RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Evaluate the dissemination of Salmonella in the environment following land application of swine manure – NPB #: 13-006

revised

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Date Submitted: 08/03/2015

Industry Summary

There is tremendous pressure on the US pork industry to ban the prophylactic and growth promotion use of antimicrobials in feed due to the generation of antimicrobial resistant (AMR) bacterial strains. An important concern is the dissemination of AMR *Salmonella* in the environment after swine manure application. The main objective of this study was to determine the potential role of lagoons and manure pits in the transmission of AMR *Salmonella* in the environment following land application of swine manure on commercial swine farms in Iowa (n=7) and North Carolina (n=6). In Iowa the manure is stored in pits and applied using an injection system while manure in NC is stored in lagoons and applied directly on the soil using a spray method. *Salmonella* prevalence was compared on these conventional swine farms at different time points including day 0 (before and after manure application) and subsequently on days 7, 14 and 28 post manure application on specific land locations at every site. The samples consisted of lagoon/manure pit and soil on day 0 while only soil samples were collected on the following sampling time points. Overall, we collected a total of 1,200 soil samples (IA=700; NC=500) and 50 lagoon and 70 manure pit samples from NC and IA, respectively. Overall *Salmonella* prevalence was 13.33% (176/1320) while the prevalence in soil and lagoon were 10.92% and 37.5%, respectively. The *Salmonella* prevalence in North Carolina (28.18%) was significantly higher than in Iowa (2.73%) ($p < 0.001$). We detected a significant decrease in prevalence of *Salmonella* from the marked areas as we moved from Day0 to Day21. We identified 12 serotypes, however, it is important to note that no serotype found in one state was detected from the other highlighting serotype association based on geographic region. For example, we detected Anatum (7.39%), Litchffield (3.98%), and Infantis (0.57%) in IA, while Altona (7.95%), Derby (3.98%), Johannesburg (3.98%), Mbandaka (1.70%), Muenster (9.09%), Rissen (0.57%), Typhimurium var5- (20.45%), Uganda (2.27%), and Worthington (5.68%) in NC. A total of 80.47% of the *Salmonella* isolates were multidrug resistant (MDR; resistance to three or more antimicrobials) with the most frequent AMR against Streptomycin (82.81%), sulfisoxazole (73.44%), and kanamycin (61.72%). PFGE genotyping revealed genotype relatedness among *Salmonella* recovered from lagoon and soil at multiple time points with relatively close geographic proximity and serotypes. Our study highlights *Salmonella* transmission in the environment in commercial swine farms is dependent on the type of manure storage and its application method. Finally, the rapid decline in the prevalence of *Salmonella* in soil samples on subsequent collection days (Days 7, 14, 21) clearly indicates the inability of this pathogen to survive in the environment for longer durations.

Keywords
Salmonella, soil, lagoon, PFGE, antimicrobial resistance

Scientific Abstract
Land application of animal manure is an important source of fertilizer. However, the presence of pathogens in soil and their occasional transmission to human and animal has become a topic of public health concern during the past few years. The objective of this study is to determine the transmission of *Salmonella* due to manure application in the environment. At the different time points of application: day 0, 7, 14, and 21, the soil and lagoon samples were collected representing swine farms in Iowa (n=7) and North Carolina (n=5). A total of 1,200 soil samples (IA=700; NC=500) and 50 lagoon and 70 manure pit samples from NC and IA, respectively. Antimicrobial susceptibility (AST) was characterized using Sensititre® with a panel of 15 antimicrobial drugs. Genotypic characterization was done using pulse field gel electrophoresis (PFGE). Overall *Salmonella* prevalence was 13.33% (176/1320). The prevalence in soil and lagoon were 10.92% and 37.5% respectively. *Salmonella* prevalence in North Carolina (28.18%) was significantly higher than in Iowa (2.73%) ($p < 0.001$). Decrease in prevalence of *Salmonella* in the area from Day0 to Day21 was observed overtime and consistently across all the farms irrespective of geographic region. We identified 12 serotypes in the study. It is important to highlight that serotypes detected in one state were not reported from the other, thereby highlighting serotype association based on manure storage and soil application method in the two regions. For example, we detected serotypes Anatum (7.39%), Litchffield (3.98%), and Infantis (0.57%) in IA, while Altona (7.95%), Derby (3.98%), Johannesburg (3.98%), Mbandaka (1.70%), Muenster (9.09%), Rissen (0.57%), Typhimurium var5- (20.45%), Uganda (2.27%), and Worthington (5.68%) were detected in NC only. Multidrug resistant (MDR; Resistance to three or more antimicrobials) *Salmonella* isolates were 80.47% with the most frequent AMR against Streptomycin (82.81%), sulfisoxazole (73.44%), and kanamycin (61.72%). According to PFGE fingerprints, we detected clonal relatedness among *Salmonella* recovered from lagoon and soil at multiple time points with relatively close geographic proximity and serotypes. The outcome of this research study provides information on the occurrence and distribution of AMR *Salmonella*, its AMR phenotypes and genotypes in swine manure which is directly applied in the field. Our study highlights that the potential of *Salmonella* transmission in the environment on swine farms is dependent on the manure storage and application method.

Introduction

The use of antimicrobials in the food animal industry for prophylaxis and treatment has proven benefits including improving the feed efficiency, disease prevention, control and resolution of disease, enhanced production and reduced burden of pathogens. However, with the growing concern of selection and transfer of antimicrobial resistant (AMR) enteric pathogens from food animals to humans, the use of antimicrobials in the food animal industry has come under great scrutiny. An important component of the swine production system is the storage, treatment and use of swine manure following the swine manure management program. Manure generated in swine farms is collected, stored and treated in anaerobic lagoons or manure pits following the best swine manure management practices before being applied as soil amendments. There are concerns that spreading of swine manure on land for use as fertilizers aids in the transmission of pathogenic bacteria which have the potential to find their way into the surface and ground water (Chee-Sanford et al., 2009). Such contamination may create a public health hazard (Tomer et al., 2010; Brooks and McLaughlin, 2009; Jindal et al., 2006; Cole et al., 2003). It is important to highlight that there is a dearth of information on the movement of AMR pathogens in commercial swine farms from the swine manure to other environmental niches.

Studies designed to determine the role of swine manure in transmission of pathogens to the environment are either conducted on a few farms (Antunes et al., 2011; Boes et al., 2005) or on experimental research stations (Holley et al., 2008; McLaughlin and Brooks, 2009; Thurston-Enriquez et al., 2005). Previous work has examined the survival of *Salmonella* in inoculated manure-amended soil under various laboratory conditions (Bech et al., 2010; You et al., 2006; Hutchinson et al., 2004). Garcia et al. (2010) observed lower *Salmonella* survival at 25°C (>6 log reduction) than 5°C (1.5-2 log reduction) in inoculated dairy cow manure applied to topsoil collected from Denmark. Bech et al. (2010) examined the effect of soil composition and depth on

Salmonella survival. After 28 days, the highest recovery rate was in the top 0.2m of soil and *Salmonella* concentrations were also higher in loamy soils compared to sandy soils (Bech et al., 2010). The authors detected *Salmonella* up to a month after application in loamy soil under cold and moist conditions (Bech et al., 2010). Limited studies have been conducted in actual field conditions on farm (Ongeng et al., 2011; Baloda et al., 2001). One study examined the survival of *Salmonella* in pig slurry applied to a Danish field and isolated *S.* Typhimurium up to 14 days after application (Baloda et al., 2001). More recently, researchers in Sub-Saharan Africa recovered *S.* Typhimurium six weeks after application of low-density inoculated manure and 14 weeks after application with high-density *Salmonella*-inoculated manure in a tropical climate (Ongeng et al., 2011).

Multidrug resistant (MDR) *Salmonella* has been among the major public health concerns worldwide. There are numerous studies reporting the prevalence of *Salmonella* and *Escherichia coli* among swine reared in the commercial production systems (Dorr et al., 2009; Rajić et al., 2004; Gebreyes et al., 2004; Hasman and Aaresterup, 2005; Patachanee et al., 2010). These nontyphoidal serovars are important reservoirs of antimicrobial resistance including multi-resistance types. AMR *Salmonella* strains, including serovars Typhimurium, Newport and Heidelberg have been reported from pigs and retail pork (Zaidi et al., 2006; Gebreyes and Thakur, 2005; Valdezate et al., 2005; Zhao et al., 2003). Studies have reported the presence of AMR genes, antimicrobial residues and pathogens in lagoons and on lands that have been exposed to the swine manure (Chee-Sanford et al., 2009; Jindal et al., 2006). However, there is no comprehensive study that has been conducted on commercial swine farms to study the dissemination and persistence of AMR *Salmonella* from swine manure systems to land after application. We do not fully understand whether the pathogens present in swine manure persist, and if yes, then for how long, following application. There is a definite need to conduct studies that look at all the variables that affect the above relationship to fully define potential impacts on the environment. The results will help producers to understand the potential for microbial contamination in the environment following manure application.

