

In-vitro* Efficacy of Ceftazidime in Combinations with Meropenem, Piperacillin/Tazobactam, Amikacin and Ciprofloxacin against Extensively Drug-Resistant *Pseudomonas aeruginosa

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Abstract.- Combination therapy is preferred against *P. aeruginosa* to make better use of available antimicrobials, subject to the in-vitro evaluation. The activity of combinations is variable depending on the organismal behaviour in different geographical areas. In this study synergistic efficacy of ceftazidime in combinations with meropenem, piperacillin/tazobactam, amikacin and ciprofloxacin was evaluated against XDR (n = 12) and SPT (n = 12) clinical strains of *P. aeruginosa*. In-vitro activity of antimicrobial combinations was determined by broth microdilution checkerboard technique. CAZ-AK combination exhibited synergism against 58.3% and 16.7% clinical strains of XDR and SPT *P. aeruginosa*, respectively. CAZ-MEM combination exhibited synergism against 8.3% clinical strains of XDR *P. aeruginosa*. CAZ-AK combination remained synergistically effective against XDR *P. aeruginosa* (p-value= 0.001).

Keywords: Antimicrobial combinations, multidrug-resistant *P. aeruginosa*, drug synergism and antagonism.

INTRODUCTION

Pseudomonas aeruginosa is the sixth most important nosocomial pathogen worldwide and is one of the most common causes of ventilator-associated pneumonia, bacteremia and urinary tract infections (Gaynes *et al.*, 2005). In cases of cystic fibrosis this organism remains on top of the list for causing respiratory tract infections (Marshall and Hazle, 2011). Hence, patients especially those on breathing machines, having in-dwelling urinary catheters, intubated, with surgical or burn wounds or cystic fibrosis are potentially at risk for serious and life-threatening infections (Defez *et al.*, 2004; Kohlenberg *et al.*, 2010). In Pakistan during 2008, *P. aeruginosa* caused 28.7% respiratory tract infections, 16.2% UTIs and 31.6% blood stream infections in ICU patients (Akhtar, 2010). The scarcity of new antipseudomonal drugs and the ability to develop resistance even during treatment leads to high morbidity and mortality. To designate extensively drug-resistant *P. aeruginosa* (XDR), the organism must be resistant to at least one agent in

six classes of antipseudomonal drugs. Emergence and spread of XDR *P. aeruginosa* is a serious global concern (Aziz *et al.*, 2006). In order to avoid resistance development and to achieve synergistic action, the use of antimicrobial combinations may have to be relied upon for therapeutic success. In-vitro evaluation of antimicrobial combination provides valuable insight into the drug interactions (White *et al.*, 1996). However, there is no general agreement about the synergistic efficacy of a combination against *P. aeruginosa*; different studies have reported different rates of synergism for the same combination against clinical strains of different geographic regions (Oie *et al.*, 2003; Fujimura *et al.*, 2009). The selection of antimicrobial combinations and the evaluation technique are the main factors that play role in the interpretation and reproducibility of the interactions (Cappelletty and Rybak, 1996). This study was aimed at exploring effects of routinely available antimicrobials in various combinations against the indigenous strains of *P. aeruginosa* in Pakistan.

MATERIALS AND METHODS

Bacterial strains

Twenty four clinical isolates of *P. aeruginosa*, urine (n= 8), blood (n= 3), sputum (n=

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0030-9923/2014/0004-1111 \$ 8.00/0

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6) and pus (n= 7) obtained from two tertiary care hospitals in Lahore, Pakistan were investigated. The strains were identified by their morphology, culture characteristics and their biochemical profile using API 20NE (bioMerieux, France). *P. aeruginosa* (ATCC® 27853) and *Escherichia coli* (ATCC® 25922) were included as standard strains. The strains were then preserved in Microbank™ vials (Pro-Lab Diagnostics, UK) at $-80\pm 5^{\circ}\text{C}$.

