RESEARCH REPORT
PORK SAFETY

Title: A Survey of Patterns and Persistence of Antimicrobial Resistance on Swine Farms Using Three Different Antimicrobial Use Strategies **- NPB #01-147**

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Abstract: This study examined the epidemiology of antimicrobial resistance on farms with different antimicrobial use strategies. One farm used no antimicrobials (NAU), one farm used limited antimicroibials (LU), and one farm used antimicrobials continuously (CU). At the time of sampling, the NAU farm had not used antimicrobials for 28 years. Resistance persisted on the NAU farm, particularly in *Campylobacter* and *E. coli*. Resistance to antimicrobials was not observed among *Salmonella* isolates from the NAU farm. The other two farms demonstrated very different resistance characteristics, particularly in the *Salmonella* isolates. On the LU farm, *Salmonella derby* was the most common serotype recovered. It was resistant to more than one antimicrobial. Ribotyping and PFGE suggested that these isolates were clonal. Further, this clone was the most common *Salmonella* recovered from the farm. Together these suggest that selective pressures exist on the farm that select for this particular clone's survival i.e., give it preference for persistence within the farm. From the CU farm, *untypable Salmonella* was the most common isolate. Ribotyping and PFGE suggest that this isolate was a clone as well. In contrast to the LU farm isolates, the CU farm isolates were predominantly sensitive to all antimicrobics tested. This suggests that, while different clones were present on the LU and CU farms, there were selective pressures present on both farms which gave preference to farm specific clones. *Campylobacter* isolates across the farms did not demonstrate this clonal relationship suggesting that if resistant *Campylobacter* are to be eliminated from farms, very different strategies may be needed when compared to *Salmonella*. Many questions remain about the effects of antimicrobial use on resistance prevalence on farms. However, if reduction in resistance prevalence on farms is the ultimate goal, management that selects for non-resistant microbe clones needs to be defined.

Introduction: It has been proposed that the use of antimicrobials in animals significantly contributes to the development of antimicrobial resistance in zoonotic pathogens and enterococci. In the U.S., multiple reviews of the research on antimicrobial resistance have been conducted by both government and private agencies. Each suggests that there is justification for concern though there is a paucity of data demonstrating an impact on human health. All studies agree that the matter needs further study. Until recently, these reports have resulted in little regulatory action to limit antimicrobial use in animals. Now the dialogue favors restricted or no access to antimicrobials. In Europe, more restrictive policy is in place. In 1971, Britain and Sweden limited the use of antibiotics in animals, particularly feed additive antibiotics, to prescription use only. In 1999, the European Union banned the used of virginiamycin, spiramycin, tylan, and bacitracin as feed additives. Two recent WHO plenary conferences on the subject called for greater monitoring and restricted use if warranted. In both the U.S. and foreign countries the greatest concern is the use of feed additive antimicrobials. In response to these concerns, FDA CVM has proposed a new framework for drug approvals. The framework calls for restricted use depending on 1) the antimicrobics perceived importance in human medicine; 2) post approval monitoring for resistance development and consequential withdrawal of approval if resistant is deemed to pose increased risks; 3) restricted use of mass treatments regimes i.e., feed additives and water medication; and 4) restricted use in cases where consequential pathogen load increases. For guidance in this debate, there is a real need for improved understanding of how farm level factors influence the character of resistance as seen on farms. Without this characterization, there will likely be greater political pressure calling for restrictions based upon the "imminent hazard provision of the Food, Drug, and Cosmetic Act". Two areas are of particular interest in antimicrobial research. One involves genotypic characterization of resistance and the second involves identification of the factor(s) affecting development of resistance persistence. Local environmental reservoirs, such as pets, soil, water, and feed are potential sources of resistance. In studies of local human populations, community based transfer is well documented. Global spread is also suggested to be a significant contributor to prevalence estimates. For example, vectors such as migratory birds have been demonstrated to spread resistant clones such as *Salmonella typhimurium DT104*. Foreign travel is also a well documented risk factor for resistance. The presence of innate or native genes is postulated to be another source of resistance. One school of thought suggests that these genes have a propensity to be self-sustainable, hidden within the genetic code of the bacteria and expressed only when placed under selective pressure. Whether or not they become submissive to normal flora when selection pressure is removed is unknown, though limited studies would suggest that this only partially occurs. Molecular technology has also improved our understanding of resistance transfer. Resistant genetic material is not only mobile within the bacteria, but is also transferrable between bacteria. It can be mobilized and transferred between bacteria by plasmids, by phage, through conjugation, and through the accumulation of unbound or free DNA. Because of the ease of gene transfer, it is postulated that clinically significant resistance is primarily acquired from other bacteria. Little is known about the presence of resistance genes in relatively innocuous bacteria such as commensals and *Campylobacter coli*, however, these organisms are likely significant and may even be primary reservoirs of resistance. There is considerable debate within the scientific community about the frequency of genetic acquiescence. In fact, this is one of the

