To the Graduate Council:

I am submitting herewith a thesis written by Felix R. Jackson entitled "Effects of Antibiotic Regimens on Bacterial Resistance." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Animal Science

Alan G. Mathew, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council

Interim Vice Provost and
Dean of the Graduate School

Effects of Antibiotic Regimens on Bacterial Resistance

A Thesis

Presented for the

Master of Science

Degree

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Felix R. Jackson

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ABSTRACT

In 2 replicated trials, 144 weaned pigs were used to test the effects of antibiotic dosing schemes on resistance in bacteria. Pigs were inoculated with the foodborne pathogen, *Salmonella enterica* var Typhimurium, prior to being treated with feed- and water- based antibiotics. Treatments included maximum label use, rotation of similar and non-similar antibiotics, increasing gradient doses, and pulse dosing of antibiotics for a period of 2 weeks following pathogen challenge. Fecal samples were obtained prior to initiation of treatments, and on various days during the treatment and throughout the grow-finish phase. The challenge organism and non-pathogenic *E. coli* were recovered from fecal samples and tested against all antibiotics used in the study to determined effects on resistance patterns. Antibiotic resistance was affected to a greater extent in non-pathogenic *E. coli* compared to *Samonella* Typhimurium. Greater ($P < .0001$) resistance occurred when similar antibiotics (apramycin, gentamicin, neomycin) were used in rotation compared to the other treatments. Significant ($P < .05$) Time by Treatment interactions also occurred during or just following rotational treatment with similar antibiotics compared to other groups. These data indicate that dosing regimens affect antibiotic resistance patterns in bacteria associated with swine.

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1. Introduction

The practice of providing therapeutic and sub-therapeutic levels of antibiotics to livestock is well established. Antibiotics are one of the few compounds used in food animals therapeutically to treat disease and sub-therapeutically to increase production performance, feed efficiency, and to modify the nutrient composition of an animal product (National Research Council, 1998). Therapeutic use generally occurs after diagnosis of a disease and treatment is governed by label instructions (National Research Council, 1998). Sub-therapeutic use of antibiotics such as sulfonamides, aminoglycosides, and tetracyclines has for many years represented the largest and most controversial category of antimicrobial use. These feed additives are believed to be contributing to or helping to establish a reservoir of drug resistant enteric microorganisms, which may be capable of transferring their drug resistance to animal or humans pathogens (Smith, 1962; Falkow, 1975).

Since 1960, studies have demonstrated the development of drug resistance and *in vivo* resistance transfer between enteric bacteria, especially for isolates from animals fed antibiotics (Mitsuhashi *et al.*, 1961; Smith, 1962; Watanabe, 1963; Smith, 1975). Bacterial resistance to antibiotics has been shown to increase not only against the antibiotics being fed but also to other related antibiotics, including drugs to which the animals and their bacteria have been never exposed (Langlois *et al*, 1978; Dawson *et al*, 1983; Langlois *et al.*, 1983; Dawson *et al*, 1984). Thus,

the objective of this research was to compare the effects of antimicrobial regimens on resistance patterns of enterotoxigenic *E. coli* and *Salmonella enterica* var Typhimurium (hereafter referred to as *Salmonella typhimurium*) in weaned pigs.

2. Literature Review

Antibiotics in Agriculture. Antimicrobial agents have been used therapeutically and sub-therapeutically since 1950 in the United States (Jukes, 1972; Guest, 1976; Hays, 1976; Kiser, 1976). In 1956 the Food and Drug Administration approved the inclusion of antibiotics in animal feeds at sub-therapeutic levels with the drugs being introduced into the rations of young animals (Franco, *et al.*, 1990). Proven advantages of such practices include an improved feed/weight gain ratio and reduced susceptibility to disease (Knothe, 1977; Hagsten *et al.*, 1980). This sub-therapeutic use, defined in the United States as the use of an antibiotic as a feed additive at less than 200 g per ton of feed, delivers antibiotics that have therapeutic effects but at dosages below those required to treat established infections (National Research Council, 1998). Novick (1978) states, "close to 50% of all the antibiotics produced in the United States (35 million pounds manufactured annually) are added directly to the feed of farm animals, chiefly poultry, pigs, and beef cattle," and it is estimated that 75% of market swine have received antibiotic treatment in some form prior to slaughter (Guthrie, 1992). The Institute of Medicine states, "40% of the total antimicrobial production in the USA is used in animal feed, much of it in swine rations" (1998).

Resistance Factors. The use and role of antibiotics in animal production have been reviewed by Hays (1976) and Kiser (1976). They concluded that antibiotics have played an important role in the

development of highly intensive and efficient cattle, poultry, and swine production systems; and a major consequence of antibiotic usage worldwide has been the emergence of resistant bacterial strains, which has limited the effectiveness of these agents in treating infectious disease. Constant exposure of organisms to antibiotics has been shown to create a large pool of resistance plasmids that can become established in the pathogenic microorganisms of animals and humans by a variety of transfer mechanisms (Dunlaney *et al.*, 1971; Falkow, 1975; Levy *et al.*, 1976).

Plasmids are self-replicating, gene-containing circular pieces of DNA. They are found mainly in bacteria but also in some eucaryotic microorganisms. Resistance factors (R factors) are plasmids of great medical importance. R factors carry genes that confer upon their host resistance to antibiotics, heavy metals, or cellular toxins (Tortora *et al.*, 1995). R factors can present significant problems for the treatment of infectious disease with antibiotics. The widespread use of antibiotics in medicine and agriculture has led to the preferential survival (selection) of bacteria that have R factors, thus populations of resistant bacteria increase over time (Tortora *et al.*, 1995).

Transfer of Resistance Elements. In the 1950's Japanese investigators established that resistance to antimicrobials can be transferred between bacteria (Mitsuhashi *et al.*, 1961). The work of Watanaba (1963) showed that genes conferring resistance to one or more antibiotics could be transferred between bacterial cells during conjugation.

Ochiai *et al.*, (1959) found that multiple antibiotic resistance carried by *shigellae* could be transferred *in vitro* to *Escherichia coli* by cytoplasmic agents now known as plasmids, and it has been shown that most multiple resistance carried by *Enterobacteriaceae* is plasmid-mediated (Ochiai *et al*, 1959). Surveys carried out by Smith and Halls (1966), Walton (1966), Smith and Armour (1966), and Lee and Lewis (1969) have shown that such transferable resistance is not limited to one bacterial genus, nor to strains from one species of animal; and they can be carried by both pathogenic and non-pathogenic *Enterobacteriaceae*.

In the 1960's scientists became aware of plasmid-mediated resistance and later learned that clinical bacterial isolates resistant to several gram-negative antimicrobial products could transfer the genetic elements encoding that resistance to other bacteria (Gustafson, 1991). This finding became the central point of the focus on feed antibiotics and human health risks. It was postulated that the use of certain antibiotics in animal feed could generate large numbers of resistance plasmids in the enteric flora of livestock, and these bacterial elements might eventually encode antibiotic resistance in human pathogens (Gustafson, 1991).

To avoid reducing the therapeutic effectiveness of antibiotics used in human medicine, certain antimicrobials are designated for use only in animals (Christie *et al.*, 1981). As an example, apramycin, an aminoglycoside antibiotic which has not been used in human medicine, was licensed only for veterinary use (Wray *et al.*, 1986). However, there

may be problems with this approach. The microbial flora of animals exposed to these agents may quickly acquire resistance to them, necessitating a constant search for new antibiotics. In addition, as indicated earlier, plasmids determining resistance to one compound often carry multiple genes for resistance to other antibiotics, including those useful in human medicine.

Human Health Perspective of Resistance. The public health significance of antimicrobic resistant pathogenic bacteria has been recognized since the early 1960s (Braude, 1978). Resistant bacteria may enter the environmental pool from which their determinants can spread to bacteria causing human disease. Petrocheilou *et al.*, (1977) demonstrated that tetracycline treatment of a woman resulted in selection of tetracycline-resistant *E. coli* strain and that the organism very quickly became the predominant coliform in her husband, who was not taking antibiotics.

Resistant bacteria from food animals are of concern to public health whether or not they are themselves pathogens. The carriage of antimicrobial resistant fecal *Escherichia coli* is common among apparently healthy humans, irrespective of previous antimicrobial use (Linton *et al.*, 1972; Degener *et al.*, 1983), and is regarded as a public health hazard because *E. coli* is a common opportunistic pathogen of people (Hartl and Dykhuizen, 1984; Phillips *et al.*, 1988). Furthermore, *E. coli* are important

reservoirs of antimicrobial resistance genes for other pathogenic gram-negative bacteria (Hartl and Dykhuizen, 1984; Hunter *et al.*, 1992).

Salmonella. The Salmonella genus was named after Daniel E. Salmon, a U. S. veterinary surgeon, in 1884. Salmonella is a rod-shaped, motile (nonmotile exceptions include *S. gallinarum* and *S. pullorum*) nonsporeforming, gram negative bacterium (Tortora *et al.*, 1995). It conforms to the Enterobacteriaceae family as a non-spore forming, oxidase negative, glucose fermenting, facultative anaerobe (Guthrie, 1992). Almost all members of this genus are potentially pathogenic. Salmonellae are common inhabitants of the intestinal tracts of many animals, especially poultry and swine. Although members of the genus *Salmonella* are commonly referred to with species-like names, such as *S. typhimurium* and *S. dublin*, no individual species are recognized. Instead, this genus is taxonomically divided into about 2000 serovars (serotypes); that is, they are differentiated by serological means (Tortora *et al.*, 1995).

Over 2 million cases of meat and poultry foodborne disease are caused by *Salmonella* or *Campylobacter* in humans in the United States each year, resulting in health-related costs approaching 1.4 billion dollars (Menning, 1988; *Salmonella* Surveillance, 1988;). In most cases for *Salmonella,* the route of entry into a host (human or animal) is oral, with the host ingesting the bacteria directly (fecal-oral) or indirectly (fecal/food-oral) (Menning, 1988).

