Journal of Microbiology (2019) Vol. 57, No. 4, pp. 271–280
DOI 10.1007/s12275-019-8421-3
eISSN 1976-3794
pISSN 1225-8873

Genomic surveillance links livestock production with the emergence and spread of multi-drug resistant non-typhoidal *Salmonella* in Mexico[§]

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(Received Aug 2, 2018 / Revised Oct 16, 2018 / Accepted Nov 1, 2018)

Multi-drug resistant (MDR) non-typhoidal *Salmonella* (NTS) is increasingly common worldwide. While food animals are thought to contribute to the growing antimicrobial resistance (AMR) problem, limited data is documenting this relationship, especially in low and middle-income countries (LMIC). Herein, we aimed to assess the role of non-clinical NTS of bovine origin as reservoirs of AMR genes of human clinical significance. We evaluated the phenotypic and genotypic AMR profiles in a set of 44 bovine-associated NTS. For comparative purposes, we also included genotypic AMR data of additional isolates from Mexico (n = 1,067) that are publicly available. The most frequent AMR phenotypes in our isolates involved tetracycline (40/44), trimethoprim-sulfamethoxazole (26/44), chloramphenicol (19/44), ampicillin (18/44), streptomycin (16/44), and carbenicillin (13/44), while nearly 70% of the strains were MDR. These phenotypes were correlated with a widespread distribution of AMR genes (i.e. *tetA*, *aadA*, *dfrA12*, *dfrA17*, *sul1*, *sul2*, *bla*-TEM-1, *bla*CARB-2) against multiple antibiotic classes, with some of them contributed by plasmids and/or class-1 integrons. We observed different AMR genotypes for betalactams and tetracycline resistance, providing evidence of convergent evolution and adaptive AMR. The probability of MDR genotype occurrence was higher in meat-associated isolates than in those from other sources (odds ratio 11.2, 95% confidence interval 4.5–27.9, $P < 0.0001$). The study shows that beef cattle are a significant source of MDR NTS in Mexico, highlighting the role of animal production on the emergence and spread of MDR *Salmonella* in LMIC.

***Keywords*:** antimicrobial resistance, *Salmonella*, genomics, beef production

Introduction

Nontyphoidal *Salmonella* are among the leading causes of foodborne diseases worldwide (WHO, 2015). In the last decade, the emergence of MDR NTS has led to increasing public health concerns at a global scale. Since MDR infections are difficult to treat and are more expensive, they may boost the already high disease burden in humans (Hoffmann *et al.*, 2012). Intense research in this field has improved our understanding of the basis of MDR. It is well established that acquired AMR in bacteria is favored by the use of antibiotics in animals and humans (McEwen and Fedorka-Cray, 2002). Moreover, antibiotic use may lead to bacterial chromosomal mutations conferring resistance to certain antibiotics (i.e. quinolones resistance in enterobacteria) (Qiu *et al.*, 2018), which is then transmitted vertically onto the progeny. However, the horizontal acquisition of foreign mobile DNA is the most relevant factor for the acquisition and dissemination of bacterial AMR. Particularly, plasmids are known to confer resistance to multiple antibiotics and they also can be transmitted by conjugation between distant bacterial species (Chang *et al.*, 2015). Hence, AMR surveillance is vital to contain the proliferation of MDR pathogens and their impact on public health.

Food animals, including beef cattle, are recognized as a possible source of MDR *Salmonella* (Antunes *et al.*, 2006; Talbot *et al.*, 2006). This has led to controversial discussions on whether the increasing AMR problem is associated or not with the use of antibiotics on farms. For instance, it has been demonstrated that livestock environments have a strong influence on the emergence and dissemination of specific MDR phenotypes among NTS isolated in the USA (An *et al.*, 2017). However, most of these isolates are of clinical origin. Although numerous studies have reported phenotypic and genotypic profiles of *Salmonella* AMR (Brichta-Harhay *et al.*, 2011; Lin *et al.*, 2015; Schmidt *et al.*, 2015; Kalambhe *et al.*, 2016), comprehensive studies combining phenotypic data with genome-wide searches for AMR determinants are still limited, especially in low and middle-income countries. This approach could help identifying novel resistance mechanisms, as well as the factors involved in AMR dissemination. Consequently, increased surveillance may facilitate depicting potential mitigation strategies to combat AMR.

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[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Recent studies conducted by our research group showed bovine-associated NTS of different serovars are genotypically close to their counterparts from vegetables, produce, and the environment (Delgado-Suárez *et al.*, 2018). Hence, the current research presents a thorough characterization of the AMR phenotypes and genotypes of a subset of 44 non-clinical NTS isolates from Delgado-Suárez *et al.* (2018). Moreover, we compared the AMR genotypes of these isolates with those isolated from multiple sources within Mexico (n = 1,067). The study reveals the proportion of MDR isolates in the studied sample is very high (≈ 70%), in contrast with that observed in isolates from other sources (< 20%). We also observed evidence of convergent evolution of isolates towards AMR to tetracycline and betalactams, suggesting heavy use of these antibiotics on farms. This highlights the role of livestock production in the emergence and spread of MDR *Salmonella* in Mexico. Although these are not disease-causing strains, the fact that they carry multiple AMR genes against several antibiotics included in the World Health Organization (WHO) list of critically and highly important antimicrobials raises concerns. Indeed, as we confirmed plasmids and class-1 integrons are significant contributors of acquired AMR genes among *Salmonella* populations, the risk of AMR dissemination among NTS and other species is a possibility. Finally, we observed AMR could also be associated with the presence of multidrug efflux pumps and/or triggered by stress response mechanisms, an area that requires further research.

Materials and Methods

Bacterial strains

We used a subset of 44 NTS isolates from a previous study (Delgado-Suárez *et al.*, 2018), for which antibiotic susceptibility data were available. The strain panel was composed of seven different serovars, collected along the beef production continuum and from distant geographical locations within Mexico, providing the basis for a thorough characterization of the resistome of bovine-associated NTS from non-clinical sources (Fig. 1). Every isolate was obtained from a different fecal, carcass, cut, or ground beef sample and some of them on different dates. Refer to Supplementary data Table S1 for strains accession numbers and metadata.

For the purpose of comparative analysis, we used additional NTS isolates from Mexico that are publicly available at NCBI and have AMR genotypic data. This comprised a subset of 65 isolates from the same serovars represented in our panel of strains, as well as the whole set of *Salmonella* isolates from Mexico in the database (n = 1,067) as of July 24, 2018. Supplementary data Table S2 contains the accessions and metadata of the additional isolates used in the study.

Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) was carried out with a panel of 17 antimicrobials included in the WHO list of critically important and highly important antimicrobials (WHO, 2017). We used the Kirby-Bauer disk diffusion method (Bauer *et al.*, 1966) with the following Bio-Rad antimicrobial susceptibility test disks and the respective concentrations: ampicillin (Amp, 10 µg), amoxicillin (Amx, 10 µg), amoxicillin-clavulanic acid (Amc, 30 µg), carbeniciline (Car, 100 µg), ceftriaxone (Cro, 30 µg), cephalothin (Cef, 30 µg), cephotaxime (Ctx, 30 µg), ciprofloxacin (Cip, 10 µg), pefloxacin (Pef, 5 µg), amikacin (Amk, 30 µg), kanamycin (Kan, 30 µg), gentamicin (Gen, 10 µg), streptomycin (Str 10 µg), netilmicine (Net, 30 µg), trimethoprim-sulfamethoxazole (Stx, 250 µg), chloramphenicol (Chl, 30 µg), and tetracycline (Tet, 30 µg). Results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012), using *Escherichia coli* ATCC 8739, *Enterococcus fecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 9027 as quality control organisms. Isolates showing resistance to ≥ 2 classes of antimicrobials were classified as MDR. Odds ratio calculations were used to test if AMR phenotypes were associated with serovar, isolation source and/or geographical location. Moreover, we estimated the association of resistance to different antibiotic classes by calculating Pearson correlation coefficients.

Whole genome sequencing, genome assembly, and annotation

Genome sequencing, assembly, and annotation were performed as previously described (Delgado-Suárez *et al.*, 2018). Raw sequences are deposited at the NCBI Sequence Read Archive (SRA) website. The SRA accessions are listed in Sup-

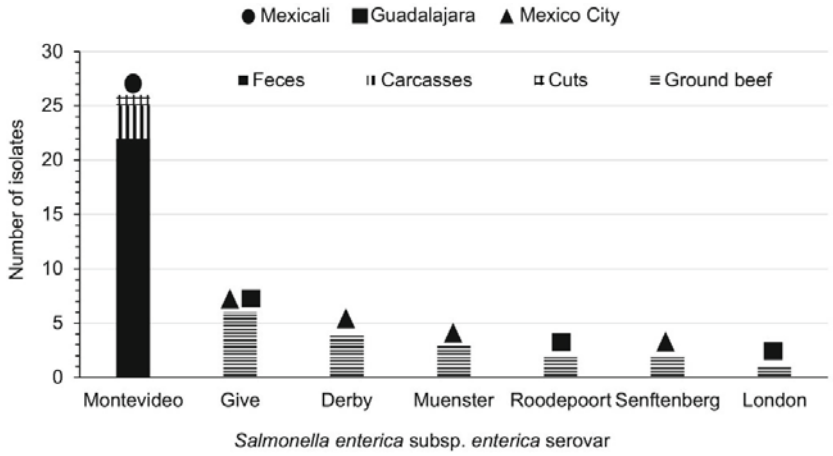


Fig. 1. *Salmonella* isolates included in the study. Isolation source is color-coded, and geographical location is mapped onto the graph with black circles, squares or triangles. For *Salmonella* Give, n = 4 in Mexico City, and n = 2 in Guadalajara. Serovars were predicted with SeqSero software (Zhang *et al.*, 2015).

plementary data Table S1, while assembly and annotation statistics are provided in Supplementary data Table S3.

Identification and comparative genomics of AMR genes

The genotypic AMR profile of our isolates was constructed based on the antibiotic resistance genes identified through genome annotation using the Comprehensive Antibiotic Resistance Database (CARD) as a reference (Jia *et al.*, 2017). We also screened the genomes for chromosomal point mutations conferring resistance to quinolones, colistin, and spectinomycin. For that purpose, we used ResFinder version 3 (Zankari *et al.*, 2012, 2017). Odds ratio was used to assess if the presence of AMR genes against a particular antibiotic class was associated with the presence of resistance genes against other antibiotic classes.

Since polymyxins resistance genes were detected in 100% of the isolates, we used the concatenated amino acid sequences of *arnC* and *arnT* genes for phylogenetic reconstruction. Both genes are part of the lipid A modification operon, which is one of the main mechanisms leading to polymyxin resistance. These sets of sequences were aligned in Seaview (Gouy *et al.*, 2010) through ClustalO (Sievers *et al.*, 2011) and curated with Gblocks (Castresana, 2000). The resulting Nexus file was used to construct a phylogenetic tree using MrBayes 3.2.6 (Ronquist *et al.*, 2012) with the following parameters: aamodelpr = mixed, samplefreq = 100, burninfrac = 0.25 in four chains and for 1 million generations. The analysis included sequences from *Salmonella bongori* (accession CP006608.1) as an outgroup.

Several multidrug efflux pump families were also present in 100% of the studied isolates. To assess their degree of conservation, we conducted a BLAST analysis using as reference the amino acid sequences of genes from the *Salmonella* Typhimurium LT2 genome (accession NC_003197.2): 1) *ydhE*, Multidrug and Toxic Compound Extrusion (MATE family), 2) STM0382, Major Facilitator Superfamily (MFS), 3) *yadG*, ATP Binding Cassette (ABC superfamily), 4) *acrA*, Resistance, Nodulation, Division (RND family), and 5) *mdtC*, *mdtABCD* multi-drug resistance cluster.

Finally, we compared the AMR genotypes of our isolates with a subset (n = 65) of additional *Salmonella* isolates from Mexico that are publicly available at NCBI (see Supplementary data Table S2). These included 18 isolates associated with livestock (bovine and porcine), as well as 47 isolates from fresh produce, vegetables, seafood, cheese, herbal tea, water, and sediment. The criteria of inclusion were: 1) isolates of the same serovars represented in our panel of strains, and 2) isolates with AMR data. In this way, we managed to get the genotypic AMR profile of additional isolates of all serovars but Roodepoort. We also analyzed the complete set of *Salmonella* isolates from Mexico that were available at NCBI (n = 1,067, as of July 24th 2018, see Supplementary data Table S2), to assess the overall diversity of AMR genes and the rate of strains with MDR genomic profiles across different sources and/or hosts. AMR genotypes were collected from the NCBI's isolate browser (https://www.ncbi.nlm.nih.gov/pathogens), which are generated through the AMRFinder tool (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder). Odds ratio and risk ratio calculations were performed to assess the influence of ecological niche and *Salmonella* serovar in the probability of isolates having MDR genotypes.

