A determination of antibiotic resistance before and after the introduction of production livestock

by

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# Table of contents

Abstract iii

Chapter 1. General Introduction
  Introduction 1
  Thesis Organization 3
  References 4
  Literature Review 6
  References 23

Chapter 2. The establishment and proliferation of antibiotic-resistant *Campylobacter* on a new dairy farm environment prior to and after the placement of dairy cattle
  Abstract 30
  Introduction 30
  Materials and Methods 33
  Results and Discussion 35
  Conclusion 42
  Acknowledgements 44

Chapter 3. A longitudinal study of the establishment and proliferation of *Enterococcus* on a dairy farm
  Abstract 50
  Introduction 50
  Materials and Methods 52
  Results and Discussion 54
  Conclusion 60
  Acknowledgements 63

Chapter 4. The establishment of *Enterobacteriaceae* and *Salmonella london* in a new dairy farm environment
  Abstract 68
  Introduction 68
  Materials and Methods 71
  Results and Discussion 83
  Conclusion 85
  Acknowledgements 86

Chapter 5. General conclusions
  General Discussion 93
  Recommendations for Future Research 97
  References 98
  Appendix of the Dairy/Animal Science Education Facility 101

Acknowledgements 102
Abstract

Bacterial antimicrobial resistance represents an important current and future problem in infectious disease public health. When reviewing longitudinal studies (research studies involving repeated observations of a location over a period of time), very little information was found regarding the proliferation and dissemination of foodborne bacteria in a new dairy farm environment. Iowa State University (ISU; Ames IA) was awarded 887 acres of land by a donor family. An academic teaching farm was later constructed, The Dairy/Animal Science Education Facility (or the ISU Dairy Farm). Prior to building the ISU Dairy Farm, the land was only used for recreational activities-no production livestock had ever been introduced. It was decided to study the bacteria microbiota Enterobacteriaceae (particularly Salmonella), Enterococcus and Campylobacter. Microbiological sampling was conducted using a variety of techniques—from phenotypic methods (e.g. detecting bacteria by using specialized media) to genotypic methods (e.g. Pulsed Field Gel Electrophoresis). Sampling took place prior to dairy cattle placement (20 October 2007 and 13 November 2007) and after placement (27 January 2008, 29 February 2008, 12 March 2008, 23 April 2008 and 28 May 2008). A literature review discussing the introduction of dairy cattle to the United States, antibiotics used in animal production, antibiotic resistance, and the “prudent” (or responsible) use of antibiotics is discussed in chapter 1. Data from the ISU Dairy Farm Studies are included in chapter 2 (the establishment and proliferation of antibiotic-resistant Campylobacter), chapter 3 (the establishment and proliferation of Enterococcus spp.) and chapter 4 (the establishment of Enterobacteriaceae and Salmonella london). Chapter 5 addresses general conclusions of this work and recommendations for future research.
Introduction

Dairy cows were first brought to the United States in the 1600s (17) and the dairy industry has changed much since then. In 2006, a total of 9.1 million cows were on 2 million farms—a considerable change from over 21 million cows on 5 million farms in 1910 (21). The rapid progress in genetics and management has created a new era in which a smaller number of dairy cows meet the growing demand for dairy products (27). Since 1970, milk production has risen by almost half, even though the numbers of dairy cows have declined by about a fourth (from about 12 million in 1970 to about 9.1 million in 2006) (22). The growth of animals in conditions of crowding often favors the appearance of infectious disease that require antimicrobial treatment (14) often at great expense to the dairy producer. Mastitis is the costliest disease in the dairy industry, generating losses of over $2 billion in the US alone (18).

There is controversy regarding the use of antibiotics in growth promotion and prophylaxis in agriculture. Some researchers believe that the use of antibiotics is implicated as a contributing source of resistant bacterial strains that can be transmitted to humans through the food chain (3, 4). Others feel that commercial livestock production in the US (especially confinement production) would be virtually impossible without antibiotics (11). To support this view they cite studies such as those demonstrating that low levels of antimicrobial drugs increase daily rates of weight gain and improve feed efficiency in livestock (which lowers feed costs) (11). Additionally, weight gain ranging from 4-60 percent has been observed in animals fed low levels of antibiotics (8, 11). In the last 25 years however, there has been a paradigm shift from the treatment of clinical illness to disease prevention using techniques such as epidemiology (to describe and quantify the interconnected risk factors that produce disease), redefining disease more broadly (to include subclinical conditions such as subclinical mastitis, ketosis and rumen acidosis), and improving the understanding of the metabolism and physiology of cows (10). Scientists are also addressing concerns about animal welfare and researching topics such as animal comfort and the physical and mental well-being of animals (25). It is hoped that these activities can contribute to the responsible use of antibiotics. Milk has a positive reputation of being a wholesome and health-promoting food, and most dairy farms are independent family farms where cows are well-cared for (27).
However, if consumers perceive milk to be unsafe for zoonotic reasons, or disapprove of animal husbandry practices, they can respond by consuming less dairy products (5, 27).

The World Health Organization (WHO) estimates that 1.5 million people died worldwide from diarrheal diseases in 2005 (2). A separate study estimates that 70% of diarrheal diseases are foodborne (2). Collectively, foodborne diseases incur enormous financial costs. In the US, the estimated financial costs for six bacterial pathogens (Campylobacter jejuni, Clostridium perfringes, Escherichia coli O157:H7, Listeria monocytogenes, Salmonella, Staphylococcus aureus) and 1 parasite (Toxoplasma gondii) were estimated at $6.5 billion to $34.9 billion dollars annually (1, 2). This is likely an underestimate of total foodborne diseases costs because there may be over 200 microbiologic agents that cause foodborne disease (2).

Salmonella infections are among the most prevalent recognized communicable disease cause by bacteria in the US (13). The vast majority of these infections are transmitted from animals to humans through food and occasionally from person to person by the fecal-oral route (13). Salmonella serotypes enteritidis, typhimurium, and newport accounted for 43% of isolates from human sources reported to the US Centers for Disease Control and Prevention (CDC) (23). Salmonella contamination is most frequently associated with eggs and poultry products, but milk and dairy products have also been implicated (23). Dairy-related foodborne outbreaks of Salmonella have been associated with consumption of unpasteurized milk as well as milk products contaminated post-processing (23). Ground beef has also been implicated in outbreaks (23).

Enterococcus spp. are a complex, diverse and important group of bacteria in terms of their interaction with humans and animals (7). They are ubiquitous and are found in the gastrointestinal tracts of humans and other animals, in soil, water, and in the foods we eat (7). For many years, Enterococcus were believed to be harmless to humans and considered unimportant medically (6). Recently, enterococci have become one of the most common hospital-acquired pathogens, with patients having a high mortality rate of up to 61% (6). Enterococcus also cause infections in animals including mastitis in cattle, diarrhea in animals such as calves and foals, as well as infections in birds (7). Enterococci are resistant to many antibiotics. In an increasing number of cases, vancomycin is the only drug that remains
effective. The emergence of vancomycin-resistant Enterococcus (VRE) is of particular concern (26).

Campylobacter is the most commonly isolated bacterial enteric pathogen of humans in the US, exceeding Salmonella (27). Until recently, little attention was paid to Campylobacter because their role as enteric pathogens were not discovered until the late 1970s (20). In addition, infection is rarely fatal and they seldom cause large outbreaks (20). The infectious dose is extremely low (about 500 organisms) and can cause a range of disease from acute diarrhea to abortion in sheep, cattle and (rarely) humans (16). In one out of 3000 cases of human campylobacteriosis, post-infection manifestations can result—including Guillain-Barré syndrome (ascending paralysis) and Miller-Fisher Syndrome (characterized by blurred vision and severe lack of muscle coordination) (15). Campylobacters are considered to be a part of the normal flora of a wide range of domestic and wild animals (24). They are widespread in the environment, where their presence suggests recent contamination with animal and avian species, agricultural runoff, and sewage effluent (9). Campylobacters are often isolated from cattle and sheep, however shedding in feces is intermittent (9, 24). Factors which encourage fecal shedding of Campylobacter include giving birth, weaning, change of pasture, movement outdoors and transport (9).

Bacterial antimicrobial resistance represents an important current and future problem in infectious disease public health (3). When reviewing longitudinal studies (a research study involving repeated observations of a location over a period of time) (19), very little information was found regarding the proliferation and dissemination of foodborne bacteria in a new dairy farm environment. Iowa State University (ISU; Ames IA) was awarded 887 acres of land by a donor family. An academic teaching farm was constructed, The Dairy/Animal Science Education Facility (or the ISU Dairy Farm). Prior to building the ISU Dairy Farm, the land was only used for recreational activities-no livestock had ever been introduced. It was decided to study the bacteria microflora Enterobacteriaceae (particularly Salmonella), Enterococcus and Campylobacter. Microbiological sampling was conducted using a variety of techniques—from phenotypic (detecting bacteria by using specialized media) to genotypic methods (Pulsed Field Gel Electrophoresis). Sampling took place prior to dairy cattle placement (20 October 2007 and 13 November 2007) and after placement (27
January 2008, 29 February 2008, 12 March 2008, 23 April 2008 and 28 May 2008). Data from these studies are included in chapter 2 (the establishment and proliferation of antibiotic-resistant *Campylobacter*), chapter 3 (the establishment and proliferation of *Enterococcus* spp.) and chapter 4 (the establishment of *Enterobacteriaceae* and *Salmonella london*). Chapter 5 addresses general conclusions of this work and recommendations for future research.

**References**


A determination of antibiotic resistance before and after the introduction of production livestock

Literature Review

Dairy cattle were first brought to the United States in the 1600s (66). The dairy industry has changed considerably since then. In 1910, more than 20 million cows were maintained on 5 million farms, averaging 4 cows per farm (64, 66). In 2006, a total of 9.1 million cows were on 2 million farms (64, 85). Despite the overall decrease in the number of animals, production of dairy products has increased substantially. Milk production increased from 66,464 million kg (146,528 million lb) in 1991 to 81,809 million kg (180,357 million lb) in 2006 (85). Consumers drink an average of 104.5 kg (230 lb) of milk and eat approximately 12 kg (26 lb) of cheese, 7.3 kg (16 lb) of ice cream and 2.3 kg (5 lb) of butter per capita annually (66). Mortality and morbidity among dairy cattle can result in increased health costs, reduced milk yield, and decreased profits for the producer. Additionally, milk can harbor a variety of microorganisms that can be an important source of foodborne pathogens (70). Foodborne diseases are estimated to cause an estimated 76 million human illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States yearly (38).

Vaccinations are a major means of controlling viral and bacterial infections in dairy herds. However diarrhea and respiratory disease are the most common illness among cattle (85). For example, clinical mastitis among US dairy cows increased from 14.7% in 2002 to 16.5% in 2008 (66). Antibiotics such as amoxicillin, erythromycin and oxytetracycline are used to treat intramammary infections (66). Antibiotics are defined as substances produced by fungi, bacteria (or by pharmaceutical chemists) that kill or inhibit the growth of other microorganisms without harm to the eukaryotic host (55, 76). They work by exploiting differences that exists in cell structure between bacterial (prokaryotic) cells and animal (eukaryotic) cells. Antibiotics are often described as “bacteriostatic” or “bactericidal” (74). The term bacteriostatic describes a drug that temporarily inhibits the growth of an organism. Once the drug is removed, the organism will resume growth. The term bactericidal refers to a drug that causes cell death (74). Bactericidal drugs are used extensively to prevent or treat microbial infections in human and veterinarian medicine (48). Antimicrobial agents have
been widely used in livestock and poultry since the 1950’s (56). Since then, food animal production includes larger farms and greater animal densities, requiring an increased need for disease management (56). According to the 2007 Census of Agriculture, the numbers of farms declined from 2.5 million to 2 million. However, during the same time period (according to NARMS)*, the average number of cattle on a dairy farm increased from 57.4 to 219.7 animals (85).

In food animal production, antibiotics are used for 4 main purposes: therapeutic use to treat sick animals, metaphylaxis (group treatment), prevention of infections at times of risk (such as transport and weaning), and growth promotion (to improve feed utilization and production) (56, 58). Growth promotion is also referred to as “improved feed efficiency” in some literature (5, 86). Growth promoters allows farmers to increase the body weight of their animals without increasing the quantity of animal feed (68). It is uncertain if weight gain is due to prevention of infection or some other physiological effect (68). In the United States, an estimated 23 million kg (51 million lb) of antibiotics are used annually; about half are provided for humans, and the remaining are manufactured for agriculture (50). About 7 million kg (15.4 million lb) of antibiotics, chiefly penicillins and tetracyclines, are used as growth promoters for food animals (50).

**Tetracycline**

Antibiotics are classified by chemical structure and mechanism of action (55). A detailed classification is beyond the scope of this review. However, the antibiotics used in this work will be discussed. In 1944, Benjamin M. Duggar, a plant physiologist, discovered the first tetracycline (chlortetracycline) while studying soil samples for antimicrobial properties. It was introduced into clinical practice in 1948 (12, 74). Tetracyclines are classified as broad-spectrum antibiotics. This group includes tetracycline, oxytetracycline, doxycycline and minocycline (76). Naturally occurring

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*NARMS is a collaboration of agencies including the Centers for Disease Control and Prevention (CDC), the Food and Drug Administration (FDA), the Center for Veterinary Medicine, United States Department of Agriculture (CVM-USDA) and state and local health departments (4). In addition to NARMS, the Foodborne Diseases Active Surveillance Network (FoodNet) conducts studies to estimate the burden and sources of specific food-borne diseases (4).
tetracyclines such as oxytetracycline are isolated from species of *Streptomyces* while other tetracycline “classes” such as glyyclcyclines are semisynthetic (18, 73). The semisynthetic classes of tetracycline are noted for resisting stomach acid and are absorbed from the intestines after oral consumption (73). Because of their slow excretion into urine, they are used to treat urinary tract infections. Amoxicillin has the added benefit of being acid-stable and does not bind to food as many antibiotics can (73). Structurally, tetracyclines are characterized by a hydronaphthacene nucleus with four fused rings (12, 55). Tetracyclines are relatively stable in acids but not in bases and form salts in both media (83). The tetracyclines are bacteriostatic or bactericidal depending on its affinity for a given target structure and its ability to cross the various structural barriers that bar access (12).

The traditional tetracyclines (including tetracycline, doxycycline, and minocycline) inhibit protein synthesis at the ribosomal level due to disruption of codon-codon interactions between tRNA and mRNA (12). “Atypical” tetracyclines such as chelocardin and anhydrochlorotetracycline act on bacteria by interfering with the electrochemical gradient of the bacterial membrane (12). This promotes lysis and cell death by stimulating autolytic enzyme activity (12). The antibacterial spectrum and activity of tetracyclines include numerous Gram-positive and Gram-negative bacteria, anaerobes, rickettsiae, mycoplasmas, chlamydiae, *Helicobacter pylori* and spirochetes (12). Certain tetracyclines are also active against a number of mycobacteria (*Mycobacterium leprae*) and protozoa such as *Plasmodium* spp. and *Toxoplasma gondii* (12). Tetracyclines can be given orally, intramuscularly or intravenously (76). Adverse effects to tetracycline ingestion include gastrointestinal problems such as epigastric burning, nausea, vomiting, and anorexia (74). Food can be taken to alleviate some symptoms but may also decrease drug absorption by 50% (74). Other side effects include hypersensitivity reactions and hepatotoxicity-which can be fatal (74).

Tetracyclines are usually not recommended for children under 8 years of age because they can cause permanent discoloration of teeth, and bone growth retardation (74). Bacterial resistance to tetracyclines are largely due to acquired resistance; resistant strains emerge from previously sensitive bacterial populations by acquisition of resistance genes (usually residing in plasmids and/or transposons) (55). Resistance to tetracyclines may be mediated by one of the following mechanisms; (a) an energy-dependent efflux of tetracycline carried out by
transmembrane proteins, which results in the reduction of the concentration of tetracycline in
the cytosol; (b) ribosomal protection, whereby the tetracyclines no longer bind productively
to the bacterial ribosome; or (c) chemical modifications requiring oxygen and NADPH as
well as catalysis by enzymes (55).

**Erythromycin**

In 1952, while examining soil samples from the Philippine archipelago, J.M. McGuire
and his colleagues at Eli Lilly discovered erythromycin, the prototype and first antibiotic in the
class of macrolides (76). The name “macrolide” is derived from “macro” (large) and “olide”
(lactone) (12, 74). The natural macrolide antibiotics (such as erythromycin) are isolated
primarily from the genus *Streptomyces* (55). Most macrolides are weak bases and are
unstable in acids (83). The action of macrolides can be bactericidal or bacteriostatic, the
effect depending on the concentration and the type of microorganism targeted by the drug
(76). Macrolides bind to the 50S subunit of the bacterial ribosome and inhibit the
transpeptidation and translocation process, causing premature detachment of incomplete
polypeptide chains (55). The antimicrobial spectrum of erythromycin is very similar to
penicillin; it has proved to be a safe and effective alternative for penicillin-sensitive patients
(76). Macrolides are effective against Gram-positive bacteria and spirochetes but not against
most Gram-negative organisms, the exceptions being *Neisseria gonorrhoeae* and
*Haemophilus influenzae* (76). *Mycoplasma pneumoniae*, *Legionella* species and some
chlamydial organisms are also susceptible (76). The macrolides are administered orally and
intravenously. They diffuse readily into most tissues, but do not cross the blood-brain barrier;
additionally there is poor penetration into synovial fluid (76). The majority of side effects
associated with macrolides are mild and transient. As a class they are generally well-tolerated
(74). The most common complaints involve the gastrointestinal tract and include diarrhea,
nausea, vomiting and abdominal pain (74). Patients may complain of an abnormal or metallic
aftertaste (74). Hepatotoxicity is a very rare but serious side effect associated with the
estolate salt of erythromycin (74). Three different mechanisms account for bacterial
resistance to the action of macrolide antibiotics; (a) target site modification (b) active efflux
and (c) enzymatic inactivation (55). Some bacterial species are able to synthesize enzymes,
encoded by a series of structurally related erythromycin-related methylase (*erm*) genes which
methyllates rRNA (55). Methylation of the 23S rRNA of the 50S subunit leads to a conformational change of the ribosome that yields broad-resistance to macrolides (55). The macrolide-specific efflux from resistant cells is driven by a membrane protein encoded by the macrolide efflux (mef) gene (55). The genes of the efflux pumps can be either acquired (such as mef), or carried intrinsically, such as acriflavin resistance AB (acrAB) (33). The clinical significance of enzymatic degradation is still being characterized (33). Members of the family Enterobacteriaceae are highly resistant to erythromycin due to erythromycin esterases encoded by the erythromycin resistant esterases, ereA and ereB (33).

**Nalidixic acid**

The quinolones, also referred to as fluoroquinolones are synthetic in origin (33). Nalidixic acid is considered a first-generation quinolone; technically nalidixic acid is not a fluoroquinolone because it is not fluorinated like other quinolones (76). Most fluoroquinolones exhibit large chemical stability (83). They are insensitive to hydrolysis and increased temperatures but are degraded by ultraviolet light (83). Nalidixic acid was introduced into clinical practice in 1963 and initially restricted to the treatment of urinary tract infections in humans (12). It has a narrow Gram-negative treatment range and limited tissue distribution when taken orally (74). Intravenous administration is ill-advised because of issues such as poor tolerance (12). Even though nalidixic acid has a limited place in therapy, it served as the prototype for new classes of fluoroquinolones (12). In the 1980s, it was discovered that the basic structure of nalidixic acid could be manipulated. This resulted in agents with a broader spectrum of antibiotic activity, better tissue distribution, and improved pharmacokinetics (the fate of pharmaceutical substances administered to a living organism) (12). These “second generation” fluoroquinolones include ciprofloxacin, ofloxacin, and enoxacin (12). Third and fourth generation fluoroquinolones were later developed (examples are gatifloxacin and gemifloxacin, respectively) (74). These antibiotics were found to expand Gram-negative and atypical pathogen coverage (74). Fourth generation fluoroquinolones further improved Gram positive and Gram-negative coverage in addition to being more effective with anaerobic pathogens than previous generations of fluoroquinolones (74). However, there were instances in which some third and fourth generation fluoroquinolones had to be withdrawn from use because of side effects such as cardiac arrest and serious liver injury (74).
Fluroquinolones can be administered orally, intravenously or by intramuscular injection (12). The drugs are well-absorbed, particularly in the kidneys, prostrate and lung (76). The mechanism of action of fluoroquinolones has not been clearly defined, but they inhibit bacterial DNA synthesis by inhibiting the enzymes DNA gyrase and topoisomerase IV (33, 74). DNA gyrase is an essential enzyme in bacterial replication and is responsible for producing a negative supercoil in DNA-permitting transcription or replication (76). Topoisomerase IV separates interlocking daughter DNA strands that form during replication. This facilitates the segregation of daughter DNA molecules into daughter cells (33). The fluoroquinolones are generally well-tolerated, although the frequency of ill effects may increase with higher dosage (74). The most common side effects are gastrointestinal (primarily nausea, diarrhea, vomiting and anorexia) (74). Adverse reactions involving the central nervous system can also take place-occurring in 1-3% of patients taking fluoroquinolones (74). Symptoms including headaches, anxiety, and tremors (74). There have been reports of cartilage damage in young animals, therefore fluoroquinolones are not recommended for use in children, pregnant women, or nursing mothers (74).