Salmonella typhimurium is the second most frequently isolated serotype of *Salmonella* from swine (Ferris *et al.*, 1985). That serovar causes enterocolitis of varying severity and is commonly involved in enzootics of diarrhea, especially in closed herds (Wilcock, 1986). Of equal importance is the fact the *S. typhimurium* is the leading cause of *Salmonella* food poisoning in human beings (*Salmonella* Surveillance, 1985), in which it also causes enterocolitis. The prevalence of this organism in swine and other meat animals constitutes a large, uncontrolled reservoir of critical importance in animals and human health (Holmerg, 1994). Through transport and at the abattoir, infected swine transmit *Salmonella* to other swine, which may lead to contamination of premises, equipment, and ultimately the final product (Woods *et al.*, 1989).

Salmonellosis is an important disease of domestic livestock and a major public health hazard. Most of the estimated 2 million human cases of food poisoning in the United States each year are attributed to consumption of infected meat products. Poultry and pork have been most frequently incriminated (Anonymous, 1998). Acute symptoms include nausea, vomiting, abdominal cramps, and diarrhea, which occur between 6 to 48 hours after infection. The duration of symptoms may last for 1 to 2 days, or longer. The severity and duration of infection depend on host's age, health, ingested dose, and strain characteristics (Anonymous, 1998).

Relevance of Salmonella to the Swine Industry. *Salmonella* are ubiquitous in nature, and have been recovered from nearly all vertebrates

(Taylor, 1969). In swine *Salmonella* are responsible for millions of dollars in lost revenue (Roof *et al.*, 1992). Of the 2,500 different serotypes described to date, only *Salmonella typhimurium* and *Salmonella choleraesuis* are important causes of clinical disease in pigs (Roof *et al.*, 1992). *Salmonella choleraesius* is a highly invasive organism commonly causing septicemic salmonellosis in weaned pigs. Clinical signs first appear 24 to 36 hours after infection (Reed, 1986). Although, distinctly different as to clinical signs, many parallels can be drawn between the two when discussing pathogenesis and immunity. Salmonellae are normally introduced orally and pass through the stomach where surviving bacteria then colonize the intestine (Roof *et al.*, 1992). Intraluminal replication varies with serotype: *Salmonella typhimurium* replicates to a greater extent intraluminally than the inherently invasive *Salmonella choleraesuis* (Wilcock, 1981).

Salmonellosis in pigs occurs mostly among intensively reared weaned pigs younger than four months of age. *Salmonella choleraesuis* is the most frequent isolate from feeder pigs. During the acute phase of the disease, pigs may shed from 10^6 and 10^7 *S. typhimurium* per gram of feces (Wilcock *et al.*, 1981). The infective dose depends on such factors as gastric acidity, animal density, stress, and susceptibility of the pigs (Wilcock *et al.*, 1981). Swine are frequently exposed to low levels of a variety of *Salmonella* serotypes from food, water, rodents, birds, and feces. *Salmonella* can establish clinically inapparent infections, which

only become important upon stress of the animal (Roof *et al.*, 1992). Very little is known about the carrier state of *Salmonella* in swine; but the ability of the organism to survive as an intracellular parasite has been suggested as a potential mechanism for persistent survival, with feces being the most likely method of transmission between animals (Wilcock *et al.*, 1981).

Woods *et al.*, (1989) documented that swine exposed to *S. typhimurium* by oral ingestion of the organism at 6 to 8 weeks of age remained infected after convalescence until at least 34 to 36 weeks of age, when the study was terminated. During that period, the organism was found most frequently and most persistently in the palatine tonsils, cecum, ileum, colon, mandibular lymph nodes, and rectum, in that order. Results of the study provided information indicating the tissues that should be targeted in efforts to reduce or eliminate the *Salmonella* carrier state.

In recent years, more attention has focused on the importance of sub-clinical salmonellosis and its possible role in human food poisoning. Wegener *et al.*, (1997) reported that "contaminated pork was estimated to account for 10% to 15% of outbreaks of salmonellosis in humans in Denmark." A similar figure was reported from the United States, where pork was responsible for 14.6% of outbreaks of salmonellosis in humans traced to the ingestion of meat (Bryan, 1980).

Salmonella infection of processed pork can occur by a variety of mechanisms. Before the slaughter process, pigs are crowded and stressed,

which may promote cross-contamination. During the slaughter process, contaminated dehairing machines, scalding tanks, and polishers can facilitate the spread of *Salmonella* (Roof *et al.*, 1992).

Escherichia coli. First described by Theodor Escherich in the late 1800's (Bertschinger *et al.*, 1992), *E. coli* are gram negative, rod-shaped bacteria belonging to the family of enterobacteriaceae. *Escherichia coli* are normal inhabitants of the alimentary tract of all mammals and are readily isolated post mortem, thus there has been considerable controversy regarding their role in enteric disease. A major problem of earlier studies was that the available techniques were not capable of differentiating between types of *E. coli*; and it was not until serological procedures developed for the classification of *Salmonellea* were applied to *E. coli* that the situation was clarified (Kauffmann, 1954).

There are currently four recognized classes of enterovirulent *E. coli* (collectively referred to as the EEC group) that cause gastroenteritis in humans. Among these are the enteropathogenic (EPEC) strains. EPEC are defined as *E. coli* belonging to serogroups epidemiologically implicated as pathogens but whose virulence mechanisms are unrelated to the excretion of typical *E. coli* enterotoxins (Bertschinger *et al.*, 1992). Source(s) and prevalence of EPEC are not well defined because foodborne outbreaks are sporadic. Humans, bovines, swine, poultry, and other species can be infected (Bertschinger *et al.*, 1992).

Relevance of *E. coli* to the Swine Industry. Neonatal diarrhea in piglets follows the proliferation of certain strains of *Escherichia coli* in the small intestine (Smith and Jones, 1963). These enteropathogenic strains synthesize enterotoxins which produce diarrhea and dehydration, frequently resulting in death of the piglets (Sellwood et al., 1975). The rapid proliferation of *E. coli* in the small intestine appears to be attributable to the ability of the bacteria to attach themselves to the intestinal epithelium (Sellwood et al., 1975) thereby avoiding removal from the small intestine by peristalsis (Dixon, 1960).

K88 *E. coli.* Sojka (1965) noted that the majority of *E. coli* strains isolated from cases of neonatal piglet diarrhea poses a surface antigen designated K88, and it has been shown that this antigen is an essential virulence determinant (Jones and Rutter, 1972) apparently because it enables K88-positive *E. coli* to attach to the intestinal lining (Jones and Rutter, 1972). K88 *E. coli*, have been implicated as a major source of diarrhea in postweaned pigs, with one study showing 72% of strains isolated from pigs greater than 24 days old being K88 positive (Wilson and Francis, 1986). Enterotoxigenic *E. coli* (ETEC) that express K88 fimbriae on their surface are known to bind the brush border of intestinal cells and cause diarrhea (Wilson and Francis, 1986). Because of their involvement in the adhesion of bacteria to brush border cells, K88 fimbriae are commonly referred to as K88 adhesins (Wilson and Francis, 1986).

Aminoglycosides. The aminoglycosides which include apramycin, streptomycin, gentamicin, kanamycin, tobramycin, amikacin, neomycin, and others have a broad antibacterial spectrum, including many gram positive as well as gram negative organisms. They are used alone, or more often, in combination with other antimicrobial drugs, to treat infections caused by bacteria (Bayley, 1993).

Aminoglycosides are protein synthesis inhibitors and act in a similar fashion as the tetracyclines, in that they cause incorrect incorporation of amino acids into bacterial peptides. Several members of this family, however, may also interfere with cellular electron transport, interfere with cell membranes, or breakdown of RNA (Davis *et al.*, 1980).

Apramycin, an aminoglycoside, which has not been used in human medicine, was licensed for veterinary use in the United Kingdom in 1980. This antibiotic has been reported as both a control and treatment of enteritis caused by gram-negative bacteria in young pigs (Bywater, 1991). Apramycin has a 28-day withdrawal period.

Neomycin is obtained from *streptomyes fradiae* and is a water-soluble basic compound. This antibiotic is similar to streptomycin chemically, pharmacologically, and in antibacterial activity. It is active against *Staphylococci*, *Streptococci*, *E. coli*, and *Proteus*. In some severe infections caused by bacteria resistant to other antibiotics, neomycin may be effective if given by intramuscular injection, intramammary injection, or applied locally. Neomycin has a 20-day withdrawal period for swine.

Gentamicin is an aminoglycoside with bactericidal action against both gram-negative and some gram-positive bacteria. This antibiotic has been reported as a treatment for enteritis caused by gram-negative bacteria in young pigs (Bayley, 1993). Susceptible bacteria are *Staphylococci*, *E. coli*, *Enterobacter*, *Salmonella*, *Proteus*, and others. Gentamicin has a 45-day withdrawal period for meat producing animals (Baryley, 1993)

Sulfonamides. The sulfonamides are derivatives of sulfanilamide, which contains the structural prerequisites for antibacterial activity. As a group, sulfonamides are quite insoluble; however they are more soluble at alkaline than at acid pH (Bayley, 1993). Certain sulfonamides are designed with low solubility, so they are slowly absorbed and are intended for use in treatment of enteric infections.

Sulfonamides are broad spectrum antimicrobial agents, inhibiting bacteria, chlamydiae, toxoplasma, and other protozoal agents such as coccidia. The MIC (minimum inhibitory concentration) of a sulfonamide for a given bacteria is imprecise and comparisons cannot readily be made among laboratories. Even within a laboratory, comparisons show variation of MIC between sulfonamides. Resistance to sulfonamides is widespread in bacteria isolated from animals, reflecting extensive use of the drug over many years. There is complete cross-resistance between the sulfonamides (Bayley, 1993).

