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KPC-Producing *Klebsiella pneumoniae* Strains That Harbor AAC(6′)-Ib Exhibit Intermediate Resistance to Amikacin

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The aminoglycoside-modifying enzyme AAC(6′)-Ib is common among carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp) strains. We investigated amikacin (AMK) activity against 20 AAC(6′)-Ib-producing CR-Kp strains. MICs clustered at 16 to 32 μg/ml. By the time-kill study, AMK (1× and 4× the MIC) was bactericidal against 30% and 85% of the strains, respectively. At achievable human serum concentrations, however, the majority of strains showed regrowth, suggesting that AAC(6′)-Ib confers intermediate AMK resistance. AMK and trimethoprim-sulfamethoxazole (TMP-SMX) were synergistic against 90% of the strains, indicating that the combination may overcome resistance.

Aminoglycosides are important options for the treatment of infections caused by carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp) strains. These agents retain potent bactericidal activity against some but not all CR-Kp strains (1). Inactivation by enzymatic modification is the most prevalent mechanism of resistance to the class (2). Indeed, 98% of CR-Kp strains studied at our center possessed at least one aminoglycoside-modifying enzyme (AME), which resulted in variable susceptibility among the aminoglycosides (3).

Amikacin (AMK) is uniquely stable against most AMEs. Of the AMEs known to affect AMK, APH(3′)-VI and ANT(4′) confer high-level resistance and are infrequently encountered (4, 5). On the other hand, AAC(6′)-Ib is common among *Enterobacteriaceae* (2), but the clinical relevance is largely unknown. AAC(6′)-Ib encodes an *N*-acetyltransferase that catalyzes acetyl coenzyme A (acetyl-CoA)-dependent acetylation of the 6′ amino group of AMK. The impact of AAC(6′)-Ib modification on the susceptibility of Gram-negative bacteria to AMK is uncertain. Published reports have identified AMK MICs above (6, 7) and below (7–9) susceptibility breakpoints. Time-kill studies of AMK against CR-Kp strains or other *Enterobacteriaceae* that produce AAC(6′)-Ib have not been published. The objective of this study was to characterize AMK MICs, time-kill responses, and interactions with other antimicrobials among AAC(6′)-Ib-producing CR-Kp strains at our center.

We selected 20 CR-Kp strains from unique patients for analysis. All strains were sequence type (ST) 258, *Klebsiella pneumoniae* carbapenemase (KPC) producers (18 producers of KPC-2 and 2 of KPC-3), and harbored the *aac(6′)-Ib* gene by PCR (3). Each strain also harbored SHV-12 and TEM-1 β-lactamase genes, but none carried NDM, IMP, VIM, or OXA-48 genes. All carried mutant *ompK35* porin genes (AA89 STOP), and 60% (12/20) had mutant *ompK36* genes. Sequence analysis revealed three *ompK36* mutant genotypes, the most common of which were an insertion at amino acid 134 to 135GD ($n = 6$) and IS*5* promoter insertions ($n = 5$). The remaining *ompK36* mutant isolate had a frameshift at nucleotide position 382. MICs were determined by standard broth microdilution methods. Median MICs of AMK, gentamicin (GEN), and tobramycin (TOB) were 32 (range, 4 to 32 μg/ml), 2 (range, 0.5 to >64 μg/ml), and 32 (range, 8 to >64 μg/ml) μg/ml, respectively. Susceptibility rates by CLSI (10) (≤4 μg/ml for GEN and TOB and ≤16 μg/ml for AMK) and EUCAST (11) (≤2 μg/ml for GEN and TOB and ≤8 μg/ml for AMK) were comparable for GEN (60% versus 55%) and TOB (0% for both) but not AMK (45% versus 5%; $P = 0.008$). Ninety-five percent (19/20) of AMK MICs were clustered at 16 and 32 μg/ml. AMK MICs did not correlate with those of GEN ($r^2 = 0.068$, $P = 0.266$) or TOB ($r^2 = 0.102$, $P = 0.171$); on the other hand, GEN and TOB MICs correlated well ($r^2 = 0.654$, $P = 0.0001$).

