Horizontal dissemination of antimicrobial resistance determinants in multiple *Salmonella* serotypes following isolation from the environment of commercial swine operations after manure application

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ABSTRACT. The aim of this study was to characterize the plasmids carrying antimicrobial resistance (AMR) determinants in multiple *Salmonella* serotypes recovered from the commercial swine farms environment after manure application on land. Manure and soil samples were collected on day0 before and after manure application on six farms in North Carolina and sequential soil samples were recollected on day7, 14, and 21 from the same plots. All environmental samples were processed for *Salmonella* and their plasmid contents were further characterized. A total of fourteen isolates including Johannesburg (n=2), Ohio (n=2), Rissen (n=1), Typhimurium var5- (n=5), Worthington (n=3), and 4,12:i:- (n=1) representing different farms were selected for plasmid analysis. Antimicrobial susceptibility test was done by broth microdilution against a panel of 14 antimicrobials on the 14 confirmed transconjugants after conjugation assays. The plasmids were isolated by the modified alkaline lysis and PCRs were performed on purified plasmid DNA to identify the AMR determinants and the plasmid replicon types. The plasmids were sequenced to further analyze, compare profiles and create phylogenetic trees. A class 1 integron with ANT(2")Ia-*aad*A2 cassette was detected in the 50-kb IncN plasmids identified in *S.* Worthington. We identified 100-kb and 90-kb IncI1 plasmids in *S.* Johannesburg and *S.* Rissen carrying the *bla*$_{\text{CMY-2}}$ and *tet*(A) genes, respectively. An identical 95-kb IncF plasmid was widely disseminated among the different serotypes and across different farms. Our study provides evidence on the importance of horizontal dissemination of resistance determinants through plasmids in multiple *Salmonella* serotypes distributed across commercial swine farms after manure application.

IMPORTANCE. The horizontal gene transfer of antimicrobial resistance (AMR) determinants located on plasmids is considered to be the main reason for the rapid proliferation and spread drug resistance. The deposition of manure generated in swine production systems into the environment is identified as a potential source of AMR dissemination. In this study, AMR gene carrying plasmids were detected in multiple *Salmonella* serotypes across different commercial swine farms in North Carolina. The plasmid profiles were characterized based on *Salmonella* serotype donors and incompatibility (Inc) groups. We found different Inc plasmids showed the evidence of AMR genes transfer in multiple *Salmonella* serotypes. We detected an identical 95-kb plasmid that was widely distributed across swine farms in NC. These conjugable resistance plasmids were able to persist on land after swine manure application. Our study provides strong evidence of AMR determinants dissemination present in plasmids of multiple *Salmonella* serotypes in the environment after manure application.

INTRODUCTION

The emergence of antimicrobial-resistance (AMR) in bacterial pathogens has threatened the sustainability of the effective global public health response to infectious diseases (1, 2). There are major gaps in our understanding of the AMR transmission within agricultural sites and their potential impacts on human, animal, and environment due to lack of studies conducted on actual commercial food animal farms (3-5). A number of studies have documented abundance of AMR pathogens associated with livestock production due to the intensive use of antimicrobials in animal husbandry practices for therapeutic and nontherapeutic purposes (6-9). However, there is limited knowledge about the effect of manure application on spread of AMR pathogens and AMR genes by the means of horizontal gene transfer (HGT) such as plasmids, transposons, and integrons upon manure spread in the environment (4, 10, 11). Exposure of bacterial pathogens to antimicrobials in the environment increases the evolution of resistance, has an influence on the abundance, distribution, and transfer of AMR genes into different bacterial species (9, 12). We recently reported the dissemination of AMR *Salmonella* in manure from commercial swine farms that were able to persist on land for at least 21 days after manure application and it was clearly observed that *Salmonella* were rarely presented in the soil before the land application (13). Given the potential risk of manure application in disseminating AMR *Salmonella* into the environment, we further characterized the plasmids that were detected in the multiple *Salmonella* serotypes isolated in our previous study.

The dissemination of undesirable AMR in gram-negative pathogenic bacteria has been mainly regarded as the acquisition of multiple plasmid-located AMR genes by HGT (14, 15). Conjugation is considered as the main mode of HGT of AMR genes among *Enterobacteriaceae* family and helps to increase bacterial genetic diversity (16, 17). Plasmids conferring resistance

have been identified as creating hindrance in antimicrobial therapy, including extended-spectrum cephalosporin and fluoroquinolone that are regarded as drug of choice for bacterial infection in human clinical cases (14, 18, 19). Studies from several parts of the world have demonstrated the distribution of plasmids harboring extended spectrum β-lactamase (ESBL; *bla*$_{CTX}$, *bla*$_{SHV}$, *bla*$_{CMY}$, *bla*$_{TEM}$) or *amp*C and plasmid-mediated quinolone resistance (PMQR; *qnr*A, *qnr*B, *qnr*S) genes in *Escherichia coli* and *Salmonella* among animal, human, and environmental sources (16, 20-22). The presence of plasmid-mediated transfer of mobile colistin resistance gene (*mcr*-1) recently identified is another example of the threat they pose to public health (23, 24). The comparative analysis of *mcr*-1-containing plasmids maintained in *Enterobacteriaceae* family revealed that it is disseminated in a broad host range, including human, animal, and food and is now being reported from different countries worldwide (25-27). Plasmids that encode carbapenem-resistant in *Enterobacteriaceae* (CRE) pose an urgent threat to public health with their global expansion (28, 29). Mollenkopf et al. (30) reported that the CRE carrying *bla*$_{IMP-27}$ plasmids were recovered from the environment of swine production area in the US. Farm environment is considered as a potential reservoir of AMR *Salmonella* strain that probably exchange AMR determinants to human and animal by plasmid horizontal transfer (13, 22, 31, 32).

The objective of this study was to determine and characterize the resistance plasmid profiles isolated from multiple AMR *Salmonella* serotypes recovered in manure and environmental samples after land application in commercial swine farms in North Carolina. To address this, we performed antimicrobial susceptibility test (AST), plasmid replicon typing, conjugation assay, and plasmid sequencing to fully understand the role of these plasmids in transferring AMR determinants in the environment.

MATERIALS AND METHODS

***Salmonella* Serotypes Selection**. One hundred and sixty-eight AMR *Salmonella* isolated from commercial swine farms environment in North Carolina area during 2013-2015 were tested for their plasmid components. The details of farms distribution, waste management system, sample collection, and *Salmonella* isolation were described in the previous study (13). Briefly, manure samples from lagoon and soil samples before and after manure spray were collected on the first day (day 0) of farm visit. The subsequent soil samples were recollected on day 7, day 14, and day 21 from the same plots as designed on day 0. The serotyping, antimicrobial susceptibility test (AST), and pulse field gel electrophoresis (PFGE) were performed for phenotypic and genotypic characterization of the *Salmonella* strains. The *Salmonella* isolates selected for plasmid characterization were chosen based on their temporal and spatial relationship, AMR profile, AMR determinants, and PFGE fingerprint profiles. Based on the above criteria, a total of 14 isolates were finalized for plasmid analysis and sequencing (Table 1). All isolates were maintained at -80°C in Brucella broth (Difco, Becton-Dickinson, USA) until further characterization.

