RESEARCH REPORT
PORK SAFETY

Title: Enumeration of *Salmonella* throughout the Pork Harvesting Process - **NPB #07-050**

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Industry Summary

This project provides comprehensive information on the levels and prevalence of *Salmonella* on pork carcasses during processing to benchmark the effectiveness of in-plant interventions on *Salmonella* reduction. During this project, 190 pork carcasses were sampled over two consecutive days in each of four seasons at a pork processing plant in the United States. Samples were collected at three steps in processing: skin (after exsanguination and before scalding), pre-evisceration carcass (after scalding, singeing, and polishing) and chilled final carcass in the cooler. Overall prevalence of *Salmonella* on skins was near 100% in all seasons with a one-day low of 85% in the winter. The range of *Salmonella* prevalence on pre-evisceration carcasses was between 10.5% and 69.5%. Final carcasses had 12.6% *Salmonella* prevalence in the summer, undetectable (0% prevalence) in the fall, ~4.2% prevalence in the winter and ~9% in the spring. The prevalence of *Salmonella* was twice as high on skins and three times higher on pre-evisceration and final carcasses as was expected. During the four seasons, the lowest enumerable level of *Salmonella* bacteria on skins was 27 cfu/100cm^2 and the highest level was 2322 cfu/100cm^2. *Salmonella* serotype and drug resistance patterns have been determined for 2,176 isolates. From this data set, 22 serotypes have been identified and the antibiotic profiles of these range from having resistance to eight antibiotics to being susceptible to all 14 antibiotics tested. When the amount of *Salmonella* on skins was high, as indicated by both a large number of enumeration positive samples and high enumeration levels, the chance of having *Salmonella* on carcasses was higher. However, the percentage of carcasses with levels high enough to enumerate and the enumeration levels were both relatively low.

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Scientific Abstract

The objective of this project was to provide a comprehensive evaluation of the levels and prevalence of *Salmonella* on pork carcasses during processing to benchmark the effectiveness of in-plant interventions on *Salmonella* reduction. Ninety five pork carcasses were sampled per day over two consecutive days in all four seasons for one year (Spring, Summer, Winter, Fall) at a pork processing plant in the United States. Using enumeration, the research quantified the number of *Salmonella* (colony forming units, cfu) at three places along the pork processing chain. Samples were obtained from the skin just after exsanguination and before scalding, the pre-evisceration carcass after scalding, singeing, and polishing, and the chilled final carcass in the cooler. Additionally, prevalence was measured to determine the overall presence of *Salmonella* on carcasses and finally the identification of serotype and antibiotic sensitivity of *Salmonella* positive samples collected from skin, pre-evisceration chilled and final carcasses was determined. Comparisons of *Salmonella* carcass prevalence at each site, or of the median skin enumeration values determined for each sample day, were made using the Kruskal-Wallis one-way ANOVA for non-parametric data and Dunn's multiple comparison post-test and *P* values of less than 0.05 were considered significantly different. Overall prevalence of *Salmonella* on skins was near 100% in all seasons with a one-day low of 85% in the winter. Pre-evisceration carcass prevalence ranged from a low of 13.6% to a high of 55%. Final carcasses had *Salmonella* prevalence as low as 0% but as high as 12.6%. Skins had higher *Salmonella* prevalence than carcasses when the data were pooled across seasons. During the four seasons, 371 out of 760 skin samples had enumerable levels of *Salmonella* ranging from 27 cfu/100cm^2 up to 2322 cfu/100cm^2. The eight sampling days from all four seasons were divided into two groups where the median *Salmonella* level of the skin samples from Group 1 was 27 cfu/100cm^2 while the level from Group 2 was 661 cfu/100cm^2. When less than 50% of the skin samples had enumerable levels of *Salmonella* (Group 1) the pre-evisceration carcasses had an average prevalence of 20%. When greater than 50% of the skin samples were enumerable (Group 2), the pre-evisceration carcasses had an average prevalence of ~51%. The total number of isolates collected from enumeration and prevalence samples on all three sample types yielded 4208 isolates. Of those isolates collected, 2176 isolates were characterized identifying 22 *Salmonella* serotypes. Many of the serotypes identified have yielded numerous antibiotic resistance profiles. Although some serotypes are susceptible to all antibiotics, 29 antibiotic resistance profiles have been identified. Although the prevalence of *Salmonella* on skins and carcasses was higher than expected, the percentage of carcasses with levels high enough to enumerate and the enumeration levels were both relatively low.

