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MECHANISMS OF RESISTANCE

Circulation of Plasmids Harboring Resistance Genes to Quinolones and/or Extended-Spectrum Cephalosporins in Multiple *Salmonella enterica* Serotypes from Swine in the United States

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ABSTRACT Nontyphoidal *Salmonella enterica* (NTS) poses a major public health risk worldwide that is amplified by the existence of antimicrobial-resistant strains, especially those resistant to quinolones and extended-spectrum cephalosporins (ESC). Little is known on the dissemination of plasmids harboring the acquired genetic determinants that confer resistance to these antimicrobials across NTS serotypes from livestock in the United States. NTS isolates ($n = 183$) from U.S. swine clinical cases retrieved during 2014 to 2016 were selected for sequencing based on their phenotypic resistance to enrofloxacin (quinolone) or ceftiofur (3rd-generation cephalosporin). *De novo* assemblies were used to identify chromosomal mutations and acquired antimicrobial resistance genes (AARGs). In addition, plasmids harboring AARGs were identified using short-read assemblies and characterized using a multistep approach that was validated by long-read sequencing. AARGs to quinolones [*qnrB15*, *qnrB19*, *qnrB2*, *qnrD*, *qnrS1*, *qnrS2*, and *aac(6')Ib-cr*] and ESC (bla_{CMY-2}, $bla_{CTX-M-1}$, $bla_{CTX-M-27}$, and bla_{SHV-12}) were distributed across serotypes and were harbored by several plasmids. In addition, chromosomal mutations associated with resistance to quinolones were identified in the target enzyme and efflux pump regulation genes. The predominant plasmid harboring the prevalent *qnrB19* gene was distributed across serotypes. It was identical to a plasmid previously reported in *S. enterica* serovar Anatum from swine in the United States (GenBank accession number KY991369.1) and similar to *Escherichia coli* plasmids from humans in South America (GenBank accession numbers GQ374157.1 and JN979787.1). Our findings suggest that plasmids harboring AARGs encoding mechanisms of resistance to critically important antimicrobials are present in multiple NTS serotypes circulating in swine in the United States and can contribute to resistance expansion through horizontal transmission.

KEYWORDS AmpC, antimicrobial drug resistance, ESBL, *Salmonella*, fluoroquinolones, plasmids, swine

Nontyphoidal *Salmonella enterica* (NTS) is a major foodborne pathogen (1). The impact of NTS is greater when strains become resistant to the antimicrobials used to treat clinical salmonellosis in humans (2). While most NTS infections are

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transient and do not require antibiotic treatment, the use of antimicrobials such as quinolones and extended-spectrum cephalosporins (ESC) is indicated in invasive infections (3).

Quinolones are bactericidal antibiotics that interfere with the uncoiling of bacterial DNA during replication by inhibiting the target enzymes DNA gyrase and topoisomerase IV. Three main genetic determinants of resistance to quinolones have been described in *Salmonella* (4): (i) chromosomal mutations or deletions in the target enzyme-encoding genes, (ii) chromosomal mutations or deletions in genes regulating efflux pumps, and (iii) acquired antimicrobial resistance genes (AARGs) harbored by plasmids. Cephalosporins are antibiotics whose bactericidal effect is mediated by alteration of the bacterial cell wall construction (5). Genetic resistance to cephalosporins in NTS is a result of plasmid-mediated AARGs (6).

Plasmids are transformable circular genetic elements of various sizes that play an important role in the dissemination of antimicrobial resistance (AMR) within and among bacterial species (7).

According to the National Antimicrobial Resistance Monitoring System (NARMS), the prevalences of resistance to ceftiofur (3rd-generation cephalosporin) in NTS isolates from poultry and swine sampled at the slaughterhouse have changed from 7.2% and 2.4%, respectively, in 2013 to 9.7% and 1.8% in 2015 (8). During the same period, no resistance to ciprofloxacin (a quinolone) was found in NTS from poultry (in which its use has been banned since 2005) (9), while ciprofloxacin resistance in NTS from swine remained low (it varied between 0.4% in 2013 and 0.2% in 2015) (8). The numbers of isolates that were resistant to ciprofloxacin and ceftriaxone (3rd-generation cephalosporin) in NTS isolates from human clinical samples in NARMS during 2014 were 9/2,172 (0.4%) and 51/2,172 (2.3%), respectively (10).

While AARGs which confer resistance to ESC were commonly found in NTS isolates from animals and environmental samples in the United States (11, 12), AARGs which confer resistance to quinolones have been reported only occasionally (13–15). However, these AARGs, along with chromosomal mutations in target enzymes and efflux pump regulation genes, were recently described in enrofloxacin-resistant *S. enterica* 4,[5],12:i:− strains isolated from Midwestern swine (16). Moreover, in contrast with NARMS reports of isolates from animals sampled at the slaughterhouse (8, 9), Hong et al. (17) found that enrofloxacin resistance has been increasing among swine clinical NTS isolates from the Midwest since 2008 and that certain serotypes had higher prevalences of resistance to enrofloxacin (a quinolone) and ceftiofur. Given the potential impact on public health from dissemination of resistance to such important antimicrobial families, we aimed to characterize the antimicrobial resistance-conferring determinants to quinolones and ESC in NTS serotypes from Midwestern swine and the plasmids contributing to their spread.

RESULTS

A maximum-likelihood tree was constructed using the core genome (3,252,309 bp, including 3,402 genes) from swine NTS isolates (between 1 and 39,174 pairwise single-nucleotide polymorphisms [SNPs], median = 26,716) (Fig. 1). The average number of within-serotype pairwise-SNP differences for serotypes represented by at least 10 isolates ranged from 18 to 399 pairwise SNPs. Overall, at least one potential resistance determinant was found in 87/89 and 65/68 of the isolates resistant to enrofloxacin and ceftiofur, respectively.

For well-represented serotypes (with at least 14 isolates resistant to either antimicrobial), phenotypic resistance to both enrofloxacin and ceftiofur (i.e., coresistance) was more frequent in *S. enterica* serovar Agona (13/22) and *S.* Heidelberg (5/14) than in *S.* 4,[5],12:i:− (5/26) and *S.* Typhimurium variant 5− (3/19) (see Table S4 in the supplemental material).

The AARGs to quinolones [*qnrB19*, *qnrB2*, *qnrB15*, *qnrD*, *qnrS1*, *qnrS2*, and *aac(6′)Ib-cr*] and ESC (bla_{CMY-2}, $bla_{CTX-M-1}$, $bla_{CTX-M-27}$, and bla_{SHV-12}) were mostly detected among phenotypically resistant isolates (Fig. 1). For quinolones, multiple mutations in target

