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Characterizing the Transfer of Bacterial Antibiotic Resistance Genes Across Generations of Swine

Kimberly Nakia Garner
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Kimberly Nakia Garner entitled "Characterizing the Transfer of Bacterial Antibiotic Resistance Genes Across Generations of Swine." 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 Mathew, Major Professor

We have read this thesis and recommend its acceptance:

Michael Smith, Ann Draughon

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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Dean of Graduate Studies

(Original signatures are on file in the Graduate Student Services Office.)

Characterizing the Transfer of Bacterial Antibiotic Resistance Genes Across Generations of Swine

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

Kimberly Nakia Garner
May 2002

DEDICATION

I would like to dedicate this thesis to my parents Horace and Gloria Garner. Your love, support, advice, and guidance played an extremely important role in obtaining this degree. Thank you.

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ABSTRACT

Sows and pigs were used to characterize the origin, transfer and persistence of bacterial resistance in swine. Effects of sow's previous exposure to antibiotics and subsequent use of antibiotics in their pigs on antibiotic resistance of *Salmonella* enterica Typhimurium, *Enterococcus faecalis*, and *E. coli* were determined. Eight pregnant sows were divided into two groups, with four sows receiving oxytetracycline and four sows receiving no antibiotics. Fecal samples were obtained from sows prior to antibiotic exposure, and at 1-week intervals until pigs were weaned. Weaned pigs were challenged with *Salmonella* Typhimurium containing a nalidixic acid. Pigs from each sow treatment group were divided equally between a subtherapeutic antibiotic treatment regimen or exclusion of antibiotics. Pigs on the antibiotic treatment received apramycin at 150 g/ton of feed, beginning 7 days postweaning and lasting for 14 days, followed by oxytetracycline at 50 g/ton throughout the grow/finish period. Fecal samples were obtained from the pigs while on the sows and at 2, 7, 14, 30, 60, 114 and 115 days postweaning. The *Salmonella* challenge organism, *E. coli* and *E. faecalis* were recovered and tested against both apramycin and oxytetracycline using a minimum inhibitory concentration (MIC) analysis. Data were analyzed using the mixed models procedure of SAS. Polymerase Chain Reaction and transformation techniques were used to characterize genetic resistance elements and determine if the location of such gene sequences. Random apramycin-resistant *E. coli* isolates (n = 110) were chosen from antibiotic treated sows and pigs, non-antibiotic treated sows and pigs and environmental manure to test through PCR,

plasmid profiling, and macrorestriction analysis. Treatments affected antibiotic resistance to the greatest extent in *E. coli*, compared to *Salmonella* Typhimurium and *Enterococcus faecalis*. The greatest resistance to apramycin occurred in *E. coli* isolates from nursing pigs on sows that had earlier exposure to tetracyclines, and from pigs treated with apramycin during the postweaning period. Resistance to oxytetracycline was consistently high throughout the study in isolates from all pigs and sows, including those with no previous exposure to that drug. Genes responsible for apramycin resistance were found in approximately 90% of resistant isolates and their location was determined to be on bacterial plasmids. It was also determined that several different types of *E. coli* contained the *aac*(*3*)-IV gene responsible for apramycin resistance. These results indicate that apramycin and tetracycline resistance in *E. coli* was affected by previous use of tetracycline in sows ($P \geq 05$). Additionally, subsequent use of antibiotics in pigs also affected ($P \leq 05$) resistance levels in *E. coli*, whereas *Salmonella* Typhimurium and *Enterococcus faecalis* were not affected by antibiotic use in sows or pigs.

Key Words: antibiotic resistance, swine, *E. coli*

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1. LITERATURE REVIEW

Antibiotic Use in Agriculture

The use of antibiotics has remained an integral element of the animal industry since the discovery of its benefits in the early 1950s (Lee et al.,1993). Livestock production utilizes approximately half of the antibiotics produced in the United States (Levy, 1986). The two primary uses of antibiotics include treatment or prevention of diseases (therapeutic) and enhancing production performance or improving feed to gain ratios (subtherapeutic). Antibiotics that are used therapeutically are generally applied after the onset of a disease condition and used according to label instructions or in accordance with a licensed veterinarian. The subtherapeutic use of antibiotics includes low doses (<200g/ton of feed) over longer periods of time (NRC, 1999). The effectiveness of subtherapeutic antibiotics lies in their ability to improve the health of an animal while enhancing their growth and production by reducing the amount of nutrients required for maintenance and reducing gut wall thickness (Cromwell, 1991).

These discoveries have opened the door to the development of intensive animal production practices, which have allowed the reduction in the number of farms while continuing to meet consumer demands (NRC, 1999). Highly intensive operations are able to manage elevated production with less labor and capital because of the use of subtherapeutic antibiotics (Hurt et al., 1992). Wade and Barkley (1992), estimated that the use of subtherapeutic drugs saved the United States swine industry approximately $2

billion per year in production costs; which translates to consumers saving approximately $0.04 per pound of pork.

Risks Associated with Antibiotic Use

The discovery of potent antimicrobial agents was one of the greatest contributions to medicine in the 20th Century (File, 1999). However, although the use of antibiotics has had a significant influence on the advancement of the animal industry, such use has been linked to the emergence and persistence of populations of animals shedding bacteria that are resistant to one or multiple antibiotics (Novick, 1981; Dawson et al, 1983; Dunlop et al., 1998). The yearly expenditures arising from drug resistance in the United States are estimated to approach $4 billion and are continuously rising (File, 1999).

Much scientific effort has been expended to address the antibiotic resistance problem. Several investigations have been conducted to study the consequences of feeding antibiotics subtherapeutically to chickens. In one study, 300 three-month old chickens were divided into either a treated group, fed 110 mg/kg of oxytetracycline, or a control group that remained on similar but antibiotic-free feed (Levy et al., 1976). Evaluation over a period of time revealed that chickens receiving oxytetracycline-supplemented feed began excreting an increasing amount of tetracycline-resistant bacteria, whereas similar organisms from the control group of chickens remained largely sensitive. Also discovered in the oxytetracycline treated group, was the presence of multiple resistant isolates within the first 3 months of the study. The control group, on the other hand did not exhibit this effect. Studies such as this demonstrate an increase in

selection of resistance elements as a result of long-term subtherapeutic antibiotic exposure.

Much concern has arisen over the development of antibiotic resistant bacteria in livestock and its relevance to human health (Wray et al., 1986; Hunter et al., 1993; van Bogaard and Stobberingh, 2000; Berends et al., 2001). Zoonotic bacteria such as *Salmonella* and *Campylobacter* are often problematic in pork and poultry products. Additionally, bacteria that are primarily non-pathogenic opportunists, such as *E. coli* and enterococcus, have the ability to transfer their resistance genes to pathogenic bacteria and thus are also of concern (Berends et al., 2001). Research has documented food animals as the source of 69% of resistant salmonella infections in humans and 46% of susceptible salmonella outbreaks (Holmberg et al., 1984). A recent study investigated gentamicin-resistant *E. coli* that were also resistant to apramycin (Hunter et al., 1993). Both drugs belong to the aminoglycoside family; however, gentamicin is used for both animal and human health whereas apramycin is used exclusively in animals. This study tested 93 gentamicin-resistant *E. coli* isolates from a local hospital for resistance to apramycin. Twenty-six percent of the isolates were determined to be resistant to apramycin. The proportion of gentamicin-resistant isolates, which were also resistant to apramycin, increased from 16% in 1981-5 to 40% in 1986-90 (Hunter et al., 1993).

Another study focused on the presence of apramycin-resistant *E. coli* in association with a stockman working on a pig farm in which pigs were treated with apramycin for outbreaks of neonatal and postweaning colibacillosis (Hunter et al., 1994). Apramycin-resistant *E. coli* isolated from both the stockman and a pig contained similar

plasmid profiles and identical antibiotic resistance patterns. This suggests that the stockman received the apramycin-resistant *E. coli* through contact with the pig. However, these findings do not eliminate the possibility that the stockman may have picked up the resistant gene through the consumption of a pork product. Therefore, more research on human contact with animal products and comparisons of resistance patterns using techniques such as plasmid profiling and DNA fingerprinting is needed to provide a clearer understanding of the problem.

Environmental Factors

Some findings suggest that factors other than antibiotic exposure may contribute to a high prevalence of antibiotic-resistant bacteria and the pervasiveness of bacteria resistant to multiple antibiotics found in animal manure (Dawson, 1984; Langlois, 1988; Mathew, 1998). The effect of age and housing location on antibiotic resistance was examined by Langlois et al. (1988). Sows used in the study were taken from herds that had not had antibiotic exposure for 126 months prior to the initiation of the experiment. Sows were raised on pasture during gestation and subsequently moved into an environmentally controlled farrowing unit. Upon weaning pigs were grown and finished on concrete flooring in a finishing unit. The proportion of resistant bacteria was generally higher in pigs 6 months of age or less. Housing also had an effect, as pigs from sows raised on pastures exhibited the greatest number of isolates that were sensitive to the 13 antibiotics used in this stud, conversely bacteria from pigs housed in the farrowing house or finishing unit expressed a higher amount of resistance.

Mechanisms of Action

The antimicrobial actions of antibiotics are diverse and involve various cellular functions and structures. Antibiotics usually operate by inhibiting an important function of the bacterial cell for survival or replication (Bryan, 1982). Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria. They act by preventing both enzymatic and non-enzymatic binding of aminoacyl-tRNA to the A site of the ribosome, thereby inhibiting protein synthesis. This is achieved through the action of one *tet* molecule, which binds strongly to the 70s ribosome preventing aminoacyl-tRNA from binding to that site. Weak binding also occurs at the 30s subunit, further preventing essential aminoacyl-tRNA binding (Bryan, 1982; Huber, 1988; Levy, 1984). Additionally, tetracyclines inhibit polypeptide chain termination by inhibition of the interaction of termination factors RF_1 or RF_2 with termination codons (Bryan, 1982).

Aminoglycosides are bactericidal antibiotics that act through the inhibition of protein synthesis. They are the only protein inhibitors with "cidal" action (Purdue, 1996). Aminoglycosides are known to create a firm bond with the structural component of the 30s ribosomal subunit to inhibit protein synthesis. The bonding of aminoglycosides is much stronger than that created by other protein synthesis inhibitors, possibly accounting for their bactericidal action (Purdue, 1996).

Mechanisms of Resistance

Bacteria have developed survival mechanisms that impede the action of antibiotics. The primary mechanism of tetracycline resistance is reduced accumulation of tetracycline by the alteration of ribosomes, preventing the binding of tetracycline (Salyers

et al., 1990). Active efflux of tetracycline is another resistance mechanism that is found in both gram-negative and gram-positive organisms and has been well documented (Salyers et al., 1990; Chopra et al., 1992; Thanassi et al., 1995; Roberts, 1996). There are eighteen *tet* genes and one *otr* gene that code for efflux pumps. These genes code for membrane-associated proteins which export tetracycline from the cell (Thanassi, 1995; Chopra and Roberts, 2001). Efflux genes associated with gram-negative bacteria are widely distributed and are usually associated with large plasmids, which are mostly conjugative. Gram-positive bacteria contain efflux genes that are primarily found on small transmissible plasmids, which occasionally become integrated into the chromosome (Chopra and Roberts, 2001). Bacteria also confer resistance to tetracyclines through ribosomal protection proteins (Taylor and Chau, 1996; Chopra and Roberts, 2001). Nine *tet* genes which code for ribosomal protection proteins have been described. These proteins protect the ribosomes from the action of tetracyclines and confer a wider spectrum of resistance to tetracyclines (Chopra and Roberts, 2001). Other mechanisms causing resistance to tetracyclines have recently been determined, but are not well unknown. For example, the *tet*(X) gene is responsible for resistance through the enzymatic alteration of tetracycline. However, this gene functions only in the presence of both oxygen and NADPH and has only been associated with *Bacteroides*, which is an anaerobic host. The *tet*(U) gene confers low levels of tetracycline resistance through the production of a small protein; however, the mechanism of action remains unknown (Chopra and Roberts, 2001).

Three primary mechanisms are associated with resistance to aminoglycosides. These include: 1) decreased transport across the cell membrane to prevent access to the ribosomes in the cytoplasm, 2) ribosomal target modification preventing antibiotic binding, and 3) expression of aminoglycoside-modifying enzymes (Mortensen et al., 1996).

Impaired transport of aminoglycosides across the bacterial cell membrane does not appear to be mediated by plasmids. Transport is an oxygen-dependent process, therefore anaerobic bacteria are resistant to aminoglycosides because they lack an oxygen-utilizing transport system. Although the clinical importance of this mechanism is unknown, it has been described as responsible for low-level resistance among facultative aerobes and enterococci (Dworzack, 1984).

Modification of the ribosomal target is also an example of nonplasmid-mediated resistance to aminoglycosides. One mutation in the ribosomal protein may cause a decrease affinity for the drug. Although this resistance mechanism is rarely encountered in Gram-negative species, it has been observed in both *E. coli* and *P. aeruginosa*. Current research has investigated the possibility of resistance to apramycin occurring through ribosomal mutations (Vasiljevic et al., 1993). In this study, it was determined that ribosomal mutations responsible for apramycin had occurred and were located in two different positions. However, more research is underway to verify this process.

The primary mechanism of resistance to aminoglycosides is the production of modifying enzymes encoded by genes often which exist on tranposons. The enzymes include three acetyltransferases (AAC) that acetylate amino groups, five

phosphotransferases (APH) that phosphorylate hydroxyl groups and four nucleotidyltransferases (ANT) that adenylate hyroxyl groups (Mortensen et al., 1996). Resistance to apramycin is a result of N-acetylation by a single enzyme of aminoglycoside acetyltransferase 3 class type IV (AAC(3)-IV). This enzyme is also capable of modifying gentamicin and tobramycin, which are important antibiotics associated with human medicine (Barnes and Hodges, 1984; Mortensen et al., 1996).

Sources of Antibiotic Resistance

Many advances have been made in the study of antibiotic resistance since the introduction of molecular biological techniques. Such knowledge has allowed researchers to follow the spread and evolution of resistance genes in various situations (Amyes, 1998). Bacteria found in nature contain multiple mechanisms for antibiotic resistance. Human and animal populations are prime examples of reservoirs of resistance genes (Baquero et al., 1998; Hooper 2001). There has been much debate as to whether bacteria developed resistance to antibiotics as a result of their selective pressure or if a random genetic drift was the influential source (Baquero et al., 1998). Current research indicates that bacteria become resistant to an antibiotic either intrinsically or through acquisition (Amyes, 1998; Hancock, 1998; Maiden, 1998; Levy, 1999).

Intrinsic resistance indicates the occurrence of natural resistance to an antibiotic by the majority of the population of bacterial species (Bryan, 1982). For example, a Gram-negative bacterium may express a permeability barrier on its outer membrane that prohibits the influx of an antibiotic into the cell. Similarly, there have also been accounts

of antibiotics failing to be transported across the cellular membrane due to the lack of a transport system (Hancock, 1998).

Populations of bacteria previously sensitive to antibiotics can develop resistance through acquisition. Two genetic processes drive acquired resistance (Bryan, 1982; Maiden, 1998; Houndt and Ochman, 2000; Berends et al., 2001). The least commonly observed mechanism outside of the laboratory is mutational resistance. This type of resistance often allows microorganisms to withstand relatively high levels of an antibiotic without an effect (Houndt and Ochman, 2000). However, sometime several mutations are required to generate an allele encoding a resistant protein. Therefore, this mechanism is relatively rare and most useful to the bacteria when combined with other mechanisms (Maiden, 1998).