Introduction

This project provides comprehensive information on the levels and prevalence of *Salmonella* on pork carcasses during processing to benchmark the effectiveness of in-plant interventions on *Salmonella* reduction. USMARC scientists recently developed methodology to rapidly enumerate *Salmonella* on hides, carcasses and raw meat products (Brichta-Harhay, et al., 2007). Using enumeration, the research quantified the number of *Salmonella* (colony forming units, cfu) per carcass sampled at three places along the pork processing chain. Additionally, the overall prevalence of *Salmonella* on carcasses was determined as well as the identification of serotype and antibiotic sensitivity of positive samples collected from skin, pre-evisceration and chilled final carcasses.

A thorough evaluation of the levels of *Salmonella* at different stages of harvest will provide the industry with better information on the need for additional carcass antimicrobial interventions. Outbreaks of foodborne illness linked to consumption of pork could have a devastating effect on profitability within the industry. Multidrug resistant *Salmonella* are viewed as emerging pathogens. By testing the antibiotic susceptibilities of the *Salmonella* identified on final carcasses, this work will provide the NPB and producers with information for use in determining *Salmonella* risk assessment.

Objectives:

Objective 1: Quantify the levels of *Salmonella* on pork carcasses during harvest processing, using an enumeration method developed at USMARC. Determine the level of *Salmonella* on pork carcasses at the following stages of processing: 1) skin soon after exsanguination and before scalding, 2) pre-evisceration carcasses (after scalding, singeing, and polishing), and 3) chilled final carcasses. Determine overall prevalence of *Salmonella*.

Objective 2: Determine the Serotype and antibiotic susceptibilities of *Salmonella* isolated from skin, pre-evisceration and chilled final carcasses in the cooler.

Materials & Methods:

Sample collection:

During each season, 190 carcasses were randomly sampled over two days at each of three sampling sites (skin, pre-evisceration carcasses and chilled final carcasses) using a prewetted sponge with 20 ml of Buffered Peptone Water. Approximately 10 up-and-down strokes on one side of the sponge and ten side-to-side strokes on the other side of the sponge were used in sampling an area of approximately 1500 cm^2 for the skin samples and approximately 4000 cm^2 for the pre-evisceration (post-scald/singe/polish) and chilled final carcasses. For final carcasses (after chilling), samples were taken from the pork carcasses at the end of the rails in the cooler and were representative of the animals slaughtered on the previous day. This sampling allowed for the greatest number of hog carcasses from multiple lots to be sampled.

Sample processing to enumerate the level of *Salmonella* on pork carcasses during harvest. All samples were enumerated for *Salmonella* as previously described (Brichta-Harhay et al., 2007). For skin samples obtained prior to scalding, 500 µl of liquid from the skin sample sponge bag was placed into a 1.5 ml micro-centrifuge tube, vortexed, allowed to settle for 3 min and then 50 µl was spiral plated onto XLDtnc medium containing tergitol (4.6 ml/L), novobiocin(15 mg/L) and cefesulodin (5 mg/L). Plates were incubated at 37ºC for 18 to 22 hr and then at room temperature for another 18 to 22 hr. Presumptive colonies were confirmed as *Salmonella* by latex test (Oxoid, Basingstoke, England) and by *invA* PCR (Rahn et al., 1992).

For pre-evisceration and chilled final carcasses, where lower numbers of *Salmonella* were expected, the more sensitive procedure using hydrophobic grid membranes was used (Brichta-Harhay et al., 2007). These samples were processed as follows: 3 ml of carcass sponge sample was mixed with 4 ml of BPW containing 1% (v/v) Tween 80. This sample was filtered through a hydrophobic grid membrane filter (HGMF) (Neogen) using a FiltaFlex HGMF filtration apparatus (FiltaFlex Ltd. Canada). The filter was placed on XLDtnc agar plate then incubated at 37ºC for 18 to 22 hr and then at room temperature for an additional 18 to 22 hr. Presumptive colonies were confirmed as described above.

