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In Vitro Activity of Ceftazidime and Meropenem in Combination with Tobramycin or Ciprofloxacin in A Clone of Multiresistant *Pseudomonas Aeruginosa*

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Abstract Therapeutic options to fight against infections caused by multiresistant (MR) *Pseudomonas aeruginosa* strains are restricted to a few antimicrobials such as colistimethate and amikacin. The purpose of this study was to compare in vitro synergy testing by epsilometric (E-test) and the checkerboard (CB) methods with time-kill analysis in MR *P. aeruginosa* clinical isolates. Four isolates belonging to a MR endemic clone were selected. Their resistance mechanisms were studied. Susceptibility to ceftazidime (CAZ) and meropenem (MEM) in combination with tobramycin (TOB) and ciprofloxacin (CIP) were tested. Synergy was consistently detected in CAZ plus TOB as well as in MEM plus TOB combinations. The E-test method was comparable to CB method. Synergy and bactericidal activity were observed at 1/4 or 1/8 MIC TOB concentration combined with 1 MIC of CAZ or 1 MIC of MEM by time kill curves, with slight differences in the two isolates tested. These findings indicate the possibility of designing therapies based on combinations of a β-lactam and an aminoglycoside as a therapeutic option in infections caused by MR *P. aeruginosa*.

Keywords *Pseudomonas aeruginosa*, Synergy, Time-kill, FICI, SPBI

1. Introduction

Pseudomonas aeruginosa is naturally resistant to a wide variety of antimicrobials and can easily become resistant to many more; this constitutes a serious and growing therapeutic challenge, particularly in hospital setting. In addition, the natural capacity of *P. aeruginosa* to survive in adverse conditions and their minimal nutritional requirements, as well as their cosmopolitan distribution, enables this bacterium to be a silent nosocomial inhabitant.

A large number of publications have pointed out the increasing resistance rates, with special concern on the increase of isolation of multiresistant and panresistant strains[1, 2, 3].

As a result, endemic situations with variable incidences have emerged in many hospitals[4, 5, 6]. The lack of pipeline antipseudomonal agents available make the situation worse in the short-term.

This has enhanced the interest in exploring the use of associations of several antibiotics and also in the rescue of old antimicrobials whose use had ceased several decades ago because of its potential toxicity; some of them have been used and assayed in several antibacterial combinations to achieve synergy[7].

The aim of this study was to assess synergistic effect of several antibiotic combinations as well as to explore bactericidal effect on *P. aeruginosa* isolates belonging to this endemic clone, thus, we explored the combinations of ceftazidime and meropenem with tobramycin and ciprofloxacin in four representative multiresistant *P. aeruginosa* isolates belonging to a nosocomial endemic clone of the Hospital del Mar, in Barcelona. Preliminary experiments to explore the mechanisms of resistance in these isolates were also

2. Materials and Methods

Bacterial strains: Four representative MR *P. aeruginosa* isolates belonging to an endemic clone from the Hospital del Mar at Barcelona (Spain) were selected.

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Table 1. Primers used to detect different genes involved in antimicrobial resistance

