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Genomic analysis of azithromycin-resistant *Salmonella* from food animals at slaughter and processing, and retail meats, 2011–2021, United States

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ABSTRACT Azithromycin, a 15-membered ring macrolide, is among the recommended antimicrobials for treating invasive salmonellosis in humans. It is not approved for use in veterinary medicine. We analyzed the U.S. National Antimicrobial Resistance Monitoring System (NARMS) culture collections (~40,700) between 2011 and 2021 from food animals at slaughter and processing and retail meats, and identified 31 azithromycin-resistant *Salmonella* spp. with the first occurrence in 2015. These isolates belonged to 12 *Salmonella* serovars and possessed one or more macrolide resistance determinants: *erm*(42), *mef*(C), *mph*(A), *mph*(E), *mph*(G), and *msr*(E) or a point mutation (*acrB*_R717L), of which *mph*(A) was dominant (61.3%). Compared with azithromycin-susceptible controls, these determinants accounted for up to 256-fold MIC increases against azithromycin with MIC_{50} and MIC_{90} increased by 32- and 8-fold, respectively. We report the first detection of an *mph*(G)-*mef*(C)-*mph*(E)-*msr*(E)-containing *Salmonella* Agona isolate with very high-level azithromycin resistance (1,024 µg/mL) and the first detection of *acrB*_R717L accounting for azithromycin resistance in nontyphoidal *Salmonella* serovars in the United States. Plasmids of diverse replicon types were identified, with 86.2% carrying multidrug resistance including azithromycin and ceftriaxone, or decreased susceptibility to ciprofloxacin. This report also highlights an emerging *mph*(A)-containing (on an IncR plasmid) *Salmonella* Newport clone of cattle/beef origin with high-level azithromycin resistance (128 µg/mL) and decreased susceptibility to ciprofloxacin (0.25 µg/mL). Further work is needed to better understand the drivers of emerging azithromycin resistance in nontyphoidal *Salmonella* associated with food animal sources.

IMPORTANCE Macrolides of different ring sizes are critically important antimicrobials for human medicine and veterinary medicine, though the widely used 15-membered ring azithromycin in humans is not approved for use in veterinary medicine. We document here the emergence of azithromycin-resistant *Salmonella* among the NARMS culture collections between 2011 and 2021 in food animals and retail meats, some with co-resistance to ceftriaxone or decreased susceptibility to ciprofloxacin. We also provide insights into the underlying genetic mechanisms and genomic contexts, including the first report of a novel combination of azithromycin resistance determinants and the characterization of multidrug-resistant plasmids. Further, we highlight the emergence of a multidrug-resistant *Salmonella* Newport clone in food animals (mainly cattle) with both azithromycin resistance and decreased susceptibility to ciprofloxacin. These findings contribute to a better understating of azithromycin resistance mechanisms in *Salmonella* and warrant further investigations on the drivers behind the emergence of resistant clones.

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Nontyphoidal *Salmonella* is a leading cause of foodborne diarrheal diseases worldwide (1). In the United States, an estimated 1.35 million *Salmonella* infections occur annually, resulting in an estimated 26,500 hospitalizations, 420 deaths, and $400 million in direct medical costs (2). Though usually self-limiting, antimicrobial therapy is necessary for extraintestinal *Salmonella* infections (1). Antibiotics such as ciprofloxacin (a fluoroquinolone drug), azithromycin (a 15-membered ring macrolide), and ceftriaxone (a third-generation cephalosporin) are sometimes used to manage severe salmonellosis (2). Resistant *Salmonella* infections can be more severe especially when resistance genes are present on plasmids bearing additional virulence mechanisms, leading to higher hospitalization rates and even deaths (2).

The use of azithromycin in treating salmonellosis is an exception to general macrolide indications, as membrane permeability barriers in Enterobacterales cause intrinsic resistance to most macrolides (3). Azithromycin, the first semisynthetic, 15-membered ring macrolide, is an azalide derived from the best known 14-membered erythromycin (4). Due to its favorable permeability, low toxicity, and broad antimicrobial activity, azithromycin is now one of the most frequently prescribed antibiotics for various Gram-negative infections in humans, including campylobacteriosis, salmonellosis, shigellosis, and traveler's diarrhea (5–7). It is not approved for use in veterinary medicine including food animals. Historically, resistance to azithromycin has been rare in *Salmonella* (8). Mechanisms of resistance, which primarily include target alteration by mutations (at 23S rRNA position A2058 or A2059) or methylations (encoded by *erm* genes), drug modification by esterases (encoded by *ere* genes) or phosphotransferases (encoded by *mph* genes), and reduced drug concentration by efflux pumps (such as those encoded by *mef* genes), are not yet fully understood (9).

Established in 1996, the U.S. National Antimicrobial Resistance Monitoring System (NARMS) collects data on antimicrobial resistance (AMR) in enteric bacteria from humans, animals, and raw retail meats (10). *Salmonella* is a major pathogen tracked by NARMS, and azithromycin has been incorporated into the NARMS Gram-negative antimicrobial susceptibility testing (AST) plate since 2011 (10). As there are no Clinical and Laboratory Standards Institute (CLSI)-defined azithromycin breakpoint for nontyphoidal *Salmonella*, NARMS has established an interpretive standard for azithromycin resistance monitoring in *Salmonella* based on CLSI's breakpoint for *Salmonella* Typhi (3). For routine genotypic AMR prediction in *Salmonella*, whole-genome sequencing (WGS) has been fully incorporated into the NARMS program (11), and long-read sequencing technologies, such as PacBio, have been used for further in-depth genomic analysis (12, 13). Out of approximately 34,200 and 6,500 *Salmonella* isolates NARMS recovered from food animals and retail meats between 2011 and 2021, respectively, we selected 31 azithromycin-resistant isolates for this study. We also included 14 azithromycin-susceptible isolates as controls: some possessed potential azithromycin resistance determinants; some had borderline MIC values for azithromycin; others were initially found to be azithromycin resistant but upon retesting were determined to be actually susceptible.

This study aimed to document the emergence of azithromycin resistance in NARMS *Salmonella* isolates from food animals and retail meats and elucidate the genetic determinants, predominantly on multidrug-resistant (MDR) plasmids, that were responsible for azithromycin resistance. For this work, we conducted AST using agar dilution with expanded azithromycin concentrations, performed short-read and long-read sequencing to identify AMR genes and their genomic contexts (surrounded by class 1 integrons and insertion sequence elements), and analyzed emerging azithromycin-resistant *Salmonella* clones using data available at the National Center for Biotechnology Information (NCBI).

RESULTS

Metadata summary

Table 1 shows key metadata for all 45 *Salmonella* isolates examined in this study. The 31 azithromycin-resistant *Salmonella* represented 12 serovars (Agona, *n* = 2; Anatum, *n* = 1; Bredeney, *n* = 1; Derby, *n* = 1; I 4,[5],12:i:-, *n* = 8, Mbandaka, *n* = 1; Meleagridis, *n* = 1; Newport, *n* = 11; Ohio, *n* = 1; Schwarzengrund, *n* = 2; Senftenberg, *n* = 1; and Typhimurium, *n* = 1), of which Newport and I 4,[5],12:i:- were predominant. Each serovar had one unique multilocus sequence typing (MLST) sequence type (ST) except I 4,[5],12:i:-, which had three STs. Of these, two (ST34 and ST3224) differed by a single *sucA* allele, and the third previously uncharacterized ST differed from ST34 by another single allele *purE*. Most isolates (*n* = 28) were recovered from food animals at slaughter [13 cattle (8 products, 4 cecal, and 1 lymph node), 11 swine (9 cecal and 2 products), 2 chicken (products), 1 sheep (cecal), and 1 turkey (product)], and 3 from retail meats (2 pork chops and 1 ground turkey). The isolation years ranged from 2015 to 2021 (no azithromycin resistance was detected from 2011 to 2014) with all *Salmonella* Newport and most (75%) *Salmonella* I 4,[5],12:i:- detected from 2018 to 2021. The isolation locations spanned 18 U.S. states, which was led by Texas (*n* = 6).

MIC distributions by azithromycin resistance determinants

Table 2 shows MIC distributions of the 31 azithromycin-resistant *Salmonella* (test group) and 14 susceptible ones (control group), and in subgroups possessing different azithromycin resistance determinant profiles. For isolates in the test group, MICs ranged from 32 to 1,024 µg/mL with MIC_{50} and MIC_{90} both at 128 µg/mL. Control group isolates had much lower MICs with MIC_{50} and MIC_{90} at 4 and 16 µg/mL, respectively. This translates to up to 256-fold MIC increases between azithromycin-susceptible and resistant ones.