key elements of the debate over agricultural antimicrobial use. Environmental and management factors further complicate the interpretation of resistance outcomes. For example, phenotypic resistance can vary depending on pig age, housing, and occurrence of stress events such as moving and transportation. It is not surprising that pigs on different antimicrobics exhibit varying phenotypic patterns. Dosage level and duration of treatment may affect phenotypic expression. Even the degree of multiple resistance can be affected by antimicrobial regime. Because of these potential confounders, it is not surprising that interpretation of phenotypic data is imprecise. However, while genotypic data provides the investigator with knowledge of the presence of resistance genes, without phenotypic information their function is unknown. There are three justifications put forth for the use of antimicrobics in livestock; for improved productivity, for improved human health, and for relief of animal pain and suffering. In pig production, antimicrobics are valuable tools, thus use restrictions have significant implications for farm productivity. Beran suggests that antimicrobial use in livestock benefits human health by decreasing the incidence of zoonotic diseases such as leptospirosis, anthrax, and ornithosis. The most obvious benefit of livestock antimicrobial use is relief of animal pain and suffering. Proponents of use restrictions suggest that with proper husbandry, similar productivity and health can be accomplished. Livestock groups agree that good husbandry is essential for health, but are concerned that good husbandry alone is insufficient, particularly when dealing with endemic diseases or acute outbreaks. Further, foregone feed efficiency and rate of gain in pig production are not likely to be recovered through good husbandry alone.

Because the industry is at risk of losing the opportunity to use antimicrobics, there is a need to better understand the epidemiology of antimicrobic resistant organisms within farms. This includes:

- The effect of complete antimicrobic withdrawal on resistance persistence.
- The relationship between level of antimicrobial use and resistance prevalence.
- The risk factors beyond antimicrobic use that are associated with persistence of resistance.
- The relationship between resistance prevalence and existence of bacterial clones within farms.

Objectives: 1) Determine if antimicrobial use impacts the development of antimicrobial resistance in *Salmonella and Campylobacter*. 2) Characterize what risk factors, including antimicrobial use, influence resistance prevalence. 3) Determine the clonal nature of isolates demonstrating resistance.

Materials & Methods: Three swine farms were selected based on their antimicrobial use practices. The farms were categorized as continuous-use (CU), limited-use (LU), or no-antimicrobial-use (NAU), based on interviews with the respective producers. The CU farm routinely used feed-based antimicrobials at subtherapeutic concentrations for the prevention and control of disease. On the LU farm, feed-based antimicrobials were only used intermittently for the control of acute disease. The NAU farm had no routine antimicrobial use for the past 28 years. The CU and LU farms are intensive production type farms with 400 to 700 sows. The NAU farm is an extensive production type farm with 60 sows.