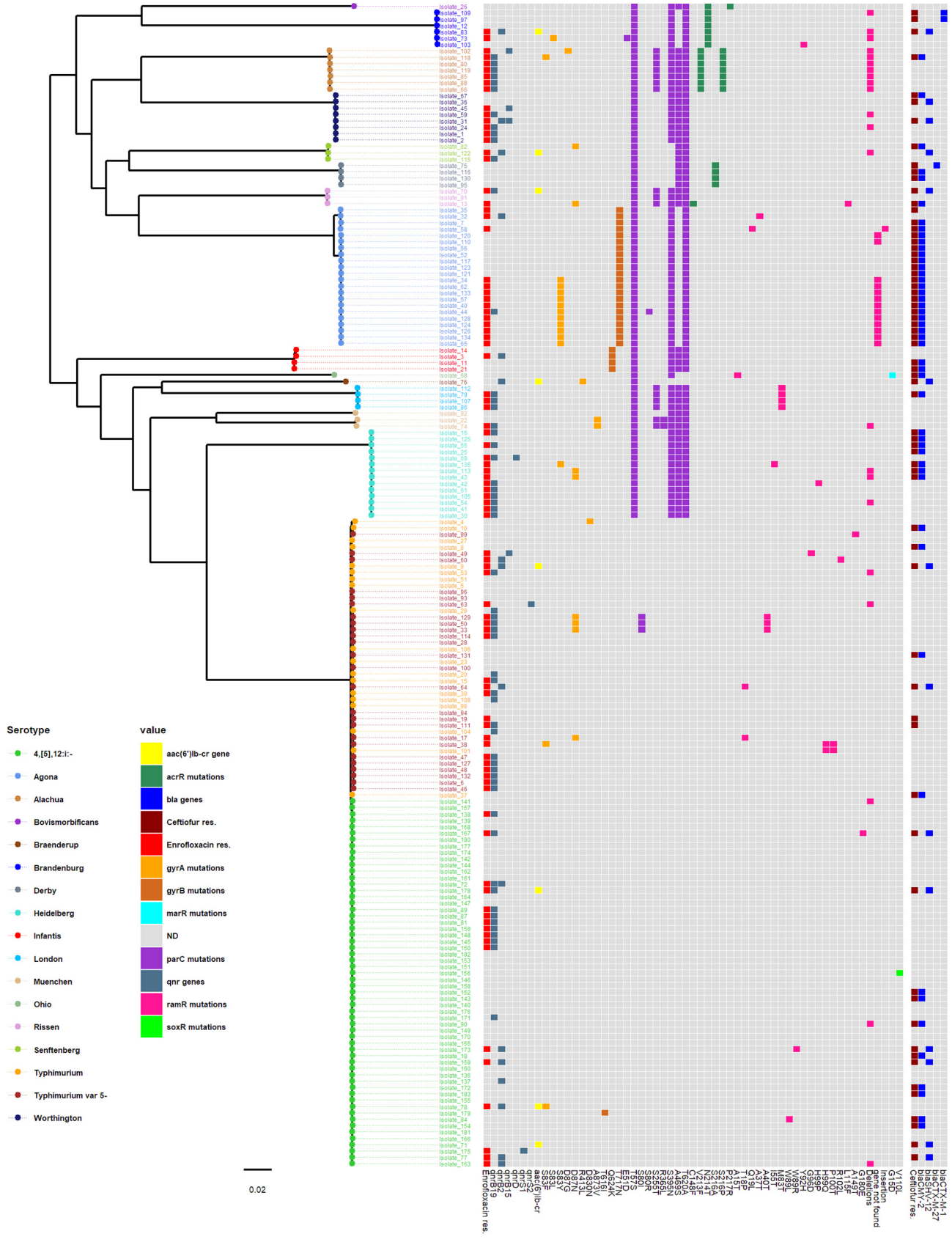

FIG 1 Maximum-likelihood tree constructed using the core-genome alignment of nontyphoidal-*Salmonella* isolates collected from Midwestern swine during the years 2014 to 2016. Two *S.* Paratyphi type A outgroup strains (GenBank accession numbers SRR3033248 and SRR3277289) were used

(Continued on next page)

TABLE 1 Potential quinolone resistance determinants identified in nontyphoidal-*Salmonella* serotypes, summarized by serotype and phenotypic resistance to enrofloxacin

Salmonella serotype	Enrofloxacin resistant[a]	Total no. of isolates	No. of isolates with indicated potential resistance determinant									
			Acquired gene		Target enzyme gene[b]				Efflux pump regulation gene[b]			
			Qnr	*aac(6′)Ib-cr*	*gyrA*	*gyrB*[c]	*parC*[c]	*parE*	*acrR*	*ramR*	*marR*	*soxR*
Agona	No	8	0	0	0	0 (8)	0 (8)	0	0	2[d]	0	0
	Yes	14	2	0	11	0 (14)	1 (13)	0	0	13[d]	0	0
Alachua	Yes	7	7	0	2	0	0 (7)	0	7	7	0	0
Bovismorbificans	No	1	0	0	0	0	0 (1)	0	1	0	0	0
Braenderup	No	1	1	1	1	0	0 (1)	0	0	0	0	0
Brandenburg	No	4	0	0	0	0	0 (4)	0	4	2	0	0
	Yes	2	1	1	1	0	1 (1)	0	2	2	0	0
Derby	No	4	0	0	0	0	0 (4)	0	4	0	0	0
Heidelberg	No	2	0	0	0	0	0 (2)	0	0	0	0	0
	Yes	12	9	0	3	0	0 (12)	0	0	5	0	0
Infantis	No	3	0	0	0	3	0 (3)	0	0	0	0	0
	Yes	1	1	0	0	1	0 (1)	0	0	0	0	0
London	No	1	0	0	0	0	0 (1)	0	0	1	0	0
	Yes	3	3	0	0	0	0 (3)	0	0	3	0	0
4,[5],12:i:-	No	41	2	1	0	1	0	0	0	3	0	1
	Yes	17	17	2	1	0	0	0	0	3	0	0
Muenchen	No	2	0	0	1	0	1 (1)	0	0	0	0	0
	Yes	1	1	0	1	0	1	0	0	1	0	0
Ohio	No	1	0	0	0	0	0 (1)	0	0	1	1	0
Rissen	No	1	0	0	0	0	0 (1)	0	0	0	0	0
	Yes	2	1	1	1	0	0 (2)	0	1	1	0	0
Senftenberg	No	1	0	0	1	0	0 (1)	0	0	0	0	0
	Yes	2	2	1	0	0	0 (2)	0	0	1	0	0
Typhimurium	No	15	4	0	1	0	0	0	0	1	0	0
	Yes	4	4	1	0	0	0	0	0	1	0	0
Typhimurium variant 5−	No	7	0	0	0	0	0	0	0	1	0	0
	Yes	18	15	0	5	0	3	0	0	9	0	0
Worthington	No	2	0	0	0	0	0 (2)	0	0	0	0	0
	Yes	6	6	0	0	0	0 (6)	0	0	2	0	0

[a]A MIC of ≥1 mg/liter was used as the cutoff for phenotypic resistance to enrofloxacin.
[b]Isolates in which nonsynonymous mutations were detected.
[c]For *gyrB* and *parC*, the number of isolates harboring nonsynonymous mutations that were found regardless of resistance to enrofloxacin and were excluded from the analysis is indicated in brackets (see text and Tables S2 and S3 for more details).
[d]Isolates in which *ramR* was not detected. These were regarded as nonsynonymous mutations.

enzyme genes (*gyrA*, *gyrB*, and *parC*), except *parE*, and in all efflux pump regulation genes (*acrR*, *ramR*, *marR*, and *soxR*), including base pair deletions of *ramR*, were found in isolates from multiple serotypes (Fig. 1, Table 1; Tables S2 and S3). In addition, *ramR* could not be detected in 13 *S.* Agona isolates (11 of which were enrofloxacin resistant)

FIG 1 Legend (Continued)
to root the tree (not included in the figure). The analysis included 122,201 variable sites in the alignment (i.e., SNPs). Tip colors indicate serotype. Data shown in heatmap include (i) resistance to enrofloxacin (MIC of ≥1 mg/liter), (ii) the presence of *qnr* and *aac(6′)Ib-cr* genes, (iii) chromosomal mutations in target enzyme genes and genes involved in the regulation of efflux pumps, (iv) resistance to ceftiofur (MIC of ≥8 mg/liter), and (v) the presence of *bla* genes.

