

# Characterization of Porin Expression in *Klebsiella pneumoniae* Carbapenemase (KPC)-Producing *K. pneumoniae* Identifies Isolates Most Susceptible to the Combination of Colistin and Carbapenems

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We characterized carbapenem resistance mechanisms among 12 *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* (referred to here as KPC *K. pneumoniae*) clinical isolates and evaluated their effects on the activity of 2- and 3-drug combinations of colistin, doripenem, and ertapenem. All isolates were resistant to ertapenem and doripenem; 75% (9/12) were resistant to colistin. Isolates belonged to the ST258 clonal group and harbored *bla*<sub>KPC-2</sub>, *bla*<sub>SHV-12</sub>, and *bla*<sub>TEM-1</sub>. As determined by time-kill assays, doripenem (8 μg/ml) and ertapenem (2 μg/ml) were inactive against 92% (11/12) and 100% (12/12) of isolates, respectively. Colistin (2.5 μg/ml) exerted some activity (range, 0.39 to 2.5 log<sub>10</sub>) against 78% (7/9) of colistin-resistant isolates. Colistin-ertapenem, colistin-doripenem, and colistin-doripenem-ertapenem exhibited synergy against 42% (5/12), 50% (6/12), and 67% (8/12) of isolates, respectively. Expression of *ompK35* and *ompK36* porins correlated with each other ( $R^2 = 0.80$ ). Levels of porin expression did not correlate with colistin-doripenem or colistin-ertapenem synergy. However, synergy with colistin-doripenem-ertapenem was more likely against isolates with high porin expression than those with low expression (100% [8/8] versus 0% [0/4];  $P = 0.002$ ). Moreover, bactericidal activity (area under the bacterial killing curve) against isolates with high porin expression was greater for colistin-doripenem-ertapenem than colistin-doripenem or colistin-ertapenem ( $P \leq 0.049$ ). In conclusion, colistin-carbapenem combinations may provide optimal activity against KPC *K. pneumoniae*, including colistin-resistant isolates. Screening for porin expression may identify isolates that are most likely to respond to a triple combination of colistin-doripenem-ertapenem. In the future, molecular characterization of KPC *K. pneumoniae* isolates may be a practical tool for identifying effective combination regimens.

Infections with *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Klebsiella pneumoniae* (referred to here as KPC *K. pneumoniae*) are widely encountered in the United States and are increasingly being reported worldwide (1–9). Mortality rates due to KPC *K. pneumoniae* infections are as high as 50% (1, 10–13), and optimal therapy is not well defined (14). Previous observational studies have shown that combinations of two or three antimicrobial agents may achieve better clinical outcomes than monotherapy (12, 14, 15), especially when the combination includes a carbapenem (16). In keeping with this clinical experience, we and others have demonstrated that the combination of colistin and doripenem is bactericidal and synergistic against KPC *K. pneumoniae* isolates *in vitro* (17–19). Doripenem was chosen to represent the carbapenems because it is more stable against hydrolysis by KPC than other agents in the same class (20). Despite these data, a subset of patients with KPC *K. pneumoniae* bacteremia does not respond to this combination clinically (21). The treatment of KPC *K. pneumoniae* infections may be further complicated by the presence of extended-spectrum β-lactamases (ESBLs) and porin mutations that impact carbapenem responses (3, 8, 9, 22), as well as the emergence of colistin resistance (18, 23, 24).

In a recent study, a double carbapenem regimen of doripenem-ertapenem rapidly reduced bacterial counts of a KPC *K. pneumoniae* isolate *in vitro* and *in vivo* (25), even though the isolate was resistant to both agents. In light of these findings and our previous doripenem-colistin data, we investigated the novel combination of colistin-doripenem-ertapenem against 12 colistin-susceptible and -resistant KPC *K. pneumoniae* isolates *in vitro*. At the same

time, we determined whether the efficacy of colistin-carbapenem combinations was impacted by the presence of specific ESBL genes or levels of porin expression by the isolates.

