To the Graduate Council:

I am submitting herewith a thesis written by Paul Ebner entitled "Effects of different antibiotic regimens on the shedding patterns of pigs infected with *Salmonella typhimurium*." 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 degrees 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:

Associate Vice Chancellor and
Dean of the Graduate School

EFFECTS OF DIFFERENT ANTIBIOTIC REGIMENS ON THE SHEDDING PATTERNS OF PIGS INFECTED WITH *SALMONELLA TYPHIMURIUM*

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Abstract

Two experiments were conducted to determine: 1) the effects of different antibiotic regimens on the shedding patterns of pigs infected with *Salmonella typhimurium*; and 2) whether the development of antibiotic resistance plays a role in shedding patterns. In experiment 1, 36 50-day-old pigs were orally and intranasally challenged with 3 mL (1.2 x 10^9 CFU/mL) of *Salmonella typhimurium* obtained from the USDA Animal Disease Control Center (Ames, IA). Pigs were blocked by litter and separated into four treatment groups including: 1) i.m. injection of ceftiofur sodium (cef) for 3 days followed by inclusion of oxytetracycline (otc) at 100g/ton of feed, 2) apramycin (apr)(150g/ton of feed) for 14 days followed by otc (100g/ton), 3) carbadox (car)(50g/ton) followed by otc (100g/ton), 4) no antibiotics (control). Treatments were initiated 48 hours post-inoculation and continued until pigs reached market weight. Rectal swab samples were collected pre-inoculation, 48 and 72 hours post-inoculation, and at weekly intervals thereafter. At $P < .05$, the overall incidence of salmonella shedding was reduced in pigs receiving cef/otc and apr/otc treatments when compared with the control group. Pigs receiving the car/otc treatment shed the salmonella in the same amount as control pigs. The second experiment involved 48 50-day-old pig. Pigs were orally and intranasally inoculated with 3 mL (1.3 x 10^9 CFU/mL) of a *Salmonella typhimurium* isolate containing a nalidixic acid resistance marker. Pigs were blocked by litter and received the same treatments as in experiment one. At $P < .05$ the overall incidence of shedding was reduced in pigs receiving the apr/otc treatment; however, no differences were observed between antibiotic treatments. Positive samples from five pigs from each treatment in experiment two were analyzed via disc-diffusion on 2, 4, 7, 21, 42 and 70 days post-inoculation. A time effect was detected indicating that the proportion of isolates resistant to at least one of the test antibiotics varied over time. Resistance was measured at 47% at 2d PI and reached a pinnacle of 89% at 70d PI. Treatment * time interactions were detected and in each treatment the percentage of isolates resistant to at least one of the test antibiotics increased significantly over time; again reaching a pinnacle at 70d PI. Similar antibiotic * time interactions were detected as the percentage of isolates resistant to each of the specific antibiotics increased over time. These data indicate that the use of antibiotics during *Salmonella typhimurium* infections results in increased proportions of antibiotic resistant *Salmonella typhimurium* isolates. Such resistance, however, does not result in increased shedding of the organism and in the case of apr/otc, while the proportion of resistant isolates increased, fecal shedding decreased.

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1. Review of the Literature

Introduction

It is estimated that between three and four million Americans are infected with salmonellosis every year through the consumption of salmonella-contaminated food (CDC, 1987; Kvenberg and Archer, 1985; Todd, 1989). Cattle, poultry and swine are reservoirs for many of the salmonellae and most cases are traced back to the consumption of contaminated meat and dairy (Edwards, et al., 1997; Jawetz, et al., 1991). Of the more than 2300 salmonella serovars (Keusch and Acheson, 1998), *Salmonella typhimurium* is ubiquitous in agricultural settings (Reed, et al., 1986) and is the most common serotype isolated from human cases of salmonellosis. (Bean and Potter, 1992; CDC, 1987).

In swine, infections of *Salmonella typhimurium* often lead to lengthy carrier states. In this asymptomatic phase of infection, animals may continue to shed the organism in feces long after clinical symptoms of disease subside (Isaacson and Kinsel, 1992). Contaminated feces can lead to cross-infection of co-habitating animals on farms (Heard and Linton, 1966; Fedorka-Cray, et al., 1995) and contamination of machinery and meat products at the abattoir (Newell and Williams, 1970; Roof, et al., 1992).

A better understanding of factors that influence the carrier state could help limit the likelihood of both endemic infections in herds and salmonella contaminated meats. Concern that use of antibiotics in livestock leads to increases in both the number of animals shedding and quantity of organisms shed has led to numerous studies examining such a relationship (Jacks, et al., 1988; Gutzmann, et al., 1976; Dealy and Moeller, 1976; DeGeeter, et al., 1976; Abou-Youssef, et al., 1979; Wilcock and Olander, 1978; Claussen,

et al., 1997). Such is the focus of the present study. It is postulated that 1) use of antibiotics during *Salmonella typhimurium* infections may result in increased proportion of antibiotic resistant *Salmonella typhimurium* in the gastro-intestinal tract; and 2) continued use of antibiotics, whether for therapy or growth promotion, may select for antibiotic-resistant organisms resulting in their proliferation and concurrent increases in bacterial shedding.

Salmonella Taxonomy

The salmonella genus is named after Dr. Salmon who in 1884 discovered the bacterium as the cause of hog cholera. It was later discovered that hog cholera is caused by virus. The bacterium Salmonella was isolated as the etiological agent of typhoid fever three years earlier by Eberth (Ziprin, 1990; Silliker and Gabis, 1988). Salmonella taxonomy remains controversial as 100 years later microbiologists fail to agree on a uniform system of salmonella nomenclature. The genus, it is agreed, falls within the family of gram-negative enterobacteriaceae (Guthrie, 1992; Silliker and Gabis, 1988). Through the years, however, different isolates of the genus have been mistakenly treated and named as separate species. Based on DNA relatedness, the amount of genetic homogeneity among serotypes should exclude considering each one as a separate species (Ziprin, 1990).

In the 1970's Salmonella taxonomy was streamlined by arranging the serotypes into three groups: *Salmonella typhi, Salmonella cholerasuis,* and *Salmonella enteriditis.* The *Salmonella typhi* and *Salmonella cholerasuis* group consist of only one host-adapted

serotype each; primarily infecting humans and swine respectively. The *Salmonella enteriditis* group is made up of the thousands of other less host-adapted serovars (Guthrie, 1992 Roof, et al., 1992; Jawetz, 1991). In the 1980's the genus was further refined into one species based on DNA homology: *Salmonella cholerasuis* (Silliker and Gabis, 1988). More recent proposals for taxonomic rearrangement divide the genus into two species: *Salmonella bongorii* and *Salmonella enteritica.* The division is based on the evolution of two distinct lineages with different virulence factors and thus pathogenesis. The *Salmonella bongorii* species consists of serotypes containing a chromosomal island of pathogenicity genes (Salmonella Pathogenicity Island 1 or SPI 1), encoding for several virulence factors mediating intestinal replication. The *Salmonella enteritica* species consists of serotypes containing both SPI 1 and a second pathogenicity island (SPI 2) which mediates the invasive phase of infection (Baumler, et al., 1998, Reeves, et al., 1989).

Salmonella typhimurium: Salmonella typhimurium, a non-host specific serotype, is perhaps the most ubiquitous of serotypes as it is repeatedly the most frequent salmonella serotype isolated from both human and animal clinical samples (Bean and Potter, 1992; CDC, 1987). It conforms to the Enterobacteriaceae family as a non-spore forming, oxidase negative, glucose fermenting, facultative anaerobe (Guthrie, 1992). It falls within the genus of Salmonella as it produces hydrogen sulfide gas detectable on triple sugar iron agar or lysine iron agar, can utilize citrate as a sole carbon source, will decarboxylate lysine and ornithine, reduces nitrates to nitrites (Ziprin, 1990; Silliker and Gabis, 1988)

and is incapable of fermenting lactose (Ziprin, 1990). Finally, being invasive it belongs to the *enteritica* species (Keusch and Acheson, 1998). Based on antigenic classification it is a member of serogroup B containing 1,4,5 and 12 somatic (O) antigens, and is serotyped by both Phase Ii and Phase II 1 and 2 flagellar (H) antigens (Jawetz, et al., 1991; Guthrie, 1992).

Salmonella Pathogenicity

Infection and Host Immunity: Infections with *Salmonella typhimurium* begin with an oral introduction of the organism to the host, primarily through contaminated feeds or feces (Jawetz, et. al., 1992; El-Gazzar and Marth, 1992). Before the organism can reach its main site of colonization it must first transverse innate host defenses of the stomach and upper small intestine, namely hydrochloric acid and digestive enzymes. Many organisms may die, however, *Salmonella typhimurium* will begin to replicate intraluminally in the distal ileum and colon under favorable conditions (Guthrie, 1992; Schwartz, 1991; El-Gazzar and Marth, 1992; Keusch and Acheson, 1998).

Salmonella typhimurium is a facultative intracellular pathogen. Invasion begins with the adhesion of the organism to host enterocytes via pili (Isaacson and Kinsel, 1992; Keusch and Acheson, 1998). In an example of induced phagocytosis, attachment provokes host cell ruffling where changes in the formation of the microfilaments of the villi act to endocytose the organism. A vacuole is formed surrounding the organism as it transverses the enterocyte. Some salmonella may be killed by antibacterial peptides

(cryptons) within enterocytes (Keusch and Acheson, 1998); those that survive are exocytosed across the basement membrane into the lamina propria (Roof, et al., 1992).

Foreign organisms entering the lamina propria are faced with several host immune responses associated with the mucosal immunity, first of which are phagocytes (namely neutrophils and macrophages) and complement (Roof, et al., 1992, Spitznagel, 1998). Phagocytes act to engulf and kill invading organisms via two mechanisms: oxygen-independent and oxygen-dependent killing. Both mechanisms involve joining of the phagosome, the vacuole formed upon endocytosis, with the lysosome, an organelle-like structure of the phagocyte loaded with antibacterials. The result is the formation of a phagolysosome. Oxygen independent killing is the result of degradation where host antibacterials, namely lysozyme, are deposited onto the organism's surface and act to degrade the cell wall. Oxygen dependent killing utilizes a reductase enzyme that bridges the membrane of the phagolysosome. Activation of the enzyme results in pumping of reactive oxygen intermediates into the phagolysosome (Spitznagel, 1998). At the same time, complement, through a series of cascade reactions, produces the membrane attack complex (MAC), a pore-forming complex which in high numbers may lyse cells. Moreover, several fragments produced in the complement reactions serve as chemotactic fragments, osponic fragments and anaphylatoxins, intensifying inflammation (Jawetz, et al., 1991; Spitznagel, 1998).

Humoral- and cell-mediated immune defenses are also stimulated; initially through B and T cells associated with the gut-associated-lymphoid-tissue (Roof, et al, 1992). Macrophage digested antigens are presented to T-cells (Roof, et al, 1992; Ziegler,

1998). Activated T-cells coordinate a host of immune responses by activating macrophages, promoting proliferation of B-cells (humoral response), and producing cytotoxic cells specific for salmonella or salmonella infected macrophages (Ziegler, 1998; Jawetz, et al., 1991). The first humoral response, IgA, is produced in the lamina propria. Upon production it tranverses the enterocyte and is secreted into the lumen to act on intraluminally replicating organisms (Roof, et al., 1992; Jawetz, et al., 1991; Ziegler, 1998). B-lymphocytes cells may produce several other immunoglobulins specific for salmonella that agglutinate invading cells, block adhesions and inactivate toxins (Roof, et al., 1992).

Salmonella Protective Mechanisms: While the host has a myriad of mechanisms designed to prevent *Salmonella typhimurium* infections, the organism also employs various mechanisms to evade host immunity. Long O-antigen polysaccharide chains make the *Salmonella typhimurium* cell hydrophilic and therefore, somewhat antiphagocytic. Moreover, the long chains hinder complement by not allowing MAC access to its membrane (Schaechter, 1998) The foremost defense of *Salmonella typhimurium* against host immunity is the organism's ability to survive inside host enterocytes and phagocytes. *Salmonella typhimurium*, upon endocytosis, have been shown to produce manganese super-oxide dismutase, catalase, and hydrogen peroxide reductase via expression of an *oxyR* gene (Roof, et al., 1992). Such enzymes allow the organism to survive inside the phagocyte by reducing reactive oxygen intermediates (Keusch and Acheson, 1998).

The ability to survive inside host phagocytes affords *Salmonella typhimurium* with a formidable advantage by shielding it from most host immune mechanisms, save cell-mediated immunity (Schaechter, 1998; Eisenstein and Engelberg, 1998). Moreover, survival inside the phagocyte aids in the dissemination of the organism throughout the body, in some cases giving rise to systemic infections (Roof, et. al., 1992; Schwartz, 1991). In swine, organisms entering lymph and blood are known to cause persistent infection of several organs, namely tonsils, lymph nodes and possibly the spleen and gall bladder. (Wood, et al., 1989; Wood, et al., 1992).

Salmonellosis: Diseases attributed to salmonellae vary from self-limited acute-gastroenteritis to enteric fever to potentially fatal septicemia (Jawetz, et al., 1991; Reed, et al., 1986; Wilcock, 1986). The wide range of disease symptoms is a result of several serotypes being very host-adapted and causing severe disease, but only in specific species. The serotypes *Salmonella typhi* (and Para-*typhi*) and *Salmonella cholerasuis* are examples of host adapted serotypes associated with humans and swine respectively (Keusch and Acheson, 1998; Roof, et al., 1992) Although rarely isolated from other species, both serovars are highly invasive and potentially fatal to their respective hosts. Conversely, infections of either species with a less host-adapted serotype, such as *typhimurium*, are usually limited to gastroenteritis (Keusch and Acheson, 1998; Roof, et al., 1992; Schwartz, 1991; Isaacson, et al., 1992).

Symptoms of bacterial diseases are either the result of damaging toxins produced by the bacterial cell or the inflammatory response of the host's immune system to a

foreign antigen (Keusch and Acheson, 1998). While *Salmonella typhimurium* have been shown to produce enterotoxins, their role in disease is uncertain (Roof, et al., 1992; Keusch and Acheson, 1998) and the symptoms associated with salmonellosis are mainly symptoms of the inflammatory response illicited by the invading cell and its associated lipopolysaccharide (i.e. LPS, endotoxin, O antigen) (Guthrie, 1992). LPS is a component of all gram-negative cell walls. It consists of a large lipid (Lipid A) embedded in the outer membrane. A glucosamine core connects to long polysaccharide chains that extend away from the cell (Jawetz, et al., 1992.). At low levels, LPS is simply inflammatory. At high levels, LPS may cause shock, disseminated intravascular coagulopathy, and/or death. However, the toxicity of LPS varies from species to species as the glucosamine core and sugars comprising the long hydrophilic chains are variable (Schaechter, 1998). The acute gastro-enteritis associated with salmonellosis caused by *Salmonella typhimurium* is a result of the host's immune response to LPS (Roof, et al. 1992). The recruitment of macrophages, neutrophils and platelets to the colon and activation of complement, T and B cells results in inflammation. The consequence is malabsorption and secretion of fluids into the lumen (Roof, et al., 1992; Keusch and Acheson, 1998). Fever is the result of LPS-induced release of pyrogenic cytokines from macrophages, namely tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) (Schaechter, 1998). Mucosal lesions and blood in feces are probably the result of complement activation and reactive oxidative intermediates released by phagocytes at the site of infection (Wilcock, 1986).

