Antibiotic resistance of Salmonella in swine

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Antibiotic resistance of *Salmonella* in swine

by

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### Affiliations
GENERAL INTRODUCTION

Introduction

There are over 2,200 serotypes of *Salmonella*, many that have the ability to infect both animals and humans. *Salmonella* is one of the most common foodborne pathogens and is responsible for acute gastroenteritis in humans. Because of the increased demand for fresh meat and dairy products, international travel, and economic incentive for higher production, an increase in foodborne disease is occurring. Additionally, new pathogens are emerging while other pathogens are reemerging. To exacerbate the problem of newly emerging, reemerging and problematic pathogens that continue to infect animals and humans, many species of bacteria are developing resistance mechanisms to evade possible treatment or prevention regimes. The development of resistance is especially problematic in human medicine if over-use of antibiotics remains unchecked.

Little research has been done to ascertain the effect(s) antibiotics have on the pathogenesis of disease *in vivo*. Most studies have been conducted *in vitro* to determine how bacteria respond to antimicrobics (i.e. growth or no growth). However, results observed for *in vivo* studies can be very different from results observed for *in vitro* studies.

The objectives of this research were twofold. First, an experiment was conducted to determine the effect of dose on persistence and shedding of *Salmonella heidelberg* in experimentally inoculated swine. A second study was designed to determine the effect of two veterinary antimicrobics on swine experimentally challenged with *Salmonella heidelberg*. 
Thesis organization

The thesis contains a general introduction, a literature review of salmonellosis with an emphasis on antimicrobial resistance, two manuscripts submitted for publication, a general summary, an appendix and acknowledgements. The master of science candidate, Heidi Lea Holcomb, is the senior author and principal investigator for the manuscripts.
CHAPTER 1. SALMONELLOSIS IN SWINE: A REVIEW OF SIGNIFICANT AREAS AFFECTING SALMONELLA AND ANTIBIOTIC RESISTANCE

*Salmonella*

**General information**

*Salmonella* was first identified over a century ago by an American veterinarian and bacteriologist, Daniel E. Salmon (Guthrie 1992). Salmonellae are Gram-negative rod-shaped facultative anaerobes in the family Enterobacteriaceae, which includes other genera such as *Escherichia, Citrobacter, Proteus, Yersinia, Klebsiella, Shigella, Providencia, Serratia, Hafnia,* and *Erwinia* (Ewing 1986). Most salmonellae are motile by peritrichous flagella and do not form spores (LeMinor 1984).

Because salmonellae are primarily intestinal pathogens, they penetrate the intestinal mucosa prior to dissemination throughout the host (Lax et al. 1995). An important feature of *Salmonella* is that it is a facultative intracellular pathogen, capable of growing in epithelial cells and phagocytic vacuoles of macrophages (Clarke and Gyles 1986). The bacterium's mode of survival and growth are important because, once inside the cell, *Salmonella* appears to be more resistant to antibiotics (Collins and Campbell 1982) and resists killing by the host's humoral and cellular immune systems.

*Salmonella*, like other enterobacteria, produce a variety of virulence factors which facilitate colonization in the host. Major virulence factors include cytotoxins, lipopolysaccharides, flagella, fimbriae and enterotoxins (Murray 1986). Fimbria and flagella
are also virulence factors responsible for attachment and mobility. The other components mediate specific events in the host. Together, these factors lead to the production of diarrhea and systemic inflammation which can severely damage tissues (Murray 1986).

Classification

Salmonellae are typically classified by the Kaufmann-White scheme which utilizes antigens present on the cell surface for serological identification (LeMinor 1984). *Salmonella typhi* strains, which are infectious to humans only, contain O and H antigens, in addition to a capsular virulence antigen, Vi. All three classes of antigens are used in primary differentiation of various *Salmonella* serotypes.

O (somatic) antigens are a part of the lipopolysaccharide layer of the outer cell wall (Guthrie 1992). The arrangement of O antigens determine whether salmonellae have a rough or smooth colony morphology when grown on agar plates. There are approximately 67 O antigens, using letters and numbers to distinguish different "species", or serotypes, of *Salmonella*. There are 8 predominant O antigens in pathogenic types of *Salmonella* (Guthrie 1992).

H (flagellar) antigens are components of bacterial flagella (LeMinor 1984). These are minor virulence factors for most *Salmonella* serotypes. Vi (capsular) antigens comprise capsule and envelope surrounding a bacterium (Guthrie 1992). These antigens are important virulence factors in *Salmonella typhi* infections.

Most investigators in the field refer to serotype as the designation of species for reporting purposes, even though there is technically only one *Salmonella* species (LeMinor
Approximately 2,200 serotypes of *Salmonella* have been identified using the Kaufmann-White scheme (LeMinor 1984). From humans or animals, however, less than 50 serotypes are frequently associated with disease (Clarke and Gyles 1986).

**Bacteriology**

*Growth and metabolism*

The simplest way to identify *Salmonella* is based on the bacteria's metabolic processes (LeMinor 1984). Salmonellae can grow aerobically or as facultative anaerobes at 8-45°C. An incubation temperature of 37°C is recommended (Ewing 1986). Aerobic conditions are thought to increase growth rate (Guthrie 1992). Most salmonellae can be killed at temperatures >65°C and are resistant to drying, making survival in dust possible (Guthrie 1992).

A majority of enterobacteria utilize citrate as the sole source of carbon (Guthrie 1992). Salmonellae produce acid and gas during fermentation of glucose and mannose. Most, however, do not ferment lactose or sucrose. During fermentation of glucose, hydrogen sulfide is usually produced (LeMinor 1984). While most salmonellae produce hydrogen sulfide, exceptions are noted (Poppe et al. 1995).

*Culture methods*

Use of enrichment media facilitates the recovery of salmonellae from a sample. A variety of media can be utilized. Pre-enrichment broths may be used initially to help bacteria recover from the stress of movement into a new environment and losses due to cell death (Guthrie 1992). Examples include peptone water or tryptone broth. Most often, selective
enrichment media is used to stimulate growth of *Salmonella* and inhibit growth of unwanted bacteria. Tetrathionate, selenite and Gram-negative (GN) broths and Rappaport-Vassialidis medium all support growth of *Salmonella* and inhibit growth of other bacteria (Ewing 1986, Vassiliadis 1983).

Plating onto solid media makes it easier to identify only salmonellae and is subsequent to culture in liquid media. Bismuth Sulfite agar has been reported to be the best at inhibiting most other enterobacteria (Guthrie 1992). Other agars include Brilliant Green, *Salmonella-Shigella*, and xylose-lysine-tergitol 4 (XLT4; a hydrogen sulfide indicator; Dusch and Altwegg 1995). Less selective agars are MacConkey, hektoen enteric, desoxycholate, and eosin-methylene blue (Guthrie 1992).

**Identification**

After selection of presumptive positive colonies from agar plates, suspect colonies are inoculated into various media to determine biotype. These include triple sugar iron agar, lysine iron agar, urease, and indole. Further identification utilizes somatic and flagellar antigens as mentioned previously (LeMinor 1984). Additional techniques for classification and analysis of significant *Salmonella* serotypes include phage typing, antibiotic susceptibility patterns and plasmid fingerprinting (Vatopoulos et al. 1994).

**Epidemiology**

While typhoid fever (a disease associated with *Salmonella typhi*) has decreased dramatically in the past 50 years, all other salmonelloses have increased (Cohen and Tauxe 1986). Salmonellosis now ranks as the fourth most infectious disease in the United States
(Anonymous 1996b). The predominant serotypes recovered from humans are *S. enteriditis*, *S. typhimurium*, *S. newport* and *S. heidelberg* (Anonymous 1996a). Order of recovery may vary slightly between years.

Host adapted strains include *S. typhi* in humans, *S. choleraesuis* in swine, *S. dublin* in cattle and *S. pullorum* in poultry (Schwartz 1990). These strains are host specific and rarely infect other hosts. However, following infection in humans with *S. choleraesuis*, severe disease and death may result (Weissbluth et al. 1981). Non-host adapted strains include *S. typhimurium*, *S. enteriditis*, and *S. heidelberg*, which comprise most *Salmonella* serotypes found today in both humans and animals (Ferris and Miller 1996).

*Salmonellae* are ubiquitous in the environment and have been recovered from nearly all vertebrates (Taylor and McCoy 1969). Outbreaks occur most frequently during the summer months and may be observed in late fall (Hook 1990). Human infections are usually foodborne and frequently cause gastroenteritis. Those most affected by salmonellosis are usually less than 5 years of age (Hook 1990), elderly (Levine et al. 1991), immunocompromised (Levine et al. 1991) or hospitalized (Baine et al. 1973). Economic losses due to salmonellosis in the United States have been estimated to be $1.1 billion (Tauxe 1991) or as high as $4 billion for 3 million human cases (Todd 1989).

**Pathogenesis in swine**

Salmonellosis is a major bacterial disease of swine, second to swine dysentery (Schwartz 1990). Salmonellosis in swine results in tremendous economic losses in the pork industry (Roof et al. 1992). Estimates range from $28 million per year in Iowa to $100
million in the United States (Schwartz 1990).

The predominant serotypes isolated from swine are *S. derby*, *S. choleraesuis*, and *S. typhimurium-copenhagen* (Ferris and Miller 1996). In Iowa, *S. choleraesuis* infections pose the biggest problem to producers, comprising >95% of the porcine salmonellosis cases (Schwartz 1990). Infection following exposure to *S. choleraesuis* is characterized as a septicemia with destruction of the pulmonary tissues. Its primary mode of transmission, like most *Salmonella*, is fecal-oral (Heard and Linton 1966) or respiratory (Wathes et al. 1988, Tannock and Smith 1971, Hardman et al. 1991). *Salmonella typhimurium*, on the other hand, is a gastroenteric pathogen causing severe diarrhea and wasting in swine. Clinical signs associated with salmonellosis in swine are fever, diarrhea, lethargy and dyspnea (Schwartz 1990).

Five main disease states have been associated with *Salmonella* infections: enterocolitis, as observed following infection with *S. typhimurium*, where salmonellae are detected in feces but not blood; bacteremia, as observed following infection with *S. choleraesuis*, isolating organisms from the blood but not the feces; localized infections following infection from a variety of salmonellae; development of a carrier state in many animals; and enteric fever (in humans only) as observed following infection with *Salmonella typhi* (Hook 1990).

The carrier state has been shown to be important in the survival and persistence of *Salmonella* in the environment (Gray et al. 1995). Following infection with *S. choleraesuis*, immune suppression has been observed in carrier pigs, compromising the host (Gray et al. 1996). Penmates can also be compromised. Gray et al. (1995) showed that naive pigs
exposed to pigs shedding 2.6 logs/gram feces will become infected and show clinical signs of disease within one day after commingling. *Salmonella* was recovered from the naive pigs up to 9 weeks after exposure. Bacteria can proliferate in the gastrointestinal tract of carrier pigs after they have been stressed (Morgan et al. 1987, Williams and Newell 1970). Carrier animals are thought to be the main source of *Salmonella* infections in other animals or humans (Wray and Sojka 1977).

Pathological observations indicate the development of lesions, enlarged lymph nodes, and hemorrhagic and inflamed intestinal epithelium (Schwartz 1990). Inflammation leads to malabsorption and fluid loss (Clarke and Gyles 1986). With *S. choleraesuis* infections, lungs can be consolidated, hemorrhagic and edematous (Schwartz 1990). A characteristic lesion is the paratyphoid nodule on the liver (Wilcock and Schwartz 1992). Severe tissue damage and necrosis, particularly in the intestine (Clarke and Gyles 1986), can result from toxins produced by *Salmonella*, all of which influence the host's immune response.

**Antibiotic Therapy**

**Background**

Antibiotics have been used to treat bacterial infections in animals and humans for decades since the inception of penicillin (Martel and Coudert 1993). Their use has expanded to include prevention of disease and growth promotion in animals (Wray et al. 1993), which results in a major economic advantage for producers. Since their inception, antibiotics have been extremely effective. Scientific research is continually expanding to produce new antibiotics to combat present and emerging bacterial pathogens.
Classes

There are many classes of antibiotics that are utilized in human and animal medicine. Some are efficacious in both practices, differing in the concentration or route of inoculation. This section will focus on those antimicrobics that apply to the field of veterinary medicine.