Objectives

This field based research study was designed to be conducted on commercial swine farms to determine the dissemination of AMR *Salmonella* uponapplication of swine manure in the environment in accordance with the farms waste management program. The objectives are: 1) To determine the dissemination of AMR *Salmonella* on land following manure application from commercial swine farms, 2) To characterize and compare the *Salmonella* isolated from different sources on farm and environment using phenotypic and genotypic approaches, and; 3) To analyze whether the different farm variables like soil type and antimicrobial use on farm impact the dissemination of AMR *Salmonella* from the farm to the environment.

This field based research study was conducted on actual commercial swine farms in North Carolina and Iowa who agreed to cooperate on this important project. The baseline data generated in this study will help us to apply for future multi institutional and multistate longitudinal studies.

Materials & Methods

1) Farm Description

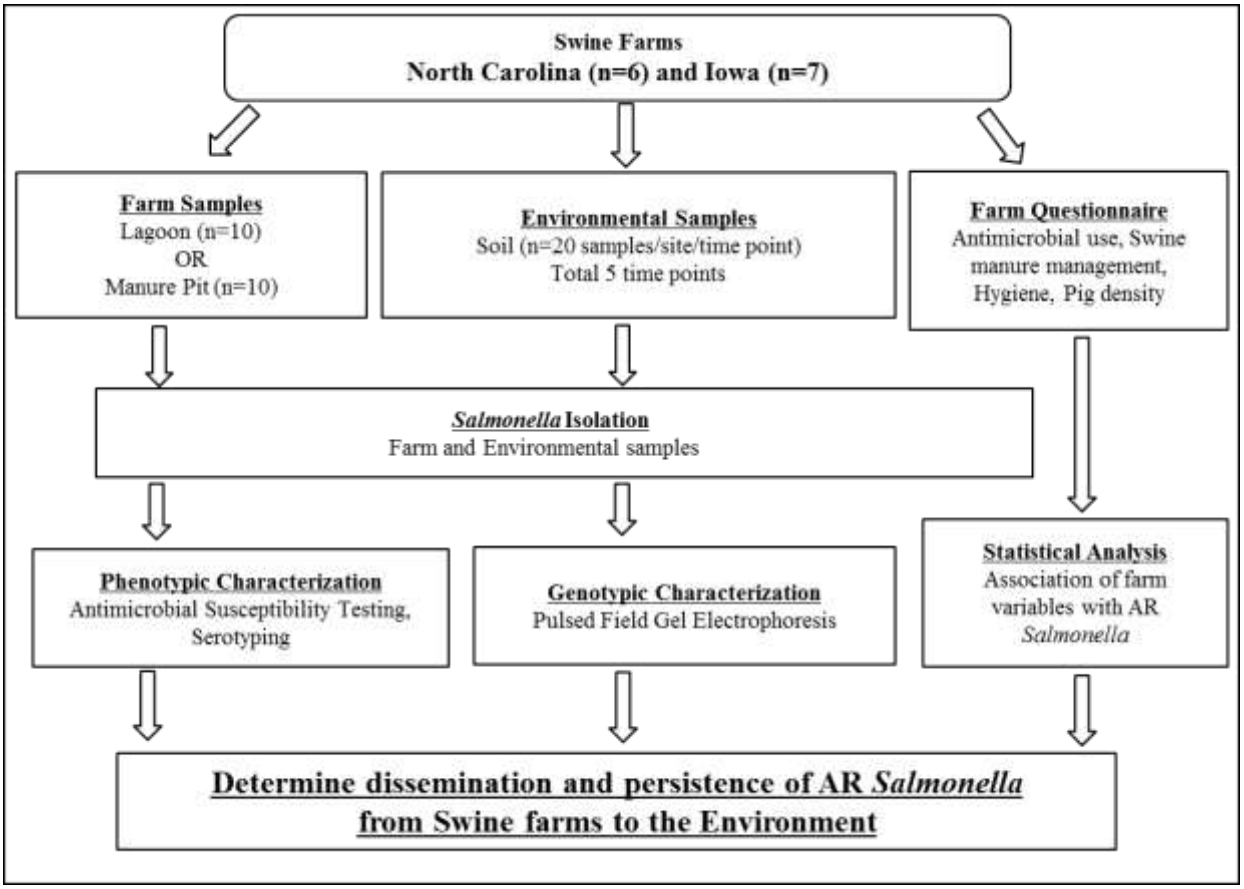
The study design scheme is illustrated in Figure 1. The sampling was conducted on a total of 13 swine farms, including seven sites in Iowa and six sites in North Carolina. We purposely identified farms that tested positive for *Salmonella* in the past. This was based on either previous *Salmonella* research conducted on these farms or information from the company veterinarian. To study the impact of soil type on pathogen transmission, we selected farms where half of the farms have a soil type that is deep sandy throughout while the other farms had a combination of sand at the top with clay loam beneath. Swine farms in North Carolina use a lagoon system for swine manure disposal which is different from the farms in Iowa which typically use a pit to store the swine manure before being applied on agricultural lands. Farms in North Carolina have a well on its property and use a flush system for manure removal from the barn to the lagoon. Farms in Iowa store undiluted manure in pits and transfer the slurry to the fields using an applicator.

2) Sample Collection Scheme

Farm environment samples were collected from a total of 13 different swine farm sites in the two states (7 sites in Iowa and 6 sites in North Carolina). The samples consisted of lagoon/manure pit and soil where the manure was applied. We worked closely with the swine producers to determine the manure application schedule and worked accordingly. Soil samples were collected from one acre size of land (88 X 55 yards) and from four different locations within plot including at a distance of 20, 40, 60 and 80 yards within the manure application area. Each location was divided into 5 grids of 1m 2 size each and soil samples were collected from within these grids. Therefore, on each sampling time point we collected a total of 20 samples (5 samples/location X 4 locations). The first set of samples were collected on day 0 (application of swine manure on land) two hours before and then two hours after manure application. This was done to determine the background status of *Salmonella* on the land before manure application and the impact immediately after manure application. Soil samples from the farm environment were collected again on days 7, 14 and 21 following deposition of swine manure. The aim of multiple sampling at different time points was to determine whether or not AMR *Salmonella* persisted in the environment 7, 14 and 21 days post manure application. The proposed sampling design (particularly, the time points) was developed, based on the study reported by Boes et al. (2005) with slight modifications.

Figure 1. Study flow to determine transmission of AR *Salmonella* from swine farms to environment.

3) Farm and



Environmental Soil Samples

The swine farm samples consisted of the following sample type (quantity; amount): a) lagoon (n=10; 25 ml including two each from the center and the four corners) OR b) swine manure pit slurry (n=10; 25 ml). The lagoon or manure pit samples was collected only once on day 0 at the start of sampling. The environment samples consisted of soil samples following the scheme described in the previous section. We identified farms with deep sandy soils (i.e. greater than 30 inches to a sandy clay loam horizon) and with soils that have a sandy surface horizon over a sandy clay loam horizon 10 to 12 inches below the surface. Samples were placed in whirl pack bags (50 gm) for transport from the farm premises. Soil samples were collected from the surface as

described previously (Boes et al., 2005). We collected five soil samples weighing 100 gm each and 10 inches deep from every grid on the land. Samples were placed in whirl pack bags for transport from the farm premises. All the samples were stored in an ice cooler at 4ºC and transported to the laboratory for further analysis. Daily maximum and minimum temperatures were recorded at every sample collection day besides measuring other environmental conditions as indicated in the questionnaire. Samples collected in North Carolina (Dr. Thakur lab) and Iowa (Late Dr. McKean Lab) were processed in NC. Further characterization at the phenotypic and genotypic levels was conducted in Dr. Thakur`s lab.

4) Questionnaire Forms

Pertinent management, facility and herd information that could possibly play a role in dissemination and persistence of AMR *Salmonella* in pigs and the environment will be collected. We are still in the process of collecting the questionnaire forms. We will collect information on the waste management program, information on manure disposal system on the farm and longevity of spray fields in use, antimicrobial used on the farm (therapeutic and prophylaxis), pig flow, stocking density, herd health, production performance, biosecurity measures and daily weather conditions.

