RESEARCHREPORT
PORK SAFETY

Title: Effect of antibiotic regimens on resistance in salmonella and other bacteria in swine **- NPB# 01-033**

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I. Abstract: To compare the effects of antimicrobial regimens on resistance of *Salmonella* Typhimurium and non-pathogenic bacteria associated with swine, 29-day-old weaned pigs, were challenged with *Salmonella* Typhimurium and divided into 6 groups and housed in identical SEW nursery rooms. Pigs were assigned by room to treatments including a control (no antibiotic); oxytetracycline in the feed for 30 days, increasing gradient application of oxytetracycline, pulse dosing with oxytetracycline, rotation with oxytetracycline, apramycin sulfate, and sodium sulfamethazine in the drinking water and carbadox in the feed, and rotation with oxytetracycline, apramycin, and neomycin sulfate in the drinking water, and neomycin sulfate in the drinking water. Fecal samples were collected prior to antibiotic treatment and throughout the grow/finish period for culture of the *Salmonella* challenge organism and *E. coli.* Isolates were tested for resistance to test antibiotics using minimum inhibitory concentration analysis. DNA-based techniques, including PCR and PFGE were used to detect resistance genes and to determine relatedness of isolates. Antibiotic regimen affected resistance of *E. coli* but not of the salmonella challenge organism. Molecular analysis indicated that the apramycin resistance gene is associated with bacterial plasmids, whereas tetracycline resistance genes are associated with the chromosome in *E. coli*.

II. Introduction: Antibiotics are commonly used in swine operations to enhance the performance of animals. Increasingly, however, bacterial resistance has caused concern among health specialists and consumer groups. Currently, only limited information is available with regard to management strategies to decrease the occurrence of resistance in bacteria associated with livestock. In order to promote increased consumption of pork, producers must demonstrate that a concerted effort is underway to develop practices that limit risks to consumers. Research that includes production components as well as microbiological and biochemical components will better address current concerns over antibiotic use in livestock operations.

Our group has been actively investigating bacterial resistance in swine for a number of years. Most recently, through the support of the National Pork Board,

we have determined how resistance patterns are affected by animal stressors and dosing regimens and we have made a number of significant observations, including that using a rotation of aminoglycosides increased resistance to apramycin, as did dosing schemes that utilized increasing dosages; whereas pulse dosing, in particular and also rotations with non-related antibiotics produced the lowest resistance among intestinal bacteria. Such data will be of great value for formulating future recommendations for the Pork Quality Assurance Program, as will the data we now have available from our work on animal stressors and associated effects on resistance. However, because resistance mechanisms, persistence of resistance genes, and strategies for control are specific to various antibiotics, it is necessary to conduct additional using other antibiotic types. Our objectives in this study then was to determine if affects describe above for aminoglycosides apply equally to tetracyclines.

III. Objectives: The objectives of this research were to: **1)** to compare the effects of various antimicrobial regimens on resistance of *Salmonella* Typhimurium and non-pathogenic (commensal) bacteria associated with swine, and **2)** compare effects of antimicrobial regimens on *Salmonella* shedding by pigs, and **3)** characterize resistance genes to determine how and in which bacterial groups they arise, and which bacterial groups act as reservoirs for such genes.

IV. Procedures: A total of 72, weaned pigs, verified to be salmonella free and with no history of antibiotic use, were challenged at 29 days of age intranasally with 10^6 colony forming units of *Salmonella* Typhimurium. The challenge strain was derived from a confirmed case of swine salmonellosis and a nalidixic acid resistance marker for subsequent isolation and confirmation. Following the challenge, pigs were randomly divided into 6 groups and each group was housed in identical SEW nursery rooms with separate environmental and waste removal systems. Pigs were provided water and a phase diet, and randomly assigned to treatments including: **1)** control, no antibiotic; **2)** oxytetracycline (50g/ton of feed) for 30 days; **3)** gradient application with oxytetracycline at 10 g/ton of feed for 10 days, then 50 g/ton for 10 days then 10 mg/lb body weight for 10 days; **4)** pulse dosing with oxytetracycline (50 g/ton of feed) for 6 days on, 6 days off, and repeating that sequence through 30 days; **5)** rotation with oxytetracycline for 10 days, followed by apramycin (150 g/ton) for 10 days and sodium sulfamethazine in drinking water (118 mg/kg body weight) for 10 days; and **6)** rotation with oxytetracycline, followed by apramycin (150 g/ton) and neomycin sulfate (22 mg/kg body weight) in the drinking water.

