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Occurrence of Antibiotic Resistance in Environmental and Amphibian E. coli Isolates Associated with Cattle and Aquatic Environments

Robin Lynn Cissell
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Robin Lynn Cissell entitled "Occurrence of Antibiotic Resistance in Environmental and Amphibian E. coli Isolates Associated with Cattle and Aquatic Environments." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Alan G. Mathew, Major Professor

We have read this thesis and recommend its acceptance:

John C. New, Jr., Gina M. Pighetti

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Anne Mayhew

Vice Chancellor and
Dean of Graduate Studies

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A Thesis Presented for the Master of Science Degree
The University of Tennessee, Knoxville

Robin Lynn Cissell
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Abstract

The widespread use of antibiotics in human medicine and livestock production has been linked to an increase in resistant bacteria, which may carry transferable resistance factors, including integrons. Foodborne pathogens, such as *Escherichia coli* and salmonella, commonly reside in livestock, including cattle, and these pathogens may acquire resistance genes as a result of routine antibiotic use. As cattle are often located in close proximity to aquatic environments, they may disperse antibiotic resistant pathogens into such environments, which may lead to contamination of aquatic wildlife. We hypothesize that class 1 integrons and/or antibiotic resistant bacteria occur more frequently in environments with cattle exposure, and resistance and class 1 integrons disperse into aquatic environments and wildlife, which in turn provides a reservoir of antibiotic resistant bacteria for cattle within that environment. We investigated the prevalence of resistance genes and class 1 integrons in *E. coli* from selected amphibian species from ponds within and adjacent to cow-calf beef production systems. *Escherichia coli* were isolated from bullfrog (*Rana catesbeiana*) and green frog (*Rana clamitans*) tadpoles, green frog metamorphs, cow manure, and pond water samples within each livestock system in an attempt to determine if transfer of resistant bacteria occurs. Integron prevalence within *E. coli* was determined by multi-plex PCR. Antibiotic resistance to tetracyclines, florfenicol, and sulfisoxazole were determined using standard microdilution broth Minimum Inhibitory Concentration technique. A selected subset of bacteria was analyzed for resistance patterns using the National Antimicrobial Resistance

Monitoring System (N.A.R.M.S.). Class 1 integrons were detected in 3% of isolates (n = 63) from pond water and in 1% of isolates (n = 123) from cow manure. Integrons were not detected in isolates (n = 1014) from tadpoles or metamorphs. Tadpole samples with isolates resistant to tetracycline, florfenicol and sulfisoxazole were more prevalent (P=0.0001, P = 0.006 and P=0.0156 respectively) from cattle-accessible ponds compared to cattle-excluded ponds. The percentage of pond water samples with tetracycline resistant *E. coli* isolates was also greater in cattle-accessible ponds (P = 0.0283) compared to isolates from cattle-excluded ponds. Antimicrobial resistance patterns were observed to differ between treatments. Information from this study will provide key information for the development of strategies to reduce the prevalence and risk of antibiotic resistant organisms.

Key words: Antibiotic resistance, Integron, *E. coli*, Salmonella, Amphibian

Preface

The terms antibiotic and antimicrobial are used interchangeably, and refer to compounds that kill or inhibit the growth of microorganisms. All figures and tables referred to in the text are located in the Appendix.

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I. A Review of Literature

A. Antimicrobial Use and Resistance in Agriculture

Antimicrobial agents have been widely used in livestock and poultry since the 1950's. In the last five decades, food animal production has intensified and infectious disease management has improved (McEwen and Fedorka-Cray, 2002). This improvement is due in part to the introduction of antimicrobials. At least 17 classes of antimicrobials are approved for use in food animals in the United States, including tetracyclines, penicillins, macrolides, lincomycin (analog of clindamycin) and virginiamycin (analog of quinupristin/dalfopristin) (Anderson et al., 2003.) Antimicrobials work in many ways including: inhibition of cell wall and cell membrane synthesis, inhibition of protein synthesis, inhibition of folate synthesis, and inhibition of DNA synthesis (Barton, 2000; Khachatourians, 1998). They may also target specific groups of organisms (e.g. Gram-positive or Gram-negative/ anaerobic or aerobic). Therefore, it is beneficial to know the causative organism before treatment. Others may be used to treat a broad spectrum of organisms when it is not possible or economically feasible to determine the causative agent.

Antimicrobials are used in food animals for four main purposes: therapeutic use to treat sick animals, control to prevent sickness, prophylactic use to prevent infections at times of risk, such as transport or weaning, and growth promotion to improve feed utilization and production (Viola and

DeVincent, 2006). In many cases it is difficult to treat individual animals in a production setting, therefore entire groups of animals may be medicated through feed or water. Also, in the presence of infection in a production setting, there may be a need for short-term mass medications termed “metaphylaxis” to treat diseased animals and prevent infection in additional animals (McEwen and Fedorka-Cray, 2002). Antimicrobials administered for growth production are usually given at “subtherapeutic” (<200g per ton for >2 weeks) levels (McEwen and Fedorka-Cray, 2002). In many cases, this occurs early in production and is discontinued as the animals mature. The total quantity of antimicrobial agents used in animals for each of these purposes and their relative contributions to antimicrobial resistance is not known with certainty. Of all purposes for antimicrobial use in animals, growth promotion has always been the most controversial (Viola and DeVincent, 2006).

1. Antimicrobial Use and Humans

Many of the antimicrobials utilized in food animal production are also used in human medicine, which is a cause of concern to many members of the medical community. This concern has arisen due to the emergence of enteropathogenic zoonotic pathogens resistant to antimicrobials (e.g. Salmonella, Campylobacter, Yersenia, and some strains of *Escherichia coli*, such as serotype 0157:H7) (McEwen and Fedorka-Cray, 2002). In nature, microorganisms have the capability to manufacture antimicrobials to protect themselves from competition. Scientists developed the antimicrobials we use

today based on the discovery of these naturally-occurring antimicrobials. Antimicrobial resistance emerged first in nature as organisms developed ways to survive antimicrobial production. As physicians began using antimicrobials in humans to treat infections, it was noted that some organisms were able to persist and still cause infection. Drug-resistant strains, including sulfonamide-resistant *Streptococcus pyogenes,* initially appeared in military hospitals in the 1930's and penicillin-resistant *Staphylococcus aureas* began to appear in London civilian hospitals in the 1940's (Levy and Marshall, 2004).

2. Reports and Recommendations Addressing Rising Resistance

The use of antimicrobials in food animal production also began to select for drug-resistant strains of organisms. One of the first reports of resistance in food animals was reported in 1951 after experimental feeding of streptomycin in turkeys (Dibner and Richards, 2005). When growth-promoting levels of antibiotics were fed to chickens, an association of resistance to tetracyclines was reported by Barnes in 1958, and Elliot and Barnes in 1959. In 1968 a committee was formed in Great Britain to consider the issue of antimicrobial resistance. The stated objectives of the committee were to:

> "Obtain information about the present and prospective uses of antibiotics in animal husbandry and veterinary medicine, with particular reference to the phenomenon of infective drug resistance, to consider the implications for animal husbandry and also for human and animal health, and to make recommendations" (Swann et al., 1969).

This committee included: Professor M.M. Swann, M.A., Ph.D., F.R.S., F.R.S.E., Dr. K.L. Baxter, Ph.D., D. Sc., F.R.S.E., H. I. Field, M. Sc., M.R.C.V.S., F.C. Path., F.R.S.A., Dr. J. W. Howie, M.D., F.C.R.P., P.C. Path., Professor I.A.M. Lucas, M. Sc., B. Sc., Dr. E.L.M. Millar, M.Sc., M.D. M.B. Ch.B., D.P.H., Professor J.C. Murdoch, B.Sc., Ph.D., Mr. J. H. Parsons, M.R.C.V.S., and Professor E.G. White, D.Sc., Ph.D., B.Sc., F.R.C.V.S. Their conclusions and recommendations concerning these issues were reported in a document commonly referred to as the Swann Report in 1969. They concluded:

- The administration of antibiotics to farm livestock, particularly at sub therapeutic levels, poses certain hazards to human and animal health which can largely be avoided and should not therefore be allowed to continue
- The dramatic increase in the number of strains of enteric bacteria of animal origin which show resistance to one or more antibiotics has resulted from the use of antibiotics for growth promotion and other purposes in farm livestock
- There is ample and incontrovertible evidence to show that man may commonly ingest enteric bacteria of animal origin
- Some enteric organisms, particularly of the salmonella group, are able to cause disease in man and also in some species of farm livestock
- Man is exposed to other risks through the ingestion of resistant enteric bacteria of animal origin, even if these bacteria are unable

to cause disease in humans, as they can transfer resistance genes to other bacteria in the human intestine

- Situations in which the treatment of human illness would be limited due to antibiotic resistance of the disease causing organism is clearly undesirable
- Evidence available does not suggest that antibiotic residues in food of animal origin pose any significant hazard to the consumer
- The usage of penicillin and tetracyclines for growth promotion has been of major importance in the development of antibiotic resistance in the enteric bacteria of the animals treated
- Similar economic benefits to the livestock industry may be secured with antibiotics which have little or no therapeutic application in man or animals (Swann et al., 1969)

The committee recommended that many of the antibiotics in use by livestock producers (e.g. tetracyclines, tylosin, penicillin, sulphonamides, nitrofuran drugs) should be available by prescription only and feed antibiotics should be controlled to only 100ppm and only used in calves up to 3 months of age and in growing pigs and poultry; and the use of antibiotics for the treatment of stress be prohibited. Among the many additional recommendations were the establishment of a surveillance program to determine prevalence of resistant bacteria of animals, animal products, and man, initiation of research into the effectiveness, feasibility and economic consequences of deliberate changes in animal husbandry in light of the current epidemiological knowledge, and in

particular, studies of the infectious diseases common to farm animals (e.g. salmonellosis) (Swann et al., 1969). The publication of this report promoted studies into the use of antimicrobials in food animals and the problem of increasing resistance of enteric bacteria in these animals.

3. Action Plan and Guidelines for Antimicrobial Use

Today many organizations have addressed the issue of increasing resistance to antimicrobials. In 1997, surveillance and educational and research initiatives to address antimicrobial resistance in foodborne pathogens were expanded due to funds provided by the US President's Food Safety Initiative (Torrence, 2001). In 1999, an interagency task force was formed, headed by the Center for Disease Control and Prevention (C.D.C.), the National Institutes of Health (N.I.H.), and the Food and Drug Administration (F.D.A.). In 2000 this committee released a Public Health Action Plan to Combat Antimicrobial Resistance. The main aspects of this plan addressed surveillance, prevention and control, and research and product development related to antimicrobial resistance (C.D.C., 2001). In 2000, the World Health Organization (W.H.O.) Department of Communicable Disease Surveillance and Response reported the W.H.O. Global Principles for the Containment of Antimicrobial Resistance in Animals Intended for Food. The goal of the W.H.O. program is to provide a framework of recommendations to reduce the overuse and misuse of antimicrobials in food animals for the protection of human health (W.H.O., 2000).