TABLE 2 Associations between phenotypic resistance to enrofloxacin and the presence of *qnr* genes or the total number of resistance determinants found among all nontyphoidal-*Salmonella* isolates[a]

Risk factor	No. of resistant isolates (MIC ≥ 1 mg/liter)/ total no. of isolates (%)		Odds ratio (95% CI)[b]	*P* value[c]
	With the risk factor	Without the risk factor		
Presence of *qnr* genes	69/76 (90.78)	20/107 (18.69)	42.88 (17.14–107.26)	<0.001
Total no. of resistance determinants higher than 2[d]	17/18 (94.44)	72/165 (43.64)	21.96 (2.85–168.89)	<0.001

[a]Univariable analyses for each risk factor were conducted with the Pearson's chi-square test.
[b]CI, confidence interval.
[c]Statistically significant when $P < 0.05/2 = 0.025$ (the *P* value was adjusted to the number of tests; Bonferroni's correction).
[d]The total number of resistance determinants is the sum of the following: (i) *qnr* genes, (ii) *aac(6')Ib-cr* genes, and (iii) target enzyme and efflux pump regulation genes with at least one nonsynonymous mutation (please refer to the manuscript for further details). The maximal number of resistance determinants was five.

using the short-read assemblies, and only 35% of the gene was detected (100% identity) in one *S*. Agona isolate (isolate 44) following long-read assembly.

The presence of *qnr* genes was significantly associated with enrofloxacin resistance in all isolates ($P < 0.025$, Pearson's chi-square test) (Table 2) but not with high MICs (MIC of >2 mg/liter) among the resistant isolates ($P > 0.025$, Fisher's exact test) (Table 3). Accordingly, in the well represented *S*. 4,[5],12:i:−, *qnr* genes were found in 17/17 and 2/41 of the enrofloxacin-resistant and -susceptible isolates, respectively. In contrast, *qnr* genes were rarely found (2/14) among *S*. Agona enrofloxacin-resistant isolates, while mutations in *gyrA* (11/14) and mutations and deletions in *ramR* (2/14 and 11/14, respectively) were common (Table 1). In addition, among isolates in which determinants of genetic resistance to quinolones were identified, 37/87 of the enrofloxacin-resistant isolates (MICs of ≥1 mg/liter) carried only AARGs [*qnr* with or without *aac(6')Ib-cr* genes] (Fig. 2). In 28 of these, *qnrB19* was the sole identified gene encoding quinolone resistance.

Isolates with higher enrofloxacin MICs harbored multiple resistance determinants (Fig. 2); for example, only 2/32 (6.2%) isolates with MICs of ≤0.5 mg/liter harbored more than one resistance determinant, while 20/22 (90.9%) isolates with MICs of >2 mg/liter harbored two or more distinct resistance determinants. This was consistent with the significant association between enrofloxacin resistance (MIC of ≥1 mg/liter) and the presence of >2 resistance determinants that was found in all isolates ($P < 0.025$, Pearson's chi-square test) (Table 2) and that remained significant when evaluated in resistant isolates while using a MIC of >2 mg/liter as a cutoff for high MICs ($P < 0.025$, Pearson's chi-square test) (Table 3).

The presence of *bla* genes was significantly higher in ceftiofur-resistant isolates (65/68) than in susceptible isolates (1/115) ($P < 0.001$, Fisher's exact test), and these genes were broadly distributed among serotypes (Fig. 1, Table 4). The AARGs $bla_{\text{CMY-2}}$ (49/66), $bla_{\text{CTX-M-27}}$ (1/66), and $bla_{\text{CTX-M-1}}$ (2/66) were found only in isolates with high MICs (MIC of >8 mg/liter). The AARG $bla_{\text{SHV-12}}$ (14/66) was found mainly in resistant isolates (MIC of 8 mg/liter) but also in one nonresistant isolate.

We were able to detect at least one plasmid group in the genome assemblies of isolates harboring all AARGs except for isolates harboring *qnrS1* (Table 5; Table S5), and

TABLE 3 Associations between high MICs to enrofloxacin and the presence of *qnr* genes or the total number of resistance determinants found among enrofloxacin-resistant nontyphoidal-*Salmonella* isolates[a]

Risk factor	No. of isolates with high MICs (>2 mg/liter)/ no. of resistant isolates (MIC ≥ 1 mg/liter) (%)		Odds ratio (95% CI)[b]	*P* value[c]
	With the risk factor	Without the risk factor		
Presence of *qnr* genes	21/69 (30.4)	2/20 (10)	3.94 (0.8–37.6)	0.084[d]
Total no. of resistance determinants is higher than 2[e]	13/17 (76.47)	10/72 (13.89)	20.15 (5.47–74.28)	<0.001

[a]Univariable analyses for each risk factor were conducted with Pearson's chi-square or Fisher's exact tests.
[b]CI, confidence interval.
[c]Statistically significant when $P < 0.05/2 = 0.025$ (the *P* value was adjusted to the number of tests; Bonferroni's correction).
[d]Fisher's exact test.
[e]The total number of resistance determinants is the sum of the following: (i) *qnr* genes, (ii) *aac(6')Ib-cr* genes, and (iii) target enzyme and efflux pump regulation genes with at least one nonsynonymous mutation (please refer to the manuscript for further details). The maximal number of resistance determinants was five.

