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Effect of Antibiotics Combination and Comparison of Methods for Detection of Synergism in Multiresistant Gram-Negative Bacteria

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Abstract

Background: Because of the rapid spread of antimicrobial resistance and the slow development of novel antimicrobials, Gram-negative infections treatments are challenging clinicians and the Public Health. We aimed to evaluate the activities of antimicrobial combination against MDR Gram-negative bacteria.

Methods: Twenty-eight *P. aeruginosa*, 20 *A. baumannii* and 17 *K. pneumoniae* carbapenem-resistant clinical isolates had MIC determined by broth microdilution. Synergistic effect was investigated using checkerboard method and time-kill assay, the gold standard method. PCR for carbapenemase genes and PFGE clonality assessment were done.

Results: All *P. aeruginosa* isolates were resistant to meropenem, but susceptible to colistin; the genes bla_{SPM} and bla_{KPC} were found in 82% and 25% of them, respectively; synergistic effect was seen only in combinations of colistin with meropenem (43%), meropenem with amikacin (36%) and colistin with amikacin (7%), by time-kill. Twenty-five *P. aeruginosa* isolates belonged to the same clonal profile. All 20 isolates of *A. baumannii* were resistant to meropenem, rifampicin, fosfomycin and harbored $bla_{OXA\text{-}51\text{-}like}$; the genes $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}143\text{-}like}$ and bla_{IMP} were found in 50%, 35% and 15% of them, respectively. Eleven in thirteen colistin-susceptible *A. baumannii* isolates showed distinct profiles and six in seven colistin-resistant isolates belonged to the same clone; synergistic effect was observed in almost all combinations by time-kill. Resistance to polymyxin B and resistance to imipenem were found in 100% and 91% of *K. pneumoniae* isolates. The bla_{KPC} gene was found in 82% of them; five clusters were identified (each one with two isolates) and other seven different isolates; synergistic effect occurred in most of three-drug combinations.

Conclusion: We demonstrated that time kill assay must be considered the gold standard method to detect synergism *in vitro*, as it allows greater dynamic assessment and higher sensitivity, when compared to the other methods. We detected that colistin combinations are frequently synergic against *A. baumannii* and *K. pneumoniae*. For *P. aeruginosa*, the results were not that optimistic.

Keywords: Multidrug resistant microorganisms; Checkerboard; Time-kill; Synergism; Inhibitory concentration; Fractional inhibitory concentration index

Introduction

Infections caused by multidrug-resistant microorganisms have emerged as serious global health problem, and therapeutic options for treatment of infections caused by these pathogens are limited [1,2]. In the last decade, polymyxins B and E (colistin) have been used to treat infections due to multidrug resistant Gram-negative bacteria [2,3], although colistin-resistant clinical isolates have been reported more recently [3-5]. Old antibiotic agents, such as fosfomycin, are now being considered potential treatment alternatives due to the lack of new antibiotics against these agents [6]. Studies have demonstrated that fosfomycin is a promising agent, particularly in combination with other agents, for the treatment of infections due to MDR Gram-negative, specially Enterobacteriaceae; however, there is concern about its use against *A. baumannii* [6,7]. There are experimental evidences that even drugs active only against Gram-positive microorganisms, such as vancomycin, may have activity against Gram-negative bacteria when combined with other antibiotics [4]. In this scenario, treatment with combination therapy, using two or more antibacterial drugs, has increased in the last years [8-10]. Some authors have considered a greater benefit the combination of three drugs for treating *K. pneumoniae* infections [11-13].

The most widely used *in vitro* methods to assess drug-drug interactions are the checkerboard technique, using fractional inhibitory concentration index (FICI) and two-well interpretation criteria, and time-kill kinetics [14]. The checkerboard method is prone to error and, by necessity, results from the checkerboard are often confirmed with the more dynamic interaction provided by the time-kill kinetic study format. However, there is no gold standard method, and few studies have evaluated pan-resistant microorganims. There is also doubt if the synergistic effect of antibiotics is related to the mechanism of resistance or to the clonality of isolates or both [14,15].

These data highlight the importance of evaluating antibiotic combinations that are effective in the treatment of infections caused by these bacteria. Thus, we evaluated the activities of some antimicrobial drugs in combination, against MDR gram-negative bacteria, including pan-resistant isolates, with different mechanisms of resistance and different clonal origins.

