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# Comparative Phenotypic and Genotypic Characterization of Temporally Related Nontyphoidal *Salmonella* Isolated from Human Clinical Cases, Pigs, and the Environment in North Carolina

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**Abstract**

Nontyphoidal *Salmonella* infections caused by antimicrobial-resistant (AMR) strains are of great public health concern. We compared the phenotypic and genotypic relationships among temporally and spatially related AMR *Salmonella* isolates ($n=1058$) representing several predominant serovars, including *Salmonella* Typhimurium, *Salmonella* Typhimurium var. 5-, *Salmonella* Derby, *Salmonella* Heidelberg, *Salmonella* Muenchen, *Salmonella* Schwarzengrund, and *Salmonella* Rissen of human clinical cases ($n=572$), pig ($n=212$), and farm environment ($n=274$) origin in North Carolina. Antimicrobial susceptibility testing was performed using the broth microdilution method, and genotypic resistance determinants, including class I and II integrons, were identified. Overall, *Salmonella* isolates exhibited the highest frequency of resistance to tetracycline (50%), followed by sulfisoxazole (36%) and streptomycin (27%). We identified 16 different antimicrobial resistance genes, including extended spectrum and AmpC $\beta$-lactamases-producing genes ($bla_{\text{TEM}}$, $bla_{\text{PSE}}$, and $bla_{\text{CMY-2}}$), in all the $\beta$-lactam- and cephalosporin-resistant *Salmonella* isolates from humans, pigs, and the environment. Class I integrons of 1-kb and 1.2-kb size were identified from all the three sources (humans, 66%; pigs, 85%; environment, 58%), while Class II integrons of 2-kb size were identified only in pig (10%) and environmental (19%) isolates. We detected genotypic similarity between *Salmonella* Typhimurium isolated from humans, pigs, and the environment while serovars Derby, Heidelberg, and Muenchen exhibited genotypic diversity. Detection of AMR *Salmonella* isolates from humans, pigs, and the environment is a concern for clinicians and veterinarians to mitigate the dissemination of AMR *Salmonella* strains.

## Introduction

Antimicrobial-resistant (AMR) *Salmonella* infections in humans and animals are of great concern in terms of national and global public health. Salmonellosis in humans is still one of the most widespread foodborne illnesses with an estimation of 1.02 million illnesses, 19,336 hospitalizations and deaths of more than 378 people each year in the United States (Scallan *et al.*, 2011). Nontyphoidal salmonellosis is generally associated with consumption of contaminated meat and other food products (Foley *et al.*, 2008; Glenn *et al.*, 2013). In addition, contact with nonfood sources such as contaminated water, as well as direct contact with farm animals and the environment also may result in salmonellosis in humans (Hendriksen *et al.*, 2004; Hoelzer *et al.*, 2011). Nontyphoidal *Salmonella (NTS) serovars including Salmonella Typhimurium, Salmonella Heidelberg, Salmonella Infantis, Salmonella Muenchen, Salmonella Anatum, and Salmonella Derby are commonly isolated from food animals, retail food products, and other environmental sources, and are responsible for* foodborne human salmonellosis in the United States (Foley *et al.*, 2007; CDC, 2007). In the United States, the role of therapeutic and prophylactic antimicrobial use in food animals, development and propagation of AMR bacterial populations, and their subsequent transmission to

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humans through the food chain is under extensive debate (Alali *et al.*, 2009; Qunitana-Hayashi *et al.*, 2012; Keelara *et al.*, 2013). It is important to conduct longitudinal-based studies to study the use of antimicrobials and subsequent development of AMR infections in food animals with potential transfer to humans along the food chain. Emergence and dissemination of drug-resistant *Salmonella* strains of human and pig origin, including those that are resistant to cephalosporins and quinolones, have been a major public health concern worldwide (Gebreyes *et al.*, 2009; Vo *et al.*, 2010). In addition, development of multidrug resistance (resistance to three or more classes of antimicrobials; MDR) among *Salmonella* isolates and their continued maintenance in the absence of known selection pressure is especially concerning (Gebreyes *et al*., 2006; Keelara *et al*., 2013).

North Carolina ranks second in pork production in the United States, contributing 14.4% of the national inventory (USDA, 2009). Several *Salmonella* outbreaks associated with pork in humans have been reported in the United States and worldwide in the past decade (CDC, 2007; Wojcik *et al*., 2012; Scavia *et al*., 2013). To the authors' knowledge, no studies have been conducted to compare temporally and spatially related NTS isolates from human clinical cases with *Salmonella* isolates arising from pigs and the environment as part of a longitudinal study in the United States. Therefore, the objective of this study was to characterize and compare *Salmonella* isolates from humans, pigs, and the environment by antimicrobial susceptibility testing, identification of AMR genes, and strain genotyping to determine whether temporally and spatially related *Salmonella* isolates from multiple sources in North Carolina were phenotypically and genotypically similar or diverse.