The Carrier State

With exception of infections in immunosuppressed individuals, Salmonellosis caused by *Salmonella typhimurium* is usually self-limiting (Ziprin, 1990; Jawetz, et al., 1991; El-Gazzar and Elmer, 1992). The intracellular nature of the organism is thought to give rise to lengthy carrier states where the organism continues to replicate in the intestines while the pig shows no apparent signs of infection (Roof, et al., 1992; Isaacson, et al, 1992.; Jawetz, et al., 1991; Wilcock, 1986). As the organism replicates in the intestine, some enterocytes are sloughed and shed in the feces along with viable bacterial cells. Infected feces are probably the main route of contamination among livestock (Heard, et al., 1966; Fedorka-Cray, et al., 1995) and the close proximity of large groups of pigs in intensive operations may favor endemic infections of herds (Schwartz, 1992). Studies investigating the length of salmonella carriage in swine report infections lasting from 28 to 72 weeks (Wood, et al., 1989; Claussen, et al., 1997; Wood and Rose, 1992).

Bacterial Shedding and Human Health: Asymptomatic shedding of *Salmonella typhimurium* by livestock poses a serious human health hazard, as the asymptomatic carrier shedding the organism at the abattoir is the principle source of salmonella contaminated meats (Newell and Williams, 1971). As a result there is a direct link between the occurrence of salmonellae in livestock and salmonellosis in humans (Silliker and Gabis, 1988.; Wray and Sojka, 1977; Edwards, et al., 1997). During pre-slaughter transportation and holding the exteriors of crowded animals invariably become

contaminated with feces leading to contamination of the machinery involved in the slaughtering and dressing processes (Schwartz, 1991). Contaminated dehairing machines, scalding tanks and polishers further facilitate the spread of salmonella to other carcasses (Schwartz, 1991; Roof, et al., 1992; Newell and Williams, 1971.).

Numerous studies have focused on decreasing the length and quantity of bacterial shedding in attempts to decrease the liklihood of contaminated animals. Two common themes have received the most attention: stress and antibiotics. It is documented that stress exacerbates shedding, as animals subclinically infected with *Salmonella typhimurium* shed the organism in greater numbers after certain activities including transportation, drop in temperature, and crowding (Williams, 1975; Williams and Newell, 1970, Linton, et al., 1970). Most likely, stress-induced immunosuppression provides a less hostile environment favoring the organism's proliferation; with proliferation, more organisms are shed.

Feeding sub-therapeutic levels of antibiotics to livestock for growth promotion has also been shown to increase shedding in sub-clinically infected animals. Williams and co-workers documented increases in shedding in pigs by treating animals infected with chlortetracycline resistant *Salmonella typhimurium* with sub-therapeutic levels of chlortetracycline (Williams, et al. 1978). Presumably the drugs offer a selection pressure in the gastro-intestinal tract that favors the growth of drug resistant microorganisms. Numerous studies using susceptible strains of *Salmonella typhimurium* showed no increase in shedding as a result of incorporating antibiotics in the diet (Claussen, et.al, 1997; Finlayson and Barnum, 1973, Gutzmann, et.al.,1976). The deftness, however, at

which *Salmonella typhimurium* obtain the genetic machinery of antibiotic resistance (Guthrie, 1992, Smith, 1972) may result in increased shedding with prolonged use of antibiotics. I postulate that use of antibiotics during infections of *Salmonella typhimurium* results in increased populations of resistant *Salmonella typhimurium* in the gastro-intestinal tract and continued use of antibiotics may select for such populations causing their proliferation and increased the length of the carrier state.

Antbiotics in Agriculture

In the early 1950's, researchers examining the growth promoting effect of vitamin B12 noticed unusually enhanced growth in birds treated with B12 from whole *Sacharomyces aureofaciens* cultures. Further examination revealed chlortetracycline in the cultures and it was soon discovered that antibiotics afforded substantial growth promotion when included in livestock feeds at seemingly very low levels (Franco, et al., 1990, Tindall and Smith, 1968). In 1956 the Food and Drug Administration approved the inclusion of antibiotics in animal feeds at sub-therapeutic levels for growth promotion and it is now a commonplace practice (Franco, et al., 1990). Indeed, over one half of the 35 million pounds of antibiotics manufactured in the United States are utilized in agriculture (Franco, et al., 1990) and it is estimated that 75% of market swine have received antibiotic treatment in some form prior to slaughter (Guthrie, 1992).

Although the mechanisms behind the growth promoting properties of antibiotics have been extensively researched, it is still uncertain as to how the drugs enhance growth. Three theories are generally agreed upon: 1) the drugs suppress microorganisms that

cause subclinical disease and reduced growth, 2) the drugs decrease the amount of microorganisms lining the intestine and the intestinal cell wall, thereby enhancing absorption of nutrients, and 3) the drugs act as a prophylaxis preventing the establishment of pathogenic organisms in the animal's gut (Tindall and Smith, 1968; Visek, 1978)

Regardless of the exact mechanisms, the inclusion of antibiotics in livestock feeds affords livestock producers a formidable management tool. In swine, the benefits of antibiotics are seen mainly in young pigs in the starter or grower phase of production. By pooling data across 800 experiments, Hays estimated that including antibiotics in the young pig's diet results in an 11-16% increase in average daily gain and 5-7% decrease in feed to gain ratios (Hays, 1985). In economic terms, the use of antibiotics in livestock spares American consumers an estimated $35 billion at the marketplace (CAST, 1981).

Antibiotic Resistance

The serendipitous discovery of antibiotics as effective growth promotants was soon followed by the ominous discovery of infectious antibiotic resistance. Following the second world war, Japan experienced repeated outbreaks of dysentery caused by the enteric pathogen *Shigella dysentereae*. Patients were treated with sulfonamide which effectively quelled outbreaks. Four years later Japanese doctors were faced with several more outbreaks of dysentery caused by strains of Shigella resistant to sulfonamide. Subsequently, newer antibiotics including streptomycin, and tetracycline were used to treat the disease. Unfortunately, after three years of therapy with the newer antibiotics,

Japanese doctors were faced with patients suffering from shigella dysentery caused by a strains of shigella resistant not only to streptomycin and tetracycline but also the original sulfonamide (Watanabe, 1963).

Japanese scientists Ochiai, Yamaka, Kimura and Sawada soon discovered that bacterial populations had unique methods of sharing genetic machinery (Watanabe, 1963). It is now agreed that the majority of antibiotic resistance is the result of infectious heredity. Plasmids, carrying extrachromosal resistance genes, are the vector by which most bacteria gain immunity to antibacterial drugs (Alexander, 1985; Willets, 1985; Dale, 1994).

Antibiotic Resistance Plasmids: Bacteria receive the bulk of their genetic material from a single, large circular chromosome (Pemberton and Don, 1981). Plasmids consist of various blocks of genes in super coiled, circular structures outside of the chromosome (Willets, 1985) and at a fraction of the size (1-2% the size of chromosomes) (Pemberton and Don, 1981). They encode for products that lend flexibility to the organism in its environment, be it a protein that blocks the uptake of an antibacterial or an enzyme that allows the bacteria to digest a new nutritional substrate (Dale, 1994).

Over 1000 plasmids have been discovered in both gram-negative and gram-positive bacteria. Crude classification divides them into two groups: non-conjugative and conjugative. The ColE1 plasmid (coligenicity plasmid) of *E. coli* can be considered a prototype of non-conjugative plasmids. It is a small plasmid (10kb) and exists in high copy number per host cell (10-15 plasmids per cell) (Willets, 1985). Being non-

conjugative, the ColE1 plasmid does not have the properties to transfer itself to another bacterial cell (Dale, 1994). Conversely, conjugative plasmids, are large but usually exist in the host in low copy numbers (1-2 plasmids per cell) (Willets, 1985). Moreover, conjugative plamsids are able to promote their own transfer to neighboring bacterial cells (Willets, 1985; Dale, 1994). Since the majority of bacterial resistance genes are carried on conjugative plasmids (Blackburn, et al., 1984) further disscussion will focus on this group of plasmids.

Infectious Heredity: Replication and conjugation of the plasmid are thought to be the two crucial components of plasmids in confering genes to unrelated bacteria (Willets, 1985). Plasmids replicate autonomously from the bacterial chromosome. Initiation of replication begins at a specific vegetative origin site (*oriV*) on the plasmid by a promoter protein *RepA*, a product of a plasmid gene (Dale, 1994; Willets, 1985). The rate at which copies are made is controlled by two genes on the plasmid, *CopA* and *CopB,* which act synergistically to inhibit the expression of the *RepA* gene (Dale, 1994). Once initiation begins, however, replication usually relies solely on host enzymes, namely DNA and RNA polymerases, ligase and gyrase (Willets, 1985).

While antibiotic resistance plasmids are present in low numbers in the cell several factors allow for their stabile association with the host and organisms which have lost such plasmids are rare (Willets, 1985). This stable association is due to a *par loci* on the plasmid which enables partitioning. Partioning distributes the plasmids evenly at host cell division ensuring that each daughter cell receives one plasmid (Dale, 1994; Willets,

1985). It is thought that the products of *par loci* expression enable the plasmid to bind to portions of the cell membrane at division (Dale, 1994). High copy number plasmids lack the *par loci* and rely on high copy number to ensure even distribution during host cell division (Willets, 1985).

The process of partioning may not be perfect however, and antibiotic resistance plasmids also rely on the process of conjugation for the direct transfer of plasmids from one cell to another (Dale, 1994). Indeed, up to 1/3 of the genes (*tra* genes) of most conjugative host cells are concerned with the conjugation process. The two major components of conjugation are formation of a pilus and the actual transfer of the DNA. Formation of the pilus is the product of one portion of the *tra loci* and allows contact and adhesion between the donor and the recipient. Upon contact, the pilus retracts, either by dissaggregating or dissolving into one the mating cell's membranes and causes the cells to be pulled into one another (Willets, 1985). The result is the formation of a conjugal tube (Pemberton and Don, 1981). Transfer begins with the nicking of a single strand of the plasmid in the donor cell (Dale, 1994) the only step in the process of conjugation that relies on plasmid gene products (Willets, 1985). Upon nicking, a single strand is unwound and transferred into the recipient cell. Recipient cell enzymes replicate the single strand creating a fully functional plasmid. The single strand remaining in the donor cell is similarly replicated (Dale, 1994; Willets, 1985).

Transposons: Early in the discovery of infectious resistance it was discovered that bacteria can quickly evolve to develop resistance to not only one but several antibiotics

(Watanabe, 1963; Anderson, 1968; Smith, 1972). With the elucidation of plasmids, it was discovered that some genes, transposons, are mobile and result in similar plasmids carrying very dissimilar genes, yet seemingly dissimilar plasmids carrying exact copies of the same genes (Dale, 1994; Levy, 1986). Transposons are small fragments of mobile DNA which carry a heritable trait and have the ability to move, or transpose, from chromosomes to plasmids, from plasmids to chromosomes, from plasmids to plasmids and from chromosomes to chromosomes (Bennett, 1985). The widespread distribution of many types of antibiotic resistance is the result of transposons. Penicillin resistance, for example, is usually a result of TEM b-lactamase, an enzyme encoded by a transposon ubiquitous among enteric bacteria carrying plasmids (Dale, 1994. Indeed, most antibiotic resistance genes are carried on transposons (Bennett, 1985).

While the mechanisms of transposition are not entirely clear, two models have been proposed. The first of which involves a transposon encoded *transposase* which mediates a recombination between a plasmid (or chromosome) carrying the transposon and a plasmid not carrying the transposon. The result is the formation of one large plasmid called the cointegrate which consists of the two plasmids fused together but with two copies of the transposons. A second transposon mediated enzyme, *resolvase*, mediates the separation or resolution of the cointegrate into two plasmids each carrying a copy of the transposon. The second model is less complicated and involves a "cut and paste" mechanism, where the transposon flanked by target sequences is cut from the donor plasmid. The transposon is then inserted into the recipient plasmid (Dale, 1994).

Stability: While conjugative plasmids are ensured stable association with the host through partioning and conjugation, the phenotypic characteristics that they express are not (Dale, 1994). Maintanance of extrachromosomal DNA and continued production of products needed for antibiotic resistance may exact a metabolic drain on the host to the point where replication time is increased in comparison to plasmid-free populations (Dale, 1994). Without the selection pressure of antibiotics, plasmid-free populations may displace plasmid-carrying populations (Dale, 1994; Edwards, 1961, Edwards, 1962).

Several key factors may help to maintain plasmid-mediated resistance among bacteria in the absence of the selective pressure of an antibiotic. In many cases, antibiotic resistance is genetically linked to a phenotypic trait such as a colonization determinant (Schlaes, et al., 1991). Such linking enables a population to maintain extrachromosomal genes simply because they are linked to genes that must be expressed for the organism to survive inside a host. Evidence of plasmid stability and maintenance was shown in English studies that found the percentage of tetracycline resistant salmonella isolates obtained from chickens has not changed since the drug was prohibited for use in poultry operations in 1971 (Guthrie, 1992) and in a recent Australian survey that found chloramphenicol resistance had actually increased among *Salmonella typhimurium* isolates obtained from cattle since 1988 when use of the drug was banned in food animals (Mackie, et al., 1996).

Products of Resistance Genes: The manner by which plasmid DNA confers antibiotic resistance to a bacterium varies with antibiotics. In some cases, the plasmid encodes for

an enzyme (eg. β-lactamases, cephalosporinases) which degrades the antibiotic rendering it inactive. In other cases the plasmid allows for the generation of proteins that alter the cell membrane of the bacterium making it less permeable to the drug (eg. Tetracyclines) (Alexander, 1985; Giovanani and Warren, 1983; Dale, 1994). Yet, in other cases bacteria are able to mutate the 30s ribosomal subunit to prevent binding of an antibiotic (eg. aminglycosides) or produce a drug-resistant protein that is able to by-pass the enzyme inhibited by the antibiotic (sulphonamides, quinolones) (Dale, 1994 Alexander, 1985).

Classification of Antibiotics

Livestock producers incorporate a wide range of antibiotics in the production of food animals (Dunlop, et al., 1998). Although antibacterial mechanisms vary among antibiotics, certain similarities in function and structure allow their grouping into families. In the interest of space, only the families of the drugs used in the present experiments will be discussed in detail.

