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Characterization of Multidrug-resistant *Salmonella enterica* subsp. *enterica* Serovar Typhimurium var. Copenhagen and Typhimurium Isolated from Feedlot Cattle

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ABSTRACT

The objective of this study was to characterize *Salmonella enterica* subsp. *enterica* Serovars Typhimurium and Typhimurium var. Copenhagen isolated from naturally infected feedlot cattle (n = 138) in North Dakota for antimicrobial resistance (AMR), presence of integrons and genotypic relatedness by use of PFGE assays. A panel of 15 selected antimicrobials and the Sensititre automated antimicrobial susceptibility test system (TREK Diagnostic Systems, Westlake, OH) were used. Class 1 and 2 integrons were targeted by PCR, using primers specific for the *intI1* and *intI2* genes. Pulsed field gel electrophoresis (PFGE) assays were performed by the *E. coli* Reference Center, Pennsylvania State University, University Park, PA. All 58 *Salmonella* isolates tested were resistant to ≥ 1 of the antimicrobials, with 56/58 (96.6%) showing multidrug resistance (resistant to ≥ 2 antimicrobials). Twenty-nine (26 of which were *Salmonella* serovars Typhimurium var Copenhagen) were positive for class 1 integron; two also tested positive for integron 2. The 58 *Salmonella* isolates were grouped into 9 distinguishable PFGE profiles, with the most prevalent genotype accounting for 46.6% (27/58) of the isolates. The predominant resistance phenotype (94.8%, 55/58) was Amox/Cla-Amp-Chl-Str-Sul-Tet. In addition, these data indicate that *Salmonella enterica* subsp. *enterica* Serovars Typhimurium and Typhimurium var. Copenhagen *Salmonella* isolated from naturally infected feedlot cattle in North Dakota showed widespread AMR, with or without presence of class 1 integron.

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INTRODUCTION

Foodborne diseases caused by nontyphoid *Salmonella* represent an important public health problem and an economic burden in many parts of the world today *(11, 18, 27)*. In the United States (US), *Salmonella* is the second most common identifiable cause of illness, and the leading cause of hospitalizations and deaths, due to foodborne bacterial infection *(17)*. Most people who suffer from *Salmonella* infections present with temporary gastroenteritis that usually does not require treatment. However, when infection becomes invasive, antimicrobial treatment is mandatory *(29)*. Traditionally, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole have been used to treat such severe cases. However, the increasing number of antimicrobial-resistant *Salmonella* strains has led to a decrease in the efficacy of these treatments *(2)*. Additionally, the frequency of isolation of *Salmonella* strains resistant to one or more antimicrobial agents has risen in the US *(7)*, and elsewhere in the world *(1)*. Fluoroquinolones and broad-spectrum cephalosporins have been employed most recently as the preferred drugs for treatment of adults and children, respectively, due to the low likelihood of resistance to them *(2, 4)*. However, the usefulness of these drugs may be diminishing, as *Salmonella* strains producing β-lactamases conferring resistance to broad-spectrum cephalosporins have been isolated from clinical patients *(6, 29)*; some of these microorganisms have been acquired from cattle *(8)*. The situation is reported to be more complex and difficult in developing countries in which there is a widespread misuse of antimicrobials in both human and veterinary medicine practices *(21)*. This uncontrolled exposure to combinations or several classes of antimicrobials has led to the emergence of multidrug-resistant (MDR) strains that may pass from food animals to humans *(7)*.

The spread of antibiotic resistance among bacteria has been associated with mobile genetic elements such as plasmids, transposons *(30)* and integrons *(19)*. Notably, MDR has been frequently linked with microbial genomic elements known as integrons, which have the ability to distribute genes encoding resistance to a number of antimicrobial drugs *(19)*. Integrons can capture genes, notably those encoding antimicrobial resistance, by a site-specific recombination system and have been located in both chromosomal and extra chromosomal DNA *(3, 12)*. The main classes of integrons are found in the family *Enterobacteriaceae*, with class 1 integrons being the most extensively studied. Class 1 integrons are characterized by the presence of two conserved segments, the 5'-conserved segment (5'-CS) and 3'-conserved segment (3'-CS) *(3)*, and are defined by an *intI* gene encoding integrase, a recombinant site *attI,* and a strong promoter. Previous studies *(30, 31)* on integrons and associated antimicrobial resistance genes in *Salmonella* revealed a predominance of gene cassettes that confer resistance to aminoglycosides and trimethoprim. The investigation of multidrug-resistance in foodborne pathogens in general and *Salmonella* in particular is essential for a more complete understanding of the epidemiology of emerging multidrug resistance in *Salmonella* serovars *(31)*. The implication of therapeutic failure in public health due to multidrug resistance is particularly important, given that *Salmonella* is the leading cause of foodborne infection in the US *(17)*.