The most often noted method of acquired resistance is through the exchange of genetic material from one bacterial species or strain to another. Plasmids and transposons are the transmission vectors in approximately 80-90% of all cases of resistant bacteria (Berends et al., 2001). Plasmids are circular DNA elements that usually carry genes for antibiotic resistance and virulence factors, thereby supplying bacteria with additional survival measures. They can become incorporated into the chromosome or they can exist as an extrachromosomal DNA. Resistance plasmids or R-plasmids can carry one or multiple genes coding for resistance to a single or several antibiotics (Bryan, 1982).

Gene transmission occurs through transduction, transformation or conjugation (Brooks et al., 1991; Burton, 1992; Berends et al. 2001). Transduction involves the carrying of genetic material from one bacterial cell to another in the process of infection

by a temperate bacteriophage (Burton, 1992; Guthrie, 1992). As the phage infects the cell, viral DNA becomes a part of the bacterial chromosome. During cell lysis, as the chromosome disintegrates, mature phages carrying fragments of the bacterial chromosome may infect other cells and introduce foreign bacterial DNA into the host. Therefore, as the virus forms and proceeds to infect other cells, genes encoding antibiotic resistance may be carried in the protective surroundings of the virus (Brooks et al., 1991; Burton, 1992; Guthrie, 1992).

The process of transformation is seen in fewer bacterial species than transduction. In transformation, DNA reaches recipient bacteria without a carrier (Guthrie, 1992). For this reason it is more difficult for DNA to become incorporated into a recipient host. DNA from a donor cell can only penetrate the cell wall of a competent recipient, which is usually during the late logarithmic growth phase. At this time, the cell has an increased permeability to DNA (Burton, 1992).

Conjugation is the most commonly observed method of gene transmission. Self-transmissible plasmids carry *tra* genes coding for transfer. Some self-transmissible plasmids can aid in the transfer of nontransmissible plasmids or portions of the chromosome (Brooks et al., 1991). In conjugation, a donor cell extends its sex pilus to form a pilus bridge connecting to a recipient cell. Genetic material is then transferred from the donor to the recipient (Burton, 1992). This method of transfer is seen very often in enteric bacteria and in the transfer of genes coding for antibiotic resistance. Bacteria of the genus *Salmonella* are among the most often associated with this transfer process (Poppe et al., 1996).

Salmonella enterica serovar Typhimurium

Salmonella are species of non-spore-forming, gram-negative, facultative intracellular bacteria first discovered in 1884, that belong to the Enterobacteriaceae family (Guthrie, 1992; Roof et al., 1992). This genus contains over 2,300 serovars, with additional serovars being added continuously. The antigens that distinguish the serovars of *Salmonella* are somatic (O), flagellar (H), and capsular (K). Currently, only two species of *Salmonella* are recognized, *Salmonella enterica* and *Salmonella bongorii*. *Salmonella enterica* is the pathogen most often studied and consists of six subspecies, each containing multiple serovars (Schaechter et al., 1999).

Infections are usually acquired through the fecal-oral route. Following ingestion, *Salmonella* must survive the acidic pH of the stomach in large numbers to set up an infection (Guthrie, 1992). Once bacteria reach the small intestine, they must attach to and penetrate the mucosa and their traveling to the midlayer of this membrane. Epithelial cells consume the organism and serve as a protective host, allowing *Salmonella* to be distributed throughout the body. Intracellular lesions may develop due to microvascular damage and the formation of blood clots (Schwartz, 1993). These invasive organisms also induce diarrhea through malabsorption and fluid leakage from the inflamed bowel. Phagocytic cells accumulate and cause tissue damage, ultimately resulting in sodium resorption and chloride secretion leading to a loss in fluids (Roof et al., 1992). Some strains can additionally produce an enterotoxin to aid in the production of diarrhea (Guthrie, 1992; Roof et al., 1992).

Salmonellosis in pigs usually occurs in high intensity production systems in pigs younger than four months of age (Roof et al., 1992). *Salmonella enterica* serovar Choleraesuis is the most frequently occurring cause of salmonellosis in swine found in the United States. However, this pathogen is host-adapted and rarely found in non-swine sources (Anderson et al., 2000). On the other hand, the second most frequent cause of salmonellosis in swine is *Salmonella enterica* serovar Typhimurium, which is a zoonotic organism and is frequently isolated from a variety of species, including humans (Wood, 1989).

Stress has been noted to cause an increase in the amount of *Salmonella* shedding occurring in pigs. Poor sanitation has also been proven to influence the shedding patterns of swine. Funk and others (1999) demonstrated that pigs housed in an environment with an extreme accumulation of manure showed a higher amount of shedding than when moved to clean pens. The stress of transport, overcrowding in holding pens and rough handling prior to slaughter have also been documented to enhance shedding of *Salmonella spp.* (Moro et al., 1998; Isaacson et al., 1999). In addition, a number of reports have indicated an increase in the shedding of antibiotic resistant isolates associated with transportation stress (Molitoris et al., 1987; Moro et al., 1998; Langlois and Dawson, 1999). Langlois and Dawson (1999) concluded that moving pigs from their housing area to a truck resulted in an increase in resistance to twelve antibiotics tested in the study. An additional 30 minutes of transport resulted in the recovery of a greater amount of resistant isolates, further showing transport is a factor in the increased shedding of antibiotic resistant *Salmonella*. Stress-related factors might alter the amount

of *Salmonella* shed, as well as the number of isolates resistant to antibiotics. This has caused concern because these bacteria may transfer antibiotic resistance to human pathogens.

Salmonellosis is the leading cause of foodborne illness in human beings worldwide (Nair et al., 1995). It is estimated that approximately 4 million people become sick and up to 4,000 people die each year because of infection (Isaacson, 1999). The most dominant effect has been noted in young children, elderly and immuno-compromised people (Poppe, 1996). Costs associated with treatment of salmonellosis have ranged from $0.69 to $3.8 billion per year, making it the most costly foodborne illness to treat (Isaacson, 1999).

Resistance of *Salmonella* to antimicrobial agents is not uncommon in environmental, human, and animal isolates and may be caused by the use of medicated feed or water (Poppe, 1996). Although salmonellae do not habitually reside in hosts treated with antibiotics and are likely to have experienced different selective pressures for resistance than commensal organisms R plasmids and other genetic elements conferring resistance can be efficiently maintained and disseminated within this species by conjugation, transformation, and transduction (Houndt and Ochman, 2000). Infections caused by antimicrobial-resistant salmonellae are increasing and have become a cause for public concern (Nair et al., 1995).

Enterococcus faecalis

Enterococci are gram-positive, ovoid and non-sporing bacteria. They can be found either singly, in pairs, or as short chains (Hardie and Whiley, 1997). Billroth

(1874), discovered chain-forming cocci in wounds and named them *streptococcos* (Hardie and Whiley, 1997). The enterococci as a group were first described in 1899 by Thiercelin, and the genus *Enterococcus* was proposed by Thiercelin and Jouhaud (1903) for gram-positive diplococci of intestinal origin (Franz et al., 1999).

In 1933, Lancefield developed a serological typing system for streptococci in which it was determined that those of fecal origin contained the group D antigen (Franz et al., 1999). It was not until 1984 that Schleifer and Kilpper-Balz discovered that D streptococci were transferred to a new genus *Enterococcus* (Hardie and Whiley, 1997).

These organisms are commensal bacteria that make up an important part of the intestinal flora in man and animals. They are among the most common bacteria found in the environment and are released through animal and human feces (Iversen, 2000). Enterococci are listed as the third cause of nosocomial infections and there has been a rapid increase of glycopeptide and high-level aminoglycoside-resistant strains (Dicuonzo et al., 2001). *Enterococcus faecalis* and *Enterococcus faecium* account for greater than 95% of enterococcal infections detected in humans (Dicuonzo et al., 2001).

Enterococci are found to be intrinsically resistant to a number of antibiotics including cephalosporins, penicillins, carbapenems, β-lactams and aminoglycosides (Morrison et al., 1997). In addition to intrinsic resistance, genetic resistance elements are responsible for resistance to all classes of antimicrobials, including chloramphenicol, tetracyclines, macrolides, streptogramins and lincosamides. Aminoglycoside-resistance stems from reduced membrane permeability (Morrison et al., 1997). As such, an

increasing number of enterococci are expressing high-level resistance genes to aminoglycosides, making it difficult to treat enterococcal infections (Sahm, 1991).

Escherichia coli

Escherichia coli (*E. coli*) was first discovered by Theodor Esherich in 1885 when it was isolated from normal infant feces. It was initially named *Bacterium coli commune*. "*B. coli*" was difficult to distinguish from *Shigella* organisms and was thought to be the cause of dysentery, although the notion was later discarded (Sussman, 1985). *E. coli* belongs to the Enterobacteriaceae family and is the lone member of the genus *Escherichia*. This organism is a short Gram-negative, facultatively anaerobic and non-sporing bacillus (Sussman, 1985).

E. coli is a member of the normal intestinal flora of man and animals and colonization takes place soon after birth. The source of infection is most often in the mother and/or the inanimate environment (Sussman, 1985). These commensal organisms may serve as a reservoir of resistance genes for potentially pathogenic bacteria as they are found to harbor several transferable R-elements. The amount of resistance conferred in these organisms is often used as an indicator for selection pressure by antibiotic use (Bogaard and Stobberingh, 2000).

Tetracyclines, which are used heavily in the livestock industry, have influenced the production of mutant *E. coli* that have become increasingly resistant to tetracyclines. Dunlop and coworkers (1998) determined that among seven antibiotics tested, the highest percentage of resistant isolates was resistant to tetracycline (approximately 70%). It has also been postulated that the use of tetracyclines in feed may encourage the occurrence

and persistence of *E. coli* resistant to other antibiotics, such as apramycin (Hunter et al., 1992).

Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a technique used to amplify a specific DNA region (Newton and Graham, 1997). The reactions require oligonucleotide primers, which are short, single stranded DNA molecules, complementary to the ends of the defined DNA template (Newton and Graham, 1997). Several variations and cycles may be used in PCR, but each protocol follows the same basic steps: denaturation, annealing, and polymerization. Denaturation is performed by heating DNA to approximately 92-95°C. The specific temperature is determined by the requirements of PCR templates, thermal cyclers, and types of tubes used. The initial step of heating causes the strands of DNA to separate to single stranded DNA (Eckert and Kunkel, 1991). The annealing step is a cooling process immediately following denaturation, allowing primers to anneal to the specific target regions. The temperature at this step is dependent upon the melting temperature of the primers as dictated by their length and G + C content. Primer extension or polymerization then takes place using Taq DNA polymerase, a thermo-stable DNA polymerase. This enzyme serves to add free dNTPs to the primers making a copy of the template. The process is usually takes only about two minutes, however longer amplicons may require additional time. During this step, the temperature is raised to approximately 70-75°C, which is the optimal temperature for Taq (Eckert and Kunkel, 1991). The cycle begins again with the denaturation step and is repeated according to the amount of amplification required.

Electroporation

Electroporation is a cell membrane phenomenon involving the use of a charge-induced mechanism as an energy source to create pores. These pores create avenues through which materials may enter the cell (Weaver, 1993). The most frequently used application of electroporation today is transfection, which involves the introduction of exogenous DNA into host cells (Weaver and Chizmadzhev, 1996). Electroporation can be utilized with a varying degree of cell types including primary cells from tissue isolates, plant protoplasts, and bacterial cells (Miesfeld, 1999). The basic steps of this technique are performed first by placing cells into glycerol or a buffered saline solution containing a small amount of DNA. This process masks the overall negative charge of cells therefore preventing the DNA elements from repelling each other. Then the suspension is placed into a special electroporation cuvette that contains positive and negative electrodes connected to a power supply. Subsequently, the cuvette is placed into a machine conferring an electric shock, which introduces pores into the cell and attracts DNA to the positive cathode. The electric field strength and length of time exposed to the electric field varies based on a particular cell type (Miesfeld, 1999). A major difference between eukaryotes and prokaryotes is the amount of voltage required to observe the most favorable results. This difference can be noted between the high efficiency electroporation of *E. coli* cells that require 2.5 kV and most mammalian cells, which require only 0.25 kV. Some DNA will enter the cells in the cuvette and become trapped on the way to the cathode.

Objectives of this research

Through the results indicated from past research it was hypothesized that the subtherapeutic use of antibiotics in animal feeds may cause an increase in the population of resistant bacteria and these bacteria may be passed through generations of animals. One objective of this study was to determine whether sow's previous exposure to antibiotics and the subsequent use of antibiotics in pigs had an effect on antibiotic resistance. Other objectives were to characterize genetic resistance elements from sows and pigs, determine the location of such gene sequences and determine a relationship between genetic resistance elements found in sows, pigs and the environment.

2. MATERIALS AND METHODS

Sow housing and treatments

Eight gilts with no prior exposure to antibiotics were purchased from the Pig Improvement Company (PIC) in Franklin, KY and transported to the University of Tennessee Johnson Animal Research and Teaching Unit (JARTU) in Knoxville, TN. Upon arrival at JARTU gilts were bred using artificial insemination procedures and housed in identical rooms with 8' x 8' finishing pens at two pigs per pen. By the use of ultrasound procedures, it was determined that four out of eight gilts conceived successfully. Three weeks prior to the expected farrowing date, four pregnant gilts with previous antibiotic exposure (tetracylines) were obtained from the University of Tennessee Blount County Experiment Station (Louisville, TN) to replace the four PIC gilts that did not conceive. Pregnant sows were separated according to antibiotic exposure and placed into two identical biosecure farrowing rooms. Each room contained four farrowing crates and separate ventilation and waste removal systems. Two weeks prior to farrowing, sows with previous antibiotic exposure received subtherapeutic concentrations of oxytetracyclines (10mg/lb body weight) via the feed, whereas the other sow group, without previous antibiotic exposure, received no antibiotics. Upon farrowing, antibiotic use was discontinued and all sows and pigs were maintained with normal production procedures.

Pig housing and treatments

Pigs were housed along with sows in farrowing crates until weaning at 21 days of age. Upon weaning, pigs were blocked by litter, grouped according to sow treatment and

moved to identical segregated early weaning nursery rooms at the JARTU Tennessee Agricultural Experiment Station of Knoxville, TN. Nursery rooms consisted of separate environmental and waste removal systems to reduce risk of cross contamination. One week post-weaning, pigs were challenged intranasally with approximately 10^7 colony-forming units (CFU) of *Salmonella enterica* serovar Typhimurium (National Animal Disease Control, USDA, Ames, Iowa). This isolate contains a naladixic acid resistance marker to assure subsequent isolation and identification. The challenge organism was prepared by innoculating XLT_4 agar (BBL, Becton Dickerson Microbiology Systems, Sparks, MD) containing naladixic acid and incubating at 37°C one day prior to the challenge. The morning of the challenge, a loopful of organism was placed into 200 mL nutrient broth (Bacto beef extract 3g/L, Bacto peptone 5g/L) containing naladixic acid (Sigma, St. Louis, MO) and incubated in a shaker at 37°C for approximately 8 hours. The culture was then maintained on ice during transportation to JARTU where the animals where held. Each pig received 2 mL of inoculate per nostril and 1 mL of inoculate orally for a total of 5 mL of *Salmonella* culture per pig.

