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Major Article

In vitro activity of antimicrobial combinations against multidrug-resistant *Pseudomonas aeruginosa*

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ABSTRACT

Introduction: *Pseudomonas aeruginosa* isolates related to nosocomial infections are often resistant to multiple antibacterial agents. In this study, antimicrobial combinations were evaluated to detect *in vitro* synergy against clinical isolates of *P. aeruginosa.* **Methods***:* Four clinical *P. aeruginosa* isolates were selected at random among other isolates from inpatients treated at the public University hospital in Ribeirão Preto, SP, Brazil. Two isolates were susceptible to imipenem (IPM-S) and several other antimicrobials, while the other two isolates were imipenem and multidrug resistant (IPM-R). The checkerboard method was used to assess the interactions between antimicrobials. **Results**: Combinations of imipenem or other anti-*Pseudomonas* drugs with complementary antibiotics, such as aminoglycosides, fosfomycin and rifampin, reached synergy rates of 20.8%, 50%, 62.5% and 50% for the two IPM-S and two IPM-R *Pseudomonas* isolates, respectively. Imipenem, piperacillin-tazobactam and ceftazidime yielded a greater synergy rate than cefepime or ciprofloxacin. Synergist combinations were more commonly observed when the complementary drug was tobramycin (65%) or fosfomycin (57%). **Conclusion**s: Some antibacterial combinations led to significant reductions of the minimum inhibitory concentrations of both drugs, suggesting that they could be clinically applied to control infections caused by multidrug-resistant *P. aeruginosa.*

Keywords: *Pseudomonas aeruginosa.* Antimicrobial synergy. Beta-lactam agents. Fosfomycin. Tobramycin. Rifampin.

INTRODUCTION

A universal tendency to bacterial resistance to antimicrobials has been observed since the beginning of antibiotic therapy. *Pseudomonas aeruginosa* is a microorganism that is particularly difficult to control because it causes opportunistic and nosocomial infections, it is non-susceptible to several antimicrobials and it develops progressive resistance to new drugs[1]. Over the last few years, carbapenemic drugs have become important therapeutic resources for the control of *P. aeruginosa* infections. However, growing resistance to imipenem and other carbapenems has been observed, and multidrug-resistance has become more common[2,3].

A therapeutic strategy against *P. aeruginosa* is the use of antimicrobial combinations to delay the selection of resistant bacterial clones and to obtain a synergistic antibacterial action[4]. The combination of beta-lactam antibiotics and aminoglycosides has been recommended for the treatment of infected patients. Antimicrobials are also combined as a method to recover the efficacy of drugs to which *P. aeruginosa* has become resistant. When the interaction is synergistic, reductions of the minimum inhibitory concentrations (MICs) of both antimicrobials occur, eventually rendering the microorganism susceptible to the levels of antimicrobials found in the blood and tissues[5].

This investigation was motivated by the high frequency of nosocomial infections caused by multidrug-resistant *P. aeruginosa* in a Brazilian emergency and trauma care hospital. The objective was to evaluate the *in vitro* susceptibility of *P. aeruginosa* by testing antimicrobials that are known to combat *P. aeruginosa*, combined with other potentially active drugs, particularly fosfomycin and rifampin.

METHODS

Pseudomonas aeruginosa was isolated from the blood (n=3) and urine (n=1) of patients who were admitted to the Emergency Unit of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil. The isolates were selected at random among other clinical isolates and were identified by the automated microbiology system Vitek (Biomérieux, Jacarepaguá, Brazil). Two isolates (8S and 34S) were susceptible to imipenem and to several other antimicrobials, while the other two isolates (46R and 72R) were imipenem and multidrug resistant. The four strains were subcultured in brain heart infusion broth (Oxoid, Hampshire,

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England), plated on Müeller Hinton agar (Oxoid, Hampshire, England) and then stored at -70°C in soy trypticase broth with 15% glycerol. Aliquots for the tests were removed from this stock, and bacterial growth was recovered by subculture in brain heart infusion broth for 24 hours.

Antimicrobial drugs

Imipenem, gentamicin, fosfomycin and polymyxin B powder were obtained from Sigma, USA(St. Louis, MO, USA) and were diluted in sterile water for the preparation of the stock solutions. Stock solutions of other drugs were prepared by dilution in sterilized water from the following commercially available medications: ceftazidime (Kefadin, ABL, São Paulo, Brazil), piperacillin-tazobactam (Taz-Pen, Cellofarm, Brazil), cefepime (ABL, São Paulo, Brazil), tobramycin (Tobramina, ABL, São Paulo, Brazil), ciprofloxacin (Ciprobacter/Isofarma, Ceará, Brazil) and rifampin (Rifaldin/Sanofi-Aventis, Paris, France). The stock solutions were stored at -70ºC, except for imipenem, which was prepared immediately before use. The various drug concentrations were obtained by successive two-fold dilutions in sterile water. The antimicrobial solutions were validated by determination of the respective MICs against *P. aeruginosa* ATCC 27853.