## Materials and Methods

### Salmonella *isolate sources*

In the present study, a total of 1058 temporally and spatially related NTS isolates from human clinical cases ($n=572$), pigs ($n=212$) and the farm environment ($n=274$) originating from multiple counties in North Carolina were characterized at the phenotypic and genotypic levels. The human NTS strains were clinical isolates received from the North Carolina State Public Health Laboratory. All samples were collected from October 2008 to December 2011. The geographical distribution of NTS isolates is represented in Table 1. The human clinical isolates ($n=572$) originated from 72 counties in North Carolina. The majority of isolates ($n=183$; 32%) came from Mecklenburg, Wake, Cumberland, and Cabarrus counties. Of

Table 1. Geographical Distribution of *Salmonella* Isolates from Humans, Pigs, and the Environment

| | *Host and county distribution* | | |
|---|---|---|---|
| *Serovars*[a] | *Humans* (n=*572*) | *Pigs* (n=*212*) | *Environment* (n=*274*) |
| *S*. Typhimurium (*N*=600; H=290; P=126; E=184) | Alamance (5), Anson (4), Brunswick (3), Cabarrus (9), Chatham (3), Columbus (3), Carven (8), Cumberland (17), Durham (14), Edgecombe (3), Forsyth (7), Franklin (5), Gaston (5), Guilford (11), Harnett (4), Johnston (6) Mecklenburg (46), Moore (5), Nash (4), Onslow (8), Orange (3), Out of State (6), Pender (3), Randolph (4), Robeson (5), Rowan (3), Stanly (9), Unknown (8), Wake (29), Wilson (4), Other[b] (46) | Bladen (7), Cumberland (6), Duplin (3), Johnston (20), Sampson (90) | Bladen (3), Cumberland (8), Duplin (10), Johnston (45), Sampson (116) |
| *S*. Typimurium Var C (*N*=137; H=135; P=2; E=0) | Alamance (5), Cabarrus (6), Chatham (3), Columbus (3), Carven (7), Cumberland (4), Durham (3), Johnston (3), Mecklenburg (11), Onslow (4), Randolph (5), Richmond (3), Rockingham (5), Robeson (3), Union (4), Unknown (7), Wake (17), Wilson (4), Other[b] (38) | N/A | N/A |
| *S*. Derby (*N*=118; H=12; P=74; E=32) | Pender (3), Other[b] (9) | Johnston (4), Sampson (70) | Johnston (26), Sampson (6) |
| *S*. Heidelberg (*N*=67; H=50; P=0; E=17) | Guilford (4), Mecklenburg (7), Out of State (3), Vance (3), Wake (3), Other[b] (30) | N/A | Johnston (16) |
| *S*. Muenchen (*N*=82; H=79; P=3; E=0) | Cabarrus (9), Forsyth (3), Mecklenburg (13), New Hanover (8), Onslow (3), Pender (4), Robeson (3), Rutherford (3), Wake (5), Other[b] (28) | Johnston (3) | N/A |
| *S*. Rissen (*N*=47; H=1; P=7; E=39) | N/A | Cumberland (6), | Cumberland (33), Sampson (6) |
| *S*. Schwarzengrund (*N*=7; H=5; P=0; E=2) | Other[b] (5) | N/A | Duplin (2) |

[a]*N*=total no. of serovars.
[b]Other represents any county with less than three isolates.
H, human, P, pig; E, environment; N/A, not applicable.

572 human clinical isolates, 173 (30%) originated from the primary pig-producing counties, including Duplin, Johnston, Sampson, and Cumberland counties. All of the pig ($n=212$) and the environmental ($n=274$) isolates originated from the major pig-producing counties mentioned above (Table 1). The *Salmonella* isolates from pigs and their environment were collected as part of longitudinal study conducted from October 2008 to December 2011 on 30 conventional farms at different stages of production from farm to slaughter in North Carolina. The environmental sampling from conventional swine farms consisted of water, feed, soil, and barn floor swabs. In addition, lagoon (repository of waste water draining from the barns), barn floor, and interfarm truck floor swab samples were also collected at conventional farms. The details of the study design, sampling methods, estimates of *Salmonella* prevalence in pigs and their environment, antimicrobial susceptibility profiles, serovars distribution, and their phenotypic and genotypic characterizations have been reported elsewhere (Keelara *et al.*, 2013).

### *Antimicrobial susceptibility testing (AST)*

AST was performed for all *Salmonella* isolates against a panel of 15 antimicrobials via the broth microdilution method in a 96-well Sensititre™ plate (CMV1AGNF, the "NARMS panel"; Trek Diagnostics, Inc., Cleveland, OH). The classes of antimicrobials tested included the following: aminoglycosides (amikacin, AMI; gentamicin, GEN; kanamycin, KAN; streptomycin, STR), $\beta$-lactam/$\beta$-lactamase inhibitor combinations (amoxicillin/clavulanic acid, AUG), penicillins (ampicillin, AMP), cephems (ceftriaxone, AXO; cefoxitin, FOX; ceftiofur, TIO), quinolones (ciprofloxacin, CIP; nalidixic acid, NAL), folate pathway inhibitors (sulfisoxazole, FIS; trimethoprim/sulfamethoxazole, SXT), phenicols (chloramphenicol, CHL), and tetracyclines (tetracycline, TET). The testing procedure was carried out as described in the previous study (Keelara *et al.*, 2013). The minimum inhibitory concentrations (MICs) were recorded and breakpoints were determined based on Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2010). The isolates exhibiting resistance to three or more classes of antimicrobials were classified as MDR.

