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Antimicrobial Susceptibility Studies

In vitro activity of Plazomicin against *Enterobacteriaceae* isolates carrying genes encoding aminoglycoside-modifying enzymes most common in US Census divisions

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ABSTRACT

Aminoglycoside-nonsusceptible isolates of *Escherichia coli*, *Klebsiella*, *Proteus*, and *Enterobacter* species (480/3675) from US hospitals collected during 2014–2015 were screened for 16S rRNA methyltransferase and aminoglycoside-modifying enzyme (AME) genes. Only 5 isolates had high aminoglycoside MICs and carried 16S rRNA methyltransferases. AME genes were observed among 89.7% (426/475) of isolates and the most common genes were *aac(3)-IIa* ($n = 270$) and *aac(6′)-Ib* ($n = 269$). Among other genes, *ant(2″)-Ia*, *aac(3)-Iva*, and *aph(3′)-VIa* were observed among 36, 23, and 3 isolates, respectively. Forty-nine (10.3%) isolates yielded negative results for the investigated AME genes. Plazomicin ($MIC_{50/90}$, 0.5/1 μg/ml) inhibited 99.3% of the AME-carrying isolates at its susceptible breakpoint while amikacin, gentamicin, and tobramycin inhibited 90.1%, 20.9%, and 18.3%, respectively. Plazomicin was approved by the US Food and Drug Administration in June 2018 for the treatment of complicated urinary tract infections when limited treatment options are available. This agent displayed activity against isolates carrying AMEs that were resistance to other aminoglycosides and comparator agents.

1. Introduction

Aminoglycosides play an important role in treating serious gram-negative infections and are often used as part of combination therapy with cell wall biosynthesis inhibition agents, such as β-lactams (Vakulenko and Mobashery, 2003). Resistance to aminoglycosides can occur by various mechanisms (Mingeot-Leclercq et al., 1999); acquired aminoglycoside-modifying enzymes (AMEs) are the most common resistance mechanism among both gram-negative and gram-positive species (Ramirez and Tolmasky, 2010). AMEs catalyze the covalent modification of amino or hydroxyl groups on aminoglycoside molecules, resulting in poor binding to the ribosome target and elevated MIC results to different aminoglycoside molecules depending on the residue modified and the type of modification (Mingeot-Leclercq et al., 1999). AME-encoding genes are often carried by mobile genetic structures often harboring β-lactamases and/or other resistance determinants that can be selected and disseminated concomitantly (Mingeot-Leclercq et al., 1999; Ramirez and Tolmasky, 2010). Studies reporting the prevalence of AMEs among contemporary gram-negative organisms are scarce and the available studies are often investigating class 1 integrons that carry AME genes as gene cassettes (Ramirez and Tolmasky, 2010) investigated to determine the presence and genetic location of β-lactamases or other resistance genes.

Another important but less prominent aminoglycoside resistance mechanism is the 16S rRNA methyltransferases. These enzymes catalyze the methylation of the 16S rRNA aminoglycoside target, rendering it inaccessible to nearly all clinically available aminoglycosides resulting in elevated MICs (Armstrong and Miller, 2010). Genes encoding 16S rRNA methyltransferases in gram-negative organisms and their prevalence have been investigated in various studies but these were primarily with limited sample sizes from a single hospital or were descriptive studies noting the presence of these genes along with carbapenemase-encoding genes (Doi et al., 2016; Galimand et al., 2005; Wachino and Arakawa, 2012; Yang et al., 2011).

Plazomicin is a semi-synthetic aminoglycoside derived from sisomicin containing structural modifications that make this molecule stable in the presence of the clear majority of AMEs (Aggen et al., 2010; Cox et al., 2018). As with most other aminoglycosides, isolates carrying 16S rRNA methyltransferases exhibit high MIC values for plazomicin. Plazomicin was approved by the US Food and Drug Administration (FDA) in June 2018 for complicated urinary tract infections when limited treatment options are available.

We evaluated the occurrence of AMEs and 16S rRNA methyltransferases among 3675 isolates collected in US hospitals during 2014 and 2015 (Castanheira et al., 2018) and analyzed the activity of plazomicin and comparator antimicrobial agents tested against clinical isolates harboring these resistance mechanisms.

