RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: *Salmonella* DIVA vaccine for cross-protection against *Salmonella* serovar I 4,[5],12:i:-: minimizing antibiotic usage and protecting swine and public health - #16-113 IPPA

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Industry Summary: In 2015, a multistate outbreak of *Salmonella* serovar I 4,[5],12:i:- was associated with pork products from Washington state and resulted in 188 illnesses including 30 individuals being hospitalized. The *Salmonella* isolates associated with the outbreak were determined to be multidrug-resistant (resistant to ≥3 antimicrobials) and ~523,380 pounds of pork products were recalled. *Salmonella* serovar I 4,[5],12:i:- has increased globally over the last 10-15 years and as of 2015 serovar I 4,[5],12:i:- was the 4th most common *Salmonella* serovar associated with human illness in the U.S. Furthermore, ~67% of serovar I 4,[5],12:i:- isolates are considered multidrug-resistant.

The Objectives were 1) Identification of unique characteristics of multidrug-resistant *Salmonella* serovar I 4,[5],12:i:-, and 2) Determine whether vaccination of swine with an attenuated *Salmonella* Typhimurium vaccine can provide protection against multidrug-resistant *Salmonella* serovar I 4,[5],12:i:- transmission.

In 2015 and 2016, the USDA, Food Safety and Inspection Service (FSIS) collected 33 isolates of *Salmonella* serovar I 4,[5],12:i:- from pork products in 15 states (CA, 2; IL, 1; IN, 2; KY, 2; MD, 1; MI, 1; NC, 2; NE, 1; NY, 3; OK, 2; PA, 1; SC, 2; SD, 1; TX, 1; and WA, 11). Thirty of the serovar I 4,[5],12:i:- isolates were multidrug-resistant including all 11 isolates from WA that were associated with the 2015 *Salmonella* outbreak. FSIS determined bacterial strain relatedness for the 33 serovar I 4,[5],12:i:- isolates using pulsed-field gel electrophoresis (PFGE) and identified 13 primary PFGE patterns. Of the 33 serovar I 4,[5],12:i:- isolates, 19 isolates share the same primary PFGE pattern JPXX01.1314, 3 isolates share the primary PFGE pattern of JPXX01.2583, and 11 isolates have unique primary PFGE patterns. The 19 serovar I 4,[5],12:i:- isolates with the primary PFGE pattern JPXX01.1314 included all of the outbreak-associated strains from WA state but also isolates from CA, IL, KY, MI, NY, SD, and TX. This data indicates that serovar I 4,[5],12:i:- isolates that are closely related to the 2015 outbreak-associated strains are present in numerous states across the U.S. We obtained the 33 isolates of *Salmonella* serovar I 4,[5],12:i:- from FSIS for our investigation. FSIS initially performed whole genome sequencing on all of the serovar I 4,[5],12:i:- isolates using the Illumina MiSeq platform. Additionally, we obtained the whole genome sequence for strain FSIS1503788 associated with the WA state outbreak using the PacBio platform to create a serovar I 4,[5],12:i:- genome scaffold and facilitate assembly of the sequencing reads previously generated by FSIS. The genome of serovar I 4,[5],12:i:- strain FSIS1503788 is 5,029,387 bp, a genome size that is similar to other sequenced *Salmonella* serovars.

Salmonella serovar designations are identified by a combination of lipopolysaccharide (LPS) and flagellar antigens. The LPS antigens for serovar Typhimurium are 4,[5],12 and the flagellar antigens are i:1,2. *Salmonella* serovars alternate expression of two flagellar antigens [*fliC* (i) and *fljB* (1,2)] resulting in a biphasic phenotype. The absence of one of the two flagellar antigens results in a monophasic phenotype. *Salmonella* serovar I 4,[5],12:i:- is a

monophasic variant of serovar Typhimurium that is missing *fljB*. The *fljB* genomic region of *Salmonella* serovar I 4,[5],12:i:- isolate FSIS1503788 is missing an ~15.7 kb portion of DNA that is present in the closely related serovar Typhimurium. However, inserted into the *fljB* genomic region of serovar I 4,[5],12:i:- isolate FSIS1503788 is an ~28 kb module encoding resistance to the metal mercury and the antimicrobials ampicillin, streptomycin, sulfisoxazole, and tetracycline (R-type ASSuT). Therefore, the 28 kb insertion in the *fljB* region of isolate FSIS1503788 confers the multidrug-resistance phenotype. The composition of this multidrug-resistance module is quite similar to an insertion in the *fljB* genomic region of multidrug-resistant serovar I 4,[5],12:i:- strain 07-2006 isolated from a lymph node of a pig in Germany.

Nucleotide sequence analysis also identified the presence of *Salmonella* Genomic Island 4 (SGI-4) in serovar I 4,[5],12:i:- isolate FSIS1503788. SGI-4 is an ~80 kb genomic island containing multiple genetic operons encoding potential resistance to copper, arsenic, and zinc. An Australian serovar I 4,[5],12:i:- strain TW-Stm6 isolated from swine feces also contains SGI-4 indicating global distribution of *Salmonella* strains related to isolates from the 2015 pork outbreak. We constructed a derivative of FSIS1503788 with a deletion of the entire 80 kb SGI-4 genomic island and therefore no longer contains the genes for metal resistance to copper, arsenic, and zinc. We performed phenotypic analysis on FSIS1503788 and its ΔSGI-4 derivative to determine microbial sensitivity to copper, arsenic, and zinc. The ΔSGI-4 mutant of serovar I 4,[5],12:i:- had increased sensitivity to arsenic and copper compounds compared to the FSIS1503788 parental wildtype. However, neither wildtype serovar I 4,[5],12:i:- isolate FSIS1503788 nor the ΔSGI-4 mutant derivative had a sensitivity to the various concentrations of zinc present in our phenotypic assay. This indicates that higher concentrations of zinc will need to be assessed to determine the level(s) for growth inhibition for these strains. The phenotypic analysis indicates that the metal resistance genes present in the SGI-4 genetic island confer increased survival for wildtype serovar I 4,[5],12:i:- isolate FSIS1503788 in the presence of copper and arsenic compared to a strain lacking these metal resistance genes. The use of copper, arsenic, and potentially zinc antimicrobials in animal and poultry production may have selected for the ~80 kb SGI-4 genetic island in *Salmonella* serovar I 4,[5],12:i:- and the presence of SGI-4 in the genome may provide a selective advantage for colonization of swine or survival in the environment when metal containing antimicrobials are used as alternatives to antibiotics during animal production.

A pathogenesis trial using *Salmonella* serovar I 4,[5],12:i:- isolate FSIS1503788 associated with the 2015 pork outbreak was performed in swine and has recently been published (Foodborne Pathog. Dis. 2018. 15(5):253-261; doi: 10.1089/fpd.2017.2378). Challenge of swine with serovar I 4,[5],12:i:- isolate FSIS1503788 significantly increased the swine body temperature, fecal moisture content, and IFN-γ levels in pigs at certain time points. Furthermore, colonization of swine with serovar I 4,[5],12:i:- resulted in the disruption of the gastrointestinal microbiota. The increases in swine body temperature, fecal moisture content, and IFN-γ levels, and the level of fecal shedding and tissue colonization for serovar I 4,[5],12:i:- following swine challenge is consistent with previous challenge experiments that we have conducted in pigs using *Salmonella* Typhimurium. Therefore, *Salmonella* serovar I 4,[5],12:i:- is pathogenic to pigs but its virulence does not appear to be greater than *Salmonella* Typhimurium strains that we have used in other swine pathogenesis trials.

A swine trial was performed to determine whether vaccination with a live, attenuated *Salmonella* Typhimurium vaccine could reduce colonization by *Salmonella* serovar I 4,[5],12:i:- isolate FSIS1503788 associated with the 2015 pork outbreak following transmission from inoculated penmates. Over the 14 day trial, fecal shedding between vaccinated (n=10) and mock-vaccinated (n=10) groups was not significantly different but serovar I 4,[5],12:i:- was significantly reduced in the cecal contents of vaccinated compared to mock-vaccinated pigs following transmission. In combination with other swine management tools, vaccination against *Salmonella* may assist in reducing *Salmonella* serovar I 4,[5],12:i:- carriage in pigs.

