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SUSCEPTIBILITY

Synergistic Activity of Colistin-Containing Combinations against Colistin-Resistant *Enterobacteriaceae*

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ABSTRACT Resistance to colistin, a polypeptide drug used as an agent of last resort for the treatment of infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacteria, including carbapenem-resistant *Enterobacteriaceae* (CRE), severely limits treatment options and may even transform an XDR organism into one that is pan-resistant. We investigated the synergistic activity of colistin in combination with 19 antibiotics against a collection of 20 colistin-resistant *Enterobacteriaceae* isolates, 15 of which were also CRE. All combinations were tested against all strains using an inkjet printer-assisted digital dispensing checkerboard array, and the activities of those that demonstrated synergy by this method were evaluated against a single isolate in a time-kill synergy study. Eighteen of 19 combinations demonstrated synergy against two or more isolates, and the 4 most highly synergistic combinations (colistin combined with linezolid, rifampin, azithromycin, and fusidic acid) were synergistic against ≥90% of strains. Sixteen of 18 combinations (88.9%) that were synergistic in the checkerboard array were also synergistic in a time-kill study. Our findings demonstrate that colistin in combination with a range of antibiotics, particularly protein and RNA synthesis inhibitors, exhibits synergy against colistin-resistant strains, suggesting that colistin may exert a subinhibitory permeabilizing effect on the Gram-negative bacterial outer membrane even in isolates that are resistant to it. These findings suggest that colistin combination therapy may have promise as a treatment approach for patients infected with colistin-resistant XDR Gram-negative pathogens.

KEYWORDS CRE, *Enterobacteriaceae*, MCR-1, NDM-1, carbapenem-resistant *Enterobacteriaceae*, checkerboard, colistin, synergy, time-kill curves

Colistin, a polypeptide antibiotic with activity against a broad range of Gram-negative pathogens, was introduced in 1949 and used widely in the following decades (1), but by the early 1980s its popularity began to wane as the extent of its nephrotoxic side effects became clear and as newer, less toxic agents were developed (2). The past 2 decades, however, have seen a resurgence in colistin use as a result of the spread of multidrug-resistant (MDR) Gram-negative bacterial pathogens, particularly carbapenem-resistant *Enterobacteriaceae* (CRE), which retain susceptibility to few, if any, antibiotics other than colistin (3). Inevitably, however, as rates of colistin use have increased, so have rates of colistin resistance (4).

Even as resistance to last-resort antibiotics continues to spread, the development of new classes of antibiotics, particularly those with activity against Gram-negative organisms, has slowed (5). In contrast, the adoption of new uses of already existing antimicrobials, a strategy that can be described as antibiotic salvage, has potential benefits, including rapid applicability, reduced expense, and greater familiarity by clinicians and

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pharmacists with side effects and other practical aspects of drug administration. Examples of the antibiotic salvage strategy include prolonged infusions of meropenem for carbapenem-resistant organisms (6) as well as combination therapy, in which two or more drugs are used together to increase or restore efficacy against a resistant pathogen.

The results of *in vitro* and *in vivo* studies evaluating combination therapy for CRE have not been entirely consistent, due in part to variability in methods and organisms (7–9) and, in the case of clinical trials, to the limitations inherent in retrospective studies (10). However, there is evidence of improved outcomes in the sickest patients when two or more drugs are used to treat CRE infections (11, 12). Fewer studies have investigated the activity of drug combinations against colistin-resistant *Enterobacteriaceae* (CoRE) (13–15), although here, too, combination therapy appears to have an advantage in very ill patients (16). In a recent study, we observed that colistin in combination with minocycline and with rifampin had strong synergistic activity against a highly colistin-resistant *Klebsiella pneumoniae* isolate (17). In order to further explore the synergistic activity of colistin-containing combinations against CoRE, we tested colistin in combination with a range of antibacterial drugs, including inhibitors of protein synthesis, RNA synthesis, and DNA replication, as well as cell wall-active agents, against a collection of 20 *Enterobacteriaceae* isolates that are resistant to colistin, as determined by phenotype (MIC), genotype (including the presence of the *mcr-1* gene), or both.

RESULTS

MICs for individual antimicrobials. MIC results for each strain were determined during checkerboard array studies through the inclusion of single-drug titrations of each drug. All isolates had MICs of >64 μg/ml for linezolid, daptomycin, and vancomycin and >32 μg/ml for clindamycin and fusidic acid. MICs are presented in Table 1; colistin MICs are the mode for 18 to 19 replicates per isolate, while the MICs of the other drugs represent a single result from the array in which that drug was tested in combination with colistin. The modal colistin MICs for strains with non-*mcr-1*-mediated colistin resistance ranged from 4 to >16 μg/ml, while for *mcr-1*-containing strains, the MICs were between 2 and 8 μg/ml. The drugs with the highest rates of activity against the strains were tigecycline (16/20 strains susceptible [80%]), ceftazidime-avibactam (14/20 strains susceptible [70%]), amikacin (12/20 strains susceptible [60%]), and minocycline (9/20 strains susceptible [45%]).