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2. Materials and methods

2.1. Bacterial isolates

A total of 3675 *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., and *Enterobacter* spp. isolates collected during 2014 and 2015 in 70 hospitals located in 61 US cities were evaluated as part of the Antimicrobial Longitudinal Evaluation and Resistance Trends (ALERT) program (Castanheira et al., 2018). This surveillance program collects key pathogens in targeted numbers (1 per patient episode) deemed to cause urinary tract infections (1131 isolates), bloodstream infections (929), pneumonia in hospitalized patients (829), skin and skin structure infections (375), intra-abdominal infections (363), or other infection sources (48 isolates). Species identification was confirmed for all isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry using the Bruker Daltonics MALDI Biotyper (Billerica, MA, USA), following manufacturer instructions.

2.2. Antimicrobial susceptibility testing

All isolates were susceptibility tested against plazomicin and comparator agents using the reference broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI, 2018a). Plazomicin breakpoints were defined as ≤2 μg/ml as susceptible, 4 μg/ml as intermediate, and ≥8 μg/ml as resistant (ZEMDRI™, 2018). Categorical interpretations for all comparator agents were found in CLSI criteria in M100 (CLSI, 2018b), EUCAST breakpoint tables (EUCAST, 2018), and/or the US FDA website (ZEMDRI™, 2018). Quality control was performed using *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853. All quality control MIC results were within acceptable ranges as published in CLSI documents.

2.3. Characterization of aminoglycoside-resistance mechanisms

Escherichia coli, *Klebsiella* spp., *Proteus* spp., and *Enterobacter* spp. isolates displaying nonsusceptible MIC values for gentamicin, amikacin, and/or tobramycin when applying the CLSI criteria and with plazomicin MIC values ≤64 μg/ml were screened for the presence of AME genes able to modify amikacin, gentamicin, and tobramycin. Three PCR assays targeting the following genes were developed using custom created primers (Supplementary Table 1): *aac(6′)-Ib* that modifies amikacin and tobramycin, *aac(3)-IIa, ant(2″)-Ia*, *aac(3)-Ia, -Ib, -Ic, -Id, -Ie*, and *aac(3)-IVa* that modifies gentamicin and tobramycin and *aph(3′)-VIa* that has variable activity against gentamicin and modifies amikacin (Ramirez and Tolmasky, 2010). Isolates yielding positive amplification were re-amplified for confirmation.

All *Enterobacteriaceae* isolates displaying plazomicin MIC results of ≥128 μg/ml were screened by PCR using custom primers for the presence of 16S rRNA methyltransferase-encoding genes, including *armA, rmtA* though *rmtH*, and *npmA*. Amplicons generated by primers that targeted 16S rRNA methyltransferase genes were sequenced on both strands, and nucleotide sequences obtained were analyzed using the Lasergene® software package (DNAStar; Madison, WI, USA) and compared to available sequences *via* NCBI BLAST search (http://www.ncbi.nlm.nih.gov/blast/).

3. Results

3.1. Occurrence of aminoglycoside resistance genes in the United States

Among 3675 isolates from selected species, 480 (13.1%) isolates displaying nonsusceptible MIC values for gentamicin, amikacin, and/or tobramycin when applying the CLSI criteria (CLSI, 2018b) were screened for the presence of 16S rRNA methyltransferase genes or select AME-encoding genes based on the plazomicin MIC result. These isolates were 240 *E. coli* (17.8% of this species), 207 *Klebsiella pneumoniae* (13.7%), 12 *Proteus mirabilis* (9.7%), 12 *Klebsiella oxytoca* (3.3%), 6 *Enterobacter cloacae* (5.8%), 2 *Proteus vulgaris* (1.7%), and 1 *Enterobacter aerogenes* (0.8%). *E. coli* and *K. pneumoniae* isolates meeting the screening criteria were observed in all US Census divisions.

Only 5 (0.1%) isolates displayed plazomicin MIC values ≥128 μg/ml and all carried genes encoding 16S rRNA methyltransferases, including *rmtE1* (2 *E. coli*), *armA* (1 *K. pneumoniae*), *rmtF1* (1 *K. pneumoniae*), and *armA* (1 *K. oxytoca*). These isolates were observed in the Middle Atlantic (2 *K. pneumoniae* and 1 *E. coli*), East North Central (1 *K. oxytoca*), and East South Central (1 *E. coli*) US Census divisions; 4 were collected during 2014 and 1 in 2015 (*armA1*).