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## MATERIALS AND METHODS

**Isolates and phenotypic and genotypic characterization.** Twelve KPC *K. pneumoniae* clinical isolates obtained from unique patients at the University of Pittsburgh Medical Center between March 2010 and June 2011 underwent testing. The isolates were stored at –80°C and passaged at least twice at 37°C before experimentation. Stock solutions of antimicrobial agents were prepared in our laboratory in sterile water, aliquoted, and stored at –70°C. MICs were determined by standard broth microdilution and disk diffusion methods (26). Breakpoints for resistance were defined according to the Clinical and Laboratory Standards Institute (27). All isolates underwent multilocus sequence typing (MLST), and full-length *bla*<sub>KPC</sub> genes were characterized by PCR and DNA sequencing (28–32).

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**TABLE 1** Bactericidal activity of single drugs and 2- and 3-drug combinations against KPC *K. pneumoniae* isolates at 24 h

Isolate	MIC ( $\mu\text{g/ml}$ )			Porin expression <sup>a</sup>		Killing activity ( $\log_{10}$ CFU/ml) <sup>b</sup>			Colistin-ertapenem	Colistin-doripenem	Doripenem-ertapenem	Colistin-doripenem-ertapenem
	Colistin	Doripenem	Ertapenem	<i>ompK36</i>	<i>ompK35</i>	Colistin	Doripenem	Ertapenem				
44	64	32	256	0.59	0.17	1.36	-3.64	-3.61	0.02	2.75	-3.84	2.82
94	4	256	>256	0.1	0.25	2.29	-3.77	-3.72	0.62	<b>3.96</b>	-3.68	<b>3.82</b>
121	4	256	>256	0.13	0.37	1.46	-3.84	-3.67	<b>4.23</b>	<b>3.24</b>	-3.73	<b>3.1</b>
404	2	64	>256	0.04	0.3	1.42	-3.57	-3.72	0.6	<b>3.99</b>	-3.67	<b>3.49</b>
25	16	32	128	2.35	0.57	-1.85	<b>3.17</b>	-3.78	2.76	<b>3.48</b>	2.65	<b>4.2</b>
83	32	64	>256	5.3	1.1	2.5	-3.48	-3.59	2.06	<b>4.27</b>	-2.81	<b>4.65</b>
89	64	128	>256	4.12	1.06	2.29	-3.19	-3.41	0.58	<b>4.39</b>	-3.32	<b>4.38</b>
155	2	512	>256	4.23	1.1	-0.05	-3.9	-4.13	2.88	2.68	-3.76	1.97
168	32	128	>256	3.02	0.52	-3.8	-3.13	-3.72	0.72	0.77	-3.29	<b>4.45</b>
175	2	64	>256	1	0.47	-0.05	-3.66	-3.67	0.17	1.26	-3.52	<b>4.56</b>
178	16	256	>256	1.23	0.78	1.11	-3.79	-3.65	0.99	1.23	-3.69	<b>3.27</b>
220	16	256	>256	1.69	0.57	0.39	-3.27	-3.43	3.17	2.62	-3.29	<b>3.23</b>

<sup>a</sup> Relative porin expression normalized to *rpoB* gene expression and compared to *K. pneumoniae* ATCC 13883.

<sup>b</sup> Degree of killing was defined as the difference between the  $\log_{10}$  of starting inoculum and the  $\log_{10}$  concentration of KPC *K. pneumoniae* after 24 h of incubation with drug(s). Negative numbers denote growth of isolates compared with starting inoculum. Positive numbers denote suppression of growth of isolates compared with starting inoculum. Bactericidal activity was defined as degrees of killing of  $\geq 3$ - $\log_{10}$  CFU/ml. Colistin was tested at concentrations of 2.5 and 1  $\mu\text{g/ml}$  against colistin-resistant (MIC > 2  $\mu\text{g/ml}$ ) and -susceptible (MIC = 2  $\mu\text{g/ml}$ ) isolates, respectively. Values that reflect bactericidal activity are in bold.