In silico plasmid profiling

The presence of plasmids replicons was predicted with the aid of PlasmidFinder 1.3 (Carattoli *et al.*, 2014). The analysis

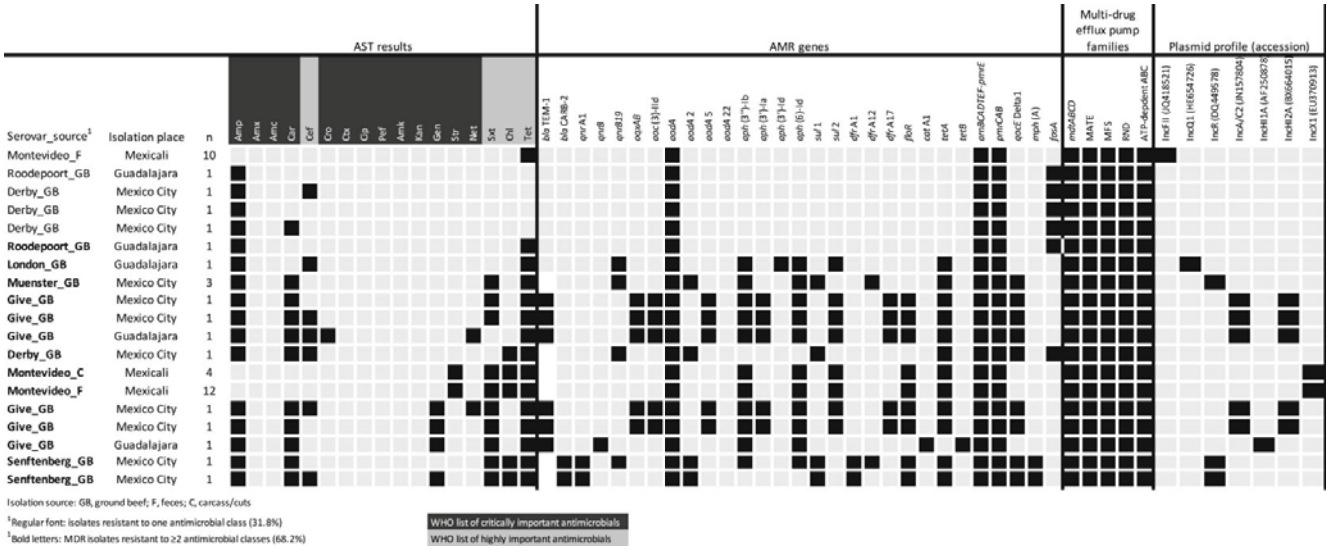


Fig. 2. Antibiotic susceptibility testing (AST), antimicrobial resistance (AMR) genes, and plasmid profile results for *Salmonella* isolates of different serovars collected along the beef production chain in Mexico. Antimicrobials used for AST included ampicillin (Amp), amoxicillin (Amx), amoxicillin clavulanic acid (Amc), carbenicillin (Car), ceftriaxone (Cro), cephalotin (Cef), cephotaxime (Ctx), ciprofloxacin (Cip), pefloxacin (Pef), amikacin (Amk), kanamicin (Kan), gentamicin (Gen), streptomycin (Str), netilmicin (Net), trimethoprim-sulfamethoxazole (Sxt), chloramphenicol (Chl), tetracycline (Tet). Results are color-coded as follows: grey cells = susceptible / AMR gene absent / plasmid replicon absent; black cells = resistant / AMR gene present / plasmid replicon present.

was carried out with the raw reads at the Center for Genomic Epidemiology web server using a threshold identity of 95%. If plasmid replicons were detected, we used the accession numbers of the prediction output to collect the plasmid's reference sequence from NCBI. The newly sequenced genomes were mapped against reference plasmids based on the identification of consecutive genes that were homologous between contigs and plasmids, as previously described (Dhanani *et al.*, 2015). If a majority of reference plasmid sequences (> 70%) were represented in the corresponding isolate's contigs, these sets of contigs were proposed to be plasmids in the newly sequenced genomes.

Results

High frequency of MDR *Salmonella*

Salmonella isolates showed resistance to all the studied antibiotic classes except quinolones (Fig. 2). The most common resistance phenotypes included tetracycline (40/44), trimethoprim-sulfamethoxazole (26/44), chloramphenicol (19/44), ampicillin (18/44), streptomycin (16/44), and carbenicillin (13/44). Conversely, resistance to third generation cephalosporins was infrequent. All isolates were susceptible to cephotaxime, while only one resisted ceftriaxone. Despite six isolates (≈ 14%) that resisted cephalotine, there was no evidence of cross-resistance with any of the 3GC. Likewise, resistance to aminoglycosides other than streptomycin was rare, while all isolates were susceptible to amikacin. Interestingly, aminoglycoside resistance was observed more likely in isolates from Mexicali City as compared to those from Mexico City and Guadalajara (odds ratio, 4.2; 95% confidence interval [CI] 1.1–15.3).

Nearly 70% of the isolates were classified as MDR *Salmonella,* regardless of isolation source, serovar or geographical location. Strikingly, among these, 70% resisted ≥ 4 antibiotic classes. The tetra-resistance pattern Chl/Str/Sxt/Tet was the most prevalent (n = 15) and was strongly associated with *Sal-*

