004. FDA approved animal drug products online database system. Available online: http://dil.vetmed.vt.edu/NadaFIrst/NADA.cfm. Accessed on 28th September 2004.

Frost, A. J. 1991. Antibiotics and animal production, A6:181-194. *In* J. B. Woolcock (ed.), World Animal Science Microbiology of Animals and Animal Products. Elsevier, New York.

Gavalchin, J., and S. E. Katz. 1994. The persistence of fecal-borne antibiotics in soil. J. Assoc. Off. Anal. Chem. Int. 77:481-485.

Gillespie, B. E., H. Moorehead, P. Lunn, H. H. Dowlen, D. L. Johnson, K. C. Lamar, M. J. Lewis, S. J. Ivey, J. W. Hallberg, S. T. Chester, and S. P. Oliver. 2002. Efficacy of extended pirlimycin hydrochloride therapy for treatment of environmental Streptococcus spp. and Staphylococcus aureus intramammary infections in lactating dairy cows. Vet. Ther. 3:373-380.

Grave, K., C. Greko, L. Nilsson, K. Odensvik, T. Mork, and M. Ronning. 1999. The usage of veterinary antibacterial drugs for mastitis in cattle in Norway and Sweden during 1990-1997. Prev. Vet. Med. 42:45-55.

Griffin, J.F.T., P. J. Hartigan, and W. R. Nunn. 1974. Nonspecific uterine infection and bovine fertility. I. Infection patterns and endometritis during the first seven weeks postpartum. Theriogenology. 1:91.

Hays, V.W. 1986. Benefits and risks of antibiotics use in agriculture, p. 74-78. *In* W. A. Moats (ed), Agricultural uses of antibiotics, Series 320 American Chemicals Society Symposium. American Chemical Society, Washington D. C.

Heinrichs, A. J., S. J. Wells, and W. C. Losinger. 1995. A study of the use of milk replacers for dairy calves in the United States. J. Dairy Sci. 78:2831-2837.

Hibbard, B., E. J. Robb, S. T. Jr. Chester, K. J. Dame, W. W. Moseley, and W. L. Bryson. 2002. Dose determination and confirmation for ceftiofur crystalline-free acid administered in the posterior aspect of the ear for control and treatment of bovine respiratory disease. Vet. Ther. 3:22-30.

Hoeben, D., C. Burvenich, and R. Heyneman. 1998. Antibiotics commonly used to treat mastitis and respiratory burst of bovine polymorphonuclear leukocytes. J. Dairy Sci. 81:403–410.

Hornish, R. E., and S. F. Kotarski. 2002. Cephalosporins in veterinary medicine - ceftiofur use in food animals. Curr. Top. Med. Chem. 2:717-731.

Hunt, E. 1985. Symposium on Calf Diarrhea. Vet. Clin. North Am. Food Anim. Pract. 1: 443.

Institute of Medicine, Forum on Emerging Infections. 1998. Antimicrobial Resistance: Issues and Options. Washington, D.C.

Institute of Medicine. 1989. Human health risks with subtherapeutic use of penicillin or tetracyclines in animal feed. Committee on human health risk assessment on using subtherapeutic antibiotics in animal feeds. National Academy Press, Washington D. C.

Jayarao, B. M., N. V. Hedge, S. R. Pillai, D. R. Wolfgang, C. M. Burns, A. A. Sawant, and L. R. Hutchinson. 2002. Raw milk: Think before you drink. National Mastitis Council Regional Meeting Proceedings. The Pennsylvania State University, University Park, PA.

Jousimies-Somer, H., S. Pyorala, and A. Kanervo. 1996. Susceptibilities of bovine summer mastitis bacteria to antimicrobial agents. Antimicrob. Agents Chemother. 40:157-160.

Kaneene, J. B., and A. S. Ahl. 1987. Drug residues in dairy cattle industry: epidemiological evaluation of factors influencing their occurrence. J. Dairy Sci. 70: 2176-2180.

Kausche, F.M., and E. J. Robb. 2003. A comprehensive review of ceftiofur sodium and hydrochloride formulations for treatment of acute bovine foot rot. Vet. Ther. 4:83-93.

Kelly, L., D. L. Smith, E. L. Snary, J. A. Johnson, A. D. Harris, M. Wooldridge, and J. G. Jr. Morris. 2004. Animal growth promoters: to ban or not to ban?; A risk assessment approach. Int. J. Antimicrob Agents. 24:205-212.

Landais, E., J. B. Coulon, J. P. Garel, and A. Hoden. 1989. Characterization of the pathology of lactating cows based on the level of lactation. Principal factors in the variation and typing of pathologic profiles of lactation. Ann. Rech. Vet. 20:277-294.

Levy, S. B., J. P. Burke, and C. K. Wallace. 1987. Antibiotic use and antibiotic resistance worldwide. Rev. Infect. Dis. Suppl. 3:9.

Lewis, G. S. 1997. Uterine health and disorders. J. Dairy Sci. 80:984-994.

Luetzow, M. 1997. Approval procedures for anti-microbial growth promoters: aspects of risk assessment and risk management. *In* The medical impact of the use of antimicrobials in food animals. World Health Organization, Geneva, Switzerland.

McEwen, S. A., A. H. Meek, and W. D Black. 1991. A dairy farm survey of antibiotic treatment practices, residue control methods and associations with inhibitors in milk. J. Food Prot. 54:454–459.

McOrist, S., A. Muller Wager, D. Kratzer, and C. G. Sjosten. 2000. Therapeutic efficacy of water-soluble lincomycin-spectinomycin powder against porcine proliferation enteropathy in a European field study. Vet. Rec. 146:61-65.

Mitchell, J. M., M. W. Griffiths, S. A. McEwen, W. B. McNab, and A. J. Yee. 1998. Antimicrobial drug residues in milk and meat: causes, concerns, prevalence, regulations, tests, and test performance. J. Food Prot. 61:742–756.

Morck, D. W., M. E. Olson, T. J. Louie, A. Koppe, and B. Quinn. 1998. Comparison of ceftiofur sodium and oxytetracycline for treatment of acute interdigital phlegmon (foot rot) in feedlot cattle. J. Am. Vet. Med. Assoc. 212:254-257.

National Animal Health Monitoring System. 1997. Antibiotic injection practices on U.S. dairy operations. United States Department of Agriculture Animal and Plant Health Inspection Service. Available online: http://www.aphis.usda.gov/vs/ceah/ncahs/index.htm. Accessed 28th September 2004.

Oliver S. P., and L. M. Sordillo. 1988. Udder health in the periparturient period. Review. J. Dairy Sci. 71:2584-2606.

Oliver, S. P., B. E. Gillespie, S. J. Headrick, H. Moorehead, P. Lunn, H. H. Dowlen, D. L. Johnson, K. C. Lamar, S. T. Chester, and W. M. Moseley. 2004. Efficacy of extended ceftiofur intramammary therapy for treatment of subclinical mastitis in lactating dairy cows. J. Dairy Sci. 87:2393-2400.

Ortman, K., and C. Svensson. 2004. Use of antimicrobial drugs in Swedish dairy calves and replacement heifers. Vet. Rec. 154:136-140.

Owens, W.E., S. C. Nickerson, P. J. Washburn, and C. H. Ray. 1991. Efficacy of a cephapirin dry cow product for treatment of experimentally induced Staphylococcus aureus mastitis in heifers. J. Dairy Sci. 74:3376-3382.

Pankey, J. W. 1989. Premilking udder hygiene. J. Dairy Sci. 72:1308-1312.

Prescott, J. F., and J. D. Baggot. 1988. Antimicrobial therapy in veterinary medicine. Blackwell Scientific Publications, Boston.

Quigley, J. D. 3rd., J. J. Drewry, L. M. Murray, and S. J. Ivey. 1997. Body weight gain, feed efficiency, and fecal scores of dairy calves in response to galactosyl-lactose or antibiotics in milk replacers. J. Dairy Sci. 80:1751-1754.

Roussel Jr. A. J., and G. W. Brumbaugh. 1991. Treatment of diarrhea of neonatal calves. Vet. Clin. North Am. Food Anim. Pract. 7:713-728.

Ruder, C. A., R. G. Sasser, R. J. Williams, J. K. Ely, R. C. Bull, and J. E. Butler. 1981. Uterine infections in the postpartum cow. II. Possible synergistic effect of *Fusobacterium necrophorum* and *Corynebacterium pyogenes*. Theriogenology. 15:573.

Sanchez, M.S., and J. L. Watts. 1999. Enhancement of the activity of novobiocin against Escherichia coli by lactoferrin. J. Dairy Sci. 82:494-499.

Shitandi, A. and A. Sternesjo. 2004. Factors contributing to the occurrence of antimicrobial drug residues in Kenyan milk. J. Food Prot. 67:399-402.

Smith, B. I., G. A. Donovan, C. Risco, R. Littell, C. Young, L. H. Stanker, and J. Elliott. 1998. Comparison of various antibiotic treatments for cows diagnosed with toxic puerperal metritis. J. Dairy Sci. 81:1555-1562.

Smith, G. W., R. Gehring, J. E. Riviere, J. L. Yeatts, and R. E. Baynes. 2004. Elimination kinetics of ceftiofur hydrochloride after intramammary administration in lactating dairy cows. J. Am. Vet. Med. Assoc. 224:1827-1830.

Sol, J., and A. Melenhorst. 1990. Efficacy of a modified formula dry cow preparation for dairy cattle containing 600 mg cloxacillin. Tijdschr. Diergeneeskd. 115:670-673.

Spicer, H. M., L. A. Goonewardene, A. O. McNeil, and W. L. Slack. 1994. Alberta dairy farm survey response. J. Dairy Sci. 77:3460-3472.

Stokstad, E. L. R., and T. H. Jukes. 1950. Further observations on the animal protein factor. Proceedings of the Society of Experimental Biology and Medicine. 73:523-528.

Talley, M. R. 1999. The national milk safety program and drug residues in milk. Vet. Clin. North. Am. Food Anim. Pract. 15:63-73.

Tenover, F. C., and J. E. McGowan. 1996. Reasons for the emergence of antibiotic resistance. Am. J. Med. Sci. 311:9-16.

U.S. Congress, Office of Technology Assessment. 1995. Impacts of Antibiotic Resistant Bacteria (OTA-H-629). Washington, D.C.

USDA/APHIS/VS/CEAH. 1998. A guide to modern calf milk replacer. Available online: http://www.aphis.usda.gov/vs/ceah/ncahs/index.htm. Accessed 20th January 2004.

U.S. International Trade Commission. 1987. Synthetic Organic Chemicals: United States Production and Sales, 1950-1986. Washington, D. C., U.S. Government Printing Office.

Virtala, A.M., G. D. Mechor, Y. T. Grohn, H. N. Erb, and E. J. Dubovi. 1996. Epidemiologic and pathologic characteristics of respiratory tract disease in dairy heifers during the first three months of life. J. Am. Vet. Med. Assoc. 208:2035-2042.

Waltner-Toews, D., S. W. Martin, A. H. Meek, and I. McMillan. 1986. Dairy calf management morbidity and mortality in Ontario Holstein herds. I: The data. Prev. Vet. Med. 4: 103-124.

Witte, W. 2000. Selective pressure by antibiotic use in livestock. Int. J. Antimicrob. Agents. 16:S19-S24.

Yancey, R. J., Jr., M. L. Kinney, B. J. Roberts, K. R. Goodenough, J. C. Hamel, and C. W. Ford. 1987. Ceftiofur sodium, a broad-spectrum cephalosporin: evaluation in vitro and in vivo in mice. Am. J. Vet. Res. 48:1050-1053.

Zwald, A. G., P. L. Ruegg, J. B. Kaneene, L. D. Warnick, S. J. Wells, C. Fossler, and L. W. Halbert. 2004. Management practices and reported antimicrobial usage on conventional and organic dairy farms. J. Dairy Sci. 87:191-201.

Chapter 3

PREVALENCE OF ANTIMICROBIAL-RESISTANT GRAM-NEGATIVE ENTERIC BACTERIA IN LACTATING DAIRY CATTLE

3.1 ABSTRACT

A study was conducted to determine the prevalence of antibiotic-resistant Gram-negative enteric bacteria (GN-EB) in the feces of calves and lactating cattle. Gram-negative enteric bacteria resistant to ampicillin, oxytetracycline, florfenicol, spectinomycin, and neomycin were isolated from feces of 31, 31, 7, 5, and 1% of 313 cows (n=33 farms), respectively. Dairy herds that fed medicated milk replacer to calves were 3.4 fold more likely to have lactating cattle which shed tetracycline-resistant GN-EB. However, a similar relationship between the use of medicated milk replacer and the presence of tetracycline-resistant, GN-EB in calves was not observed.

Escherichia coli (87%) was the most predominant of all the GN-EB species isolated from feces of healthy lactating cattle. Other species included *Citrobacter koseri, Enterobacter aerogenes*, *Klebsiella oxytoca*, *Klebsiella pneumonia*, *Kluyvera* spp.*, Morganella morganii*, *Pasturella* spp., *Providencia alcaligenes, Providencia stuartii, Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Pseudomonas* spp. *Escherichia coli* (n=229) isolates were resistant to ampicillin (60%), ceftiofur (11%), chloramphenicol (19%), gentamicin (2%), spectinomycin (22%), tetracycline (94%), ticarcillin (21%), and ticarcillin/clavulanic acid (11%). Multidrug resistance was observed in 29% of *E. coli* isolates. A total of 8 (3.5%) *E. coli* isolates encoded for shiga I and/or shiga II genes.

Gram-negative enteric bacteria resistant to ampicillin, florfenicol, neomycin, tetracycline, and spectinomycin accounted for 9, 5, 1, 14, and 10% of the total GN-EB, respectively. Under experimental conditions, a multi-drug resistant *E. coli* phenotype survived for 9 weeks at 7^{o}C in sterile water. Monte Carlo analysis revealed that multi-drug resistant *E. coli* would undergo a 3 log reduction in 126 days when held in sterile water at 7^{o}C. Using previously reported die off

constant values, a 4 log reduction of *E. coli* in dairy manure piles, soil with no organic matter, and river water was predicted to occur in 63, 42, and 28 days, respectively.

Pulsed field gel electrophoresis of 69 ampicillin-resistant and 99 tetracycline-resistant *E. coli* revealed a highly diverse genotypic population, suggesting that resistance to these antibiotics is horizontally disseminated. Interestingly, PFGE subtypes observed on one farm were not found on other farms.

The findings of the study suggest that antibiotic resistant GN-EB are widely prevalent on farms. Ampicillin- and tetracycline-resistant *E. coli* were the predominant isolates among the GN-EB species isolated. A causal relationship between use of medicated milk replacers and presence of tetracycline-resistant GN-EB was seen in lactating cows, suggestive of long term effects of selective pressure of tetracycline. Presence of multi-drug resistant *E. coli* could pose a risk to public health as these organisms have the ability to survive for long periods in the environment and can enter the food chain at various steps.

3.2 INTRODUCTION

Antibiotic usage has selected for the evolution of bacterial defenses, which result in resistance to antibiotics (Levy, 1992). The above defenses and constant exposure to antibiotics have resulted in persistence of antimicrobial resistance in bacterial populations in dairy herds.

The emergence and spread of antibiotic resistance depends on the influence of selective antibiotic pressure on bacteria. The presence of multiple antibiotics in the environment allows for the selection of bacterial variants that either use different mechanisms or optimize a single mechanism of resistance to survive under such selective pressure (Baquero et al., 1998). A

classic example of selective pressure is the use of antibiotics at subtherapeutic concentrations in feed of food producing animals (IOM, 1989).

Hospitals and farms with high rates of antibiotic use could serve as "evolutionary incubators" for emergence of antibiotic-resistant bacteria. In such environments, selection influences evolution and can help develop mechanisms that may increase the mobility of antibiotic resistant genes (Smith et al., 2002). While antimicrobial therapy is generally targeted towards specific pathogens, commensal bacteria in the treated host are also exposed to antibiotics (Levin et al., 1997). Commensal flora of the gut, nasopharynx and other habitats in the host generally consists of a number of different species and at times variants of the same species (Caugant et al., 1981). The presence of antibiotic resistance in commensal bacteria is a concern as antibiotic resistance genes and accessory genetic elements can be horizontally transmitted to pathogens like *Shigella* spp., *Salmonella* spp., *Vibrio cholerae*, and *Neisseria gonorrhoeae* (Levin et al., 1997).

From an environmental standpoint, the discharge of antibiotics and their metabolites in farm wastes could create a reservoir of resistant microorganisms in the environment. Residues of some antibiotics are known to linger in farm products (Levy et al, 1987; Tenover and McGowan, 1996; Corpet, 1996). Several antibiotics enter the aquatic and terrestrial ecosystems through the discharge of effluents from farms (Bates et al., 1994). Bioactive veterinary drug residues and antibiotic-resistant bacteria present in farm wastes when applied to the land can run into water bodies and thus serve as a potential source of antibiotic-resistant bacteria and genetic elements in the environment (Chee-Sanford et al., 2001; Austin, 1985).

It is now clear that the antibiotics to which bacterial populations are presently exposed will play a major role in deciding the type of antibiotic resistance that may emerge and spread in

bacterial populations in the future. Antimicrobial resistance in commensal flora can be used as a marker to monitor antibiotic-mediated selection in individual hosts and the general population (Levin et al., 1997). The present study attempts to determine the prevalence of antimicrobial resistance in commensal GN-EB in lactating dairy cattle and to study the relationship between antibiotic selective pressure and incidence of antimicrobial-resistance in GN-EB. The study further tries to clarify genetic relatedness among antibiotic resistant bacteria and the risks associated with survival of resistant bacteria in the environment.

3.3 MATERIAL AND METHODS

3.3.1 Dairy cattle

Fecal samples from lactating cattle (n=313) in 33 dairy herds were screened for antibiotic resistance. Within each herd, 10% of lactating cows were randomly selected and sampled. Fecal samples from calves (n=93) on 13 farms were also collected. Feces were collected from cows per rectal using a sterile disposable rectal sleeve, and from calves using a sterile disposable glove. Approximately 2-10 grams of feces were transferred to a sterile 50 ml screw-cap centrifuge tube. The tube was transported to the laboratory on ice and processed the same day as sampling.

3.3.2 Screening for antimicrobial-resistant GN-EB

One gram of feces from a thoroughly mixed sample was transferred to a 50 ml centrifuge tube containing 9 ml of sterile normal saline solution. The contents were mixed thoroughly and serially diluted ten-fold. MacConkey agar (MAC) was used for selective growth of GN-EB. From the 10^{-3} and 10^{-4} dilution, 0.1 ml was plated on a MAC control plate without antibiotics, while from the 10^{-1} and 10^{-2} dilution, 0.1 ml of the sample was plated on a MAC plate containing

ampicillin (64μg/ml), or neomycin (512μg/ml), or oxytetracycline (32μg/ml), or spectinomycin (256μg/ml) (ICN biomedicals, Aurora, Ohio,U.S.), or enrofloxacin (8μg/ml) (Baytril, Shawnee Mission, Kansas, USA), or florfenicol (16μg/ml) (Schering-Plough Animal Health, Union, NJ, USA). The concentration of antibiotics used in MAC media was one-fold higher than the recommended MIC_{90} cutoff values suggested by the National Committee for Clinical Laboratory Methods (NCCLS) document M31-A2 (2002) for bacteria isolated from animals. The inoculated plates were incubated at 37^{o}C for 24 h. The numbers of colonies on MAC without antibiotics and with antibiotics were counted and expressed as total GN-EB cfu/g of feces and antibiotic resistant GN-EB cfu/g of feces, respectively. Fecal samples from calves were used for determining the prevalence of tetracycline resistant GN-EB.

3.3.3 Species identification

Isolates (n= 23 farms) from MAC with antibiotics were identified to species level. Based on colony morphology and lactose fermentation, 2-3 colonies were selected from each plate for species identification. Colonies were tested for gram reaction, oxidase test, and IMViC battery test as described by Harley and Prescott (1993). The isolates were then identified to species level using the API-20E/NE identification kit (BioMérieux, Hazelwood, MO) as described by the manufacturer.

3.3.4 Relationship between milk replacers and tetracycline resistant GN-EB

A chi-square test of independence was applied on a 2 × 2 contingency table for evaluating the effect of feeding calves milk replacers with oxytetracycline on fecal shedding of tetracycline-resistant GN-EB in lactating cattle (n=24 farms). The chi-square test was also used to investigate if there was any significant relationship (P <0.05) between the use of tetracyclines in medicated milk replacers and the presence of tetracycline resistant GN-EB in calves on 13

farms. Epi-info-2002 (Centers for Disease Control and Prevention, Atlanta, GA), a database and statistics system for epidemiology on microcomputers, was used for performing χ^2- tests and odds ratio analysis.

3.3.5 Antimicrobial susceptibility testing

Antibiotic resistant *E. coli* isolates (n=229) were examined for susceptibility to antibiotics by disk diffusion assay. Antimicrobial disks with ampicillin (10 µg), ceftiofur (30 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), spectinomycin (100 µg), tetracycline (30 µg), ticarcillin (75 µg), and ticarcillin/clavulanic acid (75/10 µg) (Remel Inc. KS, U.S.A) were used for the disk diffusion assays. Zones of growth inhibition were measured and the results were interpreted according to criteria established by the NCCLS document M31-A2 (2002) for bacteria isolated from animals (Figure **3.3** and Table **3-5**).

3.3.6 Shiga toxin encoding genes

The presence of shiga toxins I and II were determined using primers reported by Meng et al. (1997). Isolates were screened for the presence of stxI (primer SLT I-F 5′-TGTAACTGGAAAGGTGGAGTATACA-3′ and SLT I-R 5′- GCTATTCTGAGTCAACGAA AAATAAC-3′) and stxII (primer SLT II-F 5′- GTTTTTCTTCGGTATCCTATTCC-3′ and SLT II-R 5′-GATGCATCTCTGGTCATTGTATT AC-3′) genes as described (Meng et al., 1997). The PCR was performed at 95^{o}C for 5 min for initial denaturation followed by 30 cycles of 95^{o}C for 1 min, 53^{o}C for 1 min 30 secs, and 72^{o}C for 1 min. The expected amplicon sizes were 210 bps (Shiga-I) and 484 bps (Shiga-II) respectively. The PCR assay was performed in 25µl reaction volume using puReTaq^{TM} Ready-To-Go-PCR Beads containing PCR reaction mixture (Amersham Biosciences, NJ).

3.3.7 Survival of multidrug resistant *E. coli* A92

A multidrug resistant *E. coli* isolate A92 (resistance phenotype: AMP-CHL-SPT-TET-TIC-TIM-XNL) was used for this study. Ten ml of sterile water was inoculated with *E. coli* A92 culture to obtain approximately 10^3 cfu/ml on day 0 as determined by spiral plating. The inoculum (50 µl) was spiral-plated on plate count agar at intervals of 7 days and viable cfu/ml were calculated. The water was kept at ~7^{o}C throughout the experiment. The experiment was repeated three times and the mean cfu/ml was used to calculate the die off rate constant "K" as described by Chick (1908). The constant was determined using the formula $Nt/No = 10^{-kt}$, where Nt = number of bacteria at time t, No = number of bacteria at time 0, t = time in days, k = die-off rate constant.

Monte Carlo simulation (@Risk 4.5 version, Palisade Corporation, NY) was used to determine the probability distribution of the constant `K´ value. A random `K´ value was selected from the dataset and, after defining a standard deviation to incorporate uncertainty in the data, the K value was simulated for 5000 iterations to find a probability distribution of the die-off rate constant. The mean die-off rate was used to calculate the survival curve of the *E. coli* isolate at an initial concentration of 10^5 cfu/g held at 7^{o}C.

Survival curves were calculated for *E. coli* (initial concentration of 10^5 cfu/g) using die-off rate constants reported in the literature. The predicted survival curves of *E. coli* in a dairy manure pile with temperature conditions between 2-8^{o}C (k=0.066; Jones, 1971), soil with no organic matter (k=0.097; Mallman and Litsky, 1951), and river water at 5^{o}C (k=0.144; Mitchell and Starzyk, 1975) were calculated (Figure **3.7**).

3.3.8 Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) of 99 of 113 tetracycline-resistant and 69 of 75 ampicillin-resistant *E. coli* isolates using the one day PFGE protocol described by Gautom (1997). Briefly, the bacterial plugs (known bacterial concentration encased in insert agarose gel block) were digested with *Xba*I and run on Chef Mapper^{TM} system (Bio-Rad Laboratories, CA). The running conditions were as follows: Initial switch time 2.16 secs, final switch time 35.07 secs, run time 14 hours, angle 120^{o}, gradient 6.0 V/cm, temperature 14^{o}C, and the ramping factor was kept linear. The PFGE types were first compared visually using the guidelines of Tenover et al. (1995) and subsequently by constructing a dendogram using GelDoc 2000 Molecular Analyst Fingerprinting Plus, version 6.1 Software (Bio-Rad).

3.4 RESULTS

3.4.1 Number and percent of farms and cows with antimicrobial resistant GN-EB

About 31% of the cows shed ampicillin- and tetracycline-resistant GN-EB. Ampicillin and tetracycline resistant GN-EB were observed on 61 and 64% of the farms, respectively. The prevalence of spectinomycin, florfenicol, and neomycin resistant GN-EB was lower than that observed for tetracycline and ampicillin. Enrofloxacin resistant GN-EB were not detected (Table **3-1**).

3.4.2 Relationship between resistant bacteria and total GN-EB flora

An average of 10^6 cfu/g of GN-EB was present in the feces of dairy cattle. Ampicillin- and tetracycline-resistant GN-EB were a log lower (10^5 cfu/g) than total GN-EB, while spectinomycin- and florfenicol-resistant bacteria were 2 and 3 log lower than total GN-EB,

respectively. Ampicillin-and tetracycline-resistant GN-EB on average accounted for 9.26 and 13.9% of total GN-EB, respectively.

3.4.3 Use of antibiotics on farms and prevalence of tetracycline-resistant GN-EB on farms

A significant relationship ($P < 0.05$) was observed between use of medicated milk replacers on farms and the presence of tetracycline resistant GN-EB in lactating cattle on those farms (Table **3-3**). Dairy producers who fed calves medicated milk replacers with tetracycline were 3.4 times more likely to have lactating cows shedding tetracycline-resistant GN-EB in their feces, as compared to dairy producers who did not feed milk replacers containing tetracycline. No cause and effect relationship was observed between feeding medicated milk replacers with tetracyclines to calves on farms and the prevalence of tetracycline-resistant GN-EB in calves on those farms (Table **3-3**).

3.4.4 GN-EB species isolated from MAC + antibiotic plates

Isolates of GN-EB (n=264) belonged to 13 Gram-negative bacterial species including *Citrobacter koseri, Enterobacter aerogenes, Escherichia coli*, *Morganella morganii*, *Klebsiella oxytoca*, *Klebsiella pneumonia*, *Kluyvera* spp., *Providencia alcaligenes*, *Providencia stuartii*, *Pasteurella* spp., and *Pseudomonas* spp.

3.4.5 Antimicrobial resistance among *E. coli* isolates by disk diffusion assay

Escherichia coli (n=229) isolates were screened for resistance to 9 antimicrobial agents commonly used in animal agriculture (Table **3-5** and Figure **3.3**). According to NCCLS interpretive criteria, isolates exhibiting resistance to all the antimicrobial agents except enrofloxacin were observed. The majority of the isolates were resistant to tetracycline (93.9%) followed by ampicillin (59.8%). Resistance to other antimicrobial agents was observed in less than 25% of the isolates. *Escherichia coli* isolates were resistant to ceftiofur (10.9%),

chloramphenicol (18.8%), gentamicin (2.2%), spectinomycin (21.8%), ticarcillin (20.5%), and ticarcillin and clavulanic acid (10.9%).

3.4.6 Resistance patterns of *E. coli* isolates from different MAC+antibiotics plates

The antibiotic resistant *E. coli* belonged to 26 resistance profiles (Table **3-7**). Among the 26 resistance profiles, isolates resistant to tetracycline were the single largest resistance group (36.7%). The second largest resistance profile (25.3% of the isolates) exhibited resistance to tetracycline and ampicillin. Multidrug resistance (≥3 to <9) was observed in 29% of the isolates and belonged to 18 resistance profiles. The most frequently observed multidrug resistance profile consisted of isolates resistant to ampicillin, chloramphenicol, spectinomycin, tetracycline, ticarcillin, ticarcillin and clavulanic acid, and ceftiofur. Resistance to ceftiofur, gentamicin, and ticarcillin/ clavulanic acid was observed in multi-drug resistant isolates only (Table **3-7**).

3.4.7 Shiga toxin encoding genes in antibiotic resistant *E. coli* isolates

Of the 229 isolates, a total of 8 isolates were observed to encode for shiga toxin genes. Two *E. coli* isolates encoded for both Stx_I and Stx_{II} genes, while Stx_I or Stx_{II} were observed in 2 and 4 isolates, respectively (Figure **3.4**).