This study reports on the association between the presence of integrons (Class 1 and 2) and MDR in *Salmonella* serovars isolated from naturally infected feedlot cattle housed at the North Dakota State University (NDSU) cattle feedlot research facility.

MATERIAL AND METHODS

Sample collection

Fecal samples were collected from each steer in accordance with the guidelines established by the Institute for Animal Care and Use Committee (IACUC), following a previously described protocol *(13)*. Briefly, each steer was restrained in a hydraulic chute, and about 20 g of feces was collected from the rectum. A new set of sterile polythene sleeve gloves was used for collection from each steer. The feces were put into sterile plastic cups that were placed on ice to be transported to the laboratory at NDSU for processing. In addition, a sterile dry cotton swab was used to culture the dorsal mucosa of the rectoanal junction (RAJ). Each RAJ mucosal swab was placed into a 15 ml culture tube containing 3 ml of Tryptic case Soy Broth (Difco™ Becton Dickinson & Company MD, USA) supplemented with cefixime and potassium tellurite (Dynal Biotech ASA, Oslo, Norway). Both the fecal samples and swabs were placed in iced-pack coolers before being transported to the laboratory. The sampling procedure was repeated every three weeks for the entire finishing period (March – June, 2007) and has been described in detail elsewhere *(26)*.

Isolation of *Salmonella*

Fecal samples were cultured by use of conventional culture methods optimized for the detection of *Salmonella (14)*. Briefly, a sterile swab was loaded with fecal sample, which was pre-enriched in buffered peptone water (Difco™ Becton Dickinson & Company, MD) at 37°C overnight; this was followed by immunomagnetic bead separation specific for *Salmonella* species (Dynabeads® anti-*Salmonella*, Dynal Biotech, Inc., Lake Success, NY) according to the manufacturer's instructions. After the final wash, the beads were transferred to 10 ml of Rappaport Vassiliadis R10 (RV) broth (Becton Dickinson, Sparks, MD) and incubated (with constant gentle shaking) at 42º for 24 h. Following incubation, the RV cultures were streaked onto modified brilliant green agar (mBGA) (Becton Dickinson) and mannitol lysine crystal violet brilliant green agar (MLCB) (Oxoid LTD, Basingstoke, UK). Colonies with typical *Salmonella* characteristics were stabbed and also inoculated on the surface of 10 ml triple sugar iron agar slants (Becton Dickinson), and biochemical results were read after 24 hours incubation.

Serotyping and antimicrobial susceptibility testing

Serotyping of *Salmonella* isolates was performed at the National Veterinary Laboratory Services (NVSL) at Ames, Iowa, following standard methods. The antimicrobial susceptibility test of *Salmonella* isolates was determined on a panel of 15 selected antimicrobials by use of the Sensititre automated antimicrobial susceptibility test system (TREK Diagnostic Systems, Westlake, OH) according to the manufacturer's instructions. The MIC breakpoint levels and concentrations of each antimicrobial were based on those specified by the

TABLE 1. Number (%) of *Salmonella* isolates resistant/susceptible to various antimicrobial agents tested. (Results for Ceftiofur were not interpretable)

Antibiotics	Susceptible Isolates (%)	Intermediate Isolates (%)	Resistant Isolates (%)
Amikacin (0.5–64)	58 (100.0)	-	-
Amoxicillin/clavulanic acid (1/0.5–32/16)	2 (3.5)	1 (1.7)	55 (94.8)
Ampicillin (2–32)	2 (5.3)	-	56 (94.7)
Cefoxitin (0.5–32)	58 (100.0)	-	-
Cetriaxone (0.25–64)	58 (100.0)	-	-
Ceftiofur (0.5–8)	NI	NI	NI
Chloramphenicol (2–32)	-	2 (5.3)	56 (94.7)
Ciprofloxacin (0.015–4)	58 (100.0)	-	-
Gentamycin (0.25–16)	58 (100.0)	-	-
Kanamycin (6–64)	58 (100.0)	-	-
Nalidixic acid (0.5–32)	58 (100.0)	-	-
Streptomycin (32–64)	NI	NI	56 (94.7)
Sulfizoxazole (16–512)	2 (5.3)	-	56 (94.7)
Tetracycline (4–32)	2 (5.3)	-	56 (94.7)
Trimethoprim-sulfamethoxazole (4–76)	58 (100.0)	-	-