Many of the conclusions and recommendations of the W.H.O. are very similar to those proposed in the Swann report. They also concluded that the

> "Future containment of antimicrobial resistance requires a coordinated multidimensional approach in which effective change in antimicrobial usage, infection control and epidemiologically-sound resistance surveillance are key endpoints" (W.H.O., 2000).

The F.D.A. also became involved in this issue and in 2003 released the Guidance for Industry #152. In this document a risk analysis method is outlined for evaluating new antimicrobial animal drugs in terms of the potential microbiological effects on foodborne bacteria of human health concern (F.D.A., 2003). In addition to these guidelines, the World Veterinary Association, in conjunction with the International Federation of Agricultural Producers (F.I.P.A./I.F.A.P.) and International Federation for Animal Health (I.F.A.H.) (formerly known as C.O.M.I.S.A.), released its guidelines for the prudent use of antimicrobials. In these guidelines are basic principles regarding the use or treatment of animals with antimicrobials. These include supervision of antibiotic usage by a veterinarian; bacterial diagnosis with sensitivity testing when treating an animal for therapy; following labeling instructions and restriction of off-label uses; following a specific regimen; keeping strict records; and using antibiotic alternatives where appropriate (Janssen et al., 2006; F.D.A., 2003).

4. Resistance Surveillance Programs

In the United States the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (N.A.R.M.S.-E.B.) surveillance system was established in 1996 to monitor resistance to 17 antibiotics in humans and animals. The surveillance program is coordinated by the F.D.A., the Department of Agriculture (U.S.D.A.), and the Centers for Disease Control and Prevention (C.D.C.). The 17 antimicrobials monitored were selected as representative antimicrobials used in animal and human medicine (amikacin, ampicillin, amoxicillin-clavulanic acid, apramycin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, trimethoprim-sulfamethoxazole, and ticarcillin) (Torrence, 2001). Additional surveillance programs have been formed in Sweden (the Swedish Strategic Program for the Rational Use of Antimicrobial Agents and Surveillance and Resistance: S.T.R.A.M.A.) and in Denmark (the Danish Integrated Anti-microbial Resistance Monitoring and Research Programme: D.A.N.M.A.P.) (Andreasen et al., 2005).

Throughout the last five decades it is evident that experts agree that the focus should be towards improving surveillance for emerging antimicrobial resistance problems, prolonging the useful life of antimicrobial drugs, developing new drugs, and developing new strategies (e.g. improved vaccines, diagnostics,

and infection control methods) to prevent and control antimicrobial resistance (C.D.C., 2001, Swann et al., 1969,).

5. Benefits of Antimicrobial Use in Agriculture

Many scientists are quick to report potential risks associated with using antimicrobials in food animals, but it is important to note the many benefits associated with the use of antimicrobials in food animals. In the United States, antimicrobials are widely used as feed additives to treat disease, improve carcass quality, and improve feed efficiency (Andreason et al., 2005). It is also important to note the most important human health impact of antimicrobial use in animals may be the reduction in human illnesses per year due to prudent use, leading to fewer diseased animals, more uniform slaughter weights, and lower microbial loads in processed food (Carnevale, 2005). Many bacterial diseases are not readily preventable with vaccination, and can have a commensal association with their food animal hosts, making eradication impossible. Control of subclinical disease and therapeutic intervention with antimicrobials in these instances may be the only practical approach to prevention (Phillips et al., 2004).

B. Antimicrobial Resistance Associated with Beef Cattle

More than 2 million kg of antimicrobial agents are administered to beef cattle each year (Mellon et al., 2001). Antibiotics used typically include chlortetracycline, sulfamethazine, monensin, tylosin, and virginiamycin (Inglis, 2005). Chlortetracycline or chlortetracycline plus sulfamethazine help maintain

weight gain during periods of respiratory disease challenge associated with shipping fever (Troxel and Gadberry, 2006), aid in the prevention of liver abscesses, reduce bacterial diarrhea, and prevent foot rot. The ionophore monensin inhibits the growth of Gram-positive bacteria and has been shown to increase the feed efficiency of cattle fed a high grain diet. Tylosin is given to prevent liver abscesses; and virginiamycin is used as a beef cattle feed additive (Inglis et al., 2005). Medicated feed additives are also believed to be beneficial during the weaning process of replacement heifer calves to prevent coccidiosis and increase feed efficiency (Troxel and Gadberry, 2006).

It has been shown that young animals show a higher prevalence of resistant fecal *E. coli* than older stock held on the same farm and that carriage of ampicillin-resistant *E. coli* by young calves has been shown to decline with age (Hoyle et al., 2004b). Early acquisition of resistant *E. coli* by calves could be the result of active selection. In a study performed by Hoyle and coworkers, a cohort of calves was examined for acquisition of antimicrobial resistant commensal *E. coli*. Fecal samples were collected weekly from calves over a four month period and screened for *E. coli* resistant to at least one of three antibiotics (ampicillin, apramycin and nalidixic acid). Calves were kept at pasture in a single group until the tenth week, when they were then housed. All calves had *E. coli* isolated from fecal samples which were resistant to ampicillin and *E. coli* resistant to nalidixic acid, 67% had *E. coli* resistant to apramycin. In this study it was concluded that cohort calves rapidly acquired antimicrobial resistant (e.g. nalidixic acid,

apramycin, and ampicillin) bacteria within weeks of birth (Hoyle et al., 2004a). In another study by Bradford and coworkers in 1999, upon examination of isolates of *E. coli* obtained from individual bovine calf scours cases which had failed antimicrobial therapy, it was found that all isolates were resistant to ampicillin, kanamycin, streptomycin, sulfisoxazole, and tetracycline and had reduced susceptibility to ticarcillin and piperacillin. Many were resistant to chloramphenicol, gentamicin, and trimethoprim-sulphamethoxazole and 13% of isolates were resistant to ceftiofur, an expanded spectrum cephalosporin (Bradford et al., 1999). In feedlot cattle it has been shown that subtherapeutic administration of tetracycline, alone and in combination with sulfamethazine, can select for the carriage of resistant strains of Campylobacter species (Inglis et al., 2005).

C. Bacteria Associated with Antimicrobial Resistance Gene Transfer

Foodborne pathogens (e.g. Salmonella, Campylobacter, and some strains of *E. coli*) are of special concern when considering antimicrobial resistance. The annual cost of foodborne illnesses caused by the four most common bacterial pathogens has been estimated at $6.9 billion (*Salmonella* strains, *Shigella* and *Campylobacter* species, and *E. coli*) (Allos et al., 2004). These pathogens are harbored by the host and may be passed along to humans and/or other animals to cause disease.

1. *Escherichia coli*

Escherichia coli O157:H7/NM has been recognized as an important foodborne pathogen since the first reported outbreak of the disease in the United States in 1982 (You et al 2006). Cattle have been considered to be a major reservoir of this organism (Laegreid et al., 1999). Outbreaks of *E. coli* O157:H7 have been attributed to the consumption of undercooked meat and other foods contaminated with animal feces (You et al., 2006). Initially, *E. coli* O157:H7 was considered to be sensitive to many classes of antimicrobials, but recent studies have shown the prevalence of antimicrobial resistance is increasing (Schroeder et al., 2002; Zhao et al., 2001).

2. Salmonella

Another important type of bacteria considered to be a foodborne pathogen is salmonella. There are an estimated 1.4 million cases of salmonellosis in the United States each year (C.D.C., 2002). Cattle are considered to be a natural reservoir of salmonella but rarely shed the bacteria (Beach et al, 2002), which makes it difficult to diagnose and test susceptibility. Since 1996, N.A.R.M.S. monitored the prevalence of antimicrobial resistance in non-Typhi Salmonella. Resistance has increased from 11% of test isolates being resistant to five or more drugs in 1996 to 15% in 2001 (Angulo et al., 2004). Multidrug resistant (M.D.R.) *S*. Typhimurium definitive type 104 (DT104) and M.D.R. S. Newport have both caused recent foodborne outbreaks (Angulo et al., 2004). Available information indicates that *S*. Typhimurium DT104 ACSSuT

(resistance to at least ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) spread amongst animals and then humans in the early 1990's (Ribot et al., 2002). Among human *S*. typhimurium isolates submitted to N.A.R.M.S., the resistance pattern ACSSuT was prevalent in 28% of isolates in 1999 and 2000, and in 30% of isolates in 2001 (C.D.C., 2003). Of special concern is the emergence of additional resistance in other *Salmonella* serovars, including the expression of M.D.R.-AmpC phenotype (resistant to at least ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, amoxicillin/clavulanic acid, ceftiofur, and decreased susceptibility to ceftriaxone). This resistance pattern was not detected in any serotype in 1996; whereas in 2003, 3.2% Non-Typhi, 20.7% S. Newport, and 2.2% *S*. Typhimurium in 2003 demonstrated this pattern (C.D.C., 2003). Field investigations have demonstrated an association between human S. Newport M.D.R.-AmpC infections and consumption of ground beef (C.D.C., 2002), drinking and eating unpasteurized dairy products (McCarthy et al., 2002) and living on a dairy farm (Gupta et al., 2003), suggesting that cattle are an important reservoir for S. Newport M.D.R.-AmpC (Angulo et al., 2004).

3. Campylobacter

Campylobacter species are recognized as one of the most frequent causes of acute diarrheal disease in humans in North America (Inglis et al., 2005). There are estimated to be more than 2.4 million cases of infection per year in the United States (Travers and Barza, 2002). Poultry are considered to

be the major reservoir of Campylobacter (Angulo, 2004). Many different species of Campylobacter are also shed in the feces of beef cattle (Inglis et al., 2003). When antibiotics are required for the treatment of Campylobacter gastroenteritis, erythromycin or a fluoroquinolone such as ciprofloxacin is the preferred drug (Smith et al, 1999). Recently, quinolone resistance in Campylobacter has begun to increase. This could lead to more severe illness for patients with Campylobacter gastroenteritis. The median duration of diarrhea for patients with quinolone-resistant Campylobacter infections has been shown to be 3 days longer than for quinolone-sensitive infections (Smith et al., 1999). In 2003, a total of 17.7% of Campylobacter isolates tested by N.A.R.M.S. were resistant to the quinolone ciprofloxacin, compared with 12.9% in 1997 (C.D.C., 2003). In addition, a study performed by Inglis and coworkers demonstrated that the subtherapeutic administration of tetracycline, alone and in concert with sulfamethazine, to feedlot cattle selects for the carriage of resistant strains of Campylobacter (Inglis et al., 2005), thus adding concern for an additional class of drugs.