3.4.8 Survival of multi-drug resistant *E. coli* A92 at refrigeration

An increase in *E. coli* count was observed for the first week of storage at 7^oC, followed by a gradual decline in *E. coli* count (Figure **3.5**). Viable bacteria were recovered over a 9 week period. The initial reading was ignored and data from the 1^{st} wk was used to calculate the die-off rate constant. The average die-off rate constant was observed to be 0.0229. A random die-off constant value was selected (k=0.0257, 21 days) and using MonteCarlo simulation was simulated for 5000 iterations. The mean die-off rate K was observed to be 0.0255, which was close to the mean calculated value (0.0229). The K value of 0.0255 was used to calculate the survival of

multi-drug resistant bacteria at 7^{o}C with an initial concentration of log 5 cfu/ml; under these conditions a log 3 reduction in *E. coli* would take 126 days (Figure **3.6**).

Using die-off constant values reported in the literature for *E. coli*, survival curves were predicted for a log 5 cfu/ml initial concentration of *E. coli* A92. Based on these values, it would take 63 days for a 4 log reduction in a dairy manure pile at 2-8^{o}C, 42 days in soil with no organic matter and 28 days in river water at 5^{o}C (Figure **3.7**).

3.4.9 Genotype profiling using PFGE

On dendogram analysis, a diverse collection of subtypes was observed (Figure **3.8**). The 99 tetracycline-resistant isolates belonged to 60 genotypes, while 69 ampicillin-resistant isolates belonged to 44 genotypes. A genotype detected on one farm was seen frequently on the same farm, but rarely detected on another farm (Figure **3.9** and **3.10**). Only 2 of 69 ampicillin and 4 of 60 tetracycline resistant PFGE types were shared among farms.

3.5 DISCUSSION

The study of prevalence of antibiotic resistance in commensal microflora can be very helpful in monitoring and understanding developments in antibiotic mediated selection in individual hosts as well as the general population (Levin et al., 1997). MacConkey's media supplemented with antimicrobial agents can be used for the isolation of antibiotic resistant coliforms (Langlois et al., 1984). White et al. (2000) were more successful in finding the *flo* gene in florfenicol-resistant Gram-negative bacteria with higher drug concentrations (≥16µg/ml) than the MIC_{90} recommended by NCCLS (≤ 8µg/ml). Based on the findings of Langlois et al. (1984) and White et al. (2000) I believe that using higher concentrations of antibiotics than

recommended by NCCLS, I would be able to efficiently isolate antibiotic resistant enteric bacteria.

Escherichia coli isolated from bovine diarrhea and hospitalized calves exhibiting resistance to ampicillin, florfenicol, gentamicin, spectinomycin, and tetracycline are frequently reported in the literature (Orden et al., 2000; Werckenthin et al., 2002; White et al., 2000). Most of these susceptibility studies are conducted on isolates from clinical cases, whereas our study was focused on antimicrobial- resistant GN-EB isolated from healthy lactating cows. The importance of such investigations has been emphasized by the French Institute for Public Health Surveillance. It was suggested that monitoring of resistance should not be restricted to the bacteria isolated from samples from infected individuals, but must also include the commensal bacterial flora, which could represent a pool of resistance genes (Guillemot and Courvalin, 2001).

The numbers of antimicrobial-resistant GN-EB per gram of feces was compared to the number of total GN-EB per gram of feces (Figure **3.1** and Table **3-2**). Nuru et al. (1972) observed that *E. coli* was the predominant Gram-negative species in feces of cattle and ranged from 10^4 to 10^6 cfu/g of feces with a mean of 5.6×10^5 cfu/g. *Escherichia coli* in pig feces ranged from 1.6 to 2.5×10^6 cfu/g (Van Den Bogaard et al., 2000). The findings of our study (total GN-EB 10^5 - 10^6 cfu/g) were in close agreement with that reported by Nuru et al. (1972) and Van Den Bogaard et al. (2000).

To determine the effect of withdrawal of tetracycline from swine feed, Smith (1975) conducted a study that monitored tetracycline-resistant enteric bacteria in pigs for 4 years after the ban of its use in swine in Britain. The number of tetracycline-resistant bacteria that were shed in feces of pigs showed a decrease, but the number of pigs carrying tetracycline-resistant bacteria

did not decrease. It was postulated that during the extensive use of tetracyclines, resistant strains had emerged that were now able to compete and replace gut microflora (Smith, 1975). Persistence of tetracycline resistant enteric microflora was observed for 126 months in the British swine population after withdrawal of tetracycline (Langlois et al., 1988).

A causal relationship was observed between the use of medicated milk replacers in calves and the presence of tetracycline-resistant GN-EB in lactating cows. Dairy producers who used medicated milk replacers supplemented with tetracycline were 3.4-fold more likely to have lactating cows shedding tetracycline-resistant GN-EB when compared to those dairy herds that did not use medicated milk replacers. The same relationship was not observed in calves. A study conducted by Khachatryan et al. (2004) showed the highest prevalence of resistant *E. coli* in preweaned calves. The high degree of resistance was still maintained in the absence of antimicrobial drug selection. It was suggested that the initial intake of a resistant strain by calves could be from the environment, and it could perhaps replace sensitive flora by active competition leading to the expansion of resistant bacterial populations in calves. With increase in age, the prevalence of resistant strains in the gut decreased.

Hinton et al. (1985) concluded a study which monitored the antibiotic resistance index in calves. They observed that the resistance index of fecal *E. coli* in 1-2 day old calves was low initially, but rose rapidly during the first week following weaning of the animals. The resistance index then fell from the week 3 to low levels by the time the calves were 5 months of age. The antibiotic-sensitive strains that had colonized the calves' guts in the early days of life differed from the antibiotic-sensitive strains observed after the fall in resistance index. The change was not due to the reemergence of strains, but through replenishment with newer strains, probably from the calves' environment. Such dynamic responses and changes in resistant flora of calves

under selective antimicrobial pressure could explain why we failed in the present study to observe any relationship between the use of tetracycline and tetracycline resistant GN-EB in calves.

Among GN-EB species, *Escherichia coli* was the only species that exhibited resistance to florfenicol, neomycin, spectinomycin and tetracycline. The majority of the GN-EB species exhibited resistance to ampicillin (Table **3-4**). *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* spp. are important due to their isolation from nosocomial infections in human patients (Medeiros, 1997; Livermore, 1996). Antibiotic-resistant *Klebsiella* and *Enterobacter* spp. have been observed in community and hospital acquired pneumonia, whereas cephalosporin-resistant *E. coli*, *Citrobacter koseri* (diversus) and *Klebsiella pneumoniae* have been isolated from fecal carriers in hospitals (Bouza and Cercenado, 2002; Moustaoui et al., 2004). Ampicillin-resistant *Citrobacter koseri* has been isolated as an opportunistic pathogen from urinary tract infections, and respiratory and genital tracts of human patients (Altmann et al., 1984). Most of these species have the ability to acquire resistance to newer generations of cephalosporins- and become multidrug resistant.

Resistance to the extended spectrum cephalosporin ceftiofur (10.9%) was observed only in multidrug-resistant isolates (≥4 antimicrobial agents). Ceftiofur is an expanded-spectrum, injectable cephalosporin developed solely for veterinary therapeutic use (Hornish and Kotarski 2002; Jaglan et al., 1992). Interestingly, the majority of the isolates exhibiting resistance to ceftiofur were also resistant to chloramphenicol, tetracycline, spectinomycin, and β-lactams including ampicillin, ticarcillin, and ticarcillin with clavulanic acid. Extended spectrum cephalosporin resistance including ceftiofur along with resistance to β-lactamase inhibitors like clavulanic acid, has been reported in *Escherichia coli* and *Salmonella* from food animals and

retail ground meat, and calf feces (Allen and Poppe, 2002; Zhao et al., 2001a; Hunter et al., 1993). Since ceftiofur-resistant organisms also exhibit decreased susceptibility to cephamycins and extended-spectrum cephalosporins, the use of this antimicrobial agent in food animals has come under increasing scrutiny (Zhao et al., 2001a; Winokur et al., 2000).

The multidrug resistance of ceftiofur-resistant *E. coli* isolates to other unrelated antimicrobials like tetracycline and chloramphenicol has previously been documented in *Salmonella* and *E. coli* isolated from animals (Winokur et al., 2000; White et al., 2000). Interestingly, multidrug-resistant *E. coli* isolates exhibited resistance to chloramphenicol (15%), an antibiotic that has been banned from veterinary use in food animals in the United States since the 1980s (Gilmore 1996). Florfenicol, a fluorinated structural analog of chloramphenicol approved by the Food and Drug Administration in 1996, is used for treatment of bovine respiratory pathogens. Isolates that are florfenicol-resistant can possibly exhibit resistance to chloramphenicol (White et al., 2000). There are many examples of such cross-resistance in the literature, including conservation of streptomycin resistance on genetic elements like *Tn*21-type transposons carrying integron in the absence of direct selection pressure (Chiew et al., 1998). Chiew et al. (1998) suggested that the presence of cross-resistance and association of resistance genes with versatile genetic elements can help conserve resistance to antibiotics.

Shiga toxin-producing *Escherichia coli* (STEC) have been an important cause of foodborne illness worldwide. Over 100 STEC serotypes, including O157:H7, and have been associated with human illness (Meng and Doyle, 1998). In the United States, foodborne STEC and non-STEC are estimated to annually cause approximately 94,000 and 79,000 illnesses, respectively (Mead et al., 1999). The presence of multidrug-resistance in STEC serotypes has also been reported in the literature (Zhao et al., 2001b).

In our study, antibiotic-resistant and shiga toxin positive isolates were detected in feces of healthy lactating dairy cattle, though the numbers of isolates were few (3.5%). A study conducted in southern Brazil observed the presence of STEC in the feces of healthy dairy cattle from 57 of 60 farms. Twelve out of 327 STEC isolated belonged to serogroups previously associated with cases of haemorrhagic colitis, hemolytic uremic syndrome or diarrhea in humans (Moreira et al., 2003). Recent studies have documented that *E. coli* O157:H7 and non-O157 STEC strains isolated from humans and animals have also developed antibiotic resistance, and many are resistant to the multiple antimicrobials commonly used in human and veterinary medicine (Farina et al., 1996; Kim et al., 1994; Gonzalez and Blanco, 1989, Schmidt et al., 1998). All of the 8 isolates in our study that encoded for shiga toxins were susceptible to the majority of the antibiotics we tested. On disk diffusion assay, all the isolates were resistant to ampicillin and tetracycline.

Environmental pollution with bacteria of public health significance and the likelihood of this bacteria gaining access to the food chain are the most critical areas of concern. Previous literature has mostly studied survival of pathogens like *E. coli* O157:H7 and Salmonella in water, soil, and cow manure (Kudva et al., 1998; Jiang et al., 2002; Rice and Johnson 2000; Natvig et al., 2002).

The findings of our study suggest that healthy lactating cattle on dairies can serve as a reservoir of antibiotic-resistant GN-EB. The antibiotic-resistant GN-EB could contaminate the environment and pose a public health risk. In our study, *E. coli* A92, showed an initial log period where the counts increased from 3.57 to 4.37 log cfu/ml in the first week of storage at 7^{o}C. This increase could be due to several factors, including a reduction in environmental stresses on bacteria because of dilution, lowered level of toxic compounds, and/ or increased oxygen supply

in the new environment (Moore et al., 1988). The die-off rate was therefore determined from the point where the logarithmic decrease in the numbers started (7^{th} day) using the model proposed by Chick (1908). The die-off rate constant (K = 0.0225) calculated by MonteCarlo simulation of the experimental data was used for creating the survival curve. The model predicted that 126 days would be required for a 3 log reduction (5 to 2 log cfu/ml) at 7^{o}C storage.

Using K values reported by Mitchell and Starzyk (1975), a 4 log reduction would take 28 days in river water at 5^{o}C. Rice and Johnson (2000) studied survival of *E. coli* O157:H7 in cattle drinking water at 5^{o}C and observed that 16 days were required for the count to reach log 1 cfu/ml from initial inoculum of log 3 cfu/ml. Studies conducted on survival of enteric *E. coli* in soil mixed with bovine manure indicated a survival period of at least 19 wks at 9-21^{o}C (Lau and Ingham, 2001). Using K values calculated by Jones (1971) and Mallman and Litsky (1951), a 4 log reduction was predicted to take over 63 days for *E. coli* in a dairy manure pile at 2-8^{o}C and 28 days in soil at 5^{o}C with no organic matter. Based on this data, there is a high likelihood of multidrug-resistant enteric bacteria being prevalent in the farm environment for prolonged periods of time. The significance of these observations corroborates the findings reported by Aminov et al. (2002) that the antibiotic-resistance gene pool (disseminated through fecal contamination from animal production system) persists in the environment.

The ability to characterize and determine relatedness among bacterial isolates is a prerequisite for epidemiological investigations. The degree of clonality within the natural *E. coli* population has yet to be clearly defined. The influence of antibiotic resistance on the selection of clonal lines is poorly understood. Identifying the sources of fecal contaminants in bodies of surface water, such as rivers and lakes, is of significant importance for environmental quality,

food safety, and regulatory purposes. Current DNA library-based-source-tracking approaches rely on the comparison of the genetic relatedness among the fecal contaminants (Lu et al., 2004).

Molecular fingerprinting techniques like PFGE have great value in such epidemiological analysis. This technique has now become a standard among public health agencies (Gautom, 1997). It has successfully been used in tracking diseases caused by bacterial pathogens like *E. coli* O157:H7 (Barrett et al., 1994; Bohm and Karch, 1992) and *S. aureus* (Schlichting et al., 1993). The large genotypic variation in both ampicillin- and tetracycline-resistant *E. coli* isolated indicates that resistance to these antibiotics is not associated with a particular clonal type (Figure **3.8**). Similar high strain diversity was observed in *E. coli* isolated from sewage, gulls, and dairy cattle using repetitive extragenic palindromic PCR and PFGE (McLellan et al., 2003). We share the opinion expressed by these authors that extensive isolation of strains to encompass the large diversity in *E. coli* strains is needed for genetic comparison and identifying sources of the isolates.

The sample size of our experiment perhaps was not sufficient enough to cover all the possible genotypes of antibiotic-resistant commensal *E. coli.* Interestingly the genotypes found on one farm were rarely shared by another farm and each dairy farm harbored a unique reservoir of *E. coli* genotypes (Figure **3.9** and **3.10**). Lu et al. (2004) found that using techniques which have high resolution, like PFGE, was a challenging task for tracking the source of *E. coli* in irrigation water. They observed that the PFGE patterns for the same *E. coli* changed after 8 weeks in irrigation water.

The findings of our study indicate that tetracycline- and ampicillin-resistance were the most widely prevalent antibiotic resistances in commensal enteric *E. coli* in lactating dairy cattle. The use of medicated milk replacers containing tetracyclines needs to be addressed appropriately

to prevent selection and proliferation of tetracycline-resistant enteric bacteria. Epidemiological assessments need to take into account that multidrug-resistant *E. coli* can survive for prolonged periods in various environments. The high diversity in genotypes carrying ampicillin- and tetracycline-resistance indicates the possibility of non-clonal spread in the population. Presence of ceftiofur resistance in multidrug-resistant *E. coli* signifies that the use of beta-lactams in dairy cattle requires more careful monitoring and better management.

Table **3-1**: Number and percent of farms and cows shedding antimicrobial-resistant Gram-negative enteric bacteria.

Antimicrobial agent	Farm (n=33)		Cows (n=313)	
	No.	%	No.	%
Ampicillin (64 µg/ml)	20/33	61	98/313	31
Enrofloxacin (8 µg/ml)	0/33	0.0	0/313	0.0
Florfenicol (16 µg/ml)	11/33	33	23/313	7
Neomycin (512 µg/ml)	2/33	6	4/313	1
Spectinomycin (256 µg/ml)	7/33	21	14/313	5
Tetracycline (32 µg/ml)	21/33	64	97/313	31

Table **3-2**: Number of antimicrobial-resistant and total Gram-negative enteric bacteria per gram of feces of lactating cattle belonging to 23 dairy farms.

Antibiotics	Cows	Mean total GN-EB	Mean resistant GN-EB	Ratio (Resistant GN-EB/Total GN-EB)%	
	N	cfu/g	cfu/g	Mean	Range
Ampicillin	72	3.8×10^6	3.0×10^5	9.3	0.01 - 96.5
Florfenicol	18	1.2×10^6	2.9×10^3	4.9	0.000 - 63.0
Neomycin	4	4.2×10^5	3.5×10^2	1.2	0.01 - 3.0
Spectinomycin	10	1.9×10^6	6.2×10^4	9.9	0.004 – 89
Tetracycline	89	6.9×10^6	3.9×10^5	13.9	0.01 - 100

Table **3-3**: Influence of feeding medicated milk replacers with tetracycline on the prevalence of tetracycline-resistant Gram-negative enteric bacteria in calves and lactating cattle.*

Fed milk replacers to calves	Calves			Test of significance
	Tetracycline-resistant GN-EB			
	Detected	Not-Detected	Total	
Yes	49	4	53	$\chi 2$ (P) = 2.14 (0.1443) Odds ratio (Confidence Interval): 2.60 (0.64-11.64)
No	33	7	40	
Total	82	11	93	
Fed milk replacers to calves	Lactating Cattle			$\chi 2$ (P) = 17.27 (0.00003) Odds ratio (Confidence Interval): 3.45 (1.83-6.56)
	Tetracycline-resistant GN-EB			
	Detected	Not-Detected	Total	
Yes	89	23	112	
No	56	50	106	
Total	145	73	218	

* Calves (n=93) and lactating cattle (n=218) on 13 dairy herds examined for tetracycline-resistant GN-EB.

Seven dairy herds fed milk replacers and six herds did not feed milk replacers.

Tetracycline was not used for therapeutic purposes on the 13 herds examined.

Table **3-4**: Antibiotic-resistant GN-EB species isolated from lactating cattle (n=313) on 33 dairy herds.

Species	MacConkey's agar supplemented with:				
	Ampicillin	Florfenicol	Neomycin	Spectinomycin	Tetracycline
Citrobacter koseri	14	-	-	-	-
Enterobacter aerogenes	4	-	-	-	-
Escherichia coli	75	22	6	13	113
Klebsiella oxytoca	3	-	-	-	-
Klebsiella pneumonia	-	-	-	1	-
Kluyvera spp.	-	-	-	1	-
Morganella morganii	1	-	-	-	-
Pasteurella spp.	-	2	-	-	-
Providencia alcaligenes	1	-	-	-	-
Providencia stuartii	-	-	-	1	
Pseud. aeruginosa	1	-	-	-	-
Pseud. fluorescens	5	-	-	-	-
Pseudomonas spp.	-	-	-	1	-
Total isolates	104	24	6	17	113

Table **3-5**: Antimicrobial resistance among 229 *E. coli* isolates by disk diffusion assay.

Antimicrobial agent	Resistant isolates (%)
Ampicillin	137 (59.8)
Ceftiofur	25 (10.9)
Chloramphenicol	43 (18.8)
Enrofloxacin	0 (0.0)
Gentamicin	5 (2.2)
Spectinomycin	50 (21.8)
Tetracycline	215 (93.9)
Ticarcillin	47 (20.5)
Ticarcillin/Clavulanic acid	25 (10.9)

Table **3-6**: Die off rate (constant K) of *E. coli* A92 calculated from 7th day. [a]

No cfu/ml on 7th day	Nt cfu/ml at day (t)	No/Nt	log(No/Nt)	log(No/Nt)/ t `K'	days (t)
23760	21040	1.129	0.053	0.0075	14
23760	18500	1.284	0.109	0.0078	21
23760	6870	3.459	0.539	0.0257	28
23760	3500	6.789	0.832	0.0297	35
23760	3010	7.894	0.897	0.0256	42
23760	2030	11.704	1.068	0.0254	49
23760	1220	19.475	1.289	0.0263	56
23760	260	91.385	1.961	0.0350	63
			Average	0.0229	
			k value for 21 days	0.0257 [b]	
			Uncertainty	σ (SD) =1.3	
			range	0.004-0.047	

[a] k value calculation: e.g. k value at 21 days was measured at t=28 actual days since the data used here starts from 7th day.
[b] k value selected randomly out of the above 8 values for Monte Carlo simulation.

Table **3-7**: Resistance patterns of *E. coli* (n=229) isolates.*

Resistance profiles	No. of isolates (%)
TET	84 (36.7)
CHL	1 (0.4)
AMP	10 (4.4)
SPT-TET	2 (0.9)
CHL-TET	5 (2.2)
AMP-TIC	1 (0.4)
AMP-TET	58 (25.3)
AMP-CHL	2 (0.9)
AMP-TET-TIC	7 (3.1)
AMP-SPT-TET	10 (4.4)
AMP-CHL-TET	3 (1.3)
AMP-TET-TIC-XNL	1 (0.4)
AMP-TET-TIC-TIM	1 (0.4)
AMP-SPT-TET-TIC	9 (3.9)
AMP-GEN-SPT-TET	1 (0.4)
AMP-CHL-TET-TIC	3 (1.3)
AMP-CHL-SPT-TET	4 (1.7)
AMP-TET-TIC-TIM-XNL	1 (0.4)
AMP-GEN-SPT-TET-TIC	1 (0.4)
AMP-CHL-SPT-TET-XNL	1 (0.4)
AMP-CHL-TET-TIC-TIM-XNL	2 (0.9)
AMP-CHL-SPT-TET-TIM-XNL	1 (0.4)
AMP-CHL-SPT-TET-TIC-XNL	1 (0.4)
AMP-CHL-SPT-TET-TIC-TIM	2 (0.9)
AMP-CHL-SPT-TET-TIC-TIM-XNL	15 (6.6)
AMP-CHL-GEN-SPT-TET-TIC-TIM-XNL	3 (1.3)

Antimicrobials used in disk diffusion assay: AMP, ampicillin; CHL, chloramphenicol; GEN, gentamicin; ENO, enrofloxacin; SPT, spectinomycin; TET, tetracycline; TIC, ticarcillin; TIM, ticarcillin/clavulanic acid; XNL, ceftiofur.
* Interpretive criteria: NCCLS document M31-A2 for bacteria isolated from animals (2002).

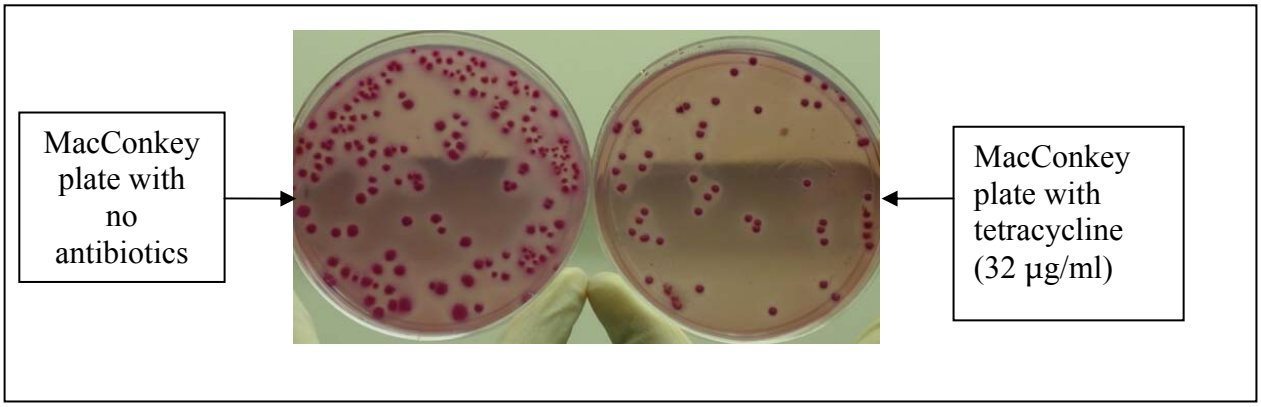


Figure **3.1**: Gram-negative enteric bacteria on control plate and plate with tetracycline (32 µg/ml) for the same fecal sample.

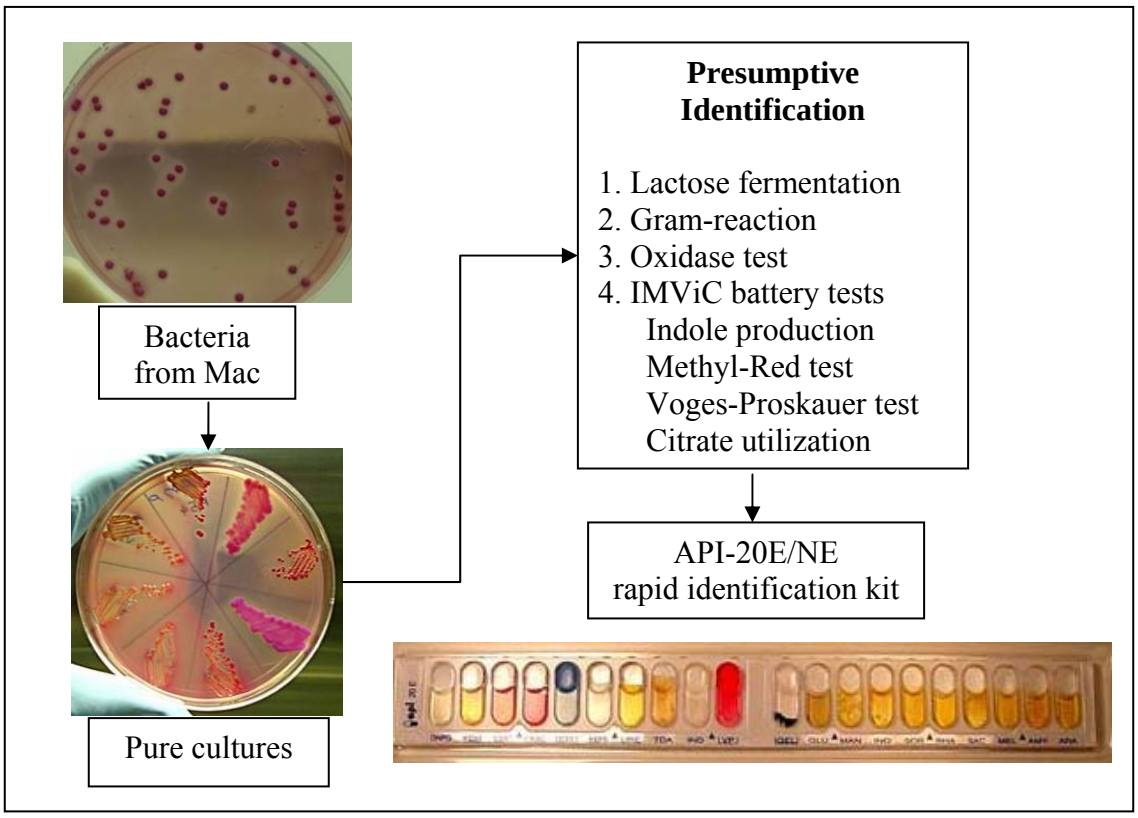
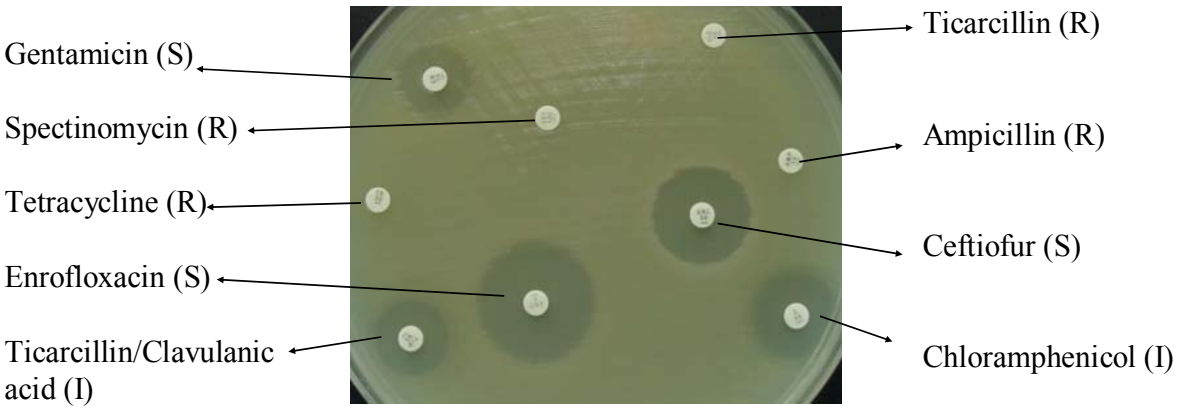


Figure **3.2**: Species identification of antimicrobial-resistant GN-EB.



Interpretive criteria: NCCLS document M31-A2 for bacteria isolated from animals (2002).

		Zone Diameter (mm)			*E. coli*	*P. aeru*
Antimicrobial agents	Disk content	S	I	R	25922	27853
Ampicillin*	10	≥ 17	14-16	≤ 13	16-22	-
Ceftiofur	30	≥ 21	18-20	≤ 17	26-31	14-18
Chloramphenicol*	30	≥ 18	13-17 (Flexible)	≤ 12	21-27	-
Enrofloxacin	5	≥ 23	17-22	≤ 16	32-40	15-19
Gentamicin*	10	≥ 15	13-14	≤ 12	19-26	16-21
Spectinomycin	100	≥ 14	11-13	≤ 10	21-25	10-14
Tetracycline*	30	≥ 19	15-18	≤ 14	18-25	-
Ticarcillin*	75	≥ 20	15-19	≤ 14	24-30	21-27
Ticarcillin-Clavulanic acid*	75/10	≥ 20	15-19	≤ 14	24-30	20-28

* Based on human interpretive criteria

Figure **3.3**: Disk diffusion assay.

