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Synergistic Activity of Colistin plus Rifampin against Colistin-Resistant KPC-Producing *Klebsiella pneumoniae*

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Infections caused by carbapenem-resistant KPC-producing *Klebsiella pneumoniae* are responsible for high rates of mortality and represent a major therapeutic challenge, especially when the isolates are also resistant to colistin. We used the checkerboard method to evaluate the synergistic activity of 10 antibiotic combinations against 13 colistin-resistant KPC-producing *K. pneumoniae* isolates (colistin MIC range of 8 to 128 mg/liter). Colistin plus rifampin was the only combination that demonstrated consistent synergistic bacteriostatic activity against 13/13 strains tested, reducing the colistin MIC below the susceptibility breakpoint (MIC ≤ 2 mg/liter) in 7/13 strains at rifampin concentrations ranging from 4 to 16 mg/liter. Bactericidal synergistic activity was also documented for 8/13 tested strains. Other antimicrobial combinations with carbapenems, gentamicin, and tigecycline showed variously synergistic results. Colistin plus rifampin also exhibited bacteriostatic synergistic activity against 4/4 colistin-susceptible KPC-producing *K. pneumoniae* isolates (colistin MIC range of 0.5 to 2 mg/liter) and 4/4 ertapenem-resistant extended-spectrum beta-lactamase (ESBL)-producing *K. pneumoniae* isolates (ertapenem MIC range of 16 to 32 mg/liter). Collectively, our data suggest that colistin plus rifampin is the most consistently synergistic combination against KPC-producing *K. pneumoniae* isolates, including colistin-resistant strains. Colistin-rifampin combinations may have a role in the treatment of multidrug-resistant *K. pneumoniae* and may possibly slow the selection of heteroresistant subpopulations during colistin therapy.

The rapid spread of carbapenem-resistant *Klebsiella pneumoniae* is quickly transforming many common health care-associated complications into infections that are untreatable with the currently available antibiotics. Carbapenem resistance is frequently caused by the production of the serine-carbapenemase KPC or OXA-48 or metallo-beta-lactamases (VIM, IMP, and NDM). AmpC-type or extended-spectrum beta-lactamases (ESBL) have weak carbapenemase activity (i.e., TEM, SHV, and CTX-M) requiring a concomitant role of other mechanisms of resistance (porins or efflux pump) (1, 2). KPC-producing strains of clonal complexes (CC) 258 and 512 (sequence types [ST] ST258 and ST512) have also emerged as the most common circulating carbapenem-resistant strains of *K. pneumoniae*, exhibiting a remarkable propensity for epidemic diffusion on a global scale (3, 4).

The gene responsible for KPC carbapenemase, bla_{KPC}, frequently resides on a large plasmid that confers resistance not only to carbapenems but also to extended-spectrum cephalosporins, aztreonam, fluoroquinolones, and some aminoglycosides (5). As a result, treatment of infections due to KPC-producing *Klebsiella pneumoniae* (KPC-Kp) is often limited to antibiotics (e.g., polymyxins, tigecycline [TIG], and fosfomycin) with significant pharmacokinetic shortcomings and limited clinical efficacy for treating severe infections, especially if used as monotherapy. The limited efficacy of these antibiotics when used alone contributes to the high crude mortality rates (>40%) observed in patients with KPC-Kp infection. Consequently, combination therapy has become a standard of care for KPC-Kp infections due to reports of significantly improved survival rates compared to the rates for patients who received monotherapy regimens for bloodstream infection (6–8).

Polymyxins are among the core antibiotics used as part of a combination treatment regimen for KPC-Kp infection. And yet, polymyxin- and, especially, colistin (CST)-resistant strains (CST-R KPC-Kp) are increasing and account for an alarming proportion of isolates in some centers, and they are associated with higher attributable mortality (9). The rapid emergence of CST resistance among KPC-Kp isolates has been attributed to the high frequency of heteroresistant isolates among circulating strains, which are rapidly selected as dominant, irreversibly resistant populations *in vitro* and *in vivo* during CST treatment (10, 11). Therefore, combination therapy is suggested to be an essential component for the effective use of colistin therapy.