Six azithromycin resistance determinant profiles were identified, namely, *mph*(A) alone (*n* = 18), *mph*(E)-*msr*(E) (*n* = 6), *erm*(42) (*n* = 3), *acrB*_R717L (*n* = 2), *mph*(A)-*mph*(E)-*msr*(E) (*n* = 1), and *mph*(G)-*mef*(C)-*mph*(E)-*msr*(E) (*n* = 1). The *mph*(A), *mph*(E), and *mph*(G) genes code for macrolide 2′-phosphotransferases and *erm*(42) encodes 23S rRNA methyltransferase, whereas others are efflux pump related: *mef*(C) in the MFS family, *msr*(E) in the ABC family, and *acrB* (R717L mutation) in the RND family (9, 14). The highest azithromycin MIC (1,024 µg/mL) was observed in a *Salmonella* Agona isolate, 18IA01PC08-S2 [Center for Veterinary Medicine (CVM) N18S0017], with the four-gene combination, whereas most *mph*(A)-containing isolates had a high MIC of 128 µg/mL (Table 2).

Three *Salmonella* controls harbored *ere*(A), *mef*(B), or *mph*(A) but were susceptible to azithromycin. Genomic analysis showed that the *ere*(A) gene in *S.* I 4,[5],12:i:- isolate 20MO07PC10-S1 (CVM N20S0154) was truncated (missing the first 62 amino acids), whereas the promoter region of *mph*(A) in *S.* Typhimurium isolate Food Safety and Inspection Service (FSIS)12032448 was also truncated by 24 bp. Both the *mef*(B) gene and its promoter in *S.* Johannesburg isolate 19MN02PC03 (CVM N19S0223) remained intact. Eleven other *Salmonella* controls were initially selected based on their phenotypic resistance to azithromycin or carrying potential macrolide resistance genes. AST and WGS performed at CVM showed they differed from the original designations. This was due to the loss of plasmids containing *mph*(A) or *ere*(A), or AST test variations for borderline isolates (azithromycin MICs around 16 µg/mL). For three isolates in the latter group, no mutations in the 23S rRNA gene were found.

Co-resistance to ceftriaxone and/or with decreased susceptibility to ciprofloxacin

As shown in Table 1, among the 31 azithromycin-resistant *Salmonella* isolates, 4 (1 *S.* Agona, 1 *S.* Mbandaka, and 2 *S.* I 4,[5],12:i:-) were co-resistant to ceftriaxone (MIC ≥4 µg/mL) and 16 (1 *S.* Bredeney, 4 *S.* I 4,[5],12:i:-, 10 *S.* Newport, and 1 *S.* Ohio) with

TABLE 1 *Salmonella* (*n* = 45) examined in this study from U.S. food animals at slaughter and processing and retail meats

Serovar	Multilocus sequence type[a]	Isolate[b,c]	Year	State	Source[d]	MIC (µg/mL)[e]		
						Azithromycin	Ceftriaxone	Ciprofloxacin
Test group (*n* = 31)								
Agona (*n* = 2)	13	18IA01PC08-S2 (CVM[f] N18S0017)	2018	IA	Pork chop bone-in	1024	16	≤0.015
		FSIS21821150[g]	2018	NC	Comminuted turkey	64	≤0.25	≤0.015
Anatum (*n* = 1)	64	FSIS1710858 (F18S049)	2017	TX	Heifer (cecal)	128	≤0.25	≤0.015
Bredeney (*n* = 1)	3806	FSIS1702037	2017	IL	Market swine (cecal)	64	≤0.25	4
Derby (*n* = 1)	40	15MN07GT04-S (CVM N58646)	2015	MN	Ground turkey	32	≤0.25	0.03
I 4,[5],12:i:- (*n* = 8)	34	17SC12PC01-S1 (CVM N17S1465)	2017	SC	Pork chop	64	≤0.25	0.03
		FSIS11809860	2018	VA	Market swine (cecal)	64	16	1
		FSIS11920112	2019	VA	Market swine (cecal)	64	≤0.25	0.03
		FSIS11922707	2019	NC	Market swine (cecal)	128	≤0.25	0.5
		FSIS21925668	2019	MI	Raw ground pork	128	≤0.25	1
	3224	FSIS31901558	2019	NY	Raw ground pork	64	≤0.25	1
	NF	FSIS1609549 (F18S030)	2016	IN	Market swine (cecal)	512	8	≤0.015
		FSIS12035116	2020	SD	Market swine (cecal)	128	≤0.25	0.06
Mbandaka (*n* = 1)	413	FSIS11814474	2018	IN	Market swine (cecal)	128	32	≤0.015
Meleagridis (*n* = 1)	463	FSIS1607675	2016	KS	Raw intact beef	64	≤0.25	≤0.015
Newport (*n* = 11)	132	FSIS11814458	2018	TX	Steer (cecal)	128	≤0.25	≤0.015
		FSIS11816184	2018	TX	Comminuted beef	128	≤0.25	0.25
		FSIS31903059	2019	UT	Raw intact beef	128	≤0.25	0.25
		FSIS12027867	2020	CA	Comminuted beef	128	≤0.25	0.25
		FSIS12034723	2020	TX	Comminuted beef	128	≤0.25	0.25
		FSIS12105828	2021	TX	Beef cow (cecal)	128	≤0.25	0.25
		FSIS12106020	2021	TN	Comminuted beef	128	≤0.25	0.25
		FSIS12142912	2021	CA	Mature sheep (cecal)	128	≤0.25	0.25
		FSIS22130757	2021	TX	Steer (lymph node)	128	≤0.25	0.25
		FSIS32104969	2021	WI	Comminuted beef	128	≤0.25	0.25
		FSIS32105981	2021	IL	Comminuted beef	128	≤0.25	0.25
Ohio (*n* = 1)	329	FSIS11808786	2018	CA	Market swine (cecal)	128	≤0.25	0.5
Schwarzengrund (*n* = 2)	96	FSIS1608447	2016	AL	Raw intact chicken	128	≤0.25	≤0.015
		FSIS1609433	2016	GA	Comminuted chicken	128	≤0.25	≤0.015
Senftenberg (*n* = 1)	14	FSIS11813680	2018	KS	Steer (cecal)	32	≤0.25	≤0.015
Typhimurium (*n* = 1)	19	FSIS11704063 (F18S031)	2017	MN	Market swine (cecal)	128	≤0.25	0.03
Control group (*n* = 14)								
I 4,[5],12:i:- (*n* = 2)	34	20MO07PC10-S1 (CVM N20S0154)[c]	2020	MO	Pork chop	4	≤0.25	0.25
		20SD07PC06-S1 (CVM N20S0157)	2020	ND	Pork chop	4	≤0.25	0.25
Infantis (*n* = 1)	NF	FSIS12033262	2020	IA	Market swine (cecal)	4	≤0.25	≤0.015
Johannesburg (*n* = 2)	471	FSIS1709981	2017	MO	Market swine (cecal)	16	≤ 0.25	0.06

(*Continued on next page*)

TABLE 1 *Salmonella* (*n* = 45) examined in this study from U.S. food animals at slaughter and processing and retail meats (*Continued*)

Serovar	Multilocus sequence type[a]	Isolate[b,c]	Year	State	Source[d]	MIC (µg/mL)[e]		
						Azithromycin	Ceftriaxone	Ciprofloxacin
19MN02PC03 (CVM N19S0223)[c]	2019	MN	Pork chop	8	≤0.25	≤0.015		
Kentucky (*n* = 2)	152	11MD11CB03-S (CVM N38232)	2011	MD	Chicken breast	4	≤0.25	≤0.015
		FSIS1609498	2016	NY	Chicken carcass	16	8	0.03
Newport (*n* = 4)	132	FSIS21924576	2019	TX	Raw intact beef	4	≤0.25	≤0.015
		FSIS12033266	2020	FL	Comminuted beef	4	≤0.25	≤0.015
		FSIS12035959	2020	TX	Steer (cecal)	4	≤0.25	≤0.015
		FSIS22106147	2021	MO	Comminuted beef	4	≤0.25	≤0.015
Reading (*n* = 1)	412	21OH06GT03-S1 (CVM N21S0376)	2021	OH	Ground turkey	4	≤0.25	≤0.015
Typhimurium (*n* = 2)	19	FSIS11815075	2018	KS	Heifer (cecal)	16	8	0.5
		FSIS12032448[c]	2020	NC	Bob veal (cecal)	4	64	≤0.015

[a]NF stands for not found, suggesting a new multilocus sequence type.
[b]Alternative IDs are provided for some isolates because historically different IDs were used at NCBI or in the literature for designating these isolates and corresponding plasmids.
[c]Three control isolates possessed nonfunctional azithromycin resistance genes.
[d]For Food Safety and Inspection Service isolates, those without parentheses were from product sample testing at slaughter. Cecal and lymph node samples are not destined for food.
[e]Breakpoints used were ≥32 mg/mL for azithromycin (a 15-membered ring macrolide) and ≥4 mg/mL for ceftriaxone (a third-generation cephalosporin). Decreased susceptibility to ciprofloxacin at ≥0.12 mg/mL was used as a marker for emerging fluoroquinolone resistance.
[f]CVM, Center for Veterinary Medicine.
[g]FSIS, Food Safety and Inspection Service.