Fluoroquinolones can be used for the treatment of nosocomially-acquired pneumonia, bronchitis, sinusitis and exacerbations of cystic fibrosis (74). However, broad use of fluoroquinolones have been followed by emergence of resistance, which is due mainly to chromosomal mutations in genes encoding subunits of the drugs’ target enzymes (DNA gyrase and topoisomerase IV), in genes that affect the expression of diffusion channels in the outer membrane, and in multidrug-resistance efflux (MDR) systems (12, 33).

Fluoroquinolone resistance resulting from multidrug efflux pumps have been found in a number of Gram-negative organisms including *Campylobacter jejuni*, *Escherichia coli* and *Salmonella typhimurium* (55).

**Penicillin**

The discovery of penicillin represented an extraordinary event in the study of infectious disease because it enabled a number of infections to be treated that previously caused chronic illness or death (12). It transformed not only medicine but also the socioeconomic events of the second half of the 20th century (12). Penicillin was derived from the fungus *Penicillium notatum* and discovered in 1928 by the Scottish microbiologist Alexander Flemming at St.
Mary’s Hospital in London, England (76). Flemming had inoculated nutrient agar with staphylococci and left for a vacation. On his return, he noted that one plate was contaminated with green fungus. Flemming became interested in the antibacterial properties of the fungus when he noticed the failure of staphylococci to grow near the mold (73). Despite Flemming’s efforts, he was not able to purify what is now known as penicillin (73). Twelve years later Howard Florey and Ernst Chain of Oxford University completed studies on the fungal metabolites of *P. notatum* and was able to purify the substance (12). Florey and Chain began to study penicillin and its clinical usefulness in treating infections. Initially, they completed tests in tissue cultures and animals (74). In February 1941, a 43-year-old Oxford policeman was the first to receive penicillin. He was diagnosed with an overwhelming staphylococcal septicemia with numerous accesses in the lungs, bone and face. Multiple doses of penicillin were given intravenously and after 5 days, the patient showed significant improvement. Unfortunately, the supply of penicillin ran out. Despite attempts at recovering penicillin from the patient’s urine and purifying it for reuse, the patient died a month later (74). Because of England’s involvement World War II, their scientists were not able to mass produce penicillin. A group of American companies developed the techniques for large scale production and later made the drug available for commercial use (73). In 1945, Flemming, Florey and Chain shared the Nobel Prize in Physiology or Medicine for the discovery and development of penicillin (73). Since the 1940s, penicillin has remained the most widely used antibiotic because of its low costs and thousands of derivatives (73).

Penicillins are characterized by a four-membered β-lactam ring fused to a five-membered thiazolidine ring containing a side chain (53). Manipulation of this side chain is important in the pharmacodynamics of penicillin (53). Penicillins are classified on the basis of antibacterial activity into subclasses including natural penicillins, penicillinase-resistant penicillins, aminopenicillins (which includes ampicillin), carboxypenicillins and ureidopenicillins/piperazine (74). The activity of penicillin is directly dependent on the β-lactam ring (83). The ring is easily cleaved in acidic and basic media (83). Aminopenicillins were the first group of penicillin antibiotics to have activity against both Gram-positive and Gram-negative bacteria (broad-spectrum activity) (74). Most penicillins can be given orally, intramuscularly, and intravenously (74). A few (such as most carboxypenicillins and
ureidopenicillins/piperazines) are given intramuscularly or intravenously (74). Penicillins are often combined with other antibiotics to enhance antibacterial chemotherapy (74). All β-lactam antibiotics interfere with the synthesis of the bacterial cell wall peptidoglycan. After attachment to binding sites on bacteria (termed penicillin binding proteins), they inhibit the transpeptidation enzyme that cross-links the peptide chains attached to the backbone of the peptidoglycan (74). Bacteria can become resistant to penicillin by the production of β-lactamases, enzymes that can be released in the external medium (in the case of Gram-positive bacteria) or in the periplasmic space (in the case of Gram-negative bacteria) (12). Reduction in permeability and a modification in target penicillin binding proteins have been described for some bacterial species such as Staphylococcus aureus and Streptococcus pneumoniae (12). A major disadvantage of penicillin use is anaphylactic reactions that appear in some patients (73). Allergic reactions such as swelling around the eyes and wrists, flushed or itchy skin, shortness of breath, and hives are signals that sensitivity exists and penicillin therapy should cease (73).

**Antibiotic Resistance**

Resistance is defined as the relative insusceptibility of a microorganism to a particular treatment under a particular set of circumstances (48). Some researchers believe that resistance is an ecological phenomenon stemming from the response of bacteria to the widespread use of antibiotics and their presence in the environment (24, 51). They believe that the rise in the frequency of antibiotic resistance among pathogens should be a “cause of great concern” and suggest “a commitment to act responsibly” (89). For example, before human patients were first treated with antibiotics (65 years ago), bacteria isolated from patients had almost no resistance genes (69). However, after each new agent became widely used, a gene expressing resistance to it ultimately emerged (69). They believe that human and veterinary medical staff, public health officials, the pharmaceutical industry and those involved in agriculture must work together to “curb the inappropriate use of antibiotics” and promote responsible prescribing (89). Others believe that there is a “concerted attack” on the use of antibiotics, and that antibiotic resistance is as ancient as bacterial organisms since bacteria are able secrete products (antimicrobials) to prevent attack from other competitive organisms (72). They believe that the actual risk of antibiotic resistance is quite small and
independent investigations “free of commercial and political influence” are necessary to study the issue (72). Additionally, mathematical models have been developed in which the effects of antibiotic resistance on human health, hospitalizations and mortality were studied (21). These models determined the use of antibiotics in food animals creates (at most) minor risks to human health (21). Despite of the ongoing debate, a majority of researchers believe that the spread of antibiotic-resistant pathogens is a problem in the US and around the world-despite the few studies that state differently (17, 19, 37, 42). A report by the World Health Organization (WHO) cited antibiotic resistance as one of the most critical human health challenges of the next century and heralded the need for a “global strategy” to contain resistance (24). For example, antibiotic resistance in *Campylobacter jejuni* is characterized as a global problem (32). In countries with lax restrictions on human and animal treatment, very high levels of antibiotic resistant *Campylobacter* were observed (32). There continues to be much debate regarding the role of antibiotics in animal husbandry and veterinary therapeutics-and the relationship of dissemination of bacterial resistance mechanisms (32).

**The Family Enterobacteriaceae**

Members of the family *Enterobacteriaceae* have earned a reputation placing them among the most pathogenic and frequently encountered organisms in microbiology (20). The name *Enterobacteriaceae* is misleading because it obscures its members’ wide variety of habitats including water, soil, food plants, trees and animals (from humans to insects) (52). Researchers have divided the family-based on their associations; (a) insect pathogens/symbionts (e.g. *Buchnera* and *Xenorhabdus*); (b) animal pathogens (e.g. *Salmonella* and *Escherichia*); (c) plant pathogens (e.g. *Erwinia* and *Samsonia*) and (d) those genera used for industrial purposes (e.g. *Alterococcus* and *Pragia*) (35). Substantial growth has occurred within the family *Enterobacteriaceae*; from 11 genera and 26 species in 1972 to 22 genera and 69 species as of 2006 (35). *Enterobacteriaceae* are Gram-negative rods, have an aerobic or facultative anaerobic metabolism, are oxidase negative, catalase positive and ferments D-glucose (35). In addition, they reduce nitrates to nitrites, do not require sodium for growth, and has a G+C content from 38-60 % (35). There are minor exceptions within species or groups, however most *Enterobacteriaceae* possess the characteristics previously listed (27). Many species are of considerable economic importance. For example, *Erwiniae*
causes blight, wilt, and soft-rot disease in crops such as corn, potatoes, pineapple and sugar cane while *Klebsiella* can cause disease in cattle, turtles, humans and lizards (27).

**Salmonella**

Salmonellae are facultative, gram-negative, motile rods (0.7-1.5 X 2.0-5.0 um) and are members of the family *Enterobacteriaceae* (10, 59). Non-typhoidal salmonellae, which includes > 2500 serotypes (or serovars) are widely distributed in nature, including the gastrointestinal tract of mammals, reptiles, birds, and insects (79). For the purpose of this work, serotypes of *Salmonella enterica*, subspecies *enterica* will be referred to as *Salmonella* accompanied by the serotype name (80); for example *S. enterica* subspecies *enterica* serotype London will be referred to as *Salmonella london*. Most salmonellae are motile, however, the serotypes *gallinarum* and *pullorum* are non-motile (10). Serotyping is based on the somatic (O) lipopolysaccharide on the external surface of the bacterial outer membrane, flagellar (H) antigen associated with the peritrichous flagella, and capsular Vi (or virulence) antigens (61). *Salmonella* serotypes may be strictly adapted to one host, ubiquitous (found in a large number of animal species) or may be of still-unknown pathogenicity (10). Serotypes adapted to humans (such as *S. typhi*) usually cause severe disease with a septicemia-typhoidic syndrome, while ubiquitous serovars are mainly responsible for food-borne infections (10).

Infection by *Salmonella enterica* is a significant health concern world-wide (26). Human salmonellosis occurs in about 1.3 million people, causes > 500 deaths and is estimated to cost the US economy a 2.3 billion to 3.6 billion each year (14, 26). Many human illnesses can be linked to the consumption of bacterially contaminated ground beef, milk, or other dairy products (14). Direct contact with infected animals can also serve as a source for *Salmonella* infection (26). Most infections on cattle farms are introduced by the purchase of infected cattle (79, 84). One study demonstrated that the introduction of newly purchased cattle to a farm increased the risk of salmonellosis, and that the period of highest risk was within one month of arrival (84). The most recent reported national dairy survey (USDA’s NARMS) indicated that 27 to 31 % of US dairy herds contained cows that shed *Salmonella* (14). *Salmonella* have been isolated from the feces of healthy cattle (where it may exist as a normal member of the gastrointestinal flora), or as a transient member of the gastrointestinal microbial population (14). Illness from salmonellosis in the bovine is seen predominantly in
young calves although it is sometimes seen in adult cattle (14). Host-specific serotypes of Salmonella (such as S. dublin) can cause diseases such as abortion or severe gastroenteritis (26). However, ubiquitous serotypes can cause sub-acute septicemia and acute enteritis. In the subclinical form of the disease, animals may either have a latent infection or become a temporary or persistent carrier (26).

In the US, infections with multi-drug resistant S. newport have emerged in recent years; the primary reservoir is thought to be bovine (79). Additionally, multi-drug resistant serotypes of Salmonella are associated with increased hospitalization and increased mortality and morbidity in many region of the world, including the United States (30). Pasteurization of milk effectively kills Salmonella, but consumption of unpasteurized milk and milk products is a well-documented risk factor for human salmonellosis (79). Furthermore, unpasteurized milk and milk products contaminated after pasteurization are recognized sources of human disease (79).

**Campylobacter**

Campylobacter is a member of the family Campylobacteraceae (81). The organism can establish long-term associations with their hosts, sometimes with pathogenic consequences (81, 90). Campylobacter cells are Gram-negative, slender and spirally curved rods (from 0.2-0.8 X 0.5-5 um) (65). Cells in old cultures may form coccoid bodies (65). Most species of Campylobacter are motile with a characteristic corkscrew-like motion by means of a single polar unsheathed flagellum at one or both ends (65). Most species are microaerophilic requiring 3-15% oxygen and 3-5% carbon dioxide for growth (59, 61). In a laboratory setting, campylobacters require selective media (32). All Campylobacter grow best at 37°C, but some Campylobacter species (C. jejuni, C. coli and C. lari) grow best at 42°C (43, 59). The organisms are more sensitive to adverse conditions (e.g. drying, heat, disinfectants, acidity) than most other enteric pathogens (59). This suggests that they are best adapted for existence in vivo.

The genus Campylobacter is capable of surviving in a wide range of environments. It has been isolated from rivers, estuarine and costal waters (43) as well as in sand, soil, and sewage (9). C. jejuni can remain dormant in water (in a viable but nonculturable or VBNC state) (61). Campylobacter is a commensal organism routinely found in cattle, sheep, swine and
avian species (61). For this reason campylobacteriosis is considered a zoonotic disease (2). In the United States, an estimated 2.4 million cases of human campylobacteriosis occur each year (6). *Campylobacter* species are one of the most common causes of bacterial diarrhea in humans worldwide (2). Two species are usually associated with most human infections, *C. jejuni* and *C. coli* (2). *C. jejuni* are estimated to cause 90% of human infections (36). The estimated rate of campylobacteriosis (number of cases/100,000 individuals) differ strongly around the world, with New Zealand as the country with the highest rate (396/100,000 persons), compared to the United States (reported as being 12.7/100,000 persons by FoodNet in 2005) (36). Doses as low as 500 organisms have been reported to cause illness (36). The most common clinical symptoms of human campylobacteriosis are fever, abdominal pain, vomiting, bloody stools and diarrhea that occur within 2-5 days of ingestion of food or water contaminated with *Campylobacter* (9, 36). The illness is usually self-limiting and is resolved within a period of 3 to 10 days; most cases do not require the use of antibiotics. When antibiotics are necessary, erythromycin (a macrolide) and fluroquinolones are usually prescribed; complications are rare (9, 36). In a small sub-group of patients, the acute phase is followed by serious sequelae: Guillain-Barre syndrome (GBS) and Miller-Fisher Syndrome (36). GBS is a serious autoimmune-mediated neurological disorder that can cause symptoms ranging from weakness of extremities to complete paralysis and respiratory distress (36). Miller-Fisher Syndrome is characterized by lack of general muscle coordination and coordinated eye movements. (11, 62, 65)

Cattle are common carriers of *Campylobacter* and the importance of raw milk as a risk factor for human campylobacteriosis has been recognized in epidemiologic studies. Consumption of unpasteurized milk has been associated with outbreaks (31). Additionally, the environmental load of *Campylobacter* in cattle manure may be a more significant factor in the transmission of infections than contaminated milk or beef (6, 31). *Campylobacter* has long been recognized as a cause of diarrhea in cattle and of septic abortion in both cattle and sheep (43, 65).

*Enterococcus*
The enterococci (family Enterococcaceae) belong to the clostridial subdivision of the Gram-positive bacteria and consists of 27 species (49) that are divided into four groups, based on tests such as 16S rRNA gene sequencing and phenotypic analyses (28). The name “entérocouque” was first used by Thiercelin in a paper published in 1899 and was proposed to emphasize the intestinal origin of this newly discovered organism (63). In 1903, Thiercelin and Jouhaud proposed the generic name “Enterococcus” with type species Enterococcus proteiformis (41). However, in 1906, Andrewes and Holder renamed the genus and species “Streptococcus faecalis”, based on its ability to form short or long chains, and the ability to form hemolytic, greening colonies on media (41). With the establishment of the Lancefield serological typing system in the 1930s, enterococci were reclassified as group D streptococci and differentiated from the non-enterococcal streptococci by distinctive biochemical characteristics (16). In 1984, Enterococcus became recognized as a separate genus from Streptococcus (1). Schleifer and Kilpper-Bälz revived the name Enterococcus when Streptococcus faecalis and S. faecium were transferred to the genus Enterococcus (46). Enterococcus are ubiquitous microorganisms, but have a predominant habitat in the gastrointestinal tract of humans and farm animals (29). Enterococcus, as members of commensal flora, are present in the human colon in numbers as high as $10^8$ colony forming units (CFUs) per gram of feces (82). Enterococci can also be found in different types of food and feed (29, 46). Some enterococcal strains are used as starter or protection cultures, feed supplements, and probiotics (46).

Traditionally enterococci are considered part of the lactic acid bacteria (LAB) group (46). LAB are Gram-positive, non-sporforming, microaerophilic/anaerobic, and catalase negative (46). They also form lactic acid and other products (ethanol and acetic acid) after fermentation of carbohydrates (46).

Enterococci are important opportunistic pathogens and now rank among the most common nosocomial agents infecting the bloodstream, surgical sites, and urinary tract (82). The role of enterococci in infective endocarditis and urinary tract infections (UTIs) are well-established (63). Enterococcus-related endocarditis accounts for 5-10% of the total bacteria endocarditis cases reported each year (82). UTIs are the most common nosocomial infections caused by enterococci; they account for about 34–46% of all
infections in hospitals and occur at a rate of 13 cases per 1000 discharges (91). Many enterococcus-related illnesses are becoming more difficult to treat because of growing resistance to antibiotics (82). Since its discovery as a human pathogen, members of the genus *Enterococcus* have distinguished themselves from other Gram-positive cocci by multiple antibiotic resistance (60). Vancomycin-Resistant *Enterococcus* (VRE) were first identified in the mid 1980s (16). VRE has become a major problem in institutions in Europe and the United States (16). Since VRE have intrinsic resistance to most of the commonly used antibiotics and the ability to acquire resistance (either by mutation or receipt of foreign genetic material), they have a selective advantage over other microorganisms in the intestinal flora and pose a major therapeutic challenge (16).

**Antibiotic Susceptibility Testing**

Antimicrobial susceptibility testing methods have been in use since 1929 to estimate the effects of antibiotic agents on microorganisms (74). Routine results are obtained by placing antibiotics and organisms together in a medium which will support growth. Methods such as agar diffusion, broth dilution, or agar dilution are frequently used in microbiology (74). Diffusion and dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents (40). The agar diffusion test is also referred to as the Kirby-Bauer Disc Method. Dried filter paper impregnated with a defined concentration of antibiotic is placed on the surface of an agar plate that has been uniformly inoculated with the bacterial organism in question (53). After incubation, the plate is reviewed for zones of inhibition. The zone diameters for individual antibiotics are translated in terms of being “susceptible”, “intermediate” or “resistant” (53). The terms “resistant” and “susceptible” are often used to express the ability (or lack of ability) of a microorganism to multiply in the presence of a given concentration of antibiotic under defined conditions (8). The “intermediate” category refers to organisms not clearly resistant or fully susceptible; this designation is considered a “buffer zone” in terms of interpretation: for example, during clinical diagnosis, a patient with an “intermediate” result may be given a higher dosage of antibiotic (53). Disc diffusion testing has the advantages of simplicity and low cost, however there can be difficulty in the
interpretation of zone diameters—especially with slow-growing bacteria (22, 53). Another version of the agar diffusion test is referred to as the E-Test (54). The E-Test is an MIC test which uses a thin plastic strip coated with a continuous antimicrobial agent on one side and a quantitative interpretative scale on the other side (54). MICs are determined by reading the antimicrobial concentration printed on the test strip at its intersection with the growth inhibitory zone (54). Because the E-Test comprises a continuous gradient, MIC values between two-fold dilutions can be obtained (84). E-Tests results are as accurate as agar dilution tests (incorporation of an antibiotic in an agar medium) (54), however unusual zone patterns can lead to MIC discrepancies for some organisms (84). Additionally, the high cost of the E-Test makes it more useful for a limited number of drugs under special circumstances (such as when quantitative susceptible data is necessary for treatment of persistent infections) (84).

The principle of broth dilution is the exposure of a given inoculum of bacteria to varying concentrations of antibiotic, each placed in an individual tube containing nutrient broth (53). After appropriate incubation, the minimal lethal concentration (MLC) is determined by identifying the tube containing the lowest concentration of antibiotic that inhibits growth (53). Standard broth dilutions are more convenient when only a few strains need to be tested against one or two drugs (74). With mechanization of the procedure, one isolate can be tested against a large number of drugs with reasonable efficiency (74). Another advantage of the broth dilution method is that minimum bactericidal concentration (MBCs) may be determined by subculturing each tube showing no visible growth to a portion of a non-antibiotic containing blood agar plate (53). Plates are then incubated and the MBC is read as the lowest concentration of drugs which show no growth on the agar plate surface (53). A major disadvantage of broth dilution is that when fastidious organisms are tested, supplements often have to be added to the broth. However, supplements can produce a cloudy medium in which microbial growth is difficult or impossible to detect (74). When a large number of isolates require testing, broth dilution can be adapted to what is called broth microdilution testing (53). Broth microdilution testing is more economical and efficient than broth dilution. For each organism being tested, as many of 12 different concentrations of 8 antimicrobials can be
tested on a specially prepared 96-well microtiter plate (53). Still, it can be difficult to find standard test panels since manufacturers may use different antibiotic formulas (39).

Agar dilution involves the incorporation of an antibiotic agent into an agar medium followed by the application of a bacterial inoculum to the agar surface of the plate (87). Stock solutions of antibiotics are prepared by weighing laboratory standard powders and dissolving them in distilled water or some other suitable diluent (7, 53). Antibiotics are aseptically added once the agar has been autoclaved and is cooling (53). The antibiotic is mixed into the molten agar and poured immediately (53). As with broth dilution, the agar dilution method provides a quantitative result in the form of an MIC, in contrast to disk diffusion that result in an indirect measure of susceptibility and provides a qualitative interpretative result (74). Some advantages offered by agar dilution include accurate determination of MICs, the ability to test many organisms against a series of a single antibiotic at the same time, the potential to extend the antibiotic concentration as far as required and adaptation to semi-automation (i.e. using automated technology such as a plate pourer to make large amounts of plates) (87). The agar dilution method is known as the “gold standard” of antibiotic susceptibility testing, however it is rarely preformed due to the large amount of manual handling that is needed (87). Agar dilution requires extensive training, is expensive and labor-intensive (especially when testing many organisms against many antibiotics) (32, 87). An agar dilution method has been standardized by the Clinical and Laboratory Standards Institute (CLSI\textsuperscript{b}, formerly the National Committee for Clinical Laboratory Standards (NCCLS) (47, 87).