Conjugation Experiment. Conjugation experiments were conducted to evaluate intra- and inter-serovar transmission of AMR genes among AMR *Salmonella* serotypes. Fourteen AMR-*Salmonella* isolates were selected to serve as donor strains and the nalidixic acid-resistant (NAL^R) *Escherichia coli* JM109 was used as recipient strain. A heat shock assay modified from Zeng et al. (33) was utilized for performing conjugation experiments. In brief, a loop full overnight culture of the donor was gently mixed in Luria-Bertani (LB) broth (Difco, Becton-

131 Dickinson, USA) with *E. coli* JM109. The donor and recipient DNA mixture were kept on ice for
132 20-30 min, given heat shock in water bath at 42°C for 30-60 sec and moved back on ice for 2
133 min. We added 250-1000 µl of LB broth and incubated the culture mix in 37°C shaking
134 incubator for 45-60 min. The culture mixtures were transferred to selective LB plates (Criterion,
135 Hardy Diagnostics, USA) containing nalidixic acid (50 µg/ml) and one of the antimicrobials
136 depending on the resistance profile of the donor strains and incubated at 37°C overnight. Trans-
137 conjugants were confirmed on non-typhoidal *Salmonella* chromogenic plates (CHROMagar,
138 Paris, France) and xylose lactose tergitol (XLT4) agar plates (Criterion,Hardy Diagnostics,
139 USA). The antimicrobials and their concentration used are as follows: ampicillin (100 µg/ml),
140 nalidixic acid (50 µg/ml), and tetracycline (20 µg/ml).

141 **Antimicrobial Susceptibility Testing.** The transconjugant AMR and their minimum inhibitory
142 concentration (MIC) profiles were determined by the broth microdilution method using the
143 gram-negative Sensititre® (CMV3AGNF) plate (Trek Diagnostic Systems, OH). The panel of 14
144 antimicrobials tested include amoxicillin/clavulanic acid (AUG2; 1/0.5-32/16 µg/ml), ampicilin
145 (AMP; 1–32 µg/ml), azithromycin (AZI; 0.12–16 µg/ml), cefoxitin (FOX; 0.5–32 µg/ml),
146 ceftiofur (XNL; 0.12–8 µg/ml), ceftriaxone (AXO; 0.25–64 µg/ml), chloramphenicol (CHL; 2–
147 32 µg/ml), ciprofloxacin (CIP; 0.015–4 µg/ml), gentamicin (GEN; 0.25–16 µg/ml), nalidixic acid
148 (NAL; 0.5–32 µg/ml), streptomycin (STR; 2–64 µg/ml), sulfisoxazole (FIS; 16–256 µg/ml),
149 trimetroprim/sulfamethoxazole (SXT; 0.12/2.38-4/76 µg/ml), and tetracycline (TET; 4–32
150 µg/ml). The MICs were determined and breakpoints were interpreted based on the Clinical and
151 Laboratory Standards Institute standards (CLSI) for broth microdilution (34, 35) and National
152 Antimicrobial Resistance Monitoring System (NARMS) (36). *E. coli* ATCC 25922 was used as a
153 quality control strain. The transconjugants with MIC in the intermediate level were categorized

154 into susceptible to avoid overestimation of resistance. The transconjugants with resistance to
155 three or more classes of antimicrobials were classified as multidrug resistance (MDR).

156 **Plasmid Isolation.** Plasmid DNA was isolated from the confirmed trans-conjugant (NAL^R *E.*
157 *coli* JM109) cultures by the modified alkali lysis method described by Sambrook et al. (37),
158 which is suitable for the isolation of both large and small sized plasmids. The purified DNA
159 concentrations of the plasmid extracts were calculated by measuring the absorbance at 260 and
160 280 nm using NanoDrop ND-2000 Spectrophotometer (NanoDrop; Wilmington, DE) and Qubit
161 3.0 Fluorometer (Invitrogen; Carlsbad, CA) to ensure that there is adequate plasmid DNA for
162 sequencing. The plasmid DNA were stored frozen at -20°C unless required.

163 **PCR Amplification of Resistance genes.** The presence of resistance genes on plasmid of
164 specific AMR *Salmonella* phenotypes were detected using PCR (31, 38). Overall, genes
165 encoding resistance to ampicillin and cephalosporin (*bla*$_{PSE-1}$, *bla*$_{TEM}$, and *bla*$_{CMY-2}$),
166 chloramphenicol (*cml*A), streptomycin (*aad*A1, *aad*A2, *str*A, and *str*B), sulfisoxazole (*sul*1 and
167 *sul*2), and tetracycline (*tet*(A), *tet*(B), *tet*(C), and *tet*(G)) were tested. Template plasmid DNA
168 were extracted by the modified alkali lysis method mentioned above. The primers, amplicon size,
169 and references used to detect the presence of the selected AMR genes are listed in Table 2. The
170 PCR condition for all resistance genes, except *cml*A and *sul*1 genes, included an initial
171 denaturation at 95°C for 4 min, followed by 30 cycles of denaturation for 1 min at 95°C,
172 annealing for 1 min at 54°C, extension for 1 min at 72°C and final extension at 72°C for 7 min.
173 For *cml*A and *sul*1 genes, the PCR condition used have been described previously (44). Briefly,
174 an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation for 45 sec at
175 94°C, annealing for 45 s at 57°C, extension for 1 min at 72°C and final extension at 72°C for 5

176 min. *Salmonella enterica* isolates carrying resistance genes and characterized in earlier studies
177 were used as positive control (31).

178 **Plasmid PCR-based Replicon Typing (PBRT).** Single and multiplex PCRs were run to identify
179 different incompatibility (Inc) groups, including FIA, FIB, FIC, HI1, HI2, I1-I_Y, L/M, N, P, W,
180 T, A/C, K, B/O, X, Y, F and FIIA. The primers and PCR running conditions have been described
181 earlier in a previous study (46). The purified plasmid DNA from the modified alkali lysis method
182 was used as template DNA. PCR running conditions used for the five multiplex-PCRs and three
183 single-PCRs included an initial denaturation for 5 min at 94°C, followed by 30 cycles of
184 denaturation for 1 min at 94°C, annealing for 30 sec at 60°C, and elongation for 1 min at 72°C,
185 with a final extension of 5 min at 72°C. The single PCR reactions for F_{prep} was performed with
186 the same amplification conditions but with an annealing temperature of 52°C. The PCR product
187 were electrophoresed on a 1.5% agarose gel in TAE buffer and UV visualized by staining with
188 ethidium bromide.