5) Sample Processing

Salmonella Isolation: *Salmonell*a will be isolated from the lagoon or pit (Dorr et al., 2009) and soil samples (Cote and Quessy, 2005) will be done as shown previously. Confirmed *Salmonella* isolates will be stored at -80˚C in Brucella broth supplemented with 20% glycerol.

Soil Analysis: Representative soil samples will be tested for their texture, pH, nutrient content, water retention and holding capacity and organic carbon mass as described previously (Sheldrick and Wang, 1993; Harris et al., 2001). This analysis will provide information on the impact of different soil characteristics on *Salmonella* transmission. Sampling by depth increments will allow an assessment of downward movement of *Salmonella* through the soil profile. Information on the water holding and retention capacity of the soil will help us to determine the impact of rainfall on *Salmonella* transmission, especially in regard to movement to shallow groundwater.

6) Phenotypic Characterization

Salmonella Serotyping: We followed the Kauffman-White scheme for serotyping *Salmonella*. The isolates were cultured overnight at 37˚C on Luria-Bertani (LB) agar (Statens Serum Institute, Denmark) and shipped to the National Veterinary Laboratory Services at Ames, Iowa for serotyping.

Antimicrobial Susceptibility Testing: We used the broth microdilution method to determine the minimum inhibitory concentration (MIC) of the *Salmonella* (Plate ID CMV1AGNF) isolates against a panel of 15 antimicrobials (Trek Diagnostic Systems, Westlake, Ohio). The MIC was determined and interpreted using the Clinical and Laboratory Standards Institute standards (CLSI) for broth microdilution (CLSI, 2010). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control organisms.

7) Genotypic Characterization by Pulsed Field Gel Electrophoresis (PFGE)

We genotyped *Salmonella* by PFGE as recommended by CDC (CDC, 2000). Briefly, overnight culture cells were mixed with an equal volume of agarose and dispensed into a mold to form agarose plugs to extract the DNA. The naked agarose embedded DNA was digested with the specific restriction enzyme. *Salmonella* Braenderup was used as the reference marker. The restriction digested DNA was then separated using CHEF-DRIII pulsed field gel electrophoresis (Biorad, CA) apparatus using the following conditions: voltage (6V/cm), initial switch time of 2.2 seconds and final switch time of 63.8 seconds for 18 hours. The gel was stained in ethidium bromide (1µg/ml). Gel Doc 2000 (Biorad) CCD camera was used to capture fingerprint images. Analysis of PFGE data was performed using Bionumerics 4.0 software (Applied Maths, Belgium) and the patterns were compared by the Dice coefficient and the UPGMA method.

8) Statistical Analysis

Analysis and interpretation of results was conducted using statistical and molecular epidemiology approaches. Analysis of variance (ANOVA) for repeated measures will be done with SigmaPlot 11.2 (Systat Software Inc., Chicago, IL) for comparing *Salmonella* prevalence and AMR profile from swine manure and the environment for the different time points. Even though we are collecting farm information through questionnaires, we will not be able to do risk factor analysis due to the small sample size. However, we will determine the strength of association between serotype and resistance pattern in *Salmonella* isolated from the swine manure and environment with the waste management program and antimicrobial use on farm using the odds ratio (OR) with a 95% confidence interval (Egret software version 2.0.3, Cytel Corp., Cambridge, MA). A value of $P < 0.05$ was considered statistically significant.

Results

1) *Salmonella* prevalence in swine farms environment in Iowa and North Carolina

A total of 1,200 soil samples (IA=700, NC=500) and 120 lagoon samples (IA=70, NC=50) were collected from the swine farm environment in Iowa and North Carolina states. It is important to mention that in Iowa swine farms, waste is collected in a slurry pit/well and not in lagoons. A total of 176 *Salmonella* isolates were recovered from the study sample population. The overall percentage of samples that tested positive for *Salmonella* was higher in North Carolina (155/550, 28.18%) when compared to Iowa area (21/770, 2.73%) ($P < 0.001$). According to table 1, only one farm in Iowa (IAF 6) was positive for *Salmonella* (21/110, 19.09%) while all 5 farms in North Carolina were positive: NCF 1: 42.73%, NCF 2: 3.64%, NCF 3: 55.45%, NCF 4: 27.27%, and NCF 5: 11.82%. Farm#6 in NC is still being processed and last two sampling (days 14, 21) are yet to be completed.

Farms*	Frequency (N)		Positive (%)
IAF 1-5,7	110 each		0
IAF 6	110		21 (19.09)
Total IAF	770		21 (2.73)
NCF 1	110		47 (42.73)
NCF 2	110		4 (3.64)
NCF 3	110		61 (55.45)
NCF 4	110		30 (27.27)
NCF 5	110		13 (11.82)
Total NCF	550		155 (28.18)
Total all	**1320**		**176 (13.33)**

Table 1. *Salmonella* isolation by farms
*IAF: Iowa Farm, NCF: North Carolina Farm

A significantly higher *Salmonella* prevalence was detected in lagoon samples from North Carolina 70.0% (35/50) compared to 14.29% (10/70) in manure pit samples from Iowa ($P < 0.01$). Soil samples were 1.57% (11/700) and 24.0% (120/500) positive for *Salmonella* in Iowa and North Carolina, respectively. The percentages of *Salmonella* isolates from two different types of sample (waste and soil) by states are categorized in table 2. The overall *Salmonella* prevalence in lagoon and manure pit samples combined (45/120, 37.5%) was significantly higher than soil samples (131/1200, 10.92%); ($P < 0.05$).

States/ Sample types	Frequency (N)	Positive (%)
Iowa	770	21 (2.73)
• Manure Pit	70	10 (14.29)
• Soil	700	11 (1.57)
North Carolina	550	155 (28.18)
• Lagoon	50	35 (70.0)
• Soil	500	120 (24.0)

Table 2. *Salmonella* isolation by geographic regions and sample types

The prevalence of *Salmonella* isolated from swine farm environment in Iowa (Fig 2) and North Carolina (Fig 3) at different time points of manure application including day 0 (before, lagoon, and after), day7, day14, and day21 were highest in day0 especially in lagoon and after-application soil samples and tended to decrease in later weeks in both IA and NC farms. Only NCF3 had a different trend in prevalence with an increase from day0 to day7. A single soil sample collected before manure application (Farm NCF4) tested positive for *Salmonella*. No *Salmonella* positives were found in day7, 14, and 21 soil samples from NCF2. Samples from IAF6 and NCF5 were positive on day0 and 7 but not on day14 and 21.

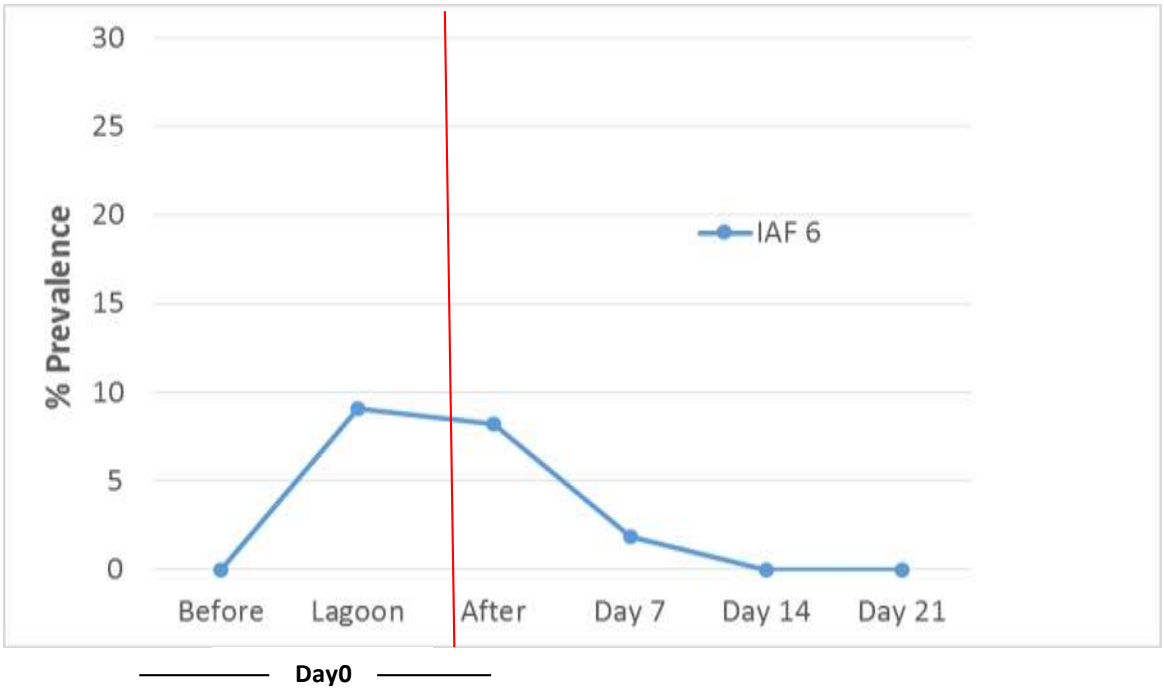


Figure 2. *Salmonella* prevalence among Iowa samples (IAF6) at different time points