**Time-kill studies.** Time-kill studies were performed using a final volume of 25 ml of cation-adjusted Mueller-Hinton broth and an initial inoculum of  $\sim 1 \times 10^6$  CFU/ml (33). The antimicrobial agent concentrations were chosen based on achievable serum levels from published pharmacokinetic data (34, 35): colistin, 2.5  $\mu\text{g/ml}$ ; doripenem, 8  $\mu\text{g/ml}$ ; and ertapenem, 2  $\mu\text{g/ml}$ . As detailed in Results, colistin-susceptible isolates were also tested against colistin (1  $\mu\text{g/ml}$ ). Flasks were incubated with shaking at 37°C, and bacterial colonies were enumerated at 0, 4, 8, 12, and 24 h. The degree of killing was defined as the difference between the  $\log_{10}$  combination of the starting inoculum and the  $\log_{10}$  concentration of KPC *K. pneumoniae* after 24 h of incubation with the agent(s). Bactericidal activity was defined as  $\geq 3$   $\log_{10}$  killing. Synergy was defined as a difference in the  $\log_{10}$  CFU/ml of isolates incubated with colistin and the  $\log_{10}$  CFU/ml of isolates incubated with combination agents of  $\geq 2$  at 24 h. Antagonism was defined as  $\geq 1$ - $\log_{10}$ -greater growth of isolates in response to combination agents than colistin alone. The area under the bacterial killing curve (AUBC) for time-kill graphs was determined using GraphPad Prism 6 software (GraphPad, San Diego, CA).

**Analysis of porin expression and levels.** DNase-treated RNA from individual isolates was obtained from late-exponential-phase cultures using a RiboPure bacterial kit (Ambion). cDNA was made using qScript cDNA mix (Quanta Biosciences). Quantitative reverse transcription PCR (qRT-PCR) for porin genes *ompK35* and *ompK36* was then performed in a TaqMan gene expression assay (ABI) using the one-step SYBR green kit (SYBR green FastMix ROX; Quanta Biosciences). The primers used in these experiments were 5'GCAATATTCTGGCAGTGGTGATC3' for the *ompK35* forward primer, 5'ACCATTTTTCCATAGAAGTCCAGT3' for the *ompK35* reverse primer, 5'TTAAAGTACTGTCCCTCCTGG3' for the *ompK36* forward primer, and 5'TCAGAGAAGTAGTGCAGACCGTCA3' for the *ompK36* reverse primer. Amplification was done in a 20- $\mu\text{l}$  final volume using the following protocol: denaturation at 95°C for 10 min, followed by 40 cycles consisting of 15 s at 95°C for denaturation, 11 s at 54°C for annealing, and 22 s at 72°C for elongation. The expression levels were expressed in cycle threshold ( $C_T$ ) units, which is the first cycle at which the signal is detected above a preset threshold. Relative quantities of mRNA from each gene of interest were determined by the comparative threshold  $C_T$ . Expression of each gene was normalized to that of the housekeeping gene *rpoB*; the primers used were 5'AAGCGAATCCAGC TTGTTTCAGC3' for the *rpoB* forward primer and 5'TGACGTTGCATG TTCGACCCATCA3' for the *rpoB* reverse primer. Data were expressed as the relative expression of *ompK35* and *ompK36* compared to that in a control isolate that is known to express both porins (*K. pneumoniae* ATCC 13883) (36). Each experiment was performed in triplicate, and the data are presented as mean  $C_T$  values from three experiments. The presence of OmpK36 porin was assessed by SDS-PAGE as previously described (37).

**Statistical analysis.** Comparisons between two groups of antimicrobial agents were made by Fisher's exact test for categorical variables and Mann-Whitney test or unpaired *t* test for continuous variables. Correlations between pairs of variables were assessed using the Spearman rank test. Receiver operating characteristic (ROC) analysis was conducted to determine optimal cutoffs for porin expression to predict synergy among 2- or 3-drug combinations. Significance was defined as a *P* value of  $\leq 0.05$  (two-tailed).

## RESULTS

**Phenotypic and genotypic characterization of KPC *K. pneumoniae*.** All isolates were resistant *in vitro* to amikacin, aztreonam, ciprofloxacin, piperacillin-tazobactam, and carbapenems. Specifically, the median MIC of doripenem was 128  $\mu\text{g/ml}$  (range, 32 to 512  $\mu\text{g/ml}$ ), and that of ertapenem was >256  $\mu\text{g/ml}$  (range, 128 to >256  $\mu\text{g/ml}$ ) (Table 1). Ninety-two percent (11/12), 17% (2/12), and 58% (7/12) of isolates were resistant to cefepime, doxycycline, and gentamicin, respectively. Seventy-five percent (9/12) of isolates were resistant to colistin, with MICs ranging from 4 to 64  $\mu\text{g/ml}$ ; the MICs against the 3 susceptible isolates were 2  $\mu\text{g/ml}$ .