Sulfamethazine is a sulfonamide that has been widely used in food producing animals and can attain effective plasma concentrations when

administered therapeutically or sub-therapeutically. Sulfamethazine has a 12-day withdrawal period (Bayley, 1993).

Antibacterial Susceptibility Testing. Antibacterial susceptibility testing may be performed reliably by either dilution or diffusion methods. The choice of methodology may be based on factors such as relative ease of performance, cost, flexibility in selection of drugs for testing, use of automated or semiautomated devices to facilitate testing, and the perceived accuracy of the methodology (Murray *et al.*, 1999).

Disk Diffusion Method. The disk diffusion method of susceptibility testing allows categorization of bacteria isolates as susceptible, resistant, or intermediately resistant to a variety of antimicrobial agents. This method measures the diameter of inhibition of growth around paper disk laced with antibiotics. Susceptibility is determined based on zone sizes according to susceptibility standards, as provided by the manufactures of each antibiotic. As the distance from the disk increases, the concentration of the antimicrobial agent decreases logarithmically, creating a gradient of drug concentrations in the agar medium surrounding each disk. Concomitant with diffusion of the drug, the bacteria that were inoculated on the surface and that are not inhibited by the concentration of antimicrobial agent continue to multiply until a lawn of growth is visible (Murray *et al.*, 1999). In areas where the concentration of drug is inhibitory, no growth occurs, forming a zone of inhibition around each disk. Using a metric ruler, zones sizes are reported

in millimeters and categorized accordingly. This method should not be used to evaluate the antimicrobial susceptibilities of bacteria that show marked strain-to-strain variability in growth rate (Murray *et al.*, 1999).

Broth Microdilution Method. The dilution susceptibility method is used to determine the minimal concentration, usually micrograms per milliliter, of an antimicrobial agent required to inhibit or kill a microorganism. Antimicrobial agents are typically tested at two-fold serial dilutions, and the lowest concentration that inhibits the visual growth of an organism is regarded as the MIC (Murray *et al.*, 1999). The use of broth microdilution trays prepared in-house (laboratory) provides a reliable standardized reference method for susceptibility testing. Inoculation and reading procedures allow relatively convenient simultaneous testing of several antimicrobial agents against individual organisms. Results of testing may be determined by visual examination or with semiautomated or automated instrumentation.

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of antibiotics that inhibits visible growth. The advantage of the broth dilution method is that it provides more quantitative information and may be applied to a wider range of isolates than the diffusion test. Another advantage is that MICs can be useful for evaluating relative degrees of susceptibility of bacteria to various antimicrobial agents and for comparing the relative activities of drugs against various organisms. The broth dilution method was used in this study because in addition to

allowing determination of resistance, this method also allowed the determination of trends or changes in susceptibility to antibiotics over the course of the test period.

3. Materials and Methods

Two experiments were conducted. In Experiment one, pig groups where housed during the study in isolated facilities where transfer of resistant bacteria between treatment groups was inhibited. In Experiment two, pigs were similarly housed until 6 days post inoculation (6th sampling period) whereupon they were moved to a common growing-finishing facility where airborne transfer of resistant organisms may have occurred between treatment groups.

Animal housing. The primary facility used for this research was a newly constructed (1998) intensive animal research facility (Johnson Animal Research and Teaching Unit) located at the University of Tennessee Agriculture Experiment Station, on the Knoxville campus. This facility was used for the first half of Experiment one, and until pigs reached market weight in Experiment two. Each room contained one 4'x 4' nursery pen, one 8' x 8' finisher pen, and associated feeding and water equipment. Each room was self contained with independent ventilation and waste removal systems, thus minimizing the chance of cross contamination of bacteria between rooms. In addition, two of the rooms were equipped with medicated treatment units for dispensing antibiotics via drinking water. The rooms and animals were cleaned on a daily basis. Cross contamination of rooms was prevented by the use of disposal boots, coveralls and disinfectant boot washes.

In Experiment two, pigs were transported via trailer to the growing-finishing facility of the University of Tennessee Experiment Station at Crossville after 6 days post inoculation. There, the pigs were maintained in their respective treatment groups in finishing pens located in a common room.

Experimental design. Because pigs in Experiment one and Experiment two were treated in the same fashion during the first 6 days post inoculation, the two experiments were treated as replicated trails during that time. For the 2 replicated trials, a total of 96, approximately 18-21 day-old weaned pigs, with no history of antibiotic use, were challenged intranasally with 10^9 colony forming units (CFU) of *Salmonella typhimurium* (Parent strain: 798-4232, National Animal Disease Control, USDA, Ames, Iowa) derived from a confirmed case of swine salmonellosis. Additionally, pigs were orally inoculated with 10^8 CFU of a hemolytic enterotoxigenic *E. coli* (K88) (*E. coli* Reference Center, Pennsylvania State University). Challenge cultures were prepared 16 hours prior to challenge. For *Salmonella typhimurium*, a 20 uL of stock solution was inoculated into 200 mL of Luria Broth (10 g/L Bacto-tryptone (Difco, Detroit, MI), 5 g/L yeast extract (Difco), 10 g/L NaCl, adjusted to pH 7.5 w/NaOH) and incubated overnight at 37°C. For K88 *E. coli*, a loopful of the original isolate, confirmed for nalidixic resistance and hemolysis, was inoculated into 300 mL of Luria Broth with 50 *u*g/mL nalidixic acid (Sigma, St. Louis, MO) and incubated overnight at 37°C.

The K88 challenge organism was used to determine the efficacy of treatments against colonization by enteric pathogens, whereas *Salmonella typhimurium* was used to determine extraneous effects of antibiotics on a foodborne pathogen.

Following the challenge (inoculation of both organisms) pigs were blocked by litter and divided into six groups of eight pigs each. Groups were then randomly assigned to treatment rooms and provided ad libitum access to feed and water. The pigs were fed an antibiotic-free diet for the first week of the trial (Table 1). The rooms were provided 12 hours light per day. Temperature in rooms was maintained at 80-85°C, and was decreased as the pigs aged. The antibiotic treatments were initiated the second week of the trial and consisted of the following: **1)** control **(C)** no antibiotic; **2)** apramycin (**LU**) (150 g/ton of feed) for 14 days (maximum label use); **3)** gradient **(G)** application with apramycin at 50 g/ton of feed for 5 days, then 100 g/ton for 5 days, then 150 g/ton for 4 days (increasing the concentration of apramycin over a 14-day period); **4)** pulse dosing (**PD**) with apramycin (150 g/ton of feed) for 3 days on, 3 days off, and repeating this sequence throughout a 14-day period; **5)** rotation with unlike antibiotics (**RU**) with apramycin for 5 days, followed by sodium sulfamethazine (118 mg/kg body weight) in drinking water for 5 days, followed by carbodox (50 g/ton of feed) for 4 days; and **6)** rotation with like antibiotics (**RL**) with apramycin in the feed for 4 days, followed by gentamicin (25 mg/gallon) in drinking water for 5 days, followed by

neomycin sulfate (22 mg/kg body weight) in drinking water for 5 days. Treatments 5 and 6 were used to determine effects of antibiotic regimens on resistance within and across antibiotic types. Upon the completion of the 14-day treatment period (apramycin has a maximum label use of 14 days) all treatment groups were placed on the second phase diet (Table 1).

Microbiological Procedures: Pigs were rectally swabbed on days 3, 6, 10, 13, 17, 31, 41, 70, 101, post challenge, using dacron fiber tipped swabs (Fisher, Houston, TX). The samples were then placed in sterilized test tubes, transported on ice to the laboratory (Brehm Hall Animal Science Building), and streaked onto lactose MacConkey Agar (Difco), for the isolation and differentiation of coliforms. Additionally, samples were streaked onto Trypticase Soy Agar with 5% sheep blood (BBL, Becton Dickerson Microbiology Systems, Cockeyville, Maryland) for determination of hemolytic bacteria. To detect K88 *E. coli*, samples were streaked onto lactose MacConkey agar containing 50 *u*g/mL nalidixic acid to inhibit non-K88 coliform growth. The three media were then incubated at 37°C for 18-24 hours. Colonies which were consistent with pink coloration and morphology of *E. coli* were selected from the lactose MacConkey Agar. Beta-hemolytic colonies on Trypticase Soy Agar with 5% sheep blood appeared as creamy to white with a distinct clear zone surrounding the colony, were determined to be hemolytic. This clearing indicates lysis (decomposition) of red blood cells caused by the bacteria.

TABLE 1. Composition of basal diet for pigs

Item	Percentage of Mix (%)	
Ingredients	Phase 1[a]	Phase 2[b]
Corn	39.98	61.36
Soybean meal (48% CP)	26.13	27.00
Whey	20.00	5.00
Fish meal	5.00	1.66
Salt	.50	.26
Fat	.60	2.23
Limestone	.60	.73
Dical phosphate	.07	1.43
Lysine-HCl	.03	.01

[a] Composition of pig diet fed 21-35 days post weaning.
Diet was formulated to contain 1.3% lysine, 22.3% protein, 8.1% fat, .85% Ca, .7% phosphate, .4% salt, 2% CP, .6% milk protein, .2% tryptophan, .9% threonine, .3% methionine, .3% histidine, 1.93% leucine, and .18% trace mineral-vitamin premix

[b] Composition of pig diet fed 34-56 days post weaning.
Diet was formulated to contain 1.1% lysine, 19.8% protein, 5.0% fat, .8% Ca, .7% phosphate, .4% salt, 2% CP, .6% milk protein, .3% tryptophan, .1% threonine, .3% methionine, .3% histidine, 1.8% leucine and .20% trace mineral-vitamin premix.

