RESEARCH REPORT
PORK SAFETY

Title: Prevalence and characterization of Salmonella from head meat and trim for ground at pork processing facilities; NPB Grant #14-203.

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Industry Summary: Recent research has reported that if superficial lymph nodes from cattle carcasses were included into ground beef, there was an increased risk of *Salmonella* contamination of ground beef. This increased risk of contamination is because *Salmonella* is often at a higher prevalence in lymph nodes compared to other tissues. Pork products, particularly the head trim and other trim destined for ground pork, may contain lymph nodes. It is not unreasonable to speculate that pork, like beef, could be at increased risk of *Salmonella* contamination if lymph nodes are present in ground pork. To date, there has been limited to no research on *Salmonella* prevalence for chops and roasts, head trim, or trim intended for ground. We had preliminary data from a pork processing plant to indicate that a high percentage (98%) of cheek meat can be contaminated with *Salmonella*. The objectives of the present study were to: 1) determine the prevalence of *Salmonella* in head meat and trim intended for ground; 2) determine the serotypes (genetics) of isolates; 3) determine the antibiotic resistance of isolates; and 4) if justified, use molecular techniques to determine the relatedness of isolates that are of the same serotype, but display differences in antimicrobial patterns.

In this study, a large pork processing plant in the United States was sampled every other month for 11 months from January to November of 2015 to determine the prevalence, seasonality, diversity of serotypes, and antimicrobial susceptibility of *Salmonella enterica* isolated from cheek meat and head trim of swine carcasses. Each cheek meat and head trim collection period (January, March, May, July, September, and November) consisted of 25 samples collected on a Monday a.m., 25 on Monday p.m., 25 on Tuesday a.m., and 25 on Tuesday p.m., for a total of 100 cheek meat and 100 head trim samples (total of 200 for each period, total of 1200 for 6 periods). Tissues were cultured for *Salmonella* by described procedures using restrictive media and enrichment techniques. *Salmonella* isolates were serotyped by the National Veterinary Services Laboratories, Ames, IA, USA.

For the six sample periods, the percentages of SE-positive sample totals were 63% for cheek meat and 66% for head trim for a total of 774 isolates. The following were the results of isolations from cheek meat and head trim: January 94%; March 80%; May 53.5%; July 58.5%; September 46.5%; and November 55%. There was a large diversity of serotypes (25) which included isolates commonly found in swine and others that have rarely seen in swine. We identified 218 isolates (99 [58.8%] cheek meat and 119 [66.8%] head trim) that were multi-drug resistant (greater than 3 classes of antibiotics). Of the multi-drug resistant isolates, 90 were the type identified by a national program (National Antimicrobial Monitoring System) as *Salmonella* (ACSSuT phenotype) of increasing concern. Additionally, increased resistance to ciprofloxacin, a broad spectrum antibiotic used in human medicine, was observed in isolates and was attributed to genetic elements within the bacteria.

The results from this study suggest that pork products from the head have a high carriage rate of *Salmonella* which includes a diverse population of serotypes with a substantial number of isolates with elevated multi-drug resistance. Based on our results, there appears to be an effect of season with increased prevalence of *Salmonella* in cooler months (Jan., Mar., Nov. = 76.3%) compared to warmer ones (May, Jul., Sept. = 52.8%). The results from this study are beneficial to the industry because now there is a knowledge base of the extent of the *Salmonella* prevalence, the level of antibiotic resistance in *Salmonella* from swine products, the potential

seasonality of *Salmonella* carriage in swine, and the wide range of serotypes and genetic diversity of *Salmonella* in swine products. With an increased knowledge of a problem comes the search for solutions. Intervention methods to reduce *Salmonella* in head products of swine processing plants are warranted and will be forthcoming. Overall pork wholesomeness will be improved and thus a "value added product".