Characterization of *Salmonella* serovar I 4,[5],12:i:- associated with a 2015 pork outbreak indicates that these strains are globally distributed and the presence of multiple antimicrobial and metal resistance genes may provide a unique and selective advantage for colonization of swine when either the antimicrobials or metals are used during animal production.

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Keywords: *Salmonella* serovar I 4,[5],12:i:-, multidrug-resistant, genome sequence, heavy metal resistance, pathogenesis, vaccination

Scientific Abstract: In 2015, a multistate outbreak of *Salmonella* serovar I 4,[5],12:i:- was associated with pork products from Washington state and resulted in 188 illnesses including 30 individuals being hospitalized. The *Salmonella* isolates associated with the outbreak were determined to be multidrug-resistant (resistant to ≥3 antimicrobials) and ~523,380 pounds of pork products were recalled. *Salmonella* serovar I 4,[5],12:i:- has increased globally over the last 10-15 years and as of 2015 serovar I 4,[5],12:i:- was the 4th most common *Salmonella* serovar associated with human illness in the U.S. We investigated 33 *Salmonella* serovar I 4,[5],12:i:- strains that were isolated by the USDA, Food Safety and Inspection Service (FSIS) during 2015-2016 from pork products in 15 states. Thirty of the serovar I 4,[5],12:i:- isolates were multidrug-resistant including all 11 isolates from WA that were associated with the 2015 *Salmonella* outbreak. Nineteen serovar I 4,[5],12:i:- isolates had the same primary PFGE pattern (JPXX01.1314) including all of the outbreak-associated strains from WA state but also isolates from CA, IL, KY, MI, NY, SD, and TX. This data indicates that serovar I 4,[5],12:i:- isolates that are closely related to the 2015 outbreak-associated strains are present in numerous states across the U.S. We analyzed the genome sequence for *Salmonella* serovar I 4,[5],12:i:- isolate FSIS1503788 associated with the 2015 pork outbreak. Two unique genomic insertions were identified in serovar I 4,[5],12:i:- isolate FSIS1503788 compared to closely related *Salmonella enterica* serovar Typhimurium. FSIS1503788 has an ~28 kb module encoding resistance to the metal mercury and the antimicrobials ampicillin, streptomycin, sulfisoxazole, and tetracycline (R-type ASSuT). The second insertion is *Salmonella* Genomic Island 4 (SGI-4) an ~80 kb DNA island that contains multiple genetic operons encoding potential resistance to copper, arsenic, and zinc. We created a derivative of serovar I 4,[5],12:i:- isolate FSIS1503788 that does not contain SGI-4 and therefore does not contain potential metal resistance genes for copper, arsenic, and zinc. A phenotypic assay was performed comparing FSIS1503788 and the SGI-4 mutant. The wildtype strain grew better in the presence of copper and arsenic compounds compared to the SGI-4 mutant indicating that genes for resistance to copper and arsenic are present on SGI-4 and enhance growth of FSIS1503788 in the presence of these metals. A pathogenesis trial was performed in swine with serovar I 4,[5],12:i:- isolate FSIS1503788 challenge. The pathogenesis of serovar I 4,[5],12:i:- was similar to other swine pathogenesis trials that have been performed with *Salmonella* Typhimurium. A transmission trial of serovar I 4,[5],12:i:- was conducted in vaccinated and mock-vaccinated swine that shared a pen with directly inoculated pigs. The colonization of serovar I 4,[5],12:i:- isolate FSIS1503788 was significantly reduced in the cecal contents of vaccinated swine compared to mock-vaccinated pigs following 14 days of exposure to donor pigs. Vaccination against *Salmonella* may provide a swine management tool to assist in reducing *Salmonella* serovar I 4,[5],12:i:- carriage in pigs. The *Salmonella* serovar I 4,[5],12:i:- strains associated with the 2015 pork outbreak are similar to serovar I 4,[5],12:i:- strains that are distributed across the U.S. and globally. These serovar I 4,[5],12:i:- strains have 2 unique genomic insertions that encode metal and antimicrobial resistance genes. The use of metal compounds as alternatives to antimicrobials in swine may select for *Salmonella* serovar I 4,[5],12:i:- isolates that contain metal resistance genes.

Introduction: The prevalence of *Salmonella* serovar I 4,[5],12:i:- has increased globally over the last 10-15 years and in 2015 a multistate pork outbreak occurred in the U.S. due to serovar I 4,[5],12:i:- (Kawakami *et al*. 2016). The outbreak was associated with pork products from Washington state and resulted in 188 illnesses including 30 individuals being hospitalized. The outbreak associated isolates of serovar I 4,[5],12:i:- were multidrug-resistant (resistant to ≥3 antimicrobials) and conferred resistance to the antimicrobials ampicillin, streptomycin, sulfisoxazole, and tetracycline (R-type ASSuT). The outbreak resulted in the recall of 523,380 pounds of pork products. The identification of unique genetic characteristics of serovar I 4,[5],12:i:- associated with the 2015 pork outbreak may provide information on the recent emergence and distribution of this *Salmonella* serovar in swine production. Genomic analysis, in vitro assays, and swine trials were performed to determine potential unique attributes of serovar I 4,[5],12:i:- that may have a benefit for this serovar in pork production environments. Furthermore, identification of swine management procedures to reduce serovar I 4,[5],12:i:- in pork production are needed. A *Salmonella* vaccine trial using a transmission model was performed to determine the potential reduction of serovar I 4,[5],12:i:- in pigs following exposure to directly inoculated swine. Vaccination of swine with an attenuated *Salmonella* vaccine demonstrated the potential to reduce serovar I 4,[5],12:i:- colonization in pigs. Genetic insertions were identified in a pork outbreak associated isolate of serovar I 4,[5],12:i:- including resistance genes for antimicrobials and metals. The use of metals as alternatives to antibiotics in animal and poultry production may not provide the expected animal health benefits when multidrug-resistant serovar I 4,[5],12:i:- is present in the production environment.

Objective 1: Identification of unique characteristics of multidrug-resistant *Salmonella* serovar I 4,[5],12:i:-.

Objective 2: Cross-protection against multidrug-resistant *Salmonella* serovar I 4,[5],12:i:- transmission with a rationally-designed Salmonella DIVA vaccine.

Materials & Methods:

Genome sequencing of *Salmonella* serovar I 4,[5],12:i:- isolates.

Acquisition of the genome sequence for *Salmonella* serovar I 4,[5],12:i:- strain FSIS1503788 was performed using the PacBio platform at Yale University. Canu was used to assemble the PacBio raw reads. Pilon was used to error correct the PacBio assembly. SPAdes was used to assemble the Illumina raw reads provided by FSIS. BWA was used to align the Illumina assembly to the PacBio assembly. Prokka was used to annotate the genome.

Deletion of SGI-4 from FSIS1503788

Salmonella Genomic Island 4 (SGI-4) an ~80 kb genetic region containing genes for resistance to heavy metals was removed from serovar I 4,[5],12:i:- isolate FSIS1503788 by recombineering. Oligonucleotides were designed with sequence homology to the genomic/SGI-4 junctions on their 5' ends and nucleotides sequences to amplify oBBI 92/93-*neo* on their 3' ends. The oBBI 92/93-*neo* fragment was PCR amplified using oBBI 528 (gatgagatccttaacgggcggaaaatgcagggtccggtcatacgatagctgaatgagtgacgtgc) and oBBI 529 (gctacggatttccattcccagaaaagcaaaaacccagccataggcatagagcagtgacgtagtcgc), run on an agarose gel, extracted, purified, and transformed into BBS 1268 (FSIS1503788 containing pKD46-Gm). Following transformation, knockout of SGI-4 in BBS 1270 was selected by growth on LB medium containing kanamycin.