Checkerboard arrays. Colistin was tested in combination with rifampin, minocycline, doxycycline, tetracycline, tigecycline, levofloxacin, meropenem, ceftazidime-avibactam, trimethoprim-sulfamethoxazole, azithromycin, erythromycin, linezolid, clindamycin, fusidic acid, amikacin, apramycin, chloramphenicol, vancomycin, and daptomycin against all 20 strains. Of the 380 trials, 17 (4.5%) had ≥2 skipped wells; on repeat testing, only 2 of these again had ≥2 skipped wells. Seventeen trials (4.5%) were repeated because the colistin MIC differed by >1 2-fold dilution from the modal colistin MIC for that strain; on repeat testing, the colistin MIC fell within ±1 2-fold dilution of the modal colistin MIC for 12 of these.

Overall, 193/373 trials with interpretable results (51.7%) demonstrated synergy. When combined with linezolid, rifampin, azithromycin, and fusidic acid, colistin had particularly high rates of synergy (90 to 95%). In 155/193 of antibiotic pair-isolate combinations where synergy occurred (80.3%), the concentration of colistin at the minimum fractional inhibitory concentration index ($FIC_{I\text{-}MIN}$) was ≤1 μg/ml, a concentration that is clinically achievable in a majority of patients (18). Eighteen of 19 combinations (94.7%) demonstrated synergy against at least 2 strains; daptomycin plus colistin did not demonstrate synergy against any strains. The rates of synergy varied among the combinations and are shown in Table 2. Each strain was susceptible to synergy by at least one drug combination. The number of combinations to which strains were susceptible varied, with intrinsically resistant species demonstrating the lowest rates of susceptibility to synergy (for *Serratia marcescens*, 6/19 combinations

TABLE 1 Strain characterization, including MICs

Isolate identifier	Species	ST[e] by multilocus sequence typing	Colistin resistance mechanism	Carbapenem resistance mechanism, if known	MIC[f] (μg/ml)														
					CST	MIN	DOX	TET	TGC	CHL	LVX	AMK	SXT[b]	MEM	CZA[c]	AZM[d]	ERY[d]	RIF[d]	APR[d]
BIDMC 18A[a]	*K. pneumoniae*	ST-258	Unknown	KPC-2	>16	2	2	2	0.5	>128	64	64	>32/640	8	1/4	>64	>64	16	>64
BIDMC 32	*K. pneumoniae*	ST-258	Unknown	KPC-3	>16	16	8	8	1	>128	>64	16	>32/640	4	2/4	32	>64	64	>64
BIDMC 34	*K. pneumoniae*	ST-258	Unknown	KPC-2	>16	4	2	1	1	>128	64	32	>32/640	8	1/4	>64	>64	16	>64
BIDMC 35	*K. pneumoniae*	ST-17	Unknown	Unknown[g]	>16	>32	>64	>64	1	>128	2	2	1/19	>32	8/4	32	>64	128	4
BIDMC 44	*S. marcescens*	[h]	Species intrinsically resistant	SME-2	>16	8	8	16	1	32	0.063	2	0.5/9.5	32	0.5/4	64	>64	64	4
BIDMC 91	*K. pneumoniae*	ST-16	Unknown	Double porin mutation	>16	32	>64	>64	2	16	>64	8	0.25/4.75	8	64/4	64	>64	32	4
FDA-CDC 0040	*K. pneumoniae*	ST-147	Unknown	VIM	4	16	32	>64	4	64	32	32	>32/640	>32	>64/4	>64	>64	32	8
FDA-CDC 0046	*K. pneumoniae*	ST-147	Unknown	VIM	4	16	32	>64	4	32	16	16	>32/640	>32	>64/4	>64	>64	32	4
FDA-CDC 0097	*K. pneumoniae*	ST-896	Unknown	KPC	16	8	4	8	2	64	>64	>128	>32/640	32	8/4	32	>64	128	32
FDA-CDC 0106	*K. pneumoniae*	ST-14	Unknown	NDM	16	16	16	16	4	>128	64	>128	>32/640	>32	>64/4	>64	>64	>128	2
FDA-CDC 0125	*K. pneumoniae*	ST-258	Unknown	KPC-3	16	4	2	2	0.5	>128	>64	32	>32/640	16	2/4	>64	>64	32	>64
FDA-CDC 0134	*Raoultella ornitholytica*	[h]	Unknown	KPC	8	1	0.5	0.25	0.25	2	0.25	2	>32/640	1	0.25/4	8	>64	16	2
FDA-CDC 0159	*P. mirabilis*	[h]	Species intrinsically resistant	NDM	>16	32	32	32	2	64	1	>128	>32/640	32	>64/4	>64	>64	8	8
FDA-CDC 0163	*Enterobacter cloacae*	ST-51	Unknown	KPC	16	64	32	>64	8	>128	32	4	>32/640	8	0.5/4	32	>64	32	4
FDA-CDC 0346	*E. coli*	[i]	*mcr-1*	None	4	4	8	>64	0.25	>128	8	>128	>32/640	0.031	1/4	32	>64	>128	8
FDA-CDC 0349	*E. coli*	ST-457	*mcr-1*	None	2	4	32	>64	1	>128	16	1	>32/640	0.031	2/4	32	>64	128	>64
FDA-CDC 0350	*E. coli*	ST-2732	*mcr-1*	None	4	1	1	1	0.125	8	16	1	0.06/1.19	0.031	0.25/4	8	64	8	4
FDA-CDC 0494	*E. coli*	ST-1485	*mcr-1*	None	8	32	32	>64	0.5	>128	8	2	>32/640	0.016	0.25/4	4	64	NA	8
FDA-CDC 0495	*E. coli*	ST-410	*mcr-1*	None	4	4	16	>64	0.25	16	32	2	>32/640	0.031	0.25/4	>64	>64	16	4
ARLG-2829 (MCR1_NJ)	*E. coli*	ST-405	*mcr-1*	NDM-5	4	4	8	>64	0.125	>128	64	2	0.25/4.75	>32	>64/4	16	>64	>128	4

[a]BIDMC strains are from the CRE genome initiative. FDA-CDC strains are from FDA-CDC Antimicrobial Resistance Isolate Bank. The ARLG strain is from the Antibiotic Resistance Leadership Group Laboratory Center Virtual Repository.