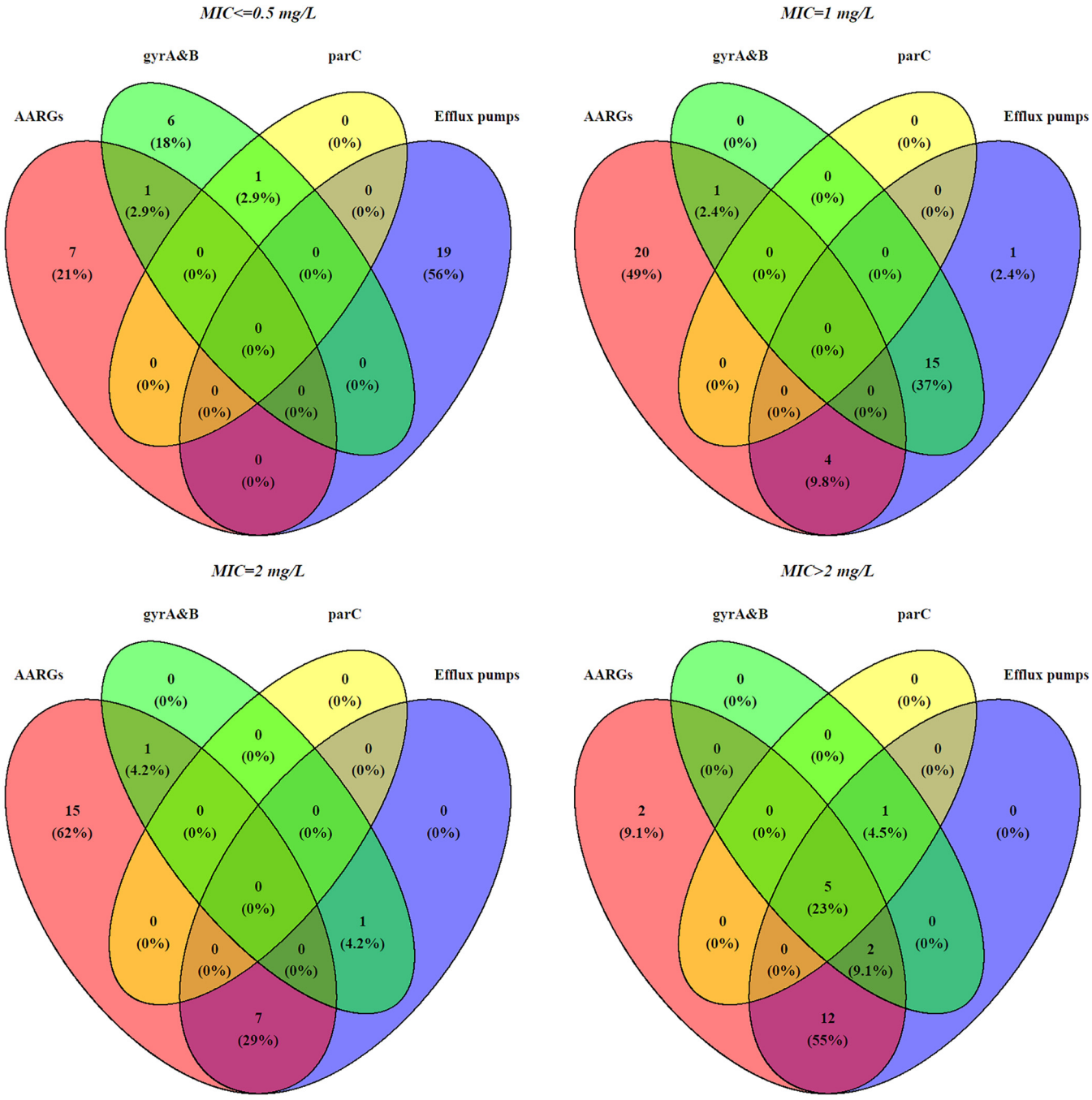

FIG 2 Venn diagrams demonstrating the degree of overlap between enrofloxacin resistance determinants and different enrofloxacin MICs. Enrofloxacin resistance determinants were grouped as follows: (i) presence of at least one AARG [*qnr* and/or *aac(6')Ib-cr*] (red); (ii) at least one mutation in *gyrA* and/or *gyrB* target genes (green); (iii) at least one mutation in *parC* target gene (yellow); and (iv) at least one mutation (including deletions and insertions) in the efflux pump regulation genes (*acrR*, *ramR*, *marR*, and/or *soxR*) (blue).

the plasmid groups identified were distributed across multiple serotypes (Fig. 3). Large plasmids (average size range, 53,880 to 324,077 bp) were detected for all *bla*, *aac(6')Ib-cr*, *qnrB15*, and *qnrB2* genes. IncQ2 plasmids (average size, 7,748 bp) were detected for *qnrS2*, and small ColRNAI and Col3M plasmids were detected for *qnrB19* and *qnrD*, respectively (Table 5).

Using long-read assemblies, we were able to identify the plasmids harboring AARGs in all 10 sequenced isolates. All plasmids detected belonged to the same incompatibility group/colicin type identified in the corresponding plasmid group identified by

TABLE 4 Presence of β-lactamase genes associated with resistance to ceftiofur, summarized by nontyphoidal-*Salmonella* serotype and resistance phenotype

Nontyphoidal-*Salmonella* serotype	Ceftiofur resistant[a]	β-Lactamase gene(s) present[b] (no. of genes detected)	No. of genes detected/ no. of isolates (%)
Agona	No		0/1 (0)
	Yes	bla_{CMY-2} (19), bla_{CMY-2}-like (1)	20/21 (95.24)
Alachua	No		0/6 (0)
	Yes	bla_{CMY-2} (1)	1/1 (100)
Bovismorbificans	No		0/1 (0)
Braenderup	Yes	bla_{SHV-12} (1)	1/1 (100)
Brandenburg	No		0/3 (0)
	Yes	$bla_{CTX-M-1}$ (2), bla_{SHV-12} (1)	3/3 (100)
Derby	No		0/1 (0)
	Yes	bla_{CMY-2} (1), bla_{CMY-2}-like (1), $bla_{CTX-M-27}$ (1)	3/3 (100)
Heidelberg	No		0/7 (0)
	Yes	bla_{CMY-2} (7)	7/7 (100)
Infantis	No		0/2 (0)
	Yes	bla_{CMY-2} (2)	2/2 (100)
London	No		0/3 (0)
	Yes	bla_{CMY-2} (1)	1/1 (100)
4,[5],12:i:-	No		0/44 (0)
	Yes	bla_{CMY-2} (8), bla_{SHV-12} (6)	14/14 (100)
Muenchen	No		0/3 (0)
Ohio	Yes	bla_{CMY-2} (1)	1/1 (100)
Rissen	No		0/1 (0)
	Yes	bla_{CMY-2} (1), bla_{SHV-12} (1)	2/2 (100)
Senftenberg	No	bla_{SHV-12} (1)	1/2 (50)
	Yes	bla_{CMY-2} (1)	1/1 (100)
Typhimurium variant 5−	No		0/21 (0)
	Yes	bla_{CMY-2} (1), bla_{SHV-12} (1)	2/4 (50)
Typhimurium	No		0/15 (0)
	Yes	bla_{CMY-2} (3), bla_{SHV-12} (1)	4/4 (100)
Worthington	No		0/5 (0)
	Yes	bla_{CMY-2} (1), bla_{SHV-12} (2)	3/3 (100)

[a]A MIC of ≥8 mg/liter was used as the cutoff for phenotypic resistance.
[b]bla_{TEM-1B} and bla_{CARB-2} were not included in this list.

short-read assembly. Overall, the sequence identity range between plasmids whose sequences were assembled using short-read technology (Illumina) and long-read technology (Pacific Biosciences [Pac-Bio]) was 99% to 100%, while the coverage ranges varied between 99% to 100% and 52% to 100% for the small (<7,555 bp) and large (>68,117 bp) plasmids, respectively (Table 5; Table S5).

In the short-read assemblies, no more than one AARG was located on the same contig (data not shown). In addition, a common plasmid (GenBank accession number CP022696.1) was only found for *aac(6')Ib-cr* and bla_{SHV-12} (Table S5). However, using the long-read assemblies, we found large plasmids harboring both *qnrB2* and bla_{SHV-12} in two isolates (isolates 69 and 77), plus *aac(6')Ib-cr* in another isolate (isolate 76). The long-read-assembly findings agreed with the significant collinearities observed only between these three AARGs (pairwise odds ratios ranged between 19 and 64).

