Characterization of integron mediated antimicrobial resistance in *Salmonella* isolated from diseased swine

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Abstract

Forty-two *Salmonella* isolates obtained from diseased swine were genetically characterized for the presence of specific antimicrobial resistance mechanisms. Twenty of these isolates were characterized as *S*. Typhimurium DT104 strains. Pulsed-field gel electrophoresis was used to determine genetic relatedness and revealed 20 distinct genetic patterns among the 42 isolates. However, all DT104 isolates fell within 2 closely related genetic clusters. Other *Salmonella* isolates were genetically grouped together according to serotype. All DT104 isolates displayed the penta-resistance phenotype to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline. Resistance to sulfamethoxazole, tetracycline, streptomycin, kanamycin, and ampicillin was most common among the non-DT104 *Salmonella* isolates. All DT104 strains contained 2 chromosomal integrons of 1000 and 1200 base pairs. The DNA sequencing revealed that the 2 integrons contained genes encoding a resistance to streptomycin and ampicillin, respectively. None of the non-DT104 strains showed the same pattern, although several strains possessed integrons of 1000 base pairs or larger. However, the majority of non-DT104 *Salmonella* strains did not possess any integrons. Two *Salmonella* isolates displayed tolerance to the organic solvent cyclohexane, indicating the possibility that they are overexpressing chromosomal regulatory genes *marA* or *soxS* or the associated multidrug efflux pump, *acrAB*. This research suggests that integrons contribute to antimicrobial resistance among specific swine *Salmonella* serotypes; however, they are not as widely disseminated among non-Typhimurium swine *Salmonella* serotypes as previously thought.

Résumé

Quarante-deux isolats de Salmonella *obtenus de porcs malades furent caractérisés génétiquement pour la présence de mécanismes spécifiques de résistance aux antimicrobiens. Vingt isolats furent caractérisés comme étant des isolats de* S. Typhimurium DT104. *L'électrophorèse en champs pulsés utilisée pour déterminer la parenté génétique entre les isolats a permis de mettre en évidence 20 profils génétiques différents parmi les 42 isolats. Toutefois, tous les isolats de DT104 se retrouvaient dans deux groupements étroitement reliés génétiquement. Les autres isolats de* Salmonella *étaient groupés génétiquement ensemble selon leur sérotype. Tous les isolats de DT104 démontraient le phénotype de quintuple résistance à l'ampicilline, au chloramphénicol, à la streptomycine, au sulfaméthoxazole et à la tétracycline. Parmi les isolats de* Salmonella *non-DT104, la résistance au sulfaméthoxazole, à la tétracycline, à la streptomycine, à la kanamycine et à l'ampicilline était le plus souvent notée. Tous les isolats de DT104 possédaient 2 intégrons chromosomiques de 1000 et 1200 paires de bases. Le séquençage de l'ADN révéla que les 2 intégrons contenaient des gènes codant, respectivement, pour la résistance à la streptomycine et à l'ampicilline. Aucun des isolats non-DT104 ne présentait ce patron, bien que plusieurs isolats possédaient des intégrons de 1000 paires de bases ou plus. Toutefois, la majorité des isolats non-DT104 ne possédaient pas d'intégron. Deux isolats de* Salmonella *démontraient une tolérance envers le solvant organique cyclohexane, indiquant ainsi la possibilité qu'ils surexprimaient les gènes chromosomiques régulateurs* marA *ou* soxS, *ou bien la pompe efflux* acrAB. *Les résultats suggèrent que des intégrons contribuent à la résistance aux antimicrobiens chez des sérotypes spécifiques de* Salmonella *porcins; toutefois, contrairement à ce que l'on pensait ils ne sont pas aussi largement disséminés parmi les sérotypes de* Salmonella *isolés de porc autres que Typhimurium.*

(Traduit par Dr Serge Messier)

Introduction

The ease with which bacteria become resistant to antimicrobial agents continues to concern clinicians, public health officials, and researchers. Antimicrobial resistance is a problem of both national and international importance, with resistance mechanisms having been described for all known antibiotics that are currently available (1). Although the spread of resistant microorganisms is disturbing, the association of resistance determinants with mobile DNA elements; such as, plasmids, transposons, and integrons, is also of concern. These

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mobile DNA elements assist in the rapid dispersion of resistance genes within bacterial species and between different species (2,3). Many antimicrobial resistance genes in *Escherichia coli* and *Salmonella* species are on large, transferable plasmids. Another type of mobile DNA element, termed the transposon, also often carries antimicrobial resistance genes. In turn, plasmids and transposons, coding multiple drug resistance, often possess another genetic element, the integron.

Integrons contain 1 or more resistance genes present as mobile gene cassettes and inserted into various arrangements between 2 conserved DNA regions, creating arrays of different antimicrobial resistance genes (3). Over 60 gene cassettes and 4 distinct classes of integrons have been identified to date (2,3). Cassette-associated genes conferring resistance to beta-lactams, aminoglycosides, trimethoprim, chloramphenicol, and quaternary ammonium compounds used as antiseptics and disinfectants have been found (2). Also, class I integrons include a sulfonamide resistance gene (*sul1*) in the backbone structure (2). Integrons were the focus of our study, since little is known about the potential contribution of integrons to maintaining multiple antimicrobial resistance among clinical swine *Salmonella* species. To date, there is limited information regarding emergence and characterization of integron-mediated, multiple antimicrobial resistance among non-Typhimurium DT104 swine *Salmonella* serotypes from North America. The majority of work published on resistance genes in *Salmonella* has been obtained from European isolates (4,5).