Sample collection. The farms were sampled quarterly for 2 years. During each farm visit approximately 150 fecal samples (30 per group) were collected from five production groups including; gestation, lactation, suckling, nursery and finishing pigs, with the exception of the NAU farm, which typically farrowed groups of 12 sows or less. The number of samples obtained from lactating sows and suckling pigs on the NAU farm was 12 or less samples per group. Samples consisted of feces collected from the rectum (gestating and lactating sows), freshly defecated feces obtained from the pen floor (nursery and finishing pigs), and rectal swabs (suckling pigs). Samples were transported on ice to the laboratory and were cultured within 24 hours of collection for *Salmonella* and *Campylobacter* spp. **Bacterial isolation and confirmation**. The procedures for culturing for *Salmonella* were as follows. Feces (1 g) and rectal swabs were incubated concurrently, in 10 mL of GN Hajna (Difco Laboratories, Detroit, MI) and Tetrathionate broth (Difco) for 18-24 and 40-48 h at 37^{o} C respectively. After the initial enrichments, an aliquot (100 µL) of each broth was transferred to 10 mL of Rappaport-Vassiliadis R10 broth (Difco) that was incubated for 18-24 h at 37^{o} C. Subsequently, an aliquot (10 µL) of Rappaport-Vassiliadis R10 broth was streaked for isolation onto Xylose-Lysine-Tergitol-4 (Difco) and BG Sulfa (Difco) agar. Plates were incubated for 18-24 h at 37^{o} C. Single colonies characteristic of *Salmonella* were inoculated into triple sugar iron and lysine iron agar slants for biochemical conformation. Presumptive positive isolates were then serogrouped by using serogroup specific antisera (Difco) and were sent to National Veterinary Services Laboratory (Ames, IA) for serotyping. *Campylobacter* were isolated by direct plating 0.1 mL of diluted fecal sample (1:4 and 1:40, in sterile phosphate-buffered saline) onto Campy-Cefex agar plates (Stern et al. 1992) that were incubated for 36-48 h at 42^{o} C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). Presumptive *Campylobacter* colonies were selected by observation of cellular morphology and motility using a wet mount under phase-contrast microscopy, and isolates were speciated using a commercial multiplex PCR (Campylobacter BAX9 PCR, DuPont Qualicon, Wilmington, DE). **Ribotyping and Pulsed-Field Gel Electrophoresis.** All *Salmonella* and a portion of the *Campylobacter* isolates, selected across collection dates and stages of production were ribotyped using an automated system (RiboPrinter9, DuPont Qualicon, Wilmington, DE) according to manufacture=s recommendations. Briefly, cell suspensions were lysed and chromasomal DNA was isolated, digested with P*vu*II (*Salmonella*) or P*st*I (*Campylobacter*), electrophoresed, and simultaneously blotted in an automated manner. Subsequently, the Southern blot was hybridized with chemiluminescently labeled 16 to 23S rRNA primer. To deduce relationships among isolates forming various ribotype clusters, a select group of *Salmonella* isolates were further characterized. DNA fragments resulting from digestion by restriction enzyme X*ba*I were separated by pulsed-field gel electrophoresis (PFGE). The resulting genomic-DNA profiles were interpreted according to established guidelines (Tenover et al., 1995). Patterns that were the same size and had the same number of bands were considered to be the same strain, while patterns that differed by four or more bands were considered to represent different strains. For both ribotype and PFGE analysis, image normalization and construction of similarity matrices were carried out using BioNumerics 2.5 (Applied Maths, Kortrijk, Belgium). Bands were assigned manually, according to densitometric curves and the accompanying TIFF images. Clustering was performed by using the unweighted pair-group method with arithmetic averages (UPGMA) based on the Dice similarity index, utilizing an

optimization parameter of zero.