### *Detection of resistance genes and integrons*

All the AMR *Salmonella* isolates from humans, pigs, and the environment were screened for the presence of all known corresponding resistance genes and class 1 and 2 integrons. Polymerase chain reaction (PCR) was performed for detection of different AMR genes using the following primers, including: the extended spectrum $\beta$-lactamases $bla_{\text{TEM}}$, $bla_{\text{PSE-1}}$ (Carlson *et al.*, 1999) and $bla_{\text{CMY-2}}$ genes (Zhao *et al.*, 2001), streptomycin resistance coding *aadA1/A2* and *strA/B* genes (Madsen *et al.*, 2000), kanamycin resistance coding *aphAI*, *Kn* genes (Frana *et al.*, 2001), chloramphenicol resistance coding *cml* gene, sulfisoxazole and trimethoprim/sulfamethoxazole resistance coding *sulI* (Briggs *et al.*, 1999) and *sulII* genes (Aarestrup *et al.*, 2003), tetracycline resistance coding *tet(A)*, *tet(B)*, *tet(C)*, and *tet(G)* genes, and class1 and class 2 integrons (Ng *et al.*, 1999). Template DNA was purified using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Amplification reactions were carried out as described in the above studies.

### *Pulsed-field gel electrophoresis (PFGE) analysis*

A subset of *Salmonella* isolates (pigs, $n=46$; environment, $n=80$; humans, $n=271$) that were purposively chosen to be representative of different sources of origin, serovar, counties, and AMR profiles were genotyped using PFGE, following the PulseNet protocol (Ribot *et al.*, 2006). BioNumerics software version 6.1 (Applied Maths, Kortrijk, Belgium) was used to analyze the PFGE images. A dendrogram was generated to determine the clonal relationship among human, pig, and environmental isolates using an unweighted-pair group method with average linkages (UPGMA), and with band position tolerance and optimization of 1.5% each.

### *Statistical analysis*

We used odds ratios (OR) with 95% confidence intervals (CI) to explore the associations between MDR and the predominant serovars detected in our study (SigmaPlot 11.2; Systat Software, Inc., Chicago, IL). A $p$-value of 0.05 or lower was considered statistically significant.

## Results

### *Distribution of* Salmonella *serovars*

The predominant serovars identified among human clinical *Salmonella* isolates ($n=572$) were *Salmonella* Typhimurium (50.6%), followed by *Salmonella* Typhimurium var. 5- (23.6%), *Salmonella* Muenchen (13.8%), and *Salmonella* Heidelberg (8.7%). Serovars from pig ($n=212$) and environmental ($n=274$) origin were predominantly represented by *Salmonella* Typhimurium (pigs, 59.4%; environment, 67.1%) and *Salmonella* Derby (pigs, 34.9%; environment, 11.6%).

### *Antimicrobial resistance profiles*

A total of 1058 *Salmonella* isolates from humans ($n=572$), pigs ($n=212$), and their environment ($n=274$) were tested against a panel of 15 antimicrobials. The overall MIC levels and frequency of AMR of *Salmonella* isolates are presented in Table 2 and Figure 1. All of the *Salmonella* isolates, irrespective of source of origin, exhibited similar $MIC_{50}$ and $MIC_{90}$ with the exceptions being the following: TET $MIC_{50}$ in humans, which was 4 $\mu$g/mL (versus pigs and environment: 32 $\mu$g/mL); FIS $MIC_{50}$ in pigs, which was 256 $\mu$g/mL (humans and environment, 64 $\mu$g/mL); and CHL $MIC_{90}$ in environmental isolates, which was 16 $\mu$g/mL (pigs and humans, 32 $\mu$g/mL). Overall, *Salmonella* isolates exhibited the highest frequency of resistance to tetracycline (TET; 50%), followed by sulfisoxazole (FIS; 36%) and streptomycin (STR; 27%). In addition, *Salmonella* isolates from human clinical cases exhibited slightly higher frequencies of resistance to cephalosporins including AXO, FOX, and TIO (3% each) compared to pigs and environmental isolates (2% each). Overall, the frequency of AMR was higher in *Salmonella* isolates of pig origin (82.6%) followed by environmental (67.6%) and human clinical isolates (28%), with the exception of AMI (0.7%) and CIP (0.3%) resistance, which were only exhibited by human clinical isolates.

### *MDR patterns identified*

The major MDR patterns identified in humans, pigs and their environment are represented in Table 3. The highest frequency of MDR patterns was detected in *Salmonella*

Table 2. Minimum Inhibitory Concentration (MIC) Levels and Frequency of Resistance of *Salmonella* Isolates from Humans, Pigs, and Environment

