Title:	Risk Factors for the Occurrence of drug resistant Salmonella spp. in commercial swine production **NPB #02-141**
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Date Received:	April 27, 2004

Abstract: We studied *Salmonella* isolates from collected from 132 Midwestern swine herds. A total of 1,558 *Salmonella* isolates were detected in lymph node samples collected at slaughter. As measured by a laboratory test, the antimicrobial drugs to which these isolates were most commonly resistant were tetracycline (52.7%), sulphamethoxazole (41.8%), streptomycin (37.7%). Less than one percent of isolates were resistant to ciprofloxacin, amikacin, ceftriaxone, trimethoprim / sulphamethoxazole, nalidixic acid and ceftiofur. Resistance to more than one antimicrobial was found for 47.4% of isolates, with 14.2% of isolates resistant to five or more antimicrobials. Tetracycline resistance was associated primarily with one gene, tet (C), and resistance to tetracycline was also associated with resistance to several other antimicrobials. Detection of this tetracycline gene was not associated with the use of tetracyclines in the particular market group of pigs studied, suggesting that tetracycline resistance genes, once acquired, are not quickly lost in a population.

Farm factors associated with increased risk of antimicrobial resistance in *Salmonella* were using more than one nursery barn to provide pigs to a grower/finisher barn and providing feed in a pelleted form. Identification of these risk factors provides guidance for further research to characterize ways to control shedding of drug resistant *Salmonella*.

Introduction: Antimicrobial resistance in bacteria from swine has been documented.[1 2] Resistance to one or more antimicrobials is common, with resistance to tetracycline most common (48%) in slaughter isolates. Also of concern is the emergence of strains that are resistance to multiple antibiotics. Recently human cases apparently associated with pork consumption have resulted in human fatality.[3]

Public awareness has also risen dramatically. Concern over the occurrence of antimicrobial resistance in bacteria from food producing animals has led some to call for the banning of subtherapeutic use of antimicrobials. In the European Union this has been scheduled, and pig producers from one leading pork producing country, Denmark, have voluntarily elected to eliminate the use of subtherapeutic antibiotics, based in part on these concerns.

Previous work in our lab has documented that Salmonellae are commonly found in U.S. pig herds. Of 4,126 samples from 70 farms, 422 samples were *Salmonella* positive from 294 pigs and 55 farms. All isolates were susceptible to Amikacin, Cefotaxime, Ceftiofur and Ciprofloxacin, drugs important for human therapy.[1] Resistance was commonly detected to tetracycline, sulfa drugs and penicillin, drugs commonly used in pork production. Herds reporting therapeutic use of tetracyclines in the final growing phase had higher proportion of resistant *Salmonella* isolates (68.3%) than did herds that did not report use of tetracyclines (48.3%) ($p=0.049$).[4] However, the statistical power to detect relationships in this dataset was limited because of the small number of herds in study. Further exploration of questions related to the route and duration of antimicrobial use was not possible for this reason.

In a second study of 141 herds, we identified 84 with *Salmonella* in lymph node at slaughter (60%). The apparent average within-herd prevalence was 9.9%.[5] Results from samples acquired from these herds are reported in the current report.

Methods to identify tetracycline resistance genes present in *Salmonella* found on swine farms were developed in our labs. Polymerase chain reaction amplification primers targeting the efflux mechanism of tetracycline resistance determinants have been developed.[6] Of 11 genes considered, only TetA and TetC genes were prevalent in *Salmonella* isolates from swine.

Objectives:

a. Describe the phenotypic (expressed) antimicrobial resistance patterns of 1,300 *Salmonella* cultured from 200 U.S. farms

b. Compare phenotypes to genotypes for tetracycline resistance among 600 *Salmonella* isolates

c. Describe the characteristics of farms with multiple resistance vs. single or no resistance to antimicrobials

Materials & Methods: Based on two completed studies, *Salmonella* isolates were identified and stored from 132 *Salmonella* positive farms of 141 Midwestern farms that were studied. In total, 1,558 isolates were identified and stored in –70F freezers. The source herds for these samples were described by a written survey. Characteristics of farms include size, hygiene and biosecurity practices, pig flow management, flooring, environmental control, feed characteristics, and therapeutic and growth promoting antimicrobial use in feed and water.

Phenotypic antimicrobial resistance patterns were tested using the Sensititre system (SensititreTM, Trek Diagnostic, Westlake, OH) with the National Antimicrobial Resistance Monitoring System (NARMS) panel of antimicrobials. Plates were custom made with 17 antimicrobics: amikacin, amoxicillin / clavulanic acid, ampicillin, apramycin, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole, tetracycline, and trimethoprim/sulphamethoxazole.

