RESEARCH REPORT
PORK SAFETY

Title: Effects of Subtherapeutic Antibiotics on Antibiotic Resistance and Virulence Gene Transfer in Swine Intestinal Bacteria – **NPB #08-002**

Investigator: Steve Carlson DVM, PhD

Institution: Iowa State University

Date Submitted: 12-21-09

Industry Summary:

Subtherapeutic antibiotics have been used for decades as growth promotants and prophylactic agents. The following antibiotics are approved for use a feed additives in swine: tetracyclines, roxarsone, hygromycin, tylosin, oleandomycin, penicillin G, sulfathiazole, carbadox, sulfamethazine, erythromycin, flavomycin, bacitracin, virginiamycin, lincomycin, apramycin, tiamulin, eftromycin, neomycin, tilmicosin, and florfenicol.

A recent study indicates that subtherapeutic chlortetracycline can be associated with an increase in multiple antibiotic resistance for swine intestinal microbes. Other studies also suggest that subtherapeutic antibiotics can lead to the propagation of an antibiotic resistant "superbug" (*Enterococcus*) in swine (Poole, *et al.*, 2001). On the other hand, some subtherapeutic antibiotics have not yet been associated with this problem (Donabedian, *et al.*, 2003). These three studies do not, however, address gene transfer events that precipitate resistance and virulence. That is, little is known about the effects of these antibiotics on gene transfer events in enteric bacteria.

The overall objective was to determine which subtherapeutic antibiotics contribute to the transfer of antibiotic resistance and virulence genes while also identifying the subtherapeutic antibiotics that do not contribute to this problem in swine. Three main objectives involved assessing various transfer events mediated by subtherapeutic antibiotics in the laboratory and in swine. These three transfer events involved determining the relative rates of transfer of two different types of antibiotic resistance gene "clusters" (plasmids and integrons) and one type of virulence gene cluster. Transfer was measured from a normal non-virulent (commensal) intestinal bacteria (*E. coli*) to six different pathogenic bacteria- *Salmonella*, *Yersinia*, *Pseudomonas*, *Shigella*, ETEC, and *Proteus*.

Results from these studies revealed that certain antibiotics were more likely to mediate gene transfer events. Our studies revealed numerous subtherapeutic antibiotic-mediated transfer events. The worst offending antibiotics were, in order of rank, lincomycin > apramycin = neomycin = > tylosin = florfenicol.

The results indicate that certain subtherapeutic antibiotics have a higher risk for mediating unwanted gene transfer into pathogenic bacteria. These six antibiotics include apramycin, lincomycin, neomycin, sulfamethazine, tylosin, and florfenicol. Other subtherapeutic antibiotics appear to have a less significant risk.

Scientific Abstract:

Subtherapeutic antibiotics have been used for decades as growth promotants and prophylactic agents. The following antibiotics are approved for use a feed additives in swine: tetracyclines, roxarsone, hygromycin, tylosin, oleandomycin, penicillin G, sulfathiazole, carbadox, sulfamethazine, erythromycin, flavomycin, bacitracin, virginiamycin, lincomycin, apramycin, tiamulin, eftromycin, neomycin, tilmicosin, and florfenicol.

A recent study indicates that subtherapeutic chlortetracycline can be associated with an increase in multiresistance for swine intestinal microbes. Other studies also suggest that subtherapeutic antibiotics can lead to the propagation of an antibiotic resistant "superbug" (*Enterococcus*) in swine (Poole, *et al.*, 2001). On the other hand, some subtherapeutic antibiotics have not yet been associated with this problem (Donabedian, *et al.*, 2003). These three studies do not, however, address gene transfer events that precipitate resistance and virulence. That is, little is known about the effects of these antibiotics on gene transfer events in enteric bacteria.

The overall objective was to determine which subtherapeutic antibiotics contribute to the transfer of antibiotic resistance and virulence genes while also identifying the subtherapeutic antibiotics that do not contribute to this problem in swine. Three main objectives involved assessing various transfer events mediated by subtherapeutic antibiotics *in vitro* and in swine. These three transfer events involved determining the relative rates of transfer of plasmids, integrons and the *Salmonella* pathogenicity island. Transfer was measured from commensal *E. coli* to six different pathogenic bacteria- *Salmonella*, *Yersinia*, *Pseudomonas*, *Shigella*, ETEC, and *Proteus*.