[b]Concentrations are expressed as the trimethoprim concentration/sulfamethoxazole concentration.

[c]Concentrations are expressed as ceftazidime concentration/avibactam concentration.

[d]Interpretive criteria for these drugs have not been established for *Enterobacteriaceae*.

[e]ST, sequence type.

[f]Dark shading indicates an MIC classified as resistant; light shading indicates an MIC classified as intermediate; no shading indicates an MIC classified as susceptible (for those drugs for which interpretive criteria for *Enterobacteriaceae* exist) (see the text for definitions). CST, colistin; MIN, minocycline; DOX, doxycycline; TET, tetracycline; TGC, tigecycline; CHL, chloramphenicol; LVX, levofloxacin; AMK, amikacin; SXT, trimethoprim-sulfamethoxazole; MEM, meropenem; CZA, ceftazidime-avibactam; AZM, azithromycin; ERY, erythromycin; RIF, rifampin; APR, apramycin; NA, no result, as two separate trials for this combination had ≥2 skipped wells.

[g]The isolate has been sequenced, but the cause of carbapenem resistance has not been elucidated (57).

[h]Multilocus sequence typing schemes are not available for these species.

[i]Isolate not yet sequenced.

TABLE 2 Rates of synergy by drug using checkerboard array

Drug tested in combination with colistin	% synergy[a] (95% confidence interval) for:	
	All strains	Strains excluding species intrinsically resistant to colistin
Linezolid	95.0 (73.1–99.7)	100 (78.1–100.0)
Rifampin	94.7 (71.9–99.7)	100 (77.1–100.0)
Azithromycin	90.0 (66.9–98.2)	100 (78.1–100.0)
Fusidic acid	90.0 (66.9–98.2)	94.4 (70.6–99.7)
Minocycline	85.0 (61.1–96.0)	88.9 (63.9–98.1)
Clindamycin	80.0 (55.7–93.4)	88.9 (63.9–98.1)
Erythromycin	80.0 (55.7–93.4)	88.9 (63.9–98.1)
Chloramphenicol	75.0 (50.6–90.4)	77.8 (51.9–92.6)
Levofloxacin	70.0 (36.4–80.0)	66.7 (41.2–85.6)
Doxycycline	60.0 (36.4–80.0)	66.7 (41.2–85.6)
Ceftazidime-avibactam	41.2 (19.4–66.5)	46.7 (22.3–72.6)
Tigecycline	25.0 (9.6–49.4)	27.8 (10.7–53.6)
Vancomycin	25.0 (9.6–49.4)	27.8 (10.7–53.6)
Tetracycline	20.0 (6.6–44.3)	22.2 (7.4–48.1)
Meropenem	15.0 (4.0–38.9)	11.1 (1.9–36.1)
Amikacin	15.0 (4.0–38.9)	16.7 (4.4–42.3)
Trimethoprim-sulfamethoxazole	15.0 (4.0–38.9)	11.1 (1.9–36.1)
Apramycin	10.0 (1.8–33.1)	11.1 (1.9–36.1)
Daptomycin	0.0 (0–22.9)	0.0 (0.0–25.3)

[a]Synergy percentages represent the results of testing of 20 isolates for each combination, except rifampin (results of testing of 19 isolates were used because 1 trial had skipped wells), daptomycin (results for testing of 17 isolates were used because 1 trial had skipped wells and 2 trials had colistin MICs $>\pm 1$ 2-fold dilution from the modal MIC), and ceftazidime-avibactam (results for 17 isolates were used because 3 trials had colistin MICs $>\pm 1$ 2-fold dilution from the modal MIC).

were synergistic; for *Proteus mirabilis*, 1/19 combinations were synergistic) and other strains being susceptible to synergy by between 7 and 15 combinations (see Table S1 and Data Set S1 in the supplemental material). Susceptibility to synergistic combinations also varied according to the type of resistance, with *mcr-1*-containing strains showing an overall 46.9% rate of susceptibility to synergistic combinations, lower than the 60.0% rate for strains with presumed chromosomal mutations ($P = 0.03$) (Tables S1 and S2). There was no significant difference in the rates of susceptibility to synergistic combinations between CRE strains, where synergy was seen in 150/279 combinations (53.8%), and non-CRE strains, where synergy was seen in 43/94 combinations (45.7%) ($P = 0.22$). For the combination of meropenem plus colistin specifically, the rates of synergy were low for both CRE isolates (2/15, 13.3%) and non-CRE isolates (1/5, 20%). Antagonism was noted in 19/373 trials with interpretable results (5.1%). Interestingly, 10/19 of these trials (52.6%) also exhibited synergy between the same two drugs at different concentrations, although these combinations were not counted as synergistic in the data analysis. Complete checkerboard synergy results for each drug combination pair against each isolate are presented in Data Set S1.