Phenotype microarray analysis

Wildtype and the SGI-4 mutant of serovar I 4,[5],12:i:- isolate FSIS1503788 were grown in Biolog Phenotype MicroArray plates PMs 9-20 to determine sensitivity to chemical compounds as per the manufacturers instructions in a Biolog OmniLog system. Microbial respiration was measured every 15 minutes for 48 hours at 33 °C. Growth curves to compare strain growth were generated in Biolog Par software.

***Salmonella* serovar I 4,[5],12:i:- pathogenesis in Swine**

Eight Yorkshire crossbred pigs from five *Salmonella*-fecal-negative sows were weaned at 16 days of age, shipped to the National Animal Disease Center, Ames, IA, and housed in a single isolation room. Pigs tested fecal-negative for *Salmonella* twice over a 6-week period as described previously (Bearson *et al.*, 2010). At 8 weeks of age, all

pigs were inoculated via the intranasal route with 1 x 10^9 colony forming units (CFU) of strain SX 240 [FSIS1503788 passaged in swine and isolated from the ileocecal lymph node (ICLN)]. The sampling interval was 0, 1, 2, 3 and 7 days post-inoculation (d.p.i.). Body temperatures were monitored using a rectal thermometer. Fecal samples were collected for microbiota analysis, moisture content evaluation, and quantitative and qualitative bacteriology analyses. Blood samples were collected from the jugular vein for serum antibody and interferon-gamma (IFN-γ) ELISA as previously described (Bearson *et al.*, 2017). One pig died during the study due to complications unrelated to *Salmonella* inoculation. At 7 d.p.i., all pigs were euthanized and necropsied to obtain samples of tonsil, cecum, cecal contents, ileo-cecal lymph nodes (ICLN), ileal Peyer's patch, and ileal mucosal scrapings at the ileal-cecal junction.

Bacteriology

Quantitative and qualitative *Salmonella* culture analyses of fecal and tissue samples were performed as previously described (Bearson *et al.*, 2016) utilizing XLT-4 medium (Becton, Dickinson and Co., Sparks, MD) supplemented with 50% tergitol, ampicillin (100 µg/ml), tetracycline (15 µg/ml), novobiocin (50 µg/ml), and streptomycin (50 µg/ml). Suspected *Salmonella* colonies were evaluated on BBL™ CHROMagar™ Salmonella (Becton, Dickinson and Co.) for mauve colonies indicative of *Salmonella*.

Fecal moisture content

To determine fecal moisture content, 1 g of feces was placed in an aluminum pan, weighed, incubated in a drying oven at 70°C for 22 h and weighed again. Percent moisture was calculated by subtracting the dry weight from the wet weight, dividing by the wet weight, and multiplying by 100.

16S rRNA gene sequencing and analysis

Fecal samples were immediately placed on ice following collection and stored at -80°C until processing. Total DNA was extracted using the PowerMag Microbiome RNA/DNA isolation kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions and adapted for use on a Biomek FX^P laboratory automation workstation (Beckman Coulter, Indianapolis, IN). Negative controls (water) were included for quality control purposes.

The 16S rRNA gene libraries were created as previously described (Allen *et al.*, 2016) with the exception that the PCR primers 515-F 5'-GTGCCAGCMGCCGCGGTAA-3' and 806-R 5'-GGACTACHVGGGTWTCTAAT-3' were used to amplify the V4 hypervariable region of the 16S rRNA gene using previously published barcodes (Kozich *et al.*, 2013). Additionally, a mock community consisting of 1 archaeal and 19 bacterial species (Allen *et al.*, 2016) was amplified in one well of each plate for quality control purposes. Libraries were sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) using the MiSeq reagent kit v2 (2 x 250).

16S rRNA gene sequences were processed using the QIIME software package v. 1.9.1 (Caporaso *et al.*, 2010b). Paired-end 16S rRNA gene reads were joined using fastq-join (Aronesty, 2013) with a minimum overlap of 200 bp and a maximum percent difference of 3. Joined reads were quality-filtered with reads being truncated following two consecutive base calls of a quality score of less than 25. Sequences with $\geq$ 85% of the original sequence length following truncation were retained. Chimeric sequences were removed using the UCHIME algorithm (Edgar *et al.*, 2011) implemented in VSEARCH V. 2.4.0 (Rognes *et al.*, 2016). Remaining sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using a de novo OTU picking method implemented in VSEARCH (Quast *et al.*, 2013). Taxonomy was assigned to OTUs using the UCLUST consensus taxonomy assigner (Edgar, 2010) and SILVA SSU database v. 128 with a minimum similarity of 0.8 and max accepts of 3. Representative sequences for each OTU were aligned using PyNast (Caporaso *et al.*, 2010a) and a phylogenetic tree was constructed using FastTree (Price *et al.*, 2010). Based on the results of the mock community OTU clustering, OTUs containing fewer than 10 sequences were excluded from further analysis, as were those classified as chloroplasts and mitochondria. Raw sequences were submitted to the NCBI Short Read Archive under BioProject PRJNA382998.

***Salmonella* invasion assays**

IPEC-J2 cells, which were derived from the small intestine of piglets (Rhoads *et al.*, 1994), were grown and maintained as previously described (Bearson and Bearson, 2008; Bearson *et al.*, 2017). Prior to use, IPEC-J2 cells were confirmed free of *Mycoplasma* contamination using the Lookout® Mycoplasma PCR Detection Kit (Sigma-Aldrich, St. Louis, MO). The ability of the MDR serovar I 4,[5],12:i:- strain SX 240 to invade IPEC-J2 cells was compared to virulent biphasic *S*. Typhimurium strain χ4232 (SX 117) (Bearson *et al.*, 2014) as previously described (Bearson and Bearson, 2008; Bearson *et al.*, 2017) with a multiplicity of infection of 10 and gentamicin concentration of 100 μg/ml to remove extracellular bacteria.

***Salmonella* serovar I 4,[5],12:i:- transmission in Swine**

Twenty-four crossbred pigs from six *Salmonella*-fecal-negative sows were weaned at 17-22 days of age, shipped to the National Animal Disease Center, Ames, IA, and siblings were housed in separate isolation rooms. Pigs tested fecal-negative for *Salmonella* twice over a 6-week period as described previously (Bearson *et al.*, 2010). At 4 weeks of age, pigs were mixed into mock-vaccinated (n=10), vaccinated (n=10), and donor (n=4) groups. The mock-vaccinated group was administered 1 ml PBS via intranasal route. The vaccinated group was administered 1 X 10^9 CFU of *Salmonella enterica* serovar Typhimurium strain BBS 866 in 1 ml PBB via intranasal inoculation. At 6 weeks of age, mock-vaccinated and vaccinated pigs were administered a booster of PBS or BBS 866 via intranasal inoculation, respectively. At 9 weeks of age, mock-vaccinated, vaccinated, and donor pigs were mixed into 2 groups consisting of 5 mock-vaccinated, 5 vaccinated, and 2 donor pigs per group and housed in separate isolation rooms. Two donor pigs in each room were inoculated via the intranasal route with 1 x 10^9 CFU of strain SX 240 in 1 ml PBS. Fecal samples were collected at 0, 1, 2, 3, 7, 10, and 14 days post-inoculation (d.p.i.) of the donor pigs. At 14 d.p.i. of the donor pigs, all pigs were euthanized and necropsied to obtain samples of cecum, cecal contents, ileo-cecal lymph nodes (ICLN), and ileal Peyer's patch.