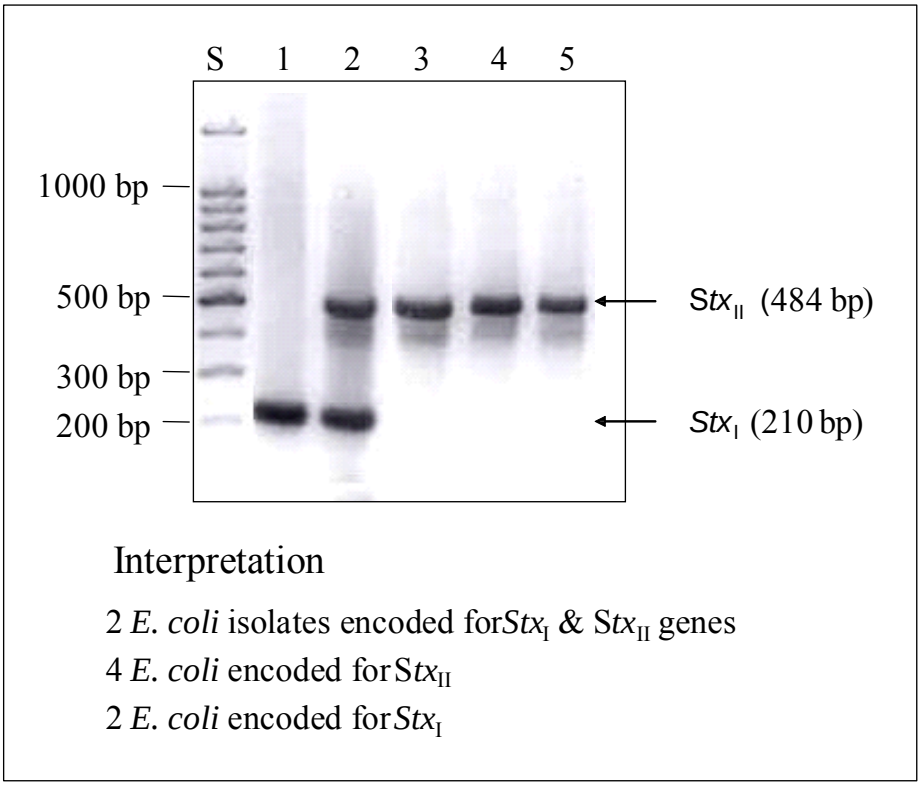


Figure **3.4**: PCR assay of shiga toxin encoding genes I and II (n=229 antibiotic-resistant *E. coli* isolates).

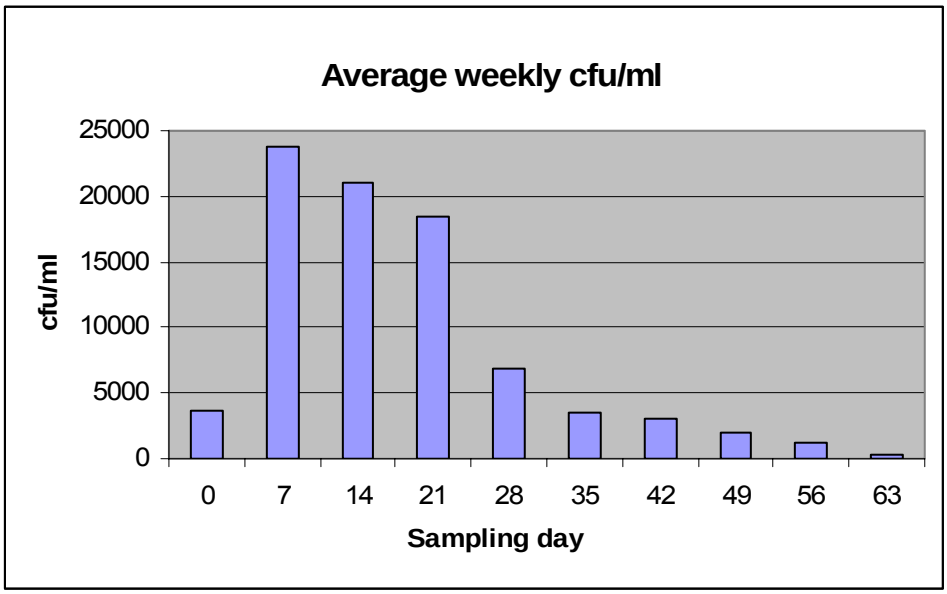
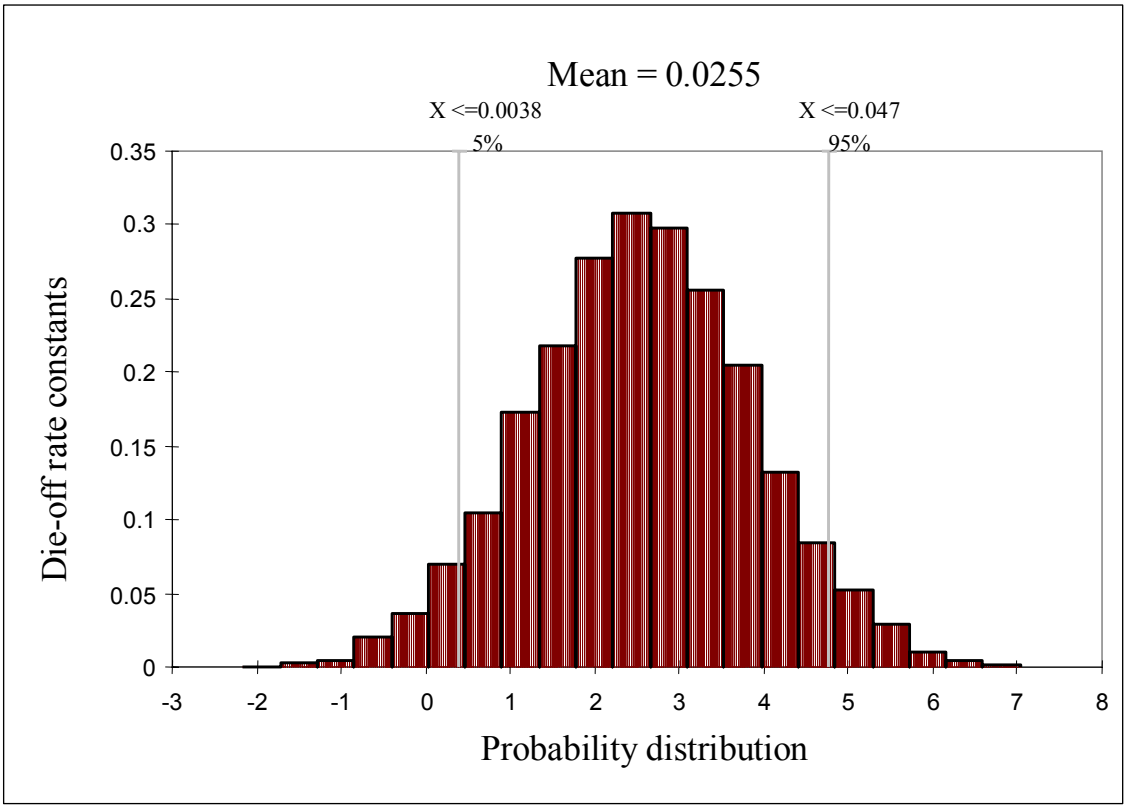


Figure **3.5**: Growth curve of A92 multidrug-resistant *E. coli* in sterile water at ~7^{o}C.



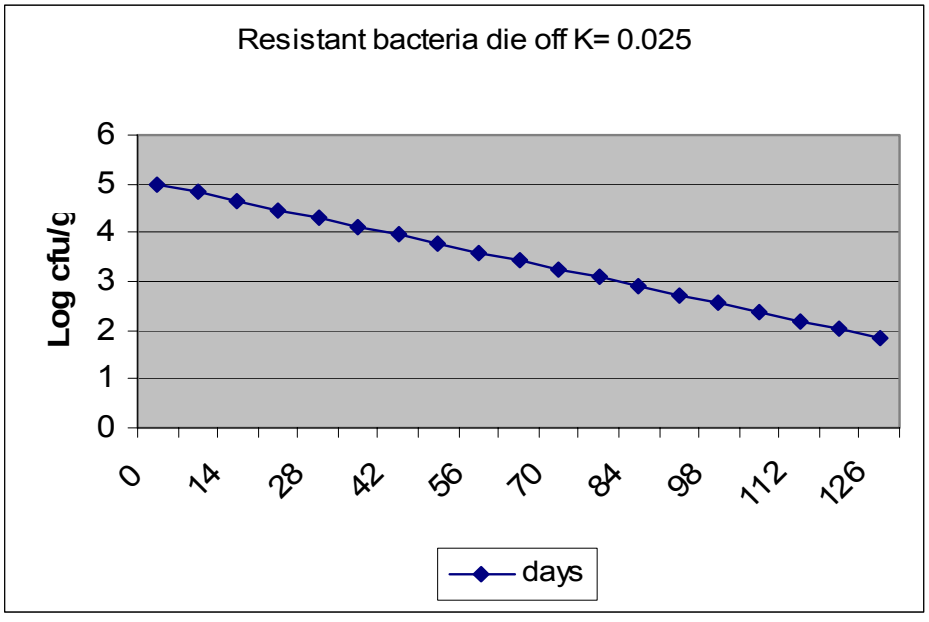
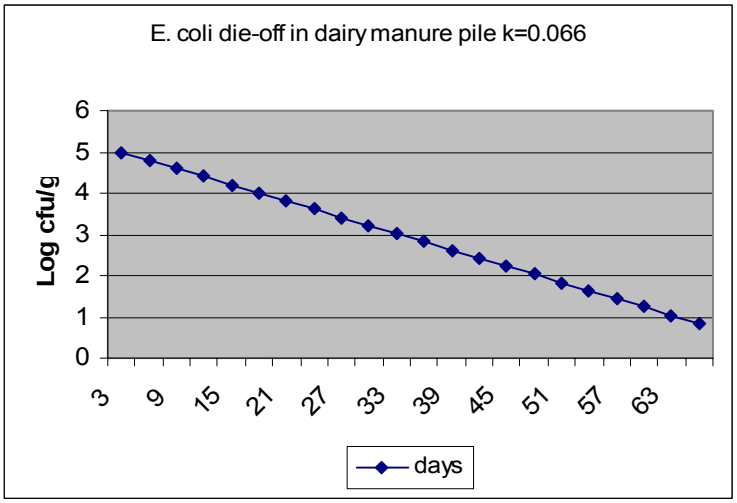
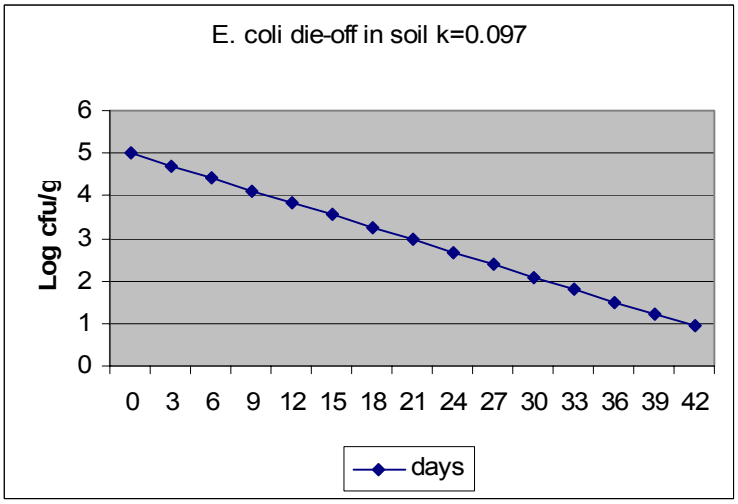


Figure **3.6**: Probability distribution of die-off rate constant using Monte Carlo simulation and predicted survival of multidrug-resistant *E. coli* in water at 7^{o}C.





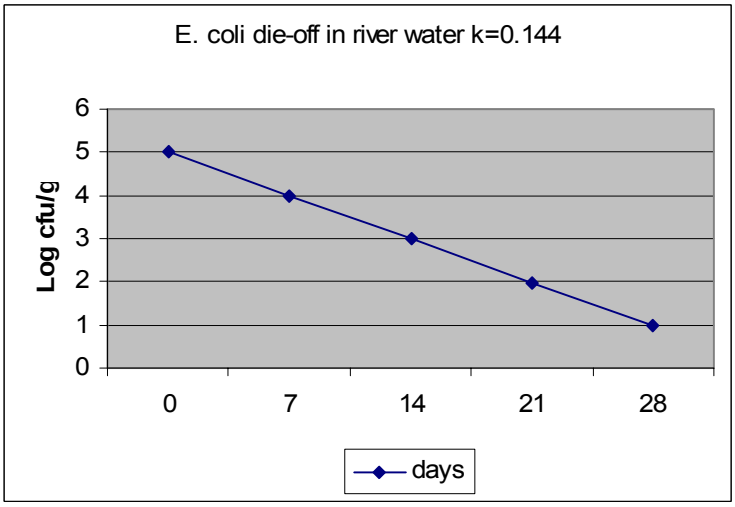


Figure **3.7**: Predicted survival of multidrug-resistant *E. coli* A92 under different conditions.

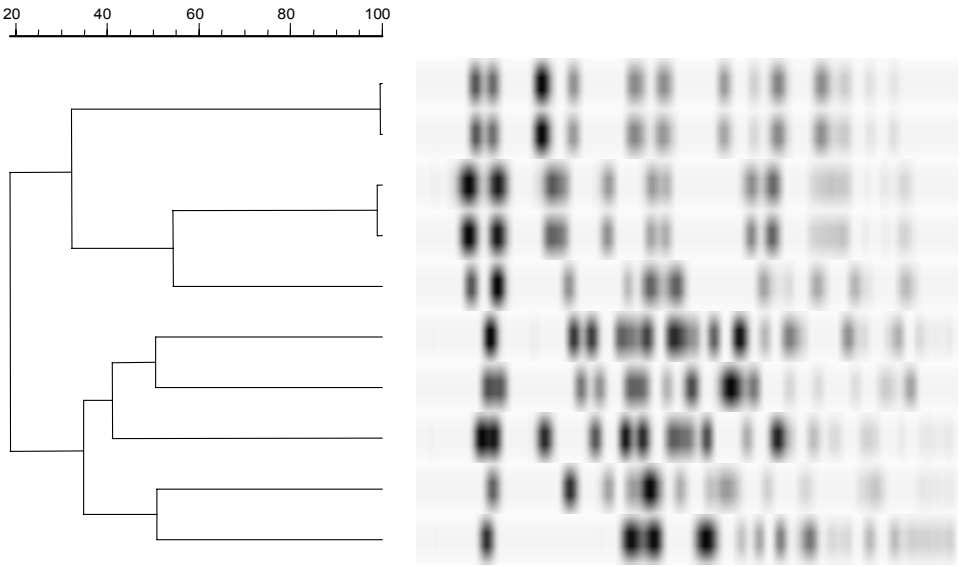


Figure **3.8**: PFGE patterns of *E. coli* isolates using dendogram analysis.

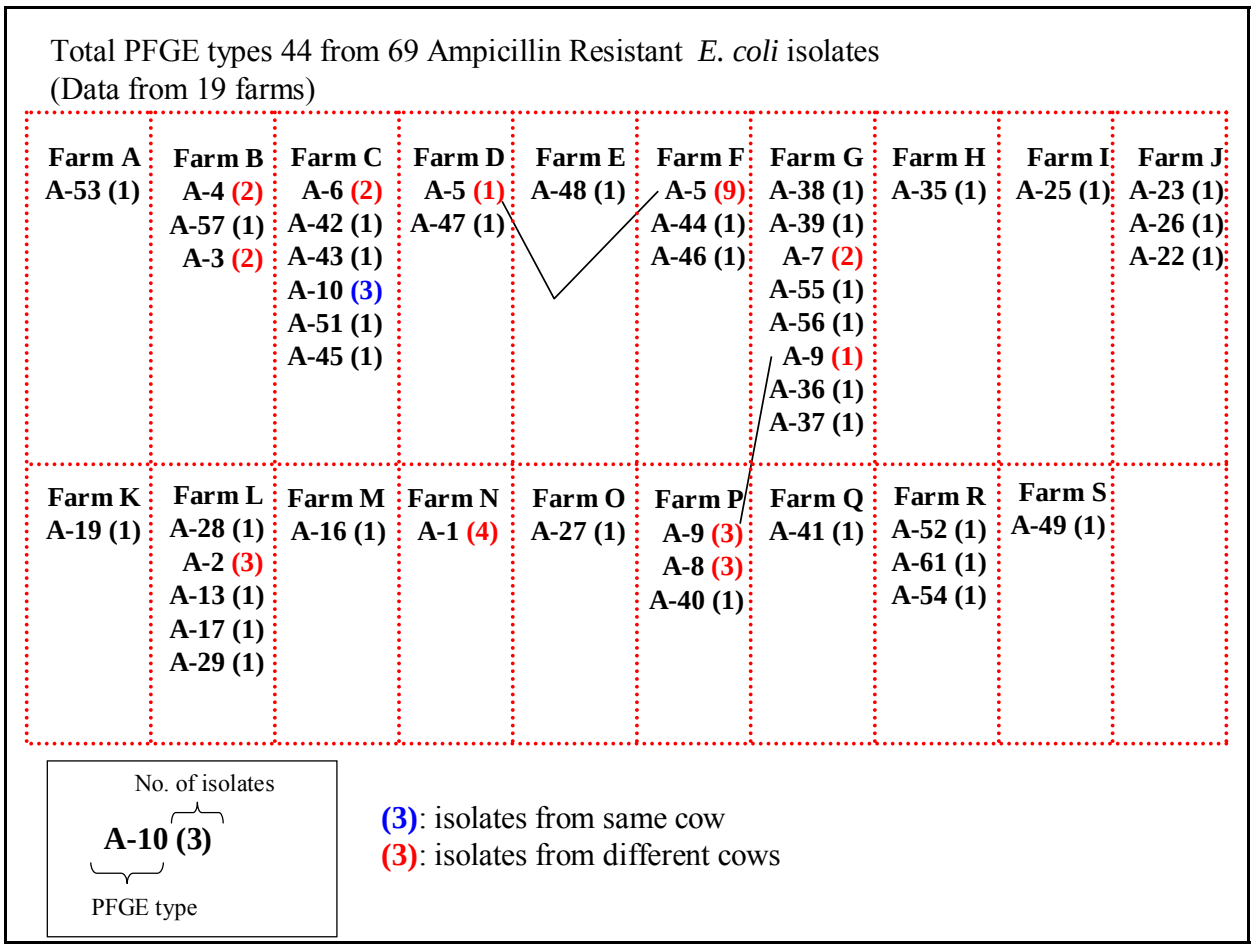


Figure **3.9**: Distribution of PFGE subtypes of ampicillin-resistant *E. coli* isolates from lactating cattle in dairy herds (lines show similar PFGE pattern observed on different farms).

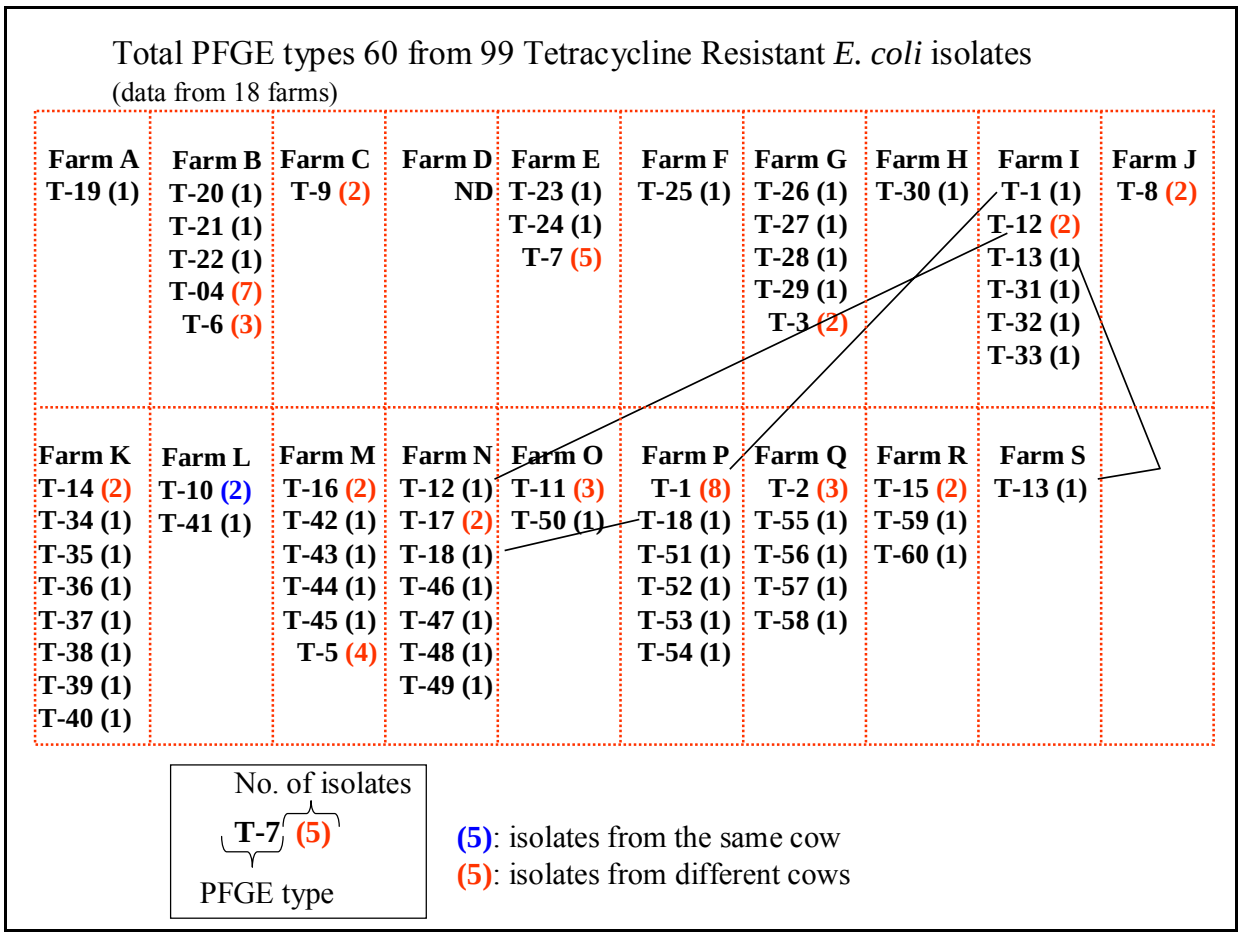


Figure **3.10**: Distribution of PFGE subtypes of tetracycline-resistant *E. coli* isolates from lactating cattle in dairy herds (lines show similar PFGE pattern observed on different farms).

3.6 REFERENCES

Allen, K. J., and C. Poppe. 2002. Occurrence and characterization of resistance to extended-spectrum cephalosporins mediated by beta-lactamase CMY-2 in *Salmonella* isolated from food-producing animals in Canada. Can. J. Vet. Res. 66:137-144.

Altmann, G., I. Sechter, I. Braunstein, and C. B. Gerichter. 1984. *Citrobacter koseri* isolated in Israel, 1972-83. Isr. J. Med. Sci. 20:1056-1060.

Austin, B. 1985. Antibiotic pollution from fish farms: effects on aquatic microflora. Microbiol. Sci. 2:113-117.

Baquero, F., M. C. Negri, M. I. Morosini, and J. Blazquez. 1998. Antibiotic-selective environments. Clin. Infect. Dis. 27 Suppl 1:S5-11.

Barrett, T. J., H. Lior, J. H. Green, R. Khakhria, J. G. Wells, B. P. Bell, K. D. Greene, J. Lewis, and P. M. Griffin.1994. Laboratory investigation of a multistage food-borne outbreak of *Escherichia coli* O157:H7 using pulse-field gel electrophoresis and phage typing. J. Clin. Microbiol. 32:3013-3017.

Bates, J., J. Z. Jordens, and D. T. Griffiths. 1994. Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. J. Antimicrob. Chemother. 34:507-514.

Bohm, H., and H. Karch. 1992. DNA fingerprinting of Escherichia coli O157:H7 strains by pulsed-field gel electrophoresis. J. Clin. Microbiol. 30:2169-2172.

Bouza, E., and E. Cercenado. 2002. *Klebsiella* and *Enterobacter*: antibiotic resistance and treatment implications. Semin. Respir. Infect. 17:215-230.

Caugant, D. A., B. R. Levin, and R. K. Selander. 1981. Genetic diversity and temporal variation in the E. coli population of a human host. Genetics. 98:467-490.

Chee-Sanford, J. C., R. I. Aminov, I. J. Krapac, N. Garrigues-Jeanjean, and R. I. Mackie. 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. Appl. Environ. Microbiol. 67:1494-1502.

Chick, H. 1908. Investigation of the laws of disinfection. J. Hyg. 8: 655.

Chiew, Y. F., S. F. Yeo, L. M. Hall, and D. M. Livermore. 1998. Can susceptibility to an antimicrobial be restored by halting its use? The case of streptomycin versus *Enterobacteriaceae*. J. Antimicrob. Chemother. 41:247-251.

Corpet, D. E. 1996. Microbiological hazards for humans of microbial growth promoter use in animal production. Rev. Med. Vet. 147:851-862.

Farina, C., A. Goglio, G. Conedera, F. Minelli, and A. Caprioli. 1996. Antimicrobial susceptibility of *Escherichia coli* O157 and other enterohaemorrhagic *Escherichia coli* isolated in Italy. Eur. J. Clin. Microbiol. Infect. Dis. 15:351-353.

Food and Drug Administration. 1996. NUFLOR ® (florfenicol). FOI summary, NADA 141-063 (original). Food and Drug Administration, Laurel, Md.

Gautom, R.K. 1997. Rapid pulsed-field gel electrophoresis protocol for typing of Escherichia coli O157:H7 and other Gram-negative organisms in 1 day. J. Clin. Microbiol. 35:2977-2980.

Gonzalez, E. A., and J. Blanco. 1989. Serotypes and antibiotic resistance of verotoxigenic (VTEC) and necrotizing (NTEC) *Escherichia coli* strains isolated from calves with diarrhoea. FEMS Microbiol. Lett. 51:31-36.

Guillemot, D., and P. Courvalin. 2001. French Working Party to Promote Research to Control Bacterial Resistance, Better Control of Antibiotic Resistance. Clin. Infect. Dis. 33:542-547.

Harley, J. P. and L. M. Prescott (eds.). 1993. *In* Laboratory exercises in Microbiology, 2nd ed. Wm. C. Brown Publishers, Dubuque, IA.

Hinton, M., A. H. Linton, and A. J. Hedges. 1985. The ecology of *Escherichia coli* in calves reared as dairy-cow replacements. J. Appl. Bacteriol. 58:131-138.

Hornish, R. E., and S. F. Kotarski. 2002. Cephalosporins in veterinary medicine - ceftiofur use in food animals. Curr. Top. Med. Chem. 2:717-731.

Hunter, J. E., J. E. Corkill, A. G. McLennan, J. N. Fletcher, and C. A. Hart. 1993. Plasmid encoded beta-lactamases resistant to inhibition by clavulanic acid produced by calf faecal coliforms. Res. Vet. Sci. 55:367-370.

Institute of Medicine. 1989. Human health risks with subtherapeutic use of penicillin or tetracyclines in animal feed. Committee on human health risk assessment on using subtherapeutic antibiotics in animal feeds. National Academy Press, Washington, D. C.

Jaglan, P. S., F. S. Yein, R. E. Hornish, B. L. Cox, T. S. Arnold, R. D. Roof , and T. J. Gilbertson. 1992. Depletion of intramuscularly injected ceftiofur from the milk of dairy cattle. J. Dairy Sci. 75:1870-1876.

Jiang, X., J. Morgan, and M. P. Doyle. 2002. Fate of *Escherichia coli* O157:H7 in manure-amended soil. Appl. Environ. Microbiol. 68:2605-2609.

Jones, JK. 1971. Fecal coliform pollution in an agricultural environment. p.122. *in* M.S. Thesis, Dept of Micro., Colorado State University.

Khachatryan, A. R., D. D. Hancock, T. E. Besser, and D. R. Call. 2004. Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. Appl. Environ. Microbiol. 70:752-757.

Kim, H. H., M. Samadpour, L. Grimm, C. R. Clausen, T. E. Besser, M. Baylor, J. M. Kobayashi, M. A. Neill, F. D. Schoenknecht, and P. I. Tarr. 1994. Characteristics of antibiotic-resistant *Escherichia coli* O157:H7 in Washington State, 1984-1991. J. Infect. Dis. 170:1606-1609.

Kudva, I.T., K. Blanch, and C. J. Hovde. 1998. Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. Appl. Environ. Microbiol. 64:3166-3174.