Enzyme	Primer name	Primer sequence	GenBank number
OXA-1t	OXA1/4	CAGCAGCGCCAGTGCATC	J02967
	OXB1/4	TCCTGTAAGTGCGGACAC	
OXA-2t	OXA-2 up	GCCAAAGGCACGATAGTTGT	X07260
	OXA-2 dn	ATAGAGCGAAGGATTGCCCG	
OXA-10t	OXA-10 up	GAGTTCTCTGCCGAAGCCG	J03427
	OXA-10 dn	GCCACCAATGATGCCCTCAC	
OXA-20t	OXA-20 up	CAGCTGTTGTACTTGTCTCTC	AF024602
	OXA-20 dn	CGGATTGAAGAATAGCACGCG	
OXA-23t	OXA-23 up	CTTGCTATGTGGTTGCTTCT	AJ132105
	OXA-23 dn	CATTACGTATAGATGCCGGC	
OXA-24t	OXA-24 up	CTCTCAGTGCATGTTCATCT	AJ239129
	OXA-24 dn	CGAATAGAACCAGACATTCC	
OXA-46	OXA-46 up	ATGGCAATCCGATTCTTCAC	AJ969237
	OXA-46 dn	TTAGTTGGGTGGCAATGCGT	
OXA-48	OXA-48 up	CGTTATGCGTGTATTAGCCTTAT	AY236073
	OXA-48 dn	TTTTTCCTGTTTGAGCACTTCTTT	
OXA-51t	OXA-51 up	ATGAACATTAAAACACTCT	AJ309734
	OXA-51 dn	TTAAGGGAGAACGCTACA	
OXA-58t	OXA-58 up	CTTGTGCTGAGCATAGTATGAG	EU642594
	OXA-58 dn	ACCAATACGTTGCAATTCAC	
GES	GES-1-up	ATGCGCTTCATTCACGCAC	GU831563
	GES-1-dn	CTATTTGTCCGTGCTCAGG	
IMI	IMI-up	GTCACTTAATGTAAAACC	U50278
	IMI-dn	TTAAGGTTATCAATTGCG	
KPC	KPC-up	TGTCACTGTATCGCCGTC	AF297554
	KPC-dn	TTACTGCCCGTTGACGCC	
NMC	NMCA-up	GTCACTTAATGTAAAGCA	Z21956
	NMCA-dn	GGTTATCAATTGCAATTC	
SME	SME-up	CAATTGCCTGAATTGCAAT	AY584237
	SME-dn	CGGCTTCATTTTTGTTTA	
GIM	GIM-up	ACTTGTAGCGTTGCCAGC	AJ620678
	GIM-dn	AATCAGCCGACGCTTCAG	
IMP	IMP up	GAAGGCGTTTATGTTCATAC	DQ842025
	IMP dn	GTAAGTTTCAAGAGTGATGC	
SPM	SPM-1 A	CTGCTTGGATTCATGGGCGC	DQ145284
	SPM-1 B	CCTTTTCCGCGACCTTGATC	
VIM	VIMB	ATGGTGTTTGGTCGCATATC	DQ489717
	VIMF	TGGGCCATTCAGCCAGATC	
ANT-2"Ia	ANT-2"Ia FW	ACGCCGTGGGTCGATGTTTGATGT	X04555
	ANT-2"Ia R	CTTTTCCGCCCCGAGTGAGGTG	
ACC-3'-IIa	AAC-3'-IIa FW	GGCAATAACGGAGGCGCTTCAAAA	X13543
	AAC-3'-IIa R	TTCCAGGCATCGGCATCTCATACG	
ACC-6'-Ia	ACC-6'-Ia FW	ATGAATTATCAAATTGTG	M18967
	ACC-6'-Ia R	TTACTCTTTGATTAAACT	
ACC-6'-Ib	ACC-6'-Ib FW	CAAAGTTAGGCATCACA	M21682
	ACC-6'-Ib R	ACCTGTACAGGATGGAC	
ACC-6'-Ic	ACC-6'-Ic FW	CTACGATTACGTCAACGGCTGC	M94066
	ACC-6'-Ic R	TTGCTTCGCCCACTCCTGCACC	
ANT-4"-IIa	ANT-4"-IIa FW	CCGGGGCGAGGCGAGTGC	M98270
	ANT-4"-IIa R	TACGTGGGCGGATTGATGGGAACC	
AAC-3'-Ia	AAC-3'-Ia FW	GCAGTCGCCCTAAAACAAA	X15852
	AAC-3'-Ia R	CACTTCTTCCCGTATGCCCAACTT	
AAC-3'-Ib	AAC-3'-Ib FW	GCAGTCGCCCTAAAACAAA	L06157
	AAC-3'-Ib R	GGATCGTCACCGTAGTCTGC	
AAC-2'-Ia	AAC-2'-Ia FW	AGAAGCGCTTTACGATTTATTA	L06156
	AAC-2'-Ia R	GACTCCGCCTTCTTCTTCAA	
parC	Ps parC up	CTATCTGAACTATTCCATGTACGT	AE004091
	Ps parC dn	ACGCGACTTCCCGCAGGTTG	
gyrA	Ps gyrA up	ATCGTCGGGCGGGCCCTGCCG	AE004091
	Ps gyrA dn	GGGGTTGTCCATCAGCGCCA	

Susceptibility tests: Susceptibility tests were done by diffusion or microdilution using the gramnegative breakpoint panel 38 for non-fermenter gramnegative bacilli of MicroScan® Walkaway system (Siemens Diagnostic Inc., CA)[Clinical Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement M100-S21. CLSI, Wayne, PA, USA, 2011. Clinical Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests -Tenth Edition; Approved Standard M02-A10. CLSI, Wayne, PA, USA, 2009.].

Clonal identification: Serotyping and pulse-field gel electrophoresis (PFGE) were done. DNA for PFGE was digested with 40 U of *xbal*.