Most antibiotics can be classified by their general action against bacteria. Penicillins, aminoglycosides, cephalosporins and cephamycins, quinolones and miscellaneous antimicrobial agents like nitrofurans are known to be bactericidal in nature (Anonymous 1991). They are capable of destroying bacteria in a host. Penicillins, cephalosporins and cephamycins (β-lactam antibiotics) act by preventing bacterial cell wall synthesis by inhibiting transpeptidase that forms cross-bridges in Gram-positive and Gram-negative organisms. They are most effective during bacterial log phase of growth, before the cell wall is fully synthesized.

Aminoglycosides are broad spectrum antibiotics that bind to membrane associated ribosomes. They inhibit protein synthesis and eventually disrupt the cell wall (Guthrie 1992). Various aminoglycosides can be very effective when used in conjunction with β-lactams. Examples include streptomycin, which is effective against Gram-negative bacteria, and kanamycin and neomycin, which are effective against both Gram-negative and Gram-positive bacteria. Aminoglycosides are not currently approved for use in food-producing animals.

Quinolones are a relatively new class of antimicrobial. They are synthetic, broad-spectrum antibiotics which have been efficacious in human medicine and have expanded to be used in animal medicine in the last 10 years. Their primary mode of action is inhibition of DNA gyrase, or topoisomerase II, which leads to improper supercoiling and cell lysis.
Quinolones are well accepted for treatment of a variety of bacterial infections. Naladixic acid has predominantly been used in humans for treatment of urinary tract infections. Quinolones are noted for their ability to inhibit intracellular pathogens, such as *Salmonella* (Guay 1992). They are favored because of their route of administration, wide tissue distribution and relative safety.

Nitrofurans are another broad spectrum, synthetic group of antibiotics, primarily used in animal medicine, especially swine health. They inhibit an enzyme required for the initiation of translation (Anonymous 1991).

Tetracyclines, chloramphenicol and congeners, macrolides, lincosamides and miscellaneous antimicrobics such as bacitracin, vancomycin, novobiocin, tiamulin, sulfonamides and sulfonamide combinations are all classified as being bacteriostatic. Tetracyclines bind to ribosomes and thus inhibit protein synthesis. They are used widely to treat disease in humans and animals. In addition to treatment, they are used as feed additives to enhance growth in food producing animals (DuPont and Steele 1987).

Chloramphenicol is a broad spectrum antimicrobial used in treating local and systemic infections in animals and humans. However, because of the possibility of sporadic hypersensitivity reactions, use is limited to non-food producing animals. It is very efficacious against Gram-positive and Gram-negative bacteria implicated in meningoencephalitis, otitis, abscesses or respiratory infections (Anonymous 1991).

Another group of antimicrobics that interferes with protein synthesis is the macrolide class of antibiotics, which include tylosin and erythromycin. They are widely distributed in tissues after administration and control pneumonia, enteritis, metritis and urinary tract

Gram-positive cocci are inhibited by lincosamides which act by altering ribosomal subunits (Guthrie 1992). Most other Gram-positive bacteria are inhibited by bacitracin and vancomycin which act against the cell wall. Novobiocin, frequently used to treat bovine mastitis, and tiamulin are also effective against Gram-positive bacteria found in veterinary medicine (Anonymous 1991).

Sulfonamides are widely used antimicrobials, especially in feed (DuPont and Steele 1987). They have a broad spectrum of activity and are relatively inexpensive. Urinary tract and intestinal infections are controlled by the use of sulfonamides. Because of their similarity to para-amino benzoic acid (PABA), they are able to inhibit a step in the synthesis of folic acid, leading to suppression of nucleic acid synthesis. Sulfonamides are more effective when combined with trimethoprim, which inhibits dihydrofolate reductase. Used alone both antibiotics are bacteriostatic but can be bactericidal when combined (Bushby 1980).

It has been observed that antibiotics can have an effect on the immune system as well as the bacteria causing the infection. Some antibiotics actually suppress the immune system during in vitro testing and include tetracycline, ampicillin and erythromycin (Van Vlem et al. 1996). More testing in vivo is recommended to augment the limited information which is available regarding immune response to antibiotic treatment in both animals and humans (Van Vlem et al. 1996).
Problems with antibiotic use

Antimicrobics are very effective in reducing numbers of bacteria in a host. They must, however, be used properly to ensure efficacy. If not used correctly the effects can be detrimental. Complications such as reinfection or long term carriage, drug induced toxicity, or a development of resistance by the pathogen often occur following improper use, (Hook 1990). Problems arise when the antibiotic is not used according to specified instructions, which includes duration of treatment, route of administration and concentration of antibiotic.

Most research has focused on treatment of human clinical illnesses with antibiotics. Few studies are available on effectiveness of antibiotics following animal bacterial infections. There is a general agreement among researchers that more work needs to be done in the area of treatment of veterinary pathogens (Hook 1990). Much of the research on antibiotic therapy has been done in vitro. Conclusions from in vitro studies are not thought to be indicative of in vivo situations (Stevens et al. 1995).

It is possible to observe no change in clinical signs of infection after a presumably effective antibiotic has been administered. Evangelisti et al. (1975) saw no response to treatment with oxytetracycline in swine inoculated with S. typhimurium. Jacks et al. (1988) observed similar results following use of efrotomycin in swine experimentally infected with S. typhimurium. In both studies, no differences were observed between treated and untreated groups.

A study involving pigs from a Salmonella-infected farm indicated no decrease in numbers of positive pigs following treatment with enrofloxacin (Dahl et al. 1996). Willson and Osborne (1985) observed no treatment effect following use of oxytetracycline, long-
acting oxytetracycline, or spiromycin (in feed) in swine chronically infected with *Actinobacillus pleuropneumonia*. Penicillin, ampicillin or ceftiofur sodium all failed to control a *Streptococcus suis* infection (Amass et al. 1996).

Failure to reduce environmental levels of the bacteria after treatment can have a detrimental effect on animals left in contact with shedding animals. Hunnemann et al. (1994) demonstrated transmission of a non-responsive *A. pleuropneumonia* (AP) to naive swine. Oxytetracycline in the feed was not able to control AP infection, nor prevent newly introduced pigs from acquiring the bacteria. This contributes to a cumulative decline in the health of animals.

Carrier animals may also be fostered during antibiotic treatment (Clarke and Gyles 1986). If the bacterium is not eliminated, it may now survive in spite of the use of an antimicrobial and persist as a latent infection within the host. Treatment can also at times cause persistent shedding of the bacteria (Hook 1990). Aserkoff and Bennett (1969) have found that some antibiotics actually prolong excretion of bacteria, especially *Salmonella*, in humans.

Another result from antibiotic use may be the induction of a more severe disease state for which the antibiotic was given. This usually arises because some antibiotics also destroy microbial intestinal flora in addition to the intended bacterial pathogen (Tauxe 1991). Destruction of natural flora can give rise to more serious infections. Reports indicate that antibiotics promote *Salmonella* infections, primarily in humans (Holmberg et al. 1984, Spika et al. 1987). Amass et al. (1996) found that after treatment failed to clear a *Streptococcus suis* infection, more severe clinical signs were evident following use of high doses of
penicillin. Additionally, resistant bacteria were observed after treatment.

The development of resistance has become a prominent issue in the application of antimicrobics (Cohen 1992, Perez-Trallero and Zigorraga 1995). Bacteria may be able to survive the selective pressure that accompanies antibiotic usage. Those that survive can become resistant to that antibiotic. Evangelisti et al. (1975) observed resistant *S. typhimurium* in chickens after treatment with oxytetracycline in feed. Smith and Tucker (1978) did not observe any decrease in infection after treatment with neomycin in chickens infected with *Salmonella*. Additionally, they noted the development of neomycin resistant *E. coli* following treatment. Ling et al. (1992) also observed the emergence of resistant bacteria after treating a previously sensitive strain of *S. typhi* with cotrimoxazole.

Antibiotic resistant organisms have been observed after use of medicated feed, which is intended for promotion of growth (DuPont and Steele 1987). Resistance develops due to the long-term use of medicated feed containing low concentrations of antibiotics (Cherubin 1984).

**Mechanisms of resistance**

Antimicrobial resistance was first observed in 1940, with coli-typhoid bacteria that produced penicillinase and were resistant to penicillin (Abraham and Chain). Throughout the era of antibiotic therapy, bacteria have become successful at surviving the destructive effects of antimicrobics. A review of the literature suggests a rapid rise in the development of resistance since the 1960's. Antibiotics are no longer considered a cure-all. Bacteria have developed ways to survive the onslaught of antimicrobial use and are developing resistance to
one or more antibiotics.

Resistance to any of the previously mentioned antibiotics can develop by altering target sites, membrane permeability or enzyme activity (Silva 1996). Enzymatic inhibition is the most common mode of resistance. Resistance that occurs following use of penicillins, aminoglycosides, cephalosporins, and chloramphenicols is based on the increase in production of an inactivating enzyme, β-lactamase, which cleaves β-lactam rings present in all of these antibiotics (Anonymous 1991). *Staphylococci* are able to develop resistance relatively quickly using this mode. Other bacteria capable of producing β-lactamase are *E.coli*, *Haemophilus*, *Klebsiella*, *Pasteurella*, *Proteus*, *Pseudomonas* and *Salmonella* spp.

Enterobacteria can also undergo chemical modifications to prevent aminoglycosides from acting on ribosomal subunits (Silva 1996). Additionally, they can produce an enzyme, chloramphenicol acetyl transferase, to inhibit chloramphenicol. Some bacteria, namely *Shigella* and *Staphylococcus aureus*, abundantly produce dihydrofolate reductase to inhibit actions of trimethoprim.

Certain microorganisms are capable of developing resistance by altering penicillin binding proteins to negate the effects penicillins and cephalosporins have on peptidoglycan. Macrolide, lincosamide and aminoglycoside resistance may develop as a result of methylation of a ribosomal subunit (Silva 1996). Bacteria may develop resistance to sulfonamides by using alternate metabolic paths or increasing synthesis of para-amino benzoic acid (PABA).

Chromosomal mediated resistance is thought to develop slowly, mediated by a slow rate of mutation. Quinolone resistance is chromosomally mediated and develops from
mutations in gyrase genes over a period of time (Wolfson et al. 1989). This slower acquisition of resistance prolongs effectiveness of treatment.

Some microorganisms, like \emph{Pseudomonas aeruginosa}, simply have impermeable cell walls (narrow porins) that will not allow the entry of antibiotics, such as penicillins, cephalosporins, aminoglycosides, tetracycline and chloramphenicol (Jacoby 1986, Trias and Nikaido 1990). Carbenicillins seem to have difficulty entering cells because of its largely hydrophobic nature and negative charge (Hancock 1997). Some antibiotics, like macrolides, are more effective against Gram-positive organisms because they are unable to penetrate the outer membrane of Gram-negatives (Anonymous 1991).

\textbf{Types of resistance}

There are many types of bacterial resistance, including natural, acquired, chromosomal, plasmid, transpositional, phenotypic, microbiological, and clinical resistance (Anonymous 1991). The most important to the animal health field are acquired resistance utilizing plasmids and chromosomes (Perez-Trallero and Zigorraga 1995).

\textit{Chromosomal resistance}

Chromosomal resistance occurs by a single step or spontaneous mutation that will in turn modify a target to decrease susceptibility to the antibiotic (Jacoby and Archer 1991). This type of resistance is not common and can only be passed on to progeny, not to other bacteria. Quinolone resistance is commonly thought to be chromosomally encoded (Guay 1992).
Plasmid resistance

Plasmids are mobile, extrachromosomal factors that can carry genes for resistance or virulence (Silva 1996). Plasmids that carry genes for resistance are also called R factors (Guthrie 1992). These can be passed on, not only to progeny, but also to other species of bacteria. Plasmid mediated resistance is more common than chromosomal resistance and is typical for β-lactam, aminoglycoside, and chloramphenicol resistance. Their presence may encourage resistance to develop and their withdrawal may enable a reversion to susceptibility.

Plasmid transfer can occur three ways: transformation (occurs in few bacteria), transduction (phage-assisted transfer) and the most sophisticated transfer, conjugation. Conjugation occurs between donors and recipients, requiring a sex pilus (Anonymous 1991). It occurs more frequently in Gram-negative bacteria than Gram-positive bacteria. Conjugation is unique in that resistance, even multiple resistance, can be transferred to different species of bacteria in one step. Many bacteria can be recipients of resistance. Salmonella bacteria are most often found to carry resistance plasmids (Blackburn et al. 1984, Poppe et al. 1995).