Knowledge of which antimicrobial agents produce reliable synergistic interactions is essential for selecting the best combination regimens. In this work, we investigated the *in vitro* activities of different antimicrobial combinations against clinical isolates of CST-R KPC-Kp. The most powerful synergistic combination, colistin plus rifampin (CST+RIF), was also tested against colistin-susceptible (CST-S) KPC-Kp isolates and *K. pneumoniae* strains expressing different resistance mechanisms.

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TABLE 1 Synergistic bacteriostatic activities of different antimicrobial combinations against 10 colistin-resistant KPC-producing *Klebsiella pneumoniae* isolates

Isolate	ST	ΣFIC[a] of[b]:									
		CST-RIF	MER-GEN	CST-GEN	CST-MER	CST-IMI	CST-TIG	IMI-GEN	GEN-TIG	MER-TIG	IMI-TIG
2018/12	ST512	0.09	0.08	0.07	0.13	0.26	2.00	0.07	2.00	2.00	2.00
2604/12	ST512	0.38	0.38	0.07	1.13	0.25	2.00	0.38	0.56	2.00	2.00
2550/12	ST512	0.08	0.63	0.07	0.16	0.38	0.13	0.13	0.63	2.00	0.56
2762/12	ST512	0.09	1.00	0.75	2.00	1.13	0.75	0.56	0.56	2.00	2.00
3031/12	ST512	0.16	0.25	0.25	2.00	2.00	0.38	2.00	1.00	2.00	2.00
3177/12	ST512	0.09	0.50	0.53	0.50	2.00	1.00	0.75	2.00	2.00	1.00
3325/12	ST512	0.28	0.16	0.19	2.00	0.63	0.31	4.00	0.63	1.00	2.00
3342/12	ST512	0.09	0.63	1.00	2.00	2.00	0.63	2.00	1.00	2.00	1.00
3434/12	ST512	0.19	0.75	0.56	1.00	3.00	0.75	2.00	2.00	2.00	2.00
3515/12	ST512	0.06	0.75	0.52	0.50	0.19	0.26	0.56	0.75	2.00	2.00
2/12	ST101	0.08	2.00	0.56	2.00	2.00	0.50	2.00	2.00	2.00	2.00
4306/11	ST101	0.12	0.75	0.53	0.50	0.50	0.56	0.56	2.00	2.00	2.00
101/11	ST101	0.09	2.00	0.75	2.00	2.00	2.00	2.00	2.00	2.00	2.00
No. (%[c]) with synergism		13 (100)	5 (38.5)	5 (38.5)	5 (38.5)	5 (38.5)	5 (38.5)	3 (23.1)	0 (0)	0 (0)	0 (0)

[a] ∑FIC = FIC-A + FIC-B (FIC, fractional inhibitory concentration; FIC-A, MIC of agent A in the presence of agent B divided by MIC of agent A alone; FIC-B, MIC of agent B in the presence of agent A divided by MIC of agent B alone).
[b] Shading indicates synergism. CST, colistin; RIF, rifampin; IMI, imipenem; MER, meropenem; GEN, gentamicin; TIG, tigecycline.
[c] $n = 13$ strains tested.

MATERIALS AND METHODS

Bacterial strains. Clinical strains of CST-R KPC-Kp ($n = 13$) were selected for testing with 10 different antibiotic combinations; 10 were ST512 and 3 ST101; 9/13 had a pulsed-field gel electrophoresis (PFGE) profile of pulsetype A. Eight CST-susceptible (CST-S) *K. pneumoniae* strains were also tested with the CST+RIF combination; 4 were KPC-Kp isolates (3 ST258 and 1 ST101) and 4 were ESBL producers with porin loss. Clinical test isolates were identified using the mini-API system (API 20E; bioMérieux) and confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Vitek-MS; bioMérieux). Antimicrobial susceptibility was determined using the reference broth microdilution method (12). MIC results were interpreted according to the EUCAST breakpoint recommendations (13). *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC BAA-1705 and BAA-1706 were used for quality controls in antimicrobial susceptibility testing.