TABLE 2 Azithromycin MIC distributions among 45 *Salmonella* isolates from U.S. food animals at slaughter and processing and retail meats

Group	Azithromycin resistance determinant	Serovar (no. of isolates)	No. of isolates with MIC (µg/mL) of[a]									MIC_{50}	MIC_{90}
			4	8	16	32	64	128	256	512	1,024		
Test	All (*n* = 31)	See below				2	7	20		1	1	128	128
	mph(A) (*n* = 18)	Newport (11), Schwarzengrund (2), Agona (1), I 4,[5],12:i:- (1), Mbandaka (1), Ohio (1), Typhimurium (1)					1	17					
	mph(E)-*msr*(E) (*n* = 6)	I 4,[5],12:i:- (6)					4	1		1			
	erm(42) (*n* = 3)	Anatum (1), Meleagridis (1), Senftenberg (1)				1	1	1					
	*acrB*_R717L (*n* = 2)	Bredeney (1), Derby (1)				1	1						
	mph(A)-*mph*(E)-*msr*(E) (*n* = 1)	I 4,[5],12:i:- (1)						1					
	mph(G)-*mef*(C)-*mph*(E)-*msr*(E) (*n* = 1)	Agona (1)									1		
Control	All (*n* = 14)	See below	10	1	3							4	16
	None (*n* = 11)	Newport (4), Kentucky (2), I 4,[5],12:i:- (1), Infantis (1), Johannesburg (1), Reading (1), Typhimurium (1)	8		3								
	ere(A)[b] (*n* = 1)	I 4,[5],12:i:- (1)	1										
	mef(B) (*n* = 1)	Johannesburg (1)		1									
	mph(A)[b] (*n* = 1)	Typhimurium (1)	1										

[a]None of the isolates had MICs from <0.125 to 2 mg/mL or >1,024 mg/mL. MIC_{50} and MIC_{90} values were not calculated for subgroups with different azithromycin resistance determinants due to low numbers of isolates.
[b]These genes were either truncated or lacking the promoter region.

decreased susceptibility to ciprofloxacin (MIC ≥0.125 µg/mL). One *S*. I 4,[5],12:i:- isolate FSIS11809860 was resistant to both azithromycin (MIC = 64 µg/mL) and ceftriaxone (MIC = 16 µg/mL) and also with decreased susceptibility to ciprofloxacin (MIC = 1 µg/mL).

The bla_{CMY-2} gene (three on IncC plasmids and one on a IncP6 plasmid) accounted for ceftriaxone resistance among the four isolates, whereas various *qnr* genes (*qnrA1*, *qnrB6*, and *qnrB19*), carried on diverse plasmid replicon types [Col440I/Col(pHAD28), IncC, IncFIB(K), IncHI2/IncHI2A, IncHI1B(R27)/IncHI1A/IncFIA(HI1), IncR, and IncY], were identified among 15 out of 16 isolates with decreased susceptibility to ciprofloxacin. The single isolate without *qnr* genes, *S*. I 4,[5],12:i:- FSIS11922707, possessed a chromosomal mutation (*gyrA*_D87N). Another isolate, *S*. Bredeney FSIS1702037, harbored both *qnrB* [on a Col440I/Col(pHAD28) helper plasmid] and a chromosomal mutation, *gyrA*_D87G, with the highest ciprofloxacin MIC of 4 µg/mL. This isolate also carried *acrB*_R717L mutation which accounted for its azithromycin resistance phenotype (64 µg/mL).

Azithromycin-resistant plasmids

All azithromycin resistance determinants were mapped to plasmids except for the *acrB*_R717L mutation in two isolates. The 29 closed plasmids belonged to various incompatibility groups (IncR, *n* = 11; IncC, *n* = 3; IncHI2/IncHI2A, *n* = 3; IncQ1, *n* = 3; others, *n* = 9), with 8 containing 2 or more replicon types (Table 3). The sizes ranged from 10,966 bp for an IncQ1 plasmid to 326,166 bp for an IncHI1B(pNDM-CIT)/IncHI1A(NDM-CIT) plasmid. All were MDR (resistant to ≥3 drug classes) except for three IncQ1 plasmids carrying *erm*(42) only and one IncP6 plasmid harboring *mph*(E)-*msr*(E). All but six plasmids contained the biocide resistance gene *qacEdelta1* (one to three copies), and one also carried *qacG2*. Twelve plasmids contained heavy metal resistance genes, with as many as 18 genes resistant to four heavy metals in one isolate.

Figure 1A shows the genomic contexts among 20 *mph*(A)-containing plasmids from food animal sources, 11 of which were *S*. Newport IncR plasmids. Not surprisingly, mobile genetic elements such as class 1 integrons and insertion sequence (IS) elements were frequently observed. Nine out of the 20 *mph*(A)-containing plasmids carried the class 1 integron integrase gene *intI1*, 8 of which had a class 1 integron proximal to *mph*(A)-containing MDR, biocide, and heavy metal resistance genes, and various IS elements. A BLAST query of pFSIS12106020 (*S*. Newport, IncR) as a representative *mph*(A)-containing plasmid revealed multiple matches with *Escherichia coli* plasmids, including CP104124 and CP043753. Of the 4 plasmids that contained the complete *mph*(A) cluster [IS*26*-*mph*(A)-*mrx*-*mphR*-IS*6100*], 1 was from *S*. Agona (pFSIS21821150) and 3 others were from *S*. Newport (pFSIS12106020, pFSIS31903059-1, and pFSIS32104969).

Figure 1B shows the genetic environments for 8 plasmids harboring *mph*(E)-*msr*(E); all except 1 (pN18S0017; Agona) were from *Salmonella* serovar I 4,[5],12:i:-. Again, IS elements were frequently observed. In 4 plasmids (pN17S1465-1, pFSIS11920112-1, pFSIS11922707-1, and pFSIS11809860-1), *mph*(E)-*msr*(E) was located between IS*15* and IS*ce29* elements, downstream from a class 1 integron. On pFSIS31901558-2, *mph*(E)-*msr*(E) was also located between IS*15* and IS*ce29* elements but was not proximal to a class 1 integron. On pFSIS21925668-1, *mph*(A)-*mph*(E)-*msr*(E) was downstream of an IS*15* element. The gene cassette *mph*(G)-*mef*(C)-*mph*(E)-*msr*(E) was upstream of IS*Vsa3* on pN18S0017, while *mph*(E)-*msr*(E) alone was upstream of IS*Vsa3* on pF18S030-2.

Similar genetic backgrounds were found among three *erm*(42)-containing plasmids from different *Salmonella* serovars recovered from food animals (Fig. 1C). All were small IncQ1 plasmids with the *erm*(42) gene upstream from an IS*Va3* element. On pF18S049, this gene was immediately downstream of another IS element, IS*Vch6*. The presence of these IS elements again suggests the mobility of this resistance gene.

Figure 2A through D depicts circular structures of 4 plasmids harboring *mph*(A), *mph*(E)-*msr*(E), *mph*(A)-*mph*(E)-*msr*(E), and *mph*(G)-*mef*(C)-*mph*(E)-*msr*(E), respectively. These MDR plasmids carried genes conferring resistance to five to seven drug classes, including beta-lactams or quinolones, as well as biocides and heavy metals. Specifically, the *S*. Agona IncHI2 plasmid (pFSIS21821150, Fig. 2A) harbored AMR genes to five drug

TABLE 3 Azithromycin-resistant *Salmonella* plasmids ($n = 29$) from U.S. food animals at slaughter and processing and retail meats