Enteric pathogens may have the ability to acquire R factors from other gut flora (Linton et al. 1981). If exposure occurs, animals or humans may acquire R factors from various sources. This suggests the potential for transfer of resistance between human and animals but is difficult to assess behavior in either host prior to acquisition of resistance. This type of transfer has been demonstrated in mice (Smith and Tucker 1978) and may lead to development of additional resistance (Aserkoff and Bennett 1969).
Transpositional resistance

A less prominent type of resistance occurs on transposons or mobile genetic elements. Transposons are gene sequences that can migrate between plasmids, chromosomes, or bacteriophages (Silva 1991). They frequently contain genes encoding resistance and can facilitate the spread of resistance (Jacoby and Archer 1991).

Problems with antibiotic resistant bacteria

Current situation

Bacterial resistance has become an important problem in treating human as well as animal infections. Pharmaceutical industries are trying to keep up with demand for novel, more efficacious antimicrobics to treat infections now caused by resistant bacteria. The general usefulness of antimicrobics has declined (Linton 1977). It seems that more bacteria are developing resistance to even novel antibiotics than before. Clearly, there is an association between antibiotic use and the development of resistant bacteria (Anonymous 1997, McCaig and Hughes 1995, Linton 1977).

Currently, the majority of antibiotics used in human medicine are for therapy, while most used in animal medicine are for prophylactic treatment and growth promotion (DuPont and Steele 1987). Half of all antibiotics used in the United States are for food producing animals (Lee et al. 1993). In the United States, 55-60% of tetracycline and penicillin is used in feed at subtherapeutic levels (Anonymous 1989a, Bennett 1980). In this way, large numbers of animals are exposed to antibiotics, increasing the rate at which resistance may develop and persist (Cohen 1992).
The majority of resistance observed in human health is a result of overuse of antibiotics directly in humans (Norrby 1996), not from transfer from animals or meat products. Animal feed has been improperly blamed for the transfer of resistance to humans after consumption of meat products from animals fed medicated feed (Cherubin 1984).

**Problems**

Progress in drug development has been slow. It has been approximately 15 years since the last class of antimicrobics has been discovered (Spratt 1996). In Europe, bacteria have already developed resistance to this latest group of antimicrobics, the fluoroquinolones (Kresken et al. 1994). Development of resistance hinders treatment as resistant bacteria are more difficult, if not impossible, to treat than susceptible ones.

Resistance can also develop in animals from low-level, long-term use in feedstuffs (DuPont and Steele 1987) which may select for resistant bacteria (Linton 1977). Resistance can be passed from animal to animal, animal to human, and human to human (Levy 1987). Olsvik et al. (1985) observed transmission of a resistant strain of *S. typhimurium* from animals to farm workers. Animals with resistant bacteria may contribute to resistance observed in humans (Ryder et al. 1980) and can cause serious illnesses (Holmberg et al. 1984). It is also likely that transfer of resistance from human to animal also occurs. In third world countries, human wastewater has been implicated in introducing drug resistant *Salmonella* to shellfish and fish (Anonymous 1989b). These modes of transfer clearly pose a threat to animals or humans in contact with the contaminated individual or animals which carry the resistant bacteria.

Problems may arise in treatment of patients with infections due to resistant bacteria.
Higher health care costs due to more hospitalizations may result from the increase in antibiotic use (Sundlof and Cooper 1996). Resistance may also lead to higher death rates in humans (Holmberg et al. 1984). High morbidity in Kenya was observed and was attributed to antibiotic resistant bacteria which were present in large numbers of immunocompromised patients (Kariuki et al. 1996).

Methods of control

Many methods to control the development of resistant bacteria have been proposed. Reducing antimicrobial levels in animal feed has been suggested as a solution to decreasing the ability for bacteria to develop resistance in the animal populations (DuPont and Steele 1987). The removal of selective pressure from antibiotics in feed is believed to decrease the numbers of resistant organisms (Levy 1987). However, this area is controversial. In the United Kingdom, a decrease in the amount of antibiotics used in feed has not had an impact on the reduction of resistant organisms (DuPont and Steele 1987). The reason for these differences is unclear.

As antibiotic use has increased, so has the need for ways to observe the impact on animals, food and the environment (Martel and Coudert 1993). Surveillance is an important tool to identify areas of concern regarding increased development of resistant microorganisms. Facilities in England and Wales have monitored *Salmonella* sensitivity in animals since 1970 (Wray et al. 1993), and susceptibility testing is common in many laboratories (Blackburn et al. 1984, Lee et al. 1993, Ryder et al. 1980). Increases in surveillance have been requested, especially on farms and in hospitals, to observe movement

It has been proposed that it may be effective to use antibiotics wisely by choosing those that have limited means of initiating resistance (Mortensen et al. 1996). Additionally, prudent and restrained use of antibiotics in humans and animals is desired for the prevention and treatment of bacterial infections (Goldmann et al. 1996, Holmberg et al. 1984, Cohen and Tauxe 1986, Cherubin 1984). Pharmaceutical companies have advocated short-term, high dose administration of antibiotics in the prevention and control of drug resistant bacteria (Sundlof and Cooper 1996). Inappropriate use is discouraged and treatment lengths should be carefully monitored and reduced if possible (Cohen 1992). In addition to decreasing indiscriminate use by doctors, patients also play a significant role in decreasing the use of antibiotics (McCaig and Hughes 1995).

Development of novel classes of antibiotics are warranted. Many studies are being conducted, targeting specific areas of bacterial structure for development of new antimicrobics (Vaara 1996). Chopra et al. (1996) introduce the idea of using analogs of past antibiotics as a new way to combat resistant microbes. Enzyme inhibitors could be incorporated with antibiotics to prevent degradation by enzymes produced by the bacteria (Chopra et al. 1996). Novel methods are desired to keep up with the development of resistant organisms.
Future Direction

Emphasis for on-farm control of salmonellosis in animals is warranted. Since *Salmonella* contamination is almost impossible to prevent and salmonellosis is very difficult to treat, an understanding of the pathogenesis and transmission of the organism is imperative.

Antibiotic therapy is becoming limited in its usefulness. Prophylactic use compounds the problem by enhancing the development of resistant bacteria. Limiting use of antibiotics in humans and animals has been desired for years. Development of new antibiotics, even if beneficial for only a short time, is desirable. However, long term control of the problem targets development of vaccines and enhancing the immune response of the host to control infectious diseases.

References


CHAPTER 2. EFFECT OF DOSE ON PERSISTENCE AND SHEDDING OF SALMONELLA HEIDELBERG IN SWINE

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ABSTRACT

The effect of inoculation dose on shedding of Salmonella heidelberg in swine was studied. Forty-five Salmonella-free pigs were placed into 4 groups. Groups 1, 2 and 3 (n=12/group) received 10^3, 10^6, or 10^9 CFU Salmonella heidelberg, respectively, intranasally at 6 weeks of age. Group 4 (n=9) served as controls. Pigs (4/group and 1 control) were necropsied at 2, 4, and 6 weeks post-challenge (PC). Tonsil and nasal swabs, rectal loops (TNR) and clinical signs were monitored throughout the study. Following challenge, only a mild to moderate cough was observed. On day 5 (D5) PC, 5/33 (15.2%) swabs and loops were positive for Group 1 compared to 22/36 (61.1%) for Group 2 and 31/33 (93.9%) for Group 3. Peak shedding levels occurred on D7 for Groups 1 and 3 (2.95 and 6.17 logs, respectively).

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respectively), while Group 2 levels peaked on D10 (4.31 logs). Shedding ceased in Group 1 by D36 PC. More tissues were positive from Group 3 than any other group at the 4 and 6 week necropsies. For all groups, the tissue colonized with the highest numbers of *Salmonella* was the ileocolic junction. These data indicate that infection with *S. heidelberg* does not result in apparent clinical disease, regardless of dose. However, a dose dependent correlation was observed with respect to shedding and number of positive tissues.

Key words: *Salmonella heidelberg*, foodborne disease, swine

**INTRODUCTION**

*S. heidelberg* is a non-host adapted serotype of swine, the fourth most frequently isolated *Salmonella* serotype from swine and humans (11, 24). It has also been recovered from cattle (21) and is a predominant serotype found in poultry (6, 11, 20).

*S. heidelberg* causes a wide variety of infections in humans, including gastroenteritis (7). Septicemia and meningitis with up to 18% case mortality have been observed in infants (1) along with atypical associations in adults like false appendicitis (27), joint infection (17) and abscesses due to bacteremia (16, 25).

Foodborne salmonellosis has been associated with consumption of meat and meat products, including pork or pork products, in addition to eggs (4). Since swine, cattle, poultry and seafood have been implicated in carriage of foodborne pathogens (2), it is likely that most foodborne disease is a result of ingestion of contaminated products. Human to human, animal to human, and contact with contaminated non-food sources are other means of initiating foodborne-related illnesses.
Much is known about host-adapted strains of *Salmonella*, such as *S. dublin* in cattle (5) and *S. choleraesuis* in swine (12, 13, 14, 23, 26). However, other than *S. typhimurium*, most non-host adapted strains have received little attention regarding their pathogenesis. Various aspects regarding the pathogenesis, carrier state, or transmission of *S. heidelberg* are relatively unknown.

Anecdotal reports implicate *S. heidelberg* in on-farm subclinical and clinical disease in swine. However, the extent of infection is unknown. Only one published report is available regarding experimental *S. heidelberg* infection in swine (22). Reed et al. (22) observed clinical manifestations resembling infection with enterotoxigenic *E. coli* following challenge with $10^{10}$ CFU *S. heidelberg*. This differs from clinical disease following challenge with *S. typhimurium* (8, 10, 30) or *S. choleraesuis* (12, 14).

The objective of this study was to determine the effect of dose on persistence and shedding of *S. heidelberg* infection in swine. The immune response was also measured.

**MATERIALS AND METHODS**

*Bacterial strains and challenge culture*   *Salmonella heidelberg* (isolated from swine) was obtained from the National Veterinary Services Laboratories, Ames, Iowa, and assigned number 25K5-7. Challenge cultures were prepared by inoculating 5 ml Luria Bertani (LB) broth with ~100 μl of a frozen (-70°C) stock culture and incubating overnight at 37°C and 150 rpm. A 1% inoculum from the overnight culture was transferred into 250 ml LB and grown for approximately 3.5 hours at 37°C and 150 rpm. The culture was centrifuged, the pellet was resuspended in 30 ml 0.1 M phosphate buffered saline (PBS, pH 7.2) and adjusted
to a final concentration of $1.01 \times 10^9$ CFU/ml in PBS ($OD_{600}=\sim 1.00$). The $10^6$ CFU/ml culture was made by diluting the $10^9$ culture 1:1000 in PBS. A subsequent dilution (1:1000) of the $10^6$ culture was used as the $10^3$ culture. Final concentrations were confirmed by plate counts on trypticase soy agar plates.

**Swine** Source sows were cultured on the farm for *Salmonella* spp. pre- and post-farrowing. Forty-five 10-14 day old pigs were weaned from *Salmonella* culture-negative swine and transported to isolation facilities at the National Animal Disease Center. Pigs were raised in isolation as described by Fedorka-Cray et al. (9). Prior to challenge, fecal pools and individual rectal loops were obtained from pigs and cultured for *Salmonella*.

**Experimental procedure** At 6.5 weeks of age, 45 *Salmonella* culture-negative pigs were randomly assigned to 4 groups. Control pigs were housed in separate isolation facilities. Within each building, all animals were housed in pens of similar size. Groups 1, 2, and 3 (n=12/group) were challenged at 6.5 weeks of age (day 0) intranasally, with 1 ml (0.5 ml in each nostril dropwise, alternating nostrils) of *S. heidelberg* at a concentration of $10^3$, $10^6$ and $10^9$ CFU/ml, respectively. Group 4 pigs (n=9) served as uninoculated controls.