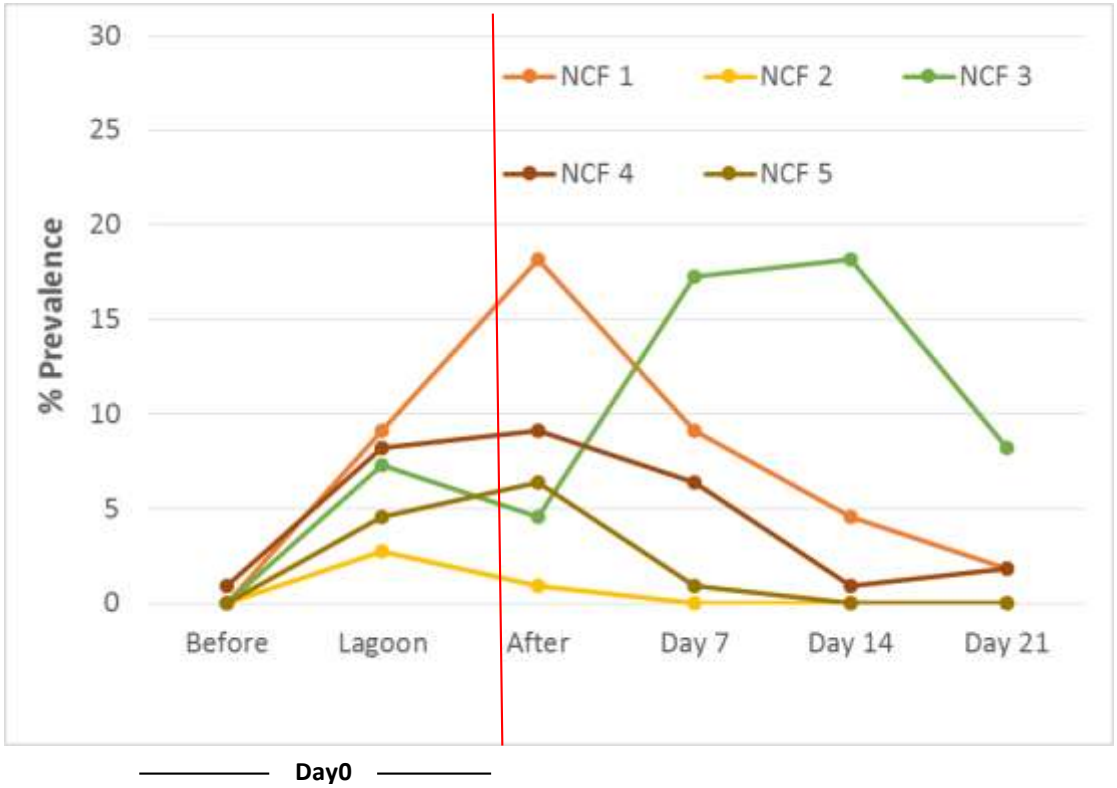


Figure 3. *Salmonella* prevalence among North Carolina samples (NCF1-5) at different time points

2) Identification and distribution of *Salmonella* serotypes

We identified 12 *Salmonella* serotypes from 176 positive samples in Iowa and North Carolina farms. Three *Salmonella* serotypes were identified in Iowa including *Salmonella* Anatum (7.39%), *S.* Litchffield (3.98%), and *S.* Infantis (0.57%). All positive manure pit samples in Iowa were identified as *S.* Anatum. In North Carolina the following serotypes were detected, *S.* Typhimurium var5- (20.45%), *S.* Muenster (9.09%), *S.* Altona (7.95%), *S.* Worthington (5.68%), *S.* Derby (3.98%), *S.* Johannesburg (3.98%), *S.* Uganda (2.27%), *S.* Mbandaka (1.70%), and *S.* Rissen (0.57%) (Table 4). *S.* Typhimurium var5- was the predominant serotype in North Carolina soil samples. One *S.* Rissen was found in a lagoon sample in North Carolina but not in the soil samples. All the serotypes detected in NC were 100% different than those isolated in IA.

***Salmonella* serotypes**	**Iowa** (n=21)		**North Carolina** (n=155)		**Total**
	Lagoon (%) n=10	**Soil** (%) n=11	**Lagoon** (%) n=35	**Soil** (%) n=120	
Altona	0	0	1 (2.86)	13 (10.83)	14 (7.95)
Anatum	10 (100)	3 (27.27)	0	0	13 (7.39)
Derby	0	0	2 (5.71)	5 (4.17)	7 (3.98)
Infantis	0	1 (9.09)	0	0	1 (0.57)
Johannesburg	0	0	4 (11.43)	3 (2.50)	7 (3.98)
Litchffield	0	7 (63.64)	0	0	7 (3.98)
Mbandaka	0	0	1 (2.86)	2 (1.67)	3 (1.70)
Muenster	0	0	5 (14.29)	11 (9.17)	16 (9.09)
Rissen	0	0	1 (2.86)	0	1 (0.57)
Typhimurium var5-	0	0	1 (2.86)	35 (29.17)	36 (20.45)
Uganda	0	0	2 (5.71)	2 (1.67)	4 (2.27)
Worthington	0	0	1 (2.86)	9 (7.50)	10 (5.68)
N/A*	0	0	17 (48.57)	40 (33.33)	57 (32.39)
Total	10	11	35	120	176

Table 3. Thedistribution of *Salmonella* serotypes from lagoon and soil samples in IA and NC (n=176)

3) Antimicrobial resistance (AMR) profile of *Salmonella*

A total of 128 *Salmonella* isolates (IA n=17, NC n=111) were tested for antimicrobial susceptibility using Sensititre with a panel of 15 antimicrobial drugs. Overall, *Salmonella* isolates showed wide spectrum of AMR with 80.47% of multidrug resistance (MDR; resistance to > 3 or more class of antimicrobials). Only 12.5% of isolates were pan-susceptible (Fig 4). Comparing the isolates from the two states, a significantly higher frequency of NC isolates were MDR than those from IA ($P < 0.05$). The highest frequencies of AMR in North Carolina were STR (83.78%), FIS (81.08%), and KAN (68.47%) while in Iowa it was STR (76.47%), XNL (29.41%), and FIS (23.53%). The percentages of resistance were highlighted in aminoglycoside class, however no IA isolate was resistance to GEN. No *Salmonella* isolates were resistance to AZI, CHL, CIP, and NAL. A squashtogram was created to compare and contrast percent of resistance and MIC distributions of *Salmonella* isolated in Iowa and North Carolina swine farm areas (Table 4). The most resistance in both states was STR which had a MIC more than 64 g/ml 76.5% in IA and 70.3% in NC.