National Committee for Clinical Laboratory Standards *(20)*. The 15 different antimicrobials used were amikacin (0.5–64 µg ml^{-1}), amoxacillin/clavulanic acid (1/0.5–32/16 µg ml^{-1}), ampicillin (2–32 µg ml^{-1}), cefoxitin (0.5–32 µg ml^{1}), ceftiofur (0.12–8 µg ml^{-1}), cetriaxone (0.25–64 µg ml^{-1}), chloramphenicol (2–32 µg ml^{-1}), ciprofloxacin (0.015–4 µg ml^{-1}), gentamycin (0.25–16 µg ml^{-1}), kanamycin (6–64 µg ml^{-1}), nalidixic acid (0.5–32 µg ml^{-1}), streptomycin (32–64 µg ml^{-1}), sulfizoxazole (16–512 µg ml^{-1}), tetracycline (4–32 µg ml^{-1}) and trimethoprim-sulfamethoxazole (0.12/ 2.4 – 4/76 µg ml^{-1}).

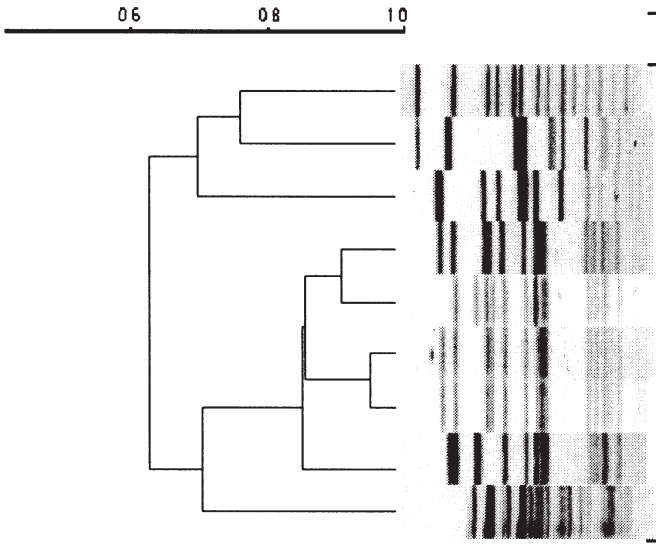
Pulsed field gel electrophoresis (PFGE)

The *Salmonella* isolates recovered from this study were sent to the *E. coli* Reference Centre, Pennsylvania State University, University Park, PA. PFGE assays were performed on 58 *Salmonella* serotypes to investigate their genotypic relatedness. The sample preparation, restriction digestion, electrophoresis, and gel staining for PFGE were accomplished following the CDC-referenced standard method. Restriction endonuclease *XbaI* (Roche Diagnostics Corporation, Indianapolis, IN) was used for restriction digestion of cDNA. The size standard used for all gels was *XbaI*-digested DNA from *Salmonella* Braenderup strain H9812 (American Type Culture Collection catalog no. BAA-664), i.e., the universal size standard used by all PulseNet laboratories. Fingerprints were analyzed by use of BioNumerics software version 3.5 (Applied Maths, Austin, Texas). Strain relatedness was done based on previously recommended criteria *(10)*, using "different bands" algorithm for clustering and the unweighted pair group for arithmetic means (UPGMA) tree-building approach with optimization of 1 and 0.5% position tolerance. Visual inspection of the patterns was performed as the final step of analysis.

PCR amplification of class 1 and 2 integrons

The bacterial DNA template preparation and the PCR conditions for the detection of class 1 and class 2 integrons were undertaken as previously described *(18)*. The screening for the presence of class 1 and class 2 integrons was carried out using PCR with primers specific for the *intI1 (13)* and *intI2 (10)*. Amplifications were performed in 10µL of 5× Taq PCR Master Mix (Promega, Madison, WI, USA), µl dntp 2pmol/L each primer, and 2 µg template DNA. Amplification specifications were as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 30 s at 72°C. PCR products were analyzed by gel electrophoresis with 2% agarose gels. Both negative and positive controls were used in the PCR reactions. In addition, a standard DNA ladder (Promega, Madison, WI, USA) was used on the gels.

