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Antimicrobial Synergy Testing By Time-Kill Methods For Extensively Drug-Resistant Acinetobacter Baumannii Isolates.

Simit Kumar[1], *Maitreyi Bandyopadhyay[2*], Mohiruddin Sk[3], Prabir Kumar Mukhopadhyay[4], Manas Kumar Bandyopadhyay[5], Anindita Chatterjee[6], Mitali Chatterjee[7]

(Department of Microbiology, R.G.Kar Medical College, Kolkata / The West Bengal University of Health Sciences, India)
**Corresponding Author: Dr. Maitreyi Bandyopadhyay*

Abstract
Introduction:*The emergence of colistin or tigecycline resistance as well as imipenem resistance in Acinetobacter baumannii poses a great therapeutic challenge. Increasing reports of polymyxin heteroresistance have suggested that rapid resistance to polymyxins can develop upon treatment with polymyxin B monotherapy, especially upon exposure to subtherapeutic polymyxin concentrations. To circumvent this phenomenon, experts have advocated that polymyxins should be used in combination with one or more antibiotics for the treatment of carbapenem resistant isolates.*
Aims & Objectives: *The aim of this study is to assess the in vitro activity of different combinations of polymyxin B, rifampicin, meropenem and tigecycline against selected clinical isolates of A. baumannii .*
Materials & Methods: *Twelve representative imipenem-resistant A.baumannii clinical isolates were included in present study. Minimal inhibitory concentration (MIC) was determined using agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guideline.Time-kill studies were performed on four antimicrobial agents and combinations of these agents according to a previously reported method.*
Results: *Only polymyxin-B was consistently effective as a single agent against all 12 isolates, but showed bacterio-static activity when used singly. Among the combinations of 0.5 x MIC antimicrobial agents, the combination of polymyxin-B and tigecycline showed synergistic or bactericidal effects against 8 of the isolates. Antimicrobial combinations are effective for killing imipenem-resistant A. baumannii isolates, even if they are simultaneously resistant to either carbapenems or tigecycline.*
Discussion & Conclusions: *The Time-kill assay method used in this study can be used for individualized treatment of patients suffering from critical infections caused by extremely drug resistant Acinetobacter isolates. The use of these lower MIC values obtained from synergy studies can be as a guide to determine effective individualized therapeutic doses can help to decrease the emergence of resistance and can also minimize the side effects associated with using a single agent at a higher dose.*

Keywords : *Synergy Testing, Time-Kill Methods, MIC, Acinetobacter baumannii, polymyxin B, Heteroresistance*

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I. Introduction

Acinetobacter baumannii has emerged as an important nosocomial pathogen, especially in intensive care units [1]. A. baumannii infections may be difficult to treat due to the pathogen's multidrug resistance. Although carbapenems, including imipenem and meropenem, have been commonly used as the mainstay of treatment for severe A. baumannii infections, carbapenem resistant isolates have emerged and disseminated worldwide in recent years. With the exception of polymyxins (such as polymyxin B and colistin) and tigecycline, few alternative therapeutic options are available [2]. However, polymyxin resistant isolates of A. baumannii have also developed,[3-4] along with tigecycline resistant isolates. Even pandrug-resistant (PDR) A. baumannii isolates, displaying resistance to all antimicrobial agents, including both polymyxins and tigecycline, have recently emerged[5-6]. In light of the futility of carbapenems against carbapenem resistant Acinetobacter baumannii isolates, clinicians are now turning to polymyxin B, an antibiotic once sidelined due to concerns about unacceptable nephrotoxicity risks, for the treatment of infections caused by carbapenem resistant isolates.[7] Unfortunately, increasing reports of polymyxin heteroresistance have suggested that rapid resistance to polymyxins can develop upon treatment with polymyxin B monotherapy, especially upon exposure to subtherapeutic polymyxin concentrations [8, 9]. To circumvent this phenomenon, experts have advocated that

polymyxins should be used in combination with one or more antibiotics for the treatment of carbapenem resistant isolates . When selecting a polymyxin B-based combination against carbapenem resistant isoaltes, one must consider the pharmacokinetic/pharmacodynamic (PK/PD) properties of the antibiotics and optimize the PK/PD target attainment of both polymyxin B and the adjuvant antibiotic employed. [10,11]