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Scientific Abstract:
Pork head meat, cheek meat, lymph nodes, and other carcass by products may become contaminated with *Salmonella* in pork slaughter facilities. In a survey, a large pork processing plant in the United States was sampled bimonthly for 11 months from January to November of 2015 to determine the prevalence, seasonality, serotype diversity, and antimicrobial susceptibility of *Salmonella enterica* (SE) isolated from cheek meat and head trim of swine carcasses. Each cheek meat and head trim collection period (January, March, May, July, September, and November) consisted of 25 samples collected on a Monday a.m., 25 on Monday p.m., 25 on Tuesday a.m., and 25 on Tuesday p.m., for a total of 100 cheek meat and 100 head trim samples (total of 200 for each period, total of 1200 for 6 periods). Tissues were cultured for SE by described procedures using restrictive media and enrichment techniques. SE isolates were serotyped by the National Veterinary Services Laboratories, Ames, IA, USA.

For the six sample periods, the percentages of SE-positive sample totals were 63% for cheek meat and 66% for head trim. The following were the results of isolations from cheek meat and head trim: January 94%; March 80%; May 53.5%; July 58.5%; September 46.5%; and November 55%. Serotypes (25) included: Derby; Heidelberg; Senftenberg; Muenchen; Typhimurium var 5-; Brandenburg; 4,12:i-; Rough_O:gst; London; Infantis; Enteritidis; Westhampton; Alachua; Ohio; Bredeney; 4,[5],12:i-; Mbandaka; Rissen; Anatum;

Typhimurium; Agona; Kentucky; Montevideo; 4,5,12:i:; and Worthington. We identified 218 isolates (99 [58.8%] cheek meat and 119 [66.8%] head trim) that were multidrug resistant (greater than 3 classes of antibiotics). Of the 45 serotype 4,[5],12:i:- *Salmonella* isolated, 26 of them exhibited the ACSSuT phenotype highlighted in the 2013 NARMS Integrated report as an increasing phenotype of concern. In addition, 97 isolates exhibited elevated ciprofloxacin MIC values (0.5 to 2 mg/L). The plasmid mediated quinolone resistance (PMQR) gene *qnrB* was found in 19 of 32 isolates sequenced. The finding of *Salmonella* isolates with elevated ciprofloxacin MIC values and PMQR genes, in addition to a multitude of other AMR genes, from pork at slaughter is concerning and warrants further investigation.

These data suggest that pork products from the head may have a relatively high carriage rate of SE which includes a diverse population of serotypes, a substantial number of isolates with elevated MDR, and based on our results, there appears to be an effect of season (increased in cooler months compared to warmer ones) on the prevalence of SE in head and cheek meat.

Introduction:

It is well-known that *Salmonella enterica* (hereafter called *Salmonella*) is shed in the feces of *Salmonella* colonized cattle (Loneragan *et al*., 2005; Loneragan *et al*., 2012; Edrington *et al*., 2004; Gragg *et al*., 2013a,b). *Salmonella*-contaminated fecal material on the hair and hide of cattle is the most common source of beef carcass contamination at slaughter. Antimicrobial intervention techniques during processing have reduced carcass contamination to less than 1% (Barkocy-Gallagher *et al*., 2003), yet *Salmonella* contamination rates in beef products remain at about 2.1% for the past ten years (FSIS, 2011). Possibly, this discrepancy could be related to the fact that beef may become contaminated when *Salmonella*-contaminated lymph nodes are incorporated into edible tissue (Arthur *et al*., 2008). This public health issue is a source of major concern to the beef industry.

It is not unreasonable to believe that lymph nodes in swine when included in edible pork could also serve as a source of *Salmonella* contamination. Swine lymph nodes such as the mandibular lymph node can become infected with *Salmonella* within three hours of exposure and then the organism can rapidly spread to other