Time-kill studies. Synergy was demonstrated in 16/18 (88.9%) of the combinations tested, and bactericidal activity was demonstrated in 6/16 of these (37.5%). Sample time-kill curves are shown in Fig. 1; the results of all time-kill studies are presented in Table 3.

DISCUSSION

As rates of resistance to last-resort antibiotics continue to rise, novel approaches to antimicrobial therapy for MDR bacteria, including CRE, will become increasingly critical. The few antibiotics that have activity against these pathogens have significant clinical drawbacks, such as toxic side effects in the case of colistin (1, 19) and a poor distribution to certain important body sites, including blood and urine, by tigecycline (20). Even novel agents, such as ceftazidime-avibactam, are vulnerable to the emergence of resistance (21). One approach to treating MDR Gram-negative pathogens is the use of combination drug regimens, but there has been little investigation to date

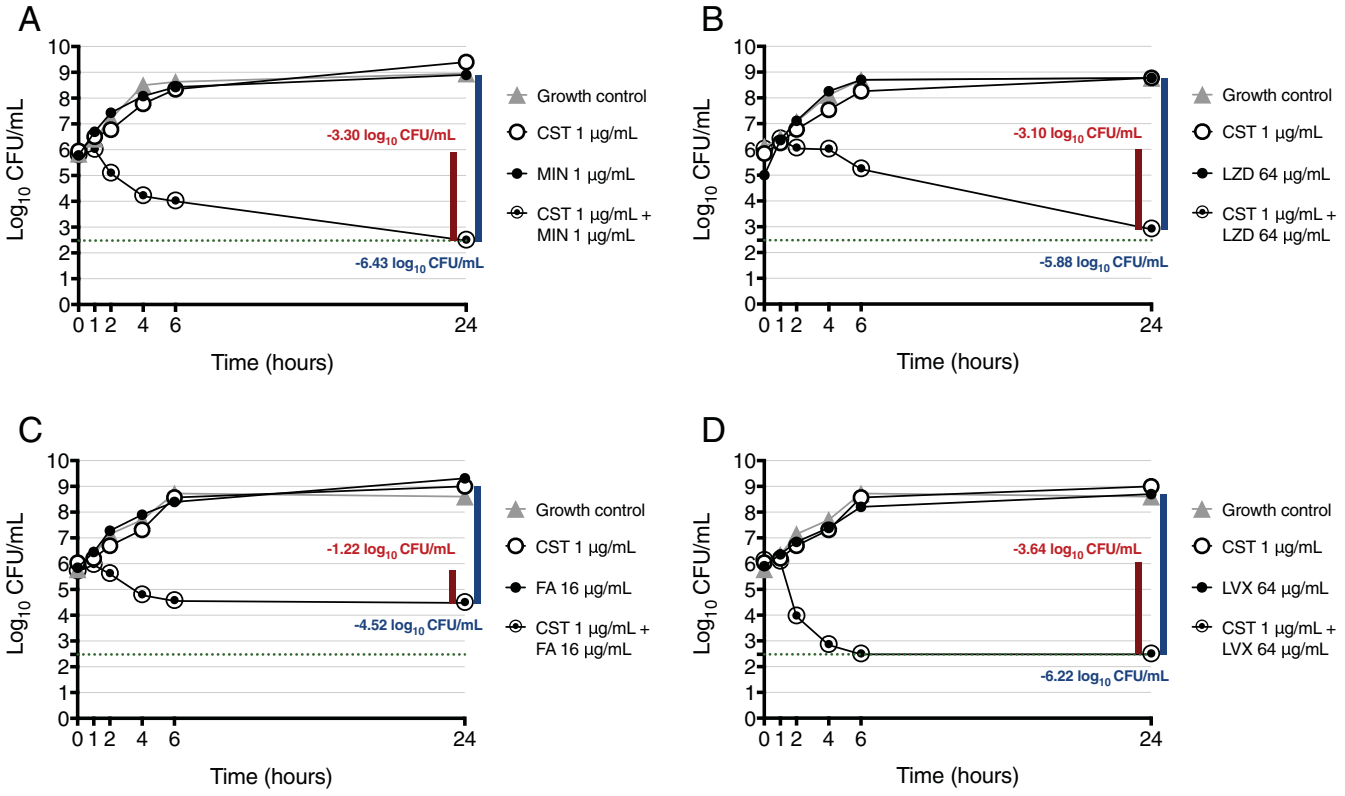

FIG 1 Time-kill synergy graphs. The activities of colistin (CST) in combination with minocycline (MIN) (A), linezolid (LZD) (B), fusidic acid (FA) (C), and levofloxacin (LVX) (D) were tested against strain BIDMC 32 (*K. pneumoniae*). Red bars and numbers indicate differences in the bacterial concentration between the starting inoculum and the drug combination at 24 h. Blue bars and numbers indicate differences in the bacterial concentration between the combination and the most active single agent at 24 h. The green dashed lines mark the lower limit of detection.

of the activity of combination regimens against CoRE (13–15). We found that colistin was synergistic with a wide range of antimicrobial drugs against CoRE by both checkerboard array and time-kill methods.