Beginning 7 days postweaning, two pig groups from each sow treatment received apramycin in the feed (150g/ton) for 14 days, followed by oxytetracycline in the feed (50g/ton) for the remainder of the experiment; whereas antibiotics were excluded from the feed of the other pig groups. The control group consisted of pigs from the non-antibiotic sow group and received no antibiotics throughout the study. At 60 days postweaning, pig rooms were further assigned to either a high sanitation (daily room cleaning) or low sanitation (no cleaning and allowing manure to accumulate) regimen

such that each of the above treatments was represented in each sanitation treatment (Table 1). At the end of the experiment, three pigs from each treatment group (n=24) were transported to a common holding facility one hour away at the Plateau Experiment Station at Crossville, TN and intermingled to simulate the effects of transport and holding stress prior to slaughter. All other pigs (n=32) remained in the original isolation facility through the final sampling period.

Sampling

Two swabs (Fisherbrand Dacron Sterile Swabs, Houston, TX) were used to collect fecal samples rectally from the sows prior to antibiotic exposure, and at 1-week intervals until the pigs were weaned. Pigs were sampled rectally (Fisherbrand) whenever the sows were sampled postfarrowing, two days following weaning (just prior to *Salmonella* challenge), 7 days postweaning (prior to assignment to antibiotic treatments), and 14, 30, 60, 114 (prior to transport of pigs), and 115 (following transport of pigs) days postweaning. Samples were obtained for the recovery of *Salmonella* Typhimurium (challenge organism), commensal *Escherichia coli*, and commensal *Enterococcus faecalis*.

Environmental samples were obtained once monthly from each treatment room. Swab samples were acquired from the floor and wall area surrounding the pens, and skin from pigs housed within the pens. Other samples were taken from feed, water, manure and air from each individual room and pen.

Disposable biohazard suits (Fisher, Suwanee, GA) and gloves (Diamond Grip Microflex, Reno, NV) were worn and changed between each room to decrease risk of

cross-contamination and as a personal safety measure. Disposable boots (Nasco, Ft. Atkinson, WI) were cleaned and disinfected between rooms by way of a footbath containing Nolvasan Solution and water. Samples were maintained on ice in sterile test tubes and containers and immediately transported to the laboratory at Knoxville, TN.

Microbiological Procedures

Upon arrival, one swab from each pig was used for the isolation of *E. faecalis*. Swabs were added to individual stomacher bags (Seward Model 80 Tekmar, Cincinnati, OH) containing 80 mL of Enterococcsel Broth (BBL, Becton Dickerson Microbiology Systems, Sparks, MD) and then incubated at 35°C for 24 hours. After 24 hours, 10μL of sample was streaked onto Streptosel agar (BBL), that contained .04% potassium tellurite (Sigma) and these plates were incubated for 48 hours at 35°C. Biochemical tests were performed using APIStrep strips (Vitek bioMerieux, Syosett, New York) to confirm that isolated bacteria were *E. faecalis*.

The second swab was streaked onto lactose MacConkey agar (Difco, Sparks, MD) and incubated at 37°C for 24 hours to isolate *E. coli*. Colonies demonstrating the characteristic pink coloration of *E. coli* were selected. Presumptive *E. coli* colonies were transferred to Trypticase Soy Agar containing 5% sheep blood (BBL) and incubated at 37ºC for 24 hours to observe for the growth of hemolytic colonies. A series of biochemical tests were conducted on randomly chosen *E. coli* colonies using API20E strips to confirm the colonies as *E.coli*. The swab was then placed back into the original tube containing 1 mL of nutrient and 1 mL of 20% glycerol (FisherScientific, Far Lawn, NJ). One milliliter of the mixture was placed into 1.5 mL microcentrifuge tubes

(Eppendorf, Brinkman Instruments, Inc., Westburg, NY) to be preserved at -80°C. The remainder of the mixture and swab was poured into a stomacher bag that contained 80 mL of Tetrathionate Broth (Difco) and incubated at 42ºC for 24 hours for enrichment of *Salmonella* Typhimurium. After 24 hours, 10µL of Tetrathionate Broth was streaked onto XLT_4 agar (BBL) that contained naladixic acid (Sigma) at 50µg/mL to assure recovery of only the resistant challenge organism. API20E strips were used to test a representative number of samples to confirm that the organism recovered was *Salmonella* Typhimurium.

Bacteria were enumerated after the first sampling to determine the amount of total aerobes and anaerobes present and to determine whether streptococcus and lactobacilli were present. Samples were also tested for the presence of Salmonella prior to the challenge.

Minimum Inhibitory Concentration Analysis (MIC)

A maximum of four confirmed bacterial colonies was chosen from each sample and tested for sensitivity to oxytetracycline and apramycin sulfate. Colonies were picked from the surface of the agar using a wire 4-mm loop and placed into sterile 16 X 120 mm glass test tubes (FisherScientific) containing 5 mL of Mueller Hinton II broth (BBL). Tubes where positioned in a shaking water bath at a temperature of 37ºC for both *Salmonella* Typhimurium and *E. coli* and at 35ºC for *E. faecalis* where they were maintained until cell concentrations where determined, by the use of a colorimeter (BioMerieux Vitex, Inc, Hazelwood, MO) to be at 0.5 McFarland standard turbidity level (approximately 10^8 CFU/mL) (NCCLS, 1997). Upon reaching the appropriate density,

25.3µL of the cell culture was added to 2.5 mL of a 1:10 dilution of Mueller Hinton and sterile water. Fifty microliters of the Mueller Hinton and bacteria mixture was added to a 96-well microtiter plate for analysis. The final bacterial concentrations were approximately 5 x 10^5 CFU/mL (NCCLS, 1997). Microtiter plates contained twelve columns and eight rows, with the twelfth row reserved for the control bacterial strain (ATCC 215922 *E.coli*, USDA, Ames, Iowa). In preparing the microtiter plate, all wells were initially filled with 50µL of Mueller Hinton II Broth. Six milliliters of Mueller Hinton II Broth and four milliliters of oxytetracycline or apramycin at the desired concentration was mixed in a sterile microdilution tray. Fifty microliters of the Mueller Hinton II Broth and antibiotic mixture was added to the top row of the microtiter plate. Two fold serial dilutions were made by pipetting from one well and adding it to the next lower well in the column. This process was continued through the seventh row, with no antibiotics being added to the last row; thus serving as a control to test for viable bacteria. Breakpoints for analysis (NCCLS) and antibiotic dilution range can be found in Table 2.

Statistical Analysis

A completely randomized design with split-split plot and repeated measures was used to compare the treatments within the experiment. Each room represented a different treatment with the individual pigs representing an experimental unit. Analysis of variance was determined using the mixed models procedures of SAS and the effects of treatment were noted (SAS Proc Mixed, 2001). Least squares means were analyzed using least squares difference at P = 0.05. MIC's were linearized to produce interpretable

least squares means. Maximum standard errors of the least squares means were also computed and compared.

Molecular Analysis

E. coli isolates found to be resistant to apramycin through MIC procedures were characterized to determine the genes responsible for resistance. For this analysis, random apramycin resistant isolates were chosen from pigs and sows of all treatment groups (111 isolates total).

Polymerase Chain Reaction Amplification (PCR)

PCR amplification was performed on genomic DNA using a primer targeting a 507 base pair sequence of a gene that encodes for apramycin resistance (AAC(3)-IV) (5'-GGCATCGCATTCTTCGCATC-3'). Fifteen apramycin-resistant *E. coli* isolates were grown overnight in 5 mL of LB (Luria) broth (Bacto tryptone 10g/L, Yeast extract 5g/l, NaCl 10g/L). DNA was prepared the next morning by lysing the cells in 0.2% Triton-X-100 solution (Mallinckrodt Specialty Chemicals Co., Paris, Kentucky). An equal volume of cells and 0.2% Triton-X-100 solution was pipetted into a sterile 1.5 mL microcentrifuge tube and boiled for five minutes. The tubes were placed into a beaker of boiling water and boiled for five minutes.

A PCR mastermix was prepared under a sterile ventilated hood away from the bench that was used for DNA preparation to reduce risk of contamination. The mastermix consisted of 1 μL Taq DNA polymerase (Promega, Madison, WI), 5 μL dNTP's (Invitrogen, US Headquarters), 1 μL primer (Operon Technologies), 10 μL 5X buffer C (Invitrogen), and 28 μL sterile water (Invitrogen). Sterile, 0.2 mL PCR tubes

(Eppendorf), were filled with 49µL of the mastermixture and 1µL of DNA. Tubes were transferred to the PCR Mastercycler Gradient (Eppendorf) and taken through a series of cycles. The cycling protocol consisted of 1 cycle at 94ºC for 2 minutes, 94ºC for 1 minute, 65ºC for 30 seconds, and 70ºC for 2 minutes; 10 cycles at 94ºC for 1 minute, 55ºC for 30 seconds, and 72ºC for 2 minutes; 24 cycles at 94ºC 1 minute, 72ºC for 5 minutes, and a final hold at 4ºC until further analysis were conducted.

DNA fragments were separated in a 1.5% agarose/0.5X TBE gel (FisherScientific, Fairlawn, NJ) by traditional electrophoresis. Ethidium bromide (3.0 µL) was added prior to solidification for visualization of DNA. PCR products were combined with loading buffer (0.5µL) into a 1.5 mL microcentrifuge tube. The mixture was then added to the agarose gel. The agarose gel was then electrophoresed in 0.5X TBE buffer for 45 minutes at 110 volts. Gels were visualized using the FisherBiotech's Electrophoresis Systems 312 nm UV Transilluminator and photographed using the MP4+ System and instant sheet film type 55 (Polaroid, Cambridge, MA). Photographs were scanned by computer (Hewlett Packard ScanJet 3300C) for further analysis.

Isolation of plasmid DNA

Plasmid DNA was prepared using the lysis solution method. Apramycin-resistant *E. coli* isolates were grown overnight in 2YT (Tryptone Peptone, Yeast Extract, and NaCl) containing 128µg/mL of apramycin sulfate. The overnight culture was diluted 1:20 into 2 mL of fresh 2YT broth and regrown for 2 to 3 hours to achieve growth at the logarithmic phase. Cells were pipetted into 2 mL centrifuge tubes and harvested by centrifugation at approximately 2,500 X g for 10 minutes. Cells were resuspended in 2

mL of TE buffer (10mMTris-Cl ph 8.0, 1mM EDTA) and recentrifuged for 10 minutes. The remaining pellet was resuspended in 40µL of TE buffer, and 600µL of lysis buffer (4% SDS in TE [pH 12.4], prepared daily) was added to each sample and mixed. Tubes (Eppendorf) were incubated at 37ºC for 20 minutes. The solution was neutralized by adding 30µL of 2.0 M Tris-HCl and tubes were mixed until a change in viscosity was evident. Immediately after neutralization, 240µL of 5 M NaCl was added to centrifuge tubes, which were subsequently incubated for 4 hours to remove chromosomal DNA. Following the 4-hour incubation, tubes were centrifuged (16,000 x *g*) for 10 minutes to sediment debris. Supernatant fluid was collected and poured into a fresh microcentrifuge tube (eppendorf) with the addition of 550µL of isopropanol (FisherScientific) to precipitate the DNA. Samples were mixed and incubated at -20ºC for 30 minutes. DNA was collected in the form of a pellet by centrifuging (16,000 x *g*) for 3 minutes. Supernatant fluid was poured off and tubes were dried under a vacuum for approximately 30 minutes to 1 hour. Remaining precipitate was resuspended using 30µL of TE and incubated overnight at 4ºC to dissolve DNA.

Thirty microliters of TE and plasmid DNA from the previous day were added to a fresh test tube because of the high accumulation of salt. Two microliters of RNase were added to remove RNA, and the plasmid and TE mixture and tubes were incubated at 37ºC for 15-20 minutes. Samples were removed from the incubator and 3.12µL of 3 M NaOAC was added, followed by the addition of 69µL of 70% ethanol. The mixture was placed on ice and incubated at -20ºC for 15-20 minutes. After incubation, tubes were centrifuged (13,000 x *g*) in a cold room for 15-20 minutes and dried under a vacuum for

approximately 30 minutes. The remaining pellet was dissolved in 30µL of TE buffer and placed at 4ºC prior to gel electrophoresis.

Electroporation

Plasmid DNA was isolated from resistant *E. coli* derived from test pigs using techniques previously described. Two microliters of total plasmid DNA was electroporated into 40µL of electrocompetent cells, which were derived from a sensitive strain of *E. coli* (JM109). Electroporated cells were grown for one hour in 1 mL SOC medium and 100µL were plated onto LB plates containing apramycin (128µg/mL). LB plates were incubated for 24 hours at 37°C. Individual colonies were obtained from the plates using a sterile 4mm wire loop and inoculated into tubes containing LB broth, which contained apramycin (128µg/mL). Apramycin sensitive cells *E. coli* (JM109) were inoculated in LB broth to serve as a negative control and all tubes were incubated overnight at 37°C. Plasmid DNA was re-isolated using an Aqua Pure plasmid DNA isolation kit (Bio-Rad Laboratories, Hercules, CA) and a plasmid profile was conducted to determine if the resistance gene was associated with plasmids.

Macrorestriction profiling

Preparation of Gel Plugs

Bacteria were grown overnight at 37°C on Tripticase Soy Agar (TSA) with 5% sheep blood agar and directly suspended using sterile cotton swabs in 2-3 ml of Cell Suspension TE buffer (100mM Tris and 100 mM EDTA pH 7.5) until 20% transmittance was obtained, as measured by a colorimeter (bioMerieux). Aliquots of 200 µL of each bacterial suspension were placed into 1.5 mL microcentrifuge tubes (Eppendorf).

Proteinase K (20 mg/mL stock) (Roche Diagnostics, Indianapolis, IN) was added at 10 μL/tube and each tube was mixed gently 5-6 times. InCert/SDS agarose mix at 1.6% (BMA, Rockland, ME) was then added at 200 μL per tube. Following mixing, the bacteria and agarose mixture was immediately dispensed into the wells of the BioRad disposable plug molds (Bio-Rad Laboratories, Hercules, CA). Plugs were allowed to solidify and then transferred to 2 mL round bottom tubes. Following this step, 1.5 mL of ES buffer (0.5 M EDTA, pH 9.0: 1% sodium-lauroyl-sarcosine) and 40 μL of proteinase K (20 mg/mL) was added. Plugs were incubated in a shaking water bath at 55°C for one hour.

Washing the Gel Plugs

After incubating in the water bath, ESP buffer was removed and plugs were transferred to pre-numbered BioRad (Hercules, CA) screen caps with two plugs from each specimen. The columns were inserted into PVC washing tubes and pre-heated sterile water (temperature 55°C) was poured into each tube. The tubes were sealed and then placed into platforms in a shaking water bath at 50°C for 15 minutes. The washing step was repeated three additional times with Plug Wash TE buffer (10mM Tris pH 7.5 and 1 mM EDTA, pH 7.5) for 15 minutes per wash. Plugs were stored in 2 mL of Plug Wash TE buffer at 4°C until the restriction digestion step was performed.