lymph nodes and tissues (Vieira-Pinto *et al*., 2005, 2012). Various lymph nodes are often found in head trim and trim for ground. The mandibular, superficial cervical, and sub-iliac lymph nodes are closely associated with head meat, shoulders, and hams, respectively. Limited research on *Salmonella* contamination of superficial lymph nodes in swine would suggest very low risk of pork contamination (Bahnson *et al.*, 2006, Wang *et al*., 2010), yet most swine studies have been conducted in the northern U.S. It is reported that *Salmonella* prevalence in cattle is increased in the southern U.S. compared to the northern U.S., and is increased in summer months compared to cooler months (Barkocy-Gallagher *et al*., 2003; Gragg *et al*., 2013a). The authors of the present study propose the same would hold true for swine. To date, there has been limited to no research on seasonality of *Salmonella* prevalence or prevalence in head meat, head trim, or other trim intended for ground. It is unknown whether head meat and trim may contribute to *Salmonella* in pork, hence the purpose of this study. However, the authors have preliminary data (Harvey et al., 2014, unpublished data) from a large commercial pork processing plant to indicate that a very high percentage (98%) of cheek meat and lymph nodes associated with cheek meat were contaminated with *Salmonella* (117 isolates); 23% had more than one serogroup isolated from the same sample; 90% of the isolates belonged to serogroup B; other isolates included serogroups E_4, E_1, C_1, and C_2; concentrations ranged from 1-3 $\log_{10}$ CFU/g; and 23% were multi-drug-resistant.

Objectives:

1) Determine the prevalence of *Salmonella* in head meat and trim intended for ground.

2) Determine serotypes of *Salmonella* isolates.

3) Determine antimicrobial susceptibilities of *Salmonella* isolates.

4) If justified, use molecular techniques to determine the genetic relatedness of isolates that are of the same serotype, but display differences in antimicrobial patterns.

Materials and Methods:

a) Sample collection. Samples at each two-day collection period consisted of 100 cheek meats and 100 trim intended for ground from a large commercial pork processing plant in the southern U.S. that services multiple grower farms. Samples were collected bi-monthly (over a two-day period) for 11 months, beginning on January

1, 2015 and continuing until November 30, 2015 (6 sample periods @ 200 per period for a total of 1200 tissues). For each two-day visit, 25 samples of each of 2 tissues (see above) were collected on the morning of day 1, 25 on the afternoon of day 1, and the process repeated on day 2 until all 200 samples had been drawn. Monthly sample dates were pre-determined to insure that the effects of sow herd/genetics and geographical locations of farms that produced the market hogs were taken into account for equal representation within collected samples.

b) Head meat and trim processing; *Salmonella* cultivation. Trim tissue and meat were processed as previously described (Brichta-Harhay *et al*., 2012). Tissues were weighed, surface sterilized by submersion in boiling water for 3-5 s, placed into sterile filtered-stomacher bags (Nasco, Fort Atkinson, WI), macerated by pounding with a rubber mallet, enriched in 80 mL of tryptic soy broth (Becton Dickinson, Sparks, MD), and homogenized for 30 s with a laboratory blender (BagMixer 400VW, Interscience Laboratories Inc., Weymouth, MA) at a medium speed (seven paddle strokes per second). The homogenized samples were incubated at 25ºC for 2 h, 42ºC for 12 h, and then held at 4ºC, for no more that 4-6 h, until further processing.

c) *Salmonella* enumeration. Immediately following homogenization in the bag described above, 1 mL of tryptic soy broth/LN/meat homogenate was plated onto Petrifilm™ Enterobacteriaceae Count Plates (EB; 3M Microbiology, St. Paul, MN) in duplicate, and incubated at 37ºC for 18-22 h. Gas-producing colonies were counted and Petrifilm™ plates were held at 4ºC until presumptive *Salmonella* enrichment results were obtained. Petrifilms™ from meat samples identified as potentially containing *Salmonella* by enrichment were selected for further confirmation.

d) Prevalence. For prevalence analysis, 1 mL from each enrichment culture was subjected to anti-*Salmonella* immunomagnetic separation (IMS). Each 1-mL aliquot received 20 µL of anti-*Salmonella* beads (Invitrogen, Carlsbad, CA), and incubated with shaking at room temperature for 15 min. The beads were extracted from the enrichment samples and washed twice in PBS-Tween 20. The beads were transferred to 3 mL of Rappaport Vassiliadis soya (RVS; Remel Products, Lenexa, KS) broth and incubated at 42ºC overnight. *Salmonella* present in these samples were detected by swabbing the RVS enrichment culture brilliant green agar (Becton