**The consequences of antibiotic resistance in humans and animals and the “prudent” use of antibiotics**

Two human health consequences of increasing antibiotic resistance are an increase in food-borne illnesses and an increase in the number of treatment failure (4). Antibiotic resistance is believed to be associated with greater mortality, morbidity, and costs than

\textsuperscript{b}The CLSI is a large, independent group of individuals representing professional societies, industries, and governmental agencies, all which have a common interest in the operation of clinical laboratories (7). CLSI is organized for the promotion and development of national and international standards that are needed for proper activity and operation of clinical laboratories (7).
infections due to susceptible organisms (15). The national costs of antimicrobial resistance for the United States have been estimated between $100 million and $30 billion dollars annually (15). The Office of Technology Assessment of Congress have estimated the minimal hospital cost associated with human nosocomial infections caused by antibiotic-resistant bacteria to be $1.3 billion per year (15).

There are significant concerns regarding MDR and the safety of the food supply—particularly with enteric bacteria such as Salmonella and Campylobacter (3, 15). Limited studies exist regarding the prevalence of antibiotic resistance among dairy herds (77). The exact reason for the emergence of drug-resistant stains of bacteria remains unknown, but selective pressure is partially to blame (25). It has been suggested that using subtherapeutic doses of antimicrobials in animal feedstuffs has led to an increase in the number of resistant strains of common and pathogenic bacteria (25). In addition, dairy and beef products have been implicated as a source of Salmonella responsible for human outbreaks. Beef and dairy herds may also represent a reservoir for transmission of antibiotic-resistant Salmonella to humans (25, 26). Milk, poultry, pork and untreated water have been linked as possible vehicles of transmission of Campylobacter (88). However most studies of Campylobacter resistance are primarily based on poultry products, especially broiler meat (23). Animals such as raccoons, rodents and birds have been implicated in transferring disease in dairy farm environments (75). Feedstuffs and factors such as animal management practices and waste management/effluent control have also been implicated (75). Denmark and Sweden decided to curb their use of antibiotics as growth promoters; this has been associated with reduced antimicrobial resistance and decreased public health risks (17). In addition, members of the United States Congress have introduced legislation to enact similar bans. (78) Conversely, there are studies in which the resistance level remained constant or even increased independent to decreasing the use antimicrobial drugs (45). Although it is not completely understood how widespread the frequency of antibiotic resistant bacteria is in the non-nosocomial community, the lack of surveillance data are especially evident in important agricultural environments, such as dairy farms (13).

The “prudent” (appropriate) use of antibiotics is an important step in decreasing antibiotic resistance. For example, extensive antibiotic resistance programs have been set up in several
European countries (Denmark, Sweden, Germany and the United Kingdom) (44, 86). The movement to restrict the use of antibiotics in livestock production is also growing in North America (57). Organizations such as the Union of Concerned Scientists (UCS) have charged that antibiotic production in the US is greatly underestimated and the Center for Science in the Public Interest (CSPI) is lobbying the Food and Drug Administration to have seven antibiotics banned from agricultural use (57). They include penicillin, tetracycline, erythromycin, tylosin, lincomycin, virginiamycin and bacitracin (34, 57). Other organizations are urging a more measured approach, including modifying prudent use guidelines as new scientific evidence on antibiotic resistance becomes available, encouraging responsible use of antibiotics and using risk assessment to determine the public health impact of antibiotic resistance (34, 71). The World Health Organization (WHO) has repeatedly concluded that data on the usage of antimicrobial agents are essential for focusing on efforts to reduce use and misuse of antimicrobial agents (67). McAllister et al. (2001) have listed important criterion to consider in order to minimize the use and misuse of antibiotics in livestock production; (a) do not use antibiotics to compensate for poor nutrition, poor hygiene, or the lack of immunization or implementation of a herd health program; (b) consider other methods of intervention (e.g. proper nutrition, stress management) prior to antibiotic therapy; (c) use antibiotics in consultation with a veterinarian; (d) select dosing rates and treatment periods in accordance with manufacturer’s recommendations; (e) maintain accurate treatment records; and (f) whenever possible, culture suspected pathogens for identification, to ensure that the selected antibiotic is targeting the causative organism (57). Such actions will require collaborative efforts by several partners, including the farming, veterinary, medical and public health communities (4). Enhanced surveillance is important; there is a particular need to establish surveillance of antibiotic usage in animals (4).

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Antimicrobial Resistance and Human Health


The establishment and proliferation of antibiotic-resistant *Campylobacter* on a new dairy farm prior to and after the placement of dairy cattle

Ginger M. Shipp and James S. Dickson

Abstract

*Campylobacter* has been recognized as the most common cause of gastroenteritis in the United States. Most cases of campylobacteriosis are self-limiting, but can be fatal in infants, young adults and the immunocompromised. Cattle contribute to human cases and outbreaks through several transmission routes, such as direct contact, environmental contamination, and milk. A longitudinal study on the antibiotic resistance of *Campylobacter* was conducted on a teaching farm (dairy) before and after the introduction of cattle. Resistance to tetracycline, erythromycin, and nalidixic acid were examined. These antibiotics are the treatment of choice for human patients and there is concern about emerging resistance. Environmental drag swab samples were taken from select farm locations over a 9-month period, plated on selective media (Preston agar) and replica plated on tryptic soy agar (TSA) containing a predetermined amount of antibiotic. Antibiotic-containing TSA plates were made following guidelines of the Clinical and Laboratory Standards Institute (CLSI) and the National Antimicrobial Research Monitoring System (NARMS). Generally, the numbers of *Campylobacter* colonies increased after placement of dairy cattle. Additionally, antibiotic-resistance profile evolved from resistance to one antibiotic (beginning in F samples) to multidrug resistance (also from F samples to the conclusion of the study). Time was more a factor in the increase in multidrug resistance-more so than factors such as temperature and humidity (however, this study was conducted over a 9-month period). Longitudinal studies are important in providing insight into the dynamics of endemic diseases of dairy herds and because of variations found in this (and other) work, it is important to continue monitoring of pathogens in the farm environment.

Key words: longitudinal study, dairy farm, erythromycin, tetracycline, nalidixic acid, drag swabs, replica plating, NARMS, CLSI.

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Since the 1970s *Campylobacter* has been recognized as the most common cause of gastroenteritis in the United States (17). Illness caused by *Campylobacter* range in severity from slightly loose stools to watery diarrhea (often with blood), lasting 3-5 days (27). Most infections are self-limiting, but occasionally are fatal in infants and young adults (4, 27). Immunocompromised patients (such as those with the Human Immunodeficiency Virus or those undergoing chemotherapy) are at a higher risk of severe illness and hospitalization (27). A small number of patients develop debilitating sequelae such as Guillain-Barré
syndrome (acute flaccid paralysis) and Miller Fisher syndrome (a variant of Guillain-Barré syndrome characterized by lack of muscle coordination, absence of neurological reflexes and lack of coordinated eye movements) (4, 36, 37). A majority of *Campylobacter* infections (~90%) are attributed to *C. jejuni* although *C. coli* is increasingly being recognized as an important pathogen (4, 46). *C. jejuni* is generally regarded as microaerophilic (growing in environments containing 3-5% CO$_2$ and 3-15% O$_2$), and thermophilic (or theromtolerant), growing in a small temperature range from 30º C to 45º C (52). Major environmental reservoirs of *Campylobacter* reside in the intestines of birds and warm-blooded mammals, where it is thought to be commensal with the gut flora, rather then pathogenic, at least in older animals (50).

Most *Campylobacter* infections are believed to result from the ingestion of contaminated food, although the role of non-food exposure in the epidemiology of sporadic campylobacteriosis is still unknown (4). The primary source of contamination is believed to be animal feces (4). Contamination of the environment by domestic and wild animal feces (and that of humans) presents an alternative exposure pathway for human infection via drinking and recreational water use (4). Humans may also be exposed to voided animal feces in the environment through outdoor activities such as camping, hiking and picnicking (4). Cattle contribute to human cases and outbreaks through several transmission routes such as direct contact, environmental contamination (via feces) and milk (4, 11). *C. jejuni* and *C. coli* commonly colonize cattle without causing symptoms and studies have reported that 40-60% of individual animals and 80% of herds shed *Campylobacter* (11). Consumption of unpasteurized or inadequately pasteurized milk have been identified as a source of campylobacteriosis and is believed to be associated with both outbreaks and sporadic cases (11). *Campylobacter* spp. are recognized as a cause of septic abortion, infectious infertility and diarrhea in cattle (48).

The emergence of strains of *Campylobacter* (and other bacterial pathogens) resistant to antibiotics used to treat disease has provoked controversy over the use of antibiotics in food production (13). Many researchers believe that antibiotic resistance is a growing concern for public and animal health (18, 30) and feel much of the antibiotic use in human and veterinary medicine is unwarranted (26, 39). Solutions range from the elimination and/or responsible
use of antibiotics (28, 35, 38) to the investigation and use of alternatives such as antibiotic efflux pump inhibitors (31), probiotics (bacterial organisms that may be antagonistic to microbial pathogens) (49), and proper animal management (age-separation, vaccination and sanitation) (35). Conversely, others believe that antibiotics produce demonstrated benefits including improved animal health, higher production and reduction in foodborne pathogens (7, 34). Mathematical models have been developed to study foodborne disease, human hospitalizations, and mortality (9). The models determined that the use of antibiotics in food animals creates (at most) minor risks to human health. Many believe that additional research is necessary before antimicrobial use is further limited or banned (43).

Antibiotic resistance in *Campylobacter* isolated from poultry production has been well-documented in studies (12, 21, 29). Additionally, there are many reports on foodborne outbreaks of *Campylobacter* (1, 24, 45). In contrast, there are few studies on antibiotic resistance in *Campylobacter* isolated from cattle farms (13). Of these studies, some involve antibiotic resistance in udder pathogens (3, 47), in lactating cattle (23), and the prevalence of *Campylobacter* in regions including northern Thailand (41), and the Midwestern and northeastern United States (20, 48). In a study of antimicrobial resistance of *Campylobacter* in the feces of US dairy cows, 47% of the isolates of *C. jejuni* were resistant to tetracycline, 4% to nalidixic acid, and 2% were resistant to erythromycin (15). Other studies demonstrate an increase in multidrug resistance in both *C. jejuni* and *C. coli* (2, 5). Mastitis is the most common condition that justifies the use of antimicrobials on dairy operations (8) and is caused by a variety of Gram positive and Gram negative organisms including *Staphylococcus aureus, Escherichia coli* and *Klebsiella* spp. (8, 16).

There is little information regarding the introduction of dairy livestock on previously unused farm land to study the establishment and proliferation of bacteria of interest in microbiology and food safety. Iowa State University (ISU; Ames IA) was awarded 887 acres of land by a donor family. ISU constructed an academic teaching farm—the Dairy/Animal Science Education Facility (henceforth referred to as the “ISU Dairy Farm”). Prior to the establishment of the ISU Dairy Farm, the facility had been used for recreational purposes; no production livestock had ever been introduced. A complex of buildings were constructed on 27 acres of land including a free-stall barn, special needs/hospital barn, maternity barn, and a
calf research barn for nutrition and husbandry research. Dairy cattle were placed beginning on 26 November 2007; the farm typically houses 450 milking cows plus a similar number of heifers, dry cows and calves. Most of the animals were moved from the former ISU teaching farm located about 20 miles north of campus (in Ankeny IA). Additionally, 50 cows were introduced to the new dairy farm from a dairy center in Calmar, IA. A map of the dairy farm is included in the appendix of this paper. A longitudinal study of antibiotic resistance of *Campylobacter* spp.-specifically resistance to tetracycline, erythromycin and nalidixic acid was conducted. Erythromycin (a macrolide) and nalidixic acid (a quinolone) are often treatments of choice for human patients with acute campylobacteriosis-particularly those who are immunocompromised (6, 46). Tetracycline is sometimes considered as an alternative therapy, however antibiotic resistance is now making tetracycline treatment less effective (46).

Environmental samples were collected at select locations on the facility prior to the introduction of livestock in to determine initial antibiotic resistance (if any). All samples were immediately processed, screened with Campylobacter Selective Agar-also known as “Preston agar” (Oxoid, Cambridge UK), and later replica plated on Tryptic Soy Agar (Becton Dickinson Company, Sparks MD) containing a predetermined amount of antibiotic, observing for growth of antibiotic resistant colonies. To confirm the presence of *Campylobacter*, colonies were surveyed for hippurate activity (hippurate discs with ninhydrin reagent, Remel, Lenxa, KS), Gram stained and analyzed by the Campylobacter Test Kit (Oxoid, Cambridge UK). Routine detection of *Campylobacter* spp. is based on culture on selective media and subsequent phenotypic identification (32, 42, 55). The sampling process was repeated after dairy cattle were introduced, duplicating the entire laboratory process. Dairy farm sampling took place on seven different dates; two prior to cattle placement and five after placement (table 1 and page 49)

**Materials and Methods:**

One sterile 3 in x 3 in drag swab moistened with 10 mL skim milk (Solar Biologics, Ogdensburg, NY) was used per sample. Briefly, a drag swab was pulled through the
environment for 60 seconds (in later sample collections, efforts were made to duplicate sampling locations). Each drag swab was placed in a sterile bag and placed in a cooler kept at 4º C. After collection, samples were taken to the laboratory and immediately processed; researchers noted that the recovery of Campylobacter is reduced by temperature storage at 4º C for 24 hours (25). Each drag swab was aseptically added to a sterile Whirl Pack 24 oz/720 mL filtered homogenizer bag (Nasco, Fork Atkinson, WI). Ten mL of buffered peptone water was added (BPW; Difco, Becton Dickinson Company, Sparks, MD) to moisten sample for pipetting. The filtered bag was homogenized for 45 seconds at 250 RPM. One ml of sample was then added to a tube containing 9 mL BPW creating a diluent concentration of 10⁻¹. The process was repeated until a diluent concentration of 10⁻⁷ was created. A quantity of 0.1 ml of the 10⁻², 10⁻⁴, 10⁻⁶ and 10⁻⁷ diluents were plated on Preston agar with supplement (Oxoid, Cambridge UK). Plates were later read as 10⁻³, 10⁻⁵, 10⁻⁷ and 10⁻⁹. Preston agar incorporates antimicrobial agents (polymyxin, rifampin, trimethoprim and cyloheximide) making the media highly selective (32, 42). Plates were incubated for 48 hours at 42 ºC under microaerophilic conditions (5 % O₂, 5% CO₂, 2% H₂ and 88% N₂ by volume) generated by a gas pack (Gas Pak™ EZ, Becton Dickinson Company, Sparks MD). Populations of campylobacter-like colonies were enumerated after incubation.

Replica plates were made according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (54) utilizing Trypitc Soy Agar (TSA). Concentrations of antibiotics used (susceptible, intermediate and resistant) were 1.5 times the amount recommended by the National Antimicrobial Research Monitoring System (NARMS)ᵃ.

ᵃNARMS was established in 1996 by the Food and Drug Administration (FDA) in collaboration with the United States Department of Agriculture (USDA) and the Centers for Disease Control and Prevention (CDC). The goals of NARMS include monitoring trends in antimicrobial resistance and foodborne illness, disseminating information, and conducting research to further understand antimicrobial resistance (51). NARMS also assists the FDA in the approval of safe and effective antimicrobial drugs for animals (51).
The CLSI has approved agar dilution as a standard susceptibility testing method for *Campylobacter* (55).

Table 2: Minimum Inhibitory Concentrations (MICs) of antibiotics used in replica plating

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>R= Resistant (ug/mL)</th>
<th>I= Intermediate (ug/mL)</th>
<th>Resistant (ug/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA with tetracycline</td>
<td>≥ 24 ug/mL</td>
<td>12 ug/mL</td>
<td>≤ 6 ug/mL</td>
</tr>
<tr>
<td>TSA with erythromycin</td>
<td>≥ 48 ug/mL</td>
<td>24 ug/mL</td>
<td>≤ 12 ug/mL</td>
</tr>
<tr>
<td>TSA with nalidixic acid</td>
<td>≥ 96 ug/mL</td>
<td>48 ug/mL</td>
<td>≤ 24 ug/mL</td>
</tr>
</tbody>
</table>

*Antibiotic Resistance 1.5 X NARMS concentrations*

Each Preston agar “master plate” was later replicated using a sterile piece of velvet and a replica plating apparatus. Each piece of velvet was lightly pressed onto a series of TSA plates containing a specified amount of antibiotic (Table 2). After incubation for 48 hours at 37°C under microaerophilic conditions (Gas Pak™ EZ, Becton Dickinson Company, Sparks MD), the populations of antibiotic-resistant bacteria were enumerated. Replica plating has been shown to be effective with large numbers of environmental samples (40) however, it requires a high level of training and expertise (19). Additionally, antibiotic-containing plates have a very short shelf-life and require frequent preparation which often takes place in longitudinal studies or clinical laboratory settings (19). Therefore antibiotic-containing replica plates were prepared and refrigerated 1-2 weeks before farm sampling. All data were analyzed by EpiInfo™ Version 3.5.1, a statistical program created by Centers for Disease Control and Prevention (Atlanta, GA) at 95% confidence limits.

**Results:**

Since there were large amounts of data collected, only the populations of resistant bacteria at 10^-5 concentration were reported (≥ 24 ug/mL tetracycline, ≥ 48 ug/mL erythromycin and ≥ 96 ug/mL nalidixic acid). Based on the available data, four locations on the ISU dairy farm were examined in more detail, as these had consistent populations of resistant bacteria; the maternity and calf barn, solids separator, the free stall barn and the special needs/hospital
barn. Overall, there were low populations of antibiotic resistant organisms (measured in colony forming units or CFUs) detected prior to cattle introduction (table 3, farm sampling A and B). After the introduction of cattle, the numbers of Preston agar “master colonies” and the antibiotic resistance colonies demonstrated some variability. However there was an overall increase in the populations of the Preston agar plates (table 3, farm sampling C-G). This also occurred with the antibiotic resistant plates (table 3, farm sampling C-G).

In Tables 4A and 4B, data are presented in graph form using a logarithmic scale. Throughout the study, the numbers of erythromycin-resistant CFUs demonstrated a steady increase (from $\log_{10} 6.1$ to $\log_{10} 7.18$). The nalidixic acid-resistant CFUs demonstrated variability in the numbers of colonies (decrease, increase then gradual increase of bacterial growth) from a low of $\log_{10} 6.3$ to a high of $\log_{10} 7.08$. Tetracycline colonies also demonstrated some variability (from a low of $\log_{10} 6.20$ to a $\log_{10} 7.48$).