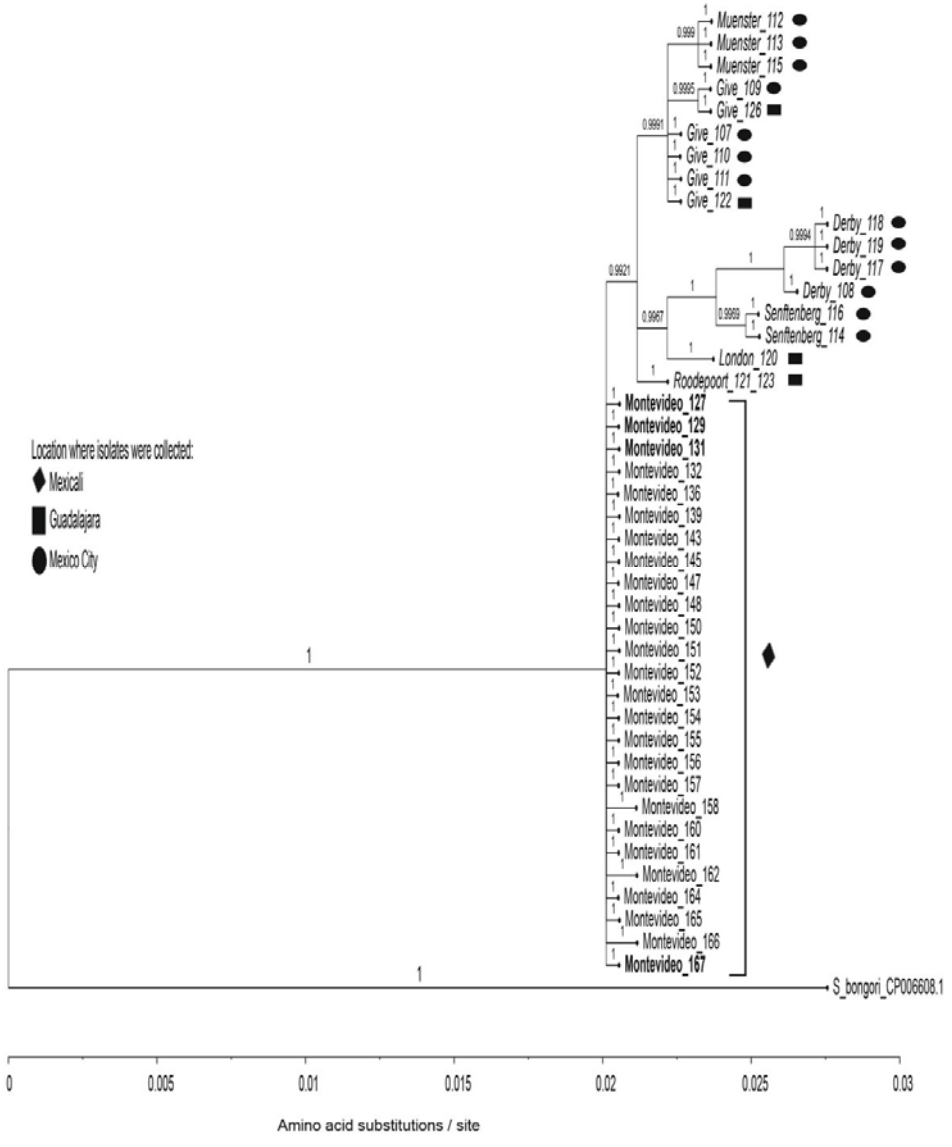
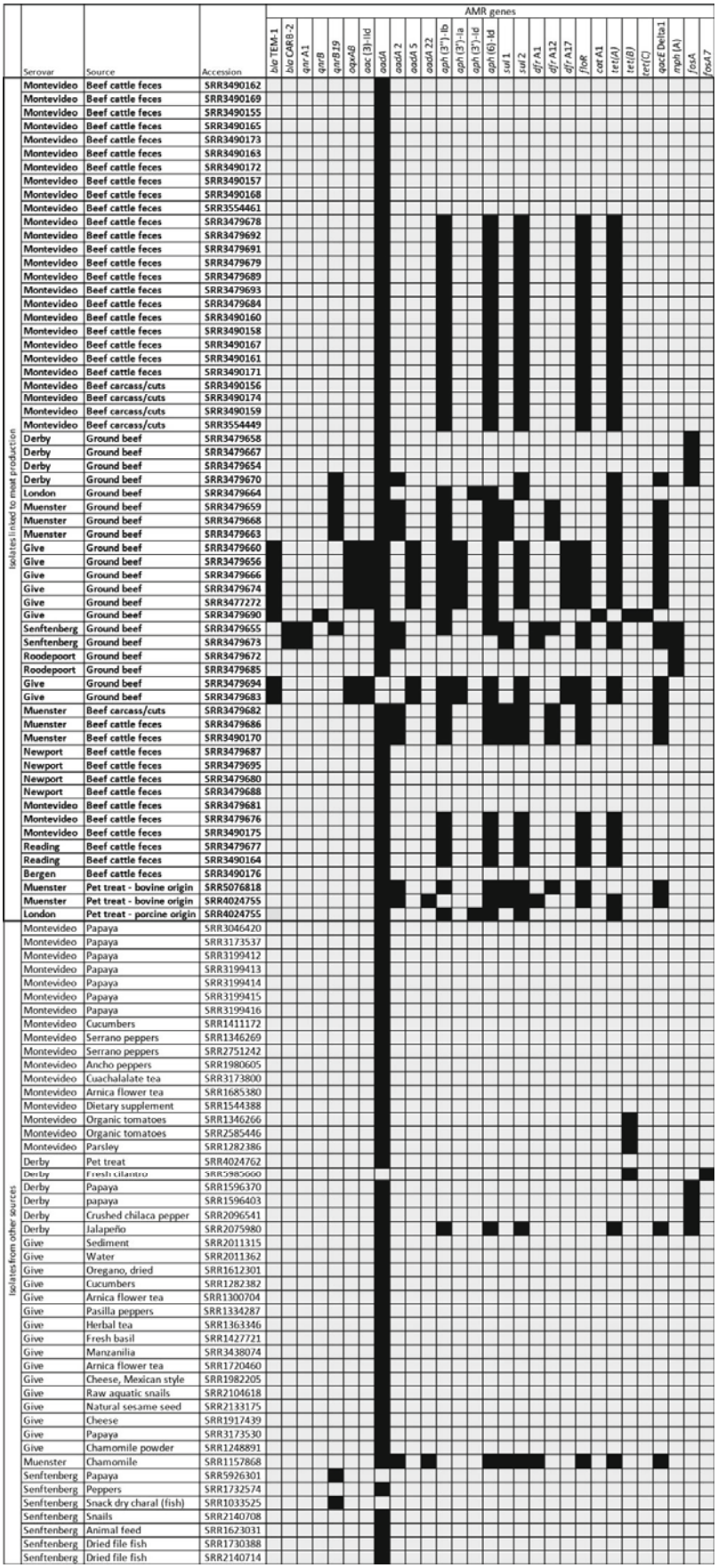


Fig. 3. Concatenated phylogenetic analysis of polymyxin resistance ArnC and ArnT proteins from NTS strains of different serovars. Isolation source is coded according to the font of serovar and sample names: italic, ground beef; bold, carcasses/cuts; regular, feces. The cities where isolates were collected are mapped onto the tree. Posterior probabilities are indicated close to each branch. Analysis conducted with MrBayes 3.2.6. For accession numbers of strains see Supplementary data Table S1.