Broth microdilution susceptibility test

The method standardized by the Clinical and Laboratory Standards Institute (CLSI)[6] was used to determine the MICs of the antimicrobials for the selected clinical isolates and for *P. aeruginosa* ATCC 27853.

Briefly, Müeller Hinton broth that was adjusted for cations (Oxoid, Hampshire, England) was added to the wells of microtiter plates (TPP, Zellkultur, Trasadingen, Switzerland). Solutions with serial dilutions of antimicrobial concentrations were added at variable ranges according to the drug and isolate tested. Finally, *P. aeruginosa* from a 24-hour subculture in Müeller Hinton broth (Oxoid) was adjusted for a turbidity equivalent to 0.5 on the McFarland scale and then diluted to obtain a final inoculum of 2 x 10^5 colony-forming units (CFU)/mL. The plates were covered with plastic film, and after 24 hours of incubation at 35ºC, the absorbance of each well of the microtiter plate was measured with a microplate reader that was adjusted for a wavelength of 490nm.

The criterion used for MIC determination was the lowest antimicrobial concentration with absorbance corresponding to ≤ 20% of the mean absorbance of the control bacterial growth wells (without the addition of the antimicrobial). CLSI-determined breakpoints[7] were used to evaluate the bacterial susceptibility to antimicrobials, with the exception of fosfomycin. Susceptibility to this drug was analyzed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criterion for *Enterobacteriaceae*[8].

Susceptibility test to combined drugs

The checkerboard method[9] was used to assess the eventual synergy between antimicrobials against the two imipenem-susceptible and the two multidrug-resistant *P. aeruginosa* isolates. Each drug combination was evaluated in duplicate with each bacterial isolate. The MIC of each separate drug was determined on different sides of the same plate. Wells were used for positive controls (only bacterial inoculum and Müeller Hinton broth) and for negative controls (only combined and separate antimicrobials and Müeller Hinton broth). The combined drugs were tested at different concentrations inside the microplate. A *Pseudomonas aeruginosa* subculture in Müller Hinton broth was adjusted to obtain a final inoculum of 2 x 10^5 CFU/mL in the well. The plates were covered and incubated at 35ºC for 24 hours, and the absorbance of each well was then measured with a microplate reader adjusted for a wavelength of 490 nm. The MIC of the separate or combined antimicrobials was defined as the lowest concentration of the drugs with an absorbance that was ≤ 20% of the mean absorbance of the bacterial growth in the control wells.

Analysis of antimicrobial interaction

Interaction was analyzed separately for each antimicrobial combination (drugs A and B) and bacterial isolate. The fractional inhibitory concentration of drug A (FIC[A]) was calculated as the following ratio: MIC obtained with drug A combined with the second drug/MIC of drug A alone. FIC[B] was obtained using a similar calculation. The FIC(A) + FIC(B) sum resulted in the FIC index (FICI), representative of the interaction of the two antimicrobials. The following criteria were adopted when the MIC value exceeded the standardized range of drug concentrations: 1) for an MIC above the range limit, the FIC was calculated with the next highest drug concentration, estimated by successive two-fold dilutions; and 2) when the MIC was equal to or below the lowest drug concentration evaluated, this value was used to calculate the FIC. The following criteria of interpretation were adopted: 1) FICI ≤ 0.5 indicates synergy between the two antimicrobials; 2) FICI > 0.5 and ≤ 4.0 indicates indifference; and 3) FICI >4 indicates antagonism[9]. The lowest FICI value obtained in the checkerboard test was considered to be representative of the interaction of the two antimicrobials against the respective *P. aeruginosa* isolate, regardless of the concentrations of the two drugs.

Statistical analysis

The chi-square test was used to analyze the differences in the synergy rates obtained between the bacterial strains and the antimicrobials tested, with the level of significance set at 0.05.

RESULTS

The two imipenem-susceptible strains (8S and 34S) exhibited susceptibility to other anti-*Pseudomonas* drugs but were resistant to fosfomycin and rifampin. The two imipenem-resistant isolates (46R and 72R) were also resistant to all antimicrobials evaluated (the MIC of the 46R strain was near the breakpoint with respect to ceftazidime and piperacillin-tazobactam). The four *P. aeruginosa* isolates were all susceptible to polymyxin B (MIC= 1 to 2µg/mL).

The FICI for the various drug combinations indicated synergic or indifferent activity, with no antagonism being

TABLE 1 - *In vitro* synergic combinations between pairs of antimicrobials against two imipenem-susceptible *Pseudomonas aeruginosa*-minimum inhibitory concentration (MIC) of the isolate or combined drugs and fractional inhibitory concentration index (FICI).