Restriction Endonuclease Digestion

One plug was removed from the storage tube and placed onto a clean sterile glass slide. Two 1 mm wide slices of the plugs were excised with a razor blade and transferred to the labeled 1.5 mL microcentrifuge tube. The remainder of the plug was saved in Plug

Wash TE buffer at 4°C until further use. Sterile water was added at 86 μL along with ten microliters of 10X appropriate enzyme buffer, one microliter of BSA, and 3 microliters (30 Units) of *Xba I* (Roche Diagnostics). The mixture was gently pipetted and incubated at 37°C in a water bath for 1 hour and 15 minutes.

Pulse-Field Gel Electrophoresis (PFGE)

After the incubation, the enzyme mixture was aspirated from the tube and replaced with 0.5 mL of Plug Wash TE buffer. The plug slices were aligned with the teeth of the comb in the appropriate order and allowed to dry. The comb was set in the gel casting mold and the 1.0% agarose (SeaKem Gold, Roche Diagnostics) was poured. The comb was removed and the wells were sealed with saved agarose. DNA was separated by PFGE using the CHEF-mapper system (Bio-Rad) with a run time of 14 hours, initial switch time of 2.16 seconds, final switch time of 35.07 seconds, angle 120°, gradient of 6.0V/cm with a linear ramping factor at 14°. After electrophoresis, the gel was stained in 500 mL of distilled water with one drop of 10 mg/mL of ethidium bromide for 20-25 minutes. Washings with distilled water followed. The gel was visualized over a UV transilluminator and photographed. Photographs were digitized for further analysis (Hewlett Packard ScanJet 3300C scanner).

Images were then analyzed using the Molecular Analyst software, version 1.6 (BioRad 1992-1998). This software was used to create dendrograms to compare profile relatedness through the Dice coefficient and clustering method of unweighted pair group method for arithmetic averages (UPGMA). The Dice coefficients were calculated using

the following formula to estimate the proportion of restriction fragments shared by two populations:

$$\frac{2n_{AB}}{n_A + n_B}$$

where n_{AB} is the number of bands common for A and B, n_A is the total number of bands in A, and n_B is the total number of bands in B (Molecular Analyst Software Manual, 1992-1996). The UPGMA clustering method is the unweighted pair group method using arithmetic averages, which operates by calculating a matrix of similarities between every pair of organisms and deducing a dendrogram from the matrix by clustering (Molecular Analyst Software Manual, 1992-1998).

3. RESULTS

I. MIC Results

IA. E. coli

Previous exposure of sows to antibiotics significantly affected resistance to apramycin and tetracycline in *E. coli* isolated from pigs (Tables 3 and 4). Isolates from pigs derived from sows that had previous antibiotic exposure had greater initial resistance to apramycin and oxytetracycline during the nursing period compared to other groups. *E. coli* isolated from pigs receiving apramycin had greater resistance following application, regardless of sow treatment (Table 3). *E. coli* isolated from the control group showed the lowest resistance to apramycin with the exception of day 28, which was the highest of all treatment groups.

Resistance to oxytetracycline remained high throughout the study in all treatment groups, and treatment effects were detected (Table 4). The addition of the sanitation treatment on day 86 did not produce an interaction with main effects of previous antibiotic exposure for either apramycin or oxytetracycline (Tables 5 and 6). *E. coli* isolated from pigs in low sanitation rooms were less resistant to oxtetracycline than *E. coli* isolated from pigs in high sanitation rooms (Table 6).

Transportation did not appear to have an influence on apramycin resistance, with the exception of day 136 in which isolates from one treatment group peaked (Table 5). Isolates from most pig treatment groups, however, experienced increased resistance to oxytetracycline following transportation (Table 6).

IB. Enterococcus faecalis

No consistent treatment effects or interactions were observed for *E. faecalis*. Resistance to both apramycin and oxytetracycline remained high in all treatment groups throughout the study (Tables 7 and 8). Isolates were however, found to be more often resistant to apramycin (Table 7). Resistance to oxytetracycline was higher on day 28, which was the same day apramycin treatment began. Sanitation treatments appeared to have no effect on resistance to either drug in any groups (Tables 9 and 10).

IC. S. Typhimurium

There was very low recovery of the salmonella challenge organism beyond two weeks post challenge. No treatment effects were noted for either apramycin or oxytetracycline (Tables 11 and 12) and resistance remained low throughout the recovery period in all treatment groups. Failure to recover salmonella in the latter stage of the study prevented the opportunity to observe sanitation and transportation stress effects.

II. Molecular Results

Results obtained through MIC testing indicated a notable interaction between sow treatment and apramycin resistant *E. coli* isolated from pigs, therefore random resistant *E. coli* isolates from both sows and pigs were chosen from days 7, 14, and 21 to represent a period of nursing through weaning. Isolates used in the genetic analysis were highly resistant to apramycin (>128μg/mL) and are shown in Tables 13 and 14.

IIA. PCR detection of aac(3)-IV gene sequences

Ninety percent (n = 111) of apramycin resistant *E. coli* from both pigs and sows contained a known gene sequence of the *aac*(*3*)-IV gene, which encodes for apramycin

resistance. Figures (1-13) show PCR products separated by gel electrophoresis of apramycin resistant isolates illustrating either the presence or non-presence of the *aac*(*3*)-IV gene. A single apramycin-resistant *S.* Typhimurium isolate (>32μg/mL) was tested and results indicated no presence of the *aac*(*3*)-IV gene (Figure12).

IIB. Plasmid Profiling

DNA profiles revealed that large plasmids were consistently present in resistant isolates from both pigs and sows (Figures 14-17).

IIC. Electroporation

Apramycin-resistant JM109 were generated via electroporation using total plasmid DNA isolated from apramycin-resistant samples (Table 15). Apramycin resistant colonies were generated with the DNA of isolates 1, 17, and 18. DNA from isolates 2 and 13 failed to produce apramycin resistant JM109. Plasmid DNA isolated from recipient apramycin-resistant JM109 cells (Figure 18) revealed the presence of large plasmids comparable to the one large plasmid previously found associated with apramycin resistant isolates. The sensitive control strain, non-transformed JM109 did not contain such plasmids (Figure 18).

IID. Macrorestriction profiling

Random apramycin resistant isolates were chosen from the non-antibiotic sow treatment group, antibiotic sow treatment group, and environmental manure samples from each treatment room to determine whether clonal relationships existed among those isolates. Figures 19 and 20 illustrate macrorestriction profiles of non-antibiotic treated sows and their pigs. Figures 21 and 22 depict macrorestriction profiles from antibiotic

treated sows, their pigs, and environmental manure samples. A dendrogram (Figure 23) based on UPGMA clusters of dice coefficients showed that although there were a few clonal isolates, many different types of *E. coli* carryed the gene coding for apramycin resistance. Isolates number 41, 37, and 50 from the antibiotic treatment group were all from sampling day 7 and were clones of one another. Isolates 34 and 29 of the antibiotic treatment group were taken from sampling day 28 and were found to be clones. A clonal relationship was found between isolates 42 and 44 from the non-antibiotic treatment group, these isolates were also from the same pig. Isolates 13 and 20 of the non-antibiotic treatment group were determined to be clones. These isolates were from the same sampling date (D7). Manure isolates from rooms 106 and 107 along with isolate 39 of the non-antibiotic treatment group were found to be clones.

4. DISCUSSION

Studies throughout the years have investigated the effect of the subtherapeutic use of antibiotics on bacterial resistance. Many have investigated the pattern of antibiotic resistance using MIC analysis and disk diffusion methods. Although these procedures provide phenotypic results, recent studies have begun to include molecular techniques such as PCR, plasmid profiling, PCR fingerprinting, PFGE and electroporation to further characterize genes coding for antibiotic resistance in hopes of solving the antibiotic resistance dilemma.

I. *E. coli*

Our results indicate that both apramycin and tetracycline resistance in *E. coli* can be affected by the use of tetracycline in sows, as indicated by elevated antibiotic resistance exhibited of bacteria isolated from pigs farrowed from sows with prior antibiotic use. One possible reason for this result is that the gene responsible for oxytetracycline resistance and the gene responsible for apramycin resistance is about the same size and therefore may reside on the same genetic cluster. Therefore, the subtherapeutic use of oxytetracycline in feed may influence the selection of resistance to other antibiotics such as apramycin. Although antibiotic use was discontinued upon farrowing, antibiotic residues may have remained in farrowing crates through fecal material and sow feed. Pigs had constant exposure to sow feces and as much as 30% of tetracycline can be excreted unchanged via fecal material (Huber, 1988). Moreover, tetracyclines can transcend the placenta and enter into fetal circulation providing pigs

with exposure to antibiotics prior to birth. Suckling pigs may have also attained contact through the sows milk (Huber, 1988).

Additionally, subsequent use of antibiotics in pigs can continuously affect resistance levels in *E. coli*. Although apramycin resistance levels remained low throughout the majority of the investigation, a peak was observed subsequent to treatment with the antibiotic. Groups not receiving antibiotics did not experience elevated resistance, thus we conclude that there is a distinct effect of apramycin use at this time. Results of this nature have been previously documented (Mathew et al., 2001; Cullen, 2001).

Isolates remained resistant to oxytetracycline through all sampling periods, consequently making it difficult to distinguish patterns or effects. On the first day of weaning, resistance levels from pig isolates recovered from the non-antibiotic treated sows decreased more than 50% and isolates recovered from the antibiotic treated sow also demonstrated lower resistance. These findings support the notion that sows have an influence on antibiotic resistance in their pigs. However, one week following weaning, isolates exhibited extremely high resistance to oxytetracyline regardless of treatment. One possibility of such a significant turnaround may have been due to challenge procedures, which incorporated the use of the same inoculation tool to dose each pig. Using the same tool between treatment rooms may have introduced pigs to common resistant bacteria. Stress may also have caused an increase in resistant organisms as nursery rooms were held at lower temperatures than farrowing rooms and lacked warming pads. Similar results were seen in an investigation by Cullen (2001), as young

pigs exposed to cold stress conditions exhibited higher resistance levels for longer periods of time.

Room sanitation did not produce interactions with previous antibiotic exposure of either sows or pigs. High and low sanitation treatments were not applied until day 81 of the experiment which ended at day 136. Therefore, perhaps there was not enough time to establish effects resulting from low sanitation. Cullen (2001), demonstrated that pigs confined in low sanitary conditions produced isolates resistant to apramycin over a longer time frame than pigs housed in a control environment with high sanitation standards.

There were also no effects identified between transport and intermingling stress and antibiotic treatment. This result is contradictory to a previous study (Langlois and Dawson, 1998) in which elevated antibiotic resistance levels were noted with all antibiotics after 30 minutes of transport. However, isolates from pigs receiving antibiotics farrowed from sows that did not receive antibiotics and housed in low sanitary conditions did have a pronounced increase in resistance to apramycin. This effect may have been caused by the fact that these pigs were among the last to be loaded onto the truck and handling procedures were more harsh at this time because the pigs had to be forced onto the truck.

II. *S.* Typhimurium

The use of antibiotics in either pigs or sows did not appear to affect resistance in the *S.* Typhimurium challenge organism. Isolates recovered from all treatment groups were highly susceptible to both oxytetracycline and apramycin. Research has shown that antibiotic resistant commensal organisms such as *E. coli* may transfer resistance genes to

Salmonella (Hunter et al., 1992). These findings were not supported by this study however, with high levels of resistance revealed in *E. coli* and low levels of resistance associated with *Salmonella*.

Low recovery of *Salmonella* was initially thought to be a result of inefficient bacteriological culture procedures. However, identical isolation techniques were used in previous studies, which showed carrier status for a longer period of time (Ebner, 1998; Mathew, 2001; Cullen, 2001). Alternate procedures were used and results were compared to those obtained through the use of laboratory methods used in past studies conducted in this laboratory. The alternate method consisted of a pre-enrichment and enrichment step rather than the single enrichment step normally used. Whole manure samples were pre-enriched in LB broth, adjusted to a pH of 7.0, and one milliliter was placed into 9 milliliters of Tetrathionate broth for additional enrichment. The remaining procedures were the same as used with the traditional laboratory method. In comparison, the alternative method of *Salmonella* isolation did not greatly enhance the amount of cells recovered and it was consequently determined that laboratory technique was not the cause of low *S*. Typhimurium recovery.

Lack of stress may have contributed to the inability of *Salmonella* to effectively colonize the intestine. Temperature was lowered in an attempt to create a stressful environment for the newly weaned pigs. However, pigs were housed in nursery rooms one week prior to inoculation with the salmonella challenge organism. The one-week period prior to inoculation allowed pigs to adjust to the temperature change and transportation stress that may occurred as a result of moving pigs into different rooms,

thus reducing adverse effects that may have otherwise by encountered in a stressful environment. One type of stress that may have an effect on the colonization of challenge organisms is transportation stress. Isaacson and co-workers (1999) examined the effect of transportation stress on the intestinal colonization of Salmonella challenge organisms and determined that transported pigs exhibited higher shedding of *Salmonella*. Therefore challenging the pigs just prior to or post transport to new rooms and withholding feed for 24 hours prior to inoculation of the challenge organism may have induced enough stress to cause an increase in the amount of gut colonization and fecal shedding of the organism. Another stressor that could have had an impact on the colonization of the challenge organism may be withholding of feed. An investigation by Balaji et al. (2000) did not use transportation as a stressor but rather allowed pigs seven days to acclimate to the new environment. However, feed was withheld for 12 hours prior to the challenge, which reduced competition in the intestine for colonization of *Salmonella*. Thus, transportation stress and withholding feed are other possible options that should be taken into consideration when challenging pigs in the future.

Salmonella was not detected beyond week 3, as a result the effect of sanitation could not be observed. However, research has shown that an accumulation of manure can serve as a reservoir aiding in the spread of bacteria from one animal to another (Funk et al., 1999). Pigs moved from poor sanitary environments into clean environments have been shown to have a higher incidence of fecal shedding of *Salmonella* organisms. The accrual of manure can also lead to the retention of genetic elements coding for resistance

to antibiotics. Animals may spread bacteria containing resistant genes to one another and subsequently, bacteria may remain in the housing area to infect future animals.

III. **E. faecalis**

Much research shows *E. faecalis* to be highly resistant to a wide variety of antibiotics (Chen and Williams, 1985; Sahm and Gilmore, 1995; Morrison et al., 1997; Franz, 2001). High levels of resistance have been associated with pathogenesis in the organism. Intrinsic resistance often aids in the survival of the organism and allowing time to acquire additional genes encoding for resistance to a wider variety of antibiotics (Sahm and Gilmore, 1995). High levels of resistance to both oxytetracycline and apramycin in *E. faecalis* were noted for all treatment groups throughout the study.

Aminoglycoside resistance of *E. faecalis* has become a great cause of concern in human medicine because they are becoming increasingly prevelent in nosocomial infections falling second only to *E. coli* (Iverson et al., 2000). *Enterococcus faecalis* was more resistant to apramycin than oxytetracycline throughout the investigation, however there was a notable peak in resistance to both antibiotics prior to antibiotic treatment. Enterococci are not susceptible to aminoglycosides because of reduced permeability and the production of aminoglycoside-modifying enzyme (Morrison et al., 1997). Research has revealed the presence of mutant apramycin resistant bacteria in humans and there is a possibility of increasing the pool of resistant gentamicin resistant isolates by continuing to use apramycin in the animal industry (Chen and Williams, 1985; Wray et al., 1986; Sahm, 1991). Wray et al. (1986) discovered that the enzyme aminoglycoside 3-N-acetyltransferase (AAC(3)IV) conferred resistance by acetylation to both apramycin,

which is used only in animal medicine, and gentamicin, which is used in humans and animals. They concluded that although it is possible for conjugal transfer to take place between apramycin resistant bacteria and gentamicin resistant bacteria, it does not occur very often because gentamicin is usually only used in hospital settings for short periods of time or by prescription use permitted by physicians. Results from studies such as this imply that there is a need for further research on the possible influence that apramycin may have on human medicine. Although time was a limiting factor in this investigation, future endeavors should include the characterization of genes encoding resistance to various antibiotics in *Enterococcus faecalis*.