Dickinson, Sparks, MD) containing sulfadiazine (80 mg/L; Sigma, St. Louis, MO). All plates were incubated at 37ºC for 18 to 20 h. After incubation, up to three suspect colonies were picked for confirmation.

e) Serotyping of *Salmonella*. Prior to serotyping, phenotypes were confirmed by traditional slide agglutination (O typing) methods, using commercial antisera (Difco/BD, Sparks, MD) following manufacturer's guidelines. Representatives within groups demonstrating the same molecular pattern were sent to the USDA-Animal and Plant Health Inspection Service, National Veterinary Services Laboratory, Ames, IA, for serotype confirmation by traditional methods.

f) Antimicrobial susceptibility testing. Susceptibility to 14 antimicrobial agents were determined by use of an automated micro-broth dilution method (Sensititre Gram Negative NARMS Plates, TREK Diagnostics Inc., Cleveland, OH) according to the manufacturer's recommendations. Isolates were classified as susceptible, intermediate, or resistant using breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2014). Isolates that were resistant to three or more classes of antimicrobials, as defined by the National Antimicrobial Resistance Monitoring System, were considered multi-drug resistant (MDR).

g) Whole genomic sequencing. Whole genome sequencing of *Salmonella* isolates was performed on an Illumina MiSeq platform using the Nextera XT Library Preparation Kit (Illumina, San Diego, California). Raw fastq files were uploaded to a suite of tools available online through the Center for Genomic Epidemiology (Technical University of Denmark, Copenhagen, Denmark) including Resfinder, PlasmidFinder, VirulenceFinder, and SeqSero to determine the presence of antimicrobial resistance genes, plasmids, and virulence genes, as well as confirm serotypes.

h) Statistical analysis. General (quantitative concentration data) and generalized (qualitative prevalence data) linear mixed models were developed to compare observed data within sample types and across time periods. To account for possible dependency in the data, observations within the same plant within the same time-point were considered clustered and an appropriate method (e.g., G-sided random intercept or R-sided over dispersion parameter) were included in the model. The independent variables of interest (month and tissue type) will be modeled as predictors of the outcome variables of interest, i.e., *Salmonella* count expressed as log10-

transformed colony-forming units/g and a binomial response variable representing prevalence were the response variable. For all models, significance was determined by alpha = 0.05.

Results:

Objective 1: Prevalence. For the six sample periods, the percentages of SE-positive sample totals were 62.8% for cheek meat and 66.3% for head trim for a total of 774 isolates (Table 1). The following were the combined isolations from cheek meat and head trim: January 94%; March 80%; May 53.5%; July 58.5%%; September 46.5%; and November 55% (Table 2). Isolations in Jan., Mar., and Nov., accounted for 76.3% of the total compared to 52.8% isolated in May, Jul., and Sep. The time of day did not appear to influence the isolation of *Salmonella* with 406 isolated in the morning versus 368 in the afternoon (Table 3). Concentrations (colony-forming units/g tissue [cfu/g]) of *Salmonella* were between 0.1 cfu/g to 1.2 cfu/g with 97.8% of isolates falling in the range of 0.1 to 0.6 cfu/g (Table 4).

Objective 2: Serotypes. There were 25 different serotypes isolated which included: Derby (20); Heidelberg (50); Senftenberg (13); Muenchen (14); Typhimurium var 5- (179); Brandenburg (15); 4,12:i- (10); Rough-_O:gst (1); London (2); Infantis (58); Enteritidis (8); Westhampton (1); Alachua (4); Ohio (18); Bredeney (13); 4,[5],12:i- (17); Mbandanka (1); Rissen (1); Anatum (32); Typhimurium (121); Agona (12); Kentucky (1); 4,5,12:i: (20); Montevideo (2) and Worthington (6). Typhimurium var 5- made up 28.92% of all serotypes whereas Typhimurium totaled 19.55%. Those two accounted for 48.47% of serotypes isolated.