Sample processing to determine prevalence of *Salmonella*.

Sponge samples from all three sampling locations within each plant were enriched with 80 ml of tryptic soy broth (TSB) incubated at 25°C for 2 hr, then at 42°C for 6 h prior to being held at 4°C overnight. Using immunomagnetic separation (IMS), 1 ml of each enrichment was subjected to anti-*Salmonella* immunomagnetic bead cell concentration (Dynal). The bacterial bead complex was selectively enriched for *Salmonella* by incubation for 24 hr at 42°C in Rappaport-Vassiliadis soya (RVS) broth. RVS broth was streaked onto XLDtnc and Brilliant Green Agar to isolate colonies of *Salmonella*. *Salmonella* isolates were confirmed by PCR (Rahn et al., 1992).

Collection and storage of *Salmonella* isolates.

From each positive sample, four colonies were picked, grown overnight in 750 microliters of TSB and mixed with glycerol to achieve a final volume of 15% glycercol then frozen at -80^{o}C and stored for future evaluations.

Serotyping of *Salmonella* recovered from skin, pre-evisceration and final carcasses.

All enumeration isolates were serotyped and the antibiotic sensitivity determined as described below. The serotypes of each confirmed *Salmonella* isolate from skin, pre-evisceration and final carcasses was determined. Isolates recovered from the prevalence determinations were screened for antibiotic resistance to Tetracycline (32 μg/ml), Ampicillin (32 μg/ml) and Kanamycin (64 μg/ml). Those isolates with either no antibiotic sensitivity or a unique combination of sensitivities were selected for serotype and drug sensitivity testing.

For the serotype analysis, an isolate was analyzed by molecular serogroup PCR as described by Herrera-Leon et al. (2007) and confirmed using Wellcolex Colour *Salmonella* Tests (Remel). Molecular serotyping also relied on the work of Herrera-Leon et al. (2004) and Echeita et al. (2002) for determining the somatic and flagellar antigens. Antisera (Remel) was used to confirm the findings of the molecular serotyping. A final determination of the serotype identity of a particular isolate was made using both the molecular and antisera based methods.

Determination of Antibiotic Sensitivity.

The Salmonella isolated from these samples were tested to determine the antibiotic susceptibility profile according to National Antimicrobial/Antibiotic Resistance Monitoring System (NARMS) protocols. Specifically, Sensititre antimicrobial susceptibility plates (CMV1AGNF) and the Sensititre automated broth micro-dilution system (TREK Diagnostic Systems, Cleveland, Ohio, USA) were used. The antibiotics in this panel are: amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, naladixic acid, streptomycin, sulfizoxazole, tetracycline, and trimethoprim/sulphamethoxazole. The antimicrobial susceptibility plates were inoculated as per the manufacturer's instructions. The plates were incubated at 37ºC for 16 to 18 h and the MIC for 15 antibiotics will be determined using a Sensititre Auto Reader (TREK Diagnostics).

Statistical analysis.

Salmonella prevalence values on skin, pre-evisceration and final carcasses were calculated by dividing the number of culture positive samples by the total number of samples collected (n=95 per sample type, per day). Percent prevalence was determined for each sample day (two sample days per season) and is reported as the mean and standard deviation (± SD) of two sample days per season (Figure 1) or eight sample days overall (Figure 2). Enumeration data determined for skin samples are shown by day in Figure 3, and the geometric mean and 95% confidence interval (95% CI) are indicated. Comparisons of *Salmonella* carcass prevalence at each site, or of the median skin enumeration values determined for each sample day, were made using the Kruskal-Wallis one-way ANOVA for non-parametric data and Dunn's multiple comparison post-test. All analyses were performed using Prism 4 GraphPad software (www.graphpad.com) and *P* values of less than 0.05 were considered statistically significantly.