Methods

Bacterial isolates

The twenty-eight isolates of *Pseudomonas aeruginosa* were obtained from bone marrow transplantation unit of Hospital das Clínicas of University of São Paulo from 2011 to 2013. The twenty *Acinetobacter baumannii* isolates belong to the bacterial collection of the Laboratory of Bacteriology (LIM-54) of the Department of Infectious Diseases of Faculty of Medicine of University of São Paulo: thirteen colistin-susceptible isolates from 2002 to 2004 and seven colistin-resistant isolates from 2011 to 2012. In addition, the seventeen isolates of *Klebsiella pneumoniae* were obtained from the Hospital Universitário of Londrina State University from 2011 to 2012.

All *P. aeruginosa* isolates were obtained from blood. The 13 isolates of colistin-susceptible *A. baumannii* were acquired from blood samples and the colistin-resistant isolates were obtained from blood (n=4), endotracheal secretion (n=1), ascites (n=1) and rectal swab (n=1). *K. pneumoniae* isolates were obtained from endotracheal secretion (n=7), blood (n=6) and urine (n=4).

Susceptibility testing

Minimum inhibitory concentrations (MIC) of colistin, polymixin B (USP Reference Standard, Rockville, MD, USA), rifampicin, imipenem, gentamycin, amikacin, tigecycline, fosfomycin, vancomycin, teicoplanin (Sigma-Aldrich, St Louis, MO, USA), and meropenem (Astra Zeneca, Cotia, SP, Brazil) were determined using the broth microdilution method in duplicate on separate days, according to Clinical and Laboratory Standards Institute (CLSI) [16]. Susceptibility, MIC50 and MIC90 were determined and interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) for polymyxin and fosfomycin [17], Food and Drug Administration (FDA) for tigecycline[18], and CLSI criteria for the other drugs [16]. *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC29213 were used as controls.

Polymerase chain reaction (PCR)

For *A. baumannii* isolates, PCR techniques for carbapenemases genes $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-24-like}}$, bla_{IMP}, bla_{SPM}, bla_{VIM}, bla_{SIM}, bla_{NDM} and $bla_{\text{OXA-143-like}}$ were performed [19,20].

For *P. aeruginosa* isolates, PCR to bla_{SPM}, bla_{VIM}, bla_{NDM} and bla_{KPC} were performed and *K. pneumoniae* isolates were submitted to PCR for bla_{KPC} [21,22].

Pulsed-field gel electrophoresis (PFGE)

Clonality of the isolates was evaluated by PFGE. The digestion of *A. baumannii* chromosomal DNA in Ultrapure Agarose (Invitrogen™, Life technologies) was performed with ApaI endonuclase (Applied Biosystems, Foster City, California, USA), using method previously described by Durmaz et al. [22]. In turn, *P. aeruginosa* and *K. pneumoniae* chromosomal DNA digestion was done using SpeI endonuclease (Applied Biosystems, Foster City, California, USA) [23].

Restriction fragments were obtained by separation using a CHEF DR®III system (Bio-Rad, Hercules, Calif., USA) with 0.5x TBE buffer for 23 h at 14°C with pulse times of 5-30 s for *A. baumannii*, 5-90 s for *P. aeruginosa* and 5-60 s for *K. pneumoniae* [22,23]

Patterns were interpreted using BioNumerics® version 7.1 (Applied Maths).

Checkerboard microdilution

Checkerboard microdilution synergism testing was performed in duplicate and evaluated after 20-24 h of incubation at 35°C. The isolates of *A. baumannii* and *P. aeruginosa* were submitted to combinations of two drugs. The FICI was then calculated, using fractional inhibitory concentration (FIC) for each drug, as FICI=FICA +FICB, where FICA=MIC of drug A in combination/MIC of drug A when alone, and FICB=MIC of drug B in combination/MIC of drug B when alone. Results were interpreted as follows: synergism if FICI ≤ 0.5, indifferent if FICI>0.5 and ≤ 4 and antagonism if FICI>4 [14,24].