| | | *Antimicrobials* | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Source* | *Range* | *AMI* | *AMP* | *AUG* | *AXO* | *FOX* | *TIO* | *CHL* | *CIP* | *FIS* | *GEN* | *KAN* | *NAL* | *STR* | *SXT* | *TET* |
| Humans ($n=572$) | MIC 50 | 2 | 1 | 1/0.5 | 0.25 | 2 | 1 | 8 | 0.02 | 64 | 0.5 | 8 | 4 | 32 | 0.12/2.38 | 4 |
| | MIC 90 | 2 | 32 | 16/8 | 0.25 | 4 | 1 | 32 | 0.02 | 256 | 0.5 | 8 | 4 | 64 | 0.12/2.38 | 32 |
| | % R | 0.7 | 18 | 2.6 | 3.4 | 3.3 | 3.1 | 12.4 | 0.3 | 19 | 1.2 | 5.9 | 1.3 | 19.5 | 0.8 | 20 |
| Pigs ($n=212$) | MIC 50 | 2 | 1 | 1/0.5 | 0.25 | 4 | 1 | 8 | 0.02 | 256 | 0.5 | 8 | 4 | 32 | 0.12/2.38 | 32 |
| | MIC 90 | 2 | 32 | 16/8 | 0.25 | 4 | 1 | 32 | 0.02 | 256 | 0.5 | 8 | 4 | 64 | 0.12/2.38 | 32 |
| | % R | 0 | 17.4 | 1.4 | 1.8 | 2.3 | 1.8 | 13.2 | 0 | 68.0 | 1.4 | 1.4 | 4.2 | 48.5 | 4.7 | 82.0 |
| Environment ($n=274$) | MIC 50 | 1 | 1 | 1/0.5 | 0.25 | 4 | 1 | 8 | 0.02 | 64 | 0.25 | 8 | 4 | 32 | 0.12/2.38 | 32 |
| | MIC 90 | 2 | 32 | 16/8 | 0.25 | 4 | 1 | 16 | 0.03 | 256 | 0.5 | 8 | 4 | 64 | 0.12/2.38 | 32 |
| | % R | 0 | 19.3 | 2.5 | 1.6 | 2.1 | 1.6 | 8 | 0 | 46 | 0 | 5.8 | 3.6 | 27.3 | 6.2 | 88.0 |

Antimicrobials: AMI, amikacin; 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/sulfamethoxazole; TET, tetracycline; TIO, ceftiofur.

% R, percent resistance.

isolated from pigs (50.9%) compared to the environment (22.6%) and human (19.2%). The most common MDR patterns identified in all the three sources were FIS-STR-TET, and AMP-CHL-FIS-STR-TET associated with *Salmonella* Derby and *Salmonella* Typhimurium, respectively. The *Salmonella* Derby serovar was significantly ($p<0.001$) associated with an MDR pattern FIS-STR-TETwith an OR of 27 (95% CI = 15.6–46.8) compared to *Salmonella* Typhimurium isolates with this MDR pattern. Specific MDR patterns were detected in particular hosts including FIS-STR-TET and AMP-AUG-AXO-FOX-TIO-TET in *Salmonella* Typhimurium isolated from pigs and environmental isolates. In contrast, the AMP-KAN-STR-TET ($n=5$) pattern was found only in *Salmonella* Heidelberg isolates of human origin (Table 3). A single human clinical *Salmonella* Typhimurium isolate was resistant to 12 antimicrobials (including quinolones and multiple cephalosporins) out of 15 tested with a MDR pattern of AMI-AXO-CHL-CIP-FOX-FIS-GEN-KAN-NAL-STR-TIO-TET.

### *Molecular characterization of AMR determinants*

Using PCR, we identified 16 different AMR genes conferring resistance to a number of classes of antimicrobials (Table 3). Ampicillin resistance was predominantly encoded by the

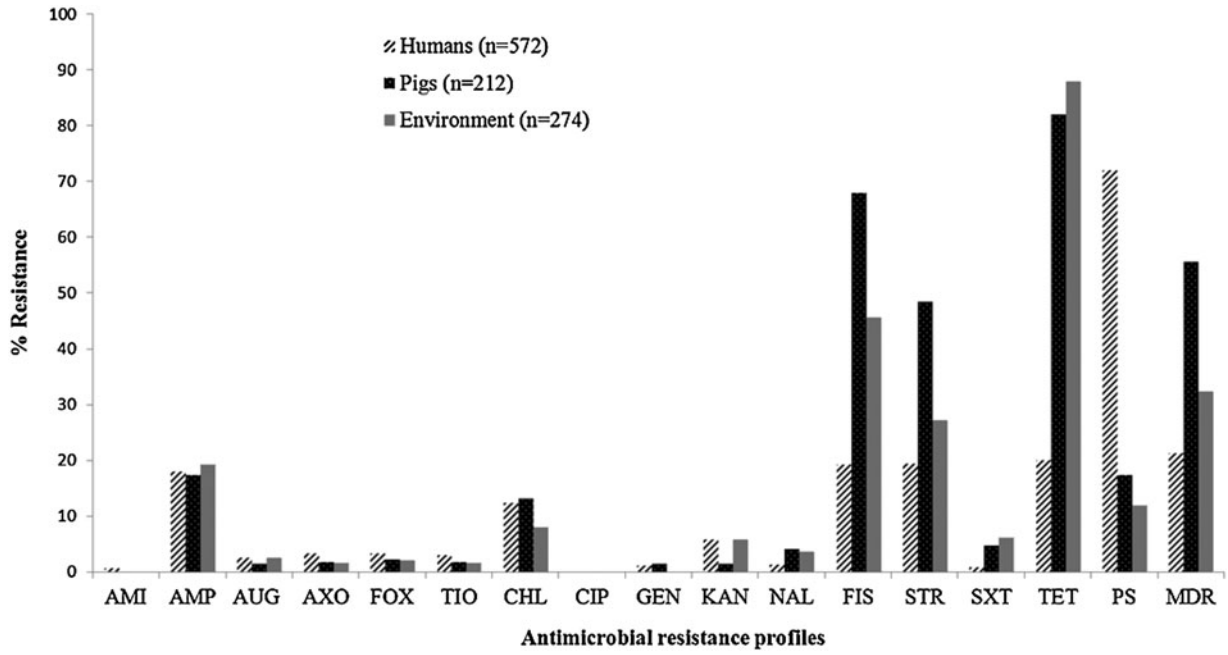


**FIG. 1.** Antimicrobial resistance profile of *Salmonella* isolates from humans, pigs, and environment. Antimicrobials: AMI, amikacin; AMP, ampicillin, AUG, amoxicillin/clavulanic acid; AXO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; FIS, sulfisoxazole; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; SXT, trimethoprim/sulfamethaxazole; TET, tetracycline; TIO, ceftiofur. MDR, multidrug resistance (resistance ≥3 classes of antimicrobials); PS (pansusceptible): susceptible to all the antimicrobials tested.