Statistical analyses

Statistical analyses of body temperature, fecal moisture content, and IFN-γ levels were performed using repeated measures ANOVA with a Dunnett's multiple comparison test with comparison to the day 0 control. Analysis of invasion assay data and the CFU of serovar I 4,[5],12:i:- isolate FSIS1503788/g of cecal contents were performed using an unpaired *t*-test. *P*-values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Alterations in the archaeal and bacterial community structure over the 7-day sampling period were assessed using weighted UniFrac distances (Lozupone *et al.*, 2007). All samples were randomly subsampled to 9,000 sequences prior to analysis to account for unevenness in sequencing depth. Weighted UniFrac distances were calculated and plotted using the R v. 3.3.3 packages phyloseq v. 1.19.1 (McMurdie and Holmes, 2013) and vegan v.2.4.2 (Oksanen *et al.*, 2017). Permutational multivariate analysis of variance (PERMANOVA) using the adonis function with 10,000 permutations in vegan was used to analyze the weighted UniFrac distances by sampling time. The homogeneity of dispersion for each sampling time was determined using the betadisper function of vegan. Linear discriminant analysis effect size (LEfSe) (Segata *et al.*, 2011) was used to identify genera with a relative abundance of greater than 0.1% that were more abundant at 0 d.p.i. compared with 1, 2, 3, and 7 d.p.i. A minimum LDA score of 4.0 was used as the threshold for classifying differentially abundant genera.

Results: In 2015 and 2016 the USDA Food Safety and Inspection Service (FSIS) collected 33 *Salmonella enterica* serovar I 4,[5],12:i:- isolates from pork products in 15 states (CA, 2; IL, 1; IN, 2; KY, 2; MD, 1; MI, 1; NC, 2; NE, 1; NY, 3; OK, 2; PA, 1; SC, 2; SD, 1; TX, 1; and WA, 11). Thirty of the serovar I 4,[5],12:i:- isolates were multidrug-resistant including all 11 isolates from WA that were associated with the 2015 *Salmonella* outbreak.

The goal of objective 1 was to identify unique characteristics of multidrug-resistant *Salmonella* serovar I 4,[5],12:i:- . We obtained the 33 serovar I 4,[5],12:i:- isolates that FSIS collected in 2015 and 2016 from pork products for our investigation. This set of strains provides the opportunity to investigate pork-associated isolates of serovar I 4,[5],12:i:- from multiple geographic regions of the U.S. FSIS determined bacterial strain relatedness for the 33 serovar I 4,[5],12:i:- isolates using pulsed-field gel electrophoresis (PFGE) and identified 13 primary PFGE patterns. Of the 33 serovar I 4,[5],12:i:- isolates, 19 isolates share the same primary PFGE pattern JPXX01.1314, 3 isolates

share the primary PFGE pattern of JPXX01.2583, and 11 isolates have unique primary PFGE patterns. The 19 serovar I 4,[5],12:i:- isolates with the primary PFGE pattern JPXX01.1314 included all of the outbreak-associated strains from WA state but also isolates from CA, IL, KY, MI, NY, SD, and TX. This data indicates that serovar I 4,[5],12:i:- isolates that are closely related to the 2015 outbreak-associated strains are present in numerous states across the U.S. FSIS performed whole genome sequencing on all of the serovar I 4,[5],12:i:- isolates using the Illumina MiSeq platform. Additionally, we obtained the whole genome sequence for strain FSIS1503788 associated with the WA state outbreak using the PacBio platform to create a serovar I 4,[5],12:i:- genome scaffold and facilitate assembly of the sequencing reads generated with the Illumina MiSeq platform. The genome of serovar I 4,[5],12:i:- strain FSIS1503788 is 5,029,387 bp, a genome size that is similar to other sequenced *Salmonella* serovars. We utilized the genome sequencing information to characterize *Salmonella* serovar I 4,[5],12:i:- strain FSIS1503788.

The minus sign "-" at the end of the antigenic formulae for serovar I 4,[5],12:i:- indicates that it is missing one of the two flagellar antigens and is therefore considered "monophasic". We compared the nucleotide sequence data for the serovar I 4,[5],12:i:- pork outbreak-associated isolate FSIS1503788 to serovar Typhimurium, a closely related serovar. Genome alignment of the serovar I 4,[5],12:i:- isolate FSIS1503788 compared to serovar Typhimurium strain UK1 indicates that the monophasic *Salmonella* strain is missing a ~15.7 kb portion of DNA in the *fljB* region of the chromosome that is visualized as a gap in the sequencing read data (Figure 1). The deletion of the *fljB* gene from the serovar I 4,[5],12:i:- genome (that corresponds to the sequencing gap in the data) accounts for the monophasic phenotype of this strain. Inserted in the *fljB* genomic region of serovar I 4,[5],12:i:- isolate FSIS1503788 is an ~28 kb module encoding resistance to the metal mercury and the antimicrobials ampicillin, streptomycin, sulfisoxazole, and tetracycline (R-type ASSuT). The composition of this multidrug-resistance module is quite similar to an insertion in the *fljB* genomic region of multidrug-resistant serovar I 4,[5],12:i:- strain 07-2006 (GenBank accession no. KR856283) isolated from a lymph node of a pig in Germany (Garcia et al. 2016.). This multidrug-resistance module is also present in serovar I 4,[5],12:i:- strain TW-Stm6 (GenBank accession no. CP019649) which was isolated from swine feces in Australia.

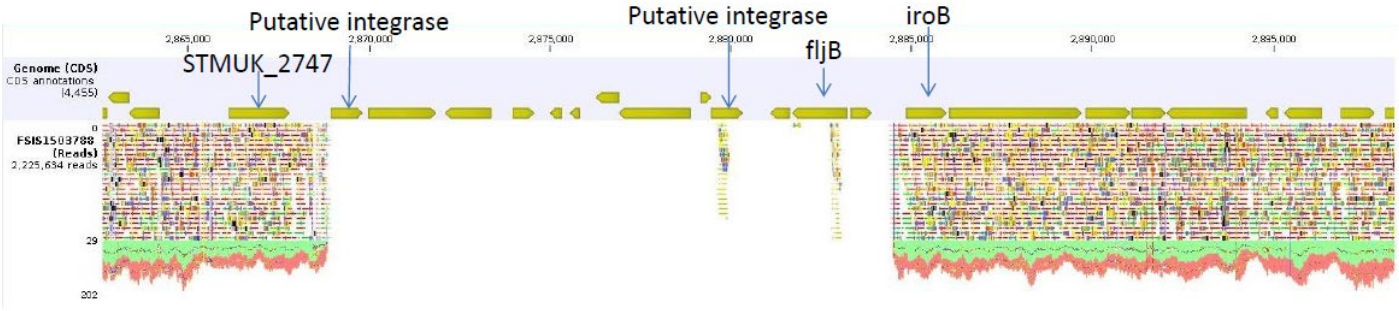

Figure 1. Comparison of nucleotide sequencing reads for the genome of serovar I 4,[5],12:i:- isolate FSIS1503788 compared to the *fljB* genomic region of *Salmonella* Typhimurium strain UK-1 (GenBank accession no. CP002614). The serovar Typhimurium strain UK-1 genes in the *fljB* genomic region are indicated in yellow in the upper portion of the figure. The nucleotide sequencing reads for the serovar I 4,[5],12:i:- isolate FSIS1503788 are multicolored in the lower portion of the figure. The gap in the sequencing read data for the serovar I 4,[5],12:i:- isolate FSIS1503788 indicates the absence of *fljB* and neighboring genes, resulting in the monophasic phenotype.

Nucleotide sequence analysis identified *Salmonella* Genomic Island 4 [SGI-4, (Petrovska et al. 2016.)] in serovar I 4,[5],12:i:- isolate FSIS1503788. This unique ~80 kb genomic island contains multiple genetic operons encoding potential resistance to copper, arsenic, and zinc. SGI-4 is also present in serovar I 4,[5],12:i:- strain TW-Stm6 from Australia. The presence of both SGI-4 and the multidrug-resistance module in the *fljB* region in serovar I 4,[5],12:i:- isolates FSIS1503788, TW-Stm6, and 07-2006 from swine related samples in the U.S., Australia, and Germany indicates that this strain type is distributed globally, respectively.