Serovar	Source	Accession
Montevideo	**Beef cattle feces**	**SRR3490162**
Montevideo	**Beef cattle feces**	**SRR3490169**
Montevideo	**Beef cattle feces**	**SRR3490155**
Montevideo	**Beef cattle feces**	**SRR3490165**
Montevideo	**Beef cattle feces**	**SRR3490173**
Montevideo	**Beef cattle feces**	**SRR3490163**
Montevideo	**Beef cattle feces**	**SRR3490172**
Montevideo	**Beef cattle feces**	**SRR3490157**
Montevideo	**Beef cattle feces**	**SRR3490168**
Montevideo	**Beef cattle feces**	**SRR3554461**
Montevideo	**Beef cattle feces**	**SRR3479678**
Montevideo	**Beef cattle feces**	**SRR3479692**
Montevideo	**Beef cattle feces**	**SRR3479691**
Montevideo	**Beef cattle feces**	**SRR3479679**
Montevideo	**Beef cattle feces**	**SRR3479689**
Montevideo	**Beef cattle feces**	**SRR3479693**
Montevideo	**Beef cattle feces**	**SRR3479684**
Montevideo	**Beef cattle feces**	**SRR3490160**
Montevideo	**Beef cattle feces**	**SRR3490158**
Montevideo	**Beef cattle feces**	**SRR3490167**
Montevideo	**Beef cattle feces**	**SRR3490161**
Montevideo	**Beef cattle feces**	**SRR3490171**
Montevideo	**Beef carcass/cuts**	**SRR3490156**
Montevideo	**Beef carcass/cuts**	**SRR3490174**
Montevideo	**Beef carcass/cuts**	**SRR3490159**
Montevideo	**Beef carcass/cuts**	**SRR3554449**
Derby	**Ground beef**	**SRR3479658**
Derby	**Ground beef**	**SRR3479667**
Derby	**Ground beef**	**SRR3479654**
Derby	**Ground beef**	**SRR3479670**
London	**Ground beef**	**SRR3479664**
Muenster	**Ground beef**	**SRR3479659**
Muenster	**Ground beef**	**SRR3479668**
Muenster	**Ground beef**	**SRR3479663**
Give	**Ground beef**	**SRR3479660**
Give	**Ground beef**	**SRR3479656**
Give	**Ground beef**	**SRR3479666**
Give	**Ground beef**	**SRR3479674**
Give	**Ground beef**	**SRR3477272**
Give	**Ground beef**	**SRR3479690**
Senftenberg	**Ground beef**	**SRR3479655**
Senftenberg	**Ground beef**	**SRR3479673**
Roodepoort	**Ground beef**	**SRR3479672**
Roodepoort	**Ground beef**	**SRR3479685**
Give	**Ground beef**	**SRR3479694**
Give	**Ground beef**	**SRR3479683**
Muenster	**Beef carcass/cuts**	**SRR3479682**
Muenster	**Beef cattle feces**	**SRR3479686**
Muenster	**Beef cattle feces**	**SRR3490170**
Newport	**Beef cattle feces**	**SRR3479687**
Newport	**Beef cattle feces**	**SRR3479695**
Newport	**Beef cattle feces**	**SRR3479680**
Newport	**Beef cattle feces**	**SRR3479688**
Montevideo	**Beef cattle feces**	**SRR3479681**
Montevideo	**Beef cattle feces**	**SRR3479676**
Montevideo	**Beef cattle feces**	**SRR3490175**
Reading	**Beef cattle feces**	**SRR3479677**
Reading	**Beef cattle feces**	**SRR3490164**
Bergen	**Beef cattle feces**	**SRR3490176**
Muenster	**Pet treat - bovine origin**	**SRR5076818**
Muenster	**Pet treat - bovine origin**	**SRR4024755**
London	**Pet treat - porcine origin**	**SRR4024755**
Montevideo	Papaya	SRR3046420
Montevideo	Papaya	SRR3173537
Montevideo	Papaya	SRR3199412
Montevideo	Papaya	SRR3199413
Montevideo	Papaya	SRR3199414
Montevideo	Papaya	SRR3199415
Montevideo	Papaya	SRR3199416
Montevideo	Cucumbers	SRR1411172
Montevideo	Serrano peppers	SRR1346269
Montevideo	Serrano peppers	SRR2751242
Montevideo	Ancho peppers	SRR1980605
Montevideo	Cuachalalate tea	SRR3173800
Montevideo	Arnica flower tea	SRR1685380
Montevideo	Dietary supplement	SRR1544388
Montevideo	Organic tomatoes	SRR1346266
Montevideo	Organic tomatoes	SRR2585446
Montevideo	Parsley	SRR1282386
Derby	Pet treat	SRR4024762
Derby	Fresh cilantro	SRR3985660
Derby	Papaya	SRR1596370
Derby	papaya	SRR1596403
Derby	Crushed chilaca pepper	SRR2096541
Derby	Jalapeño	SRR2075980
Give	Sediment	SRR2011315
Give	Water	SRR2011362
Give	Oregano, dried	SRR1612301
Give	Cucumbers	SRR1282382
Give	Arnica flower tea	SRR1300704
Give	Pasilla peppers	SRR1334287
Give	Herbal tea	SRR1363346
Give	Fresh basil	SRR1427721
Give	Manzanilla	SRR3438074
Give	Arnica flower tea	SRR1720460
Give	Cheese, Mexican style	SRR1982205
Give	Raw aquatic snails	SRR2104618
Give	Natural sesame seed	SRR2133175
Give	Cheese	SRR1917439
Give	Papaya	SRR3173530
Give	Chamomile powder	SRR1248891
Muenster	Chamomile	SRR1157868
Senftenberg	Papaya	SRR5926301
Senftenberg	Peppers	SRR1732574
Senftenberg	Snack dry charal (fish)	SRR1033525
Senftenberg	Snails	SRR2140708
Senftenberg	Animal feed	SRR1623031
Senftenberg	Dried file fish	SRR1730388
Senftenberg	Dried file fish	SRR2140714

Fig. 4. Comparison of AMR genotypes of *Salmonella* isolates associated with meat production (44 from this study and 18 additional Mexican isolates publicly available at NCBI, highlighted with bold letters); and those isolated from fresh produce, vegetables, seafood, cheese, herbal tea, water, and sediment in Mexico (n = 47, publicly available at NCBI). Results are color-coded as follows: grey cells = AMR gene absent; black cells = AMR gene present.

monella enterica ser. Montevideo isolates from Mexicali City (odds ratio 27.2, 95% CI 3.1–237.3, $P = 0.0001$). Overall, resistance to aminoglycosides had a strong positive correlation with resistance to chloramphenicol (r = 0.7286, $P < 0.0001$) and folate pathway inhibitors (r = 0.7025, $P < 0.0001$), whereas it was moderately correlated with resistance to tetracycline (r = 0.3022, $P = 0.0462$). Conversely, none of the isolates from Mexicali City showed resistance to betalactams while 100% of those from Mexico City and Guadalajara resisted at least one of the tested drugs from this class. In line with these findings, resistance to betalactams was negatively correlated with resistance to aminoglycosides (r = -0.3323, $P = 0.0275$).

