Correlation of Checkerboard Synergy Testing with Time-Kill Analysis and Clinical Outcomes of Extensively Drug-Resistant *Acinetobacter baumannii* Respiratory Infections

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We tested 76 extensively drug-resistant (XDR) *Acinetobacter baumannii* isolates by the checkerboard method using only wells containing serum-achievable concentrations (SACs) of drugs. Checkerboard results were correlated by time-kill assay and clinical outcomes. Minocycline-colistin was the best combination *in vitro*, as it inhibited growth in one or more SAC wells in all isolates. Patients who received a combination that inhibited growth in one or more SAC wells demonstrated better microbiological clearance than those who did not (88% versus 30%; $P = 0.025$). The checkerboard platform may have clinical utility for XDR *A. baumannii* infections.

Among Gram-negative organisms, extensively drug-resistant (XDR) *Acinetobacter baumannii* is considered a serious threat to public health. As *A. baumannii* infections are associated with significant morbidity and mortality, the timely administration of effective broad-spectrum antibiotics is imperative (1). In recent years, increasing resistance among *A. baumannii* isolates has limited many therapy options, requiring the administration of empirical combination therapy for serious *A. baumannii* infections (2). In an attempt to select the most effective empirical antibiotic therapy, antimicrobial stewardship programs (ASPs) annually evaluate susceptibility based on institutional, unit-specific, or combination antibiograms. However, antibiograms are simply based on percent susceptibility and do not provide clinicians with additional aspects, including synergy or antagonism between agents. This presents significant limitations for ASPs in selecting optimal combination therapy for the treatment of *A. baumannii* infections.

The comparison of specific antibiotic combinations identified in the literature provides ASPs with more definitive conclusions but does not address the heterogeneity of synergistic interactions of a specific combination against all isolates (3–6). Therefore, it may be more beneficial for ASPs to evaluate potential synergistic interactions in real time, particularly in critically ill patients infected with XDR *A. baumannii*. This study evaluated the use of a checkerboard platform to determine antibiotic combinations that are synergistic against XDR *A. baumannii* and compared the results to both time-kill assay results and clinical outcomes.

We selected 76 unique XDR *A. baumannii* clinical isolates from our center between January 2009 and October 2013. Isolates were stored at -70°C and subcultured twice prior to testing. For the checkerboard assay, we identified the susceptibilities to amikacin (Crescent Chemical, Islandia, NY), colistin (MP Biomedical, Solon, OH), doripenem (TSZChem, Framingham, MA), minocycline (EMD Millipore, Darmstadt, Germany), and tigecycline (TSZChem, Framingham, MA) by microbroth dilution. Other susceptibilities were determined by the MicroScan system (Beckman Coulter, CA). The susceptibilities (percent) to the antibiotics tested were as follows: amikacin, 2/76 (3%); ampicillin-sulbac-

tam, 2/76 (3%); cefepime, 0/76 (0%); ciprofloxacin, 0/76 (0%); doripenem, 0/76 (0%); gentamicin, 0/76 (0%); minocycline, 13/76 (17%); piperacillin-tazobactam, 0/76 (0%); tigecycline, 6/76 (8%); tobramycin, 0/76 (0%); trimethoprim-sulfamethoxazole, 1/76 (1%); colistin, 66/76 (87%). The checkerboard assay was performed in 96-well microtiter panels (Sensititre; Thermo Fisher Scientific, Cleveland, OH) with antibiotics at the concentrations listed in the footnote to Table 1. Each antibiotic combination was analyzed by utilizing nine wells with concentrations that were determined to be serum-achievable concentrations (SACs). Fifty microliters of bacterial suspension was added to each well of the panel with the Sensititre AIM device, to a final concentration of approximately 5×10^5 CFU/ml based on McFarland units. Panels were incubated for 24 h at 35 to 37°C in ambient air. The fractional inhibitory concentrations (FICs) of antibiotics in combination were determined. Synergistic, indifferent, and antagonistic activities were defined by FICs of ≤ 0.5 , 0.51 to 4.0, and > 4.0 , respectively. FICs of two-drug combinations were calculated by the equation (MIC of agent A in combination/MIC of agent A alone) + (MIC of agent B in combination/MIC of agent B alone), and FICs of three-drug combinations were calculated by the equation (MIC of agent A in combination/MIC of agent A alone) + (MIC of agent B in combination/MIC of agent B alone) + (MIC of agent C in combination/MIC of agent C alone).