Antimicrobial Agents

Six antimicrobial discs *i.e.*, piperacillin/tazobactam (TZP, 100/10 $\mu\text{g/ml}$), amikacin (AK, 30 $\mu\text{g/ml}$), ciprofloxacin (CIP, 5 $\mu\text{g/ml}$), ceftazidime (CAZ, 30 $\mu\text{g/ml}$), meropenem (MEM, 10 $\mu\text{g/ml}$) and aztreonam (ATM, 30 $\mu\text{g/ml}$) were used. The base material of antimicrobial drugs was obtained from GlaxoSmithKline and Searle Company limited Pakistan. The stock solutions of antimicrobials were prepared in recommended solvents and diluents according to CLSI and stored at $-80\pm 5^{\circ}\text{C}$.

Antimicrobial susceptibility testing

It was performed by standard Kirby-Bauer disc diffusion method. Antimicrobial discs were applied on the semi-confluent lawn of pure organism on Mueller-Hinton agar plates (Oxoid, UK) aseptically according to the “Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard.—Eleventh Edition” (M02-A11; 2012) published by Clinical and Laboratory Standards Institute (CLSI, USA). The inoculated plates were read for zones of inhibition after 18 h of incubation at $35\pm 2^{\circ}\text{C}$. The zones of inhibition were interpreted as per the breakpoints given in the guidelines of “Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement” (M100-S23; CLSI 2013). The strains of *P. aeruginosa* which were non-susceptible (intermediate or resistant) to all six antimicrobial agents were considered as XDR while those which were susceptible to these, were designated as susceptible (SPT) (Magiorakos *et al.*, 2012).

Determination of MICs

MICs of the strains for each of the

antimicrobial were determined by broth microdilution method using 96-well microtitre plates. The bacterial inoculum was prepared equivalent to 0.5 McFarland (approx. 1.5×10^8 colony forming units (CFU)/ml). The inoculum was further diluted 1:100 by cation-adjusted Mueller-Hinton broth (CA-MHB) to achieve the final inoculum (approx. 1.5×10^6 CFU/ml) and 10 μl of this was added to 100 μl of CA-MHB. The plates were incubated at $35\pm 2^{\circ}\text{C}$ for 18 h aerobically as described by “Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition” (M07-A9, 2012). The concentration ranges evaluated were 1-1024 $\mu\text{g/ml}$, 0.25-64 $\mu\text{g/ml}$, 2-512 $\mu\text{g/ml}$, 0.0625-32 $\mu\text{g/ml}$ and 2-512 $\mu\text{g/ml}$ for ceftazidime, meropenem, amikacin, ciprofloxacin and piperacillin/tazobactam, respectively. The presence or absence of turbidity in wells was noted after incubation and interpreted by MIC criteria given by CLSI supplement 2013.

Broth microdilution checkerboard technique

The activity of four antimicrobial combinations *i.e.*, ceftazidime-piperacillin/tazobactam (CAZ-TZP), ceftazidime-meropenem (CAZ-MEM), ceftazidime-ciprofloxacin (CAZ-CIP) and ceftazidime-amikacin (CAZ-AK) was evaluated by broth microdilution checkerboard technique (Verma, 2007). The concentration ranges of antimicrobial drugs tested were taken as $1/16$ times of MIC to 4 times of MIC for each clinical strain. Two fold serial dilutions of antimicrobials were prepared in separate sterile tubes and 50 μl of each drug was added to rows and columns of 96-well microtitre plates except row (A) and column (1). In row (A) and column (1) 100 μl of only one drug was added. Then 10 μl of diluted bacterial inoculum (1:100 diluted in CA-MHB) was added in each well except H12 to achieve the final inoculum (approx. 1.5×10^5 CFU/ml). The wells A1 (no drug added) and H12 (no strain added) were taken as growth and sterility control, respectively. The inoculated plates were incubated at $35\pm 2^{\circ}\text{C}$ for 18 h aerobically. After incubation the presence or absence of turbidity in wells was interpreted as growth and no growth for the drug combination and interpreted for fractional inhibitory concentration index (ΣFIC). This was

equal to the sum of the FICs for individual drugs in combination. The FIC for a drug was defined as the MIC of the drug in combination divided by the individual MIC of the drug. ΣFIC_{\min} for a drug combination is the minimum value of ΣFIC that inhibited organism while the ΣFIC_{\max} for a drug combination is the highest value of ΣFIC which inhibited the organism. ΣFIC values of ≤ 0.5 were considered synergistic, >0.5 to 4.0 were indifferent and > 4.0 were antagonistic.