Genetic determinants of antimicrobial resistance

Genotypic AMR was broadly in agreement with AST results, with about 70% of the studied population carrying a wide repertoire of AMR genes against multiple antibiotic classes (Fig. 2). However, nine isolates lacking obvious betalactamase genes still showed resistance to several of the tested betalactams. Likewise, another 11 isolates that resisted tetracycline do not carry any of the known tetracycline resistance genes. The opposite occurred with aminoglycosides, whereby 24 isolates carrying resistance genes against these drugs showed susceptible phenotypes. This is in line with the lack of point mutations related to aminoglycoside resistance in the genomes of the studied isolates. However, the most commonly found *aadA* gene was highly conserved across isolates, with an amino acid similarity above 99%, as shown by a BLAST analysis using the amino acid sequence of *Salmonella* Typhimurium LT2 (accession NP_460230.1) AadA protein as a reference.

Consistent with the observed susceptibility to quinolones, we did not detect any of the known point mutations associated with quinolone resistance. Interestingly, however, about one third of the isolates carried plasmid-mediated quinolone resistance (PMQR) genes, such as *qnrB*, *qnrB19*, and *oqxAB*. Moreover, genome annotation demonstrated the presence of resistance genes against other antimicrobials that were not included in the AST. Among these, the most relevant are the polymyxin resistance gene clusters (*pmr* and *arn* operons) which were present in the chromosome of 100% of the isolates. Although no point mutations were detected for these operons, phylogenetic analysis showed these lipopolysaccharide (LPS)-modifying genes are highly conserved across serovars, isolation sources, and geographical regions (Fig. 3), suggesting that they are subjected to strong selective pressure. Moreover, it indicates fitness advantages associated with these genes exceed fitness cost.

Another important finding is the presence of genes conferring resistance to quaternary ammonium compounds (*qacE*Delta1 gene) in ground beef isolates from distant geographical regions. Strikingly, the likelihood of isolates being MDR was greater when the *qacE*Delta1 gene was present (odds ratio, 19.3; 95% CI 1.05–354.0, $P = 0.0464$), suggesting possible co-selection of biocide and antibiotic resistance. Possibly related to this, genome annotations showed the entire set of isolates carry a vast repertoire of multi-drug efflux pumps. This included the *mdtABCD* multi-drug resistance cluster, the *acrAB–tolC* efflux system, the ATP Binding Cassette (ABC) superfamily, the Major Facilitator Superfamily (MFS), the Resistance, Nodulation, Division (RND) family, and the Multidrug and Toxic Compound Extrusion (MATE) family. These genomic features were highly conserved across isolates with 99–100% amino acid similarity.

Comparative analysis of AMR genotypes with other isolates from Mexico

We observed a higher abundance and diversity of AMR genes in isolates associated with meat production as compared to those from other sources in Mexico (Fig. 4). Aminoglycoside resistance genes were by far the most commonly present, with over 97% of isolates from all sources and serovars carrying at least one of these genes. However, meat-associated isolates exhibited a higher AMR gene diversity. For instance, 63% of these isolates carry ≥ 3 different aminoglycoside resistance genes. Conversely, in those isolates from other sources only about 4% met this criterion.

The same trend was observed for AMR genes against different antibiotic classes. Nearly 73% of isolates from meat-related sources carry resistance genes against ≥ 2 antibiotic classes, while the same was true only for 19% of isolates from other sources. In fact, the probability of *Salmonella* having

Table 1. Evaluation of risk factors for the occurrence of MDR genotypes in *Salmonella* isolates from Mexico during the period 2013–2018 (n = 1,067), based on ecological niche and serovar

Factor	n[a]	%MDR	Risk ratio	95% C. I.[b]	*P*-value
Ecological niche					
Animal production	200	32.0	2.9	2.2–3.9	< 0.0001
Vegetables & produce	652	11.2	0.5	0.4–0.7	< 0.0001
Environment	167	9.6	0.6	0.4–1.0	0.0316
Salmonella serovar					
Montevideo	45	46.7	3.5	2.4–5.0	< 0.0001
Agona	41	85.4	7.5	5.9–9.5	< 0.0001
Typhimurium	19	31.6	2.1	1.1–4.2	< 0.0001
Derby	10	90.0	6.3	4.8–8.2	< 0.0001
Others[c]	645	7.1	0.7	0.5–1.1	0.1319

[a] The sum by niche or serovar is < 1,067 for there were isolates with no isolation source or serovar indicated. Moreover, niches or serovars with < 10 isolates were excluded. Refer to Supplementary data Table S2 for accessions and metadata of additional isolates.
[b] 95% confidence interval for the risk ratio.
[c] Group of isolates from multiple serovars with an MDR genotype frequency below 10%.

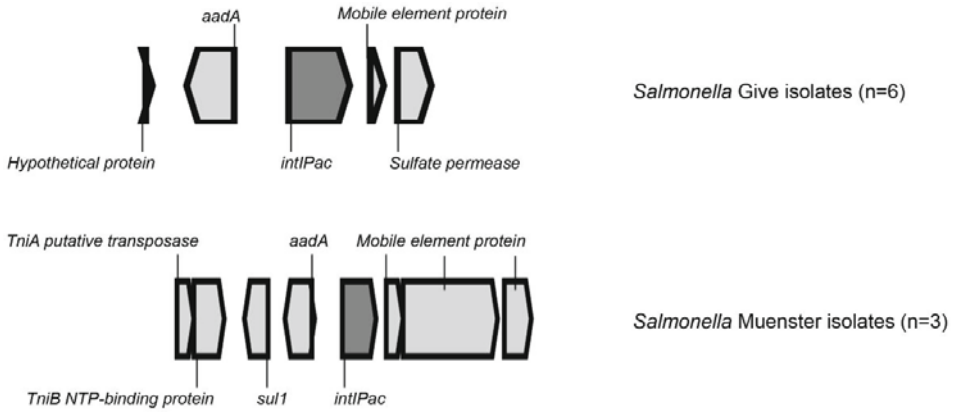


Fig. 5. Schematic representation of the genomic context of class-1 integrons (*intIPac*) detected in *Salmonella* isolates of serovar Give and Muenster.

an MDR genotype was higher in isolates associated with meat production as compared to those from fresh produce, vegetables, seafood, cheese, and the environment (odds ratio 11.2, 95% CI 4.5–27.9, $P < 0.0001$).