Overall, AME genes were found in 426 isolates of the remaining 475 (89.7%) isolates that met the screening criteria, and 263 of these isolates carried only 1 gene: 130 *aac3-IIa*, 112 *aac(6′)-Ib*, 18 *ant(2″)-Ia*, or 3 *aac(3)-IVa*. A total of 163 isolates encoded more than 1 AME gene, with *aac3-IIa* plus *aac(6′)-Ib* being the most common combination, found in 126 isolates. Interestingly, the combination of *aac(3)-IVa* plus *aac(6′)-Ib* was detected only in 15 *K. pneumoniae* from 9 US hospitals located in 4 census divisions. A combination of 3–4 AME genes was found in 9 isolates.

Isolates carrying *aac(3)-IIa* ($n = 270$; 56.8% of tested isolates) were observed in all US Census divisions, and occurrence rates were higher in the Mountain division where 17 of 20 (85.0%) isolates were positive for this gene (Fig. 1). Noteworthy, the number of isolates harboring *aac(3)-IIa* was much higher in the Middle Atlantic (22.1%; 100/453), West South Central (11.6%; 212/1834), and Pacific (8.6%; 59/686) divisions when compared to the Mountain division (Fig. 1). The *aac(3)-IIa* gene was detected among all species, except *P. vulgaris*, and included 175 *E. coli* (13.0% of collected isolates for this species; 73.5% of tested isolates for this species), 89 *K. pneumoniae* (5.9%; 43.4%), 2 *K. oxytoca*, 2 *P. mirabilis*, 1 *E. cloacae*, and 1 *E. aerogenes*.

A total of 269 (56.6% of tested isolates) isolates yielded positive results for *aac(6′)-Ib* (Table 1), including 154 *K. pneumoniae* (75.2% of tested isolates for this species), 102 *E. coli* (42.9%), 6 *K. oxytoca* (6/11 [% not used due to small numbers]), 4 *E. cloacae* (4/6), and 3 *P. mirabilis* (3/12). Isolates displaying positive results for *aac(6′)-Ib* were observed in all US Census divisions, but occurrence rates were higher in the Middle Atlantic (68.5%; 98/143), Mountain (65.0%; 13/20), and East North Central (60.0%; 42/70) divisions. Among other divisions, the occurrence of this gene ranged from 38.7% to 55.6% (9 to 35 isolates) (Fig. 1). Thirty-six isolates carried *ant(2″)-Ia* and these isolates were detected in all census divisions except the Mountain division (Fig. 1). This gene occurred among 5.2% to 16.3% of the isolates in 8 census divisions and the number of isolates per division varied from 1 to 8. The *ant(2″)-Ia* gene was observed among 14 *K. pneumoniae,* 11 *E. coli*, 8 *P. mirabilis*, 2 *K. oxytoca*, and 1 *E. cloacae* isolates.

Twenty-two of the 23 isolates harboring *aac(3)-VIa* were *K. pneumoniae* with the remaining isolate an *E. coli*. These isolates were detected mostly in the Middle Atlantic and East North Central census divisions (12 and 5 isolates; 8.4% and 7.1%, respectively), but also in the West South Central (2 isolates), Pacific (2), South Atlantic (1), and East South Central (1; 2.0% to 3.7%) divisions (Fig. 1). The *aph(3′)-IVa* gene was found in 2 *E. coli* and 1 *E. aerogenes* that originated from the Middle Atlantic, East South Central, and West South Central divisions (Fig. 1). All isolates tested yielded negative results for *aac(3)-Ia, aac(3)-Ib*, *aac(3)-Ic*, and *aac(3)-Id/e*, and 49 isolates that met the screening criteria were negative for all the AME genes tested, including 21 *E. coli*, 21 *K. pneumoniae*, 2 *E. cloacae*, 2 *K. oxytoca*, 1 *P. mirabilis*, and the 2 *P. vulgaris* isolates.

3.2. Activity of plazomicin and clinically available aminoglycosides

The activity of plazomicin against overall *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., and *Enterobacter* spp. evaluated in this study has been reported (Castanheira et al., 2018) and in this study we describe the activity of plazomin against isolates harboring AMEs and 16S rRNA methyltransferases.