MLST showed that all isolates belonged to the epidemic *K. pneumoniae* ST258 clone. Genotyping  $\beta$ -lactamase resistance by both PCR and DNA sequencing revealed that all isolates harbored *bla*<sub>KPC-2</sub>, *bla*<sub>SHV-12</sub>, and *bla*<sub>TEM-1</sub> but were negative for *bla*<sub>CTX-M</sub>, *bla*<sub>IMP</sub>, *bla*<sub>NDM</sub>, *bla*<sub>VIM</sub>, *bla*<sub>OXA-48</sub>, and *bla*<sub>AmpC</sub> (*bla*<sub>ACT-1</sub>, *bla*<sub>ACC</sub>, *bla*<sub>BIL-1</sub>, *bla*<sub>CMY</sub>, *bla*<sub>DHA</sub>, *bla*<sub>FOX</sub>, *bla*<sub>LAT</sub>, *bla*<sub>MIR-1</sub>, and *bla*<sub>MOX</sub>) (28–32). Median *ompK35* and *ompK36* expression relative to the control *K. pneumoniae* strain ATCC 13883 was 0.55 (range, 0.17 to 1.1) and 1.46 (range, 0.1 to 5.3), respectively (Table 1). Expression of *ompK35* and that of *ompK36* was directly correlated with each other ( $R^2 = 0.80$ ). The levels of OmpK36 detected in KPC *K. pneumoniae* isolates were variable and correlated with qRT-PCR findings (Fig. 1). There was no evident OmpK35 production, consistent with our qRT-PCR data and previous studies (38).

**Bactericidal activity.** As expected, ertapenem (2  $\mu\text{g/ml}$ ) had no activity against any of the isolates during time-kill studies (33) (Fig. 2). Doripenem (8  $\mu\text{g/ml}$ ) exerted no activity against 92% (11/12) of isolates; it was bactericidal against the remaining isolate (isolate 25) (Table 1). Colistin (2.5  $\mu\text{g/ml}$ ) exerted some activity against 78% (7/9) of colistin-resistant KPC *K. pneumoniae* isolates (range, 0.39 to 2.5  $\log_{10}$  killing) but did not reach bactericidal levels. Colistin had no activity against the remaining resistant iso-

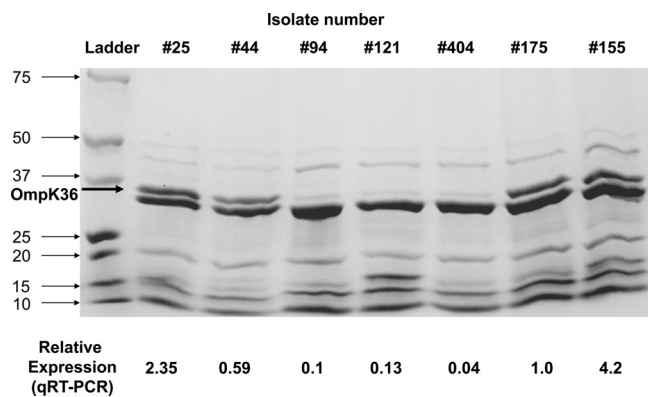


FIG 1 Outer membrane protein analysis of representative KPC *K. pneumoniae* isolates by 12% SDS-PAGE. The isolate number and relative expression of *ompK36* by real-time RT-PCR are provided for each lane. The lane marked "Ladder" contains size markers (sizes are in kDa).

lates (22%; 2/9). Colistin (2.5  $\mu\text{g/ml}$ ) was fully bactericidal against the 3 colistin-susceptible isolates at 12 and 24 h. For this reason, the colistin concentration was reduced to 1  $\mu\text{g/ml}$  (0.5 $\times$  MIC) to assess the interaction with carbapenems in subsequent combination time-kill studies of colistin-susceptible isolates.

In combination time-kill experiments, colistin-ertapenem was bactericidal against only 17% (2/12) of isolates (versus colistin alone [0%];  $P = 0.47$ ) (Table 1; Fig. 3). Median killing of any isolate at 24 h did not improve when ertapenem was added to colistin ( $P = 0.31$ ). The colistin-doripenem combination, however, was bactericidal against 50% (6/12) of isolates ( $P = 0.01$  versus colistin alone). Median killing was increased from 1.24  $\log_{10}$  with colistin alone to 3.00  $\log_{10}$  with colistin-doripenem ( $P = 0.002$ ).

