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Original Article

Determination of In Vitro Activities of Polymyxin B and Rifampin in Combination with Ampicillin/Sulbactam or Cefoperazone/Sulbactam against Multidrug-Resistant *Acinetobacter baumannii* by the E-test and Checkerboard Methods

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SUMMARY: The aim of this study was to investigate the in vitro activities of polymyxin B (PB) and rifampin (RIF) in combination with ampicillin/sulbactam (AS) or cefoperazone/sulbactam (CS) against 20 multidrug-resistant *Acinetobacter baumannii* (MDR-AB) isolates by the checkerboard and E-test methods. Fractional inhibitory concentration index (FICI) values were defined as synergy, FICI $\leq$ 0.5; additivity, 0.5 $<$ FICI $\leq$ 1.0, indifference, 1.0 $<$ FICI $<$ 4.0; and antagonism, FICI $\geq$ 4. Synergistic interaction was detected only for the RIF + AS and RIF + CS combinations. While the most frequently detected interaction type for PB + AS or PB + CS combinations was indifference, some showed antagonistic interactions. The detection rate of synergy was significantly higher by the checkerboard than by the E-test method, and the detection rate of indifference was significantly higher by the E-test than by the checkerboard method for RIF + AS combination ($P \leq 0.0001$). In addition, no statistically significant difference was detected between the checkerboard and E-test methods for the detection rates of interaction types for any of the other combinations ($P > 0.05$), except for PB + CS combination for the detection of additivity ($P = 0.018$). Owing to the high percentage of synergistic interactions between RIF and AS, we considered this combination as an effective therapeutic option for MDR-AB infections.

INTRODUCTION

Over the last 10 years, multidrug-resistant *Acinetobacter baumannii* (MDR-AB) has emerged as a serious widespread threat as nosocomial infections; these infections are often associated with higher mortality (1). Although carbapenems remain the drugs of choice for the treatment of *A. baumannii* infections, their efficacy can be compromised by the widespread use of novel class D carbapenemases. Thus, carbapenem-intermediate or resistant *A. baumannii* isolates are increasingly being reported in many countries (2). Increasing antibiotic resistance rates observed in *A. baumannii* strains and the current lack of single antimicrobial agents available to treat MDR-AB infections have forced clinicians to use combination therapies as the only viable treatment option until new antibiotics become available; consequently, empirical combination therapies have become common in clinical practice in spite of the limited microbiological and clinical evidence supporting their efficacy (3).

The fact that polymyxin B (PB) and E (colistin) are usually the only therapeutic agents available for many MDR-AB infections have given rise to the renewed use of polymyxins in clinical practice. Most studies have been associated with colistin and use of local intrathecal, intravenous, or nebulized colistin for treatment of a variety of nosocomial infections has shown good results (4–6). However, the emergence of resistance against colistin has been reported, while PB, also a polymyxin agent, remains very active against clinical *A. baumannii* isolates (7). On the other hand, it has been reported that the use of PB monotherapy may not be adequate against PB susceptible *A. baumannii* isolates; therefore, combination therapy may be the only current remaining viable therapeutic option for treatment of MDR-AB infections (8).

Although rifampicin (RIF) is generally considered a suitable antimicrobial to treat infections due to Gram-positive bacteria, in vitro studies and experimental models of infection showed that RIF alone demonstrated a bactericidal effect on MDR-AB (6,9). However, it has been shown that when used alone, bacteria can rapidly develop RIF resistance; thus, its combination with other antimicrobials is crucial (10).

The aim of this study was to investigate the in vitro synergistic activities of PB and RIF when used in combination with ampicillin/sulbactam (AS) or cefoperazone/sulbactam (CS), which are commonly preferred antimicrobial agents in antimicrobial combination ther-

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apies for the treatment of MDR-AB infections. Synergistic activities were evaluated by the checkerboard and E-test methods.

MATERIALS AND METHODS

Bacterial isolates: Twenty non-duplicate MDR-AB isolates, which were confirmed as resistant to ticarcillin, cefepime, amikacin, ciprofloxacin, imipenem, and meropenem by the disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) recommendations, were included in this study (11). These strains were isolated from the tracheal aspirate (50%) and blood (50%) cultures collected from different patients hospitalized in intensive care units between June 2009 and February 2011. All isolates were identified according to standard microbiological procedures using the BBL Crystal Identification Kit (Becton Dickinson Diagnostics, Sparks, Md., USA), subcultured in Luria–Bertani broth, and stored at −80°C until use for in vitro synergy tests. DNA macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) of the isolates was also performed for genetic characterization. This study was approved by the scientific research committee of the Suleyman Demirel University.