Langlois, B. E., K. A. Dawson, T. S. Stahly, and G. L. Cromwell. 1984. Antibiotic resistance of fecal coliforms from swine fed subtherapeutic and therapeutic levels of chlortetracycline. J. Anim. Sci. 58:666-674.

Langlois, B. E., K.A. Dawson, I. Leak, and D. K. Aaron. 1988. Antimicrobial resistance of fecal coliforms from pigs in a herd not exposed to antimicrobial agents for 126 months. Vet. Microbiol. 18:147-153.

Lau, M.M., and S. C. Ingham. 2001. Survival of faecal indicator bacteria in bovine manure incorporated into soil. Lett. Appl. Microbiol. 33:131-136.

Levin, B. R., M. Lipsitch, V. Perrot, S. Schrag, R. Antia, L. Simonsen, N. M. Walker, F. M. Stewart. 1997. The population genetics of antibiotic resistance. Clin. Infect. Dis. 24:S9-S16.

Levy, S. B., J. P. Burke, and C. K. Wallace. 1987. Antibiotic use and antibiotic resistance worldwide. Rev. Infect. Dis. (Suppl. 3):9.

Levy, S. B. 1992. The antibiotic paradox. How miracle drugs are destroying the miracle. Plenum Publishing Corporation, NY.

Livermore, D. M. 1996. Are all beta-lactams created equal? Scand. J. Infect. Dis. 101:33-43.

Lu, L., M. E. Hume, K. L. Sternes, and S. D. Pillai. 2004. Genetic diversity of *Escherichia coli* isolates in irrigation water and associated sediments: implications for source tracking. Water Res. 38:3899-3908.

Mallman, W. L., and W. Litsky. 1951. Survival of selected enteric organisms in various types of soil. Amer. Pub. Health. 41:38-44

McLellan, S. L., A. D. Daniels, and A. K. Salmore. 2003. Genetic characterization of *Escherichia coli* populations from host sources of fecal pollution by using DNA fingerprinting. Appl. Environ. Microbiol. 69:2587-2594.

Mead, P.S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P.M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. Review. 5:607-625.

Medeiros, A. A. 1997. Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. Clin. Infect. Dis. 24:S19-S45.

Meng, J., and M. P. Doyle. 1998. Microbiology of Shiga toxin-producing *Escherichia coli* in foods, p. 92-111. *In* J. Kaper and A. O'Brien (eds.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington, D.C.

Mitchell, J. R., and M. J. Starzyk. 1975. Survival of salmonella and other indicator microorganisms. Can. Jour. Microbiol. 21:1420

Moore, J. A., J. Symth, S. Baker, and J. R. Miner (eds.). 1988. Special report 817, p. 18. Evaluating coliform concentrations in runoff from various animal waste management systems. Agricultural Experiment Stations, Oregon State University, Corvallis. Oregon.

Moreira, C. N., M. A. Pereira, C. S. Brod, D. P. Rodrigues, J. B. Carvalhal, and J. A. Aleixo. 2003. Shiga toxin-producing *Escherichia coli* (STEC) isolated from healthy dairy cattle in southern Brazil. Vet. Microbiol. 93:179-183.

Moustaoui, N., A. Soukri, N. Elmdaghri, M. Boudouma, and M. Benbachir. 2004. Molecular biology of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* responsible for digestive tract colonization. J. Hosp. Infect. 57:202-208.

National Committee for Clinical Laboratory Standards. 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 2nd ed, NCCLS document M31-A2. NCCLS, Wayne, Pennsylvania.

Nuru, S., G. W. Osbaldiston, E. C. Stowe, and D. Walker. 1972. Fecal microflora of healthy cattle and pigs. Cornell Vet. 62:242-253.

Orden, J. A., J. A. Ruiz-Santa-Quiteria, S. Garcia, D. Cid, and R. De La Fuente. 2000. In vitro susceptibility of *Escherichia coli* strains isolated from diarrhoeic dairy calves to 15 antimicrobial agents. J. Vet. Med. B. Infect. Dis. Vet. Public Health. 47:329-335.

Rice, E.W. and C. H. Johnson. 2000. Short communication: survival of *Escherichia coli* O157:H7 in dairy cattle drinking water. J. Dairy Sci. 83:2021-2023.

Schlichting, C., C. Branger, J. M. Fournier, W. Witte, A. Boutonnier, C. Wolz, P. Goullet, and G. Doring. 1993. Typing of *Staphylococcus aureus* by pulsed-field gel electrophoresis, zymotyping, capsular typing, and phage typing: resolution of clonal relationships. J. Clin. Microbiol. 31:227-232.

Schmidt, H., J. von Maldeghem, M. Frosch, and H. Karch. 1998. Antibiotic susceptibilities of verocytotoxin-producing *Escherichia coli* O157 and non-O157 strains isolated from patients and healthy subjects in Germany during 1996. J. Antimicrob. Chemother. 42:548-550.

Smith, D. L., A. D. Harris, J. A. Johnson, E. K. Silbergeld, and J. G. Morris Jr. 2002. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. Proc. Natl. Acad. Sci. 30:6434-6439.

Smith, H. W. 1975. Persistence of tetracycline resistance in pig *Escherichia coli*. Nature. 258:628-630.

Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33:2233-2239.

Tenover, F. C., and J. E. McGowan. 1996. Reasons for the emergence of antibiotic resistance. Am. J. Med. Sci. 311:9-16.

Van Den Bogaard, A.E., N. London, E. E. Stobberingh. 2000. Antimicrobial resistance in pig faecal samples from the Netherlands (five abattoirs) and Sweden. J. Antimicrob. Chemother. 45:663-671.

Werckenthin, C., S. Seidl, J. Riedl, E. Kiossis, G. Wolf, R. Stolla, and O. R. Kaaden. 2002. *Escherichia coli* isolates from young calves in Bavaria: in vitro susceptibilities to 14 anti-microbial agents. J. Vet. Med. B. Infect. Dis. Vet. Public Health. 49:61-65.

White, D. G., C. Hudson, J. J. Maurer, S. Ayers, S. Zhao, M. D. Lee, L. Bolton, T. Foley, and J. Sherwood. 2000. Characterization of chloramphenicol and florfenicol resistance in *Escherichia coli* associated with bovine diarrhea. J. Clin. Microbiol. 38:4593-4598.

Winokur, P. L., A. Brueggemann, D. L. DeSalvo, L. Hoffmann, M. D. Apley, E. K. Uhlenhopp, M. A. Pfaller, and G. V. Doern. 2000. Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC beta-lactamase. Antimicrob. Agents Chemother. 44:2777-2783.

Zhao, S., D. G. White, P. F. McDermott, S. Friedman, L. English, S. Ayers, J. Meng, J. J. Maurer, R. Holland, and R. D. Walker. 2001a. Identification and expression of cephamycinase bla(CMY) genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob. Agents Chemother. 45:3647-3650.

Zhao, S, D.G. White, B. Ge, S. Ayers, S. Friedman, L. English, D. Wagner, S. Gaines, J. Meng. 2001b. Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. Appl. Environ. Microbiol. 67:1558-1564.

Chapter 4

MOLECULAR CHARACTERIZATION OF TETRACYCLINE-RESISTANT DETERMINANTS IN ENTERIC *ESCHERICHIA COLI* FROM LACTATING DAIRY CATTLE

4.1 ABSTRACT

Tetracycline-resistant *E. coli* (n=113) were analyzed for tet determinants (A-E and G) and the pathways that mediate tetracycline-resistance. Tet determinants *tet*(B) and *tet*(A) were detected in 93 and 7% of the isolates, respectively. On subculturing tetracycline-resistant isolates for several generations on antibiotic free medium, it was observed that isolates were able to retain tetracycline-resistance even in the absence of selective pressure. DNA-DNA hybridization assays revealed that *tet* determinants were located on the chromosome. A sub-genomic library of *E. coli* (T8) was created in pTrcHis. Tetracycline-resistant recombinant clones (DH5α) were sequenced. The sequenced region showed 96-99% homology to *tetR* and *tetA* (class B) genes on Tn*10* of *Shigella flexneri*. PCR amplification with primers for 5' and 3' ends of Tn*10* revealed the presence of *tet*C, *tet*D, and the right transposase gene (99% homology, *Shigella flexneri*) in wild type *E. coli* (T8). The 4632 bp sequence consisted of 5 open reading frames. The sequence has been assigned accession number AY528506 by the National Center for Biotechnology Information. This is the first report of a chromosomally located *tet* efflux pump associated with transposon Tn*10* in enteric *E. coli* isolated from lactating cattle. The results of this study show that one of the pathways through which tetracycline-resistance can be mediated through the transposable element Tn*10* that harbors the *tet*(B) determinant. The findings of this study suggest that commensal enteric *E. coli* from lactating cattle can be a significant reservoir for tetracycline-resistance determinants.

4.2 INTRODUCTION

Tetracyclines are one of the most widely used class of antibiotics in the animal industry (Chopra and Roberts, 2001). The growth promoting properties of tetracyclines were first reported by Stockstad et al. (1949), when young chicks fed with chlortetracycline showed improvement in growth rate. Following this report chlortetracycline and oxytetracycline were extensively used as animal growth promoters in swine and cattle (Gustafson and Kiser, 1985). The tetracyclines still continue to be one of the most widely used antibiotics in human medicine and animal agriculture as they are relatively cheap, can be administered orally, and has relatively few side effects (Moellering, 1990; Standiford, 1990).

Resistance to tetracyclines was first detected in the 1950s and became more apparent by the 1970s when it was widely reported among *Enterobacteriaceae*, staphylococci, streptococci, and *Bacteroides* spp. (Levy, 1984). Tetracycline-resistance determinants are now spread wide among bacterial species and have been identified in as many as 39 Gram-negative and 22 Gram-positive bacteria (Chopra and Roberts, 2001). Tetracycline-resistance is mediated through ribosomal protection proteins and through efflux mechanisms (Burdett, 1986; Roberts, 1996).

In most Gram-negative species, bacterial resistance is due to acquisition of an operon which consists of a efflux gene *tet*(A) and a repressor gene *tet*(R) that are divergently transcribed from overlapping operator regions. Nine types of efflux genes consisting of the above operon have been described so far in Gram-negative bacteria (*tet* A to E, G, and H to J) (Schnabel and Jones 1999). Spread of tetracycline efflux genes is dictated by the genes or genetic elements they are associated with. The *tet*(B) efflux can be transferred between *Actinobacillus* and *Hemophilus* species through conjugative plasmids, whereas the same gene is not mobile in *Treponema* species (Roe et al., 1995; Roberts et al., 1996). The *tet*(E) gene is associated with large plasmids

but these plasmids are neither mobile nor conjugative while *tet*(M) is associated with conjugative mobile elements in *Haemophilus ducreyi* (DePaola et al., 1988; Roberts, 1989).

A chromosomal tetracycline efflux system associated with multiple antibiotic resistance (MAR locus) has also been observed in *E. coli* (George and Levy, 1983). The resistance is mediated by mutations in the negative regulator (MarR) of the mar operon (Alekshum and Levy, 1997; Levy, 1992). A mutation in the *marR* region causes over-expression of *marA*, a transcriptional activator of a common group of promoters which increases the expression of the multiple drug efflux pump AcrAB in *E. coli* (Oethinger et al., 2000). *Escherichia coli* exposed to increasing concentrations of tetracycline or chloramphenicol may select for mutations in the *marR* region that enhances intrinsic resistance to a variety of antibiotics including penicillins, cephalosporins, rifampin, nalidixic acid, and quinolones mediated by the AcrAB efflux system (George and Levy, 1983; Levy, 1992).

The diverse ways efflux genes are associated with various mobile genetic elements of Gram-negative bacteria have great implications for the way these genes are sustained and shared in a microbial population. The objective of the present study was to determine the molecular characteristics of tetracycline-resistant determinants in enteric *E. coli* isolated from lactating cattle.

4.3 MATERIAL AND METHODS

4.3.1 Tetracycline-resistance determinants

A total of 113 tetracycline-resistant enteric *E. coli* isolated form lactating dairy cattle were screened for tetracycline genes. Specific primers used for amplifying *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G) genes are listed in Table **4-1**. The PCR assay was performed in 25 µl

reaction volume using puReTaq^{TM} Ready-To-Go-PCR Beads containing all the PCR reagents (Amersham Biosciences, NJ). The PCR conditions were similar to that reported by Ng et al. (2001).

4.3.2 Effect of subculturing on non-selective media

Tetracycline-resistant enteric *E. coli* were grown on Muller-Hinton agar without any antibiotics. The isolates were plated on a fresh Muller-Hinton agar plate without tetracycline after 24 hours of incubation at 37^{o}C. The isolates were subcultured for 8 generations on tetracycline-free medium. The isolate was then re-plated on MacConkey's with tetracycline (32 µg/ml) and incubated at 37^{o}C.

4.3.3 DNA-DNA hybridization

Small- and large-size plasmids were obtained using an alkaline lysis protocol with SDS as described by Sambrook and Russell (2001). Small-size plasmids were electrophoresed on a 0.8% agarose gel while large-size plasmids were electrophoresed on a 1% PFGE gel. Genomic DNA-PFGE was performed using one day PFGE protocol developed by Gautom (1997). The bacterial plugs were digested with *Xba*I and run on Chef Mapper^{TM} system (Bio-Rad Laboratories, CA). The running conditions for large plasmids and genomic DNA were as follows: initial switch time 2.16 secs, final switch time 35.07 secs, run time 14 hours, angle 120^{o}, gradient 6.0 V/cm, temperature 14^{o}C, and ramping factor was kept linear.

All the gels were blotted with a vacuum blotter according to the manufacturers' instructions (Bio-Rad Laboratories, CA). Positively-charged hybond-N^{+} nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) was used for blotting. Small plasmids were blotted for 90 min, while large plasmids and genomic DNA were first depurinated (0.25 N HCI for 15 min) and then blotted for 180 min.

4.3.4 Southern blotting assay

PCR amplified products *tet*(A) and *tet*(B) were purified and used as probes for the southern blotting assay. A DNA labeling and detection kit (Roche Molecular Biochemicals, Mannheim, Germany) which uses digoxigenin (DIG) to randomly label DNA probes was used. Briefly, *tet*(A) and *tet*(B) amplified products were denatured by heating in a boiling water bath (10 min) and immediately chilled on ice. Random hexanucleotides (10X, 2 µl) were added to hybridize to the denatured template. Klenow enzyme (1µl) was added to create a complementary strand to the template, using the hybridized hexonucleotides as primers and a mix of dNTPs (2µl) containing DIG labeled dUTP for elongation. The reaction mixture was incubated overnight at 37^{o}C and kept at -20^{o}C until further use. The DIG-labeled probes were used to probe blotted nucleic acids by standard southern-blot methods described by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The hybridized probes were detected with anti-digoxigenin-AP Fab-fragments and then visually detected after addition of colorimetric substrate NBT/BCIP.

4.3.5 *E. coli* isolate for *tet*(B) genomic library

A tetracycline-resistant *E. coli* isolate (T8) devoid of extrachromosomal DNA was selected for sub-genomic library construction. In this isolate the presence of *tet*(B) was confirmed by PCR. A DNA-DNA hybridization assay revealed that the *tet*(B) was located in the chromosome.

4.3.6 Sub-Genomic library construction

The genomic library was constructed following the protocols described by Ausubel et al. (1998) and Sambrook and Russell (2001). An overview of the library construction is shown in Figure **4.1**. The techniques are described in detail in Appendix B. Briefly the library was created first by a large-scale preparation of genomic DNA by a CTAB and NaCl method. The size of genomic DNA insert suitable for *tet*(B) cloning was determined to be 2.5 to 3.5 kb. Genomic DNA was digested with *Sau3A1* and DNA fragments varying from 2.5 to 3.5 kb were cut and purified from the gel.

The vector pTrcHis (Invitrogen, Carlsbad, CA.) was used for cloning the DNA library. The vector was linearized with *Bam*H I at 37^{o}C and dephosphorylated before cloning. The linearized vector ligated to genomic DNA inserts (2.5 to 3.5 kb) was transformed into electro-competent cells (DH5α) using electroporation. Transformed cells were selected on LB agar with ampicillin (50 μg/ml). Recombinant clones that were ampicillin resistant were screened for tetracycline (12 μg/ml) resistance (Figure **4.1**).

4.3.7 DNA sequencing

The recombinant plasmid from cells with tetracycline-resistance was sequenced using standard pTrc F and pTrc R primers (Invitrogen, Carsbad, California) (Table **4-1**). The whole insert was sequenced by using primers pairs F-II, R-II and F-III, R-III (Table **4-1**). Primers were designed using Oligo 6.6 software (Molecular Biology Insites, Inc, Cascade, Colorado). High stringency and T_m above 60^{o}C were the criteria used for designing primers. The sequencing and primer design was done at the Nucleic Acid Facility of the Penn State University, University Park, PA. The reverse primer R-IV was designed using the sequence of the insert DNA. This primer was used with primer IS*10*F-R for the 5' and 3' ends of Tn*10*. Another set of primers (R-

V, F-V) was designed to obtain the complete sequence (Table **4-1**). The relative location of different primers used for sequencing insert DNA from recombinant plasmid and DNA from tetracycline-resistant *E. coli* T8 is shown in Figure **4.2**.

4.4 RESULTS

4.4.1 Tetracycline-resistance determinants

On PCR analysis 105 (93%) and 8 (7%) isolates encoded for *tet*(B) and *tet*(A), respectively (Figure **4.3**).

4.4.2 Effect of subculturing tetracycline-resistant *E. coli* on non-selective media

Tetracycline-resistant *E. coli* isolates retained resistance to tetracycline even after subculturing for 8 generations on antibiotic-free medium. The isolates expressed resistance to oxytetracycline (32 μg/ml).

4.4.3 Locating presence of tetracycline-resistant determinants

Small- and large-size plasmids and genomic-DNA from 30 isolates with *tet*(B) and all 8 isolates with *tet*(A) were blotted and probed with their respective gene probes. None of the plasmids hybridized with the probes. DNA-DNA hybridization of *tet* probes with genomic DNA was detected (Figure **4.4**). Only one fragment per genomic DNA-PFGE lane exhibited color reaction indicating a single copy of *tet*(B) was present in the genome. The result showed that the efflux genes were located on the genomic DNA and not on the plasmids of the *E. coli* isolates (Figure **4.4**).

4.4.4 Genotypic characteristics of Tetracycline-resistance

Twenty nine recombinant clones from LB agar with tetracycline (12 µg/ml) were randomly collected. All the isolates showed the presence of the cloned vector. All the recombinant clones were also able to grow at 16µg/ml of tetracycline, confirming resistance to tetracycline (NCCLS, 2002). The plasmids were isolated from a randomly selected clones and 2418 bp cloned insert was sequenced using 3 primer sets (Table **4-1** and Figure **4.2**). The sequence information was used to design reverse primer (R-IV) which was paired with the IS10F-R primer. A 2274 bp of PCR product was obtained from the DNA extract of T8 isolate. The PCR product was sequenced using an additional set of primers (F-V and R-V).

All the sequences were aligned using the Basic Local Alignment Search Tool (BLAST) algorithm for pair-wise comparison of 2 sequences. Overlapping sequences were deleted and a single sequence was created which contained 4692 base pairs. The sequence was compared with available sequences in the NCBI nucleotide database by using BLAST pair-wise comparison. A 99% match was observed with *Shigella flexneri* Tn*10* (Accession no AF162223) and to open reading frames of *tet*(R), *tet*(A), *tet*(C), *tet*(D), and IS*10* transposase (*tnp*) gene. The cloned insert that expressed tetracycline-resistance was observed to encode for tetracycline repressor protein *tet*(R) and tetracycline efflux pump protein *tet*(A). Tetracycline-resistant recombinant clones carried inserts with only these 2 genes. The other genes that constituted the remaining sequence included *tet*(C), *tet*(D), and IS*10 tnp*. The presence of the open reading frame of IS*10 tnp*, a functional right transposase gene indicated that the efflux pump was associated with Tn*10* (Figure **4.5**).

.

4.5 DISCUSSION

Since the 1940s, food animal producers have used antibiotics to prevent and treat infectious diseases in livestock. Resistance to tetracycline has a history almost as long as its use. In 1953, the first tetracycline-resistant bacterium, *Shigella dysenteriae,* was isolated (Falkow, 1975; Wasteson et al., 1994). The first multi-drug resistant *Shigella* with resistance to tetracycline, streptomycin, and chloramphenicol was isolated just 2 years later, in 1955 (Akiba et al., 1960; Falkow, 1975; Lima et al., 1995). In recent years tetracycline-resistance has been observed as a part of multidrug-resistance carrying integrons in pathogens like *Salmonella enterica* serovar Typhimurium DT104 from animals as well as humans (Hosek et al., 1997; Ng et al., 1999; Threlfall et al., 1994).

In our study tetracycline-resistant commensal enteric *E. coli* were isolated from 90 lactating cattle from 23 farms in Pennsylvania. This suggests that tetracycline-resistant *E. coli* are widely distributed in dairy environments. The majority of the isolates encoded for the efflux-based *tet*(B) (Figure **4.3**). Marshall et al. (1983) also observed that the majority of lactose-fermenting coliforms from human and animal origin carried *tet*(B) (73.3%) and a subset carried the *tet*(A) (21.7%). Lee et al. (1993) observed *tet*(B) and *tet*(A) carried by plasmids in 35% and 1% of *E. coli* isolates of fecal origin from domestic pigs, respectively. Of the seven *tet* efflux reported in *E. coli,* studies have shown that no *E. coli* harbored more than one type of *tet* (Blake et al., 2003; Chopra and Roberts, 2001; Jones et al., 1992; Mendez et al., 1980). The only exception to this observation was that of Marshall et al. (1983), who showed that 3.5% of the lactose-fermenting coliforms carried 2 different *tet* genes.

Schnabel and Jones (1999) showed that five out of nine efflux determinants that encode for an efflux and a repressor protein, which included *tet*(A) and *tet*(B) determinants, were carried by plasmids of Gram-negative phylloplane bacteria in apple orchards in Michigan. They also observed that *tet*(A), *tet*(B), and *tet*(C) were associated with transposons. Others have shown that *tet* efflux genes are part of the transposons borne by plasmids in Gram-negative bacteria (Jones et al., 1992; Mendez et al., 1980). *tet*(B) has been shown to be located on the chromosome in *Haemophilus* spp. and *Moraxella catarrhalis* (Roberts et al., 1991; Roberts and Smith, 1980). *tet*(B) is not conjugative in these isolates, but can be moved by transformation using chromosomal DNA. Our study corroborates these findings, that *tet*(B) is associated with Tn*10*, and is located on the chromosome of commensal *E coli* rather than on a plasmid as frequently reported in the literature (Chopra and Roberts, 2001; Lee et al., 1993).

tet(B) is widely found in Gram-negative genera including *Escherichia*, *Enterobacter*, *Proteus*, *Salmonella, Actinobacillus*, *Haemophilus*, *Moraxella*, and *Treponema* spp. (Chopra and Roberts, 2001). It is highly likely that horizontal transfer of tetracycline-resistance occurs through the conjugation (Speer et al., 1992). Two types of conjugal elements have been described: conjugative plasmids and conjugative chromosomal elements called conjugal transposons (Salyers et al., 1990). Conjugative plasmids have undoubtedly contributed to the spread of efflux gene classes A to E within the Gram-negative bacteria and of classes K and L within Gram-positives (Hoshino et al., 1985; Lacks et al., 1986; LeBouguenec et al., 1990). The presence of *tet*(A) and *tet*(B) on the chromosome rather than on a plasmid is frequently reported in the literature was an intriguing observation, as the majority of Gram-negative efflux genes are normally associated with large plasmids, most of which are conjugative and belong to different incompatibility groups (Mendez et al., 1980; Jones et al., 1992).

Further sequence analysis of genomic DNA of the isolate indicated that *tet*(A) and *tet*(R) were associated with Tn*10* (Figure **4.5**). Tn*10* is a composite transposon flanked by inverted repeats of IS*10* that cooperate to mobilize the unique sequences (Kleckner 1989; Kleckner et al., 1996; Chalmers et al., 2000). The transposase enzyme that can mobilize either the flanking IS*10* elements or the whole of Tn*10* is encoded by the right IS*10 tnp* as the left IS*10 tnp* is defective. IS*10*/Tn*10* transposes by a non-replicative mechanism. The *tet* gene in Tn*10* is differentially regulated so that the repressor protein is synthesized before the efflux protein is expressed. The repressor protein will rebind to the DNA only when there is insufficient tetracycline (smaller than nanomolar amounts) present in the cell (Chopra, and Roberts, 2001).

Although Tn*10*s are one of the most thoroughly studied transposons, there are very few Tn*10* nucleotide sequences from different bacterial species reported in the NCBI nucleotide database. The partial sequence of Tn*10* with Tetracycline-resistance determinants showed 99% homology to Tn*10* isolated from different bacterial species. A few selected matches included plasmids from *Salmonella* Typhimurium (Accession no. AP005147) and *Serratia marcescens* (Accession no. BX664015) and chromosomally based Tn*10* associated *tet*(B) in *Salmonella* Typhi (Accession no. AY150213). High homology of our Tn*10* based *tet*(A) and *tet*(R) was observed with genomic based Tetracycline-resistance determinants of *Neisseria meningitidis* (Accession no. AB084246), but other Tn*10*-associated genes or insertion elements necessary for transposition were not observed (Takahashi et al., 2002). Truncated version of Tn*10* with only *tet*(A) and tet(R), also showed a 99% match with our sequence and was observed in *Pasteurella aerogenes* (Accession no. PAE278685) (Kehrenberg and Schwarz, 2001). In the majority of the Tn*10* sequences present in the NCBI nucleotide database, the presence of *tet*R, *tet*A, *tet*C, *tet*D, and the right transposase gene was a common feature; however, the open reading frames on the

5′ end of *tet*(R) were diverse. Association of *tet*(B) Tetracycline-resistance with Tn*10* in the chromosome of *E. coli* from the feces of lactating cattle indicate that the resistance can be maintained in these strains and also perhaps translocate to other susceptible species.

This is the first report of a Tn*10* based *tet*(B) in chromosome of *E. coli* isolated from the feces of lactating cattle. The sequence (4632 bp) can be accessed through NCBI GeneBank (accession no. AY528506, see Appendix B). The association of tet efflux genes with Tn*10* is significant as this transposable element has a broad host range and can play an important role in horizontal transmission of resistance. Once acquired, tetracycline-resistance in commensal enteric *E. coli* from lactating cattle, is likely to be conserved even in an environment free of tetracycline. The findings of this study suggest that commensal enteric *E. coli* could serve as a reservoir for tetracycline-resistance determinants in the dairy environment even in the absence of tetracycline selective pressure.

Table **4-1**: Primers used for sequence analysis.