The isolates of *K. pneumoniae* were submitted to combinations of three drugs. FICI was calculated as FICI=FICA+FICB+FICC, where FICC=MIC of drug C in combination/MIC of drug C when alone. Results were interpreted as follows: synergism if FICI<1.0, indifferent if FICI= ≥ 1.0 and ≤ 4 and antagonism if FICI>4.0 [25-27].

Time-kill assay

Time-kill assays were performed in duplicate at concentrations based on the MIC determined from checkerboard testing of isolates: drugs alone and combined at 1x MIC and 0.5x MIC. Time-kill analysis was performed according to previously published techniques by Petersen et al. [15]. Flasks containing Müeller Hinton Broth and drug were inoculated with testing organism, at a density of ~10^6 cfu/mL and final volume of 10 mL, and incubated in a shaker at 35°C in ambient air. Aliquots were removed at time 0 and 2, 4, 6 and 24 h post-inoculation and serially diluted in 0.85% sodium chloride solution. Diluted samples of 0.01 mL were plated in duplicate on Müeller Hinton agar and the colonies were counted ($\log_{10}$ cfu/mL), after 20 h of incubation at 37°C. Synergism was interpreted as ≥ $2\log_{10}$ decrease in colony count with the antimicrobial combination compared to the most active single agent; the drug combination was considered antagonistic for ≥ 2 $\log_{10}$ increase in cfu/mL and indifferent for <2 $\log_{10}$ increase or decrease in colony count with the combination compared with the most active drug alone. The results of the checkerboard method were compared to results of time-kill assay, considered as the gold standard for assessment of synergism [15].

Results

The MIC_{50} and MIC_{90} of the isolates were summarized in Table 1. All *P. aeruginosa* isolates were susceptible to colistin, resistant to meropenem and had MIC for teicoplanin>256 mg/L. The genes bla_{SPM} and bla_{KPC} were found in 82% and 25% of the isolates, respectively. The genes bla_{VIM} and bla_{NDM} were not identified.

All 20 isolates of *A. baumannii* were resistant to meropenem, rifampicin, fosfomycin, had MIC for vancomycin>256 mg/L and harbored $bla_{OXA\text{-}51\text{-}like}$. The genes $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}143\text{-}like}$ and bla_{IMP} were found in 50%, 35% and 15% of the isolates, respectively. The genes bla_{SPM}, bla_{VIM} and bla_{SIM} were not identified.

Resistance to polymyxin B, using *A. baumannii* CLSI breakpoints, and resistance to imipenem were found in 100% and 91% of *K. pneumoniae* isolates. The bla_{KPC} gene was found in 82% of them.

Twenty-five *P. aeruginosa* isolates belonged to the same clonal profile. Eleven in thirteen colistin-susceptible *A. baumannii* isolates showed distinct profiles and six in seven colistin-resistant isolates belonged to the same clone. Five clusters were identified (each one with two isolates) and other seven different isolates.

The FICI method detected indifferent effect for all *P. aeruginosa* isolates and the two-well method detected nine results of synergistic effect in meropenem with amikacin combination. The comparison between two-well and time-kill methods is shown in Table 2.

Antimicrobials	MIC_{50}	MIC_{90}	Range	CLSI % Isolates		
				R	I	S
Acinetobacter baumannii						
Colistin	2	32	0.5-64	35	0	65
Imipenem	32	128	1-256	95	0	5
Tigecycline	1	2	0.25-16	5	5	90
Gentamicin	16	64	2-128	55	5	40
Amikacin	128	256	2-512	95	0	5
Meropenem	32	128	16-128	100	0	0
Rifampicin	4	4	2-8	100	0	0
Fosfomycin[a]	128	128	32-256	100	0	0
Klebsiella pneumoniae						
Polymyxin B[a]	16	32	4-64	100	0	0
Amikacin	16	16	0.5-32	0	10	90
Imipenem	8	8	0.5-8	90	0	10
Meropenem	16	64	8-64	100	0	0
Tigecycline[b]	2	4	0.12-4	65	0	35
Pseudomonas aeruginosa						
Colistin	0.5	1	0.5-1	0	0	100
Amikacin	512	512	2-512	64	0	36
Meropenem	256	512	16-512	100	0	0

[a]The MIC50 and MIC90 were interpreted according to the European Committee on Antimicrobial Susceptibility Testing.