Results from these studies revealed that certain antibiotics were more likely to mediate gene transfer events. Animal studies revealed numerous subtherapeutic antibiotic-mediated transfer events. The worst offending antibiotics were, in order of rank, lincomycin > apramycin = neomycin = sulfamethazine > tylosin = florfenicol.. Sulfamethazine mediated an increase in transfer of both a plasmid and an integron. Lincomycin, apramycin, and neomycin caused an increase in the transfer of two different plasmids whereas sulfamethazine mediated an increase in transfer of just on plasmid type. Lincomycin mediated the highest increase in plasmid transfer.

The results indicate that certain subtherapeutic antibiotics have a higher risk for mediating unwanted

gene transfer into pathogenic bacteria. These six antibiotics include sulfamethazine, lincomycin, apramycin, neomycin, tylosin, and florfenicol. Other subtherapeutic antibiotics appear to have a less significant risk.

Introduction:

(A) Subtherapeutic Antibiotics

Subtherapeutic antibiotics have been used for decades as growth promotants and prophylactic agents. The following antibiotics are approved for use a feed additives in swine: tetracyclines, roxarsone, hygromycin, tylosin, oleandomycin, penicillin G, sulfathiazole, carbadox, sulfamethazine, erythromycin, flavomycin, bacitracin, virginiamycin, lincomycin, apramycin, tiamulin, eftromycin, neomycin, tilmicosin, and florfenicol.

A recent study indicates that subtherapeutic chlortetracycline can be associated with an increase in multiresistance for swine intestinal microbes (Funk, *et al.*, 2006). *Ex vivo* studies also suggest that subtherapeutic antibiotics can lead to the propagation of an antibiotic resistant "superbug" (*Enterococcus*) in swine (Poole, *et al.*, 2001). On the other hand, some subtherapeutic antibiotics have not yet been associated with this problem (Donabedian, *et al.*, 2003).

These three studies do not, however, address gene transfer events that precipitate resistance. That is, little is known about the effects of these antibiotics on gene transfer events in enteric bacteria.

(B) Integrons

Integrons are large (43-kb) segments of mobilizable DNA that can intercalate into the genome of bacteria. For *Salmonella*, an integron (designated as SgI-1) confers resistance to ampicillin, florfenicol, streptomycin, sulfonamides, and tetracycline. SgI-1 is also a problem since it also contains genes involved in hyperactivating virulence mechanisms for *Salmonella* (Carlson, *et al.*, 2007; Rasmussen, *et al.*, 2005). Thus SgI-1 is related to both antibiotic resistance and virulence.

SgI-1 originated in *Photobacterium piscicida* (Kim, *et al.*, 1996) and it appears to have entered *Salmonella* via a P22-like bacteriophage-mediated general transduction event (Schmieger, *et al.*, 1999). SgI-1 continues to be found in new *Salmonella* serotypes and the current list includes Agona, Albany, Newport, Meleagridis, Paratyphi B, Emek, Cerro, Derby, Dusseldorf, Indiana, Kentucky, Infantis, Kiambu, Senftenberg, Typhimurium (Mulvey, *et al.*, 2006), and Choleraesuis (unpublished observations) serotypes. SgI-1 can be transferred between *Salmonella* serotypes, between *Salmonella* and *E. coli* (Doublet, *et al.*, 2005), and it has been detected in *Proteus* (Ahmed, *et al.*, 2007). The genetic relatedness between *Salmonella*, *E. coli*, and *Shigella* suggests that the latter would also be an integron recipient. Integrons have also been detected in *Pseudomonas* (Weldhagen, 2004) thus it appears that many Gram negative bacteria can receive SgI-1.

Environmental factors that govern SgI-1 transfer are not known. The movement of integrons is a bacteriophage-mediated event and numerous studies have implicated antibiotics as activators of prophage

elements resulting in transduction. The most striking example is the carbadox-mediated transduction of virulence genes in Shiga toxin-producing *E. coli* (Köhler, *et al.*, 2000). Antibiotics can also activate bacteriophage that transduce antibiotic resistance genes in *Brachyspira hyodysenteriae* (Matson, *et al.*, 2007), the etiologic agent for swine dysentery.