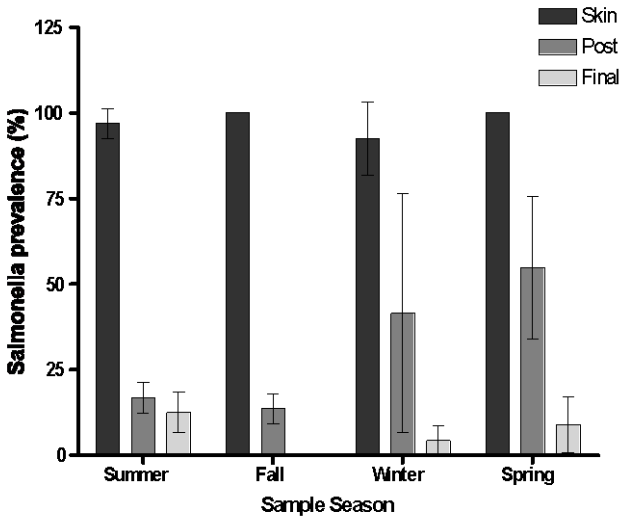
Results:

Results for Objective 1: Quantify the levels of *Salmonella* on pork carcasses during harvest processing and determine the overall prevalence on skin, pre-evisceration and chilled final carcasses.

There was no seasonal effect on skin prevalence. Overall prevalence of *Salmonella* on skins was near 100% in all seasons with a one-day low of 85% in the winter (Figure 1). In both fall and spring, *Salmonella* prevalence on skins was 100% for both days. In the summer season the prevalence was a low of 93.7% on one day and 100% on the other and in winter, the prevalence was 85 and 100% for the two days. The range of *Salmonella* prevalence on pre-evisceration carcasses was between 10.5% and 69.5% (Figure 1). No significant seasonal effect was detected for *Salmonella* prevalence on pre-evisceration carcasses. Summer and fall

prevalence on pre-evisceration carcasses was 16 and 13.6% respectively. Pre-evisceration carcasses in the winter and spring averaged 41 and 55% *Salmonella* prevalence. Final carcasses had 12.6% *Salmonella* prevalence in the summer, undetectable (0% prevalence) in the fall, 4.2% prevalence in the winter and 9% in the spring.

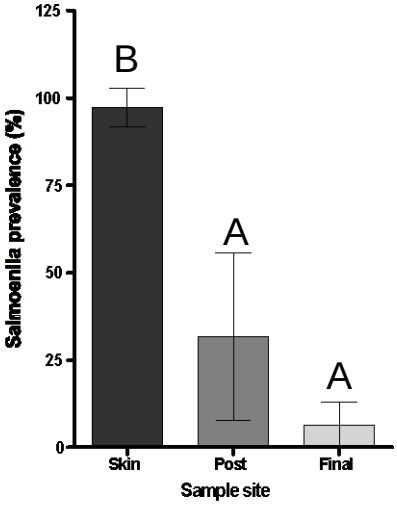
Figure 1: *Salmonella* Prevalence in all four seasons.



Each bar on the graph represents the average prevalence for that sample type taken from two sampling days in each season. A total of 95 samples per day were collected on two consecutive days per season.

A significant reduction in the prevalence of *Salmonella* on skin compared to pre-evisceration and chilled final carcasses was found when the data from all four seasons was pooled (Figure 2).

Figure 2: Reduction in *Salmonella* prevalence during processing.



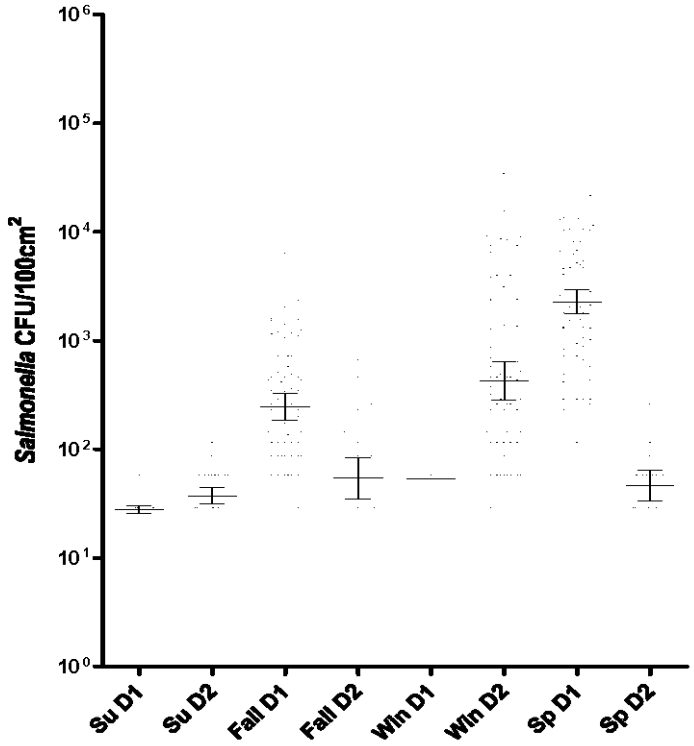
Each bar on the graph represents the average prevalence for that sample type taken from eight sampling days (n = 760 samples). Values lacking a common letter differ significantly ($P < 0.05$).