A few of our findings are particularly notable. First, colistin demonstrated high rates of synergy with linezolid, fusidic acid, and clindamycin, protein synthesis inhibitor antibiotics that individually have no activity against Gram-negative bacteria due to their limited ability to cross the outer membrane (clindamycin and fusidic acid [22, 23]) or susceptibility to efflux pump activity (linezolid [24]). These high rates of synergy between colistin and drugs active against Gram-positive bacteria suggest a likely mechanism for the synergistic activity observed throughout this study. Colistin exerts its primary antimicrobial activity by displacing Ca^{2+} and Mg^{2+} ions from the lipid A component of lipopolysaccharide molecules in the Gram-negative bacterial outer membrane, thus increasing membrane permeability and causing the leakage of cell contents and, ultimately, cell death (2, 3). Colistin resistance in *Enterobacteriaceae* results from alterations of lipid A through modification with 4-amino-4-deoxy-L-arabinose (L-ara4N) and/or phosphoethanolamine (PEtN), which reduce the net negative charge of lipid A (25). It is evident that in colistin-resistant bacteria, the drug's permeabilizing effect is attenuated such that pathogens are not inhibited. However, the synergistic activity observed between colistin and drugs active against Gram-positive bacteria suggests that colistin exerts a subinhibitory permeabilizing effect that allows increased entry into the cell of the second drug. A similar mechanism has been noted with polymyxin B nonapeptide, a polymyxin B derivative lacking a fatty acid tail which does not inhibit Gram-negative bacteria on its own but does increase the entry and activity of hydrophobic antibiotics, including rifampin, clindamycin, vancomycin, and erythromycin (26, 27); other polymyxin derivatives have shown similar effects (28, 29).

TABLE 3 Time-kill synergy results

Drug combination (concn [μg/ml])[c]	Strain	Difference in bacterial concn (no. of $\log_{10}$ CFU/ml) between:	
		Combination at 24 h and starting inoculum[a]	Combination and most active single agent at 24 h[b]
MIN (1) + CST (1)	BIDMC 32	−3.30	−6.43
DOX (1) + CST (1)	BIDMC 32	−3.48	−6.52
TET (4) + CST (1)	BIDMC 32	−1.62	−3.84
TGC (0.5) + CST (1)	BIDMC 32	−3.22	−5.88
AZM (0.5) + CST (1)	BIDMC 32	−1.56	−4.56
ERY (4) + CST (1)	BIDMC 32	2.76	0.31
AMK (16) + CST (4)	FDA-CDC 0125	−2.84	−5.62
APR (8) + CST (1)	FDA-CDC 0097	−1.32	−3.39
RIF (0.125) + CST (1)	BIDMC 32	−0.82	−3.82
CHL (128) + CST (1	BIDMC 32	−1.24	−4.18
LVX (64) + CST (1)	BIDMC 32	−3.64	−6.22
MEM (16) + CST (1)	BIDMC 44	−3.67	−7.16
CZA (2/4) + CST (1)	BIDMC 32	−2.10	−4.18
SXT (0.125/2.375) + CST (1)	BIDMC 91	0.07	−2.59
LZD (64) + CST (1)	BIDMC 32	−3.10	−5.88
CLI (16) + CST (1)	BIDMC 32	0.18	−3.05
FA (16) + CST (1)	BIDMC 32	−1.22	−4.52
VAN (64) + CST (4)	FDA-CDC 0125	1.73	−1.06

[a]Calculated as (number of $\log_{10}$ CFU per milliliter after 24 h of incubation) − (number of $\log_{10}$ CFU per milliliter of starting inoculum). Negative values indicate a decrease in the colony count from the starting inoculum. Bactericidal combinations are shaded.

[b]Calculated as (number of $\log_{10}$ CFU per milliliter of culture grown with both antibiotics after 24 h of incubation) − (number of $\log_{10}$ CFU per milliliter of culture grown with the single most active antibiotic after 24 h of incubation). Negative values indicate a lower final colony count with the combination than with the most active single agent. Synergistic combinations are shaded.

[c]MIN, minocycline; CST, colistin; DOX, doxycycline; TET, tetracycline; TGC, tigecycline; AZM, azithromycin; ERY, erythromycin; AMK, amikacin; APR, apramycin; RIF, rifampin; CHL, chloramphenicol; LVX, levofloxacin; MEM, meropenem; CZA, ceftazidime-avibactam; SXT, trimethoprim-sulfamethoxazole; LZD, linezolid; CLI, clindamycin; FA, fusidic acid; VAN, vancomycin. For ceftazidime-avibactam, the concentrations are expressed as the ceftazidime concentration/avibactam concentration, and for trimethoprim-sulfamethoxazole, the concentrations are expressed as the trimethoprim concentration/sulfamethoxazole concentration.

The hypothesis that synergy in colistin-containing combinations is mediated by outer membrane permeabilization is supported by the low rates of synergy seen when colistin is combined with amikacin and apramycin, hydrophilic aminoglycoside antibiotics able to enter the Gram-negative bacterial cell wall by active transport (30). The resistance of *Enterobacteriaceae* to aminoglycosides is usually caused by aminoglycoside-modifying enzymes (19), a mechanism not expected to be affected by outer membrane permeability. Similarly, resistance to both trimethoprim and sulfamethoxazole is most commonly due to target site modifications (31), which may explain the low rate of synergy seen between trimethoprim-sulfamethoxazole and colistin. The target of daptomycin is believed to be absent from Gram-negative bacteria (32), and as expected, this drug was not synergistic with colistin against any of the strains tested.