Tetracyclines: Tetracyclines are broad spectrum antibiotics and are among the most common antibiotics used in feeds (Einstein, et al., 1984). They are derived from the *Streptomyces* genera of fungi (*aurofaciens* or *rimosus* species) and include tetracycline, chlortetracycline and oxytetracycline (Alexander, 1985). Their name is derived from their structure as they are four fused, six sided rings. Compared to other broad spectrum antibiotics, the tetracyclines have a low toxicity and are stable in dry forms. As such they lend themselves well to incorporation into feeds (Giovanani and Warren, 1983).

Tetracyclines are bacteriostatic and inhibit bacterial growth by binding to the 30s ribosomal subunit and blocking tRNA (Giovanani and Warren, 1983; Huber, 1982). Binding to the ribosome reduces the ability of the bacterial cell to incorporate glutamate into proteins thereby blocking peptide elongation (Huber, 1983; Einstein, et al., 1984). Their effects may only be seen in rapidly growing populations (Giovanani and Warren, 1983).

A great deal of work has been done on tetracycline resistance as a model for infectious resistance, as tetracyclines are among the most commonly used antibiotics in animal feed (Einstein, et al., 1984). Resistance is caused by defective uptake of the drug by bacteria. The gene, when expressed, causes changes in the bacterial cell membrane to decrease active transport of the drug into the cytoplasm. A separate mechanism may cause an efflux of the drug out of the cell, further reducing accumulation of the drug (Huber, 1983). Cross-resistance is usually the rule with tetracyclines because resistance to one drug leads to resistance to the whole family (Alexander, 1985).

The tetracycline used in the present experiments was oxytetracycline (Terramycin ™) (Pfizer, NY, NY). It is also commonly added to swine diets as a growth promotant (Einstein, et al., 1984). The drug has a 24 hour withdrawal time (FDA-CVM, 1998).

Cephalosporins: Cephalosporins are β-lactam antibiotics derived from the mold *Cephalosporium acrimonium* (Einstein, et al., 1984). The β-lactam family all contain a β-lactam central nucleus attached to an organic acid. In the case of cephalosporins, the organic acid is 7 aminocephalosporanic acid (7 ACA). It is the B-lactam ring that is

responsible for antibacterial action, as its destruction results in loss of activity (Giovanani and Warren, 1985). The mode of action of cephalosporins resemble that of other β-lactams in blocking peptidoglycan synthesis. Peptidoglycan forms a net-like structure enclosing the periplasmic space of bacterial cells. The β-lactam ring of the drug irreversibly binds transpeptidase, the enzyme responsible for cross-linking peptidoglycan. (Huber, 1983). The result is a weakening of the cell wall and lysis of the bacterial cell in hypertonic solutions.

The original cephalosporins were considered broad spectrum antibiotics but with greater activity against gram-positive organisms (Prescott and Baggot, 1993). Through the addition of different organic acids to the R subunit of the 7-ACA base, "second" and "third generation" cephalosporins have been developed with greater activity against gram-negative organisms, namely the enterobactericeae (Giovanani and Warren, 1983.; Einstein et al., 1984; Prescott and Baggot, 1993). Until recently the use of cephalosporins has been limited in agriculture in favor of the related penicillins. The development, however, of broader spectrum cephalosporins has led to increased use. (Brander, 1986).

Resistance to cephalosporins is comparable to that of other β-lactams. In both cases enzymes called β-lactamases act to cleave the β-lactam ring and render the drug inactive. Broad spectrum β-lactamases can cleave the β-lactam ring of either cephalosporins and other β-lactam antibiotics (eg. TEM β-lactamase), thereby leading to cross-resistance among some penicillins and cephalosporins. Other enzymes produced by bacteria, however, act only on cephalosporins and are referred to as cephalosporinases (Bywater, 1991). Third generation cephalosporins were developed with the notion of

increased resistance to β-lactamases (Giovanani and Warren, 1983); however, derepression of inducible chromosomal encoded β-lactamases has been documented and can lead to rapid development of resistance (Einstein, et al., 1984).

The cepholosporin used in the present experiments is the sodium salt form of ceftiofur (Naxcel ®)(Pharmacia and Upjohn, Kalamazoo, MI, 49001). A broad-spectrum, third-generation cephalosporin, ceftiofur sodium was originally used to treat Bovine Respiratory Disease. It is now indicated for the treatment of Swine Respiratory Disease (SRD) (Salmon, et al., 1995, Pharmacia & Upjohn, Kalamazoo, MI) and is reportedly effective in the control of both gram-positive and gram-negative pathogens of veterinary importance (Gilbertson, et al., 1995). There is no withdrawal time for ceftiofur sodium (FDA-CVM, 1998).

Aminoglycosides (and Aminocyclitols): Aminoglycosides and aminocyclitols are a family of antibiotics which contain a cyclic nucleus with one or two amino sugars or amino cyclitols attached by glycosidic linkage (Bywater, 1991). They are derived from either the *micromonospora* or *streptomyces* genera of fungi (Browne and Riviere, 1989). They 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, also interfere with cellular electron transport, interfere with cell membranes, and breakdown RNA (Prescott and Baggot, 1993).

Aminoglycosides are more narrow-spectrum with activity primarily against gram-negative species (Huber, 1983). Moreover, aminoglycosides are most active against

aerobic species--anaerobic species may lack the transport system necessary for the drug to enter bacterial cells (Einstein, et al., 1984).

Resistance to aminoglycosides is well documented and can result from the development of several different mechanisms. Bacterial cells may mutate ribosomal proteins resulting in the absence of the requisite 30s subunit necessary for attachment and antibacterial activity (Einstein, et al., 1984). Decreased uptake has also been noted as a form of resistance to the drugs (Giovanani and Warren, 1983). The most common form of resistance, however, is the production of drug modifying enzymes. Such enzymes are thought to be associated with the cell wall and act to inactivate aminoglycosides (Giovanani and Warren, 1983).

Apramycin (Apralan®, Elanco, Indianapolis, IN), the aminocyclitol used in the present experiments, is indicated as both a control and treatment of enteritis caused by gram-negative bacteria in young pigs (Bywater, 1991; Einstein, et al., 1984; Elanco, Indianapolis, IN). Apramycin has a 28-day withdrawal time (FDA-CVM).

Carbadox: Carbadox is synthetic antibacterial belonging to a family of compounds classified as quinoxaline-di-N-oxides. It is a broad spectrum antibacterial (Brander, 1986) and has been used in the United States as a growth promotant for swine since 1972 (Das, 1984). Its mode of action is similar to that of the nitrofurans, in inhibiting DNA synthesis and denaturing pre-existing DNA (Prescott and Baggot, 1993.). While the exact mechanism of bacterial resistance to carbadox has not been described, resistance

has been reported in *E. coli* isolates from swine farms (Das, 1984). No cross-resistance has been seen with carbadox and other antibiotics (Huber, 1983)

Mecadox™ (Pfizer) was used in both experiments. It is indicated for the control of dysentery, bacterial enteritis, and growth promotion (FDA-CVM). The drug has also been shown to lessen the clinical manifestations of *Salmonella cholerasuis* in swine (Troutt, et al., 1974; Olson, et al., 1977).

Related Studies

Drug Resistant Salmonella: As one of the most frequently isolated food-borne pathogens, a large proportion of antibiotic resistance research has focused on the salmonella genus. The development of tetracycline resistant salmonella in agriculture serves as a useful model for studying the effects of including antibacterials in livestock feeds (Smith, 1971).

Shortly following the discovery of infectious resistance, Huey and Edwards conducted a retrospective study examining tetracycline resistance among isolates obtained from animals prior to 1948. No isolates exhibited resistance to the drug. In 1956 they found that the number had jumped to 9% (Huey and Edwards, 1958, Smith, 1971). In 1962, McWhorter and co-workers documented that 29.8% of salmonella isolates obtained from animals exhbited resistance to the drug (Smith, 1971). A 1981 survey found that the number had jumped to 48% (Blackburn, et al., 1984). Modern surveys indicate that between 50-60% of the salmonellae isolated from livestock and meat products are resistant to tetracycline (Tessi, et al., 1997; Epling and Carpenter, 1990).

In terms of other antibiotics, the list of antibiotics to which isolates of salmonella have shown resistance includes all but the newest drugs. During the period of 1984-1987, a Wales survey of 8677 isolates obtained from animals found that only 6.6% of the isolates were sensitive to all 14 antibiotics tested (Wray, et al., 1991). A smaller study of 84 isolates in Japan found that 69% of the isolates were resistant to one or more of the 12 antibiotics tested. Of note was the fact that 38 of the 58 resistant isolates were confirmed as carrying resistance plasmids. *Salmonella typhimurium* made up 95% of such isolates (Ishiguro, et al., 1980). An American survey found 935 of 1251 salmonella isolates obtained from animals to be resistant to one or more antibiotics. The highest incidences of multiple resistance were found among *Salmonella typhimurium* isolates (Pocurull, et al., 1971). Epling and Carpenter in 1990 sampling pork carcasses found that 84.3% of the 121 salmonellae isolated exhibited resistance to two or more drugs (Epling and Carpenter, 1990).

The rise in the number of single and multiple-drug resistant *Salmonella typhimurium* isolates can be attributed to the genera's ubiquity in livestock (Reed, et al., 1984) and the deftness at which the organism receives resistance plasmids (Smith, 1971). Modern surveys of food-borne pathogens in livestock show that isolates of salmonella are among the most often to carry plasmids, and the sub-species *Salmonella typhimurium* make up the majority of those isolates. (Blackburn, et.al., 1984). Coupled with the fact that antibiotics used in livestock may select for such populations (Smith, 1971), the rise in antibiotic resistant *Salmonella typhimurium* is anything but extraordinary. Of immediate concern is the emergence of strains such as *Salmonella typhimurium* DT104.

The DT104 strain is commonly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Poppe, et al., 1998). Although the organism is primarily a pathogen of cattle (Evans, 1996) it has increasingly been the source of human salmonellosis in Europe, the United States and Canada (Poppe, et al., 1998).

Studies Examining Shedding: Several similar studies exist examining the relationship between antibiotics, antibiotic resistance and the carrier state. A review of such studies reveals a wide range of experimental designs and an equally wide range of results. A common postulate exists stating that if an organism colonized in the gastro-intestinal tract is intrinsically resistant to an antibiotic, introduction of that antibiotic to the gastro-intestinal tract presents a selection pressure aiding the establishment of the resistant organism (Smith, 1971).

Perhaps the most cited study supporting this model is that of Williams and co-workers in 1978. Using a strain of *Salmonella typhimurium* containing a plasmid-mediated chlortetracycline resistance, Williams showed that treatment of infected pigs with chlortetracycline increased the quantity of *Salmonella typhimurium* shed and the duration during which pigs shed the organism (Williams, et al., 1978).

Following this model to its conclusion, antibiotics with activity towards primarily gram-positive organisms could pose the same threat in promoting growth of organisms inherently resistant to them. DeGeeter and co-workers tested such a postulate examining the effect of lincomycin on *Salmonella typhimurium* infections in pigs. Lincomycin is an aminoglycoside with activity primarily against gram-positive organisms; gram-negative

bacteria are generally not affected by the drug. Their results indicated that the presence of lincomycin did not alter the number of pigs shedding *Salmonella typhimurium*, nor did it increase the duration of shedding. Moreover, the antibiogram of the inoculating strain was unaffected by the inclusion of lincomycin in the diet (DeGeeter, et al., 1976).

Abou-Youseff and co-workers conducted similar experiments using virginiamycin, an aminoglycoside with activity similar to lincomycin. Their results mirrored those of DeGeeter and showed that treatment of *Salmonella typhimurium* infected pigs with virginiamycin did not result in increased shedding of the organism (Abou-Youssef, et al., 1979).

Gutzmann and co-workers published an experiment similar to that of Williams in examining the effects of chlortetracycline, and a combination of chlortetracycline, sulfamethazine and penicillin on the shedding patterns of *Salmonella typhimurium* infected pigs. The *Salmonella typhimurium* isolate used, however, was not resistant to any of the test antibiotics as was the case in Williams's experiment. Use of these antibiotics against such a strain did not result in any increase in the salmonella pool nor a prolonged carrier state (Gutzmann, et.al., 1976)

Wilcock and Olander published a study in 1978 examining the effects of several common antibiotic regimens in controlling *Salmonella typhimurium* infections in swine herds. While not concentrating on the effects of such antibiotic inclusion on the development of resistance, their results are noteworthy in that no antibiotic regimen prolonged the shedding period (Wilcock and Olander, 1978).

Studies examining the specific antibiotics used in the present experiment and their effects on *Salmonella typhimurium* infections are sparse. Troutt and co-workers examined the effects of carbadox only using the sub-species *Salmonella cholerasuis*. The drug was somewhat effective at reducing the severity of salmonellosis caused by *Salmonella cholerasuis* in swine (Troutt, et al., 1974). The highly invasive nature of the more host-adapted *Salmonella cholerasuis*, however, negates the possibility of accurately extrapolating the drug's effect on the *typhimurium* serovar.

An interesting study put forth by Hunter and co-workers examined apramycin resistance plasmids in *E. coli* and their transfer to *Salmonella typhimurium* in calves. During an outbreak of salmonellosis among calves and prior to treatment, apramycin resistant *E. coli* were isolated however, all *Salmonella typhimurium* isolates were sensitive to the antibiotic. Upon treatment with apramycin, the calves began shedding apramycin resistant *Salmonella typhimurium* in the feces. *In vitro* laboratory studies revealed conjugation of a plasmid encoding apramycin resistance from *E. coli* to *Salmonella typhimurium* (Hunter, et al., 1992).

2. Materials and Methods

Two experiments were conducted. The first experiment examined the effects of antibiotic regimens on fecal shedding patterns of *Salmonella typhimurium* in experimentally infected pigs. The second experiment examined shedding and the development of antibiotic resistance in an attempt to ascertain the role of resistance in bacterial shedding.

Pigs and Housing

In both experiments, pigs were obtained from the University of Tennessee Blount County Experiment Station Swine Unit. The first experiment employed 36 cross-bred pigs while the second employed 48. In both experiments, pigs of uniform size were weaned from sows in a common farrowing barn at 20-days-of-age and placed in separate nursery unit on the Blount County Experiment Station farm. The pigs were fed antibiotic-free rations and maintained in penned floors at ≤ 8 pigs per pen until 50 days of age when all pigs were inoculated with *Salmonella typhimurium*.

Immediately following inoculation, pigs in both experiments were transported via trailer to the growing-finishing facility of the University of Tennessee Experiment Station at Crossville. There, the pigs were randomly separated into the four treatment groups of groups of 9 or 12 for experiment one and two, respectively. Treatments began two days post-ionoculation.