Primer	Sequence	Target size	Reference
tet(A)F	5’ GCTACATCCTGCTTGCCTTC 3’	210 bp	Ng et al. (2001)
tet(A)R	5’ CATAGATCGCCGTGAAGAGG 3’		
tet(B)F	5’ TTGGTTAGGGGCAAGTTTTG 3’	659 bp	Ng et al. (2001)
tet(B)R	5’ GTAATGGGCCAATAACACCG 3’		
tet(C)F	5’ CTT GAG AGC CTT CAA CCC AG 3’	418 bp	Ng et al. (2001)
tet(C)R	5’ ATG GTC GTC ATC TAC CTG CC 3’		
tet(D)F	5’ AAA CCA TTA CGG CAT TCT GC 3’	787 bp	Ng et al. (2001)
tet(D)R	5’ GAC CGG ATA CAC CAT CCA TC 3’		
tet(E)F	5’ AAA CCA CAT CCT CCA TAC GC 3’	278 bp	Ng et al. (2001)
tet(E)R	5’ AAA TAG GCC ACA ACC GTC AG 3’		
tet(G)F	5’ CAG CTT TCG GAT TCT TAC GG 3’	844 bp	Ng et al. (2001)
tet(G)R	5’ GAT TGG TGA GGC TCG TTA GC 3’		
pTrc F-I	5’ GAGGTATATATTAATGTATCG 3’		Invitrogen
pTrc R-I	5’ GATTTAATCTGTATCAGG 3’		
F-II	5’ GCACCTTGCTGATGACTCT 3’		This study
R-II	5’ TCCGCATATGATCAATTCA 3’		
F-III	5’ CCAAGACCCGCTAATGAA 3’		This study
R-III	5’ AAGGCGTCGAGCAAAGC3’		
IS10F-R	5’ CTGATGAATCCCCTAATG 3’		Schnabel and Jones (1999)
R-IV	5’ TTTATCGGCAAGCTCTTT 3’		This study
F-V	5’ CTAGGAGCGGAAAACTGGA 3’		This study
R-V	5’ ATAAGCAGTTTCATACAACGG 3’		

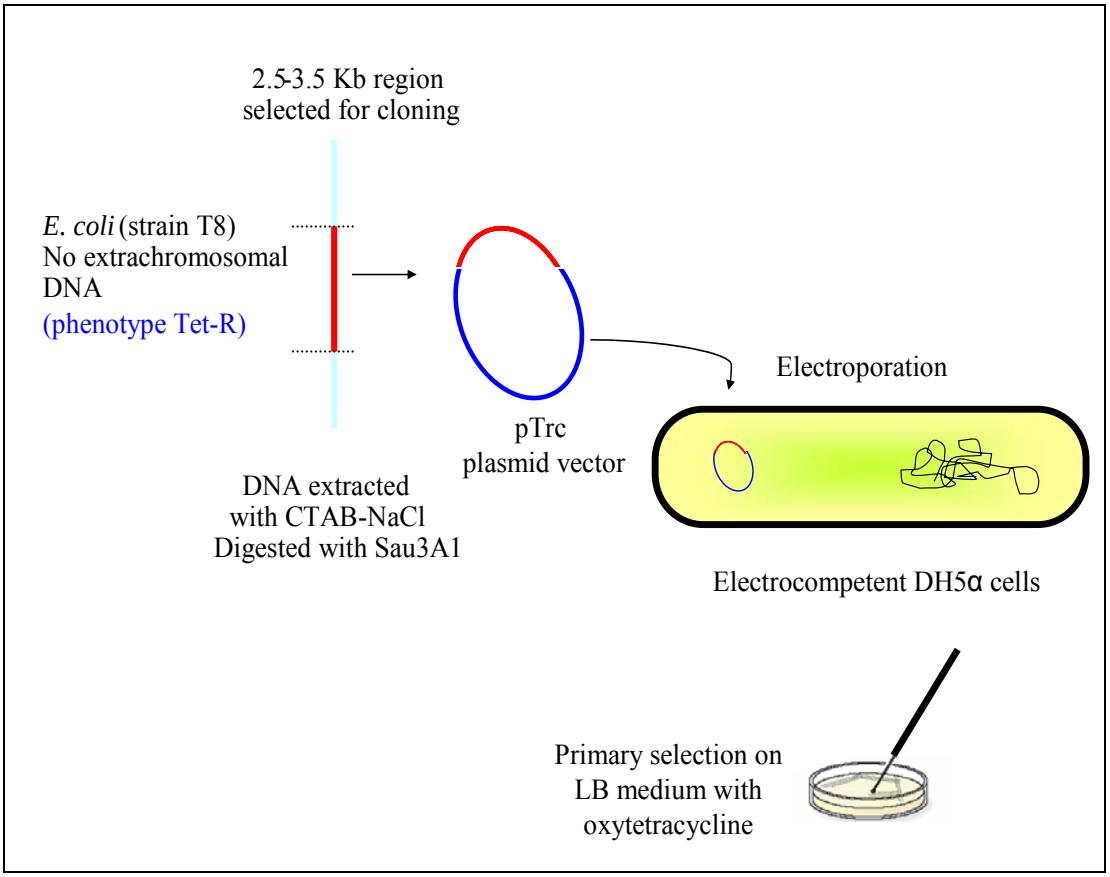


Figure **4.1**: *tet*(B) sub-genomic library construction.

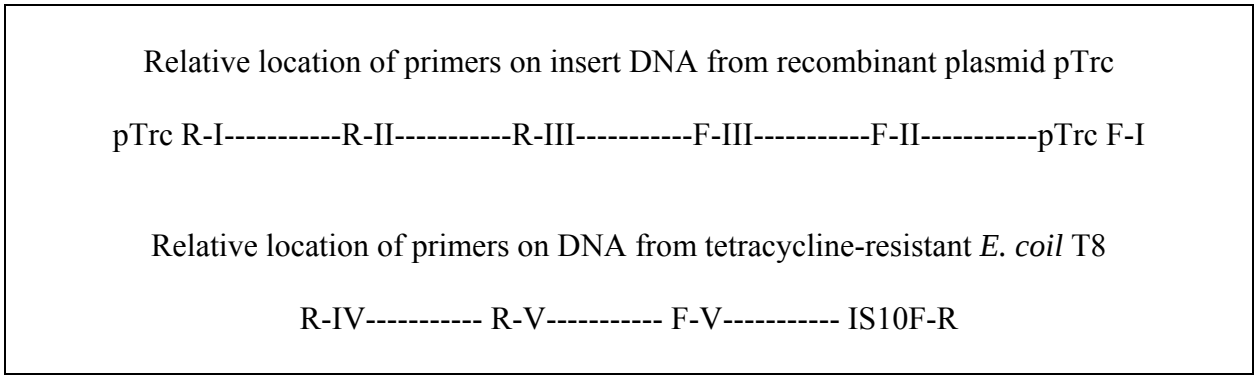


Figure **4.2**: Relative locations of different primers on insert and genomic DNA.

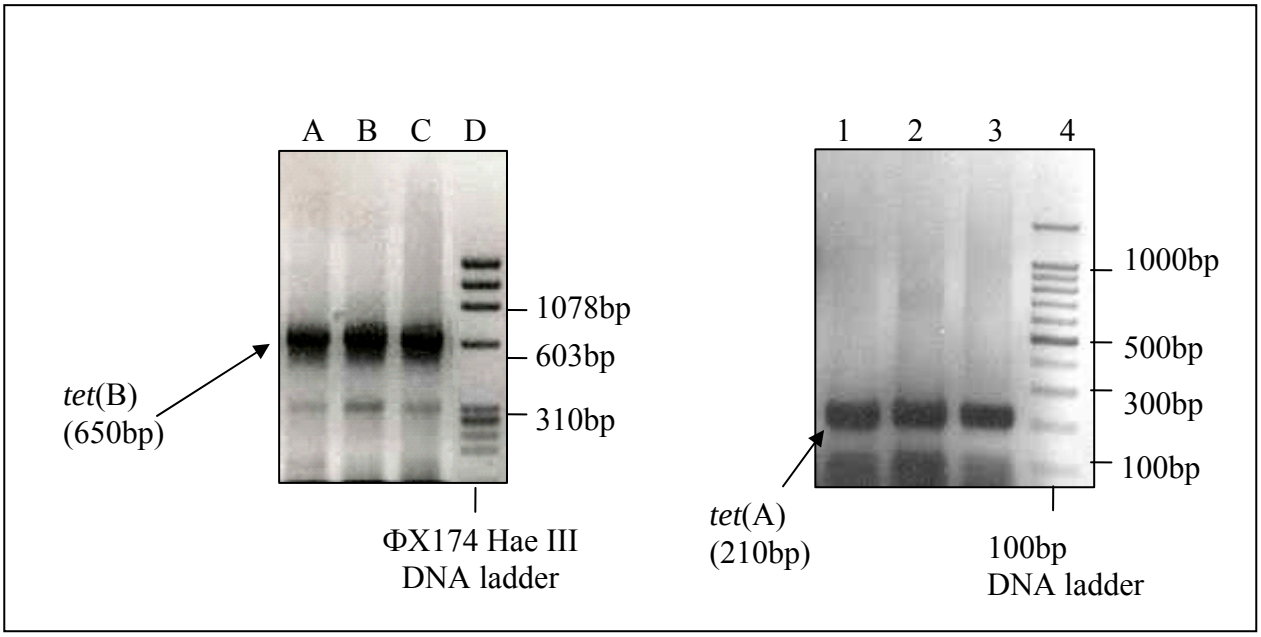
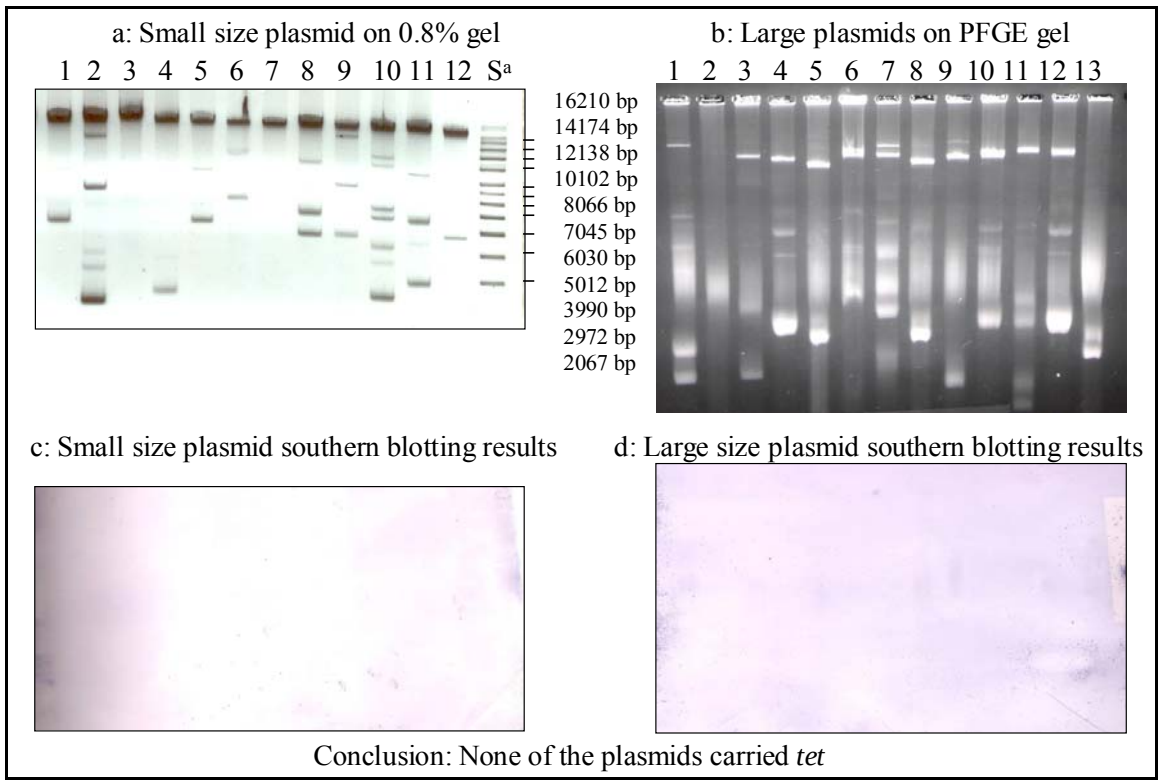


Figure **4.3**: PCR analysis of genes encoding tetracycline efflux pump based resistance. Lane A,B,and C represent PCR amplified produces of *tet*(B) gene. Lane D is ΦX174 Hae III DNA ladder. Lane 1, 2, and 3 represent PCR amplified produces of *tet*(A) gene. Lane 4 is 100bp DNA ladder.



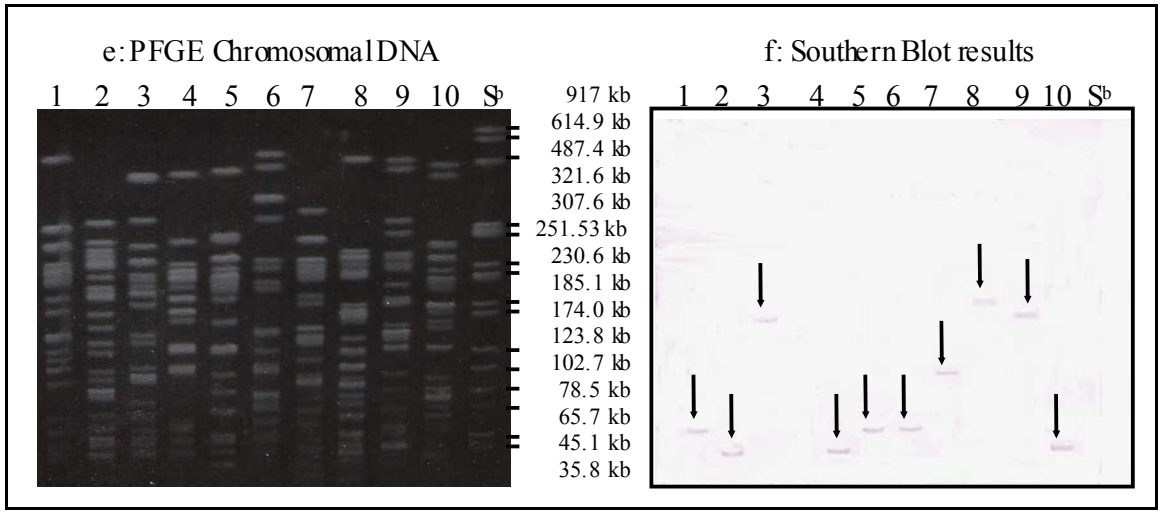


Figure **4.4**: Southern hybridization of small and large plasmids and genomic DNA. Fig. a: lanes 1 to 12 represent small size plasmids. Fig. b: lanes 1-12 represents large size plasmids on PFGE gel. Fig. c and d represent blotting results of the small and large plasmids from fig. a and b, respectively. Fig. e: lane 1-10 represent digested PFGE fragments. Fig. f: Blotting results with tet(B) probe of PFGE genomic DNA from fig. e. S^a: Supercoiled DNA ladder, S^b: *Salmonella* ser. Newport Standard strain am01144 (*Xba*I digested).

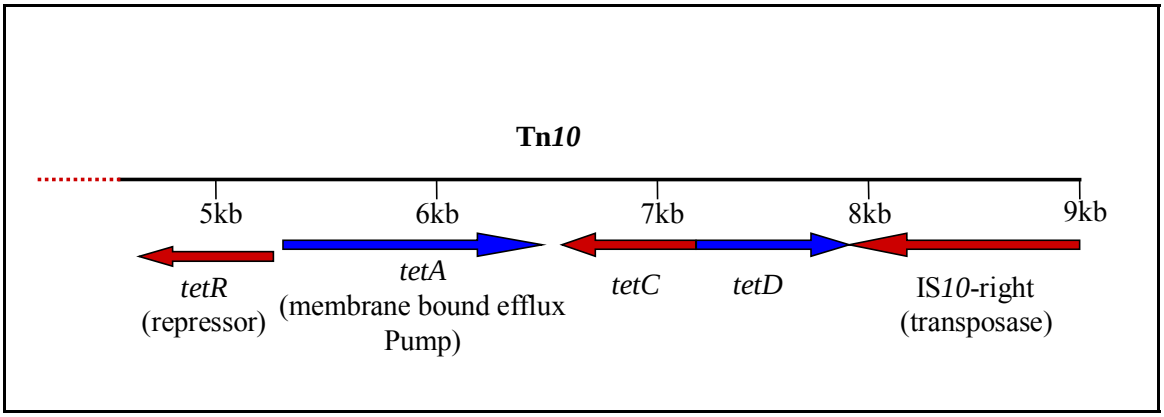


Figure **4.5**: Open reading frames of *tet* efflux pump and other Tn*10* genes.
Adapted from a figure by Lawley et al. (2000).

4.6 REFERENCES

Ausubel, F.M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhal. 1998. Current protocols in molecular biology. John Wiley and sons, Inc.

Akiba, T., K. Koyama, Y. Ishiki, S. Kimura, and T. Fukushima. 1960. On the mechanism of the development of multiple-drug-resistant clones of *Shigella*. Jpn. J. Microbiol. 4:219.

Alekshum, M. N., and S. B. Levy. 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulation. Antimicrob. Agents Chemother. 41:2067-2075.

Blake, D.P., R. W. Humphry, K. P. Scott, K. Hillman, D. R. Fenlon, and J. C. Low. 2003. Influence of tetracycline exposure on tetracycline-resistance and the carriage of tetracycline-resistance genes within commensal *Escherichia coli* populations. J. Appl. Microbiol. 94:1087-1097.

Burdett, V. 1986. Streptococcal tetracycline-resistance mediated at the level of protein biosynthesis. J. Bacteriol. 165:564-569.

Chalmers, R., S. Sewitz, K. Lipkow, and P. Crellin. 2000. Complete nucleotide sequence of Tn*10*. J. Bacteriol. 182: 2970–2972.

Chopra, I., and M. Roberts. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 65:232-260.

DePaola, A., P. A. Flynn, R.M. McPhearson, and S. B. Levy. 1988. Phenotypic and genotypic characterization of tetracycline- and oxytetracycline-resistant *Aeromonas hydrophila* from cultured channel catfish (*Ictalurus punctatus*) and their environments. Appl. Environ. Microbiol. 54:1861-1863.

Falkow, S. 1975. Infectious multiple drug resistance. Pion Ltd., London, United Kingdom.

Gautom, R.K. 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other Gram-negative organisms in 1 day. J. Clin. Microbiol. 35:2977-2980.

George, A. M., and S. B. Levy. 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: Involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. 155:531-540.

Gustafson, R. H., and J. S. Kiser. 1985. Nonmedical uses of the tetracyclines, p. 405-446. *In* J. J. Hlavka, and J. H. Boothe (ed.), Handbook of experimental pharmacology, vol. 78. Springer-Verlag KG, Berlin, Germany.

Hosek, G., D. D. Leschinksy, S. Irons, and T. J. Safranek. 1997. Multidrug-resistant *Salmonella* serotype Typhimurium. United States, 1996. Morb. Mortal. Wkly. Rep. 46:308-310.

Hoshino, T., T. Ikeda, N. Tomizuka, and K. Furukawa. 1985. Nucleotide sequence of the tetracycline-resistance gene of pTHT15, a thermophilic Bacillus plasmid: comparison with staphylococcal Tc^r controls. Gene. 37:131-138.

Jones, C. S., D. J. Osborne, and J. Stanley. 1992. Enterobacterial tetracycline-resistance in relation to plasmid incompatibility. Mol. Cell. Probes. 6:313-317.

Kehrenberg, C., and S. Schwarz. 2001. Molecular analysis of tetracycline-resistance in Pasteurella aerogenes. Antimicrob. Agents Chemother. 45:2885-2890.

Kleckner, N. 1989. Transposon Tn*10*. p. 227–268. *In* Berg, D.E. and M. M. Howe, M. M. (ed.), Mobile DNA, American Society for Microbiology, Washington, DC.

Kleckner, N., R. M. Chalmers, D. K. Kwon, J. Sakai, and S. Bolland. 1996. Tn*10* and IS10 transposition and chromosome rearrangements: mechanism and regulation *in vivo* and *in vitro* p. 49-82. *In* Saedler, H. and A. Gierl (ed.), Transposable Elements, Vol. 204, Springer, Berlin.

Lacks, S. A., P. Lopez, B. Greenberg, and M. Espinosa. 1986. Identification and analysis of genes for tetracycline-resistance and replication functions in the broad host range plasmid pLS1. J. Mol. Biol. 192:753-765.

Lawley, T. D., V. Burland, and D. E. Taylor. 2000. Analysis of the complete nucleotide sequence of the tetracycline-resistance transposon Tn10. Plasmid. 43:235-239.

LeBouguenec, C., G. de Cespedes, and T. Horaud. 1990. Presence of chromosomal elements resembling the composite structure Tn*3701* in streptococci. J. Bacteriol. 172:727-734.

Lee, C., B. E. Langlois, and K. A. Dawson. 1993. Detection of tetracycline-resistance determinants in pigs isolates from three herds with different histories of antimicrobial agent exposure. Appl. Environ. Microbiol. 59:1467-1472.

Levy, S. B. 1984. Resistance to the tetracyclines. p. 191-240. *In* L. E. Bryan (ed.), Antimicrobial drug resistance. Academic Press, Orlando, Fla.

Levy, S. B. 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. 36:695-703.

Lima, A. A. M., N. L. Lima, M. C. Pinho, E. A. Barros, Jr., M. J. Teixeria, M. C. V. Marins, and R. I. Guerrant. 1995. High frequency of strains multiply resistant to ampicillin, trimethoprim-sulfamethoxazole, streptomycin, chloramphenicol, and tetracycline isolated from patients with shigellosis in northeastern Brazil during the period 1988 to 1993. Antimicrob. Agents Chemother. 39:256-259.

Marshall, B., C. Tachibana, and S. B. Levy. 1983. Frequency of tetracycline-resistance determinant classes among lactose fermenting coliform. Antimicrob. Agents Chemother. 24: 835-840.

Mendez, B., C. Tachibana, and S. B. Levy. 1980. Heterogeneity of tetracycline-resistance determinants. Plasmid. 3:99-108.

Moellering, R. C. 1990. Principles of anti-infective therapy, p. 206-218. *In* G. L. Mandell, R. G. Douglas, and J. E. Bennett (ed.), Principles and practice of infectious disease. Churchill Livingstone, Inc., New York.

National Committee for Clinical Laboratory Standards. 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 2nd ed, NCCLS document M31-A2. NCCLS, Wayne, Pennsylvania.

Ng, L.-K., M. R. Mulvery, I. Martin, G. A. Peters, and W. Johnson. 1999. Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. Antimicrob. Agents Chemother. 43:3018-3021.

Ng, L.-K, I. Martin, M. Alfa, and M. Mulvey. 2001. Multiplex PCR for the detection of tetracycline-resistant genes. Mol. Cell Probes. 15:209-215.

Oethinger, M., W. V. Kern, A. S. Jellen-Ritter, L. M. McMurry, and S. B. Levy. 2000. Ineffectiveness of topoisomerase mutations in mediating clinically significant fluroquinolone resistance in *Escherichia coli* in the absence of the AcrAB efflux pump. Antimicrob. Agents Chemother. 44:10-13.

Roberts, M. C. 1996. Tetracycline-resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. 19:1–24.

Roberts, M.C. 1989. Plasmid-mediated Tet M in Haemophilus ducreyi. Antimicrob Agents Chemother. 33:1611-1613.

Roberts, M. C., and A. L. Smith. 1980. Molecular characterization of "plasmid-free" antibiotic resistant *Haemophilus influenzae*. J. Bacteriol. 144:476-479.

Roberts, M. C., Y. Pang, R. C. Spencer, T. G. Winstanley, B. A. Brown, and R. J. Wallace, Jr. 1991. Tetracycline-resistance in *Moraxella* (*Branhamella*) *catarrhalis* demonstration of two clonal outbreaks using pulsed-field gel electrophoresis. Antimicrob. Agents Chemother. 35:2453-2455.

Roberts, M. C., W. O. Chung, and D. E. Roe. 1996. Characterization of tetracycline and erythromycin resistance determinants in Treponema denticola. Antimicrob. Agents Chemother. 40:1690-16944.

Roe, D.E., P. H. Braham, A. Weinberg, and M. C. Roberts. 1995. Characterization of tetracycline-resistance in Actinobacillus actinomycetemcomitans. Oral Microbiol. Immunol. 10:227-232.

Salyers, A.A., B.S. Speer, and N.B. Shoemaker. 1990. New perspectives on Tetracycline-resistance. Mol. Microbiol. 4:151-156.

Sambrook and Russell. 2001. Molecular Cloning: A laboratory manual. 3rd ed. Cold Spring Harbor, New York.

Schnabel, E.L.,and A.L. Jones. 1999. Distribution of tetracycline-resistance genes and transposons among phylloplane bacteria in Michigan apple orchards. Appl. Environ. Microbiol. 65:4898-4907.

Speer, B.S., N.B. Shoemaker, and A.A. Salyers. 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin. Microbiol. Rev. 5:387-399.

Standiford, H. C. 1990. Tetracyclines and chloramphenicol, p. 284-295. In G. L. Mandell, R. G. Douglas, and J. E. Bennett (ed.), Principles and practice of infectious diseases. Churchill Livingstone, Inc., New York.

Stockstad, E. L. R., T. H. Jukes, J. Pierce, A. C. Page, and A. L. Franklin. 1949. The multiple nature of the animal protein factor. J. Biol. Chem. 180:647-654.

Takahashi, H., H. Watanabe, T. Kuroki, Y. Watanabe, and S. Yamai. 2002. Identification of tet(B), encoding high-level tetracycline-resistance, in *Neisseria meningitidis*. Antimicrob. Agents Chemother. 46:4045-4046.

Threlfall, E. J., J. A. Frost, L. R. Ward, and B. Rowe. 1994. Epidemic in cattle and humans of *Salmonella* Typhimurium DT104 with chromsomally integrated multiple drug resistance. Vet. Rec. 134:577.

Wasteson, Y., S. Hoie, and M. C. Roberts. 1994. Characterization of antibiotic-resistance in *Streptococcus suis*. Vet. Microbiol. 41:41-49.

Chapter 5

PHENOTYPIC AND GENOTYPIC ANALYSIS OF BETA-LACTAM AND EXTENDED SPECTRUM BETA-LACTAM RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM LACTATING DAIRY CATTLE

5.1 ABSTRACT

Ampicillin-resistant *E. coli* (n=94 isolates) from lactating cattle were examined for their susceptibility to other antibiotics and genetic determinants that encode for beta-lactam resistance. The majority of the *E. coli* isolates were resistant to tetracycline (88.3%) followed by spectinomycin (35.1%), ticarcillin (33%), and chloramphenicol (29.8%). Resistance was also exhibited to ticarcillin and clavulanic acid combination (23.4%) and the third generation cephalosporin ceftiofur (26.6%). All isolates were susceptible to enrofloxacin. Multidrug resistance (≥3 to 8 antimicrobial agents) was observed in 44 of 94 (46.8%) isolates, and the resistance profile of ampicillin, chloramphenicol, spectinomycin, tetracycline, ticarcillin, ticarcillin/ clavulanic acid, and ceftiofur was the most frequently observed (n=15 isolates). The isolates were also examined for susceptibility to other penicillins and extended spectrum cephalosporins. The majority of the isolates (73.4%) were resistant only to penicillins, of which resistance to ampicillin and piperacillin was predominant. Resistance to 1st, 2nd, and 3rd generation cephalosporins was observed only in isolates that were resistant to ceftiofur. Interestingly 23 of 25 (92%) ceftiofur-resistant *E. coli* were also resistant to chloramphenicol. All *E. coli* isolates were susceptible to the 4th generation cephalosporin cefepime. The extended spectrum beta-lactamases were not detected in ceftiofur-resistant isolates. All ceftiofur-resistant isolates carried the *bla*$_{CMY}$, while rest of the 69 isolates carried the *bla*$_{TEM}$. Four ceftiofur-resistant isolates carried both *bla*$_{CMY}$ and *bla*$_{TEM}$. Sequence analysis of the *bla*$_{CMY}$ from *E. coli* (n=5) isolates showed 99% to 100% identify with the plasmid encoded *bla*$_{CMY}$-2 from *Salmonella* spp. (Acc. No.AY253913, and U77414) and *K. pneumoniae* (Acc. No. X91840). Integrons of ≥1 kb were observed in 10 of 75 *E. coli* isolates. Sequence analysis revealed that the

integrons encoded for streptomycin and spectinomycin (*aadA*), and/or trimethoprim (*dfr*) resistance genes.

The findings of our study suggest that ampicillin-resistant commensal *E. coli* carry for the bla_{TEM}. Multidrug resistance was not uncommon but was more pronounced in isolates that were resistant to ceftiofur. Ceftiofur-resistant isolates carried genes for cephamycinases that were able to confer resistance to 1st, 2nd, and 3rd generations of cephalosporins. The widespread prevalence of beta-lactam resistance in the dairy environment could pose a considerable public health risk if these organisms gained access to the food chain.

5.2 INTRODUCTION

Beta-lactam based antibiotics (penicillins, cephalosporins, monobactams, and carbapenems) are used extensively in veterinary medicine. Low toxicity, a broad spectrum of activity, and reliable clinical efficacy has lead to extensive use of beta-lactam antibiotics (Mealey, 2001). Beta-lactam antibiotics can be broadly grouped as penicillins and cephalosporins.

The penicillins that are approved for use in dairy cattle include ampicillin, amoxicillin, cloxacillin, hetacillin, and penicillin. Among the cephalosporins, cephapirin and ceftiofur are approved for used in dairy cattle (FDA, 2005). The majority of penicillins are indicated for the treatment of mastitis, metritis, systemic illnesses, and respiratory diseases in dairy cattle. Cephapirin, a first generation cephalosporin is approved for mastitis treatment whereas ceftiofur, a third generation cephalosporin can be used for respiratory diseases, foot rot, and metritis

infections in dairy cattle (Hornish and Kotarski, 2002; USP Veterinary Drug Information monographs, 2003).

According to Mandell and Petri (1996) widespread use of these agents has resulted in the emergence of resistant organisms. Resistance to beta-lactam antibiotics can occur through the following pathways: 1) modification of penicillin-binding protein targets, 2) beta-lactamase production, and 3) impermeability and/ or efflux. Resistance to beta-lactam antimicrobial agents in gram-negative bacilli is primarily mediated by beta-lactamases (Livermore, 1995). Bacterial beta-lactamases are hydrolytic enzymes that catalyze hydrolysis of the amide bond of the beta-lactam ring, producing acidic derivatives with no antibacterial activity (Mealey, 2001; Livermore, 1995). The lactamase enzyme may be encoded on a plasmid (TEM-1 and SHV-1) or on the chromosome (*AmpC* types). Although a variety of beta-lactamases have been described, TEM and SHV enzymes are most frequently observed among members of the family *Enterobacteriaceae* (Bush et al., 1995; Livermore, 1995). These enzymes can hydrolyze ampicillin but are not effective against newer cephalosporins (Sanders and Sanders, 1992; Livermore, 1995; O'Callaghan, 1979).

