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Doripenem, Gentamicin, and Colistin, Alone and in Combinations, against Gentamicin-Susceptible, KPC-Producing *Klebsiella pneumoniae* Strains with Various *ompK36* Genotypes

Cornelius J. Clancy,[a,b,d] Binghua Hao,[a,b] Ryan K. Shields,[a,b] Liang Chen,[c] David S. Perlin,[c] Barry N. Kreiswirth,[c] M. Hong Nguyen[a,b]

University of Pittsburgh, Pittsburgh, Pennsylvania, USA[a]; XDR Pathogen Laboratory, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA[b]; Public Health Research Institute, New Jersey Medical School-University of Medicine and Dentistry of New Jersey, Newark, New Jersey, USA[c]; Pittsburgh VA Healthcare System, Pittsburgh, Pennsylvania, USA[d]

Gentamicin doses of 2 and 10 μg/ml were bactericidal against 64% and 100%, respectively, of gentamicin-susceptible KPC-2-producing *Klebsiella pneumoniae* strains. Treatment with the combination of doripenem (8 μg/ml) plus colistin (2 μg/ml) was inferior to treatment with gentamicin (2 μg/ml), doripenem-gentamicin, gentamicin-colistin, and doripenem-gentamicin-colistin against strains with glycine and aspartic acid insertions in OpmK36 porin at amino acid (aa) positions 134 and 135 ($n = 9$). Doripenem-colistin was comparable to other 2- or 3-drug regimens and superior to single drugs against wild-type/minor *ompK36* mutants ($n = 5$). An algorithm incorporating *ompK36* genotypes and susceptibility to gentamicin and doripenem may predict antimicrobial activity against KPC-producing *K. pneumoniae*.

Carbapenem-resistant *Klebsiella pneumoniae* strains have emerged as major nosocomial pathogens capable of causing infections that are generally unresponsive to conventional antimicrobial therapy and are associated with high mortality rates (1, 2). Observational studies from our center and others suggest that outcomes are improved with carbapenem-containing combination regimens (3). However, these findings have not been validated in clinical trials, and the optimal combinations have not been defined.

Carbapenem resistance is mediated through several mechanisms, including the production of metallo-β-lactamases and non-metallo-carbapenemases (such as *Klebsiella pneumoniae* carbapenemase [KPC] and OXA-type carbapenemase), with or without disturbances of outer membrane proteins (OMPs), such as porins. KPC subtype 2 (KPC-2)-producing sequence type 258 (ST258) *K. pneumoniae* strains predominate in U.S. hospitals and have spread worldwide. At our center and many others, ST258 *K. pneumoniae* strains carry a mutant *ompK35* porin gene, which results in a premature stop codon at amino acid (aa) position 89 (STOP-aa89) (4–6). We previously demonstrated that strains with the mutant *ompK35* gene and a wild-type or minor mutant *ompK36* gene were highly susceptible to the combination of doripenem and colistin (DOR+COL) in time-kill assays *in vitro*. In contrast, DOR+COL was inactive against strains carrying the mutant *ompK35* and major *ompK36* mutations, such as an IS*5* insertion in the promoter and a 6-bp insertion that encodes glycine and aspartic acid at amino acid positions 134 and 135 (ins aa134-135GD) (7). The ins aa134-135GD mutation is particularly important since the mutated site falls within a transmembrane β-strand loop 3 that constitutes the porin channel eyelet. The ins aa134-135GD mutant strains exhibit diminished carbapenem uptake due to porin channel constriction (8).

In an earlier time-kill study, we demonstrated that treatments with doripenem and gentamicin (DOR+GENT) were ineffective against GENT-resistant KPC-producing *K. pneumoniae* (KPC-*K. pneumoniae*) strains (9). At our center, however, ~60% of ST258 KPC-*K. pneumoniae* strains are GENT susceptible (6). The objectives of this study were to compare the *in vitro* activities of GENT, DOR+GENT, DOR+COL, GENT+COL, and DOR+GENT+COL against 14 GENT-susceptible ST258 KPC-2 *K. pneumoniae* strains with various *ompK36* genotypes. In a previous study, the strains were shown to be indistinguishable by pulsed-field gel electrophoresis typing (6).