Pigs were observed for clinical signs of disease post-challenge (PC). Rectal temperatures were taken daily from all pigs for 1 week and then once a week for 5 weeks. Tonsil and nasal swabs and rectal loops (TNR) were obtained from pigs for bacteriologic culture on days 1 through 7, 15, 22, 29, 36, and 43 PC. Fecal pools were obtained for bacteriologic culture on days 3, 7, 10, 15, 22, 29, 36 and 43 PC by mixing fresh feces from the entire pen and randomly collecting 10 g of feces from the mixed pile. Blood samples were obtained for serum on days -1, 13, 27 and 41 PC.
Four pigs from Groups 1, 2 and 3 and 1 pig from Group 4 were necropsied at 2, 4 and 6 weeks PC. Tissues were obtained using aseptic technique and included tonsil, mandibular lymph node (MLN), bronchiole lymph node (BLN), lung, liver, spleen, colonic lymph node (CLN), colon, ileocolic lymph node (ICLN), ileocolic junction (ICJ), cecal contents (Cec Cont), cecum, middle ileum (Mid II), and stomach wall (SW).

**Bacteriologic examinations** Fecal pools, TNR samples and tissues were placed into tetrathionate broth (Difco Laboratories, Inc., Detroit, MI) and incubated for 48 h at 37°C. Samples were transferred to Rappaport R10 medium (28), incubated for 24 h at 37°C and then streaked onto xylose-lysine-Tergitol-4 (XLT4) agar plates (Difco). All XLT4 plates were incubated for 24 h at 37°C. Suspect colonies were inoculated into triple sugar iron agar (Difco) and lysine iron agar (Difco) slants for biochemical confirmation. All presumptive positive colonies were serogrouped by agglutination with *Salmonella* O antiserum Group B (Difco). At least one isolate from each pig was sent to the National Veterinary Services Laboratories for serotyping.

Quantitative bacteriologic culture of fecal pools, tonsil, lung, ICJ, ICLN and Cec Cont were performed as previously described using the most probable number method (31). Results are recorded as mean values from each group at each necropsy.

**Antigen preparation** For lymphocyte blastogenesis assays, *S. heidelberg* lipopolysaccharide (LPS) antigen was prepared according to the method described by Morrison and Leive (18), lyophilized, and stored at -70°C until use. For ELISA assays, heat extract *S. heidelberg* antigen was made as described by Gray et al. (12). Briefly, frozen stock culture was scraped with a sterile swab and placed into LB broth. The culture was incubated at 37°C and 220
rpm for 16 h, centrifuged at 15,000 x g for 10 min and the supernatant was discarded. The pellet was resuspended in 1/10 volume of 0.1M PBS and placed in an erlenmeyer flask. The resuspended cells were heated in a 60°C water bath for 1 h with periodic gentle agitation, then centrifuged at 19,000 x g for 6 min. The supernatant was decanted, filtered through a Millex-GV 0.22 μm filter (Millex-GV, Millipore, Bedford, MA) and stored at -20°C until use. Protein concentration (μg/ml) was determined as described by Bradford (3) using bovine serum albumin as the standard.

**Enzyme linked immunosorbent assay** The ELISA assay was performed as described by Gray et al. (12) in 96-well microtitration plates (Immulon, Dynatech Laboratories, Inc., Chantilly, VA). Antigen concentration was adjusted to 4 μg S. heidelberg heat extract protein per well. Goat anti-swine peroxidase conjugated antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used at a 1:2000 dilution in diluent buffer. Following the addition of substrate (one component of 3,3',5,5' tetramethylbenzidine microwell substrate; Kirkegaard & Perry), the reaction was stopped after 10 minutes. Plates were read using a Dynatech MR7000 with a reference wavelength of 410 nm and a test wavelength of 450 nm. Group optical densities were determined by taking the mean experimental value/group minus the mean control value/group at each necropsy.

**Blastogenesis assay** Blastogenesis assays were performed on days -1, 13, 27 and 41 PC. Ten ml of heparinized blood was collected from each pig and diluted in 10 ml Hank's balanced salt solution (HBSS) without calcium or magnesium, then layered over Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 400 x g for 30 min. The top layers were aspirated and the dense mononuclear cell layer was removed and added to HBSS. The
mixture was centrifuged at 450 x g for 15 minutes, the supernatant was decanted and the pellet was resuspended in 10 ml HBSS and incubated with 10 ml lysing solution (0.013 M Na₂HPO₄, pH 7.2) for 40 sec. Twenty ml restoring solution (0.1 M PBS, pH 7.4) was added and the cells were centrifuged for 15 min at 450 x g. Cells were washed and centrifuged twice with HBSS at 450 x g for 15 min and resuspended at 2.5 x 10⁶ cells/ml in RPMI (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum, 25 mM HEPES (Gibco) and 50 μg/ml gentamicin (Gibco). Cells were plated in triplicate in microtiter plates.

Concanavalin A (5-10 μg/ml; Gibco) or S. heidelberg LPS (2.5-100 μg/ml) were added and the plates were incubated at 37°C with 5% CO₂ for 48 h for B cell proliferation assays. Cells were pulsed with 0.5 μCi/30 μl ³H-thymidine/well, incubated for 18 h and harvested. Values are reported as the mean ± SEM stimulation index (defined as experimental values divided by negative control values) for pigs in each group. Negative control wells contained cells plus RPMI media only.

RESULTS

**Pigs**  All pigs were Salmonella-free prior to challenge. No Salmonella was recovered from any sample taken from pigs in Group 4 during the study.

**Clinical response**  No significant increase in temperature was observed for any group. Temperatures ranged from 38.1°C (100.6°F) to 40.9°C (105.6°F), with the highest temperatures recorded on day 2 (D2). All pigs in Groups 1, 2 and 3 exhibited mild to moderate coughing beginning on D2. Within each group several pigs appeared slightly depressed and exhibited mild dyspnea or diarrhea. Clinical signs resolved by D13. Group 4
exhibited no clinical signs.

**Mortality** One pig from Group 1 was euthanized on D12 after becoming moribund; the final temperature was recorded at 40.3°C (104.6°F). At necropsy, *Salmonella* was recovered from BLN, liver, spleen, ICLN, ICJ and Mid II. Quantitative levels of *Salmonella* (log_{10} CFU/gram) in tissues were as follows: tonsil, 0.00; lung, 0.41; ICLN, 2.79; ICJ, 4.21; Cec Cont, 3.82.

One pig from Group 3 was euthanized on D8 after becoming moribund. *Salmonella* was recovered from 11/14 tissues at necropsy. Quantitative levels of *Salmonella* in tissues were as follows: tonsil, 4.84; lung, 0.00; ICLN, 4.30; ICJ, 4.02; Cec Cont, 3.20. Values from TNR samples, tissues, antibody responses and lymphocyte stimulation indeces for either of these pigs are not included in results. It should be noted that these pigs were poor doers prior to initiation of the study and were placed randomly in respective groups.

**Gross pathology** Hemorrhagic and enlarged lymph nodes, consolidation and hemorrhage in lungs, and irritation of the epithelium on the ileocolic junction were observed in tissues from several pigs in Groups 1, 2 and 3. Gross differences in pathology between groups were not observed.

**Antemortem bacteriologic results** Frequency of positive swabs and loops is shown in Table 1. All pigs in Groups 1, 2 and 3 had at least one positive swab or loop by D1. Pigs in Group 1 had less than 20% TNR samples positive throughout the study. By D5, 93.9% (31/33) of TNR samples were positive from Group 3, compared to 61.1% (22/36) samples in Group 2 and 15.2% (5/33) in Group 1. All groups had at least 1 positive swab or loop throughout the study with the exception of Group 1 pigs on D43 when no positive swabs or loops were
observed. Percent positive TNR samples are shown in Figure 1.

The magnitude of fecal shedding is shown in Figure 2. For Groups 1 and 3 shedding peaked on D7 (2.95 and 6.17 logs, respectively), whereas Group 2 shedding peaked on D10 (4.31 logs). Levels for Group 2 pigs increased slightly on D29 to 2.69 logs and subsequently decreased along with the other two groups. All groups shed greater than 1 log throughout the study until D36. Group 1 pigs ceased shedding by D36, while Groups 2 and 3 continued shedding (.723 and .401 logs, respectively) to D43 when the experiment terminated.

**Postmortem bacteriologic examination** Table 2 shows the distribution and frequency of positive tissues from each group at each necropsy. *Salmonella* was recovered from more tissues in Group 3 than any other group at 4 and 6 week necropsies. No positive tissues were recovered from pigs in Group 1 at 6 weeks PC. *Salmonella* persisted in Cec Cont, ICJ, mid-ileum, tonsil and MLN of pigs in Groups 2 and 3 through 6 weeks PC. The majority of *Salmonella* positive tissues were gut-associated (ICLN, colon, ICJ, CLN, Cec Cont, cecum, and mid-ileum) and not respiratory-associated (tonsil, MLN, BLN, and lung). From Group 3 pigs, however, *Salmonella* was recovered from more respiratory-associated tissues at 2 and 6 week necropsies.

Populations of *Salmonella* recovered from tonsil, ICJ, Cec Cont and ICLN are shown in Table 3. Although quantitative bacteriologic culture was conducted for lungs, levels were below detection limits. *Salmonella* levels were below detectable limits in Group 1 tissues at 6 weeks PC and <1 log<sub>10</sub> CFU/gram *Salmonella* were recovered from Cec Cont at 2 and 4 weeks PC. Levels of *Salmonella* were greatest in the ICLN at 2 weeks PC from pigs in Group 1. Less than 2 logs were recovered from ICLN and Cec Cont from pigs in Group 3.
Levels in the ICJ and tonsil from Group 3 pigs were greater than 3 logs throughout the study. Numbers of *Salmonella* recovered from tonsils from all groups were highest at 4 weeks PC.

**Serum antibody responses** Figures 3a and 3b show optical density values for IgM or IgG response for each group at each necropsy. Peak IgM ODs occurred at 2 weeks PC for Groups 2 and 3 and at 4 weeks PC for Group 1. Average OD values were undetectable for Group 2 and 3 at both 4 and 6 weeks PC. Peak IgG optical densities occurred at 6 weeks PC for Groups 2 and 3. Group 1 had a measurable response at 4 weeks PC but no detectable response at 2 or 6 weeks PC.

**Blastogenesis assays** Lymphocyte blastogenic responses to *S. heidelberg* LPS are shown in Figure 4. Differences between groups were not observed until D27 PC, when the average stimulation index for Group 3 was greater than the other three groups. Subsequently, a decreased response for Group 3 was noted on D43, when compared to all other groups. No differences in stimulation indices for all groups were observed with Concanavalin A.

**DISCUSSION**

Regardless of dose, clinical signs of infection were either inapparent or mild in all challenge inoculated groups. It was not surprising that clinical signs were inapparent in pigs challenged with $10^3$ CFU, however, even at high doses, pigs did not appear to be clinically ill. Mild coughing seemed to be the predominant indicator of infection, with a few pigs having sporadic diarrhea. This clinical presentation is atypical when compared to infection with *S. typhimurium* (8, 10) or *S. choleraesuis* (12, 25), which usually result in more noticeable clinical signs of infection such as diarrhea, fever, dyspnea, anorexia and lethargy.
Two explanations for the difference in clinical presentation may be attributed to route of inoculation and serotype. An intranasal inoculation has been shown to produce more severe clinical disease following challenge with *S. choleraesuis* (12). We assumed the same effect would be seen using *S. heidelberg*. In a study done by Reed et al. (22), oral challenge appeared to produce more severe disease when *S. heidelberg* was used. However, the challenge dose was higher (10\(^{10}\) CFU). This suggests that had *S. heidelberg* been administered via the oral route, different clinical disease patterns may have developed.

The only significant clinical sign we observed was coughing, possibly due to a respiratory infection brought about by intranasal inoculation. Reed et al. (22) initially reported a difference in clinical presentation and pathological changes induced by *S. heidelberg* when compared to *S. choleraesuis* or *S. typhimurium*. However, the lack of clinical signs occurred after pigs had been reinfected with 10\(^{10}\) CFU (22). Pigs in this study received only a primary challenge. Additionally, Reed et al. (22) observed fluid diarrhea 48 hours PC for three days, whereas few pigs in this study had noticeable diarrhea. Our results suggest that carriage of *S. heidelberg* in swine can be clinically inapparent which may be problematic when a carcass is presented at slaughter.