Results: Over the two-year study, 3340 fecal samples were cultured for *Salmonella* (n=1350,1200, and 790 for CU, LU and NAU farms respectively). The prevalence of *Salmonella* differed (p<.001) among farms, recovery rates were 8.5%, 5.1%, and 1.4%, for CU, LU and NAU farms respectively. Stage of production had no effect on *Salmonella* prevalence across farms, however a farm effect among stages of production was observed. Across farms, twenty-three *Salmonella* serotypes were identified, with *S. anatum* and *S. mbandaka* being the only common serotypes among farms. Seventeen serotypes were recovered from the CU farm (n=116 isolates), an untypable 4,12:i monophasic has been identified most often (63.8%) followed by *S. infantis* (8.6%) and *S. cerro* (5.2%). Isolates from the LU farm (n=61) were comprised of five serotypes; *S. derby* (67.2%), *S. london* (13.1%), *S. mbandaka* (11.5%), *S. livingstone* (3.3%) and *S. anatum* (1.6%). Six serotypes were identified from the NAU farm (n=11 isolates) including; *S. newport* (45.4%), *S. fresno* (18.2%), *S. anatum* (9.1%), *S. javiana* (9.1%), *S. mbandaka* (9.1%) and *S. typhimurium* (9.1%).

Salmonella isolates recovered from CU and LU farms expressed resistance to ten of the sixteen antimicrobials evaluated, while isolates recovered from the NAU farm were sensitive to all antimicrobials tested. No *Salmonella* isolates recovered across farms expressed antimicrobial resistance to amikacin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin or nalidixic acid. A greater proportion (p<.001) of *Salmonella* isolates recovered from the LU farm were resistant to ampicillin, kanamycin, streptomycin sulfamethoxazole and tetracycline compared to CU farm isolates. The percentage of *Salmonella* isolates expressing antimicrobial resistance differed significantly (p<.001) across farms, the percentages where 9.5%, 63.9% and 0 for CU, LU and NAU farms respectively. No differences were observed in the percentages of isolates resistant to a single antimicrobial agent across farms (1.7%, 0 and 0, for CU, LU and NAU respectively). However, a higher percentage (p<.001) of isolates from the LU farm (63.9%) were multi-resistant (expressed resistance to two or more antimicrobials) compared to CU (9.5%) and NAU (0) farms. Expression of antimicrobial resistance was distributed across several serotypes recovered from the CU farm, these included *S. derby, S. infantis, S. mbandaka, S. typhimurium* and untypeable *Salmonella* (4, 12:i monophasic) isolates. Seven different resistance patterns where observed among *Salmonella* isolates recovered from the CU farm. Two isolates were resistant to one antimicrobial, two were resistant to two antimicrobials, and seven were resistant to either three or four antimicrobials. In contrast, expression of antimicrobial resistance among isolates recovered from the LU farm was limited to *S. derby* isolates. Five resistance patterns where observed among the LU farm isolates; expression of resistance was noted with two to seven antimicrobials. Interestingly, during the second year of evaluation resistance to several aminoglycosides (amikacin, apramycin, gentamicin and kanamycin) was observed in addition to the predominant ST-SU-TC resistance pattern seen previously in the *S. derby* isolates recovered from the LU farm. The ST-SU-TC resistance pattern was the most prevalent pattern observed across farms. Stage of production had no effect on *Salmonella* antimicrobial resistance patterns among farms.

A different endemic *Salmonella* strain was isolated from CU and LU farms. Within each strain, antimicrobial resistance patterns varied dramatically. An untypable, 4,12:i monophasic *Salmonella* was the prominent type recovered from the CU farm (64%). The vast majority of these isolates (93%) were sensitive to all antimicrobials tested.