The resistance mechanisms for the strains were assessed by PCR and DNA sequencing, and the MICs of each agent were determined by broth microdilution (10–15) (Table 1). Each strain harbored the STOP-aa89 *ompK35* mutation, $bla_{\text{KPC-2}}$, $bla_{\text{SHV-12}}$, and $bla_{\text{TEM-1}}$ but was negative for $bla_{\text{CTX-M}}$, bla_{IMP}, bla_{NDM}, bla_{VIM}, $bla_{\text{OXA-48}}$, and bla_{AmpC} ($bla_{\text{ACT-1}}$, bla_{ACC}, $bla_{\text{BIL-1}}$, bla_{CMY}, bla_{DHA}, bla_{FOX}, bla_{LAT}, $bla_{\text{MIR-1}}$, and bla_{MOX}). Sixty-four percent (9/14), 21% (3/14), and 14% (2/14) of the strains had ins aa134-135GD *ompK36* mutations, wild-type *ompK36*, or minor *ompK36* mutations (a guanine insertion at nucleotide [nt] position 382 [ins nt382G], and an asparagine-asparagine-threonine-glutamic acid [NNTE] deletion at amino acid positions 84 to 87 [del aa84-87NNTE]), respectively. Since we previously showed that DOR+COL responses were similar among ins nt382G del aa84-87NNTE *ompK36* mutants and wild-type *ompK36* strains, we considered them together in this study (7). Interpretive breakpoints for colistin against *Enterobacteriaceae* have not been established by the CLSI, but we considered 21% (3/14) of the strains to be resistant (MICs, >2 μg/ml).

We performed standard time-kill assays for DOR, COL, and GENT as single agents and in two- and three-drug combinations using previously described methods (6, 7). In general, GENT is

Received 7 September 2013 **Returned for modification** 14 November 2013
Accepted 16 February 2014
Published ahead of print 24 February 2014
Address correspondence to M. Hong Nguyen, mhn5@pitt.edu.
C.J.C. and B.H. contributed equally to this work.

doi:10.1128/AAC.01949-13

TABLE 1 Killing activity of DOR, GENT, and COL alone or in combinations against 14 GENT-susceptible KPC-*K. pneumoniae* clinical strains

Strain	MIC (μg/ml) of:			*ompK36* genotype	Killing activity (log_{10} CFU/ml)[a] of:							
	DOR	GENT	COL		DOR	GENT (10 μg/ml)	GENT (2 μg/ml)	COL	DOR+COL	DOR+GENT[b]	GENT+COL	DOR+GENT+COL
216	4	0.5	8	WT[c]	3.71	−6.08	1.19	3.91	0.72	−6.09	−6.11	−6.11
347	8	2	0.25	WT	3.90	−6.03	−3.95	2.07	−4.57	−6.06	−3.74	−6.09
709	8	0.5	0.5	WT	4.06	−5.90	−5.88	−5.86	−5.87	−5.92	−5.92	−5.89
539	32	2	1	ins 382G	3.71	−6.02	1.02	−1.00	−6.08	2.10	−6.13	−6.10
41	2	1	0.25	del 84-87NNTE	3.68	−6.05	−4.79	3.60	−6.03	−6.02	−6.05	−6.03
115	32	0.5	0.125	ins aa134-135GD	3.93	−6.04	−6.14	3.75	3.65	−6.14	−6.15	−6.16
743	64	0.5	0.25	ins aa134-135GD	4.25	−5.71	1.73	1.73	−2.81	−3.86	−5.77	−5.75
155	32	1	0.25	ins aa134-135GD	3.70	−6.08	−6.08	1.68	2.77	−6.10	−6.05	−6.09
484	128	1	0.25	ins aa134-135GD	3.95	−6.02	−4.03	1.48	2.15	−4.08	−6.02	−6.02
807	64	0.5	0.5	ins aa134-135GD	3.93	−6.01	−6.05	3.89	3.83	−6.10	−6.05	−6.06
615	128	2	0.5	ins aa134-135GD	3.86	−6.06	3.92	3.93	3.87	3.69	−2.62	−0.68
436	32	2	0.5	ins aa134-135GD	3.82	−6.08	−6.11	3.79	3.78	−6.11	−6.09	−6.10
669	64	1	4	ins aa134-135GD	3.75	−6.08	−2.01	1.09	−0.08	−1.68	−6.08	−6.05
184	32	0.5	8	ins aa134-135GD	3.83	−6.08	−6.12	3.96	3.80	−6.11	−6.09	−6.13