The triple combination of colistin-doripenem-ertapenem was bactericidal against 83% (10/12) of isolates ( $P = 0.19$  versus colistin-doripenem). Median killing also improved to 3.65- $\log_{10}$  with the triple combination compared to colistin-doripenem ( $P = 0.09$ ).

To further evaluate the effects of colistin and carbapenems in combination, we calculated the area under the bacterial killing curve (AUBC) for the time-kill graphs (Table 2). The median AUBCs for colistin-ertapenem and colistin-doripenem were significantly lower than those of colistin alone ( $P = 0.03$  and  $P = 0.0005$ , respectively) (Fig. 4). The median AUBC of the triple combination was significantly lower than the AUBCs of colistin-ertapenem ( $P = 0.0002$ ) and colistin-doripenem ( $P = 0.05$ ).

**Synergistic activity.** Colistin-ertapenem, colistin-doripenem, and colistin-doripenem-ertapenem achieved synergy at 24 h against 42% (5/12), 50% (6/12), and 67% (8/12) of isolates, respectively. Antagonism was not observed with colistin-doripenem or colistin-doripenem-ertapenem but did occur with colistin-ertapenem against 25% (3/12) of isolates.

**Associations between antimicrobial activity and porin expression by isolates.** There was no correlation between doripenem MIC and *ompK35* or *ompK36* expression ( $P = 0.29$  and  $P = 0.97$ , respectively). There was also no correlation between porin expression and killing by doripenem ( $P = 0.78$  and  $0.98$  for *ompK35* and *ompK36*, respectively) or ertapenem ( $P = 0.19$  and  $0.40$  for *ompK35* and *ompK36*, respectively).

Using ROC, we identified optimal *ompK35* and *ompK36* ex-

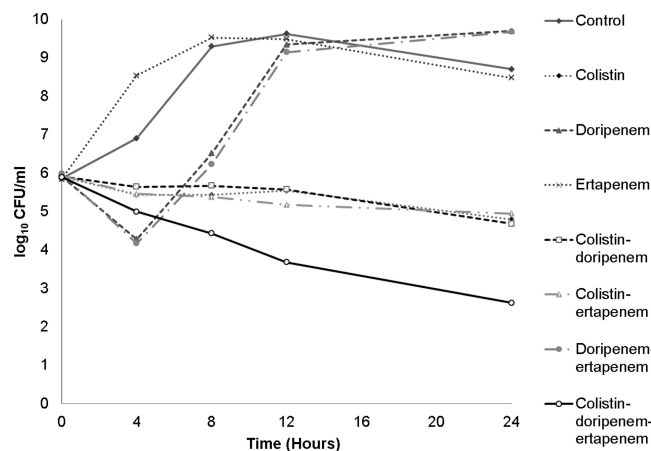


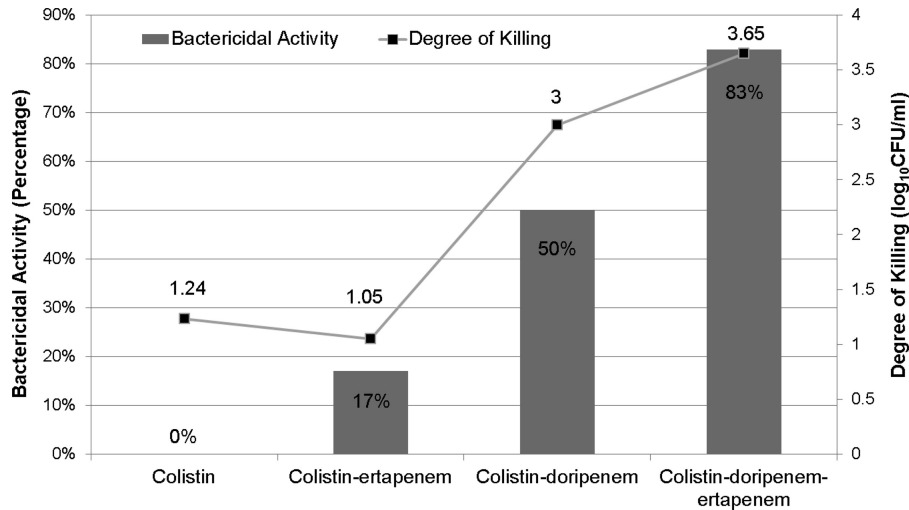
FIG 2 Time-kill curve of single drugs and 2- and 3-drug combinations against a representative KPC *K. pneumoniae* isolate. Doripenem and colistin MICs against this isolate (isolate 178) were 256  $\mu\text{g/ml}$  and 16  $\mu\text{g/ml}$ , respectively. Times on the x axis are incubation times.