Fecal samples were collected prior to antibiotic treatment, and once each during weeks 1, 2, 4, 6, 8, 10, 12, 16, and 22 following initiation of treatments, and at 24 hours following moving and mixing at the end of the study, to simulate shipment all slaughter. Samples were cultured for isolation of the *Salmonella* challenge organism, and *E. coli*. From each sample, a maximum of 4 *Salmonella* Typhimurium, 4 *E. coli* and 4 *Enterococcus faecalis* colonies were randomly selected from each pig sample (48 of each species per treatment per sampling) and each isolate was tested for resistance to all antibiotics used in the study, using a broth dilution minimum inhibitory concentration (MIC) procedure as outlined by the National Committee for Clinical Laboratory Standards (NCCLS).

To characterize location and origin of resistance genes, resistant isolates were

randomly chosen from samples obtained prior to and following antibiotic treatments. Bacterial DNA was isolated and plasmid profiles determined using standard procedures. PCR was used to detect resistance genes via methods and primers verified in our previous studies. To verify whether genes were associated with bacterial plasmids or chromosomes, plasmid DNA was isolated via standard procedures and separated via gel electrophoresis. Plasmids were then electroporated into sensitive bacteria. Analyses of antibiotic resistance patterns, plasmid profiles, and presence of known resistance genes were conducted on recipients.

For analysis of resistance data, a completely randomized design with split-split plot and repeated measures was used. Sensitivity break point data were linearized and comparisons made using Least squares means to determine treatment effects, with significance determined at $P < .05$.

V. Results: Resistance to apramycin was increased by the two drug rotation treatments, both of which included apramycin (Table 1). Resistance of *E. coli* to neomycin was also increased by the drug rotation treatments, including the rotation that did not include neomycin (Table 2). However, minimum inhibitory concentrations of neomycin did not exceed those associated with clinical resistance. Consistent with earlier studies, most *E. coli* were found to be resistant to oxytetracycline, including those derived from control pigs (Table 3). Most isolates, including those obtained from pigs prior to salmonella challenge and antibiotic treatments expressed MICs above that considered to be clinically sensitive (256 ug/mL). However, during the later stages of the study, isolates recovered from pigs on the drug rotation containing apramycin and neomycin demonstrated lower MICs compared to *E. coli* recovered from control pigs. This may have been due to the selective loss of tetracycline-resistant *E. coli* from the bacterial population as a result of this drug regimen.

Of 33 *E. coli* demonstrating resistance to both apramycin and *E. coli*, 31 were found to contain the aac(3)-IV gene which is known to encode for apramycin resistance. The gene appeared to be associated with plasmid DNA, based on results of electroporation experiments. The same gene was noted in resistance isolates obtained across various sampling days and was found in a variety of isotypes of *E. coli*, based on macrorestriction analysis, conducted via pulse-field gel electrophoresis (PFGE). Tetracycline resistance did not appear to be associated with bacterial plasmids, based on electroporation studies. As with apramycin resistance, tetracycline resistance was found to be associated with a number of *E. coli* isotypes (based on macrorestriction analysis). Lack of antibiotic resistance among recovered salmonella prevented similar analysis in that organism.

Recovery of the salmonella challenge strain decreased beginning about 35 days postchallenge, although a few pigs remained positive for the organism through the end of the study. As we have observed in previous studies, minimum inhibitory concentrations for salmonella for all tested antibiotics remained low and did not exceed those indicating clinical resistance. Drug treatments did not affect resistance patterns of the salmonella challenge organism (Tables 5 through 8).

Table 1. Sensitivity to apramycin by *E. coli* isolated from pigs[a]

Days of age	T1	T2	T3	T4	T5	T6	SEM
28	8.5	6.2	4.5	6.0	11.7	6.1	1.09
35	14.3	4.2	3.2	3.4	32.0	2.6	1.88
42	14.2	3.5	3.8	6.8	13.5	10.8	1.22
49	6.9	3.0	4.0	13.6	256*	38.1*	5.62
63	4.7	4.7	4.7	5.1	52.9*	8.2	2.42
77	4.6	3.9	6.3	4.8	166*	5.9	4.29
91	5.1	4.7	4.2	6.8	11.3	7.3	1.12
105	4.3	3.5	4.0	6.0	8.5	5.0	1.09
119	5.3	6.4	4.8	6.7	7.9	4.0	0.93
120	5.3	5.2	5.3	8.6	6.7	8.0	0.99

[1]Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter, for *E. coli* isolated from pigs prior to antibiotic exposure (28 days of age) and following administration of antibiotic treatments through 120 days of age.T1 = Control, T2 = Label use oxytetracycline, T3 = Gradient application oxytetracycline, T4 = Pulse dosing oxytetracycline, T5 = Rotation of oxytetracycline, apramycin, and sulfamethazine, T6 = Rotation of oxytetracycline, apramycin, and neomycin. SEM = Maximum standard error for Lsmeans within row.

*Indicates difference ($P < .05$) from control within day.