The USMARC enumeration method is able to determine the number of *Salmonella* in a given sample (skin limit of detection of 27 cfu/100cm^2; pre-evisceration and final carcasses limit of detection is 0.25 cfu/100cm^2). Figure 3 presents the data from 95 skin samples for each day of sampling (two per season) in all four seasons. Percentages of skin samples that had levels of bacteria at or above the limit of detection ranged from a low of 1.1% (winter Day 1) to 100% enumerable on spring day 2. The Geometric Mean (GM) of the enumerable skin samples ranged among days from 27 cfu/100cm^2 to 2278 cfu/100cm^2. This GM, representing

the level of measurable bacteria per 100cm^2, is shown at the bottom of Figure 3. In every season but summer, there is a significant difference ($p<0.01$) between days in the GM demonstrating a high day-to-day variation for *Salmonella* on hogs entering the plant.

During the summer season, 18 out of 95 skins were enumerable on day 1 and a slightly higher number of skins were enumerable on day 2 (n= 32/95). In the fall sampling period, 83/95 (day1) and 23/95 (day 2) skin samples were enumerable. Only one skin sample was enumerable on day 1 of the winter sampling period but 90/95 were enumerable on day 2. In the spring season 95/95 samples were enumerable on day 1 but only 29/95 were enumerable on day 2

Figure 3: Levels (Enumeration) of *Salmonella* on Skin in all four seasons.



GM	27	27	270	27	54	405	2322	27

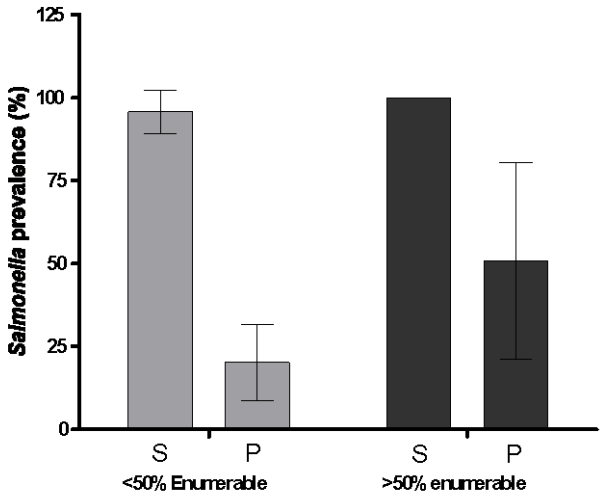
Each dot represents an individual sample and the corresponding cfu/100cm2 for that given data point. The geometric mean (GM) of the enumeration level is noted on the bottom of the graph. Seasons are denoted in some cases by abbreviations summer (Su) Fall (Fall), winter (Win) and spring (Sp) and days are noted by D1 and D2 for days 1 and 2 respectively. The number of positive enumeration samples (out of 95) per day is as follows: SuD1 = 18, SuD2 = 32, Fall D1 = 83, Fall D2 = 23, Win D1 = 1, Win D2 = 90, Sp D1 = 95 and Sp D2 = 29.

On the eight sampling days, the percentage of enumerable pre-evisceration samples ranged from undetectable to a high of 31.5%. Altogether, only sixty-four of the 760 pre-evisceration samples were enumerable (8.4%). Most of these pre-evisceration samples were enumerable at low levels with 60/64 (94%) having between 0.25 and 2.5 *Salmonella* per 100cm^2. Only four final carcasses out of 760 sampled (0.5%) had enumerable levels of *Salmonella* and those were in the range of 0.25 to 2.5 cfu/100 cm^2.