(C) Plasmids and Conjugation

While SgI-1 can confer multiresistance in one single transfer event, commensal *E. coli* also can transfer conjugative plasmids such as those conferring resistance to extended-spectrum beta-lactams like ceftiofur (Zhao, *et al.*, 2005; Zhao, *et al.*, 2001). Also of importance are conjugative plasmids encoding resistance to amikacin (Tosini, *et al.*, 1998) and fluoroquinolones (Hopkins, *et al.*, 2007). *E. coli* can conjugate with *Salmonella* (Leite, *et al.*, 2005), *Yersinia* (Strauch, *et al.*, 2003), *Pseudomonas* (Fialho, *et al.*, 1991), *Proteus*, *Shigella* (Mach, *et al.*, 1982), and many other bacteria.

As with the transduction of integrons, conjugation can be modulated by antibiotics. Tetracycline can regulate the transfer of a plasmid from *Bacteriodes* to *E. coli* (Whittle, *et al.*, 2002) while kanamycin, tetracycline, and ampicillin can regulate *E. coli* to *E. coli* conjugation (Cooper, *et al.*, 2000).

Conjugation is an orchestrated event requiring an interplay between donor and recipient bacteria. The donor strain must prepare the plasmid for export and alert the recipient strain of an incoming plasmid (Gomis-Ruth, *et al.*, 2006). This inter-bacterial signaling is dependent upon the donor bacteria releasing a peptide that will activate gene expression in the recipient strain. These gene expression changes have been identified in some bacteria (e.g. *Enterococcus* (do Carmo de Freire Bastos, *et al.*, 1998)) although the *E. coli* peptide sex pheromone and the *Salmonella* response-regulator have not been identified.

(D) Transfer of Pathogenicity Islands in *Salmonella*

SPI-1 is the main virulence determinant in *Salmonella* (Finlay, *et al.*, 1997) although little is known about the transfer events that led to the acquisition of this pathogenicity island. The size of SPI-1 (~40kb) suggests a lambda bacteriophage may have been involved in the introduction of this virulence determinant. Since *Salmonella* contain many lambda prophage elements (Slominski, *et al.*, 2007), it is possible that these elements can be recrudesced thus leading to transduction into an SPI-1 negative recipient. SPI-1(-) *Salmonella* have been identified (Ginocchio, *et al.*, 1992) and Dr. Carlson's laboratory has several including serotype Seminole.

Objectives:

The overall objective was to determine which subtherapeutic antibiotics contribute to the dissemination of antibiotic resistance and virulence genes while also identifying the subtherapeutic antibiotics that do not contribute to this problem in swine. Specific objectives are as follows:

Objective 1: To evaluate the possibility that subtherapeutic antibiotics can induce plasmid conjugation from commensal bacteria to pathogenic bacteria such as *Salmonella*. This objective addresses the hypothesis that subtherapeutic antibiotics can facilitate the conjugal transfer of antibiotic resistance plasmids from commensal to pathogenic bacteria in the swine intestine.

Objective 2: To evaluate the possibility that subtherapeutic antibiotics can activate the secretion of conjugation-related pheromones from multiple antibiotic resistant *Salmonella*. This objective addresses the hypothesis that subtherapeutic antibiotics can mediate the secretion of peptide sex pheromones that are required for the conjugal transfer of antibiotic resistance plasmids from commensal to pathogenic bacteria in the swine intestine. THIS OBJECTIVE WAS NOT COMPLETED.

Objective 3: To evaluate the possibility that subtherapeutic antibiotics can mediate integron transduction from one *Salmonella* serotype to another. This objective addresses the hypothesis that subtherapeutic antibiotics can facilitate the transduction of antibiotic resistance integrons in the swine intestine.

Objective 4: To evaluate the possibility that certain subtherapeutic antibiotics can activate the transfer of pathogenicity islands from pathogenic *Salmonella* to avirulent *Salmonella*. This objective addresses the hypothesis that subtherapeutic antibiotics can facilitate the transduction of *Salmonella* pathogenicity islands in the swine intestine.

Objective 5: Use the information from Objectives to identify "low risk" and "high risk" antibiotics. This objective addresses the hypothesis that some subtherapeutic antibiotics are less likely to cause the dissemination of antibiotic resistance and virulence genes in the swine intestine.

Materials & Methods:

Objective 1: Evaluating Conjugation

The first step of Objectives 1-4 involved surgically removing intestinal "loops" from swine. The next step in Objective 1 involved adding the following to the ligated loop: swK12-MDR, a swine-adapted commensal strain of *E. coli* (swK12) containing conjugative plasmids encoding resistance to ceftiofur (McCuddin, et al., 2006), amikacin (Carlson, *et al.*, 2002), and fluoroquinolones (Gay, *et al.*, 2006); six pathogenic bacteria (*Proteus*, *Shigella*, *Pseudomonas*, enterotoxigenic *E. coli* (ETEC), *Yersinia*, and *Salmonella*); placebo or one of fifteen subtherapeutic (we were not able to procure oleandomycin and flavomycin) antibiotics at concentrations analogous to that found in feed (1ug/ml).