Of the *Salmonella* isolates from the study, 187 were ribotyped and 45 isolates were characterized by PFGE. Overall similarity among *Salmonella* isolates by farm was 13.05%, 49.01%, and 50.52% for CU, LU, and NAU farms respectively. Riboprinting grouped the untypable *Salmonella* isolates from farm CU with S. typhimurium, and PFGE analysis of untypable 4,12:i monophasic (n=4) and S. typhimurium (n=2) CU farm isolates collected over a 6 month period where found to be identical. Ribotyping indicated approximately 90% similarity among these isolates. In contrast to the relatively antimicrobial susceptible endemic strain observed on the CU farm, 95% of the predominant serotype recovered from the LU farm (*S. derby,* 67%) expressed resistance to two or more antimicrobials. PFGE analysis of LU (n=6) and CU (n=3) farm *S. derby* isolates indicated that isolates recovered from the LU farm were closely related. There was ≥80% similarity among these isolates. LU *S. derby* isolates collected over a years time period had 0 to 3 fragment differences. While 7 to 8 fragment differences where observed between CU and LU *S. derby* isolates.

Over the two-year study, 2787 fecal samples were cultured for *Campylobacter* (n=1050,1050, and 687 for CU, LU and NAU farms respectively). All *Campylobacter* isolates across farms speciated *C. coli* by PCR. The prevalence of *Campylobacter* differed ($p<.05$) among farms; recovery was 52.1%, 41.8%, and 36.2%, for CU, LU and NAU farms respectively. *Campylobacter* isolates across farms expressed resistance to five of the eight antimicrobials tested. No *Campylobacter* isolates recovered across farms expressed antimicrobial resistance to chloramphenicol, ciprofloxacin or gentamicin. A greater proportion ($p<.001$) of *Campylobacter* isolates recovered from the CU farm were resistant to azithromycin and erythromycin compared to isolates from the LU and NAU farms, and a greater percentage ($p<.001$) of isolates recovered from the CU and LU farms were resistance to azithromycin, clindamycin, erythromycin, and tetracycline compared to isolates from the NAU farm. A greater percentage ($p<.01$) of *Campylobacter* isolates recovered from young swine (suckling, nursery and finishing) on CU and LU farms were resistance to azithromycin, clindamycin and erythromycin compared to isolates from mature sows (gestation and lactating). In addition, more LU farm isolates ($p<.001$) from young stock expressed resistance to tetracycline compared to isolates from mature sows. For young and mature swine, the percentages of isolates expressing resistance to a particular antimicrobial agent for CU were, respectively, 63.6% and 35.8% for azythromycin, 30.7% and 11.9% for clindamycin, and 63.6% and 34.3% for erythromycin. Likewise, the percentages of isolates expressing resistance from young and mature swine for the LU farm were, respectively, 28.26% and 2.3% for azythromycin, 23.1% and 0.0% for clindamycin, 29.5% and 2.3% for erythromycin, and 69.2% and 31.8% for tetracycline. Stage of production had no effect on *Campylobacter* isolates antimicrobial resistance pattern on the NAU farm. A greater percentage ($p<.001$) of the *Campylobacter* isolates recovered from CU (68.4%) and LU (63.6%) farms expressed resistance to antimicrobials tested compared to isolates recovered from the NAU farm (9.4%). The percentage of *Campylobacter* isolates expressing resistance

to a single antimicrobial differed ($p<.05$) among farms (16.1, 41.3, and 6.6% of the isolates for CU, LU and NAU respectively). Resistance to tetracycline accounted for the majority of the resistance to a single antimicrobial across farms (16.1, 39.9, and 2.8% of the isolates for CU, LU and NAU farms respectively). Differences were also observed ($p<.05$) in the proportion of multi-resistant *Campylobacter* isolates across farms (52.3%, 22.4% and 2.8% for CU, LU and NAU respectively). Ten different resistance patterns where observed among *Campylobacter* isolates recovered from CU and LU farms, with expression of resistance to as many as five antimicrobials. Only four different resistance patterns were observed among the NAU farm isolates expressing resistance to one or two antimicrobials.

Ribotyping of *Campylobacter* isolates showed greater genetic diversity among *Campylobacter* compared to *Salmonella* recovered from the same farms. Ribotyping indicated 30.15%, 21.35%, and 16.68% similarity among *Campylobacter* isolates for CU, LU, and NAU farms, respectively.