All isolates were further examined for phenotypic characteristics associated with mutation of the *marRAB* locus (6,7). The *marRAB* locus is 1 of 2 operons (the other being *soxRS*), that have been associated with chromosomal based resistance to multiple antibiotics in *E. coli* and *S*. Typhimurium (8,9). Mutations in these chromosomal loci in *E. coli* are associated with the acquisition of low-level resistance to certain antimicrobials and tolerance to certain chemicals, including cyclohexane (6,8–11). Antibiotic resistance mediated by the *soxRS* and *marRAB* regulons is directed via both the overexpression of the *acrAB*-encoded efflux pump and down-regulation of the outer membrane porin OmpF (6,7,12). There is increasing evidence that the expression of *soxS*, *marA*, and *acrAB* contributes to clinical antimicrobial resistance in *E. coli* and *Salmonella* (13-16).

Despite much research into the characterization of resistance mechanisms among *Salmonella*, the prevalence and relative contribution of both *mar*- and integron-mediated, multiple antimicrobial resistance among various veterinary *Salmonella* serotypes is unclear. Thus, our underlying goal was to advance the knowledge concerning the development and dissemination of antimicrobial resistance among bacterial pathogens important in swine husbandry and emerging as foodborne pathogens contaminating pork.

Materials and methods

Bacterial isolates

Forty-two isolates of *Salmonella* isolated from diseased swine were included in the study. *Salmonella* serotypes assayed included 20 *S*. Typhimurium DT104, 2 *S*. Typhimurium non-DT104, and 2 *S*. Typhimurium Copenhagen non-DT104 strains. The remaining *Salmonella* isolates include representatives of the Anatum (n = 3), Choleraesuis-kunzendorf (n = 4), Derby (n = 3), Heidelburg (n = 3), Infantis (n = 3), and Mbandaka (n = 2) serotypes. Isolates were obtained from the National Veterinary Services Laboratory (NVSL), in Ames, Iowa, and were representative of the most prevalent *Salmonella* serotypes submitted to NVSL from diseased swine throughout the United States during the year 2000. *Salmonella* isolates assayed were recovered from swine from 18 states with the majority submitted from North Carolina (n = 7) and Illinois (n = 6). Bacteria were grown on MacConkey agar (Difco Laboratories, Detroit, Michigan, USA) and stored in trypticase soy broth (TSB) (Difco) containing 50% glycerol at −80°C until use.

Antimicrobial susceptibility determination

Antimicrobial minimum inhibitory concentrations (MIC) of *Salmonella* isolates were determined with an automated antimicrobial susceptibility system according to the manufacturer's instructions (Sensititre automated antimicrobial susceptibility system; Trek Diagnostic Systems, Westlake, Ohio, USA) and interpreted according to the National Committee Clinical Laboratory Standards (NCCLS) standards for broth microdilution methods (17,18). *Escherichia coli* ATCC 25922 and 35218, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 were used as quality control microorganisms. A comprehensive antibiogram was determined for the *Salmonella* isolates, using a customized antimicrobial panel of 17 antibiotics with the range of concentrations employed in the National Antimicrobial Resistance Monitoring System (NARMS) established by the Center for Disease Control and Prevention (CDC), United States Department of Agriculture (USDA), and the Food and Drug Administration (FDA) (19).

Bacterial DNA preparation and PCR and DNA sequencing

To determine the extent of integron-mediated multiple antimicrobial resistance, PCR primers homologous to conserved integron sequences were used (3) to assay the swine *Salmonella* isolates. Template DNA from the *Salmonella* isolates was prepared and purified using routine procedures (5). Integron PCR primers and amplification conditions employed have been previously described (3–5). Amplicons were separated by horizontal gel electrophoresis and visualized under ultraviolet (UV) light. Appropriate amplicons were identified by size, excised from the agarose, and purified (Wizard PCR Clean Up System; Promega, Madison, Wisconsin, USA). Sequencing of PCR products was performed according to manufacturer's protocol for cycle sequencing using the cycle sequencer (Model 377; Perkin Elmer Applied Biosystems, Foster City, California, USA) at the University of Maryland Center for Agricultural Biotechnology. The DNA sequence data were analyzed using the Genetics Computer Group (GCG) suite of software (Genetics Computer Group, Madison, Wisconsin, USA), and compared, using the NCBI-BLAST program (20), with published GenBank DNA sequences.

Pulsed-field gel electrophoresis (PFGE) of *Salmonella* isolates

The PFGE was used to compare DNA fingerprinting profiles of *Salmonella* isolates. The PFGE procedure was performed according