Next, we performed time-kill assays using a standard inoculum of 1×10^6 CFU/ml. We defined susceptibility, intermediate resistance, and resistance by time-kill assay as bactericidal responses (≥3-log decrease in CFU/ml from the starting inoculum), regrowth (≥2-log increase in CFU/ml after bactericidal killing), and the lack of inhibition at any time point, respectively. AMK concentrations of 0.25×, 1×, and 4× the MIC were tested for each strain, and the surviving bacteria were enumerated at 0, 2, 4, 8, and 24 h. AMK was bactericidal against 0% (0/20), 30% (6/20), and 85% (17/20) of strains exposed to concentrations of 0.25×, 1×, and 4× the MIC, respectively (Table 1). Regrowth (intermediate resistance) occurred more commonly among strains exposed to 1× the MIC of AMK than those exposed to 4× the MIC (70% versus 15%; $P = 0.006$). Strains that demonstrated regrowth against AMK concentrations of 4× the MIC were AMK nonsusceptible and GEN susceptible (CLSI breakpoints). There were no differences in 24-h log kills among strains classified as GEN susceptible and nonsusceptible (−5.49 versus −5.55; $P = 0.56$). Likewise, there were no differences in log kills among strains with wild-type or mutant *ompK36* genotypes (−5.67 versus −5.49; $P = 0.78$). All strains demonstrated at least some inhibition at 1× and

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TABLE 1 AMK MICs and time-kill responses among CR-Kp strains

K. pneumoniae strain no.	KPC subtype	MIC (μg/ml)[a]			AMK log kill at 24 h (CFU/ml)[b]			Regrowth at 4× MIC
		AMK	GEN	TOB	0.25× MIC	1× MIC	4× MIC	
615	KPC-2	4	4	16	4.10	**−5.97**	**−5.41**	No
383	KPC-2	16	0.5	16	6.93	−0.49	**−5.28**	No
871	KPC-2	16	1	16	2.76	3.66	**−5.81**	No
930	KPC-3	16	1	8	2.95	2.71	**−5.81**	No
1	KPC-2	16	64	>64	3.80	−0.18	**−5.82**	No
136	KPC-3	16	>64	>64	3.64	3.42	**−5.46**	No
294	KPC-2	16	64	>64	3.66	0.63	**−5.56**	No
584	KPC-2	16	32	>64	3.84	**−4.00**	**−5.15**	No
928	KPC-3	16	64	>64	2.57	0.96	**−6.02**	No
184	KPC-2	32	2	32	4.05	3.20	−2.78	Yes
216	KPC-2	32	1	32	3.81	3.12	0.44	Yes
484	KPC-2	32	1	32	3.49	**−4.59**	**−5.57**	No
539	KPC-2	32	2	16	4.07	4.35	−3.12	Yes
587	KPC-2	32	1	16	3.52	−0.01	**−5.89**	No
669	KPC-2	32	2	32	3.53	3.21	**−5.85**	No
705	KPC-2	32	0.5	32	2.35	**−3.75**	**−5.77**	No
709	KPC-2	32	1	16	3.36	**−5.79**	**−5.32**	No
94	KPC-2	32	64	>64	3.59	−2.08	**−5.40**	No
649	KPC-2	32	32	32	3.94	**−5.04**	**−5.53**	No
692	KPC-2	32	64	32	3.46	3.35	**−5.78**	No

[a] AMK, amikacin; GEN, gentamicin; TOB, tobramycin.
[b] Boldface numbers indicate bactericidal (≥3-log decrease in CFU/ml from the starting inoculum) killing without regrowth.

4× the MIC, and thus, by our definition, none of the strains were fully resistant to AMK.