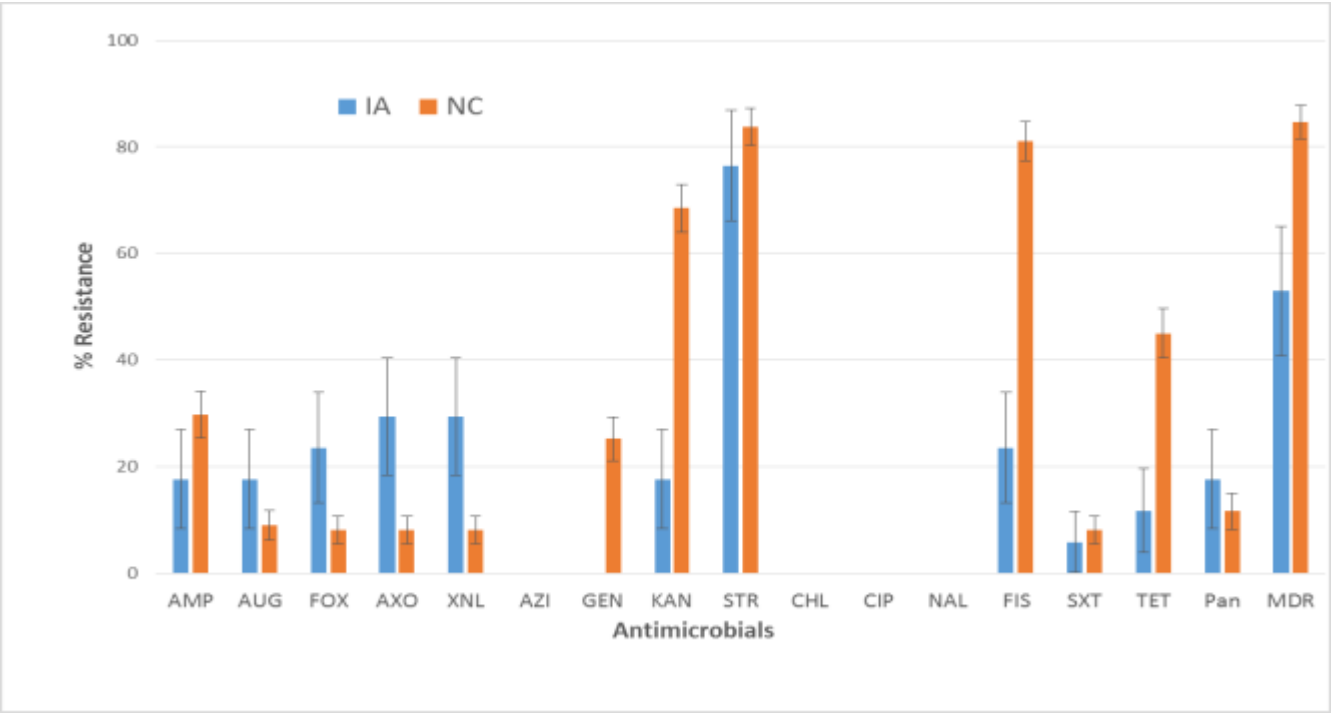


Figure 4. The percentage of antimicrobials resistance of *Salmonella* isolated from IA and NC farms

AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, XNL: Ceftiofur, TET: Tetracycline

AM	State	%R	Distribution of MICs in µg/mL (%)															
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
AMP	IA	17						29.4	0.0	41.2	5.9	0.0	5.9	17.6				
	NC	29						39.6	2.7	18.0	4.5	0.9	4.5	0.0	29.7			
AUG	IA	17						52.9	0.0	17.6	0.0	5.9	5.9	5.9	11.8			
	NC	9						47.7	0.0	3.6	1.8	0.0	36.9	5.4	4.5			
AXO	IA	29				70.6	0.0	0.0	0.0	0.0	0.0	0.0	17.6	5.9	5.9			
	NC	8				88.3	0.0	1.8	0.0	1.8	0.0	1.8	5.4	0.9	0.0			
AZI	IA	0				0.0	0.0	0.0	0.0	0.0	29.4	70.6	0.0					
	NC	0				0.0	0.0	0.0	0.0	8.1	63.1	27.0	1.8					
CHL	IA	0								0.0	23.5	82.4	11.8	0.0				
	NC	0								0.0	3.6	94.6	1.8	0.0				
CIP	IA	0	35.3	58.8	5.9	0.0	0.0	0.0	0.0	0.0	0.0							
	NC	0	67.6	30.6	1.8	0.0	0.0	0.0	0.0	0.0	0.0							
FIS	IA	23											0.0	11.8	5.9	17.6	41.2	23.5
	NC	81										4.5	0.0	3.6	8.1	2.7	0.9	80.2
FOX	IA	23						0.0	0.0	11.8	52.9	5.9	5.9	11.8	11.8			
	NC	8						0.0	0.9	27.0	55.9	4.5	3.6	6.3	1.8			
GEN	IA	0				17.6	0.0	47.1	35.3	0.0	0.0	0.0	0.0					
	NC	25				0.9	0.0	37.8	34.2	1.8	0.0	0.0	0.9	24.3				
KAN	IA	17									76.5	0.0	0.0	0.0	0.0	2.7		
	NC	68									30.6	0.0	0.0	0.9	5.4	63.1		
NAL	IA	0					5.9	0.0	0.0	0.0	88.2	5.9	0.0	0.0				
	NC	0						0.0	0.0	36.9	61.3	0.9	0.9	0.0				
STR	IA	76											23.5	0.0	0.0	76.5		
	NC	83											16.2	0.0	13.5	70.3		
SXT	IA	5			94.1	0.0	0.0	0.0	0.0	0.0	0.0	5.9						
	NC	8			55.9	0.9	22.5	12.6	0.0	0.0	0.0	1.8						
XNL	IA	29				0.0	0.0	0.0	64.7	5.9	0.0	0.0	29.4					
	NC	8				0.0	0.0	29.7	59.5	0.0	2.7	0.0	8.1					
TET	IA	11								88.2	0.0	0.0	5.9	0.0	5.9			
	NC	45								29.7	26.1	0.0	1.8	1.8	40.5			

Table 4. Comparison of resistance and MIC distribution for *Salmonella* isolated in Iowa and North Carolina (IA n=17; NC n=111)*

*The whitened areas indicate the range of dilutions tested for each antimicrobial. Shaded areas fall outside the range of tested concentrations.

4) Distribution and association of resistance patterns (R-patterns) with *Salmonella* serotypes

The MDR isolates were highly observed in both states. The most common R-patterns, which associated serotypes, and distribution are categorized in Table 5. AMP FIS KAN STR (n=18) was the most common MDR pattern that was identified in North Carolina, associated with *S.* Typhimurium var5-. Another major MDR pattern associated with *S.* Typhimurium var5- was FIS KAN STR (n=14). *S.* Muenster (n=16) isolated from North Carolina only had only 1 MDR pattern: FIS GEN KAN STR TET. Pan-susceptibility was one of the predominant R-pattern in both states. In North Carolina, *S.* Altona was associated with this pattern while in Iowa *S.* Anatum was concerned.

***Salmonella* serotypes**	**R-Patterns* (n)**	**Iowa (%)**	**North Carolina (%)**
Muenster (16)	FIS GEN KAN STR TET (16)	0	100
Typhimurium var5- (36)	AMP FIS KAN STR (18)	0	50.0
	FIS KAN STR (14)	0	38.89
Altona (14)	Pan-susceptible (11)	0	78.57
Anatum (13)	Pan-susceptible (3)	23.08	0
	STR (3)	23.08	0

Table 5. The distribution of *Salmonella* serotypes associated with predominant R-patterns.*AMP: Ampicillin, FIS: Sulfisoxazole, FOX: Cefoxitin, KAN: Kanamycin, STR: Streptomycin, TET: Tetracycline.

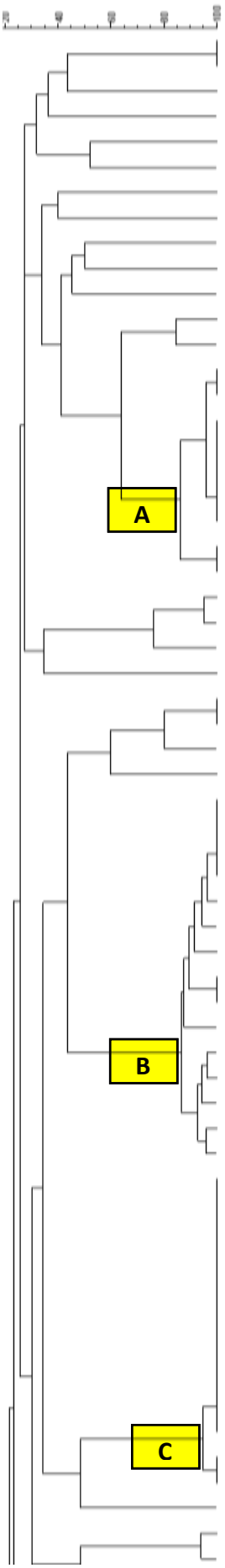
Twenty-eight antimicrobial resistance patterns (R-patterns) were identified in this study including pan-susceptible. According to table 6, the most frequent R-patterns were FIS GEN KAN STR TET (18.46%) followed by AMP FIS KAN STR (15.38%), and FIS KAN STR (13.85%). Seventeen isolates (13.03%) were counted as pan-susceptible. These data related to the result in figure 2 that a large percentage of isolates were resistant to FIS, STR, and KAN. Predominant patters are highlighted in Table 6.