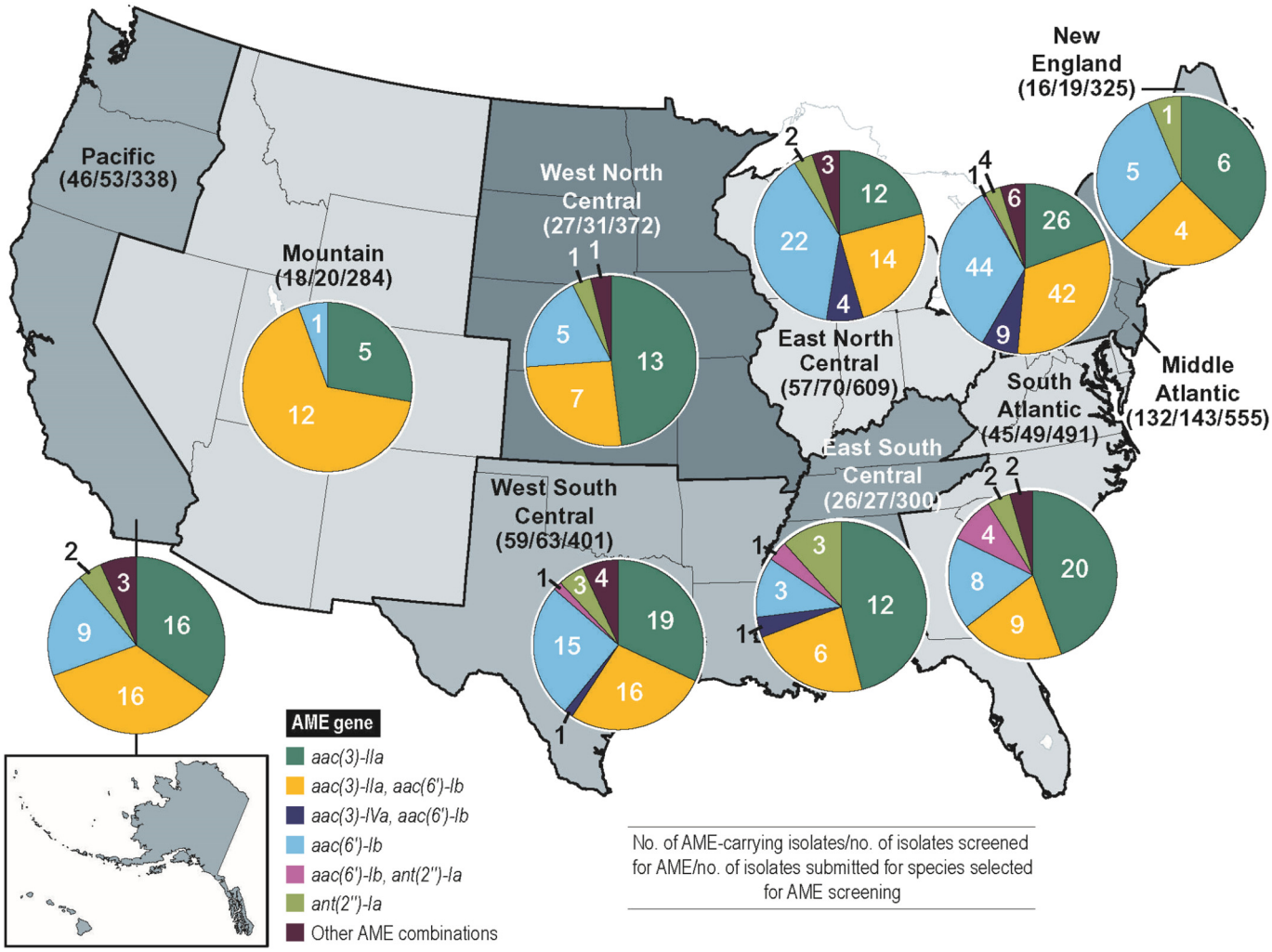

Fig. 1. Occurrence of AME genes in US Census divisions.

Overall, plazomicin was active against 99.3% of the isolates carrying AME genes at the US FDA breakpoint at 2 μg/ml and all these isolates were inhibited by this novel aminoglycoside at MICs ≤4 μg/ml (Table 1), regardless of the AME gene or number of AME genes present. Isolates not inhibited by plazomicin at the current breakpoints were 2 *P. mirabilis*, 1 *P. vulgaris* and 1 *K. pneumoniae* with plazomicin MIC values ranging from 8 μg/ml to 64 μg/ml and carrying no acquired aminoglycoside-resistance gene.