to the protocol developed by the CDC. Briefly, bacteria were grown on trypticase soy agar (TSA) blood agar (Becton Dickinson Microbiology System, Cockeysville, Maryland, USA) at 37°C for 18 h. Bacterial colonies were suspended in cell suspension buffer (100 mM Tris HCl, 100 mM EDTA, pH 8.0) and adjusted to 0.48 to 0.52 optical density (OD) (Dade MicroScan Turbidity Meter; Dade Behring Inc., West Sacramento, California, USA). The cell suspension (200 μL) was mixed with 10 μL of proteinase K (10 mg/mL) and an equal volume of melted 1% SeaKem Gold agarose (FMC BioProducts, Rockville, Maine, USA) containing 1% sodium dodecyl sulfate (SDS). The mixture was carefully dispensed into a sample mold (Bio-Rad Laboratories, Hercules, California, USA). After solidification, the plugs were transferred to a tube containing 5 mL of lysis buffer (50 mM Tris HCl, 50 mM EDTA, pH 8.0, plus 1% Sarcosyl) and 0.1 mg/mL of proteinase K. Cells were lysed overnight in a water bath at 54°C with vigorous agitation. After lysis, the plugs were washed twice with deionized water and 4 times with tris-EDTA (TE) buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) for 15 min per wash at 50°C with vigorous agitation. Agarose-embedded with DNA was digested with 50 U of *XbaI* (Boehringer Mannheim Corporation, Indianapolis, Indiana, USA) overnight in a water bath at 37°C. The plugs were then placed in a 1% SeaKem Gold agarose (FMC) gel and restriction fragments were separated by electrophoresis in 0.5 × tris-borate-EDTA (TBE) buffer at 14°C for 18 h using a Chef Mapper (Bio-Rad) with pulse times of 2.16 to 63.8 s. The gel was stained with ethidium bromide, and DNA bands were visualized with UV transillumination. The PFGE images were then analyzed (Molecular Analyst Fingerprinting Plus Software; Bio-Rad).

Organic solvent tolerance assays

Tolerance to certain organic solvents is associated with acquisition of low-level antimicrobial resistance via the overexpression of the *marRAB* and *acrAB* loci. *Salmonella* strains were grown to late logarithmic phase and diluted to a concentration of approximately 10^7 cfu/mL. A 5-μL aliquot of the bacterial suspension was spotted onto Luria-Bertani (LB) agar plates and allowed to dry. Cyclohexane, n-hexane, or n-pentane (Aldrich Chemicals, Milwaukee, Wisconsin, USA) were added to the plates at a depth of 2 to 3 mm. Plates were sealed with petroleum jelly and parafilm to prevent evaporation of solvents, and incubated at 30°C for up to 48 h (10). Plating was done in duplicate and solvent tolerance was measured as a function of bacterial growth. Growth was recorded as confluent growth (++), visible growth (<100 colonies; +), or no growth (−) after 24 h (10). Control strain *E. coli* AG100 demonstrated confluent growth under hexane and did not grow in the presence of cyclohexane whereas *E. coli* AG102 (Mar mutant) grew in the presence of both hexane and cyclohexane.

Northern blot analysis

Overnight cultures of *Salmonella* were diluted 100-fold in fresh LB broth and grown to the mid-logarithmic phase at 30°C with shaking. Total RNA was extracted from a 50-mL culture (Qiagen RNA Midiprep kit; Qiagen, Chatsworth, California, USA) and the concentration was determined spectrophotometrically at 260/280 OD. The RNA was transferred to nylon membranes (Ambion, Austin, Texas, USA) using a transfer system (Turboblotter Transfer System; Schleicher and Schuell, Keene, New Hampshire, USA) and cross-linked (Gene Linker UV chamber; Bio-Rad). Hybridization of the radiolabeled DNA probe to the membrane-bound RNA (10 μg/lane) was performed at 42°C overnight according to the specifications of the membrane manufacturer (Ambion, Austin, Texas, USA). The *marA* probe was a 387 base pairs (bp) PCR fragment containing the complete *marA* gene amplified from *E. coli* AG100 chromosomal DNA. The *marA* probe was purified with a gel extraction kit (QIAEXII gel extraction kit; Qiagen) and labeled with [α^{-32}P]dCTP (High Prime DNA labeling kit; Boehringer Mannheim). The RNA blots were washed twice with 2 × saline-sodium citrate (SSC) buffer/0.1% SDS at room temperature and twice with 0.2 × SSC/0.1% SDS at 68°C. Membranes were air dried and exposed to X-ray film (Kodak BioMax MS film; Eastman Kodak, New Haven, Connecticut, USA) for 48 h and then manually visualized.

Results and discussion

Antimicrobial resistance patterns in swine *Salmonella*

Forty-two *Salmonella* isolates recovered from diseased swine were tested for their resistance to antimicrobial agents of human and veterinary significance, using a broth microdilution method and interpreted using NCCLS standards (Table I). All *S.* Typhimurium DT104 isolates displayed the penta-resistance phenotype to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (ACSSuT). Three out of 4 non-DT104 *S.* Typhimurium strains displayed resistance to ampicillin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline. Ninety-eight percent (41/42) of *Salmonella* isolates were resistant to at least 2 different antimicrobial classes. One out of 2 *S.* Infantis isolates (CVM814) was resistant to amoxicillin, apramycin, cephalothin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline (Table I). Only 1 isolate, *S.* Mbandaka, was resistant to sulfamethoxazole and trimethoprim. Among the non-DT104 *Salmonella* isolates, resistance was most often observed to sulfamethoxazole, tetracycline, streptomycin, kanamycin, and ampicillin, as has been reported by other investigators (21). Interestingly, 95% (n = 40/42) and 88% (n = 37/42) of all *Salmonella* isolates exhibited resistance to sulfamethoxazole and tetracycline, respectively. None of the DT104 strains displayed resistance to kanamycin; however, 41% (n = 9/22) of non-DT104 *Salmonella* strains exhibited resistance to this antimicrobial. Sulfonamides, tetracyclines, and aminoglycosides are widely used in the swine production environment for treatment and prevention of disease, and for growth promotion (21-23). Therefore, it is not unexpected to see increased resistance to these antimicrobials among swine *Salmonella* serotypes.