Mortality in a Group 3 pig was not unexpected because it has been shown that high numbers (>10\(^9\)) of *Salmonella* in experimentally infected swine can cause mortality (15, 29). It seems unusual that the pig in Group 1 died unless other etiologies were present to confound a relatively low level *Salmonella* infection. As mentioned, both pigs were not thriving as well as other pigs at the start of the experiment. We suspect that regardless of placement in any group, these pigs would not have done well following challenge. If other etiologies were
not involved in mortality associated with the Group 1 pig, death would be attributed to infection with *Salmonella* alone, suggesting that low levels of *S. heidelberg* can be virulent for less than healthy animals.

A dose dependent relationship was observed for recovery of *Salmonella* from TNR samples and with fecal shedding. However, shedding patterns differed between groups. The magnitude of fecal shedding was greatest in pigs in Group 3 and lowest in Group 1 pigs. Group 1 pigs were eventually able to clear the infection after D36. However, Groups 2 and 3 were still shedding low levels up to D43 when the study terminated. A dose of $10^6$ CFU *S. heidelberg* appears to be the minimum dose required to establish an infection lasting at least 6 weeks.

Levels of *Salmonella* recovered from tissues also appeared to be dose dependent. At 6 weeks PC, high levels were recovered in ICJ from pigs in Groups 2 and 3. The ileocolic junction was the tissue of predilection. Recovery of *Salmonella* up to 6 weeks PC from the ICJ may be indicative of a progression toward the carrier state as described by Gray et al. (12).

Although a dose dependent relationship is observed, these results contrast other studies (13) in that *S. heidelberg* was recovered from pigs challenged with $10^3$ CFU for at least 36 days PC. This suggests that pigs exposed to low doses of *S. heidelberg* have the ability to become infected with and shed the bacterium in the environment, possibly contaminating other naive pigs for a longer period of time. Gray et al. (13) demonstrated that naive pigs can become infected with and shed *S. choleraesuis* following exposure to environmental levels of $\leq 2.6$ logs. In a herd, *S. heidelberg* may be difficult to monitor
because of lack of clinical disease and may be more difficult to eradicate because exposure to very low doses may result in some degree of persistence.

For Group 3, *Salmonella* continued to colonize the tonsil and ICJ throughout the experiment. Because of this persistent infection, immune function may be compromised at a challenge dose $\geq 10^9$ CFU. These results coincide with blastogenic responses to *S. heidelberg* LPS. At 6 weeks PC, some level of immunosuppression is observed in Group 3 pigs. Immunosuppression has also been suggested following challenge with *S. choleraesuis* (13).

The IgG and IgM responses were classic for all groups and confirm exposure to the antigen. A mix-ELISA to monitor a herd immune response has been proposed (19). Use of the heat extract antigen as described here may produce comparable results and warrants further investigation.

In conclusion, pigs infected with low to high doses of *Salmonella heidelberg* may not show obvious signs of disease but may become persistently infected. This makes detection of infected swine difficult and may result in presentation of swine at slaughter with an inapparent *Salmonella* infection.

**ACKNOWLEDGEMENTS**

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Administration, Center for Veterinary Medicine. Special thanks are extended to Dr. Linda Tollefson for her support.

REFERENCES


FIGURE LEGENDS

Figure 1 - Qualitative recovery of *Salmonella heidelberg* from TNR samples. (___ Group 1: 10\(^3\) CFU, _ _ Group 2: 10\(^6\) CFU, --- Group 3: 10\(^9\) CFU)

Figure 2 - Quantitative recovery of *Salmonella heidelberg* from fecal pools. See Figure 1 for legends. Reported values are CFU/gram as calculated by the most probable number method for each group.

Figure 3 - a- Serum IgM responses of swine following intranasal challenge with 10\(^3\) (Group 1), 10\(^6\) (Group 2), or 10\(^9\) (Group 3) CFU *S. heidelberg*. *Salmonella heidelberg* heat extract was used as the antigen.

b- Serum IgG responses of swine following intranasal challenge with 10\(^3\) (Group 1), 10\(^6\) (Group 2), or 10\(^9\) (Group 3) CFU *S. heidelberg*. *Salmonella heidelberg* heat extract was used as the antigen.

Figure 4 - *In vitro* blastogenic responses to *S. heidelberg* LPS from swine challenged with 10\(^3\) (Group 1), 10\(^6\) (Group 2), or 10\(^9\) (Group 3) CFU *S. heidelberg*. Group 4 represents uninoculated controls.
# Table 1 - Number of *Salmonella heidelberg*-positive tonsil and nasal swabs and rectal loops

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*a* - number positive/number sampled
Table 2 - Qualitative recovery of *Salmonella heidelberg* from the tissues of experimentally infected swine

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<td>BLN</td>
<td>1/4</td>
<td>0/4</td>
<td>0/3</td>
</tr>
<tr>
<td>Lung</td>
<td>0/4</td>
<td>0/4</td>
<td>0/3</td>
</tr>
<tr>
<td>Liver</td>
<td>0/4</td>
<td>0/4</td>
<td>0/3</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/4</td>
<td>0/4</td>
<td>0/3</td>
</tr>
<tr>
<td>ICLN</td>
<td>3/4</td>
<td>4/4</td>
<td>0/3</td>
</tr>
<tr>
<td>Colon</td>
<td>0/4</td>
<td>2/4</td>
<td>0/3</td>
</tr>
<tr>
<td>CLN</td>
<td>2/4</td>
<td>1/4</td>
<td>0/3</td>
</tr>
<tr>
<td>Cec Cont</td>
<td>0/4</td>
<td>1/4</td>
<td>0/3</td>
</tr>
<tr>
<td>Cecum</td>
<td>2/4</td>
<td>0/4</td>
<td>0/3</td>
</tr>
<tr>
<td>ICJ</td>
<td>2/4</td>
<td>2/4</td>
<td>0/3</td>
</tr>
<tr>
<td>Mid Il</td>
<td>1/4</td>
<td>1/4</td>
<td>0/3</td>
</tr>
<tr>
<td>SW</td>
<td>0/4</td>
<td>0/4</td>
<td>0/3</td>
</tr>
</tbody>
</table>

| Total pos   | 11/56 | 11/56 | 0/42 | 20/56 | 17/56 | 12/56 | 19/56 | 27/56 | 10/42 |
| % positive  | 19.6  | 19.6  | 0    | 35.7  | 30.4  | 21.4  | 33.9  | 48.2  | 23.8  |

a- weeks post-challenge  

b- number positive/number sampled
Table 3 - Mean populations of *Salmonella heidelberg* recovered from tissues

<table>
<thead>
<tr>
<th>Necropsy</th>
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<th>4</th>
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<tr>
<td>Group 1</td>
<td>tonsil</td>
<td>0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.73</td>
</tr>
<tr>
<td></td>
<td>ICLN</td>
<td>2.95</td>
<td>1.89</td>
</tr>
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<td></td>
<td>ICJ</td>
<td>1.93</td>
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<tr>
<td></td>
<td>Cec Cont</td>
<td>0.78</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>11</td>
<td>7</td>
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<tr>
<td>Group 2</td>
<td>tonsil</td>
<td>1.25</td>
<td>3.57</td>
</tr>
<tr>
<td></td>
<td>ICLN</td>
<td>2.81</td>
<td>2.35</td>
</tr>
<tr>
<td></td>
<td>ICJ</td>
<td>3.24</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td>Cec Cont</td>
<td>4.77</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Group 3</td>
<td>tonsil</td>
<td>3.42</td>
<td>5.03</td>
</tr>
<tr>
<td></td>
<td>ICLN</td>
<td>1.90</td>
<td>0.04</td>
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<tr>
<td></td>
<td>ICJ</td>
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<td></td>
<td>Cec Cont</td>
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<td>1.92</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>11</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup> values reported are log<sub>10</sub>CFU/gram  
<sup>b</sup> NQ-P - not quantifiable but positive by qualitative bacteriologic examination
Figure 3a
Figure 3b
Figure 4
CHAPTER 3. EFFECT OF NAXCEL® AND BAYTRIL® ON

SALMONELLA HEIDELBERG INFECTION IN SWINE

A paper submitted to Antimicrobial Agents and Chemotherapy

Heidi L. HolcombA, Paula J. Fedorka-CrayB* and Linda K. TollefsonC

ABSTRACT

The effect of 2 antimicrobics on Salmonella heidelberg infection in swine was studied. Forty-four Salmonella-free pigs were placed into 4 groups. Groups 1, 2 and 3 (n=12 each) received 10⁶ CFU S. heidelberg intranasally (IN) at 7 weeks of age. Group 4 (n=8) served as controls. On day 2 (D2) through D4 post-challenge (PC), Group 1 received 4 mg/kg Naxcel® (ceftiofur) intramuscularly (IM) and Group 2 received 4 mg/kg Baytril® (enrofloxacin) IM. Groups 3 and 4 did not receive antibiotics. Pigs (4/group and 1 control)

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Running Title: Effect of antibiotics on S. heidelberg infection in swine

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were necropsied at 2, 4 and 6 weeks post-challenge (PC). Tonsil and nasal swabs, rectal loops, rectal temperatures, blood and clinical signs were monitored throughout the study. Mild to moderate diarrhea and coughing were observed in Groups 1, 2 and 3, through D13. Groups 1 and 2 had 58.3% and 5.6% total positive swabs and loops on D5 PC, respectively, while Group 3 had 88.9% total positive swabs and loops. Additionally, on D5 Groups 1 and 3 were shedding 4.05 and 5.37 \(10^g\) CFU/g feces, respectively, while Group 2 was not shedding detectable numbers of \(Salmonella\). However, by D10, shedding increased to 3.42 and 1.94 \(10^{g}\) CFU/g feces for Groups 1 and 2, respectively, while Group 3 levels decreased. More tissues were positive from Group 3 than any other group at each necropsy. The tissues colonized with the highest numbers of \(Salmonella\) were the tonsil from Group 1 and ileocolic junction from Groups 2 and 3. No increase in resistance was observed for any isolate obtained throughout the study. Baytril\textsuperscript{®} appeared to be more effective than Naxcel\textsuperscript{®} in reducing \(Salmonella\) numbers. However, these data indicated that regardless of the antibiotic used, an initial decrease in numbers of \(Salmonella\) shed into the environment is followed by an increase after cessation of treatment.

keywords: antibiotic, \(Salmonella\), resistance

INTRODUCTION

\(Salmonella\) is a food-borne pathogen commonly associated with infections in swine (25). Following infection, many \(Salmonella\) serotypes are known to persist in swine, including \(S.\ typhimurium\) (8), \(S.\ choleraesuis\) (14), and \(S.\ heidelberg\) (15). Contamination of
food products, including pork and pork products, may be the result of persistently infected carrier pigs which harbor the bacteria with no outward signs of clinical disease (22, 31).

Salmonella heidelberg ranks as the fourth most frequently isolated Salmonella serotype from swine (10) and humans (2). As with other non-host adapted serotypes, with the exception of S. typhimurium, S. heidelberg has received little attention. Reed et al. (24) suggested a different clinical and pathological presentation occurs following infection with S. heidelberg when compared to infection with other serotypes. We have determined that S. heidelberg persists in swine without causing noticable clinical signs of disease regardless of infectious dose (15).

Antibiotics have been used to control the spread and reduce shedding of salmonellae in swine (29). Medicated feed has been shown to be efficacious in the reduction of bacterial infections (11, 16, 17, 30). Use of Cephamycin C reduced the febrile response and shedding levels of S. choleraesuis in swine following challenge (16).

Naxcel® (ceftiofur sodium) is a β-lactam antibiotic in the class of third generation cephalosporins. It has a broad spectrum of activity, particularly against Gram-negative bacteria and its mode of action is based on inhibition of cell wall synthesis (1). Naxcel® is primarily used to treat respiratory infections in swine caused by Actinobacillus spp., S. choleraesuis and Pasteurella spp.

Baytril® (enrofloxacin) is a fluoroquinolone antibiotic with a broad spectrum of activity against Salmonella, Mycoplasma, Brucella, E.coli, Klebsiella, Proteus, Pseudomonas aeruginosa and Staphylococci (3). It acts as a DNA gyrase inhibitor and is particularly effective against intracellular pathogens (32). In Europe, it is marketed under the trade name
Baytril® and is approved to treat salmonellosis in food producing animals.

The objective of this study was to determine the effect of two antimicrobics (ceftiofur and enrofloxacin) on *Salmonella heidelberg* infection in swine.