[b]The MIC50 and MIC90 were interpreted according to the Food and Drug Administration

Table 1: Determination of MIC_{50} and MIC_{90} of 9 antibiotics against 65 Gram-negative clinical isolates identified from 02 Brazilian hospitals.

Isolate	Resistance Mechanisms		Synergism test											
			Two-well						Time-Kill					
	SPM	KPC	Col+Mer	Col+Ami	Mer+Ami	Col+Tei	Mer+Tei	Ami+Tei	Col+Mer	Col+Ami	Mer+Ami	Col+Tei	Mer+Tei	Ami+Tei
1	+	+	I	I	I	I	I	I	SY	I	I	I	I	I
2	+	+	I	I	I	I	I	I	I	I	I	I	I	I
3	+	+	I	I	SY	I	I	I	SY	I	SY	I	I	I
4	+	+	I	I	SY	I	I	I	I	I	SY	I	I	I
5	+	+	I	I	I	I	I	I	I	I	SY	I	I	I
6	+	+	I	I	SY	I	I	I	I	I	SY	I	I	I
7	+	-	I	I	SY	I	I	I	I	I	SY	I	I	I
8	+	-	I	I	SY	I	I	I	SY	I	SY	I	I	I
9	-	-	I	I	SY	I	I	I	SY	I	SY	I	I	I
10	+	-	I	I	I	I	I	I	I	I	I	I	I	I
11	+	-	I	I	I	I	I	I	I	I	I	I	I	I
12	+	-	I	I	I	I	I	I	I	I	I	I	I	I
13	+	-	I	I	I	I	I	I	I	I	I	I	I	I
14	+	+	I	I	I	I	I	I	I	I	I	I	I	I
15	-	-	I	I	I	I	I	I	SY	I	I	I	I	I
16	-	-	I	I	I	I	I	I	I	I	I	I	I	I
17	+	-	I	I	I	I	I	I	I	I	I	I	I	I
18	+	-	I	I	I	I	I	I	I	I	I	I	I	I
19	+	-	I	I	I	I	I	I	SY	I	I	I	I	I
20	+	-	I	I	I	I	I	I	I	I	I	I	I	I
21	+	-	I	I	I	I	I	I	SY	I	I	I	I	I
22	+	-	I	I	I	I	I	I	I	I	I	I	I	I
23	-	-	I	I	I	I	I	I	I	I	I	I	I	I
24	+	-	I	I	SY	I	I	I	SY	SY	SY	I	I	I
25	+	-	I	I	SY	I	I	I	SY	SY	SY	I	I	I
26	-	-	I	I	I	I	I	I	SY	I	I	I	I	I
27	-	-	I	I	I	I	I	I	SY	I	I	I	I	I
28	-	-	I	I	SY	I	I	I	SY	I	SY	I	I	I

Table 2: Comparison between two-well and time-kill of 4 antibiotics for 28 carbapenem-resistant *P. aeruginosa* clinical isolates.

The Table 3 shows the main synergy testing results according resistance mechanisms for 20 *A. baumannii* isolates with two-well method and time-kill. According interpretation by FICI, the synergism effect was detected in ten colistin-resistant isolates in the colistin with rifampicin combination and only one isolate in the colistin with vancomycin combination. Synergistic effect was observed more frequently for fosfomycin combined with amikacin (90% of the isolates) using two-well method, and for colistin with rifampicin using FICI and two-well methods (50% and 65% of the isolates, respectively), all confirmed by time-kill assay. Antagonism was not observed for any method.