Azithromycin resistance gene	Host serovar	Plasmid	Replicon type	Size	Antimicrobial, biocide, and heavy metal resistance genes (order of appearance on plasmid)[a]
erm(42)	Anatum	pF18S049	IncQ1	12,060	*erm*(42)
	Meleagridis	pFSIS1607675	IncQ1	10,966	*erm*(42)
	Senftenberg	pFSIS11813680-1	IncQ1	10,967	*erm*(42)
mph(A)	Agona	pFSIS21821150	IncHI2	273,370	***terW, terZ, terD***, *aph(6)-Id*, *aph(3")-Ib*, *bla*$_{TEM-1}$, *aph(3′)-Ia*, *aph(6)-Id*, *aph(3″)-Ib*, *tet*(B), *mph*(A), *sul1*, *qacEdelta1*, *aac(3)-VIa*, *aadA1*,***silE, silS, silR, silC, silF, silB, silA, silP, pcoA, pcoB, pcoC, pcoD, pcoR, pcoS, pcoE***
	I 4,[5],12:i:-	pFSIS12035116-1	IncHI2A/IncN/IncHI2	244,678	***terW, terZ, terD***, *aph(3')-Ia*, *floR*, *sul3*, *lnu*(F), *aadA22*, *mph*(A), *arr*, *dfrA14*
	Mbandaka	pFSIS11814474-1	IncC	163,603	*dfrA12*, *aadA2*, *qacEdelta1*, *sul1*, *mph*(A), *aph(3')-Ia*, *qacG2*, ***merE, merD, merB, merA, merP, merT, merR***, *floR*, *tet*(A), *aph (6)-Id*, *aph(3")-Ib*, *sul2*, *bla*$_{CMY-2}$
	Newport	pFSIS12034723	IncR	53,263	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS31903059-1	IncR	53,263	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS11816184-1	IncR	46,951	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS12027867	IncR	46,951	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS32104969	IncR	46,951	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS32105981	IncR	46,951	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS12105828	IncR	46,950	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS12106020	IncR	46,950	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS12142912	IncR	46,950	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS22130757	IncR	46,950	*mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *bla*$_{CARB-2}$, *qnrA1*, *sul1*, *qacEdelta1*, *dfrA1*, *tet*(A), *floR*
		pFSIS11814458	IncR	40,637	*floR*, *tet*(A), *dfrA1*, *qacEdelta1*, *sul1*, *mph*(A)
	Ohio	pFSIS11808786-1	IncHI1B(R27)/IncHI1A/IncFIA(HI1)	210,427	*tet*(B), ***merR, merT, merP, merC***, *mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *dfrA12*, *aac (3)-IId*, *bla*$_{TEM-1}$
	Schwarzengrund	pFSIS1609433-1	IncFIA(HI1)/IncQ1/IncHI1B(R27)/IncHI1A	222,461	***arsR, arsD***, *tet*(B), ***merR, merT, merP, merC***, *dfrA12*, *aadA2*, *qacEdelta1*, *sul1*, *mph*(A), *aac(3)-IId*, *bla*$_{TEM-1}$, *sul2*, *aph(3")-Ib*, *aph(6)-Id*
		pFSIS1608447-1	IncHI1B(R27)/IncHI1A/IncQ1	183,376	*tet*(B), ***merR, merT, merP, merC***, *dfrA12*, *aadA2*, *qacEdelta1*, *sul1*, *mph*(A), *aac(3)-IId*, *bla*$_{TEM-1}$, *sul2*, *aph(3")-Ib*, *aph(6)-Id*
	Typhimurium	pF18S031-1	IncHI1B(pNDM-CIT)/IncHI1A(NDM-CIT)	326,166	*catA1*, ***merR, merT, merP, merC***, *aac(3)-Iva*, *aph(3')-Ia*, *aph(3')-Ia*, *aph(3')-Ia*, *aph(3')-Ia*, *mph*(A), *sul1*, *qacEdelta1*, *aadA2*, *dfrA12*,***arsC, terD, terZ, terW***
mph(A)-*mph*(E)-*msr*(E)	I 4,[5],12:i:-	pFSIS21925668-1	IncFIB(K)	89,414	*sul1*, *qacEdelta1*, *qnrB6*, *sul1*, *qacEdelta1*, *aadA16*, *dfrA27*, *arr-3*, *aac(6′)-Ib-cr5*, *mph*(A), *mph*(E), *msr*(E), *bleO*, *aac(3)-IId*, *aph(3′)-Ia*
mph(E)-*msr*(E)	I 4,[5],12:i:-	pFSIS11920112-1	IncHI2/IncHI2A	257,103	*sul1*, *qacEdelta1*, *aph(3')-Ia*, *mcr-9.1*, ***pcoS, arsC,*** *aac(6′)-Ie/aph(2″)-Ia*, ***terW, terZ, terD, merE, merD, merA, merT, merR,****mph*(E), *msr*(E), *armA*
		pN17S1465-1	IncHI2/IncHI2A	249,408	***arsC, terW, terZ, terD, merE, merD, merA, merT, merR***, *mph*(E), *msr*(E), *armA*, *sul1*, *qacEdelta1*, *aph(3')-Ia*, ***pcoS***

(*Continued on next page*)

TABLE 3 Azithromycin-resistant *Salmonella* plasmids ($n = 29$) from U.S. food animals at slaughter and processing and retail meats (*Continued*)

Azithromycin resistance gene	Host serovar	Plasmid	Replicon type	Size	Antimicrobial, biocide, and heavy metal resistance genes (order of appearance on plasmid)[a]
		pFSIS11922707-1	IncHI2/IncHI2A	247,657	*aac(6')-Ie/aph(2")-Ia*, ***arsC, pcoS***, *mcr-9.1*, *aac(6')-Ie/aph(2")-Ia*, *aph(3')-Ia*, *dfrA1*, *aadA5*, *qacEdelta1*, *sul1*, *armA*, *msr*(E), *mph*(E), ***terD, terZ, terW***
		pFSIS11809860-1	IncC	175,970	*bla*$_{CMY-2}$, *sul2*, *aph(3")-Ib*, *aph(6)-Id*, *tet*(A), *floR*, ***merR, merT, merP, merA, merB, merD, merE***, *sul1*, *qacEdelta1*, *aadA2*, *aph(3')-Ia*, *mph*(E), *msr*(E), *armA*, *sul1*, *qacEdelta1*, aadA5, *dfrA1*
		pFSIS31901558-2	IncY	79,718	*bleO*, *mph*(E), *msr*(E), *armA*
		pF18S030-2	IncP6	17,931	*mph*(E), *msr*(E)
mph(G)-*mef*(C)-*mph*(E)-*msr*(E)	Agona	pN18S0017	IncC	180,114	***merR, merT, merP, merA, merB, merD, merE,****bla*$_{CMY-2}$, *aadA1*, *aac(3)-VIa*, *qacEdelta1*, *sul1*, *sul2*, *mph*(G), *mef*(C), *mph*(E), *msr*(E), *tet*(D)

[a]Biocide resistance genes are underlined, whereas heavy metal resistance genes are in bold.

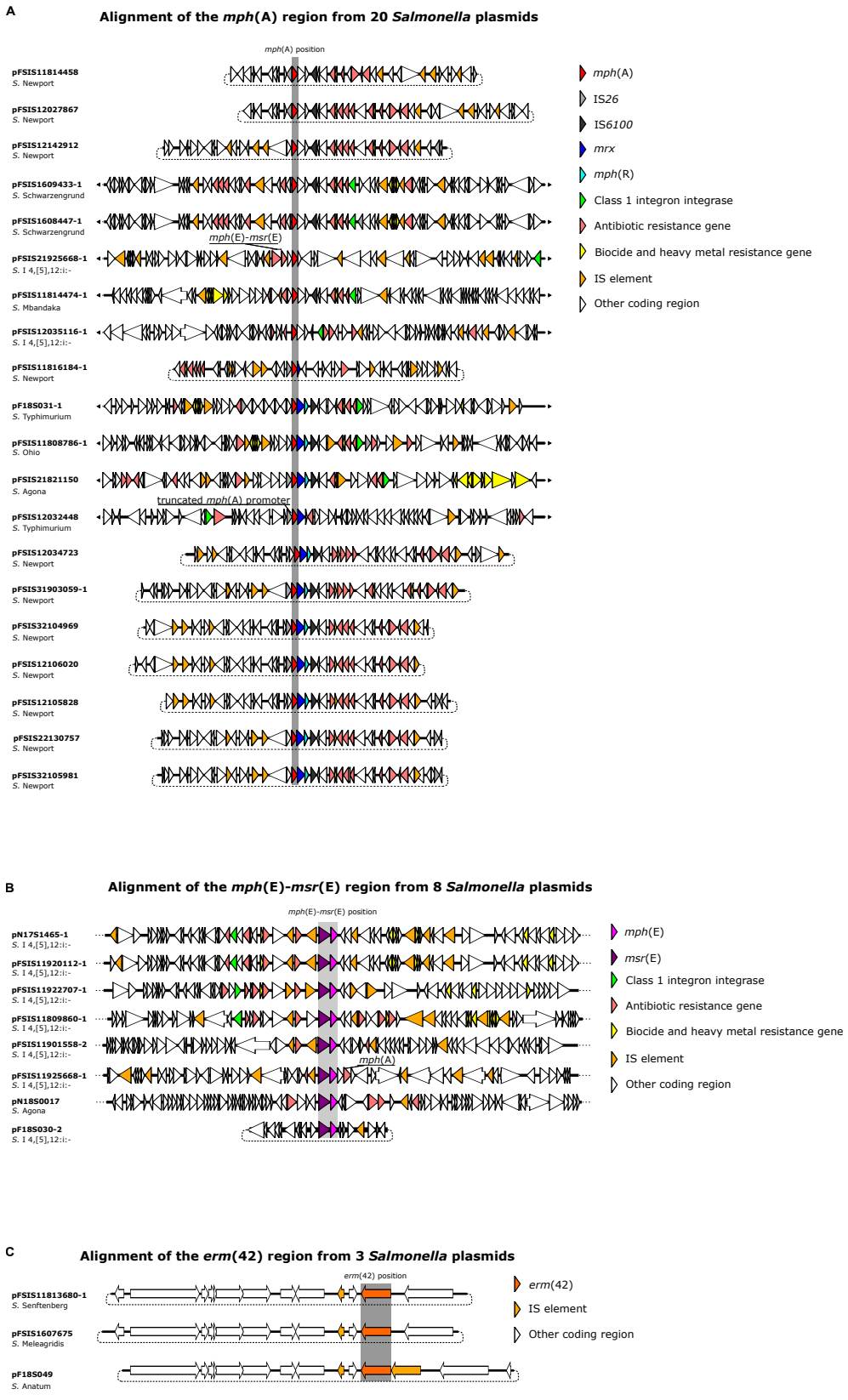

FIG 1 Genomic environments among 20 *mph*(A)-containing plasmids (A), 8 *mph*(E)-*msr*(E)-containing plasmids (B), and *3* *erm*(42)-containing plasmids (C).