Antimicrobial agents and minimal inhibitory concentration (MIC) assays: The antimicrobial agents used in combinations were in the form of standard reference powders for laboratory use and obtained from Sigma-Aldrich (Taufkirchen, Germany), except sulbactam (Zhejiang Xinhua Pharmaceutical Co. Ltd., Linhai, China). Antimicrobial stock solutions were prepared by mixing with solvents and diluents according to CLSI standards and stored at −80°C. MIC values of each antibiotic in the combinations were determined for all isolates by the broth microdilution method for the checkerboard assays and E-test strips for the E-test method. All procedures followed CLSI protocols and interpreted based on CLSI breakpoints (11). *Escherichia coli* ATCC 25922 was used as an internal quality control strain in each susceptibility test. Because no CLSI breakpoint was available for CS and RIF, the susceptibility breakpoints for these antibiotics were based on the MIC interpretive standards of CLSI for other non-*Enterobacteriaceae* and Gram-positive bacteria, respectively (11).

Checkerboard assay, E-test, and evaluation of the tests: The range of concentrations of the antibiotics used in the checkerboard assay was determined according to previously assessed MIC of each antibiotic for each isolate. The tested concentrations ranged from 0.03 × MIC to 4 × MIC of each antibiotic. The antibiotics were diluted two-fold in sterilized tubes, and 25 μL of each antimicrobial agent in each combination was added to 96-well checkerboard plates, making up a total of 50 μL in each well. Subsequently, 50 μL of 1/100-diluted 0.5 McFarland bacterial suspension of *A. baumannii* was added to each well resulting in a final concentration of the test strains of approximately 5×10^5 colony-forming units/mL. The plates were then incubated at 37°C for 20 h. Positive and negative controls were added for each combination. MICs and fractional inhibitory concentrations (FICs) were determined by examining turbidity after overnight incubation. The absence of viable cells in non-turbid wells was confirmed by the addition of Alamar blue reagent (Sigma-Aldrich), which changes the color of the wells containing active bacteria to red. The checkerboard synergy test results were interpreted by the method of Anon in which the lowest FIC index (FICI) of all non-turbid wells along the turbidity/non-turbidity interface was used (12).

In E-test method, E-test strips for each tested agent were placed at a 90° angle crossing at the MIC of each agent when tested alone. FICI was used to assess synergistic activity, which was determined by the addition of FICs of the two agents used in the combination. FIC of each agent was calculated as a ratio of MIC when used in combination and MIC when used alone. FICI results for each combination were defined as synergy for FICI ≤ 0.5, additivity for $0.5 < \text{FICI} \leq 1.0$, indifference for $1.0 < \text{FICI} < 4.0$ and antagonism for $\text{FICI} \geq 4.0$ (13).

The broth microdilution, checkerboard, and E-test methods were simultaneously performed for each isolate to prevent interday variation in the final inoculum and the length of time for which antimicrobial solutions were frozen. Each isolate was tested in duplicate against all antibiotics alone and in combination.

DNA macrorestriction analysis by PFGE and dendrogram analysis: Molecular typing by PFGE of the digested genomic DNA was performed according to modified WHO protocols as previously described (14). In this method, each isolate was separately digested with *Apa*I macrorestriction enzyme (Fermentas, Vilnius, Lithuania). All isolates were grown overnight at 37°C. Agarose plugs were prepared with 1.3% low melting agarose. Genomic DNA was digested with 20 U of ApaI restriction endonuclease for 4 h according to the manufacturer's recommendations. PFGE was performed with 1.3% agarose gels using CHEF DR II system (Bio-Rad, Hercules, Calif., USA) at 5 V/cm and 14°C in 0.5 × TBE for 24 h with switch times of 5–20 s. The Lambda Ladder PFGE Marker (catalog number: N0340S; New England Biolabs, Hertfordshire, UK; band size, 48.5–1018.5 kb) was used as molecular size marker. The gels were stained with ethidium bromide (0.2 μg/mL) and photographed under UV light. PFGE patterns were visually analyzed as described by Tenover et al. (15) and by computer assisted analysis using Gene Directory software (Syngene, Cambridge, UK). A similarity index was determined using the Dice coefficient and unweighted pair-group method average (UPGMA) with 1% band tolerance. The strains were evaluated as indistinguishable, closely related, possibly related, or unrelated according to the criteria of Tenover et al. (15).