Characterization of the mechanisms of resistance involved: Expanded spectrum ß-lactamases (ESBLs), AmpC ß-lactamases (AmpCs), oxacillinases (OXA-1t, OXA-2t, OXA-10t, OXA-20t, OXA-23t, OXA-24t, OXA-46, OXA-48, OXA-51t and OXA-58t), carbapenemases (GES, IMI, KPC, NMC, SME, GIM, IMP, SPM, VIM), aminoglycosides (ANT-2"Ia, ACC-3'-IIa, ACC-6'-Ia, ACC-6'-Ib, ACC-6'-Ic, ANT-4"-IIa, AAC-3'-Ia, AAC-3'-Ib, AAC-2'-Ia), and quinolones (parC, gyrA were determined by PCR and sequencing (Macrogen Inc, Seoul, Korea) using the primers described in table 1 or in Aragon *et al.*[8]

Outer membrane proteins (OMPs). OMP extracts obtained and electrophoresed as previously described[9]

Mechanisms of efflux. The growth inhibition assays were performed as described[10] with some modifications. *P. aeruginosa* isolates were inoculated at 1 - 2 x 10^6 cfu/ml into tubes containing TSB with antibiotics at concentrations one-fourth the previously determined MIC, either in the presence or absence of PAßN (10 mg/L final concentration). Bacteria were incubated at 37ºC, and optical density values at a wavelength of 550 nm were registered over 8 hours. The OD_{550} values were measured after 7 h.

Combination of Antibiotics: Antibiotic powders were provided by their manufacturers (Ceftazidima Combino Pharm®, Spain; Meropenem AstraZeneca, Spain; Tobramycin sulfate Fagron® Ciprofloxacino Combino Pharm, Spain).

Synergy tests: Synergy testing was performed using the checkerboard[11] and the epsilometric (E-test) methods[12]. Determinations by E-test were performed by duplicate, since the variation of concentration range on E-test strips, a MIC-to-MIC placement of the strips was easier to perform and seemed to give a more accurate diffusion of the two drugs[12]. E-test strips were placed on the bacterial lawn sequentially, the first E-test strip (strip A) was incubated for 1 h at room temperature, then removed, and the second E-test strip (strip B) was added immediately over the imprint of the strip A. Plates were incubated for 18 h at 37°C. Respective MIC strips/scales were used to read MICs by placing them in each gradient's position.

The summation operator Fractional Inhibitory Concentration Index (FICI) was calculated for each set of MICs, and the mean FICI was used to compare with the checkerboard test. High-off scale MICs (>256 mg/L) were converted to the next two-fold dilution (512 mg/L). The following formulas were used to calculate the FICI: (i) FIC of drug A = MIC of drug A in combination/MIC of drug A alone; (ii) FIC of drug B = MIC of drug B in combination/MIC of drug B alone; (iii) FICI = FIC of drug A + FIC of drug B. Synergy was defined by a FICI $\leq$ 0.5. Antagonism was defined by a FICI $\geq$4. Values of FICI between 0.5 and 1 were termed additive and those from1 to 4 indifferent[13].

The Susceptible Breakpoint Index (SBPI)[14] was also calculated. SBPI= (susceptible breakpoint A/MIC of A in combination) + (susceptible breakpoint B/MIC of B in combination). A SBPI of 2 indicates that the MICs of antimicrobials A and B in combination are either equivalent to their respective breakpoints or that the combination MIC of one of the antimicrobials is lower than its susceptible breakpoint.

Time-kill analysis: Time-kill assays were performed by the broth macrodilution technique[11] only for those antibiotic combinations showing synergy by both checkerboard and E-test. Each organism was tested against each antimicrobial agent, alone and in combination. The combinations tested against each organism were the β-lactam (CAZ or MEM) with TOB (i.e., CAZ plus TOB, MEM plus TOB). The concentrations of each antimicrobial agent tested alone or in combination were 1, 1/2, 1/4 and 1/8 of MIC values. Volumes of 10 mL tubes inoculated at 6 x 10^5 cfu/ml. were incubated at 37ºC aliquots of 0.1 ml were withdrawn from each tube at 0, 6, and 24 h, and 10-fold dilutions were prepared and inoculated onto blood agar plates. Plates were incubated for 24 h/48 h at 37ºC and colony counted, lower limit of detection was 40 cfu/ml.