Table 3. General farm trends (actual numbers)

<table>
<thead>
<tr>
<th>Sampling group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (F/C)</td>
<td>51/10.6</td>
<td>37/2.8</td>
<td>21/-6.1</td>
<td>36/2.2</td>
<td>38/3.3</td>
<td>56/13.3</td>
<td>49/9.4</td>
</tr>
<tr>
<td>Humidity (%)</td>
<td>83</td>
<td>76</td>
<td>85</td>
<td>70</td>
<td>73</td>
<td>54</td>
<td>64</td>
</tr>
<tr>
<td>Number of CFUs* (Preston)</td>
<td>0</td>
<td>2</td>
<td>132</td>
<td>17</td>
<td>213</td>
<td>89</td>
<td>332</td>
</tr>
<tr>
<td>Antibiotic resistance CFUs (Ery)</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>20</td>
<td>53</td>
<td>66</td>
<td>146</td>
</tr>
<tr>
<td>Antibiotic resistance CFUs (Nal)</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>3</td>
<td>120</td>
<td>15</td>
<td>78</td>
</tr>
<tr>
<td>Antibiotic resistance CFUs (Tet)</td>
<td>0</td>
<td>0</td>
<td>94</td>
<td>16</td>
<td>33</td>
<td>52</td>
<td>302</td>
</tr>
</tbody>
</table>

*CFUs= colony forming units, Ery=Erythromycin, Nal=Nalidixic Acid, Tet=Tetracycline
Table 4A. Erythromycin, Nalidixic Acid and Tetracycline Resistance in CFUs

<table>
<thead>
<tr>
<th>Sampling Period</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Ery 48</td>
<td></td>
<td></td>
<td>6.1</td>
<td>6.3</td>
<td>6.7</td>
<td>6.82</td>
<td>7.18</td>
</tr>
<tr>
<td>5 Nal 96</td>
<td></td>
<td></td>
<td>6.3</td>
<td>5.47</td>
<td>7.08</td>
<td>6.2</td>
<td>6.89</td>
</tr>
<tr>
<td>5 Tet 24</td>
<td></td>
<td></td>
<td>6.97</td>
<td>6.20</td>
<td>6.52</td>
<td>6.72</td>
<td>7.48</td>
</tr>
</tbody>
</table>

Ery=Erythromycin, Nal=Nalidixic Acid, Tet=Tetracycline

4B. Graph data in Log_{10} scale

Table 5. Resistance profile and number of CFUs-Campylobacter isolates (n=15)

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of antibiotics</th>
<th>Resistance profile</th>
<th>Ery 48</th>
<th>Nal 96</th>
<th>Tet 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8</td>
<td>1</td>
<td>Ery 48</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C8</td>
<td>2</td>
<td>Ery 48 Tet 24</td>
<td>5</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>C15</td>
<td>2</td>
<td>Ery 48 Tet 24</td>
<td>2</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>D10</td>
<td>2</td>
<td>Ery 48 Tet 24</td>
<td>17</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>E8</td>
<td>2</td>
<td>Ery 48 Tet 24</td>
<td>20</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>F15</td>
<td>2</td>
<td>Ery 48 Tet 24</td>
<td>9</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>E11</td>
<td>2</td>
<td>Ery 48 Tet 24</td>
<td>17</td>
<td>88</td>
<td>0</td>
</tr>
<tr>
<td>G11</td>
<td>2</td>
<td>Ery 48 Tet 24</td>
<td>6</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>C11</td>
<td>3</td>
<td>Ery 48 Nal 96 Tet 24</td>
<td>6</td>
<td>20</td>
<td>46</td>
</tr>
<tr>
<td>D15</td>
<td>3</td>
<td>Ery 48 Nal 96 Tet 24</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>E10</td>
<td>3</td>
<td>Ery 48 Nal 96 Tet 24</td>
<td>16</td>
<td>32</td>
<td>21</td>
</tr>
<tr>
<td>F10</td>
<td>3</td>
<td>Ery 48 Nal 96 Tet 24</td>
<td>50</td>
<td>13</td>
<td>39</td>
</tr>
</tbody>
</table>
Table 5. (continued)

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of antibiotics</th>
<th>Ery 48</th>
<th>Nal 96</th>
<th>Tet 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 11</td>
<td>3</td>
<td>Ery 48</td>
<td>Nal 96</td>
<td>Tet 24</td>
</tr>
<tr>
<td>G 10</td>
<td>3</td>
<td>Ery 48</td>
<td>Nal 96</td>
<td>Tet 24</td>
</tr>
<tr>
<td>G 15</td>
<td>3</td>
<td>Ery 48</td>
<td>Nal 96</td>
<td>Tet 24</td>
</tr>
</tbody>
</table>

Of the 28 sample locations (table 5), 13 had no antibiotic resistance profile (all A and B sampling locations, D8, D11, and E15 which are not included in table 5). In one sampling location (F8, the maternity/calf barn), resistance was detected to one antibiotic (erythromycin). Resistance to two antibiotics (erythromycin and tetracycline) were detected in 7 locations; C8 and E8, (the maternity/calf barn and free stall barn); D10, the solids separator; E11 and G 11 (free stall barn); as well as C15 and F15, the hospital/special needs barn). Resistance to all three antibiotics (erythromycin, nalidixic acid and tetracycline) occurred at locations E10, F10, and G10 (solids separator), C11 and F11 (free stall barn) as well as D15 and G15 (hospital and special needs barn). In order to review individual changes in sampling groups over time, the location and number of antibiotic-resistant profiles were diagrammed. (table 6)

Table 6. Locations and antibiotic resistance profiles

<table>
<thead>
<tr>
<th>Sample (time)</th>
<th>Zero</th>
<th>Resistance to 1 antibiotic</th>
<th>Resistance to 2 antibiotics</th>
<th>Resistance to 3 antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8</td>
<td>A8 (MC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10</td>
<td>A10 (SS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A11</td>
<td>A11 (FSB)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A15</td>
<td>A15 (HSN)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td>B8 (MC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10</td>
<td>B10 (SS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B11</td>
<td>B11 (FSB)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B15</td>
<td>B15 (HSN)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>C8 (MC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10</td>
<td>C10X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C11</td>
<td>C11 (FSB)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C15</td>
<td>C15 (HSN)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D8</td>
<td>D8 (MC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D10</td>
<td>D10 (SS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D11</td>
<td>D11 (FSB)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
All of the A and B samples had an antibiotic profile of zero (prior to introduction of dairy cattle). The C samples varied from an antibiotic profile of 2 (C15, the special needs/hospital barn) to an antibiotic profile of 3 (C11, Free Stall barn). No antibiotic resistance was detected in locations D8 and D11 (maternity/calf barn and free stall barn, respectively). However in locations C15 and D10 (the solids separator and hospital/special needs barn) there were antibiotic profiles of 2. In the E sampling group, locations E8, E10 and E11 (the maternity/calf barn, solids separator, and free stall barn) had a resistance of profile of 2 or 3 (table 6). All locations in the F group had an antibiotic resistance profile. The location of F8 (solids separator) had an antibiotic resistance profile of 1; location F15 (the hospital/special needs barn, respectively) had a resistance profile of two. F10 and F11 (the solids separator and free stall barn) had an antibiotic profile of 3. Location G11 (free stall barn) had an antibiotic profile of 2, and locations G10 and G15 (the solids separator and hospital/special needs barn, respectively) had an antibiotic profile of 3. Table 7 depicts the percentages of antibiotic resistant colonies per sampling location. Each location is listed according to the resistance (R) profile-therefore all locations having a “zero” profile are grouped together, those with an antibiotic resistance profile of 1 are grouped together, and so on. Additionally, the numbers of total colonies grown on each Preston Agar “master plate” versus the
percentage of colonies that grew on antibiotic-containing replica plates were determined (table 7).

Table 7: Percentage of antibiotic resistant colonies

<table>
<thead>
<tr>
<th>Farm Location</th>
<th>ID Number</th>
<th>R*</th>
<th>COP</th>
<th>**P Ery48</th>
<th>**P Nal96</th>
<th>**P Tet 24</th>
<th>Locations</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Maternity/calf barn</td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Solids separator</td>
</tr>
<tr>
<td>A</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Free stall barn</td>
</tr>
<tr>
<td>A</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Special/hospital</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Maternity/calf barn</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Solids separator</td>
</tr>
<tr>
<td>B</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Free stall barn</td>
</tr>
<tr>
<td>B</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Special/hospital</td>
</tr>
<tr>
<td>D</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Maternity/calf barn</td>
</tr>
<tr>
<td>D</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Free stall barn</td>
</tr>
<tr>
<td>G</td>
<td>11</td>
<td>0</td>
<td>128</td>
<td>5</td>
<td>0</td>
<td>14</td>
<td>Free stall barn</td>
</tr>
<tr>
<td>F</td>
<td>8</td>
<td>1</td>
<td>8</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>Maternity/calf barn</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>2</td>
<td>28</td>
<td>9</td>
<td>0</td>
<td>23</td>
<td>Special/hospital</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>2</td>
<td>10</td>
<td>170</td>
<td>0</td>
<td>130</td>
<td>Solids separator</td>
</tr>
<tr>
<td>E</td>
<td>8</td>
<td>2</td>
<td>45</td>
<td>44</td>
<td>0</td>
<td>27</td>
<td>Maternity/calf barn</td>
</tr>
<tr>
<td>E</td>
<td>11</td>
<td>2</td>
<td>108</td>
<td>16</td>
<td>81</td>
<td>0</td>
<td>Free stall barn</td>
</tr>
<tr>
<td>F</td>
<td>15</td>
<td>2</td>
<td>28</td>
<td>32</td>
<td>0</td>
<td>18</td>
<td>Special/hospital</td>
</tr>
<tr>
<td>G</td>
<td>11</td>
<td>2</td>
<td>128</td>
<td>5</td>
<td>0</td>
<td>14</td>
<td>Free stall barn</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>3</td>
<td>10</td>
<td>10</td>
<td>33</td>
<td>77</td>
<td>Free stall barn</td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>3</td>
<td>7</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>Special/hospital</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
<td>3</td>
<td>60</td>
<td>27</td>
<td>53</td>
<td>35</td>
<td>Solids separator</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>3</td>
<td>43</td>
<td>116</td>
<td>30</td>
<td>91</td>
<td>Solids separator</td>
</tr>
<tr>
<td>F</td>
<td>11</td>
<td>3</td>
<td>10</td>
<td>60</td>
<td>20</td>
<td>80</td>
<td>Free stall barn</td>
</tr>
<tr>
<td>G</td>
<td>10</td>
<td>3</td>
<td>60</td>
<td>80</td>
<td>117</td>
<td>122</td>
<td>Solids separator</td>
</tr>
<tr>
<td>G</td>
<td>15</td>
<td>3</td>
<td>144</td>
<td>64</td>
<td>6</td>
<td>156</td>
<td>Special/hospital</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>TNTC</td>
<td>TNTC</td>
<td>TNTC</td>
<td>TNTC</td>
<td>122</td>
<td>Solids separator</td>
</tr>
<tr>
<td>E</td>
<td>15</td>
<td>TNTC</td>
<td>TNTC</td>
<td>TNTC</td>
<td>TNTC</td>
<td>Special/hospital</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>8</td>
<td>TNTC</td>
<td>TNTC</td>
<td>TNTC</td>
<td>TNTC</td>
<td>Maternity/calf barn</td>
<td></td>
</tr>
</tbody>
</table>

COP=colonies on each Preston plate  TNTC=Too Numerous To Count  *R=Number resistance profile  **P=percent

Medical Treatment and Sanitizers Used
Medications were used to treat conditions such as mastitis, bovine respiratory disease and wound infections (tables 9A and 9B). The medications were prescribed by an ISU faculty veterinarian who disclosed uses and dosages given. Ill cattle were separated and treated individually (there were no group treatments). In addition, detailed protocols of treatment regimens were strictly followed.

Table 8A: List of antibiotics used on the ISU Dairy Farm (name, class, and mode of action)

<table>
<thead>
<tr>
<th>Antibiotic(s)</th>
<th>Class</th>
<th>Treatment(s)</th>
<th>Mode of action (2, 7, 8, 33, 53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquamycin</td>
<td>Tetracycline</td>
<td>Pneumonia (shipping fever), scours, wound infections</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Draxxin, Tylan</td>
<td>Marcolide</td>
<td>Draxxin-Bovine Respiratory Disease</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tylan-shipping fever, pneumonia, foot rot, calf diphtheria</td>
<td></td>
</tr>
<tr>
<td>Nuflor</td>
<td>Chloramphenicol</td>
<td>Bovine Respiratory Disease</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Pirsue</td>
<td>Lincosamide</td>
<td>Clinical and subclinical mastitis</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Excenel, Spectramast LC, Spectramast DC, Naxcel</td>
<td>Cephalosporin</td>
<td>Clinical and subclinical mastitis</td>
<td>Inhibits cell wall synthesis</td>
</tr>
<tr>
<td>Polyclinic</td>
<td>Penicillin</td>
<td>Broad spectrum antibiotic, shipping fever, pneumonia</td>
<td>Inhibits cell wall synthesis</td>
</tr>
</tbody>
</table>

Table 8B: Additional medications used on the ISU Dairy Farm

<table>
<thead>
<tr>
<th>Medication</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banamine</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
</tr>
<tr>
<td>Sterile Saline, Hypertonic Saline Solution</td>
<td>Intravenous therapy</td>
</tr>
</tbody>
</table>

BAC-STOP udder predip (Esteam Manufacturing Ltd., Calgary, Alberta) and Transcend udder postdip (IBA, Millbury, MA.) were used as sanitizers prior to and after milking. FC-98 Udder Wash (IBA, Millbury, MA.) was also used a boot sanitizer.

Discussion
It appears that the length of time cattle were housed at the ISU Dairy Farm directly correlated to the detection of antibiotic resistance in on the ISU dairy farm; more so than changes in farm temperature and humidity (although this was a 9-month study). Even though there were variability in the populations of *Campylobacter* detected on Preston agar and on the antibiotic-containing TSA agar plates (nalidixic acid and tetracycline), the number of CFUs present on Preston and TSA plates increased overall (table 3). There were also sizeable increases in the populations of antibiotic-resistant bacteria that grew on antibiotic-containing plates between sampling locations F and G (table 3).

When reviewing antibiotic resistance profiles (table 5), 7 out of 15 locations (47%) possessed the Ery48 Nal96 Tet24 resistance profile and 7 out of 15 locations (47%) possessed the Ery48 Tet24 profile. The MDR profiles began to appear during sampling group C (27 January 2008) after dairy cattle placement. When the resistance profiles were viewed according to sampling date (e.g. all A samples were graphed together, all B samples were grouped together, and so on; table 6), there was a general trend toward MDR as time progressed. Multi-drug resistance (2 agents, n=7) were divided between the maternity/calf barn (location #8), the solids separator (location # 10), the free stall barn (location # 11) and the hospital/special needs barn (location # 15). Resistance to three antibiotics tested (n=7) included the solids separator (location # 10), the free stall barn (location # 11) and the hospital/special needs barn (location # 15). Also, several sampling locations had TNTC totals (C10, solids separator; E15, hospital/special needs barn; and G8, maternity/calf barn; table 8); it is probable that multidrug resistance could have been detected at those sampling locations.

When examining the percentages of antibiotic resistance (when compared to the initial Preston plate colony counts), there was some variability, but percentages increased with progression of time (Table 7). For example, when examining location # 10 (solids separator), no bacterial populations were detected in either A and B sampling periods. However, in sampling period C, a TNTC plate was detected. In summary, the percentages of antibiotic-resistant populations increased once production animals were introduced to the facility.

**Conclusions**
The prevalence of antibiotic resistance in *Campylobacter* has fluctuated in various studies. In a 1999-2000 study of antimicrobial resistance patterns of *Campylobacter* from feedlot cattle, 51.6%, 12.1% and 0.9% of isolates (*C. jejuni* and *C. coli*) were resistant to tetracycline, nalidixic acid and erythromycin, respectively (14). This study focused on feedlots with 1000 head or higher (which held over 80% of US dairy cattle as of 1999). In a 2001 survey of lactating dairy cattle (720 cows from farms in the Northeast, Pacific and Southwestern United States), a lower overall prevalence of antibiotic resistance was determined; 53% of isolates were resistant to tetracycline, but isolates were susceptible to other antibiotics (including erythromycin and ampicillin) (22). In a 2006 survey, fecal samples were taken from 1435 dairy cows (representing 96 dairy operations in 21 US states) (15). Of the 735 *Campylobacter* isolates tested, 49.4% were resistant to tetracycline, 4.5% were resistant to nalidixic acid and 1.3% were resistant to erythromycin. In this study, it is important to recall that the amounts of antibiotics used in making TSA replica plates were 1.5 times the NARMS amount stated for antibiotic resistance; e.g. antibiotic-resistant CFUs formed on plates containing very high amounts of antibiotics. Other studies have also utilized higher MIC breakpoints than cited by NARMS in determining antibiotic resistance (53). This is the first study to our knowledge that studied the establishment and proliferation of bacteria (of interest in microbiology and food safety) before and after the introduction of production livestock. Prior to cattle placement, the numbers of *Campylobacter* identified as well as antibiotic resistance profiles were low. However, the numbers of detected *Campylobacter* increased once production animals were introduced to the facility. Additionally, resistance to one (or multiple) antibiotics was detected immediately after production animal placement.

Caution should be used when comparing prevalence between studies due to factors such as differences in microbiological culture methods and sampling protocols (10). For example in one study, environmental samples were transported for laboratory analysis within 24 hours of collection (22) while in this study, environmental samples were immediately processed after collection and transport to the lab (all materials needed for experiments were organized prior to collection). In addition, there were large amounts of samples collected over an extended period of time in some research (15) while other studies collected fewer samples over a
shorter period of time (22). Duration of sample collection periods varied; some were short (22) while other collection periods covered several years (14).

Longitudinal studies are important in providing insight into the dynamics of endemic diseases of dairy herds (44). A detailed understanding of topics such as the infection process and mechanisms for persistence are essential to controlling infections and lowering their prevalence in dairy cattle (44). A study by Pradham et al. (2009) reviewed the prevalence of Campylobacter in fecal samples collected from dairy cows on three farms in the states of New York, Vermont and Pennsylvania. Samples were taken seven times in a 30 month period (from Feb 2004 to May 2007). The range of Campylobacter spp. varied from a low of 0% to a high of 80.8%. The study was important in demonstrating different prevalence of zoonotic pathogens (such as Campylobacter) within and between farms (44). It is believed that factors such as farm management, genetics, nutrition, hygiene and manure management practices may be involved (44). In this longitudinal study, there was also some variation which may be related to previously mentioned factors. Continued monitoring of pathogens such as antibiotic resistance Campylobacter is important in reducing the prevalence of infections in animals and in humans.

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28. **Levy, S. B.**
31. **Mahamoud, A., J. Chevalier, S. Alibert-Franco, W. V. Kern, and J. M. Pages.**
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33. **Mascaretti, O. A.**
34. **Mathew, A. G., R. Cissell, and S. Liamthong.**


Appendix: The Iowa State University's Dairy/Animal Science Education and Discovery Facility

1) Feed Storage Area (FSA)
2) Open Field NE (OF)
3) Heifer Barn 9-15 months (H9)
4) Heifer Barn 2-8 months (H2)
5) Heifer Barn 16-22 months (H16)
6) Maternity/Calf Barn (MC 6)
7) Maternity/Calf Barn (MC 7)
8) Maternity/Calf Barn (MC 8)
9) Dry Cow/Transition Barn (DC)
10) Solids Separator (SS)
11) Free Stall Barn (FS11)
12) Free Stall Barn (FS12)
13) Free Stall Barn Walkway (FW)
14) Holding Area Walkway (HW)
15) Holding Area/Milking (HM)
16) Special Needs/Hospital Barn (SH)
17) Parking Main Entrance (PM)
18) Parking/Employee/Showers (PS)
19) Equipment Storage (ES)
20) Machine Storage Area (MS)
A longitudinal study of the establishment and proliferation of *Enterococcus* on a Dairy Farm

Ginger M. Shipp and James S. Dickson

**Abstract**

*Enterococci* are Gram-positive, facultative anaerobic cocci. They occur in a remarkable array of environments, because of their ability to grow and survive under harsh conditions (22). *Enterococci* are also found in a large variety of foods including milk and dairy products, vegetables, plants, cereals and meats. *Enterococcus* spp. are considered commensal organisms, but can also be opportunistic pathogens associated with morbidity and mortality of humans and animals. There is little information regarding the introduction of dairy livestock (and bacterial organisms) on previously unused farm land. A longitudinal study of antibiotic resistance of *Enterococcus* (to ampicillin, erythromycin and tetracycline) was conducted on an academic teaching farm. Environmental samples were collected by drag swabs at select locations prior to and after the introduction of livestock to determine antibiotic resistance. All samples were initially processed and screened with specialized media (Enterococcosel agar) then later replica plated on Tryptic Soy Agar (TSA) containing a predetermined amount of antibiotic. Both the numbers and the percentages of bacterial and antibiotic-resistant colonies increased as cattle were placed at the facility. Most antibiotic profiles of resistant organisms that grew on $10^{-3}$ TSA plates had the profile of combined erythromycin and tetracycline resistance. There were no Too Numerous to Count (TNTC) Enterococcosel agar plates prior to dairy cattle placement. However after cattle placement, TNTC plates began to appear. The numbers of TNTC plates were not time-dependant and appeared consistently in sampling periods C-G (after placement of cows). There is little information on the prevalence and epidemiology of antibiotic resistance of *Enterococci* outside of the hospital setting, including on dairy farms. In this (and other studies cited), the percentage of antibiotic resistance varied. Longitudinal studies are important in providing insight into the dynamics of establishment and proliferation of bacteria and of antibiotic resistance.

Key words: longitudinal study, dairy farm, ampicillin, erythromycin, tetracycline, drag swabs, replica plating, NARMS, CLSI.

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*Enterococci* are Gram-positive, facultative anaerobic cocci that occur singly, in pairs or as short chains (16). The term ‘enterococcus’ originates from the Greek *enteron* meaning ‘the gut or intestines’ and *kokkos* meaning ‘a berry or kernel’ (33). The enterococci as a group were first described by Thiercelin (1899) and the genus *Enterococcus* was proposed by Thiercelin and Jouhaud (1903) for Gram-positive diplococci of intestinal origin (13). *Enterococci* were later placed in the genus *Streptococcus* in the 1930s, with the establishment
of the Lancefield serological typing system (22). The genus Enterococcus was described by Shleifer and Kilpper-Bälz (1984) who demonstrated that Streptococcus faecalis and Streptococcus faecium were distinct enough from other streptococci to warrant their transfer to the genus Enterococcus (13). Enterococcus is member of the lactic acid bacteria (LAB) group of organisms. LABs are found in a large variety of foods including milk and dairy products, vegetables, plants, cereals and meats. LAB are used to manufacture and preserve foods or deliberately added as “starter cultures” in order to execute the fermentation process (13, 29). Enterococcus are also used as probiotics (cultures of live microorganisms which, when given to animal or humans, beneficially affect the hosts by improving properties of indigenous flora) (13). There is some controversy regarding the use of Enterococcus in food products. Unlike other LAB, enterococci are not considered “Generally Recognized as Safe” (or GRAS) organisms because they are considered emerging pathogens, are sometimes implicated in food spoilage, and can indicate fecal contamination (30).

Enterococci occur in a remarkable array of environments, because of their ability to grow and survive under harsh conditions. They are considered commensal organisms, but can also be opportunistic pathogens associated with significant morbidity and mortality of humans and animals (27). They usually cause infections in patients who have severe underlying disease, are immunocompromised, or are elderly (15, 30). In the mid 1980s, reports of high-level vancomycin resistance were documented, particularly in hospitals (21, 33). Mortality is generally high (from 42-68%) in patients with enterococcal bacteremia and usually occur in persons who are debilitated, have underlying disease or have received medical instrumentation (13, 27, 43).

Enterococci can cause many economically important veterinary diseases such as bovine mastitis and diarrhea (16, 35). Mastitis in cattle causes significant losses in the dairy industry (44) causing cows to produce less milk, produce milk that can not be sold, or cause farmers to cull sick cows rather than treat them due to the cost of treatment (17). The epidemiology of Enterococcus in bovine mastitis has not been totally clarified but enterococci are generally associated with infections related to poor hygiene (16).