[a] Killing activity was defined as the difference between the log_{10} concentration of KPC-*K. pneumoniae* after 24 h of incubation with drug(s) and the log_{10} of starting inoculum. Positive numbers denote growth of strains compared with starting inoculum.
[b] Gentamicin concentration was 2 μg/ml during combination testing.
[c] WT, wild type.

administered once a day in order to exploit its concentration-dependent bactericidal activity against Gram-negative bacteria. For life-threatening infections, the targeted peak and trough serum concentrations are 8 to 10 and 1 to 2 μg/ml, respectively (16, 17). In single-drug time-kill studies, we first used a GENT concentration of 10 μg/ml. GENT exerted bactericidal activity (≥3-log_{10} reduction in CFU/ml) within minutes against each of the 14 strains, which were maintained without regrowth through 24 h (Table 1). Since these high-level activities limited our ability to show additional effects with the addition of DOR or COL, we performed subsequent time-kill assays with GENT at 2 μg/ml. DOR (8 μg/ml) and COL (2 μg/ml) concentrations that are achieved in human sera were used in the single-drug and combination studies (7, 9).

Kill curves showing the median kills by the single drugs and the 2- and 3-drug combinations are presented in Fig. 1. GENT (2 μg/ml) achieved bactericidal activity and complete kills at 24 h against 64% (9/14) and 36% (5/14) of the strains, respectively (Table 1). COL exerted bactericidal activity against only one strain (7%). DOR was not active against any of the strains. To compare the activities of the drugs over the 24-h period, we measured areas under the bactericidal curves (AUBCs); greater killing resulted in lower AUBCs. GENT (median AUBC, 53-log_{10} CFU/ml · h) was more bactericidal than COL (115-log_{10} CFU/ml · h; $P = 0.0008$) and DOR (221.3-log_{10} CFU/ml · h; $P < 0.0001$). COL was more bactericidal than DOR ($P < 0.0001$).

The addition of DOR to GENT or COL improved the rates of bactericidal activity from 64% (9/14) to 79% (11/14) and 7% (1/14) to 29% (4/14), respectively ($P = 0.02$ for DOR+GENT versus DOR+COL). GENT+COL and DOR+GENT+COL each had bactericidal activity against 93% (13/14) of the strains ($P = 0.0013$ versus DOR+COL). DOR+GENT, DOR+COL, and GENT+COL were synergistic against 21% (3/14), 36% (5/14), and 36% (5/14) of the strains, respectively (≥2-log_{10}-greater kills at 24 h with the combinations than with the best single agent). DOR+GENT+COL did not provide synergy compared to the most active two-drug combination.

DOR+COL exerted greater bactericidal activity against the wild-type/minor *ompK36* mutants than the ins aa134-135GD mutants ($P = 0.0002$) (Fig. 2 and 3). DOR+COL did not differ significantly from GENT (2 μg/ml) alone, DOR+GENT, GENT+COL, or DOR+GENT+COL against the wild-type/minor *ompK36* mutants. However, DOR+GENT, GENT+COL, DOR+GENT+COL, and GENT alone were significantly more bactericidal than DOR+COL against the ins aa134-135GD mutants; for these strains, the activity of DOR+COL did not differ from that of COL alone. Note that the GENT+COL combination was synergistic against 40% (2/5) and 33% (3/9) of the wild-type/minor *ompK36* and ins aa134-135GD mutants, respectively ($P = 1.0$).