Integron mediated antimicrobial resistance in swine *Salmonella*

As was expected, all 20 swine DT104 strains exhibiting the penta-resistance phenotype, possessed 2 integron PCR amplicons, of 1000 bp and 1200 bp in size (Figure 1). The DNA sequencing revealed that the 1000 bp integron contained a gene encoding resistance to streptomycin (*aadA2* aminoglycoside 3′ adenyltransferase), whereas the 1200 bp integron contained a gene encoding

Table I. Antimicrobial susceptibility patterns of swine *Salmonella* spp[a] (μg/mL)

Isolate	ST[b]	Amk[c]	Amo	Amp	Apr	Cef	Cet	Cep	Cml	Cip	Ffc	Gen	Kan	Nal	Str	Sul	Tet	Tri
S. Typhimurium DT104[a]																		
CVM790[d]	CO	≤4	16	>32[a]	4	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	0.50	≤16	≤4	128[a]	>512[a]	>32[a]	0.25
CVM800[d]	IA	≤4	16	>32[a]	4	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	0.50	≤16	8	128[a]	>512[a]	>32[a]	0.50
CVM801[d]	IA	≤4	16	>32[a]	4	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	0.50	≤16	≤4	64[a]	>512[a]	>32[a]	0.25
CVM785[d]	IN	≤4	16	>32[a]	4	≤0.50	≤0.25	2	>32[a]	≤0.01	>16	0.50	≤16	≤4	256[a]	>512[a]	>32[a]	0.25
CVM793[d]	IN	≤4	16	>32[a]	4	≤0.50	≤0.25	8	>32[a]	≤0.01	>16	0.50	≤16	≤4	128[a]	>512[a]	>32[a]	0.25
CVM798[d]	IL	≤4	16	>32[a]	4	2	≤0.25	8	>32[a]	0.06	>16	0.50	≤16	8	64[a]	>512[a]	>32[a]	0.25
CVM799[d]	IL	≤4	16	>32[a]	4	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	0.50	≤16	≤4	128[a]	>512[a]	>32[a]	0.25
CVM789[d]	MN	≤4	16	>32[a]	≤2	≤0.50	≤0.25	8	>32[a]	≤0.01	>16	0.50	≤16	≤4	256[a]	>512[a]	>32[a]	0.25
CVM787[d]	NC	≤4	16	>32[a]	4	≤0.50	≤0.25	2	>32[a]	≤0.01	>16	0.50	≤16	≤4	64[a]	>512[a]	>32[a]	0.25
CVM803[d]	NC	≤4	16	>32[a]	4	≤0.50	≤0.25	2	>32[a]	≤0.01	>16	0.50	≤16	≤4	64[a]	>512[a]	>32[a]	0.25
CVM786[d]	OH	≤4	16	>32[a]	≤2	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	0.50	≤16	≤4	64[a]	>512[a]	>32[a]	0.50
CVM795[d]	OH	≤4	16	>32[a]	4	≤0.50	≤0.25	2	>32[a]	≤0.01	>16	0.50	≤16	≤4	128[a]	>512[a]	>32[a]	0.25
CVM804[d]	OH	≤4	16	>32[a]	≤2	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	0.50	≤16	≤4	64[a]	>512[a]	>32[a]	0.25
CVM792[d]	OK	≤4	16	>32[a]	4	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	0.50	≤16	≤4	128[a]	>512[a]	>32[a]	≤0.12
CVM797[d]	OK	≤4	16	>32[a]	4	≤0.50	≤0.25	8	>32[a]	≤0.01	>16	1	≤16	≤4	64[a]	>512[a]	>32[a]	0.25
CVM802[d]	OK	≤4	16	>32[a]	4	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	0.50	≤16	≤4	64[a]	>512[a]	>32[a]	≤0.12
CVM791[d]	PA	≤4	16	>32[a]	4	≤0.50	≤0.25	2	>32[a]	0.03	>16	0.50	≤16	≤4	64[a]	>512[a]	>32[a]	0.25
CVM796[d]	SC	≤4	16	>32[a]	4	≤0.50	≤0.25	2	>32[a]	≤0.01	>16	0.50	≤16	≤4	64[a]	>512[a]	>32[a]	0.25
CVM788[d]	VA	≤4	16	>32[a]	4	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	0.50	≤16	≤4	128[a]	>512[a]	>32[a]	0.25
CVM794[d]	ND	8	16	>32[a]	8	≤0.50	≤0.25	4	>32[a]	≤0.01	>16	2	≤16	≤4	256[a]	>512[a]	>32[a]	0.25
S. Typhimurium non-DT104[a]																		
CVM806	MN	≤4	8	>32[a]	4	≤0.50	≤0.25	16	16	≤0.01	4	0.50	>64[a]	≤4	256[a]	≤512[a]	≤32[a]	0.25
CVM808	NC	≤4	1	16	8	≤0.50	≤0.25	4	16	≤0.01	4	0.50	≤16	≤4	64[a]	≤512[a]	8	≤0.12
S. Typhimurium – copenhagen[a]																		
CVM807	NC	≤4	8	>32[a]	≤2	≤0.50	≤0.25	4	8	≤0.01	4	0.50	>64[a]	≤4	256[a]	>512[a]	>32[a]	0.25
CVM805[d]	WI	≤4	16	>32[a]	4	2	≤0.25	16	16	0.06	16	4	>64[a]	16	256[a]	>512[a]	>32[a]	0.50
S. Anatum																		
CVM811	IL	≤4	4	>32[a]	≤2	≤0.50	≤0.25	8	8	0.03	4	0.50	≤16	8	≤32	>512[a]	>32[a]	≤0.12
CVM810	NC	≤4	1	≤2	8	1	≤0.25	2	8	0.03	4	≤0.25	≤16	8	≤32	>512[a]	32[a]	≤0.12
CVM813	TX	≤4	1	≤2	≤2	≤0.50	≤0.25	4	8	0.50	4	≤0.25	≤16	8	≤32	>512[a]	>32[a]	≤0.12