Pathogenic bacteria will be fluorescently labeled with tetramethyl rhodamine isothiocynate in order to distinguish them from native background flora and from swK12. Dr. Carlson's laboratory has experience with ceftiofur (McCuddin, et al., 2006) and amikacin plasmids (Carlson, et al., 2002), with all six of the pathogens (Carlson, *et al.*, 1999) including fluorescently-labeled *Salmonella* (Carlson, *et al.*, 2002), and with ligated loops (Wu, et al., 2002).

The next step involved removing the mixture and incubate it on media that selects for each pathogen and for each antibiotic resistance encoded by the conjugative plasmids. Selective media (all available from Becton Dickinson or MG Scientific) will entail CHROMagar Orientation agar (*Proteus*), Chromogenic Shigella spp. agar (*Shigella*), Pseudomonas Aeruginosa Selective agar (*Pseudomonas*), TSB-GN (a 1:1 mixture of trypticase soy broth and GN Hajna broth for ETEC), BIN agar (*Yersinia*), and brilliant green sulfa agar (*Salmonella*) each containing ceftiofur, amikacin, or enrofloxacin at breakpoint concentrations (CLSI, 2001). Fluorescent CFUs were enumerated using these selective media.

The next step involved determining the frequency of conjugation for each of the three variables, i.e. pathogen recipient (n=6), subtherapeutic antibiotic or placebo (n=21), and resistance (n=3). For each of the variables, frequency was determined by dividing the number of transconjugates recovered by the number of recipient CFUs added to the assay. Statistical analysis entailed an analysis of variance using Scheffe's F test for multiple comparisons. This allowed for comparisons between pathogens, between subtherapeutic antibiotics, and between resistances.

Objective 2: Identifying Sex Peptide Pheromones

The subtherapeutic antibiotic with the highest conjugation frequency was used in a proteomic-based search for novel peptides secreted by *E. coli*. Specifically, *E. coli* swK12 and lincomycin were incubated with explants and secreted proteins (secretome) were recovered from the mixture in the lumen of the explant. Two-dimensional polyacrylamide gel electrophoresis was used to separate the proteins based on their isoelectric focusing points and molecular weights. Specifically, the mixture were centrifuged in order to pellet bacteria so that cell-free supernatants can be analyzed. Supernatants will then be TCA-precipitated (as per (Penheiter, *et al.*, 1997)) followed by resolution on 4-20 % SDS-PAGE gels. The gels were stained with Sypro Ruby and found to be unusable because of the abundance of proteins in both principle and control samples.

Objective 3: Evaluating SgI-1 Transfer

The first step in this phase involved surgically removing intestinal loops from swine. The next step involved adding the following to the ligated loop: *Salmonella* spp. containing SgI-1; six pathogenic bacteria (*Proteus*, *Yersinia*, and a mixture *Salmonella* serotypes with no history of SgI-1); and placebo or one of the fifteen subtherapeutic antibiotics at concentrations analogous to that found in feed (1ug/ml). Pathogenic bacteria were fluorescently labeled with tetramethyl rhodamine isothiocynate in order to distinguish them from native background flora.

The next step involved removing the mixture and incubate it on media that selects for each pathogen and two (ampicillin (50ug/ml) and florfenicol (25ug/ml)) of the five resistances encoded by SgI-1. Selective enumeration was performed as described in Objective 1. Recovered bacteria were subjected to a PCR assay that detects SgI-1 (Carlson, et al., 1999).

The next step involved determining the frequency of transduction for both variables, i.e. six recipients and 16 treatments (subtherapeutic antibiotic or placebo). For each of the variables, frequency was determined by dividing the number of transductants recovered by the number of recipient CFUs added to the assay.

Objective 4: Evaluating SPI-1 Transfer

The first step in this phase was to surgically remove intestinal loops from swine. The next step involved adding the following to the ligated loop: *Salmonella* spp. containing SPI-1; *Salmonella betiocky* that lacks SPI-1; and placebo or one of the fifteen subtherapeutic antibiotics at concentrations analogous to that found in feed (1ug/ml). *Salmonella betiocky* was fluorescently labeled with tetramethyl rhodamine isothiocynate in order to distinguish from the donor *Salmonella* containing SPI-1.