A. baumannii isolates are associated with bloodstream infection, nosocomial-acquired pneumonia or ventilator-associated pneumonia in critically ill patients and those receiving inappropriate treatment are associated with higher mortality. However, with very few, new antimicrobials effective against A. baumannii in the pipeline, the use of combinations of two or more agents has drawn attention as an option for treating multidrug-resistant (MDR) A. baumannii infections, although the effectiveness of such combinations remains controversial . In addition to increasing eradication efficacy, combination therapy may also help to prevent the emergence of resistant populations. So far, several combinations, such as imipenem and ampicillin-sulbactam, rifampicin and polymyxin B, imipenem and polymyxin B, and colistin and rifampicin, have been reported to be effective in vitro against carbapenem-resistant A. baumannii. However, studies on the effects of these combinations against colistin- or tigecycline-resistant A. baumannii isolates are very limited .[12]The combination drugs treatment are used to 1) widen antibacterial activity of treatment, 2) reduce the probability of selection of resistant mutant, 3) to obtain the advantage of synergy of different antibacterial drugs, which might be helpful in reducing toxic effects associated with large doses of drugs when used alone. The different methods commonly employed for synergy testing are 1) Checkerboard dilution assays :- measure of the inhibitory activity 2) Time kill curve methods :- assesses bactericidal activity 3) Multiple combination bactericidal testing (MCBT) 4) Synergy testing using E (epsilometer) tests.[13]In this study, we investigated the synergistic and bactericidal effects of combinations of antimicrobial agents by in vitro time-kill analysis using a microdilution method against imipenem-resistant A. baumannii clinical isolates that were also resistant to either polymyxin B or tigecycline.

The use of an individualized antibiotic combination which are selected on the basis of the results of in vitro combination testing was associated with significantly lower rates of infection-related mortality in patients with XDR GNB infections.[14]

II. Materials And Methods

Twelve isolates of A. baumannii were collected from different patients admitted to our centre and suffering from nosocomial infections during that period. These isolates were responsible for documented wound infection (5 isolates), endotracheal tube tip (3 isolates),central line tip (1 isolates) and urinary tract infection (1 isolate), pleural fluid(2 isolates). Isolates were diagnosed and identified using the conventional methods in the microbiology laboratory, which included identification of colony morphology on solid media and stained smears, cytochrome oxidase testing, catalase testing, indole testing, motility testing, citrate utilization testing, urease testing and triple sugar iron testing. [15]

2.1 Antimicrobial susceptibility testing

Susceptibility patterns of A. baumannii isolates were tested using a panel of antibiotics including piperacillin-tazobactam, ceftriaxone, imipenem, meropenem, cefotaxime, amikacin, doxycycline, lomefloxacin, levofloxacin, and trimethoprim-sulphmethoxazole, cefoperazone-sulbactam, tigecycline, aztreonam, azithromycin, polymyxin, shown in Table 1, and this was done using a modified Kirby Bauer method following the CLSI guidelines of 2017.[16]

2.2 Determination of MIC.

Minimum Inhibitory Concentrations (MICs) of meropenem, polymyxin B, rifampicin and tigecycline were tested using the agar dilution method according to the Clinical and Laboratory Standards Institute protocol. Breakpoints were interpreted according to CLSI 2017 [17]. No breakpoints for rifampicin and tigecycline are available in the CLSI guidelines; therefore CLSI criteria recommended for Staphylococci were applied to rifampicin (resistant > 4 mg l^{-1}), and the criteria of the United States Food and Drug Administration for Enterobacteriaceae were used for tigecycline (intermediate 4 mg l^{-1} ; resistant >8 mg l^{-1}). Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29213, and Pseudomonas aeruginosa ATCC 27853 were used as control strains. Mueller–Hinton (MH) agar considered the reference medium.[18]