Table 2. Sensitivity to neomycin by *E. coli* isolated from pigs*

Days of age	T1	T2	T3	T4	T5	T6	SEM
28	2.2	2.2	2.3	2.8	4.5	4.1	0.50
35	3.9	2.0	2.2	2.1	6.4*	2.0	0.62
42	2.8	2.3	2.1	2.5	3.3	3.5	0.45
49	2.7	2.4	2.3	4.0	9.5*	8.0*	1.46
63	2.4	2.3	2.3	2.8	5.1	26.4*	1.20
77	2.2	2.3	2.8	3.0	10.2*	6.6*	0.78
91	2.5	2.2	2.2	3.4	3.4	11.8*	0.94
105	2.6	2.3	3.0	2.7	2.9	3.2	0.46
119	2.4	2.5	2.1	4.7	2.6	3.0	0.47
120	2.6	3.0	2.2	4.3	2.3	2.4	0.42

[1]Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter, for *E. coli* isolated from pigs prior to antibiotic exposure (28 days of age) and following administration of antibiotic treatments through 120 days of age.T1 = Control, T2 = Label use oxytetracycline, T3 = Gradient application oxytetracycline, T4 = Pulse dosing oxytetracycline, T5 = Rotation of oxytetracycline, apramycin, and sulfamethazine, T6 = Rotation of oxytetracycline, apramycin, and neomycin. SEM = Maximum standard error for Lsmeans within row.

*Indicates difference ($P < .05$) from control within day.

Table 3. Sensitivity to oxytetracycline by *E. coli* isolated from pigs*

Days of age	T1	T2	T3	T4	T5	T6	SEM
28	362.0	410.8	424.4	476.6	367.8	458.4	5.21
35	476.6	332.0	391.0	365.0	487.4	517.2	5.87
42	466.1	421.0	498.0	446.7	403.4	389.2	5.67
49	379.8	458.6	399.4	364.4	485.8	252.7	9.31
63	412.3	435.0	482.5	424.6	356.8	292.7	6.14
77	433.7	480.1	337.6	418.5	453.6	109.1*	5.82
91	435.2	444.3	315.4	415.7	374.9	181.5*	5.35
105	287.7	400.8	330.6	392.5	267.0	76.2*	5.60
119	236.0	325.9	195.8	269.4	326.4	196.7	4.81
120	186.4	408.8	385.6	336.2	259.3	82.7*	5.37

[1]Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter, for *E. coli* isolated from pigs prior to antibiotic exposure (28 days of age) and following administration of antibiotic treatments through 120 days of age.T1 = Control, T2 = Label use oxytetracycline, T3 = Gradient application oxytetracycline, T4 = Pulse dosing oxytetracycline, T5 = Rotation of oxytetracycline, apramycin, and sulfamethazine, T6 = Rotation of oxytetracycline, apramycin, and neomycin. SEM = Maximum standard error for Lsmeans within row.

*Indicates difference ($P < .05$) from control within day.

Table 4. Sensitivity to sodium sulfamethazine by *E. coli* isolated from pigs[1]

Days of age	T1	T2	T3	T4	T5	T6	SEM
28	261.6	256.2	275.2	324.2	385.6	424.4	5.09
35	408.7	234.8	483.3	350.8	684.6	946.8	7.94
42	437.9	388.0	861.1	484.6	514.7	664.0	7.57
49	309.5	237.3	410.7	163.3	278.5	271.4	9.96
63	285.3	332.1	325.7	307.4	373.3	758.1	7.10
77	197.9	175.6	201.1	329.2	341.7	519.4	5.88
91	256.3	318.4	257.3	327.2	214.0	392.5	5.99
105	284.6	163.2	248.1	224.2	278.1	224.5	5.04
119	185.9	44.4	163.4	448.0	268.1	204.9	4.63
120	167.6	132.3	467.7	259.5	242.0	200.2	5.85

[1]Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter, for *E. coli* isolated from pigs prior to antibiotic exposure (28 days of age) and following administration of antibiotic treatments through 120 days of age.T1 = Control, T2 = Label use oxytetracycline, T3 = Gradient application oxytetracycline, T4 = Pulse dosing oxytetracycline, T5 = Rotation of oxytetracycline, apramycin, and sulfamethazine, T6 = Rotation of oxytetracycline, apramycin, and neomycin. SEM = Maximum standard error for Lsmeans within row.