FIGURE 1. Dendrogram generated from the *Xbal* patterns of the 58 *Salmonella* isolates using UPGMA clustering analysis with the BioNumerics software. A positive tolerance of 1.5% was chosen.



PFGE Profile	SS	Isolates (No)	SP- 1 (No)	SP-2 (No.)	SP- 3 (No.)
I	STC	1	-	-	1
II	STC	1	1	-	-
III	STC	1	1	-	-
IV	STC	4	4	-	-
V	STC	28	8	17	2
VI	STC	15	6	9	-
VII	STC	2	1	1	-
VIII	STC	2	-	2	-
IX	ST	4	3	-	1

SS= *Salmonella* Serovar; SP= Sampling points; STC= *Salmonella* Typhimurium serovar Copenhagen; ST= *Salmonella* Typhimurium.

RESULTS

Salmonella serotypes and antimicrobial susceptibility testing

A total of 54 of 58 (93.1%) of the *Salmonella* species belonged to the serotype Typhimurium vars Copenhagen. The rest (4/58, 6.9%) were *Salmonella* Typhimurium. AMR testing showed that all isolates were resistant to more than one of the antibiotics (Table 1). All but two of the isolates (ID 26 and 30) were resistant to more than two of the antibiotics tested, with 96.6% (56 of 58) of the isolates being MDR. All isolates tested were susceptible to amikacin, cefoxitin, cetriaxone, ciprofloxacin, gentamycin, nalidixic acid, and trimethoprim-sulfamethoxazole. Almost all of the isolates recovered from this study had a similar antimicrobial resistance pattern.

Presence of integrons

Regardless of sampling points (1, 2, or 3), 29 were positive for class I integron (280 bp product) while only two of the isolates showed a 233-bp PCR product by use of primers *intI2,* suggesting the presence of class 2 integron. Both of the two isolates also carried the class 1 integron.

PFGE analysis

The PFGE analysis identified 9 distinguishable *Salmonella* genotypes. Of the 9 PFGE profiles, STC and ST accounted for 94.8% (55 of 58) and 5.2 % (3 of 58) of the isolates, respectively (Fig. 1). Type IV, V, VII, VIII, and IX derived from cattle at two sampling periods showed 100% similarity. Type X (1 isolate from sampling 2), which is the most distant strain, showed only 73% similarity in PFGE banding patterns. Genotype V was the most prevalent (28/58, 48.3%) of the isolates, followed by types VI (15/58, 25.9%), IV and IX (both 3/58, 5.2%). Additionally, genotypes I, II, II, and X each represented 1.7% (1 of 58) of the genotypes (Fig. 1). Isolates 49 and 65, which were positive for both *Int* 1 and 2, belonged to genotypes I and IV, respectively. The relationship between molecular types, integron carriage and resistance phenotypes of the *Salmonella* Typhimurium serovars Copenhagen is shown in Table 2.

DISCUSSION

In this study, all but two of the *Salmonella* isolates were resistant to more than two of the antimicrobials tested, with 96.6% of the isolates showing multidrug resistance. The widespread AMR of *Salmonella* isolated from cattle in North Dakota has been reported before *(23)*, with most animal strains showing more multidrug resistance than human *Salmonella* isolates show, possibly due to a difference in antimicrobial selection pressure exerted on the microorganisms in the two populations.

The emergence and dissemination of MDR among *Salmonella* isolates from healthy cattle have potential adverse implications to public health. Since the first description of class 1 integron by

TABLE 2. Relationship between molecular types, integron carriage and resistance phenotypes of the *Salmonella* Typhimurium serovar Copenhagen and *Salmonella* Typhimurium. STC = *Salmonella* Typhimurium serovar Copenhagen; ST = *Salmonella* Typhimurium

PFGE genotype	No. of isolates	Serovars	*Int I* No. (%)	*Int 2* No. (%)	Resistance phenotypes
I	1	STC	1 (100)	-	Amox/Cla-Amp-Chl-Str-Sul-Tet
II	1	STC	1 (100)	-	Amox/Cla-Amp-Chl-Str-Sul-Tet
III	1	STC	1 (100)	-	Amox/Cla-Amp-Chl-Str-Sul-Tet
IV	4	STC	3 (75)	1 (33)	Amox/Cla-Amp-Chl-Str-Sul-Tet
V	26	STC	11 (42.3)	-	Amox/Cla-Amp-Chl-Str-Sul-Tet
V	1	STC	-	-	Susceptible
V	1	STC	-	-	Amp-Chl-Str-Sul-Tet
VI	14	STC	6 (42.9)	-	Amox/Cla-Amp-Chl-Str-Sul-Tet
VI	1	STC	-	-	Susceptible
VII	2	STC	2 (100)	-	Amox/Cla-Amp-Chl-Str-Sul-Tet
VIII	2	STC	2 (100)	1 (50)	Amox/Cla-Amp-Chl-Str-Sul-Tet
IX	4	STC	2 (50)	-	Amox/Cla-Amp-Chl-Str-Sul-Tet
Total	**58**	**29**	**(50)**	**2 (3.4)**	