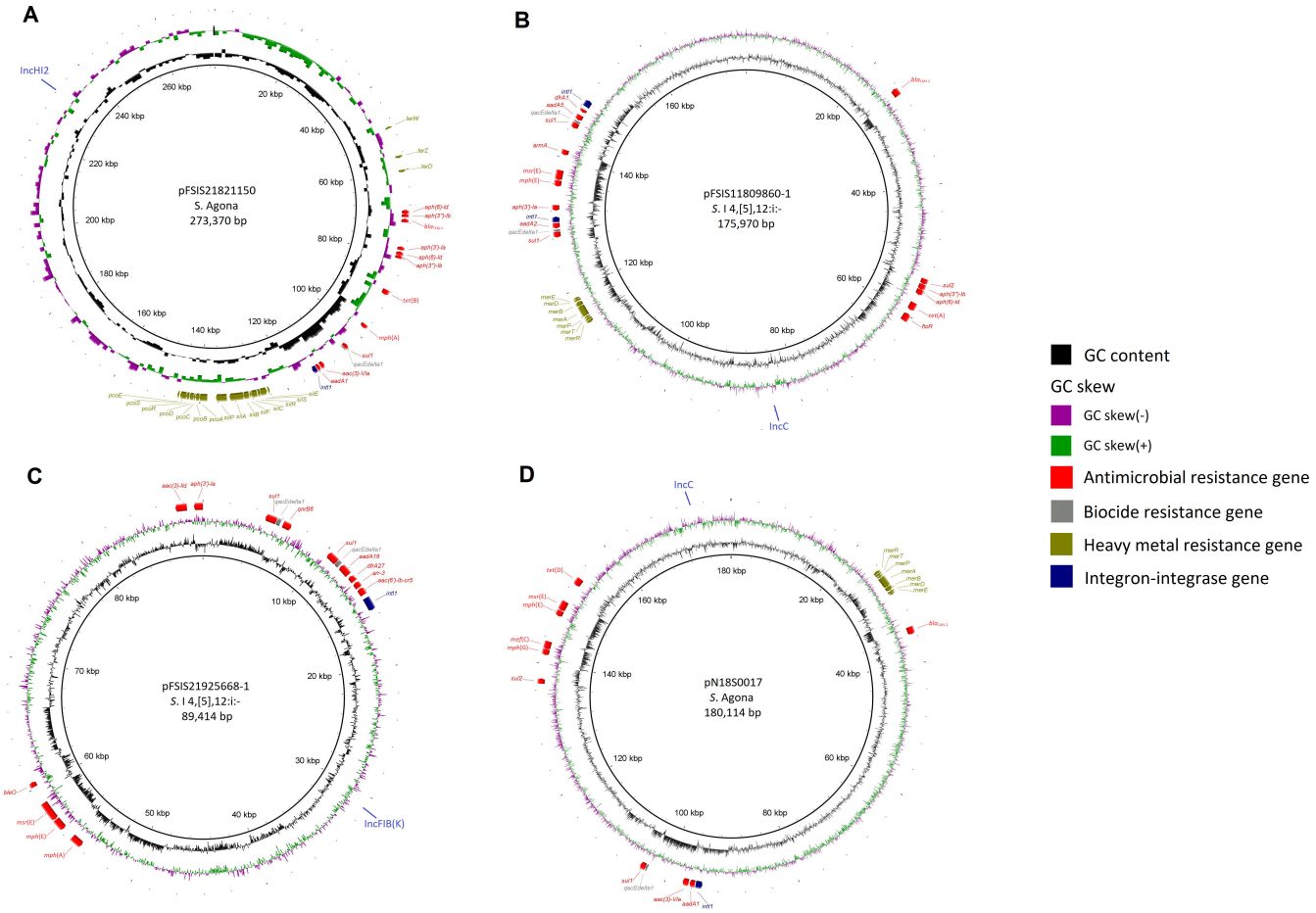

FIG 2 Structures of 4 MDR plasmids harboring *mph*(A) [pFSIS21821150 (A)], *mph*(E)-*msr*(E) [pFSIS11809860-1 (B)], *mph*(A)-*mph*(E)-*msr*(E) [pFSIS21925668-1 (C)], and *mph*(G)-*mef*(C)-*mph*(E)-*msr*(E) [pN18S0017 (D)], respectively.

classes, including beta-lactams (bla_{TEM-1}), *qacEdelta1*, eighteen heavy metal resistance genes, and *intI*. The *S.* I 4,[5],12:i:- IncFIB(K) plasmid (pFSIS21925668-1, Fig. 2C) contained AMR genes to seven drug classes, including fluoroquinolones [*aac*(*6'*)-*Ib-cr5* and *qnrB6*], two copies of *qacEdelta1*, and *intI*. The 2 IncC plasmids, from *S.* I 4,[5],12:i:- (pFSIS11809860-1; Fig. 2B) and *S.* Agona (pN18S0017, Fig. 2D), both carried bla_{CMY-2} conferring resistance to third-generation cephalosporins, seven mercury resistance genes, and *intI*. Notably, the *S.* I 4,[5],12:i:- isolate FSIS11809860 also carried *qnrB19* on a small plasmid (not shown), conferring decreased susceptibility to ciprofloxacin.

Emerging MDR *S.* Newport and *S.* I 4,[5],12:i:- clones of cattle and swine origins, respectively

Of the 11 azithromycin-resistant *S.* Newport isolates, 10 were from cattle and 1 from sheep. Ten harbored MDR IncR plasmids (a representative one pFSIS12106020 shown in Fig. 3) that carried AMR genes for seven drug classes, conferring resistance to aminoglycosides (streptomycin), beta-lactams (ampicillin), folate pathway inhibitors (sulfisoxazole and trimethoprim), macrolides (azithromycin), phenicols (chloramphenicol), quinolones (decreased susceptibility to ciprofloxacin), and tetracyclines (tetracycline). These *S.* Newport isolates were found in a large NCBI single nucleotide polymorphism (SNP) cluster PDS000007781.898 [*n* = 3,060; 94% clinical and 86.4% carrying *mph*(A)]. Clinical isolates were from the United States (*n* = 2,398), United Kingdom (*n* = 82), Mexico (*n* = 72), Canada (*n* = 15), and Chile (*n* = 5). Among 160 environmental isolates, 82 were from the United States; 72 were from Mexico; and 5 were from Chile. All but 14 U.S. isolates

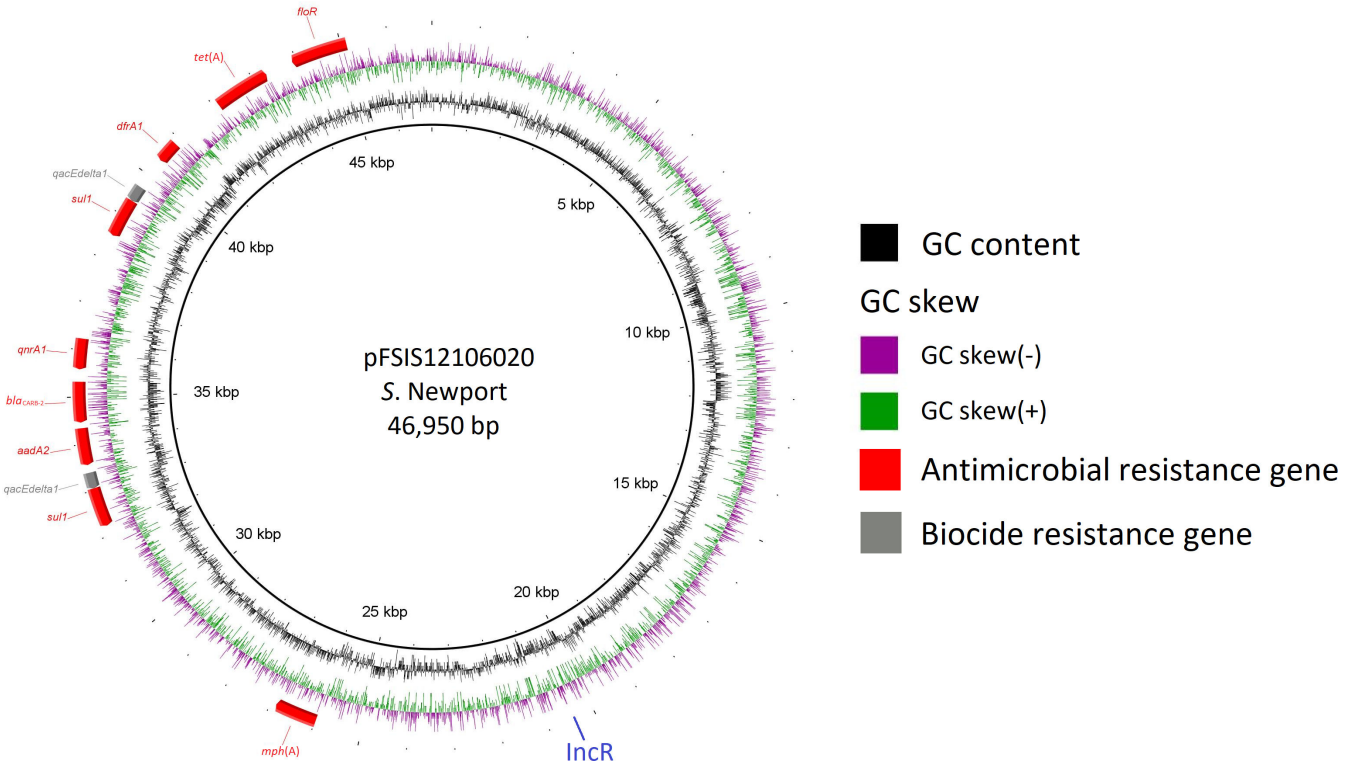

FIG 3 The structure of a representative MDR IncR plasmid (pFSIS12106020) from the emerging *Salmonella* Newport clone of cattle/beef origin.