Synergy was defined as decreases $\geq 10^2$ cfu/ml ($\geq$2-log 10) at 6 or 24 h in the combination compared with that of the most active single agent. Indifference as a $\leq$10-fold change in colony count at 6 or 24 h in the combination compared with that of the most active single agent and Antagonism as a 100-fold increase in colony count at 6 or 24 h in the combination compared with that of the most active drug alone. Bactericidal activity was defined as a $\geq$ 3 log10 cfu/ml decrease in the starting inoculum. *P. aeruginosa* ATCC 27853 was used as quality control strain in all susceptibility tests by microdilution technique, in all checkerboards, time-kill tests, and in every lot of E-tests strips . ATCC 27853 is a susceptible strain (CAZ 1 µg/ml; MEM 0.25 µ/ml; TOB 0.5µg/ml and CIP 0.25 µg/ml)

3. Results

In order to determine phenotypical susceptibility of the isolates studied, MicroScan® determination of MIC values (mg/L) gave the following results: Aztreonam 16; Ceftazidime 16 and >16; Cefepime 16; Piperacilline + tazobactam 32 and 64; Imipenem 8 and >8; CIP >2;

Gentamicin >8; TOB >8; Amikacine 8 and 16; Colistine ≤2 mg/L.

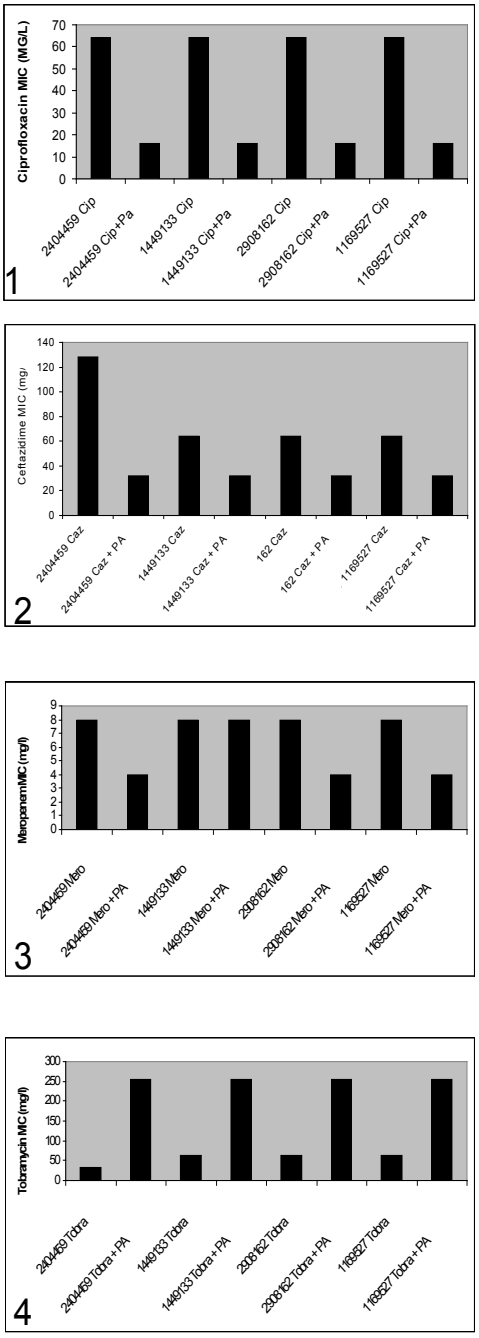

Figure 1. Effect of inhibition of efflux by the efflux pump inhibitor (EPI) PAßN on (1)ciprofloxacin susceptibility; all the strains were tested over the same range of ciprofloxacin concentrations (0.125 to 128 µg/ml), and showed 4-fold decrease in MIC with the EPI; (2) ceftazidime the strains were tested over the same range of ceftazidime concentrations (0.125 to 128 µg/ml), and showed 2 and 4-fold decrease in MIC with the EPI; (3) meropenem susceptibility, strains were tested over the same range of meropenem concentrations (0.125 to 128 µg/ml), and showed 0 and 2-fold decrease in MIC with the EPI and (4) tobramycin susceptibility. All the strains were tested over the same range of tobramycin concentrations (0.125 to 128 µg/ml), in this case the antagonism between tobramycin and the EPI does not allow to measure the effect