Randomly selected colonies were subjected to an API20E test (BioMerieux Vitek, Inc., 595 Anglum Drive Hazelwood, MO). The API20E consists of a series of biochemical tests used to identify *Enterobacteriaceae* and other gram negative bacteria.

Isolation of Salmonella. The procedures used to isolate the *Salmonella typhimurium* challenge organism involved three steps: **1)** enrichment in tetrathionate broth (Difco), **2)** plating on xylose-lysine-tergitol-4 (XLT4) (Difco), and **3)** biochemical confirmation using lysine iron agar (Difco) and sugar iron slants (Difco), as indicated in details below.

After the swabs had been streaked on the respective media, the tips were removed and placed in glass tubes, to which 5 mL of Cryo-Pro solution (100 mL H_2O, 3 g T-soy, 14 mL horse serum, 28 mL glycerol) was added. The tubes were then vortexed and 2.5 mL was pipetted into a Stomacher bag (Fisher) containing 80-100 mL of tetrathionate broth (Difco). The Stomacher bags were then sealed and incubated at 42°C for 18-24 hours.

Following enrichment in tetrathionate broth the cultures were plated on XLT4 medium (Difco), which is a highly selective medium for non-typhi salmonella (Difco manual). Preparation of XLT4 was preformed according to manufacturers instructions. In addition, nalidixic acid was added to a concentration of 50*ug*/mL (after media cooled to approximately 80 °C) to exclude the growth of salmonellae other than the

challenge isolate. The stomacher bags were removed from the incubator, and using a sterile sharp scalpel, small incisions were made across top of each bag. A 4 mm wire loop was used to transfer a loopful of culture from the bag to the XLT4 medium (previously poured into 100 x 15mm petri plates). In order to promote distinct and individual colonies, three streaks were made across the top of the plate. Then three streaks were drawn from the original. This sequence was repeated twice until four sets of streaks were drawn on the plate. Plates were incubated at 37°C for 18-24 hours. Non-typhi salmonella appeared on the medium as black or black centered colonies with pink outer layer. The presumed colonies were then biochemically tested using Triple Sugar Iron or TSI (Difco) and Lysine Iron Agar or LIA (Difco) to confirm them as salmonella.

Triple Sugar Iron medium was used for the differentiation of gram negative enteric bacilli, based on carbohydrate fermentation and the production of hydrogen sulfide (Draughon, 1998 personal communication). TSI contains three sugars (dextrose, lactose, and sucrose) and phenol red as an acid based indicator of fermentation. The agar was dispensed into a glass tube (slanted position until solidified) streaked and inoculated (stabbed) with presumed isolates, then incubated for 18-24 hours at 37°C. Confirmed salmonella isolates produced an acid butt (yellow) from fermentation of dextrose, alkaline slant (red) and hydrogen sulfide (black section in middle of tube).

Lysine Iron Agar medium was used to differentiate salmonella based on fermentation of sugars, lysine decarboxylation, and hydrogen sulfide. Bromocresol purple was used as a pH indicator in this medium. Organisms producing lysine decarboxylase produce an alkaline condition (purple color in bottom of tube) (Draughon, 1998 personal communication). The medium was dispensed into glass tubes, streaked, and stabbed, then incubated for 18-24 hours at 37°C. Confirmed isolates produced an acid reaction (yellow) and an alkaline reaction (red) on both the slant and butt of the tube.

Antibiotic Sensitivity Testing. From two of the media (MacConkey and XLT4 with nalidixic acid) four isolates (individual colonies) were obtained from each plate (*E. coli* from lactose MacConkey and Salmonella from XLT4). Using a 4 mm wire loop, the colonies were transferred to a sterile test tube filled with 5 mL Mueller-Hinton II broth (BBL), placed in racks at a 45° angle in a shaking water bath (Lab-Line Instrument Melrose Park, ILL) and incubated at 37°C. Cell cultures were grown to a concentration matching a .5 McFarland standard turbidity level (approximately 10^8 CFU/mL) (NCCLS, 1976). Mueller-Hinton II broth (cation adjusted) is recommended as the medium for susceptibility testing of commonly isolated rapidly growing pathogens (NCCLS, 1976). The incubation time for *E.coli* in the Mueller-Hinton II broth ranged from 10-15 minutes, whereas for *Salmonella* incubation time ranged from 20-30 minutes. After cultures reached the appropriate turbidity level they were

removed and placed on ice to prevent further growth. If cultures exceeded the turbidity level they were diluted to the standard with additional sterile Mueller-Hinton II broth. A 1:10 dilution was then made in sterile water (approximately 10^7 CFU/mL) and 5 *uL* of this material was added to 45 *u*L of Mueller-Hinton II broth (approximately 10^6 CFU/mL). Fifty microliters of this material was then added to the microtiter plate wells (described below) containing 50 *u*L antibiotic dilution in Mueller-Hinton broth (5×10^5 CFU/ml).

Microdilution Tray Preparation. The antibiotics used in the susceptibility test were as follows: gentamicin (ICN Biochemical, Inc., Aurora, OH); apramycin (Eli Lilly and Company, Indianapolis, IN); neomycin (Sigma); and sulfamethazine (Sigma). Stock solutions were stored at -80°C until used. Sterile microdilution trays (Costar, Corning NY) containing eight rows and 12 columns (total of 96 wells) were used for susceptibility testing. All wells were filled with 50 *u*L of Mueller-Hinton II broth. Fifty microliters of antibiotic stock solution was then added to the top row and serial dilutions were made by taking 50 uL from the top well, and transferring it to well immediately below it. This procedure was repeated for each successive well. The last row was left without antibiotics to serve as a control for testing the growth of isolates. The completed trays were then sealed and stored at -80°C. Before use the trays were allowed to thaw to room temperature and labeled appropriately.

The final concentration of bacteria per well was approximately 5×10^5 CFU/mL.

Statistical Analysis. A randomized block design, blocked on 2 replicates with repeated measures was used to analyze data pertaining to antibiotic resistance (SAS Proc Mixed, 1997). The experimental units for diets (6 different diets) were pens and the experimental unit for day was pig. In Experiment one, sample days 1 through 6 from both reps were combined and analyzed for changes in antibiotic resistance over time. Experiment two involved sample days 7 through 10, for which the two reps were analyzed separately due to different management practices. Break point (apramycin, neomycin, gentamicin, and sulfamethazine) data were linearized (eg. if breakpoint was <2 then =0; if 2 then = 1; if 4 then =2; if 8 then =3; if 16 then =4; if 32 then =5; etc.) and comparisons were made using Least squares means. In determining treatment and time effects, resistance was defined as the percentage of *Salmonella* or *E. coli* isolates resistant to the antibiotics listed in table 2.

TABLE 2. Minimum inhibitory concentration (ug/mL) interpretive standards of susceptibility and concentration range used in the microtiter plate

Antibiotic	Susceptible	Resistant	Antibiotic Concentration Range
Apramycin	≤8	>8	0-128
Gentamicin	≤4	>4	0-128
Neomycin	≤4	>4	0-128
Sulfamethazine	<256	>256	0-128

Minimum Inhibitory Concentration (MIC) breakpoints as determined by susceptibility standards outlined by NCCLS.

4. Results

Experiment One. *K88 E. coli.* From the combined replicates, a total of 11 confirmed colonies of K88 were isolated. Because recovery was very low an effective statistical analysis was not possible.

Non-pathogenic *E. coli.* Treatment effects ($P < .0001$) were noted for resistance to apramycin (Figures 1 and 2), gentamicin (Figures 3 and 4), neomycin (Figures 5 and 6), and sulfamethazine (Figures 7 and 8). Time x treatment interactions ($P < .0001$) were noted for *E. coli*, with the greatest resistance generally occurring during or following antibiotic treatment.

RL produced the greatest percentage of resistant isolates to apramycin, neomycin, and gentamicin. Within this treatment, an increase in percentage of resistant *E. coli* developed on day 10 post challenge and resistance was maintained until day 31 post challenge (Figures 1 through 6). This trend was also observed for all antibiotics except for sulfamethazine.

RU antibiotics produced the greatest percentage of resistant *E. coli* to sulfamethazine. For this treatment, an increase occurred on day 10 post challenge and decreased on day 31 post challenge. Although this trend was observed for apramycin, neomycin, and gentamicin, MIC's did not exceed the breakpoint indicating resistance. All *E. coli* isolates from all treatment groups were susceptible to sulfamethazine.

Compared to controls, rotation treatment with either like or unlike antibiotics increased ($P < .0001$) the proportion of *E. coli* resistant to apramycin, gentamicin, and sulfamethazine; furthermore, the proportion of bacteria resistant to apramycin, neomycin, and sulfamethazine was greater in pigs treated with like antibiotics compared to unlike antibiotics (Figure 1 through 8).

The percentage of organisms resistant to apramycin, gentamicin, neomycin, and sulfamethazine was greatest ($P < .0001$) for pigs receiving LU treatment compared to the G, PD, and C treatments.

The C, and PD treatments produced MICs within a susceptible range for gentamicin, and neomycin (MIC <4 ug/mL) (Figures 4 and 6), and sulfamethazine (MIC < 256 ug/mL) (Figure 8).

Salmonella typhimurium. Main effects of treatments were not observed for susceptibility of salmonella or for the percentage of resistant isolates. Resistance to all antibiotics remained low in salmonella throughout this study and MICs did not increase to resistance levels (Figure 9 through 16).