IV. PCR

PCR analysis was only used to test apramycin resistant isolates from *E. coli* and a single *S.* Typhimurium isolate. We found only one *S.* Typhimurium isolate to be resistant to apramycin (32μg/mL) therefore this was the only isolate to used in PCR analysis. Tetracycline resistant isolates were not tested because there are many genes coding for tetracycline resistance and there was not enough time for sufficient analysis. Isolates were chosen from days 0, 7, 14, and 28. Day 0 represents the latest date sows were receiving antibiotics prior to farrowing. Day 7 characterizes when pigs were a week old and nursing. Day 14 represents a time frame prior to weaning, but after maximum contact between sows. Lastly, day 28 represents post weaning and the initiation of treatment with antibiotics in pigs. Isolates were taken from both antibiotic and non-antibiotic treated sows. A greater number of isolates were tested from antibiotic treated sows than from sows which were not exposed to antibiotics, primarily because there were

more isolates that were resistant to apramycin recovered from the antibiotic group in the days indicated above.

PCR analysis confirmed that the *aac*(*3*)-IV gene sequence was present in a majority (90%) of the samples. The resistant *S.* Typhimurium did not contain the *aac*(*3*)-IV gene. All of the *E. coli* isolates tested were resistant at 128μg/mL or greater, whereas the *S.* Typhimurium was only resistant at 32 μg/mL, which is considered breakpoint resistance. Therefore, it is possible that the *S.* Typhimurium isolate was a mutant that did not contain the typical gene coding for apramycin resistance. Another possibility is that *E. coli* may have had several more copies of the gene than *S.* Typhimurium thus allowing easier detection.

V. Plasmid Profiling

Plasmid profiling was used to detect the presence of plasmids and to determine a possible pattern associated with apramycin resistant *E. coli*. Many of the isolates that contained the *aac*(*3*)-IV apramycin resistance gene revealed large plasmids (approximately 25 kb). Fagarasan et al. (1997) used plasmid profiles to aid in the characterization of antibiotic resistant *Salmonella*. Thirty-eight isolates of *Salmonella enterica* serovar Typhimurium and 19 isolates of *Salmonella enterica* serovar Enteritidis were obtained from hospitalized children in Cluj-Napoca during the period of 1995-1997. *S.* Typhimurium was highly resistant to penicillins, tetracycline, streptomycin, tobramycin and trimethoprim-sulfamethoxazole. The incidence of plasmids and antibiotic resistance was shown to be very high, however there was no correlation between resistance and plasmid profiles. Therefore, plasmid profiling may only be

accurate at detecting plasmids present and not relationships between plasmids present and antibiotic resistance.

VI. Electroporation

Through plasmid profiling it was determined that most of the isolates contained a large plasmid. The *aac*(*3*)-IV gene is often found to be associated with large plasmids, therefore it was hypothesized that the gene would be plasmid-borne. Plasmid DNA isolated from resistant *E. coli* derived from the test pigs was electroporated into a sensitive strain of *E. coli* (JM109). Apramycin resistant colonies were generated with the DNA from isolates 7, 17, and 18. Plasmid DNA isolated from apramycin resistant JM109 revealed the presence of a large plasmid similar to those found in the original isolates. Consequently, the gene encoding resistance to apramycin was assumed to be present on the plasmid.

Similar experiments with oxytetracycline proved unsuccessful, as there were no oxytetracycline colonies generated. It was therefore determined that either the gene coding for resistance to oxytetracycline was not located on a plasmid or there was an error in the methodology.

Steele et al. (1994) conducted a study on the effect of different antibiotics on the efficiency of transformation of bacteria by electroporation. They discovered that electroporation produced fewer tetracycline resistant bacteria than ampicillin resistant bacteria. This led to speculation that antibiotics causing cell wall damage may decrease transformation efficiency since electroporation itself has damaging effects on the membrane. Those researchers also decided that it was possible that the mechanism of

drug resistance encoded by the plasmid may have an effect. The difference between ampicillin resistance and tetracycline resistance may occur because ampicillin resistance is mediated by the enzyme lactamase that hydrolyzes the antibiotic, whereas resistance to tetracycline is due to a protein that either decreases transport of the antibiotic into the cell or leads to its transport out of the cell (Steele et al. 1994). It was thought that because transport proteins are located on the inner cytoplasmic membrane, when the membrane is damaged through electroporation the proteins may be compromised, thus leading to the inability to recover tetracycline-resistant colonies. These theories may also be applied to the results of this study which produced apramycin-resistant JM109 but no tetracycline-resistant JM109 colonies. The primary mechanism of apramycin resistance is similar to the mechanism of ampicillin resistance in that resistance is mediated by a cellular enzyme. Therefore, electroporation may be more successful in producing apramycin resistant bacteria than with the production of oxytetracycline resistant bacteria.

VIII. Macrorestriction profiling

A number of isolates (n = 44) from both non-antibiotic and antibiotic sow groups and manure from environmental sampling were tested through PFGE, with computer analysis offering the opportunity compare the results. Isolates 41, 37, and 50, all of the antibiotic sow and pig treatment group, were also from the same sampling day (D7) and exhibited a relationship of 100%. Isolates 41 and 50 were from the same pig on the same sampling date. Isolate number 37 was derived from a pig born from the same sow, as were the other two isolates. It is therefore possible that these pigs were exposed to *E. coli* from the same source. Other isolates determined to be 100% related were numbers 34

and 29 both from the antibiotic treatment group and from sampling day 28. These isolates were obtained from pigs housed in the same treatment group during the early post-weaning period. During this time, these pigs may have also obtained *E. coli* from the same source. Isolates number 13 and 20, both taken from sampling day 7, were from the non-antibiotic treatment group and were 100% correlated. These bacteria were obtained from different pigs housed in the same treatment group post weaning, thus it is possible that contact may have been made with the same source. Another relationship of 100% was found between environmental manure samples from rooms 106, 107, and a pig from the non-antibiotic treatment group. The pig was housed in room 107; therefore, the manure that was collected may have been from that pig or another pig with the same strain of *E. coli*. Rooms 106 and 107 both housed pigs derived from non-antibiotic treated sows; therefore, pigs from each of these rooms were farrowed from the same sows. Hence, pigs from these rooms may have obtained the same strain of *E. coli* derived from a common source. Interestingly, a 96% relationship was found among isolates from a pig in the non-antibiotic sow treatment and a pig from the antibiotic sow treatment. These isolates were also from different sampling dates.

Although there were clones and strong relationships observed, the ultimate result is that most of the *E. coli* were different or had very weak relationships. Therefore, it can be said that may different types of *E. coli* served as a reservoir for the apramycin resistance gene. With more time available, future studies should test more isolates and more species of bacteria.

Conclusion

The growing use of antibiotics in agriculture as well as human medicine has increased public awareness of antibiotic resistant organisms. Concern has risen among consumers and health experts and the need for more research in this area has been heightened. Today's advanced molecular techniques can provide new information with regard to the sources of genetic resistance elements and mechanisms by which bacteria become resistant to antibiotics. The purpose of this study was to determine whether the use of antibiotics in sows or housing environment had an influence on the development of resistant bacteria in pigs. Results indicate that the subtherapeutic use of antibiotics by sows does have an influence on their pigs prior to weaning, as those animals exhibited greater resistance than did pigs from sows that did not receive antibiotics. It was also determined through pulse-field gel electrophoresis that several pigs farrowed from the same sow had identical *E. coli* macrorestriction profiles, indicating the possibility that *E. coli* were derived from the same source. The results from this study indicate that the subtherapeutic use of antibiotics during the period of gestation may affect pigs prior to weaning and therefore should be used practically.

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APPENDIX

Table 1. Pig dietary and housing treatments

Treatment 1 (n = 7)	Sows received antibiotics (10mg oxytetracycline/lb body weight)/ Pigs received antibiotics (50g/ton oxytetracycline via the feed)/ Optimal housing conditions with daily manure removal
Treatment 2 (n = 7)	Sows did not receive antibiotics/ Pigs received antibiotics (50g/ton oxytetracycline via the feed)/ Optimal housing conditions with daily manure removal
Treatment 3 (n = 7)	Sows received antibiotics (10mg oxytetracycline/lb body weight)/ Pigs did not receive antibiotics/ Optimal housing conditions with daily manure removal
Treatment 4 (n = 7)	Sows did not receive antibiotics/ Pigs did not receive antibiotics/ Optimal housing conditions with daily manure removal
Treatment 5 (n = 7)	Sows received antibiotics (10mg oxytetracycline/lb body weight)/ Pigs received antibiotics (50g/ton oxytetracycline)/ Daily access to manure
Treatment 6 (n = 7)	Sows did not receive antibiotics/ Pigs received antibiotics (50g/ton oxytetracycline)/ Daily access to manure
Treatment 7 (n = 7)	Sows received antibiotics (10mg oxytetracycline/lb body weight)/ Pigs did not receive antibiotics/ Daily access to manure
Treatment 8 (n = 7)	Sows did not receive antibiotics/ Pigs did not receive antibiotics/ Daily access to manure

*** n represents number of pigs per treatment**

Table 2. Antibiotic Dilutions and Breakpoints (NCCLS)

Antibiotic	Antibiotic Concentratin Range (μg/mL)	Breakpoint
Apramycin Sulfate	2-128/*8-512	≥32 ≥512*
Oxytetracycline	8-512	≥16

***E. faecalis* resistance testing only**

Table 3. MIC to apramycin for *E. coli* isolated from pigs derived from sows with or without previous exposure to antibiotics

Days of age	SW-PW	SW-P0	S0-PW	S0-P0 (control)	SEM
7	9.1	39.0*	10.9	8.5	3.12
14	46.0*	46.0*	4.8	4.6	3.28
21	5.5	27.6*	7.2	4.4	3.06
23	4.0	5.2	2.9	4.6	1.14
28	6.7	6.2	5.5	10.6	2.17
35	7.8	5.3	3.2	2.8	2.05
51	227.9*	19.6	209.4*	2.5	9.93

Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *E. coli* isolated from pigs prior to and following weaning, through 51 days of age.
SW= sows with previous exposure to antibiotics; S0= sows without antibiotic exposure; PW= pigs treated with antibiotics; P0= pigs not treated with antibiotics; SEM = maximum standard error for Lsmeans within row. Comparisons are within row. * Indicates difference from control within day
Treatment effect, P < .05.

Table 4. MIC to oxytetracycline for *E. coli* isolated from pigs derived from sows with or without previous antibiotic exposure

Days of age	SW-PW	SW-P0	S0-PW	S0-P0 (Control)	SEM
7	123.6*	125.8*	64.5	43.8	4.67
14	123.9*	129.6*	87.4	69.1	4.55
21	111.3*	127.9*	21.3	39.3	5.27
23	413.1*	712.5*	367.1	210.9	10.89
28	622.0*	684.4*	485.7*	289.2	13.72
35	844.2	653.2*	498.0*	892.4	17.41
51	326.4	308.3	335.5	386.2	10.31

*Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *E. coli* isolated from pigs prior to and following weaning, through 51 days of age.
SW= sows with previous exposure to antibiotics, S0= sows without antibiotic exposure, PW= pigs treated with antibiotics, P0= pigs not treated with antibiotics. SEM = maximum standard error for Lsmeans within row. Comparisons are within row. * Indicates difference from control within day
Treatment effect, P < .05.

Table 5. MIC to apramycin for *E. coli* isolated from pigs exposed to high or low room sanitation

Days of age	SW-PW-HS	SW-P0-HS	SW-PW-LS	SW-P0-LS	S0-PW-HS	S0-P0-HS (Control)	S0-PW-LS	S0-P0-LS	SEM
81	6.2	3.5	2.3	4.5	3.5	3.4	4.2	3.6	1.37
135	3.6	3.0	3.3	2.9	3.1	5.6	9.6	33.0*	3.30
136	5.8	2.5	3.0	2.3	3.1	3.9	49.0*	2.7	4.66

Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *E. coli* isolated from growing pigs. SW= sows with previous exposure to antibiotics, S0= sows without antibiotic exposure, PW= pigs treated with antibiotics, P0= pigs not treated with antibiotics, HS= High sanitation, LS= Low sanitation. SEM = maximum standard error for Lsmeans within row. Comparisons are within row. * Indicates difference from control within day
Treatment effect, P < .05.

Table 6. MIC to oxytetracycline for *E. coli* isolated from pigs exposed to high or low room sanitation

Days of age	SW-PW-HS	SW-P0-HS	SW-PW-LS	SW-P0-LS	S0-PW-HS	S0-P0-HS (Control)	S0-PW-LS	S0-P0-LS	SEM
81	433.5	455.1	596.3	256.0	526.4	501.5	439.6	948.8	12.82
135	689.8	144.0*	596.3	342.5*	512.0	1021.0	347.3*	44.0*	23.62
136	792.3	786.9	396.2*	390.7*	643.6	982.3	467.9*	249.0*	28.18

Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *E. coli* isolated from growing pigs. SW= sows with previous exposure to antibiotics, S0= sows without antibiotic exposure, PW= pigs treated with antibiotics, P0= pigs not treated with antibiotics, HS= High sanitation, LS= Low sanitation. SEM = maximum standard error for Lsmeans within row. Comparisons are within row. * Indicates difference from control within day
Treatment effect, P < .05.

Table 7. MIC to apramycin for *Enterococcus faecalis* isolated from pigs derived from sows with or without previous exposure to antibiotics

Days of age	SW-PW	SW-P0	S0-PW	S0-P0 (Control)	SEM
7	128.6*	60.4*	278.5	404.4	6.19
14	150.6*	130.8*	313.4	309.3	3.68
21	411.1	260.4	500.6	375.5	5.59
23	200.3	138.2	302.8	257.9	3.91
28	316.5	326.1	398.6	289.5	5.65
35	174.0*	129.1*	474.1	505.6	6.93
51	--------	313.2	300.9	389.1	5.75

Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *Enterococcus faecalis* isolated from pigs prior to and following weaning, through 51 days of age. SW= sows with previous exposure to antibiotics, S0= sows without antibiotic exposure, PW= pigs treated with antibiotics, P0= pigs not treated with antibiotics. SEM = maximum standard error for Lsmeans within row. * Indicates difference from control within day. Treatment effect, P < .05.

Table 8. MIC to oxytetracycline for *Enterococcus faecalis* isolated from pigs derived from sows with and without previous antibiotic exposure

Days of age	SW-PW	SW-P0	S0-PW	S0-P0 (Control)	SEM
7	55.9	42.2	61.4	42.6	2.87
14	60.1	78.1	76.9	61.7	2.50
21	93.1	90.7	64.8	73.3	2.97
23	52.1	81.7	103.5	88.7	3.30
28	202.0	133.7	170.1	179.4	4.50
35	52.9	68.3	81.5	69.6	3.46
51	--------	61.5	74.7	100.4	4.76

Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *Enterococcus faecalis* isolated from pigs prior to and following weaning, through 51 days of age. SW= sows with previous exposure to antibiotics, S0= sows without antibiotic exposure, PW= pigs treated with antibiotics, P0= pigs not treated with antibiotics. SEM = maximum standard error for Lsmeans within row. Comparisons are within row. * Indicates difference from control within day Treatment effect, $P < .05$.