Isolates	Resistance Mechanisms				Synergism test													
					Two-well							Time-kill[a]						
	OXA51	OXA23	OXA143	IMP	Col +Imi	Col +Van	Col +Mer	Col +Rif	Imi +Gen	Fos +Gen	Fos +Ami	Col +Imi	Col +Van	Col +Mer	Col +Rif	Imi +Gen	Fos +Gen	Fos +Ami
1	+	-	-	-	I	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY
2	+	-	-	-	I	SY	I	SY	I	SY	SY	SY	SY	SY	SY	SY	SY	SY
3	+	-	-	-	I	I	I	SY	I	SY	SY	SY	SY	SY	SY	SY	SY	SY
4	+	-	+	-	I	I	SY	SY	I	SY	SY	SY	SY	SY	SY	SY	SY	SY
5	+	-	-	-	I	SY	I	SY	I	I	SY	SY	SY	SY	SY	SY	SY	SY
6	+	-	+	+	I	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY
9	+	-	-	+	SY	SY	I	I	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY
11	+	+	+	-	I	SY	I	I	SY	I	SY	SY	SY	SY	SY	SY	SY	SY
13	+	-	+	-	I	I	I	I	I	SY	SY	SY	SY	SY	SY	SY	SY	SY
14	+	-	+	+	I	I	I	I	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY
15	+	-	-	-	I	SY	I	I	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY
18	+	+	+	-	SY	SY	I	I	I	I	SY	SY	SY	SY	SY	SY	SY	SY
20	+	+	+	-	I	SY	I	I	SY	SY	SY	SY	SY	SY	SY	SY	SY	SY
22	+	+	-	-	SY	SY	SY	SY	I	I	SY	SY	SY	SY	SY	SY	SY	SY
23	+	+	-	-	SY	SY	SY	SY	I	I	SY	SY	SY	SY	SY	SY	SY	SY
24	+	+	-	-	SY	SY	SY	SY	I	I	SY	SY	SY	SY	SY	SY	SY	SY
25	+	+	-	-	SY	SY	SY	SY	I	SY	SY	SY	SY	SY	SY	SY	SY	SY
26	+	+	-	-	SY	SY	SY	SY	SY	I	SY	SY	SY	SY	SY	SY	SY	SY
27	+	+	-	-	SY	SY	SY	SY	SY	SY	I	SY	SY	SY	SY	SY	SY	SY
28	+	+	-	-	SY	SY	SY	SY	I	SY	I	SY	SY	SY	SY	SY	SY	SY

Col: Colistin; Imi: Imipenem; Van: Vancomycin; Mer: Meropenem; Gen: Gentamycin; Ami: Amikacin; Rif: Rifampicin; Fos: Fosfomycin; I: Indifference; SY: Synergism; +: Presence; -: Absent. [a]Results with 1xMIC and 0.5xMIC.

Table 3: Synergy testing results according resistance mechanisms against 20 *Acinetobacter baumannii* clinical isolates.

Isolates	Resistance Mechanisms		Synergism test		
	KPC	Drug Combination	Two-well	FICI	Time-Kill[a]
2	+	PB+Tig	I	I	SY
11	+	PB+Ami	I	I	SY
12	+	PB+Mer	I	I	I
13	+	PB+Tig	SY	I	I
14	+	PB+Mer	I	I	I
15	+	PB+Imi	I	I	SY

PB: Polymyxin B; Tig: Tigecycline; Ami: Amikacin; Mer: Meropenem; I: Indifferent; SY: Synergism. [a]Results with 1xMIC and 0.5xMIC

Table 4: Comparison between FICI, two-well and time-kill for *K. pneumoniae.*

Six *K. pneumoniae* isolates were tested with two drug combinations (Table 4). The FICI method detected indifferent effect for all isolates and two-well method for five isolates; time-kill detected three isolates with synergistic effects (polymyxin B with tigecycline, polymyxin B with amikacin, and polymyxin B with imipenem). Eleven *K. pneumoniae* isolates were tested in three-drug checkerboard microdilution method, and synergism was detected for two isolates by FICI and time-kill assay (Table 5). Antagonism was observed in one isolate by FICI, however synergic result by time-kill.