We constructed a derivative of FSIS1503788 (BBS 1270) by recombineering with a deletion of the entire 80 kb SGI-4 genomic island that no longer contains the genes for metal resistance. We performed Biolog Phenotype

Microarrays on FSIS1503788 and BBS 1270 to determine microbial sensitivity to various chemical compounds. The BBS 1270 ΔSGI-4 mutant of serovar I 4,[5],12:i:- had increased sensitivity to arsenic and copper compounds compared to the FSIS1503788 parental wildtype (Figure 2). Neither wildtype serovar I 4,[5],12:i:- isolate FSIS1503788 nor BBS 1270 (ΔSGI-4 mutant) had a sensitivity to the various concentrations of zinc present in the Biolog Phenotype Microarray. This indicates that higher concentrations of zinc will need to be assessed to determine the level(s) for growth inhibition for FSIS1503788 and its ΔSGI-4 derivative. The phenotype microarray analysis indicates that the metal resistance genes present in the SGI-4 genetic island confer increased survival for wildtype serovar I 4,[5],12:i:- isolate FSIS1503788 in the presence of copper and arsenic compared to BBS 1270 (ΔSGI-4 mutant). The use of copper, arsenical, and potentially zinc antimicrobials in animal and poultry production may have selected for the ~80 kb SGI-4 genetic island in *Salmonella* serovar I 4,[5],12:i:- and the presence of SGI-4 in the genome may provide a selective advantage for colonization of swine when metal containing antimicrobials are used during animal production.

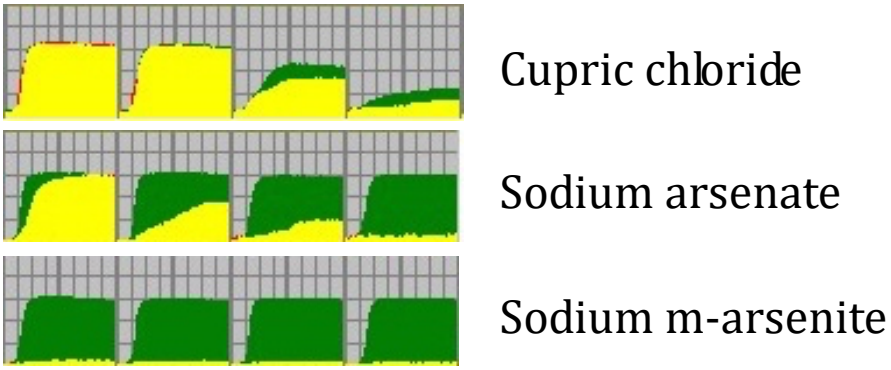

Figure 2. Increased metal sensitivity of the serovar I 4,[5],12:i:- isolate FSIS1503788 ΔSGI-4 mutant missing the ~80 kb metal resistance island. Growth of *Salmonella* serovar I 4,[5],12:i:- strains FSIS1503788 and BBS 1270 in 4 increasing concentrations of cupric chloride, sodium arsenate, and sodium m-arsenite in Biolog Phenotype microarrays. Wildtype serovar I 4,[5],12:i:- isolate FSIS1503788 growth curves shown in green. The growth curves of the serovar I 4,[5],12:i:- strain derivative BBS 1270 (ΔSGI-4 mutant) is shown in red. An overlap in bacterial growth is indicated in yellow.

A pathogenesis trial using serovar I 4,[5],12:i:- isolate FSIS1503788 from the 2015 pork outbreak was performed in swine and has recently been published (Foodborne Pathog. Dis. 2018. 15(5):253-261; doi: 10.1089/fpd.2017.2378). At 8-weeks of age, pigs were challenged via the intranasal route with 1 X 10^9 CFU of serovar I 4,[5],12:i:-; the following observations were made. First, the mean swine body temperature was significantly increased at day 2 post-challenge with serovar I 4,[5],12:i:- compared to day 0 (Figure 3). Two, the evaluation of moisture content in swine feces is a quantitative assessment of fecal consistency for clinical symptoms of diarrhea. At day 2 post-challenge with serovar I 4,[5],12:i:-, the mean moisture content of swine feces was significantly increased compared to day 0 (Figure 4). Three, the porcine immune response produces the cytokine interferon-gamma (IFNγ) in response to infection with viral and intracellular bacterial pathogens. The mean IFN-γ level was significantly increased at days 2 and 3 post-challenge with serovar I 4,[5],12:i:- compared to day 0 (Figure 5). Four, the fecal shedding of serovar I 4,[5],12:i:- at days 1, 2, 3, and 7 following challenge is shown in Figure 6. Five, tissue colonization (tonsil, ileocecal lymph node, cecum, cecal contents, Peyer's Patch, and ileal mucosal scrapings) of serovar I 4,[5],12:i:- following necropsy at day 7 of challenge is shown in Figure 7. Six, the presence of antibodies in swine serum against *Salmonella* LPS was determine using the IDEXX HerdChek® Swine *Salmonella* Test Kit. All pigs were negative prior to challenge (Day 0) but positive at day 7 following challenge with serovar I 4,[5],12:i:- for serum antibodies to *Salmonella* LPS. This indicates that the pigs had not been previously exposed to *Salmonella* but upon challenge with serovar I 4,[5],12:i:-, the LPS from this isolate induced an immune response. Furthermore, the absence of the FljB flagellar antigen does not inhibit production of an immune response against serovar I 4,[5],12:i:- LPS. Lastly, analysis of the 16S rRNA gene sequences demonstrated that the fecal microbiota was significantly altered following MDR serovar I 4,[5],12:i:- inoculation, with the largest shift

observed between 0 and 7 d.p.i. (Figure 8). Taken together, the increased swine body temperature, fecal moisture content, and IFN-γ levels in pigs challenged with serovar I 4,[5],12:i:- indicate that this isolate associated with the 2015 pork outbreak is pathogenic to swine including the disruption of the gastrointestinal microbiota. Furthermore, the level of fecal shedding and tissue colonization for serovar I 4,[5],12:i:- following swine challenge is consistent with previous challenge experiments that we have conducted in pigs using *Salmonella* Typhimurium.

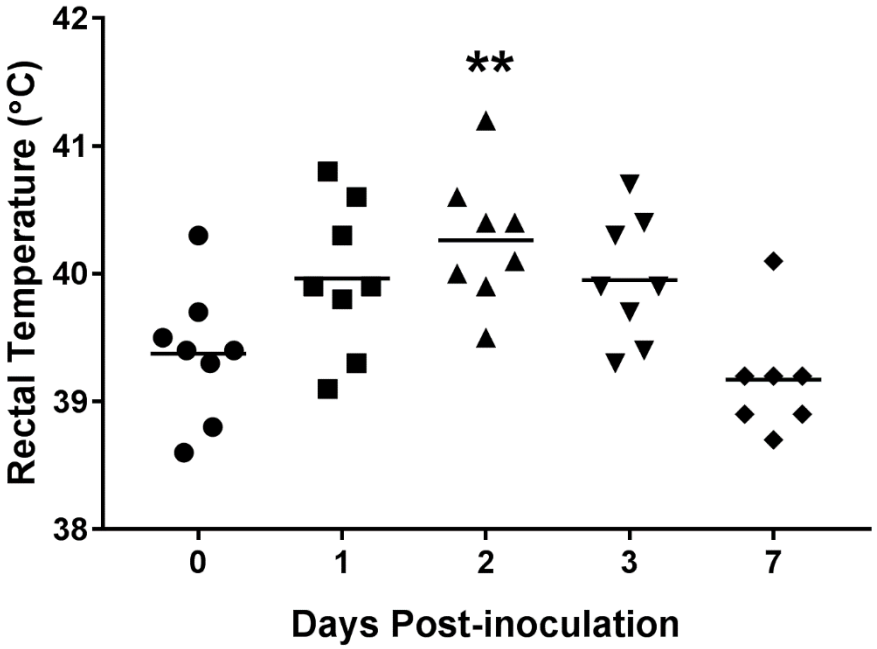

Figure 3. Body temperature is significantly increased in pigs inoculated with MDR serovar I 4,[5],12:i:-. At 8 weeks of age, pigs were inoculated with MDR serovar I 4,[5],12:i:-, and rectal temperatures (fever) were recorded at 0, 1, 2, 3 and 7 d.p.i. Each data point represents the temperature of an individual pig with the horizontal line representing the mean for each time point. **Significant difference ($P < 0.01$) when compared to day 0.