Statistical analysis

Statistical analysis of different combinations was done by Chi square using IMB SPSS 20 software.

RESULTS

The values of MIC_{90} of ceftazidime, amikacin, meropenem, piperacillin/tazobactam and ciprofloxacin for XDR *P. aeruginosa* strains were 1024, 128, 256, 128 and 32 $\mu\text{g/ml}$, respectively while for susceptible *P. aeruginosa* strains were 4, 0.5, 2 and 0.5 $\mu\text{g/ml}$, respectively. The values of MIC range, MIC_{50} , and mode for XDR and SPT clinical strains of *P. aeruginosa* (Table I).

Values of ΣFIC_{\min} and ΣFIC_{\max} for clinical strains of XDR and SPT *P. aeruginosa* are given in table II and describe the spectrum of activity of drug combinations. Among XDR *P. aeruginosa*, CAZ-AK combination exhibited synergism in seven (58.3%) clinical strains while the CAZ-MEM combination exhibited synergism in one (8.3%). CAZ and AK inhibited synergistically at concentration ranges of 4 to 256 $\mu\text{g/ml}$ and 4 to 32 $\mu\text{g/ml}$, respectively. Among the SPT *P. aeruginosa* strains, the CAZ-AK combination produced synergism in two clinical strains (16.7%). The combinations of ceftazidime with ciprofloxacin and piperacillin/tazobactam produced no synergism for XDR or SPT clinical strains of *P. aeruginosa*. None of the combinations exhibited antagonism in either group (Table II).

CAZ-AK combination produced statistically significant synergism against clinical strains of XDR *P. aeruginosa* ($p\text{-value} = 0.001$). No other combination was statistically significant against

clinical strains of XDR or SPT *P. aeruginosa* ($p\text{-value} = 0.234$) (Table III). The synergism produced by CAZ-AK combination against clinical strains of XDR *P. aeruginosa* was statistically insignificant than that of produced against clinical strains of SPT *P. aeruginosa* ($p\text{-value} = 0.089$) (Table IV).

DISCUSSION

The broth microdilution checkerboard (not needed) is generally a convenient, reliable and an economical method. There is a 44% to 88% agreement of checkerboard with the time kill assay (White *et al.*, 1996).

Among the four combinations evaluated, CAZ-AK resulted in moderately high rates of synergism in XDR and SPT clinical strain of *P. aeruginosa*. This in-vitro efficacy of β -lactams and aminoglycosides correlates well with other studies (Mayer and Nagy, 1999; Fujimura *et al.*, 2009; Dundar and Otkun, 2010). However, the CAZ-AK combination was more synergistic for XDR than for SPT clinical strains. The synergism was independent of whether the organism was individually resistant or susceptible to the drugs in combination ($p\text{-value} = 0.089$). It has been reported by many researchers (Dundar and Otkun, 2010).

Combination of ceftazidime with other β -lactams *i.e.*, piperacillin/tazobactam and meropenem remained synergistically less effective and only low rate of synergism was observed with the later. Many studies failed to report any significant rate of synergism with β -lactam- β -lactam combinations yet Fujimura *et al.* (2009) reported 14.3% antagonism against MDR *P. aeruginosa*.

β -lactam and fluoroquinolones combination (CAZ-CIP) could not exhibit any synergism against clinical strains of XDR or SPT *P. aeruginosa* as reported by others (Song *et al.*, 2003). Interestingly Fish *et al.* (2002) observed that the CAZ-CIP combination exhibited synergism in 80 % of strains of *P. aeruginosa*. This very high rate of synergism is probably attributed to the use of Time kill assay (Fish *et al.*, 2002).

Although in-vitro synergistic combinations have good correlation with clinical outcome yet clinically achievable plasma level of the drugs is an important limitation for their therapeutic use. The

Table I.- Values of MIC₅₀, MIC₉₀, MIC range and mode for clinical strains.