were from beef sources (82.9%). Isolates from Mexico were mainly from river/canal/pond (68.1%) and beef (27.8%), whereas all five Chilean isolates were from river samples. Except for 75 isolates recovered from 2016 to 2017, the remaining 2,985 isolates in this SNP cluster were from 2018 onward (15).

All 8 azithromycin-resistant *S.* I 4,[5],12:i:- isolates were of swine origin and carried MDR plasmids of six incompatibility groups [IncC and IncFIB(K) plasmids shown in Fig. 2B and C, respectively]. Five isolates were found in a large NCBI SNP cluster PDS000159488.2 (n = 13,464; 78% clinical and 1.9% carrying various azithromycin resistance genes). The majority (66.3%; 1,465 out of 2,211) of environmental isolates in the cluster were from swine/pork sources. Both clinical and environmental isolates spanned globally and temporally with the earliest isolation in 2001 (15).

DISCUSSION

Both World Health Organization and U.S. Food and Drug Administration (FDA) have classified macrolides as critically important antimicrobials for human medicine, along with third-generation cephalosporins and quinolones/fluoroquinolones (16, 17). All three drug classes are also considered critically important for veterinary medicine, though the widely used 15-membered ring macrolide azithromycin in humans is not approved for use in veterinary medicine (18). We report here the detection of *Salmonella* serovars in food animals and retail meats with co-resistance to ceftriaxone (a third-generation cephalosporin) or decreased susceptibility to ciprofloxacin (a fluoroquinolone) and provide insights into the underlying genetic mechanisms and genomic contexts on MDR plasmids. Further, the report highlights the emergence of an MDR *S.* Newport clone in food animals (mainly cattle, also sheep) in the United States with azithromycin resistance and decreased susceptibility to ciprofloxacin.

Though NARMS food animal and retail meat arms started routine azithromycin AST for *Salmonella* in 2011, occurrence of resistance was first detected in 2015 in an *S.* Derby isolate from ground turkey that had the *acrB*_R717L mutation (Table 1). During 2016, the

S. I 4,[5],12:i:- clone with *mph*(E)-*msr*(E) was first detected, in addition to one *erm*(42)-containing *Salmonella* Meleagridis and two *mph*(A)-containing *Salmonella* Schwarzengrund isolates. In 2018, we detected the highly resistant *S*. Agona isolate harboring *mph*(G)-*mef*(C)-*mph*(E)-*msr*(E) and additional serovars (Agona, Mbandaka, Newport, and Ohio) containing *mph*(A). This study clearly documents the sequential detection of azithromycin resistance in *Salmonella*, including the *S*. Newport clone of cattle/beef origin in 2018. This trend can also be observed in the real-time display of NARMS monitoring data accessible through the NARMS Now dashboard online (19).

As noted earlier, NARMS monitoring of azithromycin resistance in nontyphoidal *Salmonella* was based on the CLSI's breakpoint (≥32 µg/mL) for *Salmonella* Typhi. Our study (Table 2) and others (unpublished data) provided further evidence that *Salmonella* MICs to azithromycin do not differ among serovars. This breakpoint also agrees with the European Committee on Antimicrobial Susceptibility Testing's epidemiological cut-off value, which distinguishes *Salmonella* likely resistant to azithromycin from a wild-type susceptible population (20).

The predominance of *mph*(A) in our study isolates corroborates with numerous reports on azithromycin resistance mechanisms in Enterobacterales (9, 21–23). Four *S*. Newport isolates (FSIS21924576, FSIS12033266, FSIS12035959, and FSIS22106147) have lost the *mph*(A)-containing plasmids [searching up these isolates in NCBI pathogen detection isolates browser (15) still shows the presence of this gene as those reflect original testing results], suggesting plasmid instability/mobility. The finding of one *mph*(A)-harboring *Salmonella* Typhimurium isolate with a truncated promoter region is interesting, suggesting a new mechanism accounting for the lack of azithromycin resistance phenotype in *Salmonella* harboring *mph*(A). A recent in-depth characterization of two *Salmonella* conjugative plasmids showed the complete genetic makeup of the *mph*(A) cluster [IS*26*-*mph*(A)-*mrx*-*mphR*-IS*6100*], and the deletion of *mphR* restored azithromycin susceptibility (24). Four study isolates (three *S*. Newport and one *S*. Agona) had the complete *mph*(A) cluster and adjacent class 1 integron, suggesting the transferability of this gene cluster.

The *mph*(E) gene was co-located with *msr*(E) among all study isolates, supporting previous reports that they are part of a common operon in Gram-negative bacteria of different origins, including livestock (9, 25, 26). These two genes are often found on plasmids flanked by IS*26* sequences associated with Tn1548::*armA* transposon, which facilitates the spread (25, 26). Agreeably, *armA* was found in five (out of eight) *S*. I 4,[5],12:i:- containing *mph*(E)-*msr*(E). Further, we detected one *S*. I 4,[5],12:i:- isolate from swine containing *mph*(E)-*msr*(E) with adjacent *mph*(A) on an IncFIB(K) plasmid and one *S*. Agona isolate harboring the *mph*(G)-*mef*(C)-*mph*(E)-*msr*(E) cluster on an IncC plasmid. The MIC to azithromycin in the latter isolate was eightfold higher than those harboring *mph*(A) alone. A recent study reported the first detection of Shiga toxin-producing *E. coli* in France carrying plasmid-borne *mef*(C)-*mph*(G) (27). Another report showed that *mef*(C)-*mph*(G) was carried on various vectors including plasmids and integrative and conjugative elements (ICEs) among marine and wastewater bacteria in Asian countries (28), and yet another study reported the occurrence of *mef*(C)-*mph*(G) in bacteria from the aquatic environment (29). The latter study also showed that *mef*(C) alone did not influence azithromycin susceptibility of *E. coli*, but *mph*(G) alone did, with more dramatic increases when both were introduced, suggesting a synergistic effect (29). To the best of our knowledge, ours is the first report on *mph*(G)-*mef*(C)-*mph*(E)-*msr*(E)-containing *Salmonella* with high-level azithromycin resistance.

The contribution of *erm*(42) to azithromycin resistance in *Salmonella* has been confirmed recently by direct cloning using an IncFIB/IncHI1B plasmid from *Klebsiella pneumoniae* (30). This gene has been detected earlier on a type 2 IncA/C2 plasmid from two *Salmonella* serovars (Ohio and Senftenberg) from swine in Australia (31). Two very recent studies in Taiwan reported an *erm*(42)-carrying ICE in 26.4% of MDR *Salmonella* Albany from human salmonellosis (32) as well as in *Salmonella* Enteritidis and *S*. Typhimurium IS*26* composite transposons in the chromosomes (33). To our best

knowledge, the genetic context of the three *Salmonella* serovars containing *erm*(42) reported in this study (on IncQ plasmids) is novel. Mutation in the RND-type transporter gene (*acrB*_R717L/Q), which has been reported mostly in *S.* Typhi and *S.* Paratyphi A, has been confirmed recently to confer azithromycin resistance in *E. coli* by cloning (34, 35). The first Arg717 substitution was predicted to have emerged around 2010 with travel-related R717Q mutant found in the United Kingdom (36). To our best knowledge, this is the first report of *acrB*_R717L in nontyphoidal *Salmonella* in the United States.

Whether, and to what degree, the wide use of other 15-membered ring macrolides such as gamithromycin and tulathromycin, and 14-membered or 16-membered ring macrolides in veterinary medicine (specifically food animals) selects for resistance or confers cross-resistance to macrolides used in human medicine, such as azithromycin, remains largely unknown. As *Salmonella* is intrinsically resistant to 14- and 16-membered ring macrolides, further cloning studies are needed to better evaluate the contribution of azithromycin resistance determinants such as those identified in this study to cross-resistance to those macrolides.