D. Mechanisms of Antimicrobial Resistance

Microbial populations develop resistance to antimicrobials through several mechanisms. The rate at which an individual gene mutates to express an antimicrobial resistance phenotype involves the environment, cell physiology, bacterial genetics, and population dynamics (Martinez and Baquero, 2000).

Antimicrobial resistance in bacteria may also be acquired laterally or horizontally through gene transfer. There are several processes through which this may occur.

1. Mutation

Although not the most common method of antimicrobial resistance development, it is possible for bacteria to spontaneously mutate in the presence of an antimicrobial to allow survival. This process has allowed for bacteria to survive in the presence of naturally occurring antimicrobials for centuries. Bacteria such as E. coli have been reported to spontaneously mutate to streptomycin resistance at a rate of 0.00004 mutations per 100,000 gametes (Russell, 2002). Another bacterium, *Diplococcus pneumoniae*, is reported to spontaneously mutate to penicillin resistance at a rate of 0.01 mutations per 100,000 gametes (Russell, 2002).

2. Transformation

Transformation, the uptake of naked DNA from the immediate surrounding, involves specific recognition sequences in order for the new DNA to be taken up by the bacteria (Roe and Pillai, 2003). The bacteria must also be "competent", in the appropriate physiological state, in order to acquire the exogenous DNA. Bacteria such as Campylobacter are believed to be naturally competent (Roe and Pillai, 2003).

3. Conjugation

Another mechanism by which bacteria can exchange and acquire antimicrobial resistance is through conjugation. In this process, plasmids or self-replicating extra-chromosomal DNA are transferred through physical contact between cells via a pillus. This allows the DNA to be transferred between donor and recipient cells (Russell, 2002). An example of a gene transferred in this manner is the *floR* gene, which encodes florfenicol resistance in *E. coli* and has been found in cattle isolates (Cloeckaert et al., 2000).

4. Transduction

Transduction, a third process of gene transfer, is facilitated by bacteriophages. In this process genetic material is introduced when the virus attaches and injects its own nucleic acids into the bacterium. In some cases this material can be integrated into the bacterial genome (Russell, 2002).

5. Transposons

Transposons, genetic elements conferring a selectable phenotype flanked by two insertion sequences, are involved in horizontal gene transfer events between bacteria (Roe and Pillai, 2003). They are unique in that they have the ability to remove themselves from one genetic locus and move to another within the same bacteria or within bacteria in other taxa (Roe and Pillai, 2003). Transposons can be transferred via transformation, conjugation or transduction and they play a major role in the development of antimicrobial resistance

because often they contain antimicrobial resistance mediating gene sequences termed integrons (Stokes and Hall, 1989). Integrons are believed to play a major role in the rapid dissemination of multiple-antimicrobial resistance among bacteria (Ochman et al., 2000).

6. Integrons

Stokes and Hall first identified integrons in 1989. These gene elements are now considered to be a primary means by which bacteria acquire antimicrobial resistance (Roe and Pillai, 2003). Integrons possess two conserved segments separated by a variable region. This variable region includes integrated antibiotic resistance genes or genes of unknown function. The 5' conserved segment contains the *int* (integrase) gene, which encodes a polypeptide of 337 amino acids shown to be homologous to other members of the integrase family (Ouellette and Roy, 1987). The complimentary strand contains a common promoter region (*P1-P2*), which is directed toward the site of integration (Levesque et al., 1995). The 3' conserved region contains the *qacEΔ1* gene, which confers resistance to ethidium bromide and quaternary ammonium compounds (Paulsen et al., 1993), a *sul1* gene, which confers resistance to sulfonamides, and an open reading frame, (ORF) orf5 (Stokes and Hall, 1989). The incorporation of the resistance gene and its expression from the integron promoter results from a site-specific recombination event between the attachment site and a recombination site, known as the 59 base element, located downstream of the promoterless resistance gene (Ochman et al., 2000).

Resistance genes without promoters are referred to as gene cassettes. Gene cassettes code for a wide range of antimicrobial resistance determinants (e.g. aminoglycosides, trimethoprim, chloramphenicol, penicillins and cephalosporins) (Hall, 1997). They are exchanged between bacteria and linearized by the integrase enzyme before being incorporated at the integration site (Roe and Pillai, 2003). Multiple gene cassettes can be inserted into the integron to confer a multiple antibiotic resistant phenotype to the bacteria (Hall and Collis, 1995).

Four different classes of integrons exist and are designated as class 1, class 2, class 3, and class 4, with each having distinctive traits (Mazel et al., 1998; Hall and Collis, 1995). The primary difference between the four classes is the sequence of the integrase gene. The amino acid sequence of the integrase genes of class 2 (*intI2*) and class 3 (*intI3*) integrons are only 45% and 60% homologous to class 1 (*intI1*) integrons, respectively (Hall and Collis, 1995). Class 1 integrons are the most common family of integrons (Hall, 1997). In a study performed by Singh and coworkers (2005), 274 Shiga toxin producing *E. coli* (STEC) isolated from poultry, cattle, swine and humans, were screened for antimicrobial resistance and class 1 integrons. Class 1 integrons were detected in 43 (16%) of the 274 isolates. In this case, transfer of integrons between strains of *E. coli* conferred resistant phenotypes for ampicillin, chloramphenicol, cephalothin, gentamicin, tetracycline, trimethoprim, sulfamethoxazole, and streptomycin (Singh et al., 2005). In another study, 104 *E. coli* were isolated from swine with diarrhea in Korea. A high percentage (64.2%) contained class 1

integrons and all isolates were resistant to at least 3 antimicrobials (Kang et al., 2005). Class 1 integrons have also been shown to occur at high frequency in *E. coli* isolated from dairy cows with mastitis, conferring resistance to tetracycline, streptomycin, and sulfonamide resistance (Murinda et al., 2005; Lanz, et al., 2003).

Class 1 integrons and antimicrobial resistance genes can be exchanged indiscriminately between bacteria of different taxa (Roe and Pillai, 2003). When considering the presence of commensal enteric bacteria, this raises much concern. Commensal bacteria are naturally occurring in host animals. In the gastrointestinal system they may persist for only a few days, or may persist for many years (Smith et al., 2002). If commensal bacteria are exposed to antimicrobials, resistant bacteria may develop, and they may share genes (Angulo et al., 2004). Small increases in the prevalence of antimicrobial resistance in commensal bacteria can potentially initiate large epidemics (Smith et al., 2002). Resistant commensal bacteria of food animals might contaminate meat products and reach the intestinal tract of humans (van de Bogaard and Stobberingh, 2000). As the population of resistant bacteria increases, the resistance gene population (e.g. plasmids, transposons, integrons) becomes larger and may allow for the more frequent transfer of resistance to pathogenic bacteria such as Salmonella and Shigella (Angulo, et al., 2004). Antimicrobial resistant *E. coli* can be isolated from the intestines of healthy animals and humans (Singh et al., 2005). Studies have shown that *E. coli* readily transfer

resistance genes to other *E. coli* and to other strains of bacteria (Johnson et al., 1994; Zhao et al., 2001). In a study performed by Saenz and coworkers, 17 multiple antimicrobial resistant nonpathogenic (commensal) *E. coli* isolates from food products and healthy animals and humans were analyzed for the presence of class 1 and class 2 integrons by detection of the *qacEΔ1*, *intI1* and *intI2* genes. One sample contained both integrase genes, whereas 11 others contained genes for class 1 integrons, and 3 contained genes for the class 2 integrons (Saenz et al., 2004). It is evident that the ability of bacteria to acquire resistance genes from organisms that constitute the normal bacterial flora of humans and animals, especially under the selective pressure of antimicrobial agents, should not be underestimated (Tenover, 2001).

E. Antimicrobial Resistance in Aquatic Environments

1. Contamination by Livestock

Livestock, such as beef and dairy cattle, swine, and poultry, are major sources of fecal contamination of surface and ground waters (Parveen et al., 2006). The contamination of surface waters with antimicrobial-resistant bacteria due to fecal contamination is an emerging concern. More than 100 million tons of dry livestock manure are produced annually in the United States (Waggoner et al., 1995). In a study conducted by Parveen and coworkers, more than 2000 *E. coli* isolates were collected from water retention ponds (swine, poultry, beef and dairy) and composite manure pits (beef) from farms in south, central, and north

Florida and analyzed for multiple antimicrobial resistance. Resistance to at least one antimicrobial was detected in 84% of isolates tested (Parveen et al., 2006). Runoff from retention ponds could spread antimicrobial resistance into nearby waterways and ground water. In a study performed by Ash and coworkers in 2002, antimicrobial resistant bacteria were isolated from 16 US rivers. More than 40% of resistant isolates contained plasmids (Ash et al., 2002). Plasmid transfer has been demonstrated in many different aquatic environments (Seveno et al., 2002). Bacterial activity and gene transfer is enhanced in sediments and water surfaces that provide higher nutrient input and favorable temperatures (Wellington and van Elsas, 1992). Aquatic environments in or near livestock systems (e.g. ponds, streams) provide high nutrient inputs due to fecal contamination, making them ideal locations for gene transfer. In a study performed by Biyela and coworkers in 2004, 80% of *E. coli* isolated from a river near agricultural activities were resistant to 3 antimicrobials (rifampicin, cephalothin, and novobiocin) (Biyela et al., 2004).

2. Wildlife

Enteric microflora in wildlife in or near aquatic environments within livestock systems have the potential to acquire resistance genes from resistant bacteria present via fecal contamination. Cole and coworkers in 2005 analyzed antimicrobial resistance phenotypes from *E. coli* isolated from Canada Geese living in an area of agricultural production and an area where no apparent contact of livestock wastes was evident. Of *E. coli* isolates tested near agricultural

production, 72% exhibited resistance to more than 1 antimicrobial. In contrast, only 19% of *E. coli* isolated from the non-agricultural locations exhibited resistance, and those were resistant only to β-lactam antimicrobial agents (Cole et al., 2005). Also, of all isolates tested, the class 1 integrase gene was located only in those with agricultural exposure (9/25) (Cole et al., 2005).

3. Amphibians

Amphibians, in particular frogs, live in ponds and dig into mud and soils very rich in microbes. Hird and coworkers (1983) isolated 29 species of *Enterobacteriaceae*, including *E. coli* and *Salmonella arizonae* from frogs and tadpoles (Hird et al., 1983). Also, Gram-negative bacteria known to cause illness in humans (*Klebsiella pneumoniae, Proteus vulgaris, Aeromonas hydrophila, and Enterobacter agglomerans 2*) have been isolated from frogs, and resistance to nalidixic acid, rifampicin, and streptomycin was identified in those isolates (Boman, 2000). The intestinal flora of frogs and tadpoles may acquire antimicrobial resistance genes from aquatic environments. In nature, frogs and tadpoles are coprophagic (feces eating), as feces increases the length of time food is resident in the intestinal tract, allowing for some microbial digestion to occur (McDiarmid and Altig, 1999; Minette, 1984). Frogs and tadpoles in aquatic environments within livestock systems thus may be able to ingest and maintain antimicrobial resistant bacteria from animal feces. Antimicrobial resistant bacteria could then be passed back into the environment (e.g. pond) where the

livestock have the potential to ingest these organisms. To date, no studies have been performed to test this possibility.

