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Diversity of High-Level Aminoglycoside Resistance Mechanisms among Gram-Negative Nosocomial Pathogens in Brazil

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Studies on high-level aminoglycoside resistance (HLAR), especially pertaining to 16S rRNA methyltransferases (16S-RMTases), have mostly involved *Enterobacteriales*, whereas comparable data on glucose-nonfermenting Gram-negative bacilli (NFGNB) remain scarce (1, 2). HLAR in Gram-negative bacilli (GNB) may also be conferred by the production of multiple aminoglycoside-modifying enzymes (AMEs) or increased efflux (3–5). The aim of this study was to elucidate the mechanisms of HLAR among Gram-negative nosocomial pathogens in Brazil, including NFGNB.

Gram-negative bacterial isolates identified from cerebrospinal fluid, blood, and urine of patients in three states in Brazil during 2007 to 2014 and resistant to oxyimino-cephalosporins and/or aztreonam were investigated ($n = 107$). Disk diffusion and broth microdilution MIC testing were performed (6). MIC testing of amikacin and gentamicin was also performed in the presence and absence of 50 μg/ml phenylalanine-arginine β-naphthylamide (PAβN) (7), and a minimal 4-fold reduction in the MIC values in the presence of PAβN was considered to be efflux mediated. PCR and sequencing for detection of 16S-RMTase genes were performed as described previously (8–10).

Twenty-six isolates were resistant to gentamicin, amikacin, and tobramycin, and 19 of them presented MICs of >128 μg/ml, 10 of which were positive for *rmtD* or *rmtG* by PCR accounting for the HLAR phenotype, as observed in other studies (10, 11). The remaining 9 HLAR isolates were negative for any 16S-RMTase gene (Table 1). By an efflux inhibition assay, only 1 of the 9 isolates (*Acinetobacter baumannii* 874/13) presented a 4-fold MIC reduction with amikacin-PAβN, suggesting minimal involvement of efflux pumps in the resistance phenotype (Table 1).

To examine the correlation between arbekacin resistance and 16S-RMTase production, 11 isolates with MICs of >128 μg/ml for gentamicin, amikacin, and tobramycin were tested for arbekacin susceptibility. Four *Pseudomonas aeruginosa* and four *A. baumannii* aminoglycoside-susceptible clinical isolates were also included for comparison. Among the 19 isolates, only 3 showed arbekacin MIC values of >256 μg/ml and an absence of an inhibition zone by disk diffusion testing. These were the 16S-RMTase-producing isolates as determined by PCR/whole-genome sequencing (WGS). The remaining isolates, both aminoglycoside resistant and susceptible, showed arbekacin MICs values of ≤256 μg/ml and an inhibition zone of >6 mm. Hence, both an arbekacin

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TABLE 1 Phenotypic and genotypic characteristics of aminoglycoside susceptibility and resistance among *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* isolates included in this study

Isolate[a]	Origin[b]	Susceptibility testing[c]								Aminoglycoside resistance gene(s)[d]
		Zone of inhibition (mm)				MIC (μg/ml)				
		AMK	GEN	TOB	ARB	AMK	GEN	TOB	ARB	
P. aeruginosa ATCC 27853		22	20	24	27	4	1	0.25	0.5	ND
Escherichia coli ATCC 25922		25	23	22	25	2	0.5	1	2	ND
A. baumannii 360/10	São Paulo, Brazil	11	6	6	8	>128	>128	>128	256	*aacA4*, *aacA1*, *aphA7*
A. baumannii 874/13	São Paulo, Brazil	8	6	6	9	>128[e]	>128	>128	128	*aacA4*, *aac(3)-I*, *aphA7*
A. baumannii 143/14	São Paulo, Brazil	6	6	6	7	>128	>128	>128	256	*aacA4*, *aacC1*, *aphA7*, *aadB*, *aadA1*, *aphA6*
P. aeruginosa HC402/07	São Paulo, Brazil	6	6	6	15	>128	>128	>128	32	*aacA4*, *aph(3′)-IIb*, *aphA6*, *aadA6*
P. aeruginosa HC408/07	São Paulo, Brazil	6	6	6	22	>128	>128	>128	4	*aacA4*, *aph(3′)-IIb*, *aphA6*, *aadB*, *aadA6*
P. aeruginosa HC305/07	São Paulo, Brazil	6	6	6	18	>128	>128	>128	8	*aacA4*, *aph(3′)-IIb*, *aphA6*, *aadB*, *aadA6*
P. aeruginosa 463/12	São Paulo, Brazil	12	6	6	14	>128	>128	>128	32	*aacA4*, *aph(3′)-IIb*, *aadB*, *aadA6*, *strA*, *strB*
P. aeruginosa 1206/13	São Paulo, Brazil	6	6	6	17	>128	>128	>128	8	*aacA4*, *aph(3′)-IIb*, *aphA6*, *aadA2*
P. aeruginosa 9me/14	São Paulo, Brazil	6	6	6	6	>128	>128	>128	>256	*aacA4*, *aph(3′)-IIb*, *aadA7*, *rmtD1*
P. aeruginosa 862/07	São Paulo, Brazil	6	6	6	6	>128	>128	>128	>256	*rmtD*
P. aeruginosa HC367/07	São Paulo, Brazil	6	6	6	6	>128	>128	>128	>256	*rmtD*
P. aeruginosa HC103/07	São Paulo, Brazil	6	6	6	7	>128	>128	>128	>256	*rmtD*
P. aeruginosa HC84/07	São Paulo, Brazil	6	6	6	6	>128	>128	>128	128	*rmtD*
P. aeruginosa HC313/07	São Paulo, Brazil	6	6	6	6	>128	>128	>128	>256	*rmtD*
P. aeruginosa HC58/07	São Paulo, Brazil	6	6	6	6	>128	>128	>128	>256	*rmtD*
P. aeruginosa 883/07	São Paulo, Brazil	6	6	6	6	>128	>128	>128	>256	*rmtD*
P. aeruginosa 979/09	São Paulo, Brazil	6	6	6	6	>128	>128	>128	>256	*rmtD*
K. pneumoniae 931/08	São Paulo, Brazil	6	6	6	6	>256	>256	>256	>256	*rmtG*[f]
K. pneumoniae 1180/11	São Paulo, Brazil	6	6	6	6	>256	>256	>256	>256	*rmtG*[f]
P. aeruginosa 102	United States	26	22	26	25	4	1	0.25	2	*aph(3′)-IIb*
P. aeruginosa 104	United States	26	23	27	26	4	1	0.25	2	*aph(3′)-IIb*
P. aeruginosa 105	United States	30	22	25	25	4	1	0.25	1	*aph(3′)-IIb*
P. aeruginosa 106	United States	21	18	22	22	8	4	0.5	2	*aph(3′)-IIb*
A. baumannii 162	United States	26	26	25	27	1	0.5	0.25	0.25	—
A. baumannii 165	United States	26	27	26	28	1	1	0.25	0.25	—
A. baumannii 172	United States	25	26	25	27	4	1	0.5	1	—
A. baumannii 176	United States	25	23	24	25	2	1	0.5	2	—