The third step involved washing the lumen of the explant in order to remove non-invasive bacteria. The explant was then be homogenized and fluorescent *Salmonella* were isolated using brilliant green sulfa agar. These potentially invasive *S. betiocky* subclones will then be subjected to a florfenicol protection assay that measures invasion of *Salmonella* into eukaryotic cells (Carlson, et al., 2000). Subclones were designated as invasive if the rate of cellular entry exceeds 10% for BHK cells.

Objective 5: Ranking Antibiotics

Each subtherapeutic antibiotic will be ranked based on the frequency of individual transfer events. In order to ascribe an overall risk to each subtherapeutic antibiotic, each antibiotic was ranked based on the number transfer events mediated. Enhanced transfer was ascribed when the frequency of transfer increased greater than 10-fold relative to antibiotic-free controls.

Results:

③Objective 1:

As shown in Table 1, plasmid transfer was exacerbated by four different subtherapeutic antibiotics in *Yersinia* and *Proteus*. For both pathogens, the transferred plasmid contained a gene encoding an extended-spectrum beta-lactamase (ESBL). No antibiotic had an effect on plasmid transfer in the other four pathogens or the other two plasmids.

Recipient Strain	Plasmid(s)	Subtherapeutic Antibiotic(s)	Transfer Frequency (% of control)
Salmonella, Pseudomonas, ETEC, and Shigella	All plasmids	All antibiotics	13-110%
Yersinia	Amikacin resistance plasmid	All antibiotics	55-103%
	ESBL plasmid	Sulfamethazine, lincomycin, apramycin, and neomycin	52,000-156,000%
	qnr plasmid encoding fluoroquinolone resistance	All antibiotics	77-112%
Proteus	plasmid encoding amikacin resistance	All antibiotics	16-98%
	ESBL plasmid	lincomycin, apramycin, and neomycin	104,000-156,000%
	ESBL plasmid	other antibiotics	50-123%
	qnr plasmid encoding fluoroquinolone resistance	All antibiotics	34-108%

③Objective 2: We were unable to identify any sex peptide pheromones.

③Objective 3:

As shown in Table 2, SgI-1 transfer was exacerbated by three different subtherapeutic antibiotics in *Yersinia*. No antibiotic had an effect on SgI-1 transfer in the other five pathogens.

Recipient Strain	Subtherapeutic Antibiotic(s)	Transfer Frequency (% of control)
Salmonella, Proteus, Pseudomonas, ETEC, and Shigella	All antibiotics	5-144%
Yersinia	Tylosin, sulfamethazine, and florfenicol	1,900-2,200%
	Other antibiotics	56-118%

③Objective 4:

No antibiotic had an effect on SPI-1 transfer in *Salmonella*.

③Objective 5:

As shown in Table 3, six antibiotics were ranked based on the transfer events mediated by each drug. Rankings were based on the number of transfer events exacerbated by the drug.

Subtherapeutic Antibiotic	**ESBL plasmid transfer**	**SgI-1 transfer**	**Overall Risk**
Apramycin	*Yersinia* and *Proteus*		High
Bacitracin			Low
Carbadox			Low
Erythromycin			Low
Florfenicol		*Yersinia*	Moderate
Hygromycin			Low
Lincomycin	*Yersinia* and *Proteus*		High
Neomycin	*Yersinia* and *Proteus*		High
Penicillin			Low
Roxarsone			Low
Sulfamethazine	*Yersinia*	*Yersinia*	High
Sulfathiazole			Low
Tetracycline			Low
Tylosin		*Yersinia*	Moderate
Virginiamycin			Low

Discussion:

Our findings indicate that certain subtherapeutic antibiotics can enhance gene transfer events in pathogenic bacteria present in the swine intestine. Apramycin, lincomycin, neomycin, and sulfamethazine were the most likely to mediate these events. Sulfamethazine mediated two gene transfer events (plasmid and SgI-1) and exhibited the highest increase in gene transfer. The other three antibiotics mediated plasmid transfer in two different pathogens. Florfenicol and tylosin were deemed as moderate inducers since these drugs enhanced the transfer of only SgI-1 in one pathogen. The other nine antibiotics did not mediate any of the gene transfer events.