Statistical analysis: Differences between rates of detected interaction types depending on the synergy test methods (checkerboard and E test) used for the combinations in this study were tested by chi-square analysis, and a probability value (P) of ≤ 0.05 was considered statistically significant.

RESULTS

Sources of the study isolates, isolation times, PFGE types, MIC values, and susceptibility profiles of each antibiotic alone for each isolate as determined by the broth microdilution method are shown in Table 1. All

Table 1. Specimen sources, isolation dates, PFGE patterns of 20 clinical *A. baumannii* isolates and MICs and susceptibility profiles of each antibiotic alone for each isolate with broth microdilution method

Isolate no.	Isolation date	PFGE pattern	Specimen source	MICs and susceptibility profiles of each antibiotic alone (mg/L)			
				AS	CS	PB	RIF
1	May 10	A	Blood	32 (R)	64 (R)	0.0156 (S)	2 (I)
2	Jun 10	D	TA	64 (R)	256 (R)	0.0156 (S)	2 (I)
3	Jun 09	A	Blood	32 (R)	64 (R)	0.0156 (S)	2 (I)
4	May 09	B	TA	32 (R)	64 (R)	0.0078 (S)	2 (I)
5	May 09	B	Blood	32 (R)	64 (R)	0.031 (S)	4 (R)
6	May 09	B	Blood	32 (R)	64 (R)	0.0156 (S)	2 (I)
7	May 09	D	Blood	128 (R)	512 (R)	0.0156 (S)	2 (I)
8	May 09	B	TA	32 (R)	64 (R)	0.0078 (S)	2 (I)
9	Nov 10	C	Blood	32 (R)	128 (R)	0.0156 (S)	2 (I)
10	Nov 10	E	Blood	32 (R)	256 (R)	0.125 (S)	2 (I)
11	Nov 10	A	TA	32 (R)	64 (R)	0.125 (S)	4 (R)
12	Dec 10	A	TA	64 (R)	128 (R)	0.0156 (S)	4 (R)
13	Dec 10	C	Blood	32 (R)	128 (R)	0.0156 (S)	2 (I)
14	Jan 11	F	TA	128 (R)	512 (R)	0.0156 (S)	16 (R)
15	Jan 11	G	TA	32 (R)	32 (R)	0.0156 (S)	4 (R)
16	Jan 11	A	Blood	64 (R)	128 (R)	0.031 (S)	4 (R)
17	Jan 11	A	TA	32 (R)	128 (R)	0.0156 (S)	2 (I)
18	Feb 11	H	TA	32 (R)	128 (R)	0.0156 (S)	2 (I)
19	Feb 11	I	TA	64 (R)	128 (R)	0.0156 (S)	2 (I)
20	Feb 11	A	TA	32 (R)	256 (R)	0.0156 (S)	4 (R)
MIC_{50}				32 (R)	128 (R)	0.0156 (S)	2 (I)
MIC_{90}				64 (R)	256 (R)	0.031 (S)	4 (R)

TA, tracheal aspirate; R, resistant; I, intermediate; S, susceptible.

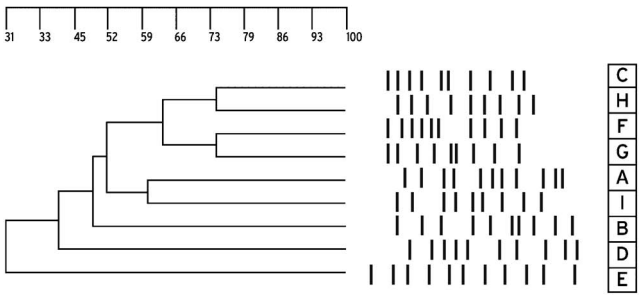


Fig. 1. Dendogram of PFGE patterns of *A. baumannii* strains yielded after digestion with macrorestriction enzyme *Apa*I, using Dice coefficient and unweighted pair-group method (UPGMA) with a 1.0% tolerance limit (strains 1, 3, 11, 12, 16, 17, and 20 belonged to Cluster A, strains 4, 5, 6, and 8 belonged to Cluster B, strains 9 and 13 belonged to Cluster C, and strains 2 and 7 belonged to Cluster D).

isolates displayed MIC values within the resistance range for AS (MIC range, 32–128 mg/L) and CS (MIC range, 32–512 mg/L), whereas they were susceptible to PB (MIC range, 0.0078–0.031 mg/L). Thirteen isolates (65%) demonstrated intermediate MIC values (2 mg/L), and the MIC values of the other 7 isolates (35%) were in the resistance range (≥4 mg/L) for RIF.