Gentamicin and tobramycin had limited activity against isolates carrying AME genes, and these aminoglycosides inhibited 20.9% and 18.3% of the isolates applying CLSI breakpoints, respectively (Table 2). Amikacin inhibited 90.1% of these isolates applying the current CLSI interpretative criteria. AME-harboring isolates displayed low susceptibility rates against ceftazidime, ceftriaxone, and levofloxacin (38.5%, 30.8%, and 29.3%, respectively) and meropenem and piperacillin-tazobactam inhibited 81.5% and 64.1% of these isolates, respectively

Table 1
Activity of plazomicin against carrying aminoglycoside resistance mechanisms.

Isolate group (no. of isolates)	No. of isolates at MIC (μg/ml; cumulative %)													MIC_{50}	MIC_{90}
	≤0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>128	>		
All isolates carrying AME genes (426)	5	24	85	171	111	27	3							0.5	1
	1.2	6.8	26.8	66.9	93.0	99.3	100.0								
Isolates carrying *aac(3)-IIa* (270)	2	13	49	111	80	13	2							0.5	1
	0.7	5.6	23.7	64.8	94.4	99.3	100.0								
Isolates carrying *aac(3)-IVa* (23)	0	3	6	6	6	2								0.5	1
	0.0	13.0	39.1	65.2	91.3	100.0									
Isolates carrying *aac(6′)-Ib* (269)	5	22	63	109	59	10	1							0.5	1
	1.9	10.0	33.5	74.0	95.9	99.6	100.0								
Isolates carrying *ant(2″)-Ia* (36)	0	3	8	11	6	7	1							0.5	2
	0.0	8.3	30.6	61.1	77.8	97.2	100.0								
Isolates carrying *aph(3′)-VIa* (3)		0	1	1	1									0.5	
		0.0	33.3	66.7	100.0										
Isolates carrying RNA methylase genes (5)											0	1	4	>128	
											0.0	20.0	100.0		

(Table 2). Colistin and tigecycline were the only non-aminoglycosides that inhibited >90.0% of the AME-carrying isolates (Table 2).

Plazomicin inhibited 97.2% to 100.0% of the isolates carrying *aac(3)-IIa* ($n = 270$), *aac(6′)-Ib* ($n = 269$), *aac(3)-IVa* ($n = 23$), *ant(2″)-Ia* ($n = 36$), and *aph(3′)-VIa* ($n = 3$). Other aminoglycosides, β-lactams, and levofloxacin had variable activity against these isolates (Table 2).

As expected, the activity of all aminoglycosides, including plazomicin, was reduced against isolates carrying genes encoding 16S rRNA methylases (Tables 1 and 2).

4. Discussion

In this study, we screened 480 isolates for the presence of AMEs or 16S rRNA methyltransferase-encoding genes. Five isolates displayed resistance to all clinically available aminoglycosides and highly elevated plazomicin MIC values (≥64 μg/ml) and, as expected, all 5 isolates encoded a 16S rRNA methyltransferase. The occurrence of 16S rRNA methyltransferases is low in US isolates (0.1%), but monitoring isolates carrying these genes is prudent since they are resistant to virtually all aminoglycosides, including plazomicin.

The remaining 475 isolates were tested for the presence of AME genes that encode enzymes that modify and inactivate aminoglycosides used to treat serious infections, such as amikacin, gentamicin, and tobramycin. The literature data are scarce about the current prevalence of AME genes, and this information is pivotal when evaluating plazomicin to demonstrate the differential activity of this agent in the presence of AMEs. Our results confirmed that plazomicin is active against a large collection of contemporary *Enterobacteriaceae* carrying 1 to 3 AME genes collected in 70 US hospitals. These isolates mainly harbored *aac3-IIa* or *aac (6′)-Ib,* which included *aac(6′)-Ib-cr* that is able to modify ciprofloxacin and modify amikacin (Robicsek et al., 2006) and *ant(2″)-Ia, aac(3)-IVa,* and *aph(3′)-VIa.* All isolates carrying AMEs alone or in combination displayed plazomicin MIC values at ≤4 μg/ml, and this new agent inhibited 99.3% of the isolates at the US FDA-defined susceptibility breakpoint (≤2 μg/ml).