pression cutoffs of 0.45 and 1.0, which identified isolates against which the triple combination of colistin-doripenem-ertapenem was synergistic. At these cutoffs, all isolates with high *ompK35* expression had high *ompK36* expression, and isolates with low *ompK35* expression had low *ompK36* expression. From this point forward, we refer to high *ompK35/ompK36* expression as "high porin expression" and low *ompK35/ompK36* expression as "low porin expression." Synergy with colistin-doripenem-ertapenem was achieved against 100% (8/8) of isolates with high porin expression but none (0/4) of the isolates with low porin expression ( $P = 0.002$ ). Synergy between colistin and doripenem was achieved against 63% (5/8) and 25% (1/4) of isolates with high and low porin expression, respectively ( $P = 0.5$ ).

We also evaluated associations between porin expression and AUBCs (Fig. 5). For isolates with high porin expression, the triple combination had lower median AUBC than colistin-doripenem (74.45 versus 104.45;  $P = 0.049$ ). However, for isolates with low porin expression, median AUBCs of the triple combination and colistin-doripenem were not significantly different (83.95 versus 89.25;  $P = 0.49$ ). Colistin-doripenem had a lower median AUBC than colistin alone, regardless of the level of porin expression ( $P = 0.03$  and  $0.01$ ).

## DISCUSSION

There are three particularly notable findings from this study. First, the data corroborate previous observations by our group and others that the colistin-doripenem combination is more effective than colistin-ertapenem or any of the agents alone against KPC *K. pneumoniae* isolates, exhibiting greater killing and more often achieving bactericidal activity and synergy (17–19). In fact, the beneficial effects of colistin-doripenem were evident with isolates that were highly resistant to both agents, as determined by MICs. Second, the addition of ertapenem to colistin-doripenem further enhanced killing, bactericidal activity, and synergy, despite the fact that the colistin-ertapenem combination itself was no more effective than colistin alone. Third, the enhanced activity of colistin-doripenem-ertapenem was observed exclusively with KPC *K. pneumoniae* isolates with high levels of *ompK35* or *ompK36* expression. In contrast, the



**FIG 3** Bactericidal activity and degree of killing by single drugs and 2- and 3-drug combinations against 12 KPC *K. pneumoniae* isolates. The line graph represents the median degree of killing, and the bar graph represents the percentage of bactericidal activity of each drug regimen against the 12 KPC *K. pneumoniae* isolates. There was a stepwise improvement in bactericidal activity from colistin only and colistin-ertapenem to colistin-doripenem to colistin-doripenem-ertapenem. Bars (left y axis) show bactericidal activity, and the line (right y axis) shows the degree of killing by single drugs and 2- and 3-drug combinations.

benefits of colistin-doripenem over colistin alone were independent of porin expression. Our findings are consistent with a recent clinical observation that treatment with colistin-carbapenem combinations led to favorable survival among patients with KPC *K. pneumoniae* bacteremia (15). Moreover, they suggest that the triple combination of colistin-doripenem-ertapenem may have a role in the treatment of at least some KPC *K. pneumoniae* infections, and screening for porin expression may identify isolates that are most susceptible to this combination. Indeed, the study offers a model by which molecular characterization of difficult-to-treat microbial isolates can be used to identify effective antimicrobial regimens.

To our knowledge, this is the first study to evaluate the activity of antimicrobial combinations against KPC *K. pneumoniae* in the context of molecular mechanisms of resistance. In this regard, our most important finding is that “one-size-fits-all” approaches to identifying optimal antimicrobial regimens against resistant pathogens are not likely to be effective, even for isolates from a single center that share similar genetic backgrounds. Indeed, all isolates in the study belonged to the ST258 international clone and carried *bla*<sub>KPC-2</sub> carbapenemase, *bla*<sub>SHV-12</sub> ESBL, and *bla*<sub>TEM-1</sub> β-lactamase genes. Nevertheless, the range of porin expression and responses to antimicrobial agents were quite heterogeneous and could not be predicted solely based on MICs of individual

agents. Of course, the molecular characteristics of isolates from other centers may differ from ours. As such, the efficacy of colistin-doripenem with or without ertapenem in our experience may not be representative of isolates from other centers or for clonal groups that carry different KPC and β-lactamase genes. Therefore, we must acknowledge that our findings need to be confirmed by follow-up studies elsewhere.