Table 3. Distribution of Predominant Multidrug Resistance (MDR) Patterns and Antimicrobial Resistance (AR) Genes in Humans, Pigs, and Environment

| *Serovars* | *MDR patterns*[a] | *Humans (%), AR genes*[b] | *Pigs (%), AR genes* | *Environment (%), AR genes* |
|---|---|---|---|---|
| *Salmonella* Typhimurium (H = 132, P = 59, E = 60) | FIS-STR-TET | 0<br>N/A | 9 (15)<br>*aad A1/A2*, *strA/B*, *sul*2, *tet* (A), class I integrons | 7 (12)<br>*aad A1/A2*, *sul*2, *tet* (A), class I integrons |
| | AMP-AUG-AXO-FOX-TIO-TET | 0<br>N/A | 3 (5)<br>$bla_{TEM}$, $bla_{CMY-2}$, *tet* (C) | 5 (8)<br>$bla_{TEM}$, $bla_{CMY-2}$, *tet* (C) |
| | AMP-CHL-FIS-STR-TET | 43 (32)<br>$bla_{TEM}$ / $bla_{PSE}$, *cmlA*, *aadA1/A2 and tet* (G) | 21 (35)<br>$bla_{TEM}$ / $bla_{PSE}$, *cmlA*, *aadA1/A2 and tet* (G) | 20 (33)<br>$bla_{TEM}$ / $bla_{PSE}$, *cmlA*, *aadA1/A2 and tet* (G) |
| *Salmonella* Heidelberg (H = 29, P = 0, E = 12) | AMP-KAN-STR-TET | 5 (17)<br>$bla_{TEM}$, *str A/B*, *tet* (C) | 0<br>N/A | 0<br>N/A |
| *Salmonella* Derby (H = 6, P = 57, E = 17) | FIS-STR-TET | 6 (100)<br>*aad A1/A2*, *sul*2, *tet* (A), class I *Int* | 56 (98)<br>*aad A1/A2*, *sul*2, *tet* (A), class I *Int* | 16 (98)<br>*aad A1/A2*, *sul*2, *tet* (A), class I *Int* |

[a]Antimicrobials: AMP, ampicillin; AUG, amoxicillin/clavulanic acid; AXO, ceftriaxone; CHL, chloramphenicol; FIS, sulfisoxazole; FOX, cefoxitin; KAN, kanamycin; STR, streptomycin; TET, tetracycline; TIO, ceftiofur.

[b]$bla_{TEM}$/$bla_{PSE}$, genes encoding ampicillin; *cmlA*, chloramphenicol; *aadA1/A2* and *str A/B*, streptomycin; *tet* (A) (C), tetracycline; $bla_{CMY-2}$, extended-spectrum β-lactamases.

H, MDR *Salmonella* isolates representing humans; P, MDR *Salmonella* isolates representing pigs; E, MDR *Salmonella* isolates representing environment.

extended-spectrum β-lactamase–producing $bla_{TEM}$ gene (humans, 71%; pigs, 49%; environment, 81%), followed by the $bla_{PSE-1}$ gene (humans, 17%; pigs, 49%; environment, 11%). All the *Salmonella* isolates from humans, pigs, and the environment that were resistant to third-generation cephalosporins carried the AmpC β-lactamases–producing $bla_{CMY-2}$ gene. Tetracycline-resistant isolates from pigs and the environment harbored four different genes, predominantly *tet(A)* (pigs, 72%; environment, 68%) followed by *tet(B)* (pigs, 10%; environment, 19%), *tet(C)* (pigs, 4%; environment, 5%), and *tet(G)* (pigs, 15%; environment, 9%) genes. Among *Salmonella* isolates from humans, tetracycline resistance was predominantly encoded by *tet(G)* (58%), *tet(A)* and *tet(C)* (each at 17%), and with the notable absence of the *tet(B)* gene. The *Salmonella* Typhimurium penta-resistant (AMP-CHL-FIS-STR-TET) isolates from humans, pigs, and the environment harbored similar resistant genes, including $bla_{TEM}$/$bla_{PSE}$, *cml*A, *aadA1/A2* and *tet(G)*, encoding resistance to AMP-CHL-STR-TET, respectively (Table 3). The FIS encoding gene *sul2* was only detected in the isolates with FIS-STR-TET resistance pattern (Table 3). We detected class I integrons of 1 kb and 1- and 1.2-kb size (humans, 66%; pigs, 85%; environment, 58%) and class II integrons of 2 kb (humans, 0%; pigs, 10%; environment, 19%) in MDR *Salmonella* isolates from pigs and the environment. All the penta-resistant *Salmonella* Typhimurium from each source exhibited class I integrons of 1- and 1.2-kb size.