Some drugs that had high rates of synergy with colistin are not, however, agents for which outer membrane impermeability constitutes the primary barrier to intracellular accumulation in Gram-negative organisms. Minocycline, for example, is a tetracycline antibiotic that crosses the outer membrane through porin channels (33), while efflux pump activity is the primary mechanism of exclusion of linezolid from Gram-negative bacterial cells (24, 34). Perhaps by increasing the rate of entry of these drugs into the cell through outer membrane permeabilization, colistin has the effect of producing an overall increase in the entry-to-exit ratio of antibiotics with which it acts in synergy. This accords with the finding that laboratory strains of *Escherichia coli* are modestly sensitized to linezolid by polymyxin derivative-induced membrane permeabilization, although not to nearly as marked a degree as by inactivation of the AcrAB-TolC efflux pump (29, 32).

The activity of the combinations that we identified against strains containing bla_{NDM} carbapenemases, metallo-β-lactamase enzymes not susceptible to the activity of new β-lactam–β-lactamase inhibitor drugs, such as ceftazidime-avibactam and meropenem-vaborbactam (35, 36), indicates that these combinations may have a particular role in

the treatment of colistin-resistant NDM-expressing strains, for which therapeutic options are otherwise extraordinarily limited. We would especially highlight the activity of the combinations that we tested against ARLG-2829, an *E. coli* isolate originally reported as MCR1_NJ (37), which contains both *mcr-1* and a bla_{NDM-5} carbapenemase (see Data Set S1 in the supplemental material). Minocycline, for example, was synergistic with colistin against this strain at a minocycline concentration of 0.5 μg/ml and a colistin concentration of 0.25 μg/ml, which are clinically achievable concentrations (18, 38). This combination was also bactericidal and synergistic against the strain with which it was tested in a time-kill study (Table 3).

We observed a low rate of antagonism of 5.1% (Data Set S1). In more than half the instances in which a combination was antagonistic against an isolate at one or more combinations of concentrations, it was also synergistic against the isolate at a different set of concentrations. This variability within a given combination-strain pairing underscores the importance of investigations into the pharmacokinetic and pharmacodynamic parameters of combination therapy and suggests that simplified checkerboard array arrangements, in which only a limited set of concentration combinations is tested (39), risk providing false reassurance that a combination is not antagonistic.

By their nature, *in vitro* investigations of antimicrobial synergy are limited in their ability to predict *in vivo* efficacy. Our use of a single strain for each time-kill experiment is also a specific potential limitation, although the role of the time-kill experiments in this study was primarily as a method to provide complementary supportive data for the higher-volume checkerboard array results. Future studies in which the combinations identified here are tested in animal models, pharmacokinetic/pharmacodynamic studies, and, ultimately, human subjects will be essential in assessing the clinical applicability of our findings. We did, however, take certain steps to increase the robustness of our results within the confines of *in vitro* methods by testing 20 separate isolates in the checkerboard array and by further investigating each combination that demonstrated synergy through the use of a time-kill study; in 16/18 cases, synergy was also present by time-kill methods, and in 6 of these cases, the synergistic activity was bactericidal (Table 3). It is notable that three of the drugs that were synergistic and bactericidal in combination with colistin are drugs that are bacteriostatic rather than bactericidal when used alone (minocycline, doxycycline, and linezolid [40, 41]), suggesting that the use of these drugs in combination may not only restore but also even potentially enhance their activity.

Our findings demonstrate that colistin-containing combinations, particularly those in which colistin is combined with a protein or RNA synthesis inhibitor, demonstrate high rates of synergistic activity against CoRE. The recent emergence of transferable colistin resistance in *E. coli* isolates from humans and food animals in the form of the plasmid-borne *mcr-1* gene, which encodes a PEtN transferase, has raised the specter of a rapid worldwide spread of colistin resistance (42, 43). As extensively drug-resistant (XDR) Gram-negative pathogens, such as CoRE, increase in prevalence and in the number of agents to which they are impervious, novel therapeutic strategies such as combination therapy will become increasingly essential to the management of patients infected with these organisms. Our results suggest that colistin, long considered a drug of last resort for the treatment of such pathogens but now threatened by emerging resistance, may yet be salvageable for use in combination.

MATERIALS AND METHODS

Bacterial strains. We evaluated 20 *Enterobacteriaceae* isolates. Six of these were deidentified clinical isolates that were collected at Beth Israel Deaconess Medical Center under Institutional Review Board-approved protocols and sequenced through the carbapenem-resistant *Enterobacteriaceae* genome initiative at the Broad Institute (Cambridge, MA), 13 isolates were obtained from the U.S. Food and Drug Administration (FDA)-CDC Antimicrobial Resistance Isolate Bank (Atlanta, GA), and 1 isolate was obtained from the Antibiotic Resistance Leadership Group Laboratory Center Virtual Repository. Information on these strains is presented in Table 1. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Klebsiella pneumoniae* ATCC 700603 were obtained from the American Type Culture Collection (Manassas, VA). All strains were colony purified, minimally passaged, and stored at −80°C in tryptic soy broth

(BD Diagnostics, Franklin Lakes, NJ) with 50% glycerol (Sigma-Aldrich, St. Louis, MO) prior to use in this study.