The protocol was that used by the USDA Agricultural Research Service for the NARMS system, and was modified from a published protocol.[7] Briefly, isolates are streaked onto a blood agar plate and incubated overnight. On day two, after observing for signs of pure growth, each clone was inoculated onto a 96-well test plate with standardized amount of an antimicrobial in each well. Plates are produced by a commercial vendor (TREK Diagnostics, Inc.) and have been used in other agricultural food safety research.[8] These plates are designed to provide decreasing amounts of the antimicrobial in a series of wells. A minimum inhibitory concentration has been defined elsewhere to indicate sensitivity / resistance of the isolate to the specific antimicrobial.[9]

Isolates that are able to grow in wells that have in excess of this concentration are classified resistant to that particular antimicrobial. Two to six colonies were selected, then a standard turbidity (0.5 McFarland Turbidity Standard) was achieved in sterile water tubes. Ten microliters of inoculated water was then transferred to its corresponding Mueller Hinton broth tube containing a substrate strip. Ten microliters was then transferred to each of the 96 wells in the antimicrobial test plate. The plate was sealed, then incubated overnight (18-24 hours) and read using the Sensititre reader. Data were compiled into an electronic database for further analysis.

A survey of farm characteristics, herd management factors, and use of antimicrobials in feed and water during the growing and finishing stages of production were assessed by a written survey. The 14 page survey consisted of questions covering husbandry, facilities, pig management and flow, and antimicrobial use.

Data from the prior risk factor assessments have already been stored electronically and identified to the farm and date of sample collection. Data from antimicrobial resistance phenotypes was be coded by antimicrobial agent and individual strain. These data were summarized by the prevalence of organisms resistant to one or more antimicrobials, and by the number strains that are resistant to more than one antimicrobial. Special attention was drawn to isolates that are "penta-resistant" to the five antimicrobials tetracycline, sulfonamide, chloramphenicol, penicillin. The associations between the specific antimicrobial usage and the occurrence of resistance among *Salmonella* isolates were assessed by conditional logistic regression, adjusting for the clustering effect of herd of origin.

Tetracycline resistance can be coded by a group of genes regulating tetracycline efflux pumps. These genes are found almost exclusively in gram-negative bacteria. Polymerase chain reaction (PCR) based methods were used to identify these genes.[6] PCR amplification primers targeting genes in this family have been developed for a number of genes, including TetA, TetB, TetC, TetD, TetE, TetG, TetH, TetJ, TetY, TetZ and Tet30. After testing for these genes on several hundred samples, three, namely tet(A), tet(B) and tet(C) were found to account for nearly all the genes of this class detected in *Salmonella* isolated from Midwestern pig farms. All *Salmonella* samples were tested for the presence of these three.

The associations between genes coding for tetracycline resistance and observed (phenotypic) resistance to tetracycline were examined for each gene detected. Linkages between tetracycline genes, the *in vitro* expression of antimicrobial resistance (i.e., the antimiogram resistance phenotype) and the use of antimicrobials were investigated. Conditional logistic regression, adjusted for the clustering of samples within herds, was completed for analysis linking herd-level risk factors to the *Salmonella* isolate genes and antibiogram phenotypes (Egret, Cytel Software, Inc., Cambridge, Massachusetts, U.S.A). Associations are reported only for those factors with a statistical p-value <0.05.

Results:

Objective 1: Describe the phenotypic (expressed) antimicrobial resistance patterns of 1,300 Salmonella cultured from 200 U.S. farms

A total of 1588 isolates were assayed. At least one isolate was found resistant to each of the antimicrobials tested. Those with 5% or more of isolates resistant were tetracycline (52.7%), sulphamethoxazole (41.8%), streptomycin (37.7%), ampicillin (16.6%), chloramphenicol (9.1%), and kanamycin (8.6%). Less than one percent of isolates were resistant to ciprofloxacin, amikacin, ceftriaxone, trimethoprim / sulphamethoxazole, nalidixic acid and ceftiofur. Resistance to more than one antimicrobial was found for 47.4% of isolates, with 14.2% of isolates resistant to five or more antimicrobials. Resistance to the five antimicrobial combination Ampicillin, Chloramphenicol, Sulphamethoxazole, streptomycin, and tetracycline, ACSuST, a pattern reported commonly in a virulent strain of *Salmonella* typhimurium, phage type DT 104, accounted for 6.4% of all isolates. A second penta resistant pattern previously reported in swine isolates, resistance to amikacin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline, was detected in 5.4% of isolates.

Of the 1598 isolates, serotypes had been determined for 922 isolates, based on funding obtained elsewhere. Antimicrobial resistance patterns varied by serotype. Among the serotypes with ten or more isolates, the serotype with the highest proportion of resistance to five or more of any combination of antimicrobials was *S. London* (52% of *S. London* isolates), followed by *S. typhimurium (Copenhagen)* (48% of *S. typhimurium (Copenhagen)* isolates) and *S. Muenchen* (42% of *S. Muenchen* isolates). Other serotypes with more than 10% of isolates resistant to five or more antimicrobials were *S. typhimurium* and *S. Heidelberg*.