189 **Plasmid Sequencing, Assembly, and Annotation.** Isolated plasmid DNA libraries were
190 prepared for sequencing using the Nextera XT kit (Illumina, San Diego, CA). Multiplexed
191 sequencing of these libraries were done with a single run on an Illumina MiSeq using 2*250- or
192 2*300-bp paired end reads (MiSeq Reagent Kit v3). Following demultiplexing, sequences were
193 analyzed using CLC Genomic Workbench 10 (Qiagen, Valencia, CA). For analyzing plasmid
194 content, *de novo* assembly of unused reads into new contigs were applied. The initially
195 assembled contigs were analyzed using the National Center for Biotechnology's Basic Local
196 Alignment Search Tool (BLAST). In addition, individual sequence reads were mapped back to
197 the assembled plasmids to confirm that there are continuous overlapping reads over the entire
198 length of the assembled plasmid. Following completion of plasmid assembly, the plasmid

sequences were run through BLAST individually and compared to GenBank sequences. The open reading frame (ORF) of each gene in plasmid contigs were identified and the particular genes of interest were annotated using Geneious R10 software (BioMatters, New Zealand). Manual trimming and editing of terminally redundant contig ends generated circular plasmid genomes. The complete plasmid sequence were visualized using plasmid mapping in CLC and deposited in GenBank with prospective accession numbers.

Comparative Genotypic Analysis. To further characterize the plasmids and compare their profiles, we mapped the PCR primers described by Carattoli et al. (46) to the assembled plasmid sequences with BLAST configured for short reads. Based on the annotations and BLAST output, the plasmids were assessed for the presence of known AMR genes, plasmid transfer (*tra*) genes, and mobile genetic elements, including Class I integrons and transposons. The assembled plasmid sequences submitted to BLAST were compared to previously sequenced plasmids in GenBank. We identidfied 14 plasmid sequences and analyzed them for variation using Geneious R10 software (BioMatters, New Zealand) global alignment with 70% similarity to construct neighbor joining trees with Tamura-Nei genetic distance model. The sequencing output of the 14 *Salmonella* plasmids was submitted to the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA293224. Individual plasmid sequence reads have been deposited in the Sequence Read Archive (SRA) as BioSample numbers SAMN07345795-SAMN07345807. In addition, all 14 plasmid sequences were typed by pMLST as previously described (47) and assigned to STs at the www.pubmlst.org/plasmid/ site for the ST prevalence analysis.

RESULTS

***Salmonella* Serotypes and Plasmid Characterization.** A total of 14 different *Salmonella* serotypes isolated from commercial swine farms in North Carolina were selected to determine whether the AMR genes were located on transmissible plasmids. We also wanted to find out whether dissemination of AMR *Salmonella* through manure application assists in the transmission of genes via plasmids to other susceptible bacterial populations. *Salmonella* isolates collected from the swine farm environment after manure application were selected from each farm based on types of sample (lagoon and soil), sampling day, serotype, and resistant phenotype (Table 1). All the 14 *Salmonella* donor harbored at least one large plasmid of larger than 40 kb in size and their plasmid profiles were dependent on farm origin and donor *Salmonella* serotype. PCR-replicon typing (PBRT) revealed four plasmid replicons (FI, FII, I1, and N) among the 14 isolates carrying plasmids (Table 1). IncN plasmids (n=3) of 50 kb in size were found in *Salmonella* Worthington isolated from both lagoon and soil samples in NC farm1. In NC farm3, 100-kb IncI1 (n=2) plasmids were isolated from *S.* Johannesburg while *S.* Typhimurium var5- which was the predominant serotype in this farm and contained IncFII plasmids (n=4) of 95 kb in size. Furthermore, IncFII plasmids were also found in *S.* Typhimurium var5- from NC farm5 and *S.* 4,12:i:- from NC farm6. A single *S.* Rissen isolate from lagoon sample in NC farm6 carried IncI1 plasmid of 90 kb in size. The heterogeneous IncF group was the predominant replicon type detected in this study. Within the IncF group, we detected the subgroups: FIA, FIB, FIC, FIIA, and F_{rep}, with IncFIC and F_{rep} the most prevalent subgroup. The IncFI plasmid group found in 10 *Salmonella* isolates (Table 1) was determined to be a small-size plasmid (less than 40 kb in size). However, the plasmids identified in our study were represented by more than one replicon family in each isolate.

Antimicrobial Resistance Phenotypes. To determine the AMR phenotypes and MIC level of all 14 confirmed transconjugants NAL^R *E. coli* and the 14 AMR *Salmonella* donor isolates from the environmental source, we conducted antimicrobial susceptibility testing using broth microdilution. The results of transconjugant AST correlated with the AMR profiles and the MIC levels of the *Salmonella* donor isolates confirming the successful transfer of plasmids from the donors to the recipient strains (Table 3). The NAL^R was detected in all 14 transconjugants since NAL^R *E. coli* JM109 strain was used as recipient for plasmid transfer. Five out of 14 plasmids were considered as multidrug resistance (MDR; resistance to more than 3 classes of antimicrobial) including pS6 (*S.* Worthington donor), pS9-pS10 (*S.* Johannesburg donor), pS24 (*S.* 4,12:i:- donor) and pS27 (*S.* Typhimurium var5- donor) (Table 1). The plasmid pS6 had the MDR pattern: FIS GEN STR TET, while plasmid pS7 and pS8 had the different R-pattern: FIS STR TET. These three transconjugants were successfully transferred to the recipient *E. coli* from *S.* Worthington donors recovered from NC farm1, but only transconjugant pS6 had 100% AMR profile that matched with the donor isolate. Two plasmids, pS9 and pS10, were isolated from transconjugants of *S.* Johannesburg in NC farm3 representing identical MDR pattern: AMP AUG2 AXO FOX. However, the ceftiofur (XNL) resistance represented in *S.* Johannesburg isolates was not detected in the transconjugants (Table 3). The plasmid pS27 from *S.* Typhimurium var5- recovered from NC farm5 had the MDR pattern: AMP CHL FIS STR TET. Unlike pS12, pS13, pS14, and pS15 isolated from transconjugants of *S.* Typhimurium var5- in farm 3, they had the R-pattern: AMP FIS. The plasmid from NC farm6, pS24 with MDR patrrern: AMP FIS STR TET was isolated from *S.* 4,12:i:- transconjugant. Another plasmid in farm 6 (pS20) from *S.* Rissen was resistant to only TET. All the transconjugants with AMP-

266 resistant were selected on the LB plates with AMP and NAL as the marker, while the rest of the
267 transconjugants were selected on NAL and TET marker LB plates.

268 **Determination of Antimicrobial Resistance Genes.** Following the conjugation experiment and
269 AST, 14 AMR-encoding genes were tested using PCR-based method (Table 2). Only eight of
270 these marker genes, including *bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM}}$, *sul*1, *sul*2, *aad*A, *aad*A2, *tet*(A), and *tet*(B) were
271 detected in plasmids. The *bla*$_{\text{CMY-2}}$ gene was detected in 100-kb IncI1 plasmid (pS9). The *bla*$_{\text{TEM}}$
272 gene was found in IncFII plasmid (pS27). We detected *tet*(A) or *tet*(B) in plasmids that encoded
273 tetracycline resistance. In plasmids with streptomycin resistance, *aad*A1 and *aad*A2 were found.
274 The *sul*1 gene was the most prevalent among plasmids which were resistant to the antimicrobial
275 sulfisoxazole. Plasmid pS14 and pS15 did not test positive for any AMR genes which were
276 tested in this study. The resistance genotypes of all 14 plasmids were tabulated in Table 1.

