

Original Article

In vitro efficacy of synergistic antibiotic combinations in imipenem resistant *Pseudomonas aeruginosa* strains

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Abstract:

The increase in antibiotic resistance coincided with the decline in production of new antibiotics. Combination antibiotic treatment is preferred in nosocomial infections caused by multidrug resistant *Pseudomonas aeruginosa*. In vitro synergism test by agar dilution method were used to choose the combinations which might be used in clinic. The aim of this study was to investigate the synergistic efficacy of antibiotic combinations in imipenem resistant *P. aeruginosa* strains. Carbapenem resistance (imipenem and meropenem) was determined by disk diffusion method. Among isolated *P. aeruginosa* 44.9% were carbapenem resistant. The MIC of drugs among 25 imipenem resistant isolates ranged from ≥ 256 mg/L to ≤ 8 mg/L for imipenem, ≥ 1024 mg/L to ≤ 64 mg/L for ceftriaxone, ≥ 256 mg/L to ≤ 8 mg/L for amikacin, ≥ 16 mg/L to ≤ 2 mg/L for colistin, ≥ 512 mg/L to ≤ 16 mg/L for piperacillin/tazobactam. Among antibiotic combinations, piperacillin/tazobactam-amikacin was most effective with 80% synergism next to which was imipenem-amikacin with 60% synergism, then imipenem-colistin with 50% synergism, imipenem-ceftriaxone with 30% synergism. Only one combination (piperacillin/tazobactam -imipenem) showed 20% antagonism. All these combinations had considerable proportion of additive effect which is also desirable for these multi drug resistant isolates

Key Words: Antagonistic combinations, Antibiotic combination, Imipenem resistance, MIC, *P. aeruginosa*, Synergistic combinations.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) are among the major nosocomial pathogens and are able to demonstrate particularly all known enzymatic and mutational mechanism of bacterial resistance. In addition, *P. aeruginosa* is able to acquire other drug-resistance determinants by horizontal transfer of mobile genetic elements coding for class B carbapenamases, called metallo- β -lactamases or MBLs, which hydrolyze all β -lactams except aztreonam.¹ Carbapenems are often used as last resort against multi-drug resistant bacteria. Resistance to carbapenem is due to decreased outer membrane permeability, increased efflux system, alteration of penicillin binding proteins and carbapenamases.² Treatment of infections caused by these resistant bacterial pathogens relies on two therapeutic modalities: development of new antimicrobials and combination of available antibiotics. Until better antibiotics are being developed, novel antibiotic combination that yield some in vitro activity are perhaps the

best resources. Combination antibiotic treatment provide larger spectrum antimicrobial effect, prevent the rapid emergence of resistance strains, decrease dose-related toxicity by using reduced dose of both drugs, enhanced inhibition of microorganisms.³ The aim of this study was to determine the in vitro effects of some antimicrobial drug combinations on imipenem resistant *P. aeruginosa*.

Materials and methods

Bacterial Isolates

Study isolates were chosen from nosocomial *P. aeruginosa* isolates collected from July 2011 to December 2012 from burn unit of Dhaka Medical College Hospital, Dhaka, Bangladesh. A total of 98 non-duplicate isolates of *P. aeruginosa* were included in the study. The isolates were cultured from burn wound and stored at -70° C and studied after being sub-cultured on MacConkey agar media. Approval was obtained from research review committee (RRC) and ethical review committee (ERC) of Dhaka Medical College. Forty four isolates were resistant to carbapenem (imipenem or meropenem) by disk diffusion technique (according to Clinical and Laboratory Standards Institute [CLSI] guidelines). Among them ten imipenem resistant *P. aeruginosa* were randomly chosen for combination study.