Bacteria Preparation and Inoculation

Salmonella typhimurium #4232 was used as the inoculate in all experiments and was provided by the USDA Animal Disease Control Center in Ames, Iowa. It is a mutation of the parent strain Salmonella typhimurium 798 carrying a nalidixic acid resistance marker (Fedorka-Cray, et al., 1995). The parent strain (798) is known to persistenly infect pigs (Isaacson, and Kinsel, 1992; Wood and Rose, 1991) Twelve hours prior to inoculation, one loopful (1ml) of the original stock was inoculated into 100 mL of nutrient broth (3g beef extract, 5g peptone/ 1L H_20) and incubated overnight at 37C with agitation. The resulting culture was enumerated via serial dilution and plating on nutrient agar (Difco, Detroit, MI). Enumeration revealed a culture of 1.3×10^9 CFU/ml in the first experiment and 1.2×10^9 CFU/ml. Confirmation was made biochemically using API 20E (BioMerieux Vitch, Inc., Hazelwood, MO).

Failure to add nalidixic acid to nutrient broth in the preparation of the inoculate in Experiment One resulted in loss of the nalidixic acid resistance marker. As such, isolates obtained from pigs post-inoculation failed to grow on agar plates containing nalidixic acid. Preparation of the inoculate in the second experiment mirrored that of the first, however, nalidixic acid as added at 50ug/ml to the nutrient broth to ensure that the nalidixic acid resistance marker stayed intact (Fedorka-Cray, et al., 1995).

The flask containing the inoculate was placed on ice and taken to the aforementioned nursery unit on the Blount County Experiment Station farm. Pigs in both experiments were individually inoculated with 3ml of culture intranasally and orally by

placing a small rubber tube on the end of a 10cc syringe filled with 3ml of culture. The plastic tube was then placed into one nostril and 1ml of culture was discharged. Similarly, a second milliliter was discharged into the opposite nostril. A third milliliter was discharged into the most posterior portion of the oral cavity.

Treatments

Pigs were blocked by litter and randomly assigned to four treatments groups: 1) Intramuscular injection of ceftiofur sodium (Naxcel™, Pharmacia, Upjohn, Kalamazoo, MI) for three days followed by the inclusion of oxytetracycline (Terramycin™, Pfizer, NY, NY) at 100g/ton of feed, 2) apramycin (Apralan™, Elanco, Indianapolis, IN) at 150g/ton of feed for 14 days followed by oxytetracycline at 100g/ton of feed, 3) carbadox (Mecadox™, Pfizer, Groton, CT) at 50g/ton of feed until pigs reached 35 kg followed by oxytetracycline at 100g/ton of feed and finally 4) no antibiotics (control). All feeds were mixed at the East Tennessee Farmers Cooperative in La Vergne, TN and aside from containing different levels of antibiotics were identical.

Sample Collection

Fecal samples were collected on the day of inoculation, 2 days post-inoculation (dPI), 4dPI, 7dPI, 14dPI, 21dPI, 28dPI, 42dPI, 56dPI, 70dPI, and 84dPI. Fecal samples were obtained by placing two non-absorbable swabs (MacroPur™) (PurFybr, Inc., Munster, IN) into the pig's rectum and with a swift circular motion were withdrawn. Swabs were then placed in individual test tubes and stored on ice for transport to the

laboratory in Brehm Animal Science Building on the Knoxville campus.

Isolation of Salmonella

Numerous techniques exist for the isolation of salmonella however, no method is universally accepted among microbiologists. Proof of the existence of salmonella in heavily contaminated samples usually requires a multifactorial isolation process that can successfully isolate the challenge organism from other more numerous associated microorganisms. Traditional methods employ four steps: 1) pre-enrichment in a non-selective mediu to repair or resuscitate damaged and dormant cells, 2) enrichment in a selective broth which will facilitate the growth of salmonella while suppressing competitors, 3) plating on a selective or differential medium and 4) serological or biochemical confirmation of presumptive colonies (Galton, et.al., 1967, Feng, 1992).

The isolation technique used in both experiments is a small variation upon this common theme. The traditional pre-enrichment step was excluded in accordance with the FDA's Biological Analytical Methods recommendation that highly contaminated samples (eg. feces) be directly enriched in a selective broth so as not to subject any salmonella present to exclusion via the overgrowth of competing species (Andrews, 1985, FDA, 1978). The technique used was a three step process: 1) enrichment in both tetrathionate (Difco, Detroit, MI, 48118) and selenite cystine (Difco, Detroit, MI, 48118) broths, 2) plating on xylose-lysine-tergitol-4 plating medium (Difco) and 3) biochemical confirmation using lysine iron agar (Difco) and triple sugar iron slants (Difco).

Step One: Enrichment: It is generally agreed that the more methods and media employed, the greater the success in isolating salmonellae. Moreover, one type of medium may prove more effective in one trial yet less effective in another (Fagerberg and Avens, 1974, McCullough and Byrne, 1952). Both tetrathionate and selenite cystine were used as selective enrichment media in the hope that inadequacies of one could be overshadowed by the other. Moreover, they are the most commonly used enrichment media for isolating salmonellae (Andrews, 1985).

The tetrathionate broth used in all experiments is a Difco (Detroit, MI) product containing a base of 5g Bacto Proteose Peptone, 1 g Bacto bile Salts, 30g sodium thiosulfate ($Na_2S_2O_3$) and 10 g calcium carbonate. Tetrathionate ($Na_2S_4O_6$) is produced upon addition of iodine (6 g) dissolved in potassium iodide (5g) according to the following the reaction: $2Na_2S_2O_3 \bullet 5H_2O + I_2 + [KI] \rightarrow Na_2S_4O_6 \bullet 2H_2O + 2NaI + [KI]$ (Palumbo and Alford, 1970). The tetrathionate ion is believed to be the main inhibitory agent in the broth, however, the exact mechanism of inhibition is unclear. Moreover, thiosulfate and iodides are believed to play a role, as inhibition is enhanced with their addition. Bile salts are added to inhibit the growth of many gram-negative and non-enteric bacteria (Palumbo and Alford, 1970).

The selenite cystine broth used in all experiments is also a Difco product containing 5 g Bacto tryptone, 4 g Bacto lactose, 10 g disodium phosphate, 4 g sodium acid selenite and 0.01 g L-cystine. The selective agent of selenite cystine is the sodium acid selenite (Sveum and Kraft, 1981). Inhibition of selenite-susceptible organisms

results in retarded growth and is accompanied by reduction of selenite to metallic selenium (Weiss, et al., 1965). Although the exact mechanisms of inhibition are not completely understood, Tuve and Williams (1961) proposed two mechanisms that are generally agreed upon. First, selenite may react with sulfhydryl groups of enzymes, proteins, etc., rendering them inactive. Reduction of selenite to metallic selenium allows its incorporation into sulfur containing amino acids. Incorporation of the seleno-amino acids into proteins may inactivate the protein (Weiss, et al. 1965; Fagerberg and Avens, 1974). It is believed that the salmonellae and perhaps a few other genera are innately resistant to levels of selenite toxic to other genera (Weiss, et al., 1965).

In the enrichment step, the tips were cut off of both fecal swabs samples. One tip was placed in a Stomacher bag (Stomacher Lab System) containing 100 ml tetrathionate broth while the other was placed in a bag containing 100ml selenite cystine broth. Incongruence exists as to the efficacy of elevated temperatures and enhanced salmonella recovery (Andrews, 1984). As a compromise, Stomacher bags containing tetrathionate broth were sealed and incubated at 42°c for 18-24 hours while Stomacher bags containing selenite cystine were sealed and incubated at the traditional 37C for 18-24 hours.

Step 2: Plating : XLT4 plating medium (Difco, Detroit, MI) is a highly selective medium for non-typhi salmonellae (Difco manual). It is a slight modification of the traditional XLD and relies on a few properties indigenous to salmonellae, namely xylose fermentation, lysine decarboxylation and hydrogen sulfide (H_2S) production (Fagerberg and Avens, 1974). Three fermentable sugars (xylose, sucrose and lactose) are included

along with phenol red as a pH indicator. Salmonella readily ferment xylose, causing an acid (yellow) reaction. Upon exhaustion of xylose, salmonella decarboxylate lysine, causing a return to alkaline (red) pH. Most other contaminants do not revert back to alkaline pH, as they are likely able to ferment sucrose and lactose into acidic metabolites (Fagerberg and Avens, 1974). Hydrogen sulfide indicators, sodium thiosulfate and ferric ammonium citrate, further distinguish salmonellae, as the genus generally will produce black centered colonies (Miller, et al., 1991). Tergitol 4 is included as another selective agent to prevent growth of Proteus colonies which can appear similar to salmonellae on the traditional XLD media (Feng, 1991). Miller and co-workers concluded that salmonella was essentially the only genus capable of forming pink colonies with black centers within 24 hours on XLT4 thus allowing for exceptionally easy identification (Miller, et al., 1991).

Preparation of XLT4 medium in the first experiment followed the label guidelines. The nalidixic acid resistance marker was apparently lost from the original inoculate, therefore nalidixic acid was not added to the medium. The preparation of XLT4 in the second experiment included the addition of 50ug nalidixic acid/ml to the XLT4 medium as a selective agent excluding the growth of salmonellae other than the original isolate (Fedorka-Cray[a], et al., 1995). The stomacher bags were removed from the incubator and using a sterile scalpel small incisions were made across the top of each bag. One loopful (1ml) of culture was removed and transferred to a plate containing approximately 9ml xylose-lysine-tergitol-4 plating medium. In an attempt to promote the growth of distinct and individual colonies, three streaks were made across the top of the

plate. Three streaks were then drawn from the original. This procedure was repeated twice until four sets of three streaks were drawn on the plate. Plates were incubated at 37C for 24 hours. Black or black centered colonies with pink outer shells appearing within 24 hours were considered presumptive salmonella colonies, to be confirmed biochemically.

Step 3: Biochemical Confirmation: Use of triple sugar iron (TSI) (Difco) and lysine iron agar (LIA) (Difco, Detroit, MI) are useful biochemical rather than serological methods of further differentiating salmonellae and are routinely recommended by government, industrial and clinical laboratories (Cox and Williams, 1990). Both media differentiate *salmonella* spp. based on certain fermentation, lysine-decarboxylation and hygdrogen sulfide production properties indigenous to the genus. Triple Sugar Iron incorporates the three sugars, dextrose, sucrose and lactose. Fermentation is detected by acid production as indicated by phenol red pH indicator. Ferrous sulfate and sodium thiosulfate are included as substrates for hydrogen sulfide production. When incorporated into a slant tube, inoculation with salmonella isolates produces an acid butt (yellow) from the fermentation of dextrose and an alkaline slant (reversion back to red) and hydrogen sulfide (black)(Cox and Williams, 1990). LIA medium works similarly to XLT4 plating medium, differentiating salmonella based on fermentation of sugars, lysine decarboxylation and hydrogen sulfide. Inoculation of LIA slants with salmonella initially produces an acid (yellow) reaction from the fermentation of dextrose. Salmonellae, however, are able to decarboxylate lysine in an alkaline reaction, reverting the tube back

to red (both butt and slant) (Cox and Williams, 1990). Ferric ammonium citrate and sodium thiosulfate allow for hydrogen sulfide production turning the majority of the tube black.

Presumptive colonies were individually inoculated into LIA and TSI slants using a sterile wire loop. Confirmations were made using the aforementioned system of chemical and color reactions. A pig was confirmed as shedding the isolate if colonies isolated on XLT4+nalidixic acid from either selenite cystine or tetrathionate broth cultures conformed to the set of chemical reactions indicative of salmonella on LIA and TSI.

Antibiotic Sensitivity Testing

The technique used in the third experiment, Bauer-Kirby disc diffusion, to determine antibiotic susceptibility of isolates from shedding pigs is a more standard and accepted procedure. The procedure involves placing discs (BBL Sensi-disc™, St. Louis, MO) containing specified concentrations of each antibiotic on the surface of Mueller-Hinton agar (Beckton-Dickinson, Cockeysville, MD,) inoculated with pure culture. Zones of inhibition surrounding each disc are measured and compared to a standard to determine the efficacy of an antibiotic against a single isolate (BBL Sensi-Disc Manual, 1997; Bauer, et al., 1967).

Five pigs from each treatment group in the second experiment were randomly selected for antibiotic resistance testing. A portion (250 uL) of the tetrathionate and selenite cystine broth cultures from each shedding-positive pig was frozen in a mixture of 250ul nutrient broth and 500ul 10% sterile saline in a 2ml eppedorf tube and frozen at

-70C. Frozen cultures from day one, 2dPI, 4dPI, 7dPI, 14dPI, 21dPI, 42dPI and 70dPI were thawed and enriched once again in tetrathionate broth containing 50ug/ml nalidixic acid. One loopful of enrichment culture was streaked onto plates containing XLT4 (9ml) with 50ug/ml nalidixic acid. Nine individual and uniform colonies were chosen from each XLT4 plate. Individual colonies were inoculated into tubes containing 5ml LB broth (10g Bacto-tryptone, 5g yeast extract, 10g NaCl / 1L H_20) and grown at 37C with agitation to meet a .5 Mcfarland standard in accordance with National Committee for Clinical Laboratory Standards (NCCLS, 1976). Once the Mcfarland standard was reached 200ul of the broth was inoculated onto Mueller-Hinton agar plates and spread using a sterile glass pipette for confluent growth. Plates were allowed to dry for 15-10 minutes. Discs containing Apramycin (15ug) (prepared by our lab), Ceftiofur Sodium (30ug) (Sigma Chemical Co., St. Louis, MO), Carbadox (20ug) (Sigma Chemical Co.), and Oxytetracycline (Sigma Chemical, Co) were placed in the four corners of a single plate. Plates were incubated at 37C for 24hrs. Measurements of zone sizes were taken using a metric ruler and recorded in millimeters. Susceptibility was based on zone sizes according to susceptibility standards as provided by the manufacturers of each disc, as indicated below:

	Zone sizes (mm)	
Antibiotic	Susceptible	Resistant
Apramycin	≥12	≤11
Carbadox	≥12	≤11
Ceftiofur Sodium	≥21	≤20
Oxytetracycline	≥19	≤18

Zones sizes as ascribed by the manufacturers of each disc include an intermediate susceptibility zone, however, we considered intermediate resistance to be a degree of

resistance relative to the present study, therefore, the two zone sizes were considered as one.

Zone sizes were tested for accuracy using an ATCC *E.Coli* strain (24567 "Coast"). This strain proved susceptible to all antibiotics and verified zone sizes. Moreover, the *salmonella* strain used to inoculate pigs in both experiments was tested against these zones sized and proved susceptible to all antibiotics.

Statistical Methods

Average daily gain data were analyzed using SAS GLM. Comparisons of differences in the least square means were made using T-tests. SAS Proc-Mixed for repeated measures was used to analyze data pertaining to shedding and antibiotic resistance (SAS, 1985). Pigs were considered shedding if presumptive isolates on XLT4 medium from either tetrathionate or selenite cystine broth were biochemically confirmed on TSI and LIA slants. Shedding data were assigned real numbers (eg. shedding = 1, no shedding = 0) and comparisons were made of the least square means (model: shedding = treatment time treatment * time / predicted). Fisher's Exact Test was used to test the accuracy of the model. Similarly, resistance data were assigned whole numbers (eg. resistant = 0, susceptible = 1) and comparisons were made of the least square means of such numbers (model: zone size = antibiotic | treatment | time / predicted). In determining treatment and time effects, resistance was defined as the percentage of isolates resistant to at least one test antibiotic. Antibiotic effects refer to the specific antibiotics.