Objective 3: Antimicrobial susceptibility. When an automated system was used to test 582 *Salmonella* isolates for susceptibility to 14 antimicrobial agents, 494 were resistant to at least one antibiotic, whereas 88 were pan-susceptible. Most isolates (62.9%) were resistant to three or more antimicrobials and were classified as multi-drug resistant (MDR) (Table 5). In addition, 97 isolates exhibited reduced susceptibility to ciprofloxacin (MIC values: 0.5 to 2 µg/ml). There were no differences in antimicrobial resistance patterns when cheek meat isolates were compared to those of head trim (data not shown). Ninety (15.5%) of 582 isolates demonstrated an ACSSuT pattern (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline)

(Table 6). This pattern would encompass five of the nine classes of antimicrobials. Five isolates of 582 (0.86%) demonstrated a MCRampC pattern (data not shown), which includes ACSSuT resistance plus resistance to ceftiofur and augmentin. This pattern would encompass seven of the nine classes of antimicrobials.

Objective 4: Genetic factors/whole genomic sequencing. Whole genomic sequencing of select *Salmonella* serotype 4,[5],12:i:- isolates that were ACSSuT revealed the presence of *tetA, tetB, tetC, tetD, tetG, sul1, sul2, strA, strB, floR, dfrA, bla*$_{CMY}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ antimicrobial resistance genes among the isolates. In isolates that exhibited increased MIC values to ciprofloxacin, the plasmid mediated quinolone resistance (PMQR) gene *qnrB* was found in 19 of 32 isolates sequenced.

Discussion

In the authors' opinions, the prevalence of *Salmonella* in tissues of this study (>60%) appeared unusually high. Previous swine studies have shown that cheek meat can be contaminated with *Salmonella* at 30-98% prevalence (Frederick *et al*., 1994; Norman *et al*., 2016;[Harvey *et al*., 2014, unpublished data]). The present study suggests that *Salmonella* isolations may be more prevalent in cooler months of the year when compared to warmer months, and this agrees with the results of a study on swine carcass isolations in Germany (Meyer *et al*., 2010). On the other hand, cattle studies suggest just the opposite in that higher prevalence occurs during warmer months (Barkocy-Gallagher *et al*., 2003; Gragg *et al*., 2013a). In the present study, there were no differences in isolation rates between cheek meat and head trim, nor were there differences on the basis of time of day. Concentrations of *Salmonella* in the present study ranged from 0.1 to 1.2 cfu/g with 97.8% of isolates falling in the range of 0.1 to 0.6 cfu/g. This is considerably lower than that of $\log_{10}$ 3.5 cfu reported for swine cheek meat; however in that study, concentrations were determined by cfu/cm^2 compared to ours of cfu/g tissue (Frederick *et al*., 1994).

In a recent study in Mexican abattoirs, swine tonsils and mandibular lymph nodes, which are associated with head meats, had *Salmonella* prevalence rates of 12-40% (Chaves *et al*., 2017). In another study, chorizo, a sausage which incorporates cheek meat, had a *Salmonella* prevalence of 78-88% (Escartin *et al*., 1999). These

findings and that of the present study are important in that *Salmonella*-contaminated cheek meat and head trim can find their way into retail foods and thus pose a human health risk. Indeed, chorizo was implicated in a salmonellosis outbreak in 2013 that sickened over 294 people after consuming cooked chorizo from a restaurant in Las Vegas, NV (Food Poisoning Bulletin, 2013). The serotype responsible was I:4,5,12:i-. In the present study, we had 17 isolates of that serotype.

In our study, we had 25 different serotypes out of 774 *Salmonella* isolates with Typhimurium and Typhimurium var 5- composing approximately 50% of all serotypes. These serotypes are the predominant ones nationwide from human salmonellosis cases associated with pork (CDC 2012). This is important in that many of these Typhimurium serotypes were multi-drug-resistant, thus potentially adding risks of human exposure to pathogens. Most of the serotypes of the present study have been reported in swine previously (CDC 2012); however, some such as serotype Alachua have never or rarely been reported in swine.