Isolate	ST[b]	Amk[c]	Amo	Amp	Apr	Cef	Cet	Cep	Cml	Cip	Ffc	Gen	Kan	Nal	Str	Sul	Tet	Tri
S. Choleraesuis – kunzendorf[a]																		
CVM824	IL	≤4	8	>32[a]	8	1	≤0.25	8	8	0.03	4	0.50	≤16	8	>256[a]	>512[a]	>32[a]	0.50
CVM823	KS	≤4	1	≤2	4	≤0.50	≤0.25	2	8	0.03	4	0.50	≤16	16	>256[a]	>512[a]	≤4	0.50
CVM816	MO	≤4	16	>32[a]	≤2	1	≤0.25	16	≤4	0.03	4	1	≤16	≤4	>256[a]	>512[a]	>32[a]	≤0.12
CVM821	NE	≤4	1	≤2	4	≤0.50	≤0.25	2	≤4	0.03	4	0.50	>64[a]	8	>256[a]	>512[a]	>32[a]	0.25
S. Derby																		
CVM817[d]	OH	≤4	1	≤2	4	≤0.50	≤0.25	8	8	≤0.01	4	0.50	32	≤4	256[a]	>512[a]	>32[a]	0.25
CVM819	MN	≤4	1	≤2	≤2	1	≤0.25	2	16	0.03	8	0.50	≤16	≤4	128[a]	>512[a]	>32[a]	0.50
CVM820	MO	≤4	1	≤2	4	1	≤0.25	2	8	0.03	8	0.50	≤16	≤4	≤32	≤128	≤4	≤0.12
S. Heidelburg																		
CVM822	IL	≤4	1	≤2	≤2	≤0.50	≤0.25	4	8	0.03	4	0.50	>64[a]	≤4	128[a]	>512[a]	>32[a]	≤0.12
CVM815	NC	≤4	8	≤2	≤2	≤0.50	≤0.25	2	16	0.03	8	0.50	>64[a]	≤4	64[a]	>512[a]	>32[a]	≤0.12
CVM825	OK	≤4	1	≤2	≤2	≤0.50	≤0.25	2	8	≤0.01	4	≤0.25	>64[a]	≤4	128[a]	≤128	>32[a]	≤0.12
S. Infantis																		
CVM812	ID	≤4	1	8	≤2	1	≤0.25	4	8	0.03	8	≤0.25	≤16	≤4	≤32	>512[a]	≤4	0.25
CVM818	IL	≤4	1	≤2	8	1	≤0.25	4	8	≤0.01	8	≤0.25	≤16	≤4	≤32	>512[a]	≤4	0.25
CVM814	ND	≤4	>32[a]	16	>32[a]	1	≤0.25	>32[a]	>32[a]	≤0.01	16	>16[a]	>64[a]	≤4	128[a]	>512[a]	>32[a]	0.25
S. Mbandaka																		
CVM809	IN	≤4	1	≤2	4	1	≤0.25	4	16	0.03	8	1	≤16	≤4	≤32	>512[a]	>32[a]	≤0.12
CVM826	NC	≤4	1	≤2	4	≤0.50	≤0.25	2	8	≤0.01	8	1	>64[a]	8	128[a]	>512[a]	>32[a]	>4

[a] Indicates resistance. The MIC's determined via broth-microdilution methods according to NCCLS standards (17,18)

[b] State of origin: CO, Colorado; IA, Iowa; ID, Idaho; IN, Indiana; IL, Illinois; KS, Kansas; MN, Minnesota, MO, Missouri; NE, Nebraska; NC, North Carolina; ND, North Dakota; OH, Ohio; OK, Oklahoma; PA, Pennsylvania; SC, South Carolina; TX, Texas; VA, Virginia; WI, Wisconsin

[c] Amk, Amikacin; Amo, amoxicillin/Clavulanic acid; Amp, Ampicillin; Apr, Apramycin; Cef, Ceftiofur; Cet, Ceftriaxone; Cep, Cephalothin; Cml, chloramphenicol; Cip, ciprofloxacin; Ffc, Florfenicol; Gen, Gentamicin; Kan, Kanamycin; Nal, Nalidixic acid; Str, Streptomycin; Sul, Sulfamethoxazole; Tet, Tetracycline; Tri, Trimethoprim/Sulfamethoxazole

[d] Indicates strains possessing integrons

E. coli ATCC 25922, *E. coli* ATCC 35218, *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 were used as controls in MIC determinations