277 **Plasmid Sequencing and Analysis.** The incompatibility (Inc) group and resistance genes of
278 plasmid were confirmed using sequencing (Table 1). Plasmid sequencing was able to identify the
279 replicon families of each individual plasmid. BLASTN comparison revealed that 95-kb IncF
280 plasmids from different farms and serotypes (pS9, pS10, pS12, pS13, pS14, pS15, pS27; Table
281 1) were identical to another fully sequenced plasmid pSTY1-H2662 previously isolated from *S.*
282 Typhimurium from human stool (accession no. CP014980) (48). A class 1 integron was
283 identified in plasmid pS6-pS8 isolated from *S.* Worthington using *in silico* method. This integron
284 comprised of 5'conserved segment, variable part, and 3'conserved segment (Fig 1; pS7). The
285 unusual variable part contained ANT(2")Ia-*aad*A2 gene cassette which is responsible for
286 aminoglycoside resistance while *sul*1 gene was always found in the 3'CS responsible for
287 sulfonamide resistance. In addition, plasmid sequence analysis revealed the presence of VirB-
288 family type IV secretion systems (T4SS) in all the 14 plasmids, together with multiple *tra* genes

including *tra*C, *tra*F, *tra*G, *tra*I, *tra*J, *tra*O, and *tra*U. The evolutionary tree of 14 plasmid sequences was created using Geneious R10 software (Fig 2). At 70% similarity, the plasmids from the same *Salmonella* donor were clustered together including pS6, pS7, pS8 (from *S.* Worthington) and pS28, pS29 (from *S.* Ohio). The plasmid with distinct size, 100-kb pS9 and 90-kb pS20 were separated from other group. Plasmid pS24 was not included in the analysis because of the incomplete sequencing output. The pMLST database revealed that three 50-kb IncN plasmids isolated from *S.* Worthington were belonged to ST5. The IncI1 plasmid (pS9) isolated from *S.* Johannesburg was designed to ST12 and CC-12 while another IncI1 plasmid (pS20) isolated from *S.* Rissen was typed in ST155 but clonal complex (CC) was not defined (Table 1).

DISCUSSION

The aim of the study was to characterize the plasmids identified in different AMR *Salmonella* serotypes isolated from commercial swine farms environment after land application of manure. We also wanted to determine the plasmid role in the dissemination of AMR genes to other potential bacterial recipients in the environment. The results potentially addressed the key role played by plasmids in the horizontal gene transfer that lead to the rapid proliferation of AMR genes in the environment. It is important to stress that our study were conducted in the commercial swine farms not a research station in NC which is the top two leading pork producing states in the US. The *Salmonella* serotypes with multiple plasmids are common in the *Enterobateriaceae* family (49). However, we focused on large (defined as being ≥40 kb in size) plasmids which are abundant in *E. coli* and *Salmonella* and comprise important pools of adaptive

and transferable genetic information, especially AMR corresponding genes, in these bacteria (49, 50). The large sized plasmid, in the range of 40-200 kb, have been suggested to be the necessary markers for extended spectrum β-lactamases (ESBL), β-lactamase encoding gene, plasmid-mediated quinolone resistance (PMQR) (14, 16, 51). In our study, five out of 14 plasmids that we detected were 95-kb in size and were isolated from Typhimurium var5- serotype (n=5). The plasmid profiles of these five isolates were similar though they were recovered from different farms and time points, indicating the persistence of this plasmid in this serotype in the environment after manure deposition. The results correlated with a previous study that reported that *Salmonella* plasmids were conserved and primarily serotype-specific, including Typhimurium and Heidelberg serotypes, and they tended to persist for a long period in environment (49). These plasmids were in contrast to *E. coli* plasmids which were more variable and not specific to particular strains (22, 49). The pS24 plasmid isolated from *S.* 4,12:i:- had similar profile as Typhimurium plasmids and the parent strain was also isolated from different swine farm environment. During the last decade, *S.* 1,4,12:i:-, *S.* 1,4,[5],12:i:- and *S.* 4,12:i;- have emerged around the world and frequently reported from human, animal, agricultural production, and environment (52-54). These serotypes are believed to be a mosaic variant of *S.* Typhimurium and are related to plasmid-mediated colistin resistance encoded by the *mcr*-1 gene. (45, 47, 48). We detected one *S.* Rissen plasmid of approximately 90 kb in size that had a tetracycline resistance marker on it. This is in comparison to our previous report where we identified a 90-100 kb plasmid in a tetracycline-resistant *S.* Rissen from farm environment in North Carolina containing the *tet*(A) gene (31). This serotype is not common in the US agriculture system and was identified for the first time in North Carolina swine farms in 2009 (56).

333 Plasmid incompatibility (Inc), the inability of two plasmids of the same family to co-exist in the
334 same host cell, classifies the plasmids based on their stability during conjugation (50, 51). This
335 classification helps to categorize plasmids into clusters and relies on their phylogenetic
336 relatedness, distribution in host cell and environment, and their evolutionary origin (57, 59).
337 Currently, 27 Inc groups are identified among the *Enterobacteriaceae* family (46, 57). On the
338 basic of PCR-based replicon typing (PBRT) method, 18 Inc groups were detected in our study.
339 We used total plasmid DNA from each isolate in conducting PBRT, so the results did not
340 differentiate individual plasmids in multi-plasmid isolates. Most of the isolates were positive for
341 more than one replicon family either because that isolate contained multiple plasmids from
342 different incompatibility groups or because a single plasmid encoded replication or partitioning
343 genes from more than one incompatibility group. However, we were able to identify the exact
344 replicon families after assessing the plasmid sequencing data (Table 1). We did not differentiate
345 the heterogeneous IncF plasmids into individual group because of their partitioning of replication
346 genes (49) and the small-size (<40 kb in size) plasmids were not characterized in this study.

347 Particular plasmid Inc families, including IncN, IncI1, and IncF, are more frequently associated
348 with the dissemination of AMR genes (14). These 3 plasmid Inc families have been associated
349 with specific *Salmonella* serotypes and geographic farm area in our study (49). The IncN family
350 was detected in *S.* Worthington which was consistently isolated from NC farm1, while IncI1 was
351 detected in *S.* Johannesburg isolated from NC farm3. Both families are associated with large-size
352 plasmids related to MDR phenotypes (Table 1). IncF family was detected in multiple serotypes
353 and farms (NC farm3, 5, and 6). These findings are in accordance with the previous studies that
354 IncF and IncI1 are the most prevalent replicon types distributed among *Enterobacterceae* (14,

355 49). The IncI and IncF plasmids generally recovered from *E. coli* and *Salmonella* from human
356 and animal sources are considered as the source of several ESBL genes (14, 20, 23).