		MIC (μg/mL)		
	drugs	alone	combined	
Strain	(A/B)	(A/B)	(A/B)	FICI
8S	IPM/TOB	2/0.5	0.5/0.13	0.500
8S	IPM/FOSF	1/512	≤0.13/16	0.161
8S	IPM/RIF	2/32	0.5/≤4	0.375
8S	PIP-T/TOB	4/0.5	0.5/≤0.13	0.375
8S	PIP-T/FOSF	4/512	1/16	0.281
34S	IPM/TOB	2/1	0.5/0.25	0.500
34S	IPM/FOSF	1/512	0.25/64	0.375
34S	IPM/RIF	2/32	0.5/8	0.500
34S	PIP-T/GEN	16/2	2/≤0.5	0.375
34S	PIP-T/TOB	16/1	≤0.13/≤0.06	0.068
34S	PIP-T/FOSF	8/512	≤0.13/64	0.141
34S	CEFTA/GEN	4/2	0.25/0.5	0.313
34S	CEFTA/TOB	4/2	1/≤0.5	0.500
34S	CEFTA/FOSF	8/>512	1/32	0.156
34S	CEFTA/RIF	8/64	1/≤8	0.250
34S	CIP/FOSF	0.25/>512	0.06/16	0.256
34S	TOB/RIF	2/64	0.5/16	0.500

IPM: imipenem; PIP-T: piperacillin-tazobactam; CEFTA: ceftazidime; TOB: tobramycin; GEN: gentamicin; CIP: ciprofloxacin; FOSF: fosfomycin; RIF: rifampin.

observed. The MIC value of an antimicrobial generally decreased when a second drug was present in subinhibitory concentrations, but in general, the MIC reductions were discrete. For the synergic antimicrobial combinations, the initial and the lowest MIC values obtained in the presence of another drug acting jointly are listed in **Table 1** (imipenem-susceptible isolates) and **Table 2** (imipenem-resistant isolates). Some drug combinations led to a great MIC reduction, such that the new MIC of both drugs was reduced below the respective breakpoint. **Table 3** shows that there was synergy in 46% of the combinations of all drugs for the four isolates tested. Tobramycin (65%) and fosfomycin (57%) were more effective as the second drug of the combinations than gentamicin (25%) and rifampin (36%) in terms of synergistic action against *P. aeruginosa*. Synergy with these drugs was obtained more frequently when imipenem, piperacillin-tazobactam and ceftazidime were used as the first anti-*Pseudomonas* drugs ($p < 0.05$). The rate of synergy obtained with all antimicrobial combinations for isolates 8S, 34S, 46R and 72R reached 20.8% (5/24), 50% (12/24), 62.5% (15/24) and 50% (12/24), respectively ($p < 0.05$).

TABLE 2 - *In vitro* synergic combinations between pairs of antimicrobials against two imipenem-resistant *Pseudomonas aeruginosa*-minimum inhibitory concentration (MIC) of the isolate or combined drugs and fractional inhibitory concentration index (FICI).

		MIC (μg/mL)		
	dugs	alone	combined	
Strain	(A/B)	(A/B)	(A/B)	FICI
46R	IPM/TOB	32/>256	2/64	0.188
46R	IMP/FOSF	32/256	4/32	0.250
46R	PIP-T/GEN	64/>512	16/256	0.500
46R	PIP-T/TOB	128/256	4/≤8	0.063
46R	PIP-T/FOSF	128/256	16/≤8	0.156
46R	PIP-T/RIF	64/16	16/≤4	0.500
46R	CEFTA/TOB	8/256	2/32	0.375
46R	CEFTA/FOSF	16/512	4/64	0.375
46R	CEFTA/RIF	16/32	4/≤8	0.500
46R	CPM/GEN	>512/512	32/≤8	0.047
46R	CPM/TOB	>512/256	≤1/≤8	0.033
46R	CPM/FOSF	256/512	≤1/16	0.035
46R	CPM/RIF	>512/32	32/≤4	0.156
46R	TOB/FOSF	512/>256	128/32	0.313
46R	TOB/RIF	512/32	16/≤4	0.156
72R	IPM/GEN	512/128	2/8	0.066
72R	IPM/FOSF	>512/128	256/32	0.500
72R	IPM/RIF	512/32	128/≤4	0.375
72R	PIP-T/TOB	>512/256	64/32	0.188
72R	PIP-T/FOSF	>512/256	4/64	0.254
72R	CEFTA/TOB	>512/>256	128/32	0.188
72R	CEFTA/FOSF	>256/512	128/128	0.500
72R	CPM/TOB	512/256	64/32	0.250
72R	CIP/TOB	16/256	4/16	0.313
72R	CIP/FOSF	16/256	2/16	0.188
72R	TOB/FOSF	>512/256	16/32	0.141
72R	TOB/RIF	512/32	8/≤4	0.141

IPM: imipenem; PIP-T: piperacillin-tazobactam; CEFTA: ceftazidime; TOB: tobramycin; GEN: gentamicin; CIP: ciprofloxacin; FOSF: fosfomycin; RIF: rifampin.