There is little information regarding the introduction of dairy livestock on previously unused farm land to study the establishment and proliferation of bacterial organisms of
interest in microbiology and food safety. Iowa State University (ISU; Ames IA) was awarded 887 acres of land by a donor family. ISU constructed an academic teaching farm—the Dairy/Animal Science Education Facility (henceforth referred to as the “ISU Dairy Farm”). Prior to the establishment of the facility, it had been used for recreational purposes; no livestock had ever been introduced. A complex of buildings were constructed on 27 acres including a free-stall barn, special needs/hospital barn, maternity barn, and a calf research barn for nutrition and husbandry research. Dairy cattle were introduced on the facility beginning on 26 November 2007; the farm typically houses 450 milking cows plus a similar number of heifers, dry cows and calves. Most of the animals were moved from the former ISU teaching farm located about 20 miles north of campus (in Ankeny IA). Additionally, 50 cows were introduced to the new dairy farm from a dairy center in Calmar, IA. A map of the dairy farm is included in the appendix of this paper.

It was decided to conduct a longitudinal study of antibiotic resistance of \textit{Enterococcus} spp.—specifically resistance to ampicillin, erythromycin and tetracycline. Ampicillin is the first aminopenicillin introduced into clinical use (28) and is one of the most prescribed antibiotics in the world (4). Ampicillin (along with penicillin) is indicated for enterococcal infections other than endocarditis in non-allergic cases (16). Erythromycin (a macrolide) has a broad spectrum and is active in vitro against most Gram-positive and some Gram negative bacteria (28), however reported macrolide resistance among enterococci from non-human sources have been noted in several studies (16). The tetracyclines were once widely used to treat bacterial infections (including \textit{Enterococcus}), however, use has declined as the incidence of acquired bacterial resistance has increased and there are currently more active and better tolerated antimicrobial agents (12, 23).

\textbf{Materials and Methods}

Environmental samples were collected at select locations on the ISU Dairy Farm prior to the introduction of livestock in order to determine initial antibiotic resistance (if any). All samples were immediately processed, screened with specialized media (Enterococcosel Agar, Becton Dickinson Company, Sparks MD)\textsuperscript{a}, and later replica plated on Tryptic Soy Agar (Becton Dickinson Company, Sparks MD) containing a predetermined amount of antibiotic, observing for growth of antibiotic resistant colonies. (8). The sampling process was repeated
after dairy cattle were introduced, duplicating the entire laboratory process. Dairy farm sampling took place on seven different dates; two prior to cattle placement and five after Placement (table 1)

Table 1. ISU Dairy Farm Sampling Dates

<table>
<thead>
<tr>
<th>Prior to introducing dairy cattle</th>
<th>After introduction of dairy cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 October 2007 (A)</td>
<td>27 January, 2008 (C)</td>
</tr>
<tr>
<td>13 November 2007 (B)</td>
<td>29 February 2008 (D)</td>
</tr>
<tr>
<td>12 March 2008 (E)</td>
<td></td>
</tr>
<tr>
<td>23 April 2008 (F)</td>
<td></td>
</tr>
<tr>
<td>28 May 2008 (G)</td>
<td></td>
</tr>
</tbody>
</table>

One sterile 3 cm x 3 cm drag swab moistened with 10 mL skim milk (Solar Biologics, Ogdensburg, NY) was used per sample. Briefly, a drag swab was pulled through the environment for 60 seconds (in later collections, efforts were made to repeat sampling from previously examined locations). Each drag swab was placed in a sterile bag and stored in a cooler kept at 4°C. After collection, samples were taken to the laboratory and immediately processed. Each drag swab was aseptically added to a sterile Whirl Pack 24 oz/720 mL filtered homogenizer bag (Nasco, Fork Atkinson, WI). Ten mL of buffered peptone water was added (BPW; Difco, Becton Dickinson Company, Sparks, MD) to moisten sample for pipetting. The filtered bag was homogenized for 45 seconds at 250 RPM. One ml of sample was then added to a tube containing 9 mL BPW and creating a diluent concentration of 10⁻¹. The samples were serially diluted until a concentration of 10⁻⁷ was created. A quantity of 0.1 ml of the 10⁻², 10⁻⁴, 10⁻⁶ and 10⁻⁷ diluents were plated on Enterococcus agar. Plates were incubated for 48 hours at 37 °C. Colonies on each plate were later read as 10⁻³, 10⁻⁵, 10⁻⁷ and 10⁻⁸ after incubation populations of Enterococcus were enumerated after incubation.

Replica plates were made according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (47) utilizing Tryptic Soy Agar (TSA). Tetracycline plates (susceptible, intermediate and resistant) were made at 1.5 times the amount recommended by

*Enterococcus agar relies on the inhibitory properties of oxgall (a concentrated bile salt) against Gram-positive organisms other than Enterococci (8, 49). The growth of Gram-negative species are reduced by sodium azide (8, 49). Esceuletin reacts with an iron salt, ferric ammonium citrate, to form a dark brown or black complex (49). Enterococci produce brown-black zones beneath brown-black colonies.
the National Antimicrobial Research Monitoring System (16). Ampicillin plates were also made 1.5 times the NARMS standards for susceptible and resistant concentrations (12 and 24 ug/mL, respectively). *Enterococci* is becoming increasingly resistant to ampicillin (40) therefore, TSA plates containing 48 ug/mL of ampicillin were included in this study. *Enterococcus* is considered broadly resistant to erythromycin (3) therefore, TSA plates containing (higher) intermediate and resistant concentrations of erythromycin (6 and 12 ug/mL, respectively) were also used.

Table 2. Concentrations of antibiotics used in replica plating

<table>
<thead>
<tr>
<th></th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA with ampicillin</td>
<td>≥ 48 ug/mL</td>
<td>24 ug/mL</td>
<td>≤ 12 ug/mL</td>
</tr>
<tr>
<td>TSA with erythromycin</td>
<td>≥ 48 ug/mL</td>
<td>12 ug/mL</td>
<td>≤ 6 ug/mL</td>
</tr>
<tr>
<td>TSA with tetracycline</td>
<td>≥ 24 ug/mL</td>
<td>12 ug/mL</td>
<td>≤ 6 ug/mL</td>
</tr>
</tbody>
</table>

Each Enterococcus agar “master plate” was later replicated using a sterile piece of velvet and a replica plating apparatus. A single piece of velvet was pressed onto a series of TSA plates containing a specified amount of antibiotic (table 2). After incubation for 24 hours at 37º C under aerobic conditions, the populations of antibiotic-resistant bacteria were enumerated. Replica plating is effective with large numbers of environmental samples (32) however, it requires a high level of training and expertise (19). Antibiotic-containing plates have a very short shelf-life and require frequent preparation—which often takes place in longitudinal studies or clinical laboratory settings (19). Therefore antibiotic-containing replica plates were prepared and refrigerated 1-2 weeks before sampling. All data were analyzed by EpiInfo™ Version 3.5.1, a statistical program created by Centers for Disease Control and Prevention (Atlanta, GA) at 95 % confidence interval.

**Results**
Since there were large amounts of data collected, only the populations of “resistant” bacteria \(10^{-3}\) and \(10^{-5}\) were reported (> 24 ug/mL tetracycline, > 48 ug/mL erythromycin and > 48 ug/mL ampicillin). The dairy farm locations are diagrammed below in table 3 and on page 67.

Table 3. ISU Dairy Farm Sampling Locations

<table>
<thead>
<tr>
<th>Farm Location</th>
<th>Farm Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Feed Storage Area (FSA)</td>
<td>11) Free Stall Barn (FS 11)</td>
</tr>
<tr>
<td>2) Open Field NE (OF)</td>
<td>12) Free Stall Barn (FS 12)</td>
</tr>
<tr>
<td>3) Heifer Barn 9-15 months (H9)</td>
<td>13) Free Stall Barn Walkway (FW)</td>
</tr>
<tr>
<td>4) Heifer Barn 2-8 months (H2)</td>
<td>14) Holding Area Walkway (HW)</td>
</tr>
<tr>
<td>5) Heifer Barn 16-22 months (H16)</td>
<td>15) Holding Area Milking (HM)</td>
</tr>
<tr>
<td>6) Maternity/Calf Barn (MC 6)</td>
<td>16) Special Needs/Hospital Barn (SH)</td>
</tr>
<tr>
<td>7) Maternity/Calf Barn (MC 7)</td>
<td>17) Parking Main Entrance (PM)</td>
</tr>
<tr>
<td>8) Maternity/Calf Barn (MC 8)</td>
<td>18) Parking/Employee/Showers (PS)</td>
</tr>
<tr>
<td>9) Dry Cow/Transition Barn (DC)</td>
<td>19) Equipment Storage (ES)</td>
</tr>
<tr>
<td>10) Solids Separator (SS)</td>
<td>20) Machine Storage Area (MS)</td>
</tr>
</tbody>
</table>

When assessing ampicillin concentration (48ug/mL) and antibiotic resistance, the numbers of CFUs growing on \(10^{-3}\) TSA plates were as follows:

Table 4. Total colonies and ampicillin-resistant colonies \(10^{-3}\)

<table>
<thead>
<tr>
<th>Farm Location</th>
<th>Log(_{10}) values (total colonies)</th>
<th>Total colonies (E)*</th>
<th>Log(_{10}) Values (CFUs Resistance*)</th>
<th>CFUs Resistance*</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.38</td>
<td>24</td>
<td>0</td>
<td>1</td>
<td>4%</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>10</td>
<td>0.70</td>
<td>5</td>
<td>50%</td>
</tr>
<tr>
<td>C</td>
<td>2.15</td>
<td>142</td>
<td>1.38</td>
<td>24</td>
<td>17%</td>
</tr>
<tr>
<td>D</td>
<td>2.35</td>
<td>227</td>
<td>2.36</td>
<td>4</td>
<td>2%</td>
</tr>
<tr>
<td>E</td>
<td>2.60</td>
<td>407</td>
<td>2.15</td>
<td>142</td>
<td>35%</td>
</tr>
<tr>
<td>F</td>
<td>2.10</td>
<td>127</td>
<td>1.20</td>
<td>16</td>
<td>13%</td>
</tr>
<tr>
<td>G</td>
<td>1.91</td>
<td>82</td>
<td>---</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

* CFUs Resistant= grown on TSA with antibiotic E=Enterococcus Agar

Resistance ranged from none detected (G) to 50% (B). In examining the numbers of *Enterococcus* colonies and erythromycin resistance:

Table 5. Total colonies and erythromycin-resistant colonies \(10^{-3}\)

<table>
<thead>
<tr>
<th>Farm Group</th>
<th>Log(_{10}) values (total colonies)</th>
<th>Total Colonies (E)*</th>
<th>Log(_{10}) Values (CFUs Resistance*)</th>
<th>CFUs Resistance*</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.38</td>
<td>24</td>
<td>---</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>10</td>
<td>1.54</td>
<td>35</td>
<td>350%</td>
</tr>
<tr>
<td>C</td>
<td>2.15</td>
<td>142</td>
<td>1.20</td>
<td>16</td>
<td>11%</td>
</tr>
</tbody>
</table>
Resistant colonies that grew on TSA containing erythromycin ranged from none (A) to 350 % (B).

When identifying numbers of tetracycline-resistant colonies during the study, there were no resistant colonies detected before dairy cattle introduction. However, after cattle introduction, the percentages of resistant colonies growing on antibiotic-containing TSA plates were high-ranging from 74 % (G) to 183 % (F):

Table 6. Total colonies and tetracycline-resistant plates ($10^{-3}$)

<table>
<thead>
<tr>
<th>Farm Group</th>
<th>Log$_{10}$ values (total colonies)</th>
<th>Total Colonies (E)*</th>
<th>Log$_{10}$ Values (CFUs Resistance*)</th>
<th>CFUs Resistant*</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.38</td>
<td>24</td>
<td>---</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>10</td>
<td>---</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>C</td>
<td>2.15</td>
<td>142</td>
<td>2.10</td>
<td>126</td>
<td>89 %</td>
</tr>
<tr>
<td>D</td>
<td>2.35</td>
<td>257</td>
<td>2.40</td>
<td>249</td>
<td>97 %</td>
</tr>
<tr>
<td>E</td>
<td>2.60</td>
<td>407</td>
<td>2.52</td>
<td>338</td>
<td>83 %</td>
</tr>
<tr>
<td>F</td>
<td>2.10</td>
<td>127</td>
<td>2.36</td>
<td>232</td>
<td>183 %</td>
</tr>
<tr>
<td>G</td>
<td>1.91</td>
<td>82</td>
<td>1.79</td>
<td>61</td>
<td>74 %</td>
</tr>
</tbody>
</table>

* CFUs Resistant= grown on TSA with antibiotic  E=Enterococcosel Agar

When examining $10^{-5}$ concentrations of dairy farm diluents, the total number of CFUs detected were lower overall (see diagrams). Antibiotic resistant colonies were also lower overall-except for tetracycline:

Table 7. Ampicillin 48 ug/mL ($10^{-5}$)

<table>
<thead>
<tr>
<th>Farm Group</th>
<th>Log$_{10}$ values (total colonies)</th>
<th>Total Colonies (E)*</th>
<th>Log$_{10}$ Values (CFUs Resistance*)</th>
<th>CFUs Resistant*</th>
<th>Percentages</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>---</td>
<td>0</td>
<td>---</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>B</td>
<td>---</td>
<td>0</td>
<td>---</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>C</td>
<td>1.89</td>
<td>77</td>
<td>0.30</td>
<td>2</td>
<td>3 %</td>
</tr>
<tr>
<td>D</td>
<td>1.62</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>E</td>
<td>1.46</td>
<td>29</td>
<td>0</td>
<td>1</td>
<td>3 %</td>
</tr>
<tr>
<td>F</td>
<td>1.76</td>
<td>58</td>
<td>0</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>G</td>
<td>1.28</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>0 %</td>
</tr>
</tbody>
</table>

* CFUs Resistant= grown on TSA with antibiotic  E=Enterococcosel Agar
Table 8. Erythromycin 48 ug/mL (10^{-5})

<table>
<thead>
<tr>
<th>Farm Group</th>
<th>Log_{10} values (total colonies)</th>
<th>Total Colonies (E)*</th>
<th>Log_{10} Values (CFUs Resistance*)</th>
<th>CFUs Resistant*</th>
<th>Percentages</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>---</td>
<td>0</td>
<td>---</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>B</td>
<td>---</td>
<td>0</td>
<td>---</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>C</td>
<td>1.89</td>
<td>77</td>
<td>1.26</td>
<td>18</td>
<td>23 %</td>
</tr>
<tr>
<td>D</td>
<td>1.62</td>
<td>42</td>
<td>1.26</td>
<td>18</td>
<td>43 %</td>
</tr>
<tr>
<td>E</td>
<td>1.46</td>
<td>29</td>
<td>0.60</td>
<td>4</td>
<td>14 %</td>
</tr>
<tr>
<td>F</td>
<td>1.76</td>
<td>58</td>
<td>1.58</td>
<td>38</td>
<td>66 %</td>
</tr>
<tr>
<td>G</td>
<td>1.28</td>
<td>19</td>
<td>1.78</td>
<td>60</td>
<td>32 %</td>
</tr>
</tbody>
</table>

* CFUs Resistant= grown on TSA with antibiotic  E=Enterococcosel Agar

Table 9. Tetracycline 24 ug/mL (10^{-5})

<table>
<thead>
<tr>
<th>Farm Group</th>
<th>Log_{10} values (total colonies)/Total Colonies (E)*</th>
<th>Total Colonies (E)*</th>
<th>Log_{10} Values (CFUs Resistance*)</th>
<th>CFU Resistant*</th>
<th>Percentages</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>---</td>
<td>0</td>
<td>---</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>B</td>
<td>---</td>
<td>0</td>
<td>---</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>C</td>
<td>1.89</td>
<td>77</td>
<td>2.12</td>
<td>133</td>
<td>172 %</td>
</tr>
<tr>
<td>D</td>
<td>1.62</td>
<td>42</td>
<td>1.62</td>
<td>42</td>
<td>100 %</td>
</tr>
<tr>
<td>E</td>
<td>1.46</td>
<td>29</td>
<td>1.68</td>
<td>48</td>
<td>166 %</td>
</tr>
<tr>
<td>F</td>
<td>1.76</td>
<td>58</td>
<td>2.34</td>
<td>223</td>
<td>384 %</td>
</tr>
<tr>
<td>G</td>
<td>1.28</td>
<td>60</td>
<td>2.12</td>
<td>132</td>
<td>220 %</td>
</tr>
</tbody>
</table>

* CFUs Resistant= grown on TSA with antibiotic  E=Enterococcosel Agar

It was interesting to note in the 10^{-5} group, antibiotic resistance was not detected before placement of dairy cattle (keep in mind that resistance was detected in some of the 10^{-3} diluent samples prior to the introduction of dairy livestock). Also of note, even though the numbers of CFUs formed on Enterococcusel agar were lower (as expected, when compared to 10^{-3} diluent concentration), the percentages of colonies resistant to tetracycline remained high.

The antibiotic profiles of both 10^{-3} and 10^{-5} dilutions were examined in detail. The data were analyzed and placed in tables (tables 10 and 11) for ease of interpretation.
The majority of isolates plated on TSA with tetracycline grew (which is consistent with the high amounts of antibiotic resistance in table 9).

When examining the numbers of antibiotic-resistance colonies on Enterococccsl agar, it is important to consider plates too numerous to count (TNTC). Below is a table citing where TNTC plates were found (farm group and farm number):

### Table 12: TNTC Locations

<table>
<thead>
<tr>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>N=number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>1 (FSA)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2 (OF)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3 (H9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4 (H2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5 (H16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6 (MC 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>7 (MC 7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>8 (MC 8)</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>9 (DC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10 (SS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>5</td>
</tr>
<tr>
<td>11 (FS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>11 (FSS)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 12. (continued)

<table>
<thead>
<tr>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>N=number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>12 (FS 12)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>13 (FW)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>14 (HW)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>15 (HM)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>16 (SH)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>17 (PM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>18 (PS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>19 (ES)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>20 (MS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>N (group)</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>34</td>
</tr>
</tbody>
</table>

**Medical Care and Sanitizers**

Dairy cattle who presented with medical conditions such as diarrhea, upper respiratory disease, or general weakness were reported to farm administrators and treated according to the directives of the farm veterinarian. Additionally, the farm veterinarian made frequent “rounds” on the dairy farm to personally assess animal condition. During meetings with the dairy farm staff and the veterinarian, protocols were used that detailed the care (and isolation, if necessary) of sick cattle. A summary of medications used are included in table 13. It is important to note that there was no “group treatment” of animals; each sick cow was treated individually.

Table 13: Medications used on the ISU Dairy Farm

<table>
<thead>
<tr>
<th>Medication</th>
<th>Medical conditions</th>
<th>Class of Drug (1, 6, 28, 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin SQ</td>
<td>Navel infection, foot rot</td>
<td>β-lactam</td>
</tr>
<tr>
<td>Excenel</td>
<td>Navel infection, diarrhea, pneumonia, metritis, foot rot, mastitis,</td>
<td>β-lactam</td>
</tr>
<tr>
<td>Nuflor SQ</td>
<td>Pneumonia, upper respiratory disease</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>Draxxin SQ</td>
<td>Upper respiratory disease, pneumonia</td>
<td>Marcolide</td>
</tr>
<tr>
<td>Sulfadimethoxine</td>
<td>Pneumonia, upper respiratory disease</td>
<td>Sulfonamide</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>Pneumonia, foot rot, hairy wart</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Polyflex</td>
<td>Metritis</td>
<td>β-lactam</td>
</tr>
<tr>
<td>Spectramast LC</td>
<td>Mastitis</td>
<td>β-lactam</td>
</tr>
<tr>
<td>Flunixinime IV</td>
<td>Pneumonia, upper respiratory disease</td>
<td>Non-steroidal Anti-inflammatory drugs (NSAIDs)</td>
</tr>
</tbody>
</table>
Table 13 (continued)

<table>
<thead>
<tr>
<th>Medications</th>
<th>Medical conditions</th>
<th>Class of Drug (1, 6, 28, 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lutalyse</td>
<td>Pyometra</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>Estrumatae</td>
<td>Pyometra</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>Oral Calcium, IV Calcium</td>
<td>Milk fever, retained placenta</td>
<td>Mineral Supplement</td>
</tr>
<tr>
<td>Dextrose</td>
<td>Metritis, ketosis</td>
<td>Monosaccharide (energy, metabolism)</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>Retained placenta</td>
<td>Pharmaceutical solvent</td>
</tr>
</tbody>
</table>

BAC-STOP udder predip (Esteam Manufacturing Ltd., Calgary, Alberta) and Transcend udder postdip (IBA, Millbury, MA.) were used as sanitizers prior to and after milking. FC-98 Udder Wash (IBA, Millbury, MA.) was also used a boot sanitizer.

**Discussion**

In this longitudinal study, it was interesting to note the populations of bacteria that were resistant prior to and after the placement of dairy cattle. The numbers and percentage of bacterial and antibiotic-resistant colonies increased as cattle were placed at the facility. This is to be expected since many bacterial organisms (including pathogens) originate in the ruminant intestinal tract (25, 31, 36). However, in this study, antibiotic-resistant *Enterococcus* grew on TSA plates containing very high levels of ampicillin and erythromycin. The amounts of tetracycline and erythromycin incorporated into TSA (both 48 ug/mL) were much higher than the amount deemed resistant by CLSI (≥ 24 ug/mL for ampicillin and ≥ 12 ug/mL for erythromycin). Even at the highest concentrations, there were growth of antibiotic-resistant organisms, particularly after the introduction of dairy cattle-and more so with erythromycin when compared to ampicillin (tables 7 and 8). In addition, there were very high percentages of resistant populations on TSA plates containing tetracycline (tables 6 and 9). One and one half times the amount of antibiotic deemed resistant by NARMS was incorporated into TSA (24 ug/mL) as opposed to 16 ug/mL (the recommended amount). Also, significant growth occurred after the placement of dairy cattle. None of the populations of enterococci in both A and B groups detected were resistant to tetracycline.