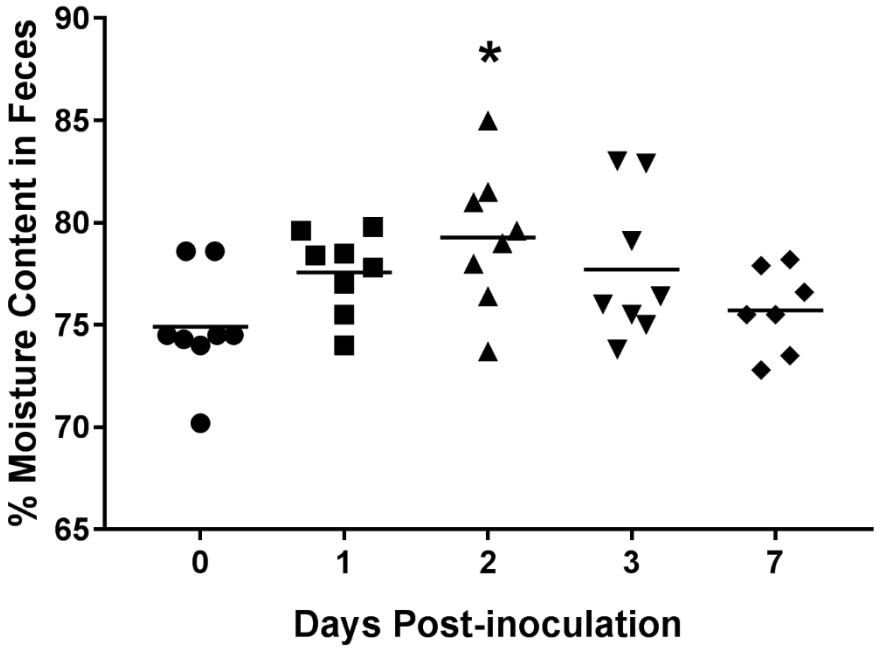

Figure 4. Significantly increased moisture content in swine feces following inoculation with MDR serovar I 4,[5],12:i:-. Moisture content (diarrhea) of pig feces collected at 0, 1, 2, 3 and 7 d.p.i. was determined. Each data point represents the fecal moisture content of an individual pig with the horizontal line representing the mean for each time point. *Significant difference ($P < 0.05$) when compared to day 0.

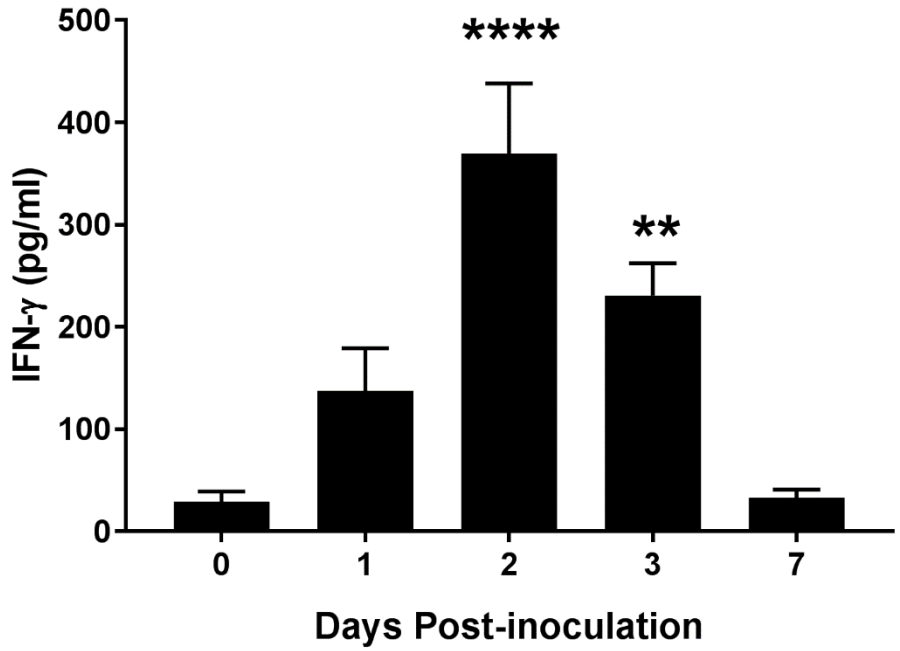

Figure 5. Significantly increased circulating IFN-γ levels in swine inoculated with MDR serovar I 4,[5],12:i:-. Serum IFN-γ levels at 0, 1, 2, 3 and 7 d.p.i. were determined by ELISA. Data are expressed as arithmetic means with error bars indicating the standard experimental mean.
****$P \leq 0.0001$, **$P < 0.01$ when compared to day 0.

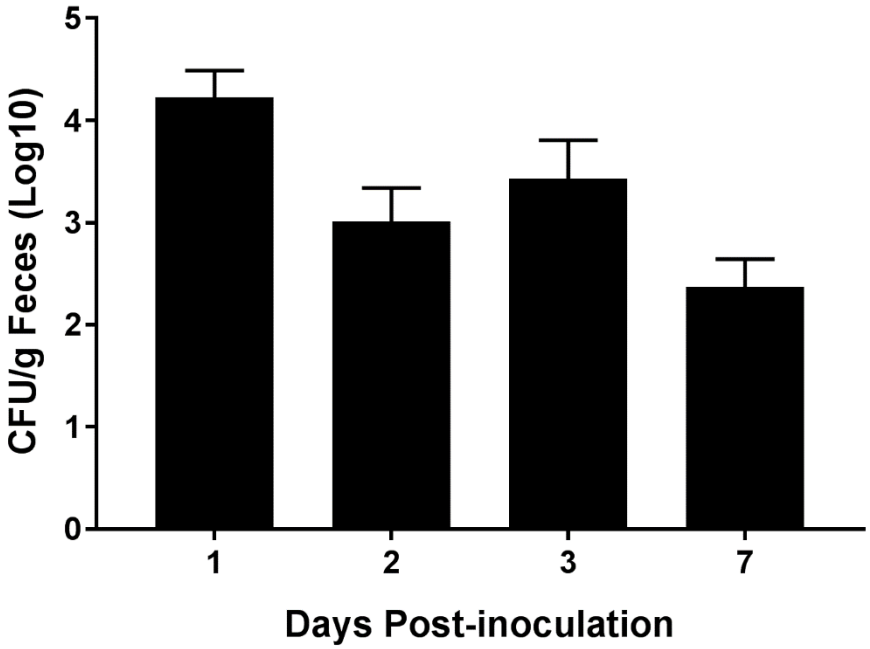

Figure 6. Fecal shedding of MDR serovar I 4,[5],12:i:- in pigs. Following inoculation with MDR serovar 1,4,[5],12:i:-, fecal shedding at 1, 2, 3 and 7 d.p.i. was measured. Data are expressed as arithmetic means with error bars indicating the standard experimental mean.