Discussion: The effects of antimicrobial use appears variable among *Salmonella* serotypes in selection of resistant populations. Susceptible and resistant *Salmonella* strains persisted over the course of this study on two of the farms. Resistance profiles also differed dramatically between bacterial spp.; Sherley et al. 2000, reported a similar observation in bacteria recovered from wild animals. The lack of antimicrobial resistance observed among *Salmonella* isolates from the NAU farm may be attributed to lack of selective pressures and the relatively low recovery of *Salmonella*. *Campylobacter* isolates from the NAU farm demonstrate that resistant populations can exist and persist for extended periods in the absence of antimicrobial use. In general, resistance profiles tended to follow antimicrobial use patterns among farms. Quinolones are not used in swine, and no isolates resistant to ciprofloxacin were observed across farms. Furthermore, among farms using antimicrobials, a greater proportion of isolates were resistant to antimicrobials that have been used for treatment of disease and growth promotion for several decades (streptomycin, sulfonamides, and tetracyclines). Particularly among *Campylobacter* isolates, resistance to macrolides, tended to correspond to usage. The CU farm fed tylosin in the grower and finisher diets (40 and 120 g/ton, respectively) and exhibited the highest proportion of macrolide resistant isolates. The LU farm fed pigs tylosin for 5 d or less, entering the nursery to control diarrhea resulting in significantly less resistance among *Campylobacter* isolates. Resistance to tetracycline was observed on all three farms. The NAU farm has not used antimicrobials for 28 years, yet 5.7 % (6 of 106) of the *Campylobacter* isolates tested expressed resistance to tetracycline. Tetracycline resistance in Campylobacter is thought to be associated with a plasmid. In the absence of selective pressure, unless the plasmid carries other factors of benefit, retention of the plasmid is of little value to the organism.

Regardless, of antimicrobial use (continuous vs limited) *Salmonella* clones appear to become established within farms. Further, these clones persisted over a period of two years and appeared, at least on one farm, not to be associated with susceptibility. The source of these clones is unknown. At least on one farm, the predominant pan-sensitive (>90%) *Salmonella* persisted even with antimicrobial use, which suggests that factors beyond antimicrobic use are associated with fitness selection. In comparison to *Salmonella*, *Campylobacter* was more

genotypically dissimilar and appeared to be less clonal.

This study suggests that the elimination or even reduction of antimicrobic use on farms may not have the desired effect of resistance elimination within farms, though surely the prevalence rate of resistance will be diminished. Further, risk factors for resistance prevalence appear to be farm specific and the targeted elimination of farm specific clones may decrease the prevalence of resistance, particularly in the case of *Salmonella*. The results also lead us to speculate that in some farms, the presence of a pan-sensitive clone may reduce resistance prevalence. Because *Campylobacter* resistance is typically plasmid related and because *Campylobacter* appear less familial in nature, resistance reduction through *Campylobacter* reduction may need to more broadly addressed rather than targeting a specific clone.

Lay Interpretation: Antimicrobial resistance expression at the farm level is proposed to adversely impact human health, though this relationship is poorly understood. This work suggests that prevalence of antimicrobial resistance within farms is determined to varying degrees by factors other than antimicrobic use and is at least partially a function of a particular clone's ability to survive in a given farm environment. Antimicrobic use may, however, contribute to resistance prevalence as a selective pressure should the particular clone possess resistance traits (genes). Withdrawing antimicrobics from a farm, even over a prolonged time, does not completely eliminate resistance expression, particularly in cases such as *Campylobacter* where resistance is plasmid mediated. If the objective is to eliminate resistance transfer through the food supply, then this work suggests that strategies are needed to eliminate resistant carrier clones from farms. Further, there is need to determine what other factors drive susceptible clone selection on farms.

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