When reviewing antibiotic profiles of the populations of resistant organisms that grew on TSA plates at a 10^{-3} dilution (table 10), most had the profile of combined erythromycin and tetracycline resistance (n=18). Nine plates were resistant to only tetracycline (n=9) and five...
were resistant to the three antibiotics tested (erythromycin, ampicillin and tetracycline). In table 11 ($10^{-5}$ dilution), nine plates were resistant to ampicillin and tetracycline; eight TSA plates exhibited resistance to erythromycin and tetracycline; and two were resistant to the three antibiotic tested (erythromycin, ampicillin and tetracycline). Of the populations of enterococci plates that were resistant, 25 (or 66%) of the $10^{-3}$ plates were multi-drug resistant (table 10) and 19 (or 76%) of the $10^{-5}$ plates were multi-drug resistant (table 11). This is significant because multi-drug resistance is becoming more prevalent (14) and is compromising the treatment of disease in both humans and animals (24, 39).

In table 12, TNTC Enterococcus plates are diagrammed (they were not subsequently tested for antibiotic resistance). In locations A and B (before dairy cattle placement), there were no estimated counts. However, immediately after the placement of cattle, large populations of enterococci began to appear. The numbers of TNTC plates were consistent throughout the sampling periods; C (n=8), D and G (n=7) as well as E and F (n=6). When reviewing specific dairy farm locations, there were 5 TNTC plates at the solids separator (location 10), free stall barn (location 12) and free stall walkway (location 13). There were 4 TNTC plates at the free stall barn (location 11), holding area walkway (location 14) and the holding/milking area (location 15). It is interesting to note that there was only one TNTC plate at the special needs/hospital barn (location 16). Since the TNTC Enterococcus plates could not be replicated, the actual numbers of bacterial CFUs and the antibiotic resistant profiles (derived from antibiotic-containing plates) are likely much higher and more extensive.

One possible limitation of this study is that slight differences in making specialized media may have influenced microbial counts. To account for this, a staff member who has more than twenty years experience making media and reagents assisted in media/reagent preparation. Additionally, when colonies were counted, additional staff persons verified colony counts. Since this study took place over a 9-month period, it would be appealing to continue sampling over a longer period of time. The information in this (and other) studies can be helpful in studying antibiotic resistance in livestock. Measures could then be implemented to reduce conditions leading to antibiotic resistance, and limit the spread of resistant infectious bacterial organisms in dairy farm environments (5).
Conclusion

There is little information on the prevalence and epidemiology of antimicrobial resistance in Enterococci outside the hospital setting, including on dairy farms (20). Makovec et al. (2003) conducted a study in which over 8900 milk samples were tested. Of the Enterococci identified (n=405), erythromycin resistance increased from over time from 29% to 47% (26). Additionally, over 49% of isolates were resistant to tetracycline and 38% were resistant to ampicillin (26). In a study of central California dairies (38), 326 environmental samples indentified as Streptococcus uberis (39.9%), Streptococcus dysgalactiae (42.2%) and Enterococcus spp. (11.1%) were tested to determine the prevalence of antibiotic susceptibility and resistance. Enterococcus spp. were the most resistant organisms tested. Anderson et al. (2008) compared antibiotic-resistant Enterococci in American bison and pastured cattle in northeastern Kansas. Cattle isolates were more resistant to erythromycin (12.7% versus 4% in bison) and tetracycline (42.9% versus 8% in bison) (3). Both the cattle and bison isolates demonstrated low resistance to ampicillin. In the African country of Botswana, the prevalence of antibiotic resistant enterococci in meat, beef and chicken products were reviewed (7). Of the 415 E. faecalis isolates, 400 were resistant to antibiotics (7). Ninety six percent were resistant to ampicillin and 18% were tetracycline resistant. Ten percent were resistant to both ampicillin and tetracycline. Of note, a few isolates demonstrated MDR to vancomycin, cephalothin and teicoplanin. This is significant because the antibiotics used in this study represent the major groups of antibiotics used in health care facilities (7). Additionally, E. faecium isolates were highly resistant to the same drugs.

There have been many studies of antibiotic resistance in food items such as cheeses, meats and fermented foods such as yogurt (45). Increased resistance of enterococci in foods is of interest because the ability of these organisms to infect immunocompromised hosts and cause serious medical conditions in humans (45). Dairy cows that develop infections caused by diseases such as mastitis and post-parturient disease may become chronically infected and be sent to slaughter prematurely (26, 41). A number of researchers advocate spending less time collecting antimicrobial use data citing numerous studies determining the rate of disease in dairy cattle (41). They also believe data could be estimated from existing datasets to develop reasonable estimates without the expense of additional surveys (41). However, other
researchers feel that it is not completely understood how widespread the frequency of antibiotic-resistant bacteria are in the non-nosocomial community and the lack of surveillance data is especially evident in important agricultural environments such as dairy farms (5). When completing a literature review concerning antibiotic resistance in a new dairy farm environment (analysis of bacteria prior to, and after placement of dairy cattle), no information was found at the time of this writing. To our knowledge, this is the first study of the establishment and proliferation of bacteria (and antibiotic resistance) before and after the introduction of production livestock on previously unused farm land.

While there may be an abundance of information regarding treatment of conditions in dairy cattle such as mastitis and post-parturient disease (10, 34, 41, 48), the role that non-human sources and reservoirs (other than hospitalized patients) may play in the spread of Enterococcus is controversial and poorly understood (20). Most antimicrobial studies have focused on bacteria such as Salmonella, enterotoxigenic Escherichia coli, and bacteria isolated from clinical cases (39). While there are studies that question the reported risks of using antibiotics in animal production (9, 42), the general consensus is that increased surveillance of bacteria (such as Enterococcus) and compliance with appropriate use of antibiotics would be beneficial to human and animal health (2, 9, 37). Minimizing antibiotic resistance requires a multidisciplinary approach (14). Efforts such as creating new antibiotics (24) completing research that minimize bacterial infections of animals (and of humans) (3, 7, 26), improving diagnostic skills of laboratory workers (11, 18), using antibiotics in a responsible (or “prudent”) fashion (37), and better farm management (41) are useful in addressing the issue of antibiotic resistance among humans and animals.

Acknowledgements: We thank Kay Christiansen, Joe Detrick, Bruce Leuschen, DVM and Leo Timms, Ph.D. for their assistance.

References


Appendix: The Iowa State University’s Dairy/Animal Science Education and Discovery Facility

1) Feed Storage Area (FSA)
2) Open Field NE (OF)
3) Heifer Barn 9-15 months (H9)
4) Heifer Barn 2-8 months (H2)
5) Heifer Barn 16-22 months (H16)
6) Maternity/Calf Barn (MC 6)
7) Maternity/Calf Barn (MC 7)
8) Maternity/Calf Barn (MC 8)
9) Dry Cow/Transition Barn (DC)
10) Solids Separator (SS)
11) Free Stall Barn (FS11)
12) Free Stall Barn (FS12)
13) Free Stall Barn Walkway (FW)
14) Holding Area Walkway (HW)
15) Holding Area/Milking (HM)
16) Special Needs/Hospital Barn (SH)
17) Parking Main Entrance (PM)
18) Parking/Employee/Showers (PS)
19) Equipment Storage (ES)
20) Machine Storage Area (MS)
The establishment of *Enterobacteriaceae* and *Salmonella london* in a new dairy farm environment.

Ginger M. Shipp and James S. Dickson

**ABSTRACT**

*Salmonella* spp. are important zoonotic pathogens in humans and animals (77). A longitudinal study was conducted on the Iowa State University’s campus (at the Dairy/Animal Science Education and Discovery Facility) to observe change in *Enterobacteriaceae* (specifically *Salmonella*) before and after the placement of dairy livestock. To our knowledge, this is the first study that evaluated environmental changes of Gram-negative organisms in a new dairy farm environment. Environmental samples were taken using drag swabs and immediately processed in the laboratory using phenotypic methods (replica plating, the BBL Crystal Identification System for enteric/nonfermenter organisms, and plating on specialized media/broths). Genotypic methods were also used (the BAX PCR system™ and PFGE). Organisms identified as *Salmonella* were sent to the National Veterinary Services Laboratory (Ames, IA) for confirmatory serotyping. Resistance to antibiotics (ampicillin, nalidixic acid and tetracycline) was determined from replica plating of *Enterobacteriaceae* and *Salmonella* isolates using the guidelines of the National Antimicrobial Resistance Monitoring System (NARMS) and Clinical and Laboratory Standards Institute (CLSI). The microbiota of *Enterobacteriaceae* changed as cattle were introduced and as time progressed. Additionally, multi-drug resistant (MDR) isolates began to appear immediately after cattle were introduced (MDR isolates were rare prior to introduction of livestock). Variables such as temperature and humidity did not affect the proliferation of bacterial organisms; however this study was completed over a 9-month period. Seventeen *Salmonella* isolates were identified as *S. london* and three isolates as *S. montevideo*. Based on PFGE-generated dendograms, it is likely that the 17 *S. london* isolates are clonal and the 3 *S. montevideo* isolates are clonal.

Key words: longitudinal study, dairy farm, ampicillin, nalidixic acid, tetracycline, drag swabs, replica plating, NARMS, CLSI, PFGE, PCR

A manuscript to be submitted to *Foodborne Pathogens and Disease*

*Salmonella* spp. are important zoonotic pathogens in humans and animals (77). In the United States, human salmonellosis (caused by non-typhi *Salmonella*) occurs in about 1.3 million people, causes > 500 deaths and is estimated to cost the US economy 2.3 billion to 3.6 billion each year (10). Most cases of salmonellosis are caused by a single species, *S. enterica* which consists of over 2500 serotypes (39). Although *S. enterica* serotypes are closely related, there are important differences between them. Some serotypes are “generalists” that infect a wide variety of animals. For example, *S. typhimurium* infects hosts such as humans, mice and chickens (21). Other serotypes are highly host-adapted infecting
one or only a few species (21). For example, *S. pullorum* predominantly infects chickens and *S. dublin* largely infects cattle (11, 41). In one study involving serotypes infecting humans, only 10-100 ingested organisms were required to cause illness (6).

Although the majority of human *Salmonella* infections result in asymptomatic or self-limiting diarrheal illness, life-threatening bacteremias and other infections do occur—particularly in immunocompromised hosts, neonates and the elderly (82). Outbreaks of non-typhoidal *Salmonella* infections and sporadic illness have been associated with foods of animal origins, fruits and vegetables contaminated with manure, and contact with animals, including reptiles (77). For example, salmonellosis on dairy farms can be costly to dairy producers because of treatment costs, increased culling rates, decreased weight gain, and decreased milk production (32).

Bacteria are an important part of the soil microbiota because of their abundance, their species diversity, and the multiplicity of their metabolic activities (56). Traditional microbial culture is considered the established method for the examination of environmental microorganisms (33). In microbial culture, samples are collected, transported to the laboratory, and dispersed on various media having the required nutrients and physiochemical conditions for growth (33, 66). After a suitable period of incubation, colonies are subcultured and identified according to variables such as colony and cellular morphology, Gram-stain pattern and biochemical characteristics (33). Culture methods can detect very small number of cells, and approaches such as formulating the medium to target the growth of specific bacteria (while inhibiting others) can be implemented for more sophisticated and specific measurements (13). Cultural detection also allows for quantification of microorganisms with considerable precision (81). However, culturing can be time-consuming and take up to 7 days to complete (65). In addition, culturing can be inefficient for epidemiological studies involving the low prevalence of bacterial organisms due to large number of samples that are typically required (65). Bacteria can also be uncultivable, and viable cells that lose the ability to form colonies may not be detected (13, 36). Plate counts assume that during the process of plating, individual colonies are separated and will form discreet colonies; if cells are aggregated, underestimation may occur (13). Despite the disadvantages, the ability of
microbiologists to cultivate bacteria is still considered extremely important to obtain a thorough understanding and evaluation of microbial communities (23, 56).

During the last 25 years, molecular techniques have increased in prominence, allowing individual bacterial species to be identified and sometimes quantified from environmental samples (13, 23). Rapid enumeration of bacteria is completed using a variety of approaches. Polymerase Chain Reaction (PCR) technology is used to decrease the time and increase the sensitivity of detection of bacteria in food, environmental, and clinical samples (65). During PCR, large quantities of a specific gene fragment can be synthesized during repeated cycles of denaturation, primer annealing and DNA polymerase-catalyzed elongation (23). PCR is a specific and sensitive technique used to detect nucleic acids; however the presence of inhibitory compounds may affect the PCR reaction and give a false positive result (16). Suboptimal reaction conditions (inappropriate primers, improper temperature conditions, incorrect Mg\(^{2+}\) concentration) may also influence the outcome of PCR (81).

Another DNA-based technology that is used to identify bacterial species and their relatedness is Pulsed-Field Gel Electrophoresis (PFGE). PFGE “fingerprints” reflects the structural organization of bacterial chromosome (55). In PFGE, suspended bacterial cells are embedded in an agarose “plug” and chromosomal DNA is digested with restriction enzyme(s) (which cuts the chromosome in fragments of varying size). The digested plugs are then placed in larger agarose gel and positioned in an electrophoresis chamber. Electrical pulses are applied to the gel resulting in size-dependent movement of DNA fragments (smaller fragments migrate faster through agarose than larger fragments). Contour-clamped Homogeneous Electrical Field (CHEF) electrophoresis uses a complex electrophoresis chamber with multiple electrodes to achieve a uniform electrophoretic field resulting in better resolution of DNA fragments (4). PFGE is the current “gold standard” for highly discriminatory subtyping of most bacterial pathogens (27, 66) including *Salmonella* spp. The disadvantages of PFGE are complex and time-consuming DNA preparation and electrophoresis (85), costly reagents and the costs of electrophoresis equipment (46). Furthermore, certain organisms such as *Clostridium difficile* may not be typeable by PFGE because their DNA can not be extracted intact (71).
The Iowa State University’s Dairy/Animal Science Education and Discovery Facility (henceforth referred to as the “ISU Dairy Farm”) is located on an 887-acre site three miles south of ISU’s central campus. The farm houses 450 milking cows—plus a similar number of heifers, dry cows, and calves. A complex of buildings were constructed on 27 acres of land including a free-stall barn, special needs/hospital barn, maternity barn, and a calf research barn for nutritional and husbandry studies. The land the ISU Dairy Farm now occupies was donated to the university by a donor. The farm had been recreational and no production livestock had ever been introduced. Dairy cattle were placed on the farm beginning on 26 November 2007 (a map of the dairy farm is located in the appendix of this paper). Most of the animals were moved from the former ISU teaching farm located about 20 miles north of campus (in Ankeny IA). Additionally, 50 cows were introduced to the new ISU Dairy Farm from a dairy center in Calmar, IA.

A longitudinal study was conducted to observe the change in environmental microorganisms before and after the placement of dairy livestock (paying particular attention to Enterobacteriaceae and Salmonella spp.) Phenotypic and genotypic methods (such as biochemical requirements of organisms and PFGE profiles, respectively) were utilized to identify and classify organisms. Research described in this paper was completed during seven sampling periods (table 1).

**Materials and methods**

One sterile 3 cm x 3 cm drag swab moistened with 10 mL skim milk (Solar Biologics, Ogdensburg, NY) was used per sample. Briefly, a drag swab was pulled through the farm environment (table 2 and page 92) for 60 seconds. Each sample was placed in a cooler kept at 4º C.

Table 1. ISU Dairy Farm sampling dates

<table>
<thead>
<tr>
<th>Prior to introducing dairy cattle</th>
<th>After the introduction of dairy cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 October 2007 (A)</td>
<td>27 January 2008 (C)</td>
</tr>
<tr>
<td>13 November 2007 (B)</td>
<td>29 February 2008 (D)</td>
</tr>
<tr>
<td></td>
<td>12 March 2008 (E)</td>
</tr>
<tr>
<td></td>
<td>23 April 2008 (F)</td>
</tr>
<tr>
<td></td>
<td>28 May 2008 (G)</td>
</tr>
</tbody>
</table>
Table 2. ISU Dairy Farm Sampling Locations

<table>
<thead>
<tr>
<th>Location Description</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Feed Storage Area (FSA)</td>
<td></td>
</tr>
<tr>
<td>2) Open Field NE (OF)</td>
<td></td>
</tr>
<tr>
<td>3) Heifer Barn 9-15 months (H9)</td>
<td></td>
</tr>
<tr>
<td>4) Heifer Barn 2-8 months (H2)</td>
<td></td>
</tr>
<tr>
<td>5) Heifer Barn 16-22 months (H16)</td>
<td></td>
</tr>
<tr>
<td>6) Maternity/Calf Barn (MC 6)</td>
<td></td>
</tr>
<tr>
<td>7) Maternity/Calf Barn (MC 7)</td>
<td></td>
</tr>
<tr>
<td>8) Maternity/Calf Barn (MC 8)</td>
<td></td>
</tr>
<tr>
<td>9) Dry Cow/Transition Barn (DC)</td>
<td></td>
</tr>
<tr>
<td>10) Solids Separator (SS)</td>
<td></td>
</tr>
<tr>
<td>11) Free Stall Barn (FS 11)</td>
<td></td>
</tr>
<tr>
<td>12) Free Stall Barn (FS 12)</td>
<td></td>
</tr>
<tr>
<td>13) Free Stall Barn Walkway (FW)</td>
<td></td>
</tr>
<tr>
<td>14) Holding Area Walkway (HW)</td>
<td></td>
</tr>
<tr>
<td>15) Holding Area Milking (HM)</td>
<td></td>
</tr>
<tr>
<td>16) Special Needs/Hospital Barn (SH)</td>
<td></td>
</tr>
<tr>
<td>17) Parking Main Entrance (PM)</td>
<td></td>
</tr>
<tr>
<td>18) Parking/Employee/Showers (PS)</td>
<td></td>
</tr>
<tr>
<td>19) Equipment Storage (ES)</td>
<td></td>
</tr>
<tr>
<td>20) Machine Storage Area (MS)</td>
<td></td>
</tr>
</tbody>
</table>

After collection, samples were taken to the laboratory and immediately processed. Each drag swab was aseptically added to a sterile Whirl Pack 24 oz/720 mL filtered homogenizer bag (Nasco, Fork Atkinson, WI) containing 10 mL of buffered peptone water (BPW; Difco, Becton Dickinson Company, Sparks, MD) to add moisture for easier pipetting. The sample was homogenized for 45 seconds at 250 rpm. One ml of sample was added to a tube containing 9 mL BPW creating a dilutent concentration of $10^{-1}$. The samples were then serially diluted to a $10^{-4}$ concentration. A quantity of 0.1 ml $10^{-4}$ diluent was plated on XLT-4 agar and incubated for 48 hours at 37 °C. (results were recorded as $10^{-5}$). Each plate was later screened for presence or absence of black colonies, characteristic of *Salmonella* spp. and populations of typical salmonella colonies were enumerated after incubation. The BBL Crystal Identification Systems Enteric/Nonfermenter ID kit (Sparks, MD) was used to further identify colonies to the genus and species level.

Resistance to antibiotics was also investigated over time via replica plating. Replica plating has been used widely since its invention by Lederberg and Lederberg (48). It involves identifying and counting bacterial colonies on a master plate then later “replicate” (or stamp) the colonies from the master plate onto several different antibiotic-containing plates (51). This method makes it possible to determine the frequency of resistant and susceptible strains in each sample (51). In this study, colonies from XLT-4

*XLT-4 is a selective culture medium and is used for identification of salmonellae from food and environmental samples. XLT-4 utilizes the detergent tergitol to obtain a nearly complete inhibition of *Proteus* spp.. Additionally, sodium thiosulfate from H$_2$S production reacts with ammonium sulfate (incorporated in the agar) to form black colonies, characteristic of salmonellae (19). *Salmonella* is differentiated on XLT-4 agar from other organisms such as *Escherichia coli* (growth of yellow colonies) and *Shigella* species (growth of red colonies). (14, 19)
media were replica plated (using a replica plating cylinder covered with velvet) onto Tryptic Soy Agar (Difco, Becton Dickinson Company, Sparks, MD) containing ampicillin (48 ug/mL; Sigma, St. Louis, MO), nalidixic acid (48 ug/mL; Sigma, St. Louis, MO) and tetracycline (24 ug/mL; MP Biomedicals, Solon OH); these quantities were at least 1.5 times the concentrations recommended by the National Antimicrobial Resistance Monitoring System (NARMS). Replica plating has been shown to be effective with large numbers of environmental samples (51), however it requires a high level of training and expertise (29). Additionally, antibiotic-containing plates have a very short shelf-life and require frequent preparation which often takes place in longitudinal studies or in clinical laboratory settings (29). Therefore, all antibiotic-containing replica plates were prepared using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and refrigerated 1-2 weeks before sampling was carried out.

The BAX PCR system™ (Qualicon Inc., Wilmington, DE, USA) was used to screen for *Salmonella*. The BAX system uses real-time PCR to detect and confirm the presence of target organisms (such as *Salmonella*) within 24 hours (57). After a standard enrichment, cells were lysed to release DNA and placed in the BAX system for detection (12). BAX PCR is sensitive, accurate and rapid (results can be known in as little as 13-24 hours), however, prolonged enrichment is required to detect lower levels of bacterial contamination (42). Additionally, false positive and false negative results have been reported (31, 74). In this study, a 5 ul aliquot of 10⁻¹ BPW diluent (taken from specified locations on the dairy farm) was enriched and later added to 200 ul of lysis buffer. After incubation at 37 °C for 20 minutes and 95°C for 10 minutes, the lysate was cooled in a cooling block. Fifty microliters (uL) of the lysate was combined with a tablet containing PCR reactants (provided in the screening kit) and subjected to real-time PCR in a BAX System Q7 Thermocycler (Applied Biosystems, Wilmington DE, USA) using the thermocycling...