357 IncFI group including FIA, FIB, and FIC, together with IncFIIA subtype, were the most
358 frequently detected replicon types in this study. All the 14 *Salmonella* isolates carried at least one
359 IncF plasmids. Our result supports the view that IncF (both FI and FII) family was well adapted
360 and commonly distributed in *E. coli* and *Salmonella* (14, 15, 49, 60). Wang et al. (14) reported
361 that IncFIIA was only detected in serotype Typhimurium which correlates with our findings,
362 however, we also detected the FIIA type in the serotype 4,12:i:-. IncF family plasmids have been
363 reported to contribute to the spread of AMR in *Enterobacteriaceae* and have been associated
364 with specific genes conferring resistance to aminoglycosides, β-lactams, and quinolones (57, 60,
365 61).

366 Conjugative plasmids with IncI1 replicon type were usually associated with multiple resistance
367 compounds, especially extended-spectrum cephalosporinase of both CTX-M and CMY types
368 (61-63). The IncI1 plasmids carrying TEM-52 have been identified in *E. coli* and *Salmonella*
369 cultured from human, chicken and turkey product in EU (64-66). The *bla*$_{CMY}$-IncI1 plasmids
370 linked to poultry, ground beef, and tomato sources have been identified to be responsible for
371 ceftriaxone-resistant *Salmonella* outbreaks in US during 2011-2012 (18). They reported that
372 *Salmonella* serotype Heidelberg, Infantis, Typhimurium, and Newport were associated to IncI
373 plasmids carrying *bla*$_{CMY}$ gene. Similar to our study, IncI plasmids with *bla*$_{CMY}$ were identified
374 in ceftriaxone-resistant serotype Johannesburg from commercial swine farms environment
375 sampled in our study.

376 IncN plasmids are the major vehicles for the dissemination of multiple AMR genes, the PMQR
377 or ESBL genes including *bla*$_{\text{CTX-M}}$ (22, 67, 68). In contrast to our study, IncN plasmids were
378 identified only in *S.* Worthington transconjugants and exhibited resistance to sulfisoxazole,
379 streptomycin, and tetracycline but not quinolones and ampicillin. Thus, characterization based on
380 plasmid profiling and corresponding Inc group using the PBRT technique are essential tools for
381 plasmid epidemiological surveillance enhancing discrimination between *Salmonella* serotypes
382 and tracing the spread of AMR genes (14, 16).

383 Multiple MDR coding genes were found in plasmids. We detected plasmids carrying *sul*1 and
384 *sul*2 genes conferring sulfisoxazole resistance, while plasmids with streptomycin resistance
385 contained the *aad*A and *aad*A2 genes. Similarly, the *tet*(A) and (B) genes were found in
386 plasmids in *Salmonella* strains that were resistant to tetracycline. β-lactamase-encoding (*bla)*
387 genes, including *bla*$_{\text{TEM}}$ and *bla*$_{\text{CMY,}}$ were detected in the plasmids which encoded for the
388 resistance of ampicillin and cephalosporin group antimicrobials. Several mechanisms are
389 available for *bla* genes to support HGT between bacteria, thereby ensuring the spread of these
390 markers to new hosts and the environment (14, 69). The heavy use of specific antimicrobials
391 such as tetracycline play a key role in plasmid dissemination and allows for the selection and
392 enrichment of bacteria with multiresistant plasmids (22, 70, 71)

393 The class 1 integron with ANT(2")Ia-*aad*A2 gene cassette was detected in plasmids pS6-pS8
394 retrieved from *S.* Worthington (Fig. 1; pS7). The integron had an unusual organization, with an
395 ANT(2")Ia gene cassette which is responsible for resistance against gentamicin (72). The
396 gentamicin resistance was not identified in pS7 but in S7 (pS7 donor) and pS6 (Table 1 and 3).
397 After BLAST analysis at NCBI, pS6-8 had genetic relatedness to *Klebsiella pneumoniae* MDR
398 IncN plasmid reported from Japan (73). However, the *K. pneumoniae* plasmid harbored different

399 resistance genes than what we detected in the *Salmonella* serotypes from our study. The
400 integrons are able to locate on either chromosome or mobile genetic element such as plasmids
401 (74). Several studies have stated that the integrons with *aad*A or variant of *aad*A genes are
402 common among *Salmonella* species (75-78). The variable parts of integrons might be composed
403 of variants of *aad*, *dfr*, or *bla* genes that contribute to aminoglycoside, sulfonamide, and
404 cephalosporin resistance, respectively (75, 76). Serotype Worthington detected in our study are
405 commonly found in poultry, poultry product, and environment in several parts of the world and
406 harbor integrons either in their chromosome or the plasmids (77, 79-81). The presence of genetic
407 elements such as integrons, transposons and plasmids has been consequently associated with
408 multi-resistance phenotypes among *Salmonella* isolates (76). Our study reports an emerging
409 multi-resistant clone isolated from *Salmonella* serotypes in commercial swine farm environment
410 carrying a large conjugative plasmid with an ANT(2")Ia-*aad*A2 gene cassette located on an
411 integron.

412

413 Though the *Salmonella* plasmids were transferred to *E. coli* JM109 recipient in laboratory
414 conditions, the presence of VirB-family type 4 secretion systems (T4SS) and *tra* genes in our
415 study confirms HGT by conjugation that is likely to occur in the environment. The T4SSs in
416 gram-negative bacteria functionally encompass the conjugation system and the effector
417 translocators for interbacterial transfer of AMR genes, virulence determinants, and genes
418 encoding other traits beneficial to its host (82). IncN plasmids (pS6-8) and IncI1 (pS9;100k,
419 pS20) employed TraJ which has ability to conjugate and the conjugation process could be
420 stimulated approximately 100-fold, demonstrating functional conservation of a significant
421 regulatory feature of F-like conjugation modules (83).

422 The phylogenetic tree of 14 plasmids (Fig. 2) at 70% similarity suggested that the plasmids
423 analyzed in our study were clustered based on the *Salmonella* donor serotypes such as *S.*
424 Worthington cluster (pS6-8) and *S.* Ohio cluster (pS28-29). Within these three Inc groups (IncI1,
425 IncN, and IncF), the phylogenetic analysis also suggested the existence of Inc group that is
426 serotype-specific plasmid subgroups which mentioned previously (49). Comparing to pMLST
427 database, all IncN (pS6-8) plasmids which were specific to serotype Worthington were belonged
428 to the same ST5. These results were in accordance to the BLAST output of individual plasmid
429 and the *Salmonella* clustering done by PFGE in our previous study (13).