R-patterns	Isolates (n)	Percentage
AMP AUG AXO FIS FOX GEN STR XNL TET	2	1.54
AMP AUG AXO FIS FOX KAN STR XNL	3	2.31
AMP AUG AXO FIS FOX KAN STR XNL TET	1	0.77
AMP AUG AXO FIS KAN STR XNL TET	1	0.77
AMP AUG FIS KAN STR	1	0.77
AMP AXO FIS FOX GEN STR XNL TET	2	1.54
AMP AUG AXO FOX KAN STR XNL	1	0.77
AMP AXO FIS STR XNL	2	1.54
AMP FIS KAN STR	20	15.38
AMP FIS STR	1	0.77
AMP FOX	1	0.77
AUG AXO FOX FIS KAN STR XNL TET	1	0.77
AUG AXO FOX KAN STR XNL	1	0.77
AXO FOX XNL	1	0.77
FIS GEN KAN STR SXT TET	1	0.77
FIS GEN KAN STR TET	24	18.46
FIS KAN STR	18	13.85
FIS KAN STR TET	3	2.31
FIS SXT STR TET	4	3.08
FIS SXT	3	2.31
FIS STR	4	3.08
FIS STR TET	4	3.08
KAN STR SXT TET	1	0.77
STR	4	3.08
STR SXT TET	1	0.77
STR TET	7	5.38
TET	1	0.77
Pan-susceptible	17	13.08

Table 6. Distribution of Antimicrobial resistance patterns of *Salmonella* isolates (n=130).

5) Pulse fielded gel electrophoresis (PFGE)

Salmonella isolates from Iowa and North Carolina were characterized for genotypic relatedness using PFGE. A total of four distinct clusters (A-D) were identified among 87 *Salmonella* isolates (NC n=70; IA n=17) at the 74% genetic relatedness (see Figure 5). Each cluster was grouped based on geographic origin and *Salmonella* serotypes. Most isolates in each cluster presented the related AMR patterns which is similar to the clusters in NC PFGE dendrogram (Figure 6). However, the IA PFGE dendrogram (Figure 7) was comprised of sporadic isolates which cannot group into any clusters. The dendrogram representing genotypic similarity in the same geographic region. All serotypes were identified from the same farm in North Carolina. *S.* Altona isolated from lagoon and soil in NCF1 were 100% similar to each other and either in *S.* Altona (cluster C) found in day 0, day 7, day 14, and day 21. Almost of the genotypically similar pan-susceptible *S.* Altona isolates were grouped in the same cluster. The fingerprint profiles of *S.* Muenster (cluster B) isolated from NCF1 at different time points and sources were grouped in the same cluster with closed genetic relatedness and shared the same MDR pattern.



Dendrogram	PFGE	Farms	Samples	Day	ID	Serotypes	R-patterns
		IA6	Soil	D0	A10	Anatum	FIS STR
		IA6	Soil	D7	19	Anatum	STR
		NC2	Soil	D7	5	N/A	FIS STR SXT TET
		IA6	Lagoon	D0	L2	Anatum	Pan-susceptible
		NC1	Soil	D7	17	Uganda	AMP AUG AXO FOX FIS GEN STR XNL
		IA6	Lagoon	D0	L8	Anatum	AMP AUG AXO FOX KAN STR XNL
		NC3	Soil	D21	1	Derby	FIS STR TET
		NC2	Soil	D7	10	N/A	FIS STR SXT TET
		NC3	Lagoon	D0	L5	Rissen	STR TET
		NC3	Lagoon	D0	L9	N/A	FIS STR TET
		NC3	Lagoon	D0	L4	N/A	AMP FIS KAN STR
		NC3	Lagoon	D0	L8	Typhimurium var 5-	AMP FIS KAN STR
		NC3	Soil	D14	19	Typhimurium var5-	AMP FIS KAN STR
		NC3	Soil	D21	2	Typhimurium var5-	FIS KAN STR
		NC3	Soil	D21	4	Typhimurium var5-	FIS KAN STR
		NC3	Soil	D21	12	Typhimurium var5-	FIS KAN STR
		NC3	Soil	D21	15	Typhimurium var5-	FIS KAN STR
		NC3	Soil	D21	17	Typhimurium var5-	FIS KAN STR
A		NC3	Soil	D21	6	Typhimurium var5-	FIS KAN STR
A		NC3	Soil	D21	7	Typhimurium var5-	FIS KAN STR
A		NC3	Soil	D21	3	Typhimurium var5-	FIS KAN STR
A		NC3	Soil	D21	9	Typhimurium var5-	FIS KAN STR
		IA6	Soil	D0	A8	Litchfield	STR SXT TET
		IA6	Soil	D0	A13	Litchfield	STR
		IA6	Soil	D7	10	Litchfield	STR TET
		IA6	Soil	D0	A3	Litchfield	STR
		NC1	Lagoon	D0	L7	Uganda	Pan-susceptible
		NC1	Lagoon	D0	L9	Uganda	Pan-susceptible
		NC1	Soil	D0	A6	Uganda	AMP AUG AXO FOX FIS GEN STR XNL
		NC3	Lagoon	D0	L2	Johannesburg	AMP AUG2 AXO FOX FIS KAN STR XNI
		NC1	Soil	D0	A7	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D0	A18	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D14	10	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D14	20	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D7	5	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D0	A14	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D0	A16	Muenster	FIS GEN KAN STR TET
		NC1	Lagoon	D0	L1	Muenster	FIS GEN KAN STR TET
		NC1	Lagoon	D0	L3	Muenster	FIS GEN KAN STR TET
		NC1	Lagoon	D0	L6	Meunster	FIS GEN KAN STR TET
B		NC1	Lagoon	D0	L8	Muenster	FIS GEN KAN STR TET
B		NC1	Soil	D14	3	Muenster	FIS GEN KAN STR TET
B		NC1	Soil	D7	13	Muenster	FIS GEN KAN STR TET
B		NC1	Lagoon	D0	L5	Muenster	FIS GEN KAN STR TET
B		NC1	Soil	D0	A1	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D0	A2	Altona	Pan-suscetible
		NC1	Soil	D0	A8	Altona	AMP AXO FOX FIS GEN STR XNL TET
		NC1	Soil	D0	A15	Altona	Pan-susceptible
		NC1	Soil	D0	A17	Altona	Pan-susceptible
		NC1	Soil	D7	15	Altona	TET
		NC1	Soil	D7	19	Altona	Pan-susceptible
		NC1	Soil	D7	20	Altona	Pan-susceptible
		NC1	Soil	D14	4	Altona	Pan suscetible
		NC1	Soil	D14	14	Altona	Pan-susceptible
		NC1	Soil	D21	5	Altona	Pan-susceptible
C		NC1	Soil	D21	20	Altona	Pan-susceptible
C		NC1	Soil	D7	4	Altona	Pan-susceptible
		NC1	Lagoon	D0	L10	Altona	Pan-susceptible
		NC1	Soil	D0	A3	Altona	AMP FOX
		IA6	Lagoon	D0	L4	Anatum	FIS STR
		IA6	Lagoon	D0	L9	Anatum	AMP AXO FIS STR XNL