3. Results

Shedding

Experiment One: By 48 hours post-inoculation, the majority of pigs were actively shedding Salmonella via the feces. The loss of the nalidixic acid resistance marker limits the accuracy in stating that the salmonellae shed were *Salmonella typhimurium,* however no pigs tested positive for shedding salmonella prior to inoculation and the pattern of shedding mirrored that of experiment in which the nalidixic acid resistance marker stayed intact. Some pigs exhibited watery and discolored stool as supporting proof of infection, however, such clinical symptoms disappeared by the second week post-inoculation. None of the antibiotic regimens affected growth performance as measured by average daily gain (Figure 1, Table 1)*. Both the ceftiofur/oxytetracycline (cef/otc) and apramycin/oxytetracycline (apr/otc) treatment were effective in reducing bacterial shedding when compared to the control ($P < .05$) (Figure 3, Table 3). Significant differences in the incidence of shedding were observed beginning one week post-inoculation and continued until pigs reached market weight. No salmonella were isolated from pigs receiving cef/otc beyond 70 days post-inoculation. Conversely, both carbadox/oxytetracycline (car/otc) and the control continually shed *Salmonella typhimurium* throughout the study.

***Experiment Two*:** The rate of infection as measured by bacterial shedding mirrored that of Experiment 1, however, clinical symptoms of infection were sporadic and less

* All tables and figures can be found in the appendix

obvious. Again, no differences in growth performance were detected between antibiotic treatments and the control (Figure 2, Table 2). The apr/otc treatment again reduced both the incidence and duration of bacterial shedding when compared to the control. However, in contrast to the first experiment, the cef/otc treatment did not significantly reduce the number of shedding pigs. Moreover, no differences were detected between antibiotic regimens (Figure 4, Table 4).

Antibiotic Resistance

Antibiotic Effect: The percentages of isolates resistant to apramycin, carbadox, ceftiofur sodium and oxytetracycline regardless of time or treatment was 65%, 75%, 50% and 45% respectively (Figure 5, Table 5). Incidence of resistance to carbadox was greater than resistance to the other drugs ($P < .05$). Incidence of resistance to apramycin was lower than that of carbadox but greater than ceftiofur and oxytetracycline ($P < .05$)

Treatment Effect: Resistance (i.e. the percentage of isolates resistant to at least one antibiotic) was greatest in pigs receiving the car/otc treatment (61.1%) when compared to resistance in pigs receiving the cef/otc treatment (41.3%) and the control (50.6%). No differences in resistance were detected among pigs receiving apr/otc (54.4%) (Figure 7, Table 7).

Time Effect: Time effects were detected throughout the experiment and indicated significant increases in resistance (percentage of isolates resistant to at least one antibiotic

regardless of treatment) with time across all sample dates ($P < .05$). At three days post-inoculation resistance was measured at 36%. At five days post-inoculation, resistance decreased significantly to 26.9% ($P < .05$). From seven days post-inoculation to 70 days post-inoculation, however, resistance rose significantly with 89.3% of the isolates obtained displaying resistance to at least one antibiotic on the last sampling day (Figure 6, Table 6).

Antibiotic X Time Effect: Antibiotic X time interactions were detected throughout the experiment indicating that changes in the incidence of resistance over time varied among antibiotics ($P < .05$). The incidence of resistance through 4dPI was very low for ceftiofur sodium and oxytetracycline compared to apramycin and carbadox; however, by 70d PI the incidence of resistance was very high not different among antibiotics.

Treatment X Time Effect: Treatment X Time interactions were also detected indicating that the incidence of resistance (percentage of isolates resistant to at least one antibiotic) among treatments varied over time every treatment (Figures 9-12, Tables 9-12) ($P < .05$). While development of resistance in each treatment followed a similar crescendo pattern in each treatment group, resistance was greatest in pigs receiving the carbadox/OTC treatment on three days post-inoculation, five days post-inoculation and 42 days post-inoculation.

Treatment X Antibiotic Effect: Resistance to carbadox was significantly greater in pigs receiving the car/otc treatment (88.6%) compared with the cef/otc (60%) and apr/otc treatments (64%) but not when compared to the control (79.3%) ($P < .05$). No other Treatment X Antibiotic effects were detected. (Figure 13, Table 13).

Treatment X Time X Antibiotic Effect: Few Treatment X Time X Antibiotic interactions were detected. On days five and seven post-inoculation the amount of resistance due to carbadox resistance was greater ($P < .05$) in the treatment group receiving car/otc compared to both the groups receiving cef/otc and apr/otc (data not shown). The difference was not significant when compared to the control. Such interactions disappeared past 21 days post-inoculation. Similar differences were detected in the amount of apramycin resistance among isolates from pigs receiving the car/otc treatement compared to the cef/otc at five days post-inoculation. No differences were detected, however, when compared to the other treatment groups.

4. Discussion

As salmonellosis caused by *Salmonella typhimurium* makes up a large portion of the food-borne infections in humans, antibiotic-induced proliferation of shedding of the organism in animals would constitute a serious health hazard. These data indicate that the use of antibiotics whether for therapy or growth promotion increases the proportion of antibiotic resistant salmonella in the pig, however such resistance does not result in increases in shedding of the organism.

Examination of the time effect indicates that the proportion of isolates resistant to at least one antibiotic increased with time. The percentage of resistance at two days post-inoculation (36%) can be interpreted as a basal level of resistance as treatments began at two days post-inoculation and the sample was taken just prior to inclusion of antibiotics in the feed. At four days post-inoculation the percentage of resistance as measured by the time effect decreased significantly from the basal level to 26.9%. Four days post-inoculation, however, could be viewed as the pinnacle of infection. At this time the organism is may be replicating much faster than it can acquire the genes needed for antibiotic resistance. Sampling at this date would show a higher quantity of salmonella being shed with a lower proportion of the isolates exhibiting antibiotic resistance. The proportion of resistant isolates increased from four days post-inoculation; reaching a pinnacle of 89.3% on the last day of sampling

In terms of specific antibiotics, antibiotic x time interactions were detected throughout the sampling period indicating that the changes in the incidence of resistance over time varied among antibiotics. Through four days post-inoculation, the proportion

of isolates resistant to ceftiofur sodium and oxytetracycline was significantly lower compared to both apramycin and carbadox. By 70 days post-inoculation, however, the proportion of isolates resistant to the former antibiotics increased to point of being similar to both apramycin and carbadox.

Examination of the treatment x time effect indicates that the proportion of isolates resistant to at least one antibiotic varied over time within every treatment. Again, the amount of resistance at two days-post inoculation could be viewed as a basal level of resistance. Mirroring the patterns seen in the time effect, resistance decreased significantly at four days post-inoculation in the cases of ceftiofur sodium/oxytetracycline, apramycin/oxytetracycline, and the control treatments. The proportion of resistant isolates increased in every case to a pinnacle at 70 days post-inoculation. In each treatment, the proportion of isolates resistant to at least one antibiotic was significantly greater at 70 days post-inoculation compared to two days post-inoculation.

Very few treatment x antibiotic interactions were detected indicating that the proportion of isolates resistant to the specific antibiotics varied little within each treatment. The exception was the proportion of isolates resistant to carbadox which was greatest in isolates obtained from pigs receiving the carbadox/oxytetracyline treatment when compared to isolates obtained from pigs receiving both the ceftiofur sodium/oxytetracyline treatment and the apramycin/oxytetracyline treatment. However, the level of carbadox resistance was not different in pigs receiving carbadox/oxytetracyline compared to control pigs.

Treatment x time x antibiotic interactions are perhaps the most revealing measures of the pattern of resistance development and would indicate if the incidence of resistance to specific antibiotic varied over time within treatments. Unfortunately, few such interactions were detected indicating that resistance to specific antibiotics developed at similar patterns regardless of whether the pigs received the specific antibiotics. The lack of such effects could be explained in the design of the room in which pigs were housed. The experiment was designed to mirror general standards of the modern swine industry and while pigs were never in direct contact with pigs of other treatment groups, cross-contamination from individuals taking samples and farm workers was possible. Contamination in this manner would obscure treatment x antibiotic x time interactions by possibly introducing pigs of different treatment groups to organisms with slightly different resistance profiles.

Nevertheless, the antibiotic x time, treatment x time and antibiotic x treatment interactions all indicate that resistance developed to each of the drugs over time and in the cases of apramycin, carbadox and ceftiofur sodium, continued after use of the drugs was discontinued.

It is clear, however, that the development of resistance did not result in an increase in bacterial shedding. Shedding data from both Experiment One and Experiment Two indicates that in no case did pigs receiving antibiotics shed *Salmonella typhimurium* at a greater frequency than pigs not receiving antibiotics. In the cases of ceftiofur sodium/oxytetracycline in Experiment One and aprmycin/oxytetracycline in both

Experiment One and Experiment Two, the incidence of shedding was actually significantly decreased in pigs receiving antibiotics.

These data seem to indicate that the acquisition of antibiotic resistance by *Salmonella typhimurium* in the gastro-intestinal tract is probably not a very strong virulence factor. Fuller and co-workers, in 1960, were among the first to demonstrate that including penicillin and chlortetracycline in the diets of swine resulted in dramatic increases in the percentage of resistant lactobacilli, streptococci and coliforms isolated form the feces. These are among the normal microflora of the pig's gastro-intestinal tract. If, as was the case in Fuller's study, the use of antibiotics resulted in a large percentage of the residential flora becoming resistant, the presence of the antibiotic would offer very little selection pressure to favor the growth of one organism over the other.

In a study similar to the present experiments, Claussen and co-workers (1997) examined the strength of antibiotic resistance as a virulence factor but reported different results. Pigs infected with a tetracycline resistant strain of *Salmonella anatum* and treated with tetracycline shed the isolate in greater numbers than pigs infected with a tetracycline susceptible strain of *Salmonella typhimurium* and treated with tetracyline. It should be noted, however, that *Salmonella anatum* was shed in equal numbers between groups treated with tetracycline and non-medicated groups. The difference in shedding among medicated pigs inoculated with tetracycline resistant *Salmonella anatum* and tetracycline susceptible *Salmonella typhimurium* probably has more to do with the difference in pathogenesis between the two serotypes rather than their antibiotic resistance profiles.

The present data, however are supported by a the several studies that examined the effect of gram-postive spectrum antibiotics on the shedding patterns of pigs infected with *Salmonella typhimurium* (Abou-Youssef, et al., 1979; Gutzmann, et al., 1976). *Salmonella typhimurium*, being gram-positive, would be intrinsically resistant to gram-positive spectrum antibiotics such as virginiamycin and lincomycin. However, there exist several other gram-negative genera that normally inhabit the gastro-intestinal tract of swine. In no case did inclusion of narrow-spectrum antibiotics exhibit a selection pressure great enough to cause an increase in bacterial shedding.

Moreover, several authors postulate that the lengthy carrier state of pigs infected with *Salmonella typhimurium* is more a function of the intracellular nature of the organism. Unfortunately, most information concerning pathogenesis of *Salmonella typhimurium* in swine is borrowed from studies using mice or human cell culture as a model (Roof, et al., 1992). Wood and co-workers (1989) and Wood and Rose (1992), however, both established that the organism can persist for several months inside several key organs and tissue of the pig, namely tonsils, lymph nodes and intestinal cells while the pig shows no signs of clinical infection. If lengthy carrier states are the result of the ability of the organism to survive for long periods inside host cells, antibiotic resistance would again not afford much virulence as many antibiotics lack the capacity to permeate mammalian cell membranes. Failure to permeate the membrane would negate the selection pressure of an antibiotic.

It should be noted that in the cases of ceftiofur sodium/oxytetracycline and apramycin/oxytetracycline the amount of shedding was decreased. While most salmonellae are invasive, the speed at which the organisms are able to invade host cells varies to degree. *Salmonella typhimurium* replicate intraluminally before invasion to a greater degree than the more host adapted *Salmonella cholerasuis* (Roof, et al., 1992). The ability of the ceftiofur sodium/oxytetracycline and apramycin/oxytetracycline treatments to reduce shedding is probably a result of the antibiotics acting on the organism as it replicates intraluminally, thereby decreasing the load of organisms able to invade and persist in the animal. The implications of antibiotic resistant *Salmonella typhimurium*, however, may be much greater than the scope of the present experiments therefore use of these drugs in the control of *Salmonella typhimurium* may not be prudent. IgA, however secreted into the lumen would act in a similar fashion by reducing the load of organisms able to invade host cells. A vaccine that properly stimulates IgA and T-cell production could help control shedding by both decreasing the initial load of organisms in the gastro-intestinal tract (IgA) and attacking salmonella infected cells (cell mediated immunity) without producing antibiotic resistant organisms (Roof, et al., 1992). Further studies should focus on how the organism behaves *in vivo* and the particulars of the pig's immune response to the organism. Armed with such knowledge, effective vaccines could be developed that would limit incidence and duration of shedding in pigs.

Eradication of *Salmonella typhimurium* from swine herds may prove impossible, however, controlling factors that may result in increased shedding would limit the likelihood of salmonella contaminated meat. These data indicate that use of antibiotics

during *Salmonella typhimurium* infections does not result in increased shedding of the organism in the feces. In some cases, antibiotics may decreased shedding.

5. Conclusions

Controversy has surrounded the use of antibiotics in the livestock industry for several decades. There exists little agreement on whether such use of antibiotics poses a threat to public health. In terms of the effects of antibiotics on infections of *Salmonella typhimurium* in swine, the present experiments indicate that while use of the drugs favors the development of antibiotic resistance, such resistance does not affect the duration of bacterial shedding. It follows that the use of antibiotics should not result in an increased incidence of *Salmonella typhimurium* contaminated pork and pork products.