The genetic relationship between four selected representative multiresistant *P. aeruginosa* explored by PFGE. show identical pattern. All belonged to the O:4 serotype. In all of the isolates same resistance mechanisms were detected: bla-OXA-1-type, bla-OXA-2-type, ant(2")-Ia and ant(4")-IIb. Moreover two mutations on parC (Leu87Trp and Leu168Gln) one in gyrA (Asn87Asp) as well as an important reduction of OmpD porin expression were detectedThe mechanisms of resistance active in Gramnegative bacteria involve different biochemical machineries. In general multidrug-resistant isolates are characterized by over-expression of efflux pumps. Efflux pumps are unspecific, and can act pumping out several antimicrobials and other foreign molecules. Thus, we have explored the presence and activity of extrusion machineries in our isolates. Fig 1 shows the effect of inhibition of efflux by the efflux pump inhibitor (EPI) PAßN on (1) CIP susceptibility showing 4-fold decrease in MIC with the EPI; (2) CAZ susceptibility to which 2 and 4-fold decrease in MIC with the EPI were measured; (3) MEM susceptibility demonstrating 0and 2-fold decrease in MIC with the EPIand finally (4) TOB in this case the antagonism between TOB and the EPI does not allow to measure the effect.

By both checkerboard and E-test, combinations of CAZ and MEM with TOB resulted to be synergistic against the isolates (Table 2). The combinations with CIP resulted to be additive or indifferent by the two methods, in the two isolates tested (Table 2). SBPI values were in concordance with FICI interpretation except in MEM + TOB combination.

Time-kill experiments done showed in two of the isolates synergy in the TOB-β-lactam combinations. CAZ+TOB as well MEM+TOB combinations showed synergy and bactericidal activity at different concentrations (Table 3).

4. Discussion

In our hospital a prolonged endemism of MR *P. aeruginosa* has been observed. Colistine resistant or intermediate isolates were encountered, albeit in low number; the treatment of infections caused by such isolates is difficult and nowadays restricted to colistine and amikacine.

On the other hand we tried to validate synergy testing by E-test as a rapid and easier tool to demonstrate synergy compared with the CB. Studied isolates showed resistance against all available antipseudomonal antibiotics except amikacin and colistine. Antibiotics for synergy testing were selected after reviewing the literature on the potentially active and less toxic antibiotic combinations for MR *P. aeruginosa*.

The resistance pattern and the lack of carbapenemases and extended spectrum betalactamases suggested that resistance was mainly due to a derepresion of AmpC enhanced by OXA type betalactamases , up-regulation efflux system showed by the pump inhibitor effect of PAßN, reduction of OprD expression and alteration of topoisomerases and two aminoglycoside-modifying enzymes encoding resistance to kanamycin, gentamycin and tobramycin.

Table 2. In vitro interaction between ceftazidime and meropenem with tobramycin and ciprofloxacin, expressed by means of FICI (checkerboard and E-test methods) and SBPI. FICI: Synergy ≤ 0.5; Additive >0.5 -1 Indifferent: >1 - <4; Antagonism ≥4; b: SBPI: Susceptible Breakpoint Index: SBPI CLSI/ SBPI EUCAST if different values were present; ND: Not done

Isolate number	CAZ+TOB			MEM+TOB			CAZ+CIP			MEM+CIP		
	E-test		Check. test	E-test		Check. test	E-test		Check. test	E-test		Check. test
	[a]FICI	[b]SBPI	FICI	FICI	SBPI	FICI	FICI	SBPI	FICI	FICI	SBPI	FICI
1449133	0.36	2.12	0.34	0.35	1.33/0.83	0.31	1-1.5	0.69/0.67	1	>1	≤0.19/≤0.17	1
2404459	0.25	2.12	0.25	0.41	1.12/0.62	0.5	ND	ND	ND	ND	ND	ND
1169527	0.31	1.83	0.37	0.25	1.66/0.99	0.37	2	0.26/0.25	1	1-1.5	0.19/0.17	1
2908162	0.29	2.33	0.37	0.5	1.12/0.62	0.37	ND	ND	ND	ND	ND	ND

Synergy, was observed in combinations of CAZ and MEM with TOB both by E-test and checkerboard test. In the present study, as stated by other authors[12] , checkerboard and E-test methods yielded equivalent results, making the E-test, by its simplicity, a suitable method in clinical laboratories for synergy studies. The SBPI values were in concordance with FIC index in all combinations but for MEM+TOB. In some combinations SPBI values were higher when CLSI susceptibility breakpoints instead EUCAST[http://www.eucast.org/clinical_breakpoints] were used. The SBPI was more discriminatory than FIC because it uses the susceptible breakpoint MICs and likely has more clinical relevance[14].