Our data highlight how molecular characterization of antimicrobial-resistant isolates can provide insights into the possible mechanisms and extent of antimicrobial synergy. Colistin exerts its bactericidal effects by permeabilizing the outer membrane of Gram-negative bacteria (39–41). Porins are outer membrane channels that allow molecules, including β-lactams, to diffuse into their periplasmic active sites (42). Absence or reduced expression of *ompK35* and *ompK36* porins, which typically results from mutations within promoter regions or coding sequences, can contribute to carbapenem resistance (36, 42–46). The lack of correlation between the levels of porin expression by our isolates and carbapenem MICs suggests that this mechanism may be less relevant in the face of high-level resistance due to KPC as well as other β-lactamases and mechanisms. The synergy we found between colistin and doripenem is consistent with a model in which membrane permeabilization by the former agent facilitates increased access of the latter

**TABLE 2** Area under the bacterial killing curve (AUBC) of 2- or 3-drug combinations against 12 KPC *K. pneumoniae* isolates

Drug(s)	Median AUBC (log <sub>10</sub> CFU/ml · h) (interquartile range) <sup>a</sup>	P value	
		Vs colistin <sup>b</sup>	Vs colistin-doripenem <sup>c</sup>
Colistin	123.9 (114.1–128.1)	NA	0.0005
Colistin-ertapenem	112.8 (98.3–120.9)	0.03	0.07
Colistin-doripenem	93.0 (84.1–107.5)	0.0005	NA
Colistin-doripenem-ertapenem	82.8 (73.0–91.8)	<0.0001	0.05

<sup>a</sup> Calculated using GraphPad Prism 6 software. A more effective drug regimen yields a lower AUBC.

<sup>b</sup> The AUBCs of colistin-ertapenem, colistin-doripenem, and colistin-doripenem-ertapenem were significantly lower than the AUBC of colistin alone.

<sup>c</sup> The AUBC of colistin-doripenem-ertapenem was significantly lower than the AUBC of colistin-doripenem. NA, not applicable.



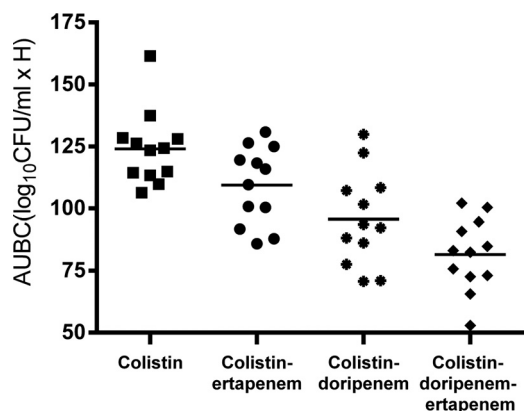


FIG 4 Area under the bacterial killing curves (AUBC) of single drugs and 2- and 3-drug combinations against 12 KPC *K. pneumoniae* isolates. The median AUBC was significantly lower for colistin-doripenem-ertapenem than colistin with a carbapenem (colistin-ertapenem,  $P = 0.0002$ ; colistin-doripenem,  $P = 0.05$ ) and colistin only ( $P < 0.0001$ ).

to its penicillin-binding protein targets, allowing it to overcome its hydrolysis by KPC (47–49). In the time-kill studies, colistin (2.5  $\mu\text{g}/\text{ml}$ ) was active against the majority of colistin-resistant KPC *K. pneumoniae* isolates, indicating that subinhibitory concentrations still induced some degree of membrane permeabilization. The fact that synergy between colistin and doripenem was independent of the level of porin expression suggests that doripenem's inhibitory effects were maximized in the presence of colistin; either high expression of porin channels did not appreciably increase doripenem access, or further access did not enhance doripenem's effects. Along these lines, there are two possible explanations for the correlation between high levels of porin expression and additional synergy with the addition of ertapenem to colistin-doripenem. First, the enhanced access of both carbapenems through porins may allow ertapenem to act as a suicide substrate that binds to KPC and permits doripenem to be freely available at the penicillin-binding site (25). Alternatively, the effects may not be

specific to either agent but rather stem from increased cumulative carbapenem concentrations that saturate KPC.