F. Hypothesis

The primary hypothesis for our study is that class 1 integrons and/or antibiotic resistant bacteria occur more frequently in environments with cattle exposure, and resistance and class 1 integrons disperse into aquatic environments and wildlife, which in turn provides a reservoir of antibiotic resistant bacteria for cattle within that environment.

II. Materials and Methods

A. Antibiotic Use Information

Antibiotic usage information at the Plateau Research Center and the Grasslands Research Center was obtained from health records kept by the animal caretakers of these centers from 1995-2005.

B. Treatments

Two treatments (cattle-accessible and cattle-excluded) were determined based upon previous cattle-use criteria for 9 aquatic environments at the University of Tennessee Plateau Research and Education Center Crossville, TN. Cattle-accessible ponds (n=5) were exposed to livestock operations (Cow/calf production system) for greater than 10 years (some maintained the presence of cattle at all times, others had cattle rotated). Cattle-excluded ponds (n=4) were not exposed to cattle for greater than 10 years. A satellite photograph demonstrating the location of these aquatic environments is provided in the appendix (Figure 1).

C. Animals

At the time of the first sampling date there were 147 yearlings, 26 bulls, 68 2-year-olds, and 65 calves in the livestock system. In March, 61 yearlings and

26 bulls were sold. In September, the 68 2-year-olds were moved to a different station, and 19 bulls and 124 calves were brought into the system.

D. Sample Collection

American bullfrog (*Rana Catesbeiana*) and green frog (*Rana clamitans*) larvae, pond water samples and cow manure samples of selected cattle-accessible and cattle-excluded environments were collected on February 15, 2005, June 15, 2005 and October 12, 2005. Green frog metamorphs were obtained from pit fall traps (large buckets placed into the ground with their lids removed) (Dodd and Scott, 1994) over a one week period from June 10 to June 15.

Tadpoles were caught using seine nets and dip nets. Dip nets were used to search around the perimeter of the pond in vegetation and seine nets were used to search in open water for the presence of bullfrog or green frog tadpoles. If all accessible areas of the pond were searched and less than the desired amount (5 per species) were obtained, collection attempts were ceased. Captured tadpoles were rinsed thoroughly on-site with sterile water then placed in individual jars of sterile water and transported to a laboratory at the University of Tennessee. Tadpoles were in the sterile water jars for no less than three hours, and up to 12 hours (long enough for a fecal sample to be voided). Fecal samples were obtained from jars using a pipette to remove debris and 0.5 ml of sample was saved in 0.5 ml of 20% glycerol and stored at -80^{o}C for future use. A

section of GI tract was removed from euthanatized metamorph individuals and placed in a tube of sterile water, then vortexed to remove fecal samples. Euthanatization was performed by Dr. Debra Miller, DVM of the University of Georgia, using IACUC approved methods. Pond water samples were obtained by using a 12 ml pipette to remove water at three locations from each pond. Samples from individual ponds were combined and debris was removed for bacterial isolation. Cattle manure samples were obtained from random locations surrounding each individual pond using sterile swabs. Swabs were then placed in tubes of sterile water to form a slurry, which was easier to use for isolation purposes.

E. Bacterial Isolation:

1. Escherichia coli

One hundred μl of preserved sample was spread onto MacConkey agar (BD/Difco, Sparks, MD ref# 212122) plates and incubated at 37°C for 18-24 hours. Up to 10 colonies with bright reddish-purple color were picked from each plate and inoculated into Nutrient Broth (BD/Difco, Sparks, MD ref#234000). Inoculated Nutrient Broth tubes were incubated either overnight or on a shaker for 3-4 hours at 37°C. One-half ml of each sample was saved in 0.5 ml of 20% Glycerol and stored at -80°C for future use.

2. Salmonella

Approximately 2 ml of fecal/water sample was placed into Secure T sterile stomacher bags (Fisher*brand,* Suwannee, GA). Sixty ml of Tetrathionate Broth (BD/Difco, Sparks, MD ref# 210420) was added, bags were sealed and samples were incubated at 42°C for 18-24 hours. One hundred microliters of sample was then plated onto XLT4 Agar and incubated for 18-24 hours at 37°C. Zero to 10 colonies were picked from each plate and inoculated into Nutrient Broth. Black colonies picked from agar that remained red were chosen. Tubes were incubated either overnight or on a shaker platform for 3-4 hours at 37°C. One-half ml of each sample were saved in 0.5 ml of 20% glycerol and stored at -80°C for future use.

F. Integron Analysis

Integron presence was detected using a multiplex polymerase chain reaction (MP-PCR) analysis, performed by targeting three conserved sequences of class 1 integrons (*qacEΔ1*, *intI1* and *sul1*) as described by Ebner (Ebner, 2003). Primer pairs were designed using published sequences (GenBank accession no. AF161825) and manufactured by Operon, Inc. (Alameda, CA) (Table 1).

Total DNA was prepared by boiling 0.5 ml of overnight cultures in 2xYT broth (BD/Difco, Sparks, MD ref# 244020) in an equal volume of 0.2% (wt/vol)

Triton X-100 (Mallinckrodt, Paris, KY) for five minutes [Khan, et al., 2000]. Boiled cultures were cooled on ice for 5 min and used immediately for PCR. PCR reagents, excluding template DNA, were combined in a master mix prior to aliquoting. The final reaction volumes for each aliquot included: 1) 1 µl of each primer pair (50pmol [each primer] μl^{-1}); 2) 1 µl of *Taq* DNA polymerase (0.5U μl^{-1}; Promega, Madison, WI); 3) 10 µl reaction buffer (12.5mM $MgCl_2$, pH 8.5; Invitrogen, Carlsbad, CA); 4) 5 µl dNTPs solution (2.5mM of each dNTP, pH 8.0; Invitrogen); and 5) 32 µl sterile H_20. Sample DNA (1 µl) was then added to each aliquot. Reactions were conducted in a Mastercycler Gradient thermocycler (Eppendorf, Westbury, NJ) with the following conditions: 1) 1 cycle of 94°C for 4 min; 2) 10 "touchdown" cycles of 94°C for 1 min, 65°C for 30s (decreasing 1°C/cycle), 70°C for 2 min; 3) 24 cycles of 94°C for 1 min, 55°C for 30s, 70°C for 2 min; and 4) 1 final cycle of 70°C for 5 min. *Salmonella enterica* Typhimurium DT104 (provided by Dr. Timothy Barrett of the Centers for Disease Control and Prevention), known to contain two class 1 integrons [Ng et al., 1999], was used as a positive control. A blank containing only PCR reagents and Triton X-100 was used as a negative control. Reaction products were separated by conventional electrophoresis in 1.5% agarose and stained with ethidium bromide for visualization.

G. Antibiotic Susceptibility Testing

Minimum Inhibitory Concentration of isolates to tetracycline, florfenicol, and sulfisoxazole was determined using the microbroth dilution technique

described by the CLSI (CLSI, 2002). Antibiotic plates were made as diagrammed in Figures 2 and 3. All *E. coli* isolates were grown for 18 to 24 hours on MacConkey agar at 37°C, and tubes containing 5 ml of Mueller Hinton II broth (BD/Difco, Starks, MD ref# 212322) were inoculated with each sample and grown to a 0.5 McFarland Standard. Twenty-three □l of each of bacterial culture was then added to 2.5 ml of diluted Mueller Hinton II broth (2.27 ml of MHII broth and 0.227 ml of sterile water per sample). Fifty □l of sample was then added to each well of the antibiotic plate (96 wells, 8 wells per sample). Plates were incubated for 18 to 24 h at 37°C then read for susceptibility/resistance value. Inhibitory concentration was determined by a complete clearance of bacteria growth for tetracycline and florfenicol, and by an 80% reduction in growth for sulfamethoxazole. Isolates were considered resistant to tetracycline if inhibition of growth occurred at ≥ 16 □l/ml and florfenicol resistance was determined if inhibition of growth occurred at ≥ 8 □l/ml. Resistance to sulfisoxazole was determined if an 80% reduction in growth (relative to the control well H) occurred at ≥ 512 microliters/ml. Integron-harboring *E. coli* isolates and samples representing pond water, manure, and tadpole samples from one pond of each treatment from two months of sampling (n=35 total samples, 21 cattle-accessible isolates, 14 cattle-excluded) were screened for resistance using the broth dilution method according the guidelines published by the National Antimicrobial Resistance Monitoring System (N.A.R.M.S., 1997). N.A.R.M.S. veterinary Gram-negative panels (#CMV1AGNF), which included standardized dilutions of amikacin, amoxicillin/clavulanic acid, ampicillin,

cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciproflaxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulfamethoxazole were used to determine multi-antibiotic resistance patterns.

H. Statistical Analysis

Statistical analysis was performed using the mixed model analysis of variance model in SAS 9.13 (SAS, 2002). A Completely Randomized Block Design (CRD) factorial was used with pond treatment (cattle-accessible and cattle-excluded) and sample type (tadpole, pond water, and metamorph) as the treatment factors. An additional analysis was added to analyze month (February, June, and October) as a treatment factor.

III. Results

Antibiotic treatments used in the cow-calf production system in our study included florfenicol, sulfa-drugs, tetracyclines, and penicillin G. Antibiotics were used for therapeutic or prophylactic purposes. A list of all antibiotics on record utilized for treatment since 1995 was obtained (Tables 2, and 3).

A. Antibiotic Use for Animals Present During the Span of the Study

Between January and November 2005, antibiotics were used to treat pink eye, foot rot, and scours in individual animals. In the months of January and February, 1 animal was treated topically with penicillin G for ocular ulcers (9 doses) and 4 animals were treated orally with oxytetracycline for scours. In June, one animal was treated for foot rot with injectible penicillin G (6 doses). Calves brought in during September were maintained at the Grasslands Research and Education Center near Crossville. In August and early September, prior to transport, seven animals from this center were treated individually with injectible oxytetracycline**,** 3 for foot rot and 4 for pink eye (7 total doses). In 2005, a mineral supplement (Bob's range mineral) containing chlortetracycline (1.12gm/lb) was present at all pastures at the Plateau Research and Education Center. A weaning diet (Purina Preconditioning/Receiving Chow CTSM 3152) containing chlortetracycline (70gm/ton) and sulfamethazine (0.0077%) was fed to calves at the Plateau Center and the Grasslands Center for 7-14 days during the month of September.