Antimicrobial agents. Chloramphenicol, doxycycline, tetracycline, azithromycin, erythromycin, and levofloxacin were obtained from Sigma-Aldrich (St. Louis, MO). Colistin sulfate was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and from Alfa Aesar (Tewksbury, MA). Amikacin was obtained from Santa Cruz Biotechnology. Apramycin was obtained from Alfa Aesar. Vancomycin was obtained from MP Biomedicals (Santa Ana, CA). Minocycline, sulfamethoxazole, linezolid, fusidic acid, and ceftazidime were obtained from Chem Impex International (Wood Dale, IL). Meropenem was obtained from Ark Pharm (Libertyville, IL). Rifampin was obtained from Fisher Scientific (Pittsburgh, PA). Daptomycin was obtained from ApexBio (Houston, TX). Tigecycline was obtained from Biotang Inc. (Lexington, MA). Trimethoprim was obtained from Research Products International (Mt. Prospect, IL). Avibactam was obtained from MedChemExpress (Monmouth Junction, NJ).

Antibiotic stock solutions used for the digital dispensing method (DDM) were dissolved either in dimethyl sulfoxide (DMSO) (chloramphenicol, rifampin, tetracycline, azithromycin, erythromycin, trimethoprim and sulfamethoxazole, linezolid, fusidic acid, daptomycin) or in sterile water (all other antimicrobials) with the addition of 0.3% polysorbate 20 (P-20; Sigma-Aldrich, St. Louis, MO); as recommended by CLSI, ~5% NaOH was added to the levofloxacin stock solution and anhydrous sodium carbonate at 10% ceftazidime weight was added to the ceftazidime stock solution (44). The solvents described were used for DDM testing because the HP D300 digital dispenser instrument (HP, Inc., Palo Alto, CA) requires stock solutions prepared in either DMSO or water with surfactant for proper fluid handling. The final concentrations of surfactant ranged from 4.7×10^{-7}% to 0.0024%. (Of note, a different surfactant, polysorbate 80 [P-80], at a concentration of 0.002% has been noted to lower the colistin MICs for organisms with colistin MICs of <2 μg/ml in standard broth microdilution [BMD] assays [45, 46].) Previous research from the Kirby laboratory has demonstrated that P-20 at concentrations up to 0.0015% had no effect on D300 instrument-assisted MIC results in comparison with those obtained by the reference BMD method (47). In the assays in the present study, P-20 was introduced at concentrations of $>$0.002% in 3 assay wells for only 3 combinations (minocycline, doxycycline, and vancomycin at 64 μg/ml combined with colistin at 4, 8, and 16 μg/ml); in all of these wells, the fractional inhibitory concentration index (FIC_i; see below) was >0.5, so that any theoretical enhanced inhibitory effect of P-20 on colistin activity in these wells could not have resulted in false synergy calls. The antibiotic stock solutions used for time-kill studies were dissolved in sterile water (colistin, minocycline, doxycycline, meropenem, levofloxacin [with addition of NaOH], clindamycin, amikacin, apramycin, vancomycin, tigecycline, ceftazidime [with addition of anhydrous sodium carbonate], avibactam) or 95% ethanol (azithromycin, erythromycin, chloramphenicol) according to CLSI recommendations (44) or in DMSO if they were not soluble in CLSI-recommended solvents at the concentrations needed (tetracycline, trimethoprim, sulfamethoxazole, linezolid, fusidic acid, daptomycin, rifampin). All antibiotic stock solutions were quality control (QC) tested with *E. coli* ATCC 25922, *S. aureus* ATCC 29213, and/or *K. pneumoniae* ATCC 700603 using the D300 dispensing method described below (for stocks to be used for checkerboard arrays) or the standard BMD technique using the direct colony suspension method (48) (for stocks to be used for time-kill experiments) prior to use in synergy studies. Stocks were used only if they produced an MIC result within the accepted QC range according to CLSI guidelines (44) or, in the case of apramycin, for which CLSI does not provide QC guidelines, according to published criteria (49). Antimicrobials were stored as aliquots at -20°C and discarded after a single use.

Selection of interpretive breakpoints. For purposes of strain set characterization, CLSI-recommended interpretive breakpoints for *Enterobacteriaceae* were used for all antibiotics for which such interpretive criteria are available (doxycycline, minocycline, tetracycline, amikacin, chloramphenicol, levofloxacin, trimethoprim-sulfamethoxazole, meropenem) (44). U.S. Food and Drug Administration (FDA) breakpoints were used for tigecycline (50), for which CLSI has not established interpretive criteria. In the case of colistin, for which CLSI has not established breakpoints for *Enterobacteriaceae*, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint (where an MIC of ≤ 2 μg/ml is susceptible and an MIC of >2 μg/ml is resistant) was used (51).

Checkerboard array testing. To create checkerboard arrays, serial 2-fold dilutions of 2 antibiotics were dispensed in orthogonal titrations by the D300 instrument using the DDM previously developed by our group (17, 47, 52). Titrations consisted of 11 2-fold dilutions of colistin (0.016 to 16 μg/ml) and 13 2-fold dilutions of all other antibiotics.