TABLE 5 General characteristics of the plasmid groups detected in short-read (Illumina) assemblies that were identified with the corresponding plasmids detected using long-read (Pac-Bio) assembly[a]

	Plasmid detected using Illumina reads						Plasmid identified using hybrid assembly				
AARG	Plasmid group[b] (no. of isolates)	Incompatibility group/ colicin type	Size range (median) (bp)	Mobility[c]	AMR[c]	Heavy metals[c]	Isolate ID(s)	Size in bp [range (median)]	Circular	% identity[d]	% coverage [range (median)][e]
aac(6′)Ib-cr	I (8)	IncHI2A, IncHI2, TrfA[f]	318,782–328,945 (324,503)	+	+	+	64, 76, 77[g]	225,754–309,825 (307,579)	Yes	99	59–94 (82)
qnrB15	I (1) ND (3)	IncN	54,641	+	+	−					
qnrB19	I (11)	ColRNAI	2,617–2,826 (2,699)	−	−	−					
	II (39)	ColRNAI[f]	3,071–3,082 (3,071)	−	−	−	33, 44, 61, 69[h]	3,071 (3,071)	Yes	99	99–100 (99)
	III (1)	ColRNAI	2,989 (2,989)								
	ND (5)										
qnrB2	I (15)	IncHI2A, IncHI2, TrfA[f]	261,310–339,962 (263,138)	+	+	+	64, 76, 77[g]	225,754–309,825 (307,579)	Yes	99	62–99 (84)
	ND (1)										
qnrD	I (1)	Col3M[f]	2,682–2,683 (2,683)	−	−	−	69[i]	2,683	Yes	100	100 (100)
qnrS1	ND (1)										
qnrS2	I (1)	IncQ2[f]	7,748	+	−	−	63[j]	7,555	Yes	100	99 (99)
bla_{CMY-2}	I (36)	IncA/C2[f]	152,216–199,469 (166,161)	+	+	+	44[k]	179,765	No	99	80–98 (90)
	II (8)	IncI1[f]	99,184–109,170 (104,177)	NA	NA	NA	116[l]	98,867	No	99	99 (99)
	ND (5)										
$bla_{CTX-M-27}$	I (1)	IncFII[f]	68,117–78,962 (73,540)	+	+	−	75[m]	68,088	Yes	99	99 (99)
$bla_{CTX-M-1}$	I (1)	IncN	43,265–44,494 (43,880)	NA	−	NA					
bla_{SHV-12}	I (14)	IncHI2A, IncHI2, TrfA[f]	314,137–328,945 (321,541)	+	+	+	64, 76, 77[g]	225,754–309,825 (307,579)	Yes	99	59–94 (81.5)

[a]See Table S5 for detailed information and references.
[b]Plasmid groups detected in the Illumina short-read genome assemblies of isolates harboring the AARGs; different plasmid groups are indicated by roman numerals. ND, not detected (isolates in which an AARG was found and yet a plasmid group was not detected).
[c]Independent mobility of the plasmids, presence of multiple AMR genes, and presence of genes associated with resistance to heavy metals on the plasmids according to the literature (see Table S5 for details). NA, not available.
[d]Minimal percent identity of the plasmid identified in the long-read (Pac-Bio) assembly with the plasmids identified in the short-read (Illumina) assembly, determined by megablast (through NCBI).
[e]Percent coverage range and median value (when applicable) of the plasmid identified in the long-read (Pac-Bio) assembly and the plasmids identified in the short-read (Illumina) assembly, determined by megablast (through NCBI).
[f]The same incompatibility group/colicin type was found in the plasmid detected using the long-read (Pac-Bio) assembly.
[g]Isolates 64 and 77 harbored a plasmid carrying both bla_{SHV-12} and *qnrB2* (accession numbers in GenBank of the plasmids detected using long-read assembly are MK191841 and MK191844, respectively). In isolate 76, *aac(6′)Ib-cr* was also found on the same plasmid (the accession number in GenBank of the plasmid detected using long-read assembly is MK191835).
[h]The plasmid accession numbers in GenBank are MK191837, MK191838, MK191839, and MK191842 for isolates 33, 44, 61, and 69, respectively.
[i]The accession number in GenBank of the plasmid detected using long-read assembly is MK191843.
[j]The accession number in GenBank of the plasmid detected using long-read assembly is MK191840.
[k]The accession number in GenBank of the plasmid detected using long-read assembly is MK191845.
[l]The accession number in GenBank of the plasmid detected using long-read assembly is MK191846.
[m]The accession number in GenBank of the plasmid detected using long-read assembly is MK191836.

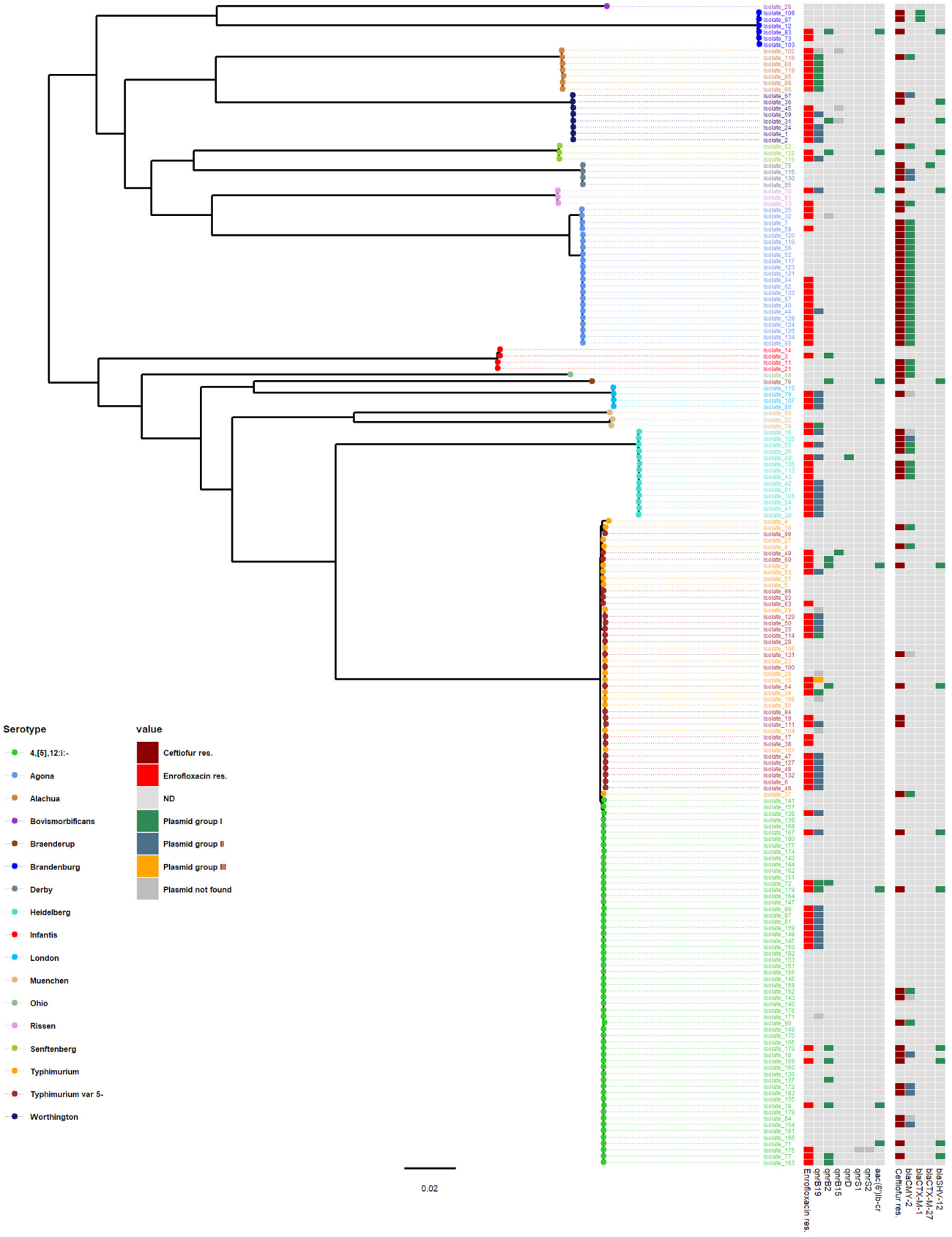

FIG 3 Maximum-likelihood tree constructed using the core-genome alignment of nontyphoidal-*Salmonella* isolates collected from Midwestern swine during the years 2014 to 2016. Two *S.* Paratyphi type A outgroup strains (GenBank accession numbers SRR3033248 and SRR3277289) were used to root