### *Pulsed-field gel electrophoresis (PFGE)*

Genotyping of temporally and spatially related *Salmonella* isolates ($n = 397$) by PFGE with the *Xba*I restriction enzyme distributed them into 74 major clusters consisting of isolates with similar fingerprint profiles, and another 118 unique PFGE patterns represented by a single isolate each (data not shown). *Salmonella* Muenchen isolates from each source had more diversified fingerprint profiles compared to other study serovars. All the *Salmonella* Heidelberg isolates were closely related based on fingerprint profiles and distributed into one large cluster with 80% genotypic similarity.

We created individual dendrograms, one each for *Salmonella* Derby and *Salmonella* Typhimurium, to represent relationship between counties and AMR patterns. Identical fingerprint profiles were found among *Salmonella* Derby isolates of pig and environmental origin from Cumberland, Johnston, and Sampson counties (cluster 3–5; Fig. 2). All the *Salmonella* Derby isolates with MDR pattern FIS-STR-TET from the three sources were clustered closely in the dendrogram (cluster 2–6; Fig. 2). Even though the *Salmonella* Derby from humans (ID HS749 and HS289) had an identical resistance pattern (FIS-STR-TET) to those that originated from pigs and the environment in the same county (Sampson and Cumberland), they had different fingerprint profiles (Fig. 2). Genotypic (100%) and phenotypic similarity based on MDR pattern (AMP-CHL-FIS-STR-TET) among isolates of human, pig, and environmental origin was detected only among the *Salmonella* Typhimurium serovar (cluster 1 and 2; Fig. 3). Overall, the isolates from human, pigs, and the environment exhibited higher levels of genotypic diversity among *Salmonella* Derby, *Salmonella* Heidelberg, and *Salmonella* Muenchen isolates and much more genotypic similarity in *Salmonella* Typhimurium isolates.

## Discussion

The objective of this study was to compare temporally and spatially related NTS isolates from humans, pigs, and the environment based on their AMR phenotypes and genotypic

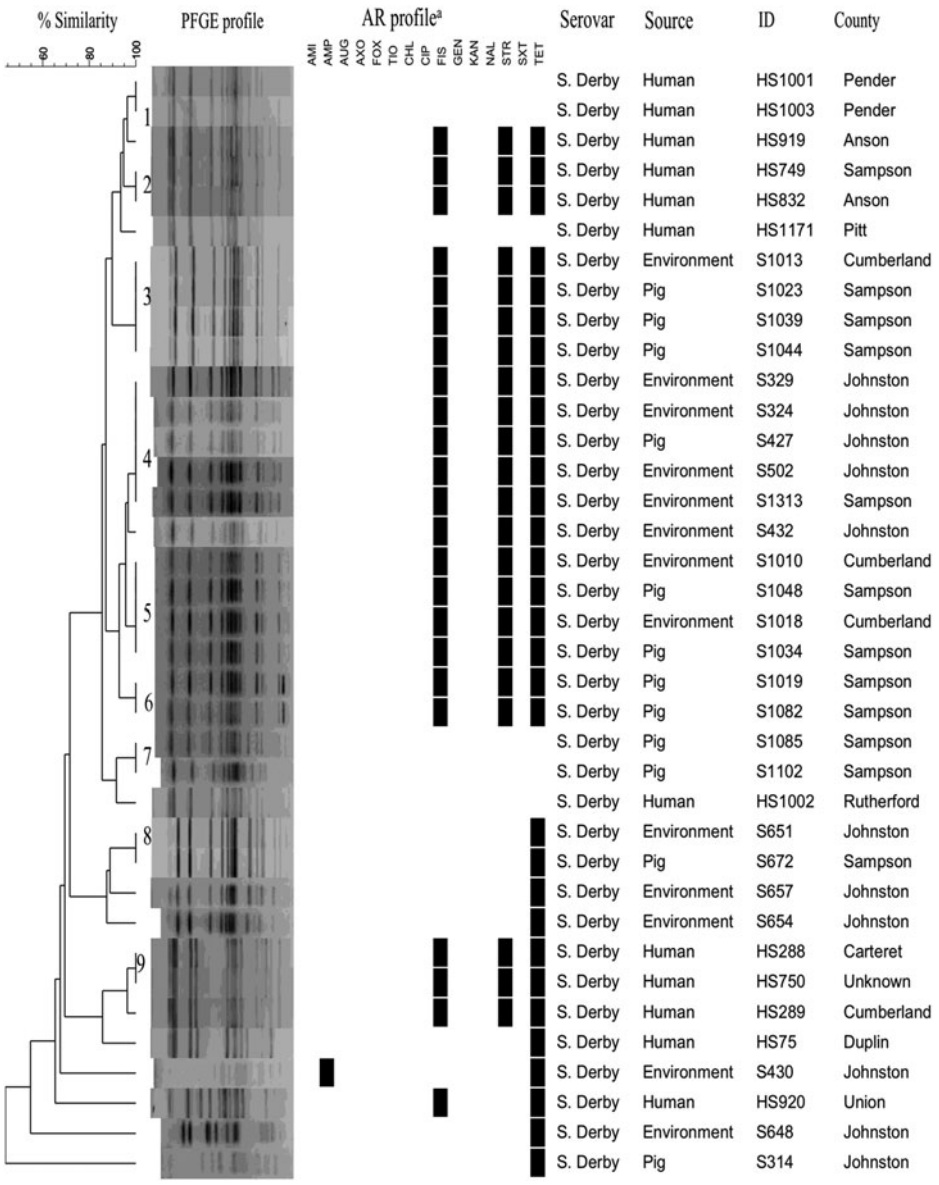


**FIG. 2.** Dendrogram showing genotypic similarity among *Salmonella* Derby isolated from humans, pigs, and environment. PFGE, pulsed-field gel electrophoresis; AR, antimicrobial resistance. [a]AMI, amikacin, AMP, ampicillin; AUG, amoxicillin/clavulanic acid; AXO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; FIS, sulfisoxazole; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TIO, ceftiofur.