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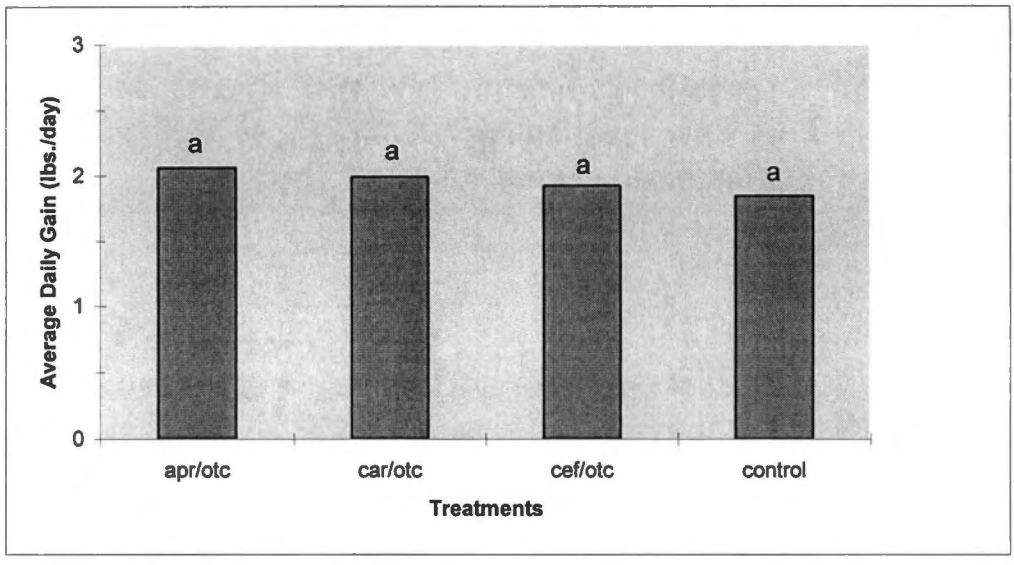
Appendix

Figure 1: Effect of different antibiotic regimens on the average daily gain in pigs following challenge with 10^9 CFU of *Salmonella typhimurium* (Experiment One)



SEM = 0.12

n = 9 pigs/treatment; averages represent least square means; bars with different superscripts are significantly different at P < .05; apr/otc = apramycin/oxytetracyline; car/otc = carbadox/oxytetracycline; cef/otc = ceftiofur/sodium; control = no antibiotics.

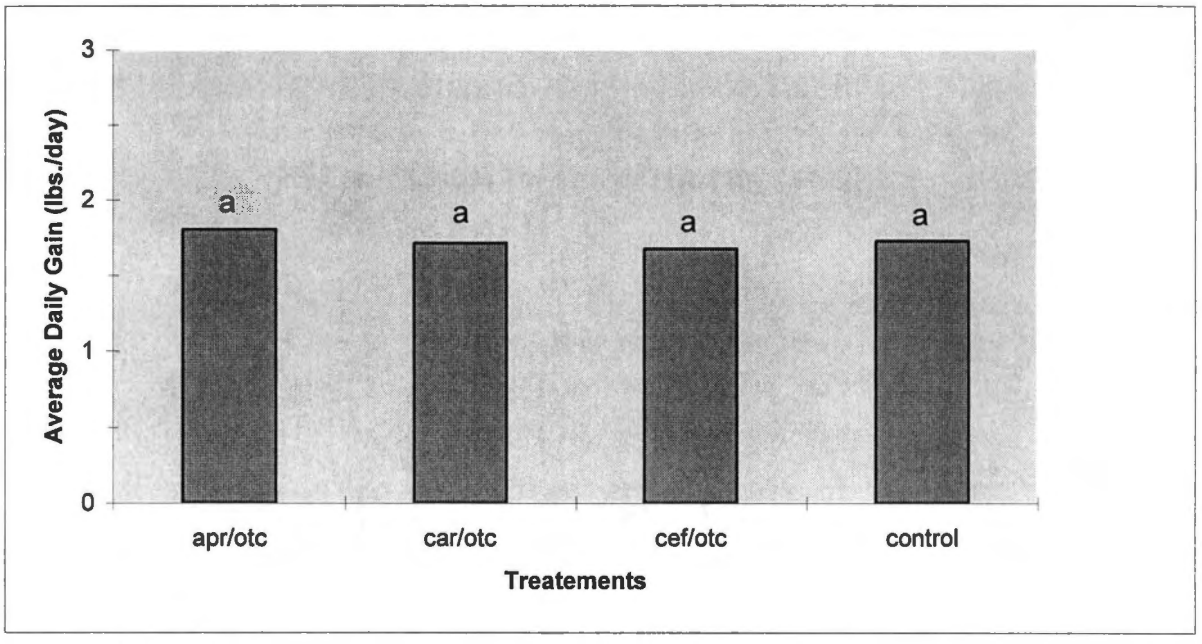
Table 1: Least square means of average daily gain in pigs following challenge with 10^9 CFU of *Salmonella typhimurium* (Experiment One).

Treatment	ADG
apr/otc	2.06[a]
car/otc	1.99[a]
cef/otc	1.92[a]
control	1.84[a]

SEM = 0.12

n = 9 pigs/treatment; ADG = average daily gain (lbs./day); means with different superscripts are significantly different at P < .05; apr/otc = apramycin/oxytetracyline; car/otc = carbadox/oxytetracycline; cef/otc = ceftiofur/sodium; control = no antibiotics.

Figure 2: Effect of different antibiotic regimens on the average daily gain in pigs following challenge with 10^9 CFU of *Salmonella typhimurium* (Experiment Two).



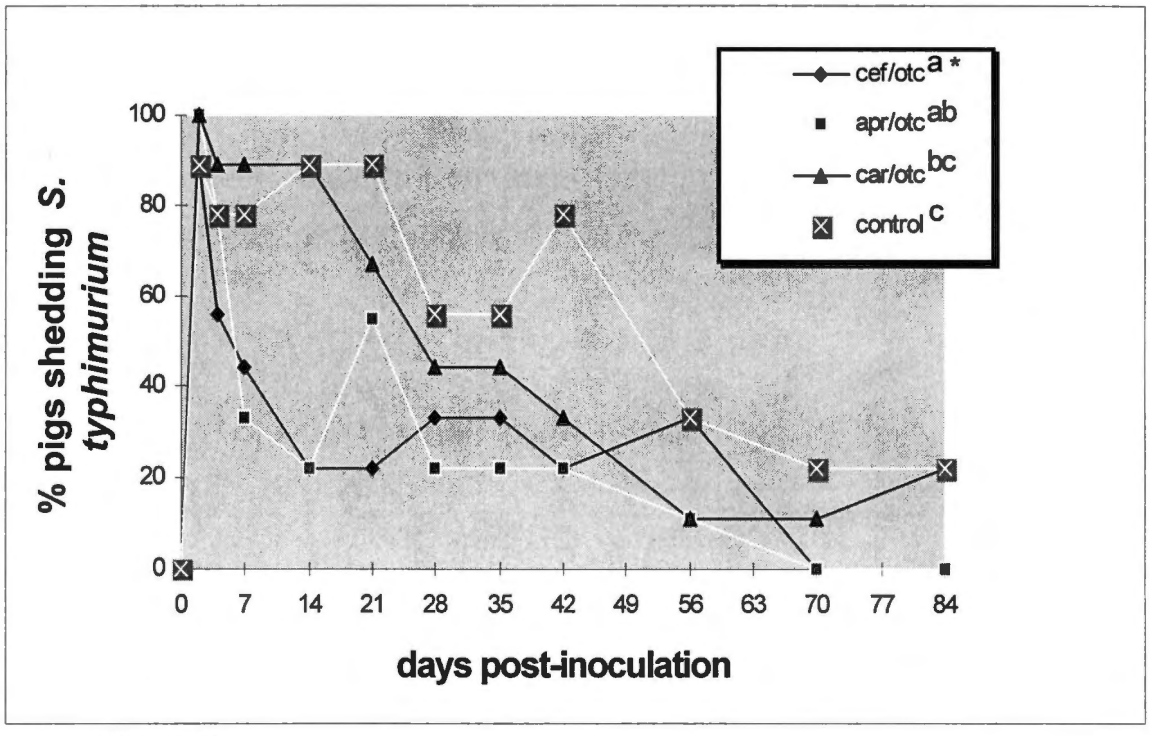
SEM = 0.12
n = 12 pigs/treatment; averages represent least square means; bars with different superscripts are significantly different at P < .05; apr/otc = apramycin/ oxytetracyline; car/otc = carbadox/oxytetracycline; cef/otc = ceftiofur/sodium; control = no antibiotics.

Table 2: Least square means of average daily gain in pigs following challenge with 10^9 CFU of *Salmonella typhimurium* (Experiment Two).

Treatment	ADG
apr/otc	1.80^a
car/otc	1.71^a
cef/otc	1.67^a
control	1.72^a

SEM = 0.12
n = 12 pigs/treatment; ADG = average daily gain (lbs./day); means with different superscripts are significantly different at P < .05; apr/otc = apramycin/oxytetracyline; car/otc = carbadox/oxytetracycline; cef/otc = ceftiofur/sodium; control = no antibiotics.

Figure 3: Effect of different antibiotic regimens on bacterial shedding in pigs challenged with 10^9 CFU of *Salmonella typhimurium* (Experiment One).



SEM = 0.33

n = 8 pigs/treatment group; cef/otc = ceftiofur sodium/oxytetracycline treatment; apr/otc = apramycin/ oxytetracycline treatment; car/otc = carbadox/oxytetracycline treatment; control = no antibiotics; different superscripts indicate differences in the overall incidence of shedding across treatment groups at $P < .05$.

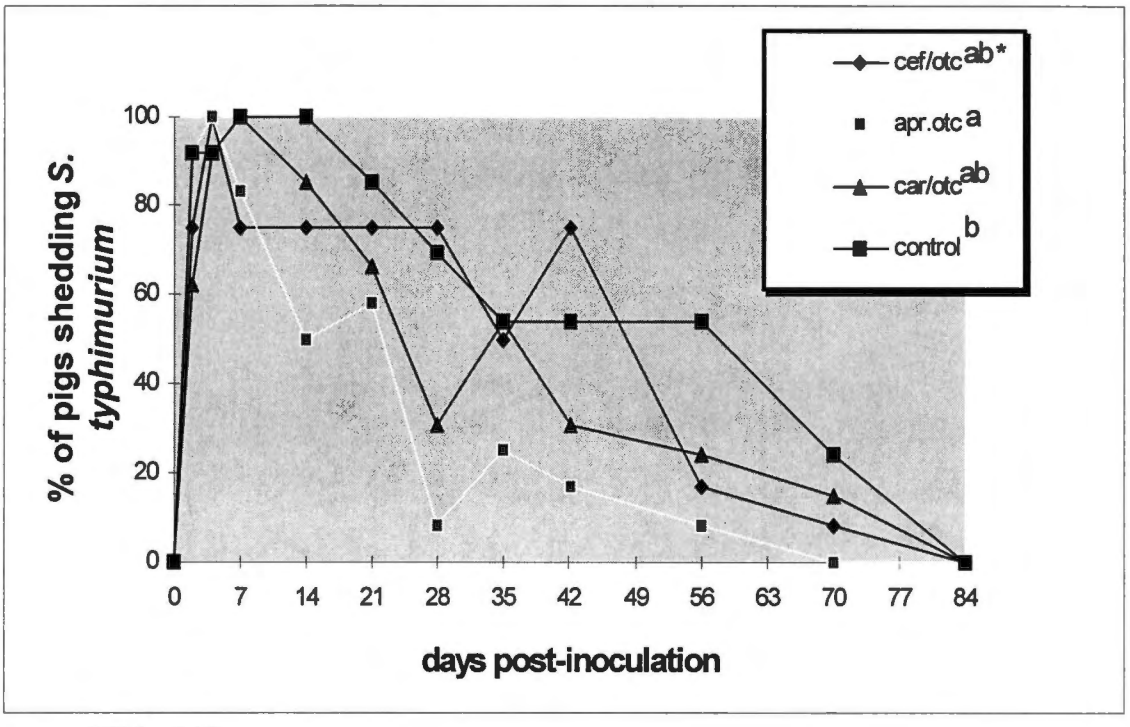
Table 3: Effect of different antibiotic regimens on bacterial shedding in pigs challenged with 10^9 CFU of *Salmonella typhimurium* (Experiment One).

	Number of Pigs Shedding *Salmonella typhimurium*											
Treatment	0d PI*	2d PI	4d PI	7d PI	14d PI	21d PI	28d PI	35d PI	42d PI	56d PI	70d PI	84d PI
apr/otc	0/9 ^{a*}	8/9 ^{a}	4/9 ^{a}	5/9 ^{b}	3/9 ^{b}	2/9 ^{b}	3/9 ^{a}	3/9 ^{a}	2/9 ^{b}	3/9 ^{a}	0/9 ^{a}	0/9
car/otc	0/9 ^{a}	9/9 ^{a}	6/9 ^{a}	4/9 ^{b}	3/9 ^{b}	4/9 ^{ab}	2/9 ^{a}	2/9 ^{a}	2/9 ^{b}	1/9 ^{a}	0/9 ^{a}	0/9
cef/otc	0/9 ^{a}	9/9 ^{a}	7/9 ^{a}	8/9 ^{a}	7/9 ^{a}	5/9 ^{ab}	5/9 ^{a}	4/9 ^{a}	3/9 ^{b}	1/9 ^{a}	1/9 ^{a}	2/9
control	0/9 ^{a}	8/9 ^{a}	6/9 ^{a}	7/9 ^{ab}	7/9 ^{a}	7/9 ^{b}	5/9 ^{a}	4/9 ^{a}	6/9 ^{a}	3/9 ^{a}	2/9 ^{a}	2/9

SEM = 0.33 or 2.64/8

d PI = days post-inoculation; n = 8 pigs/treatment group; numerator = number of pigs shedding *Salmonella typhimurium*; denominator = total number of pigs receiving treatment; cef/otc = ceftiofur/oxytetracyline; apr/otc = apramycin/oxytetracycline; car/otc = carbadox/oxytetracyline; control = no antibiotics; different superscripts indicate differences in the overall incidence of shedding across treatment groups at $P < .05$; comparisons are within days post-inoculation.

Figure 4: Effect of different antibiotic regimens on bacterial shedding in pigs challenged with 10^9 CFU of *Salmonella typhimurium* (Experiment Two)



SEM = 0.32

n = 12 pigs/treatment group ; cef/otc = ceftiofur sodium/oxytetracycline treatment; apr/otc = apramycin/ oxytetracycline treatment; car/otc = carbadox/oxytetracycline treatment; control = no antibiotics; different superscripts indicate differences in the overall incidence of shedding across treatment groups at $P < .05$.

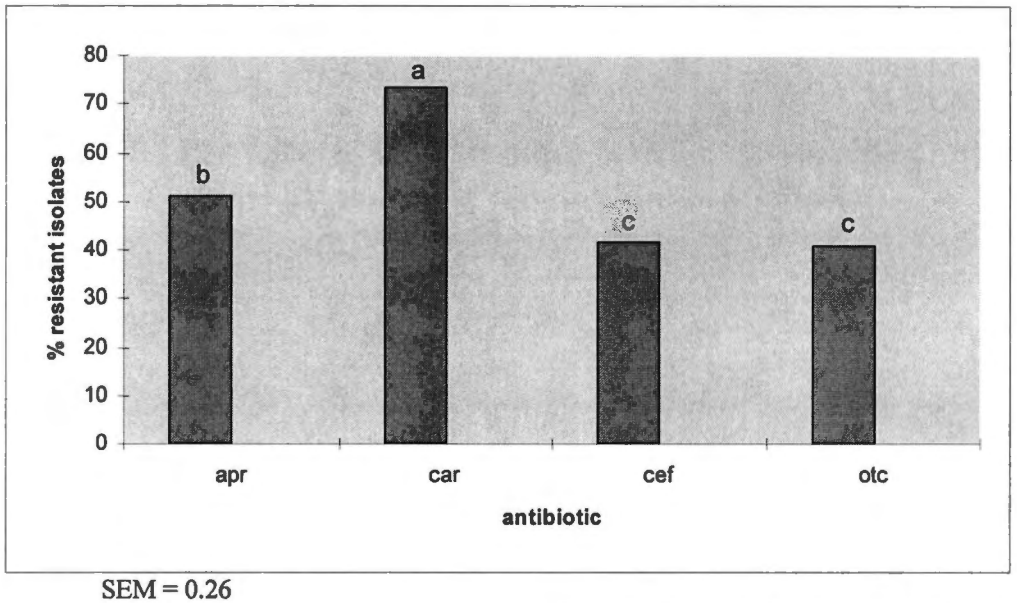
Table 4: Effect of different antibiotic regimens on bacterial shedding in pigs challenged with 10^9 CFU of *Salmonella typhimurium* (Experiment Two).