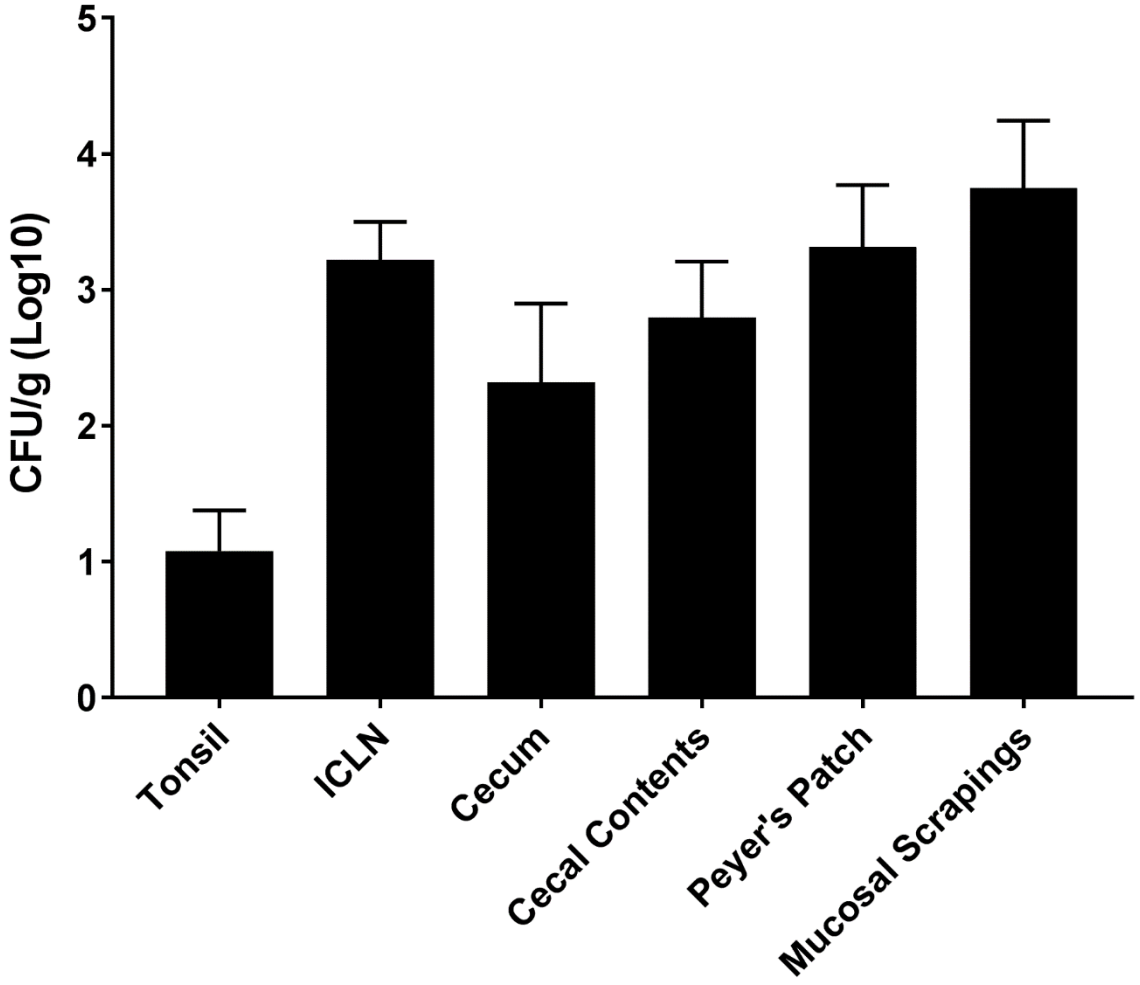

Figure 7. Tissue colonization of MDR serovar I 4,[5],12:i:- in pigs. Bacteriological analysis of MDR serovar I 4,[5],12:i:- (CFU/g) recovery from pig tissues at 7 d.p.i. was determined. Data are expressed as arithmetic means with error bars indicating the standard experimental mean.

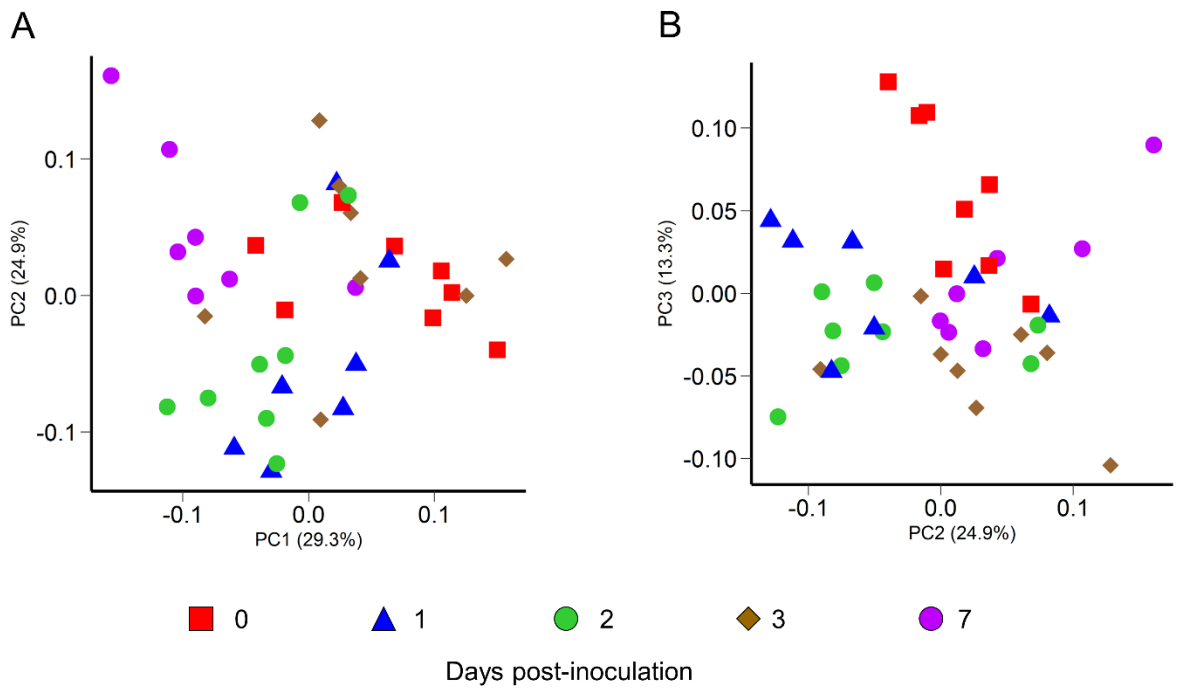

Figure 8. Alterations in the swine fecal microbiota following inoculation with MDR serovar I 4,[5],12:i:-. Principal coordinates analysis (PCoA) plot of the weighted UniFrac distances for each fecal sample by sampling time. A) principal coordinate (PC) 1 vs. PC2 and B) PC2 vs. PC3. The numbers in parentheses represent the percentage of variation explained by each axis. Each data point indicates the fecal microbiota for an individual pig at a single time point assessed using 16S rRNA gene sequencing. All swine fecal samples collected on a particular day share a common colored symbol.

Objective 2 was to determine cross-protection against multidrug-resistant *Salmonella* serovar I 4,[5],12:i:- transmission with a rationally-designed *Salmonella* DIVA vaccine. Previous investigations demonstrated that swine vaccinated with an attenuated *Salmonella* Typhimurium strain BBS 866 had reduced intestinal colonization, fecal shedding, and disease severity due to direct inoculation with virulent *Salmonella* Typhimurium UK-1 (Bearson et al. 2017). In our current investigation, we used a transmission model to determine protection against multidrug-resistant *Salmonella* serovar I 4,[5],12:i:- colonization of vaccinated and mock-vaccinated swine.

Piglets were vaccinated (n=10) twice with attenuated *Salmonella* Typhimurium strain BBS 866 or mock-vaccinated with PBS (n=10) at 4 and 6 weeks of age in separate isolation rooms. At 9 weeks of age, 5 BBS 866-vaccinated and 5 mock-vaccinated pigs were co-housed in one isolation room and the remaining 10 pigs (vaccinated and mock-vaccinated) were co-housed in another isolation room. Two additional pigs per room, from the original age-matched cohort, were inoculated with *Salmonella* serovar I 4,[5],12:i:- and co-housed with vaccinated and mock-vaccinated pigs. The swine body temperature of vaccinated and mock-vaccinated pigs was monitored at 0, 1, 2, 3, 7, and 14 d.p.i. There was not a significant difference in body temperatures comparing vaccinated and mock-vaccinated pigs at any time point in the study, nor was there a significant difference in body temperatures comparing time points 1, 2, 3, 7, or 14 d.p.i. for each group (vaccinated or mock-vaccinated) to 0 d.p.i. (data not shown). Fecal shedding of *Salmonella* serovar I 4,[5],12:i:- was determined at 1, 2, 3, 7, 10, and 14 d.p.i. A significant difference in fecal shedding was not seen comparing mock-vaccinated and vaccinated groups. At 14 d.p.i. all pigs were euthanized and necropsies performed to harvest gastrointestinal tissues. A significant reduction of *Salmonella* serovar I 4,[5],12:i:- in the cecal contents (P=0.0306) was seen comparing vaccinated to mock-vaccinated pigs (Figure 9).