Acinetobacter baumannii strains after digestion with the *Apa*I restriction enzyme generated 4 PFGE clusters (A–D) and 5 individual patterns (E–I). Cluster A consisted of 7 isolates, while clusters B, C, and D consisted of 4, 2, and 2 isolates (with 100% band homology), respectively (Fig. 1). PFGE fingerprint patterns of *A. baumannii* strains are shown in Fig. 2 and Fig. 3.

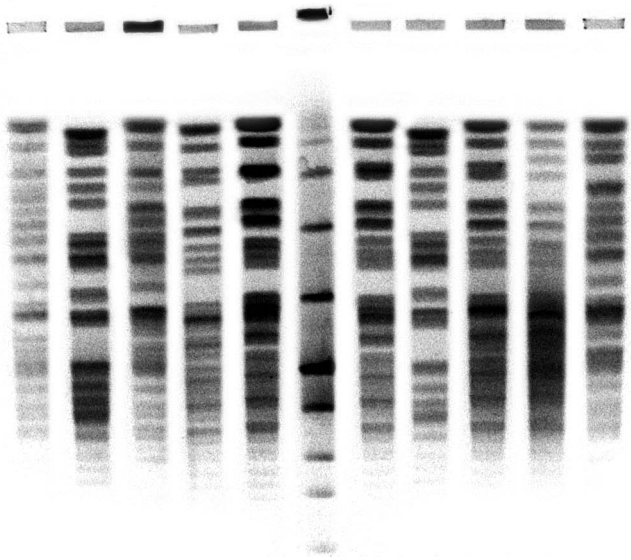


Fig. 2. PFGE fingerprint patterns of *A. baumannii* strains 1, 2, 3, 4, 5, M, 6, 7, 8, 9, 10 after restriction with *Apa*I enzyme. M, lambda ladder PFGE marker (N0340S; band size; 48.5–1018.5 kb)

Table 2 shows FICIs of the used combinations determined by the checkerboard and E-test methods. Table 3 shows the rates of interaction types (synergy/additivity/indifference and antagonism) for all tested combinations among study isolates. While synergy and additivity were detected among 85% (17/20) and 15% (3/20) of the isolates for the RIF + AS combination by the

checkerboard assay, respectively; the interaction rates were 5% (1/20) and 40% (8/20), respectively, for the same combination by the E-test method. The most frequently detected interaction type was synergy (85%) for the RIF + AS combination by the checkerboard assay, whereas indifference was detected more frequently (55%) by the E-test method for the same combination. This combination showed statistically different ($P \leq 0.0001$) synergy and indifference detection rates depending on the synergy test methods used. On the other hand, there was no significant difference between the checkerboard and E-test methods for additivity and antagonism detection rates for this combination (Table 3).

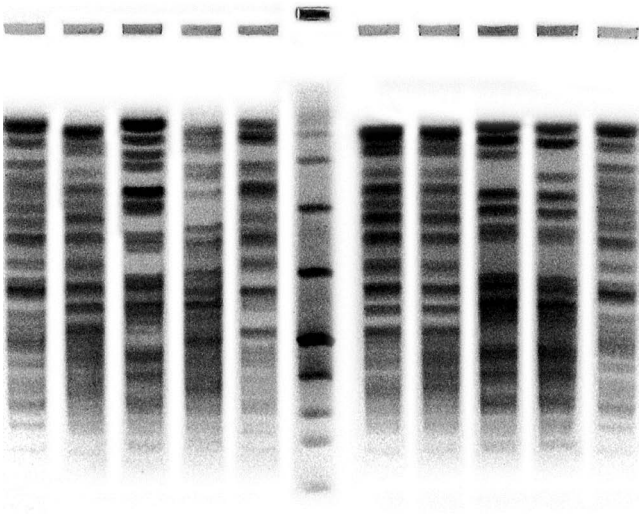


Fig. 3. PFGE fingerprint patterns of *A. baumannii* strains 11, 12, 13, 14, 15, M, 16, 17, 18, 19, 20 after restriction with *Apa*I enzyme. M, lambda ladder PFGE marker (N0340S; band size; 48.5–1018.5 kb)

For the RIF + CS combination, additivity was the most frequently detected interaction type both by the checkerboard (60%) and E-test methods (60%). Antagonistic interactions were not detected for the RIF + AS and RIF + CS combinations by either method. While the most frequently detected interaction type was indifference for the PB + AS (checkerboard, 80%; E-test, 70%) and PB + CS (checkerboard, 75%; E-test, 60%) combinations, synergistic interactions were not detected for these combinations by either method. Antagonistic interactions were detected for the PB + AS and PB + CS combinations, particularly by the checkerboard method (PB + AS, 10%; PB + CS, 20%). In addition, statistically significant differences were not detected between the checkerboard and E-test methods for the detection rates of interaction types for any of the other combinations ($P > 0.05$), except for PB + CS for the detection of additivity ($P = 0.018$).