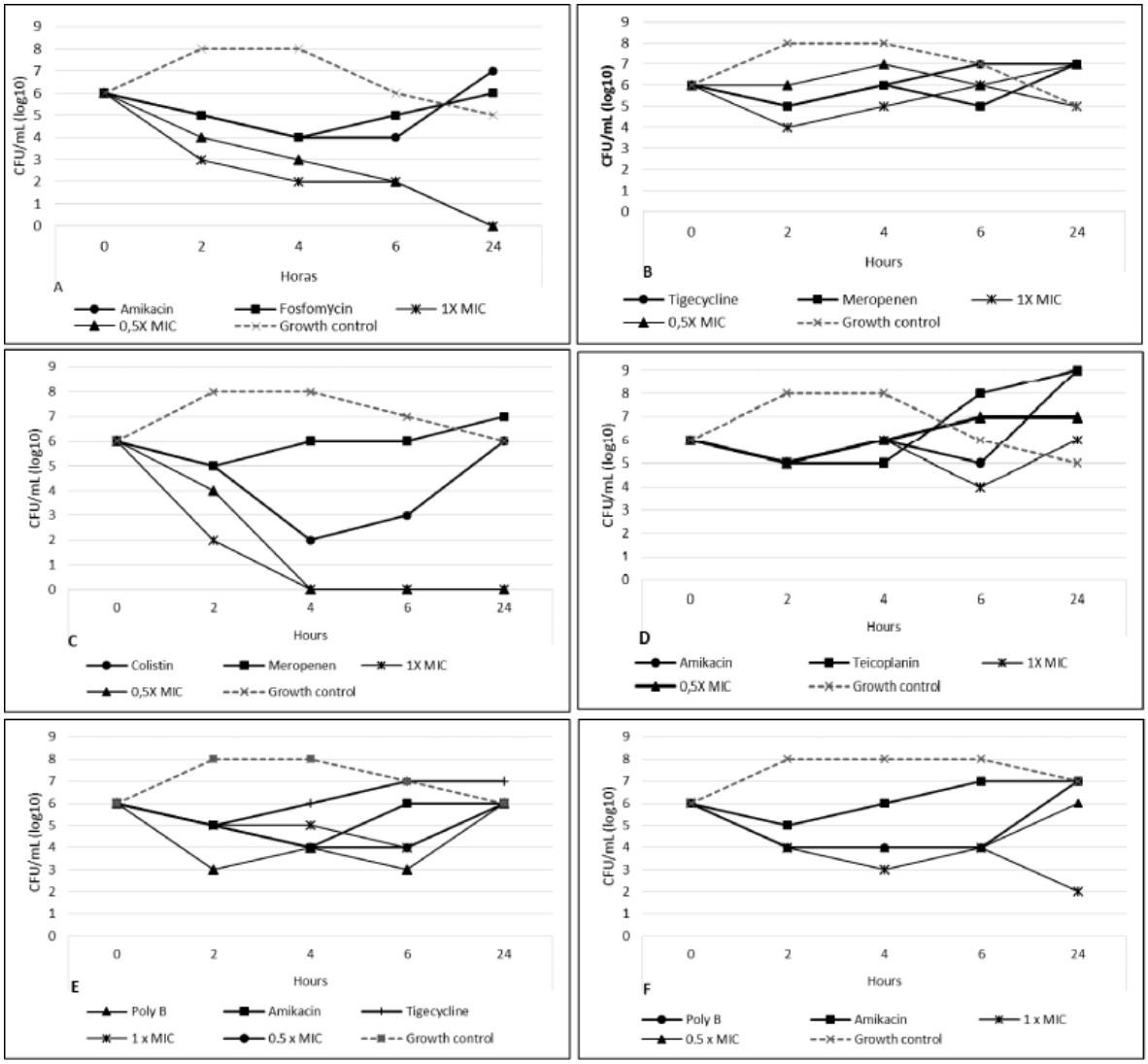

Figure 1: Time-kill assay using drugs alone and in combination to 1x MIC and 0.5x MIC. *A. baumannii* isolates: (A) Fosfomycin+Amikacin (B) Tigecycline+Meropenen. *P. aeruginosa* isolates: (C) Colistin+Meropenen (D) Amikacin+Teicoplanin. *K. pneumoniae* isolates: (E) Polymyxin B+Tigecycline+Amikacin (F) Polymyxin B+Amikacin.

Time-kill analysis was performed for all antimicrobial combinations. Colony counts of the isolates previously exposed for 0, 2, 4, 6 and 24 h to these combinations were then compared, after 20h of incubation, to the most active single agent (Figure 1). For *P. aeruginosa* isolates, the synergistic effect was observed in combinations between colistin with meropenem, colistin with amikacin and meropenem with amikacin (43%, 7% and 36% of the isolates, respectively). The use of two antibiotics in combination against *A. baumannii* showed synergistic effect for the most of the microorganisms, except for combinations with tigecycline, that showed regrowth before 24 hours. Combinations of fosfomycin with gentamicin, fosfomycin with meropenem, fosfomycin with vancomycin and imipenem with vancomycin showed regrowth in 20%, 50%, 45% and 45% of the isolates, respectively. At 1x MIC, two and three combined drugs showed synergism in 100% and 91% of the *K. pneumoniae* isolates, respectively. However, at 0.5x MIC, none of the isolates had synergistic effect in two-drug combinations and 64% of the isolates tested had synergistic effect when three drugs were combined.