We observed similar results when analyzing the whole dataset of isolates from Mexico at NCBI. Again, aminoglycoside resistance genes were the most commonly found in the genomes of NTS of all isolation sources and serovars (95.4%, n = 1,067). Likewise, the risk of MDR-genotype occurrence was nearly 3 times higher in isolates linked to animal production ($P < 0.0001$) as compared to those from vegetables, produce, and the environment (Table 1). It should be noted that isolates of human origin were not included in the analysis because there were only 10 isolates from this ecological niche, with 50% of them having MDR genotypes. Regarding *Salmonella* serovars, the frequency of MDR genotypes was generally low, except for Derby, Agona, Montevideo, and Typhimurium, where the risk of finding MDR genotypes was significantly higher ($P < 0.0001$).

Plasmid profiling

We detected replicons from six different resistance plasmids in over 86% of isolates, except those of *S. enterica* ser. Derby and Roodepoort (Fig. 2). Moreover, several *S. enterica* ser. Montevideo isolates (n = 10) carry replicons of a virulence plasmid of the IncFII incompatibility group. Read mapping of reference plasmids against the newly sequenced genomes suggested these genetic elements are a significant mechanism of acquired AMR. For instance, the entire sequence of the IncQ1 plasmid, predicted in the *S. enterica* ser. London strain, was fully represented in this isolate's genome (see Supplementary data Fig. S1). This plasmid carry aminoglycoside (*strB*, *aph(3")-lb*) and sulphonamide (*sul2*) resistance genes. Likewise, over 90% of the IncHI1A plasmid sequence, which carries a tetracycline resistance gene cluster (*tetRACD*), was present in the genome of one *S. enterica* ser. Give strain (Supplementary data Fig. S2). Moreover, most of IncHI2A plasmid sequence (73.6%) was represented in the genome of *S. enterica* ser. Give strains (Supplementary data Fig. S3). This plasmid carries chloramphenicol (*cat*), tetracycline (*tetRAC*), and aminoglycoside (*aphA*) resistance genes. There was also a group of 16 isolates of *S. enterica* ser. Montevideo that showed 70% sequence identity to the IncX1 plasmid, which carries PMQR (*oqxAB*) and betalactamases (blaTEM) genes (Supplementary data Fig. S4).

We observed a lower representation of plasmid IncA/C2 sequences in the genomes of *S. enterica* ser. Give isolates (55%), as well as of plasmid IncR sequences in the contigs of *S. enterica* ser. Muenster (58%) and Senftenberg (33.3–46.6%), respectively (Supplementary data Figs. S5–S6). However, isolates carrying replicons of either plasmid encode class-1 integrons and multiple AMR genes in their chromosomes (Fig. 2), which may have contributed to their MDR phenotypes. Nevertheless, most AMR genes in these isolates were outside the integron structure, which harbors only one or two resistance gene cassettes against aminoglycosides and/or sulphonamides (Fig. 5).

Discussion

Our results show that there is a high rate of MDR *Salmonella* among isolates collected from various non-clinical sources of bovine origin in Mexico. The MDR prevalence observed here is much higher than that reported 5–7 years ago in isolates from cattle and other food-producing animals in Africa (Sibhat *et al.*, 2011), Asia (Van *et al.*, 2012), or Latin America (Perez-Montaño *et al.*, 2012; Junod *et al.*, 2013). Although AMR of isolates usually vary across time and regions, these results show the emergence and spread of MDR pathogens is far from being controlled.

The most common resistance patterns (i.e. tetracycline, folate pathway inhibitors, amphenicols, betalactams, aminoglycosides) are similar to those reported previously in *Salmonella* isolated from beef sources (Varela-Guerrero *et al.*, 2013; Schmidt *et al.*, 2015; Quesada *et al.*, 2016). These findings are consistent with the current approval of these antibiotic classes for use in livestock in Mexico (SAGARPA, 2018). Conversely, we observed that resistance to third generation cephalosporins was low in isolates from the studied regions as compared to that observed in recent studies (Schmidt *et al.*, 2015; Mir *et al.*, 2016; Quesada *et al.*, 2016). Although these are not disease-causing strains, they carry multiple resistance determinants against several drugs of the WHO list of critically important and highly important antimicrobials (WHO, 2017). Hence, they may act as reservoirs of AMR genes of human clinical significance, posing a serious public health risk. Moreover, the significant correlation of resis-

tance to unrelated antibiotic classes led us to speculate a possible role of epistatic interactions leading to MDR phenotypes, an area that requires further research.

The extensive distribution of MDR isolates indicates animal production likely favors the emergence of MDR *Salmonella* genotypes in farm environments, mainly in the form of adaptive and acquired AMR. In that sense, our results showed the presence of different genetic determinants associated with the same resistance phenotype (i.e. tetracycline and betalactam resistance), which provides evidence of the convergent evolution of isolates as a result of environmental pressure. Overall, these findings implicate that several antibiotics are still heavily used in some livestock production settings in Mexico. Such practices put huge selective pressure on AMR genes. Consequently, optimizing the use of antimicrobials at the farm level is vital to reduce the risk of AMR gene dissemination, as well as human exposure to MDR *Salmonella*.

Comparative genomics showed there is a higher abundance and diversity of AMR genes in meat-associated and animal-associated isolates as compared to those collected from plant sources. While we did not find data on the consumption of antibiotics in either animal or plant production in Mexico, the use of antibiotics is known to be heavier in animals (OECD, 2016). Hence, these results suggest there is a link between the more intensive use of antibiotics in livestock and a stronger AMR genotypic profile of *Salmonella* collected from animal sources. Although the number of isolates included in the analysis is large (n = 1,067), we only included publicly available genomes. Therefore, it would be interesting to test whether this trend remains as more isolates from both sources are sequenced.

