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In Vitro Antibiotic Synergistic Activity against Multidrug Resistant Isolates in a Tertiary Care Hospital by Checkerboard Method

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ABSTRACT

Purpose

To perform invitro synergy testing of antibiotics against multidrug resistant isolates by checker board method and to determine whether the antibiotic combination is synergistic, indifferent or antagonistic.

Method

Gram negative bacterial isolates collected from clinical samples are tested for multidrug resistance i.e isolates that exhibit resistant to atleast three classes of antibiotics (cephalosporins, aminoglycosides and fluoroquinolones) by Kirby Bauer disk diffusion method. Pseudomonas isolates were tested against antibiotic combination amikacin and ceftazidime by checker board assay. Acinetobacter, E.coli and Klebsiella isolates were tested against antibiotic combination colistin and amikacin by checkerboard assay. The effect of antibiotic combinations on the isolates was determined as synergy, additive/ indifferent or antagonist.

Result

83% of Acinetobacter isolates, 66% of Klebsiella and E.coli isolates showed synergy to amikacin and colistin antibiotic combination.80% of Pseudomonas isolates showed synergy to ceftazidime and amikacin antibiotic combination. Additive effect is seen in 17% of Acinetobacter ,20% pseudomonas and 24% of klebsiella and E.coli isolates. No antagonism was seen among the isolates.

Conclusion

Synergy testing has emphasized the importance of combination therapy not only to increase the efficacy of antibiotics but also to minimize the development of resistance especially against drug-resistant strains. Our study results supports the concept of antibiotic combination synergy by using checker board assay. This can be implemented to test MDR pathogens especially in critically ill patients in the tertiary hospital setting.

Keywords

Antibiotic synergy,checkerboard assay, Multidrug resistant organisms(MDRO), Combination antibiogram.

INTRODUCTION

Multidrug resistant(MDR)infections are currently considered as a major public health problem and they pose a major threat to the critically ill patients globally (*Roca et al.,2015*)[1].Though multidrug resistant organisms(MDRO) is a global concern, they pose increased risk in lower and middle income countries where the lower drug cost, unregulated sale of antibiotics led to the higher rates of antibiotic resistance. The mortality increased by 2-3 times with the MDRO associated infections in lower and middle income countries (*Gandra et al.,2019*)[2]. The development of new classes of antibiotics has not kept up with the various emerging resistant mechanisms by the multidrug resistant organisms(*Bush et al.,2010*)[3]. One approach to combating MDR infections is combination of two or more antimicrobial drugs during a treatment regimen which is a better alternative to the introduction of newer antibiotics to which these organisms are eventually developing resistance. Invitro combination testing provides information if the antibiotic combination incur good clinical outcome (*Worthington and Melander et al.,2013*)[4].

The rationale for the synergy testing include (i) Extension of antimicrobial spectrum (ii)Minimization of toxicity and (iii) to reduce the possibility of development of resistance (*Eliopoulos et al.,1988*)[5].Various methods are available for invitro synergy testing which include: Time kill assay, checkerboard assay and E-test. Time kill assay is the standard reference assay and measures bactericidal effect of combination. But it is time-consuming and labour intensive and only limited concentrations can betested.E-test is easy to perform but its is limited to few drug combinations only. Checkerboard assay is easy to perform and multiple concentrations and multiple combinations of drug can be tested (*Laishram et al.,2017*) [6]. Checkerboard assay correlated well with time-kill assay in demonstrating synergy for two-drug combinations (*Sopirala et al.,2010*)[7].A combination antibiogram assist the physicians to access the highly effective empiric coverage of a particular combination that may be particularly useful in settings with problematic multidrug-resistant Gram-negative pathogens (*Klinker et al.,2018*)[8].In view of the above context our study is aimed to determine the effect of antibiotic combinations on the multidrug resistantgram negative isolates collected from clinical samples in our hospital using checkerboard method.