B. February 15, 2005

A total of 40 bullfrog tadpoles were captured from cattle-accessible and cattle-excluded ponds on February 15, 2005 (n=20 per treatment). *Escherichia coli* were isolated from 60% of fecal samples obtained from tadpoles captured from cattle-accessible ponds, yielding 93 isolates for analyses. Forty percent of tadpoles from cattle-excluded ponds were found to contain *E. coli* in fecal samples, providing 71 isolates for study. Salmonella were not recovered from any sample. Pond water and manure samples were not collected at this time,

C. June 2005

During the second sampling period of June 15, 2005, 50 green frog tadpoles (n=30 for CA n=20 for CE) and 42 bullfrog tadpoles (n=20 for CA and n=22 for CE) were captured from cattle-accessible and cattle-excluded ponds. Of samples taken from cattle-accessible ponds, *E. coli* were isolated from 100% (n=5) of water samples, 100% (n=5) of cattle manure samples, 90% of fecal samples from green frog tadpoles, and 100% (n=20) of fecal samples from bullfrog tadpoles. The total count of *E. coli* isolates obtained from these samples included 27 from water samples, 36 from cattle manure, 123 from green frog tadpoles, and 101 from bull frog tadpoles. From cattle-excluded ponds, *E. coli* were isolated from 100% (n=4) of water samples, 85% of fecal samples from green frog tadpoles, and 82% of fecal samples from bullfrog tadpoles. No cattle manure samples were present at cattle-excluded ponds. *Escherichia coli* totals from cattle-excluded ponds included 21 from water samples, 91 from green frog

tadpole samples, and 81 from bullfrog tadpole samples. No salmonella were found in any of the samples.

Over the period of June 10 through June 15, 2005, 39 green frog metamorphs (n=19 for CA, n=20 for CE) were captured. *Escherichia coli* were isolated from 89% of green frog metamorph fecal samples obtained from cattle-accessible ponds and 100% of fecal samples from green frog metamorphs obtained from cattle-excluded ponds. In all, 70 *E. coli* isolates from cattle-accessible ponds and 113 isolates from cattle-excluded ponds were obtained. Salmonella were not isolated from any of the samples.

D. October 12, 2005

On October 12, 2005, 21 bullfrog tadpoles (n=1 for CA and n=20 for CE) and 74 green frog tadpoles (n=49 for CA and n=25 for CE) were captured. Twenty cattle manure samples were also taken. *Escherichia coli* were isolated from 100% (n=4) of water samples, 95% of cattle manure samples, 63% of fecal samples from green frog tadpoles and 100% of fecal samples from bullfrog tadpoles obtained from cattle-accessible ponds. In all, 36 *E. coli* isolates were obtained from water samples, 87 were obtained from cattle manure samples, 135 were obtained from green frog tadpole samples and 5 were obtained from bull frog tadpole samples from cattle-accessible ponds. For cattle-excluded ponds, *E. coli* were isolated from 100% (n=3) of water samples, 84% of green frog tadpole fecal samples and 85% of fecal samples from bullfrog tadpoles. No

manure samples were present at cattle-excluded ponds. *Escherichia coli* totals from cattle-excluded ponds included 28 from water samples, 69 from green frog tadpole samples, and 62 from bullfrog tadpole samples. Salmonella were not isolated from any of the samples. A table with a combined total of bacteria isolated from all samples is included in the appendix (Table 4).

E. Class 1 Integrons

Class 1 integrons were detected in 3% of isolates (n = 63) from cattle-accessible pond water and 1% of isolates (n = 123) from cattle manure (Figures 4, 5 and 6).

F. Antibiotic Resistance

Fifty-Two percent of *E. coli* isolates from cow manure were resistant to tetracycline, 88% were resistant to florfenicol, and 11% were resistant to sulfisoxazole (Figure 7). The percentage of tadpole and water samples with tetracycline resistant *E. coli* isolates was greater in cattle-accessible (CA) ponds (P = 0.0001 for tadpoles and P = 0.0283 for water samples) compared to isolates from cattle-excluded (CE) ponds (29% from CA isolates for tadpoles vs. 11% from C-E isolates and 19% from CA isolates for water samples vs. 0% for CE isolates). No difference was detected between treatments for metamorph isolates (Figure 8). Isolates resistant to florfenicol was more prevalent (P = 0.006) in tadpole samples from cattle-accessible ponds compared to cattle-excluded ponds (73% from CA isolates vs. 56% from CE isolates). However, no

significances were observed with respect to pond water of metamorph samples (Figure 9). There was also greater (P = 0.0156) resistance to sulfisoxazole in samples taken from tadpoles obtained from cattle-accessible ponds compared to samples taken from tadpoles obtained from cattle-excluded ponds (8% from CA isolates vs. 2% from CE isolates) No difference was detected between treatments for pond water or metamorph samples (Figure 10).

When date of sampling was added to the model, a significant difference (P < 0.0001) was noted across months in the prevalence of tadpole isolates resistant to tetracycline from cattle-accessible ponds (Figure 11). A difference (P<0.0001) was also noted across sampling dates with regard to the prevalence of florfenicol resistant isolates from tadpoles taken from cattle-accessible ponds. No significant difference for sampling date was detected in any other sample types.

Of isolates selected for N.A.R.M.S. panel testing, multi-resistance patterns were also observed to differ between sample sources (Table 5). All isolates from cattle-accessible ponds (n=21) were resistant to at least one antimicrobial compared to 57% (n=14) of cattle-excluded isolates. None of the isolates selected from cattle-excluded ponds were resistant to sulfisoxazole, and only isolates (n=2) from cattle accessible ponds were resistant to more than three antimicrobials. A summary table of all results is included in the appendix (Table 7, A and B).

IV. Discussion

Our primary hypothesis for this research was that class 1 integrons and/or antibiotic resistant bacteria occur more frequently in environments with cattle exposure, and resistance and class 1 integrons disperse into aquatic environments and wildlife, which in turn provides a reservoir of antibiotic resistant bacteria for cattle within that environment. It should be noted however that cow-calf production systems do not typically use large amounts of antibiotics, particularly growth promoting feed-based antibiotics often associated with intensive livestock operations such as modern swine and poultry systems (McEwen and Fedorka-Cray, 2002).

E. coli is a member of the normal intestinal flora of ruminants, and colonization of the gut takes place soon after birth. The mother and/or inanimate environment is the most frequent source of colonization (Sussman, 1985). Mature cattle may serve as a reservoir of antimicrobial resistance bacteria (Schroeder et al., 2002). Thus if bacteria associated with a cow harbored resistance genes, those genes could easily be transferred to its calf via direct transfer of bacteria or through gene transfer mechanisms. This theory was demonstrated in sows and their young by Mathew and coworkers (2005). In this study it was demonstrated that pigs whose sows had been treated with oxytetracycline had consistently greater percentages of antimicrobial resistant (apramycin and oxytetracycline) *E. coli* isolates that pigs derived from untreated sows. This idea is relevant to our study, as calves were brought in from another

station (Grasslands Research Center) for weaning at the Plateau Research Center.

It is important to explain the lack of salmonella isolated from the samples obtained in this experiment. Some reports indicate that salmonella are difficult to isolate from cattle, as those organisms are not consistently shed in their feces. Shedding of salmonella by adult beef cattle has been shown to be as low as 1% when no stressor is present (Beach et al, 2002). It is unknown why salmonella were not isolated from the amphibians captured in this study. It is possible that salmonella could have been present but were not isolated due to the initial incubation temperature used in our study. As amphibians are cold-blooded animals, their body temperatures would have been similar to that of the pond water, and in turn any salmonella associated with the amphibians may have been adapted to those temperatures. In contrast, we used a temperature regimen typical for recovery of salmonella from warm blooded animals (42^{o}C) for incubation. Thus, it may have been beneficial to lower the incubation temperature of the samples to 29^{o}C, as opposed to 42^{o}C or even 37^{o}C. However, as these organisms are typically pathogens of livestock, humans and other warm-blooded hosts, it would seem that our incubation temperatures should have been tolerated by the salmonella and conducive for their growth. It is probable that prevalence of salmonella was quite low or even non-existent in the cattle of our study. In general, the farm was well maintained; the cattle

appeared healthy and were maintained in a good environment with low animal densities.

The minimal usage of antimicrobials for therapeutic purposes within this system could explain the low prevalence of class 1 integrons within our cattle-accessible isolates. Integrons cannot move between bacteria on their own, therefore they are primarily located on transposons, which are usually incorporated into plasmids (Levesque et al., 1995). Through conjugation, plasmids may move freely between Gram-negative and some Gram-positive bacteria, and may be lost from the cell when not needed (Inoue, 1997). It is speculated that increased selective pressure in the form of high antimicrobial usage would be needed for bacteria in this system to maintain and spread integrons. Although the prevalence was low, discovery of class 1 integrons is important, as this shows they are present in and surrounding the aquatic environment.

The most probable sources of antibiotic resistant bacteria to the intestinal flora are food and water (Witte, 2000). Cattle at the Plateau Research and Education Center routinely eat around and drink from ponds contaminated with cattle manure. Livestock drinking water heavily contaminated with enteric bacteria could also serve as a common source of exposure to such resistant *E. coli* (LeJeune, et al, 2001). As shown in our data, even without high usage of antimicrobials, cattle manure exhibited a substantial prevalence of antimicrobial resistant *E. coli*. These isolates may be dispersed into the aquatic environment,

where resistance genes have been shown to spread over a variety of different microbial species over long distances (Biyela et al, 2004). The coprophagic habit of amphibians would provide an additional opportunity for them to become infected with bacteria resistant to antimicrobials through ingestion of cattle feces in their environment (Minette, 1984). As bullfrog tadpoles can remain in the environment for 2 years and green frog tadpoles for 1 year, they are likely candidates for resistance gene proliferation and dissemination into the environment.

Tetracyclines have been used for many years in managing infectious disease in food animals due to their low cost, broad antimicrobial activity, ease of administration, and general effectiveness (Prescott et al., 2000). They are utilized as the primary antibiotic for treatment at both the Plateau and the Grasslands Research and Education Centers. They have also been present in a low level form (chlortetracycline, 1.12gm/lb) as a free fed mineral supplement to all cattle within both systems for many years (although the Grasslands Center changed to one without chlortetracycline in 2005). The weaning diet which is fed to all heifer and bull calves brought to the Plateau Center, as well as to all steer calves weaned at the Grasslands Center, also contains chlortetracycline in low level amounts (70gm/ton). The therapeutic and dietary use of tetracycline could explain the apparent difference in antibiotic resistance between isolates from water and tadpole samples at cattle-accessible ponds and isolates at the cattle-excluded ponds. A second potential mechanism relates to the potential excretion

of tetracycline in cattle feces. Up to 30% of tetracyclines can be excreted virtually unchanged in the feces (Huber, 1988). Tetracycline excretion into the surrounding environment could provide the selective pressure needed to spread and maintain resistance genes in environmental isolates, as low concentrations of oxytetracycline via fecal contamination have been reported to stimulate conjugative transfer of transposons in environmental isolates (Salyers and Shoemaker, 1996). Though the usage of tetracycline was minimal during the time of this study, it is plausible that excretion of tetracycline by treated animals could provide enough selective pressure for tetracycline resistance genes to be maintained in the environment, and calves could be exposed to resistant organisms, even without treatment of that antimicrobial product. Another possibility is that calves were exposed to tetracyclines *in utero*, as tetracyclines have been reported to pass through the bovine placenta and enter fecal circulation (Huber, 1988).