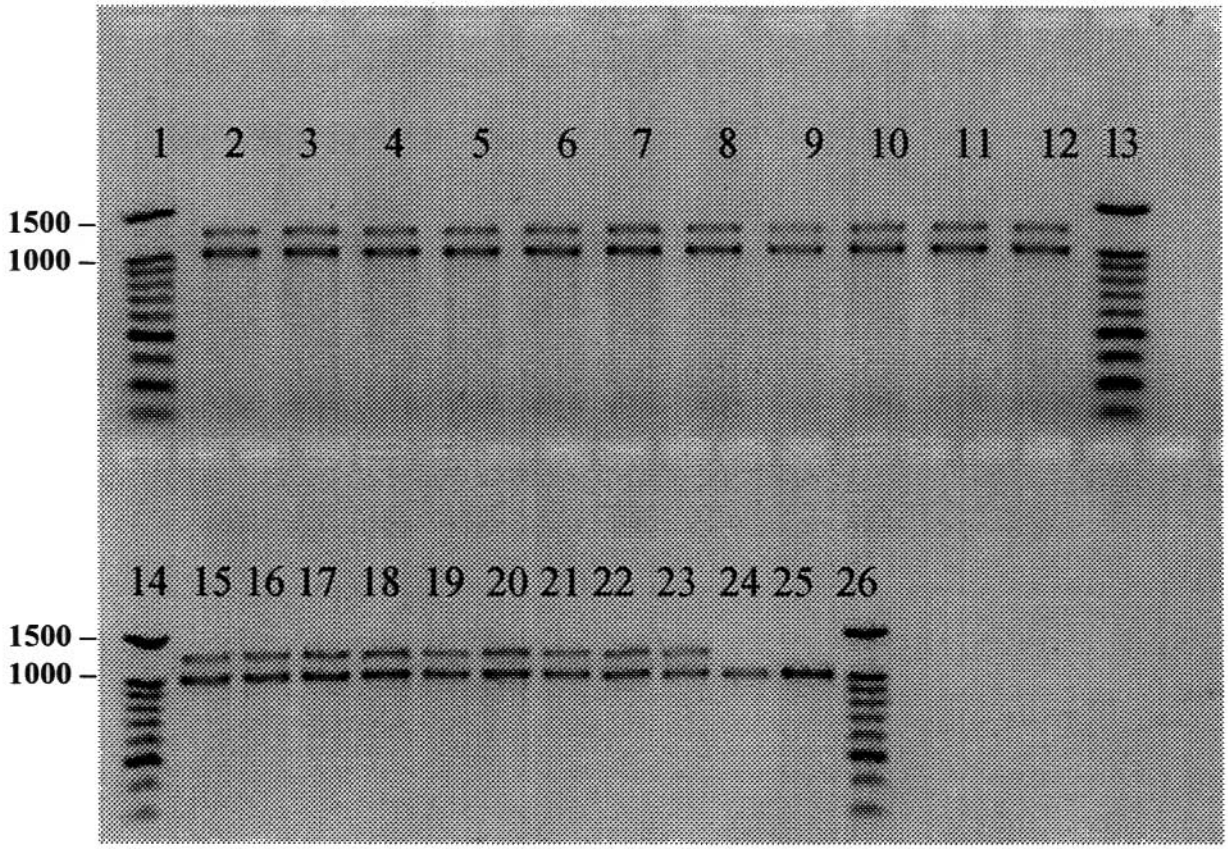

Figure 1. Polymerase chain reaction (PCR) amplification of integrons among swine *Salmonella* isolates. The PCR products generated using integron specific primers (5) were run on a 1% agarose gel. A 100 base pairs (bp) ladder (Promega) was used as the molecular size standard in lanes 1,13,14, and 26. Lanes 2 to 12 and 15 to 23 are *S.* Typhimurium DT104 isolates (CVM785 and 804, respectively). Lanes 24 to 25 are *S.* Typhimurium var Copenhagen (CVM805) and *S.* Derby (CVM817).

resistance to ampicillin (*bla* $_{PSE\text{-}1}$ β-lactamase). These have been previously described by other investigators and have been shown to be located on the chromosome rather than on transmissible plasmids (4,5,24,25). None of the non-DT104 strains showed this same pattern although 2 strains (CVM805; *S.* Typhimurium var Copenhagen and CVM817; *S.* Derby), produced a 1000 bp integron upon PCR amplification (Figure 1). The DNA sequencing revealed that the 1000 bp integron that recovered from CVM805-*S.* Typhimurium var Copenhagen contained the *aadA* gene, encoding resistance to streptomycin and spectinomycin. Whereas the 1000 bp integron amplified from CVM817-*S.* Derby contained the *aadA2* gene as described previously, also encoding resistance to streptomycin and spectinomycin. Integrons containing streptomycin resistant determinants have been previously described in a wide range of *Salmonella* serotypes including *S.* Enteritidis, *S.* Derby, *S.* Anatum, *S.* Typhimurium non-DT104, and numerous others (24,26–28). It is interesting to note that many of the *Salmonella* isolates were resistant to streptomycin but did not possess integrons suggesting the presence of alternative resistance mechanisms for this antimicrobial. The majority of non-*S.* Typhimurium strains did not possess integrons as determined by PCR (Table I), suggesting that integrons are not as widely disseminated among non-*S.* Typhimurium swine *Salmonella* serotypes, as had been previously thought. Therefore, it is of interest to continually screen for these DNA elements as part of any future epidemiological investigation aimed at characterizing the dissemination of antimicrobial resistance among *Salmonella* serotypes.