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TABLE 1 Results of synergy tests of multiple antibiotic combinations via the checkerboard method against 76 *A. baumannii* isolates^a

Interaction	% of wells								
	Minocycline-colistin	Tigecycline-amikacin	Minocycline-amikacin	Minocycline-doripenem	Doripenem-tigecycline	Tigecycline-colistin	Doripenem-colistin	Doripenem-colistin-minocycline	Doripenem-colistin-tigecycline
Synergistic	6.6	2.6	1.3	2.6	3.9	0.0	5.3	6.6	2.6
Indifferent	93.4	97.4	97.4	97.4	96.14	86.8	94.7	93.4	97.4
Antagonistic	0.0	0.0	1.3	0.0	0.0	13.2	0.0	0.0	0.0
Inhibition of growth in any SAC well	100	58	67	62	66	0	93	100	97
Inhibition of growth in all SAC wells	51.3	0.0	10.5	15.8	0.0	0.0	7.9	68.4	23.7

^a The concentrations of each drug in combination were as follows: amikacin, 1 to 4 µg/ml; colistin, 0.5 to 2 µg/ml; doripenem, 1 to 4 µg/ml; minocycline, 1 to 4 µg/ml; tigecycline, 0.5 to 2 µg/ml.

By the checkerboard method, the minocycline-colistin and doripenem-colistin combinations displayed synergy against 6.6 and 5.3% of the isolates, respectively (Table 1). The best triple combination was doripenem-colistin-minocycline, which displayed synergy against 6.6% of the isolates. Tigecycline-colistin was the only combination that did not display a synergistic effect against any isolate. The combination was antagonistic against 13.2% of the isolates tested. Additionally, tigecycline-colistin was the only combination not to inhibit growth in any of the nine SAC wells in all isolates tested. Minocycline-colistin and doripenem-colistin-minocycline inhibited growth in at least one SAC well in all isolates tested.

Next, we performed time-kill assays with all of the isolates against which synergistic activity was demonstrated by the checkerboard method. Time-kill assays were performed according to previously published techniques (3). Ten milliliters of Mueller-Hinton broth (Remel, Lenexa, KS) containing concentrations of the antibiotics minocycline, doripenem, tigecycline, amikacin, and colistin equal to the FIC demonstrating synergy by the checkerboard method was inoculated to a concentration of approximately 10⁶ CFU/ml. Cultures were incubated at 35 to 37°C with aeration with ambient air. One-hundred-microliter aliquots were obtained at 0, 6, and 24 h, and colony counts were determined by serially diluting aliquots in 0.9% saline and plating them on Trypticase soy blood agar plates (Remel, Lenexa, KS). Bactericidal activity was defined as a >3-log decrease in the number of CFU per milliliter at 24 h. Synergistic activity of a combination was defined as a >2-log decrease in the number of CFU per milliliter compared to that achieved with the most active agent alone. Regrowth was defined as a >3-log decrease in the number of CFU per milliliter and a subsequent >2-log increase in the number of CFU per milliliter at 24 h (7).

By time-kill analysis, the combinations of minocycline-colistin and doripenem-colistin were bactericidal against 100 and 50% of the isolates tested, respectively (Table 2). Similar to doripenem-colistin, the triple antibiotic combinations were not consistently bactericidal. In contrast, isolates exposed to tigecycline-amikacin or doripenem-tigecycline demonstrated growth or stasis in the time-kill assay.