**MATERIALS AND METHODS**

**Swine.** Source sows were cultured for *Salmonella* pre- and post-farrowing. At approximately 14 days of age, 44 pigs were weaned from culture-negative sows and transported to isolation facilities at the National Animal Disease Center in Ames, Iowa. Pigs were raised in isolation as previously described (9). Rectal loops and fecal pools were collected from pigs weekly for 4 weeks prior to challenge.

**Bacterial strain and culture methods.** A clinical isolate of *S. heidelberg*, 2SKS-7, was obtained from the National Veterinary Services Laboratories and used as the challenge strain (15). The resistance pattern of the isolate was determined by using a custom made panel for the Sensititre system (Table 1; Accumed, Westlake, OH).

The challenge culture was made by inoculating 5 ml of Luria Bertani (LB) broth with ~100 µl of frozen stock and incubating the broth stationary and overnight, at 37°C. A 1% inoculum was transferred to 250 ml of fresh LB and incubated at 37°C and 150 rpm for approximately 3.5 hours. The culture was centrifuged at 10,000 x g for 20 minutes and the pellet was resuspended in 30 ml of 0.1M phosphate buffered saline (PBS, pH 7.2). The culture was adjusted to a final concentration of 1.43 x 10⁹ CFU/ml (OD₆₀₀=1.053). Final concentration was confirmed by plate counts on trypticase soy agar.

**Experimental Procedure.** Forty-four pigs were randomly assigned to 4 groups (Groups 1, 2
and 3, n=12/group; Group 4, n=8) at 7 weeks of age (day 0) and weighed. Pigs were challenged intranasally (IN) with 1 ml (0.5 ml in each nostril dropwise, alternating nostrils) of $10^9$ CFU/ml *Salmonella heidelberg*. Pigs in Group 1 were treated intramuscularly (IM) one time daily for 3 days beginning on D2 PC with 4 mg/kg Naxcel®, while pigs in Group 2 were treated IM for 3 days beginning on D2 PC with 4 mg/kg Baytril®. Group 3 pigs served as challenge controls and Group 4 pigs (n=8) served as uninoculated negative controls.

After challenge, pigs were observed for clinical signs of infection. Tonsil and nasal swabs, rectal loops (Midwest Veterinary Supply, Des Moines, IA; TNR) and rectal temperatures were obtained daily for the first week and then weekly thereafter. Fecal pools were collected for quantitative bacteriologic culture on days 2, 5, 7, 10, 13, 20, 29, 36 and 43 PC. Pools were obtained by gathering fresh feces in each pen, homogenizing the entire amount and randomly selecting approximately 10 grams. Blood was collected for serum on days 11, 27 and 41 PC.

Necropsies were conducted on 4 pigs from each challenge group and one pig from the control group at 2, 4 and 6 weeks PC. Aseptic technique was used in the collection of tissues which included tonsil, mandibular lymph node (MLN), bronchiole lymph node (BLN), lung, liver, spleen, colon, colonic lymph node (CLN), ileocolic lymph node (ICLN), ileocolic junction (ICJ), cecal contents (Cec Cont), cecum, middle ileum (Mid II) and stomach wall (SW).

**Bacteriologic culture.** Bacteriologic culture was performed as previously described (15). Briefly, all TNR samples, fecal pools and tissues were cultured in tetrathionate broth (Difco Laboratories, Inc., Detroit, MI) for 48 hours at $37^\circ$C, then transferred to Rappaport R10
medium (28; Difco). After incubation at 37°C overnight, samples were streaked onto xylose-
lysine-Tergitol 4 (XLT4) agar plates (Difco) and incubated 24 hours at 37°C. All colonies
typical of *Salmonella* were inoculated into triple sugar iron agar (Difco) and lysine iron agar
(Difco) for biochemical confirmation. Presumed positive colonies were serogrouped by
agglutination using *Salmonella* O antisera Group B (Difco). Throughout the study at least
one positive sample per pig was sent to the National Veterinary Services Laboratories for
serotyping.

Tonsil, lung, ICJ, Cec Cont and ICLN were obtained at each necropsy for most
probable number analysis (33). Results are recorded as mean values from each group.

**Antigen preparation.** *Salmonella heidelberg* lipopolysaccharide (LPS) antigen was
prepared as described by Morrison and Leive (21) and lyophilized for use in lymphocyte
blastogenesis assays. Heat extract antigen was prepared for use in ELISA assays as described
by Gray et al. (12). Briefly, ~100µl of frozen (-70°C) stock culture was inoculated into 1L
LB broth and incubated at 37°C and 220 rpm. After 16 hours, the culture was centrifuged at
15,000 x g for 10 min. The pellet was resuspended in 1/10 volume of 0.1M PBS (pH 7.2)
and heated in a 60°C water bath for 1 hour with periodic agitation. Cells were centrifuged at
19,000 x g for 6 minutes and the supernatant was filtered through a Millex-GV 0.22 µm filter
(Millex-GV, Millipore, Bedford, MA) and stored at -70°C until use. Protein concentration
was determined as described by Bradford (5) using bovine serum albumin as the standard.

**Lymphocyte proliferation assay.** Serum from pigs was obtained on days 11, 27 and 41 PC
for blastogenesis assays and performed as previously described (15). Microtiter plates
(Dynatech) containing concanavalin A (5-10 µg/ml; Gibco, Gaithersburg, MD) or S.
*heidelberg* LPS (2.5-100 μg/ml) were inoculated with cells in triplicate and incubated with 5% CO₂ at 37°C for 48 hrs and then pulsed with 0.5 μCi/30 μl ³H-thymidine/well. Cells were harvested after 18 hrs. Values are reported as mean ± SEM stimulation index (experimental values divided by control values) for pigs in each group. Wells containing RPMI media plus cells served as negative controls.

**Enzyme linked immunosorbent assay.** ELISA assays were performed as described by Gray et al. (12). Antigen concentration was adjusted to 4 μg *S. heidelberg* protein/well. Conjugate, goat anti-swine peroxidase IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) or IgG (Kirkegaard & Perry), was diluted 1:2000. The substrate was 3,3′5,5 tetramethylbenzidine microwell substrate (Kirkegaard & Perry) and the reaction was stopped by the addition of 1% sulfuric acid after 10 minutes. Plates were read using a Dynatech MR7000 plate reader (Dynatech Laboratories, Inc., Chantilly, VA) at a reference wavelength of 410 nm and a test wavelength of 450 nm. Optical densities were determined by subtracting the mean control value from the mean experimental value for each group.

**RESULTS**

**Swine.** All pigs were culture negative for *Salmonella* prior to challenge. Group 4 pigs remained culture negative throughout the study.

**Clinical signs.** Temperatures remained normal throughout the study with the exception of D2 in which 1 pig from Group 1 had a temperature of 41.4°C (106.5°F) and 1 pig from Group 3 had a temperature of 41.8°C (107.3°F). Mild to moderate diarrhea was observed in several pigs in each challenge group through D7 PC. Slight coughing was detected in all groups.
through D13 PC. Dyspnea was infrequently observed in several pigs in Groups 1 and 2 through D9 PC. No clinical signs were observed in Group 4 pigs.

**Pathology at necropsy.** Hemorrhagic and consolidated lungs, enlarged and hemorrhagic lymph nodes, reddened intestinal epithelia, fibrinous adhesions on lungs or peritoneal cavity and hemorrhages in liver and spleen were observed in several pigs from all groups. Pigs in Group 4 did not have visible pathological manifestations.

**Antemortem bacteriologic results.** Figure 1 and Table 2 show distribution of positive TNR samples for each group. On D5 PC, Group 3 pigs had 88.9% positive samples, while pigs in Groups 1 and 2 had 58.3% and 5.6% positive samples, respectively. Percent positive samples from both treated groups increased after D5 PC and peaked on D7 PC for Group 1 and D13 PC for Group 2. After a decline in numbers of positive samples on D29 PC, an increase was observed on D36 PC to 50% in Groups 1 and 2. At least one positive sample was recovered from each group on D43 PC.

The magnitude of fecal shedding is shown in Figure 2. On D5 PC, Groups 1 and 3 shed greater than 4 logs while no *Salmonella* was detected from Group 2 fecal pools. An increase in fecal shedding was observed after D5 PC for Group 2 pigs while Group 1 pigs began shedding more *Salmonella* after D7 PC. By D36 PC, levels were low (0.06 logs) in Group 2 pigs, while Groups 1 and 3 were shedding 0.80 and 0.97 log_{10} CFU/g feces, respectively, through D43. No salmonellae were recovered from feces of pigs in Group 2 on D43 PC.

**Postmortem bacteriologic results.** Frequency of recovery of positive tissues at each necropsy is shown in Table 3. At 6 weeks PC, Groups 1 and 3 had more tissues that were
positive (35.7% and 42.9%, respectively) than Group 2 (12.5%). Group 1 showed a slight increase in numbers of positive tissues at 6 weeks (35.7%), while numbers of positive tissues for weeks 4 and 6 were the same for Group 3 (42.9%). Group 3 had the highest percentages of respiratory-associated (54.2%; tonsil, MLN, BLN, lung) and gut-associated tissues (65.5%; CLN, colon, ICLN, ICJ, Cec Cont, cecum, Mid II) that were positive. All three groups had more numbers of positive tissues from gut-associated tissues than respiratory-associated tissues.

Quantitative analyses of tonsil, lung, ICLN, ICJ and Cec Cont are shown in Table 4. Greater than 2.5 \( \log_{10} \) CFU/g of tissue were recovered from the tonsil and ICJ from all groups throughout the study. The ICJ was the tissue of predilection in Groups 2 and 3, while Group 1 had the highest numbers of \( \text{Salmonella} \) in tonsil. No salmonellae were recovered from the ICLN of any group at 6 weeks PC, while \( \text{Salmonella} \) numbers remained greater than 3 \( \log_{10} \) CFU/g of tissue in the ICJ at each necropsy. Numbers of \( \text{Salmonella} \) in cecal contents increased at 6 weeks PC for Groups 1 and 2 (1.27 \( \log_{10} \) CFU/g of tissue and 4.01 \( \log_{10} \) CFU/g of tissue, respectively), while levels decreased in Group 3 pigs (1.22 \( \log_{10} \) CFU/g of tissue). Numbers of \( \text{Salmonella} \) in the lungs increased at 4 weeks in Groups 1 and 3 but declined at 6 weeks PC.

**Serum antibody results.** IgM responses for Group 1, 2 and 3 to \( S. \text{heidelberg} \) heat extract antigen are shown in Figure 3a. Optical densities were greatest at 2 weeks PC and decreased at 4 and 6 weeks PC for all groups. IgG responses were similar for all groups at 2 and 4 weeks PC (Figure 3b). However, at 6 weeks PC, Groups 1 and 2 had lower average optical densities than Group 3.
Lymphocyte blastogenic responses. An decreased response was observed in Group 2 pigs at 6 weeks PC when compared to Group 4 pigs (Figure 4). Stimulation increased for Group 1 pigs throughout the experiment.

DISCUSSION

Following treatment with either antimicrobial, an initial reduction in numbers of *S. heidelberg* shed into the environment was observed by D5 PC for Groups 1 and 2. Treatment protocol was indicative of regular veterinary applications with ceftiofur, and a 3 day duration for enrofloxacin was considered to be appropriate. For pigs, calves and poultry, the typical range for treatment length for enrofloxacin is 3 to 5 days (3, 4). Reductions in numbers of *Salmonella* following use of antimicrobics have been reported after treatment with 112.5 mg Cephamycin C (16) or after using neomycin and oxytetracycline in feed (30). Merkt et al. (20) did not detect *Salmonella* after a treatment length of 10 days with Baytril in feed at a concentration of 200 ppm. In contrast to our study, for all of these studies, treatment length was typically longer than 3 days in order to maximize antimicrobial effect.

Although we observed an initial decrease in numbers of *Salmonella* recovered, following cessation of treatment numbers rapidly increased indicating that elimination from the host had not occurred. Persistance of *Salmonella* in the host has also been reported following use of other antimicrobics. After 26 days, Jacks et al. (16) isolated *S. choleraesuis* from the ICLN after treating experimentally infected swine with Cephamycin C (337.5 mg) for 10 days. Additionally, use of medicated feed has not been totally effective in eliminating *Salmonella*. After more than 28 days of treatment, *S. typhimurium* was still recovered from
tissues (17, 30) and feces (7, 17) following treatment with efrotomycin or oxytetracycline.