In contrast to a previous study showing that the doripenem-ertapenem combination rapidly reduced KPC *K. pneumoniae* bacterial counts in *in vitro* chemostat and mouse thigh infection models, we did not observe any synergy with this combination (25). The discrepancies between our findings and the earlier data offer further evidence that results from particular centers or with particular isolates may not be broadly applicable. Other noteworthy findings were consistent with the previous literature. Colistin-ertapenem was significantly less active than colistin-doripenem, which was expected since ertapenem has preferential affinity for KPC enzymes compared to other carbapenems and is more susceptible to hydrolysis (50). As mentioned earlier, colistin MICs were poorly associated with activity by time-kill assays, as some degree of killing was evident even against resistant isolates. The limitations of colistin MICs are well described and present challenges in defining resistance and accurately identifying resistant isolates (51–53).

In conclusion, our data demonstrate that not all KPC *K. pneumoniae* isolates are created equal. In the future, effective treatment regimens will likely have to be defined based on a constellation of factors, including the molecular biology of specific isolates, types of infection being treated, underlying diseases, host factors, and pharmacokinetic/pharmacodynamic parameters. It is imperative to accurately identify patients for whom combination antimicrobial regimens are most likely to be effective in order to both optimize the outcomes and minimize the potential for toxicity. Colistin, for example, is among the most toxic antimicrobials currently in use, and combinations of carbapenems have the potential for synergistic neurotoxicity and other untoward events. Therefore, minimizing exposure to such regimens if they are unlikely to be of benefit will be as important as identifying cases in which they may be useful. Along these lines, the development of rapid molecular tests that identify isolates that are susceptible or resistant to antimicrobial agents or combinations of agents in real time is a top priority for the field. The data from this study attest to the potential feasibility of such tests. Follow-up *in vitro*, animal model, and

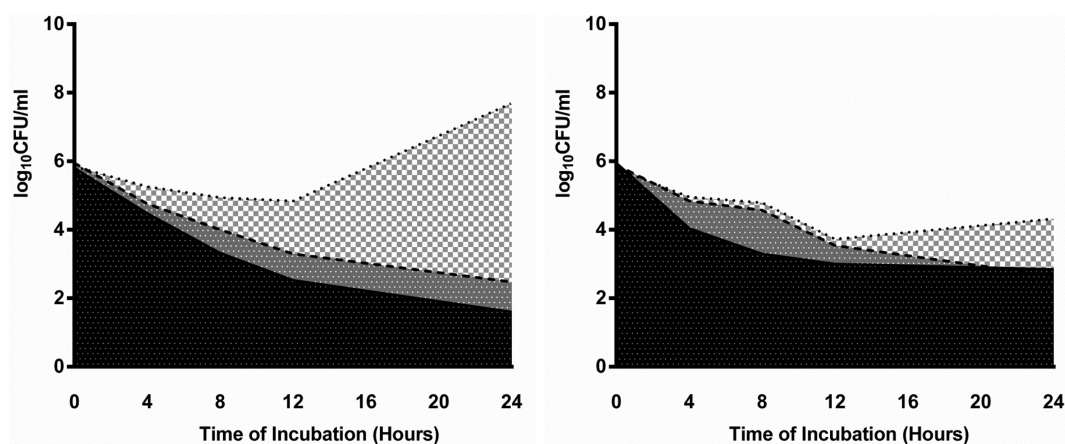


FIG 5 AUBCs of representative KPC *K. pneumoniae* isolates with high porin expression (isolate 25 [left]) and low porin expression (isolate 121 [right]). The dotted line, dashed line, and solid line represent time-kill curves of colistin, colistin-doripenem, and colistin-doripenem-ertapenem, respectively. Black, AUBC of colistin-doripenem-ertapenem; dark gray, difference in AUBC between colistin-doripenem and colistin-doripenem-ertapenem; light gray, difference in AUBC between colistin and colistin-doripenem.

clinical studies to corroborate our findings for KPC *K. pneumoniae* and other difficult-to-treat pathogens are urgently needed.

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