The fact that plasmids and class-1 integrons contributed resistance genes confirms they play a major role in the dissemination of acquired AMR. Class-1 integrons are frequently present in *Salmonella* isolates. This feature allows the pathogen to acquire resistance genes and disseminate them across its own and other bacterial species. In line with previous studies, we observed aminoglycoside and sulphonamide resistance genes are among the most common gene cassette arrays associated with class-1 integrons (Meng *et al.*, 2011; Van *et al.*, 2012). Thus, the considerable proportion of resistant isolates to both antimicrobials in the studied samples is not surprising. This could also be one of the factors contributing to the widespread distribution of aminoglycoside resistance genes observed in the whole set of *Salmonella* isolates from food sources in Mexico that are publicly available at NCBI.

In this research, all strains carrying resistance plasmid replicons were MDR. This is further supported by the fact that all of these isolates carry toxin-antitoxin systems (i.e. *ccdAB*, *stbED*), which ensures that all of the progeny inherits the plasmid, stabilizing its fitness. Although the very nature of plasmids makes them attractive antimicrobial targets, it has been difficult to design feasible strategies to combat AMR through plasmid elimination. Interventions aimed at disrupting plasmid propagation, as well as toxin-antitoxin systems were deemed as promising strategies about a decade ago (Lujan *et al.*, 2007; Williams and Hergenrother, 2008). However, the little progress that has been achieved in this area suggests that this is a strategic field of research that needs to be promoted.

The widespread distribution of highly-conserved multidrug efflux pumps among the studied isolates suggests they are strong contributors to the observed AMR phenotypes. For instance, the *mdfA* gene, which belongs to the MFS efflux system, is known to confer resistance to a broad spectrum of drugs, including rifamycins and tetracycline (Edgar and Bibi, 1997). Likewise, upregulation of the *acrAB-tolC* efflux system by the *marRAB* operon confers resistance to ciprofloxacin, tetracycline and chloramphenicol (Swick *et al.*, 2011). Given these facts, it is reasonable to consider multidrug efflux pump genes as potential targets for the development of novel antimicrobials.

Notably, our genotype data suggests that the pathogens may have an even stronger resistance phenotype than that suggested by AST. For instance, nearly one third of the isolates carry PMQR genes, which usually provide low-level quinolone resistance, below the CLSI breakpoint (Strahilevitz *et al.*, 2009). Hence, these isolates are usually susceptible to quinolones when subjected to AST. However, PMQR genes could lead to higher resistance phenotypes if the isolates are exposed to quinolones at therapeutic levels. Despite the lack of point mutations associated with quinolone resistance in the studied genomes, the presence of multiple PMQR genes (i.e. *oqxAB*, *qnrA*, *qnrB*) is worrisome since it has been linked to the emergence of ciprofloxacin resistant *Salmonella* (Karczmarczyk *et al.*, 2010; Lin *et al.*, 2015). Furthermore, their presence in mobile genetic elements is an additional factor contributing to dissemination of fluoroquinolone resistance genes among bacterial pathogens of different species.

Along the same lines, considering the complex regulation of AMR responses in bacteria, isolates carrying aminoglycoside resistance genes that were susceptible in the AST may also show resistance in a human clinical setting. This is further supported by the BLAST analysis showing a high degree of conservation of the *aadA* gene, which was the most common aminoglycoside resistance gene among isolates. Similarly, the presence of macrolide, phosphomycin, and polymyxin resistance genes further implicates the significant source of therapeutic complications pose by these strains. Although the studied isolates did not carry mutations in these genes, it has been demonstrated that colistin resistance in *Salmonella* does not correlate with point mutations (Quesada *et al.*, 2015). Fortunately, the risk of horizontal transfer of these particular AMR genes appears to be low, considering none of them seemed to be plasmid-borne. At least for polymyxins, there is an increasing prevalence of plasmid-carried resistance determinants (i.e. *mcr-1* gene) in Gram-negative bacteria across the globe, while reports in *Salmonella enterica* are still limited to some European countries (Baron *et al.*, 2016). Additional research involving gene expression under varied growing conditions may help complement our understanding of the actual risks associated with the AMR of environmental isolates.

Apart from antimicrobials, bacterial pathogens also deal with numerous stresses both inside the hosts and in the environment. These stresses can trigger various adaptive responses in the cell leading to changes in their antimicrobial susceptibility. For instance, resistance to betalactams and polymyxin B activates when the cell is exposed to cell wall-

active agents (Poole, 2012). In this study, the likelihood of isolates being MDR was greater when the *qacE*Delta1 gene was present. Probably, the membrane damage stress exerted by quaternary ammonium compounds promotes the recruitment of resistance determinants, leading to MDR phenotypes. Thus, further research on the influence of different stresses on the pathogen antimicrobial susceptibility, as well as the potential use of stress-response genes as therapeutic targets may lead to significant advances in AMR mitigation strategies.

In summary, this research showed that beef cattle represent an important reservoir of AMR genes of significant connotation for human health. It also provides comprehensive AMR data from a middle-income country, highlighting the role of animal production in these nations as a significant contributor to the emergence and spread of MDR *Salmonella*. Moreover, it emphasizes the need for continued AMR surveillance of *Salmonella* strains circulating in apparently healthy animals to improve public health protection. Of particular interest is the widespread distribution of highly conserved multidrug efflux systems in *Salmonella* populations, which likely contributes to MDR phenotypes. We believe this is a promising research topic that may help to fight bacterial resistance more efficiently. Our results also emphasize the relevance of class-1 integrons and plasmids in promoting acquired AMR. In that sense, it is important to develop feasible plasmid propagation control strategies to reduce the spread of AMR genes among bacterial populations. Finally, further research on stress-induced AMR may shed additional light on the dissemination of AMR in food-associated isolates.

Acknowledgements

This work was partially funded by the National Autonomous University of Mexico, grant number PAPIIT IN212817. Whole genome sequencing of isolates was funded by the US Food and Drug Administration (FDA) through the GenomeTrakr Program. The authors are grateful for the technical assistance with experiments provided by professionals from the Center for Food Safety and Applied Nutrition, College Park, Maryland, U.S.A., and from the Infectious Diseases Molecular Epidemiology Laboratory, College of Veterinary Medicine, Ohio State University.

Conflict of Interest

The authors declare no conflict of interest.

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