This study is the latest in a series of studies from our group to identify effective antimicrobial regimens against KPC-*K. pneu-*

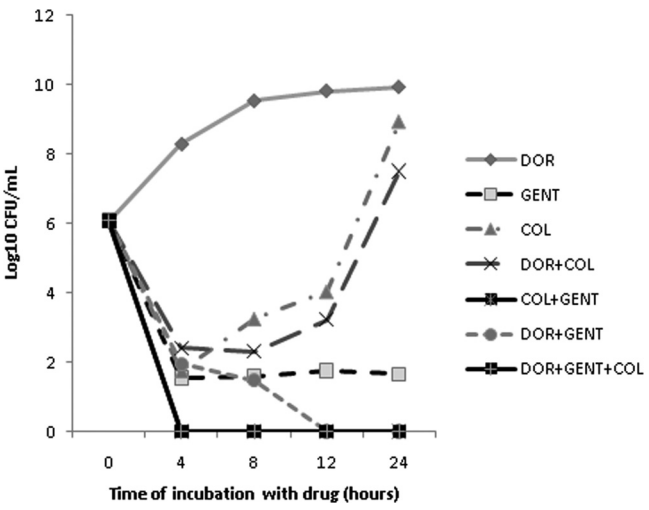

FIG 1 Kill curves of KPC-*K. pneumoniae* strains by single drugs and combinations. Data represent the median log_{10} CFU/ml for specific drug regimens over time. There were no differences in growth at 12 and 24 h between strains incubated with DOR and those with no drug control. Note that the kill curves for COL+GENT and DOR+GENT+COL overlap.

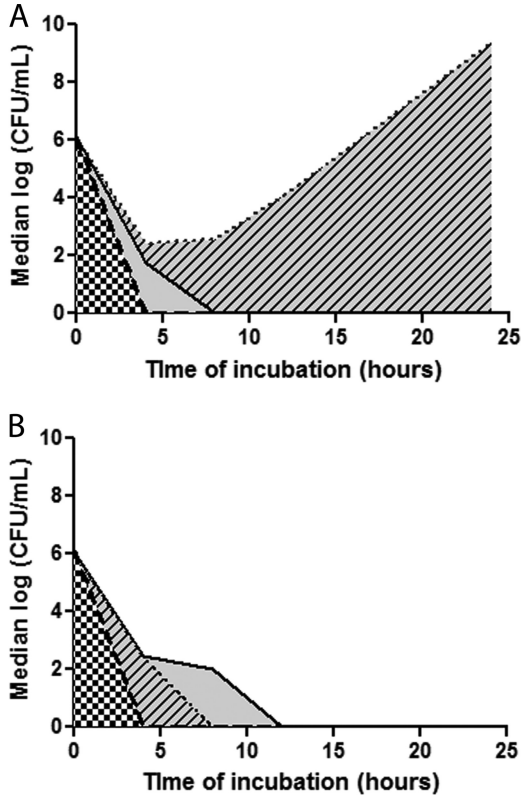

FIG 2 Median AUBCs for 2-drug combinations against KPC-*K. pneumoniae* isolates, stratified by *ompK36* genotypes. Shown are time-kill results against ins aa134-135GD mutants (A) and wild-type/minor mutants (B). Data represent the median $\log_{10}$ CFU/ml for specific combinations over time. The black dotted line represents median time-kills by DOR+COL. The solid black line and the thick broken black lines represent median time-kills by DOR+GENT and GENT+COL, respectively. Therefore, the AUBCs highlighted by black-and-white checkers depict the activity of GENT+COL, and AUBCs highlighted by solid gray and gray with black diagonal lines depict the differences observed with DOR+GENT and DOR+COL, respectively.