In this study, persistence may be attributed to either challenge with a high infectious
dose or a relatively short treatment length. Use of enrofloxacin for more than 3 days may be
indicated to significantly lower numbers of *Salmonella* and prevent reinfection.
Additionally, a lower infectious dose may have been more likely to be cleared. Comparable
results are noted in other studies using a $10^9$ CFU or greater dose of *Salmonella* (7, 16, 17,
30). However, Gray et al. (13) demonstrated that the clinical response and persistence of
*Salmonella* in pigs naturally exposed to infected pigs shedding less than 3 logs per gram of
feces parallels a high challenge dose. It is likely, then, that regardless of dose, *Salmonella*
will persist following treatment with antimicrobics.

Although numbers of *Salmonella* increased after D5 PC, Baytril® appeared to have a
greater effect on reducing numbers of bacteria than Naxcel®. At D5 PC, numbers of
*Salmonella* were reduced 6 logs in Group 2 compared to a 1.3 log reduction in Group 1 pigs.
This dramatic decrease of 6 logs may be attributed to the ability of enrofloxacin to act on
salmonellae which had been phagocytosed. In contrast to Naxcel®, Baytril® has been shown
to be very effective against a variety of intracellular pathogens at relatively low
concentrations (3).

Despite intracellular efficacy, persistence of *Salmonella* was observed in tissues of
Group 2 pigs. Interestingly, Group 1 and 3 pigs had low numbers of *Salmonella* in the Cec
Cont while Group 2 numbers increased by 2.8 logs at 6 weeks PC. No salmonellae were
detected in fecal pools from Group 2 at 6 weeks while average numbers of *Salmonella* in Cec
Cont were high ($4.01 \log_{10}$ CFU/g feces) and at least one pig had 1 positive rectal loop at 6
weeks. This suggests that numbers of *Salmonella* being shed may approach zero while tissue levels increase, creating a problem for presentation of animals at slaughter. Use of Naxcel was less effective in reducing numbers of *Salmonella* and persistence was observed through 6 weeks. The tissue most often cultured at the slaughter plant for *Salmonella* is the ICLN. Numbers of *Salmonella* in the ICLN from all three groups were not detected at 6 weeks PC based on quantitative analysis, while numbers in the Cec Cont remained high. Culture of the ICLN only at slaughter may lead to false negative results which suggests that both the ICLN and Cec Cont should be cultured.

Serum antibody responses observed in this study are comparable to those reported by others (12). Blastogenic responses may indicate some level of immunosuppression in Group 2 by 6 weeks PC which has been previously described (12). Antimicrobics have been shown to have both immunoenhancing and immunodepressing properties (27). The immune suppression observed in this study does not appear as marked for the challenge control group suggesting that the observed effect was due to the antimicrobial and not the infectious dose. Immunosuppression following treatment with ciprofloxacin (a fluoroquinolone) has been reported (23).

The opposite effect appears to be occurring with Naxcel in that an immunostimulation is observed at 6 weeks PC for Group 1. Since immunostimulation has been reported following treatment with cephalosporins, cefotaxime (19) or cefodizime (18, 19, 26) and an immunosuppression has been observed following exposure to *S. choleraesuis* (12), the effect observed for Group 1 may be attributed to use of the antimicrobial and not the infection.

These data indicate that while antimicrobics may initially decrease the amount of
Salmonella shed into the environment, the numbers of Salmonella shed will increase when treatment stops. Both antibiotics failed to clear a 10⁹ CFU S. heidelberg infection although Baytril® appeared to be more effective than Naxcel® at reducing overall numbers. Further in vivo studies using antimicrobics and different Salmonella serotypes are warranted.

ACKNOWLEDGEMENTS

The authors wish to thank Scott Ladely, Ruth Willson, Kathy Foss, Brad Chriswell, Aaron Lehmkuhl and Melissa Lehmkuhl for their excellent technical assistance.

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LITERATURE CITED


FIGURE LEGENDS

Figure 1 - Qualitative recovery of *Salmonella heidelberg* from combined tonsil, nasal, and rectal samples. (___ Group 1: Naxcel® treated, _ _ Group 2: Baytril® treated, --- Group 3: untreated)

Figure 2 - Quantitative recovery of *Salmonella heidelberg* from fecal pools. See Figure 1 for legends. Reported values are CFU/gram as calculated by the most probable number method for each group.

Figure 3 - a - Serum IgM responses of swine following treatment with Naxcel® (Group 1), Baytril® (Group 2) or untreated (Group 3), to *S. heidelberg* heat extract antigen. Reported values are the mean ELISA IgM specific OD.

b - Serum IgG responses of swine following treatment with Naxcel® (Group 1), Baytril® (Group 2) or untreated (Group 3), to *S. heidelberg* heat extract antigen. Reported values are the mean ELISA IgG specific OD.

Figure 4 - Two-day *in vitro* blastogenic responses to *S. heidelberg* LPS of peripheral blood lymphocytes from swine treated with Naxcel® (Group 1) or Baytril® (Group 2) or untreated (Group 3). Group 4 consisted of negative control pigs. Assays were performed on days 11, 27 and 41 PC. Error bars represent the standard error of the mean.
Table 1 - Resistance pattern of #25K5-7, swine isolate, *S. heidelberg*

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<th>Pattern</th>
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<td>= 1</td>
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\(^a\) susceptible  
\(^b\) resistant  
\(^c\) values reported in µg/ml
Table 2 - Number of *S. heidelberg*-positive tonsil and nasal swabs and rectal samples

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Table 3 - Qualitative results from tissues of pigs challenged with *S. heidelberg*

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<td>0/4</td>
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</table>

a-weeks post-challenge
b-number positive/number sampled
Table 4 - Mean populations of *S. heidelberg* recovered from tissues

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<th>Necropsy</th>
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<td></td>
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<td>ICJ</td>
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<tr>
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<td>1.22</td>
</tr>
</tbody>
</table>

a-data are expressed as log_{10} CFU/gram tissue
b-not quantifiable but positive by bacteriology
Figure 1
Figure 2
Figure 3a
Figure 3b
Figure 4

Group 1  Group 2  Group 3  Group 4

stimulation index (SI)

0  10  20  30  40  50  60  70

11  27  41
day
SUMMARY

These experiments were designed to study the pathogenesis of *Salmonella heidelberg* infections in swine. Since few studies have been published regarding *S. heidelberg* in swine, we attempted to determine the effect of different doses on shedding and persistence in swine. Additionally, we attempted to determine the effect of antibiotics on *S. heidelberg* infection in a second study.

In the first study, three groups of pigs inoculated with different concentrations of *S. heidelberg* were monitored for clinical signs of infection, qualitative analysis of tonsil and nasal swabs and rectal samples (TNR) and tissues, and quantitative analysis of shedding and tissues. Necropsies were performed three times during the study, ending at six weeks post-challenge. Our data indicated a dose dependent relationship regarding shedding, numbers of positive tissues and TNR samples. Results were atypical when compared to *Salmonella* infections with *S. typhimurium* or *S. choleraesuis*. Pigs infected with $10^3$ CFU *S. heidelberg* were still shedding by D36, whereas pigs infected with *S. typhimurium* or *S. choleraesuis* typically can clear an infection in less time.

Additionally, *S. heidelberg* produced inapparent or mild disease regardless of dose. Typically at two days post-challenge, clinical signs such as a febrile response, diarrhea, dyspnea and lethargy can be observed in pigs infected with *S. typhimurium* or *S. choleraesuis*. The differences observed in this study are problematic to the industry in that salmonellae can persist in a herd, without giving rise to clinical signs of illness, resulting in presentation of animals at slaughter that harbor salmonellae.
In the second study, pigs were inoculated with $10^9$ CFU *Salmonella heidelberg* and treated for 3 days with ceftiofur sodium or enrofloxacin after clinical signs were first observed. Similar parameters were monitored following challenge. We initially observed a reduction in numbers of *Salmonella*, with enrofloxacin most effective until D5. This may be attributed to the intracellular efficacy of enrofloxacin when compared to ceftiofur. However, when treatment ceased, shedding and numbers of positive TNR samples increased. Additionally, tissues continued to be colonized throughout the experiment, regardless of treatment. High numbers were present in tissues, primarily the ICJ and tonsil. These increases may be due to the inability of the host to clear a high dose or treatment length not being long enough to clear the infection. Additionally, it has been reported that many antimicrobics have an immunosuppressing or immunoenhancing effect on the host, thus affecting the host's ability to clear an infection.

These two studies indicate that *S. heidelberg* is capable of persisting in a host and resisting treatment, even following use of an intracellular antibiotic.
APPENDIX

Bradford Protein Assay


I. Solutions

A. 0.9% Saline (pH 7.0)

\[
\begin{align*}
\text{NaCl} & \quad 9.0 \text{ L} \\
\text{dd H}_{2}\text{O} & \quad 1.0 \text{ L}
\end{align*}
\]

B. Protein Dye Reagent Concentrate (BioRad Cat # 500-0006)

Store at 4°C. Discard after 1 year.

C. Bovine Serum Albumin Frac V Standard (1 mg/ml)

BSA (Sigma #A4503) 250 mg
0.9% Saline 250 ml

Distribute 1.0 ml aliquots in 1.5 ml microfuge tube. Store at -20°C.

II. Protocol

1. Prepare working solution of BSA by diluting frozen BSA Standard (solution C, above) 1:10 with 0.9% saline.

2. Prepare a BSA standard curve as follows:

<table>
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<th>ml of working solution</th>
<th>ml of normal saline</th>
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</table>

NOTE: Only glass test tubes may be used in this procedure.

3. Dilute test samples in normal saline so that the protein concentration will lie within the linear range of the standard curve. Dilutions of 1:10, 1:100 and 1:1000 are usually sufficient. Test samples are usually made so that the final volume is 1.0 ml.

4. Using a glass pipette, add 1 ml Protein Dye Reagent Concentrate to each dilution of the standard curve. Only 0.25 ml of dye concentrate is required for the 1.0 ml test samples. Mix. Allow color to develop for 5 min.

5. Read absorbance at 595 nm within 1 hour.

6. Plot standard curve and calculate protein concentration.
Endotoxin Preparation

by Thomas J. Stabel


1. Pellet 2 L of cells at 7,000 rpm for 15020 min at 4°C. Note: Record empty weight of each centrifuge bottle before using.

2. Resuspend bacteria in cold 0.85% saline at a concentration of 250 g/L (eg. 62.10 g - 56.61 g (empty bottle weight) = 5.49 g pellet; 250 g/1000 ml = 5.49 g/x ml, x = 21.96 ml saline).

3. Add equal volume of H₂O-saturated 1-butanol (top layer); stir 15 min.

4. Centrifuge at 35,000 x g (16,000 rpm) for 20 min.

5. Separate aqueous phase (lower) from upper phase and save aqueous phase.

6. Twice re-extract upper phase (1-butanol) and insoluble precepitate (i.e. everything else) with half the initial volume of saline. Combine aqueous extracts and centrifuge.

7. Add non-specific protease (from Streptomyces griseus; Boehringer Mannheim) in 0.2 M Na₂HPO₄ buffer, pH 7.0, to aqueous extract for a final enzyme concentration of 20 µg/ml (eg. prepare 2 mg protease in 1 ml buffer; add 0.1 ml of 2 mg/ml solution per 10 ml aqueous extract). Incubate overnight at 37°C.

8. Remove white flocculent precepitate at 10,000 x g for 40 min. Carefully remove thin insoluble layer on top of centrifuge tube by aspiration.

9. Concentrate extract by ultrafiltration with an Amicon pressure cell and a XM-100a filter (76 mm #14243; Amicon), pressure = 25-30 psi.

10. Wash extract in the Amicon cell with saline at a constant volume until effluent shows no absorbance at 260 nm. Collect effluent in 50 ml conicals (eg. started with 60 ml extract; conc. to 30 ml and then repeatedly added 30 ml volumes).

11. Dialyze extensively against H₂O, lyophilize and store at -20 to -70°C.
Lymphocyte Blastogenesis Assay

Reagents: by Thomas J. Stabel

A. Phosphate buffered saline (PBS), pH 7.2
   32 g NaCl
   4.6 g Na₂HPO₄
   0.8 g KH₂PO₄
   0.8 g KCl
   3.5 L dd H₂O
   Adjust pH to 7.2 and q.s. to 4 L with dd H₂O. Autoclave and store at 4°C.