Discussion

In this study, we evaluated the *in vitro* synergistic effects of different antimicrobial combinations against MDR *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* using checkerboard and time-kill assay.

Using any interpretation criteria, synergistic effect was found infrequently in *P. aeruginosa* isolates in the present study. Meropenem with amikacin combinations had synergistic effect in only 36% of the isolates. Previous studies using multi-susceptible isolates reported frequent synergistic effect of meropenem with amikacin, reaching close to 60% of strains tested, in contrast to few studies that showed that this combination was less active against *P. aeruginosa* [28,29].

More recently, despite reports that suggest an advantage of combination therapy over monotherapy, clinical data are scarce, and well-designed randomized trials with synergistic effect are needed to better elucidate the efficacy of the various combination regimens [30]. For colistin with meropenem combination, synergistic effect was observed in 43% of the isolates. One study showed that combined therapy of colistin with carbapenem in *P. aeruginosa* is more active when compared to the monotherapy in susceptible and resistant isolates to the drugs used. Another study conducted by the same author showed that the best activity of combination therapies was achieved with colistin against *P. aeruginosa* isolates [31,32].

Isolates	Resistance Mechanisms		Synergism test		
	KPC	Drug Combination	ΣFICI	FICI	Time-Kill[a]
1	+	PB+Tig+Ami	2	I	SY
3	+	PB+Tig+Ami	1.75	I	I
4	-	PB+Tig+Ami	1.18	I	SY
5	+	PB+Tig+Ami	2.25	I	SY
6	+	PB+Tig+Ami	0.5	SY	SY
7	-	PB+Tig+Ami	2	I	SY
8	+	PB+Tig+Ami	0.87	SY	SY
9	+	PB+Tig+Mer	4.4	A	SY
10	+	PB+Tig+Imi	3	I	SY
16	-	PB+Ami+Imi	1.9	I	SY
17	+	PB +Ami+Mer	1.73	I	SY

PB: Polymyxin B; Tig: Tigecycline; Ami: Amikacin; Mer: Meropenem; I: Indifferent; SY: Synergism. [a]Results with 1x MIC and 0.5x MIC

Table 5: Summary of antimicrobial testing for three drugs for *Klebsiella pneumoniae.*

To our knowledge, this the first study that evaluated the synergistic effect of fosfomycin with others antibiotics using time-kill assay, against well characterized *A. baumannii* isolates. Only few previous studies evaluated synergistic effect of fosfomycin against MDR *A. baumannii*, and reported low synergistic effect results [33,34]. In our study, all clinical isolates of *A. baumannii* harbored $bla_{\text{OXA-51-like}}$, a gene that naturally exists in *A. baumannii*. The carbapenemase gene $bla_{\text{OXA-23-like}}$ was found in 50% of the isolates; this gene has been reported as an important carbapenem resistance mechanism in many countries, including Brazil [35]. It is important to notice that seven of ten isolates that harbored $bla_{\text{OXA-23-like}}$ were colistin-resistant. All isolates that harbored $bla_{\text{OXA-143-like}}$ showed a synergistic effect with fosfomycin and amikacin and 86% presented synergism with tigecycline and amikacin. Among the isolates harboring $bla_{\text{OXA-23-like}}$, 100%, 80% and 80% presented a synergistic effect when exposed to the combination of colistin with vancomycin, colistin with imipenem and fosfomycin with amikacin. Discrepancies in resistance mechanisms between isolates from different centers may explain, at least in part, why combination regimens reported as synergic were not successful among our assays.