430 Our study demonstrated that the identical plasmids were recovered from different *Salmonella*
431 serotypes isolated either from the same of different farm environment. Our findings provide
432 evidence of a single large 95kb IncF plasmid being distributed across the swine production
433 systems in NC among different serotypes of *Salmonella*. In addition, we found that AMR
434 plasmids were able to persist in swine farm environment after manure application for a minimum
435 period of 21 days (final sampling time point). The AMR determinants on these plasmids were
436 transferable among *Salmonella* serotypes and underlined the fact that manure deposition enriches
437 the environmental resistome. We recommend conducting longitudinal based studies on
438 commercial food animal farms and determine the role of manure deposition on the
439 environmental dissemination of AMR genes.

440

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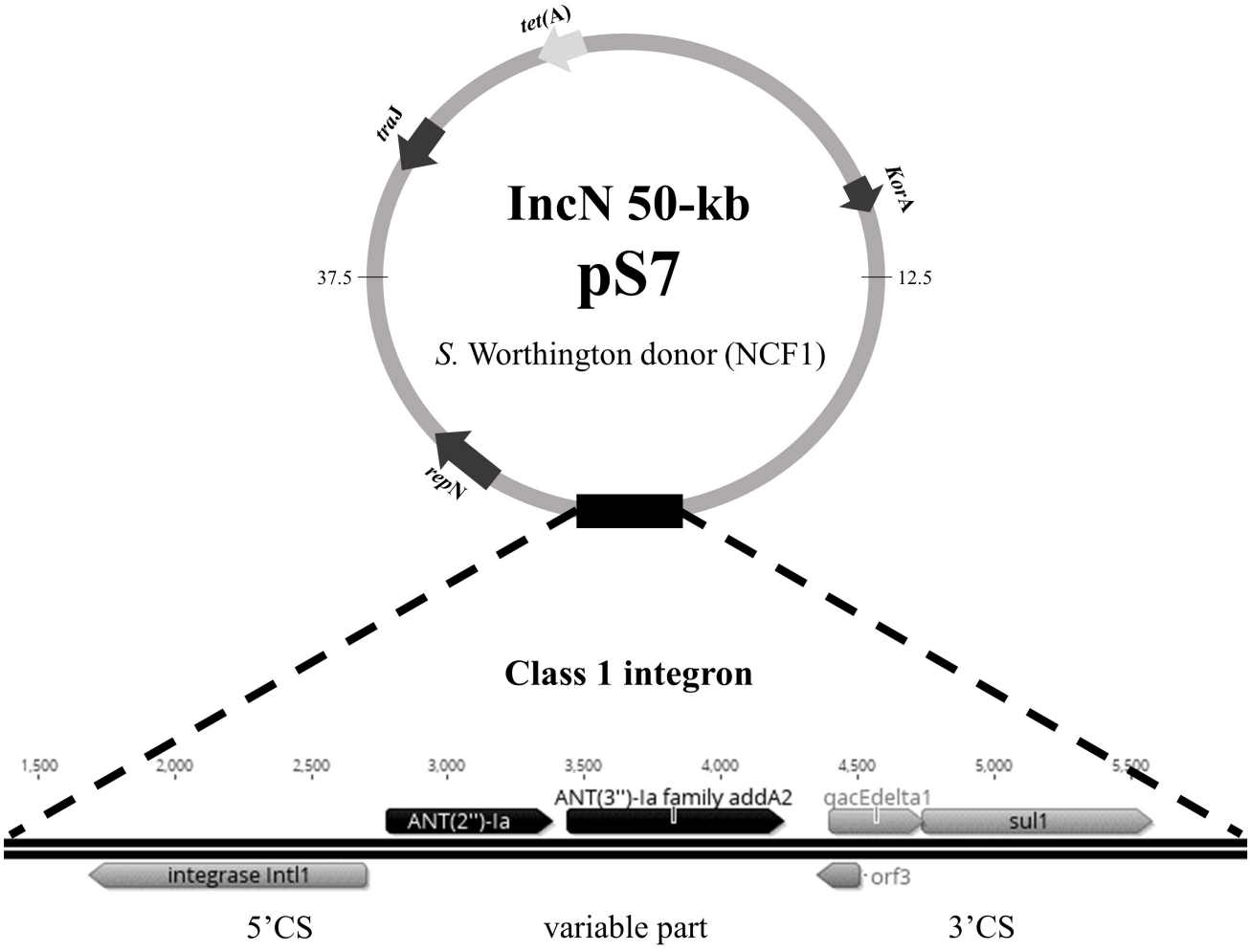
FIG 1 Schematic representation of a class 1 integron in 50-kb IncN plasmid pS7: [5'conserved segment] intl1: integrase gene, [variable region] ANT(2")-Ia: producing aminoglycoside resistance enzyme; *aad*A2: ANT(3")-Ia family *aad*A2 gene producing streptomycin resistance, [3'conserved segment] qacEΔ1: partially deleted gene that encodes quaternary ammonium compound resistance; *sul*1: sulphonamide resistance; orf3: unknown function, on the gene cassette recognised by the integrase, and arrows indicate the direction of coding sequence.

FIG 2 Phylogenetic diversity for sequences of 14 plasmids acquired from environmental *Salmonella*. Evolutionary distances between plasmids were computed using the neighbor-joining algorithm. The distance was obtained from pairwise alignments with 70% similarity and no outgroup. The plasmid label names related to data in Table 1. Phylogenetic analyses were conducted in Geneious R10.