fingerprint profiles. The frequency of AMR was higher in *Salmonella* isolates from pigs (82.6%) and their environment (67.6%) compared to human clinical *Salmonella* isolates (28%). In addition, we observed a higher $MIC_{50}$ (32 $\mu$g/mL) for TET in pig and environmental isolates compared to the $MIC_{50}$ (4 $\mu$g/mL) for human isolates. One reason could be that the pig and environmental isolates were from conventional production systems that routinely use antimicrobials for prophylaxis (tetracycline and macrolides as growth promoter) and therapeutic purposes, thereby contributing to an increased frequency of resistance and MIC to antimicrobials (Keelara *et al*., 2013).

Resistance to $\beta$-lactams, including ampicillin and cephalosporins, was observed at low levels in human, pig, and environmental isolates.

Human clinical *Salmonella* isolates exhibited resistance to CIP (0.3%), which was never observed in pig or environmental isolates. AMR trend to CIP in *Salmonella* isolates of humans appears to be increasing every year based on the National Antimicrobial Resistance Monitoring System (NARMS) annual report on AMR of NTS isolates from 1999 to 2010, as well as other studies (Patchanee *et al.*, 2008; Dorr *et al.*, 2009; NARMS, 2010; Boxstale *et al.*, 2012). The use of ciprofloxacin for treating severe bacterial infections could potentially explain the increase in AMR to this important antimicrobial. AMR among *Salmonella* to ciprofloxacin and third-generation cephalosporins is concerning since they are the drugs of choice for treating human invasive *Salmonella* infections.

A higher frequency of MDR isolates was observed in *Salmonella* isolates of pigs (50.9%) and their environment (22.6%)

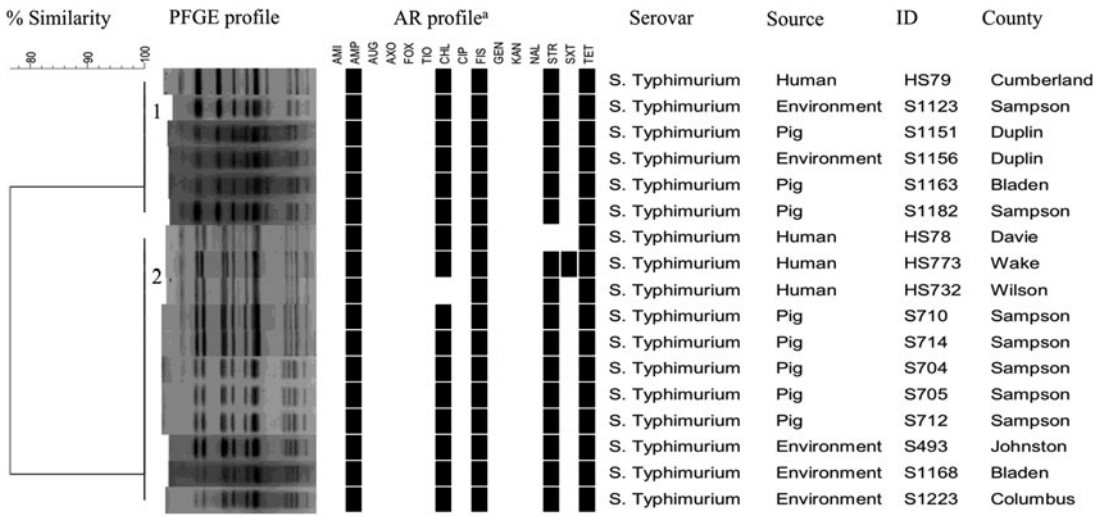


**FIG. 3.** Dendrogram showing genotypic similarity among *Salmonella* Typhimurium isolated from humans, pigs, and environment. PFGE, pulsed-field gel electrophoresis; AR, antimicrobial resistance. [a]AMI, amikacin; AMP, ampicillin; AUG, amoxicillin/clavulanic acid; AXO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; FIS, sulfisoxazole; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TIO, ceftiofur.

compared to humans (19.2%). According to the NARMS executive report (1999–2010), the number of reported NTS MDR isolates in humans dropped to 9% from 2007 to 2010. Interestingly, in our study the frequency of MDR isolates from human clinical NTS isolates was higher (19.2%) compared to recent NARMS data (NARMS, 2010). This variation could be attributed to regional differences in the definition of MDR used, reporting practices, human antimicrobial use, or regional strain variation. The two most common patterns observed in this study among all the three sources were FIS-STR-TET and AMP-CHL-FIS-STR-TET. The *Salmonella* Derby serovar was 27 times (odds ratio) more likely to be associated with the FIS-STR-TET pattern than *Salmonella* Typhimurium, which suggests establishment of this serovar with a specific MDR pattern in this region. Emergence of *Salmonella* Derby with this specific MDR pattern is concerning, as it is the top serovar isolated from pigs (NARMS, 2010). Importantly, all of the three classes of antimicrobials to which it is resistant are currently considered critically important by the World Health Organization (WHO, 2012).

