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* _KPC-2_, *bla* _SHV-12_, and *bla* _TEM-1_. 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 log₁₀) 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 (R² = 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 ≤ 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.

(Preliminary data were presented at the 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 9 to 12 September 2012.)

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* _KPC_ 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

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colistin (µg/ml)</th>
<th>Doripenem (µg/ml)</th>
<th>Ertapenem (µg/ml)</th>
<th>ompK36</th>
<th>ompK35</th>
<th>Colistin</th>
<th>Ertapenem</th>
<th>Doripenem</th>
<th>Colistin-doripenem</th>
<th>Ertapenem</th>
<th>Doripenem-doripenem</th>
<th>Ertapenem</th>
<th>Doripenem-doripenem</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>64</td>
<td>256</td>
<td>&gt;256</td>
<td>0.59</td>
<td>0.17</td>
<td>1.36</td>
<td>−3.64</td>
<td>−3.61</td>
<td>0.02</td>
<td>2.75</td>
<td>−3.84</td>
<td>2.82</td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>4</td>
<td>256</td>
<td>&gt;256</td>
<td>0.1</td>
<td>0.25</td>
<td>2.29</td>
<td>−3.77</td>
<td>−3.72</td>
<td>0.62</td>
<td>3.96</td>
<td>−3.68</td>
<td>3.82</td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>4</td>
<td>256</td>
<td>&gt;256</td>
<td>0.13</td>
<td>0.37</td>
<td>1.46</td>
<td>−3.84</td>
<td>−3.67</td>
<td>4.23</td>
<td>3.24</td>
<td>−3.73</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>404</td>
<td>2</td>
<td>64</td>
<td>&gt;256</td>
<td>0.04</td>
<td>0.3</td>
<td>1.42</td>
<td>−3.57</td>
<td>−3.72</td>
<td>0.36</td>
<td>3.99</td>
<td>−3.67</td>
<td>3.49</td>
<td></td>
</tr>
</tbody>
</table>

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 ≤0.05 (two-tailed).

RESULTS
Phenotypic and genotypic characterization of KPC K. pneumoniae. All isolates were resistant in vitro to amikacin, aztreonam, ciprofloxacin, pipercillin-tazobactam, and carbapenems. Specifically, the median MIC of doripenem was 128 µg/ml (range, 32 to 512 µg/ml), and that of erapenem was >256 µg/ml (range, 128 to >256 µg/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 µg/ml; the MICs against the susceptible isolates were 2 µg/ml.

MLST showed that all isolates belonged to the epidemic K. pneumoniae ST258 clone. Genotyping β-lactamase resistance by both PCR and DNA sequencing revealed that all isolates harbored blaKPC-2, blaSHV-12, and tetM, but were negative for blaCTX-M, bladNDM, blava_48, and blablACo. The presence of OmpK35 and OmpK35 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 OmpK35 was directly correlated with each other (R² = 0.80). The levels of OmpK35 detected in KPC K. pneumoniae isolates were variable and correlated with qRT-PCR findings (Fig. 1). There was no evidence of OmpK35 production, consistent with our qRT-PCR data and previous studies (38).

Bactericidal activity. As expected, erapenem (2 µg/ml) had no activity against any of the isolates during time-kill studies (33) (Fig. 2). Doripenem (8 µg/ml) exerted no activity against 92% (11/12) of isolates; it was bactericidal against the remaining isolate (isolate 25) (Table 1). Colistin (2.5 µg/ml) exerted some activity against 87% (7/9) of collagen-resistant KPC K. pneumoniae isolates (range, 0.39 to 2.5 log₁₀ killing) but did not reach bactericidal levels. Colistin had no activity against the remaining resistant iso-
Colistin (2.5 μg/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 μg/ml (0.5× 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-ertapenem combination, however, was bactericidal against 50% (6/12) of isolates (\(P = 0.01\) versus colistin alone). Median killing was increased from 1.24 log₁₀ with colistin alone to 3.00 log₁₀ with colistin-ertapenem (\(P = 0.002\)).

The triple combination of colistin-ertapenem-ertapenem was bactericidal against 83% (10/12) of isolates (\(P = 0.19\) versus colistin-ertapenem). Median killing also improved to 3.65-log₁₀ with the triple combination compared to colistin-ertapenem (\(P = 0.09\)).

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 not significantly different (83.95 versus 89.25; \(P = 0.03\) and \(P = 0.01\)). 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 \(P = 0.01\)).

**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 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).

**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 μg/ml and 16 μg/ml, respectively. Times on the x axis are incubation times.
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*KPC-2 carbapenemase, *bla*SHV-12 ESBL, and *bla*TEM-1 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 drug(s).

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

<table>
<thead>
<tr>
<th>Drug(s)</th>
<th>Median AUBC (log$_{10}$ CFU/ml · h) (interquartile range)</th>
<th>$P$ value</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$V_s$ colistin</td>
<td>$V_s$ colistin-doripenem</td>
</tr>
<tr>
<td>Colistin</td>
<td>123.9 (114.1–128.1)</td>
<td>NA</td>
<td>0.0005</td>
</tr>
<tr>
<td>Colistin-ertapenem</td>
<td>112.8 (98.3–120.9)</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>Colistin-doripenem</td>
<td>93.0 (84.1–107.5)</td>
<td>0.0005</td>
<td>NA</td>
</tr>
<tr>
<td>Colistin-doripenem-ertapenem</td>
<td>82.8 (73.0–91.8)</td>
<td>&lt;0.0001</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* a Calculated using GraphPad Prism 6 software. A more effective drug regimen yields a lower AUBC.

* b The AUBCs of colistin-ertapenem, colistin-doripenem, and colistin-doripenem-ertapenem were significantly lower than the AUBC of colistin alone.

* c The AUBC of colistin-doripenem-ertapenem was significantly lower than the AUBC of colistin-doripenem. NA, not applicable.
to its penicillin-binding protein targets, allowing it to overcome its hydrolysis by KPC (47–49). In the time-kill studies, colistin (2.5 μg/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 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 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).

**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. pneumoniae and other difficult-to-treat pathogens are urgently needed.

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