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Genetic background of neomycin resistance in clinical *Escherichia coli* isolated from Danish pig farms

Prabha Subramani,[1,2] Gaia Menichincheri,[2] Mattia Pirolo,[1] Gabriele Arcari,[2] Egle Kudirkiene,[1] Riccardo Polani,[2] Alessandra Carattoli,[2] Peter Damborg,[1] Luca Guardabassi[1]

AUTHOR AFFILIATIONS See affiliation list on p. 15.

ABSTRACT Neomycin is the first-choice antibiotic for the treatment of porcine enteritis caused by enterotoxigenic *Escherichia coli*. Resistance to this aminoglycoside is on the rise after the increased use of neomycin due to the ban on zinc oxide. We identified the neomycin resistance determinants and plasmid contents in a historical collection of 128 neomycin-resistant clinical *E. coli* isolates from Danish pig farms. All isolates were characterized by whole-genome sequencing and antimicrobial susceptibility testing, followed by conjugation experiments and long-read sequencing of eight selected representative strains. We detected 35 sequence types (STs) with ST100 being the most prevalent lineage (38.3%). Neomycin resistance was associated with two resistance genes, namely *aph(3″)-Ia* and *aph(3′)-Ib*, which were identified in 93% and 7% of the isolates, respectively. The *aph(3″)-Ia* was found on different large conjugative plasmids belonging to IncI1α, which was present in 67.2% of the strains, on IncHI1, IncHI2, and IncN, as well as on a multicopy ColRNAI plasmid. All these plasmids except ColRNAI carried genes encoding resistance to other antimicrobials or heavy metals, highlighting the risk of co-selection. The *aph(3′)-Ib* gene occurred on a 19 kb chimeric, mobilizable plasmid that contained elements tracing back its origin to distantly related genera. While *aph(3″)-Ia* was flanked by either Tn*903* or Tn*4352* derivatives, no clear association was observed between *aph(3′)-Ib* and mobile genetic elements. In conclusion, the spread of neomycin resistance in porcine clinical *E. coli* is driven by two resistance determinants located on distinct plasmid scaffolds circulating within a highly diverse population dominated by ST100.

IMPORTANCE Neomycin is the first-choice antibiotic for the management of *Escherichia coli* enteritis in pigs. This work shows that *aph(3″)-Ia* and to a lesser extent *aph(3′)-Ib* are responsible for the spread of neomycin resistance that has been recently observed among pig clinical isolates and elucidates the mechanisms of dissemination of these two resistance determinants. The *aph(3″)-Ia gene* is located on different conjugative plasmid scaffolds and is associated with two distinct transposable elements (Tn*903* and Tn*4352*) that contributed to its spread. The diffusion of *aph(3′)-Ib* is mediated by a small non-conjugative, mobilizable chimeric plasmid that likely derived from distantly related members of the *Pseudomonadota* phylum and was not associated with any detectable mobile genetic element. Although the spread of neomycin resistance is largely attributable to horizontal transfer, both resistance determinants have been acquired by a predominant lineage (ST100) associated with enterotoxigenic *E. coli*, which accounted for approximately one-third of the strains.

KEYWORDS AMR, neomycin, enterotoxigenic *Escherichia coli*, clonal diversity, conjugation, plasmids

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Address correspondence to Luca Guardabassi, lg@sund.ku.dk.

Prabha Subramani and Gaia Menichincheri contributed equally to this article. Author order was determined based on increasing seniority.

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Pathogenic *Escherichia coli,* mainly enterotoxigenic *E. coli* (ETEC), is a leading cause of porcine enteritis, resulting in significant economic losses in pig production. Traditionally, colistin and zinc oxide were widely used for the treatment of porcine enteritis, but restrictions on the use of these two antimicrobials imposed in the European Union (EU) and other parts of the world have made antibiotic treatment of this disease difficult (1, 2). Currently, neomycin (NEO) is one of the first choices for this indication in view of its excellent antimicrobial activity against *E. coli* and its favorable pharmacological properties for the treatment of enteritis (i.e., low gastrointestinal absorption) (3). In Denmark, NEO was used for the treatment of post-weaning diarrhea in pigs until its withdrawal in 2008 and subsequently was reintroduced in 2017. This resulted in a gradual rise in the prevalence of NEO resistance by up to 20% in 2020 among clinical *E. coli* isolates from Danish pig farms (4, 5). Recently, we demonstrated an association of NEO resistance in clinical isolates from weaners with NEO use at the farm level and the presence of F4 and F18 fimbriae, which are typically associated with ETEC (6).

NEO resistance is mediated by aminoglycoside-modifying enzymes such as aminoglycoside 3′ O-phosphotransferases (*aph-3′*) (7, 8). The *aph(3′)-Ia* gene (previously *aphA1*) is the most commonly reported NEO/kanamycin resistance gene in commensal and pathogenic *E. coli* isolated from pigs (9–11). Other aminoglycoside phosphotransferase-encoding genes previously reported in porcine *E. coli* include *aph(3′)-Ib, aph(3′)-IIa,* and *aph(3′)-IIIa* (12, 13). NEO resistance genes are usually located on transposons or other mobile genetic elements, facilitating their horizontal movement between different clonal lineages and bacterial species (10, 14). Previous studies described the mobilization mechanism of the *aph(3′)-Ia* gene in Tn*4352,* a compound transposon bounded by IS*26*, identified on an IncA/C2 plasmid (15). Tn*4352* is widely diffused, often in association with other relevant resistance determinants, such as the *tet*(X7) gene, conferring high-level tigecycline resistance identified in *E. coli* from chickens, or *mcr-1.1* conferring colistin resistance in *E. coli* from swine, poultry, and bovine sources (16, 17). The *aph(3′)-Ia* gene has also been reported as part of Tn*903* in *E. coli* (18). However, the genetic environment of NEO resistance genes in pig pathogenic *E. coli* is largely unknown.

The objective of this study was to identify the NEO resistance determinants and assess their genetic location and mobility in a historical collection of 128 NEO-resistant *E. coli* isolates collected from Danish pig farms between 1992 and 2020. All isolates were analyzed by whole-genome sequencing (WGS), followed by conjugation experiments and long-read sequencing of selected strains for plasmid characterization.

RESULTS

Antimicrobial resistance

Antimicrobial susceptibility testing (considering the intermediate category as resistance) showed that 122 isolates (95.3%) were resistant to three or more antimicrobial classes tested in addition to NEO. A single isolate was susceptible to all tested antimicrobials. The highest proportions of resistance were observed for streptomycin (89.8%), sulfamethoxazole (85.9%), ampicillin (83.6%), and tetracycline (78.9%). Resistance proportions were the lowest for cefotaxime (5.5%), colistin (3.1%), and ciprofloxacin (no resistance detected). NEO MICs ranged from 64 to ≥4,096 mg/L, with most of the isolates (n = 105, 82%) displaying MICs between 128 and 512 mg/L (Fig. 1). Significant differences in the prevalence of antimicrobial resistance (AMR) were observed between toxigenic and non-toxigenic isolates with ETEC displaying lower proportions of resistance to ampicillin, chloramphenicol, sulfamethoxazole, and streptomycin (Table 1).