In a recent study evaluating the presence of AMEs among *Enterobacteriaceae* isolates collected from Europe and adjacent countries (Castanheira et al., 2018), we demonstrated that all 4 AME genes observed in the United States were also present; however, *aac(6′)-Ib* was slightly more common than *aa(3)-IIa* (583 and 453, respectively, among 728) and 1 isolate carried *aac(3)-Ia* that was not observed in this study. Additionally, the occurrence of 16S rRNA methyltransferases was higher among European countries when compared to this study (60 *versus* 5 isolates). In a study evaluating the presence of AMEs among *Enterobacter* spp. isolates from a hospital in the United States (Haidar et al., 2016), 22 out of 65 (34%) isolates carried 1 or more AME genes and isolates carrying ≥2 AME-encoding genes were highly resistant to gentamicin and/or tobramycin. The same investigators evaluated the prevalence of AMEs among 50 carbapenem-resistant *K. pneumoniae* (Almaghrabi et al., 2014) and, similar to our study, observed that *aac(6′)-Ib* was the most common AME detected in this collection followed by *aph(3)-Ia*, *aac(3)-IV*, and *ant(2′)-Ia* (in this order). In all studies, plazomicin was very active against isolates harboring AME genes. A slightly different scenario of AME occurrence was noted by Spanish investigators when analyzing amoxicillin-clavulanate-resistant *E. coli* isolates. Although *aac(6′)-Ib* was still the most common AME, approximately the same number of isolates harboring *aph(3′)-Ia*, *ant(2″)-Ia,* and *aac(3)-IIa* was noted among the isolates.

Overall, AME-carrying isolates displayed low susceptibility rates (29.3% to 38.5%) against non-aminoglycoside antimicrobial agents, including cephalosporins, levofloxacin, and piperacillin-tazobactam. Furthermore,18.5% of these isolates were nonsusceptible to meropenem. However, the susceptibility rates were lower among isolates carrying *aac(6′)-Ib* that were prevalent and *aac(3)-IVa*. Aminoglycosides are often used in combination therapy for the treatment of infections caused by MDR organisms, including carbapenem-resistant *Enterobacteriaceae*, and it is important to understand the dissemination and susceptibility profiles of these isolates among the United States and other regions.

Plazomicin is not modified by the most common AMEs detected in clinical isolates (Armstrong and Miller, 2010) and was more potent than other aminoglycosides (amikacin, gentamicin, and tobramycin) against isolates carrying AME genes. Isolates carrying 16S rRNA methyltransferases exhibited elevated plazomicin MIC values, but these isolates are uncommon in US hospitals. These results suggest that plazomicin is a valuable agent for the treatment of *Enterobacteriaceae* isolates collected in the United States, regardless of widely disseminated AMEs present.

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Table 2
Activity of plazomicin and comparator agents tested against isolates carrying aminoglycoside resistance mechanisms.

Isolate group (no. of isolates)	Susceptibility %[a]										
	Plazomicin	Amikacin	Gentamicin	Tobramycin	Ceftazidime	Ceftriaxone	Meropenem	Piperacillin-tazobactam	Tigecycline	Levofloxacin	Colistin
All isolates carrying AME genes (426)	99.3	90.1	20.9	18.3	38.5	30.8	81.5	64.1	98.4	29.3	91.2
Isolates carrying *aac(3)-IIa* (270)	99.3	96.7	4.4	25.6	48.1	38.9	95.6	78.1	99.3	32.6	97.8
Isolates carrying *aac(3)-IVa* (23)	100.0	56.5	8.7	0.0	0.0	0.0	26.1	8.7	100.0	8.7	65.2
Isolates carrying *aac(6′)-Ib* (269)	99.6	84.8	32.7	4.5	20.4	13.0	72.5	50.2	98.5	22.3	90.6
Isolates carrying *ant(2″)-Ia* (36)	97.2	97.2	0.0	27.8	47.2	44.4	94.4	66.7	91.7	47.2	73.5
Isolates carrying *aph(3′)-VIa* (3)	100.0	100.0	0.0	66.7	100.0	100.0	100.0	66.7	100.0	66.7	100.0
Isolates carrying RNA methylase genes (5)	0.0	0.0	0.0	0.0	20.0	20.0	80.0	80.0	100.0	40.0	100.0

[a] Susceptible based on criteria as published by CLSI 2018 except for plazomicin, tigecycline (FDA breakpoints) and colistin (EUCAST).

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A.W. Serio was an employee of Achaogen at the time of the study and K.M. Krause is an employee of Achaogen; both contributed with the design of the study and review of the manuscript, but not with analyzing or interpreting the results.

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