	Number of Pigs Shedding *Salmonella typhimurium*											
Treatment	0d PI*	2d PI	4d PI	7d PI	14d PI	21d PI	28d PI	35d PI	42d PI	56d PI	70d PI	84d PI
apr/otc	$0/12^{a}$	$9/12^{a}$	$12/12^{a}$	$8/12^{a}$	$8/12^{ab}$	$8/12^{ab}$	$8/12^{ab}$	$6/12^{a}$	$8/12^{a}$	$2/12^{b}$	$1/12^{a}$	0/12
car/otc	$0/12^{a}$	$11/12^{a}$	1212^{a}	$10/12^{a}$	$6/12^{b}$	$6/12^{b}$	$6/12^{b}$	$3/12^{a}$	$2/12^{b}$	$1/12^{b}$	$0/12^{a}$	0/12
cef/otc	$0/12^{a}$	$7/12^{b}$	$11/12^{a}$	$12/12^{a}$	$10/12^{a}$	$10/12^{a}$	$7/12^{ab}$	$6/12^{a}$	$4/12^{b}$	$3/12^{ab}$	$2/12^{a}$	0/12
control	$0/12^{a}$	$11/12^{a}$	$11/12^{a}$	$12/12^{a}$	$12/12^{a}$	$12/12^{a}$	$10/12^{a}$	$6/12^{a}$	$6/12^{ab}$	$6/12^{a}$	$3/12^{a}$	0/12

SEM = 0.32 or 2.56/12

d PI = days post-inoculation; n = 12 pigs/treatment group; numerator = number of pigs shedding *Salmonella typhimurium*; denominator = total number of pigs receiving treatment; cef/otc = ceftiofur/oxytetracyline; apr/otc = apramycin/oxytetracycline; car/otc = carbadox/oxytetracyline; control = no antibiotics; means with different superscripts are significantly different at $P < .05$; comparisons are within days post-inoculation.

Figure 5: The percentage of isolates resistant to specific antibiotics across all sampling days (Antibiotic Effect).



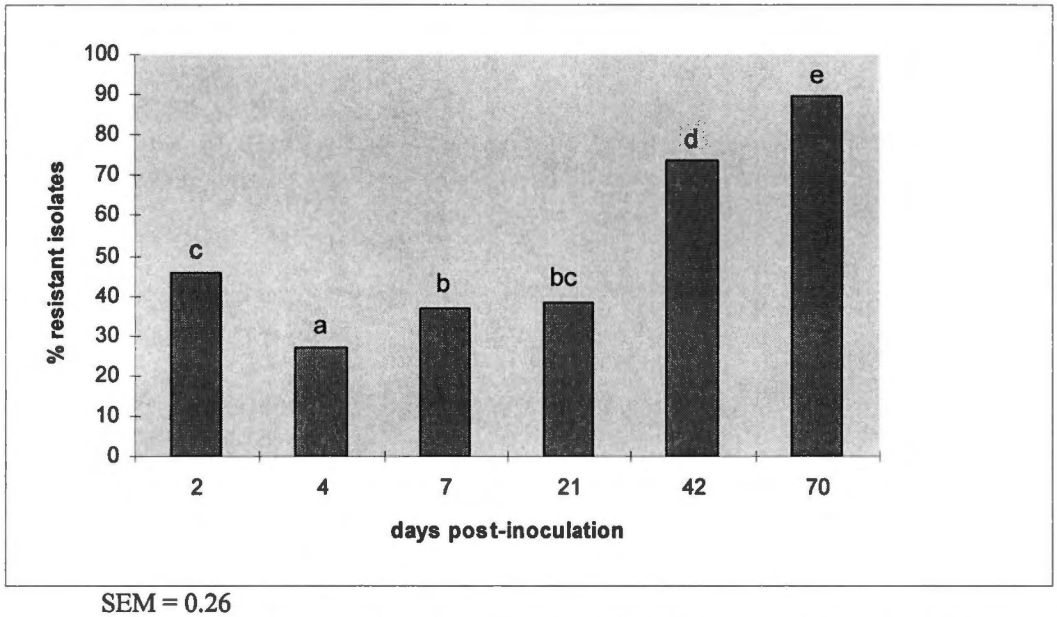
SEM = 0.26
n = 873 isolates; apr = apramycin; car = carbadox; cef = ceftiofur sodium; otc = oxytetracyline; bars with different subscripts are different at $P < .05$.

Table 5: The percentage of isolates resistant to specific antibiotics across all sampling days (Antibiotic Effect).

Antibiotic	% isolates resistant
Apramycin	51.3^{b}
Carbadox	73.6^{a}
Ceftiofur Sodium	41.8^{c}
Oxytetracyline	40.8^{c}

SEM = 0.26
n = 873 *Salmonella typhimurium* isolates; apr = apramycin; car = carbadox; cef = ceftiofur sodium; otc = oxytetracyline; means with different superscripts are significantly different at $P < .05$).

Figure 6: Change in the percentage of resistance over time across all treatments.



SEM = 0.26

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance is measured as the percentage of isolates resistant to at least one antibiotic (apramycin, carbadox, ceftiofur sodium, oxytetracycline) regardless of treatment; bars with different superscripts are significantly different at $P < .05$).

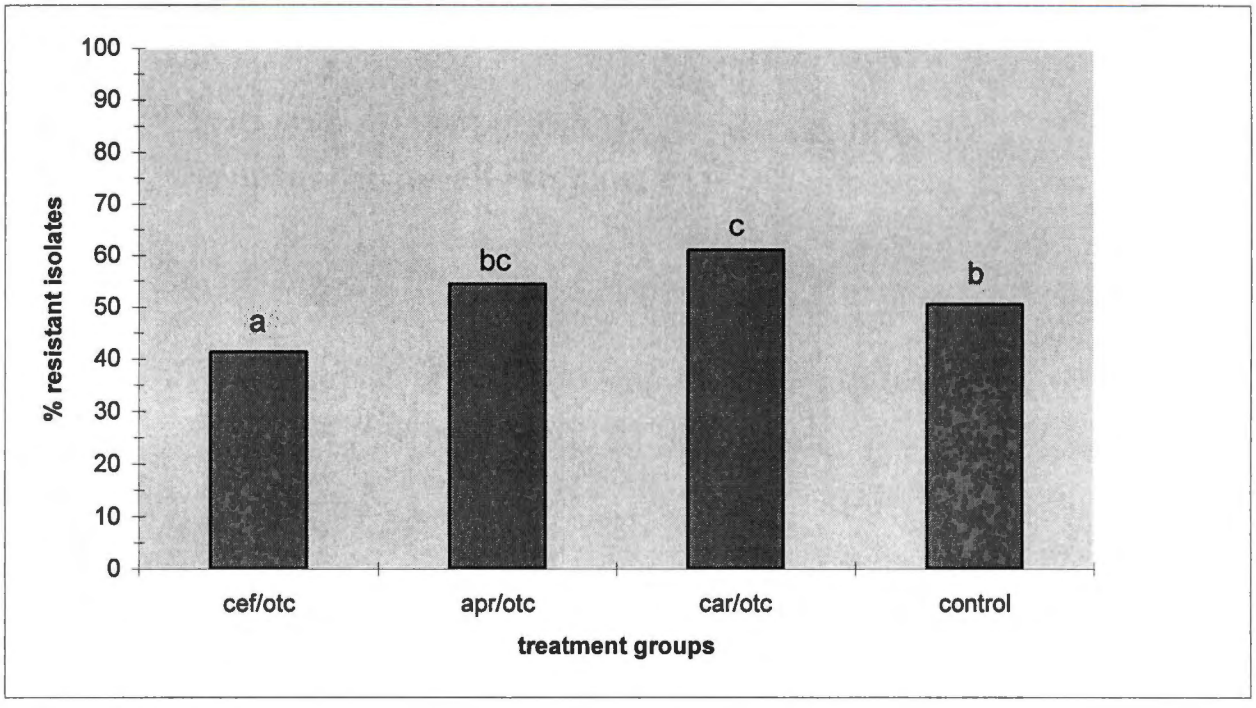
Table 6: Change in the percentage of resistance over time across all treatments.

Days Post-inoculation	% resistant isolates
2	46.0^{c}
4	26.9^{a}
7	36.7^{b}
21	38.5^{bc}
42	73.7^{d}
70	89.4^{e}

SEM = 0.26

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance is measured as the percentage of isolates resistant to at least one antibiotic (apramycin, carbadox, ceftiofur sodium, oxytetracycline) regardless of treatment; means with different subscripts are significantly different at $P < .05$.

Figure 7: The percentage of resistant isolates within different treatments across all sampling days.



SEM = 0.26

n = 873 *Salmonella typhimurium* isolates; cef/otc = ceftiofur sodium/oxytetracycline; apr/otc = apramycin/oxytetracycline; car/otc = carbadox/oxytetracycline; control = no antibiotics; resistance refers to the percentage of isolates resistant to at least one antibiotic; bars with different superscripts are significantly different at $P < .05$.

Table 7: The percentage of resistant isolates within different treatments across all sampling days.

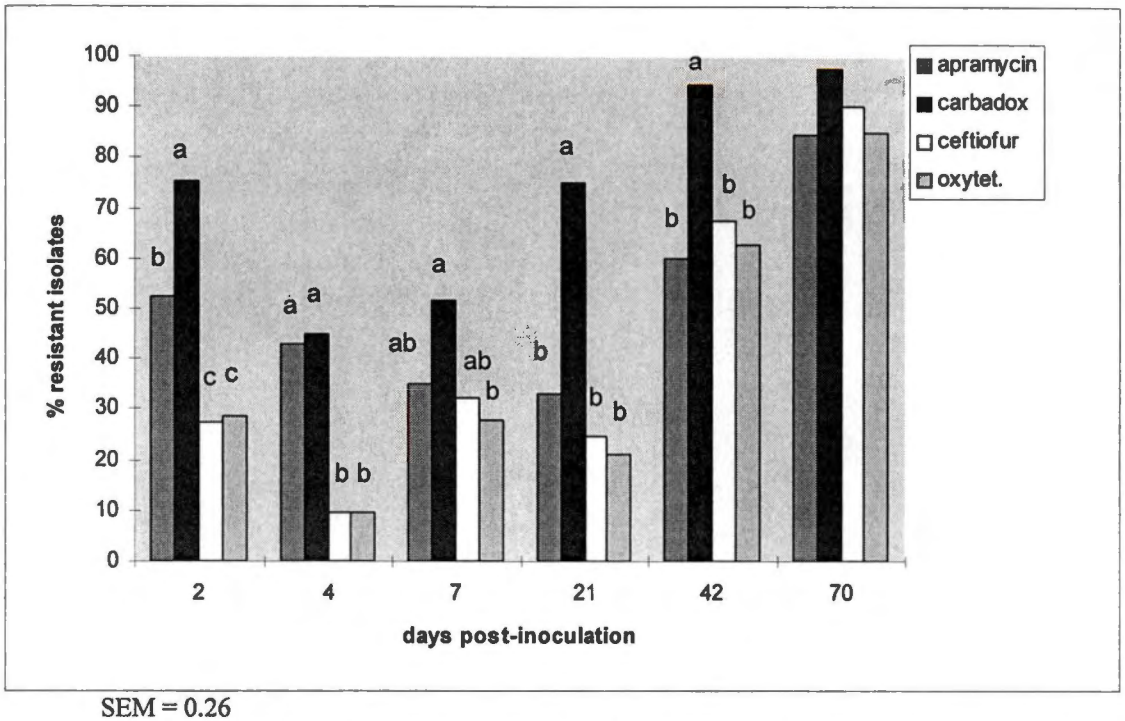
Treatment	% Resistant Isolates
cef/otc	41.3^{a}
apr/otc	54.4^{bc}
car/otc	61.1^{c}
control	51.6^{b}

SEM = 0.26

n = 873 *Salmonella typhimurium* isolates; cef/otc = ceftiofur sodium/oxytetracycline; apr/otc = apramycin/oxytetracycline; car/otc = carbadox/oxytetracycline; control = no antibiotics; resistance refers to the percentage of isolates resistant to at least one antibiotic; means with different superscripts are significantly different at $P < .05$.

Figure 8: Change in the percentage of resistance to specific antibiotics

over time.



SEM = 0.26

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance is measured as the percentage of isolates resistant to the specific antibiotics regardless of treatment; refer to table 8 for letter comparisons; car = carbadox; apr = apramycin; cef = ceftiofur sodium; otc = oxytetracycline; bars with different superscripts are significantly different at $P < .05$; comparisons are within days post-inoculation.

Table 8: Changes in the percentage of resistance to specific antibiotics over time.