Florfenicol is recommended for the treatment of bovine respiratory disease, as several disease causing agents, including *Pasteurella spp*. and *Haemophilus spp.* are highly susceptible to this drug (Prescott et al., 2000). This antibiotic is on record as being utilized for treatment at the Plateau and Grasslands centers from 1998 until 2001. The use of florfenicol at the recommended dosage of 20 mg/kg for respiratory disease or other infections caused by highly susceptible bacteria would not be expected to significantly inhibit enteric bacteria such as *E. coli* (Prescott et al., 2000). This may have led

to the development of resistant *E. coli* during previous florfenicol use, as cattle would have been exposed to a lower dose of the antimicrobial when utilized for treatment of respiratory disease than would have been effective against the enteric bacteria. This most likely led to greater recovery of florfenicol resistant bacteria in cattle-accessible areas. Resistant isolates from tadpole samples were significantly higher in cattle-accessible areas (P=0.006). In a study performed by White et al., (2000), 92% of *E. coli* (n=48) isolated from bovine diarrheal cases were resistant to florfenicol. Data from this study supports this finding, as 88% of our isolates from cattle manure were resistant to florfenicol.

Florfenicol was difficult to dissolve in a stock solution, and though the control bacteria (ATCC 29522) were eliminated within the control range (2-8μg/ml), its MIC was generally at the highest end of the range (8 μg/ml). This may indicate that the concentration of the florfenicol solution may not have been as high as required, allowing false identification of resistant isolates. Another explanation was suggested by Singer and coworkers in 2004. When 1,987 *E. coli* isolates were analyzed for resistance using MIC micobroth dilution technique, a bimodal pattern was observed with the MIC distribution. The MIC's for all isolates were either ≤16 or ≥256 μg/ml. Singer and coworkers proposed that research studies might overestimate florfenicol resistance if they were to use the MIC breakpoint of 8μg/ml for *E. coli* isolates, and suggested an alternate breakpoint value of ≥32μg/ml (Singer et al., 2004).

Isolates from tadpoles of cattle-accessible ponds showed a significantly higher resistance to sulfisoxazole compared to those from cattle-excluded ponds. Although sulfonamides are no longer frequently used therapeutically for treatment at either station, they are still occasionally utilized for the treatment of scours at the Grasslands Center. Sulfamethazine (0.0077%) is also present in the weaning diet utilized by both stations as a prophylactic treatment to help prevent scours in calves during this highly stressful period of time. Sulfonamides are extensively metabolized in the animal body, and following absorption are eliminated via urine, feces, bile, milk, sweat and tears (Huber, 1988). Elimination through urine or feces may provide a route of exposure to bacteria within the environment, leading to development of resistance. Many sulfonamides have a long duration of action because their non-ionized forms are highly lipid soluble and undergo extensive reabsorption (Huber, 1988). The persistence of sulfonamides in a cow's system may help to maintain resistant bacteria in the gut. Use of sulfonamides also can cause changes in the rumen microflora by inhibiting growth of the normal flora (Huber, 1988), and resistant microorganisms may remain and share genes with other commensal bacteria such as *E. coli.* As cows range in age from 2-13 years at the Grasslands Center, it is also speculated that these animals may harbor bacteria with resistance to this class of antimicrobial and these bacteria may be shed into the environment.

There are several possible explanations for the presence of antibiotic resistant bacteria in tadpoles, metamorphs and pond water when cattle did not have access to those areas. One possibility could be due to contamination of the aquatic environments with resistant bacteria from the watershed of the adjacent livestock containing systems. This method has been proposed by many researchers (Witte, 2000; Seveno, 2002; Ash, 2002; Biyela, 2004). Another potential explanation is that resistant microbes were already present in the soil. Reisenfeld et al., in 2004 found resistance genes present in soil microbes which have not previously been cultured using DNA isolated from those microbes. They identified nine clones expressing resistance to aminoglycoside antibiotics and one expressing tetracycline resistance and determined that soil bacteria are a reservoir of antibiotic resistance genes with greater genetic diversity than previously accounted for (Reisenfeld et al., 2004). This establishes soil microbes as a possibility for resistance genes which have not been previously discovered, as most resistance genes have been discovered via culturable microbes. Yet another possibility which may explain the prevalence of resistant *E.coli* in the cattle-excluded environments is the transfer of organisms through fecal contamination by wildlife. Cole in 2005 showed that geese from environments with agricultural activity demonstrated a higher prevalence of *E. coli* resistant to antimicrobials than those not exposed to agricultural activity. As the aquatic environments at the Plateau center were in close proximity, wildlife such as geese or other birds may expose adjacent systems with antimicrobial resistant enteric bacteria.

V. Conclusions

A low prevalence of class 1 integrons was noted in *E. coli* recovered from cattle- accessible ponds, and no integrons were detected in *E. coli* from amphibians from those ponds. However, resistance to tetracycline, florfenicol, and sulfisoxazole was noted in isolates from all samples, including those from areas not containing cattle. We conclude from this work that antibiotic resistance is widespread in *E. coli* from environments within and adjacent to cattle production systems, however, such resistance does not appear to be associated with class 1 integrons. Additional studies will be needed to determine what, if any, risks are associated with antibiotic resistance transfer between livestock and adjacent aquatic environments.

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Appendix

Figures

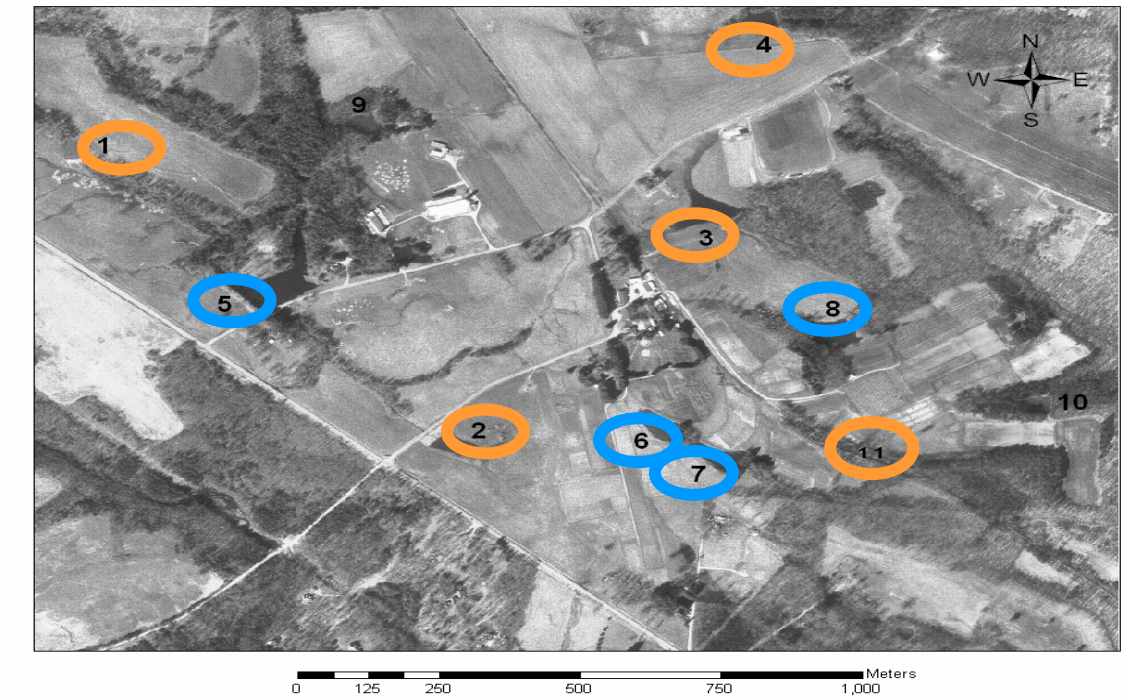


Figure 1. Aerial view of aquatic environments used for this study. Cattle-accessible environments are indicated with an orange circle (1, 2, 3, 4, 11), cattle-excluded are indicated with a blue circle (5, 6, 7, 8). Ponds 9 and 10 were not used.

A	128	128	128	128	128	128	128	128	128	128	128	128
B	64	64	64	64	64	64	64	64	64	64	64	64
C	32	32	32	32	32	32	32	32	32	32	32	32
D	16	16	16	16	16	16	16	16	16	16	16	16
E	8	8	8	8	8	8	8	8	8	8	8	8
F	4	4	4	4	4	4	4	4	4	4	4	4
G	2	2	2	2	2	2	2	2	2	2	2	2
H	0	0	0	0	0	0	0	0	0	0	0	0

Figure 2. Antibiotic dilutions for M.I.C. procedure: tetracycline and florfenicol. Dilutions were used for 96 well plates. Concentrations are in μg/ml.

A	512	512	512	512	512	512	512	512	512	512	512	512
B	256	256	256	256	256	256	256	256	256	256	256	256
C	128	128	128	128	128	128	128	128	128	128	128	128
D	64	64	64	64	64	64	64	64	64	64	64	64
E	32	32	32	32	32	32	32	32	32	32	32	32
F	16	16	16	16	16	16	16	16	16	16	16	16
G	8	8	8	8	8	8	8	8	8	8	8	8
H	0	0	0	0	0	0	0	0	0	0	0	0

Figure 3. Antibiotic dilution concentrations for MIC: sulfisoxazole. Dilutions were used for 96 well plates. Concentrations are in μg/ml.