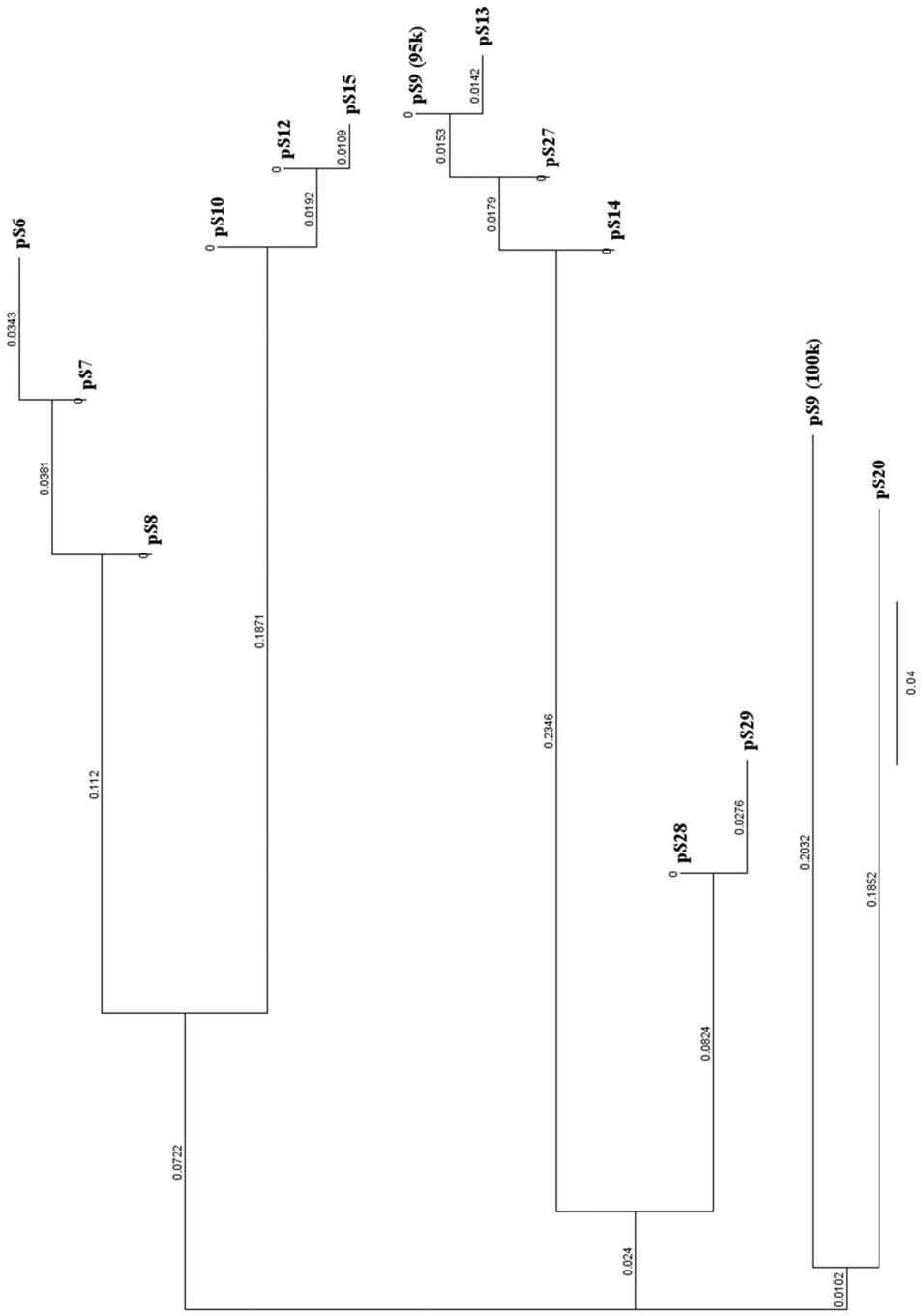


TABLE 1 Conjugative resistance plasmids content of 14 environmental isolates harboring AMR gene recovered from *Salmonella* donor isolates after manure application in commercial swine farms, NC.

Salmonella donor isolate				Plasmid					
Farm[a]	Source	Day	Serotype	ID	Size (kb)	Inc group[b]	pMLST[c]	R-pattern[d] (MIC; µg/ml)	AMR gene
NCF1	Lagoon	Day0	Worthington	pS6	50	N(50k), FI	N:ST5	FIS(>256), GEN(16), STR(64), TET(>32)	*sul*1, *aadA*2, *tet*(A)
NCF1	Soil	Day0	Worthington	pS7	50	N(50k), FI	N:ST5	FIS(>256), STR(64), TET(>32)	*sul*1, *aadA*2, *tet*(A)
NCF1	Soil	Day7	Worthington	pS8	50	N(50k), FI	N:ST5	FIS(>256), STR(64), TET(>32)	*sul*1, *aad*A2, *tet*(A)
NCF3	Lagoon	Day0	Johannesburg	pS9	100, 95	I1(100k), FI(95k)	I1:ST12	AMP(>32), AUG2(32/16), AXO(16), FOX(32)	bla_{CMY-2}
NCF3	Soil	Day7	Johannesburg	pS10	95	I1, FI(95k)	-	AMP(>32), AUG2(32/16), AXO(16), FOX(32)	bla_{CMY-2}
NCF3	Lagoon	Day0	Typhimurium var5-	pS12	95	FII, FI	-	AMP(>32), FIS(>256)	*sul*1
NCF3	Soil	Day7	Typhimurium var5-	pS13	95	FII, FI	-	AMP(>32), FIS(>256)	*sul*1
NCF3	Soil	Day14	Typhimurium var5-	pS14	95	FII	-	AMP(>32), FIS(>256)	-
NCF3	Soil	Day21	Typhimurium var5-	pS15	95	FII	-	AMP(>32), FIS(>256)	-
NCF6	Lagoon	Day0	Rissen	pS20	90	I1(90k), FI	I1:ST155	TET(>32)	*tet*(A), *tet*(B)
NCF6	Soil	Day0	4,12:i:-	pS24	95	FII, FI	-	AMP(>32), FIS(>256), STR(>64), TET(>32)	bla_{TEM}, *sul*2, *aad*A
NCF5	Lagoon	Day0	Typhimurium var5-	pS27	95	FII, FI	-	AMP(>32), CHL(>32), FIS(>256), STR(32), TET(32)	*sul*1, *aad*A2
NCF5	Lagoon	Day0	Ohio	pS28	40	FI	-	TET(>32)	*tet*(A)
NCF5	Soil	Day0	Ohio	pS29	40	FI	-	TET(>32)	*tet*(A)

[a] NCF=North Carolina farm

[b] Incompatibility group based on PBRT (46).

[c] Sequence type (ST) based on pMLST: www.pubmlst.org/plasmid/ (47).

[d] The plasmid R-pattern: Nalidixic acid (NAL) resistance was removed from NAL^R *E. coli* JM109 transconjugant R-pattern. amoxicillin/clavulanic acid (AUG2; 1/0.5-32/16 µg/ml; breakpoint≥32/16), ampicillin (AMP; 1-32 µg/ml; breakpoint≥32), cefoxitin (FOX; 0.5-32 µg/ml; breakpoint≥32), ceftriaxone (AXO; 0.25-64 µg/ml; breakpoint≥4), chloramphenicol (CHL; 2-32 µg/ml; breakpoint≥32), gentamicin (GEN; 0.25-16 µg/ml; breakpoint≥16), streptomycin (STR; 32-64 µg/ml; breakpoint≥32), sulfisoxazole (FIS; 16-256 µg/ml; breakpoint≥512), and tetracycline (TET; 4-32 µg/ml; breakpoint≥16).