Lastly, we retrospectively evaluated the outcomes of patients who had pneumonia (*n* = 17) or bacteremia (*n* = 1) and received ≥48 h of an antibiotic combination analyzed by the checkerboard analysis. Pneumonia was defined as having signs and symptoms of respiratory tract infection (a cough, a body temperature of >38°C, or a white blood cell count of <4,000 or >12,000, and an infiltrate

identified on a chest radiograph (8). Bacteremia was defined as a positive blood culture and the presence of signs and symptoms of systemic inflammation (9).

Demographic and clinical outcome data were collected from

TABLE 2 *A. baumannii* time-kill assay responses of isolates against which drug combinations were synergistic by the checkerboard method

Combination and isolate	24-h log killing ^a	Regrowth	24-h synergy
Minocycline-colistin			
12	−6.1	No	No
30	−5.9	No	Yes
31	−5.5	No	Yes
34	−6	No	Yes
40	−6	No	Yes
Tigecycline-amikacin			
18	2.1	No	No
31	0.5	No	No
Minocycline-amikacin, 17			
	−1.1	Yes	Yes
Minocycline-doripenem			
17	−2.6	No	No
52	−1.1	No	Yes
Doripenem-tigecycline			
17	−2.1	No	Yes
31	0.1	No	No
53	1.4	No	No
Doripenem-colistin			
24	−5.9	No	Yes
31	−3.9	No	No
33	−1.2	No	No
69	−1.4	No	Yes
Doripenem-colistin-minocycline			
2	−1.3	No	No
8	−0.8	No	No
12	−3.3	No	Yes
24	−6.1	No	Yes
28	−0.9	No	Yes
Doripenem-colistin-tigecycline			
12	−0.3	No	No
69	−5.7	No	Yes

^a Bold values indicate a bactericidal effect.

TABLE 3 Correlation of checkerboard test results with clinical outcomes among 18 XDR *A. baumannii* isolates

Parameter	Growth inhibition in any SAC well	Growth in all SAC wells	<i>P</i> value ^a
No. of patients/total (%) with:			
Clinical cure	4/8 (50)	3/10 (30)	0.63
Microbiological clearance	7/8 (88)	3/10 (30)	0.02
30-day all-cause mortality	3/8 (38)	6/10 (60)	0.63
Median APACHE II score	20	20.5	0.29
Median Charlson score	4	5	0.32
Median time (h) to targeted therapy	87	67	0.23

^a Fisher's exact and Mann-Whitney U tests (GraphPad Software, Inc.) were used to compare categorical and continuous variables, respectively.

the patients' electronic medical records. On the basis of checkerboard results, patients were placed into one of two groups for study analysis. Group 1 patients received a combination that inhibited growth in one or more SAC wells, while group 2 patients received a combination that demonstrated growth in all nine SAC wells by checkerboard assay. Clinical outcomes included clinical cure, microbiologic eradication, and 30-day all-cause mortality rate. Clinical cure and microbiologic eradication were defined as previously described (10). Clinical cure and failure were assigned by a trained infectious disease physician.

For the demographic and clinical characteristics of the patients and the antimicrobials used, see Tables S1 and S2 in the supplemental material. There were no differences in the acute physiology and chronic health evaluation II (APACHE II) score, the Charlson score, or the time to targeted therapy between groups 1 and 2 (Table 3). Colistin-tigecycline (9/18) was used most frequently. Interestingly, there was better microbiological clearance in group 1 than in group 2 (88 versus 30%; *P* = 0.025). Group 1 patients demonstrated microbiological clearance by follow-up culture (4/5) or presumed microbiologic eradication (3/3). Group 2 patients demonstrated microbiological clearance by follow-up cultures (1/5) or presumed microbiologic eradication (2/5).