A further analysis of the enumeration data found that when skins entered the plant with high levels of *Salmonella*, carcasses were more likely to have *Salmonella* (Figure 4). *Salmonella* positive skins samples from the eight sampling days from all four seasons were divided into two groups. Group 1 had data from five days in which less than 50% of the skins sampled had enumerable *Salmonella*. The second group had three days where skin samples were >50% enumerable. The median *Salmonella* level of the skin samples from Group 1 was 27

cfu/100cm^2 while the level from Group 2 was significantly higher at 661 cfu/100cm^2. Additionally, it is worth noting from data presented in Figure 4 that when the skin samples were less than 50% enumerable (Group 1) the pre-evisceration carcasses had an average prevalence of 20%. When the skin samples were greater than 50% enumerable (Group 2), the pre-evisceration carcasses had an average prevalence of ~51%. This demonstrates the downstream affect of having hogs with highly contaminated skins enter the plant.

Figure 4: *Salmonella* prevalence on skins and pre-evisceration carcasses as a function of incoming *Salmonella* levels on skin.



Five out of the eight sample collection days (Group 1) had less than 50% of the skin samples being enumerable (<50% Enumerable) and the remaining three days (Group 2) had >50% of the skin samples being enumerable.

Results for <u>Objective 2:</u> Determine the serotype and antibiotic susceptibilities of *Salmonella* isolated from skin, pre-evisceration and final carcasses.

Preliminary estimates of the number of *Salmonella* isolates that would be recovered for this project were based on previously published work. We estimated that ~416 samples (40% of skin samples and 6% of post-singe and final carcasses) would be positive for *Salmonella*. We greatly underestimated the number of *Salmonella* positive samples as well as the diversity of serotypes that would be detected. A total of 740 (97%) skin samples and 19% of pre-evisceration and final carcasses were positive. The total number of isolates collected from enumeration and prevalence samples on all three sample types yielded 4208 isolates of which 2176 were characterized and a summary of information from these isolates is provided in Tables 1 and 2. A total of 22 *Salmonella* serotypes have been identified (Table 1).

Table 1: *Salmonella* serotypes identified in Pork

Serotype	Where serotype was found.
Agona	Skin
Altona	Pre-evisceration
Anatum	Skin, Pre-evisceration
Cerro	Skin, Final
Cubana	Skin
Derby	Skin, Pre-evisceration
Djugu	Skin
Infantis	Skin,
Johannesburg	Skin, Final
Kentucky	Skin
London	Skin, Pre-evisceration
Mbandaka	Skin
Montevideo	Skin
Muenster	Skin
Nontypeable	Skin
Putten	Skin
S. II	Pre-evisceration
Senftenberg	Skin
Thompson	Skin
Typhimurium 5-	Skin, Pre-evisceration, Final
Typhimurium 5+	Skin, Pre-evisceration, Final
Worthington	Skin

Many of the serotypes identified have yielded numerous antibiotic resistance profiles. Based on preliminary screening of all isolates using Tryptic Soy Agar plates containing either Tetracycline (32 μg/ml), Ampicillin (32 μg/ml) or Kanamycin (64 μg/ml), a select number of these isolates (n=468) were examined in detail for antibiotic resistance. Although some serotypes were susceptible to all antibiotics, 29 antibiotic resistance profiles have been identified (Table 2). Of those resistance profiles identified, (Am)ApCSSuTe is the most common profile and is found in ~33% of antibiotic resistant isolates (Table 2).

Table 2: Antibiotic Resistance Profiles of a select number of resistant *Salmonella* from Pork.