DISCUSSION

The high rate of synergism found for RIF in combination with AS in this study suggested that this combination may present an alternative therapeutic option for the treatment of MDR-AB infections. In accordance with our findings, high synergy rates have been reported in many previous studies investigating in vitro synergistic activities of RIF with sulbactam-containing agents against MDR-AB (5,6,16,17). Synergistic interactions for the RIF + AS combination appeared to be present for all PFGE types except for PFGE type H, which displayed an additive interaction with an FICI value of

Table 2. Fractional inhibitory concentration indices (FICIs) of used combinations with checkerboard (CB) and E-test (ET) methods for each isolate

Isolate no.	Isolation date	PFGE pattern	RIF combination with				PB combination with			
			AS		CS		AS		CS	
			CB	ET	CB	ET	CB	ET	CB	ET
1	May 10	A	0.53	1.75	0.62	1.00	2.03	1.50	4.03	1.10
2	Jun 10	D	**0.50**	2.00	1.00	1.50	1.50	2.00	2.03	2.00
3	Jun 09	A	**0.37**	**0.50**	0.56	1.00	1.03	0.75	2.06	0.75
4	May 09	B	**0.37**	1.16	1.00	0.87	1.06	2.00	3.00	4.90
5	May 09	B	**0.28**	0.87	0.53	0.75	1.50	1.00	2.00	0.75
6	May 09	B	**0.31**	0.70	1.03	0.87	1.50	1.10	4.03	0.88
7	May 09	D	**0.50**	1.16	1.00	2.00	1.03	2.00	1.03	2.00
8	May 09	B	**0.50**	0.87	1.00	1.00	4.06	1.40	4.12	2.00
9	Nov 10	C	**0.50**	2.00	1.03	1.00	1.03	0.67	2.03	1.15
10	Nov 10	E	**0.50**	0.87	0.53	1.12	0.62	1.25	0.58	0.88
11	Nov 10	A	**0.25**	1.00	**0.50**	1.00	2.01	2.00	4.03	2.00
12	Dec 10	A	**0.28**	1.00	0.53	1.25	1.03	2.00	2.03	0.88
13	Dec 10	C	**0.37**	2.00	1.03	2.00	2.25	4.03	2.03	0.88
14	Jan 11	F	**0.28**	2.00	0.75	2.00	1.03	2.00	1.12	1.25
15	Jan 11	G	**0.28**	1.58	0.53	0.78	0.53	1.30	1.03	1.17
16	Jan 11	A	**0.28**	0.70	0.53	0.84	1.03	0.84	1.25	1.10
17	Jan 11	A	0.53	1.16	1.03	1.00	2.03	1.41	2.03	1.10
18	Feb 11	H	0.53	1.25	1.03	2.00	4.03	1.25	2.03	2.00
19	Feb 11	I	**0.31**	2.00	1.03	0.62	1.03	1.40	2.03	1.15
20	Feb 11	A	**0.25**	1.00	**0.37**	1.25	2.03	0.83	1.50	1.00

FICIs written in bold type show synergistic interactions.

Table 3. Interactions for all combinations with checkerboard and E-test methods

		Synergy	Additivity	Indifference	Antagonism
RIF + AS	Checkerboard	**85%**	15%	0%	0%
	E-test	5%	40%	**55%**	0%
	P	**≤0.0001**	0.077	**≤0.0001**	NA
RIF + CS	Checkerboard	10%	**60%**	30%	0%
	E-test	0%	**60%**	40%	0%
	P	0.147	1.00	0.507	NA
PB + AS	Checkerboard	0%	10%	**80%**	10%
	E-test	0%	25%	**70%**	5%
	P	NA	0.212	0.465	0.548
PB + CS	Checkerboard	0%	5%	**75%**	20%
	E-test	0%	35%	**60%**	5%
	P	NA	**0.018**	0.311	0.151

NA, not applicable.
The highest rates of interaction types detected for each combination with each method and *P* values showing statistically significance are written in bold type.