Experiment Two. A noticeable difference between pigs from the two facilities on the RL treatment was observed for apramycin sensitivity (Table 3). Resistance was greatest in isolates from pigs housed in isolated rooms compared to those from the open facility. For both housing treatments, MICs did not exceed 32 ug/mL. For gentamicin and

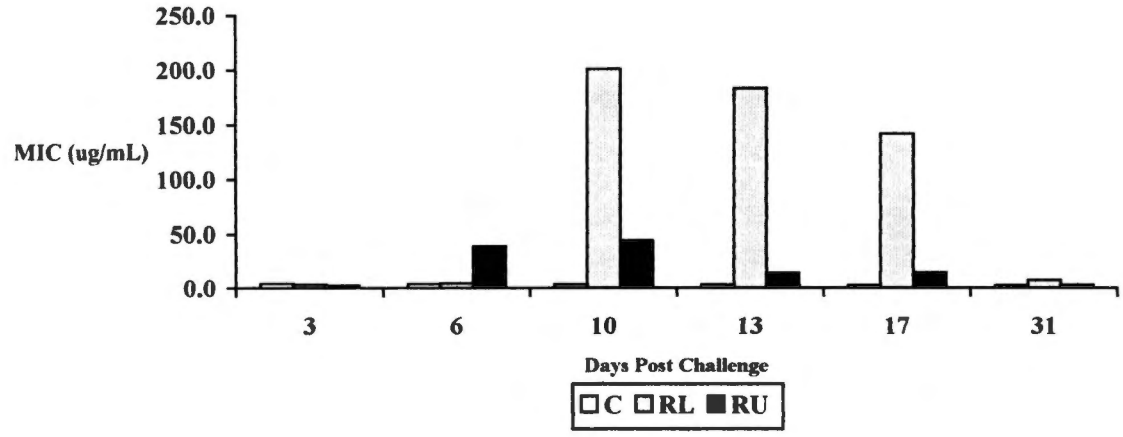

Figure 1. Apramycin sensitivity in *E. coli* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 360 *E. coli* isolates
Treatment effect, P < .0001
Treatment x time effect, P < .0001
C = control, RL = rotation with like antibiotics, RU = rotation with unlike antibiotics

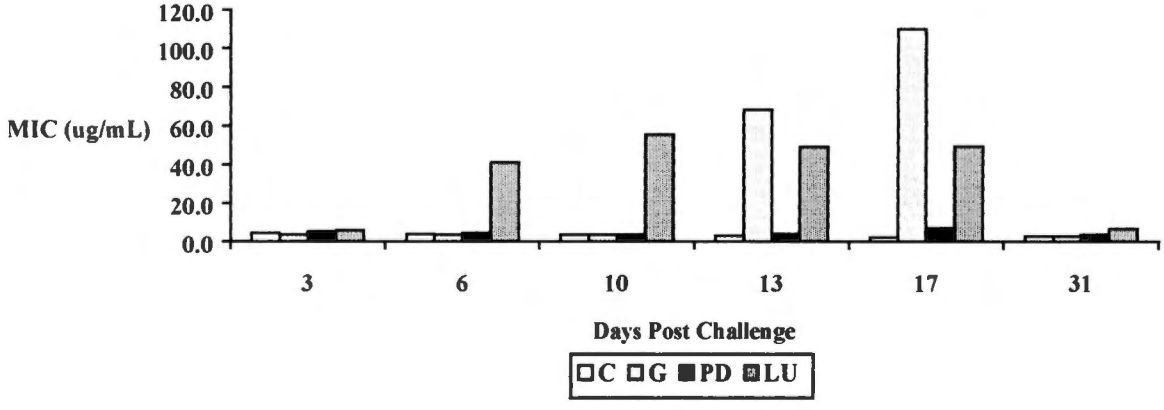

Figure 2. Apramycin sensitivity in *E. coli* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 360 *E. coli* isolates
Treatment effect, P < .0001
Treatment x time effect, P < .0001
C = control, G = gradient, PD = pulse dosing, L = label use

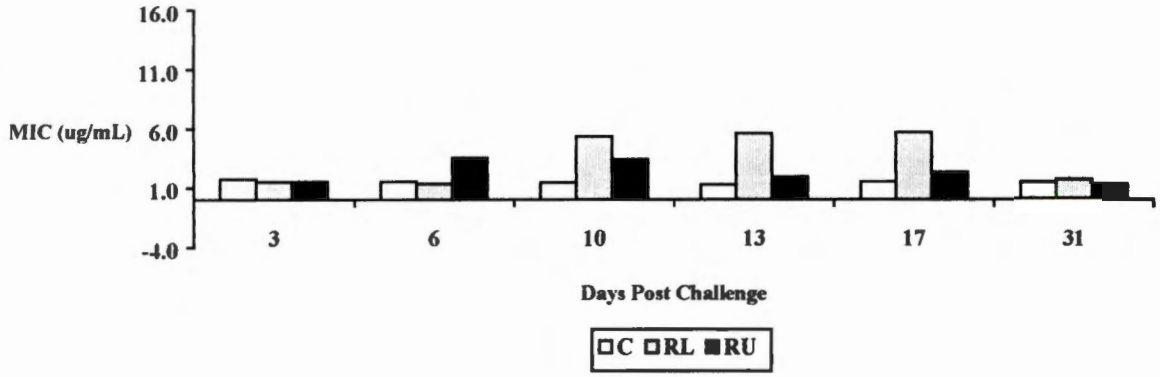

Figure 3. Gentamicin sensitivity in *E. coli* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 360 *E. coli* isolates
Treatment effect, P < .0001
Treatment x time effect, P < .0001
C = control, RL = rotation with like antibiotics, RU = rotation with unlike antibiotics

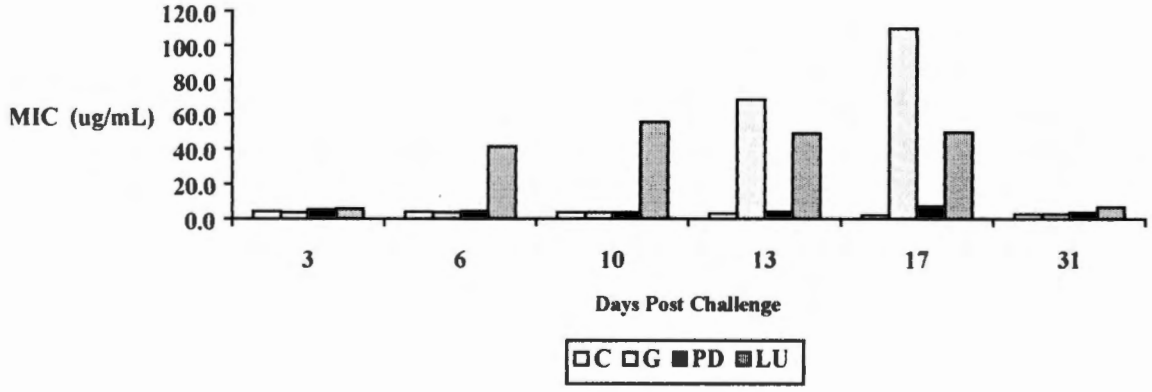

Figure 4. Gentamicin sensitivity in *E. coli* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 360 *E. coli* isolates
Treatment effect, P < .0001
Treatment x time effect, P < .0001
C = control, G = gradient, PD = pulse dosing, L = label use

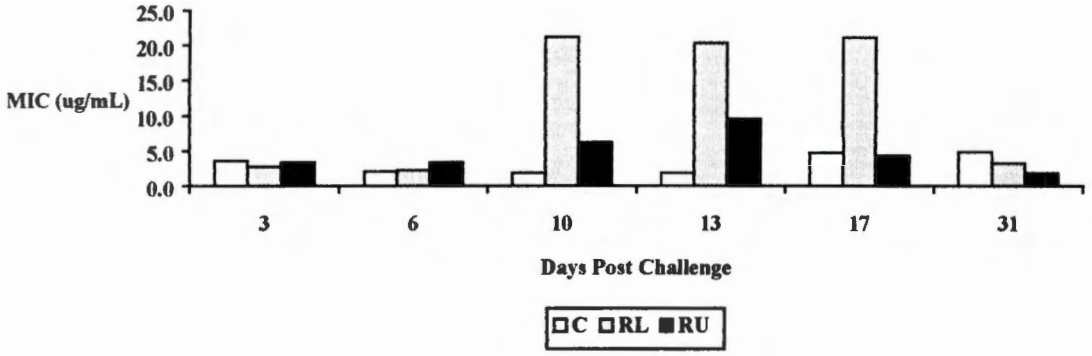

Figure 5. Neomycin sensitivity in *E. coli* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 360 *E. coli* isolates
Treatment effect, P < .0001
Treatment x time effect, P < .0001
C = control, RL = rotation with like antibiotics, RU = rotation with unlike antibiotics

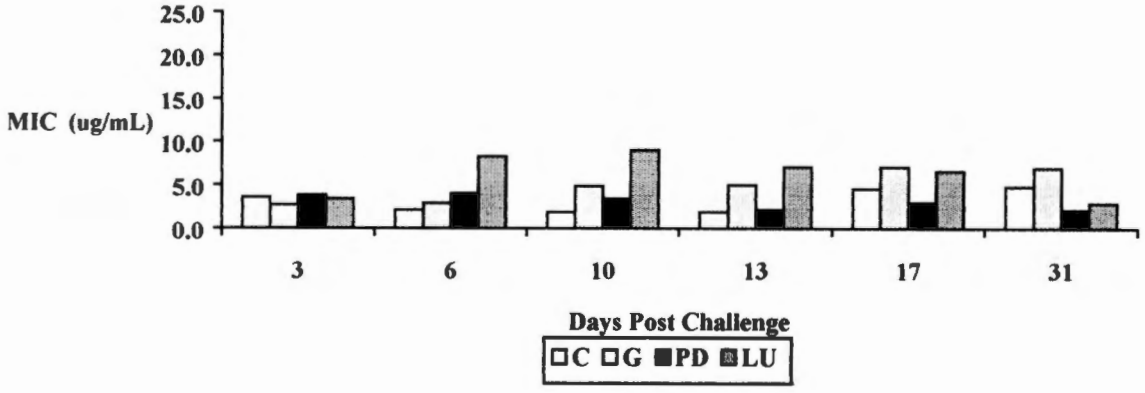

Figure 6. Neomycin sensitivity in *E. coli* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 360 *E. coli* isolates
Treatment effect, P < .0001
Treatment x time effect, P < .0001
C = control, G = gradient, PD = pulse dosing, L = label use