Antibiotic resistance profiles	% of isolates tested that have this pattern	Total # antibiotic resistances in the pattern shown (does not include intermediate resistances)
(Am)ApCSSuTe	33.55	5
SSuTe	24.79	3
SuTe	9.62	2
AmApCSSuTe	7.69	6
(Am)ApCSuTe	5.98	4
Te	4.27	1
(C)SSuTe	2.14	3
ApCSSuTe	1.92	5
AmApCSuTe	1.50	5
AmApFT(Ax)(C)SSuTe	1.28	7
ApSSuTe	1.28	4
AmApFT(Ax)SSuTe	0.64	7
ApKSSuTe	0.64	5
ApSuTe	0.64	3
SSu	0.64	2
(C)Te	0.43	1
ApCSuTe	0.43	4
(Am)Ap(F)CSSuTe	0.21	5
(Am)ApCKSSuTe	0.21	6
(Am)ApSuTe	0.21	3
(C)	0.21	0 (1 intermediate)
(C)KSSuTe	0.21	4
(C)SuTe	0.21	2
AmApF	0.21	3
AmApFT(Ax)(C)SSuTeSxt	0.21	8
AmApFT(Ax)CNSuSxt	0.21	8
AmApFT(Ax)SuTe	0.21	6
Ap	0.21	1
STe	0.21	2

( ) indicates intermediate resistance for that particular antibiotic. Am = Amoxicillin/Clavulanic Acid, Ap = Ampicillin, F = Cefoxitin, T = Ceftiofur, Ax = Ceftriaxone, C = Chloramphenicol, K = Kanamycin, N = Nalidixic Acid, S = Streptomycin, Su = Sulfisoxazole, Te = Tetracycline and Sxt = Trimethoprim/Sulphamethoxazole. To compile this data, a sampling of 468 isolates was tested using the antibiotic susceptibility profile according to National Anitmicrobial/Antibiotic Resistance Monitoring System (NARMS) protocols.

Discussion:

Enumeration methodology recently developed at USMARC made it possible to quantify the number of *Salmonella* (colony forming units, cfu/100cm^2) at three places along the pork processing chain. Overall prevalence of *Salmonella* on skins was near 100% in all seasons suggesting that based on this data set, no seasonal effect occurs. The range of *Salmonella* prevalence on pre-evisceration carcasses was between 10.5% and 69.5%. Final carcasses had *Salmonella* prevalence as low as 0% and up to 12.6% among the different

seasons. A review of the literature suggested that only 40% of skin samples and 6% of pre-evisceration and final carcasses would be positive for *Salmonella* yet this work found ~97% of skin and 19% of pre-evisceration and final carcasses positive. Possibly the methodology, which samples a larger carcass area than most protocols and the sensitivity of the immunomagnetic separation method used for isolation of *Salmonella* contributed to these higher than anticipated prevalence numbers.

Enumeration of *Salmonella* was possible on 371/760 skin samples, 64/760 pre-evisceration and 4/760 final carcasses. During the four seasons, the lowest enumerable level of *Salmonella* bacteria on skins was 27 cfu/100cm^2 and the highest level was 2322 cfu/100cm^2. The *Salmonella* enumeration levels on pre-evisceration carcasses ranged from 0.25 cfu/100cm^2 to >10.1 cfu/100cm^2. Enumerable Salmonella on chilled final carcasses was low with all 4 samples being ~0.25 cfu/100cm^2.

The enumeration data suggest that when the *Salmonella* load on incoming skins is high, carcasses were more likely to be *Salmonella* positive. Furthermore, day-to-day variation in the load of *Salmonella* on skins indicates source of hogs may be just as important as the lairage environment for skin contamination with *Salmonella*. Although 49/760 (6.4%) of chilled final carcasses were positive for the presence of *Salmonella*, only four had high enough levels detectable by the enumeration procedure. The remaining 45 needed an enrichment strategy to allow for detection of *Salmonella*.

The analysis of the serotype and antimicrobial resistances presents a complex mixture of serotypes and antibiotic resistant patterns. Twenty-two serotypes combine to have 29 antibiotic resistance profiles.

Several outcomes from this research should be particularly useful to producers and processors. When the amount of *Salmonella* on skins was high, as indicated by both a large number of enumeration positive samples and high enumeration levels, the chance of having *Salmonella* on carcasses was higher. Although the prevalence of *Salmonella* on skins and carcasses was higher than expected, the percentage of final carcasses with levels high enough to enumerate and the enumeration levels were both relatively low. Finally, the number of different *Salmonella* serotypes and different antibiotic resistance patterns was relatively high.