In our study, all azithromycin resistance genes were carried on plasmids, with 86.2% being MDR. This is not surprising owing to the significant role plasmids play in the evolution and acquisition of AMR genes in *Salmonella* (12). The finding of an *S.* I 4,[5],12:i:- isolate resistant to macrolides, third-generation cephalosporins, and with decreased susceptibility to fluoroquinolones, and an emerging MDR *S.* Newport clone of cattle origin (also sheep) resistant to macrolides with decreased susceptibility to fluoroquinolones is concerning. This clone has been involved in a 2018–2019 outbreak linked to beef in the United States and soft cheese obtained in Mexico (37). Notably, clinical isolates dominate this NCBI SNP cluster, whereas environmental isolates are mainly from water sources. We acknowledge that this SNP analysis was limited as it was solely based on publicly available data at NCBI. To contain the evolution and spread of azithromycin resistance in *Salmonella*, a One Health approach focused on monitoring and infection control, and a strong commitment to better understand the drivers of azithromycin resistance in *Salmonella* associated with food animal sources is warranted.

MATERIALS AND METHODS

Azithromycin-resistant *Salmonella* isolates and controls

We examined NARMS food animal and retail meat culture collections (approximately 34,200 and 6,500 *Salmonella* isolates, respectively) between 2011 and 2021 for potential azithromycin-resistant *Salmonella* isolates. For the food animal sources, collected during slaughter and processing, the U.S. Department of Agriculture's FSIS and Agriculture Research Service tested cecal and lymph node samples (not destined for food) and product samples [regulatory/hazard analysis and critical control point (HACCP) program] from chickens, turkeys, cattle, swine, sheep, lamb, and goat. For the retail meat arm, U.S. FDA's CVM, in partnership with state and local public health and agricultural departments and universities, tested retail raw meat samples from chicken, turkey, beef, and pork collected from grocery stores (10). AST was conducted using broth microdilution with NARMS Gram-negative plates (CMV2AGNF, from 2011 to 2013; CMV3AGNF, 2014–2015; CMV4AGNF, 2016–2019; and CMV5AGNF, 2020–2021) on the Sensititre Complete Automated AST System (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions and CLSI guidelines (38). Breakpoints established by CLSI were adopted for MIC interpretation wherever available (3). For azithromycin, a resistant breakpoint of ≥32 µg/mL (CLSI breakpoint for *S.* Typhi) was used. Thirty-one azithromycin-resistant *Salmonella* and 14 susceptible controls were selected based on initial phenotypic and genotypic data (Table 1).

Agar dilution

Besides the broth dilution mentioned above, AST against an expanded range of azithromycin concentrations (0.125–1,024 µg/mL) for all 45 *Salmonella* isolates was performed by agar dilution following CLSI guidelines (38). *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 29213 were used as quality control organisms, with QC ranges from CLSI where available (3, 39). MIC differences between azithromycin-resistant isolates (n = 31) and susceptible controls (n = 14) were compared, including fold changes, MIC_{50}, and MIC_{90}.

Short-read and long-read WGS

Short-read WGS was performed at NARMS partner labs and CVM following manufacturers' instructions. The procedure used at CVM has been described previously (40). Briefly, genomic DNA was extracted using the QIAamp 96 DNA QIAcube HT kit in the automated QIAcube HT instrument (QIAGEN, Germantown, MD). Libraries were prepared with the Nextera XT DNA library preparation kit (Illumina, San Diego, CA) in the automated Sciclone G3 liquid handling workstation (PerkinElmer, Santa Clara, CA). Sequencing was performed on the MiSeq platform using the MiSeq reagent kit v3 chemistry with the 600-cycle option (Illumina). Raw reads were *de novo* assembled using the CLC genomics workbench 10 (QIAGEN).

Long-read sequencing was also performed at CVM as described previously (41) with slight modifications. Briefly, genomic DNA libraries with an average insert size of 10 kb or larger were prepared using the SMRTbell template prep kit 1.0 (PacBio, Menlo Park, CA). Sequencing was performed on the Sequel platform with the Sequel sequencing kit 3.0, and reads were assembled using the microbial assembly pipeline in SMRT link v11.0 (PacBio).

Bioinformatic analysis and data visualization

Salmonella serovars were determined by SeqSero2 (42), whereas genetic determinants for AMR, biocide resistance, and heavy metal resistance were identified with AMRFinder-Plus v3.10 (14). Plasmid replicon typing was conducted using PlasmidFinder 2.1 (43), and IS elements flanking the resistance genes were located using ISfinder (44). MLST was performed with MLST 2.0 (45).

Alignments of three *Salmonella* isolates possessing *ere*(A), *mef*(B), and *mph*(A) with reference *E. coli* genes (GenBank accession no. NG_047765.1, NG_047978.1, and NG_047985.1, respectively) were performed with BLAST (46). Similarly, three *Salmonella* isolates with 16-µg/mL azithromycin MIC were examined for 23S rRNA gene mutations against a reference *E. coli* genome (GenBank accession no. NC_004431.1).

For data visualization, alignments of AMR gene-encoding regions were performed using Easyfig v2.2.2 (47). Circular structures of select azithromycin-resistant plasmids were drawn with BLAST ring image generator (48). The clustering of azithromycin-resistant *Salmonella* isolates to clinical and environmental *Salmonella* isolates deposited at NCBI was observed using the NCBI pathogen detection isolates browser (15).

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DATA AVAILABILITY

MiSeq short-read and PacBio long-read WGS data were deposited to the NCBI short-read archive under BioProject accession no. PRJNA290865 and PRJNA292661, respectively.

REFERENCES

1. WHO. 2018. Salmonella (non-Typhoidal) fact sheet. Available from: https://www.who.int/news-room/fact-sheets/detail/salmonella-(non-typhoidal)
2. CDC. 2019. Antibiotic resistance threats in the United States, 2019. Available from: https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf
3. CLSI. 2023. Performance standards for antimicrobial susceptibility testing. In CLSI supplement M100, 33rd ed. Clinical and Laboratory Standards Institute, Wayne, PA.
4. Mutak S. 2007. Azalides from azithromycin to new azalide derivatives. J Antibiot (Tokyo) 60:85–122. https://doi.org/10.1038/ja.2007.10
5. Hicks LA, Taylor TH, Hunkler RJ. 2013. U.S. outpatient antibiotic prescribing. N Engl J Med 368:1461–1462. https://doi.org/10.1056/NEJMc1212055
6. CDC. 2023. CDC yellow book 2024: Health information for International travel. Available from: https://wwwnc.cdc.gov/travel/page/yellowbook-home
7. Committee on Infectious Diseases American Academy of Pediatrics; Kimberlin DW, Barnett ED, Lynfield R, Sawyer MH. 2021. Salmonella infections. Available from: https://publications.aap.org/redbook/book/347/Red-Book-2021-2024-Report-of-the-Committee-on
8. Sjölund-Karlsson M, Joyce K, Blickenstaff K, Ball T, Haro J, Medalla FM, Fedorka-Cray P, Zhao S, Crump JA, Whichard JM. 2011. Antimicrobial susceptibility to azithromycin among *Salmonella enterica* isolates from the United States. Antimicrob Agents Chemother 55:3985–3989. https://doi.org/10.1128/AAC.00590-11
9. Gomes C, Martínez-Puchol S, Palma N, Horna G, Ruiz-Roldán L, Pons MJ, Ruiz J. 2017. Macrolide resistance mechanisms in Enterobacteriaceae: Focus on azithromycin. Crit Rev Microbiol 43:1–30. https://doi.org/10.3109/1040841X.2015.1136261
10. FDA. 2023. The National antimicrobial resistance monitoring system. Available from: https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/about-narms
11. McDermott PF, Tyson GH, Kabera C, Chen Y, Li C, Folster JP, Ayers SL, Lam C, Tate HP, Zhao S. 2016. Whole-genome sequencing for detecting antimicrobial resistance in nontyphoidal *Salmonella*. Antimicrob Agents Chemother 60:5515–5520. https://doi.org/10.1128/AAC.01030-16
12. Li C, Tyson GH, Hsu CH, Harrison L, Strain E, Tran TT, Tillman GE, Dessai U, McDermott PF, Zhao S. 2021. Long-read sequencing reveals evolution and acquisition of antimicrobial resistance and virulence genes in *Salmonella enterica*. Front Microbiol 12:777817. https://doi.org/10.3389/fmicb.2021.777817
13. Zhao S, Li C, Hsu CH, Tyson GH, Strain E, Tate H, Tran TT, Abbott J, McDermott PF. 2020. Comparative genomic analysis of 450 strains of *Salmonella enterica* isolated from diseased animals. Genes 11:1025. https://doi.org/10.3390/genes11091025
14. NCBI. 2023. Reference gene catalog. Available from: https://www.ncbi.nlm.nih.gov/pathogens/refgene
15. NCBI. 2023. Pathogen detection isolates Browser. Available from: https://www.ncbi.nlm.nih.gov/pathogens