Our study is one of the first to assess the correlation between an *in vitro* checkerboard method and clinical outcomes among XDR *A. baumannii* isolates. In our study, patients who received a combination that inhibited growth in a SAC well by the checkerboard method had better microbial clearance than those who did not. The patients included were critically ill, as demonstrated by their APACHE II scores and Charlson comorbidity indices. Among group 2 patients, tigecycline-colistin was frequently utilized. A recent study showed that the tigecycline-colistin combination was associated with a higher 14-day mortality rate when the tigecycline MIC was >2 µg/ml than that achieved with the combination of a carbapenem and colistin (11). We confirm their results and may give a plausible explanation for these findings. The tigecycline-colistin combination was primarily indifferent and in some cases antagonistic in our checkerboard analysis. Indeed, when we evaluated the patients in our clinical cohort who received this combination, colistin by itself had an MIC of 1 µg/ml in eight of nine patients and when it was used in combination with tigecycline, there was visible growth in all SAC wells (greatest concentration, 2 µg/ml of tigecycline and colistin). This suggests that the addition of tigecycline to colistin may be harmful, considering the removal

of susceptible-range wells and poor clinical outcomes seen in patients who received that combination. Our study correlates with a study by Cikman and colleagues that demonstrated a high rate of antagonism with tigecycline-colistin against XDR *A. baumannii* (12). Taken together, the results show that utilizing drug combinations that inhibit growth in SAC wells may be a more appropriate alternative than utilizing a combination that does not when treating XDR *A. baumannii* pneumonia. At our center, we discourage the use of tigecycline-colistin for XDR *A. baumannii* infections.

While a carbapenem-colistin combination is one of the most commonly reported synergistic combinations against *A. baumannii* (13, 14), we found minocycline-colistin and doripenem-colistin-minocycline to have the best *in vitro* results. Importantly, minocycline-colistin inhibited the growth of 100 and 51.3% of the isolates in at least one SAC well and all of the SAC wells, respectively. Additionally, minocycline-colistin was the only combination to have a bactericidal effect against all of the isolates tested by time-kill analysis. It is thought that polymyxins disrupt the efflux pumps, and therefore, an increased intracellular concentration of minocycline is obtained, leading to a synergistic interaction (15). Unfortunately, few of our patients received minocycline-colistin to treat an *A. baumannii* infection during this time period. However, minocycline-colistin at our center (10) and others (16) has shown promising clinical and microbiological success against drug-resistant *A. baumannii* infections. Currently, at our center, minocycline-colistin is used as empirical therapy for XDR *A. baumannii* infections.

Our checkerboard platform was specifically designed to evaluate SACs and therefore would avoid obtaining a synergistic result with an antimicrobial concentration above that which is achievable in serum. To determine the utility of a real-time analysis, we limited each combination to nine wells; thus, the lowest concentration of colistin was 0.5 µg/ml. Therefore, an organism for which the colistin MIC is 1 µg/ml could not show a synergistic interaction by definition, as a 2-fold dilution decrease in each antimicrobial is required. As a consequence, many of our combinations that inhibited all SAC wells could have been synergistic but, because of limitations of our platform, were not designated as such. Similar to all retrospective studies, ours was limited to the data available in the electronic medical records. Additionally, we did not assign infection-related mortality; however, XDR *A. baumannii* likely contributed to death, considering the elevated APACHE II scores of the patients evaluated. Finally, the small number of patients in our clinical cohort prevented us from performing multivariable predictor logistic regression analysis for independent predictors of treatment outcomes.

In conclusion, minocycline-colistin was the most active antibiotic combination against XDR *A. baumannii* in both the checkerboard and time-kill assays. We identified a checkerboard platform that could be utilized and may have a clinical benefit, as patients who received a combination that inhibited growth in a SAC well demonstrated improved microbiological clearance. Prospective evaluations of the checkerboard platform for XDR *A. baumannii* infections are needed.

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