Based on WGS data, a total of 67 AMR genes were detected by ResFinder, including genes encoding aminoglycoside resistance mediated by *aadA* adenylyltransferases (*aadA1, aadA2, aadA2b, aadA3, aadA5, aadA7, aadA8b, aadA11, aadA12, aadA13, aadA17,* and *aadA22*), *aph* phosphotransferases [*aph(3′)-Ia, aph(3′)-Ib, aph(3″)-Ib, aph(4)-Ia, and aph(6)-Id*], and *aac* acetyltransferases [*aac(3)-IV* and *aac(3)-IId*]. Among those, two phosphotransferase-encoding genes were previously associated with NEO resistance, namely *aph(3′)-Ia*, which was detected in 119 isolates (93%), and *aph(3′)-Ib*, which was

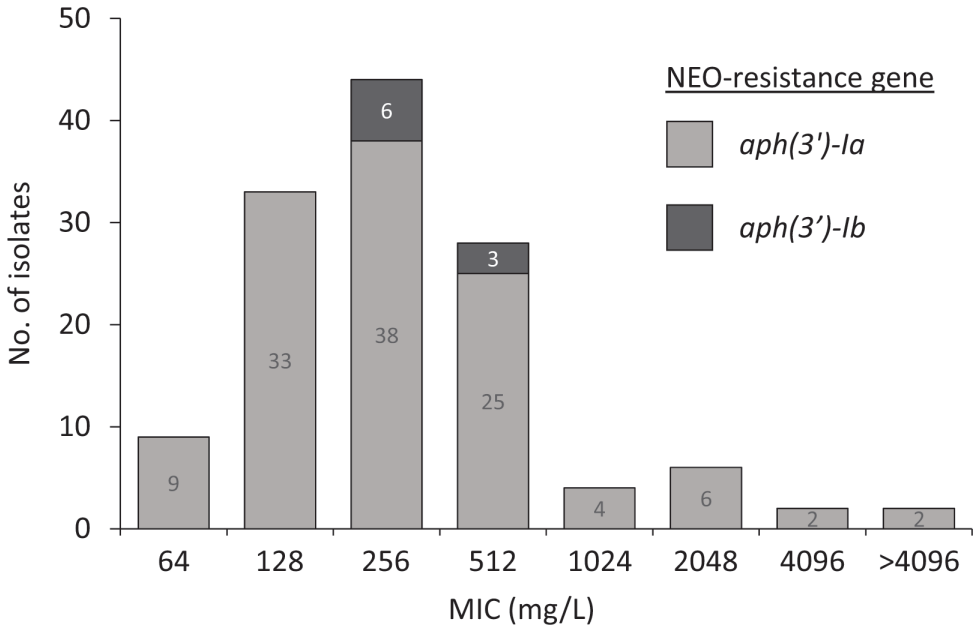

FIG 1 Neomycin MIC distribution of 128 porcine *E. coli* strains. NEO MIC was tested in the range from 64 to ≥ 4,096 mg/L.

found in nine ETEC isolates (7%). The occurrence of genes encoding resistance to antimicrobial classes other than aminoglycosides is presented in Table 1. ETEC harbored more frequently *aph(3′)-Ib* and less frequently *sul2, aph(3″)-Ib, tet*(B)*, dfrA5, dfrA12,* and *tet*(M) genes in comparison with non-ETEC isolates (Table 1). Statistical analysis revealed a strong agreement between the presence of resistance genes and phenotypic resistance based on MIC testing. The kappa value ranged between 0.62 and 1.00 for all tested antimicrobials, except for nalidixic acid and spectinomycin, which displayed kappa values of 0.03 (slight agreement) and 0.62 (substantial agreement), respectively. No known colistin resistance determinants were identified in the four strains defined as colistin resistant by phenotypic testing.

Strain diversity

Multilocus sequence typing (MLST) analysis of the 128 NEO-resistant isolates revealed the presence of 35 sequence types (STs), including 20 singleton STs and 10 clonal complexes (CC): CC10, CC12, CC23, CC42, CC86, CC101, CC155, CC156, CC165, and CC469. In total, 38 strains did not belong to a CC. The most common lineage was ST100/CC165, which accounted for 49 strains (38.3%), followed by ST10/CC10 (22.7%), CC23 (7.8%), and CC42 (6.3%). Among the 128 isolates tested, phylogroup A was the most common (64.1%), followed by B1 (14.1%), B2 (0.8%), C (7.8%), D (7.8%), E (3.1%), G (0.8%), or undetermined phylogroups (1.6%). The SerotypeFinder tool identified a total of 31 O-types and 23 H-antigens, leading to 41 O:H combinations (see also Data set S1 in the supplemental material). The most common serotype was O149:H10 (28.9%) followed by O141:H4 (6.3%) and O138:H14 (3.9%).

A phylogenetic tree alignment, based on 1,989 core genes (defined as present in 99% of the strains), was performed to assess the genetic relationships among the 128 Illumina-sequenced strains (Fig. 2). Coherently with MLST results, a midpoint-rooted visualization of the tree displayed several branches, the most populated one consisting of 49 genomes belonging to ST100 and two genomes with unassigned STs. All but one of the other 76 genomes clustered in multiple clades (Fig. 2). Associations between CCs and phylogroups were observed (Fig. 2). Namely, the two major CCs, CC165 and CC10, were clearly associated with phylogroup A (95.8% and 96.5% isolates, respectively) and all CC23 isolates belonged to phylogroup C. On the contrary, phylogroup B1 exhibited

TABLE 1 Prevalence of AMR phenotypes and genotypes identified in 128 clinical *E. coli* isolates from Danish pig farms, including 86 enterotoxigenic (ETEC) and 42 non-ETEC isolates[c]

Antimicrobial class	Drug	Prevalence of AMR phenotype (%)			AMR gene	Prevalence of AMR genotype (%)			Kappa[b]
		Total	ETEC	non-ETEC		Total	ETEC	non-ETEC	
Aminoglycosides	APR	24.2	27.9	16.7	*aac(3)-IV*	20.3	24.4	11.3	0.81
	GEN	24.2	25.6	21.4	*aac(3)-IId*	3.1	1.2	7.1	0.87
	SPT	68.5	66.3	66.7	*aph(3")-Ib*[a]	71.0	61.6	90.5	0.62
	STR[a]	89.8	84.9	100	*aph(6)-Id*	71.9	62.8	90.5	0.92
					aph(4)-Ia	19.5	23.3	11.3	
					aadA11	1.6	2.3	0	
					aadA13	2.3	3.5	0	
					aadA14	0.8	1.2	0	
					aadA2	14.8	12.8	19	
					aadA5	3.1	2.3	4.8	
					aadA7	2.3	3.5	0	
					ant(3")-Ia	57	62.8	45.2	
					aph(3')-Ia[a]	93	89.5	100	
					aph(3')-Ib[a]	7.0	10.5	0	
Beta-lactams	AMC	7.8	4.6	14.3	bla_{TEM-1A}	6.3	8.1	2.4	0.85
	AMP[a]	83.6	76.7	97.6	bla_{TEM-1B}	66.4	61.6	76.2	0.89
					bla_{TEM-30}[a]	3.1	1.2	7.1	
					Other *bla* genes	4.7	3.5	7.1	
	CTX	5.5	7.0	2.4					ND
Macrolides					*mph*(A)	22.7	17.4	33.3	ND
					mph(B)[a]	8.6	11.6	2.4	ND
					mph(G)	1.6	1.2	2.4	ND
					erm(B)	21.9	17.4	31.0	ND
Phenicols	CHL[a]	24.2	17.4	38.1	*catA1*	4.7	2.3	9.5	0.72
					catB2	0.8	0	2.4	
					cmlA1	12.5	10.5	16.7	
	FLO	15.6	11.6	23.8	*floR*	14.0	10.5	21.4	0.68
Polymyxins	CST	3.1	3.5	2.4					ND
Quinolones	CIP	0	0	0					ND
	NAL	14.0	18.6	4.8	*qnrS1*	3.1	3.5	2.4	0.02
Folate pathway inhibitors	SUL[a]	85.9	79.0	100	*sul1*	57.0	60.4	50.0	0.94
					sul2[a]	53.9	43.0	76.2	
					sul3	14.0	11.6	19.0	
	TMP	74.2	72.1	78.6	*dfrA1*	63.4	67.4	54.8	0.97
					dfrA5[a]	8.6	3.5	19.0	
					dfrA12[a]	12.5	8.1	21.4	
					dfrA14	3.1	3.5	2.4	

(*Continued on next page*)

TABLE 1 Prevalence of AMR phenotypes and genotypes identified in 128 clinical *E. coli* isolates from Danish pig farms, including 86 enterotoxigenic (ETEC) and 42 non-ETEC isolates[c] (*Continued*)

Antimicrobial class	Drug	Prevalence of AMR phenotype (%)			AMR gene	Prevalence of AMR genotype (%)			Kappa[b]
		Total	ETEC	non-ETEC		Total	ETEC	non-ETEC	
					dfrA17	3.1	2.3	4.8	
					others (*dfrA8/10/16*)	3.9	2.3	3.5	
Tetracyclines	TET	78.9	77.9	80.9	*tet*(A)	60.1	64.0	52.4	0.93
					tet(B)[a]	24.2	17.4	38.1	
					tet(D)	0.8	1.2	0	
					tet(G)	0.8	1.2	0	
					tet(M)[a]	3.1	0	9.5	
					tet(X)	3.1	3.5	2.4	
Miscellaneous					*mdf*(A)	93.0	93.0	92.9	
					oqxA	0.8	1.2	0	

[a]Significant difference (p-value < 0.05) between ETEC and non-ETEC in the prevalence of resistant isolates as determined by pheno- or genotyping testing.
[b]Kappa values indicate slight agreement (between 0.01-0.20), fair agreement (0.21-0.40), moderate agreement (0.41-0.60), substantial agreement (0.61-0.80), or perfect agreement (0.81-1.00) between phenotypic and genotypic resistance. For resistance phenotypes associated with multiple genes, the Kappa value is shown in correspondence with the first gene listed in the table.
[c]Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; APR, apramycin; CHL, chloramphenicol CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; FLO, florfenicol GEN, gentamicin; NAL, nalidixic acid; SPT, spectinomycin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; ND, the Kappa value was not determined because no relevant resistance genes were detected or the relevant drug was not tested (e.g., macrolides).

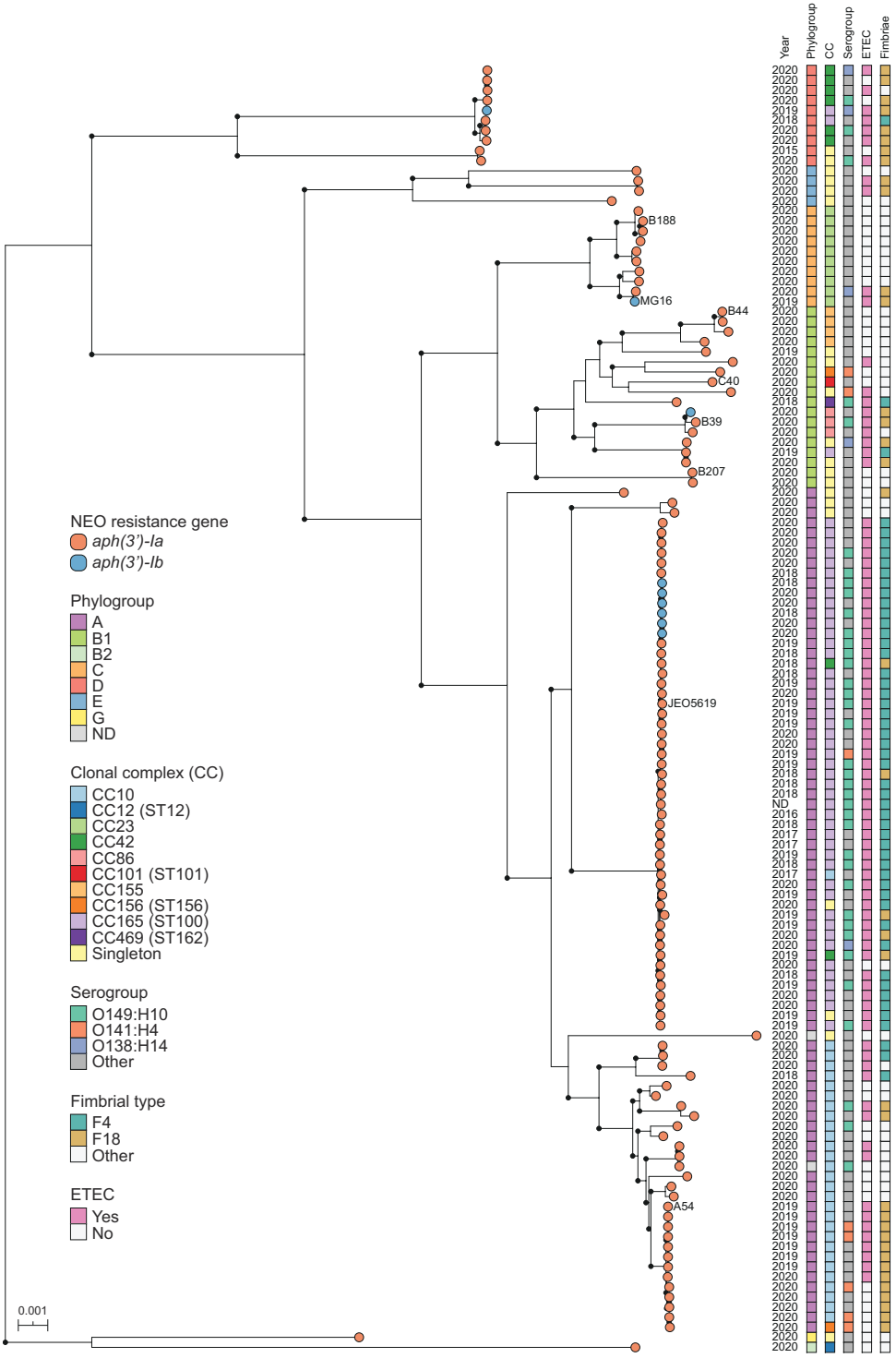

FIG 2 Mid-point rooted phylogenetic tree for the 128 sequenced strains using the core-genome alignment. For each strain, the year of isolation and the traits phylogroup, clonal complex, serogroup, and porcine pathotype are indicated. ST enclosed in brackets denotes that there was only a single ST within that CC. Branches with strain ID indicate strains selected for Oxford Nanopore Technologies long-read sequencing. The scale bar indicates the expected number of substitutions per site. Bootstrapping was performed on 1,000 ultrafast replicates, and bootstrap values above 70% are illustrated by filled circles at the ends of the branches.

a high level of heterogeneity as it comprised six CCs and seven singleton STs. No associations were observed between serogroups and clades (Fig. 2).

While *aph(3″)-Ia* occurred in a variety of genetically distinct *E. coli* lineages, *aph(3″)-Ib* showed a less heterogeneous distribution. Out of the nine strains carrying this NEO resistance gene, six strains were obtained from pigs in three different Danish regions, with a mean distance on the core genome of 13 single-nucleotide polymorphisms (SNPs; range: 7–19 SNPs) and were localized in the ST100 branch (SNP distance matrix is provided in Data Set S2). The three remaining strains did not show any core genome-based relationship.

Distribution of virulence genes

The most common virulence genes detected in the 128 sequenced isolates were curlin major subunit (*csgA*, 99.2%), lipoprotein precursor (*nlpI*, 96.9%), avian *E. coli* hemolysin gene (*hlyE,* 95.3%), followed by the presence of fimbrial cluster genes (*yehA/B/C/D*, 91.4%). In all, 86 (67.2%) isolates were ETEC, defined as *E. coli* with one or more enterotoxin genes (6), namely *estA, estB* (heat-stable enterotoxin A and B; STa and STb), and *elt* (heat-labile enterotoxin; LT). The most common enterotoxin gene was *estB* (60.2%), followed by *elt* (51.6%) and *estA* (24.2%). The most prevalent enterotoxin combination was *estB:elt* (40.6%). The *astA* gene encoding enteroaggregative *E. coli* heat-stable enterotoxin was present in 60.9% of isolates. Two isolates were identified as hybrid ETEC/STEC (shiga toxin-producing *E. coli*), as they exhibited the presence of both shiga toxin (*stx2e*) and enterotoxins (*estA/B*), along with F18 fimbria (*fedA*).

Fimbrial genes were widely distributed, with the most common types being *fimH* type 1 fimbriae (50.8%), followed by *faeG* encoding F4 (41.4%) and *fedA* encoding F18 (29.7%), which was present as two subunits *fedAac* (n = 33) and *fedAab* (n = 5). Other fimbrial genes, including *afa*, *focC*, *sfaD*, *yfcV*, *papA_F16, papC_F48,* and *f17G* (F17), were detected in single isolates. ETEC isolates harbored *faeG* (52.3%), *fedA:fimH* (25.6%), *faeG:fimH* (6.9%), *fedA* (6.9%), *fimH* (6.9%), while one isolate (1.2%) had no fimbriae-encoding genes. Accordingly, 59.3% and 32.5% of ETEC isolates could be classified as ETEC:F4 and ETEC:F18, respectively (Table S1).

In vitro conjugative transfer of neomycin resistance

PlasmidFinder performed on WGS data demonstrated high variability of replicons within the 128 NEO-resistant strains. In all, 32 strains were selected for conjugation experiments based on the phylogenetic tree of Fig. 2 (see also Data Set S1 in supplemental material). All donor strains were positive for more than one replicon (Table 2). NEO resistance was transferable to *E. coli* K12 from 25 out of the 32 selected donor strains. Each of the 25 transconjugants harbored either *aph(3″)-Ia* (n = 23) or *aph(3″)-Ib* (n = 2), and at least one of the replicon types from the respective donor (Table 2). Based on PCR-based replicon typing (PBRT), the most prevalent replicon type associated with the transfer of *aph(3″)-Ia* was I1(α) (14/23 donor strains). Overall, this replicon type was identified in 80 of the 119 strains carrying *aph(3″)-Ia*. Other replicon types commonly associated with *aph(3″)-Ia* in the transconjugants were FII (n = 8), HI2 (n = 5), and X1 (n = 3), which occurred in 79, 20, and 49 *aph(3″)-Ia*-positive strains, respectively. The two transconjugants with *aph(3″)-Ib* carried I2 and HI2 plasmids, respectively (Table 2). Some transconjugants did not transfer only one single plasmid but were positive for multiple (up to five) replicon types, such as X1, X4, I2, FIB, FIA, HI1, HI2, and FII.

Plasmid identification

To better characterize the plasmids carrying the *aph(3″)-Ia* and *aph(3″)-Ib* genes, eight donor strains, used for the conjugation experiments and displaying unique plasmid profiles by PBRT, were selected for long-read sequencing (indicated by arrows in Fig. 2; see also Data Set S1 in the supplemental material). The selected strains belonged to ST10, ST23, ST58, ST88, ST100, ST101, ST2952, and ST12193. Table 3 shows the plasmid profiles of these strains that were sequenced by Oxford Nanopore Technologies (ONT).

TABLE 2 Characteristics of the 32 NEO-resistant strains selected for bacterial conjugation, including ST, CC, MIC of NEO, NEO resistance gene, transfer of NEO resistance by conjugation, and PBRT in both donor and transconjugant strains

Strain ID	ST/CC (no.)	NEO MIC (mg/L)	NEO resistance gene	Conjugative	PBRT in donor	PBRT in transconjugant
JEO5619[a]	CC165 (49)	4,096	*aph(3')-Ia*	Yes	I1α, FIB, X1, FII	I1α
AV1	CC165 (49)	256	*aph(3')-Ib*	Yes	I2, FIB, I1Ɣ, FIIS, FII	I2(δ)
B188[a]	CC23 (10)	512	*aph(3')-Ia*	Yes	I1α, FIB, X1, FII	I1α
MG16[a]	CC23 (10)	256	*aph(3')-Ib*	Yes	HI2, FIB, FIIS, FII	HI2
A73	CC86 (3)	256	*aph(3')-Ia*	Yes	I2, BO, FIB, FIIS, FII	BO, FII
C71	ST641 (2)	256	*aph(3')-Ib*	No		
AV54[a]	CC10 (29)	64	*aph(3')-Ia*	Yes	I1α, BO, FIIS, X1, FII	I1α
A34	CC156 (2)	256	*aph(3')-Ia*	Yes	I1α, FIB, FIA, FII	I1α
B44[a]	CC155 (4)	256	*aph(3')-Ia*	Yes	I1α, FIB, FII	I1α
C40[a]	CC101 (1)	256	*aph(3')-Ia*	Yes	N, FIB, FII	N, FII
A126	CC12 (1)	256	*aph(3')-Ia*	Yes	BO, FIB, FII	BO
MG12	CC469 (1)	128	*aph(3')-Ia*	No		
A102	Unknown CC (20)	256	*aph(3')-Ia*	Yes	HI2, I1α, X1, FII	HI2, I1α, FII
MG9	CC42 (8)	128	*aph(3')-Ia*	Yes	I1α, FIB, FII	I1α
C4	ST117 (2)	512	*aph(3')-Ia*	Yes	I1α, FIB, FII	I1α
C66	ST542 (2)	128	*aph(3')-Ia*	Yes	FIB, FII	FIB, FII
C134	ST760 (1)	256	*aph(3')-Ia*	Yes	I1α, X1, FII	I1α
C109	ST763 (3)	256	*aph(3')-Ia*	No		
C147	ST772 (3)	256	*aph(3')-Ia*	Yes	HI2, I1α, I2, FIB, FII	HI2
B61	ST1629 (1)	2,048	*aph(3')-Ia*	Yes	I1α, FIB, FIA, X1, FII	I1α, FIB, FIA, FII
C16	ST2177 (1)	512	*aph(3')-Ia*	No		
B209	ST2521 (1)	128	*aph(3')-Ia*	Yes	I2, FIB, FIA, X1, FII	X1
C74	ST2602 (1)	128	*aph(3')-Ia*	Yes	HI2, I1α, FIB, X1, FII	HI2, FII
Z6	ST2628 (1)	128	*aph(3')-Ia*	Yes	I1α, FIA, FIB, FII	I1α
B138	ST2675 (1)	512	*aph(3″)-Ia*	Yes	HI2, I1α, X1, FII, X4	I1α
B207[a]	ST2952 (2)	128	*aph(3″)-Ia*	Yes	HI1, I1α, I2, FIB, X1, FII, X4	HI1, I1α, I2, FIB, X4
C52	ST3524 (2)	256	*aph(3')-Ia*	No		
B218	ST3525 (1)	1,024	*aph(3')-Ia*	Yes	HI1, I1α, I2, FIB, X1, FII	X1, FII
C30	ST7651 (1)	256	*aph(3')-Ia*	No		
B24	ST10333 (4)	128	*aph(3')-Ia*	No		
B39[a]	ST12193 (1)	128	*aph(3')-Ia*	Yes	HI2, I1α, FIIS, N2, X1, FII	HI2
B146	ST11407 (1)	128	*aph(3')-Ia*	Yes	HI2, I1α, X1, FII	I1α, X1, FII

[a]Strains selected for Oxford nanopore technologies sequencing.

The *aph(3″)-Ia* was located on (i) an IncI1α plasmid (pAV54_I, pB188_I, pB44_I; 110 kb) that was conjugated from three distinct donor strains belonging to ST10 (CC10), ST88 (CC23), and ST58 (CC55); (ii) an IncN plasmid (pC40_N; 76.7 kb) transferred from an ST101 (CC101) donor; (iii) an IncHI1 plasmid (pB207_H; 205 kb) from singleton ST2952; (iv) an IncHI2 plasmid (pB39_H; 264 kb) from singleton ST12193; and (v) a 5.9 kb ColRNAI plasmid from ST100 (CC165). This plasmid was estimated at 60 copies per cell. Various genes encoding resistance to other antimicrobials [*aadA1, aadA2, aadA12, dfrA1, dfrA12, ΔqacE, aph(3″)-Ib, aph(6)-Id, ant(3″)-Ia, aac(3)-IId, bla*$_{\text{TEM-1B}}$*, bla*$_{\text{TEM-214}}$*, sul1, sul2, tet*(A), *tet*(B), *erm*(B), *mph*(A)] or heavy metals such as mercury (*merT*) and tellurite (*terW*) were detected in all these NEO resistance plasmids except the small ColRNAI plasmid (Table 3). In one of the three IncI1α plasmids analyzed (pB44_I), the *aph(3″)-Ia* on Tn*903* showed a distinct localization along the plasmid backbone (Fig. S1). As for *aph(3″)-Ib*, the prototype MG16 strain harbored this gene on a 19-kb chimeric plasmid characterized by a RepA protein not detected by PlasmidFinder (here named RepMG16), whose gene shared a 98.8% nucleotide identity with the one from pAX22 plasmid (HF679279.1) (19). Yet, the BLASTn research performed on the entire plasmid indicated similarities with additional plasmid backbones, such as plasmids pROUE1 (MK047608.1) (20) and pLM16A1 (KM659090.1) (21) found, respectively, in a *Pseudomonas putida* strain, isolated

TABLE 3 Main features of the eight completely sequenced strains, including plasmid replicon type, size, virulence genes, and resistance genes

Strain ID (ST)	Plasmid	Replicon type	Size (kb)	Virulence genes on plasmid	Resistance genes on plasmid	NCBI Acc. no.
JEO5619 (ST100)	pJEO5619_Col	ColRNAI	5.9	–[a]	*aph(3″)-Ia*	OQ401023
	pJEO5619_F	IncFIB/FII	117.8	*astA, eltA, eltB*	*aadA1, dfrA1, mph*(B), *ΔqacE, sul1*	OQ401022
	pJEO5619_FSE11	IncFII(pSE11)	83.1	*faeC, faeD, faeE, faeF, faeH, faeI, faeJ* (K88 pili/F4 fimbriae)	–	OQ401021
	pJEO5619_V	UT[b]	65.6	*hlyA, hlyB, hlyC, hlyD, astA*	–	OQ401019
	pJEO5619_X	IncX1	33.5	–	*aadA1, dfrA1, mph*(B), *ΔqacE, sul1*	OQ401018
	pJEO5619_I	IncI1α	101.4	–	*aph(3″)-Ib, aph(6)-Id*	OQ401020
AV54 (ST10)	pAV54_I	IncI1α	107.0	–	*aadA1, aph(3')-Ia, dfrA1, ΔqacE, sul1, aph(3″)-Ib, aph(6)-Id,* bla_{TEM-1B}, *sul2, tet*(A), *tet*(R), *Mer* locus	OQ420467
	pAV54_F	IncFII	82.6	*esta*	–	OQ344286
	pAV54_FX	IncFII/IncX1	76.4	*hlyA, hlyB, hlyC, hlyD*	–	OQ420466
	pAV54_BO	IncB/O	116.1	–	*Mer* locus	OQ420465
B188 (ST88)	pB188_I	IncI1(α)	112.5	–	*aadA2, aph(3')-Ia, dfrA12, erm*(B), *mph*(A), *ΔqacE, sul1, Mer* locus	OQ420475
	pB188_F	IncFIB	171.6	*iroB, iroC, iroD, iroN, iutA, iucA, iucB, iucC, iucD*	*aph(3″)-Ib, aph(6)-Id, sitABCD, sul2, tet*(A), *arsD, Mer* locus	OQ420474
	pB188_p0111	p0111	92.2	–	–	OQ420476
	pB188_X	IncX1	41.3	–	*aadA5, dfrA17,* bla_{TEM-1B}, *ΔqacE*	OQ420477
MG16 (ST23)	pMG16	pMG16	19.6	–	*aph(3')-Ib*	OQ401017
	pMG16_H[c]	IncHI2	ND	ND	ND	
B44 (ST58)	pB44_I	IncI1α	101.4	–	*aph(3')-Ia,* bla_{TEM-1B}	OQ420464
C40 (ST101)	pC40_N	IncN	76.7	–	*ant(3'')-Ia, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id,* bla_{TEM-1B}, *ΔqacE, dfrA1, mph*(A), *aadA1, sul1, Mer* locus	OQ401024
	pC40_Col	Col440II	5.4	–	–	OQ401025
B207 (ST2952)	pB207_H	IncHI1	205.2	–	*aac(3)-IId, dfrA12, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id,* bla_{TEM-1B}, $bla_{TEM-214}$, *sul2, tet*(B), *locus Mer*	OQ401029
	pB207_I	IncI1α/X1	153.2	*hlyA, hlyB, hlyC, hlyD*	bla_{TEM-1B}, bla_{TEM-1B}	OQ401028
	pB207_FF	IncFIB/FII	97.2	–	–	OQ420463
	pB207_I2	IncI2	59.6	–	–	OQ401027
	pB207_X	IncX4	31.8	–	–	OQ401026
	pB207_F	IncFII	68.9	–	*aadA12,* bla_{TEM-1B}, *erm*(B), *mph*(A), *ΔqacE, sul1*	OQ420462
B39 (ST12193)	pB39_H	IncHI2	264.4	–	*aph(3')-Ia, tet*(B), *ter*(W)	OQ420469
	pB39_V	UT	260.7	–	–	OQ420472
	pB39_C	IncC	93.3	*fedA*	*ant(3″)-Ia, aph(3″)-Ib, aph(6)-Id, dfrA1, erm(42), floR, mph*(B), *ΔqacE, sul1*	OQ420468
	pB39_I	IncI1α	110.3	–	*aadA1, ant(3″)-Ia, cmlA1, dfrA12, sul3*	OQ420470
	pB39_X	IncX1	64.4	*hlyA, hlyB, hlyC, hlyD, esta*	–	OQ420473
	pB39_p0111	p0111	64.5	–	–	OQ420471

[a]Negative for virulence and/or resistance determinants.
[b]UT, untypable replicon by PlasmidFinder version 2.1.
[c]pMG16_H plasmid sequence was not completely closed. Therefore size is not determined (ND) and virulence/resistance genes identified in WGS cannot be attributed to this plasmid that remains as undetermined.

from the hospital setting, and a member of the *Achromobacter* genus, isolated from an environmental source (Fig. 3). Despite sharing similar backbones and replicase genes, these plasmids did not carry the *aph(3″)-Ib* gene. The MG16 plasmid showed 100% nucleotide identity in a region of 2.5 kb flanking the NEO resistance gene *aph(3″)-Ib* with pUZ8002 plasmid (MN602278.1) isolated from an *E. coli* in Germany. This region carries genes encoding for the protein *traB, ΔtraA, aph(3″)-Ib*, and a toxin/antitoxin system. None

of the sequenced plasmids carrying NEO resistance determinants was associated with virulence genes.

Mobile elements

The mobile element containing NEO resistance genes was analyzed in the eight strains selected for ONT. The *aph(3″)-Ia* gene was found on different mobile elements. Plasmids pJEO5619_Col and pB44_I were characterized by Tn*903* (TnCentral acc. no.: V00359.1). The transposon of pB44_I displayed an inversion in one of the two genes encoding for the TnpA transposase (Fig. 4, panel A). In plasmids pB188_I, pB207_H, pAV54_I, pB39_H, and pC40_N, *aph(3″)-Ia* was found in Tn*4352* derivatives, in all but one (pB39_H) integrated in proximity (1–6.5 kb) of the locus *Mer* (Fig. 4, panel B).

In the *aph(3″)-Ib* plasmid pMG16, there was no clear association between the NEO resistance gene and a transposable element (Fig. 3).

DISCUSSION

In Denmark, NEO was reintroduced in 2017 for the treatment of porcine enteritis resulting in an increase in the proportion of NEO resistance in clinical *E. coli* (6). This study demonstrates that this rise in NEO resistance is attributable to the spread of two resistance determinants, *aph(3″)-Ia* and, to a lesser extent *aph(3″)-Ib*, across distinct clonal lineages. These two NEO resistance determinants were previously reported among pig pathogenic *E. coli* in Denmark (11) and limited to *aph(3″)-Ia*, also in Spain (22), Switzerland (9), and Australia (10). However, their genetic environment and mechanism of dissemination had not been extensively explored prior to this study. Based on our genomic analysis of a comprehensive collection of porcine clinical *E. coli* isolates, it appears that the spread of *aph(3″)-Ia* in Danish pig production is attributable to multiple acquisitions of at least five distinct conjugative plasmids of variable size (76–264 kb) by *E. coli* ST100 or other less common lineages. A high prevalence (67.2%) of the I1-I(α) replicon was observed in our strain collection, whereas the other replicons associated with this gene were less frequent, suggesting that IncI1-I(α) plasmids are the main carrier of this NEO resistance gene. Plasmid insertion of *aph(3″)-Ia* was associated with two different transposable elements (Tn*903* and Tn*4352*), and insertion by Tn*4352* occurred at different sites on the plasmid backbone, highlighting the complexity of the evolutionary process leading to the widespread occurrence of this NEO resistance determinant among Danish pathogenic *E. coli*, which involves a variety of host lineages, plasmids, transposable elements, and insertion sites. Upon analyzing the data available on the NCBI database, it is evident that the distribution of Tn*4352* and Tn*903* is heterogeneous across multiple species and isolation sources. Both transposons have been previously associated with *aph(3″)-Ia* in *E. coli* and other Gram-negative bacteria (18, 23).

In contrast to *aph(3″)-Ia*, the dissemination of *aph(3″)-Ib* is mediated by the small non-conjugative plasmid pMG16 (19 kb) that has been acquired by four phylogenetically distinct *E. coli* lineages, that is, ST100, ST641, ST42, and ST23. The gene encoding for RepMG16 was found in all the nine strains carrying *aph(3″)-Ib*, indicating that the spread of this NEO resistance determinant is strongly associated with pMG16. This plasmid is non-conjugative, as it lacks part of the genes encoding mating pair formation (20). The plasmid content was assessed in the transconjugants of the AV1 and MG16 strains (data not shown), suggesting that even if pMG16 is non-conjugative, it can be mobilized by conjugative plasmids, presumably I2(δ) and HI2 in these donor strains, respectively (Table 2). Based on nucleotide sequence identity, this is a chimeric plasmid, with the scaffold shared with plasmids isolated from the *Achromobacter* and *Pseudomonas* genera and the region flanking the *aph(3″)-Ib* gene in common with plasmids found mainly in *E. coli*. The mechanism by which *aph(3″)-Ib* was inserted into pMG16 remains unknown since no mobile elements were detected in the region flanking this second NEO resistance determinant.

Despite the high diversity observed in our collection of NEO-resistant strains, the results indicate that ST100 (CC165) is the main lineage responsible for NEO resistance

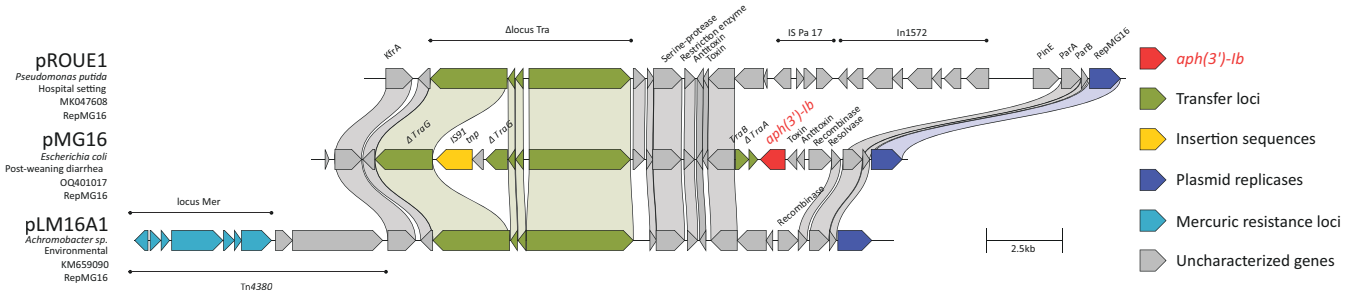

FIG 3 Map of the pMG16 plasmid containing the *aph(3″)-Ib* gene. Arrows indicate open reading frames deduced from the sequence of the pMG16 plasmid (OQ401017) in comparison with two plasmids sharing similar backbone retrieved from the GenBank Database, namely pROUE1 (MK047608.1) from *Pseudomonas putida* and pLM16A1 (KM659090.1) from *Achromobacter* sp.

in Danish pig production. This lineage was almost exclusively composed of ETEC strains (44/49 strains), harbored both NEO resistance determinants, usually exhibited resistance to three or more antimicrobial classes (44/49 strains), and occurred throughout the period covered by the strain collection, namely 1992 and 2016–2020. ST100 has previously been reported as prevalent among clinical porcine ETEC strains from the United States (24), Korea (25), and Denmark (11). The second most common lineage was ST10 (CC10), which occurred in both ETEC and non-ETEC strains and was previously associated with porcine ETEC strains in Spain (26), the United States (27), and China (28). ST10 has also been described as responsible for recurring *E. coli* outbreaks with high mortality levels in Danish broiler production (29) and is one of the global extraintestinal pathogenic lineages of increasing importance in human infections (30).

NEO-resistant strains displayed high proportions of resistance to alternative antibiotics that can be used to manage porcine ETEC enteritis, such as spectinomycin (89.8%), sulfamethoxazole (85.9%), and tetracycline (78.9%) (Table 1). This result highlights the lack of effective alternatives to NEO for the treatment of this common disease in pig production. ONT sequence analysis of the plasmids mediating NEO resistance revealed that *aph(3″)-Ia* was usually located on plasmids carrying AMR genes conferring resistance to other antimicrobials such as tetracyclines [*tet*(A), *tet*(B)], sulfonamides (*sul1*, *sul2*), trimethoprim (*dfrA*), and macrolides [*mph*(A)]. Co-resistance was particularly evident in the scaffolds of IncI1α, IncHI1, and IncN plasmids, which carried additional resistance genes (Fig. 4), providing evidence that NEO resistance may be co-selected by the use of other antimicrobials and vice versa. Indeed, NEO resistance might also be selected by exposure to mercury and tellurite, as suggested by the occurrence of genes conferring resistance to these heavy metals on NEO resistance

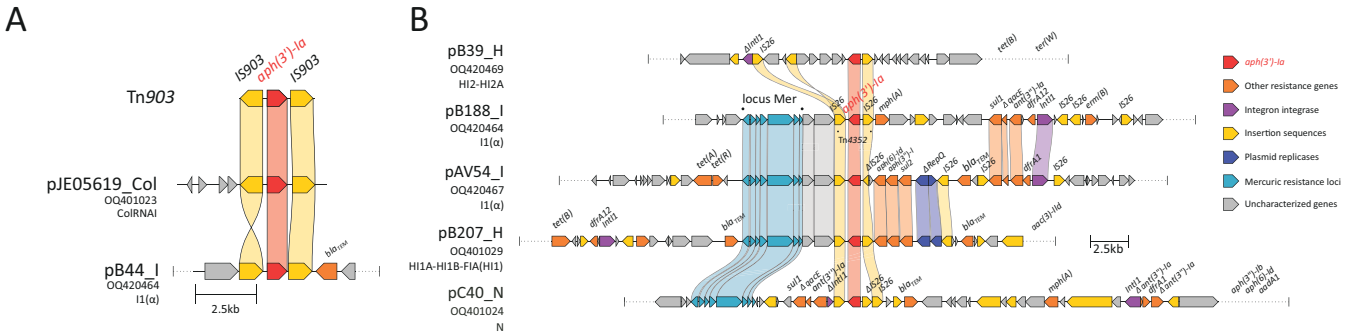

FIG 4 Maps showing the genetic environment surrounding the *aph(3″)-Ia* gene. (A) Map of *aph(3″)-Ia* associated with Tn*903* transposons. Comparison among the Tn*903* reference sequence (TnCentral acc. no. : V00359.1) and the open reading frames deduced from the sequences of pJEO5629_Col (OQ401023) and pB44_I (OQ420464) plasmids from the study. (B) Map of *aph(3″)-Ia* associated with Tn*4352* transposons with open reading frames deduced from the sequences of the pB39_H (OQ420469), pB188_I (OQ420475), pAV54_I (OQ420467), pB207_H (OQ401029), and pC40_N (OQ401024) plasmids from the study.

plasmids (Table 3). Notably, fimbrial, hemolysin, and enterotoxin genes associated with ETEC occurred on IncF plasmids that do not carry NEO resistance genes, excluding possible co-selection of virulent strains by NEO usage as hypothesized in a previous study (6).

There was an excellent agreement between AMR phenotype and AMR gene content, except for nalidixic acid, spectinomycin, and colistin. The low agreement between the phenotype and genotype of nalidixic acid resistance is attributable to the fact that resistance to this quinolone is frequently not conferred by transferrable resistance genes but by point mutations in the drug target (DNA polymerase). In all, 16 strains harbored *aadA1* or *ant(3″)-Ia* and displayed spectinomycin MICs ≥ 32 mg/L but were not classified as resistant by phenotypic testing because their MIC fell below the EUCAST epidemiological cutoff value (R ≥ 128 mg/L), indicating that strains harboring these spectinomycin resistance genes may be categorized as susceptible using this cutoff. It is unclear why no colistin resistance determinants were identified in the four strains defined as colistin resistant by phenotypic testing. This apparent discrepancy between phenotype and genotype could be due to the presence of mutations in genes associated with the modification of the lipid A of LPS, the primary target of colistin, or other not yet characterized mechanisms of resistance.

We acknowledge some limitations of our study. First, the distribution of isolates over time was uneven, and only eight strains were selected for ONT sequencing, which may have led to a biased representation of the NEO-resistant *E. coli* population and overlooked genetic variations and plasmids present in the remaining strains. To mitigate this risk, we selected eight prototype strains representing the whole collection and its salient features based on their diverse localization on the phylogenetic tree as well as on their plasmid profiles, NEO MICs, and NEO resistance genes. In addition, the study only examined NEO-resistant *E. coli* from pig farms in Denmark, and therefore our findings cannot be generalized to pig production in other countries.

In conclusion, the population of NEO-resistant *E. coli* strains that cause enteric disease in Danish pig farms is highly diverse and consists of numerous clonal lineages, with ST100 accounting for approximately one-third of the strains sequenced in this study. The spread of neomycin resistance that has been recently observed in association with the increased use of this aminoglycoside is driven by two resistance determinants, *aph(3′)-Ia* and, to a lesser extent *aph(3′)-Ib*. While *aph(3′)-Ia* is located on different conjugative plasmid scaffolds and associated with two distinct transposable elements (Tn*903* and Tn*4352*) that contributed to its spread, diffusion of *aph(3′)-Ib* is mediated by a small non-conjugative, mobilizable chimeric plasmid (pMG16) and the mobile element that permitted its integration into this plasmid remains unknown.

MATERIALS AND METHODS

Bacterial strains

A total of 128 clinical porcine NEO-resistant *E. coli* isolates were included in the study. Except for a single isolate from 1992, all isolates were collected from 2015 to 2020 [2015 (n = 1), 2016 (n = 1), 2017 (n = 3), 2018 (n = 15), 2019 (n = 26), and 2020 (n = 81)]. The isolates from 2020 comprised all NEO-resistant *E. coli* isolates obtained from routine diagnostic samples submitted to the SEGES Laboratory for swine diseases that year (6). The remaining isolates were previously obtained from this laboratory as part of various research projects. Most isolates originated from small intestinal content obtained during post-mortem examination (n = 75), followed by feces (n = 24), rectal swabs (n = 9), liver (n = 9), spleen (n = 2), kidney (n = 1), and unknown origin (n = 8). Metadata for all isolates are provided in Data Set S1 in the supplemental material.

Antimicrobial susceptibility testing

All isolates were tested by broth microdilution using custom-made Sensititre plates (ThermoFisher Scientific, Waltham, USA) containing the following antimicrobials:

amoxicillin/clavulanic acid, ampicillin, apramycin, cefotaxime, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, nalidixic acid, spectinomycin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim (6). Results were interpreted according to CLSI clinical breakpoints (CLSI M100, 2018; CLSI VET08 4th ed., 2018), EUCAST CBPs (EUCAST, v 8.1 Breakpoint Tables) (31–33), or according to epidemiological cutoffs (ECOFFs) available on EUCAST.org or proposed by Tian et al. (34).

Breakpoints used for each drug are reported in Table S1. In addition, minimum inhibitory concentrations of NEO were tested by broth microdilution using concentrations ranging from 8 to 4,096 mg/L and the reference strain *E. coli* ATCC 25922 as a quality control strain (35).

Illumina sequencing

DNA was extracted using the Maxwell RSC Cultured Cells DNA Kit (Promega, Wisconsin, USA), following the manufacturer's instructions in the Maxwell RSC machine (Promega). The quality and quantity of extracted DNA were determined using NanoDrop-1000 (Thermo Fischer Scientific, Massachusetts, USA) and agarose gel electrophoresis. DNA libraries were constructed using the Nextera XT Library Preparation Kit (Illumina, California, USA) following the manufacturer's sequencing protocol on MiSeq (Illumina).

Bacterial conjugation

Based on the results of genome sequence analysis, one strain per NEO resistance gene (*aph(3″)-Ia/Ib*) and per clonal complex based on MLST analysis was selected as donors for bacterial conjugation (n = 32 strains; Table 2). Lactose-positive, rifampicin-resistant *E. coli* K12 JEO432 was used as the recipient strain. Both donor and recipient strains were grown in Luria Bertani (LB) broth up to an OD_{600} = 0.5, followed by centrifugation at 5,000 rpm for 3 min and resuspension of the pellet in fresh LB medium. Bacterial conjugation was performed on LB agar plates with filters (0.22 µM, Millipore, Copenhagen, Denmark) at 37°C. Donor and recipient were mixed in a 1:1 ratio to a final volume of 100 µL on the filters. After overnight incubation at 37°C, bacterial material was washed off from the filters by vortexing using 0.9% isotonic NaCl. Dilution series were made, and 100 µL of bacterial suspensions was plated on MacConkey agar plates containing 32 mg/L NEO and 50 mg/L rifampicin for transconjugant selection. NEO resistance genes were confirmed in presumptive transconjugants by multiplex colony PCR targeting *aph(3″)-Ia/Ib* using primers and PCR conditions previously published by Miró et al. (36). Transconjugants confirmed by PCR were stored at −80°C for further analysis.

Plasmid-based replicon typing

Plasmid replicon types in donors and transconjugants were determined using the PBRT 2.0 Kit (Diatheva, Fano, Italy) following the manufacturer's instructions, as previously described (37). The amplified products were analyzed by 2.5% agarose gel electrophoresis.

Nanopore sequencing

In total, 32 donor strains were chosen for bacterial conjugation, and as a result, 25 *E. coli* K12 strains acquired NEO resistance. Eight of these 25 NEO donor strains were selected for further sequencing using ONT. These strains were selected based on unique plasmid profiles revealed by PBRT of both donors and transconjugants.

High molecular weight DNA extraction was performed using the Monarch HMW DNA Extraction Kit for Tissue (New England Biolabs, Massachusetts, USA) following the manufacturer's instructions. Nanopore library preparation was performed using the Rapid Barcoding Kit 96, SQK-RBK110-96 (Oxford Nanopore Technologies, Oxford, UK). Sequencing was performed on an Mk1C MinION platform on a Flow Cell R9.4.1 (Oxford Nanopore Technologies).

Bioinformatics analysis

Illumina raw sequencing reads were assembled using SPAdes Genome Assembler (v.3.13.1) (38), and quality was checked on QUAST (v.5.0.2) (39). The 128 assemblies were annotated using Prokka (40), and the resulting .gff files were used to determine a core genome alignment using the Roary tool (41). The IQ-TREE tool (42) was deployed for phylogenetic tree construction, using 1,000 ultrafast bootstraps. The tree exhibits multiple clades, wherein each clade represents a branch within the cladogram that encompasses a single presumed ancestor and all its descendant lineages. The visualization was carried out using microreact (43), and the figure was adjusted using the InkScape software (https://inkscape.org/). SNP distance among isolates was estimated using the snp-dists tool (https://github.com/tseemann/snp-dists). Assembled genomes were screened for resistance determinants using ABRicate v1.0.1 (https://github.com/tseemann/abricate) against the ResFinder database (44), and alignment results with identity scores greater than 95% were selected as positive matches. Raw sequences were analyzed using MLST 2.0 (Achtman scheme), and CC clustering was performed by Phyloviz v2.0 (45). Strains sharing seven identical alleles were grouped and given the same ST. A CC was defined as a group of STs in which every ST shares at least five of the seven alleles with the other STs in the complex. To further describe the relationship between the strains, we used the term "clonal lineage" to refer to a group of phylogenetically related strains originating from a common ancestor and belonging to the same CC, even if they do not necessarily share the same ST. Serogroups and phylogroups were determined using assembled genomes in the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org) (SerotypeFinder) (46) and ClermonTyping (47), respectively. VirulenceFinder (48) and PlasmidFinder (49) available at the CGE database were used to identify the proportion of virulence genes and plasmids.

Long-read assembly was performed using Flye (50). Illumina reads and ONT assemblies were integrated by the Unicycler tool version 0.4.8.0 (51) using a bold bridging mode. In the cases in which the Unicycler tool could not yield full-length, circular plasmids in the strains subjected to ONT (i.e., pAV54_BO, pAV54_F, pAV54_FX, pAV54_I, pB188_X, pB207_I, and pJEO5619_Col), the circular Flye assembly was used as scaffold for the reconstruction using the Unicycler partial assemblies by BLASTn. AMR and replicon genes were identified using the ResFinder and PlasmidFinder tools, respectively, on staramr (Galaxy Version 0.9.1+galaxy0) (https://github.com/phac-nml/staramr). All these tools are available on the GalaxyEU Server (https://usegalaxy.eu/). Plasmid annotation was performed on the RAST Server (https://rast.nmpdr.org/). Transposon identification was performed using the TnCentral database (https://tncentral.ncc.unesp.br/) (52). Gene cluster comparison figures were realized using the clinker tool (53), and figures were adjusted using the open-source InkScape software.

Statistical analysis

Cohen's kappa statistical analysis was used to calculate the discrepancies between the phenotype and genotype resistance of the tested antimicrobials (SPSS, version 26, IBM, USA) (54). Correlation between these factors was interpreted based on kappa value; 0.01–0.20, slight agreement; 0.21–0.40, fair agreement 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–1.00, perfect agreement. Fisher's exact test was used to assess whether there was an uneven distribution of AMR and resistance genes between ETEC and non-ETEC strains [JASP Team (2023), JASP (Version 0.17.1) (Linux)]. Statistical significance was considered for a P-value <0.05.

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AUTHOR AFFILIATIONS

[1]Department of Veterinary and Animal Sciences, Section for Veterinary Clinical Microbiology, University of Copenhagen, Frederiksberg C, Denmark
[2]Department of Molecular Medicine Sapienza, University of Rome, Rome, Italy

AUTHOR ORCIDs

Mattia Pirolo http://orcid.org/0000-0002-9814-1500
Luca Guardabassi http://orcid.org/0000-0002-2389-5084

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DATA AVAILABILITY

Illumina WGS data were previously submitted to the NCBI Sequence Read Archive submitted under BioProjects PRJEB38608 (*n* = 23 strains) (11) and PRJNA849907 (*n* = 60 strains) (55). WGS data for 30 strains were submitted to NCBI SRA under BioProject PRJNA934822. The remainder of WGS data (*n* = 15 strains) are available upon request. Complete sequences of 30 plasmids were deposited in the NCBI GenBank database under the accession numbers: pAV54_F (OQ344286); pAV54_BO (OQ420465); pAV54_FX (OQ420466); pAV54_I (OQ420467); pB188_F (OQ420474); pB188_I (OQ420475); pB188_p0111 (OQ420476); pB188_X (OQ420477); pB207_F (OQ420462); pB207_FF (OQ420463); pB207_H (OQ401029); pB207_I (OQ401028); pB207_I2 (OQ401027); pB207_X (OQ401026); pB39_C (OQ420468); pB39_H (OQ420469); pB39_I (OQ420470); pB39_p0111 (OQ420471); pB39_V (OQ420472); pB39_X (OQ420473); pB44_I (OQ420464); pC40_ColI (OQ401025); pC40_N (OQ401024); pJEO5619_ColI (OQ401023); pJEO5619_F (OQ401022); pJEO5619_FSE11 (OQ401021); pJEO5619_I (OQ401020); pJEO5619_V (OQ401019); pJEO5619_X (OQ401018); and pMG16 (OQ401017).

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplementary Figure S1 (AEM00559-23-S0001.pdf). Supplementary Figure 1: The aph(3')-Ia resistance regions in IncI1α plasmids.
Supplemental Dataset S1 and S2 (AEM00559-23-S0002.xlsx). Supplemental Data Set 1: Description of 128 neomycin-resistant Escherichia coli strains.Supplemental Data Set 2: SNP-distance matrix
Supplementary Table S1 (AEM00559-23-S0003.pdf). Supplementary Table 1: Breakpoints used for antimicrobial susceptibility testing.

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