Table 5. Sensitivity to apramycin by *Salmonella* Typhimurium isolated from pigs[1]

Days of age	T1	T2	T3	T4	T5	T6	SEM
35	4.3	4.8	3.8	5.2	3.8	4.5	0.30
42	6.1	5.3	5.6	5.6	3.8	5.4	0.41
49	8.0	6.2	5.9	7.2	12.9	12.4	0.83
63	7.9	5.9	n/a	10.7	13.0	16.0	0.93
77	6.7	n/a	31.9	4.9	9.0	5.2	2.61
91	7.0	9.5	6.4	6.4	6.7	n/a	1.43
105	5.7	n/a	n/a	n/a	4.9	5.7	1.10
119	4.6	n/a	n/a	4.0	3.5	6.8	0.70
120	n/a	n/a	n/a	11.3	n/a	2.5	1.10

[1]Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *S.* Typhimurium isolated from pigs prior to administration of antibiotic treatments (28 days of age) through 120 days of age. T1 = Control, T2 = Label use oxytetracycline, T3 = Gradient application oxytetracycline, T4 = Pulse dosing oxytetracycline, T5 = Rotation of oxytetracycline, apramycin, and sulfamethazine, T6 = Rotation of oxytetracycline, apramycin, and neomycin. SEM = Maximum standard error for Lsmeans within row.

Table 6. Sensitivity to neomycin by *Salmonella* Typhimurium isolated from pigs[1]

Days of age	T1	T2	T3	T4	T5	T6	SEM
35	2.1	2.1	2.1	2.1	2.3	2.1	0.10
42	2.1	2.1	2.1	2.0	2.0	2.0	0.12
49	2.4	2.3	2.6	2.8	2.6	2.9	0.16
63	2.6	2.5	n/a	2.4	3.1	2.5	0.18
77	2.2	n/a	4.0	2.6	2.0	2.1	0.40
91	2.8	3.4	2.0	2.0	2.4	n/a	0.37
105	2.0	n/a	n/a	n/a	2.2	2.0	0.29
119	2.2	n/a	n/a	2.0	2.3	2.0	0.21
120	n/a	n/a	n/a	2.6	n/a	2.0	0.29

[1]Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *S.* Typhimurium isolated from pigs prior to administration of antibiotic treatments (28 days of age) through 120 days of age. T1 = Control, T2 = Label use oxytetracycline, T3 = Gradient application oxytetracycline, T4 = Pulse dosing oxytetracycline, T5 = Rotation of oxytetracycline, apramycin, and sulfamethazine, T6 = Rotation of oxytetracycline, apramycin, and neomycin. SEM = Maximum standard error for Lsmeans within row.

Table 7. Sensitivity to oxytetracycline by *Salmonella* Typhimurium isolated from pigs[1]

Days of age	T1	T2	T3	T4	T5	T6	SEM
35	2.4	2.5	2.3	2.6	3.1	2.2	0.27
42	3.1	2.4	2.6	3.9	5.1	5.3	0.42
49	4.4	4.0	4.0	3.7	4.0	4.0	0.49
63	5.8	2.0	n/a	12.7	4.3	3.7	1.00
77	2.0	n/a	38.1	2.2	3.6	2.3	3.00
91	10.4	2.8	4.0	3.2	3.4	n/a	1.11
105	2.8	n/a	n/a	n/a	2.8	2.6	0.82
119	2.0	n/a	n/a	2.0	4.0	2.0	0.49
120	n/a	n/a	n/a	3.1	n/a	8.0	1.37

[1]Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *S.* Typhimurium isolated from pigs prior to administration of antibiotic treatments (28 days of age) through 120 days of age. T1 = Control, T2 = Label use oxytetracycline, T3 = Gradient application oxytetracycline, T4 = Pulse dosing oxytetracycline, T5 = Rotation of oxytetracycline, apramycin, and sulfamethazine, T6 = Rotation of oxytetracycline, apramycin, and neomycin. SEM = Maximum standard error for Lsmeans within row.

Table 8. Sensitivity to sodium sulfamethazine by *Salmonella* Typhimurium isolated from pigs[1]

Days of age	T1	T2	T3	T4	T5	T6	SEM
35	772.7	748.9	539.6	688.4	856.1	992.2	2.94
42	667.4	599.4	619.5	703.5	571.7	560.7	2.77
49	715.7	619.5	775.2	703.5	824.6	776.1	4.45
63	771.2	512.0	n/a	574.7	1024.0	824.6	4.45
77	1024.0	n/a	512.0	819.5	1024.0	980.6	7.01
91	939.0	861.1	812.8	1024.0	1024.0	n/a	9.91
105	512.0	n/a	n/a	n/a	512.0	683.5	7.01
119	558.3	n/a	n/a	645.1	512.0	512.0	5.18
120	n/a	n/a	n/a	724.1	n/a	512.0	7.01

[1]Data are Least squares means of minimum inhibitory concentrations (MIC) measured in micrograms per milliliter for *S.* Typhimurium isolated from pigs prior to administration of antibiotic treatments (28 days of age) through 120 days of age. T1 = Control, T2 = Label use oxytetracycline, T3 = Gradient application oxytetracycline, T4 = Pulse dosing oxytetracycline, T5 = Rotation of oxytetracycline, apramycin, and sulfamethazine, T6 = Rotation of oxytetracycline, apramycin, and neomycin. SEM = Maximum standard error for Lsmeans within row.