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*NARMS is a collaborative effort between the Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and the Centers for Disease Control and Prevention (CDC) (58). NARMS monitors changes in antimicrobial susceptibility of enteric bacteria from human and animal sources (58).*
program recommended by the manufacturer. Positive and negative controls were used
(Salmonella spp., Enterococcus faecalis and Listeria monocytogenes) during PCR and each
$10^{-1}$ diluent was processed for PCR (for a total of
140 PCR reactions completed). Once BAX samples were identified as Salmonella positive,
they were later cultured with additional specialized media to identify salmonellae for PFGE
and serotyping (table 3). Diluted samples were also plated on specialized media (in addition
to XLT-4) because there may have been overgrowth of Salmonella by Enterobacteriaceae
such as E. coli and Shigella. Data were analyzed by EpiInfo (version 3.5), a public domain
software program created by the CDC’s PulseNet Program (Atlanta GA) at 95% confidence
limits.

### PFGE

Chromosomal DNA was prepared following the PFGE protocol developed by the CDC
PulseNet program (60) with some modifications; agarose plugs were incubated overnight at
37° C using 10 uL of Xba I (5000 U, 10 U/mL, Roche Diagnostics, Mannheim, Germany).
PFGE was carried out with a CHEF III Mapper System (Bio-Rad Laboratories, Hercules,
CA). The running conditions were as follows; initial switch time, 2.2 s, final switch time,
63.8 s, at 6 volts. The induced angle was 120° and run time, 19 h. Fragments were estimated
by comparison with a lambda ladder (Bio-Rad Laboratories, Hercules, CA) as a molecular
marker. The gels were stained with ethidium bromide and visualized under a UV light using
a Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA.). PFGE patterns were analyzed by the
BioNumerics software program, version 2.0 (Applied Maths, Kortrijk, Belgium).

Dendograms were generated using the Dice coefficient for similarity and unweighed pair
groups method with arithmetic mean (UPGMA) clustering.

<table>
<thead>
<tr>
<th>Table 3. Additional media used for identification of Salmonella spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bismuth Sulfite agar (Acumeida, Baltimore, MD.)</td>
</tr>
<tr>
<td>Eosin methylene blue agar (Difco, Becton Dickinson, Sparks, MD)</td>
</tr>
<tr>
<td>Hecot-Enteric agar (Remel, Lenexa, KS.)</td>
</tr>
<tr>
<td>MacConkey agar (Difco, Becton Dickinson, Sparks, MD)</td>
</tr>
<tr>
<td>Salmonella-Shigella agar (Difco, Becton Dickinson, Sparks, MD)</td>
</tr>
<tr>
<td>Tetrathionate Broth (Difco, Becton Dickinson, Sparks, MD)</td>
</tr>
<tr>
<td>Rappaport-Vassiliadis Broth (Difco, Becton Dickinson, Sparks, MD)</td>
</tr>
<tr>
<td>Terrific Broth (Difco, Becton Dickinson, Sparks, MD)</td>
</tr>
</tbody>
</table>
Results

Environmental microflora results

There were a variety of microbiota detected prior to and after the placement of dairy cattle (table 4). The family Enterobacteriaceae represent a large and diverse taxonomic
group (34). Some genera are very uncommon and cause more plant disease than human illness (Pantoea) while others are common and important in human hospital-acquired infections (Proteus). There are genera that rarely cause human disease but can cause illness and death in the immunocompromised (Morganella, Citrobacter and Hafnia). Conversely, Klebsiella is a cause of mastitis in cattle (35) and has been implicated in outbreaks (28). In humans, Klebsiella is a frequent cause of illnesses such as urinary tract infections and respiratory disease; Klebsiella has also been identified in outbreaks at intensive care facilities (34). A recent discovered genus (Cedeca, named in honor of the CDC) is increasingly implicated in human wound infections. All Enterobacteriaceae are characterized by their ubiquitous nature and the ability to form niches where they can grow and proliferate (34).

**Salmonella Serotypes**

All environmental samples were enriched to identify salmonellae using specialized agar media (table 3). Colonies phenotypically identified as Salmonella were sent to the National Veterinary Services Lab (NVSL) for confirmatory serotyping. Twenty were identified as

| Before Introduction of Cattle | Morganella morganii, Enterobacter cloacae |
| A group | Morganella morganii, Enterobacter cloacae |
| B group | Citrobacter freundii, Enterobacter cancerogenus, E. cloacae, Klebsiella oxytica, Stenotropomonas mattophilia |
| After Introduction of Cattle | Acinetobacter iwatii, Escherichia coli |
| C group | E. coli, Pantoea agglomerans, Cedeca lapagei, Proteus mirabilis, Klebsiella pneumoniae |
| D group | Citrobacter amalonaticus, E. cancerogenus, |
| E group | Pseudomonas putida, C. freundii, E. coli, Hafnia alvei |
| F group | C. freundii, E. coli, Hafnia alvei |
Salmonella spp. (table 5); of those, seventeen were serotyped as S. london and remaining three were identified as S. montevideo.

Table 5 Serotyping results (group and farm location)

<table>
<thead>
<tr>
<th>Salmonella london (n=17)</th>
<th>Salmonella montevideo (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D11 (FS11), D13 (FW), E10 (SS), E11 (FS11), E12 (FS12), E13 (FW), E14 (HW), F6 (MC6), F10 (SS), F11 (FS11), F12 (FS12), F13 (FW), F14 (HW), F15 (HM), F16 (SH), G10 (SS), G13 (FW)</td>
<td>D10 (SS), D14 (HW), D15 (HM)</td>
</tr>
</tbody>
</table>

All salmonellae were found after dairy cattle placement. S. montevideo has been identified as one of the most common Salmonella isolates found on dairy farms (76, 79) and has been implicated in outbreaks caused by cheese made from raw milk (17) and bean sprouts (78). Outbreaks have also take place among sheep and wild birds (59). In a study of antibiotic susceptibility of Salmonella and U.S. dairy farms by the USDA Animal and Plant Health Inspection Service (APHIS), S. montevideo isolates were sensitive to streptomycin and ampicillin (34% and 9%, respectively), but resistant to tetracycline (76). However, little is known about the rare (but emerging) serotype S. london. Most human cases have been reported in Korea and are typically related to the consumption of food products including powdered milk and other dairy products (37, 53). In early reports regarding the antimicrobial susceptibility of S. london, all isolates tested were sensitive to drugs such as chloramphenicol (63). However, S. london is beginning to spread world-wide and has been implicated in products other than dairy-related items (7, 62). Pet chews, pig meat, meat products, and environmental wastewater have been found to harbor S. london (7, 83, 84). In addition, there is much variation in the antibiotic resistance of S. london isolates. In a 2006 study of wastewater in Spain, S. london isolates were sensitive to all antibiotics tested (including ampicillin, nalidixic acid and tetracycline) (25). In a New Zealand study, Wong et al. (2009) found that three isolates of S. london discovered on pet treats were resistant to ampicillin, but sensitive to a panel of antibiotics (including nalidixic acid and tetracycline) (84). In a 2009 U.S. study on the prevalence of salmonellae in ground meats, S. london was found to be sensitive to tetracycline but resistant to antibiotics such as ampicillin and nalidixic acid (7).
In this study, the numbers of *Salmonella* colonies identified on $10^{-5}$ XLT-4 plates (plated directly from the farm environment to XLT-4 agar) were extremely low. Of all the XLT-4 plates inoculated with environmental diluents (20 $10^{-5}$ plates per sampling date, 7 sampling dates, for a total of 140 plates), only three XLT-4 plates were found depicting colonies phenotypically consistent with *Salmonella*; F10 (SS; solids separator), F13 (FW; free stall walkway), and F16 (SH; special needs/hospital barn) (tables 2 and 5). All phenotypically-appearing *Salmonella* colonies were identified in the F group (after dairy cattle were introduced). Black colonies were marked on the XLT-4 plates while replica plating and antibiotic resistance profiles were determined from those colonies (table 6). The isolates studied were resistant to ampicillin and tetracycline, but susceptible to nalidixic acid.

Table 6. Specific antibiotic resistance of XLT-4 identified salmonellae from $10^{-5}$ dilutions*

<table>
<thead>
<tr>
<th>*Farm Location</th>
<th>XLT (5) (black)</th>
<th>$\log_{10}$ total numbers</th>
<th>Amp 48</th>
<th>Nal 48</th>
<th>Tet 24</th>
<th>Per Amp 48</th>
<th>Per Nal 48</th>
<th>Per Tet 24</th>
<th>ARP</th>
</tr>
</thead>
<tbody>
<tr>
<td>F10 (SS)</td>
<td>3</td>
<td>0.48</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>33</td>
<td>0</td>
<td>33</td>
<td>AT</td>
</tr>
<tr>
<td>F13 (FW)</td>
<td>6</td>
<td>0.78</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>17</td>
<td>0</td>
<td>100</td>
<td>AT</td>
</tr>
<tr>
<td>F16 (SH)</td>
<td>7</td>
<td>0.85</td>
<td>3</td>
<td>0</td>
<td>9</td>
<td>43</td>
<td>0</td>
<td>129</td>
<td>AT</td>
</tr>
</tbody>
</table>

* Number XLT (5) (number of colonies growing on XLT-4 $10^{-5}$ plates), Amp=ampicillin, Nal=nalidixic acid, Tet=tetracycline, Per= percent, ARP= antibiotic resistance profile.

Most colonies that grew on XLT-4 plates were phenotypically consistent with *Enterobacteriaceae* such as *Escherichia coli* (production of yellow colonies). Other Gram-negative bacteria that will grow on XLT-4 plates includes *Citrobacter*, *Proteus*, and *Enterobacter* (14). When XLT-4 plates were replica plated on TSA agar containing a predetermined amount of antibiotic, the numbers, percentages and antibiotic resistance profile of each location was recorded (table 5). When analyzing isolates using the CDC’s EpiInfo program, there were a total of 2061 colonies that grew on XLT-4 plates during the course of this experiment. Most colonies (667) grew in the E group (sampling date 12 March 2008); the fewest number of colonies (9) were detected during the first sampling period (A, 30 November 2007). Of those, 667 were resistant to at least one antibiotic (32% of colonies). When reviewing antibiotics and the numbers of colonies resistant during all sampling periods, there were 791 isolates (38%) resistant to ampicillin, 27 isolates (or 1.3%) resistant
to nalidixic acid, and 170 (8%) resistant to tetracycline. Additionally, there was a more detailed review of antibiotic resistance by groups (A-G) and location (1-20) by both the numbers of colonies and resistance (table 7). BAX PCR™ results (indicative of *Salmonella* spp.) are also listed by group and location. Prior to the introduction of cattle, ampicillin resistance was detected in locations A5 and A9 (heifer barn, 16-22 months and the dry cow/transition barn, respectively). Antibiotic resistance was not detected in sampling period B (13 November 2007). After the animals were introduced (sampling periods C-H), there was an increase in the number of isolates resistant to one antibiotic (ampicillin or tetracycline) as well as MDR. Interestingly, resistance is spread throughout the dairy farm and not limited to certain expected locations (such as the free stall barn and special needs/hospital barn). Resistance to ampicillin only (n=9) occurs in many of the sampling periods—with the exceptions of B and F (27 January 2008 and 23 April, 2008). Both tetracycline and nalidixic acid/tetracycline resistance were detected after the placement of cattle (table 7). A combination of ampicillin and nalidixic acid resistance (n=4) were found in locations D and F; in D3, the heifer barn (H9); in F1, the free stall area (FSA); in F5, the heifer barn-16-22 months (H16); and in F17, the main parking lot entrance (PM). No ampicillin/tetracycline resistance was detected before the introduction of cows, however, after introduction, this antibiotic resistance profile (n=13) was detected in the remaining sampling periods (C-G) all being indoor locations such as the maternity and calf barns (D8 or MC8); free stall barns (E11 or FS11) and special needs/hospital barns (SH). Likewise, the ampicillin/nalidixic/tetracycline profile were identified after the

<table>
<thead>
<tr>
<th>Farm Location</th>
<th>Number XLT4</th>
<th>Amp 48</th>
<th>Nal 48</th>
<th>Tet 24</th>
<th>Per Amp 48</th>
<th>Per Nal 48</th>
<th>Per Tet 24</th>
<th>ABP</th>
<th>BAX PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 5 (H16)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>(-)</td>
</tr>
<tr>
<td>A 9 (DC)</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>(-)</td>
</tr>
<tr>
<td>A 12 (FS12)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>(+)</td>
</tr>
<tr>
<td>C 3 (H9)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>(-)</td>
</tr>
<tr>
<td>C 14 (HW)</td>
<td>21</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>38</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>(+)</td>
</tr>
</tbody>
</table>

Table 7. XLT-4 Colony Growth, Antibiotic Resistance and Pattern, BAX *
Table 7. (continued)

<table>
<thead>
<tr>
<th>Farm Location</th>
<th>NumberXLT</th>
<th>Amp 48</th>
<th>Nal 48</th>
<th>Tet 24</th>
<th>Per Amp 48</th>
<th>Per Nal 48</th>
<th>Per Tet 24</th>
<th>ABP</th>
<th>BAX PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>D 12 (FS12)</td>
<td>110</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>73</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>(+)</td>
</tr>
<tr>
<td>E 8 (MC8)</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>(+)</td>
</tr>
<tr>
<td>G 17 (PM)</td>
<td>35</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>(+)</td>
</tr>
<tr>
<td>G 19 (ES)</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>(+)</td>
</tr>
<tr>
<td>C 9 (DC)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>T</td>
<td>(-)</td>
</tr>
<tr>
<td>G 16 (SH)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>T</td>
<td>(-)</td>
</tr>
<tr>
<td>D 11 (FS11)</td>
<td>14</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>7</td>
<td>57</td>
<td>NT</td>
<td>(+)</td>
</tr>
<tr>
<td>D 3 (H9)</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>133</td>
<td>33</td>
<td>0</td>
<td>AN</td>
<td>(-)</td>
</tr>
<tr>
<td>F 1 (FSA)</td>
<td>22</td>
<td>25</td>
<td>2</td>
<td>0</td>
<td>113</td>
<td>9</td>
<td>0</td>
<td>AN</td>
<td>(-)</td>
</tr>
<tr>
<td>F 5 (H16)</td>
<td>13</td>
<td>18</td>
<td>3</td>
<td>0</td>
<td>138</td>
<td>23</td>
<td>0</td>
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<td>(-)</td>
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<tr>
<td>F 17 (PM)</td>
<td>5</td>
<td>7</td>
<td>1</td>
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<td>140</td>
<td>20</td>
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<td>C 16 (SH)</td>
<td>10</td>
<td>13</td>
<td>0</td>
<td>9</td>
<td>130</td>
<td>0</td>
<td>90</td>
<td>AT</td>
<td>(+)</td>
</tr>
<tr>
<td>D 8 (MC8)</td>
<td>155</td>
<td>96</td>
<td>0</td>
<td>57</td>
<td>62</td>
<td>0</td>
<td>37</td>
<td>AT</td>
<td>(-)</td>
</tr>
<tr>
<td>D 13 (FW)</td>
<td>110</td>
<td>13</td>
<td>0</td>
<td>49</td>
<td>12</td>
<td>0</td>
<td>45</td>
<td>AT</td>
<td>(+)</td>
</tr>
<tr>
<td>D 15 (HM)</td>
<td>81</td>
<td>12</td>
<td>0</td>
<td>44</td>
<td>15</td>
<td>0</td>
<td>54</td>
<td>AT</td>
<td>(+)</td>
</tr>
<tr>
<td>E 5 (H16)</td>
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<td>0</td>
<td>4</td>
<td>29</td>
<td>0</td>
<td>57</td>
<td>AT</td>
<td>(-)</td>
</tr>
<tr>
<td>E 11 (FS11)</td>
<td>33</td>
<td>5</td>
<td>0</td>
<td>21</td>
<td>15</td>
<td>0</td>
<td>64</td>
<td>AT</td>
<td>(+)</td>
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<tr>
<td>E 16 (SH)</td>
<td>248</td>
<td>7</td>
<td>0</td>
<td>72</td>
<td>3</td>
<td>0</td>
<td>29</td>
<td>AT</td>
<td>(+)</td>
</tr>
<tr>
<td>F 6 (MC6)</td>
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<td>0</td>
<td>1</td>
<td>100</td>
<td>0</td>
<td>50</td>
<td>AT</td>
<td>(-)</td>
</tr>
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<td>F 7 (MC7)</td>
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<td>40</td>
<td>0</td>
<td>52</td>
<td>75</td>
<td>0</td>
<td>98</td>
<td>AT</td>
<td>(-)</td>
</tr>
<tr>
<td>G 6 (MC 6)</td>
<td>4</td>
<td>9</td>
<td>0</td>
<td>7</td>
<td>225</td>
<td>0</td>
<td>175</td>
<td>AT</td>
<td>(+)</td>
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<tr>
<td>G11 (FS11)</td>
<td>60</td>
<td>6</td>
<td>0</td>
<td>9</td>
<td>10</td>
<td>0</td>
<td>15</td>
<td>AT</td>
<td>(+)</td>
</tr>
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<td>G 12 (FS12)</td>
<td>35</td>
<td>3</td>
<td>0</td>
<td>27</td>
<td>9</td>
<td>0</td>
<td>77</td>
<td>AT</td>
<td>(+)</td>
</tr>
<tr>
<td>G 16 (SH)</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>33</td>
<td>0</td>
<td>33</td>
<td>AT</td>
<td>(-)</td>
</tr>
<tr>
<td>C 8 (MC8)</td>
<td>20</td>
<td>17</td>
<td>1</td>
<td>25</td>
<td>85</td>
<td>5</td>
<td>125</td>
<td>ANT</td>
<td>(-)</td>
</tr>
<tr>
<td>D 6 (MC6)</td>
<td>49</td>
<td>39</td>
<td>1</td>
<td>41</td>
<td>80</td>
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<td>84</td>
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<td>(-)</td>
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<td>6</td>
<td>225</td>
<td>175</td>
<td>150</td>
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<td>(-)</td>
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<tr>
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<td>6</td>
<td>67</td>
<td>4</td>
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<td>152</td>
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<td>(+)</td>
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<td>49</td>
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<td>71</td>
<td>29</td>
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<td>(-)</td>
</tr>
<tr>
<td>F 16 (SH)</td>
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<td>2</td>
<td>15</td>
<td>44</td>
<td>22</td>
<td>167</td>
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<td>(-)</td>
</tr>
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<td>G 2 (OF)</td>
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<td>39</td>
<td>11</td>
<td>7</td>
<td>144</td>
<td>41</td>
<td>26</td>
<td>ANT</td>
<td>(-)</td>
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<tr>
<td>G 4 (H2)</td>
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<td>88</td>
<td>1</td>
<td>48</td>
<td>34</td>
<td>1</td>
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<td>G 5 (H16)</td>
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<td>27</td>
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<td>3</td>
<td>85</td>
<td>3</td>
<td>9</td>
<td>ANT</td>
<td>(-)</td>
</tr>
<tr>
<td>G 8 (MC8)</td>
<td>64</td>
<td>77</td>
<td>13</td>
<td>9</td>
<td>120</td>
<td>20</td>
<td>14</td>
<td>ANT</td>
<td>(-)</td>
</tr>
<tr>
<td>G 9 (DC)</td>
<td>12</td>
<td>10</td>
<td>1</td>
<td>3</td>
<td>83</td>
<td>8</td>
<td>25</td>
<td>ANT</td>
<td>(-)</td>
</tr>
</tbody>
</table>

Amp=ampicillin, Nal=nalidixic acid, Tet=tetracycline, Per= percent, ARP= antibiotic resistance profile.
introduction of cattle and is spread throughout the farm environment—one (G2) being detected in an open field (OF). Overall, the percentages of nalidixic acid (only) resistant colonies were small (when compared to ampicillin and tetracycline). Also, it was noted that the numbers of XLT-4 colonies (and antibiotic-resistant colonies) would change as sampling progressed. Often the numbers would drop, then percentages would rise—and later decrease. When examining tetracycline resistance only, the few colonies that grew exhibited high antibiotic resistance (50 and 100%, respectively). There were similar results in both ampicillin/nalidixic acid-resistant colonies (increase then decrease) as with the ampicillin/nalidixic/tetracycline-resistant colonies. Overall, there were no steady increases (as would be expected) as Enterobacteriaceae became more established.

When examining the BAX PCR™ results, there was a relationship between the number of detected “Salmonella positive” isolates (n=42) and the dairy farm location;

Figure 1: ISU Dairy Farm locations and BAX PCR results

Most of the BAX positive results were in locations 12, the free stall barn (FS n=6); locations 11 and 13, the free stall barn, and the free stall barn walkway (FS11 and FW n=5); and locations 10, 14 and 15, the solids separator, holding area walkway, and the holding area milking (SS, HW and HM respectively, n=4). The special needs/hospital barn (SH16) reported only 2 BAX Salmonella-positive results. Location 2, open field north east (OF),
location 5, heifer barn 16-22 months (H16) and location 9, the dry cow/transition barn (DC) reported negative BAX PCR results during the study.