Table 9. MIC to apramycin for *Enterococcus faecalis* isolated from pigs exposed to high or low room sanitation

Days of age	SW-PW-HS	SW-P0-HS	SW-PW-LS	SW-P0-LS	S0-PW-HS	S0-P0-HS (Control)	S0-PW-LS	S0-P0-LS	SEM
81	215.3	512.0	512.0	472.1	675.6	430.5	455.1	699.4	10.35
135	292.0	407.3	280.1	724.1	296.1	352.1	362.0	442.6	8.29
136	146.0	181.0	304.4	256.0	186.1	215.3	219.8	198.1	5.37

Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *Enterococcus faecalis* isolated from growing pigs. SW= sows with previous exposure to antibiotics, S0= sows without antibiotic exposure, PW= pigs treated with antibiotics, P0= pigs not treated with antibiotics, HS= High sanitation, LS= Low sanitation. SEM = maximum standard error for Lsmeans within row. Comparisons are within row. No statistical differences from control identified.

Table 10. MIC to oxytetracycline for *Enterococcus faecalis* isolated from pigs exposed to high or low sanitation

Days of age	SW-PW-HS	SW-P0-HS	SW-PW-LS	SW-P0-LS	S0-PW-HS	S0-P0-HS (Control)	S0-PW-LS	S0-P0-LS	SEM
81	32.0*	144.0	128.0	64.0	130.7	76.6	65.8	49.9	5.59
135	70.0	80.4	76.1	49.5	108.4	67.6	135.3*	35.3*	4.45
136	146.0	99.0	107.6	128.0	128.0	140.1	118.6	50.2*	4.63

Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *Enterococcus faecalis* isolated from growing pigs. SW= sows with previous exposure to antibiotics, S0= sows without antibiotic exposure, PW= pigs treated with antibiotics, P0= pigs not treated with antibiotics, HS= High sanitation, LS= Low sanitation. SEM = maximum standard error for Lsmeans within row. Comparisons are within row. * Indicates difference from control within day

Table 11. MIC to apramycin for *Salmonella* Typhimurium isolated pigs derived from sows with or without previous exposure to antibiotics

Days of age	SW-PW	SW-P0	S0-PW	S0-P0 (Control)	SEM
28	2.6	3.7	3.0	2.7	0.34
35	4.8	6.0*	3.8	3.9	0.39
51	--------	2.0	2.0	2.4	0.25

Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *Salmonella* Typhimurium isolated from postweaned pigs through 35 days of age. SW= sows with previous exposure to antibiotics, S0= sows without antibiotic exposure, PW= pigs treated with antibiotics, P0= pigs not treated with antibiotics. SEM = maximum standard error for Lsmeans within row. Comparisons are within row. * Indicates difference from control within day
Treatment effect, $P < .05$.

Table 12. MIC to oxytetracycline for *Salmonella* Typhimurium isolated from pigs derived from sows with or without previous exposure to antibiotics

Days of age	SW-PW	SW-P0	S0-PW	S0-P0 (Control)	SEM
23	3.4	4.1	3.8	3.8	0.13
28	4.0	4.1	3.8	3.8	0.13
35	--------	2.0	2.0	2.1	0.10

Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *Salmonella* Typhimurium isolated from postweaned pigs through 35 days of age.
SW= sows with previous exposure to antibiotics, S0= sows without antibiotic exposure, PW= pigs treated with antibiotics, P0= pigs not treated with antibiotics. SEM = maximum standard error for Lsmeans within row. Comparisons are within row. No statistical differences from control identified.

Table 13. *E. coli* isolates used in PCR detection obtained from sows that did not receive antibiotics and their pigs with confirmed resistance to apramycin (>128μg/mL)

PCR#	Pig/Sow and Isolate Number	Sampling day
1	069-1 (Sow)	D14
2	069-2 (Sow)	D14
3	169-1 (Sow)	D14
4	169-2 (Sow)	D14
5	169-3 (Sow)	D14
6	169-4 (Sow)	D14
7	061-1 (Sow)	D7
8	37-1 (Pig)	D7
9	38-1 (Pig)	D7
10	38-2 (Pig)	D7
11	38-3 (Pig)	D7
12	38-4 (Pig)	D7
13	39-2 (Pig)	D7
14	39-3 (Pig)	D7
15	39-4 (Pig)	D7
16	42-2 (Pig)	D7
17	51-4 (Pig)	D7
18	53-1 (Pig)	D7
19	53-2 (Pig)	D7
20	53-3 (Pig)	D7
21	56-3 (Pig)	D7
22	57-4 (Pig)	D7
23	59-1 (Pig)	D7
24	60-1 (Pig)	D7
25	27-2 (Pig)	D7
26	27-3 (Pig)	D7
27	28-2 (Pig)	D7
28	28-3 (Pig)	D7
29	30-1 (Pig)	D7
30	30-3 (Pig)	D7
31	30-4 (Pig)	D7
32	31-1 (Pig)	D7
33	31-2 (Pig)	D7
34	31-3 (Pig)	D7
35	31-4 (Pig)	D7
36	26-4 (Pig)	D28

Table 13. Continued

PCR#	Pig and Isolate Number	Sampling day
37	28-1 (Pig)	D28
38	28-2 (Pig)	D28
39	28-3 (Pig)	D28
40	28-4 (Pig)	D28
41	27-1 (Pig)	D28
42	27-2 (Pig)	D28
43	27-3 (Pig)	D28
44	27-4 (Pig)	D28
45	31-1 (Pig)	D28
46	31-2 (Pig)	D28
47	169-1 (Sow)	D0
48	169-4 (Sow)	D0
49	202-1 (Sow)	D0
50	202-2 (Sow)	D0
51	202-4 (Sow)	D0

PCR# represents a simplified numbering scheme for each random isolate tested. Pig number refers to the ear tag number each pig was assigned and isolate number refers to an isolated colony of *E. coli* as four colonies from each pig were tested in the MIC analysis. Example:15-1, 15 being the pig's assigned ear tag number and 1 being the first isolate of four tested. D= day

Table 14. *E. coli* isolates used in PCR detection obtained from sows receiving antibiotics via the feed and their pigs with confirmed resistance to apramycin (128μg/mL)

PCR#	Pig/Sow and Isolate Number	Sampling day
1	OR51-2 (Sow)	D7
2	OR51-3 (Sow)	D7
3	Y20-3 (Sow)	D7
4	OR51-4 (Sow)	D14
5	Y20-1 (Sow)	D14
6	Y20-2 (Sow)	D14
7	Y20-3 (Sow)	D14
8	OR58-1 (Sow)	D0
9	OR58-2 (Sow)	D0
10	OR58-3 (Sow)	D0
11	OR58-4 (Sow)	D0
12	Y20-4 (Sow)	D0
13	15-1 (Pig)	D28
14	15-2 (Pig)	D28
15	19-1 (Pig)	D28
16	19-2 (Pig)	D28
17	22-2 (Pig)	D28
18	1-1 (Pig)	D7
19	1-3 (Pig)	D7
20	4-2 (Pig)	D7
21	4-4 (Pig)	D7
22	8-1 (Pig)	D7
23	5-3 (Pig)	D7
24	12-1 (Pig)	D28
25	12-2 (Pig)	D28
26	18-1 (Pig)	D28
27	18-2 (Pig)	D28
28	21-1 (Pig)	D28
29	21-3 (Pig)	D28
30	6-1 (Pig)	D7
31	8-4 (Pig)	D7
32	18-1 (Pig)	D7
33	12-3 (Pig)	D28
34	12-4 (Pig)	D28
35	15-3 (Pig)	D28

Table 14. Continued

PCR#	Pig and Isolate Number	Sampling day
36	4-1 (Pig)	D7
37	6-3 (Pig)	D7
38	7-3 (Pig)	D7
39	11-2 (Pig)	D7
40	13-3 (Pig)	D7
41	2-1 (Pig)	D7
42	6-2 (Pig)	D7
43	7-1 (Pig)	D7
44	7-2 (Pig)	D7
45	3-2 (Pig)	D7
46	3-4 (Pig)	D7
47	15-4 (Pig)	D28
48	18-4 (Pig)	D28
49	2-2 (Pig)	D7
50	2-3 (Pig)	D7
51	2-4 (Pig)	D7
52	4-3 (Pig)	D7
53	6-4 (Pig)	D7
54	1-4 (Pig)	D14
55	16-1 (Pig)	D14
56	16-3 (Pig)	D14
57	18-3 (Pig)	D14
58	18-4 (Pig)	D14
59	20-2 (Pig)	D14
60	25-1 (Pig)	D14
61	25-2 (Pig)	D14
62	22-1 (Pig)	D14
63	22-2 (Pig)	D14

PCR# represents a simplified numbering scheme for each random isolate tested. Pig number refers to the ear tag number each pig was assigned and isolate number refers to an isolated colony of *E. coli* as four colonies from each pig were tested in the MIC analysis. Example:15-1, 15 being the pig's assigned ear tag number and 1 being the first isolate of four tested. D= day

Table 15. *E. coli* Isolates used for electroporation

PCR#	Pig and Isolate Number	Sampling day	Number of colonies after electroporation
2	OR51-3	D7	No growth @ 20 hrs.
7	Y20-3	D14	>100 colonies
13	15-1	D28	No growth @ 20 hrs.
17	22-2	D28	>100 colonies
18	1-1	D7	>100 colonies

PCR# represents a simplified numbering scheme for each random isolate tested. Pig number refers to the ear tag number each pig was assigned and isolate number refers to an isolated colony of *E. coli* as four colonies from each pig were tested in the MIC analysis. Example:15-1, 15 being the pig's assigned ear tag number and 1 being the first isolate of four tested. D= day

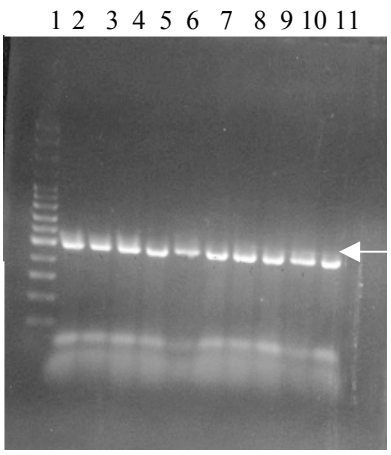

Figure 1. Detection of *aac*(*3*)-IV gene in *E. coli* isolates 1-9 from antibiotic treated sows and pigs via PCR.
PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the arrows. Lanes 2-10 contain isolates 1-9 respectively and can be found in Table 14. Lane 11 contains isolate 19 and Lane 1 is the molecular standard.

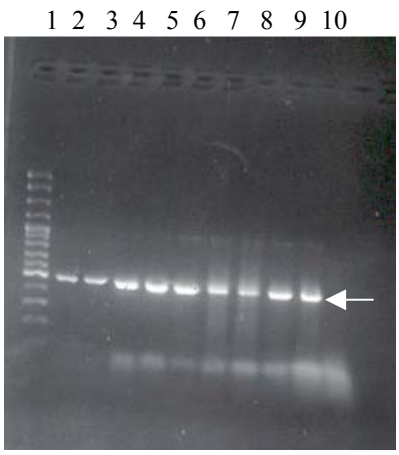

Figure 2. Detection of *aac*(*3*)-IV gene in *E. coli* isolates 10-18 from antibiotic treated sows and pigs via PCR.
PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the arrows. Lanes 2-10 contain isolates10-18 respectively and can found in Table 14. Lane 1 is the molecular standard.

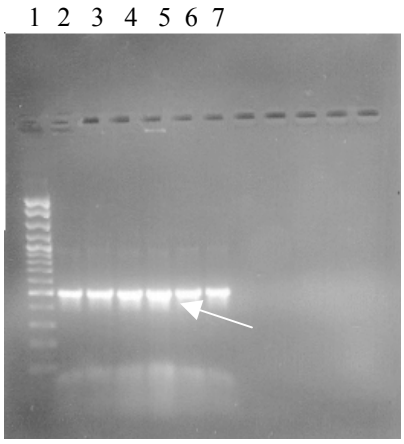

Figure 3. Detection of *aac*(*3*)-IV gene in *E. coli* isolates 20-25 from antibiotic treated sows and pigs via PCR.
PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the arrows. Lanes 2-7 are from isolates 20-25. Lane 1 is the molecular standard.

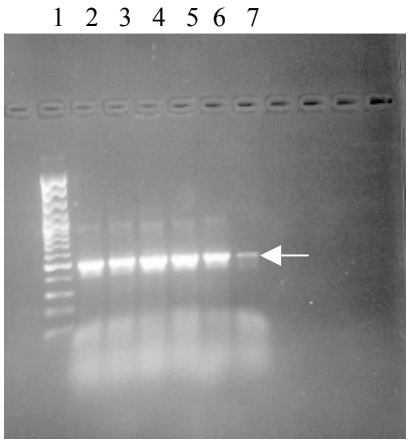

Figure 4. Detection of *aac*(*3*)-IV gene in *E. coli* isolates 26-30 from antibiotic treated sows and pigs via PCR.
PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the Lanes 2-7 are from isolates 26-30. Lane 1 is the molecular standard.

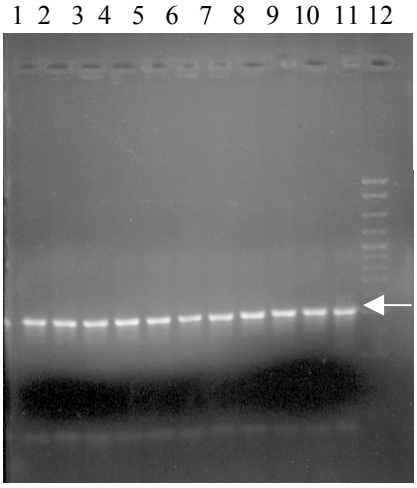

Figure 5. Detection of *aac*(*3*)-IV gene in *E. coli* isolates 31-41 from antibiotic treated sows and pigs via PCR.
PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the arrows. Lanes 1-11 contain isolates 31-41 respectively. Lane 12 is the molecular marker.

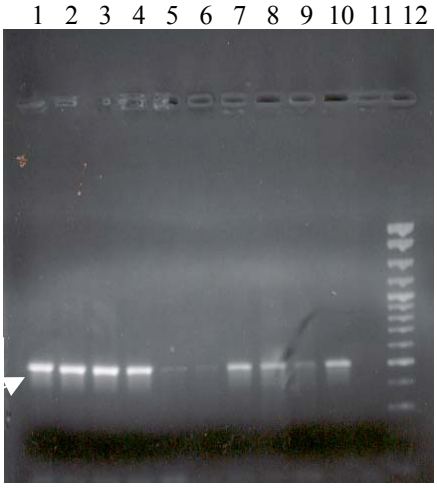

Figure 6. Detection of *aac*(*3*)-IV gene in *E. coli* isolates 42-51 from antibiotic treated sows and pigs via PCR.
PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the Lanes 1-11 have isolates 42-51. Isolates 46, 47 and 50 show weak amplification. Lane 12 is the standard.