0.53 (close to the break point for synergy). Notably, although some of the study isolates generated particular clusters (PFGE patterns A, B, C, and D) with 100% band homology, the combination tests did not reveal the same results for all strains. In addition, although all of the combination test results revealed the same interaction types for strains in clusters C and D, those in clusters A and B did not exhibit the same type of interactions, particularly for combinations with PB. This discrepancy may be explained by the fact that although clonal relatedness among the strains may continue for long periods, their drug susceptibility profiles are changeable, and clonally related strains of *Acinetobacter* that differ in susceptibility patterns may coexist within a single hospital, depending on selective pressure related to antibiotic exposure (18,19).

The synergy rate for the RIF + CS combination was not as high as that for the RIF + AS combination; however, there was a considerable number of isolates displaying an FICI value ≤1 for the RIF + CS combination by both the checkerboard and E-test methods. Because there are various reports defining synergism as FICI ≤1, we suggest that the RIF + CS combination also warrants attention as a choice in future studies concerning combination therapies against MDR-AB isolates (20,21). A promising finding was that antagonism was not detected with these combinations. Since carbapenems are frequently administered to treat MDR-AB infections, most in vitro studies on antimicrobial combinations are performed with a carbapenem and a polymyxin in combination (10,21–23). However, there is limited data regarding the efficacy of PB with AS or CS, which are usually suggested agents in combination therapies (17). In our study, no synergistic activity was observed among any of the tested strains for PB + AS or PB + CS combinations, and the most frequently detected interaction type was indifference by both methods. Although a two-fold reduction in the MIC was detected for AS and CS in combination with PB among some of the isolates, the calculated FICI values were >1.0, which caused these isolates to be evaluated as displaying indifferent interactions for these combinations. This result may have occured because the isolates used in this study were susceptible to PB with very low MIC values (≤0.125 μg/mL).

Typically, if a bacterial isolate is susceptible to one of the agents in the combination, it would be preferable to administer a monotherapy instead of a combination therapy. However, the use of polymyxin as a single agent may be inadequate particularly in *A. baumannii* pneumonia because of its poor diffusion into the fluid lining of the lung epithelium. In addition, it has been reported that the increased use of polymyxin B as a single agent for the treatment of *A. baumannii* infections have caused an increase in the incidence of *Pseudomonas aeruginosa* isolates resistant to polymyxins (24). It has also been suggested that even in the situation of no statistically significant differences in the clinical outcomes between mono and combination therapies, the use of combination therapy is preferable to prevent the emergence of heteroresistance. Thus, we should not underestimate the reduction that was detected in the MIC values of AS and CS in combination with PB among most of the isolates, despite the absence of synergistic or additive interactions. In fact, most FICI values were <2.00 for these combinations, suggesting that MICs of the combined agents decreased, albeit with no synergism.

Although the time-kill and checkerboard methods are considered as standard testing methods for antimicrobial combination studies, the E-test method has been suggested as an alternative and convenient method for synergy testing because this method is less labor intensive and relatively easy to perform, while it appears to be more conservative than either the checkerboard or time-kill tests for detecting synergistic activities (e.g., when synergy is observed by either the checkerboard or time-kill tests, additivity/indifference is observed by the E-test method). Our synergy test results by the E-test and checkerboard methods were similar to previously published comparisons of the synergy methods (3). In fact, the detection rate of synergy was significantly higher by the checkerboard than by the E-test method, and the detection rate of indifference was significantly higher by the E-test than by the checkerboard method for the RIF + AS combination in our study ($P \leq$

0.0001). In addition, we found that when antagonism was observed by the checkerboard method, additivity/indifference was detected by the E-test method.

In conclusion, owing to the high percentage of synergistic interactions between RIF and AS, we propose that this combination presents an effective therapeutic option for MDR-AB infections. However, these in vitro test results should be used with extreme caution in empirical therapy because synergistic activity may depend on the bacterial strains and susceptibility testing methods. We must emphasize that in vitro synergy testing of drug combinations is required prior to starting any combination therapy for the treatment of MDR-AB infections with the awareness that there may not be an exact agreement in the qualitative interpretation of results by different synergy testing methods. Finally, we should also take in to consideration the fact that in vitro synergy may or may not always translate into in vivo benefits, so it is also necessary to combine in vitro findings with additional pharmacokinetic and pharmacodynamic data to provide more meaningful predictions of the in vivo efficacy of synergistic combinations in clinical practice.

Conflict of interest None to declare.

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