Time-kill results showed synergy in most of the combinations. Bactericidal activity was also observed in a lesser extent. Synergy and bactericidal activities observed with TOB concentrations lower than MIC values when combined with CAZ or MEM at 1 MIC. The bactericidal activity at 6 hours but not at 24 hours observed in most of meropenem combinations could be the consequence of meropenem degradation and the low TOB concentration that can't prevent regrown.

In *P. aeruginosa*, synergistic activities between β-lactam and aminoglycosides were described previously[15, 16], but variable synergy rates were described depending on the β-lactam antibiotic and on the characteristics of the *P. aeruginosa* strains included (susceptible, resistant or multiresistant strains). The mechanism of synergy between β-lactam and aminoglycosides is believed to be due to the increase of aminoglycoside penetration due to the activity of the β-lactam and, in turn, to the increase of entry of the β-lactam due to the cationic displacement caused by aminoglycosides[17]. The mechanisms of resistance have a critical role in the interaction of the different antibiotics and the absence of synergy observed in the ciprofloxacin combinations, unlike other studies, may be due to the presence of a resistance mechanism in our isolates[18]. It should be noticed that in our experiments synergy with antibiotics whose resistance mechanisms are chromosomally encoded resulted to be less evident that for those antibiotics whose resistance mechanisms are related to acquisition of genetic material.

Meropenem is commonly prescribed in nosocomial infections when the presence of *P. aeruginosa* is suspected. A peak plasma concentration of 53-62 mg/L were yielded after a dose of 1g meropenem in ICU patients[19], which is higher than all MIC values observed in the present study. Meropenem shows a time-dependent killing above MIC activity and its administration in continuous infusion could improve clinical results. Both strategies have shown concentrations above MIC in clinical studies[20]. Only maximum serum concentrations of ceftazidime administered in intermittent intravenous administration in human experiences[21] were in excess of the all MIC values of the studied isolates.

Table 3. Time-kill results at 24 h. Antibiotic combinations expressed as MIC's fractions Synergy: S; Bactericidal activity: B; No synergy: NS; No Bactericidal: NB. Microdilution MIC values (mg/L): 1449133 CAZ 64, MEM 8, TOB 128; 1169527: CAZ 16, MEM 8, TOB 64; Synergy: ≥2-log $_{10}$ decrease in colony count in the combination compared with that of most active single agent; Bactericidal: ≥ 3 log $_{10}$ cfu/ml decrease in the starting inoculum. a: Synergy at 6 h; b: Bactericidal at 6 h

Antibiotic combinations	Isolate number			
	1449133		1169527	
1 TOB + 1 CAZ	S	B	S	B
1/2 TOB + 1 CAZ	S	B	S	B
1/4 TOB + 1 CAZ	S	B	S	B
1/4 TOB + 1/2 CAZ	S	NB	S	NB
1/8 TOB + 1 CAZ	S	B	S	NB
1 TOB + 1 MEM	S	B	S	B
1/2 TOB + 1 MEM	S	NB	S	B
1/4 TOB + 1 MEM	NS[a]	NB	S	NB[b]
1/8 TOB + 1 MEM	NS	NB	S	NB[b]

The pharmacodynamic profile of the aminoglycosides has been characterized both in vitro and in vivo. Since these antibiotics eliminate bacteria more rapidly when their concentrations are above the MIC of the bacteria, their killing activity is referred to as concentration or dose-dependent bactericidal activity[22]. Concentrations of

8 to 10 times MIC value have been proposed to achieve the optimal bactericidal activity of aminoglycosides[23]. Once-daily dose of nearly 7mg/Kg tobramycin has been associated with peak concentrations of 30 mg/L in adult and pediatric patients with cystic fibrosis[24, 25].

This concentration matches with 1/2, 1/4 and 1/8 MIC values of tobramycin included in our in vitro study. Synergy and bactericidal activity against 1449133 P. aeruginosa isolate was mainly observed with these MIC tobramycin values combined with 1 MIC ceftazidime at 6h and 24h assay. However, tobramycin and meropenem combinations showed better results at 6 h, possibly, as stated above, as a consequence of meropenem degradation.

Recently, a mathematical simulation in combination therapy based on quantitative methods in a neutropenic murine pneumonia model have shown high consistence with the predictions of this *in vitro* model[26] .

Other in vitro studies as antibiotic degradation, more antibiotic combinations and also more isolates with different resistance mechanisms are in progress before initiating the studies in animal models in order to find a combination active on *P. aeruginosa* MR.

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