Detection of KPC and of CTX-M beta-lactamase genes and characterization of the genes encoding the OmpK35 and OmpK36 outer membrane proteins was carried out by PCR and sequencing as described previously (14). Multilocus sequence typing (MLST) was performed according to the protocols on the *K. pneumoniae* MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). Genotyping by PFGE after restriction of genomic DNA with the XbaI restriction endonuclease was carried out as described for *E. coli* (http://www.cdc.gov/pulsenet/pathogens/index.html).

Antimicrobial combination susceptibility testing. Antibiotic powders were purchased from Sigma-Aldrich and prepared as fresh stock solutions in sterile distilled water or medium on the day of testing. Rifampin was dissolved in ethanol and then diluted in water. The following 10 combinations of antimicrobials were tested against the 13 CST-R KPC-Kp isolates: CST+RIF, CST plus imipenem (CST+IMI), CST plus meropenem (CST+MER), CST plus tigecycline (CST+TIG), CST plus gentamicin (CST+GEN), TIG+IMI, TIG+MER, TIG+GEN, IMI+GEN, and MER+GEN.

The activities of the antimicrobial combinations were determined in duplicate for each isolate with the checkerboard method (15) using fresh cation-adjusted Mueller-Hinton broth (BBL; Becton, Dickinson, Sparks, MD) prepared on the day of the experiment and 96-well microtiter plates (Sarstedt, Inc., Newton, NC). Each well was inoculated with 100 μl of a suspension of 5×10^5 CFU/ml in a final volume of 200 μl. Inocula were prepared by direct suspension in Mueller-Hinton broth of bacteria grown overnight on MacConkey medium. The checkerboard plates were then incubated for 24 h at 35°C. The total fractional inhibitory concentration (∑FIC) for each combination was calculated according to EUCAST definitive document E.Def 1.2 (16) as follows: ∑FIC equals FIC of agent A plus FIC of agent B, where the FIC of agent A or B is the MIC of agent A or B in the presence of the other divided by the MIC of agent A or B alone.

Results were interpreted as follows: a ∑FIC value of ≤0.5 indicated synergism, a ∑FIC of 0.5 to 4 indifference, and a ∑FIC of >4 antagonism. The minimum bactericidal concentration (MBC) and total fractional bactericidal concentration (∑FBC) for each combination were calculated in a similar fashion after subculturing the broth (50 μl) from nonturbid wells onto antibiotic-free medium. Bactericidal activity was defined as a 3-$\log_{10}$ reduction of the initial inoculum.

RESULTS

The 13 KPC-producing CST-R strains were isolated from blood ($n = 6$), abdominal drainage ($n = 2$), sputum ($n = 2$), a vascular prosthesis ($n = 1$), urine ($n = 1$), and feces ($n = 1$). CST MICs were 8 mg/liter ($n = 1$), 16 mg/liter ($n = 1$), 32 mg/liter ($n = 3$), 64 mg/liter ($n = 7$), and 128 mg/liter ($n = 1$). The RIF MICs were 8 mg/liter ($n = 1$), 16 mg/liter ($n = 7$), and 32 mg/liter ($n = 5$). All strains were resistant to carbapenems (MICs ranging from 128 to 256 mg/liter). Resistance to tigecycline was documented in 4/10 strains; only one strain was resistant to gentamicin.

Among the antimicrobial combinations tested, only CST+RIF exhibited synergistic activity against all 13 CST-R strains. Synergistic inhibitory activity was observed in 5/13 strains with CST+GEN, MER+GEN, CST+MER, CST+IMI, and CST+TIG and in 3/13 strains with IMI+GEN. No synergy was observed with the TIG+MER, TIG+IMI, or TIG+GEN combination. Antagonism was not observed with any antibiotic combination (Table 1).