Antimicrobial drugs	XDR <i>P. aeruginosa</i> (µg/ml)			SPT <i>P. aeruginosa</i> (µg/ml)		
	MIC ₅₀	MIC ₉₀	Mode	MIC ₅₀	MIC ₉₀	Mode
CAZ	128	1024	128	2	4	4
AK	64	128	64	4	4	4
MEM	16	256	16	0.5	1	0.5
TZP	32	128	32	2	8	2
CIP	32	32	32	0.5	1	0.5

CAZ, ceftazidime; AK, amikacin; MEM, meropenem; TZP, piperacillin/tazobactam; CIP, ciprofloxacin; MIC₅₀: MIC that inhibits 50 % of strains, MIC₉₀: MIC that inhibits 90 % of strains.

Table II.- Comparative values of ΣFIC for clinical strains.

Drug combinations	XDR <i>P. aeruginosa</i> (n= 12)		SPT <i>P. aeruginosa</i> (n= 12)	
	ΣFIC _{min}	ΣFIC _{max}	ΣFIC _{min}	ΣFIC _{max}
CAZ-AK	0.25	1.0	0.50	0.75
CAZ-MEM	0.50	1.5	0.75	2.12
CAZ-TZP	0.56	1.0	0.56	1.0
CAZ-CIP	0.56	1.5	0.62	2.0

ΣFIC, fractional inhibitory concentration index

For other abbreviations see Table I.

Table III.- Comparative synergistic efficacies of various combinations against XDR and SPT clinical strains of *P. aeruginosa*

Clinical strains	Activity	CAZ-AK	CAZ-TZP	CAZ-CIP	CAZ-MEM	<i>p</i> -value
		n	n	n	n	
XDR <i>P. aeruginosa</i> (n = 12)	Synergism	7	0	0	1	0.001
	Indifference	5	12	12	11	
SPT <i>P. aeruginosa</i> (n = 12)	Synergism	2	0	0	0	0.234
	Indifference	10	12	12	12	

For abbreviations see Table I.

Table IV.- Association of CAZ-AK Combination with XDR and SPT *P. aeruginosa*.

Synergistic combination	Activity	XDR <i>P. aeruginosa</i> (n=12)	Susceptible <i>P. aeruginosa</i> (n=12)	<i>p</i> -value
		CAZ-AK	Synergism	
	Indifference	5	10	

For abbreviations see Table I.

peak plasma concentration of ceftazidime after 1g intravenous dose is 107µg/ml (Song *et al.*, 2003). In our study, ceftazidime inhibited 58.3% of XDR

clinical strains with a concentration range of 4µg/ml to 64µg/ml in synergistic combinations with amikacin. The synergistic concentration range of

ceftazidime is well below its peak plasma concentration. However, in β -lactam drugs the in-vivo bactericidal effect, being time-dependent, is achieved when the drug concentration is over MIC for the strain. But due to the short half-life of 2 h for ceftazidime, the levels above MIC are not sustainable. The peak plasma concentration of amikacin is 56 $\mu\text{g/ml}$ to 64 $\mu\text{g/ml}$ for a 15mg/kg intravenous dose (Song *et al.*, 2003). The amikacin in combination with ceftazidime synergistically inhibited all XDR strains with concentration range of 4 $\mu\text{g/ml}$ to 16 $\mu\text{g/ml}$. In our study, amikacin concentrations are achievable in plasma. However, aminoglycosides exhibit concentration-dependent bactericidal activity and 8 to 10 times of MIC was required to demonstrate maximum killing activity (Segura *et al.*, 2013).

It is concluded that the CAZ-AK combination has demonstrated an effective in-vitro synergism against extensively drug-resistant *P. aeruginosa* by broth microdilution checkerboard method. It is further concluded that the synergism demonstrated by CAZ-AK combination is independent of whether the clinical strains of *P. aeruginosa* are XDR or SPT. However, combinations of ceftazidime with piperacillin/tazobactam, meropenem and ciprofloxacin are concluded as indifferent for XDR clinical strains of *P. aeruginosa*.

ACKNOWLEDGEMENTS

We are grateful to University of Health Sciences, Lahore, Pakistan for funding this research and GlaxoSmithKline and Searle Company Limited Pakistan for the provision of base materials. We are grateful to the Prof. Dr. Mateen Izhar, Sheikh Zayed Hospital Lahore, Pakistan and Dr. Farhan Rasheed, Combined Military Hospital, Lahore, Pakistan for the provision of the clinical strains.

Conflict of interest

There is no conflict of interest.

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(Received 26 April 2014, revised 22 May 2014)