(Continued on next page)

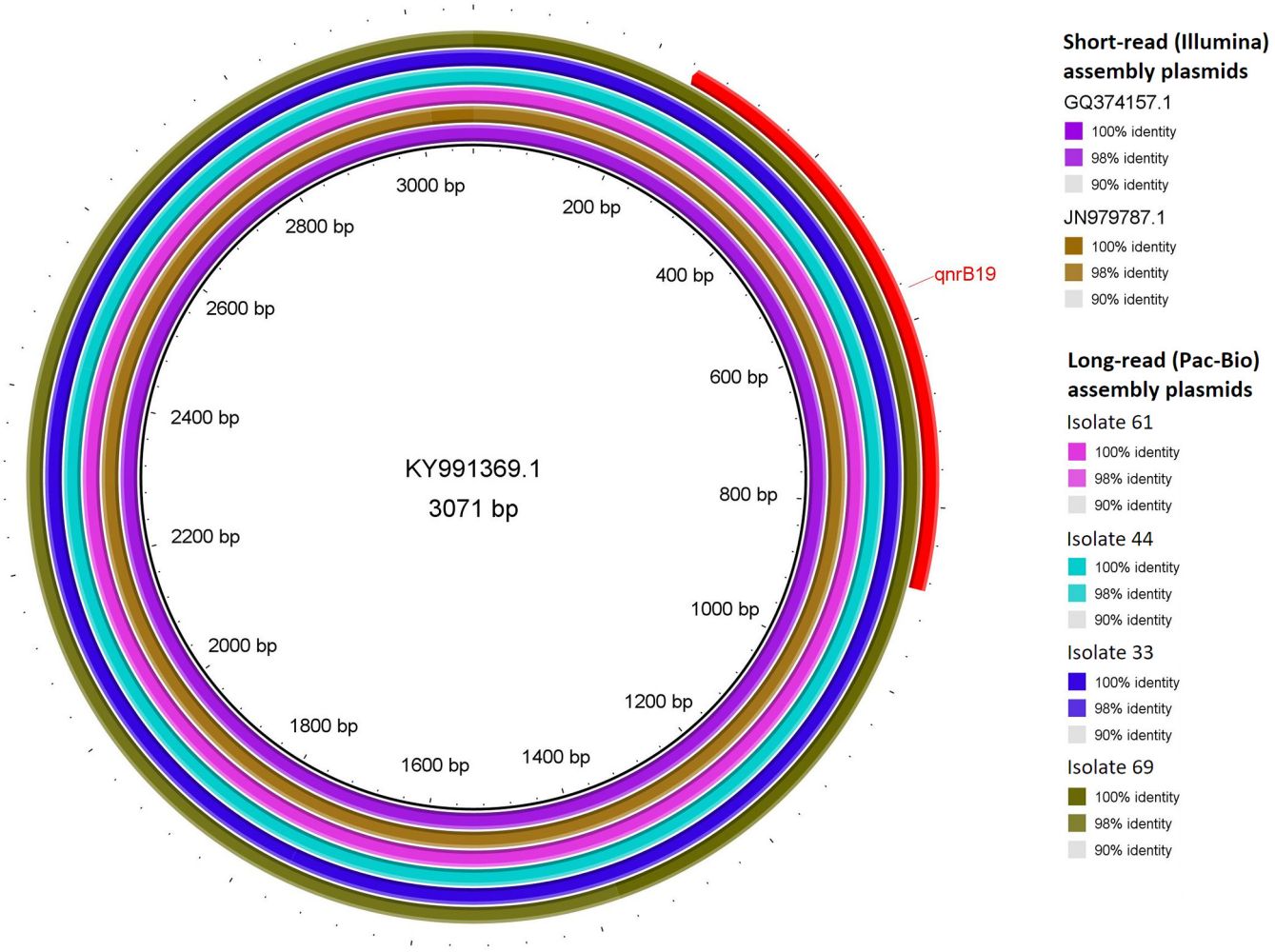

FIG 4 A BLAST ring alignment of the two plasmids detected in short-read (Illumina) assemblies (GenBank accession numbers GQ374157.1 and JN979787.1) and four long-read (Pac-Bio) assembly plasmids (isolates 33, 44, 61, and 69) identified for the predominant group of plasmids harboring the *qnrB19* gene (group II), with the short-read-assembly-identified plasmid with GenBank accession number KY991369.1 as a reference. The percentage of identity with the reference for each aligned sequence is indicated in the key. The location of the *qnrB19* gene is indicated in red.

Circular plasmids harboring *qnrB19* were found in four isolates (isolates 33, 44, 61, and 69) by using long-read assemblies. Three were identical and one was highly similar (one SNP difference) to a short-read-identified plasmid of group II whose GenBank accession number is KY991369.1 (Fig. 4, Table 5; Table S5). Similar plasmid groups were identified in 19 *Salmonella* isolates of swine origin harboring the *qnrB19* gene in the FDA NARMS Now database (32). These were categorized as harboring plasmids of group I ($n = 3$) or group II ($n = 15$), while in one isolate, the plasmid could not be identified.

DISCUSSION

The whole-genome sequencing (WGS) analysis of NTS isolates from swine clinical samples revealed the presence of multiple plasmid-mediated genetic determinants conferring resistance to quinolones or ESC. Each of the AARGs detected was harbored on only a few plasmid groups that were distributed among serotypes, suggesting

FIG 3 Legend (Continued)
the tree (not included in the figure). The analysis included 122,201 variable sites in the alignment (i.e., SNPs). Tip colors indicate serotype. Data shown in the heatmap include (i) resistance to enrofloxacin (MIC of ≥1 mg/liter), (ii) the plasmid groups demonstrated in short-read assemblies for each *qnr* and *aac(6′)Ib-cr* gene, (iii) resistance to ceftiofur (MIC of ≥8 mg/liter), and (v) the plasmid groups obtained in short-read assemblies for each *bla* gene.

potential horizontal spread of resistance genes between and within serotypes. In addition, we found multiple known and novel mutations in target enzymes and efflux pump regulation genes. These novel mutations (which are likely to be identified more often given the increasing use of WGS) in the genes involved in the quinolone resistance mechanism may potentially lead to reduced susceptibility. This potential was demonstrated in isolate 58, which was resistant to enrofloxacin (MIC of 1 mg/liter) and did not harbor AARGs, and the only mutation that was found in this isolate was a nonsynonymous mutation in *ramR*. However, given the complexity of such mechanisms, further molecular studies are essential to determine the contribution of such novel mutations to phenotypic resistance.