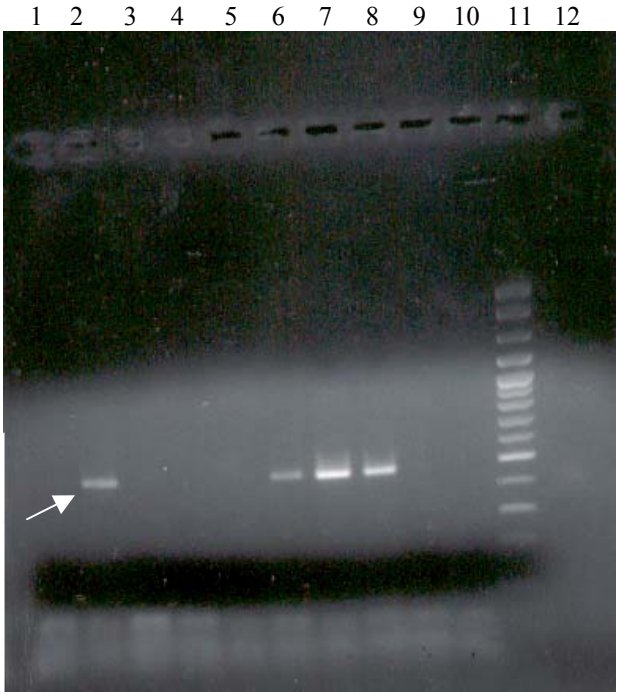

Figure 7. Detection of *aac*(*3*)-IV gene in *E. coli* isolates 52-60 from antibiotic treated sows and pigs via PCR.
PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the arrows.Lanes 1-9 contain isolates 52-60 respectively (table 14). Lane 10 is a negative control. Lane 11 is standard.

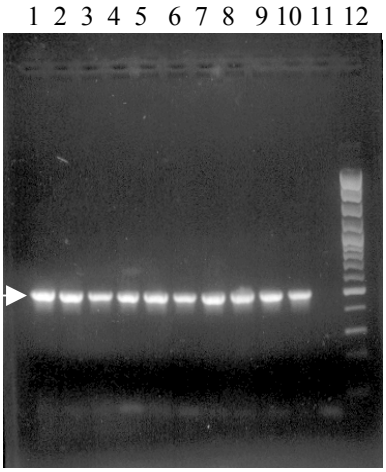

Figure 8. Detection of the *aac*(*3*)-IV gene in *E. coli* isolates 2-11 from non-antibiotic treated sows and pigs via PCR. PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the arrows. Lanes 1-9 contain isolates 2-11 (Table 13). Lane 10 is an apramycin positive control. Lane 11 is an apramycin negative control Lane 12 is the molecular weight marker.

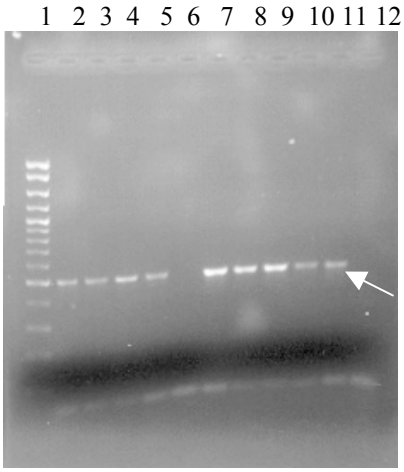

Figure 9. Detection of the *aac*(*3*)-IV gene in *E. coli* isolates 12-21 from non-antibiotic treated sows and pigs via PCR. PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the Lanes 2-10 contain isolates 12-21 (Table 13). Lane 11 is an apramycin positive control. Lane 12 is an apramycin negative control. Lane 1 holds The molecular weight marker.

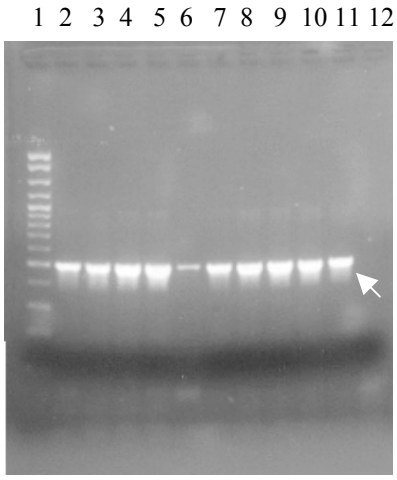

Figure 10. Detection of the *aac*(*3*)-IV gene in *E. coli* isolates 22-30 from non-antibiotic treated sows and pigs via PCR. PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the arrows. Lanes 2-12 are from isolates 22-30 (Table 13). Lanes 11 and 12 contain the apramycin positive and negative controls respectively.

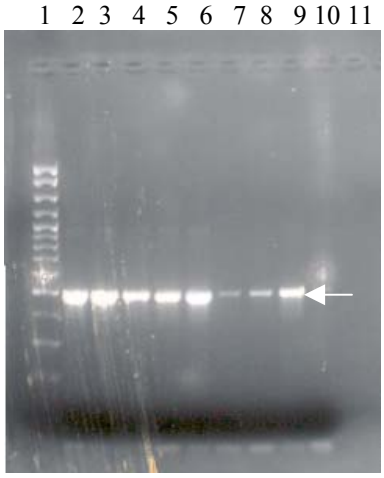

Figure 11. Detection of the *aac*(*3*)-IV gene in *E. coli* isolates 31-38 from non-antibiotic treated sows and pigs via PCR. PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the Lanes 2-9 are from isolates 31-38 (Table 13). Lanes 10 and 11 contain the apramycin positive and negative controls respectively.

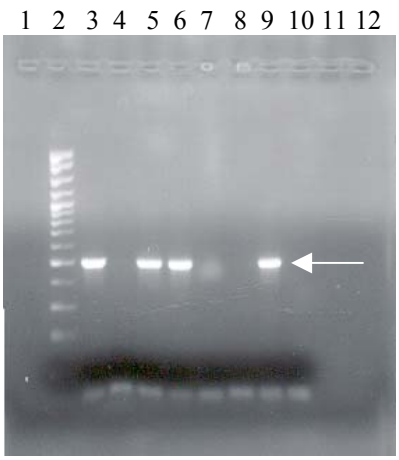

Figure 12. Detection of the *aac*(*3*)-IV gene in *E. coli* isolates 39-42 from non-antibiotic treated sows and pigs via PCR.
PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the arrows. Lanes 3-10 contain isolates 39-42 (Table 13). Lanes 7 and 8 contain apramycin-resistant *S*. Typhimurium. Lanes 9 and 10 are positive and negative Controls and Lane 2 is the molecular ladder.

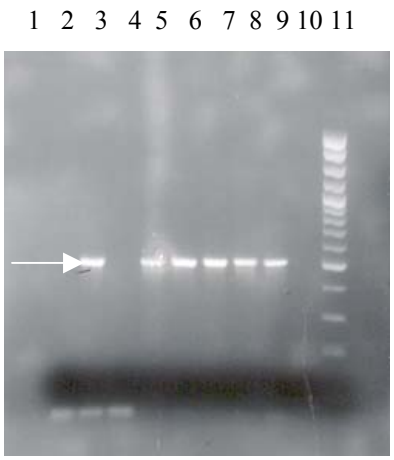

Figure 13. Detection of the *aac*(*3*)-IV gene in *E. coli* isolates 43-50 from non-antibiotic treated sows and pigs via PCR.
PCR products separated by gel electrophoresis in 1.0% agarose are stained with ethidium bromide for visualization. A fragment of 507 bp is indication of the presence of the *aac*(*3*)-IV gene in each lane as shown by the arrows. Lanes 1-7 contain isolates 43-50 (Table 13). Lanes 9 and 10 are positive and negative controls and lane 11 is the molecular ladder.

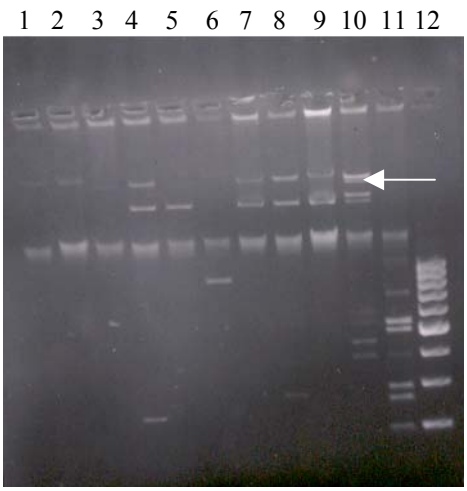

Figure 14. Plasmid profile of apramycin-resistant *E. coli* (1-10) from antibiotic treated sows and their pigs.
DNA separated by traditional gel electrophoresis and stained with ethidium bromide for visualization. Lanes1-10 contain isolates 1-10 (Table 14) and lane 11 contains a sensitive control strain. Lane 12 contains the molecular ladder. The white arrow indicates a 25 kb plasmid common to many of the apramycin-resistant isolates.

Figure 15. Plasmid profile of apramycin-resistant *E.coli* (11-20) from antibiotic treated sows and their pigs.
DNA separated by traditional gel electrophoresis and stained with ethidium bromide for visualization. Lanes 1-10 contain isolates 11-20 (Table 14) and lane 11 contains a sensitive control strain. Lane 12 contains the molecular ladder. The white arrow indicates a 25 kb plasmid common to many of the apramycin-resistant isolates.

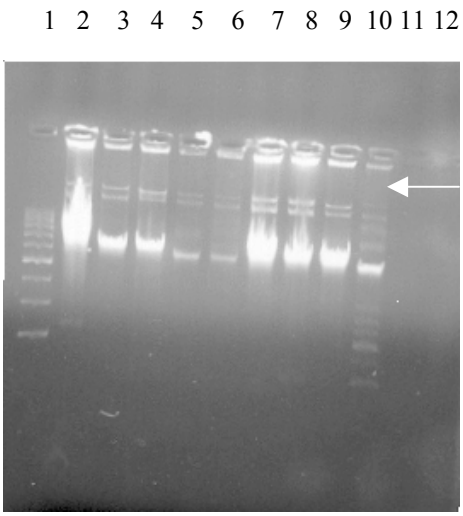

Figure 16. Plasmid profile of apramycin-resistant *E. coli* (1-8) from non-antibiotic treated sows and their pigs.
DNA separated by traditional gel electrophoresis and stained with ethidium bromide for visualization. Lanes 2-9 contain isolates 1- 8 respectively (Table 13). Lane 10 contains a sensitive control strain. Lane1 is the molecular ladder. The white arrow indicates a 25 kb plasmid common to many of the apramycin-resistant isolates.

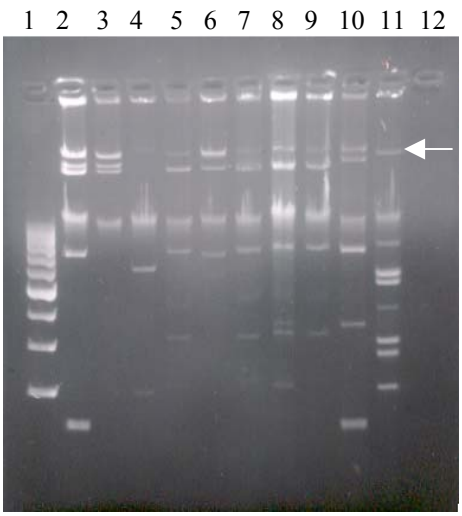

Figure 17. Plasmid profile of apramycin-resistant *E.coli* (26-35) from non-antibiotic treated sows and their pigs.
DNA separated by traditional gel electrophoresis and stained with ethidium bromide for visualization. Lanes 2-11 contain isolates 26-35 respectively (Table 13). Lane 12 contains a sensitive control strain. Lane 1 is the molecular ladder. The white arrow indicates a 25 kb plasmid common to many of the apramycin-resistant isolates.

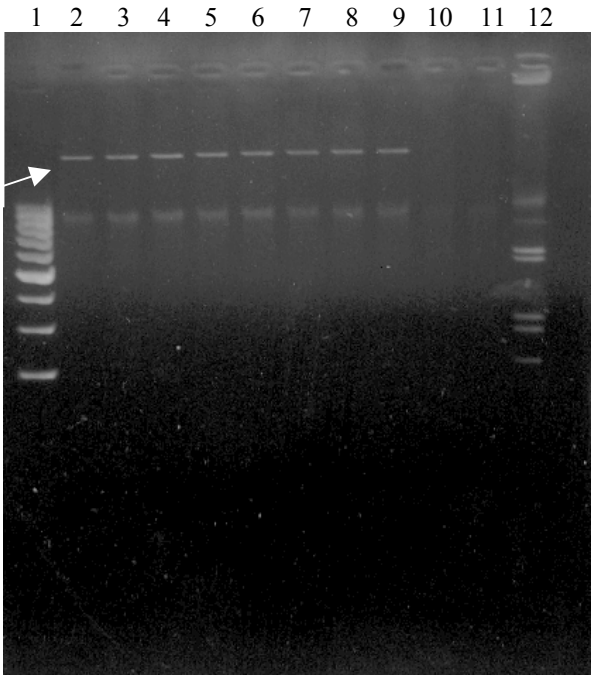

Figure 18. Electroporation of a sensitive *E. coli* strain.
DNA separated using traditional gel Electrophoresis and stained with ethidium bromide for visualization. The white arrow indicates the presence of large plasmids in the previously sensitive JM109 *E. coli* strain. These plasmids are comparable to those previously found associated with apramycin-resistant isolates. Lanes 2-9 contain electroporated JM109. Lanes 10 and 11 show the original JM109 (sensitive to apramycin) and lane 12 shows *E. coli* control strain V517 which served as a ladder.