Over the last two decades the frequency of isolation of bacteria that produce extended spectrum beta-lactamases (ESBL) has increased considerably. ESBLs are variants of TEM-1, TEM-2, and SHV-1 type due to point mutations which produce 1 to 4 amino acid substitutions. These mutations allow the ESBLs to inactivate many newer cephalosporins and monobactams (Jacoby and Medeiros, 1991; Livermore, 1995; Philippon et al., 1989). The presence of ESBLs poses a serious clinical problem because of difficulty in identifying their presence. Karas et al. (1996) described cephalosporin treatment failure for septicemia caused by a *K. pneumonia* strain which was found to be susceptible to cephalosporins on disc diffusion and MIC tests. This isolate

was later found to produce an ESBL. The numbers of reported ESBL variants has been growing and currently more than 100 different natural ESBL variants have been reported (Gniadkowski, 2001).

Another category of extended spectrum beta-lactamases that has gained importance is the plasmid-based *AmpC*-like beta-lactamase. Plasmid borne *AmpC*-like resistance was first detected in 1989 in an isolate of *Klebsiella pneumoniae*. The plasmid encode for beta-lactamase that exhibited resistance to extended spectrum beta-lactams similar to ESBLs (Bauernfeind et al., 1989). The beta-lactamase was named cephamycinase (bla_{CMY}) and was genotypically different from TEM based ESBLs (Bauernfeind et al., 1989). The cephamycinase gene shares extensive homology to chromosomal *AmpC* beta-lactamases. The CMY-1 is more closely related to *AmpC* of *Pseudomonas aeruginosa* while there is close relationship between *CMY-2* and *AmpC* of *Citrobacter freundii*. This suggests that plasmidic cephamycinases may have evolved from their corresponding chromosomal counterparts (Bauernfeind et al., 1996). Plasmid-borne *AmpC* beta-lactamases have been frequently reported in Gram-negative bacteria including *E. coli*, *Salmonella* spp., *P. mirabilis* and *C. freundii* (Bauernfeind et al., 1998).

With the exception of cephapirin and ceftiofur approved for use in dairy cattle, most of the approved cephalosporins have been developed for use in humans. Zhao et al. (2001b) recovered *Salmonella* and *E. coli* isolates from food animals and retail ground meat that contained bla_{CMY} and exhibited resistance to extended spectrum cephalosporins included ceftiofur. The presence of this type of resistance in food-borne pathogens is of concern to public health. The focus of this study was to characterize commensal ampicillin-resistant *E. coli* with respect to; 1) resistance to other antibiotics and cephalosporins, and 2) genetic determinants that encode for penicillin and cephalosporin resistance.

5.3 MATERIAL AND METHODS

5.3.1 Ampicillin-resistant *E. coli*

A total of 94 ampicillin-resistant *E. coli* isolated from feces of lactating dairy cattle (n=73) from 33 farms in Pennsylvania were used for this study.

5.3.2 Antimicrobial susceptibility testing

Disk diffusion assay: Disks containing ampicillin (10 µg), ceftiofur (30 µg) chloramphenicol (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), spectinomycin (100 µg), tetracycline (30 µg), ticarcillin (75 µg), or ticarcillin/clavulanic acid (75/10 µg) (Remel Inc. KS, U.S.A) were used for the disk diffusion assay. The isolates were screened for susceptibility according to the NCCLS disk diffusion assay protocol. The results were interpreted according to criteria established by NCCLS document M31-A2 (2002) for bacteria isolated from animals.

Pasco MIC gram-negative panel: The Pasco MIC gram-negative panel (Becton Dickinson, Sparks, Maryland, USA) was used for evaluating susceptibility to penicillins and 1st, 2nd, 3rd, and 4th generation cephalosporins. The isolates were screened according to the manufacturer's instructions. The minimum inhibitory concentration (MIC) results obtained from Pasco test panels were interpreted based upon NCCLS MIC interpretive standards (interpretive worksheet provided by manufacturer). Results of susceptibility to 20 of 27 antimicrobials are presented in this study.

5.3.3 Integron assay

Ampicillin-resistant *E. coli* (n=75) isolates were screened for the presence of integrons by PCR analysis using 5' and 3' end conserved primers for class 1 integrons as described by Tosini et al. (1998). Integrons ≥ 1 kb were sequenced at the Nucleic Acid Facility at Penn State

University. Integron sequences were compared with the gene-bank database using the BLAST program available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

5.3.4 Screening for Extended Spectrum Beta-Lactamases (ESBLs)

ESBL production in *E. coli* was determined by using the NCCLS (1999) method for screening and confirming the presence of ESBLs. Disks impregnated with ceftazidime and cefotaxime alone and with clavulanic acid were used for identification of ESBL phenotypes (Sensi-Disc. ESBL confirmatory test disks, BD BBL, Sparks, MD, USA). Isolates that showed a ≥ 5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone were considered phenotypically confirmed ESBL-producing isolates.

5.3.5 PCR analysis of beta-lactamase genes

Isolates were screened for the presence of bla_{TEM}-1 and bla_{CMY} by PCR analysis, conducted as described by Vahaboglu et al. (2001) and Zhao et al. (2001a), respectively. The PCR amplification products for bla_{TEM}-1 and bla_{CMY} were 861 bp and 1 kb, respectively. The bla_{CMY} amplified product was sequenced at the Nucleic Acid Facility at Penn State University, and was compared with the gene-bank database using the BLAST program available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

5.4 RESULTS

5.4.1 Resistance profiles of ampicillin-resistant *E. coli* isolates to antimicrobial agents -disk diffusion assay

Nearly 88.3% of ampicillin-resistant *E. coli* were resistant to tetracycline. Resistance was also observed to chloramphenicol, spectinomycin, and ticarcillin in 28 (29.8%), 33 (35.1%), and 31 (33.0%) isolates, respectively (Table **5-1**). Isolates with resistance to ticarcillin and clavulanic acid combination (23.4%) and ceftiofur (26.6%) were also observed. Few isolates showed resistance to gentamicin (5.3%), and resistance to enrofloxacin was not observed.

Ampicillin-resistant *E. coli* belonged to 18 antibiotic resistance profiles (Table **5-2**). Nearly 42% of the isolates exhibited resistance to ampicillin and tetracycline. Multidrug-resistance (≥ 3 antibiotics) was observed in 44 isolates. All of the 25 isolates that were resistant to ceftiofur exhibited multidrug resistance to other unrelated antimicrobial agents (Table **5-2**).The most common multidrug resistance profile (n=15) showed resistance to ampicillin, chloramphenicol, spectinomycin, tetracycline, ticarcillin, ticarcillin/ clavulanic acid, and ceftiofur (Table **5-2**).

5.4.2 Resistance profiles of ampicillin-resistant *E. coli* isolates to other penicillins and extended spectrum cephalosporins

The Pasco MIC Gram-negative panel was used to study resistance to cephalosporins approved for use in human medicine. The panel included first, second, third, and fourth generation cephalosporins. A total of 27 resistance profiles were observed (Table **5-3**). A large number of isolates (n=42) showed resistance to ampicillin and piperacillin. All ceftiofur-resistant isolates (n=25) were resistant to 6 or more antimicrobial agents and included resistance to 1st, 2nd, and 3rd generations cephalosporins (profile no. 12 to 27; Table **5-3**). Interestingly, 23 of 25

ceftiofur-resistant isolates showed resistance to chloramphenicol, a drug prohibited for use in food-producing animals. All isolates were susceptible to the fourth generation cephalosporin, cefepime.

5.4.3 Integron assay of 75 ampicillin-resistant *E. coli* isolates

PCR-amplified DNA fragments of integron 1 (1,000-1600 bp) were obtained using the set of primers for conserved regions (5'CS and 3'CS). Integron 1 was observed in 10 *E. coli* isolates. A total of 5, 1, and 4 isolates showed the presence of 1 kb, 1.2 kb, and 1.6 kb of amplified product, respectively (Figure **5.1**). On sequence analysis, PCR-amplified DNA of ~1000 bp (A92) showed 95% sequence match to *aadA23*, which encodes for streptomycin and spectinomycin resistance from *Salmonella* Agona (AJ809407). Amplified DNA of ~1200 bp (A73) showed 99% match with *dfrA* which encodes resistance to trimethoprim from *E. coli* (AB161449). An integron sequence of ~1600 bp (A107) matched 99% to both *dfr17* and *aadA5* from *Salmonella* spp. (AY263739) (Figure **5.2**).

5.4.4 Extended-Spectrum Beta-Lactamases (ESBLs)

The ceftiofur and extended-spectrum cephalosporin-resistant isolates (n=25) were considered as potential candidates for ESBLs screening. The isolates failed to show any clavulanic acid effect. Based on this observation, these isolates were not classified as ESBL producers (Figure **5.3**).

5.4.5 Beta-lactamase genes

All the ceftiofur-resistant isolates contained the bla_{CMY} gene. The remaining 69 isolates carried bla_{TEM}-1 (Figure **5.1**). Four ceftiofur-resistant isolates carried both bla_{CMY} and bla_{TEM}-1 (Figure **5.1**). The bla_{CMY} genes from 5 *E. coli* isolates were sequenced. Sequence analysis

showed 99% to 100% match with plasmid-borne *bla*$_{CMY}$-2 from *Salmonella* spp. (AY253913 and U77414) and *K. pneumoniae* (X91840) (Figure **5.4**).

5.5 DISCUSSION

Nonpathogenic, multidrug resistant *E. coli* in the intestine are probably an important reservoir of antibiotic resistance genes (Levy, 1978; Levy et al., 1988; Marshall et al., 1990**;** Österblad et al., 2000). Oppegaard et al. (2001) indicated that *E. coli* is a major carrier of resistance traits in the coliform flora of both humans and animals. They observed that 30 of 39 *E. coli* were resistant to ampicillin, tetracycline, streptomycin, trimethoprim, and sulfonamide. DeFrancesco et al. (2004) studied antibiotic resistance in commensal *E. coli* isolated from cows and calves from farms with a history of multidrug resistant (MDR) *Salmonella* infections. They hypothesized that high antimicrobial pressure on farms was responsible for the prevalence of MDR salmonellosis. The prevalence of MDRs in *Salmonella* was measured by studying MDRs in commensal *E. coli*. A significantly higher percentage of *E. coli* isolates from farms with a history of MDRs salmonellosis showed resistance to ampicillin, chloramphenicol, sulfadiazine, trimethoprim, and gentamicin compared those farms with no outbreaks of salmonellosis.

Antibiotic resistance among commensal *E. coli* can serve as an indicator of antimicrobial selection pressure on farms and can be useful for estimating selection pressure (van den Bogaard and Stobberingh, 2000 and DeFrancesco et al., 2004). Studies of neonatal diarrhea in calves due to E. coli have resulted in high resistance (from 23 to 50 %) to ampicillin, neomycin, kanamycin, spectinomycin, chloramphenicol, sulphadimethoxine, and trimethoprim (Orden et al., 2000). Schroeder et al. (2003) observed *E. coli* isolates from retail meats displaying high resistance to

tetracycline (59%), sulfamethoxazole (45%), streptomycin (44%), cephalothin (38%) and ampicillin (35%). Resistance was also observed, but to a lesser extent, to gentamicin (12%), nalidixic acid (8%), chloramphenicol (6%), ceftiofur (4%) and ceftriaxone (1%). They observed multidrug resistance (≥ 3 antimicrobial agents) in 49% of the isolates. An interesting observation in previous studies and our data is the resistance to chloramphenicol in a large number of multidrug resistant *E. coli* isolates (Table **5-2**), an antibiotic that has been banned from veterinary use in food animals in the United States since the 1980s (Gilmore 1996). This can be explained by the fact that bovine-origin florfenicol-resistant *E. coli* isolates exhibited cross-resistance to chloramphenicol (White et al., 2000). Though chloramphenicol is banned, florfenicol, a related analog of chloramphenicol, approved by the FDA for treating bovine respiratory diseases, was widely used on the farms that we surveyed and could have selected for chloramphenicol-resistant isolates (Table **2-3** and **A-8**).

A wide range of resistance profiles were observed in the *E. coli* isolates on Pasco MIC Gram-negative panel. Though resistance to extended-spectrum beta-lactams was observed, a sizable number of isolates were only resistant to ampicillin, piperacillin, and narrow-spectrum cephalosporins. Brinas et al. (2002) studied beta-lactam resistant *E. coli* from foods, fecal samples of humans, and healthy animals, and observed nearly half (48%) of the ampicillin-resistant *E. coli* isolates were resistant only to aminopenicillins and not to other beta-lactams or beta-lactamase inhibitors. These results can be attributed to beta-lactamases that are most frequently associated with ampicillin resistance in *E. coli* but also confer low level resistance to first generation cephalosporins (Medeiros, 1997; Wu et al., 1994).

Extended-spectrum cephalosporin-resistant *E. coli* have been previously isolated from diarrhoeal disease in cattle, clinical cases of cattle, swine, and humans (Bradford et al., 1999;

Brinas et al., 2003; Winokur et al., 2001). In our study we were able to isolate extended-spectrum cephalosporin-resistant *E. coli* from the feces of healthy lactating cattle. Most of the isolates that were resistant to extended-spectrum cephalosporins were also resistant to beta-lactamase inhibitor combinations like ampicillin/sulbactam and ticarcillin/clavulanic acid. In our study only 3 isolates were resistant to the piperacillin/tazobactam combination (Table **5-2** and **5-3**). Our data agrees with the findings of Vanjak et al. (1995) who studied susceptibility of 300 amoxicillin resistant *E. coli* isolates to beta-lactamase inhibitors. They reported 62% of the strains had low susceptibility to penicillins, cephalothin, or ampicillin/sulbactam and ticarcillin/clavulanic acid combinations except for piperacillin/tazobactam. Of this 23.3% isolates exhibited resistance to different cephalosporins. In our study multidrug resistance to non beta-lactam antibiotics was observed in extended-spectrum cephalosporin-resistant *E. coli* (Tables **5-2** and **5-3**). Previous studies have observed resistance to chloramphenicol, sulfa drugs, streptomycin, and tetracycline in extended-spectrum cephalosporin-resistant *E. coli* and *Salmonella* (Navarro et al., 2001; Winokur et al., 2000).

A novel system to which multiple-drug resistance and its dissemination is regularly attributed is bacterial integrons (Hall, 1997). Integrons have the ability to carry antibiotic-resistance gene cassettes that can possess resistance genes for beta-lactams, aminoglycosides, trimethoprim, chloramphenicol, streptothricin and quaternary ammonium compounds (Hall, 1997). Integrons have been reported in *E. coli* isolated from swine, poultry, and cattle (Sunde and Sorum, 1999; Bass et al., 1999; Zhao et al., 2001a). Resistance to beta-lactams and extended-spectrum cephalosporins was not located within class 1 integrons found in the ampicillin-resistant *E. coli* isolates from our study. These integrons carried genes that encoded

resistance to streptomycin, spectinomycin, and trimethoprim. Similar results were reported by Winokur et al. (2001).

The majority of ampicillin-resistant *E. coli* isolates we studied carried the $bla_{\text{TEM-1}}$. The TEM-1 beta-lactamase has been seen in ampicillin-resistant *E. coli* from animal and human origin and is located on plasmids (Brinas et al., 2002; Thomson and Amyes, 1993). *Escherichia coli* that exhibit resistance to extended-spectrum cephalosporins is commonly observed in strains that are ESBL producers or if they encode for *AmpC*-like genes (Shuttleworth, 2004; Mammeri et al., 2004). As none of the extended-spectrum cephalosporin-resistant *E. coli* isolates were observed to be ESBL producers, these isolates were screened for bla_{CMY}.

All of the ceftiofur-resistant *E. coli* isolates carried the bla_{CMY}. Sequence comparison using the NCBI gene bank database revealed that it shared extensive homology with plasmid borne bla_{CMY}-2 isolated from species like *Salmonella* Choleraesuis, *K. pneumoniae* and *Salmonella* Senftenberg. The sequence data indicates that plasmid borne bla_{CMY}-2 is globally spread across species and even the host that inhabit them. Zhao et al. (2001b), while studying bla_{CMY} in *E. coli* and *Salmonella* found that this resistance is most likely horizontally disseminated via large, broad-host-range plasmids or mobile transposons. Winokur et al. (2001) has provided conclusive evidence that plasmid borne bla_{CMY}-2 (*AmpC* like beta-lactamase) can be transferred between *E. coli* and *Salmonella* isolates.

Most of the studies conducted on antibiotic resistance study in animal agriculture have primarily been directed towards pathogenic bacteria (Winokur et al., 2001; Bradford et al., 1999). The findings of this study provide a unique perspective on the role of commensal *E. coli* as potential reservoirs of genetic determinants for beta-lactams. The importance of monitoring resistance in commensal bacteria such as *E. coli* is essential as they might gain access to the food

chain. Zhao et al. (2001b) recently showed extended-spectrum cephalosporin-resistant *E. coli* and *Salmonella* present in retail ground meat, signifying the public health importance of this issue. The high risk of transfer of beta-lactam genetic determinants from commensal *E. coli* to pathogenic bacteria is likely to occur within the animal intestinal tract (Winokur et al., 2001; Blake et al., 2003). This can be an important mechanism for acquiring antibiotic resistance in pathogenic bacteria that pose a challenge for effective antibiotic therapy. The findings of our study suggest that commensal *E. coli* can perhaps play a dynamic role in the ecology of beta-lactam resistance in the dairy environment.

Table **5-1**: Resistance of 94 ampicillin-resistant *E. coli* to other antimicrobial agents using disk diffusion assay

Antimicrobial resistance	No of isolates (%)
Ampicillin	94 (100)
Chloramphenicol	28 (29.8)
Gentamicin	5 (5.3)
Enrofloxacin	0 (0.0)
Spectinomycin	33 (35.1)
Tetracycline	83 (88.3)
Ticarcillin	31 (33.0)
Ticarcillin/clavulanic acid	22 (23.4)
Ceftiofur	25 (26.6)

Table **5-2**: Resistance profiles of 94 ampicillin-resistant *E. coli* isolates on disk diffusion assay

No.	Antimicrobial resistance patterns	No. of isolates
1	AMP	10
2	AMP-TET	39
3	AMP-TIC	1
4	AMP-CHL-TET	1
5	AMP-SPT-TET	9
6	AMP-TET-TIC	3
7	AMP-CHL-SPT-TET	1
8	AMP-CHL-TET-TIC	3
9	AMP-GEN-SPT-TET	1
10	AMP-TET-TIC-XNL	1
11	AMP-CHL-SPT-TET-XNL	1
12	AMP-TET-TIC-TIM-XNL	1
13	AMP-GEN-SPT-TET-TIC	1
14	AMP-CHL-SPT-TET-TIC-XNL	1
15	AMP-CHL-SPT-TET-TIM-XNL	1
16	AMP-CHL-TET-TIC-TIM-XNL	2
17	AMP-CHL-SPT-TET-TIC-TIM-XNL	15
18	AMP-CHL-GEN-SPT-TET-TIC-TIM-XNL	3

Antimicrobial agents used in the study: AMP, Ampicillin; CHL, Chloramphenicol; GEN, Gentamicin; SPT, Spectinomycin; TET, Tetracycline; TIC, Ticarcillin; TIM, Ticarcillin/clavulanic acid; XNL, Ceftiofur

Table **5-3**: Resistance profiles of 94 ampicillin-resistant *E. coli* isolates to extended spectrum beta-lactams

No.	Antimicrobial resistance patterns [a, *]	No. of isolates
1	AMP	6
2	AMP-PIP	42
3	AMP-SAM	1
4	AMP-SAM-PIP	9
5	AMP-PIP-GEN	1
6	AMP-PIP-CHL	2
7	AMP-PIP-CEF	3
8	AMP-PIP-CEF-CHL	2
9	AMP-SAM-PIP-CEF	1
10	AMP-SAM-PIP-CHL	1
11	AMP-SAM-PIP-TOB-GEN	1
12	AMP-SAM-CEF-FOX-CPD-CHL	1
13	AMP-CFZ-CEF-FOX-CPD-CHL	3
14	AMP-SAM-CFZ-CEF-FOX-CPD-CHL	1
15	AMP-SAM-PIP-CFZ-CEF-CXM-FOX-CPD	1
16	AMP-SAM-CFZ-CEF-CXM-FOX-CPD-CHL	4
17	AMP-SAM-CFZ-CEF-CXM-FOX-CPD-CAZ-CHL	1
18	AMP-SAM-PIP-CFZ-CEF-CXM-FOX-CPD-CHL	1
19	AMP-SAM-CFZ-CEF-CXM-FOX-CPD-CAZ-CHL	4
20	AMP-SAM-CFZ-CEF-CXM-FOX-CRO-CPD-CAZ-CHL	1
21	AMP-SAM-CFZ-CEF-CXM-FOX-CPD-CAZ-ZOX-CHL	1
22	AMP-SAM-PIP-CFZ-CEF-CXM-CTT-FOX-CPD-CAZ	1
23	AMP-SAM-PIP-CFZ-CEF-CXM-CTT-FOX-CPD-CAZ-CHL	2
24	AMP-SAM-PIP-TZP-ATM-CFZ-CEF-CTT-FOX-CRO-CPD-CFP-CAZ-ZOX-CHL	1
25	AMP-SAM-PIP-ATM-CFZ-CEF-CXM-CTT-FOX-CRO-CPD-CFP-CAZ-ZOX-CHL	1
26	AMP-SAM-PIP-TZP-ATM-CFZ-CEF-CXM-FOX-CRO-CPD-CFP-CAZ-CTX-ZOX-CHL	1
27	AMP-SAM-PIP-TZP-ATM-CFZ-CEF-CXM-CTT-FOX-CRO-CPD-CFP-CAZ-ZOX-CHL	1

[a] Antimicrobial agents: AMP, Ampicillin; SAM, Amp/Sulbactam; PIP, Piperacillin; TZP, Pipera/tazobactam; ATM, Aztreonam; CFZ, Cefazolin; CEF, Cephalothin; CXM, Cefuroxime; CTT, Cefotetan; FOX, Cefoxitin; CRO, Ceftriaxone; CPD, Cefpodoxime; CFP, Cefoperazone; CAZ, Ceftazidime; CTX, Cefotaxime; ZOX, Ceftizoxime; GEN, Gentamicin; TOB, Tobramycin; CHL, Chloramphenicol; NIT, Nitrofurantoin.

1st gen. cephalosporins 2nd gen. cephalosporins 3rd gen. cephalosporins

* Isolates from pattern 12 thorough 27 were resistant to ceftiofur on disk diffusion assay.

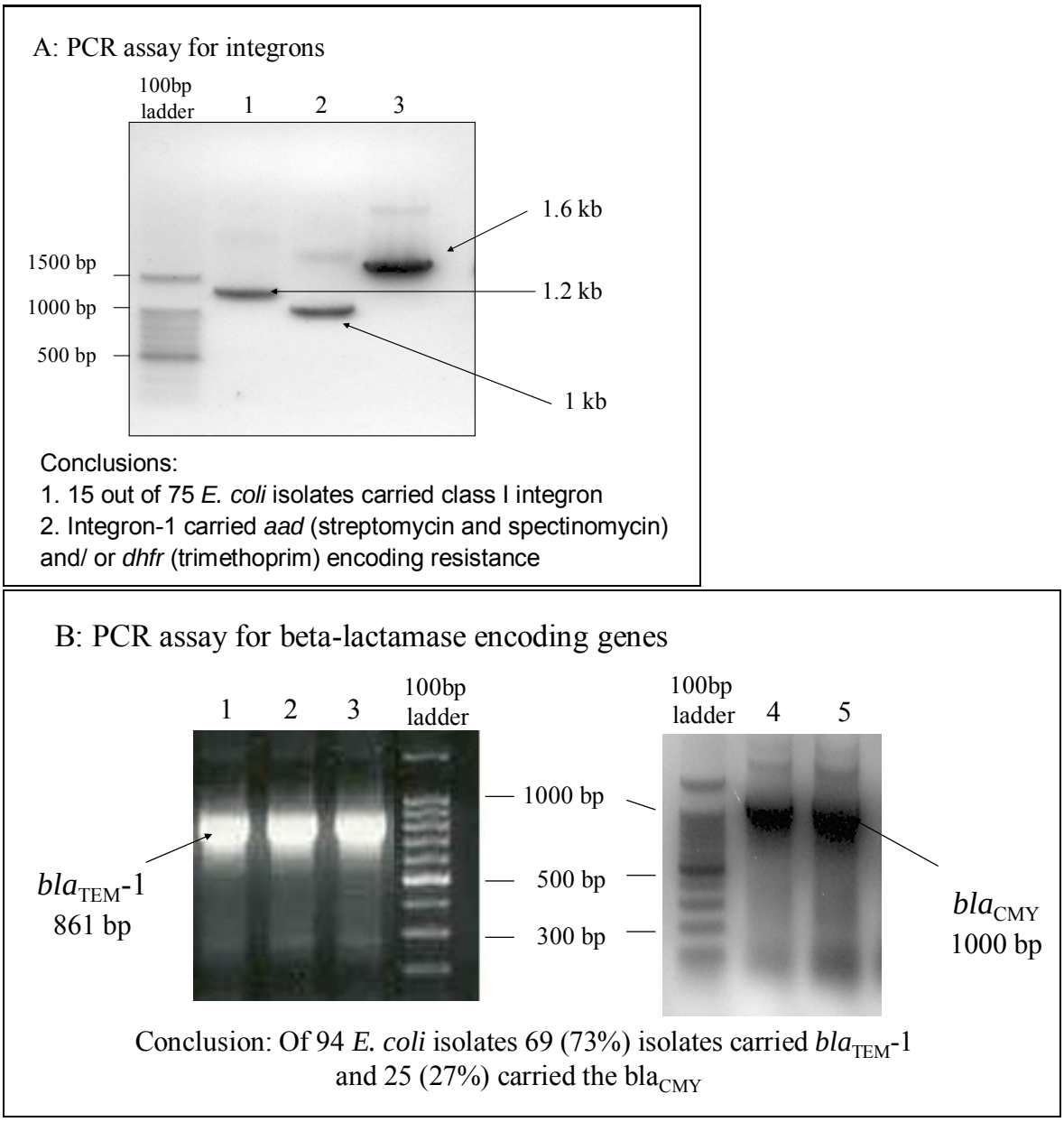


Figure **5.1**: PCR analysis for integrons and beta-lactamases. Fig. A: lane 1 to 3 represent isolates with integrons of different sizes. Fig. B: lane 1, 2, and 3 represent isolates with *bla*$_{TEM}$-1 and lane 4 and 5 represent isolates with *bla*$_{CMY}$

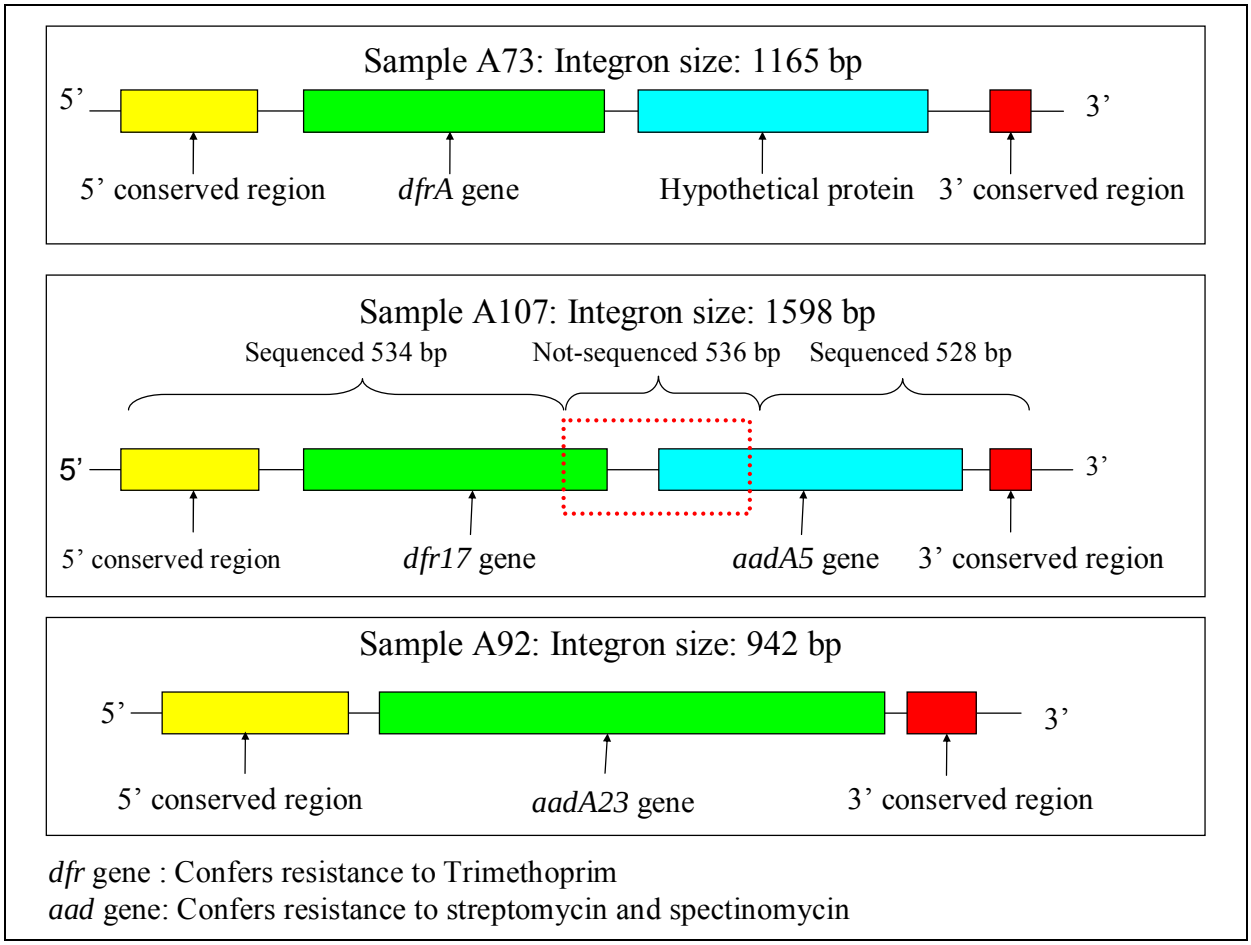


Figure **5.2**: Genes carried by the class 1 integrons isolate A73, A107, and A92 on the basis of sequence match with NCBI database sequences

Results: An ≥ 5mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone with tested alone = ESBL producer.