TABLE 2 Primer use for PCR detection resistance genes

Genes	Forward oligonucleotide sequence (5'to 3')	Reverse oligonucleotide sequence (5' to 3')	Expected Size (bp)	References
bla$_{CMY-2}$	GACAGCCTCTTTCTCCACA	TGGAACGAAGGCTACGTA	1015	39
bla$_{PSE-1}$	TTTGGTTCCGCGCTATCTG	TACTCCGAGCACCAAATCCG	150	40
bla$_{TEM}$	GCACGAGTGGGTTACATCGA	GGTCCTCCGATCGTTGTCAG	860	41
*aad*A	GTGGATGGCGGCCTGAAGCC	AATGCCCAGTCGGCAGCG	528	42
*aad*A2	CGGTGACCATCGAAATTTCG	CTATAGCGCGGAGCGTCTCGC	250	43
*str*A	CCTGGTGATAACGGCAATTC	CCAATCGCAGATAGAAGGC	548	42
*str*B	ATCGTCAAGGGATTGAAACC	GGATCGTAGAACATATTGGC	509	42
*sul*1	CGGACGCGAGGCCTGTATC	GGGTGCGGACGTAGTCAGC	591	44
*sul*2	GCGCTCAAGGCAGATGGCATT	GCGTTTGATACCGGCACCCGT	285	41
*cml*A	TGGACCGCTATCGGACCG	CGCAAGACACTTGGGCTGC	641	44
tet(A)	GCTACATCCTGCTTGCCTTC	CATAGATCGCCGTGAAGAGG	210	45
tet(B)	TTGGTTAGGGGCAAGTTTTG	GTAATGGGCCAATAACACCG	659	45
tet(C)	CTTGAGAGCCTTCAACCCAG	ATGGTCGTCATCTACCTGCC	418	45
tet(G)	CAGCTTTCGGATTCTTACGG	GATTGGTGAGGCTCGTTAGC	844	45

TABLE 3 Antimicrobial susceptibilities with minimum inhibitory concentration (MIC) of AMR environmental *Salmonella* isolates and corresponding *E. coli* transconjugants.

Salmonella isolate or transconjugant[a]	MIC[b] (μg/ml)													
	AMP	AUG2	AXO	AZI	CHL	CIP	FIS	FOX	GEN	NAL	STR	SXT	XNL	TET
S6	<1	<1/0.5	<0.25	4	8	<0.015	**>256**	4	**16**	2	**64**	<0.12/2.38	1	**>32**
TC-S6	<1	2/1	<0.25	2	8	0.06	**>256**	4	**16**	**>32**	**64**	<0.12/2.38	0.5	**>32**
S7	<1	<1/0.5	<0.25	4	8	<0.015	**>256**	4	**16**	2	**>64**	<0.12/2.38	0.5	**>32**
TC-S7	2	2/1	<0.25	2	8	0.12	**>256**	4	8	**>32**	**64**	<0.12/2.38	0.5	**>32**
S8	<1	<1/0.5	<0.25	4	8	<0.015	**>256**	4	**>16**	2	**64**	<0.12/2.38	0.5	**>32**
TC-S8	2	2/1	<0.25	2	8	0.12	**>256**	2	8	**>32**	**64**	<0.12/2.38	0.5	**>32**
S9	**>32**	**32/16**	**16**	8	8	0.03	256	**32**	0.5	4	4	<0.12/2.38	**>8**	<4
TC-S9	**>32**	**32/16**	**16**	2	8	0.12	<16	**32**	<0.25	**>32**	4	<0.12/2.38	4	<4
S10	**>32**	**32/16**	**16**	8	8	0.03	256	**32**	0.5	4	8	<0.12/2.38	**>8**	<4
TC-S10	**>32**	**32/16**	**16**	2	8	0.25	<16	**>32**	0.5	**>32**	4	<0.12/2.38	4	<4
S12	**>32**	8/4	<0.25	4	8	<0.015	**>256**	2	0.5	4	8	<0.12/2.38	0.5	<4
TC-S12	**>32**	8/4	<0.25	4	8	0.12	**>256**	4	0.5	**>32**	8	<0.12/2.38	1	<4
S13	**>32**	8/4	<0.25	4	8	<0.015	**>256**	2	0.5	4	8	0.25/4.75	0.5	<4
TC-S13	**>32**	8/4	<0.25	4	8	0.12	**>256**	4	0.5	**>32**	8	<0.12/2.38	1	<4
S14	**>32**	8/4	<0.25	4	8	<0.015	**>256**	2	0.5	4	8	<0.12/2.38	1	<4
TC-S14	**>32**	8/4	<0.25	4	8	0.12	**>256**	2	0.5	**>32**	8	<0.12/2.38	1	<4
S15	**>32**	<1/0.5	<0.25	4	8	0.25	**>256**	2	0.5	4	8	<0.12/2.38	1	<4
TC-S15	**>32**	8/4	<0.25	4	8	0.25	**>256**	2	0.5	**>32**	16	0.25/4.75	1	<4
S20	<1	<1/0.5	<0.25	8	8	0.03	64	4	0.5	4	4	<0.12/2.38	1	**>32**
TC-S20	2	2/1	<0.25	4	8	0.12	<16	4	<0.25	**>32**	4	<0.12/2.38	<0.12	**>32**
S24	**>32**	4/2	<0.25	8	8	0.03	**>256**	2	0.5	8	**>64**	<0.12/2.38	1	**>32**
TC-S24	**>32**	8/4	<0.25	4	8	0.12	**>256**	2	0.5	**>32**	**>64**	<0.12/2.38	0.5	**>32**
S27	**>32**	**32/16**	**8**	8	**>32**	<0.015	**>256**	16	0.5	4	**>64**	<0.12/2.38	**8**	**>32**
TC-S27	**>32**	8/4	<0.25	4	**>32**	0.12	**>256**	2	<0.25	**>32**	**32**	<0.12/2.38	0.5	**32**
S28	<1	<1/0.5	<0.25	8	8	<0.015	64	2	<0.25	2	4	<0.12/2.38	1	**>32**
TC-S28	2	2/1	<0.25	8	8	0.12	<16	8	<0.25	**>32**	4	<0.12/2.38	0.5	**>32**
S29	<1	<1/0.5	<0.25	4	8	<0.015	64	2	0.5	4	8	<0.12/2.38	1	**>32**
TC-S29	2	2/1	<0.25	4	8	0.12	<16	2	<0.25	**>32**	<2	<0.12/2.38	0.5	**>32**

[a] *E. coli* transconjugants are indicated by designations beginning TC. *Salmonella* isolates begin with letter S.

[b] amoxicillin/clavulanic acid (AUG2; 1/0.5-32/16 μg/ml; breakpoint≥32/16), ampicilin (AMP; 1-32 μg/ml; breakpoint≥32), azithromycin (AZI; 0.12-16 μg/ml; breakpoint≥32), cefoxitin (FOX; 0.5-32 μg/ml; breakpoint≥32), ceftiofur (XNL; 0.12-8 μg/ml; breakpoint≥8), ceftriaxone (AXO; 0.25-64 μg/ml; breakpoint≥4), chloramphenicol (CHL; 2-32 μg/ml; breakpoint≥32), ciprofloxacin (CIP; 0.015±4 μg/ml; breakpoint≥4), gentamicin (GEN; 0.25-16 μg/ml; breakpoint≥16), nalidixic acid (NAL; 0.5-32 μg/ml; breakpoint≥32), streptomycin (STR; 32-64 μg/ml; breakpoint≥32), sulfisoxazole (FIS; 16-256 μg/ml; breakpoint≥512), trimetroprim/sulfamethoxazole (SXT; 0.12/2.38-4/76 μg/ml; breakpoint≥4/76), and tetracycline (TET; 4-32 μg/ml; breakpoint≥16)

Bold letters indicate the resistance of *Salmonella* isolates or transconjugants in each antimicrobial.