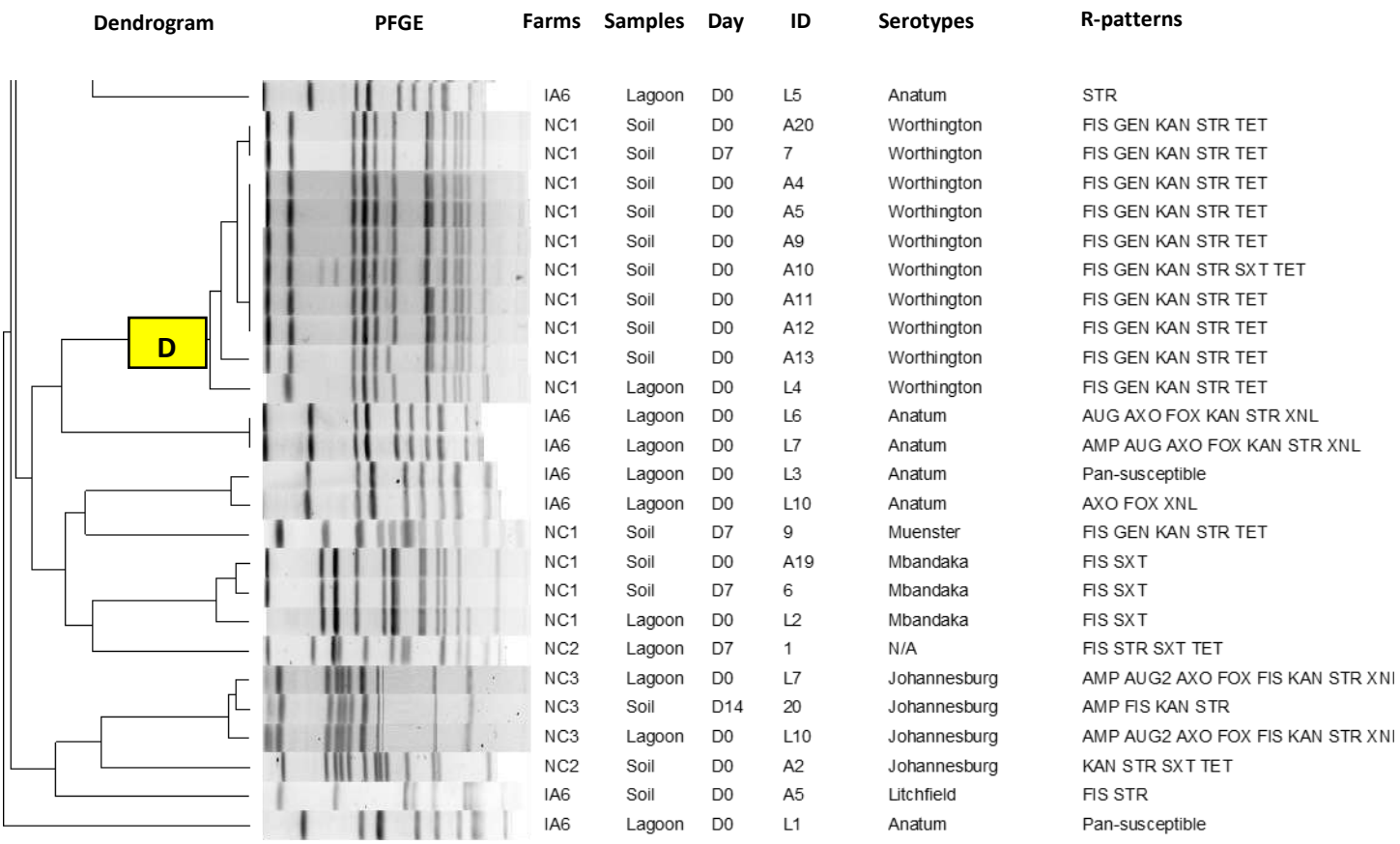
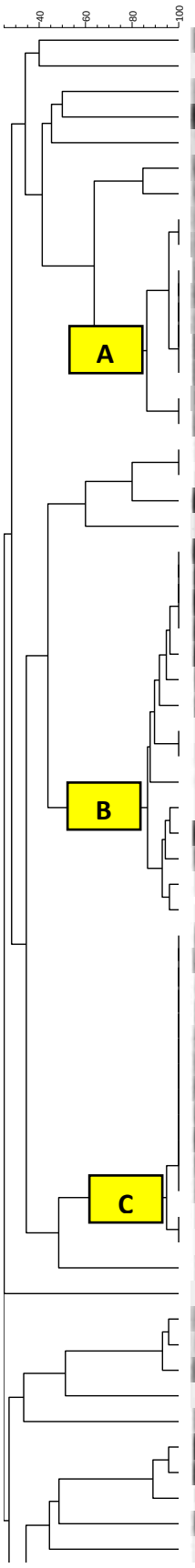


Figure 5. Dendrogram showing genotypic similarity among *Salmonella* isolated from Iowa (IAF6) and North Carolina farms (NCF1-NCF3)



Farms	Samples	Day	ID	Serotypes	R-patterns
NC3	Soil	D21	1	Derby	FIS STR TET
NC2	Soil	D7	10	N/A	FIS STR SXT TET
NC3	Lagoon	D0	L5	Rissen	STR TET
NC3	Lagoon	D0	L9	N/A	FIS STR TET
NC3	Lagoon	D0	L4	N/A	AMP FIS KAN STR
NC3	Lagoon	D0	L8	Typhimurium var 5-	AMP FIS KAN STR
NC3	Soil	D14	19	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D21	2	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	4	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	12	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	15	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	17	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	6	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	7	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	3	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	9	Typhimurium var5-	FIS KAN STR
NC1	Lagoon	D0	L7	Uganda	Pan-susceptible
NC1	Lagoon	D0	L9	Uganda	Pan-susceptible
NC1	Soil	D0	A6	Uganda	AMP AUG AXO FOX FIS GEN STR XNL TET
NC3	Lagoon	D0	L2	Johannesburg	AMP AUG2 AXO FOX FIS KAN STR XNL
NC1	Soil	D0	A7	Muenster	FIS GEN KAN STR TET
NC1	Soil	D0	A18	Muenster	FIS GEN KAN STR TET
NC1	Soil	D14	10	Muenster	FIS GEN KAN STR TET
NC1	Soil	D14	20	Muenster	FIS GEN KAN STR TET
NC1	Soil	D7	5	Muenster	FIS GEN KAN STR TET
NC1	Soil	D0	A14	Muenster	FIS GEN KAN STR TET
NC1	Soil	D0	A16	Muenster	FIS GEN KAN STR TET
NC1	Lagoon	D0	L1	Muenster	FIS GEN KAN STR TET
NC1	Lagoon	D0	L3	Muenster	FIS GEN KAN STR TET
NC1	Lagoon	D0	L6	Meunster	FIS GEN KAN STR TET
NC1	Lagoon	D0	L8	Muenster	FIS GEN KAN STR TET
NC1	Soil	D14	3	Muenster	FIS GEN KAN STR TET
NC1	Soil	D7	13	Muenster	FIS GEN KAN STR TET
NC1	Lagoon	D0	L5	Muenster	FIS GEN KAN STR TET
NC1	Soil	D0	A1	Muenster	FIS GEN KAN STR TET
NC1	Soil	D0	A2	Altona	Pan-suscetible
NC1	Soil	D0	A8	Altona	AMP AXO FOX FIS GEN STR XNL TET
NC1	Soil	D0	A15	Altona	Pan-susceptible
NC1	Soil	D0	A17	Altona	Pan-susceptible
NC1	Soil	D7	15	Altona	TET
NC1	Soil	D7	19	Altona	Pan-susceptible
NC1	Soil	D7	20	Altona	Pan-susceptible
NC1	Soil	D14	4	Altona	Pan suscetible
NC1	Soil	D14	14	Altona	Pan-susceptible
NC1	Soil	D21	5	Altona	Pan-susceptible
NC1	Soil	D21	20	Altona	Pan-susceptible
NC1	Soil	D7	4	Altona	Pan-susceptible
NC1	Lagoon	D0	L10	Altona	Pan-susceptible
NC1	Soil	D0	A3	Altona	AMP FOX
NC2	Soil	D7	5	N/A	FIS STR SXT TET
NC3	Lagoon	D0	L7	Johannesburg	AMP AUG2 AXO FOX FIS KAN STR XNL
NC3	Soil	D14	20	Johannesburg	AMP FIS KAN STR
NC3	Lagoon	D0	L10	Johannesburg	AMP AUG2 AXO FOX FIS KAN STR XNL T.
NC2	Soil	D0	A2	Johannesburg	KAN STR SXT TET
NC1	Soil	D7	17	Uganda	AMP AUG AXO FOX FIS GEN STR XNL TET
NC1	Soil	D0	A19	Mbandaka	FIS SXT
NC1	Soil	D7	6	Mbandaka	FIS SXT
NC1	Lagoon	D0	L2	Mbandaka	FIS SXT
NC2	Lagoon	D7	1	N/A	FIS STR SXT TET
NC1	Soil	D7	9	Muenster	FIS GEN KAN STR TET

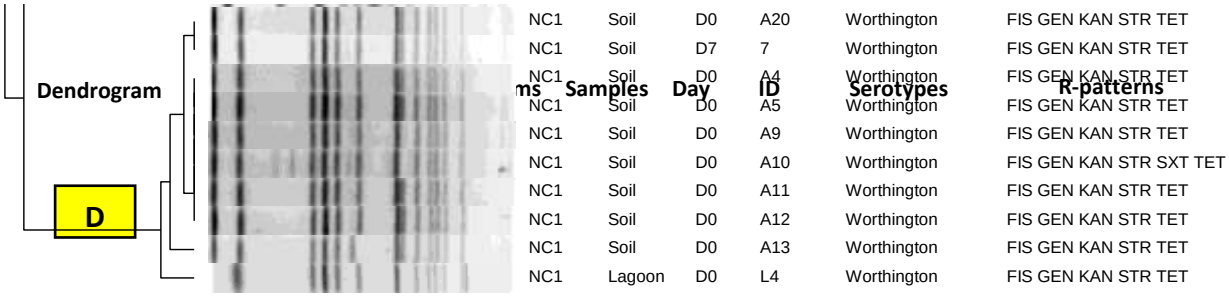


Figure 6. Dendrogram showing genotypic similarity among *Salmonella* isolated from North Carolina farms (NCF1-NCF3)