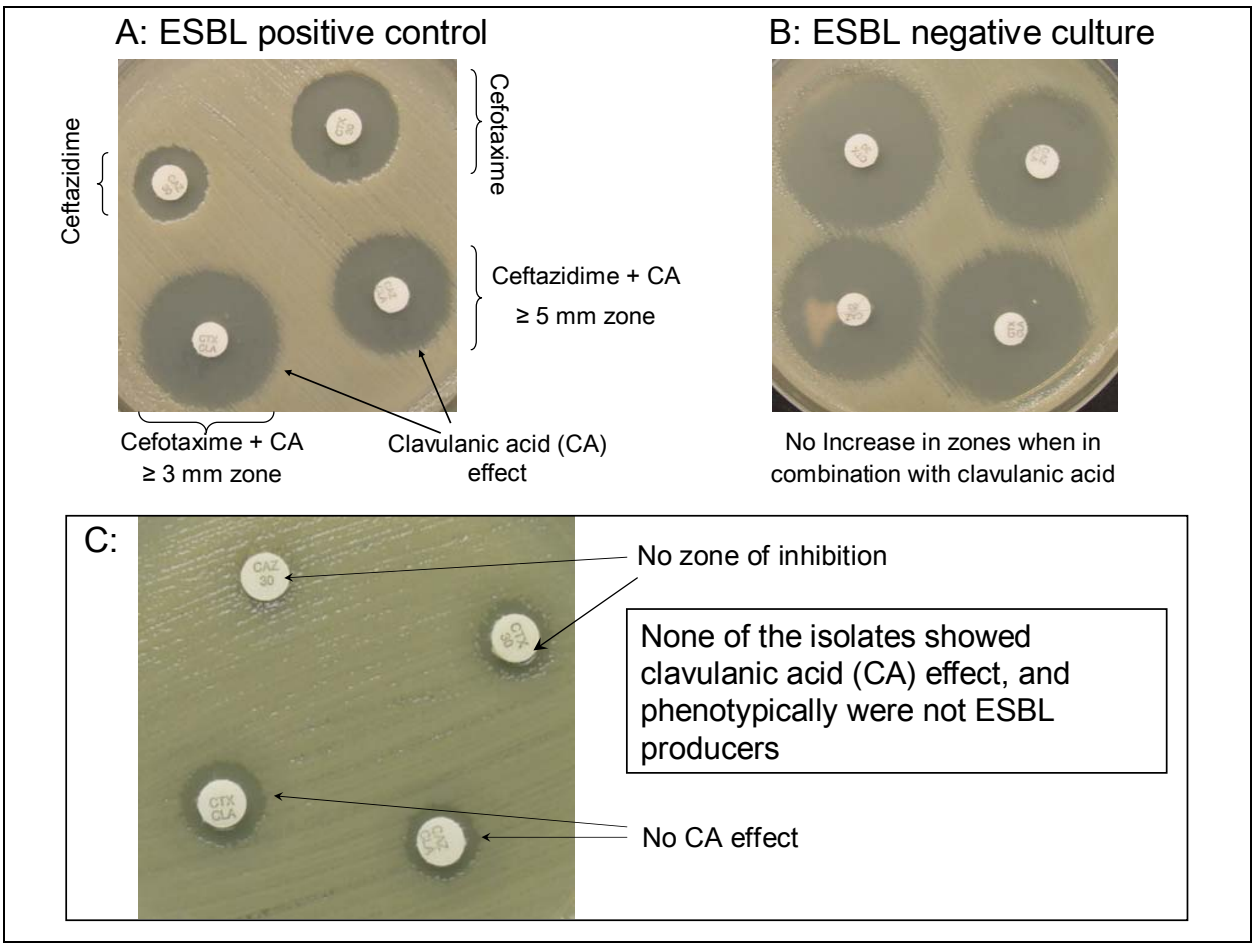


Figure **5.3**: Phenotypic confirmation of potential ESBL producers.

Fig. A: ESBL producer *K. pneumoniae* ATCC 700603 showing ≥ 3 mm increase in cefotaxime zone diameter; ≥ 5 mm increase in ceftazidime zone diameter. Fig. B: Negative ESBL producer *E. coli* ATCC 25922 showing ≤ 2 mm increase in zone diameter for antimicrobial agent tested alone versus its zone diameter when tested in combination with clavulanic acid. Fig. C: Results of ESBL analysis of 25 ceftiofur and extended-spectrum cephalosporin-resistant *E. coli*.

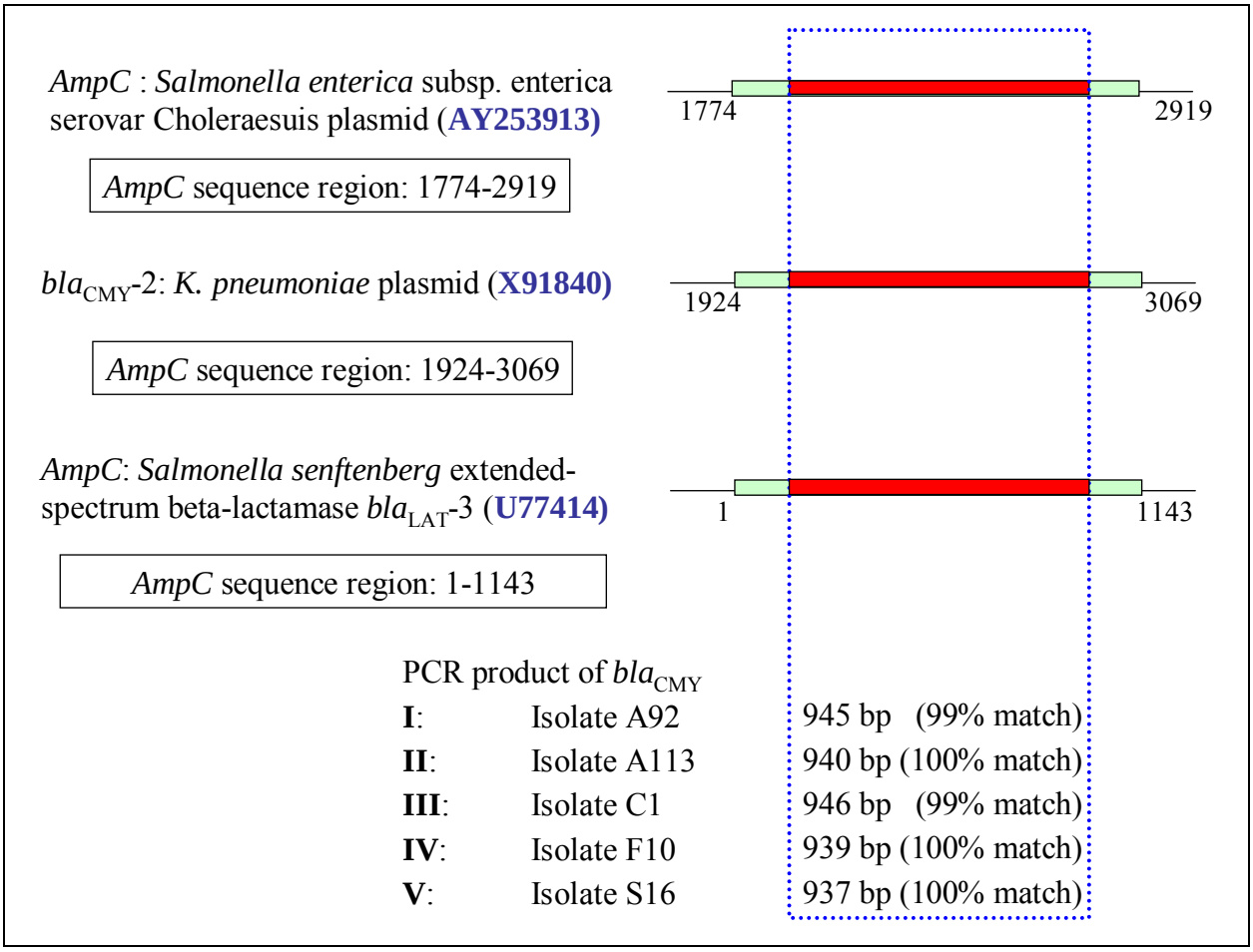


Figure **5.4**: Sequence match results of PCR amplicons of *bla*$_{CMY}$ carried by ceftiofur and extended spectrum cephalosporin (A92, A113, C1, F10, S16) resistant *E. coli* with the NCBI database. The figure represents 3 gene sequence matches (99-100%) from NCBI genebank database (Accession nos. AY253913, X91840, and U77414) for all the 5 *bla*$_{CMY}$.

5.6 REFERENCES

Bass, L., C. A. Liebert, M. D. Lee, A. O. Summers, D. G. White, S. G. Thayer, and J. J. Maurer. 1999. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance in avian *Escherichia coli*. Antimicrob. Agents Chemother. 43:2925-2929.

Bauernfeind, A., Y. Chong., and S. Schweighart. 1989. Extended broad spectrum beta-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. Infection. 17:316-321.

Bauernfeind, A., I. Stemplinger., R. Jungwirth., R. Wilhelm, and Y. Chong. 1996. Comparative characterization of the cephamycinase bla_{CMY}-1 gene and its relationship with other beta-lactamase genes. Antimicrob. Agents Chemother. 40:1926-1930.

Bauernfeind, A., Y. Chong., and K. Lee. 1998. Plasmid-encoded *AmpC* beta-lactamases: how far have we gone 10 years after the discovery? Yonsei Med. J. 39:520-525.

Blake, D. P., K. Hillman, D. R. Fenlon, and J. C. Low. 2003. Transfer of antibiotic resistance between commensal and pathogenic members of the *Enterobacteriaceae* under ileal conditions. J. Appl. Microbiol. 95:428-436.

Bradford, P. A., P. J. Petersen, I. M. Fingerman, and D. G. White. 1999. Characterization of expanded-spectrum cephalosporin resistance in *E. coli* isolates associated with bovine calf diarrhoeal disease. J. Antimicrob Chemother. 44:607-610.

Brinas, L., M. Zarazaga, Y. Saenz, F. Ruiz-Larrea, and C. Torres. 2002. Beta-lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. Antimicrob. Agents Chemother. 46:3156-3163.

Brinas, L., M. A. Moreno, T. Teshager, M. Zarazaga, Y. Saenz, C. Porrero, L. Dominguez, and C. Torres. 2003. Beta-lactamase characterization in *Escherichia coli* isolates with diminished susceptibility or resistance to extended-spectrum cephalosporins recovered from sick animals in Spain. Microb. Drug Resist. 9:201-209.

Bush, K., G. A. Jacoby, and A. A. Medeiros. 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. 39:1211-1233.

DeFrancesco, K. A., R. N. Cobbold, D. H. Rice, T. E. Besser, and D. D. Hancock. 2004. Antimicrobial resistance of commensal *Escherichia coli* from dairy cattle associated with recent multi-resistant salmonellosis outbreaks. Vet. Microbiol. 98:55-61.

Food and Drug Administration: Centre of Veterinary Medicine. 2005. FDA approved animal drug products (Greenbook). Available Online at www.fda.gov/cvm. Accessed on 26th January 2005.

Gniadkowski, M. 2001. Evolution and epidemiology of extended-spectrum beta-lactamases (ESBLs) and ESBL-producing microorganisms. Clin. Microbiol. Infect. 7:597-608.

Gilmore, A. 1996. Chloramphenicol and the politics of health. Can. Med. Assoc. J. 134:423-435.

Hall, R. M. 1997. Mobile gene cassettes and integrons: moving antibiotic resistance genes in gram-negative bacteria. Ciba Found. Symp. 207:192-202.

Hornish, R.E. and S. F. Kotarski. 2002. Cephalosporins in veterinary medicine - ceftiofur use in food animals. Curr. Top. Med. Chem. 2:717-731.

Jacoby, G. A., and A. A. Medeiros. 1991. More extended-spectrum β-lactamases. Antimicrob. Agents Chemother. 35:1697-1704.

Karas, J. A., D. G. Pillay, D. Muckart, and A. W. Sturm. 1996. Treatment failure due to extended-spectrum beta-lactamase. J. Antimicrob. Chemother. 37:203-204.

Levy, S. B. 1978. Emergence of antibiotic-resistant bacteria in the intestinal flora of farm inhabitants. J. Infect. Dis. 137:689-690.

Levy, S. B., B. Marshall, S. Schluederberg, D. Rowse, and J. Davies. 1988. High frequency of antimicrobial resistance in human fecal flora. Antimicrob. Agents Chemother. 32:1801-1806.

Livermore, D. M. 1995. β-lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. 8:557-584.

Mammeri, H., H. Nazic, T. Naas, L. Poirel, S. Leotard, and P. Nordmann. 2004. *AmpC* beta-lactamase in an *Escherichia coli* clinical isolate confers resistance to expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. 48:4050-4053.

Mandell, G. L. and W. A. Petri. 1996. Penicillins, cephalosporins, and other β-lactam antibiotics. p. 1073-1101. *In* Hardman J. G. and L. E. Limbird. eds. Goodman and Gilman's the pharmacological basis of therapeutics. New York: McGraw-Hill Book Co.

Marshall, B., D. Petrowski, and S. B. Levy. 1990. Inter- and intraspecies spread of *Escherichia coli* in a farm environmental in the absence of antibiotic usage. Microbiology. 87:6609-6613.

Mealey, K. L. 2001. Penicillins and beta-lactamase inhibitor combinations. J. Am. Vet. Med. Assoc. 218:1893-1896.

Medeiros, A. A. 1997 Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. Clin. Infect. Dis. 24:S19-S45.

National Committee for Clinical Laboratory Standards. 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 2nd ed, NCCLS document M31-A2. NCCLS, Wayne, Pennsylvania.

National Committee for Clinical Laboratory Standards. 1999. Performance standards for antimicrobial susceptibility testing. Ninth information supplement, M100-S9. National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania.

Navarro, F., E. Perez-Trallero, J. M. Marimon, R. Aliaga, M. Gomariz, and B. Mirelis. 2001. CMY-2-producing *Salmonella enterica, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis* and *Escherichia coli* strains isolated in Spain (October 1999-December 2000). J. Antimicrob. Chemother. 48:383-389.

O'Callaghan, C. H. 1979. Description and classification of the newer cephalosporins and their relationships with the established compounds. J. Antimicrob. Chemother. 5:635-671.

Oppegaard, H., T. M. Steinum, and Y. Wasteson. 2001. Horizontal transfer of a multi-drug resistance plasmid between coliform bacteria of human and bovine origin in a farm environment. Appl. Environ. Microbiol. 67:3732-3734.

Orden, J. A., J. A. Ruiz-Santa-Quiteria, S. Garcia, D. Cid, and R. De La Fuente. 2000. In vitro susceptibility of *Escherichia coli* strains isolated from diarrhoeic dairy calves to 15 antimicrobial agents. J. Vet. Med. B. Infect. Dis. Vet. Public Health. 47:329-335.

Österblad, M., A. Hakanen, R. Manninen, T. Leistevuo, R. Peltonen, O. Meurman, P. Huovinen, and P. Kotilainen. 2000. A between-species comparison of antimicrobial resistance in enterobacteria in fecal flora. Antimicrob. Agents Chemother. 44:1479-1484.

Philippon, A., R. Labia., and G. Jacoby. 1989. Extended-spectrum beta-lactamases. Antimicrob. Agents Chemother. 33:1131-1136.

Sanders, C. C., and W. E. Sanders. 1992. Beta-Lactam resistance in gram-negative bacteria: global trends and clinical impact. Clin. Infect. Dis. 15:824-839.

Schroeder, C. M., D. G. White, B. Ge, Y. Zhang, P. F. McDermott, S. Ayers, S. Zhao, and J. Meng. 2003. Isolation of antimicrobial-resistant *Escherichia coli* from retail meats purchased in Greater Washington, DC, USA. Int. J. Food. Microbiol. 85:197-202.

Shuttleworth, A. 2004. The rising incidence of antibiotic-resistant ESBL-producing *E. coli*. Nurs. Times. 100:30-31.

Sunde, M., and H. Sorum. 1999. Characterization of integrons in *Escherichia coli* of the normal intestinal flora of swine. Microb. Drug Resist. 5:279-287.

Thomson, C. J., and S. G. Amyes. 1993. Molecular epidemiology of the plasmid-encoded TEM-1 beta-lactamase in Scotland. Epidemiol. Infect. 110:117-125.

Tosini, F., P. Visca, I. Luzzi, A. M. Dionisi, C. Pezzella, A. Petrucca, and A. Carattoli. 1998. Class 1 integron-borne multiple-antibiotic resistance carried by IncFI and IncL/M plasmids in *Salmonella enterica* serotype Typhimurium. Antimicrob. Agents Chemother. 42:3053-3058.

United States Pharmacopeia. Available on line: http://www.usp.org/veterinary/ monographs/ main.html. Accessed 8th February, 2005.

Vahaboglu, H, M. Fuzi, S. Cetin, S. Gundes, E. Ujhelyi, F. Coskunkan, and O. Tansel. 2001. Characterization of extended-spectrum beta-lactamase (TEM-52)-producing strains of *Salmonella enterica* serovar Typhimurium with diverse resistance phenotypes. J. Clin. Microbiol. 39:791-793.

Van den Bogaard, and E. E. Stobberingh. 2000. Epidemiology of resistance to antibiotics. Links between animals and humans. Int. J. Antimicrob. Agents. 14:327–335.

Vanjak, D., C. Muller-Serieys, B. Picard, E. Bergogne-Berezin, and N. Lambert-Zechovsky. 1995. Activity of beta-lactamase inhibitor combinations on *Escherichia coli* isolates exhibiting various patterns of resistance to beta-lactam agents. Eur. J. Clin. Microbiol. Infect. Dis. 14:972-978.

White, D. G., C. Hudson, J. J. Maurer, S. Ayers, S. Zhao, M. D. Lee, L. Bolton, T. Foley, and J. Sherwood. 2000. Characterization of chloramphenicol and florfenicol resistance in *Escherchia coli* associated with bovine diarrhea. J. Clin. Microbiol. 38:4593-4598.

Winokur, P. L., A. Brueggemann, D. L. DeSalvo, L. Hoffmann, M. D. Apley, E. K. Uhlenhopp, M. A. Pfaller, and G. V. Doern. 2000. Animal and human multidrug-resistant, cephalosporin-resistant salmonella isolates expressing a plasmid-mediated CMY-2 *AmpC* beta-lactamase. Antimicrob. Agents Chemother. 44:2777-2783.

Winokur, P. L., D. L. Vonstein, L. J. Hoffman, E. K. Uhlenhopp, and G. V. Doern. 2001. Evidence for transfer of CMY-2 *AmpC* beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. 45:2716-2722.

Wu, P. J., K. Shannon, and I. Phillips. 1994. Effect of hyperproduction of TEM-1 beta-lactamase on in vitro susceptibility of *Escherichia coli* to beta-lactam antibiotics. Antimicrob. Agents Chemother. 38:494-498.

Zhao, S., D. G. White, B. Ge, S. Ayers, S. Friedman, L. English, D. Wagner, S. Gaines, and J. Meng. 2001a. Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. Appl. Environ. Microbiol. 67:1558-1564.

Zhao, S., D. G. White, P. F. McDermott, S. Friedman, L. English, S. Ayers, J. Meng, J. J. Maurer, R. Holland, and R. D. Walker. 2001b. Identification and expression of cephamycinase bla(CMY) genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob. Agents Chemother. 45:3647-3650.

Chapter 6

CONCLUSIONS AND RECOMMENDATIONS

Although the use of antibiotics was part of a routine on the dairy farms studied, current management practices are not structured for prudent use of antibiotics. It is important to educate the dairy producers about prudent antibiotic use and its implications for public health and that antibiotics are precious weapons in our fight against disease causing bacteria. Protocols on antibiotic usage should, therefore, be made an integral part of on-farm quality assurance programs.

Commensal Gram-negative bacteria are resistant to a variety of approved antibiotics, however the type and level (number of antibiotics) of resistance is highly variable. Multidrug-resistant commensal *E. coli* could survive for long periods in the environment. Monitoring of commensal antibiotic-resistant bacteria is essential as it will provide a more realistic picture of the potential issues that could emerge as a result of antibiotic usage. Using tetracyclines in medicated milk replacers fed for calves could influence the number of lactating cows shedding tetracycline-resistant Gram-negative enteric bacteria. Studies are needed to be directed toward addressing the influence of antibiotic selective pressure, in particular longitudinal studies to determine the effect of withdrawal of antibiotic usage on the dynamics of antibiotic-resistant bacteria in dairy cattle.

The role of *Tn*10 as the reservoir of *tet*(B) determinants in commensal *E. coli* and the frequency of transfer of resistance within a given species and between species needs to be assessed. This data would be valuable in developing risk assessment models that address transfer and maintenance of resistance in bacterial populations. It is recommended to elucidate whether or not antibiotic treatment protocols (dose and frequency) have a significant influence on treatment outcomes.

The prevalence of multidrug-resistance and extended-spectrum cephalosporin-resistance in commensal *E. coli* is an important public heath concern. This study warrants the necessity for continued monitoring of multidrug- and cephalosporin-resistance in dairy environments. Cephalosporins, such as ceftiofur, are the latest and most valuable antimicrobial agents available to the dairy industry and attempt to judiciously use this antibiotic need to be widely promoted.

Appendix A

SURVEY QUESTIONNAIRE

Table **A-1**: Herd information

FILE NO:					
1	**Date**				
2	**Extension Educator**				
3	**County**				
4	**Herd Code**	A B C (circle one)			
	Size	A: < 100	B: < 199	C :> 200	
5	**Farm Name**				
6	**Owner**				
7	**Address**				
8	**E-mail**				
9	**Telephone**				
10	**Fax**				
		Name	**Telephone**	**Address**	**E-mail**
11a-b-c-d	**Veterinarian**				
12a-b-c-d	**Milk Cooperative**				
13a-b-c-d	**Local Sanitarian**				
14	**Herd ID**				
15	**Herd DHIA Code #**				
16	**Other**				

Table **A-2**: Herd history

	Category	**Reported Number**	**Ideal ratios**	**Comments**
17	**Number of animals (as of today, or last month)**			
17a	Calves			
17b	Unbred heifers			
17c	Bred heifers			
17d	Milk cows			
17e	Dry cows			
17f	Bulls			
17g	Total			
18	**Breed**			
18a	Holsteins		X	
18b	Jersey		X	
18c	Other		X	
19	**Number of times the cows are milked**			
19a	2 times		X	
19b	3 times		X	
19c	4 times		X	
29	**Milk Production**	**Avg. per cow/day**	**Yearly avg.**	**Total milk shipped**
29	2001-2002			
30	**Milk Premiums**		**Score**	
30a	2 of 6 Months		1	
30b	3 of 6 Months		2	
30c	4 of 6 Months		3	
30d	5 of 6 Months		4	
30e	6 of 6 Months		5	

Table **A-3**: Practices Associated with Antibiotic Usage on Farm

		Yes	No	Score	Cum. Score
31a-b	Does the farm keep written records of all antibiotic treatments?				
32a-b	Do you have written plans for treating sick animals with antibiotics?				
33	Do you seek your veterinarian's advice before administering antibiotics?				
33a	Always (9-10)				
33b	Most of the time (6-8)				
33c	Sometimes (1-5)				
34	Other than your veterinarian, who administers the antibiotics to animals?				
34a	Owner				
34b	Manager / Herdsman				
34c	Milker / Farm worker				
35	Following administration of an antibiotic, is the course of treatment completed?				
35a	Always (9-10)				
35b	Most of the times (6-8)				
35c	Sometimes (1-5)				
36a-b	Is extra label usage of medication done only based on the orders and written guidelines from a veterinarian?				
37a-b	Are treated cows always visibly marked as "treated"?				
38a-b	Are treated cows physically separated from other milking cows?				
39a-b	Are treated cows milked last?				
40a-b	Are treated cows milked with a separate milking unit?				
41a-b	Are dry cows treated with antibiotics visibly marked as dry cow treated?				
42a-b	Were there any antibiotic residue violations in the past 6 months?				
43a-b	Are cows routinely screened after freshening for antibiotics with an antibiotic residue detection kit?				

Table **A-4**: Practices Associated with Antibiotic Usage on Farm

	Category	**Reported/ Observed**	**Ideal ratios**	**Assigned value**	**Score**	**Cum. Score**	
44	How many cases of clinical mastitis did this farm have in the past one year?						
45	Number of cases of clinical mastitis in a month? (Month_____?)						
46	How many cows have you treated with antibiotics in the last 30 days?						
47a-f	In your observation, when do the majority of mastitis cases occur? (circle one)	3 to 7 days before calving	7 days post calving	30 days into milk	mid lactation	late lactation	3-5 days post drying off
48a-e	In which group of animals do you experience most of your clinical mastitis? (circle one)	Dry cows	Fresh cows	First calf heifers	High producers	Others	

Table **A-5**: Records on Antibiotic Usage

CALVES					
	Antibiotic	**1**	**2**	**3**	**4**
Scours	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Pneumonia	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Abscesses Injuries Navel ill	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Eye Infections	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Milk Replacer	Name of Antibiotic				
	Route	oral			
	Tetracycline	Yes/No			
	Neomycin	Yes/No			
	Coccidiostat	Yes/No			
	Product Name				
Other	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				

Table **A-6**: Records on Antibiotic Usage

UNBRED HEIFERS					
	Antibiotic	1	2	3	4
Scours	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Pneumonia	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Foot rot	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Abscesses/Injuries	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Other_____	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Other_____	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				

Table **A-7**: Records on Antibiotic Usage

HEIFER MASITIS					
	Antibiotic	1	2	3	4
Lactation Preparation	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
	CMT				
	Pre-dip				
	Post-dip				
	Gloves				
Dry Cow Preparation	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
	CMT				
	Pre-dip				
	Post-dip				
	Gloves				
Other_____	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Other_____	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				

Table **A-8**: Records on Antibiotic Usage

LACTATING COWS					
	Antibiotic	1	2	3	4
Scours	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Pneumonia	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Foot rot	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Metritis	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Other_____	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Other_____	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				

Table **A-9**: Records on Antibiotic Usage

LACATING COW MASTITIS					
	Antibiotic	1	2	3	4
Lactation Preparation	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
	CMT				
	Pre-dip				
	Post-dip				
	Gloves				
Dry Cow Preparation	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
	CMT				
	Pre-dip				
	Post-dip				
	Gloves				
Other_____	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				
Other_____	Name of Antibiotic				
	Route				
	Amount				
	Frequency				
	How many per year				

Table **A-10**: List of antibiotics approved for use in dairy animals

Antibiotic	**Route of administration**	**Condition**
Amoxicillin	Intramammary Systemic	Lactating cow mastitis Respiratory diseases, foot rot
Ampicillin	Systemic	Respiratory diseases
Ceftiofur	Intramuscular/subcutaneous	Metritis, foot rot, respiratory diseases
Cephapirin 1.Sodium 2.Benzathine	 Intramammary Intramammary	 Lactating cow mastitis Dry cow mastitis
Cloxacillin 1.Sodium 2.Benzathine	Intramammary	 Lactating cow mastitis Dry cow mastitis
Chlortetracycline Oxytetracycline	Systemic Milk replacer	Enteritis, weight gain, pneumonia, foot rot
Erythromycin	Intramammary	Lactating and dry cow mastitis
Florfenicol	Systemic	Pneumonia and foot rot
Hetacillin	Intramammary	Lactating cow mastitis
Neomycin	Medicated feed Milk replacer	Bacterial enteritis
Novobiocin	Intramammary	Lactating and dry cow mastitis
Penicillin G	Intramammary Systemic	Lactating and dry cow mastitis Black leg, rhinitis, pneumonia, metritis
Penicillin G and Novobiocin	Intramammary	Lactating and dry cow mastitis
Penicillin G and Streptomycin	Intramammary	Dry cow mastitis
Pirlimycin	Intramammary	Lactating cow mastitis
Spectinomycin	Systemic	Pneumonia
Sulfadimethoxine	Systemic	Shipping fever complex, bacterial pneumonia, calf diphtheria, and foot rot
Compiled from 1. United States Pharmacopeia (http://www.usp.org/veterinary/monographs/main.html). 2. Food and Drug Administration-Center for Veterinary Medicine HFV-12, 301/594-1755, Infectious Mastitis Preparations (http://www.fda.gov/cvm/index/memos/cvmm34.html). 3. The Code of Federal Regulations (CFR) (http://www.gpoaccess.gov/cfr/index.html).		