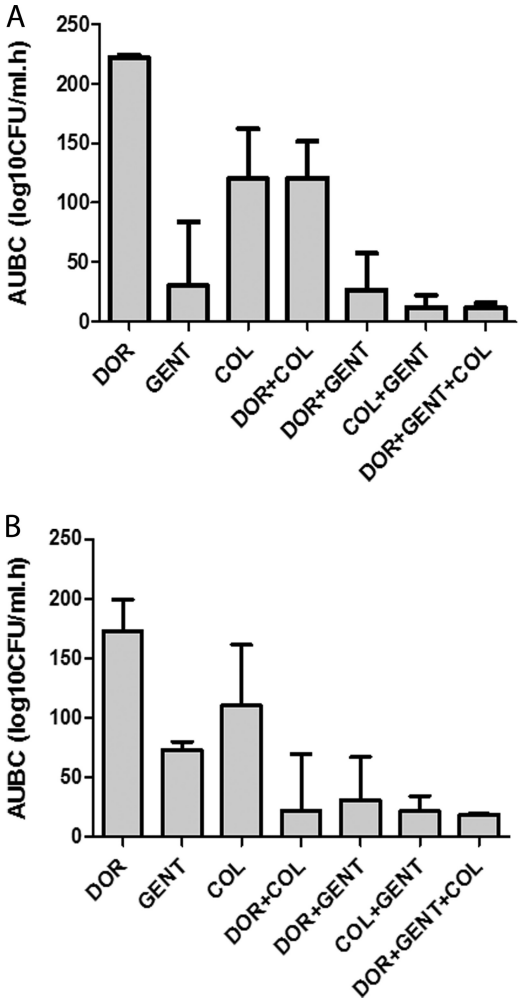

FIG 3 AUBCs for single drugs and combinations against KPC-*K. pneumoniae* strains, stratified by *ompK36* genotypes. Shown are time-kill results against ins aa134-135GD (A) and wild-type/minor (B) mutants. Data represent median AUBCs with interquartile ranges for specific regimens over time. For ins aa134-135GD mutants, the median AUBCs were higher for DOR+COL than for GENT ($P = 0.007$), DOR+GENT ($P = 0.004$), GENT+COL ($P = 0.002$), and DOR+GENT+COL ($P < 0.001$). For wild-type/minor mutants, the median AUBCs were not significantly different between DOR+COL, DOR+GENT, GENT+COL, and DOR+GENT+COL.

moniae strains (7, 9, 12). We demonstrated that GENT and GENT-containing combination regimens were highly effective in time-kill assays against GENT-susceptible KPC-2-producing ST258 strains. GENT concentrations of 10 and 2 μg/ml were rapidly bactericidal against 100% (14/14) and 63% (9/14) of the strains, respectively. The activity of GENT as a single agent limited our ability to demonstrate positive interactions with other drugs. Nevertheless, DOR+GENT (2 μg/ml) was synergistic against 3 strains, achieved bactericidal rather than bacteriostatic activity against 2 strains, and suppressed the regrowth of 2 strains (data not shown). GENT+COL was even more effective than DOR+GENT *in vitro*, but the clinical utility of this combination may be limited by the nephrotoxicity of both agents. The use of DOR+GENT+COL did not offer advantages over two-drug combinations, which may reflect the strong activity of GENT+COL and the limited activity of DOR by itself. These data suggest that GENT is a useful treatment option against infections caused by GENT-susceptible KPC-*K. pneumoniae*.

DOR+COL was inferior to GENT alone against GENT-susceptible ins aa134-135GD *ompK36* mutant strains *in vitro*. Since porin channel constriction resulting from the ins aa134-135GD mutation results in diminished carbapenem uptake, DOR+GENT is unlikely to offer much advantage over GENT alone in treating infections by these strains. For strains with minor *ompK36* mutations or wild-type *ompK36*, DOR+COL, GENT, and DOR+GENT were equally bactericidal in the time-kill studies. Therefore, the DOR+COL combination remains an option for treating infections caused by these strains. In such cases, we have insufficient data to conclude that GENT, DOR+GENT, or DOR+COL is superior. We feel that DOR+GENT is the preferred regimen for two reasons. First, the combination may achieve synergy against at least some GENT-susceptible wild-type/minor mutant *ompK36* strains. Second, GENT by itself exerts significantly greater activity against GENT-susceptible strains than does COL. However, clinicians may consider DOR+COL for the treatment of infections in which GENT is potentially limited by pharmacokinetic considerations. In diseases like pneumonia or in cases of abscesses, for example, the efficacy of GENT