CST+RIF showed synergistic inhibitory activity (∑FIC ≤ 0.5) in the presence of RIF concentrations ranging from 1 to 16 mg/liter. RIF reduced the CST MIC of CST-R KPC-Kp strains below

TABLE 2 Synergistic bactericidal activities of different antimicrobial combinations against 10 colistin-resistant KPC-producing *Klebsiella pneumoniae* isolates

Isolate	ST	ΣFBC[a] of[b]:									
		CST-RIF	MER-GEN	CST-TIG	CST-GEN	CST-IMI	GEN-TIG	IMI-GEN	CST-MER	MER-TIG	IMI-TIG
2018/12	ST512	0.16	0.50	NA	2.00	0.19	NA	0.31	1.25	NA	NA
2604/12	ST512	0.52	0.38	NA	0.28	0.63	0.31	2.00	0.75	NA	NA
2550/12	ST512	2.00	0.63	0.02	2.00	2.00	2.00	0.53	0.52	NA	0.53
2762/12	ST512	2.00	0.75	2.00	2.00	2.00	2.00	2.00	NA	NA	NA
3031/12	ST512	0.14	2.00	0.31	2.00	NA	NA	NA	NA	NA	NA
3177/12	ST512	0.31	2.00	2.00	2.00	NA	NA	2.00	1.00	NA	2.00
3325/12	ST512	2.00	2.00	2.00	2.00	2.00	2.00	NA	NA	0.56	NA
3342/12	ST512	0.31	2.00	2.00	2.00	NA	2.00	NA	NA	NA	2.00
3434/12	ST512	2.00	1.00	2.00	0.75	NA	NA	NA	NA	NA	NA
3515/12	ST512	0.09	0.75	2.00	0.50	2.00	2.00	0.75	2.00	NA	NA
2/12	ST101	0.37	NA	2.00	2.00	NA	NA	NA	NA	NA	NA
4306/11	ST101	0.37	2.00	0.25	0.75	0.5	NA	2	0.5	NA	NA
101/11	ST101	0.07	NA	NA	2.00	NA	NA	NA	NA	NA	NA
No. (%[c]) with synergism		8 (61.5)	2 (15.4)	3 (23.1)	2 (15.4)	2 (15.3)	1 (7.7)	1 (7.6)	1 (7.6)	0 (0)	0 (0)

[a] ∑FBC = FBC-A + FBC-B (FBC, fractional bactericidal concentration; FBC-A, MBC of agent A in the presence of agent B divided by MBC of agent A alone; FBC-B, MBC of agent B in the presence of agent A divided by MBC of agent B alone; MBC, minimum bactericidal concentration).
[b] Shading indicates synergism. CST, colistin; RIF, rifampin; IMI, imipenem; MER, meropenem; GEN, gentamicin; TIG, tigecycline; NA, not applicable.
[c] $n = 13$ strains tested.

the breakpoint of susceptibility (2 mg/liter) for 7 of 13 (53.8%) isolates at RIF concentrations of 4 to 16 mg/liter.

Synergistic bactericidal activity (∑FBC ≤ 0.5) was documented in 8/13 CST-R KPC-Kp isolates with CST+RIF, 3/13 strains with CST+TIG, 2/13 strains with MER+GEN, IMI+GEN, and CST+GEN, and 1/13 strains with IMI+GEN, CST+MER, and GEN+TIG, while no synergistic bactericidal activity was observed for MER+TIG or IMI+TIG (Table 2).

The CST+RIF combination showed bacteriostatic synergism against all 4 CST-S KPC-Kp strains and the 4 CTX-M beta-lactamase producers with porin loss and bactericidal synergism against 1/4 CST-S KPC-Kp strains.