Finally, checkerboard analysis was used to investigate the change in AMK MIC when combined with a second antimicrobial agent. Colistin (COL), doripenem (DOR), tigecycline (TGC), and trimethoprim-sulfamethoxazole (TMP-SMX) were selected for analysis as agents that may have preserved activity against CR-Kp. Fractional inhibitory concentrations (FIC) were calculated for each combination using the following equation: (MIC of agent A in combination/MIC of agent A alone) + (MIC of agent B in combination/MIC of agent B alone). FIC values of ≤0.5 were classified as synergistic and 0.51 to 4 as indifferent. Rates of synergy between AMK and COL, DOR, TGC, or TMP-SMX were 30%, 35%, 0%, and 90%, respectively (Table 2). AMK and COL were synergistic among COL-resistant (MIC, >2 μg/ml) strains exclusively and 67% (6/9) of such strains overall. Among the remaining COL-susceptible strains, AMK and COL were indifferent (FIC range, 0.625 to 2). AMK and TMP-SMX were synergistic against all but 2 strains; both were TMP-SMX susceptible (MIC, <2/38 μg/ml). MICs for AMK in combination with TMP-SMX (median, 4 μg/ml; range, 2 to 8 μg/ml) were significantly lower than MICs for AMK alone (median, 32 μg/ml; range, 4 to 32 μg/ml; $P < 0.001$).

Taken together, the data suggest that AAC(6′)-Ib confers intermediate resistance to AMK against CR-Kp strains. AMK MICs were clustered between 16 and 32 μg/ml, classifying 95% of strains nonsusceptible to AMK by EUCAST criteria and 55% by CLSI criteria. Strains demonstrated suboptimal time-kill responses when exposed to AMK. In fact, 70% exhibited regrowth in the presence of AMK at concentrations equal to the MIC. At 4× the MIC, AMK was bactericidal against 85% of strains, which is not surprising given the concentration-dependent nature of the aminoglycosides. Nevertheless, achieving the corresponding serum concentrations of AMK in patients is unlikely with conventional dosing of 15 mg/kg body weight/day. AMK doses exceeding 25 mg/kg/day may achieve serum drug levels of ≥60 μg/ml, which approaches concentrations of 4× the MIC for strains with MICs of 16 but not 32 μg/ml (12–14).

AMK is unique among the aminoglycosides in that a side chain protects the drug from most AMEs by steric hindrance or folding (15). Nevertheless, it remains susceptible to inactivation by AAC(6′)-Ib, an enzyme that is frequently found among Gram-negative bacteria (2), including CR-Kp strains (3). Strains producing AAC(6′)-Ib are often classified as susceptible to AMK based on the current CLSI breakpoints; however, by time-kill assay, responses are suboptimal and at least intermediate levels of resistance are present. Given this, our data suggest that GEN or the newest aminoglycoside plazomicin is likely to be a more reliable therapeutic option than AMK against CR-Kp strains that produce AAC(6′)-Ib in the absence of 16S rRNA methyltransferases (16). Although we did not screen for 16S rRNA methyltransferases in the present study, our high rates of AMK and GEN susceptibility suggest their presence among strains at our center is unlikely. In the same light, we did not test for other AMEs that may affect AMK susceptibility, namely, APH(3′)-VI and ANT(4′), which are rare and confer high-level AMK resistance (2). Finally, mutations in the porin gene *ompK36*, which attenuate carbapenem susceptibility *in vitro* (17), predictably had no effect on AMK activity.