MATERIALS AND METHODS

The study was carried out on the 47 multidrug resistant gram negative isolates collected from the clinical samples during a period of 6 months. The isolates which are resistant to atleast three out of the six classes of antibiotics (i.ebetalactam+betalactamase inhibitors, ampicillin sulbactum, aminoglycosides,

carbapenems, fluoroquinolones and cephalosporins) were included in the study[9].

The study included 11 isolates of Acinetobacter,17 isolates of Pseudomonas,11 isolates of Klebsiella species and 8isolates of Escherichia coli isolated by conventional bacterial culture and showing multidrug resistance identified by Kirby Bauer disk diffusion method. Checkerboard assay was performed based on the protocol given by *Lorian et al.,2005*[10] and the standard microbroth dilution guidelines given in CLSI M07[11].The clinical breakpoints for colistin, amikacin and ceftazidime for the isolates were considered from the CLSI M100 [12].Three to four colonies of overnight incubated culture of the isolate isadded to CAMHB and turbidity adjusted to 0.5 McFarland.The suspension is further diluted to achieve the final standard inoculum size of $5x10^5$ CFU/ml. Amikacin ,ceftazidime and colistin were obtained as powders. Stock solutions of the antibiotics were prepared to achieve a final concentration of 1mg/ml as per the guidelines in CLSI M07[11] and stored at -20^0C until further use. The working stock solution of the drug was prepared by diluting the stock solution to achieve a concentration of the drug which is four times the required concentration in the assay. Serial two fold dilutions of the working stock solution was made and used in the checkerboard assay.The range of concentrations of the drug included were taken as 4xMIC to ¼ MIC for amikacin and ceftazidime and 8xMIC to 1/8x MIC for colistin.

A 90 well microtitre plate is used to test combination of two antibiotics against a single isolate.Each well is filled with 25µl of each antibiotic and 50µl of inoculum ,the total volume being 100µl.The schematic diagram of microtitre plate showing the checkerboard protocol (Fig1) is given.

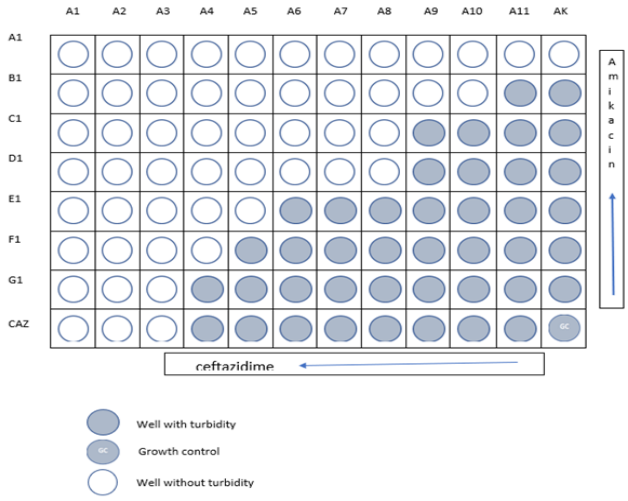
25µl of two fold dilutions of antibiotic A is dispersed from column 1 to 11. 25µl of antibiotic B is dispersed from row A1 to G1.The last column is filled with only antibiotic B and the last row is filled with only antibiotic A. The 50µl of isolate is filled in all wells.

2.1 Fractional inhibitory concentration index (FICI) is calculated using the formula[13]:

FICI = FIC of Colistin + FIC of Meropenem

FICI =MIC of colistin in combination/MIC of colistin alone + MIC of Meropenem in combination/MIC of meropenem alone.