B. Hanks Balanced Salt Solution (HBSS; w/o calcium or magnesium)
   8.0 g NaCl
   0.4 g KCl
   0.06 g Na₂HPO₄
   0.06 g KH₂PO₄
   1.0 g dextrose
   0.35 g NaHCO₃
   Adjust pH to 7.4, q.s. to 1 L with dd H₂O and filter sterilize.

C. Heparin (10,000 units/ml) dilute in PBS, pH 7.2, and filter sterilize. Store at 4°C.

D. Ficoll-Paque (Pharmacia, Cat. #17-0840-02) warmed to room temperature

E. Gentamicin solution (5 mg/ml)
   4 ml gentamicin stock (50 mg/ml; Sigma, Cat. #G-1397)
   36 ml PBS, pH 7.2 (sterile)
   Dispense 1 and 5 ml aliquots and store at -20°C.

F. Culture media (RPMI 1640 containing 25 mM HEPES, L-Glutamine, supplemented with 10% FBS and 50 µg/ml gentamicin)
   50 ml FBS (Hyclone; heat-inactivated 56°C, 30 min)
   5 ml gentamicin solution (Reagent E)
   500 ml RPMI 1640 (Gibco, Cat. #380-2400AJ; already containing HEPES and L-Gln)

G. Lysing solution (0.013 M phosphate buffered H₂O)
   80 ml 0.15 M Na₂HPO₄
   1500 ml dd H₂O
   Adjust pH to 7.2 with 0.15 M KH₂PO₄ (~4 ml/L), q.s. to 2000 ml with dd H₂O. Autoclave and store at 4°C.

H. Restoring solution (PBS, 2.7%)
   80 ml 0.15 M Na₂HPO₄
   1500 ml dd H₂O
   54 g NaCl
   Adjust pH to 7.4 with 0.15 M KH₂PO₄ (~4 ml/L), q.s. to 2000 ml with dd H₂O, Autoclave and store at 4°C.
I. $^3$H-thymidine solution (33.34 μCi/ml)  
 48.34 ml culture media (Reagent F)  
 1.66 ml $^3$H-thymidine (1 μCi/ml; Amersham Cat#TRA.120)  
Store in a clearly labeled 50 ml Blue Max tube at 4°C.

Note: Withdraw tritium from sterile duoseal vial with 1 ml tuberculin syringe and 25-g needle. All work should be done in laminar flow hood on disposable bench paper clearly labeled with radioactive tape. Dispose of solid waste in $^3$H solid waste can.

Peripheral Blood Lymphocyte (PBL) Isolation:
1. At necropsy, collect porcine whole blood in sterile 10 ml Vacutainer tubes with heparin.  
Note: At non-terminal bleeding dates, collect whole blood by jugular venapuncture using a 15 ml draw heparin Vacutainer tube with 20 gauge/1.5" needle (three tubes/animal).
2. Dilute 30 ml of whole blood (1:1) with 10 ml HBSS (w/o Ca$^{2+}$ or Mg$^{2+}$). Layer 20 ml of diluted blood over 9 ml Ficoll-Paque per 50 ml Blue Max tube (two tubes per animal).
3. Centrifuge at 400 x g (1400 rpm using Sorvall 6000R; 1300 rpm using IEC PR 7000; 1350 rpm using Beckman TJ-6, 3.8) for 30 min at 18°C with acceleration and brake "off".
4. Aspirate off and discard enough of the upper plasma layer to allow transfer of the "Buffy Coat" (i.e. PMNs) with a 10 ml pipet to a clean 50 ml Blue Max tube. Fill remainder of tube with HBSS (w/o Ca$^{2+}$ or Mg$^{2+}$).
5. Centrifuge at 450 x g (1474 rpm in Sorvall 6000R; 1400 rpm in IEC PR 7000; 1450 rpm in Beckman TJ-6, 4.0) for 10-15 min at 18°C.
6. Pour off supernatant and resuspend cells in 10 ml HBSS (w/o Ca$^{2+}$ or Mg$^{2+}$).
7. Add 10 ml lysing solution, mix, and let set for 40 sec.
9. Wash cells 2x with HBSS (w/o Ca$^{2+}$ or Mg$^{2+}$). Centrifuge cells as in Step #6.
10. Resuspend cells in ~2 ml media and count using Celltrak-3B Cell Counter. (Optional: Do Trypan Blue viability test.)

Cell Proliferation Assay:
1. Dilute porcine cells to 2.5 x 10$^6$ cells/ml in media and dispense in 96-well microtiter plates (100 μl/well; in triplicate) containing 100 μl/well of appropriately diluted mitogens and/or antigens (done in triplicate).

Examples:  
  ConA (Sigma, Cat #C-5275)  
  PHA-P (Sigma, Cat #L-9132)  
  PWM (Sigma, Cat #L-9379)  
  Salmonella endotoxin

Note: Remember to prepare mitogens and antigens 2x more concentrated than desired final concentration. As a control include media and cells.
2. Incubate plates in a 37°C, 5% CO$_2$ moisture incubator for 48 hours (Note: For T-cell proliferation studies incubate 5 days).
3. Pulse cells with 0.5 μCi/30 μl $^3$H-thymidine (Reagent I) per well. Incubate 18 h and harvest.
ELISA

Antigen Preparation by Gray and Cray 1995

<table>
<thead>
<tr>
<th>Salmonella serotypes</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhimurium</td>
<td>798</td>
<td>NADC</td>
</tr>
<tr>
<td>S. choleraesuis</td>
<td>3246pp</td>
<td>NADC/Curtiss</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>21108</td>
<td>ATCC</td>
</tr>
<tr>
<td>S. anatum</td>
<td>281-43</td>
<td>NAHMS COFE '94</td>
</tr>
<tr>
<td>S. heidelberg</td>
<td>25K5-7</td>
<td>NAHMS Swine '95</td>
</tr>
</tbody>
</table>

Rotors: SS-34 (small) - #5; GSA (large) - #10

Materials:

Luria Bertani broth 5% Tween 20
10 g tryptone 5 ml Tween 20
5 g yeast 95 ml dd H2O
10 g NaCl
Add 950 ml dd H2O, adjust to pH 7.0, q.s. to 1 L
Autooclave

Diluent Buffer
0.5 g BSA 200 μl 5% Tween 20

Phosphate Buffered Saline (0.1 M PBS) 100 ml 0.1 M PBS
2.28 g Na2HPO4 (dibasic) Block
0.46 g NaH2PO4 (monobasic) 1 g BSA
9.00 g NaCl 100 ml 0.1 M PBS
1 L dd H2O
Adjust to pH 7.2

Conjugate
Goat anti-swine IgG or IgM peroxidase conjugate
KPL, Gaithersburg, MD

Wash Buffer
1 L 0.1 M PBS Stop Solution (0.18 M)
2 ml 5% Tween 20 1 ml 18 M sulfuric acid

Substrate
TMB microwell peroxidase substrate 99 ml dd H2O
KPL, Gaithersburg, MD

To make heat extracted antigen from each strain:
1. Scrape top of -70°C frozen stock culture with a sterile cotton swab.
2. Place swab in 1 L of LB broth and incubate at 37°C, 220 rpm for 16 h.
3. Centrifuge culture at 15,000 x g (12,000 rpm) for 10 min. at 4°C and resuspend pellet in 1% original volume with 0.1 M PBS.
4. Heat resuspend cells in an erlenmeyer flask, 1 h at 60°C (5.2 on dial).
5. Centrifuge heated cells at 30,000 x g (19,500 rpm) for 6 min at 4°C. Decant and save supernatant.
6. Filter supernatant through a 0.22 μm MillexGV (Millipore, Bedford, MA) syringe filter.
7. Perform Bradford protein assay on filtered supernatant, store at -20°C until use.

Coat plates:
1. Adjust antigen concentration so that
   a. all (4) strains will be equally mixed in 0.1 M PBS
   b. 4 μg protein per well divided equally between strains (1 μg each strain/well)
   c. 5 ml/plate or 50 μl/well
2. Add the adjusted mixed antigen to Immulon 4 plates (Dynatech, Chantilly, VA)
3. Let plates stand overnight 4°C in humidor.
4. Wash plates 3x in 0.1 M PBS
5. Block plates with 100 μl 1% BSA in 0.1 M PBS, 30 min at 26°C (room temperature).
6. Wash plates 3x in 0.1 M PBS, leave last PBS wash in wells until plates are used.

Assay for optical density:
1. In a separate dilution plate, add 120 μl of diluent/well.
2. Add 5 μl of respective sera in triplicate to diluent wells.
3. Empty PBS out of coated Immulon 4 plates.
4. Mix sera and diluent 6x in the wells of the dilution plates and transfer 50 μl to coated plates using a multichannel pipettor, changing tips between rows.
5. Incubate plates at 37°C 1 h in humidor.
6. Wash 3x with 150 μl wash buffer with 1 min incubations for each wash.
8. Incubate plates at 37°C 1 h in humidor.
9. Wash 3x with 150 μl wash buffer with 1 min incubations for each wash.
10. Add 30 μl substrate to each well.
11. Incubate 10 min at 26°C.
12. Add 30 μl stop solution.
13. Read on advanced program 8 - Dynatech MR7000
   Test wavelength = 450 nm
   Reference wavelength = 410 nm
   + control SC pig = pig hypered with killed 3246pp
   - control = a known negative pig (culture)
   Blank = no serum

Program 8 - pos = F10-12
   neg = G10-12
   blank = H10-12
Program 11 - blank = F10-12
   pos = G10-12
   neg = H10-12
Susceptibility Testing - Broth Microdilution Method

Materials:

- LB broth
- LB + 30% glycerol
  - 1. autoclave glycerol 1 h
  - 2. cool
  - 3. add 30 ml to 70 ml LB
  - 4. mix well
- sterile dd H₂O (5 ml)
- cryovials, 2 ml internal thread
- blood agar plates, 5% bovine or sheep blood
- 1 μl sterile inoculating loops
- cotton swabs

The following are purchased from Sensititre:

- 10 μl sterile transfer loops cat. no.: E251586
- Mueller-Hinton broth tubes cat. no.: T3462
- substrate strips cat. no.: E1012
- dose heads cat. no.: E3010
- microtiter plates

Procedure:

A. Isolating clones
1. Streak sample onto blood agar plates for isolation
2. Incubate plates 37°C overnight
3. Pick one well-isolated colony and streak onto new blood agar plate
4. Incubate plates 37°C overnight

B. Screening for resistance
(calibrate nephelometer with each use)
1. Set up a rack with 1 water tube and 1 Mueller-Hinton (M-H) tube for each isolate
2. Add 1 substrate strip to each M-H tube for a minimum of 15 min prior to inoculation
   Note: Once substrate strips are added, broth must be used within 1 h.
3. Pick 2-6 colonies from plate using sterile cotton tipped swab and inoculate water tube
4. Vortex and adjust density according to nephelometer reading
5. Transfer inoculum from water tube to M-H tube using 10 μl loop
6. Vortex and inoculate microtiter plate using autodiluter as per manufacturer's instructions
7. Incubate plates 37°C for 18-20 h
   Note: do not stack plates more than 2 high
8. Read plates after 18 h according to manufacturer's instructions.
   Note: do not read plates after 20 h.

C. Freezing clones
1. Label cryovials with appropriate information - study, isolate number, date inoculated.
2. Add 1 ml LB + glycerol to cryovial
3. Pick 4-8 colonies from last blood agar plate using sterile 1 μl inoculating loops
4. Shake loop in media to dislodge bacteria and screw cap tightly
5. Freeze cryovials at -70°C
Susceptibility Testing - Disk Diffusion Method

Materials:

- blood agar plates, 5% sheep or bovine blood
- Mueller-Hinton agar plates
- water tubes, 5 ml
- cotton swabs
- antibiotic disks (Difco)
- wooden sticks

Procedure:

1. Swab top of frozen clone and streak onto blood agar plate
2. Incubate plates 37°C overnight
3. Pick colonies from plate with swab and inoculate water tube
4. Adjust density according to nephelometer
5. Dip swab in tube to soak with inoculum
6. Streak plate to achieve a confluent lawn, usually 3-4 times around plate
7. Place disks on plate with autodispenser or forceps
8. Press disks firmly and evenly to agar using wooden stick
9. Incubate plates, agar side up, 37°C for 18-24 h
10. Read using susceptibility testing sheets depending on antibiotic
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