Objective 2: Compare phenotypes to genotypes for tetracycline resistance among 600 Salmonella isolates.

Although we agreed to include 600 isolates in our project, a total of 1431 *Salmonella* isolates were tested for tetracycline resistance genes in a separately funded project .[10] Since these were a subset of the 1,558 samples studied for objective 1, we were able to compare all geno- and phenotypes for all 1431 isolates. Tetracyline resistance was observed in 50.9% of these isolates, while 1.4% were intermediate in sensitivity. The tet(A), tet(B) and tet(C) genes were detected in 18.6%, 1.6% and 66.4% of isolates, respectively. When broken down by genetic pattern, resistance / intermediate resistance was found in the following proportion of isolates: A-,B-,C-, 23.0%; A+, B-, C-, 93.1%; A+, B-, C+, 94.7%; and A-, B-, C+, 69.7%. The percentage of bacteria with resistant phenotypes, but for which no resistance gene was found was 6.2%. The percentage of isolates with one or more gene detected but with susceptible phenotypes was 29.4%. Because of the small proportion of isolates with tet(B) detected, further analysis was not conducted for this gene. The odds of expressing the tetracycline resistance phenotype were higher for isolates with the tet(A) (OR = 15.4) and tet(C) (OR = 3.8) genes, when compared to isolates where the genes were not detected. There was no statistical evidence of interaction between the genes. [11]

Objective 3: Describe the characteristics of Salmonella positive farms with multiple resistances vs. single or no resistance to antimicrobials.

Survey respondents classified the use of each antimicrobial administered as either therapeutic or growth promoting. Where respondents also provided the dose (g/ton feed or mg/lb body weight) of the antimicrobial provided, this dose was compared with manufacturer label claims. (Table 1) If a discrepancy was found between the label claim (therapy vs. growth promotion) and the survey response, the application was reclassified to match the label claim.

Table 1. Label claims for antimicrobials provided in swine feeds.

Antimicrobial	*Application*	*Dose ppm (g/ton)*
Bacitracin	Growth promotion	11-33 ppm (10-30 g/ton)
	Therapy	275 ppm (250 g/ton)
Carbadox	Growth promotion	11-27.5 ppm (10-25 g/ton)
	Therapy	55 ppm (50 g/ton)
Chlortetracycline	Growth promotion	11-55 ppm (10-50 g/ton)
	Therapy	55-110 ppm (50-100 g/ton)

		11 ppm (10 g/ton)[1]
		22 mg / kg bw (10 mg/lb)
Oxytetracycline	Growth promotion	11-55 ppm (11-50 g/ton)
		22 mg / kg bw (10 mg / lb bw)
	Therapy	55-165 ppm (50-15 g/ton)
Lincomycin	Growth promotion	22 ppm (10 g/ton)
	Therapy	44 or 110 ppm (40 or 100g/ton)
Tylosin	Growth promotion	11-44 ppm (10-40 g/ton)
	Therapy	110 ppm (100 g/ton)
Bambermycins	Growth promotion	2.2-4.4 ppm (2-4 g/ton)
Virginiamycin	Growth promotion	5.5-11 ppm (5-10 g/ton)
	Therapy	110 ppm (100 g/ton)

Valid survey responses to questions on antimicrobial use were returned for 108 herds. Antimicrobials were used in the feed or water during the finishing phase in 106 herds. The most commonly used antimicrobials in feed and water were bacitracin, tylosin and chlortetracycline. These drugs were provided for 58.1%, 50.7%, and 48.2% of groups of pigs, respectively.

Among *Salmonella* positive farms, combining pigs from more than one nursery room to a single growing-finishing barn was associated with increased detection of resistance to one or more antimicrobials (OR = 18). Resistance to one or more antimicrobials was not associated with the current use of antimicrobials. Use of several antimicrobials was associated with increased risk of finding *Salmonella* resistant to more than one antimicrobial (multiple resistance) at the univariate level. However, these associations were not statistically significant when pelleting of feed was included in the model. Pellets were associated with an increased risk of finding multiply resistant *Salmonella* isolates (OR = 6.8). Finding resistance to five or more antimicrobials was associated multiple nurseries (OR = 7.7) and pelleting feed (OR = 13.3). In addition, inclusion of tylosin at a therapeutic dose was associated with a reduced risk of resistance to five or more antimicrobials (OR = 0.2)

Discussion: *Salmonella* bacteria are commonly found on commercial pig farms. Resistance to antimicrobials was common among *Salmonella* isolated from the pigs studied pigs. Since *Salmonella* can cause foodborne infection in humans by transmission down the pork chain, finding a high proportion of resistant *Salmonella* increases the risk of transmitting resistant infections to humans. However, most of the resistance detected was to antimicrobials that are not of critical importance in human medicine, but are commonly used in pig production. Tetracyclines and sulfa drugs are commonly used on farms, and many *Salmonella* isolates were resistant to these. In contrast, *Salmonella* isolates were nearly all susceptible to drugs more critical drugs for human therapy, including ciprofloxacin and cefatriaxone. As a consequence, these organisms are susceptible to and should be treatable by these human-only use drugs, should human infection with pork derived strains occur.