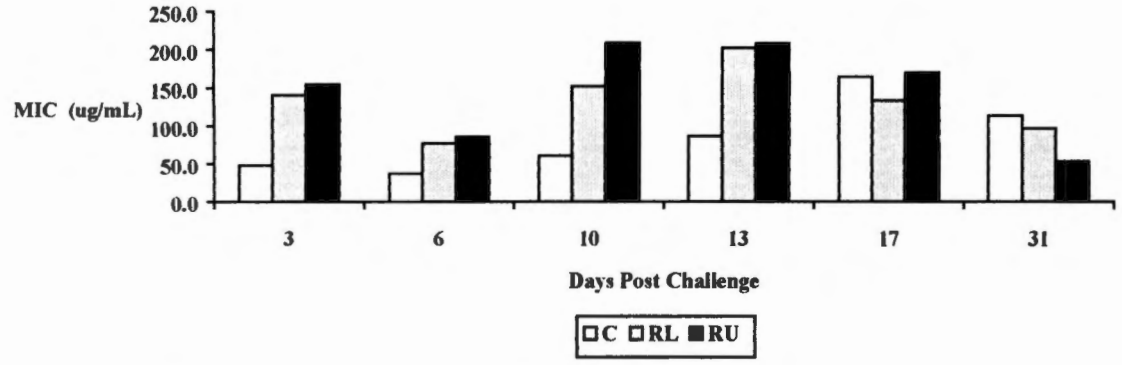

Figure 7. Sulfamethazine sensitivity in *E. coli* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 360 *E. coli* isolates
Treatment effect, P < .0001
Treatment x time effect, P < .0001
C = control, RL = rotation with like antibiotics, RU = rotation with unlike antibiotics

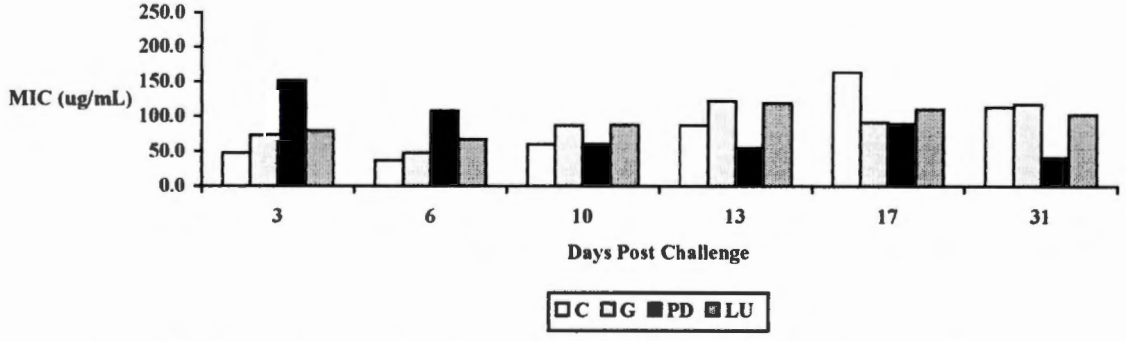

Figure 8. Sulfamethazine sensitivity in *E. coli* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 360 *E. coli* isolates
Treatment effect, P < .0001
Treatment x time effect, P < .0001
C = control, G = gradient, PD = pulse dosing, L = label use

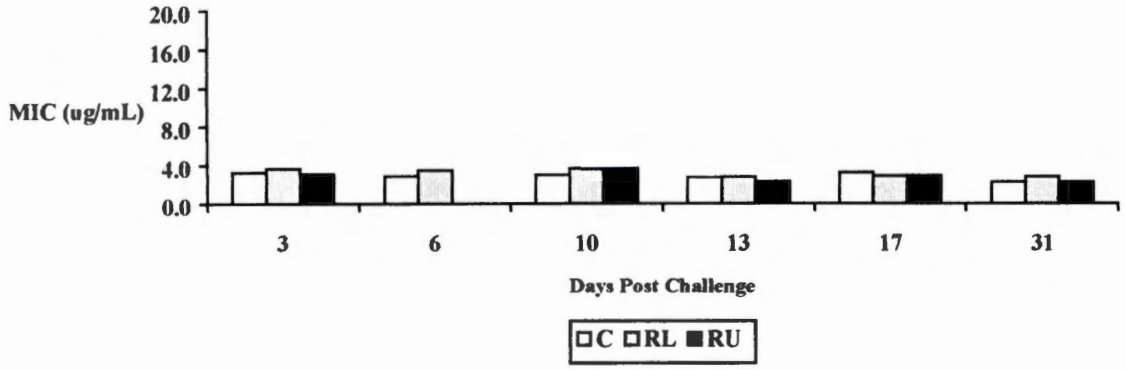

Figure 9. Apramycin sensitivity in *S. typhimurium* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 335 *S. typhimurium* isolates
Treatment effect, P < .7093
Treatment x time effect, P < .5205
C = control, RL = rotation with like antibiotics, RU = rotation with unlike antibiotics

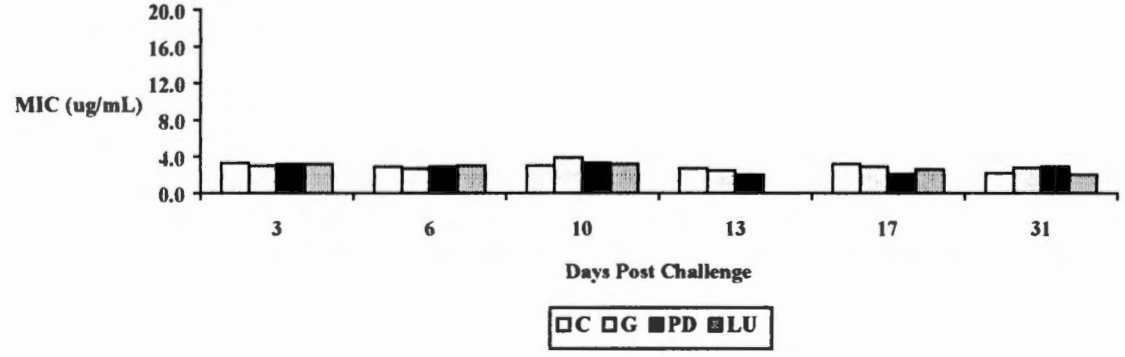

Figure 10. Apramycin sensitivity in *S. typhimurium* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 335 *S. typhimurium* isolates
Treatment effect, P < .0001
Treatment x time effect, P < .0001
C = control, G = gradient, PD = pulse dosing, L = label use

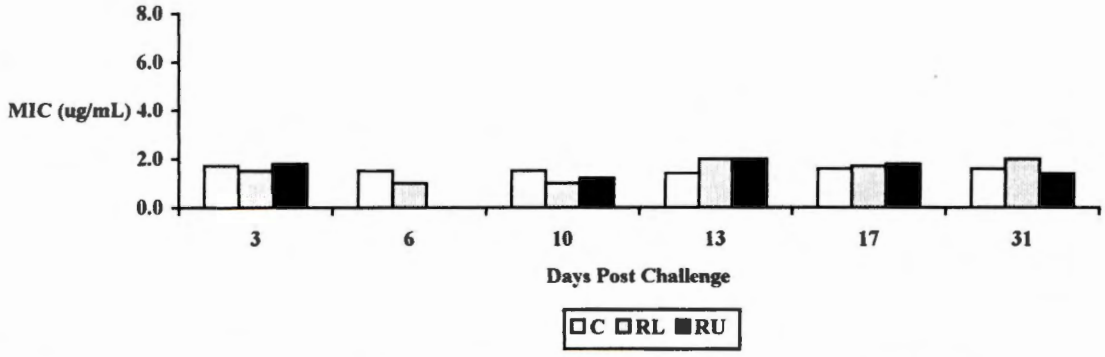

Figure 11. Gentamicin sensitivity in *S. typhimurium* isolated from pigs over time, Experiment 1. Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 335 *S. typhimurium* isolates
Treatment effect, $P < .90$
Treatment x time effect, $P < .02$
C = control, RL = rotation with like antibiotics, RU = rotation with unlike antibiotics

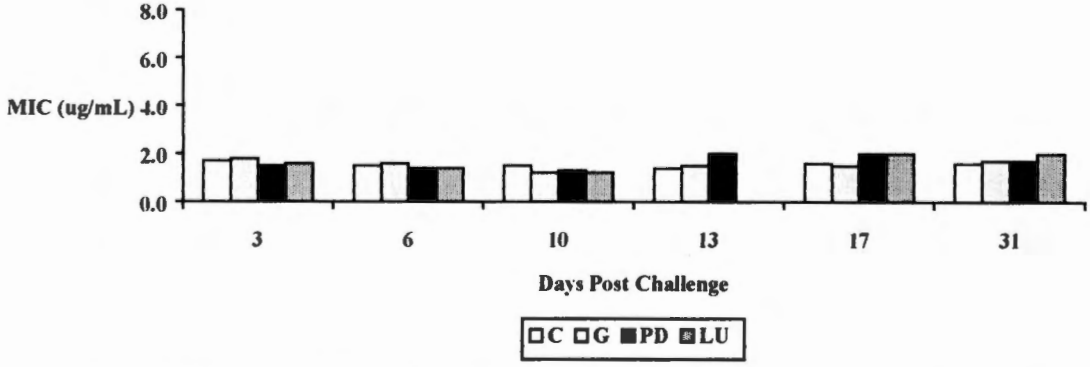

Figure 12. Gentamicin sensitivity in *S. typhimurium* isolated from pigs over time, Experiment 1. Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 335 *S. typhimurium* isolates
Treatment effect, $P < .90$
Treatment x time effect, $P < .02$
C = control, G = gradient, PD = pulse dosing, L = label use