One possible mechanism to overcome intermediate resistance to AMK is combination therapy. To our knowledge, this is the first study to evaluate and identify high rates of synergy between AMK and TMP-SMX among CR-Kp strains. The combination demonstrated higher rates of synergy (90%) than any other combination tested (35%; $P = 0.0008$ by McNemar's chi-square test) and lowered median AMK MICs by 2- to 4-fold. Future studies exploring the interactions between these agents are warranted. The next most synergistic combination was AMK and DOR, which was evaluated previously and shown to result in higher rates of

TABLE 2 Results of checkerboard synergy testing among CR-Kp strains

K. pneumoniae strain no.	MIC (μg/ml)[a]					FIC[b]			
	AMK	COL	DOR	TGC	TMP-SMX	AMK-COL	AMK-DOR	AMK-TGC	AMK–TMP-SMX
615	4	>64	128	1	>128	1	0.75	2	0.25
383	16	0.125	8	2	>128	0.75	0.5	0.75	0.375
871	16	0.125	16	2	>128	1	0.625	0.75	0.375
930	16	0.25	32	1	0.125	1	0.375	0.75	2
1	16	>64	64	0.25	>128	0.265	0.5156	0.75	0.25
136	16	>64	64	2	16	0.375	0.5	0.75	0.5
294	16	0.5	4	0.25	>128	0.75	0.5	0.75	0.25
584	16	64	>128	2	128	0.375	0.5156	2	0.5
928	16	32	4	1	>128	0.375	0.25	1	0.3125
184	32	16	64	0.25	>128	0.75	1	2	0.375
216	32	16	4	0.25	>128	0.5	0.75	0.5625	0.25
484	32	0.5	>128	2	>128	0.75	0.5	0.5625	0.5
539	32	0.25	64	0.5	>128	1.5	1	2	0.3125
587	32	0.25	64	0.5	>128	0.75	1	2	0.375
669	32	0.25	128	2	1	1	0.5	0.75	0.56
705	32	0.125	128	0.5	>128	0.675	0.75	1	0.375
709	32	0.125	16	2	>128	1	0.5625	0.75	0.375
94	32	64	128	2	>128	0.5	1	0.5625	0.3125
649	32	64	>128	1	>128	0.625	0.75	0.625	0.375
692	32	0.25	8	2	>128	2	0.75	0.75	0.3125

[a] AMK, amikacin; COL, colistin; DOR, doripenem; TGC, tigecycline; TMP-SMX, trimethoprim-sulfamethoxazole.

[b] The following concentrations were tested for each agent: AMK, 2 to 128 μg/ml; COL, 0.06 to 64 μg/ml; DOR, 0.125 to 128 μg/ml; TGC, 0.06 to 64 μg/ml; TMP-SMX, 0.125 to 128 μg/ml. TMP and SMX were tested at a fixed ratio of 1:19 in accordance with CLSI susceptibility testing guidelines (10). The percentages of synergy (FIC of ≤0.5) were as follows: AMK-COL, 30%; AMK-DOR, 35%; AMK-TGC, 0%; and AMK–TMP-SMX, 90%.

synergy and prolonged survival in mice than combinations of AMK with rifampin or levofloxacin (18). Nevertheless, responses were not universal among our strains, a finding that is consistent with our previous studies evaluating DOR and GEN activity (1). Finally, AMK and COL demonstrated synergy, but exclusively against COL-resistant CR-Kp strains. Previous studies among COL-resistant CR-Kp strains identified combinations of COL plus rifampin (19, 20) or doxycycline (19) as potentially synergistic. These combinations may be more attractive for subsequent studies given the potential additive toxicities of AMK and COL combination therapy.

In conclusion, the presence of AAC(6′)-Ib may not be sufficient to render CR-Kp strains fully resistant to AMK by standard susceptibility testing. However, our data suggest that AAC(6′)-Ib-carrying CR-Kp strains exhibit at least intermediate AMK resistance. AMK concentrations of 4× the MIC are associated with bactericidal killing of CR-Kp, but obtaining such serum concentrations in patients is not feasible if MICs are >16 μg/ml. To achieve 4× serum drug concentrations against strains with MICs of 16 μg/ml, daily doses of AMK that exceed current recommendations must be used. Therefore, AMK may best be reserved for infections caused by CR-Kp strains that test susceptible to this agent and do not harbor AAC(6′)-Ib. The novel combination of AMK with TMP-SMX is an attractive target for future studies.

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