Colistin is frequently used to treat carbapenem-resistant infections and considered the last treatment option, because it is often the only agent with *in vitro* activity. Few studies, however, have evaluated the synergism of colistin with other drugs as a potential option to treat infections due to colistin-resistant *A. baumannii* [32-34]. In our study, the combination of colistin with rifampicin showed the highest synergistic effect against colistin-resistant *A. baumannii*, followed by colistin with vancomycin. Colistin and rifampicin combination has already been suggested for treatment of MDR *A. baumannii*, by both *in vitro* and *in vivo* studies, mainly series of cases, and the combination of colistin with vancomycin has been reported in some studies against a few isolates of *A. baumannii* [33,34]. According to these authors, the colistin would disrupt the outer membrane and could facilitate the glycopeptide penetration across the outer membrane and expose the target site of the cell wall. A synergistic effect of colistin and vancomycin is an interesting result since, in the intensive care setting, the empiric combination for septic patient is a beta-lactam plus vancomycin and, in hospitals with high MDR rates, polymyxin is added [34-36].

Time-kill kinetics confirmed the synergistic effect demonstrated by the checkerboard method in our study and this was also shown by other studies, moreover, the high synergistic effect showed by our study was restricted for some combinations; these results were observed in seven in 25 antimicrobial combinations tested. Tan et al. evaluated the synergistic effect with polymyxin B and tigecycline, polymyxin B and rifampicin and tigecycline and rifampicin combinations; synergism was present in 40% of combinations by the method of time-kill, 17% by checkerboard and 2% by Etest, there was no agreement between time-kill and Etest methods for synergism testing [37]. In our study, although the high rate of susceptibility to tigecycline (95% of the isolates), few antibiotic combinations using tigecycline showed synergistic effect. The best synergistic tygecicline combinations results were obtained by time-kill interpretation criteria; in contrast, a previous study reported by Petersen et al. [15] showed as frequent as 56% of synergistic effect in tigecycline with amikacin combination by checkerboard, but not by time-kill.

It is important to emphasize that *K. pneumoniae* isolates used in this study were resistant to polymyxin B. It is increasingly frequent the dissemination of this microorganism, with such sensitivity profile, in hospitals in developing countries [11,12]. This phenomenon hinders the possibility of further treatment of infections caused by this pathogen, which usually harbors carbapenemases encoding genes in its genome, which confers resistance to carbapenems, commonly used drugs for the treatment of nosocomial infections. The use of two or three-drug combination therapy in carbapenemase-producing *K. pneumoniae* has been reinforced by some authors. In addition to the scarce clinical research, several *in vitro* studies have shown the benefit of the drug combination of use of strains of *K. pneumoniae* [11,38].

In this study, we noticed frequent synergism in regimens containing polymyxin B (50% of the isolates in two-drug combinations and 91% in three-drug combinations). These data are similar to the results found in a recent study, when synergistic or bactericidal activity was demonstrated for double and triple-antibiotic combinations with colistin, using time-kill experiment in two VIM- and two NDM-producing *K. pneumoniae* strains; in this study, however, all strains tested were susceptible to colistin, unlike the strains tested in our study [38]. In other studies with colistin-resistant *K. pneumoniae* strains, antibiotic combinations with colistin showed potential therapeutical

options against infections caused by multidrug-resistant carbapenemase-producing *K. pneumoniae* isolates [39,40].

A variety of methods for interpreting the results can be used and these methods may, lead to different results and conclusion, even the checkerboard technique that is well standardized. A study conducted by Bonapace et al. [15] that used different methods to interpret the results by checkerboard showed that the poorest agreement was found with FICI and time-kill assay. Standardization of interpretation would be desirable to decrease discordant results. It is a big challenge to perform synergism testing routinely in the clinical microbiology laboratory, especially due to the lack of accepted standards. The methods available are laborious, time-consuming, and require expertise in the specific procedures.

In conclusion, our study demonstrates that time kill assay must be considered the gold standard method to detect synergism *in vitro*, as it allows greater dynamic assessment and higher sensitivity, when compared to the other methods. We detected that colistin combinations are frequently synergic against *A. baumannii* and *K. pneumoniae*, and probably not related to the clonality of isolates, and that fosfomycin combined with amikacin or with gentamicin were surprisingly active against *A. baumannii*. For *P. aeruginosa*, the results were not that optimistic. However, in the present scenario of multi-resistance in which the choices of active drugs are scarce, combination therapy is an option to treat these infections, and *in vitro* experience is needed for the development of future clinical studies.

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