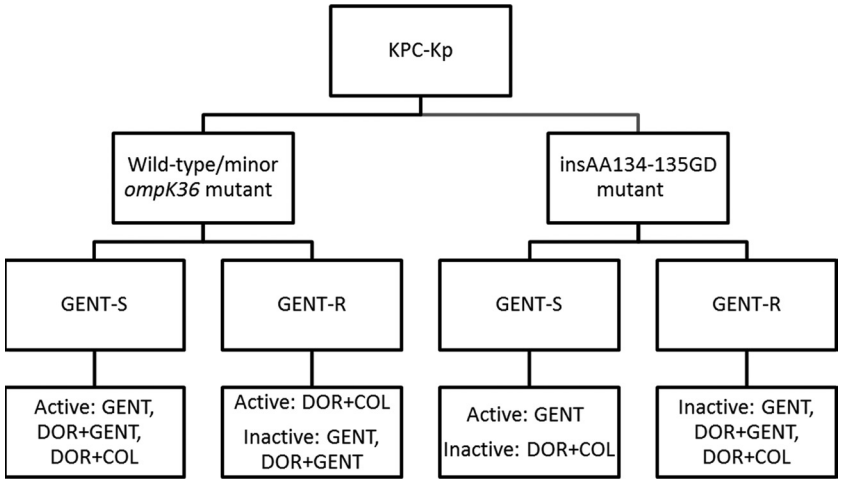

FIG 4 Proposed algorithm for predicting active antimicrobial regimens against KPC-*K. pneumoniae* strains. A doripenem MIC of ≤8 μg/ml can be used as a proxy for the presence of wild-type/minor *ompK36* mutation, and a doripenem MIC of >8 μg/ml can be used as a proxy for the presence of an ins aa134-135GD mutation. DOR+GENT may offer synergy against some GENT-susceptible wild-type/minor *ompK36* mutant strains, but it is not likely to offer synergy over GENT alone against GENT-susceptible ins aa134-135GD mutant strains, since constriction of the porin channel will restrict DOR uptake.

may be compromised by suboptimal penetration into infected lung tissue or reduced activity at an acidic pH, respectively (18, 19). In certain cases of severe infection, clinicians may choose to initiate treatment with DOR+GENT+COL as they await MIC data. This strategy will increase the likelihood that both GENT-susceptible and GENT-resistant strains are covered. GENT or COL can be discontinued when data become available, which may ameliorate the increased risk of nephrotoxicity with the agents combined.

Considering the current data and our previous findings that GENT and DOR+GENT were ineffective against GENT-resistant KPC-2-producing ST258 *K. pneumoniae* strains (9), we propose a paradigm that uses GENT susceptibility data and *ompK36* genotypes to identify antimicrobial combinations that are likely to be active (Fig. 4). DOR MICs may be incorporated into the paradigm as proxies for *ompK36* genotypes, since *ompK36* genotypes determine the level of carbapenem resistance. Our data must be corroborated and extended using KPC-*K. pneumoniae* strains with various genetic backgrounds from different centers and geographical locations. All *in vitro* findings should be validated in animal models of KPC-*K. pneumoniae* infections as a prelude to testing treatment paradigms in human clinical trials. The present study and others from our group further support the development of molecular assays that can rapidly identify the antimicrobial regimens that are best-suited to treat infections due to KPC-*K. pneumoniae* or other extensively resistant bacteria. Improved outcomes among patients with these infections will require multifaceted strategies that integrate advances in molecular biology and informatics, optimized pharmacokinetics and pharmacodynamics of antimicrobials, new drugs, and judicious antimicrobial usage.

ACKNOWLEDGMENTS

This study was fully supported by the University of Pittsburgh Medical Center XDR Pathogen Laboratory.

R.K.S. is supported by the National Center for Advancing Translational Sciences of the National Institutes of Health under award KL2TR000146.

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

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