As described before for NTS (4, 18), the mutations found here varied between serotypes, and target enzyme mutations were not restricted to the quinolone resistance-determining region (QRDR) only, as opposed to mutations described in *Escherichia coli* (4). Hopkins et al. (4) postulated that the mutations' locations outside the QRDR in *Salmonella* suggest that these mutations confer resistance by different mechanisms than mutations within the QRDR. However, additional molecular studies are essential to support this theory.

The two antimicrobials studied here, ceftiofur and enrofloxacin, have been licensed in the United States for use in swine since 1992 and 2008, respectively (new animal drug application [NADA] application numbers 141-068 [enrofloxacin] and 140-338, 141-235, 141-288, and 200-420 [ceftiofur] [19]). According to NARMS reports, the prevalence of resistance to ESC in swine samples from the slaughterhouse has decreased and stabilized following the ban on extra-label use in livestock in 2012 (13) and the prevalence of resistance to quinolones remained low ($<$1%) between 2013 and 2015 (8). However, higher levels of resistance have been reported in clinical isolates from swine (17). Comparison of genotypic and phenotypic resistance in this study revealed significant associations between enrofloxacin resistance (MIC of $\geq$1 mg/liter) and the presence of *qnr* genes, as well as the number of resistance determinants. The presence of multiple resistance determinants was also significantly associated with high MICs (enrofloxacin MIC of $>$2 mg/liter) among enrofloxacin-resistant isolates, consistent with a cumulative effect of these genetic determinants. This has been described previously (4, 20), yet to our knowledge, not as part of a comprehensive comparison of all genetic determinants as conducted here. Resistance to ceftiofur was predominantly mediated by $bla_{\text{CMY-2}}$, commonly found in farm animals in the United States (11, 12), and the presence of this gene has recently been linked with the occurrence of ESC-resistant *S.* Heidelberg strains in Europe (21). This AmpC β-lactamase confers extended resistance to cephalosporins similar to that provided by extended-spectrum β-lactamases (ESBL), but due to its additional resistance to clavulanic acid (6), it may constitute an even higher limitation upon options for medical treatment. The presence of this gene and the ESBL bla_{CTX} genes, which are commonly found in Europe (22) but not in the United States (23), resulted in high MICs (ceftiofur MIC of $>$8 mg/liter).

Our multistep approach to detect and characterize plasmid groups using the short-read assemblies enabled the characterization of plasmid groups for almost all AARGs, which was further confirmed through long-read sequencing. Still, we had limited ability to identify the simultaneous presence of multiple AARGs on large ($>$200 kbp) plasmids, which is inevitable given the limitations imposed by the short length of the contigs assembled (24). Overall, the approach taken here could be a useful method to determine whether certain AARGs are distributed by similar plasmids (especially those smaller than 7,555 bp) in NTS across serotypes and/or host species (e.g., human and swine). This knowledge should advance the understanding of the dynamics behind the increasing prevalence of resistant strains and inform the potential design of mitigation measures.

The plasmid with GenBank accession number KY991369.1, present in all four isolates harboring *qnrB19* and subjected to Pac-Bio sequencing, was first identified in the United States in an *S.* Anatum isolate from swine cecal samples in 2014 (14). This small plasmid is highly similar to plasmids in isolates from humans in Bolivia (2005) (25) and Argentina (2008) (26), and we have characterized it as part of the *qnrB19*-bearing

plasmid group II. This group was identified in 39/57 and 15/19 of the isolates harboring *qnrB19* in this study and in the NARMS Now data (32), respectively, suggesting it may be widely distributed in the United States. The presence of such a plasmid across multiple serotypes may have been attributed by the emergence of serotypes like *S.* 4,[5],12:i:− harboring *qnrB19* genes (16). This finding is salient, given that our data and other recent studies (14, 16) suggest that *qnrB19* alone may be sufficient to confer phenotypic resistance to quinolones, in contrast with previous reports (4, 20).

Among the plasmids harboring *qnrB19* whose sequences were assembled using short-read technology (Illumina), the plasmids with GenBank accession numbers GQ374156.1 (25) and KU674895.1 (27) from plasmid group I and the plasmid with GenBank accession number GQ374157.1 (25) from plasmid group II demonstrated mobility via transformation *in vitro*. In addition, the plasmid with GenBank accession number FN428572.1 from plasmid group I lacked the mobilization system, and its horizontal spread was suggested to occur only through phage transduction, fusion with conjugative replicons, or transformation of naked DNA (28). These findings may suggest that the plasmids detected in *Salmonella* isolates from swine in this study are able to spread horizontally to other bacteria. However, further molecular studies for determination of the conjugation ability and plasmid transfer frequency are required for better evaluation of their potential for natural transmissibility and the risk for public health.

Coresistance to antimicrobials due to the presence of multiple AARGs in the same plasmid may underpin the persistence of resistance in a bacterial population even after eliminating the use of one of the antimicrobials (29). In addition, such multiresistant pathogens may lead to higher costs of treatment and to increased use of carbapenems or tigecycline in human clinical cases (3). In this study, the detection of large plasmids harboring *qnrB2*, bla_{SHV-12}, and occasionally *aac(6′)Ib-cr* indicates the possible spread of such coresistance in swine. This may impose an additional challenge for mitigating these resistant phenotypes. Interestingly, in *S.* Agona, in which phenotypic coresistance was abundant, these plasmids were not found and cooccurrence of AARGs against quinolones and ESC was rare, suggesting that other mechanisms not identified in this study may be involved in coresistance.

Higher prevalences of resistance to enrofloxacin and ceftiofur in serotypes 4,[5],12:i:− and Agona described previously in the Midwestern swine clinical samples (17) led us to hypothesize that the same genetic determinants could contribute to resistance in other serotypes. However, we found different resistance determinants in these serotypes: in *S.* 4,[5],12:i:−, resistance to ceftiofur was mediated by the presence of either bla_{CMY-2} or bla_{SHV-12}, while resistance to enrofloxacin was mediated by the presence of *qnrB2* and *aac(6′)Ib-cr* (potentially in the same plasmid as bla_{SHV-12}) or by the presence of *qnrB19*. In contrast, resistance to ceftiofur in *S.* Agona was associated mainly with bla_{CMY-2}, while enrofloxacin resistance was mediated mainly by an S83Y mutation (a change of the amino acid Serine to Tyrosine in position 83) in *gyrA* previously described in *Salmonella* and associated with resistance (18). In addition, in the same *S.* Agona isolates, *ramR* was not detected in the short-read assemblies and a deletion of 375 bp in the gene sequence was identified in the long-read assembly. Akiyama and Khan (30) described a 315-bp deletion in *ramR* from *S.* Schwarzengrund isolates that resulted in the overexpression of *ramA* and led to reduced susceptibility to quinolones, and therefore, a similar effect could be hypothesized here.

Overall, these findings demonstrate that diverse determinants contribute to resistance to ESC and quinolones in NTS serotypes in swine in the United States, and the serotype-specific genotypes described here highlight the importance of serotype-specific AMR surveillance. Due to the importance of these antimicrobials in human medicine, the potential of transmission of such resistant strains to humans is of concern, especially given the presence of plasmids harboring AARGs that could spread horizontally and potentially be transmitted to other bacteria.