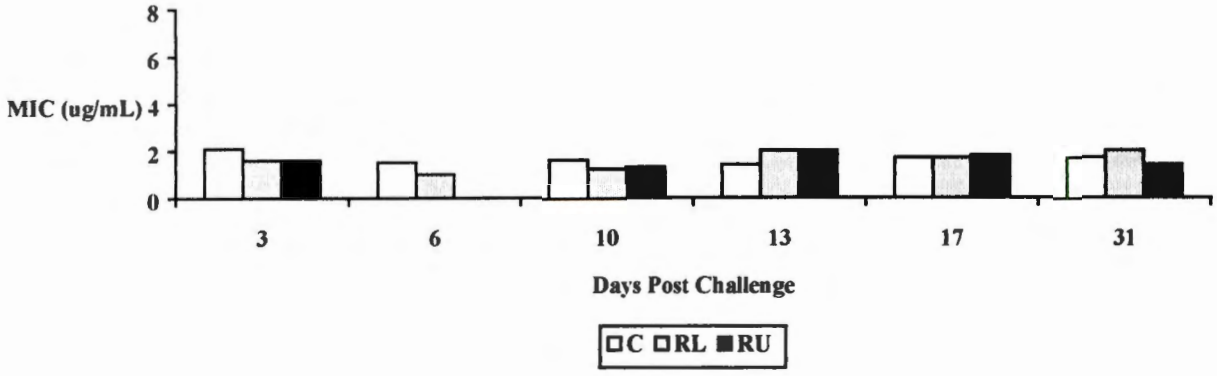

Figure 13. Neomycin sensitivity in *S. typhimurium* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 335 *S. typhimurium* isolates
Treatment effect, P < .8899
Treatment x time effect, P < .0498
C = control, RL = rotation with like antibiotics, RU = rotation with unlike antibiotics

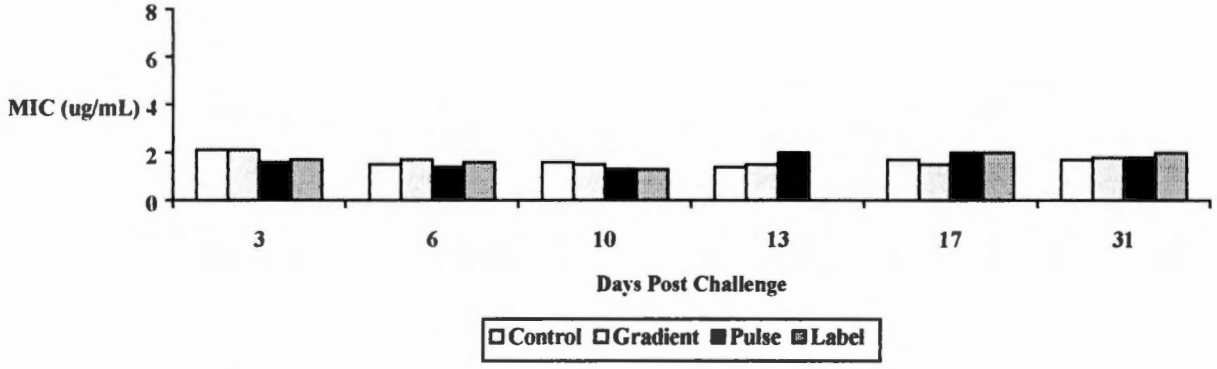

Figure 14. Neomycin sensitivity in *S. typhimurium* isolated from pigs over time, Experiment 1.
Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 335 *S. typhimurium* isolates
Treatment effect, P < .8899
Treatment x time effect, P < .0498
C = control, G = gradient, PD = pulse dosing, L = label use

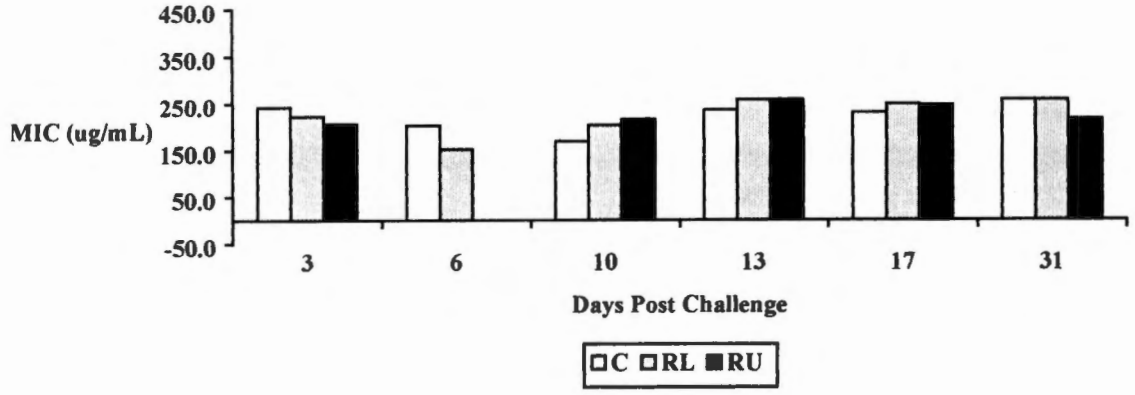

Figure 15. Sulfamethazine sensitivity in *S. typhimurium* isolated from pigs over time, Experiment 1. Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 335 *S. typhimurium* isolates
Treatment effect, P < .0660
Treatment x time effect, P < .0004
C = control, RL = rotation with like antibiotics, RU = rotation with unlike antibiotics

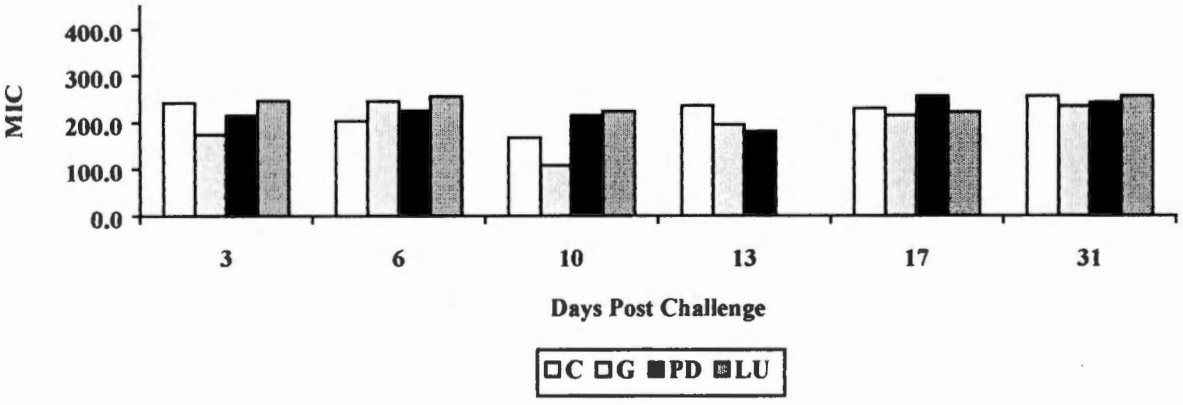

Figure 16. Sulfamethazine sensitivity in *S. typhimurium* isolated from pigs over time, Experiment 1. Data are Least Squares Means representing Minimum Inhibitory Concentration (MIC) measured in micrograms per milliliter
N = 335 *S. typhimurium* isolates
Treatment effect, P < .0660
Treatment x time effect, P < .0004
C = control, G = gradient, PD = pulse dosing, L = label use

TABLE 3. Sensitivity to Apramycin by *E. coli* isolated from pigs housed in isolation verses common facility

	Treatment											
	C		L		G		P		RL		RU	
	Is	Co	Is	Co	Is	Co	Is	Co	Is	Co	Is	Co
Days post weaning												
41	2.3	2.9	3.5	2.7	3.3	2.5	3.4	2.8	15.5	2.7	2.5	2.7
70	2.6	0.0	4.6	2.5	2.5	2.1	2.8	2.6	2.0	2.5	3.4	2.6
101	2.1	0.0	2.8	2.0	2.0	4.0	2.5	2.8	4.5	2.0	2.4	2.4

Data are Least Squares Means of MICs pooled over all sampling days.
Is = isolated; Co= confined;
C = control; L= label use; G = gradient; PD = pulse dose; RL= rotation with similar antibiotics;
RU = rotation with unrelated antibiotics; Experiment 2.

neomycin, a slight increase in MICs was noted for all treatments over time, in isolates from pigs housed in the open facility compared to isolates from pigs that remained isolated during the time period (Table 4 and 5). For both the RL and RU treatments, the greatest MICs to sulfamethazine were found in the open facility, with MICs exceeding 256 ug/mL, compared to isolates from pigs in isolation (Table 6).

All treatment groups produced salmonella with high sensitivity to apramycin, gentamicin, and neomycin. MICs for these three antibitoics did not exceed 4 ug/mL. RU produced the greatest MICs to sulfamethazine.

TABLE 4. Sensitivity to Gentamicin by *E. coli* isolated from pigs housed in isolation verses common facility

	Treatment											
	C		L		G		P		RL		RU	
	Is	Co	Is	Co	Is	Co	Is	Co	Is	Co	Is	Co
Days post weaning												
41	2.1	1.0	2.0	1.0	2.0	1.0	2.1	1.0	3.1	1.0	2.1	1.0
70	2.0	0.0	3.3	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
101	2.0	0.0	2.0	4.0	2.4	2.0	2.1	2.0	2.1	2.0	2.0	2.0

Data are Least Squares Means of MICs pooled over all sampling days.
Is = isolated; Co= confined;
C = control; L= label use; G = gradient; PD = pulse dose; RL= rotation with similar antibiotics;
RU = rotation with unrelated antibiotics; Experiment 2.