2.3 Time-kill analysis.

Time-kill studies were performed on four antimicrobial agents (meropenem, polymyxin-B rifampicin and tigecycline) and six combinations of these agents (meropenem and polymyxin, tigecycline and polymyxin, polymyxin and rifampicin, meropenem and rifampicin, tigecycline and meropenem,tigecycline and rifmpicin.) according to a previously reported method .[18] Time-kill assays were performed in duplicate using concentrations of 0.5 and 1x MIC in both single-agent and combination studies. Bacterial growth was quantified after 0, 2, 4, and 24 h incubation at 37 ^{0}C by plating 10-fold dilutions on sheep blood agar. Antimicrobials were

considered bactericidal when a > 3 log10 decrease in c.f.u. ml^{-1} was reached compared with the initial inocula. Synergy of the antimicrobial combination was defined as a >2 log10 decrease in c.f.u. ml^{-1} as compared to use of a single agent.[19]

III. Results

In vitro susceptibilities The MICs for meropenem, , polymyxin B, tigecycline and rifampicin of the 12 A. baumannii isolates are presented in [Table 1]. All isolates were resistant to the carbapenems, imipenem and meropenem. Additionally, all were resistant to levofloxacin, cefoperazone, ceftriaxone, cefoperazone-sulbactam, amikacin, piperacillin-tazobactam, doxycycline and cotrimoxazole ,aztrreonam, azithromycin. The MIC results of individual drugs has been shown in Table 2. The isolates that were resistant to carbapenems showed a high MIC ranging from 64-256 µg/ml, while those showing susceptibility to tigecycline and rifampicin showed MIC's in the higher range.

3.1 Single-agent studies

Only meropenem was bactericidal against all twelve A. baumannii isolates tested. Polymyxin B was bactericidal against only one isolates . Although 1x MIC of tigecycline initially decreased growth in all A. baumannii isolates after 2 and 4 h of incubation, regrowth was observed in five isolates. None of the A. baumannii isolates used in this study were completely killed by tigecycline as a single regimen at either 0.5x or 1x MIC. Rifampicin also had no bactericidal effect against any of the *A. baumannii* isolates tested.

3.2 Combination studies

With a combination of 1x MIC meropenem and polymyxin, tigecycline[7] and polymyxin B, exerted bactericidal effects on all twelve A. baumannii isolates tested (Table 3). However, treatment with 0.5x MIC meropenem plus polymyxin B was bactericidal against only eight isolates. A. baumannii isolates were also not detected in incubations with the combination of 1x MIC meropenem and tigecycline except one and with polymyxinB and tigecycline. (Table 3). The combination of 1x and 0.5x MIC of polymyxin B and meropenem showed complete bactericidal activity within 8 h after incubation (figure 1). Treatment with a combination of 1x MIC polymyxin B and tigecycline was also bactericidal against all A. baumannii isolates (Table 3). Treatment with 0.5 x MIC Polymyxin B plus tigecycline (figure 2) was bactericidal or synergistic against seven isolates: one showed regrowth after 12 h of incubation with 0.5 x MIC polymyxin B plus tigecycline. Treatment with the combination of tigecycline and rifampicin was the least effective against the A. baumannii isolates tested (Table 3). Although all isolates reached undetectable levels in incubations with 1 x MIC tigecycline plus rifampicin, removal took longer (12–24 h) than for the other combinations. The 0.5 x MIC of tigecycline and rifampicin was synergistic against only seven isolates.

Table 1. Antibiogram Of Studied *A. Baumannii* Isolates

Isolate Antibiogram	1	2	3	4	5	6	7	8	9	10	11	12
CFS	R	R	R	R	R	R	R	R	R	R	R	R
AT	R	R	R	R	R	R	R	R	R	R	R	R
PB	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
AZM	R	R	R	R	R	R	R	R	R	R	R	R
TCC	R	R	R	R	R	R	R	R	R	R	R	R
LE	R	R	R	R	R	R	R	R	R	R	R	R
LOM	R	R	R	R	R	R	R	R	R	R	R	R
DO	R	R	R	R	R	R	R	R	R	R	R	R
AK	R	R	R	R	R	R	R	R	R	R	R	R
COT	R	R	R	R	R	R	R	R	R	R	R	R
IPM	R	R	R	R	R	R	R	R	R	R	R	R
PIT	R	R	R	R	R	R	R	R	R	R	R	R
TIG	**S**	**S**	**S**	**S**	R	R	**S**	R	R	**S**	**S**	**S**
CTR	R	R	R	R	R	R	R	R	R	R	R	R
CPZ	R	R	R	R	R	R	R	R	R	R	R	R

S: sensitive R: resistant.