MATERIALS AND METHODS

Study population. A subset of 183 NTS isolates comprising 17 serotypes recovered from Midwest swine clinical samples at the Minnesota Veterinary Diagnostic Laboratory during 2014 to 2016 were selected and sent for whole-genome sequencing (WGS) using Illumina platforms. Isolates were (i) resistant to either enrofloxacin ($n = 56$) or ceftiofur ($n = 35$) or both ($n = 33$) or (ii) susceptible to both ($n = 59$). Among these, the WGS information and resistance phenotypes of 48 *S.* 4,[5],12:i:− isolates were available from a previous study (16).

Clinical and Laboratory Standards Institute methodology (31) and clinical breakpoints for resistance were adopted (16) to determine phenotypic resistance to enrofloxacin (MIC of ≥1 mg/liter) and ceftiofur (MIC of ≥8 mg/liter). For this purpose, isolates with intermediate MICs (i.e., MICs above 0.25 and lower than 1 mg/liter and above 2 and lower than 8 mg/liter for enrofloxacin and ceftiofur, respectively) were regarded as susceptible.

In addition, the slaughterhouse findings of the U.S. Food and Drug Administration (FDA) NARMS Now surveillance program (32) were screened (using free-text search) to identify *Salmonella* isolates recovered from swine cecal samples between 2013 and 2015 that harbored the *qnrB19* gene. Their raw reads were downloaded and subjected to the same analysis for plasmid characterization (see below).

Data analysis. *De novo*-assembled contigs created using the SPAdes assembler (version 3.12.0) (33) were used to (i) perform core-genome alignment and phylogenetic analysis (see below), (ii) determine the presence of nonsynonymous mutations (herein simply "mutations") in known target enzyme genes and genes involved in the regulation of efflux pumps (Table S1) using a local BLAST (version 2.4.0+) (34), and (iii) determine the presence of AARGs, plasmid replicons, and multilocus sequence types using the bacterial analysis pipeline (with the default settings, i.e., threshold cutoffs for gene detection were set to at least 90% identity and more than 60% coverage of the query sequence) at the Center for Genomic Epidemiology (CGE; https://cge.cbs.dtu.dk/services/).

In addition, the *Salmonella In Silico* Typing Resource (SISTR) platform (version 1.0.2) (35) was used to determine the serotypes (see the supplemental material for further details).

Core-genome alignment and phylogeny construction. The *de novo* assemblies were annotated using Prokka (version 1.13.3) (36), and a core genome (including the outgroup strains; see below) was extracted using Roary (version 3.12.0) (37). The core-genome alignment was used for the construction of the maximum-likelihood trees using RAxML (version 8.2.10) (38) with the generalized time-reversible with gamma (GTR+Γ) substitution model. Trees were rooted using *S.* Paratyphi type A as an outgroup (GenBank accession numbers SRR3033248 and SRR3277289). Support for nodes on the trees was assessed using 5,000 bootstrap replicates. The packages ape (version 5.0) (39) and ggtree (version 1.10.5) (40) in R (version 3.4.3) (41) were used for visualization.

Identification of the plasmids harboring AARGs. Following the detection of AARGs using the CGE website, local BLAST queries were used to identify the contigs containing the AARGs. These contigs were then BLASTed (online) against the NCBI repository (NCBI nt), and the first 10 matches (i.e., top matches sorted [ascending] by their E values) obtained for each contig were recorded (overall coverage and identity ranged from 6% to 100% and 90.89% to 100%, respectively). For each AARG, a matrix of contigs (each representing an isolate, as none of the isolates harbored more than a single copy of an AARG) and BLAST matches was generated. Then, the NCBI GenBank records from matches identified in at least 10% of the isolates were screened (using free-text search) to include only matches indicated as plasmids and in which the AARG in question or resistance to its antimicrobial family (quinolones/cephalosporins) were indicated. The sequences of these plasmids were then downloaded and used as a reference to align (using Bowtie2 version 2.3.4.1) (42) the raw reads of the isolates in which a given AARG was found. In these alignments, a conservative approach was taken and a base coverage depth of zero was assigned to locations with fewer than 8 bases aligned. The breadth of coverage percentile [breadth of coverage percentile = 100 × (reference genome length − number of positions with zero coverage)/reference genome length], the absolute breadth of coverage [absolute breadth of coverage = (breadth of coverage percentile × reference genome length)/100], and the number of single-nucleotide polymorphisms (SNPs) were then calculated for each alignment.

The plasmid references for which the alignments had the highest percent breadth of coverage (top two) or absolute breadth of coverage (top two) were selected out of the alignments that had at least 60% breadth of coverage (the same cutoff used as the default setting for gene detection in ResFinder [version 2.1]) (43) and fewer than 200 SNPs (arbitrarily selected to reduce the number of possible matches). The plasmids selected following this multistep filtering process were grouped based on similarity and were considered the most likely plasmids containing the AARG.

Pac-Bio sequencing. Ten isolates were selected for Pacific Biosciences (Pac-Bio) long-read sequencing based on their plasmids identified in short-read assemblies. Hybrid assemblies of the Pac-Bio long reads with the Illumina short reads were created using Unicycler (version 0.4.4) (44). Bandage (version 0.8.1) (45) was used to visualize assemblies and to identify the plasmids harboring the AARGs in long-read assemblies. Pac-Bio-identified plasmids were compared with the plasmid groups identified in the previous step using NCBI nucleotide megablast, and the BLAST Ring Image Generator (BRIG version 0.95) (46) was used for alignment visualization.

Data summarization and statistical analysis. Data were summarized using Microsoft Excel and R (version 3.43) (41). Associations between phenotypic enrofloxacin resistance and (i) the presence of *qnr* genes or (ii) the total number of potential resistance determinants (including AARGs and mutations) were evaluated in separate univariable analyses. The analyses were conducted in (i) all the isolates using a MIC of ≥1 mg/liter as a cutoff value for resistance and (ii) resistant isolates using a MIC of >2 mg/liter as a cutoff for high MICs. In addition, the association between the presence of *bla* genes and phenotypic

resistance to ceftiofur was assessed. All associations were estimated in separate univariable analyses with Pearson's chi-square or Fisher's exact tests using the WinPEPI statistical package (47). A P value of <0.05 was considered to indicate statistical significance, and when necessary, it was adjusted for multiple comparisons using Bonferroni's correction.

The relationships between the presence of resistance determinants and the MICs found for enrofloxacin were visualized (including only isolates in which resistance determinants to quinolones were identified [$n = 121$]) in Venn diagrams using the VennDiagram package (version 1.6.18) (48) in R. In addition, collinearity between AARG pairs was assessed following the method of Dohoo et al. (49).

For the purpose of this analysis, the chromosomal mutations detected (Table S3 in the supplemental material) were defined as potentially contributing to resistance to quinolones. However, the chromosomal mutations T717N in *gyrB* and T57S, S255T, S395N, A469S, and T620A in *parC* were found in multiple serotypes regardless of the enrofloxacin resistance phenotype and were excluded from the analyses (Fig. 1; supplemental material and Tables S2 and S3).

For further details on materials and methods, see the supplemental material.

Accession number(s). The raw reads from Illumina and Pac-Bio sequencing were deposited at the NCBI under BioProject accession numbers PRJNA215333 and PRJNA505665. In addition, the plasmids harboring AARGs that were detected using the long-read assemblies were uploaded to GenBank under accession numbers MK191835 to MK191846.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02602-18.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.03 MB.

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The authors declare no conflict of interests.

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