Characterization of *Salmonella* Mar mutants

Salmonella isolates were assayed for growth in the presence of the organic solvent cyclohexane. Cyclohexane tolerance in *E. coli* and *Salmonella* has been linked to increased antibiotic resistance due to upregulation of efflux pump mechanisms via overexpression of the *marA* and *soxS* regulatory genes (10,11). Cyclohexane tolerance was observed in 1 DT104 strain and 1 *S.* Typhimurium Copenhagen non-DT104 isolate (CVM798 and 805, respectively). Interestingly, these 2 strains differed from other *Salmonella* strains tested in that they displayed decreased susceptibility to ceftiofur and ciprofloxacin (minimal inhibitory concentration (MIC) of 2 μg/mL and 0.06 μg/mL to ceftiofur and ciprofloxacin, respectively) (Table I). This decrease in susceptibility to ciprofloxacin is especially troublesome since some researchers have noted that DT104 strains may acquire resistance to this drug (29,30). Emergence of resistance to ciprofloxacin in *Salmonella* is potentially disturbing since it is often the drug of choice for treating human salmonellosis (29).

Expression of *marA* was evaluated by Northern blot analysis in isolates that demonstrated increased organic solvent tolerance. The RNA from the 2 isolates (CVM798 and 805), displaying increased organic solvent tolerance and wild type *E. coli* AG100 and Mar mutant *E. coli* AG102, were probed with an *E. coli*-derived *marA* gene