Table 2 . Acinetobacter baumannii isolates respective MICs as determined by agar dilution method against the four antibiotics used

Isolate	Meropenem MIC	Polymyxin B MIC	Rifampicin MIC	Tigecycline MIC
1	128(R)	1(S)	2(S)	2(S)
2	64(R)	.5(S)	.5(S)	1(S)
3	64(R)	.5(S)	4(R)	2(S)
4	64(R)	1(S)	1(S)	2(S)
5	256(R)	2(S)	4(R)	4(R)

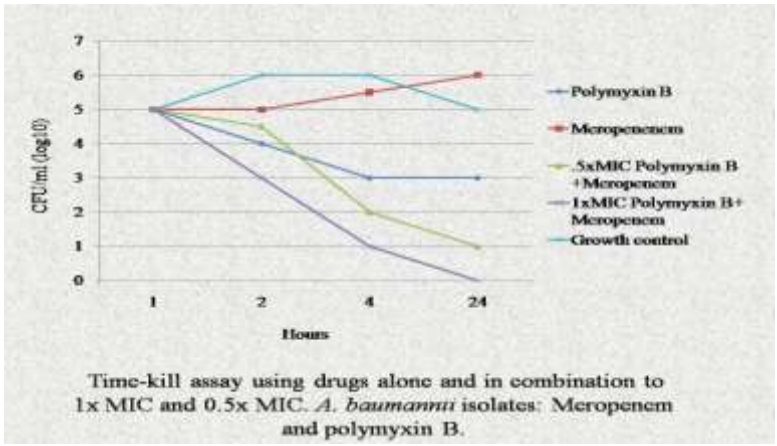
6	128(R)	1(S)	2(S)	4(R)
7	64(R)	.5(S)	4(R)	1(S)
8	128(R)	1(S)	2(S)	4(R)
9	256(R)	2(S)	2(S)	4(R)
10	128(R)	1(S)	2(S)	1(S)
11	64(R)	1(S)	4(R)	2(S)
12	128(R)	.5(S)	4(R)	2(S)

to test synergy.

Table .3: Synergistic effects of antimicrobial combinations against imipenem-resistant *A.baumannii* isolates

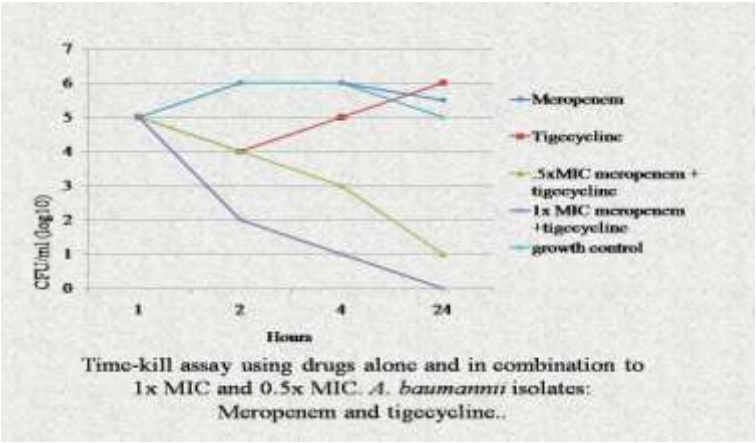
Antimicrobial combination	**Meropenem +Polymyxin B**		**Tigecycline+ Polymyxin B**		**Tigecycline+ Meropenem**		**Tigecycline+Rifampicin**		**Meropenem +Rifampicin**	
Isolate	.5xMIC	1xMIC	.5xMIC	1xMIC	.5xMIC	1xMIC	.5xMIC	1xMIC	.5xMIC	1xMIC
1	S	S	S	S	S	S	S	S	S	S
2	S	S	S	S	S	S	S	S	S	S
3	S	S	S	S	S	S	S	S	S	S
4	S	S	S	S	S	S	S	S	S	S
5	S	S	S	S	NS	S	NS	S	NS	NS
6	NS	S	S	S	NS	S	NS	NS	NS	NS
7	NS	S	S	S	S	S	S	S	S	S
8	NS	S	S	S	S	NS	NS	S	NS	S
9	NS	S	S	S	S	S	NS	S	NS	NS
10	S	S	S	S	S	S	S	S	S	S
11	S	S	S	S	NS	S	S	S	NS	S
12	S	S	S	S	S	S	NS	S	NS	S

S,synergistic(when > 2log10 decrease in c.f.u. ml^{-1} as compared to use of a single agent);NS,non-synergistic.