[a]*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as negative controls for all experiments. *A. baumannii* 360/10, 874/13, and 143/14 and *P. aeruginosa* HC402/07, HC408/07, HC305/07, 463/12, 1206/13, and 9me/14 were subjected to WGS.

[b]City and/or country of origin.

[c]A value of 6 indicates the absence of a zone of inhibition. The concentrations of amikacin (AMK), gentamicin (GEN), tobramycin (TOB), and arbekacin (ARB) disks were 30, 10, 10, and 10 μg, respectively. Arbekacin disks were purchased from Eiken Chemical (Tokyo, Japan) and provided by Meiji Seika Kaisha Ltd.

[d]Aminoglycoside resistance genes were identified by whole-genome sequencing; 16S-RMTase genes were also identified by PCR. Dashes indicate that no gene was detected. ND, not determined.

[e]Isolate that showed a 4-fold MIC reduction with amikacin in combination with PAβN in an efflux assay.

[f]These two isolates were not included for WGS, and the 16S-RMTases were detected by PCR.

MIC of >256 μg/ml and the absence of an inhibition zone were highly sensitive and specific in predicting the presence of a 16S-RMTase gene, corroborating the utility of these arbekacin cutoff values in predicting 16S-RMTase production by Gram-negative bacteria, including NFGNB.

All 9 HLAR isolates without any 16S-RMTase gene detected by PCR were subjected to WGS using Illumina NextSeq 250-bp paired-end sequencing. *De novo* assembly was accomplished using CLC Genomics Workbench 10.1.1, and antimicrobial resistance genes were predicted using ResFinder (12). In addition, to rule out 16S-RMTase homologues, BLAST was optimized for low-similarity sequences using the available option (https://blast.ncbi.nlm.nih.gov/Blast.cgi). As a result, 13 AME genes were identified [*aacA4*, *aacA1*, *aacC1*, *aphA6*, *aphA7*, *aph(3′)-IIb*, *aadB*, *aadA1*, *addA2*, *aadA6*, *aadA7*, *strA*, and *strB*] (Table 1). Besides, *rmtD1* was identified in one *P. aeruginosa* isolate, which had been missed by PCR previously. None of the remaining isolates carried any known 16S-RMTase gene. However, the combinations of AMEs could explain HLAR among these isolates. For instance, the combination of *aacA*, *aphA6*, *aphA7*, *aacC*, and *aadB* genes was consistent with the aminoglycoside resistance phenotype. *aacA* genes confer resistance to amikacin and tobramycin, while *aphA6* and *aphA7* are responsible for amikacin resistance (1). Furthermore, *aacC* confers resistance to gentamicin, and *aadB* confers resistance to tobramycin and gentamicin. Other studies have also re-

ported an abundance of AME genes among aminoglycoside-resistant *A. baumannii* and *P. aeruginosa* isolates (13, 14).

In summary, HLAR among GNB in Brazil is due to the production of 16S-RMTase or a combination of multiple AMEs, while the involvement of efflux appears to be minimal. A combination of AMEs was particularly common among *P. aeruginosa* and *A. baumannii*, leading to the HLAR phenotype. High-level resistance to arbekacin could be used as a marker to differentiate the two resistance mechanisms among these species.

Accession number(s). This BioProject has been deposited at the DDBJ/ENA/GenBank database under accession number PRJNA431093.

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