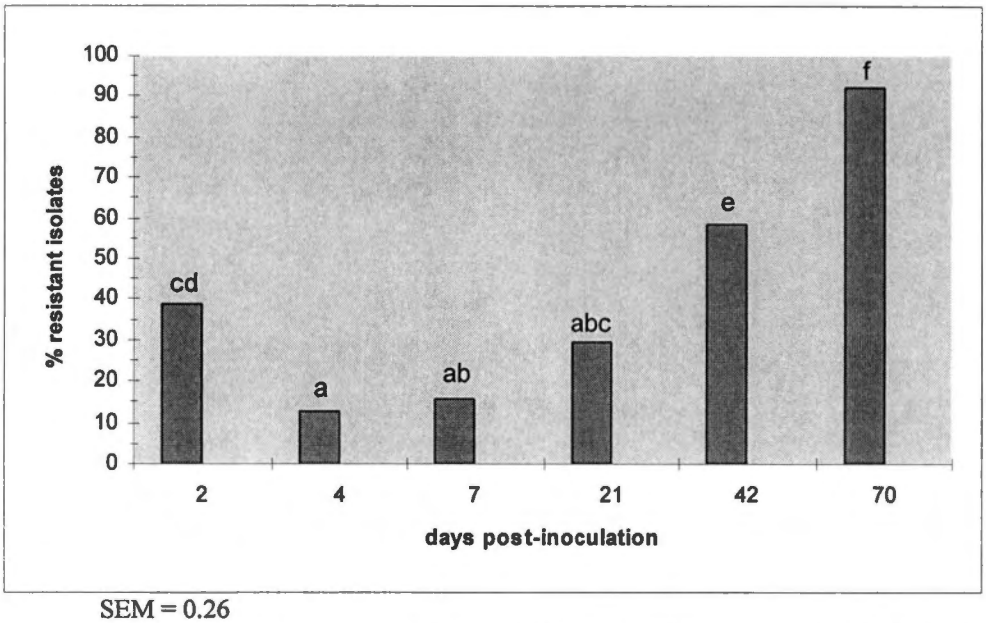
% o Resistant Isolates						
Antibiotic	**2dPI**	**4dPI**	**7dPI**	**21dPI**	**42dPI**	**70dPI**
Apramycin	52.3^{b}	42.8^{a}	35.0^{ab}	32.8^{b}	60.0^{b}	84.5^{a}
Carbadox	75.2^{a}	45.0^{a}	51.7a	75.0^{a}	94.5^{a}	97.8^{a}
Ceftiofur	27.3^{c}	9.5^{b}	32.3^{ab}	24.5^{b}	67.3^{b}	90.0^{a}
Oxytet.	28.4c	9.5^{b}	27.8^{b}	21.2^{b}	02.8^{b}	85.0^{a}

SEM = 0.26

141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); letter comparisons are within days post-inoculation; means with different superscripts are significantly different at $P < .05$; comparisons are within days post-inoculation.

Figure 9: Changes over time in the percentage of resistant *Salmonella*

***typhimurium* isolated from pigs receiving the ceftiofur sodium/oxytetracycline treatment.**



SEM = 0.26

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance refers to the percentage of *Salmonella typhimurium* isolated from pigs receiving ceftiofur sodium/oxytetracycline showing resistance to at least one antibiotic; bars with different superscripts are significantly different at $P < .05$.

Table 9: Changes over time in the percentage of resistant *Salmonella typhimurium* isolated from pigs receiving the ceftiofur sodium/oxytetracycline treatment.

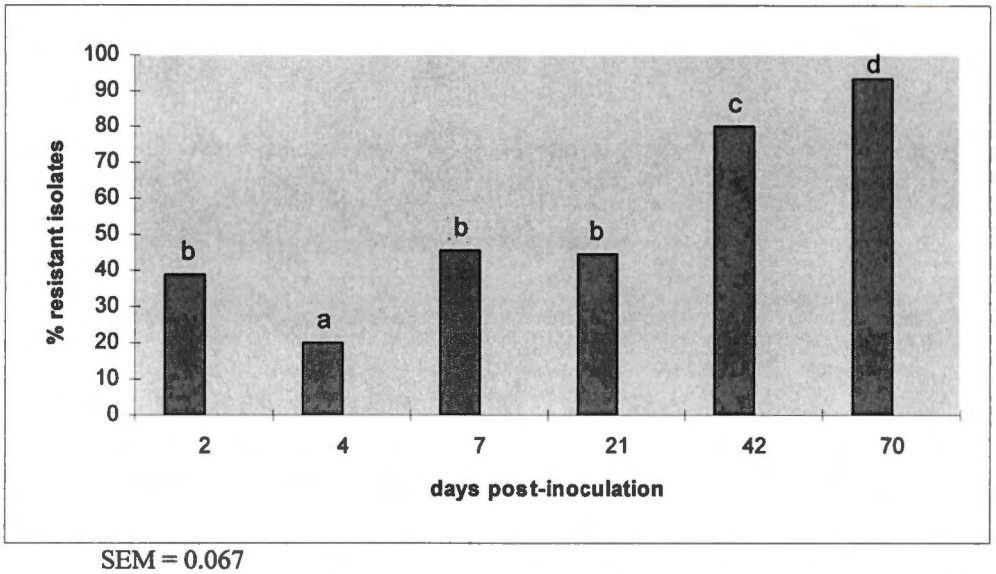
Days Post-inoculation	% resistant isolates
2	38.9^{cd}
4	12.8^{a}
7	15.6^{ab}
21	29.5^{abc}
42	58.4^{e}
70	92.3^{f}

SEM = 0.26

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance refers to the percentage of *Salmonella typhimurium* isolated from pigs receiving ceftiofur sodium/oxytetracycline showing resistance to at least one antibiotic; means with different superscripts are significantly different at $P < .05$.

Figure 10: Changes over time in the percentage of resistant *Salmonella*

***typhimurium* isolated from pigs receiving the apramycin/ oxytetracycline treatment.**



SEM = 0.067

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance refers to the percentage of *Salmonella typhimurium* isolated from pigs receiving apramycin/oxytetracycline showing resistance to at least one antibiotic; bars with different superscripts are significantly at $P < .05$.

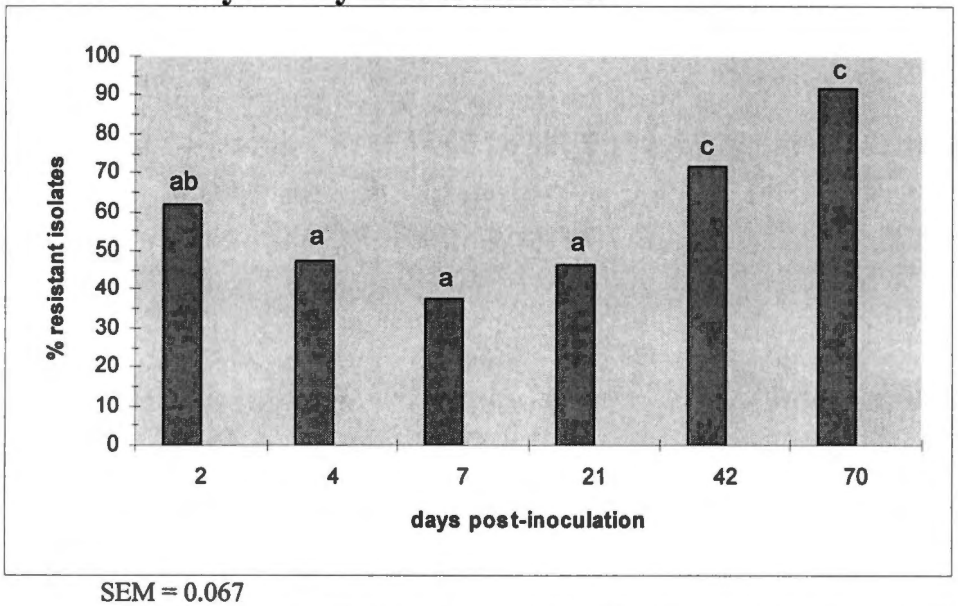
Table 10: Changes over time in the percentage of resistant *Salmonella typhimurium* isolated from pigs receiving the apramycin/ oxytetracycline treatment.

Days Post-inoculation	% Resistant Isolates
2	38.9^{b}
4	20.0^{a}
7	45.6^{b}
21	44.5^{b}
42	80.0^{c}
70	93.3^{d}

SEM = 0.067

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance refers to the percentage of *Salmonella typhimurium* isolated from pigs receiving apramycin/oxytetracycline showing resistance to at least one antibiotic; means with different superscripts are significantly different at $P < .05$

Figure 11: Changes over time in the percentage of resistant *Salmonella typhimurium* isolated from pigs receiving the carbadox/ oxytetracycline treatment.



SEM = 0.067

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance refers to the percentage of *Salmonella typhimurium* isolated from pigs receiving carbadox/oxytetracycline showing resistance to at least one antibiotic; bars with different superscripts are significantly different at $P < .05$.

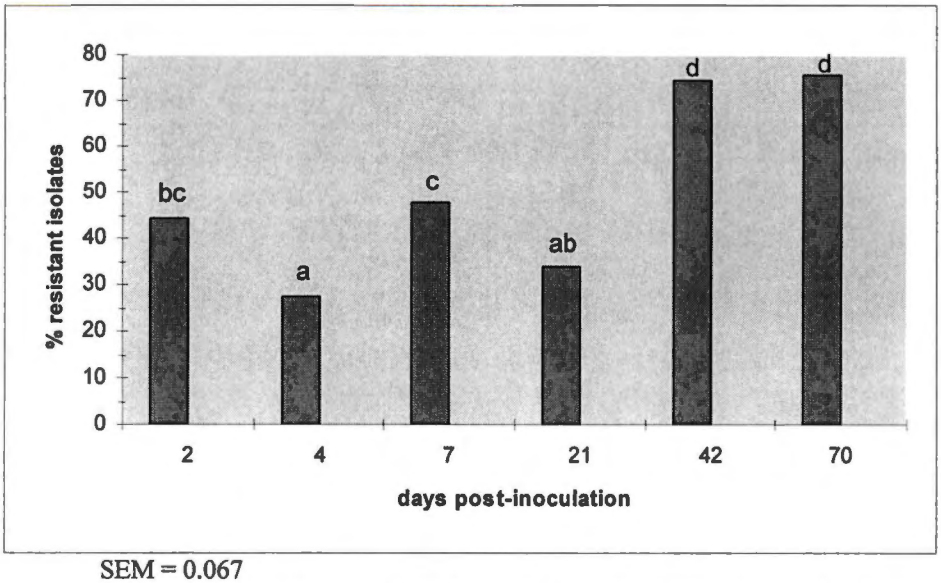
Table 11: Changes over time in the percentage of resistant *Salmonella typhimurium* isolated from pigs receiving the carbadox/ oxytetracycline treatment.

Days Post-inoculation	% Resistant Isolates
2	61.7^{ab}
4	47.3^{a}
7	37.8^{a}
21	46.2^{a}
42	71.7^{c}
70	91.7^{c}

SEM = 0.067

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance refers to the percentage of *Salmonella typhimurium* isolated from pigs receiving carbadox/oxytetracycline showing resistance to at least one antibiotic; means with different superscripts are significantly different at $P < .05$.

Figure 12: Changes over time in the percentage of resistant *Salmonella typhimurium* isolated from pigs not receiving antibiotics (control).



SEM = 0.067

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance refers to the percentage of *Salmonella typhimurium* isolated from pigs in the control showing resistance to at least one antibiotic; bars with different superscripts are significantly different at $P < .05$.

Table 12: Changes over time in the percentage of resistant *Salmonella typhimurium* isolated from pigs not receiving antibiotics (control).

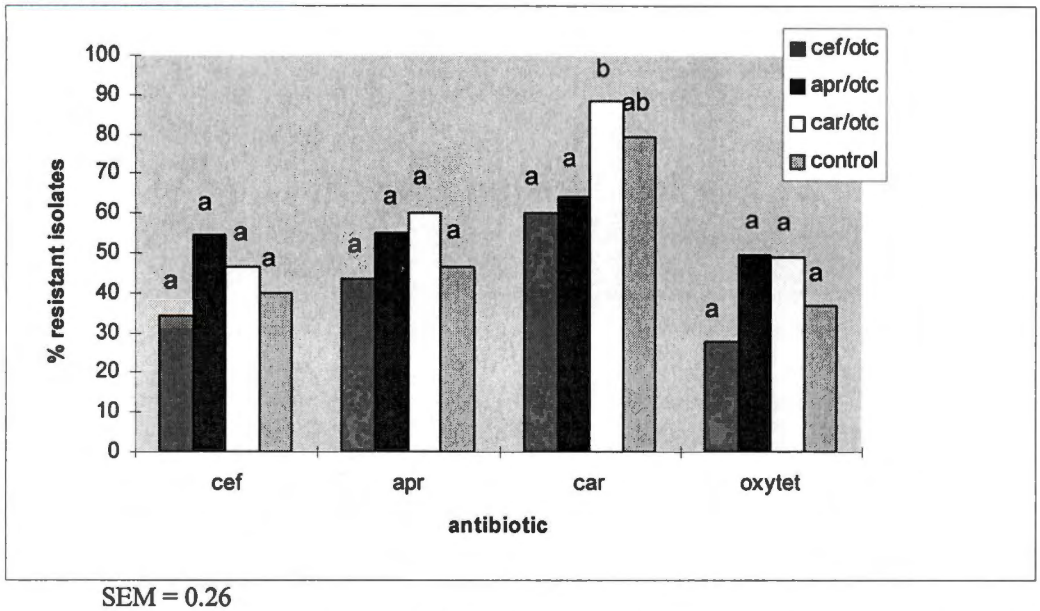
Days Post-inoculation	% Resistant Isolates
2	44.5^{bc}
4	27.3^{a}
7	47.8^{c}
21	33.9^{ab}
42	74.5^{d}
70	75.6^{d}

SEM = 0.067

$P < .05$

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); resistance refers to the percentage of *Salmonella typhimurium* isolated from pigs in the control showing resistance to at least one antibiotic; means with different superscripts are significantly different at $P < .05$.

Figure 13: Percentage of *Salmonella typhimurium* resistant to specific antibiotic across different antibiotic regimens.



SEM = 0.26

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); cef/otc = ceftiofur sodium/oxytetracycline treatment; apr/otc =apramycin/oxytetracycline treatment; car/otc = carbadox/oxytetracycline treatment; control = no antibiotics; letter differences refer to differences within antibiotic; bars with different superscripts are significantly different at P < .05; comparisons are within antibiotic.

Table 13: Percentage of *Salmonella typhimurium* resistant to specific antibiotic across different antibiotic regimens.

	% Resistant Isolates			
Treatment	**ceftiofur sodium**	**apramycin**	**carbadox**	**oxytetracyline**
cef/otc	34.1[a]	43.4[a]	60.0[a]	27.8[a]
apr/otc	54.7[a]	54.9[a]	64.3[a]	49.7[a]
car/otc	46.7[a]	60.0[a]	88.6[b]	48.9[a]
control	39.7[a]	46.7[a]	79.3[ab]	36.7[a]

SEM = 0.26

n = 141 isolates (2d PI), 164 isolates (4d PI), 218 isolates (7d PI); 163 isolate (21d PI), 107 isolates (42d PI), 80 isolates (70d PI); cef/otc = ceftiofur sodium/oxytetracycline treatment; apr/otc =apramycin/oxytetracycline treatment; car/otc = carbadox/oxytetracycline treatment; control = no antibiotics; letter differences refer to differences within antibiotic; means with different superscripts are significantly different at P < .05.; comparisons are within antibiotic.

Vita

Paul Ebner was born and raised in Livonia, Michigan. He attended Livonia Stevenson High School, Waseda University (Tokyo, Japan) and graduated from Kalamazoo College in 1993 with a Bachelor of Arts degree in Political Science. Paul spent one year working at Civic Searchlight, a voter information service in Detroit, Michigan and the following two years as a volunteer in the United States Peace Corps in Paraguay. He began his studies in Animal Science at the University of Tennessee in 1996. In 1999 Paul married Paige Cooper and is currently pursuing a Ph.D in animal science.