Time-kill assay using drugs alone and in combination to 1x MIC and 0.5x MIC. *A. baumannii* isolates: Meropenem and polymyxin B.

Figure 1: Time-kill assay using drugs alone and in combination to 1x MIC and 0.5x MIC. *A. baumannii* isolates: Meropenem and polymyxin B.



Time-kill assay using drugs alone and in combination to 1x MIC and 0.5x MIC. *A. baumannii* isolates: Meropenem and tigecycline..

Figure 2: Time-kill assay using drugs alone and in combination to 1x MIC and 0.5x MIC. *A. baumannii* isolates: Meropenem and tigecycline..

IV. Discussion & Conclusion

A. baumannii infections have traditionally been treated with broad-spectrum cephalosporins, β-lactams and β -lactamase inhibitors, and carbapenems .[20] Management of MDR-AB infection is a medical concern because of the limited therapeutic options available and the tendency of infection to occur in critically ill hosts with limited physiologic reserve. In this scenario, combination therapy has [21] become the ultimate resource to treat MDR and pan-resistant A. baumannii infections. Monotherapy with polymyxin B may be problematic for the treatment of polymyxin B heteroresistant A. baumannii infections [22].Various in vitro studies showed variation in results using different methods like checker board matrix and E tests. Synergy testing methods are not standardized for reproducibility and interpretation, and therefore, it is extremely difficult to compare these results from different studies [9]. Time-kill assay was used to detect in vitro synergy in our study. Although time-consuming and cumbersome, the time-kill assay (TKA) provides a dynamic picture of antibiotic action over time; however, it is too labor intensive for use in routine diagnostic laboratories and is unlikely to provide results in a clinically relevant time frame [11]. In the time-kill assay for synergy, drug concentrations are fixed and do not decrease over time as they would in vivo. Additionally, there are no standard concentrations at which antibiotics are tested. The inoculum size and time frame of the time-kill assay add more variability to the test. The time parameter of 24 h can limit or alter the results of the experiment if regrowth occurs with one or both antibiotics. Regrowth can be caused by use of sub-inhibitory concentrations of antibiotics. Emergence of resistant subpopulations may also account for the regrowth, or regrowth may be due to bacteria that adhere to the surface of the bottle and are subsequently released into the medium. Another factor affecting regrowth is inactivation of the antibiotics in vitro [21]. Looking at the tigecycline and polymyxin combination, synergy was found in 12/12 (100%) isolates using the TKA. To make a useful clinical application of these results, we can use the MIC values measured together with the half-life or the dose interval to calculate the highest concentrations reached in plasma by the studied drug. The results also highlight the fact that the polymyxin B might exhibit bacterio-static action when used singly, which might be due to the development of hetero-resistance as proposed by Cai *et al*.[14]

The data obtained in this study together with other calculated pharmacological data, such as volume distribution and systemic availability, will help calculate the best dose that can be used to decrease the unnecessary use of higher doses that can favor the emergence of resistance and also be associated with the unwanted side effects.

As mentioned previously, in vitro testing has several limitations related to the relatively constant nature of study parameters under test tube conditions and also the absence of interactions between the antibiotic at the tested concentration, the bacterial population and the physiology of the living system. The use of an individualized antibiotic combination which are selected on the basis of the results of in vitro combination testing was associated with significantly lower rates of infection-related mortality in patients with XDR GNB infections.[14]This study recommends the use of tigecycline and polymyxin combination for the treatment of *Acinetobacter* infection. This can be translated into a useful therapeutic strategy by combining the results of lower proven effective MIC values with additional pharmacokinetic and pharmacodynamic data to calculate the effective therapeutic dose of such a synergistic combination in clinical practice in collaboration with the treating clinician. This will help in decreasing the emergence of resistance as a result of the previously used improper doses, which are empirical and are not based on laboratory-measured MIC, and it can also decrease the side effects associated with the use of single agents at higher doses.

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