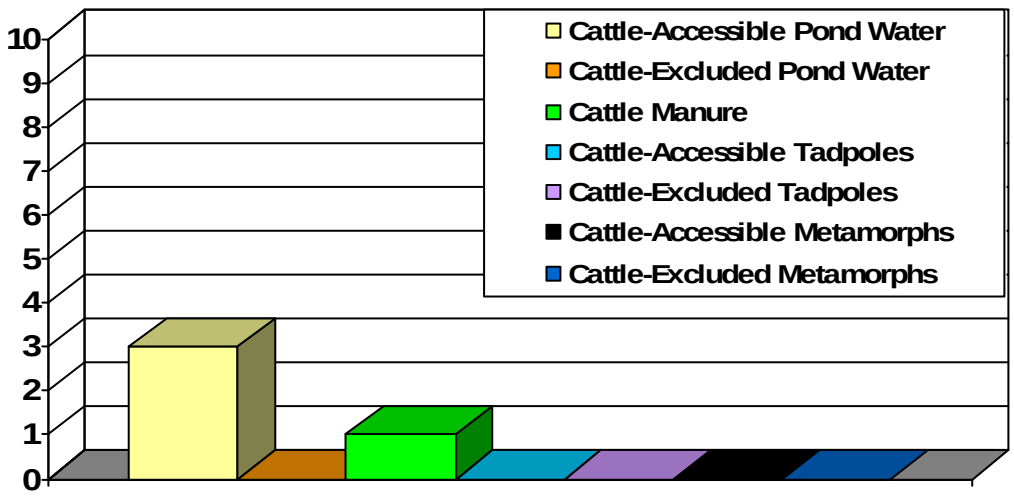
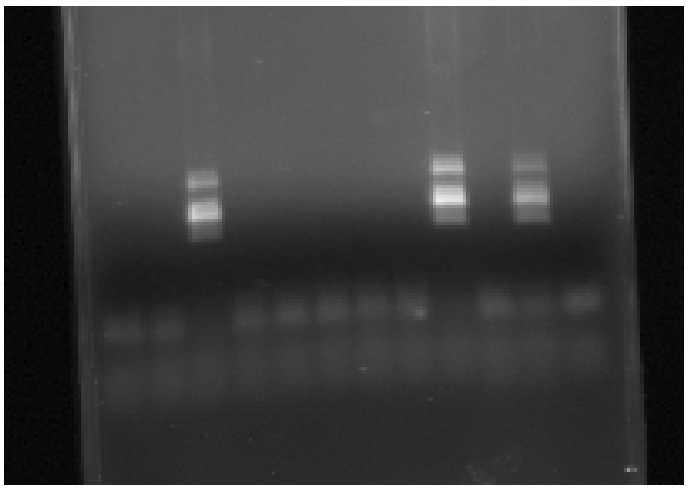
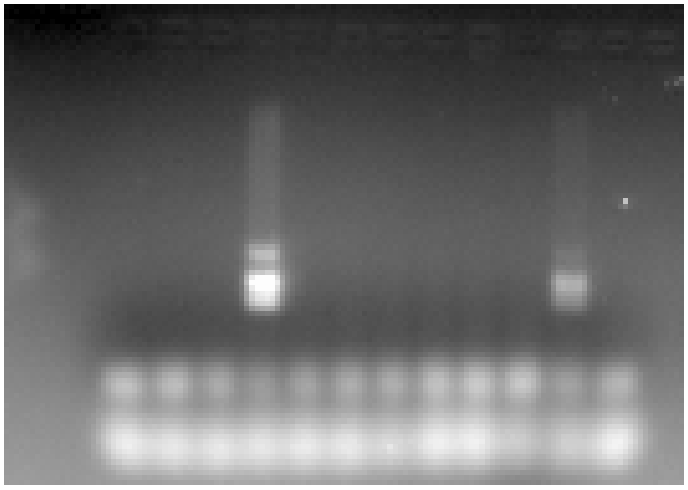


Figure 4. Prevalence of class 1 Integrons (% of positive isolates).



Lane#: 1 2 3 4 5 6 7 8 9 10 11 12

Figure 5. Integron positive isolates from pond water samples of cattle-accessible ponds. Lanes 3 and 9 were integron positive samples, lane 11 was the positive control.



Lane#: 1 2 3 4 5 6 7 8 9 10 11 12

Figure 6. Integron positive isolates from cattle manure samples of cattle-accessible ponds. Lane 4 is the positive sample, lane 11 was the positive control.

For the following figures, the designation of A and B on each graph indicates whether samples within the same type were considered to be the same, or to differ (e.g. A:A does not differ significantly, A:B does)

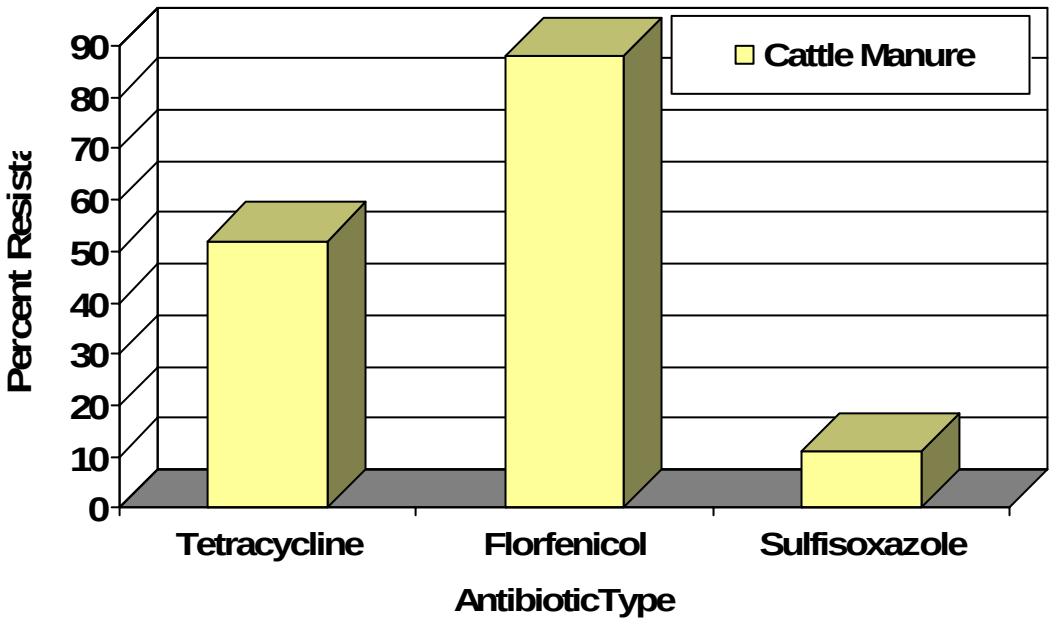


Figure 7. Resistance detected in cattle manure isolates.

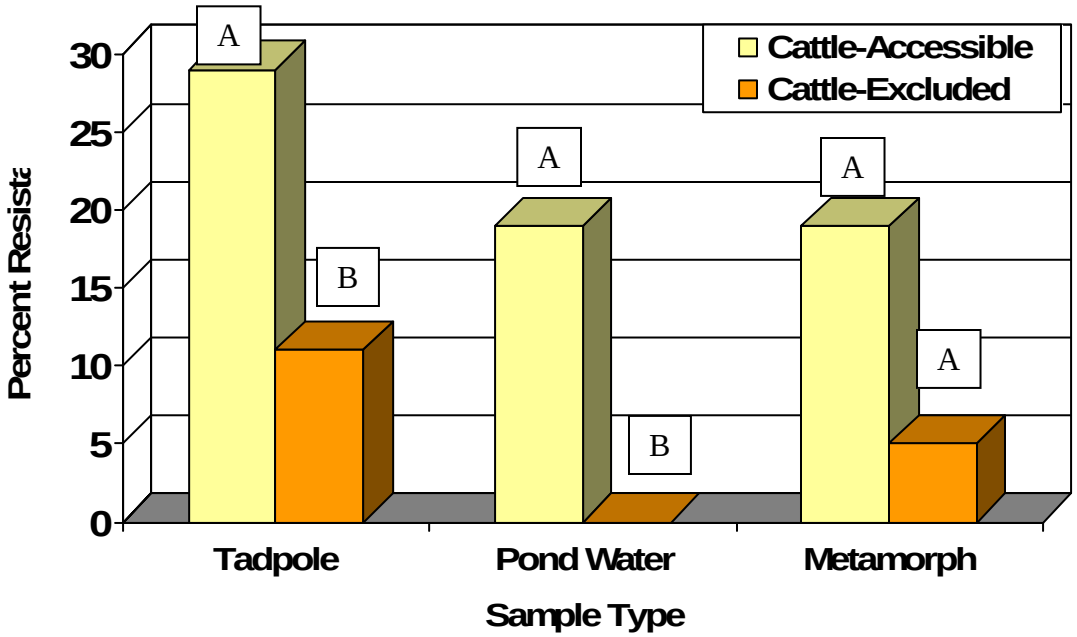


Figure 8. Percentage of samples with isolates resistant to tetracycline. Both tadpole isolates and pond water isolates differed (p=0.0001 and p=0.0283 respectively).

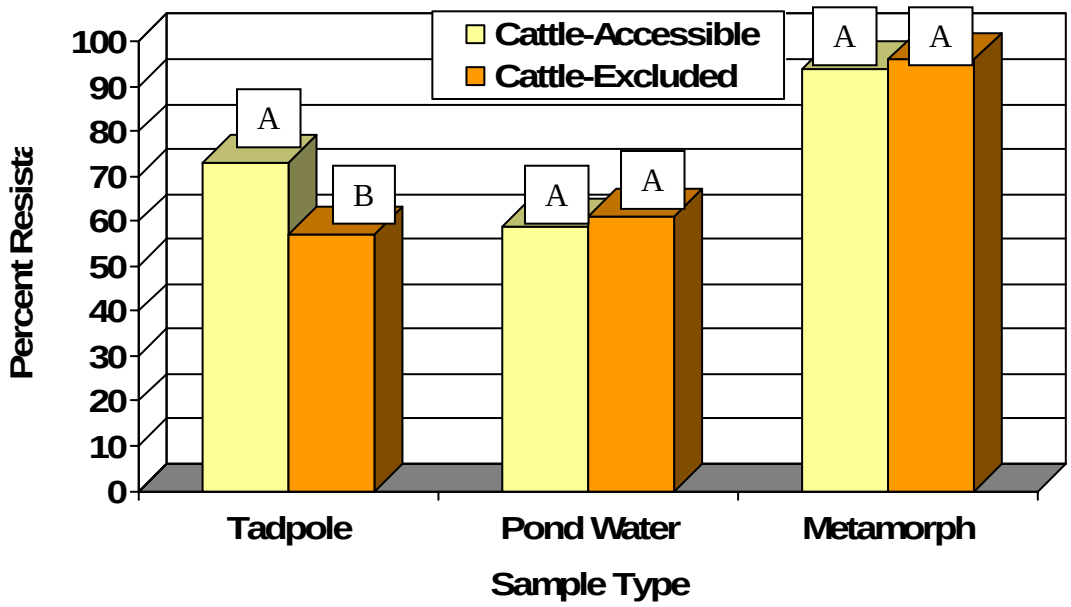


Figure 9. Percentage of samples with isolates resistant to florfenicol. Tadpole isolates differed (p=0.006).