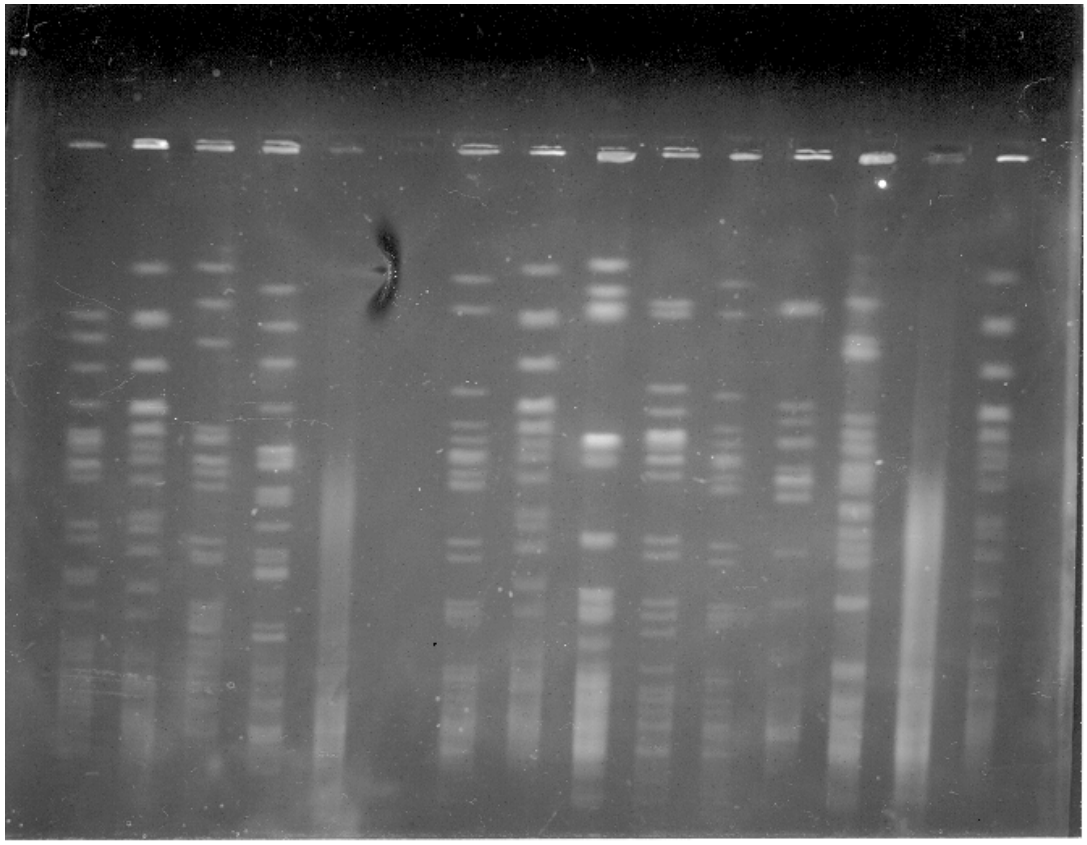

Figure 19. Macrorestriction profiles of apramycin resistant *E. coli* isolated from non-antibiotic treatment sows and their pigs.
DNA was digested with *Xba I* and separated in 1.0% agarose by PFGE. Lanes 2, 8, and 15 contain *E. coli* O157:H7 control. Lane 1 is d14 009-1, lane 3 is d14 169-2, lane 4 is d7 37-1, lane 5 is d7 38-3, lane 6 is blank, lane 7 is d7 39-2, lane 9 is d7 39-4, lane 10 is d7 51-4, lane 11 is d7 53-3, lane 12 is d7 56-3, lane 13 is d7 59-1, and lane 14 is d7 27-2.

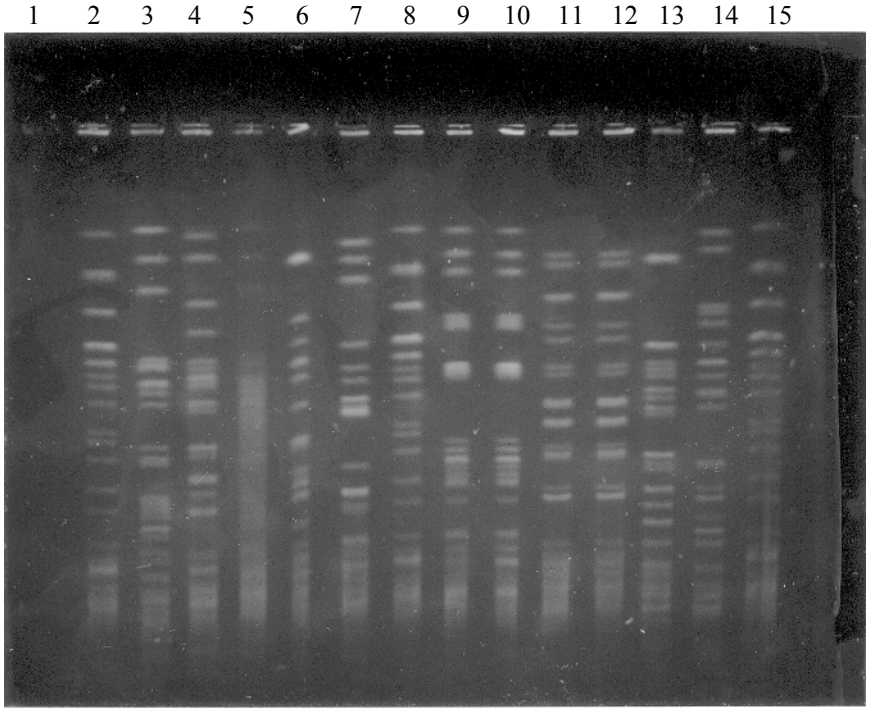

Figure 20. Macrorestriction profiles of apramycin resistant *E. coli* isolated from non-antibiotic treatment sows and their pigs.
DNA was digested with *Xbe I* and separated in 1.0% agarose by PFGE. Lane 1 is blank. Lanes 2, 8, and 15 contain the *E. coli* O157:H7 control. Lane 3 is d7 28-2, lane 4 is d7 30-1, lane 5 is d7 31-1, lane 6 is d7 31-3, lane 7 is d28 26-4, lane 9 is d28 28-2, lane 10 is d28 28-3, lane 11 is d28 27-2, lane 12 is d28 27-4, lane 13 is d0 202-1, and lane 14 is d0 202-2.

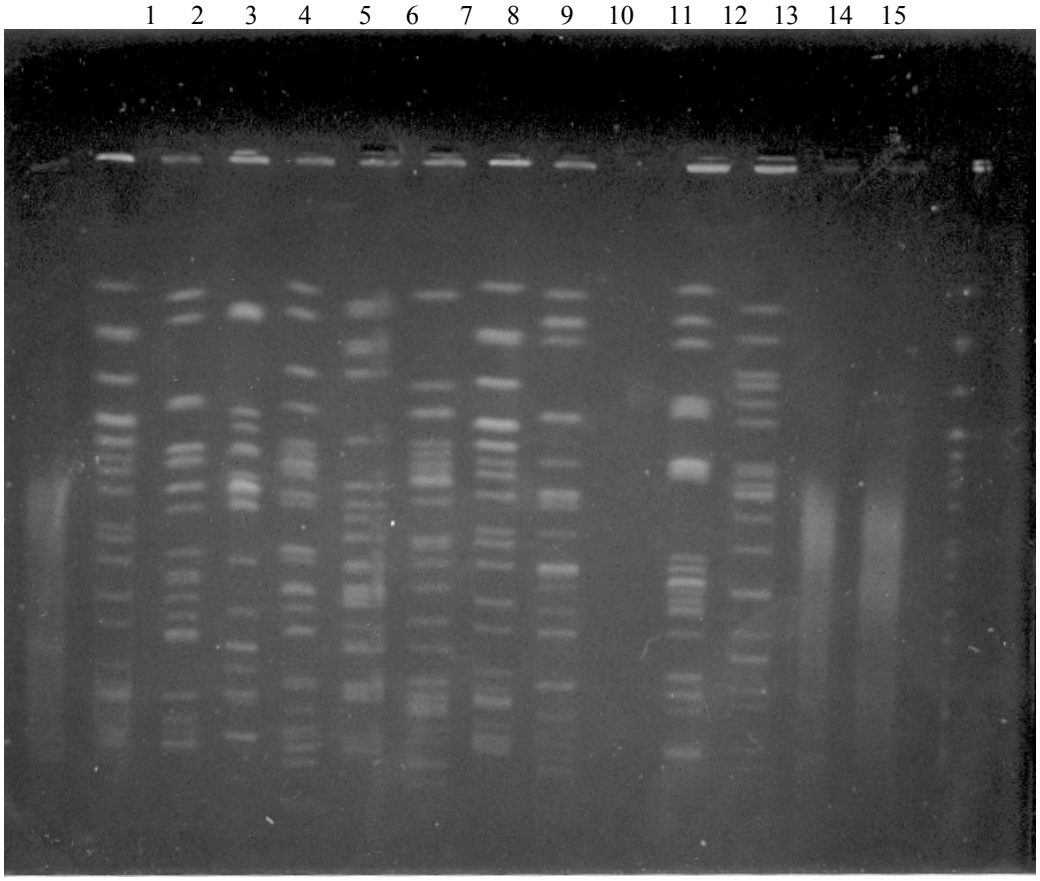

Figure 21. Macrorestriction profiles of apramycin resistant *E. coli* isolated from antibiotic treatment sows and their pigs.
DNA was digested with *Xba I* and separated in 1.0% agarose by PFGE. Lanes 2, 8, and 15 contain the *E. coli* O157:H7 control. Lane 1 is d7 Y20-3, lane 3 is d14 Y20-3, lane 4 is d0 OR58-4, lane 5 is d28 19-1, lane 6 is d7 1-3, lane 7 is d7 5-3, lane 9 is manure room 104-1, lane 10 is blank, lane 11 is manure room 106-1, lane 12 is manure room 117-2, lane 13 is manure room 116-2, and lane 14 is manure room 114-3.

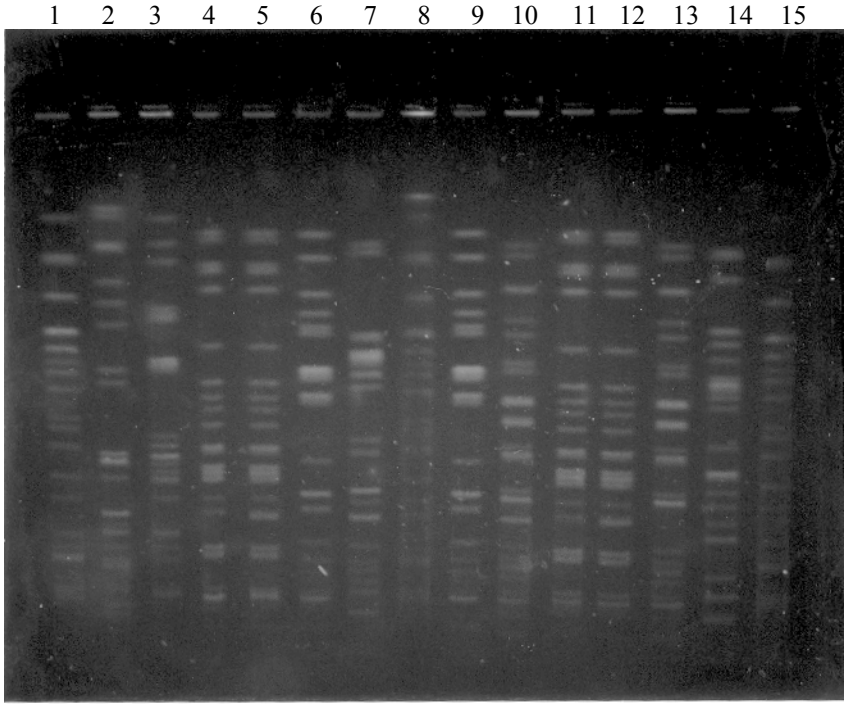

Figure 22. Macrorestriction profiles of apramycin resistant *E. coli* isolated from antibiotic treatment sows and their pigs.
DNA was digested with *Xba I* and separated in 1.0% agarose by PFGE. Lanes 1, 8, and 15 contain the *E. coli* O157:H7 control. Lane 2 is manure room 113-2, lane 3 is manure room 107-1, lane 4 is d7 2-1, lane 5 is d7 6-3, lane 6 is d28 12-4, lane 7 is d7 6-1, lane 9 is d28 21-3, lane 10 is d14 20-2, lane 11 is d7 4-3, lane 12 is d7 2-3, lane 13 is d28 15-4, and lane 14 is d0 7-2.

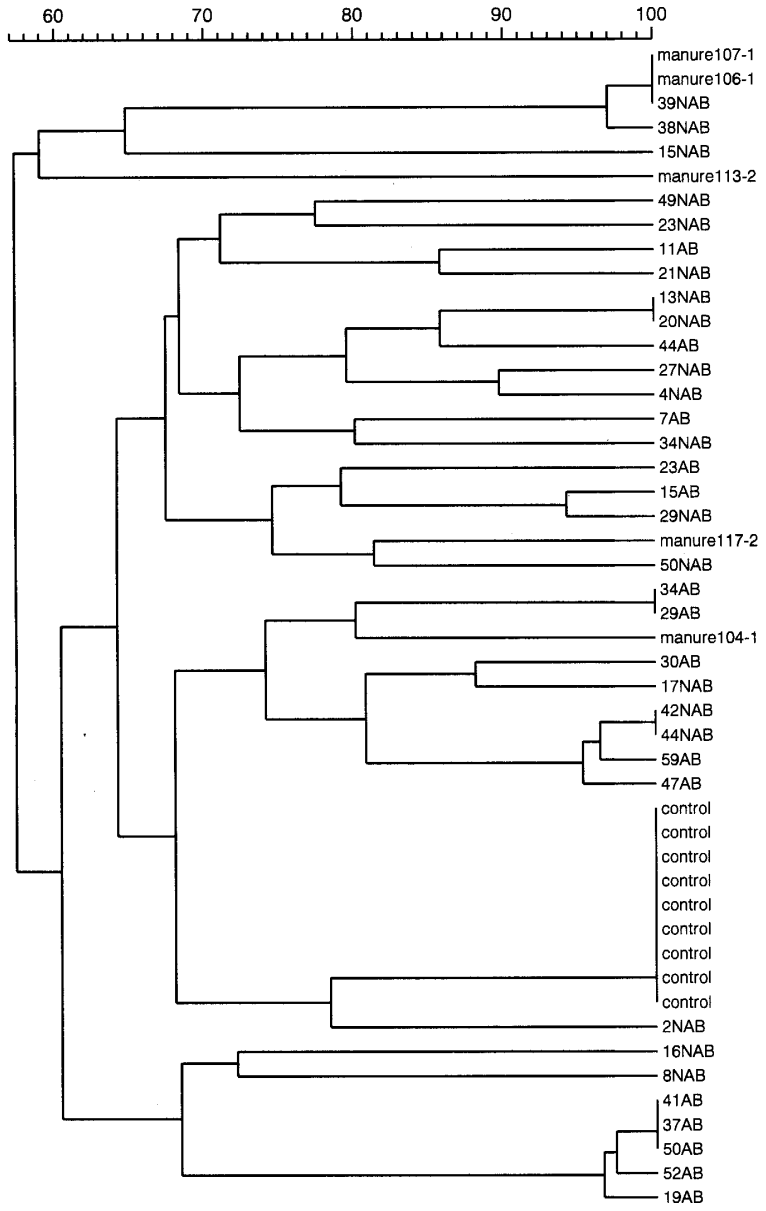

Figure 23. Dendrogram generated by Molecular Analyst Software for PFGE on non-antibiotic sows and pigs, antibiotic sows and pigs, and environmental manure samples from each treatment room. UPGMA clusters were made based on dice coefficients, and 2% tolerance in band position difference was used. The scale at the top represents % correlation between bands. NAB represents isolates from the non-antibiotic treatment group and AB represents isolates from the antibiotic treatment group. Explanations of isolate numbers are found in Tables 13 and 14. The control strain was *E. coli* O157:H7.

Vita

Kimberly Nakia Garner was born in Davenport, Iowa on August 20, 1977. However, two years later she moved with her family to Huntsville, AL where she currently considers home. Upon graduating from J. O. Johnson High School in 1995, she attended Tennessee State University in Nashville, TN where she obtained her Bachelor of Science degree in Agricultural Science in August 1999. Upon obtaining her Bachelors degree, Kim attended the University of Tennessee, Chattanooga for one semester where she was a part of the Environmental Science Masters program. While attending U.T. Chattanooga, she also worked with the Tennessee Department of Environment and Conservation as a laboratory technician. In January 2000, Kim was accepted into the Department of Animal Science at the U.T. Knoxville as a graduate research assistant under the leadership of Dr. Alan Mathew. Upon obtaining her Masters degree she would like to pursue a career in microbiological research.