DISCUSSION

Combinations of colistin plus rifampin exhibited the most reliable synergistic inhibitory activity *in vitro* against all CST-R KPC-Kp strains tested in our study. Perturbation of the outer bacterial cellular membrane by colistin may favor the uptake of rifampin, allowing the drug to reach sufficient intracellular concentrations to inhibit protein synthesis. For 53.8% of CST-R KPC-Kp strains, the MICs in the presence of rifampin were reduced below the current colistin susceptibility breakpoint. These *in vitro* data suggest that use of CST+RIF combinations may be advantageous for CST-R KPC-Kp strains, particularly if the CST MICs are elevated only a few dilutions above the susceptibility breakpoint. Further animal and clinical studies are needed to clarify the pharmacodynamics of this synergistic interaction *in vivo* and the relative limits of this synergism for restoring colistin activity against KPC-Kp strains.

Synergistic bactericidal activity was also observed against 8/13 CST-R KPC-Kp strains, which could be of benefit in treating severe infections. The bacteriostatic and bactericidal synergistic contributions of rifampin were present *in vitro* at concentrations easily obtained in serum and tissues with currently utilized doses (600 to 900 mg/day).

Molecular typing revealed that 10 of the 13 tested strains were clonally related (ST512), despite the different resistance phenotypes and various patterns of antimicrobial synergy observed during combination testing. Three strains belonging to the rare ST101 complex had different resistance phenotypes but displayed similar patterns of synergistic activity with the CST+RIF combination. However, our data suggest that antimicrobial susceptibility and synergy cannot be inferred from genotype alone, and testing should be considered for each patient isolate to support the selection of an optimal combination regimen during the treatment of KPC-Kp.

Other investigators have reported synergistic activity of rifamycin-polymyxin combinations against KPC-Kp strains; Elemam et al. reported synergistic interactions of rifampin and polymyxin B against all 12 polymyxin B-resistant KPC-Kp isolates that they tested (17, 18). Additional synergy studies have been reported for multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains (19–21). However, previous findings of synergy were not consistent, utilized different methods, and were reported in strains that were not characterized genotypically (22), making it difficult to generalize the results. Our study is the largest describing consistent synergistic activity with CST-RIF combinations against KPC-Kp of clonal complex ST512 that included CST-resistant strains. The applicability of our findings to the ST101 clonal strains is less certain, however, given the limited number of strains that were available for testing.

To our knowledge, this is the first report comparing CST+IMI or CST+MER against CST-R KPC-Kp strains. Both carbapenems displayed the same degree of synergistic interactions with colistin *in vitro*. However, meropenem is likely to be preferred in clinical practice because of its improved safety at higher doses (23). Jernigan et al. reported synergistic activity in 50% of KPC-Kp isolates tested with a combination of colistin plus doripenem, with higher rates of bactericidal activity for doripenem-colistin combinations than we observed with colistin plus meropenem or imipenem

(24). Conversely, CST+TIG combinations were synergistic in 38.5% of strains tested in our series, compared to only 8% of isolates tested against colistin-doxycycline by Jernigan et al. (24). In addition, we also observed much higher rates of synergy with CST+GEN combinations, which were synergistic in 38.5% of CST-R KPC-Kp strains tested in our series. Interestingly, gentamicin alone was bactericidal against KPC-Kp at clinically achievable concentrations. This activity may explain why gentamicin has been reported to eradicate gastrointestinal carriage of KPC-Kp (25). The clinical utility of CST+GEN combinations, however, must be weighed against the disadvantages of using two nephrotoxic agents in combination.

Traditional laboratory reporting for KPC-Kp isolates as simply "resistant" to all antimicrobials tested does not provide guidance for clinicians treating these difficult infections. Although the optimal therapy for KPC-KP is not well defined, combination therapy appears to be associated with improved survival in patients with KPC-Kp bacteremia (6). Therefore, an empirical combination regimen containing CST+RIF pending more definitive susceptibility results or synergy testing may be an appealing initial regimen for many patients, especially in institutions with increasing rates of CST-R KPC-Kp.

In conclusion, our data identify CST+RIF as the most consistently active antimicrobial combination against KPC-producing *K. pneumoniae*, especially for colistin-resistant strains. The synergistic activity of this combination might also help to prevent the selection of heteroresistant populations when treating CST-susceptible KPC-producing *K. pneumoniae*.

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