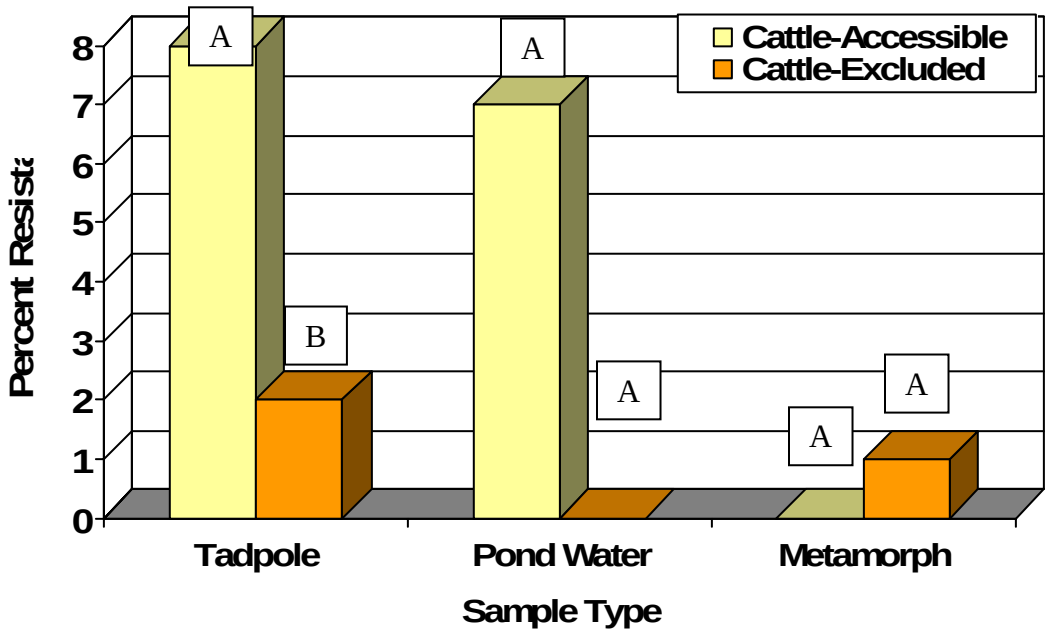


Figure 10. Percentage of samples with isolates resistant to sulfisoxazole. Tadpole isolates differed (p=0.0156)

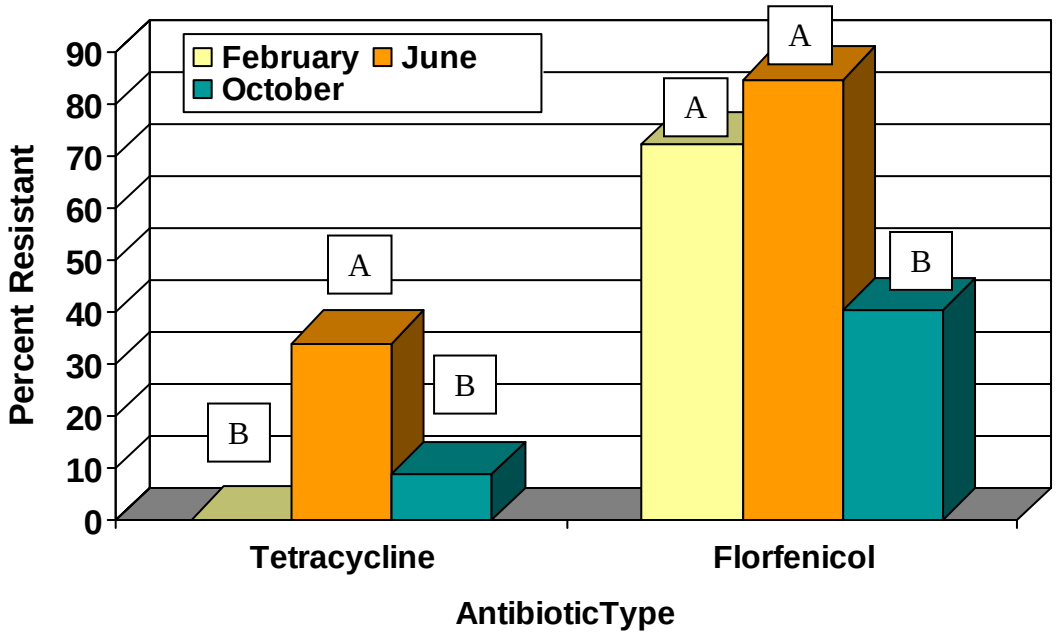


Figure 11. Effect of sampling date on prevalence of resistant bacteria. Tetracycline resistance was higher ($p<0.0001$) for tadpole samples in June than in any other month regardless of treatment type. Resistance of tadpole samples to florfenicol was higher ($p<0.0001$) in February and June than in October.

Tables

Table 1. Primer pairs used in PCR experiments.

Name	Sequence	Target	PCR product size (bp)
1) s407 (f)	atcagacgtcgtggatgtcg	*sulI1*	346
s753 (r)	cgaagaaccgcacaatctcg		
2) i965 (f)	ccttcgaatgctgtaaccgc	*intI1*	254
i1219 (r)	acgcccttgagcggaagtatc		
3) q024 (f)	gagggctttactaagcttgc	*qacEΔ1*	200
q224 (r)	atacctacaaagccccacgc		

Table 2. Antibiotics Used at Grasslands Research Center 1995-2005.

Year:	Therapeutic Antibiotics	Prophylactic Antibiotics
1995	Panmycin 500 (Tetracycline HCL) LA-200 (Oxytetracycline) Calfspan (Sulfamethazine) Albon (Sulfadimethoxine)	Bob's range mineral (with chlortetracycline 1.12 gm/lb) Preconditioning/Receiving chow CTSM 3152 (with chlortetracycline 70 gm/ton and sulfamethazine 0.0077%)
1996	Panmycin 500 LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
1997	None listed	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
1998	Nuflor (Florfenicol) LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
1999	Nuflor Sulfasure (Sulfamethazine) LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2000	Panmycin 500 Nuflor	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2001	Nuflor LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2002	LA-200 Sustain III (sulfamethazine)	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2003	None Listed	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2004	LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2005	LA-200 Sustain III	Preconditioning/Receiving Chow CTSM 3152

Preconditioning/Receiving Chow was utilized for weaning purposes only. Mineral supplement was provided on a free feed basis at locations at each pasture.

Table 3. Antibiotics Used at Plateau Research Center 1995-2005.

Year:	Therapeutic Antibiotics	Prophylactic Antibiotics
1995	Panmycin 500 (Tetracycline HCL) LA-200 (Oxytetracycline) Calfspan (Sulfamethazine) Albon (Sulfadimethoxine)	Bob's range mineral (with chlortetracycline 1.12 gm/lb) Preconditioning/Receiving chow CTSM 3152 (with chlortetracycline 70 gm/ton and sulfamethazine 0.0077%)
1996	Panmycin 500 LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
1997	None listed	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
1998	Nuflor (Florfenicol) LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
1999	Nuflor Sulfasure (Sulfamethazine) LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2000	Panmycin 500 Nuflor	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2001	Nuflor LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2002	LA-200 Sustain III (sulfamethazine)	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2003	LA-200	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2004	LA-200 Terramycin tablets (Oxytetracycline)	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152
2005	LA-200 Penicillin G Terramycin tablets	Bob's range mineral Preconditioning/Receiving Chow CTSM 3152

Preconditioning/Receiving Chow was utilized for weaning purposes only. Mineral supplement was provided on a free feed basis at locations at each pasture.

Table 4. Totals of *E. coli* isolated from each sample type with all results from all sample dates combined.

Sample Type	Cattle-Accessible	Cattle-Excluded
Cattle Manure	123	N/A
Pond Water	63	49
Bullfrog Tadpole	199	214
Green Frog Tadpole	258	160
Green Frog Metamorph	70	113

Table 5. Antibiotic resistance patterns of isolates selected for N.A.R.M.S.

analysis.

Resistance Pattern	Cattle-Accessible	Cattle-Excluded
Susceptible	0	4
FIS	1	0
TET	10	8
STR	0	1
FIS-TET	5	0
STR-TET	1	0
STR-FIS-TET	2	0
AUG-AXO-STR	0	1
KAN-STR-FIS-TET	1	0
CHL-STR-FIS-TET	1	0

FIS: Sulfisoxazole, TET: Tetracycline, STR: Streptomycin, AUG: Amoxicillin/ Clavulanic acid, AXO: Cefoxitin, KAN: Kanamycin, CHL: Chloramphenicol. Integron-positive isolates were resistant to FIS-TET (2), and FIS.

Table 6. Summary Table of Results.

A. Totals of samples taken and % of E. coli isolated from those samples.

		Sample Type								
M		**CAW**	**CEW**	**CAB**	**CEB**	**CAG**	**CEG**	**CAM**	**CEM**	**CM**
F	# samples taken:	0	0	20	20	0	0	0	0	0
E	% containing E. coli:	0	0	60	40	0	0	0	0	0
B	# of isolates:	0	0	93	71	0	0	0	0	0
J	% containing E. coli:	5	4	20	22	30	20	19	20	5
U	# samples taken:	100	100	100	82	90	85	89	100	100
N	# of isolates:	27	21	93	71	123	91	70	113	36
O	# samples taken:	4	3	1	20	49	25	0	0	20
C	% containing E. coli:	100	100	100	85	63	84	0	0	36
T	# of isolates:	36	28	5	62	135	69	0	0	36

B. Percentage of Isolates positive for class 1 integron presence and resistant to tetracycline, florfenicol, and sulfisoxazole.

	Sample Type						
	CAW	**CEW**	**CAT**	**CET**	**CAM**	**CEM**	**CM**
%Integron Positive:	3	0	0	0	0	0	1
%Resistant to **Tet:**	19	0	29	11	19	5	52
%Resistant to **Flor:**	59	61	73	57	94	96	88
%Resistant to **Sul:**	7	0	8	2	0	1	11

M= Time Sample Was Taken (February, June, or October), **CAW=** Cattle-Accessible Pond Water, **CEW=** Cattle-Excluded Pond Water, **CAB=** Cattle-Accessible Bullfrog Tadpole, **CEB=** Cattle-Excluded Bullfrog Tadpole, **CAG=** Cattle-Accessible Green Frog Tadpole, **CEG=** Cattle-Excluded Green Frog Tadpole, **CAM=** Cattle Accessible Green Frog Metamorph, **CEM=** Cattle-Excluded Green Frog Metamorph, **CM=** Cattle Manure, **CAT=** Cattle-Accessible Tadpole (Species were combined for analysis), **CET=** Cattle-Excluded Tadpole (Species were combined for analysis), **Tet=** Tetracyclines, **Flor=** Florfenicol, **Sul=** Sulfisoxazole

Vitae

Robin Lynn Cissell was born in Lebanon, KY on September 28, 1978. She lived in Bardstown, KY until the age of ten, when she moved to Elizabethton, TN. Upon graduation from Unaka High School in 1996, she attended the University of Tennessee, Knoxville where she graduated with a Bachelor of Science degree in Agriculture in May, 2001. Robin spent three years employed outside of the field of Animal Science, and in Fall of 2004 was accepted into the Department of Animal Science as a graduate research assistant under the tutorial of Dr. Alan Mathew. Upon obtaining her Master of Science degree in Animal Science, she would like to pursue a Doctor of Philosophy degree in the field of Infectious Disease.