16. WHO. 2019. Critically important antimicrobials for human medicine, 6th revision. Available from: https://apps.who.int/iris/rest/bitstreams/1217095/retrieve
17. FDA. 2023. GFI #152: Evaluating the safety of antimicrobial new animal drugs with regard to their Microbiological effects on bacteria of human health concern. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cvm-gfi-152-evaluating-safety-antimicrobial-new-animal-drugs-regard-their-microbiological-effects
18. WOAH. 2021. OIE list of antimicrobial agents of veterinary importance. Available from: https://www.oie.int/app/uploads/2021/06/a-oie-list-antimicrobials-june2021.pdf
19. FDA. 2023. NARMS now: Integrated data. Available from: https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/narms-now-integrated-data
20. EUCAST. 2023. Antimicrobial wild type distributions of microorganisms. Available from: https://mic.eucast.org
21. Dinos GP. 2017. The macrolide antibiotic renaissance. Br J Pharmacol 174:2967–2983. https://doi.org/10.1111/bph.13936
22. Gomes C, Ruiz-Roldán L, Mateu J, Ochoa TJ, Ruiz J. 2019. Azithromycin resistance levels and mechanisms in *Escherichia coli*. Sci Rep 9:6089. https://doi.org/10.1038/s41598-019-42423-3
23. Faccone D, Lucero C, Albornoz E, Petroni A, Ceriana P, Campos J, Viñas MR, Francis G, AZM-R-Group, Melano RG, Corso A. 2018. Emergence of azithromycin resistance mediated by the *mph*(A) gene in *Salmonella* Typhimurium clinical isolates in Latin America. J Glob Antimicrob Resist 13:237–239. https://doi.org/10.1016/j.jgar.2018.04.011
24. Xie M, Chen K, Chan E-C, Chen S. 2022. Identification and genetic characterization of two conjugative plasmids that confer azithromycin resistance in *Salmonella*. Emerg Microbes Infect 11:1049–1057. https://doi.org/10.1080/22221751.2022.2058420
25. Dolejska M, Villa L, Poirel L, Nordmann P, Carattoli A. 2013. Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance-nodulation-cell division/multidrug efflux pump. J Antimicrob Chemother 68:34–39. https://doi.org/10.1093/jac/dks357
26. Du XD, Li DX, Hu GZ, Wang Y, Shang YH, Wu CM, Liu HB, Li XS. 2012. Tn1548-associated *armA* is co-located with *qnrB2*, *aac(6')-Ib-Cr* and $bla_{CTX-M-3}$ on an IncFII plasmid in a *Salmonella enterica* subsp. *enterica* serovar Paratyphi B strain isolated from chickens in China. J Antimicrob Chemother 67:246–248. https://doi.org/10.1093/jac/dkr407
27. Bizot E, Cointe A, Bidet P, Mariani-Kurkdjian P, Hobson CA, Courroux C, Liguori S, Bridier-Nahmias A, Magnan M, Merimèche M, Caméléna F, Berçot B, Weill F-X, Lefèvre S, Bonacorsi S, Birgy A. 2022. Azithromycin resistance in Shiga toxin-producing *Escherichia coli* in France between 2004 and 2020 and detection of *mef*(C)-*mph*(G) genes. Antimicrob Agents Chemother 66:e0194921. https://doi.org/10.1128/AAC.01949-21
28. Sugimoto Y, Suzuki S, Nonaka L, Boonla C, Sukpanyatham N, Chou HY, Wu JH. 2017. The novel *mef*(C)-*mph*(G) macrolide resistance genes are conveyed in the environment on various vectors. J Glob Antimicrob Resist 10:47–53. https://doi.org/10.1016/j.jgar.2017.03.015
29. Nonaka L, Maruyama F, Suzuki S, Masuda M. 2015. Novel macrolide-resistance genes, *mef*(C) and *mph*(G), carried by plasmids from *Vibrio* and *Photobacterium* isolated from sediment and seawater of a coastal aquaculture site. Lett Appl Microbiol 61:1–6. https://doi.org/10.1111/lam.12414
30. Liu X, Yang X, Ye L, Chan E-C, Chen S. 2022. Genetic characterization of a conjugative plasmid that encodes azithromycin resistance in Enterobacteriaceae. Microbiol Spectr 10:e0078822. https://doi.org/10.1128/spectrum.00788-22
31. Harmer CJ, Holt KE, Hall RM. 2015. A type 2 A/C2 plasmid carrying the *aacC4* apramycin resistance gene and the *erm*(42) erythromycin resistance gene recovered from two *Salmonella enterica* serovars. J Antimicrob Chemother 70:1021–1025. https://doi.org/10.1093/jac/dku489
32. Hong Y-P, Chen Y-T, Wang Y-W, Chen B-H, Teng R-H, Chen Y-S, Chiou C-S. 2023. Integrative and conjugative element-mediated azithromycin resistance in multidrug-resistant *Salmonella enterica* serovar Albany. Antimicrob Agents Chemother 65:e02634-20. https://doi.org/10.1128/AAC.02634-20
33. Chiou C-S, Hong Y-P, Wang Y-W, Chen B-H, Teng R-H, Song H-Y, Liao Y-S. 2023. Antimicrobial resistance and mechanisms of azithromycin resistance in nontyphoidal *Salmonella* isolates in Taiwan, 2017 to 2018. Microbiol Spectr 11:e0336422. https://doi.org/10.1128/spectrum.03364-22
34. Hooda Y, Sajib MSI, Rahman H, Luby SP, Bondy-Denomy J, Santosham M, Andrews JR, Saha SK, Saha S. 2019. Molecular mechanism of azithromycin resistance among Typhoidal salmonella strains in Bangladesh identified through passive pediatric surveillance. PLoS Negl Trop Dis 13:e0007868. https://doi.org/10.1371/journal.pntd.0007868
35. Zwama M, Nishino K. 2022. Proximal binding pocket Arg717 substitutions in *Escherichia coli* AcrB cause clinically relevant divergencies in resistance profiles. Antimicrob Agents Chemother 66:e0239221. https://doi.org/10.1128/aac.02392-21
36. Sajib MSI, Tanmoy AM, Hooda Y, Rahman H, Andrews JR, Garrett DO, Endtz HP, Saha SK, Saha S. 2021. Tracking the emergence of azithromycin resistance in multiple genotypes of typhoidal *Salmonella*. mBio 12:e03481-20. https://doi.org/10.1128/mBio.03481-20
37. Plumb ID, Schwensohn CA, Gieraltowski L, Tecle S, Schneider ZD, Freiman J, Cote A, Noveroske D, Kolsin J, Brandenburg J, Chen JC, Tagg KA, White PB, Shah HJ, Francois Watkins LK, Wise ME, Friedman CR. 2019. Outbreak of *Salmonella* Newport infections with decreased susceptibility to azithromycin linked to beef obtained in the United States and soft cheese obtained in Mexico - United States, 2018-2019. MMWR Morb Mortal Wkly Rep 68:713–717. https://doi.org/10.15585/mmwr.mm6833a1
38. CLSI. 2018. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. In CLSI standard M07, 11th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
39. CLSI. 2018. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. In CLSI standard VET08, 4th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
40. Domesle KJ, Young SR, Ge B. 2021. Rapid screening for *Salmonella* in raw pet food by loop-mediated isothermal amplification. J Food Prot 84:399–407. https://doi.org/10.4315/JFP-20-365
41. Tate H, Li C, Nyirabahizi E, Tyson GH, Zhao S, Rice-Trujillo C, Jones SB, Ayers S, M'ikanatha NM, Hanna S, Ruesch L, Cavanaugh ME, Laksanalamai P, Mingle L, Matzinger SR, McDermott PF. 2021. A national antimicrobial resistance monitoring system survey of antimicrobial-resistant foodborne bacteria isolated from retail veal in the United States. J Food Prot 84:1749–1759. https://doi.org/10.4315/JFP-21-005
42. Zhang S, den Bakker HC, Li S, Chen J, Dinsmore BA, Lane C, Lauer AC, Fields PI, Deng X. 2019. Seqsero2: Rapid and improved salmonella Serotype determination using whole-genome sequencing data. Appl Environ Microbiol 85:e01746-19. https://doi.org/10.1128/AEM.01746-19
43. Carattoli A, Hasman H. 2020. PlasmidFinder and *in silico* pMLST: Identification and typing of plasmid replicons in whole-genome sequencing (WGS). Methods Mol Biol 2075:285–294. https://doi.org/10.1007/978-1-4939-9877-7_20
44. Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: The reference centre for bacterial insertion sequences. Nucleic Acids Res 34:D32–D36. https://doi.org/10.1093/nar/gkj014
45. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L, Sicheritz-Pontén T, Ussery DW, Aarestrup FM, Lund O. 2012. Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol 50:1355–1361. https://doi.org/10.1128/JCM.06094-11
46. NCBI. 2023. Basic local alignment search tool. Available from: https://blast.ncbi.nlm.nih.gov/Blast.cgi
47. Sullivan MJ, Petty NK, Beatson SA. 2011. Easyfig: A genome comparison visualizer. Bioinformatics 27:1009–1010. https://doi.org/10.1093/bioinformatics/btr039
48. Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA. 2011. BLAST ring image generator (BRIG): Simple prokaryote genome comparisons. BMC Genomics 12:402. https://doi.org/10.1186/1471-2164-12-402