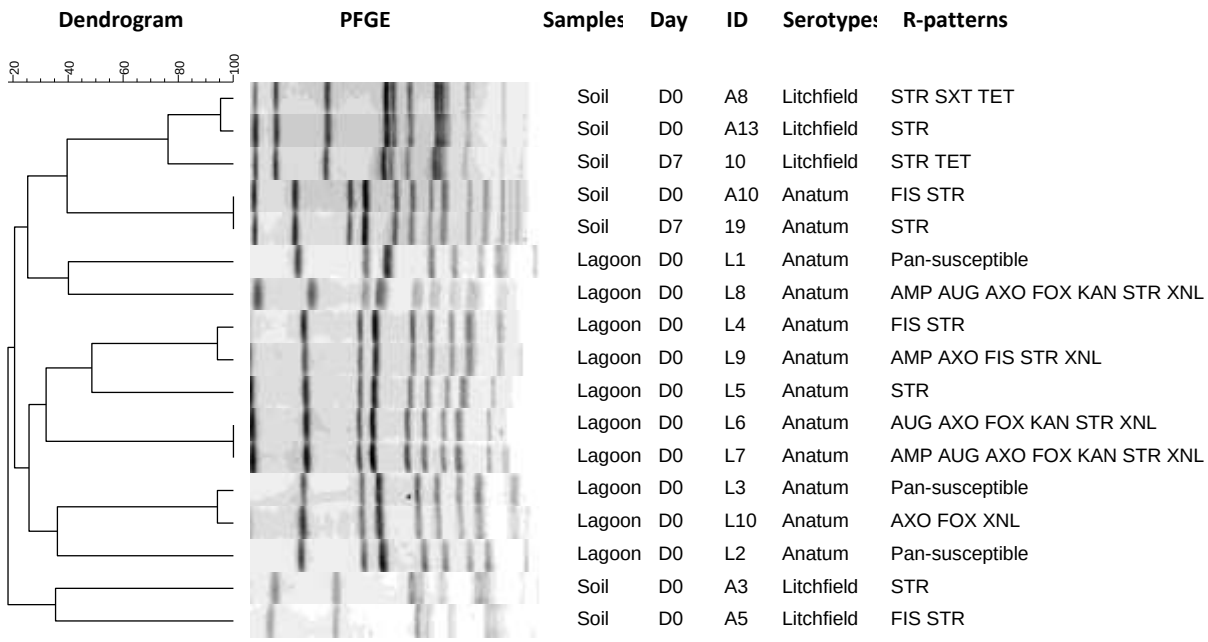


Figure 7. Dendrogram showing genotypic similarity among *Salmonella* isolated from Iowa farm (IAF 6)

Discussion:

In this study, we identified and determined the dissemination of AMR *Salmonella* isolated in the environment following manure application from commercial swine farms and then compared the *Salmonella* isolates isolated from different sources by geographic region using phenotypic and genotypic characterization. The *Salmonella* prevalence in Iowa was significantly lower than the prevalence in North Carolina. One possible reason concerning the significantly different prevalence is the swine manure management programs in two states. In Iowa, a deep pit slurry system is utilized to treat swine manure while an anaerobic lagoon system is widely used to store swine manure in North Carolina. The lagoon system need a large surface area and the required anaerobic treatment volume allows facultative bacteria to thrive in the first few feet of lagoon liquid (ASAE EP403.2, 1998). On the other hand, the pit system is located under the ground and keeps most manure solids in suspension making them more easily removed when the pit is drained. The prevalence of *Salmonella* was also significantly higher in lagoon (NC) and manure pit (IA) samples than in soil samples in both two states which applied particular manure management systems. We observed a decrease in prevalence of *Salmonella* in different time point of sampling date (day0, day7, day14, and day21). This is an important finding and it clearly indicates that *Salmonella* survival in the environment decreases with increasing time. It can be assumed that *Salmonella* can persist on land at least until day21 after manure application, however, only a few studies have been published to study this in more detail. According to Chao et al. (1987), *Salmonella* can be widely disseminated in soil and sediment, even in the absence of active fertilization. *Salmonella* has been isolated ubiquitously in environmental soil samples collected from agricultural and recreational areas (Thomason et al., 1977). There is a report that *Salmonella* can persist in soil for 54 days at maximum (Cote and Quessy, 2005) and can survive and multiply for at least 1 year in the ecosystem (Thompson et al., 1977; Davies et al., 1995).

Several *Salmonell*a serotypes were identified in our study, however, none of the serotypes from one state were detected in the other. The small number of farms sampled and the sampling size could be one potential reason for this outcome. The most common serotypes isolated in North Carolina were Typhimurium var5-. Meunster, and Altona. Previous study in NC reported that the predominant serotypes isolated from swine farms were Typhimurium followed by Infantis, Derby, and Anatum (Keelara et al., 2013). However, in our study, *S.* Anatum was not identified in NC but in IA in which it ranked the first place. Abley et al. (2013) reported that the top three *Salmonella* serotypes in three swine producing states (Iowa, North Carolina, and Minnesota) were Typhimurium (42%), Derby (25%) and Adelaide (5%). According to the CDC annual surveillance (2011), the most frequent *Salmonella* serotypes isolated from porcine source were *S.* Typhimurium, *S.* Derby, *S.* Agona, *S.* Infantis, and *S*. Heidelberg (CDC, 2013). One *S.* Rissen was isolated from a lagoon sample in NC. This serotype was not well-known until the outbreak in California 2008-2009 that resulted from white ground pepper imported from Asia. Unlike in the US, Rissen are the most frequent serotype presented in Asia and its prevalence in those countries has remained high (Dorn-in et al., 2009; Galanis et al., 2006). However, *S.* Rissen was identified for the first time in pigs and the environment in NC in late 2009 (Keelara et al., 2013).

Salmonella isolates from our study in both Iowa and North Carolina were resistant to various classes of antimicrobials. Overall, *Salmonella* isolates had the highest frequency of resistance against STR (82.82%), FIS (73.44%), KAN (61.72%), and TET (40.63%). High frequency of resistance to STR and FIS is in agreement with previous reports (Abley et al., 2013; Keelara et al., 2013). The most common R-patterns were FIS GEN KAN STR TET (18.46%), AMP FIS KAN STR (15.38%), and FIS KAN STR (13.85%). The aminoglycoside class (GEN, KAN, STR) was highlighted. STR and KAN showed the high percentage of resistance in both states while no KAN resistant isolate was observed in IA. However, the number of positive samples in IA included in the study was low (n=17) and belonged to only one farm. We also detected high MDR in isolates from both states. PFGE fingerprinting was applied to analyze the genotypic relatedness among *Salmonella* isolates from Iowa and North Carolina. PFGE is used by the Centers for Disease Control and prevention (CDC) PulseNet surveillance program and is considered as the gold standard typing technique for foodborne bacteria including *Salmonella* (Garaizar et al., 2000). Therefore, we used this method to determine the similarity of *Salmonella* isolates, isolated atdifferent time points and in different areas. Our study states that *Salmonella* isolates were clustered based on geographic origin, farms, and serotypes. We recognize that the isolates in the same cluster shared similar phenotypes in AMR pattern. However, we detected sporadic isolates isolated from Iowa which could not be grouped into cluster. We did fine evidence, based on phenotypic and genotypic similarity, that isolates recovered from swine manure were 100% similar to isolates recovered from soil on days 7, 14 and 21 of sampling.

Based on phenotypic and genotypic characterization, our study showed the potential dissemination of *Salmonella* transmission after application of swine manure in the environment. In such studies, it is important to acknowledge the fact that the pathogen of interest (*Salmonella* in this case) is already present in the land where manure is applied. From this result, it can be assumed that *Salmonella* from swine manure when spread on land can persist for at least 21 days in soil. However, it is important to highlight that the study was conducted on limited number of commercial swine farms and will have limited internal and external validity. It will be important to conduct longitudinal based studies to make valid interpretations that will eventually benefit the swine production system

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