**PFGE Results**

When reviewing the PFGE macrorestriction patterns of *S. montevideo* using the criteria of Tenover et al. (70), there was a one-fragment difference between cluster 1 (F10, F15) and cluster 2 (F14) which is consistent the gain of a DNA restriction site. When examining the PFGE macrorestriction patterns of *S. london*, again using the criteria of Tenover, et al. (also see figure 2, lanes 6-9 and 11-13), the patterns appear to be identical, indicating that isolates are indistinguishable from one another (69). When examining Bionumerics-generated dendograms of the PFGE patterns of *S. montevideo* (partial data, figure 2 lanes 3 and 4), there were two clusters. The members of cluster 1 included D10, the solids separator (SS) and D15, the holding/milking area (HM); the only member of cluster 2 was D14 the holding area walkway (HW). The PFGE profiles of *S. london* (figure 3) resulted in two distinct clusters; cluster 1 (F16, G10, G13, F12, F14, F15, F13, F11 and F10) and cluster 2 (D11, E11, E10, D13, E13, E12, F6 AND E14) both with at least 85% similarity (also see figure 3 for farm locations). All *Salmonella* isolates were discovered after cattle were introduced on the dairy farm which suggests that the source of the isolates were likely cattle (43, 68) although there can be other

Figure 2. Example of a PFGE gel with *S. montevideo* and *S. london* isolates (lanes on next page)
sources of the organism (see discussion section). In addition, in PFGE analysis, a 1 or 2 band difference may not indicate a difference in strains. Researchers have suggested that isolates with such minor profile differences are clonally related (defined as isolates recovered from different sources, locations, and at different times demonstrating many identical phenotypic and genotypic traits) (22, 72). When examining the information within both clusters, most of cluster 1 isolates (7/9 or 78%) were collected on 23 April 2008 within the large complex that housed adult animals (free stall barn, special needs/hospital barn, holding areas). The majority of cluster 2 isolates (5/8 or 63%) were collected on 12 March 2008, again within the complex that housed adult animals.

Figure 3. Dendrogram of relatedness of DNA profiles of PFGE strains (S. london) in study

Medications and Sanitizers:
Medications were used to treat conditions such as mastitis, bovine respiratory disease and wound infections (tables 8 and 9). The medications were prescribed by an ISU faculty veterinarian who disclosed uses and dosages given. Sick cattle were treated individually. In addition, a detailed protocol for treatment was strictly followed by the ISU Dairy Farm staff.
BAC-STOP udder predip (Esteam Manufacturing Ltd., Calgary, Alberta) and Transcend udder postdip (IBA, Millbury, MA.) were used as sanitizers prior to and after milking. FC-98 Udder Wash (IBA, Millbury, MA.) was also used a boot sanitizer.

**Discussion:**

When evaluating *Enterobacteriaceae* isolated before and after dairy cattle placement, there was a change from the detection of organisms that rarely cause disease in humans and animals (*Morganella* and *Citrobacter freundii*) (40, 49) to organisms frequently causing illness (*E. coli* and *Klebsiella*) (38, 67). It is likely that pathogenic organisms (including salmonellae) were introduced by cattle (43, 61, 68, 75). There is also the possibility that pathogenic bacterial organisms could have been introduced by other

<table>
<thead>
<tr>
<th>Antibiotic(s)</th>
<th>Class</th>
<th>Treatment(s)</th>
<th>Mode of action (1, 8, 44, 80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquamycin</td>
<td>Tetracycline</td>
<td>Pneumonia (shipping fever), scours, wound infections</td>
<td>Inhibits protein synthesis</td>
</tr>
</tbody>
</table>

**Table 8:** List of antibiotics used on the ISU Dairy Farm (name, class, and mode of action)

<table>
<thead>
<tr>
<th>Antibiotic(s)</th>
<th>Class</th>
<th>Treatment(s)</th>
<th>Mode of action (1, 8, 44, 80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Draxxin, Tylan</td>
<td>Marcolide</td>
<td>Draxxin-Bovine Respiratory Disease, Tylan-shipping fever, pneumonia, foot rot, calf diphtheria</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Nuflor</td>
<td>Chloramphenicol</td>
<td>Bovine Respiratory Disease</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Pirsue</td>
<td>Lincomamide</td>
<td>Clinical and subclinical mastitis</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Excenel, Spectrumast LC, Spectrumast DC, Naxcel</td>
<td>Cephalosporin</td>
<td>Clinical and subclinical mastitis</td>
<td>Inhibits cell wall synthesis</td>
</tr>
<tr>
<td>Polyflex</td>
<td>Penicillin</td>
<td>Broad spectrum antibiotic, shipping fever, pneumonia</td>
<td>Inhibits cell wall synthesis</td>
</tr>
</tbody>
</table>
Table 9: Additional medications used on the ISU Dairy Farm

<table>
<thead>
<tr>
<th>Medication</th>
<th>Purpose (1, 8, 44, 80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banamine</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
</tr>
<tr>
<td>Sterile Saline, Hypertonic Saline Solution</td>
<td>Intravenous therapy</td>
</tr>
</tbody>
</table>

animals and insects attracted to the facility after dairy cattle placement (competing with cattle for shelter and food, for example) (18, 50) or by materials brought into the dairy farm such as hay, bedding and mattresses (47).

BAX PCR™ was much more sensitive at detecting salmonellae in environmental samples (table 7 and figure 1) than sampling with XLT-4 (table 6) or using specialized media to recover *Salmonella* in isolates (table 3). In a Swedish study (24) comparing BAX PCR™ to other methods of detection such as modified semisolid Rappaport-Vassiliadis agar (MSRV) and the selective Enzyme-Linked Immunoabsorbent Assay (ELISA), MSRV performed slightly better than BAX (93% accuracy and 88% sensitive for BAX versus 99% accuracy and 98% sensitivity for MSRV). The selective ELISA was only 78% accurate and 63% sensitive. Swedish researchers used fecal samples “spiked” with *Salmonella*, artificially contaminated poultry swine, and other fecal samples. Other studies have also attested to the high accuracy and sensitivity of the BAX method (5, 73, 74).

The percentage of antibiotic resistance of *Enterobacteriaceae* (table 7) did not depend on factors such as temperature and humidity also referred to “seasonality” in some literature (30); however, this study was done over a 9-month period. Additionally, the lack of seasonality has been supported by studies in the US (54) and internationally (64).

Conversely, there are studies that depicted the seasonality of *Salmonella* (20, 30). In the ISU Dairy Farm study, antibiotic-resistant colonies were discovered throughout the sampling periods. However, MDR colonies appeared after cattle were introduced (e.g. ampicillin/nalidixic acid resistance first appeared at the dry cow/transition barn (D9); ampicillin/tetracycline resistance initially appeared at the special needs/hospital barn (C16); and ampicillin/nalidixic acid/tetracycline resistance initially appeared in the maternity/calf barn (C8). Of note, the percentages of antibiotic resistance varied during sampling periods as
in other studies (54). There were also intermittent levels of *Salmonella* (repeated decreases and increases in bacterial levels) during the ISU Dairy farm study, again similar to results in other studies (52).

Although animals can be infected clinically or subclinically, *Salmonella* often persists in the subclinical form, making detection of infected animals difficult (52). Carrier animals may also play an important role in the spread of infections in herds and consequently serve as sources of food contamination and human infection (86). Emergence of MDR *Salmonella* reduces therapeutic options in cases of infection in humans and animals. MDR resistance is an increasingly important issue world-wide; examples include studies in the US (2) China (15) and Brazil (26).

**Conclusion:**

From this experiments (and others cited in this article), there is value in continued monitoring of changes in bacteria in farming environments-particularly changes in antimicrobial susceptibility and resistance. To our knowledge, this is the first study that evaluated the change of *Enterobacteriaceae* (and specifically *Salmonella*) before and after the placement of production animals (dairy cattle). This work has demonstrated that organisms of medical importance such as *Salmonella* have the ability to quickly establish and proliferate on dairy facilities. It would be interesting to continue longitudinal studies to survey ongoing changes in environmental microbiota. In addition, additional antibiotics could be used to obtain more extensive antibiotic-resistance profiles. However, more extensive replica plating experiments and costs would likely be prohibitive.

Surveillance programs are helpful in documenting (and preventing) the spread of pathogenic bacteria. However, there is concern that passive laboratory-based surveillance is likely to underreport the true incidence of diseases such as salmonellosis in livestock (3, 86). Surveillance activities can be combined with additional approaches to decrease the effects of antibiotic resistance as reported by researchers such as McAllister et al. (2001) and Oliver et al. (2009). They include 1) not using antibiotics to compensate for poor nutrition, poor hygiene, or the lack of immunization; 2) using antibiotics in consultation with a veterinarian; 3) minimizing as much as possible the use of antibiotics considered important for treating human diseases; 4) using probiotics to compete with potentially pathogenic bacteria (a
Probiotic is defined as a feed product containing viable, defined organisms which alters the microbiota in a host and exerts beneficial health effects (45, 50). Additionally research such as those reviewing the association between management type (organic versus conventional farms) is now taking place (3). The US has the safest food supply in the world, however foodborne pathogens can be a significant threat to human health (9). Therefore it is important to study the epidemiology of antibiotic resistance within both animal and human populations.

Acknowledgements: The authors thank Joe Detrick, Kay Christiansen, Bruce Leuschen, DVM, and Orhan Sahin, Ph.D. for their assistance.

References


Appendix: The Iowa State University’s Dairy/Animal Science Education and Discovery Facility

1) Feed Storage Area (FSA)
2) Open Field NE (OF)
3) Heifer Barn 9-15 months (H9)
4) Heifer Barn 2-8 months (H2)
5) Heifer Barn 16-22 months (H16)
6) Maternity/Calf Barn (MC 6)
7) Maternity/Calf Barn (MC 7)
8) Maternity/Calf Barn (MC 8)
9) Dry Cow/Transition Barn (DC)
10) Solids Separator (SS)
11) Free Stall Barn (FS11)
12) Free Stall Barn (FS12)
13) Free Stall Barn Walkway (FW)
14) Holding Area Walkway (HW)
15) Holding Area/Milking (HM)
16) Special Needs/Hospital Barn (SH)
17) Parking Main Entrance (PM)
18) Parking/Employee/Showers (PS)
19) Equipment Storage (ES)
20) Machine Storage Area (MS)
General Discussion and Recommendations for Future Research

For most of the twentieth century, the predominant sentiment about the treatment, control, and prevention of infectious disease was optimism, both in human and animal health (4). For instance, in 1969, (then) Surgeon General William Stewart told the United States Congress that “it was time to close the book on infectious disease” (4). To be fair, there were many significant advances in disease control and prevention, including the development of antibiotics (termed “wonder drugs”) that increased human life expectancy—from 47 years in 1900 to 68 years in 1950 (22). Public health measures such as protection of food and water supplies, improved nutrition, and personal hygiene also increased lifespan (22). Animal health also benefited from scientific advances. LeBlanc et al. (2006) noted that in the 1940s, antibiotics revolutionized the treatment of common animal diseases, and in the 1960s there was a shift from reactive medicine (treating illness) to proactive medicine (disease prevention) (21). However, the over-optimism and complacency of the 1960s was tempered with the reality of recently discovered disease (such as AIDS) and zoonotic diseases (22). For example, *Escherichia coli* O157:H7 (first identified as a pathogen in 1982) causes severe diarrhea and hemolytic uremic syndrome in humans; the same organism is a cause of mastitis in dairy cattle (21, 25). *Campylobacter jejuni* (discovered in the 1970s), can result in severe diarrheal illness in humans and abortion in cattle (13, 29).

Antibiotics have become indispensable tools for decreasing morbidity and mortality associated with infectious diseases in humans and animals (24). Yet in the past 10-15 years, there has been rapid acceleration in the emergence of MDR pathogens including those of importance in food microbiology (28, 34). At the same time, there are also more individuals susceptible to disease (4). Aging, increases in underlying (typically immunosuppressive) diseases, and technological advances in health care are all factors (4). Additionally, there is currently little consensus on proper antibiotic use in a (including in a farm environment). There are those who believe that antibiotics are a vital part of the livestock industry (16) and antibiotics are important in treating diseases, increasing weight gain, and improving feed efficiency (14). Supporters concede that commercial livestock production in the United States, especially confinement production would be virtually impossible without antibiotic
drugs (23). The American Veterinary Medical Association (AVMA) opposes US Congressional legislation amending the Federal Food, Drug, and Cosmetic Act to eliminate the non-therapeutic use of antibiotic drugs considered important for human health (30). Non-therapeutic use refers to “…use of a drug as a feed or water additive (in the absence of any clinical disease) for growth promotion, feed efficiency, weight gain, routine disease prevention or other purposes” (30). The AVMA has also cited their cooperation with the Centers for Disease Control and Prevention (CDC), the Food and Drug Administration (FDA) and others agencies to promote “prudent and careful therapeutic use of antimicrobials (24, 30).

Conversely, there are researchers that believe excess amounts of antibiotics are used; not only in human medicine and animal agriculture-but in numerous preparations (including cleaning products and over-the-counter preparations) (34). While the evidence is considered “complex” (Dryden et al. 2009), it is generally accepted that increased antibiotic use is associated with greater bacterial resistance (26). The American Society for Microbiology (ASM) Task Force on Antibiotic Resistance believes there is an urgent need for more prudent use of antibiotics in both human and veterinary medicine, especially as it relates to food production (24). Groups such as the World Health Organizations (WHO), the Institute of Medicine (IOM) of the National Academies and the Alliance of Prudent Use of Antibiotics (AUPA) have petitioned to discontinue the use of antimicrobial agents in food animals for growth promotion that are also used in human medicine (2). Several European countries (and the European Union) have taken steps toward banning certain antibacterial agents as growth promoters in farm animals (2). They cite studies in which the discontinuation of antimicrobial agents as growth promoters have resulted in a decrease in antibiotic resistance in animals, food products and humans (2).

There is also a growing consensus that alternative research is necessary in areas such as livestock production and veterinary/human medicine regarding antibiotic use. Possibilities include creating alternatives to antimicrobial growth promoters (16), developing new antibiotics (11), improving disease control on farms (using methods such as epidemiology, nutrition, and improved design of animal housing) (21), improving human and veterinary diagnostic laboratories (24) and increasing the number of studies to decrease the
“information gaps” that exist in diverse areas such as microbial ecology, bacterial pathogenesis, and food safety (5, 21, 24, 27).

There is very little research regarding the (longitudinal) establishment and proliferation of bacterial organisms on a new dairy farm environment. Most studies focus on bacterial contamination of foods including ground beef and unpasteurized milk (12), eggs (9), fresh produce (1) and meat products (17). The studies on environmental contamination tend to focus on areas such as the survival of zoonotic pathogens in soil, water and animal excrement (manure) (3). When assessing the research that took place on the ISU Dairy farm there was one important conclusion that applied to all collected data. Temperature and humidity did not affect the proliferation of bacterial organisms (also referred to “seasonality” in some literature) (15). The length of time that dairy cattle resided on the farm was more of a factor (even though there were circumstances when the number of CFUs of bacteria would repeatedly increase and decrease; one example of this can be found in the Enterococcus 10⁻³ and 10⁻⁵ data, tables 4-9).

When reviewing the Enterococcus data, it was noted that antibiotic resistance colonies grew on TSA plates with very high levels of antibiotics (48 ug/mL of ampicillin, 48 ug/mL erythromycin, and 24 ug/mL tetracycline). The antibiotic concentration was much higher than recommended by the Clinical and Laboratory Standards Institute (CLSI). This is significant because of the increasing difficulty of treating enterococcal infections due to multidrug resistance (10, 20) Additionally, vancomycin (considered the “last line of defense” in the treatment of Enterococcus) is becoming less effective, resulting in increased mortality and morbidity of human patients (18). In terms of establishment and proliferation of enterococci, it became evident that the placement of dairy cattle directly correlated with establishment and proliferation. In tables 4-9 of the Enterococcus data, low levels of CFUs (or no CFUs) were recovered prior to dairy cow introduction. After dairy cow introduction, the numbers of CFUs dramatically increased-except for ampicillin (table 7). Again, it is notable the number of CFUs were cyclic in nature.

Since there were substantial amounts of data collected while studying Campylobacter, four locations were reviewed in detail; the maternity and calf barn, solids separator, free stall barn and the special needs/hospital barn. Antibiotic resistance was not detected prior to dairy
cattle introduction. After placement of dairy cattle, there were increases in the numbers of CFUs as well as resistance to a single drug/multidrug resistance (tables 3, 6 and 7 of *Campylobacter* data). The Tryptic Soy Agar (TSA) plates were made with higher concentrations of antibiotic recommended by CLSI. Additionally, with the progression of time, multidrug resistance was detected throughout the farm environment. Location was not a significant factor; multidrug resistance was eventually detected throughout the farm (*Campylobacter* data, tables 6 and 7). There was some fluctuation in the presence of antibiotic resistance—which also occurred in other *Campylobacter* studies (7, 8).

When evaluating data from the *Entrobacteriaceae* experiments (table 4 of *Enterobacteriaceae/Salmonella* data), it appears likely that the introduction of dairy cattle was directly related to changes in the genera and species of Gram-negative bacteria present. The types of recovered organisms changed from those that rarely cause zoonotic disease to those causing disease-some which are of concern due to increasing antibiotic resistance. Recovery of *Salmonella* on XLT-4 media was minimal (plating of environmental samples directly from serial dilutions to XLT-4 media; table 6 *Enterobacteriaceae/Salmonella* data). Typically there are enrichment steps that are followed when detecting *Salmonella* on specialized media; for that reason, it may take 4-7 days to recover organisms (32). The BAX PCR System™ is more sensitive than conventional plating method. In table 6 (*Enterobacteriaceae/Salmonella* data), there were only 3 locations that were *Salmonella* positive using XLT-4 (with no enrichment); F10 (the solids separator); F13 (the free stall walkway); and F16, (the special needs/hospital barn). However, BAX PCR detected 43 environmental locations that were *Salmonella*-positive (figure 1 *Enterobacteriaceae/Salmonella* data). Additional *Salmonella*-positive data (table 5) were derived by enrichment on specialized media (listed on table 3 of *Enterobacteriaceae/Salmonella* data). All *Salmonella*-positive plating results were sent to the NVSL for confirmatory serotyping.

Two serotypes of *Salmonella* were detected during the study; *S. montevideo* (3 isolates) and *S. london* (17 isolates). The presence of *S. london* was initially detected in location D11 (the free stall barn) after placement of dairy cattle and continued to be recovered until the conclusion of the study (table 5 *Enterobacteriaceae/Salmonella* data). Until recently,
presence of *S. london* was restricted to far eastern countries such as Korea but is now beginning to appear worldwide (33, 36, 37). Researchers have advocated for additional work with *S. london* due to varying (but increasing) resistance to antimicrobials (36), the severe illness that can be caused by this serotype (19) and its uncertain etiology (the study of causation or origination). The PFGE patterns of *S. London* from this study were clustered into two distinct groups. Most of the isolates from the first group were collected on 23 April 2008 (G) and the majority of the isolates from the second group were collected on 12 March 2008 (E). Despite the two groups, all of the isolates may be clonal in origin. Even though the BioNumerics program divided the isolates into two groups, some suggest that isolates with such minor profile differences (figure 3 *Enterobacteriaceae/Salmonella* data) are actually clonally related (of common origin) (6, 35). As mentioned, the etiology is uncertain although it is probable that the source of the isolates on the ISU Dairy Farm were cattle-given the (limited) evidence where milk products have been implicated in past *S. london* infections (19, 31). It is uncertain if the isolates came from one animal or from multiple animals.

There is very little research regarding the establishment and proliferation of bacterial organism in a new dairy farm environment. Future research could include continued studies at the ISU Dairy Farm regarding the establishment of bacteria of interest in food safety (e.g. *Salmonella, Campylobacter* and *Enterococcus*) and antibiotic resistance. Baseline information (collected from this study) is now known. Additional antibiotics could be added (incorporated into TSA media)-especially antibiotics important in human and animal health (such as vancomycin in humans and Excenel/cephalosporin in cattle). Other tests involving antibiotic resistance-such as agar disc diffusion and broth microdilution-can ease the preparation time and expense of future studies. Individual dairy cows could also be followed to ascertain what types of bacteria are being shed in the farm environment. Certain categories of animals could be studied at length such as sick animals, animals that may be stressed (from calving), and new animals. Molecular studies should continue with *S. london*, since it is apparent that it may be an emerging serotype (36). It is hoped that studies such as this could give additional insight into topics involving dairy cattle production and food safety.
References


The Iowa State University’s Dairy/Animal Science Education and Discovery Facility

1) Feed Storage Area (FSA)  
2) Open Field NE (OF)  
3) Heifer Barn 9-15 months (H9)  
4) Heifer Barn 2-8 months (H2)  
5) Heifer Barn 16-22 months (H16)  
6) Maternity/Calf Barn (MC 6)  
7) Maternity/Calf Barn (MC 7)  
8) Maternity/Calf Barn (MC 8)  
9) Dry Cow/Transition Barn (DC)  
10) Solids Separator (SS)  
11) Free Stall Barn (FS11)  
12) Free Stall Barn (FS12)  
13) Free Stall Barn Walkway (FW)  
14) Holding Area Walkway (HW)  
15) Holding Area/Milking (HM)  
16) Special Needs/Hospital Barn (SH)  
17) Parking Main Entrance (PM)  
18) Parking/Employee/Shower (PS)  
19) Equipment Storage (ES)  
20) Machine Storage Area (MS)
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