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Identification of species among the imipenem resistant isolates

Samples collected from burn wound were inoculated on MacConkey agar media and blood agar media. From the lactose non fermenting colonies on MacConkey agar media, isolates were identified as *P. aeruginosa* if they were (i) oxidase positive (ii) a triple sugar iron (TSI) agar reaction of alkaline over no change (iii) motile, indole and urease negative in motility-indole-urea (MIU) agar media (iv) citrate utilized in simmons citrate agar media and v) grew at both 37°C and 42°C. Additional bacterial characteristics including its Gram stain, colony morphology, hemolytic criteria and pigment production were also used to identify the species.

Antimicrobial agents

Antibiotic powders were obtained from manufacturers as follows: Ceftriaxone (Square pharmaceuticals, Dhaka, Bangladesh), imipenem (Reneta Limited, Dhaka, Bangladesh), amikacin injection (ACI Limited, Bangladesh), colistin (Forest Laboratories UK Limited), piperacillin/tazobactam (Popular Pharmaceuticals Limited, Tongi, Bangladesh). Stock solutions were prepared using sterile distilled water and stored at -20°C until use.

Susceptibility test

Following CLSI guidelines,⁴ the antimicrobial susceptibility pattern was determined by disk-diffusion technique using commercially available antibiotic disks (Oxoid, Hampshire, UK) (CLSI, 2010). *P. aeruginosa* ATCC 27853 was used for quality control. The minimum inhibitory concentration of ceftriaxone, imipenem, amikacin, colistin, piperacillin/tazobactam were determined by agar dilution method,⁵ and CLSI criteria were used in the interpretations of the results. Serial two fold dilutions, ranging from 64 to 1024 mg/L for ceftriaxone, 8 to 256 mg/L for imipenem, 16 to 256 mg/L for amikacin, 0.5 to 16 mg/L for colistin, 16 to 512 mg/L for piperacillin/tazobactam were prepared in Mueller Hinton agar media. The inoculum was prepared by bacterial suspension of each isolate in normal saline, adjusted to a turbidity equivalent to 0.5 McFarland standard and diluted 10 times to give a final concentration of 10⁴ cfu/spot. One µl of 10 times diluted inoculum was placed on Mueller Hinton agar plate and incubated at 37°C overnight. MIC was defined as the lowest concentration of antibiotic to completely inhibit visible growth.

Synergy studies

In vitro interactions of ceftriaxone-imipenem, amikacin-imipenem, colistin-imipenem, piperacillin/tazobactam-amikacin were investigated by agar dilution method. For each plate 25 ml Mueller Hinton

medium was prepared. For each sample four plates were prepared. The first plate contained two fold higher dilutions than the MIC of the two drugs in combination for that isolate, the second plate contained MIC of antibiotics in combination, the third plate contained two fold lower dilutions than the MIC of both antibiotics, and fourth plate contained fourfold lower dilution than the MIC of both antibiotics for that sample. After incubation at 37°C overnight, synergy was present by agar dilution method when there was a fourfold or greater reduction in MICs of both antibiotics. A reduction of less than fourfold in MICs of both antibiotics was considered additive. Indifference was considered when neither drug exhibited a decrease in MIC, and an increase in MIC was considered antagonism.

Statistical method

Data were analyzed by using Microsoft Excel (2007) software (Microsoft, Redmond, WA, USA).

Result

A total 98 *P. aeruginosa* strains were isolated from patients over a 1.5 year period from burn unit of Dhaka Medical College Hospital, 44 (44.9%) of them was carbapenem resistant identified by disk diffusion test.

The MIC of drugs among 25 imipenem resistant isolates ranged from ≥ 256 mg/L to ≤ 8 mg/L for imipenem, ≥ 1024 mg/L to ≤ 64 mg/L for ceftriaxone, ≥ 256 µg/ml to ≤ 8 mg/L for amikacin, ≥ 16 mg/L to ≤ 2 mg/L for colistin, ≥ 512 to ≤ 16 mg/L for piperacillin/tazobactam.