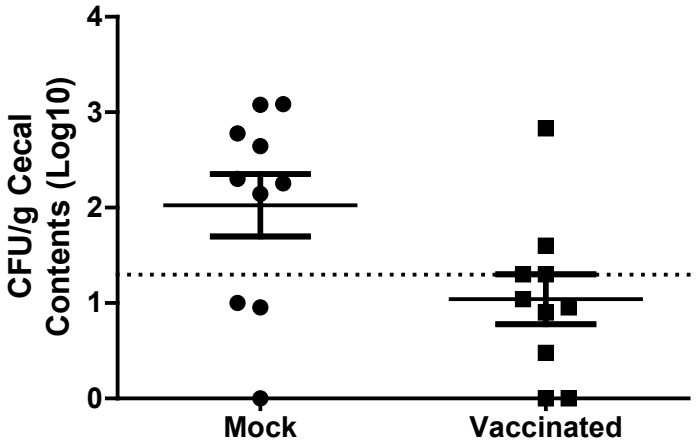

Figure 9. Comparison of *Salmonella* serovar I 4,[5],12:i:- CFU/g cecal contents in mock-vaccinated and vaccinated pigs following 14 days exposure to donor pigs. Three and 6 mock-vaccinated and vaccinated pigs were quantitatively negative for *Salmonella*, respectively. The dotted line indicates the detection limit for quantitation of *Salmonella*. Two and 4 mock-vaccinated and vaccinated pigs were *Salmonella* positive by enrichment, respectively.

Discussion: *Salmonella* serovar I 4,[5],12:i:- has emerged as the 4th most common *Salmonella* serovar to cause human illness in the U.S. and ~67% of serovar I 4,[5],12:i:- isolates are multidrug-resistant. A multistate outbreak of multidrug-resistant *Salmonella* serovar I 4,[5],12:i:- was associated with pork in 2015. We obtained 33 serovar I 4,[5],12:i:- isolates that USDA, FSIS had collected from slaughter plants in 2015 and 2016. Eleven of the 33 serovar I 4,[5],12:i:- isolates were associated with the 2015 pork outbreak. Strain comparisons demonstrate that the outbreak-associated isolates from WA were closely related to FSIS isolates from CA, IL, KY, MI, NY, SD, and TX. This indicates that serovar I 4,[5],12:i:- isolates related to the 2015 pork outbreak are distributed across the U.S. and all have similar antimicrobial resistance patterns.

We demonstrated that the pathogenesis of *Salmonella* serovar I 4,[5],12:i:- isolate FSIS1503788 in swine is similar to other serovar Typhimurium isolates that we have characterized in pigs and altered parameters include the induction of a transient increase in body temperature, fecal moisture content, and IFN-γ levels. Furthermore, colonization of swine with serovar I 4,[5],12:i:- results in a disruption of the gastrointestinal microbiota which may impact production efficiency. Vaccinated swine exposed to donor pigs directly inoculated with *Salmonella* serovar I 4,[5],12:i:- isolate FSIS1503788 demonstrated significantly reduced colonization of the cecal contents by serovar I 4,[5],12:i:- following 14 days of exposure compared to mock-vaccinated swine. This data suggests that a reduction in gastrointestinal colonization should correspond to reduced fecal shedding of *Salmonella* at later time points.

The presence of two unique genomic insertions differentiates *Salmonella* serovar I 4,[5],12:i:- isolates from serovar Typhimurium. An ~28 kb insertion is present in the *fljB* region and contains genes conferring resistance to the metal mercury and the antimicrobials ampicillin, streptomycin, sulfisoxazole, and tetracycline (R-type ASSuT). The gene arrangement and composition of the multidrug-resistance module is similar to an insertion in the same genomic region of multidrug-resistant serovar I 4,[5],12:i:- strains 07-2006 and TW-Stm6 which were isolated from a lymph node of a pig in Germany (Garcia et al. 2016) and swine feces in Australia, respectively. The second genomic insertion is an ~80 kb genomic island named *Salmonella* Genomic Island 4 (SGI-4) (Petrovska et al. 2016). SGI-4 contains genes for resistance to metals including zinc, copper, and arsenic. *Salmonella* serovar I 4,[5],12:i:- isolate TW-Stm6 from Australia also contains SGI-4 indicating not only the presence of this strain in the U.S. but also the global distribution of serovar I 4,[5],12:i:- associated with the 2015 pork outbreak. Deletion of SGI-4 from serovar I 4,[5],12:i:- isolate FSIS1503788 and phenotypic analysis in the presence of copper and arsenic compounds indicated that genes in the SGI-4 region conferred increased metal tolerance to the wildtype strain. A phenotypic difference was not seen comparing wildtype FSIS1503788 and its SGI-4 mutant during growth in the presence of zinc. This indicates that both strains grew in the presence of zinc and higher concentrations of zinc will need to be assessed to determine whether

the SGI-4 genes for zinc resistance provide a growth advantage to the wildtype strain. We were unable to determine whether the mercury resistance genes present in the antimicrobial resistance module confer increased metal tolerance as we do not have any mercury containing compounds for analysis due to toxicity.

The presence of multiple antimicrobial resistance genes on the same DNA fragment allows for the co-selection of the other antimicrobial resistance genes whenever the bacterial strain is exposed to an antimicrobial to which one of the genes confers resistance. Therefore, *Salmonella* serovar I 4,[5],12:i:- exposure to one antimicrobial such as chlortetracycline would select for resistance genes for mercury, ampicillin, streptomycin, and sulfisoxazole. Likewise, exposure to copper would select for resistance genes to zinc and arsenic in *Salmonella* serovar I 4,[5],12:i:-. Furthermore, although the antimicrobial resistance module and SGI-4 are not in close proximity on the chromosome, the exposure to either an antimicrobial or a metal would select for resistance genes in the other region because they are present on a single, large DNA molecule. Therefore, the exposure of *Salmonella* serovar I 4,[5],12:i:- to either antimicrobials or metals provides selective pressure to maintain this resistance gene composition in the genome.

Although the presence of antimicrobial and metal resistance genes on the genome of *Salmonella* serovar I 4,[5],12:i:- associated with the pork outbreak is clear, this investigation was not designed to determine the selective events that have culminated in the acquisition of the antimicrobial and metal resistance genes in *Salmonella* serovar I 4,[5],12:i:- over time. However, the use of antimicrobials or metals to which *Salmonella* serovar I 4,[5],12:i:- is resistant may select for this strain if it is present in the environment. For example, the use of zinc and copper in swine diets as alternatives to antibiotics may not have the intended animal health outcome if *Salmonella* serovar I 4,[5],12:i:- is present on a farm. Further research is needed to determine whether *Salmonella* serovar I 4,[5],12:i:- containing metal resistance genes can efficiently colonize swine that are fed diets containing copper and zinc. For now, swine producers need to be aware that multidrug-resistance *Salmonella* serovar I 4,[5],12:i:- strains with metal resistance genes are circulating in the U.S. and the use of diets containing zinc and copper may have unintended consequences.

The global emergence of multidrug-resistant *Salmonella* serovar I 4,[5],12:i:- over the last 10-15 years and serovar I 4,[5],12:i:- being the 4th most common *Salmonella* serovar to cause human illness in the U.S. may be related to its unique combination of chromosomal-encoded resistance genes including 4 antimicrobial classes and multiple metals. If inclusion of zinc and copper in swine diets as alternatives to antibiotics provides a colonization advantage for *Salmonella* serovar I 4,[5],12:i:- in pigs, a management strategy may need to be implemented to cycle diets with and without copper and zinc to reduce pressure for colonization selection.

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