DISCUSSION

This study revealed that various antimicrobial combinations could act synergistically *in vitro* against multidrug-resistant Brazilian *P. aeruginosa* isolates. Polymyxins and fosfomycin are old antibiotics that have been retrieved in an attempt to control

TABLE 3 - *In vitro* synergistic combinations of antimicrobials against four strains of *Pseudomonas aeruginosa.*

	Number of strains with synergy								
	Drug A							Synergistic/all	
Drug B	IPM	PIP-T	CEFTA	CPM	CIP	GEN	TOB	tests n (%)	
GEN	1	2	1	1	0			5/20	25.0
TOB	3	4	3	2	1			13/20	65.0
FOSF	4	4	3	1	2	0	2	16/28	57.0
RIF	3	1	2	1	0	0	3	10/28	36.0
Synergistic/all tests n (%)	11/16 (69.0)	11/16 (69.0)	9/16 (56.0)	5/16 (31.0)	3/16 (19.0)	0/8 (0.0)	5/8 (63.0)	44/96	46.0

IPM: imipenem; PIP-T: piperacillin/tazobactam; CEFTA: ceftazidime; CPM: cefepime; GEN: gentamicin; TOB: tobramycin; FOSF: fosfomycin; RIF: rifampin; CIP: ciprofloxacin. $p<0.05$

nosocomial infections caused by this microorganism[10-11-12], but isolates with high MICs for these drugs have already been detected[1-13-14]. The increasing number of infections related to multidrug-resistant *P. aeruginosa* in different areas of the world[4-15-16] has stimulated the investigation of the synergy of antimicrobial combinations. The checkerboard method is widely employed for this purpose and was modified in this study to measure the growth in microplates as absorbance units. The results obtained with this instrumental technique show a good correlation with the visual reading and provide a better, more objective definition of the intermediate zone of growth and therefore, more precise MIC determinations for combined drugs.

The overall rate of synergy observed in this study was 46%. The combinations of imipenem, piperacillin-tazobactam and ceftazidime with a second drug often resulted in synergy. Combinations of these antibiotics with tobramycin exhibited synergy in 83% of the tests performed with the four *P. aeruginosa* isolates. In another study assessing multidrug-resistant *P. aeruginosa,* ceftazidime plus tobramycin and piperacillin-tazobactam plus tobramycin combinations were evaluated, and synergy ratios of 67% and 50%, respectively, were observed[17]. With respect to fosfomycin, synergistic interactions with other antibacterial drugs were verified in 57% of the tests, a rate similar to that reported previously for multidrug-resistant *P. aeruginosa*[18]. Fosfomycin enhances the active transport of tobramycin in *P.aeruginosa*[19]; *in vitro* synergic actions were also demonstrated for polymyxin E[10], imipenem[20], ceftazidime[20] and ciprofloxacin[21]. Previous studies showed that rifampin had *in vitro* synergism with other antimicrobials[22-25], whereas in this investigation, synergism was more frequently demonstrated with imipenem and aminoglycosides.

The clinical application of the *in vitro* synergy results must be considered with caution in view of the variable susceptibilities of *P. aeruginosa* isolates to combined drugs and the pharmacokinetic characteristics of the antimicrobials. As observed in other studies[26, 27], the rate of synergy of antibacterial combinations varies according to isolate and is not strictly associated with susceptibility or resistance to imipenem. Comparison of the two multidrug-resistant *P. aeruginosa* isolates revealed more frequent and significant drug MIC reductions for the 46R isolate than for the 72R isolate. Thus, it is advisable to test each multidrug-resistant isolate with the different drugs in combination[17,28].

Among the synergy results, only a few antibacterial combinations have led to sufficient MIC reductions that reach the breakpoint and the usual plasma level of the drugs, which is essential if a synergistic action in a clinical setting is going to take place[5,28]. For the 72R isolate, the only antibacterial combinations that would likely be synergic *in vivo* are imipenem plus gentamicin, piperacillin-tazobactam plus fosfomycin, ciprofloxacin plus fosfomycin, tobramycin plus fosfomycin and tobramycin plus rifampin.

In conclusion, antimicrobial synergy was observed against clinical isolates of imipenem-susceptible or imipenem-resistant *P. aeruginosa*. Some drug combinations resulted in sufficient MIC reductions, which suggests that these combinations may be of clinical use for infections of multidrug-resistant *P. aeruginosa* as an alternative to antibiotic therapy with polymyxins.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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