Appendix B

SUBGENOMIC LIBRARY

Sub-Genomic library construction (protocols: Ausubel et al., 1998; Sambrook and Russell, 2001)

Isolation of genomic DNA with CTAB and NaCl method (large scale preparation)

A 100 ml of overnight culture of *E. coli* T8 in LB broth was spun down in 50 ml tubes to get a pellet. The pellet was re-suspended in 9.5 ml of TE buffer, 0.5 ml of 10% SDS, and 50 µl of 20 mg/ml proteinase K and incubated for 1hr at 37^{o}C. To this viscous solution, 1.8 ml of 5M NaCl was added and mixed thoroughly. A total of 80µl of prewarmed CTAB/NaCl was added, mixed, and incubated for 10 min in water bath at 65^{o}C. An equal ratio of chloroform/isoamylalcohol was added to the extract and centrifuged (10 min at 10,000 rpm). The aqueous phase was treated with an equal ratio of phenol/chloroform/isoamylalcohol and spun (10 min at 10000 rpm). The aqueous phase was transferred to a fresh tube and DNA was precipitated with 0.6 volume of isopropanol, then washed with 70% ethanol. The supernatant was decanted and the pellet was air dried and re-suspended in 100 µl of TE buffer.

Preparation of insert DNA from genomic DNA

Complete enzyme digestion was done with *Sau3A1*. Genomic DNA was incubated with *Sau3A1* (5´…▼GATC▲…3´) for variable lengths of time (0, 3, 6, 9 min….) until the genomic DNA was completely digested. Once the correct time and enzyme concentration was determined, the reaction was scaled up to get enough digested insert for cloning. The digested DNA was then treated with phenol/chloroform to remove the enzyme, followed by chloroform wash. The DNA

was precipitated at -20°C with final concentration of 0.3 M sodium acetate and equal volume of 100% chilled ethanol. The DNA pellet was washed with 70% ethanol and air dried. The digested DNA was electrophoresed through low melting point agarose gel. The size of genomic DNA suitable for *tet*(B) cloning was determined to be 2.5 to 3.5 kb region. All the DNA fragments with sizes between 2.5 to 3.5 kb were cut out from the gel and purified.

Transformation of chemically competent DHFα cells with pTrcHis vector

Plasmid pTrcHis vector (50 ng) (Invitrogen, Carlsbad, California) was added to 200 µl of $CaCl_2$ –treated DHFα cell suspension (Invitrogen, Carlsbad, California) in a sterile, pre-chilled microcentrifuge tube. The tube contents were mixed by swirling gently and stored on ice for 30 minutes. The tube was then transferred to a preheated 42°C circulating water bath and held for 90 seconds. After 90 seconds the tube was rapidly transferred to an ice bath for 1-2 minutes. To this tube 800 ul of LB broth was added and the tube was incubated for 1 hour at 37°C on a rotary shaker. Approximately 200µl were inoculated on a LB plate with ampicillin (50 µl/ml) and incubated at 37°C for 20 hours.

Midi-preparation of plasmid pTrcHis by alkaline lysis method

Ten ml of LB broth containing ampicillin (50µl/ml) was inoculated with a single colony of DHFα cells containing pTrcHis vector. The culture was incubated overnight at 37°C with vigorous shaking. The culture was centrifuged and the bacterial pellet was re-suspended in 200ul of ice-cold alkaline lysis solution I by vigorous vortexing. To this mixture 400µl of freshly prepared alkaline solution lysis solution II was added, the tube was inverted several times to mix the contents and then placed on ice. To the tube 300µl of alkaline solution lysis solution III was added, the tube was inverted several times and stored on ice for 3-5 minutes. The bacterial cell lysate was centrifuged at 14,000 rpm for 5 min and the supernatant transferred to a fresh tube. To

this an equal volume of phenol:chloroform was added and the organic and aqueous phase vortexed, the emulsion centrifuged at 14,000 rpm for 10 min and the aqueous upper layer transferred to a fresh tube. An equal volume of chloroform/isoamylalcohol was added, mixed, and centrifuged for 10 min at 14,000 rpm. The plasmids were precipitated from the supernatant with 0.8 volume of isopropanol at room temperature by mixing and left to stand for 2 minutes at room temperature. The precipitated nucleic acid was collected by centrifugation for 5 minutes, the supernatant removed and 1 ml of 70% ethanol added to the pellet, followed by centrifugation for 5 minutes. The supernatant was removed and tube was left to air dry. The plasmid was re-suspended in 100µl of TE buffer containing 20µl/ml of DNase-free RNase. The plasmid was gel purified by electrophoresing through low melting temperature agarose gel similar to the extraction of the genomic DNA digested fragments from gel slices (refer to paragraph 2 of material and methods of chapter 4).

Dephosphorylation of plasmid

The plasmid pTrcHis was linearized by *Bam*H I (5′-G↓ GATC-3′, site compatible with *Sau3A1*) at 37^{o}C for 2 hours. To prevent self-ligation and circularization of plasmid DNA, alkaline phosphatase enzyme was added and the mix was incubated at 37^{o}C for 1 hour followed by phenol-chloroform extraction to inactivate the enzyme.

Ligation of insert DNA to vector DNA

A number of small-scale ligations were performed using a set amount of vector, and varying amount of insert DNA using T4 DNA ligase within a reaction volume of 20µl. Various molar ratios of insert/vector (5:1, 2:1, 1:1, 0.5:1, and 0.2:1) were used. A control ligation of vector with no insert was used to determine the background of non-recombinant clones. The samples were incubated at 12^{o}C overnight. The samples were subjected to drop dialysis for 30

minutes to remove salts to prevent arcing of the electrical discharge. The samples were then used for electroporation.

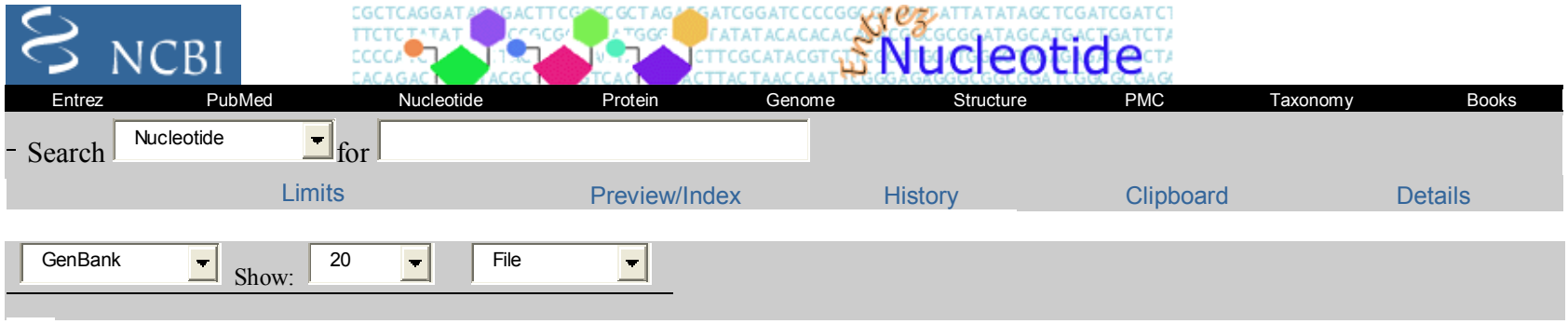
Electroporation

Electrocompetent cells (40 µl) were thawed and kept on ice. DNA (1-10 µg/ml) to be electroporated was added to the electrocompetent cells and kept on ice. The electroporation apparatus (MicroPulser, Bio-Rad, Hercules, CA) was used to deliver an electric pulse. The electroporation conditions were pre-programmed in to the MicroPulser, program E2 (V=2.5 kV) and the bacterial setting was used. The DNA/cell mixture was pipetted into an ice-cold electroporation cuvette (0.2 cm). The condensation and moisture from the outside of the cuvette was wiped off and the cuvette was placed in the electroporation device, an electric pulse delivered to the cells for ~5 milliseconds. Once the DNA/cell mixture was pulsed, immediately 1ml of LB broth was added at room temperature and the cells were then transferred to a 1.5ml centrifuge tube. The tubes were then gently rotated on a rotary for 1 hour at 37^{o}C. Approximately 200µl were inoculated on a LB plate with ampicillin (50µl/ml) to estimate the transformation efficiency. The ratio of DNA to vector that gave the best transformation efficiency was selected and the reaction was scaled up to 10 transformations. The transformed cells were plated on LB agar (150mm plates) with ampicillin (50µg/ml) and incubated at 37^{o}C for 24 hrs. The colonies growing on the plates with ampicillin were harvested and pooled and plated on LB agar with tetracycline (12µg/ml) and incubated at 37^{o}C for 24 hrs.

Screening for tetracycline resistance determinants

Midi plasmid extraction from clones growing on tetracycline (12µg/ml) was done with an alkaline lysis method. The cloned plasmids were then gel purified. The purified plasmids were sequenced with primers pTrc F 5'-gaggtatatattaatgtatcg-3' and pTrc R 5'-gatttaatctgtatcagg-3'

(Invitrogen, Carsbad, California). A clone was selected for sequencing the entire insert with 2 more sets of primers (F-II, R-II and F-III, R-III) that were designed using the initial sequencing data. Primers were designed using Oligo 6.6, Molecular Biology Insites, Inc, Cascade, Colorado. High stringency and Tm above 60^{o}C were the criteria used for designing primers. The sequencing and primer designing was done at the Nucleic Acid Facility, 210 Wartik Laboratory, Penn State University, University Park, PA.



1: AY528506. Escherichia coli ...[gi:42494905]

```
LOCUS       AY528506                4632 bp    DNA     linear   BCT 15-FEB-2004
DEFINITION  Escherichia coli transposon Tn10 tetracycline repressor protein
            (tetR), tetracycline efflux pump protein (tetA), TetC (tetC), and
            TetD (tetD) genes, complete cds, and insertion sequence IS10
            transposase protein (tnp) gene, complete cds.
ACCESSION   AY528506
VERSION     AY528506.1  GI:42494905
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 4632)
  AUTHORS   Sawant,A.A., Hegde,N.V. and Jayarao,B.M.
  TITLE     Partial sequence of Tn10 transposon associated with tetracycline
            resistance in Escherichia coli from feces of dairy cattle
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 4632)
  AUTHORS   Sawant,A.A., Hegde,N.V. and Jayarao,B.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-JAN-2004) Veterinary Science, Pennsylvania State
            University, 115 Henning Building, University Park, PA 16802, USA
```

Figure **B.1**: Partial sequence of *Tn*10 with *tet* determinants on NCBI database

```
FEATURES             Location/Qualifiers
     source          1..4632
                     /organism="Escherichia coli"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:562"
     repeat_region   <1..>4632
                     /transposon="Tn10"
     gene            complement(224..850)
                     /gene="tetR"
     CDS             complement(224..850)
                     /gene="tetR"
                     /codon_start=1
                     /transl_table=11
                     /product="tetracycline repressor protein"
                     /protein_id="AAS17729.1"
                     /db_xref="GI:42494906"
                     /translation="MMSRLDKSKVINSALELLNEVGIEGLKTRKLAQKLGVEQPTLYW
                     HVKNKRALLDALAIEMLDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHL
                     GTRPTEKQYETLENQLAFLCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERE
                     TPTTDSMPALLRQAIELFEHQGAEPAFLFGLELIICGLEKQLKCESGS"
     gene            929..2134
                     /gene="tetA"
     CDS             929..2134
                     /gene="tetA"
                     /codon_start=1
                     /transl_table=11
                     /product="tetracycline efflux pump protein"
                     /protein_id="AAS17730.1"
                     /db_xref="GI:42494907"
                     /translation="MNSSTKIALVITLLDAMGIGLIMPVLPTLLREFIASEDIANHFG
                     VLLALYALMQVIFAPWLGKMSDRFGRRPVLLLSLIGASLDYLLLAFSSALWMLYLGRL
                     LSGITGATGAVAASVIADTTSASQRVKWFGWLGASFGLGLIAGPIIGGFAGEISPHSP
                     FFIAALLNIVAFLVVMFWFRETKNTRDNTDTEVGVETQSNSVYITLFKTMPILLIIYF
                     SAQLIGQIPATVWVLLAENRFGWNSMMVGFSLAGLGLLHSVFQAFVAGRIATKWGEKT
                     AVLLGFIADSSAFAFLAFISEGWLVFPGLILLAGGGIAIPALQGVMSIQTKSHQQGAL
                     QGLLVSLTNATGVIGPLLFAVIYNHSLPIWDGWIWIIGLAFYCIIILLSMTFMLTPQA
                     QGSKQETSA"
```

```
gene            complement(2247..2915)
                /gene="tetC"
CDS             complement(2247..2915)
                /gene="tetC"
                /codon_start=1
                /transl_table=11
                /product="TetC"
                /protein_id="AAS17731.1"
                /db_xref="GI:42494908"
                /translation="MFLRLSRPHHHDTINILTVCYFKSIMENKNHQQENFKSTYQSLV
                NSARILFVEKGYQAVSIDEISGKALVTKGAFYHHFKNKKQLLSACYKQQLIMIDAYIT
                TKTDLTNGWSALESIFEHYLDYIIDNNKNLIPIQEVMPIIGWNELEKISLEYITGKVN
                AIVSKLIQENQLKAYDDDVLKNLLNGWFMHIAIHAKNLKELADKKGQFIAIYRGFLLS
                LKDK"
gene            2928..3344
                /gene="tetD"
CDS             2928..3344
                /gene="tetD"
                /codon_start=1
                /transl_table=11
                /product="TetD"
                /protein_id="AAS17732.1"
                /db_xref="GI:42494909"
                /translation="MYIEQHSRYQNKANNIQLRYDDKQFHTTVIKDVLLWIEHNLDQS
                LLLDDVANKAGYTKWYFQRLFKKVTGVTLASYIRARRLTKAAVELRLTKKTILEIALK
                YQFDSQQSFTRRFKYIFKVTPSYYRRNKLWELEAMH"
```

```
gene            complement(2247..2915)
                /gene="tetC"
CDS             complement(2247..2915)
                /gene="tetC"
                /codon_start=1
                /transl_table=11
                /product="TetC"
                /protein_id="AAS17731.1"
                /db_xref="GI:42494908"
                /translation="MFLRLSRPHHHDTINILTVCYFKSIMENKNHQQENFKSTYQSLV
                NSARILFVEKGYQAVSIDEISGKALVTKGAFYHHFKNKKQLLSACYKQQLIMIDAYIT
                TKTDLTNGWSALESIFEHYLDYIIDNNKNLIPIQEVMPIIGWNELEKISLEYITGKVN
                AIVSKLIQENQLKAYDDDVLKNLLNGWFMHIAIHAKNLKELADKKGQFIAIYRGFLLS
                LKDK"
gene            2928..3344
                /gene="tetD"
CDS             2928..3344
                /gene="tetD"
                /codon_start=1
                /transl_table=11
                /product="TetD"
                /protein_id="AAS17732.1"
                /db_xref="GI:42494909"
                /translation="MYIEQHSRYQNKANNIQLRYDDKQFHTTVIKDVLLWIEHNLDQS
                LLLDDVANKAGYTKWYFQRLFKKVTGVTLASYIRARRLTKAAVELRLTKKTILEIALK
                YQFDSQQSFTRRFKYIFKVTPSYYRRNKLWELEAMH"
```

```
gene            complement(3354..4562)
                /gene="tnp"
CDS             complement(3354..4562)
                /gene="tnp"
                /codon_start=1
                /transl_table=11
                /product="transposase protein"
                /protein_id="AAS17733.1"
                /db_xref="GI:42494910"
                /translation="MCELDILHDSLYQFCPELHLKRLNSLTLACHALLDCKTLTLTEL
                GRNLPTKARTKHNIKRIDRLLGNRHLHKERLAVYRWHASFICSGNTMPIVLVDWSDIR
                EQKRLMVLRASVALHGRSVTLYEKAFPLSEQCSKKAHDQFLADLASILPSNTTPLIVS
                DAGFKVPWYKSVEKLGWYWLSRVRGKVQYADLGAENWKPISNLHDMSSSHSKTLGYKR
                LTKSNPISCQILLYKSRSKGRKNQRSTRTHCHHPSPKIYSASAKEPWVLATNLPVEIR
                TPKQLVNIYSKRMQIEETFRDLKSPAYGLGLRHSRTSSSERFDIMLLIALMLQLTCWL
                AGVHAQKQGWDKHFQANTVRNRNVLSTVRLGMEVLRHSGYTITREDLLVAATLLAQNL
                FTHGYALGKL"
repeat_region   complement(<3354..>4562)
                /insertion_seq="IS10"
```

```
   1 atcacattaa gtggtattca atattttcat gaaatgggaa ttgacgttcc ttccaaacat
  61 tcacgtaaaa tctgttgtgc gtgtttagat tggagtgaac gccgtttcca tttaggtggg
 121 tacgttggag ccgcattatt ttcgctttat gaatctaaag ggtggttaac tcgacatctt
 181 ggttaccgtg aagttaccat cacggaaaaa ggttatgctg cttttaagac ccactttcac
 241 atttaagttg tttttctaat ccgcatatga tcaattcaag gccgaataag aaggctggct
 301 ctgcaccttg gtgttcaaat aattcgatag cttgtcgtaa taatgccggc atactatcag
 361 tagtaggtgt ttccctttct tctttagcga cttgatgctc ttgatcttcc aatacgcaac
 421 ctaaagtaaa atgccccaca gcgctgagtg catataatgc attctctagt gaaaaacctt
 481 gttggcataa aaaggctaat tgattttcga gagtttcata ctgtttttct gtaggccgtg
 541 tacctaaatg tacttttgct ccatcgcgat gacttagtaa agcacatcta aaacttttag
 601 cgttattacg taaaaaatct tgccagcttt ccccttctaa agggcaaaag tgagtatggt
 661 gcctatctaa catctcaatg gctaaggcgt cgagcaaagc ccgcttattt tttacatgcc
 721 aatacaatgt aggctgctct acaccaagct tctgggcgag tttacgggtt tttaaacctt
 781 cgattccgac ctcattaagc agctctaatg cgctgttaat cactttactt ttatctaatc
 841 tagacatcat taattcctaa tttttgttga cactctatca ttgatagagt tattttacca
 901 ctccctatca gtgatagaga aaagtgaaat gaatagttcg acaaagatcg cattggtaat
 961 tacgttactc gatgccatgg ggattggcct tatcatgcca gtcttgccaa cgttattacg
1021 tgaatttatt gcttcggaag atatcgctaa ccactttggc gtattgcttg cactttatgc
1081 gttaatgcag gttatctttg ctccttggct tggaaaaatg tctgaccgat ttggtcggcg
1141 cccagtgctg ttgttgtcat taataggcgc atcgctggat tacttattgc tggcttttc
1201 aagtgcgctt tggatgctgt atttaggccg tttgctttca gggatcacag gagctactgg
1261 ggctgtcgcg gcatcggtca ttgccgatac cacctcagct tctcaacgcg tgaagtggtt
1321 cggttggtta ggggcaagtt ttgggcttgg tttaatagcg gggcctatta ttggtggttt
1381 tgcaggagag atttcaccgc atagtccctt ttttatcgct gcgttgctaa atattgtcgc
1441 tttccttgtg gttatgtttt ggttccgtga aaccaaaaat acacgtgata atacagatac
1501 cgaagtaggg gttgagacgc aatcgaattc ggtatacatc actttattta aaacgatgcc
```

```
1561 cattttgttg attatttatt tttcagcgca attgataggc caaattcccg caacggtgtg
1621 ggtgctatta gccgaaaatc gttttggatg gaatagcatg atggttggct tttcattagc
1681 gggtcttggt cttttacact cagtattcca agcctttgtg gcaggaagaa tagccactaa
1741 atggggcgaa aaaacggcag tactgctcgg atttattgca gatagtagtg catttgcctt
1801 tttagcgttt atatctgaag gttggttagt tttccctgga ttaattttat tggctggtgg
1861 tgggatcgct atacctgcat tacagggagt gatgtctatc caaacaaaga gtcatcagca
1921 aggtgcttta cagggattat tggtgagcct taccaatgca accggtgtta ttggcccatt
1981 actgtttgct gttatttata atcattcact accaatttgg gatggctgga tttggattat
2041 tggtttagcg ttttactgta ttattatcct gctatcgatg accttcatgt taacccctca
2101 agctcagggg agtaaacagg agacaagtgc ttagttattt cgtcaccaaa tgatgttatt
2161 ccgcgaaata taatgaccct cttgataacc caagagggca ttttttacga taaagaagat
2221 ttagcttcaa ataaaaccta tctattttat ttatctttca agctcaataa aaagccgcgg
2281 taaatagcaa taaattggcc ttttttatcg gcaagctctt ttaggttttt cgcatgtatt
2341 gcgatatgca taaaccagcc attgagtaag tttttaagca catcatcatc ataagcttta
2401 agttggttct cttggatcaa tttgctgaca atggcgttta ccttaccagt aatgtattca
2461 aggctaattt tttcaagttc attccaacca atgataggca tcacttcttg gatagggata
2521 aggtttttat tattatcaat aatataatca agataatgtt caaatatact ttctaaggca
2581 gaccaaccat ttgttaaatc agtttttgtt gtgatgtagg catcaatcat aattaattgc
2641 tgcttataac aggcactgag taattgtttt ttatttttaa agtgatgata aaaggcacct
2701 ttggtcacca acgcttttcc cgagatctca tctattgaaa cagcttgata gccttttca
2761 acaaacaata ttcgtgctga gttaaccagt gattgatagg tactcttaaa attttcttgt
2821 tgatgatttt tattttccat gatagattta aaataacata ccgtcagtat gtttatggta
2881 tcatgatgat gtggtcgtga caatcttaag aacatttagg ttattttatg tatattgaac
2941 agcattctcg ctatcaaaat aaagctaata acatccaatt aagatatgat gataagcagt
```

```
3001 ttcatacaac ggttatcaaa gatgttctat tatggattga acataattta gatcagtctt
3061 tactgcttga tgatgtggcg aataaagcgg gttataccaa gtggtatttt cagcggctgt
3121 tcaaaaaagt aacaggggtc acactggcta gctatattcg tgctcgtcgt ttgacgaaag
3181 cggctgttga gttgaggttg acgaaaaaaa ctatccttga gatcgcatta aaatatcaat
3241 ttgattccca acaatctttt acacgtcgat ttaagtacat ttttaaggtt acaccaagtt
3301 attatcggcg taataaatta tgggaattgg aggcaatgca ctgagagatc ccctcataat
3361 ttccccaaag cgtaaccatg tgtgaataaa ttttgagcta gtagggttgc agccacgagt
3421 aagtcttccc ttgttattgt gtagccagaa tgccgcaaaa cttccatgcc taagcgaact
3481 gttgagagta cgtttcgatt tctgactgtg ttagcctgga agtgcttgtc ccaaccttgt
3541 ttctgagcat gaacgcccgc aagccaacat gttagttgaa gcatcagggc gattagcagc
3601 atgatatcaa aacgctctga gctgctcgtt cggctatggc gtaggcctag tccgtaggca
3661 ggacttttca agtctcggaa ggtttcttca atctgcattc gcttcgaata gatattaaca
3721 agttgtttgg gtgttcgaat ttcaacaggt aagttagttg ctagaaccca tggctccttt
3781 gccgacgctg agtagatttt aggtgacggg tggtgacaat gagtccgtgt cgagcgctga
3841 ttttttcggc ctttagagcg agatttatac aatagaattt ggcatgagat tggattgctt
3901 ttagtcagcc tcttatagcc taaagtcttt gagtgactag atgacatatc atgtaagttg
3961 ctgataggtt tccagttttc cgctcctagg tctgcatatt gtacttttcc tcttactcga
4021 cttaaccagt accaacccag cttctcaacg gatttatacc atggcacttt aaagccagca
4081 tcactgacaa tgagcggtgt ggtgttactc ggtagaatgc tcgcaaggtc ggctagaaat
4141 tggtcatgag ctttctttga acattgctct gaaagcggga acgctttctc ataaagagta
4201 acagaacgac cgtgtagtgc gactgaagct cgcaatacca taagtcgttt ttgctcacga
4261 atatcagacc agtcaacaag tacaatgggc atcgtattgc ccgaacagat aaagctagca
4321 tgccaacggt atacagcgag tcgctctttg tggaggtgac gattacctaa caatcggtcg
4381 attcgtttga tgttatgttt tgttctcgct ttggttggca ggttacggcc aagttcggta
4441 agagtgagag ttttacagtc aagtaatgcg tggcaagcca acgttaagct gttgagtcgt
4501 tttaagtgta attcggggca gaattggtaa agagagtcgt gtaaaatatc gagttcgcac
4561 atcttgttgt ctgattattg atttttcgcg aaaccatttg atcatatgac aagatgtgat
4621 ccacctaact aa
```

Appendix C

ABBREVIATIONS

AMP	: Ampicillin
ATM	: Aztreonam
BLAST	: Basic Local Alignment Search Tool
CA	: Clavulanic acid
CAZ	: Ceftazidime
CDC	: Centers for Disease Control and Prevention
CEF	: Cephalothin
CFP	: Cefoperazone
CFZ	: Cefazolin
CHL	: Chloramphenicol
CPD	: Cefpodoxime
CRO	: Ceftriaxone
CTT	: Cefotetan
CTX	: Cefotaxime
CXM	: Cefuroxime
DHIA	: National Dairy Herd Improvement Association
ENO	: Enrofloxacin
ESBL	: Extended Spectrum Beta Lactamases
FDA-CVM	: Food and Drug Administration-Center for Veterinary Medicine
FOX	: Cefoxitin
GEN	: Gentamicin
GEN	: Gentamicin
GN-EB	: Gram-Negative Enteric Bacteria
MAC	: MacConkey's medium
MDR	: Multi Drug Resistant
NARMS	: National Antimicrobial Resistance Monitoring System
NCBI	: National Center for Biotechnology Information
NCCLS	: National Committee for Clinical Laboratory Standards
NIT	: Nitrofurantoin.
PBP	: Penicillin Binding Protein
PCR	: Polymerase Chain Reaction
PFGE	: Pulse Field Gel Electrophoresis
PIP	: Piperacillin
SAM	: Ampicillin and Sulbactam
SPT	: Spectinomycin
STEC	: Shiga Toxin Producing *Escherichia coli*
TET	: Tetracycline
TIC	: Ticarcillin

TIM : Ticarcillin and Clavulanic acid
TOB : Tobramycin
TZP : Piperacillin and tazobactam
USDA : United States Department of Agriculture
XNL : Ceftiofur
ZOX : Ceftizoxime

VITA

Ashish Sawant

- B.V.Sc & A.H. 1995 Bombay Veterinary College, India.
- M.V.Sc. 1998 Food Hygiene and Veterinary Public Health, BVC.
- Ph. D. 2005 Pathobiology, Pennsylvania State University, PA.

Awards and Honors

2004 1st place award, Annual Environmental Chemistry Student Symposium
2004 4th place award, Gamma Sigma Delta- College of Agricultural Sciences
2003 Gerald Gentry Award for Excellence in Graduate Research
2001 3rd place award, Annual Graduate Exhibition in Health and Life Sciences

Publications

- **Sawant, A. A.**, S. R. Pillai, and B. M. Jayarao. 2001. Evaluation of five selective media for isolation of catalase negative gram positive cocci from raw milk. J. Dairy Sci. 85: 1127-1132.
- Jayarao B. M., S. R. Pillai, **A. A. Sawant**, D. R. Wolfgang, and N. V. Hegde. 2004. Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. J. Dairy Sci. 87:3561-3573.
- **Sawant A. A.,** N. V. Hegde, and B. M. Jayarao. 2004. Partial sequence of Tn10 transposon associated with tetracycline resistance in *Escherichia coli* from feces of dairy cattle. Sequence Accession No: 528506.
- **Sawant A. A.,** and B. M. Jayarao. 2002. Milk replacers and bacterial antibiotic resistance. Herd Health Memo. December. 4-5.

Grants: Jayarao, B. M., **A. A. Sawant** (Collaborator), B. C. Love, and N. V. Hedge. 2003. Study on the prevalence of hoof infections in dairy cattle with special reference to "Super Foot Rot" in Pennsylvania. PDA grant. Funding: $18,000.

Select presentations

- **Invited Guest speaker**. 2004. Food Microbiology Short Course, PSU, Reading, PA
- **Poster presentation**. 2004. NMC 43rd Annual meeting, Charlotte, North Carolina
- **Oral presentation**. 2003. ADSA-ASAS and MAAP meeting. Phoenix, Arizona.
- **Oral presentation**. 2002. ADSA-ASAS and CSAS meeting. Quebec City, Canada

Master's Thesis

Improvement in microbiological quality and shelf life of processed meat products employing low dose gamma irradiation. 1998. Bombay Veterinary College, India.