The MDR pattern AMP-CHL-FIS-STR-TET was detected at a higher frequency among the human *Salmonella* Typhimurium isolates when compared to pigs and their environment. This penta-resistant pattern is specific to *Salmonella* Typhimurium phage type DT104 and is commonly associated with clinical salmonellosis and human foodborne outbreaks worldwide (Helms *et al*., 2005; Gebreyes *et al*., 2009; Medalla *et al*., 2013). This penta-resistant phenotype is often a component of higher-order MDR patterns that include resistance elements against the critically important fluoroquinolones and third- and fourth-generation cephalosporins. As an example, a single *Salmonella* Typhimurium isolate from a clinical human case exhibited resistance to 12 antimicrobials: very importantly, including resistance against both quinolones and multiple cephalosporins. This pattern in *Salmonella* Typhimurium has been previously reported from human clinical cases (Patchanee *et al*., 2008; Glenn *et al*., 2013).

The molecular characterization of these isolates using PCR to detect the presence of genes encoding resistance to various antimicrobials was consistent among the *Salmonella* isolates from all three sources. Detection of the AmpC $\beta$-lactamases producing gene $bla_{\text{CMY-2}}$ among all the *Salmonella* isolates resistant to third-generation cephalosporins, including AXO and TIO, was in agreement with previous reports (Zhao *et al*., 2001; Van *et al*., 2012). This is the first report of AmpC $\beta$-lactamases producing ($bla_{\text{CMY-2}}$) *Salmonella* from this region. Presence of this gene is concerning because *Salmonella* isolates carrying these genes are at an increased risk of acquiring resistance to other classes of antimicrobials (Hamilton *et al*., 2012; Glenn *et al*., 2013).

The majority of penta-resistance in *Salmonella* Typhimurium was encoded by resistance genes carried on class I integrins, and these are believed to play an important role in the dissemination of AMR among susceptible populations of *Salmonella* both in humans and animals (Aarestrup *et al*., 2008). This was evidenced by detection of class I integrons of 1 and 1.2 kb in all the penta-resistant (AMP-CHL-FIS-STR-TET) *Salmonella* Typhimurium isolates of human, pig, and the environment. All of these isolates carried similar resistance genes, including $bla_{\text{TEM}}/bla_{\text{PSE}}$, *cml*A, *aadA1/A2*, and *tet(G)*. All of the *Salmonella* Derby with FIS-STR-TET pattern carried a Class I integron of 1-kb size, suggesting too that this serovar could play a potentially important role in transmission of AMR to a susceptible population as for *Salmonella* Typhimurium. We identified the rarely reported Class II integrons of 2-kb size in pig and environmental *Salmonella* isolates. To the best of our knowledge, this is the first report of Class II integrons in *Salmonella* in this region. Further analysis of the class II integrons detected in our study will be performed at a later date to identify additional resistant gene cassettes that may be located on them.

A subset of *Salmonella* isolates from humans, pigs, and the environment exhibiting similar AMR patterns and originating from pig-producing and non-pig-producing counties were

genotyped using PFGE. We identified 74 major clusters consisting of isolates with similar fingerprint profiles and an additional 118 unique profiles. Isolates of *Salmonella* Muenchen from human clinical cases that originated from the same county were grouped into two different clusters. However, the majority of *Salmonella* Muenchen isolates from humans had a unique fingerprint profile, which has previously been reported in *Salmonella* Muenchen and other *Salmonella* serovars (Gebreyes *et al*., 2005; Patchanee *et al*., 2008). This diversity indicates that there are numerous clones circulating for serovar Muenchen in this region. Similarly, *Salmonella* Derby isolates from humans were more diverse compared to pig and environmental isolates, even though they had similar resistance patterns and were isolated from counties adjacent to major pig-producing counties; this indicates that different genotypes of *Salmonella* Derby are prevalent in North Carolina. We identified 100% genotypic similarity only in penta-resistant *Salmonella* Typhimurium isolates from humans, pigs, and the environment originating from the major pig-producing counties of North Carolina (cluster 1 and 2; Fig. 3). It is worth mentioning that the human *Salmonella* Typhimurium isolates in these clusters were reported from counties (Cumberland and Wake) adjacent to the major pig-producing counties of North Carolina. However, it is important to mention that no human *Salmonella* outbreak associated with pork has been reported from this area.

## Conclusions

In conclusion, this is the first report to compare temporally and spatially related NTS isolated from humans, pigs, and the environment from this region of the United States. Detection of similar MDR patterns, Class I and II integrons in *Salmonella* of pigs, their environment, and of human origin is a growing public health concern. Even though the human isolates were not directly linked to pigs/farm environment, identical fingerprint profiles suggest that the same strains are circulating in North Carolina. However, it is important to mention that this study cannot predict the direction of *Salmonella* transmission. Further studies to determine the role played by different reservoirs in determining the occurrence and dissemination of AMR *Salmonella* in the food chain will be key to identifying these sources.

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## Disclosure Statement

No competing financial interest exists.

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