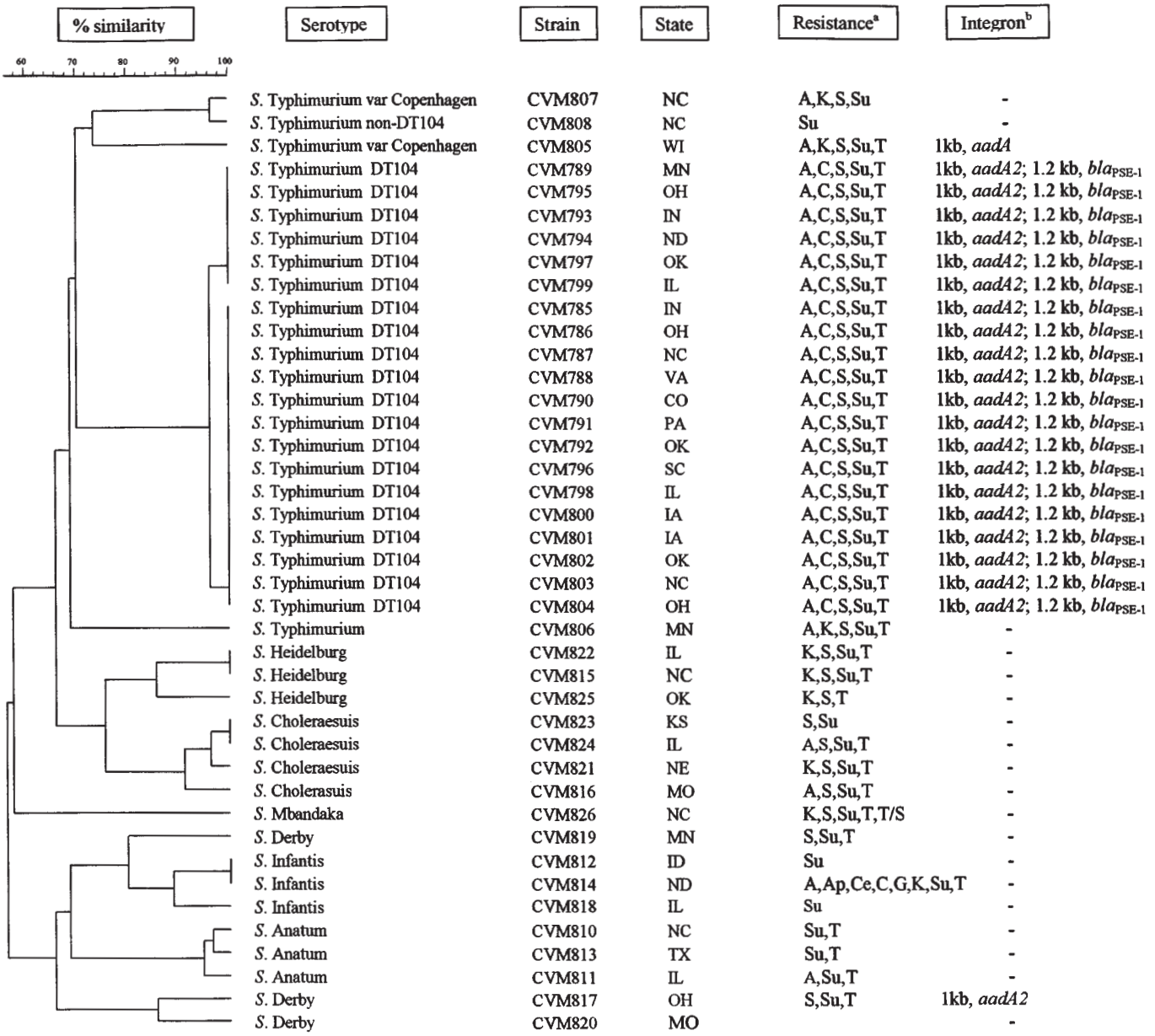

Figure 2. Genetic relatedness among swine *Salmonella* isolates. Dendrogram of pulsed-field gel electrophoresis (PFGE) patterns of *Salmonella* isolates recovered from diseased swine, and their association with serotype, state of origin, antimicrobial resistance profile, and integron content.
The PFGE patterns of *Salmonella* species were cleaved with restriction enzyme *Xba1*. The percent similarity among the tested *Salmonella* isolates is represented by a dendrogram generated using Molecular Analyst Fingerprinting Plus Software (Bio-Rad). The percent similarity between PFGE types is shown at the top of the figure; scale at 100 means identical.
[a] A, ampicillin; Ap, apramycin; Ce, cephalothin; C, chloramphenicol; G, gentamicin; K, kanamycin; S, streptomycin; Su, sulfamethoxazole; T, tetracycline; T/S, trimethoprim/sulfamethoxazole.
[b] Size of integron/resistance gene: *aadA*, aminoglycoside adenyltransferase; *aadA2*, aminoglycoside adenyltransferase; bla_{PSE-1}, beta-lactamase.

probe. The *marA* gene encodes a transcriptional activator of the *marRAB* operon (14,31). The *marA* gene was not overexpressed in these 2 isolates (data not shown). Failure to detect *marA* expression in organisms exhibiting a mar-like phenotype suggests that these 2 *Salmonella* isolates may be overexpressing other genes; such as, *soxS*, the efflux pump *acrAB*, or both (13,15). Further studies are needed to determine the molecular basis of the observed Mar phenotype in these 2 isolates.

Pulsed-field gel electrophoresis (PFGE) profiles

To assess genetic relatedness among the swine *Salmonella* isolates, pulsed-field gel electrophoresis (PFGE) was used. The PFGE revealed 20 distinct genetic patterns among the 42 isolates (Figure 2). All *S*. Typhimurium DT104 isolates fell within 2 closely related genetic clusters, confirming other studies which have demonstrated the highly clonal nature of *S*. Typhimurium DT104 (24,32,33).

No correlation was observed between the state of origin of the DT104 isolate and genetic clustering. Two out of the 3 *S*. Heidelburg isolates (CVM822 and 815) possessed both identical PFGE and antimicrobial resistance patterns (kanamycin, streptomycin, sulfamethoxazole, and tetracycline), but were isolated from diseased swine in Illinois and North Carolina, respectively (Figure 2). Identical PFGE patterns with different antimicrobial susceptibility patterns were observed between 2 out of 4 *S*. Choleraesuis isolates and 2 out of 3 *S*. Infantis isolates. In both cases, however, isolates originated from diseased swine in different states; Kansas and Illinois for *S*. Choleraesuis, and Idaho and North Dakota for *S*. Infantis (Figure 2). One out of the 2 *S*. Infantis isolates (CVM814) that shared identical PFGE patterns also displayed multiple resistance to amoxicillin-clavulanic acid, streptomycin, apramycin, cephalothin, chloramphenicol, gentamicin, kanamycin, sulfamethoxazole, and tetracycline. The other *S*. Infantis isolate (CVM812) that shared the same PFGE pattern as this multidrug resistant strain was only resistant to sulfamethoxazole. Since both *S*. Infantis isolates that shared the same PFGE pattern were negative for integrons, this suggests that CVM814 has acquired the multidrug resistance phenotype from some type of mobile DNA element, most likely a plasmid. Overall, PFGE typing grouped the majority of isolates according to serotype, with 1 *S*. Mbandaka strain being untypeable by PFGE and 1 *S*. Derby (CVM819) appearing to be more related to the *S*. Infantis cluster than the other Derby cluster (Figure 2). This data supports the findings of other investigators that PFGE, using *XbaI* restriction endonuclease, is a sensitive method for fingerprinting diverse *Salmonella* serotypes (32,34,35). As has been reported with isolates from other animals, our results show that *S*. Typhimurium DT104 isolates from diseased swine in the United States are clonal in origin and geographically widely distributed. Although only a small number of isolates were analyzed in this study, our preliminary data suggests that there are specialized swine pathogenic clones of other *Salmonella* serotypes (for example, Heidelburg, Choleraesuis, and Infantis) circulating among diseased swine in the United States.

In summary, this research adds to the growing body of knowledge concerning antimicrobial resistance among swine *Salmonella* and demonstrates that multiple mechanisms, including integrons, contribute to resistance. Although we did not detect overexpression of *marA*, a known regulator of multi-drug efflux pumps, our data imply the presence of an active efflux in 2 multi-resistant strains (CVM 798 and 805), as evidenced by their tolerance to organic solvents. Large differences in susceptibility profiles between strains with identical PFGE patterns suggests the involvement of multi-drug resistance plasmids. Unlike plasmid-mediated resistance, which may disappear in the absence of continued selective pressure, chromosomally mediated resistance is often maintained; thus, it is necessary to implement measures to eliminate the resistant strain to prevent transfer among animals and between animals and humans. In an effort to reduce the prevalence of multi-drug resistant *Salmonella* in swine, additional preventive animal health management factors, besides antimicrobial use, should be considered; such as, increased biosecurity, administration of probiotics, prebiotics, and vaccination (36).

Because our findings are based on a limited sample size, further research with a larger number of non-DT104 strains is needed to better assess both the clonality of geographically dispersed isolates and the array of resistance mechanisms operative in swine *Salmonella*.

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