Bacterial inocula were prepared by suspending colonies in cation-adjusted Mueller-Hinton broth (CAMHB; BD Diagnostics, Franklin Lakes, NJ), measuring the optical density at 600 nm (OD_{600}), and diluting the colonies in CAMHB to an OD_{600} of 0.0003 ($\sim 5 \times 10^5$ CFU/ml). For daptomycin, CAMHB was supplemented to 50 μg/ml calcium (44, 48), and for tigecycline, CAMHB was prepared fresh on the day of testing (44, 48). Antimicrobial stocks were dispensed in appropriate volumes (0.013 to 461 nl) into empty, flat-bottomed, untreated 384-well polystyrene plates (Greiner Bio-One, Monroe, NC) by the D300 instrument. Immediately after addition of antimicrobials, the wells were inoculated with 50 μl of bacterial suspension and the plates were incubated for 16 to 20 h at 35°C in ambient air. After incubation, bacterial growth was quantified by measurement of the OD_{600} using a Tecan Infinite M1000 Pro microplate reader (Tecan, Morrisville, NC). An OD_{600} reading of 0.07 or greater (concordant with visual growth assessment) was considered indicative of bacterial growth.

MICs for each antibiotic were determined from wells in the array containing only that drug. For each well containing both antibiotics in which growth was inhibited, the fractional inhibitory concentration (FIC) for each antibiotic was calculated by dividing the concentration of the antibiotic in that well by the MIC of the antibiotic. The FIC index (FIC_I) was determined by summing the FICs of the two drugs. If the

MIC of an antibiotic was off scale, the highest concentration tested was assigned an FIC of 0.5 to permit FIC_I calculation. A minimum FIC_I ($FIC_{I\text{-}MIN}$) of ≤0.5 was considered indicative of synergy, an $FIC_{I\text{-}MIN}$ of >4.0 was considered indicative of antagonism, and intermediate values were considered indicative of indifference (53). When a skipped well occurred (i.e., inhibition of growth at a given FIC_I with growth at the next highest FIC_I), the higher FIC_I was used to avoid false-positive synergy interpretations. If ≥2 skipped wells occurred in a single grid or if the colistin MIC was >1 2-fold dilution above or below the modal colistin MIC for that strain, the results were not used and the test was repeated with a new inoculum. If the same error recurred, the antibiotic pair-isolate combination was excluded from further analysis.

Time-kill synergy experiments. Time-kill studies were performed using strain BIDMC 32, a KPC3-producing *K. pneumoniae* isolate, for all combinations that had demonstrated synergy against this strain in the checkerboard array. For combinations not synergistic against BIDMC 32 in the checkerboard array, a strain against which they had demonstrated synergy was chosen instead. Daptomycin plus colistin, which had not demonstrated synergy against any isolates in checkerboard studies, was not tested by the time-kill method. Antibiotic stocks were prepared as described above and diluted in 10 ml of CAMHB in 25- by 150-mm glass round-bottom tubes to the desired starting concentrations. For tigecycline, CAMHB was prepared fresh on the day of testing (44, 48). Concentrations were chosen on the basis of the $FIC_{I\text{-}MIN}$ results from checkerboard array studies. If the concentrations tested did not demonstrate synergy in the time-kill study, the concentration of the noncolistin antibiotic was doubled and the experiment was repeated; if synergy was not demonstrated under these circumstances, no further concentrations were tested.

To prepare a starting inoculum for the time-kill studies, 100 μl of a 0.5 McFarland standard suspension of colonies from an overnight plate was added to 5 ml of CAMHB and incubated on a shaker in ambient air at 35°C until it reached log-phase growth (approximately 4 h). The culture was then adjusted to the turbidity of a 1.0 McFarland standard in CAMHB, and 100 μl was added to each of the antimicrobial solutions. A growth control and a negative control were run in parallel with each experiment. Cultures were incubated on a shaker in ambient air at 35°C.

Aliquots from the culture were removed at 0, 1, 2, 4, 6, and 24 h. A 10-fold dilution series was prepared in 0.9% sodium chloride. A 10-μl drop from each dilution was transferred to a Mueller-Hinton plate (Thermo Fisher, Waltham, MA) (54, 55) and incubated overnight in ambient air at 35°C. The colonies within each drop were counted; drops containing 3 to 30 colonies were considered countable. For countable drops, the cell density of the sample was calculated; if more than one dilution for a given sample was countable, the cell density of the two dilutions was averaged. If no drops were countable, the counts for consecutive drops above and below the countable range were averaged. The limit of detection was 300 CFU/ml. The antibiotic carryover effect was not observed at the concentrations tested. A combination was considered synergistic if it resulted in a ≥2-$\log_{10}$ reduction in the number of CFU per milliliter at 24 h compared to the number of CFU per milliliter achieved with the most active agent alone. Bactericidal activity was defined as a reduction of ≥3 $\log_{10}$ CFU/ml at 24 h compared to the starting inoculum (39, 56).

Data analysis. Statistical analysis was performed using R (v3.1; R Foundation, Vienna, Austria) and Microsoft Excel (Microsoft Corporation, Redmond, WA) software. The chi-squared test was used for comparison of proportions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00873-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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