In the present study, 63% of *Salmonella* isolates were MDR (resistant to 3 or more antibiotics) and 15.5% were ACSSuT. This pattern in any species of bacteria is of high interest to the National Antimicrobial Resistance Monitoring System (NARMS, 2014) because it signifies resistance to five of the nine classes of antibiotics. Although ACSSuT in the present study is 15%, other studies in Spain and Minnesota show swine ACSSuT isolates at 61% and 54%, respectively (Agustin *et al.*, 2005; Wedel, *et al*, 2005). As of 2014, the prevalence of ACSSuT in human *Salmonella* isolates has been steadily dropping to 3.1% in the United States (NARMS 2014), which makes our prevalence five times that level. The presence of MCRampC resistance (ACSSuT plus ceftiofur and augmentin resistance) is even more alarming because it demonstrates resistance to seven classes of antibiotics, many of which are important lines of defense in human medicine (Garcia *et al.*, 2016). Decreased susceptibility to ciprofloxacin (fluoroquinolone resistance) in isolates of our study is of concern because it is a broad spectrum antibiotic of importance in human medicine (Casas *et al*., 2016). Nationally, a trend of ciprofloxacin resistance is emerging in human and cattle *Salmonella* isolates (NARMS 2014) and the results of our study appear to agree with this trend. The presence of MDR in meat products is important in that it shows the existence of MDR bacteria in food animals which through bacterial interspecies transfer could increase the

prevalence of MDR in the environment (Garcia *et al*., 2016). Additionally, MDR in food products puts humans at risk for acquiring MDR microflora in their intestinal tract.

Genetic factors of resistance in the present study reveal that a number of phenotypic resistance patterns are genetically generated (Casas *et al*., 2016; Garcia *et al*., 2016). The presence of a number of resistance genes suggests that bacteria can readily acquire resistance through transfer of genetic material. This is true even for commensal (beneficial) bacteria the human digestive tract. These genetic data merely support and strengthen the findings of the antimicrobial susceptibility testing in this study.

Conclusions

The results from this study suggest that pork products from the head have a high carriage rate of *Salmonella*, a seasonal trend towards increased prevalence during cooler months of the year, a diverse population of serotypes, a substantial number of isolates with elevated multi-drug resistance, and genetic factors that induce drug resistance and potentially enhance resistance transfer. All of these findings are cause for concern with regard to foodborne disease. With increased awareness of a problem comes a search for solutions. Intervention methods to reduce *Salmonella* in head products of swine processing plants are warranted.

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***Salmonella* Isolated from Swine Cheek Meat and Head Trim NPB #14-203**

I. Prevalence of *Salmonella*

Table I

Tissue	#Positive	Percent Positive
Cheek	377	62.8
Head	397	66.3

Table 2

Month	#Positive	Percent Positive
Jan.	187	94.0
Mar.	160	80.0
May	107	53.5
Jul.	117	58.5
Sep.	93	46.5
Nov.	110	55.0

Table 3

Sample Time of Day	# Positive	Percent Positive
Morning	406	67.7
Afternoon	368	61.4

II. Concentration (colony forming units [cfu]/g tissue) of *Salmonella*

Table 4

Concentration (cfu)	Frequency	Percent
0.1	625	82.45
0.2	35	4.62
0.3	20	2.64
0.4	19	2.51
0.5	24	3.17
0.6	18	2.37

III. Antimicrobial Resistance of *Salmonella*

Table 5

# Antibiotic Resist	# Isolates	Percent Isolates
0	88	15.12
1	35	6.01
2	93	15.98
3	167	28.69
4	35	6.01
5	122	20.96
6	18	3.09
7	9	1.55
8	2	0.34
9	5	0.86
10	3	0.52
11	5	0.86
12	0	0.0
13	0	0.0
14	0	0.0

Table 6

ACSSuT*	Frequency	Percent
Negative	492	84.54
Positive	90	15.46

*ACSSuT = combined resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline