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Aminoglycoside-modifying enzyme and 16S ribosomal RNA methyltransferase genes among a global collection of Gram-negative isolates

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ABSTRACT

Objectives: The prevalence of genes encoding aminoglycoside-modifying enzymes (AMEs) and 16S rRNA methyltransferases among 200 Gram-negative clinical isolates resistant to different aminoglycosides and collected worldwide during 2013 was evaluated.
Methods: Selected AMEs and 16S rRNA methyltransferase genes were screened by PCR/sequencing among 49 *Acinetobacter* spp., 52 *Pseudomonas aeruginosa* and 99 Enterobacterales.
Results: In total 72 isolates carried *aac(6′)-Ib* variants (36.0% overall; 55.6% Enterobacterales): 30 *aac(6′)*-Ib-cr, 21 *aac(6′)*-Ib and 21 *aac(6′)*-Ib-like displaying substitutions L119S (alone or in combination with V71A or R173K) or S100G. Ten *aph(3′)*-VI variants were detected among 35 isolates (46.9% of *Acinetobacter* spp.). Nineteen isolates carried variants of *aac(3)-I*, with *aac(3)-Ia* ($n = 13$, mostly *Acinetobacter* spp.) being the most prevalent. Other AME genes detected were *ant(3″)*-Ia ($n = 41$), *ant(2″)*-Ia ($n = 24$), *aac(3)-IIe* ($n = 23$), *aac(3)-IId* ($n = 21$), *aac(6′)*-Im ($n = 13$, mostly *P. aeruginosa*), *aacA8* ($n = 3$), *aac(3)-IIf* ($n = 1$) and *aac(3)-IVa* ($n = 1$). Among 42 isolates resistant to amikacin, gentamicin and tobramycin tested for 16S rRNA methyltransferase genes, 21 (50.0%) tested positive; *armA* was most common ($n = 14$), but 4 isolates carried *rmtB1*, 2 *rmtF1* and 1 new variant *rmtB4*. Over 60 gene combinations, consisting of one to four AMEs and 16S rRNA methyltransferases, were observed. Cloning genes not previously characterised revealed diverse aminoglycoside resistance patterns for some AMEs, but expected results for *rmtB4*.
Conclusions: Studies broadly evaluating these aminoglycoside resistance genes are needed. Using agents stable in the presence of these resistance genes might help overcome resistance.

1. Introduction

Aminoglycosides play an important role in the treatment of aerobic, Gram-negative infections, usually in combination with β-lactam agents. In the USA, the most commonly prescribed aminoglycosides for the treatment of serious infections include gentamicin, tobramycin and amikacin [1]. These and other aminoglycoside agents act by binding at the A-site of the 30S ribosomal subunit where codon–anticodon accuracy is assessed; the binding interferes with the function of the 16S rRNA ribosomal subunit and inhibits protein synthesis [2]. Many bacterial species have developed resistance mechanisms to aminoglycosides, including antimicrobial modification, ribosomal alteration and decreased permeability.

In the clinical setting, resistance to aminoglycosides is primarily mediated by aminoglycoside-modifying enzymes (AMEs) [3,4]. AMEs have significant clinical importance because the genes encoding these enzymes can be disseminated by plasmids or transposons and are often detected as part of gene cassettes carried by integrons that usually harbour other resistance markers, including metallo-β-lactamases, facilitating their selection [4]. AMEs inactivate aminoglycosides by catalysing the modified amino or hydroxyl groups through the process of acetylation (AAC), phosphorylation (APH) and/or adenylation (ANT) [4]. Each enzyme has a unique resistance phenotype owing to its varying effects on particular aminoglycosides [2].

Previous studies have noted that aminoglycoside resistance in *Acinetobacter baumannii* is usually encoded by APH and ANT and in

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Pseudomonas aeruginosa by AAC [5]. Because the AMEs in *A. baumannii* and *P. aeruginosa* are often associated with transferable plasmids and integrons, these genes can be shared with other Gram-negative species through horizontal gene transfer [5–7]. It has also been observed that the prevalence of aminoglycoside resistance mechanisms in Enterobacterales (previously Enterobacteriaceae) isolates correlates with aminoglycoside usage and changes with time and location [5].

Although less common, methylation of the 16S ribosomal subunit through the action of plasmid-mediated methyltransferases is a serious threat to the aminoglycoside class of antimicrobials. These enzymes methylate specific nucleotides of the ribosomal target sites that restrict binding of the aminoglycoside and result in high-level resistance to almost all aminoglycosides [8]. Currently, ten 16S rRNA methyltransferase enzymes have been identified (RmtA–H, ArmA and NpmA). The genes encoding these enzymes have all been shown to be plasmid-mediated [8] and are often associated with β-lactamases, including carbapenemases, which can facilitate their selection and dissemination [8–10].

The aim of this study was to evaluate the prevalence of common aminoglycoside resistance genes, including genes encoding AMEs and 16S rRNA methyltransferases, among 200 Gram-negative clinical isolates composed of *Acinetobacter* spp., *P. aeruginosa* and Enterobacterales species collected worldwide in 2013 displaying distinct resistance patterns for amikacin, tobramycin and gentamicin.

2. Materials and methods

2.1. Bacterial isolates

A total of 200 Gram-negative isolates, including 49 *Acinetobacter* spp., 52 *P. aeruginosa* and 99 Enterobacterales, were selected from isolates collected in 2013 from hospitals in North America (65 isolates), Europe (72 isolates), Latin America (39 isolates) and Asia-Pacific (24 isolates) as part of the SENTRY Antimicrobial Surveillance Program. Enterobacterales isolates included 23 *Klebsiella pneumoniae*, 19 *Escherichia coli*, 8 *Providencia stuartii*, 9 *Proteus mirabilis*, 8 *Enterobacter cloacae* species complex, 8 *Citrobacter freundii* species complex, 7 *Serratia marcescens*, 5 *Klebsiella oxytoca*, 5 *Morganella morganii*, 3 *Proteus vulgaris* group, 2 *Enterobacter aerogenes*, 1 *Providencia rettgeri* and 1 *Providencia* spp. The *Acinetobacter* species included 47 *A. baumannii–calcoaceticus* species complex, 1 *Acinetobacter. nosocomialis* and 1 *Acinetobacter pittii*. Only one isolate per patient was included in the study. Selection was based on resistance patterns to amikacin, tobramycin and gentamicin (Table 1). Species identification was confirmed for all selected isolates by matrix-associated laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) using a MALDI Biotyper® (Bruker Corp, Billerica, MA) following the manufacturer's instructions.

2.2. Antimicrobial susceptibility testing

All isolates were tested for antimicrobial susceptibility using the reference broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) [11] and susceptibility interpretations were performed according to the CLSI [12]. Quality control was performed using *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. All quality control results were within specified ranges as published in CLSI documents [12].

2.3. PCR screening and sequencing of aminoglycoside resistance genes

All isolates were screened for the presence of AME genes, whereas only isolates showing resistance to amikacin, tobramycin and gentamicin were screened for 16S rRNA methyltransferases ($n = 42$). PCR screening for AMEs and 16 s rRNA methyltransferases was performed using five multiplex reactions and three singleplex reactions (Supplementary Table S1). Primers for amplification of *aac(3)-Ia*, *aac(3)-Ib*, *aac(3)-Ic*, *aac(3)-Id*/*e*, *aac(3)-IIa*, *aac(3)-IIc*, *aac(3)-IId*, *aac(3)-IIe*, *aph(3′)-VIa*, *aac(6′)*-Ib (aacA4), *ant(2″)*-Ia, *aac(3)-IVa*, *aac(6′)*-Il (*aacA7*), *ant(3″)-Ia* (*aadA1*), *rmtA–H*, *armA* and *npmA* were designed from sequences deposited in the NCBI GenBank database or were selected from the literature (Supplementary Table S1) [9,13,14].

All positive amplicons were sequenced on both strands of the nucleotide and the deduced amino acid sequences were analysed using Lasergene software (DNASTAR, Madison, WI). Primers for sequencing were designed for *aac(6′)*-Ib, *armA* and *rmtB* from sequences deposited in the NCBI GenBank database or were selected from the literature (Supplementary Table S1) [15].

2.4. Cloning aminoglycoside-modifying enzyme and 16S rRNA methyltransferase genes

The genes *aac(6′)*-Ib, *aac(3)-II*, *aph(3′)*-VI and *rmtB4* from one isolate carrying each new variant were amplified using primers targeting the full gene, designed based on either the sequence deposited in the NCBI GenBank database or sequences obtained by amplifying and sequencing the integron harbouring the *aac(6′)*-Ib variants (Supplementary Table S1). Amplicons were sequenced on both strands and the full nucleotide sequences for all new gene variants were submitted to the NCBI GenBank database. Amplicons were cloned using a CloneJET™ PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA) and were transformed into One Shot® TOP10 Electrocomp™ *E. coli* (Thermo Fisher Scientific) for *rmtB4* or into ElectroMAX™ DH5α-E Competent Cells (Invitrogen, Carlsbad, CA) for all other genes following each manufacturer's instructions. Transformants and recipient strains were tested for antimicrobial susceptibility using the reference broth microdilution method as described in Section 2.2.

Table 1
Isolate distribution according to aminoglycoside resistance profile.

Bacterial group	No. of isolates resistant to aminoglycosides when applying CLSI breakpoints[a]					
	GEN	GEN + AMK	GEN + TOB	TOB	TOB + AMK	GEN + TOB + AMK
Overall (200)	36	16	45	29	19	42
Acinetobacter spp. (49)	6	8	7	5	5	11
Enterobacterales (99)[b]	3	1	27	19	8	16
Pseudomonas aeruginosa (52)	7	7	11	5	6	15

CLSI, Clinical and Laboratory Standards Institute; GEN, gentamicin; AMK, amikacin; TOB, tobramycin.
[a] Isolates resistant to amikacin only were not very common and thus were not included.
[b] Enterobacterales isolates included 23 *Klebsiella pneumoniae*, 19 *Escherichia coli*, 10 *Providencia* spp., 9 *Proteus mirabilis*, 8 *Citrobacter freundii*, 8 *Enterobacter cloacae*, 7 *Serratia marcescens*, 5 *Klebsiella oxytoca*, 5 *Morganella morganii*, 3 *Proteus vulgaris* group and 2 *Enterobacter aerogenes*.

3. Results

3.1. Detection of aminoglycoside resistance genes

Among 25 038 Gram-negative clinical isolates collected during 2013 as part of the SENTRY Program, 200 isolates were randomly selected for testing based on their aminoglycoside resistance profile (Table 1). A total of 164 (82.0%) of the 200 tested isolates carried 16S rRNA methyltransferase- and/or AME-encoding genes. Most Enterobacterales isolates carried these resistance genes (93/99; 93.9% of this group), whereas only 79.6% of *Acinetobacter* spp. (39/49) and 61.5% *P. aeruginosa* (32/52) yielded positive results for these resistance mechanisms (Table 2). Resistance to ceftazidime and/or ceftriaxone was noted among 44/99 Enterobacterales isolates, and carbapenem resistance was observed among 11/99 (9 *K. pneumoniae*), 18/52 and 37/49 Enterobacterales, *P. aeruginosa* and *Acinetobacter* spp., respectively.

Overall, *aac(6′)*-Ib variants were the most prevalent AME gene and were detected in a total of 72 isolates (36.0%), including 55 Enterobacterales, 9 *P. aeruginosa* and 8 *Acinetobacter* spp. (Table 2). Among the *aac(6′)*-Ib variants, *aac(6′)*-Ib-cr was the most common (29 Enterobacterales and 1 *P. aeruginosa*), followed by *aac(6′)*-Ib detected among 21 isolates (18 Enterobacterales and 3 *P. aeruginosa*), and 1 *P. aeruginosa* isolate that carried both genes. Variants of *aac(6′)*-Ib harbouring mutations leading to amino acid alteration were detected in 21 isolates and included L119S (17 isolates, including 7 *Acinetobacter* spp., 7 Enterobacterales and 3 *P. aeruginosa*) alone or in combination with substitutions V71A (1 *P. aeruginosa*) or R173K (1 *P. aeruginosa* and 1 *K. pneumoniae*) (Table 2). The substitution S100G was also observed in the AAC(6′)-Ib-encoding gene of 1 *P. aeruginosa* isolate (Supplementary Fig. S1). Isolates carrying *aac(6′)*-Ib variants were observed in all geographic regions: 23 isolates in Europe (11 countries), 21 in the USA, 17 in Latin America (8 countries) and 11 in Asia-Pacific (4 countries). In addition to *aac(6′)*-Ib variants, less common *aac(6′)*-I genes were detected and included *aac(6′)*-Im noted among 12 *P. aeruginosa* isolates and 1 *P. stuartii* and *aacA8* among 3 *P. aeruginosa* isolates from Europe (all different hospitals). Isolates harbouring *aac(6′)*-Im were not observed in Asia-Pacific but were present in the remaining regions.

A total of 41 isolates carried *ant(3″)*-Ia, also known as *aadA1*, and this was the second most prevalent gene detected in this collection. Isolates carrying these genes included 23 Enterobacterales, 14 *Acinetobacter* spp. and 4 *P. aeruginosa* (Table 2).

Genes belonging to the AAC(3)-II group were also common. A total of 21 Enterobacterales isolates harboured *aac(3)-IId* and another 21 carried *aac(3)-IIe* that was also detected in 2 *Acinetobacter* spp.

One *Acinetobacter* spp. isolate harboured a new variant, named AAC(3)-IIf, which shows 98.6% amino acid sequence homology to AAC(3)-IIe (Supplementary Fig. S2). *aac(3)-IIa* was not detected in any of the isolates tested.

A total of 19 isolates carried *aac(3)-I* variants: 11 *Acinetobacter* spp. and 2 *S. marcescens* (from Europe, different countries) harboured *aac(3)-Ia*, whilst *aac(3)-Ib*, *aac(3)-Ic* and *aac(3)-Id/e* were detected in *P. aeruginosa* exclusively. One occurrence of *aac(3)-IVa* in a *K. pneumoniae* isolate from Latin America was observed (Table 2). During this study, we noticed that the sequences deposited for *aac(3)-Id* (GenBank accession nos. **AB114632** and **AY458224**) [16,17] and *aac(3)-Ie* (**AY463797** and **DQ520937**) [18,19] are identical; therefore, this gene is referred to as *aac(3)-Id/e* in this study.

Ten variants of *aph(3′)*-VI were detected among 35 isolates. These isolates were mostly *Acinetobacter* spp. (23/49; 46.9% of the total for this group), but also included 10 Enterobacterales (10.1%) and 2 *P. aeruginosa* (3.8%) (Table 2). Variants detected included *aph(3′)*-VIa (5 *Acinetobacter* spp. and 1 *P. mirabilis*), *aph(3′)*-VIc (10 *Acinetobacter* spp.), *aph(3′)*-VId (2 Enterobacterales species), *aph(3′)*-VIe (1 *Acinetobacter* spp.), *aph(3′)*-VIf (2 *P. aeruginosa*), *aph(3′)*-VIg (3 *Acinetobacter* spp.), *aph(3′)*-VIh (1 *Acinetobacter* spp.), *aph(3′)*-VIi (5 Enterobacterales and 1 *Acinetobacter* spp.), *aph(3′)*-VIj (1 *Acinetobacter* spp.) and *aph(3′)*-VIk (2 *Enterobacter* spp.), but no occurrences of *aph(3′)*-VIb were found. Two *Acinetobacter* spp. strains harboured two different versions of *aph(3′)*-VI; we were unable to differentiate these variants for one isolate, thus the result is *aph(3′)*-VIa-like (Table 3).

A total of 24 isolates harboured *ant(2′)*-Ia, including 12 *P. aeruginosa*, 7 Enterobacterales and 5 *Acinetobacter* spp. These isolates were detected in all geographic regions.

Among the 42 isolates that were non-susceptible to all three aminoglycosides tested (gentamicin, tobramycin and amikacin), genes encoding 16S rRNA methyltransferases were detected in 21 isolates (50.0%) (Table 3). The most common gene was *armA*, which occurred in 14 isolates (7 *Acinetobacter* spp., 6 Enterobacterales and 1 *P. aeruginosa*), followed by *rmtB1* observed in 4 Enterobacterales isolates. RmtB1 was detected among two *E. coli* and one each of *K. pneumoniae* and *P. stuartii*. Two isolates carried *rmtF1* (one *P. aeruginosa* and one *K. pneumoniae*) (Table 3). One *P. mirabilis* isolate harboured a new gene, named *rmtB4* [20]. RmtB4 shows 99.2% homology in its deduced protein sequence compared with RmtB1 and possesses an A229T substitution (Supplementary Fig. S3). Nineteen isolates displaying resistance to amikacin, gentamicin and tobramycin that were negative for 16S rRNA methyltransferase genes carried AME genes and only two *P. aeruginosa* exhibited negative results for all genes tested (Table 3).

A total of 36 isolates (20 *P. aeruginosa*, 10 *Acinetobacter* spp. and 6 Enterobacterales) did not yield a positive result for any of the aminoglycoside resistance genes that were tested in this study.

Combinations of multiple AME genes or combinations of AMEs plus a 16S rRNA methyltransferase were observed among 123 isolates, including 56 Enterobacterales isolates (Table 3).

3.2. Susceptibility profiles of aminoglycoside-modifying enzyme genes not previously described and of RmtB4

Among *aph(3′)*-VI variants observed in this study, nine variants [*aph(3′)*-VIc, *aph(3′)*-VId, *aph(3′)*-VIe, *aph(3′)*-VIf, *aph(3′)*-VIg, *aph(3′)*-VIh, *aph(3′)*-Vii, *aph(3′)*-VIj and *aph(3′)*-VIk] have either been published or match unpublished whole-genome sequence data deposited in the GenBank database [13,21–29] and have not been characterised for their aminoglycoside resistance profile. These genes have been reported as either *aph(3′)*-VIa or *aphA6* despite encoding heterogeneous proteins (Supplementary Fig. S4). Similarly, *aac(3)-IIf* and the *aac(6′)*-Ib S100G and V17A plus L119S have not been fully characterised. These genes were cloned and expressed in a recipient *E. coli* strain and each variant showed a two-fold increase in the minimum inhibitory concentration (MIC) to at least one aminoglycoside agent tested compared with the recipient alone without the plasmid vector (Table 4).

Genes belonging to the APH(3′)-VI group have been reported to encode resistance to amikacin and kanamycin, among others. The *aph(3′)*-VI variants cloned displayed a slight increase (two-fold) in amikacin MICs, and kanamycin MICs increased two- to four-fold except for *aph(3′)*-VIk. This gene showed a two-fold increase in apramycin MIC results. The susceptibility profile of *aac(3)-IIf* displayed an increase in gentamicin, tobramycin and netilmicin MICs compared with the recipient strain (Table 4).

The *aac(6′)*-Ib-like genes cloned demonstrated a distinct profile. The gene carrying an alteration S100G exhibited a 4-fold increase in amikacin MIC and a 32-fold increase in tobramycin, kanamycin and netilmicin MICs. The *aac(6′)*-Ib gene displaying alterations V17A plus L119S displayed highly elevated MIC results for all

Table 2
Distribution of 16S rRNA methyltransferase and aminoglycoside-modifying enzyme (AME) genes among 200 Gram-negative clinical isolates.

Geographic region/bacterial group	Total	No. of isolates positive for each aminoglycoside resistance gene																													AME and/or 16S rRNA methyltransferase negative
		16S rRNA methyltransferases				AMEs																										
		armA	*rmtB1*	*rmtB4*	*rmtF1*	*aac(3)-Ia*	*aac(3)-Ib*	*aac(3)-Ic*	*aac(3)-Id/e*	*aac(3)-IId*	*aac(3)-IIe*	*aac(3)-IIf*	*aac(3)-IVa*	*aacA8*	*aac(6′)-Ib*	*aac(6′)-Ib-cr*	*aac(6′)-Ib-like* [a]	*aac(6′)-Im*	*ant(2″)-Ia*	*ant(3″)-Ia*	*aph(3′)-VIa*	*aph(3′)-VIa-like* [b]	*aph(3′)-VIc*	*aph(3′)-VId*	*aph(3′)-VIe*	*aph(3′)-VIf*	*aph(3′)-VIg*	*aph(3′)-VIh*	*aph(3′)-VIi*	*aph(3′)-VIj*	*aph(3′)-VIk*	
Overall	**200**	**14**	**4**	**1**	**2**	**13**	**1**	**2**	**3**	**21**	**23**	**1**	**1**	**3**	**21**	**30**	**21**	**13**	**24**	**41**	**6**	**1**	**10**	**2**	**1**	**2**	**3**	**1**	**6**	**1**	**2**	**36**
Acinetobacter spp.	49	7				11					2	1					8		5	14	5	1	10		1		3	1	1	1		10
Enterobacterales	99	6	4	1	1	2				21	21		1		18	29	8	1	7	23	1			2					5		2	6
Pseudomonas aeruginosa	52	1			1		1	2	3					3	3	1	5	12	12	4						2						20
Asia-Pacific	**24**	**3**	**2**	**1**	**1**	**4**				**4**	**1**				**1**	**4**	**6**		**5**	**5**		**1**				**1**						**3**
Acinetobacter spp.	6	1				4											2			4		1										1
Enterobacterales	10	1	2	1						4	1				1	4	1		2													
P. aeruginosa	8	1			1												3		3	1						1						2
Europe	**72**	**9**	**2**			**7**		**2**	**2**	**7**	**10**	**1**		**3**	**5**	**11**	**7**	**9**	**6**	**21**	**1**		**7**	**2**	**1**	**1**			**3**	**1**		**8**
Acinetobacter spp.	18	4				5						1					3			6	1		7		1				1	1		3
Enterobacterales	36	5	2			2				7	10				5	11	3	1	1	13				2					2			2
P. aeruginosa	18							2	2					3			1	8	5	2						1						3
Latin America	**39**	**1**								**1**	**9**		**1**		**6**	**7**	**4**	**3**	**3**	**8**	**4**		**1**				**2**		**3**		**2**	**4**
Acinetobacter spp.	12	1									2						2		1	3	3		1				2					2
Enterobacterales	20									1	7		1		4	6	2			4	1								3		2	
P. aeruginosa	7														2	1		3	2	1												2
USA	**65**	**1**			**1**	**2**	**1**		**1**	**9**	**3**				**9**	**8**	**4**	**1**	**10**	**7**	**1**		**2**				**1**	**1**				**21**
Acinetobacter spp.	13	1				2											1		4	1	1		2				1	1				4
Enterobacterales	33				1					9	3				8	8	2		4	6												4
P. aeruginosa	19						1		1						1		1	1	2														13

[a] Isolates carrying *aac(6′)-Ib*-like had the following alterations: L119S (17 isolates, including 7 *Acinetobacter* spp., 7 Enterobacterales and 3 *P. aeruginosa*), V71A + L119S (1 *P. aeruginosa*), L119S + R173K (2; 1 Enterobacterales and 1 *P. aeruginosa*) and S100G (1 *P. aeruginosa*).
[b] One of the *aph(3′)*-VIa genes sequenced for one *Acinetobacter* spp. had double peaks, suggesting that at least two variants of this gene were present.

Table 3
Combinations of aminoglycoside resistance genes detected among 200 Gram-negative clinical isolates.

Combination of aminoglycoside resistance genes	Total	No. of isolates by species			No. of isolates by region			
		Enterobacterales	*Acinetobacter* spp.	*Pseudomonas aeruginosa*	Asia-Pacific	Europe	Latin America	USA
Isolates carrying 16S rRNA methyltransferases	21	12	7	2	7	11	1	2
aac(6′)-Ib-like, *ant(3″)-Ia*, *armA*	4	2	2			3	1	
aac(6′)-Ib-like, *armA*	2		1	1	1	1		
aac(3)-Ia, *aac(6′)*-Ib-like, *ant(3″)*-Ia, *armA*	1		1		1			
aac(3)-IId, *armA*	1	1				1		
aac(3)-IId, *rmtB1*	1	1			1			
aac(3)-IIe, *rmtB1*	1	1			1			
aac(6′)-Ib-cr, *armA*	1	1			1			
aac(6′)-Ib-cr, *rmtF1*	1	1						1
aac(6′)-Ib-like, *ant(3″)*-Ia, *aph(3′)*-VIc, *armA*	1		1			1		
aac(6′)-Ib-like, *aph(3′)*-VIc, *armA*	1		1					1
aac(6′)-Ib-like, *rmtF1*	1			1	1			
aac(6′)-Im, *ant(3″)*-Ia, *rmtB1*	1	1				1		
ant(2″)-Ia, *rmtB4*	1	1			1			
aph(3′)-VIc, *armA*	1		1			1		
aph(3′)-VIi, *armA*	1	1				1		
armA	1	1				1		
rmtB1	1	1				1		
Isolates carrying only AMEs	143	81	32	30	14	53	34	42
aac(3)-IId	12	12			1	3	1	7
aac(6′)-Ib	10	9		1		3	1	6
aac(6′)-Ib-cr	10	10			1	3	3	3
aac(3)-IIe, *aac(6′)*-Ib-cr	9	9				4	2	3
ant(2″)-Ia	9	1	3	5	1	2		6
aac(6′)-Ib-like	6	2	1	3	2	1	2	1
aph(3′)-VIc	6		6			4	1	1
aac(3)-IId, *aac(6′)*-Ib-cr	5	5			2	2		1
aac(3)-IIe	5	3	2			2	3	
aac(3)-Ia, *ant(3″)*-Ia	4	2	2		2	2		
aac(6′)-Ib, *ant(2″)*-Ia	4	3		1	1		1	2
aac(6′)-Ib-like, *ant(3″)*-Ia	3	2	1		1			2
aac(3)-Id/*e*, *aac(6′)*-Im	3			3		2		1
aac(6′)-Ib, *ant(3″)*-Ia	3	3					2	1
ant(2″)-Ia, *ant(3″)*-Ia	3	1		2	1	1		1
aac(3)-Ia	2		2			1		1
aac(3)-Ia, *ant(3″)*-Ia, *aph(3′)*-VIa	2		2			1		1
aac(3)-IIe, *aac(6′)*-Ib-cr, *ant(3″)*-Ia	2	2				2		
aac(6′)-Im	2			2		1	1	
aac(6′)-Im, *aacA8*	2			2		2		
aac(6′)-Im, *ant(2″)*-Ia	2			2		1	1	
ant(2″)-Ia, *aph(3′)*-VIg	2		2				1	1
ant(3″)-Ia	2	1		1		1		1
ant(3″)-Ia, *aph(3′)*-VIa	2		2				2	
ant(3″)-Ia, *aph(3′)*-VIi	2	2				1	1	
aph(3′)-VIi	2	2					2	
aac(3)-Ia, *ant(3″)*-Ia, *aph(3′)*-VIc	1		1			1		
aac(3)-Ia, *ant(3″)*-Ia, *aph(3′)*-VIe	1		1			1		
aac(3)-Ia, *ant(3″)*-Ia, *aph(3′)*-VIi	1		1			1		
aac(3)-Ia, *aph(3′)*-VIa-like	1		1		1			
aac(3)-Ib	1			1				1
aac(3)-Ic	1			1		1		
aac(3)-Ic, *aac(6′)*-Im	1			1		1		
aac(3)-IId, *aac(6′)*-Ib, *ant(2″)*-Ia, *ant(3″)*-Ia	1	1				1		
aac(3)-IId, *ant(3″)*-Ia	1	1						1

aac(3)-IIe, *aaq(6′)*-Ib	1		1																			1			
aac(3)-IIe, *aaq(6′)*-Ib, *ant(3″)*-Ia	1		1																		1				
aac(3)-IIe, *aaq(6′)*-Ib-like, *ant(3″)*-Ia	1		1																			1			
aac(3)-IIe, *ant(3″)*-Ia	1		1																		1				
aac(3)-IIe, *aph(3′)*-VIa	1		1																			1			
aac(3)-IIe, *aph(3′)*-VIk	1		1																			1			
aac(3)-IIf	1				1																1				
aac(3)-IVa, *aac(6′)*-Ib-cr	1		1																			1			
aac(6′)-Ib, *aac(6′)*-Ib-cr	1						1															1			
aac(6′)-Ib-like, *ant(3″)*-Ia, *aph(3′)*-VId	1		1																	1					
aac(6′)-Im, *aacA8*, *ant(2″)*-Ia	1						1													1					
aac(6′)-Im, *ant(3″)*-Ia	1						1															1			
ant(2″)-Ia, *aph(3′)*-VIf	1						1																		
ant(3″)-Ia, *aph(3′)*-VId	1		1																	1					
aph(3′)-VIa	1				1															1					
aph(3′)-VIf	1						1															1			
aph(3′)-VIg	1				1																				
aph(3′)-VIh	1				1																	1			
aph(3′)-VIj	1				1															1					
aph(3′)-VIk	1		1																	1					
Negative screening	36		6		10		20				3									8		4			21

AME, aminoglycoside-modifying enzyme.

aminoglycosides tested (8- to 16-fold), except for streptomycin for which a slight increase was noted (2-fold).

In addition, *rmtB4* was cloned and when this gene was expressed in an *E. coli* background, elevated MICs for amikacin, gentamicin, tobramycin and netilmicin, but not apramycin or neomycin, were noted (Table 4).

GenBank accession numbers: *aph(3′)*-VId (**KU922933.1**); *aph(3′)-VIi* (**KU922932.1**); *aph(3′)-VIf* (**KU922931.1**); *aph(3′)-VIk* (**KU922930.1**); *aph(3′)-VIj* (**KU922928.1**); *aph(3′)-VIg* (**KU922925.1**); *aph(3′)-VIe* (**KU922929.1**); *aph(3′)-VIh* (**KU922927.1**); *aph(3′)-VIc* (**KU922926.1**); *aac(3)-IIf* (**KU956932.1**); *aac(6′)*-Il (**KU745734.1**); *aac(6′)-Ib*-like (ST100 alteration; **KU745733.1**); and *rmtB4* (**KM999534.1**).

4. Discussion

Aminoglycoside resistance can be caused by various resistance mechanisms that include AME-modified aminoglycoside molecules, target modifications by spontaneous mutations or 16S rRNA methyltransferases, and efflux and permeability issues. In this study, 200 Gram-negative isolates were screened for AME- and 16S rRNA methyltransferase-encoding genes. Over 60 profiles consisting of combinations of one to four AME and/or 16S rRNA methyltransferase genes were observed among selected Enterobacterales, *Acinetobacter* spp. and *P. aeruginosa* isolates displaying different resistance profiles to gentamicin, tobramycin and amikacin (Table 3).

Genes encoding 16S rRNA methyltransferases encode elevated resistance levels to virtually all aminoglycosides. In this study, a subset of isolates resistant to all three aminoglycosides tested (gentamicin, tobramycin and amikacin) were selected for screening of 16S rRNA methyltransferase genes; however, less than one-half (47.6%) of the isolates carried these resistance determinants. Other aminoglycoside agents such as arbekacin, fortimicin, kanamycin and netilmicin, as well as neomycin and apramycin for NpmA [15], that are needed to infer the high-level phenotype indicative of producing 16S rRNA methyltransferases were not tested in this study and it is possible that some isolates in this subset do not show this characteristic resistance profile.

AME genes were observed among 164 isolates tested, including 21 isolates carrying 16S rRNA methyltransferase genes. These genes were often detected in combination, which makes it difficult to predict the aminoglycoside resistance profile based on the presence of a single gene. Furthermore, many of the genes have not been fully characterised, making these determinations more troublesome. We attempted to characterise the AME genes observed in this study that were detected as part of this study and/or that have not been previously characterised.

There are more than 45 versions of AAC(6′)-Ib in the NCBI database and at least 32 versions have identical names but different amino acid sequences. It is important to differentiate these variants of AAC(6′)-Ib as one or two amino acid substitutions have been shown to result in an altered substrate phenotype of the enzyme [30]. The protein encoded by *aac(6′)*-Ib-cr possesses only two alterations compared with AAC(6′)-Ib (W104 and D181Y) that are known to also confer resistance to some fluoroquinolone antimicrobial agents [31]. Variants of AAC(6′)-Ib with a serine at position 119 in place of a leucine have been shown to confer increased susceptibility to amikacin and resistance to gentamicin, displaying a phenotype more similar to AAC(6′)-II enzymes that normally possess a serine at position 119 [32,33]. Among *P. aeruginosa* isolates, AAC(6′)-I genes were the most prevalent, with 46.2% of isolates carrying either *aac(6′)*-Im, *aac(6′)*-Ib variants or *aacA8*. Genes for the AAC(6′)-I family of AMEs were also the most common mechanism of aminoglycoside resistance among Enterobacterales, with 55.6% carrying *aac(6′)*-Ib variants. The V71A and

Table 4
Aminoglycoside susceptibility testing results for genes encoding AMEs or 16S rRNA methyltransferases not previously characterised.

AME or 16S rRNA methyltransferase genes expressed in *Escherichia coli* background[a]	Fold change in MIC							
	AMK	GEN	TOB	KAN	NET	NEO	APR	STR
aph(3′)-VIc	2	ND[b]	ND	2	ND	ND	2	ND
aph(3′)-VId	2	ND	ND	4	ND	ND	2	ND
aph(3′)-VIe	2	ND	ND	4	ND	ND	2	ND
aph(3′)-VIf	2	ND	ND	2	ND	ND	ND	ND
aph(3′)-VIg	2	ND	ND	2	ND	ND	ND	ND
aph(3′)-VIh	2	ND	ND	4	ND	ND	ND	ND
aph(3′)-VIi	2	ND	ND	2	ND	ND	ND	ND
aph(3′)-VIj	2	ND	ND	2	ND	ND	2	ND
aph(3′)-VIk	ND	ND	ND	ND	ND	ND	2	ND
aac(3)-IIf	ND	32	2	ND	8	ND	ND	ND
aac(6′)-Ib-like (S100G)	4	ND	32	32	32	ND	ND	ND
aac(6′)-Ib-like (V71A, L119S)	8	8	16	16	16	8	16	2
rmtB4	64	≥64	16	32	≥64	0.5	ND	NT[c]

AME, aminoglycoside-modifying enzyme; MIC, minimum inhibitory concentration; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; NET, netilmicin; NEO, neomycin; APR; apramycin; STR, streptomycin.
[a] pJET and transformed into One Shot® TOP10 Electrocomp™ *E. coli* for *rmtB4* or ElectroMAX™ DH5α-E Competent Cells for AME genes.
[b] No difference (ND) in MIC or lower MIC was noted.
[c] Not tested (NT) since One Shot® TOP10 Electrocomp™ *E. coli* possesses a STR-resistant phenotype.

S100G substitution had not been reported previously so the whole gene sequence along with surrounding gene sequences were submitted to the GenBank database (GenBank accession nos. **KU745733** and **KU745734**). Cloning experiments indicated that expression of AAC(6′)-Ib with V71A and L119S may cause decreased susceptibility to amikacin, gentamicin, tobramycin, kanamycin, netilmicin, neomycin, apramycin and streptomycin. and the production of AAC(6′)-Ib with S100G may result in increased MICs for amikacin, tobramycin, kanamycin and netilmicin (Table 4). A DNA hybridisation study found *aac(3)-IIa* to be the most common AME gene carried by isolates with an AAC(3)-II phenotype [34]. In the current study, 46.7% and 51.1% of AAC(3)-II genes were *aac(3)-IId* and *aac(3)-IIe*, respectively, but *aac(3)-IIa* was not detected in any of the isolates tested. It is possible that *aac(3)-IId* and *aac(3)-IIe* were misidentified as *aac(3)-IIa* owing to a sequence homology of 97.0% and 96.2%, respectively, in previous studies using DNA hybridisation [35]. One *A. baumannii* isolate harboured a new variant, *aac(3)-IIf*; preliminary cloning experiments for *aac(3)-IIf* showed increases in gentamicin, tobramycin and netilmicin MICs compared with the recipient strain (Table 4).

APH(3′)-VI is commonly associated with *Acinetobacter* spp. [7], which was confirmed in the isolates tested in this study: 46.9% of *A. baumannii* isolates carried *aph(3′)*-VI variants, which included two isolates harbouring two *aph(3′)*-VI variants [36–38].

A total of 36 isolates did not give a positive result for any genes that were tested in this study. Among these isolates, 2 *P. aeruginosa* isolates showed resistance to gentamicin, tobramycin and amikacin and 18 displayed resistance to one or two aminoglycosides. Only the most common AMEs were screened for so it is possible that these isolates produce other AMEs; however, resistance is likely caused by the hyperexpression of MexXY–OprM efflux [3,34].

In conclusion, aminoglycoside resistance genes are genetically and structurally divergent and were observed among most Enterobacterales and various *Acinetobacter* spp. and *P. aeruginosa* isolates tested. Scarce data are available on the distribution of AMEs, but the importance of understanding the occurrence of these genes became clear with the development of new agents that have increased activity against isolates carrying these genes compared with other aminoglycosides, such as plazomicin [39].

Lastly, many AME gene variants were reported and we noticed during the process of designating names for these variants that there are issues regarding the nomenclature of AMEs and their genes that should be addressed by developing a consensus on a uniform approach to nomenclature, which is necessary to alleviate confusion regarding AME names and their genes [40]. A curated database that is central and easily accessible is highly desirable. It was noted that a proportion of the isolates displayed an uncharacteristic aminoglycoside resistance profile. Only a scarce number of contemporary surveillance studies with broad genetic evaluation have been performed; therefore, many aminoglycoside resistance genes that may be harboured by strains displaying unexpected aminoglycoside resistance profiles remain uncharacterised. A more complete understanding of AMEs and other methods of aminoglycoside resistance will allow clinicians to use aminoglycosides more wisely in treatment regimens.

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None.

Competing interests

JMI Laboratories was contracted to perform services in 2017 for Achaogen, Allecra Therapeutics, Allergan, Amplyx Pharmaceuticals, Antabio, API, Astellas Pharma, AstraZeneca, Athelas, Basilea Pharmaceutica, Bayer AG, BD, Becton, Dickinson and Co., Boston Pharmaceuticals, CEM-102 Pharma, Cempra, Cidara Therapeutics, Inc., CorMedix, CSA Biotech, Cutanea Life Sciences, Inc., Entasis Therapeutics, Inc., Geom Therapeutics, Inc., GSK, Iterum Pharma, Medpace, Melinta Therapeutics, Inc., Merck & Co., Inc., MicuRx Pharmaceuticals, Inc., N8 Medical, Inc., Nabriva Therapeutics, Inc., NAEJA-RGM, Novartis, Paratek Pharmaceuticals, Inc., Pfizer, Polyphor, Ra Pharma, Rempex, Riptide Bioscience Inc., Roche, SCYNEXIS, Shionogi, SinSa Labs Inc., Skyline Antiinfectives, Sonoran Biosciences, Spero Therapeutics, Symbiotica, Synlogic, Synthes Biomaterials, TenNor Therapeutics, Tetraphase, The Medicines Company, Theravance Biopharma, VenatoRx Pharmaceuticals, Inc., Wockhardt, Yukon Pharma, Zai Lab and Zavante Therapeutics, Inc.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2018.10.020.

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