Table1: MIC ranges, MIC50, MIC90 of the 25 imipenem resistant strains

Antibiotic	Imipenem resistant strain (n=25)		
	MIC (mg/L)		
	Range	50%	90%
IPM	8-256	32	256
CRO	64-1024	512	1024
AK	8-256	32	128
CT	0.5-16	4	8
PIP/TAZ	16-512	64	256

IPM, Imipenem; CRO, Ceftriaxone; AK, Amikacin
CT, Colistin; PIP/TAZ

Table 2 shows four fold or two fold reduction of MICs (synergy or additive effect) or no reduction of MIC (indifference) and an increase in MIC (antagonism) among 10 imipenem resistant isolates by the combination of two drugs among the five drugs. While combining imipenem with ceftiaxone, 30% showed synergism, combination of imipenem with amikacin showed 60% synergism, combination of imipenem with colistin showed 50% synergism, combination

of piperacillin/tazobactam with amikacin showed 80% synergism, combination of piperacillin/tazobactam with imipenem showed 40% synergism. Antagonism (20%) was observed only in piperacillin/tazobactam and imipenem combinations. The synergism of piperacillin/tazobactam plus amikacin was significantly higher ($p < 0.05$) than ceftriaxone plus imipenem.

Table 2: Agar dilution synergy results for different antimicrobial combination

Antibiotic	Imipemem resistant strain (n=10)			
	Synergistic n %	Additive n %	Indifferent n %	Antagonism n %
CRO +IPM	30%	50%	20%	-
AK + IPM	60%	30%	10%	-
CT +IPM	50%	30%	20%	-
PIP/TAZ + AK	80%	10%	10%	-
PIP/TAZ+ IPM	40%	10%	30%	20%

CRO, Ceftriaxone; PIP/TAZ, Piperacillin/Tazobactam; AK Amikacin; CT, Colistin, IPM, Imipenem

Antibiotic concentration at which synergistic interaction were observed in imipenem resistant strains are shown in Table 3. Concentration which provide synergy in resistant strain can predict clinically achievable limits of the drugs in combination.

Table 3: Synergistic antibiotic concentrations observed in imipenem resistant strains

Strain No. (n=10)	Concentration mg/L			
	CRO-IPM	AK-IPM	CT-IPM	PIM/TAZ-AK
D ₁	128-32	-	-	16-2
D ₂	256-64	16-64	1-64	16-16
D ₃	32-64	8-64	-	64-8
D ₄	-	32-16	0.25-16	-
D ₅	-	16-8	-	32-16
D ₆	-	11-16	4-16	64-16
D ₇	-	8-4	1-4	-
D ₈	-	-	2-32	64-16
D ₉	-	-	-	16-8
D ₁₀	-	-	-	32-32

CRO, Ceftriaxone; PIP/TAZ, Piperacillin/Tazobactam; AK Amikacin; CT, Colistin; IPM, Imipenem

Discussion

The world is facing a growing threat from multidrug-resistant Gram-negative "superbugs," such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*.^{6,7,8} This problem is compounded by a lack of novel antimicrobial agents in the drug development pipeline.^{7,8,9} Combination of antibiotics acting by different mechanisms is used for the treatment of MDR bacterial