TABLE 5. Sensitivity to Neomycin by *E. coli* isolated from pigs housed in isolation verses common facility

	Treatment											
	C		L		G		P		RL		RU	
	Is	Co	Is	Co	Is	Co	Is	Co	Is	Co	Is	Co
Days post weaning												
41	3.3	1.0	2.1	2.0	2.9	1.2	2.1	1.0	8.5	9.2	6.8	9.2
70	2.8	0.0	5.2	2.0	2.1	8.0	2.7	2.0	2.0	2.0	2.4	2.0
101	5.4	0.0	2.7	2.0	3.2	2.0	2.7	11.3	2.7	2.0	2.4	2.0

Data are Least Squares Means of MICs pooled over all sampling days.
Is = isolated; Co= confined;
C = control; L= label use; G = gradient; PD = pulse dose; RL= rotation with similar antibiotics;
RU = rotation with unrelated antibiotics; Experiment 2.

TABLE 6. Sensitivity to Sulfamethazine by *E. coli* isolated from pigs housed in isolation verses common facility

	Treatment											
	C		L		G		P		RL		RU	
	Is	Co	Is	Co	Is	Co	Is	Co	Is	Co	Is	Co
Days post weaning												
41	111	130	40	72	59	131	62	89	86	105	62	216
70	134	0	152	117	94	103	119	168	128	118	65	83
101	152	0	153	64	224	64	147	117	169	128	256	143

Data are Least Squares Means of MICs pooled over all sampling days.
Is = isolated; Co= confined;
C = control; L= label use; G = gradient; PD = pulse dose; RL= rotation with similar antibiotics;
RU = rotation with unrelated antibiotics; Experiment 2.

5. Discussion

We postulated that the lack of K88 *E.coli* recovery was due to competition from the challenge strain of Salmonella which successfully colonized. This hypothesis may be supported by the dosage of Salmonella (10^9 CFU) and route of entry compared to that of the K88 (10^8 CFU). In addition, Rutters (1975) stated, "in order to multiply in the small intestine, enterotoxigenic strains must compete successfully for an ecological niche." This means that there are a number of barriers such as gastric acidity, composition of bile salts or production of antagonistic factors that could prevent or modify bacterial colonization of the gut, and there is little evidence that K88 *E.coli* can overcome these barriers more effectively than non-pathogenic strains (Rutters, 1975). Although these are reasonable explanations regarding the lack of infection by K88 *E. coli*, the actual reasons are clearly unknown and more research is needed in this area.

In evaluating *S. typhimurium*, it was postulated that low resistance to the test antibiotics by this organism was due to housing the animals in a new facility where no pigs had been previously housed. This is in contrast to an earlier study by our group, which took place in a finishing unit built in the 1970's and was used frequently thereafter. In that study, *Salmonella* quickly acquired resistance when pigs were treated with antibiotics (Ebner 2000). This may indicate that resistance elements from *Salmonella* were pre-existing in the building. It thus appears that in the new facility *E. coli*

may have a greater ability to mutate to resistance or acquired pre-existing resistance elements, compared to *Salmonella.*

The recovery rate of *S. typhimurium* has been a topic of controversy for many years. This controversy is centered on the notion that the route of inoculation and dose affects the duration or carrier state of this organism. Nnalue (1991) states, "route of inoculation has been shown to affect the rate of bacterial growth and bacteria survival." Interestingly, despite the controversy of dosage, researchers seem to agree that intranasal is the preferred route of inoculation because of the involvement of the upper respiratory tract and the nasopharyngeal lymphoid (Fedorka-Cray, 1995). In addition, Nnalue (1991) has shown that the use of intranasal inoculation in mice affected the survival and multiplication of *Salmonella* in vivo. Therefore, it would not be unreasonable to speculate that transmission involving the nasal passage may lead to the development of a long term carrier state.

In the present study, after the 6^{th} sampling period (8 weeks post inoculation), the number of isolates recovered decreased considerably. Although, scours and low appetite were observed during the first 48 hours post challenge, it might be postulated that the infective dose (10^9 CFU), given intranasally, was not high enough to induce sufficient shedding over a longer period of time. Wood et al. (1989) documented that swine exposed to greater than 10^{10} CFU of *S. typhimurium* at 6 to 8 weeks of age by oral ingestion remained infected after convalescence until at least 34 to

36 weeks of age, at which point the study was terminated. This is in agreement with Kampelmacher et al. (1969), who demonstrated clearance of *S. typhimurium* from infected swine following a 10^2 to 10^{10} CFU oral challenge. It was found that *S. typhimurium* was recovered from more tissues as the dose increased. In contrast, Wood et al. (1989), criticized this study by stating, "none of the exposure doses bring forth a clinical response, suggesting that the virulence of different strains may be even more important than exposure dose."

A total of 1,124 isolates of non-pathogenic *E. coli* were subjected to apramycin, gentamicin, neomycin, and sulfamethazine to determine the effects of anitmicrobial regimens on resistance patterns. This study found that the use of similar antibiotics (RL) produced the greatest percentage of *E. coli* resistant to apramycin (28.9%), gentamicin (2.9%), and neomycin (7.5%) across all treatments. This cross-resistance within the aminoglycoside group is common (Tortora, 1995). Neomycin and gentamicin, share more commonalities in structure and function than with apramycin (Mathew et al, 1999). Studies by Nijsten, criticized the use of these aminoglycosides, suggesting that the use of apramycin in animals may lead to gentamicin resistant isolates in hospital patients (1993).

A study by Mathew et al. (1999) observed that the majority of apramycin and neomycin resistance generally occurred as part of a multiple resistance pattern. Other researchers have suggested that the use of apramycin, leads to the appearance of *E. coli* resistant not only to the

antibiotic itself, but also to other agents as well as other aminoglycosides. This phenomenon is due to the possibility that plasmids are selected that code for multiple resistance (Tortora, 1995). In fact, several studies by Hedges and Shannon have identified transmissible plasmids conferring apramycin-resistance to *E. coli* (1984). Although the mechanisms of cross resistance are complex it can be concluded from this study as well as others, that drug combinations of similar antibiotics could result in multiple resistance.

Rotation with similar and dissimilar antibiotics produced the greatest MICs in *E. coli* to sulfamethazine (127.9 and 131.1 respectively). These data are not surprising given that sulfonamides have been used widely at subtherapeutic and therapeutic concentrations in the livestock industry since the 1930's. MICs for sulfamethazine are inherently greater than other antibiotics used in this study, thus while numerically high compared to apramycin, gentamicin, and neomycin these MICs do not indicate greater clinical resistance to sulfonamides.

Rotation with unlike antibiotics caused considerably lower resistance compared to RL. As stated earlier, this rotation consisted of apramycin, sulfamethazine, and carbadox. Although sulfamethazine and carbadox have been linked to the emergence of resistant *E. coli*, when used in this combination, low resistance to apramycin, gentamicin, and neomycin occurred. As a result, it might be postulated that short term use

of certain unrelated antibiotics for the treatment of infection will not lead to cross resistance, as was observed in other studies of related antibiotics.

Interestingly, the LU treatment also produced the greatest resistance to apramycin, gentamicin, and neomycin. This occurrence is difficult to explain since the treatment ended within the 14-day period considered maximum use by the manufacturer. The resistance was noted during the first week of the antibiotic treatment of pigs that were not previously exposed to any antibiotics. Also, in the G treatment, we noted an increase in resistance as the concentration of apramycin increased. These occurrences could be examples of "acquired resistance," meaning that an organism is initially sensitive to the drug, but acquires resistance during the treatment of an animal. This could be true for all treatments that increased resistance.

An analysis of data was conducted pertaining to the effect of different housing of pigs on antibiotics resistance. Surprisingly, the RL treatment produced the greatest percentage of resistant *E. coli* to apramycin, gentamicin, and neomycin in pigs housed in the isolated rooms in contrast to pigs in a common room facility. In comparing the housing conditions of both facilities, it might be noted that pigs that were housed in isolated rooms were in constant contact with fecal material, being raised on solid flooring; whereas, flooring in the open room facility was slatted to allow fecal material to pass through. Langlois et al. observed that housing conditions in intensive pig units, which allow high fecal contact,

are probably important factors to consider when treating animals for infection (1988). Other studies suggested that pigs that are in constant contact with feces, which they ingest, are continuously exposed to contamination by fecal bacteria and antibiotic resistance genes (Tortora, 1995).

Although this is a logical hypothesis, this experiment was conducted approximately six weeks after the antibiotic treatments and only the last three sampling days were taken into account. Although, it would have been preferable to have started this study before the introduction of antibiotic treatment, the data collected nevertheless support conclusions made earlier regarding drug combinations with similar antibiotics. These conclusions indicate that with improved management schemes, treatments will be effective in reducing the number of resistant organisms while maintaining the economic benefits of antimicrobials.

6. Implications

Many antibiotics currently used have a range of recommended dose rates for different food-animal species. However, with a continued increase of therapeutic and subtherapeutic antibiotics applied to swine, significant levels of antibiotic resistance by bacteria will develop. To decrease the numbers of resistant organisms, it is recommended that swine producers reassess their use of antibiotics. If antibiotics are necessary, a producer can help decrease resistance by alternating the use of several unrelated drugs to replace the constant use of a single antibiotic, however, this may promote multiple resistance. Our data also indicate treatment schemes with short term exposure (pulse dose) to antibiotics compared to long term (label use) reduces incidence of resistant bacteria. Ultimately, the hope is that the safety of the food supply will be improved by reducing the adverse consequences of antibiotic overuse, while maintaining high standards of animal welfare, production, and food quality.

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