Stokes and Hall *(25)*, integron-mediated resistance has been reported in clinical isolates of various organisms, including *K. pneumoniae, K. oxytoka, Pseudomonas aeroginosa, E. coli, C. fruedii* and *V. cholerae (23, 24).* It has been reported *(5)* that classes 1 and 2 are most common in resistant bacteria, and the mobility of these integrons was undoubtedly important in facilitating their spread into many different bacterial species.

In this study, although the prevalence of class 1 and 2 integrons were 50% (29/58) and 3.5% (2/58), respectively, more than 90% of the isolates were multidrug resistant. The commonest resistance phenotype was Amox/Cla-Amp-Chl-Str-Sul-Tet (94.8%, n = 55/58). The observed lower frequency of class 2 than class 1 integrons in this study cannot be fully explained, but reasons could vary from lower exposure to selective pressure of antibiotics among the isolates *(31)*. Additionally, two isolates positive for class 1 integrons also had class 2 integrons. These isolates belonged to genotypes I and IV (Table 1 and 2) and showed only about 67% genomic similarity (Fig. 1). They were recovered from different sampling points (points one and two, respectively).

It is important to note that all 29 isolates with class 1 integrons were susceptible to amikacin, cefoxitin, cetriaxone, ciprofloxacin, gentamycin, kanamycin, nalidixic acid, and trimethoprim-sulfamethoxazole (not shown on table). Reasons for this are not known but could vary from lack of exposure to possibly presence of non-functional integrons, as reported in a previous study *(15)*. Classes 1 and 2 integrons were not detected in 29 isolates (n = 29) and yet 93% (27/29) of these were MDR. Similarities in the drug resistance phenotypes between integron positive and negative isolates indicate that AMR may or may not be integron related. This observation is similar to what has been reported in a previous study in which class 1 integron was not always involved in the resistance of *E. coli* isolates to antimicrobial agents *(15)*. However, integrons have been often associated with broad antibiotic resistance, even if they do not encode multiple drug resistant determinants *(30)*. This was also evident in our study, as not all integron bearing strains expressed resistance to antibiotics. Predominance of resistance phenotype Amox/Cla-Amp-Chl-Str-Sul-Tet across different PFGE profiles and integron carriage is strongly suggestive of influence of farm or microbial environment. Isolation of *S*. Typhimurium vars Copenhagen as the major *Salmonella* serovar (95% of the isolates) supports previous reports of the predominance of genotypes of some serotypes circulating among the steers. Clonality (both PFGE and antimicrobial resistance profiles) of *S*. Typhimurium vars Copenhagen was reported in a study that characterized *Salmonella* isolates from feedlot cattle *(14)*, humans, and ready-to-eat-turkey produce *(22)*. This strongly suggests the existence of well-established strains or similarities in antimicrobial usage in the farm environments. Therefore, sur-

veillance of AMR and their determinants in bacteria isolated from food animals and their products is critical to stem the risk they present to humans.

Variations in MDR profiles and integron carriage in *Salmonella* isolated from healthy feedlot steers in this study further support previous reports that MDR may or may not be integron related *(17, 27)*. Additionally, different genotypes were observed to share similar antimicrobial resistance profiles, a strong suggestion of the influence of common environmental (farm) exposure. This phenomenon, which has been observed before by other studies *(8)*, calls for inclusion of farm environments and their antimicrobial drug usage in future studies.

CONCLUSION

This study highlighted the genotypic and phenotypic variations in *Salmonella* isolated in healthy feedlot steers. A common farm environment seemed to influence observed similarities in the resistance profiles of the different genotypes isolated. Although the AMR observed in the majority of *Salmonella* isolates was not matched with presence of integrons, an indication that AMR in *Salmonella* may be explained by determinants or mechanisms other than integrons warrants further research.

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