infections. Combination therapy is recommended for the treatment of *P. aeruginosa* infections in order to ensure synergistic action and decrease the risk of development of resistance. The agar dilution method for susceptibility testing is regarded as the golden standard for all other susceptibility testing methods. It is extremely important to prepare agar plates in such a way that the obtained antimicrobial concentration in the plates is exactly or very close to the desired concentrations. Comparison of result from different combination is difficult due to variation in microbiology test materials, methods and synergy definitions.¹⁰ b-lactam and aminoglycoside combinations are most frequently used for the treatment of *P. aeruginosa*.¹¹ Synergistic interactions of these combinations have been reported in many studies.^{12,13,14} The present study detected 60% synergy, 30% additive and 10% indifference while combining imipenem and amikacin. In agreement with the present findings, earlier study observed 58% synergism, 40% additive effect, 2% indifference while combining meropenem with amikacin.¹⁵ Higher synergy (100%) was detected by Le et al¹⁶ by time-kill assay against four *Klebsiella pneumoniae* carbapenemase (KPC) strains. Piperacillin/tazobactam plus amikacin combination showed 80% synergism, 10% additive effect, and 10% indifference in the present study. Fujimura *et al*¹⁷ who performed checkerboard synergy test, also showed 95.9% synergism and 4.1% indifference in earlier study. Therefore, the combination of b-lactam and aminoglycoside is worth considering for imipenem resistant *P. aeruginosa*. Aminoglycoside permeabilization of the outer membrane increases b-lactam uptake thus increasing effectiveness of b-lactam aminoglycoside combinations.¹⁸ Colistin has significant *in vitro* antibacterial activity against gram negative "superbugs".^{19,20,21} Plasma colistin concentration are sometimes suboptimal with recommended dose regimen,^{22,23} increasing dose may cause nephrotoxicity.^{22,24,25} Colistin activity can be enhanced when combined with antibiotics with different action as carbapenems, rifampicin, ceftazidime.^{26,27,28} Present study observed 50% synergism, 30% additive effect, 20% indifference while combining colistin with imipenem. In accordance with the present study Souli *et al*²⁹ reported 56.3% synergism and 43.7% indifference. Combination of two b-lactam is less frequently reported but it may have broad antibacterial spectrum against gram negative bacilli.³⁰ In addition it may reduce incidence of nephrotoxicity.³¹ This study observed combination of ceftriaxone and imipenem showing, 30% synergism, 50% additive effect, 20% indifference. However Pasticci *et al*³² reported 60% synergism while combining ceftriaxone and ampicillin against *Enterococcus faecalis*. Combination of imipenem with piperacillin/tazobactam showed 40%

synergism, 10% additive, 30% indifference and 20% antagonism. Fujimura *et al*¹⁷ reported 10.2% synergism, 57.1% indifference and 32.7% antagonism which correlate with present findings. The antagonism might be due to fact that combination of two β -lactams might have induced β lactamase which might have accounted for antagonism³³. Bertam and young³⁴ demonstrated β -lactamase was induced in 21 of 28 strains which showed antagonism but could not establish the association. Present study observed 80% synergism with piperacillin/tazobactam plus amikacin combination and 30% with imipenem plus ceftriaxone, this difference is statistically significant ($p < 0.05$). All the other differences of synergistic effect were not statistically significant. In earlier studies Yamashiro *et al*³⁵ reported combination of piperacillin plus amikacin was more effective than combination of imipenem plus amikacin. β -lactam and aminoglycoside combinations were shown to be most effective combinations against imipenem resistant *P. aeruginosa*. MIC at which synergy was achieved can predict the clinically achievable plasma concentration of drugs in combination. Generally a margin of safety of ten times the MIC is desirable to ensure successful treatment of the disease.³⁶ In present study synergy was considered when there was two fold reduction of MICs, further lowering of MICs of both antibiotics was not done, so it will predict probable achievable rather actual achievable limit.

Conclusion

Pseudomonas aeruginosa was an important pathogen in burn wound infection among which carbapenem resistance is prevalent. Among the antibiotic combinations, piperacillin/tazobactam plus amikacin combination was most effective next to which is imipenem-amikacin combination, then imipenem-colistin and imipenem-ceftriaxone combination. All these combinations had considerable proportion of additive effects which is also desirable for these drug resistant isolates. Furthermore, there may not be a correlation between *in vitro* synergy and clinical efficacy. Therefore, additional *in vivo* studied to asses clinical efficacy of combinations are needed. Moreover, antimicrobial combination must be based on a sound knowledge about the effect of two or more drugs in combination to avoid possible untoward effect like antagonism.

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