The lack of association between antimicrobial use and the most commonly detected tetracycline resistance genes suggests that tetracycline resistance, one acquired, appears not to rapidly be lost. The prior history of tetracycline use on these farms was not described, so it is not known how long it had been since an antibiotic was used on a farm. However, tetracyclines were commonly used among these farms. It is

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[1] Therapeutic and growth pomotin label doses overlap for this drug. For these analyses, 11 ppm (10 g/ton) was considered growth promoting.

possible that farms which at the time of the study were not using tetracycline may have used them in the past.

The presence of the most commonly found tetracycline resistance gene, tet(C), was associated not only with resistance to tetracycline, but also a number of other antimicrobials. This suggests that the tet(C) gene may be closely linked to genes coding for resistance to other antimicrobials. If this were the case, administering tetracycline would be expected to increase the selective pressure favoring *Salmonella* strains resistant to multiple antimicrobials. Antimicrobial resistance to tetracyclines and the tet(C) gene is common in growing pigs. The tet(C) gene is associated with phenotypic resistance to multiple antimicrobials. Current antimicrobial use is not an important predictor of tetracycline resistance genes.

Pelleting of finisher diets was most consistently associated with increased risk of finding resistant *Salmonella* isolates. Although pelleted diets have previously been associated with increased risk of shedding generic *Salmonella*, this is the first report of increased risk of antimicrobial resistance.

Introducing pigs from more than one source or from an outside source appears to increase the risk of spread and/or multiplication of antimicrobial resistant *Salmonella*. Having more than one source of pigs for a growing / finishing barn may increase the diversity of *Salmonella* strains introduced to a barn. It is also likely that the immune status of two groups housed separately would differ. Mixing these groups may then allow strains common in one group to spread to the other. It may be that these production systems also differ on other, unmeasured factors that could contribute to increased shedding of antimicrobial resistant *Salmonella*.

Lay Interpretation: Resistance to two antimicrobial drugs frequently used in swine production, tetracycline and sulfa drugs, was common among *Salmonella* isolates from commercial farms. Since these isolates were from apparently healthy pigs, this resistance may not be important for therapy of disease in pigs. However, administering these drugs could result in increased prevalence of these strains, and potentially increase the number of *Salmonella* in slaughtered pigs. The presence of antimicrobial resistant strains probably increases the risk of passing antimicrobial resistant strains down the food chain, especially if the farms administer antibiotics to which these strains are resistant.

Very few isolates, less than one percent, were resistant to antimicrobials more important for human therapy, including ciprofloxacin, amikacin, ceftriaxone, and trimethoprim / sulphamethoxazole. Thus, the human infections by these strains should be responsive to therapy, should foodborne infection occur.

The presence of one gene accounted for the majority of tetracycline resistance. However, this gene was apparently linked to resistance genes or factors for a number of antimicrobials. Therefore, the use of tetracycline has the potential to select for *Salmonella* strains resistant to multiple antimicrobials. Farmers could, therefore, inadvertently select for multi-resistant strains when using only one antimicrobial, such as tetracycline. This could potentially compromise therapy on farms and pose an increased risk to pork food safety.

Increased mixing of pigs appears to increase the risk of resistant strains, since multiple nurseries flowing to a single grower / finisher increased the risk of finding resistant *Salmonella* strains. The biological reasons for this cannot be determined by this research method, but bears further research. One possible cause is simply from mixing of pigs with more diverse *Salmonella* strains from separate populations. Other causal factors, not recorded by the survey, may also be associated with these farm types.

Increased barn size, but not increased farm size, was associated with increased risk of antimicrobial resistant *Salmonella*. The presence of larger numbers of animals in a single barn may result in an increased variety of strains of bacteria at placement, increasing the likelihood of starting with more resistant strains. In addition, larger barns may be managed in different ways from smaller barns. These associated management factors could cause increased spread of antimicrobial resistant strains.

Feeding pelleted feed was associated with antimicrobial resistance. Pelleted feed has been also associated elsewhere with increased risk of *Salmonella* shedding. Coupled with the findings here, additional research on the mechanisms by which pelleted diets might cause (or be associated with) these increased risks is warranted.

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