Figure 1: Schematic diagram representing protocol of checkerboard assay:



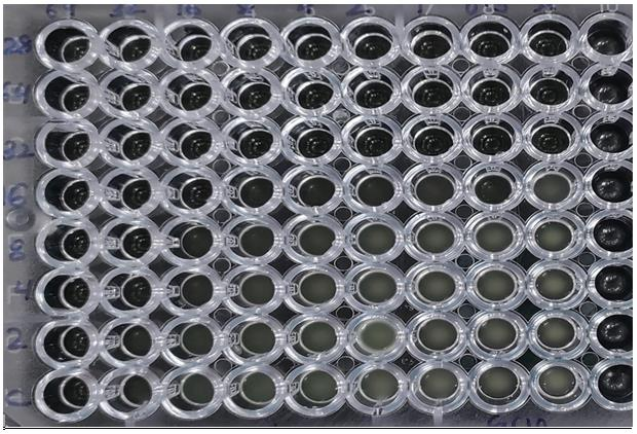
2.2 INTERPRETATION:

FICI ≤0.5 – SYNERGY

FICI >0.5– 4 - Additive or indifference

FICI > 4 Antagonist

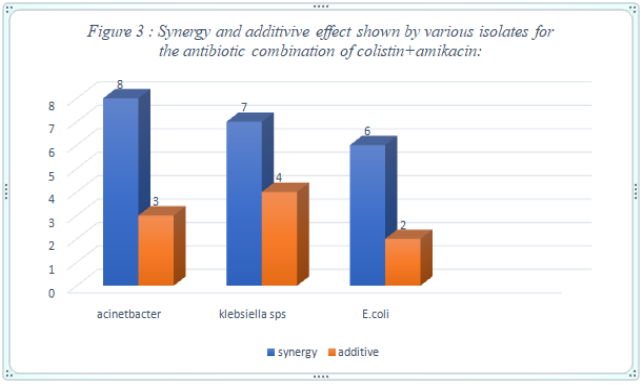
Figure 2: The checkerboard assay microtitre plate in which anitibiotic combination ceftazidime+amikacin was tested against pseudomonas:



RESULTS

Among the 47 isolates identified as multidrug resistant in our study,12 were isolated from blood sample, 12 from sputum sample,18from urine sample and 5 were isolated from pus sample. 80% of these MDR organisms were isolated from paediatric patients between 0-7 years present in the intensive care unit.

The antibiotic combination colistin and amikacin was synergistic and additive in 72.7% and 27.3% of Acinetobacter(n=11) where as in Klebsiella(n=11) isolatesit is 63.63% and 36.3% respectively. The same combination has shown synergy in75% of Escherichia coli(n=8) isolates. The antibiotic combination amikacin and ceftazidime was synergistic and additive in 70.6% and 29.4% of Pseudomonas (n=17) isolates respectively. None of the isolates showed antagonism.8 out of 11 acinetobacter isolates and 2 out of 11 Klebsiella isolates showed four fold reduction in MIC of colistin in combination with amikacin.4 out of 17 pseudomonas isolated showed four fold reduction in MIC of ceftazidime in combination with amikacin.



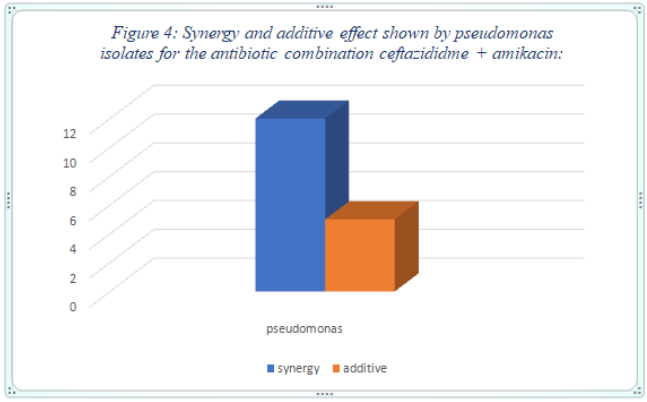


Table 1: Synergy (%) showed by the MDR isolates from various sites of infection:

Site of infection	Colistin +Amikacin			Ceftazidime +Amikacin
	Acinetobacter (n=11)	Escherichia coli(n=8)	Klebsiella species(n=11)	Pseudomonas species(n=17)
Urine(n=18)	100%	100%	33.3%	100%
Blood(n=12)	66.6%	50%	0%	-
Sputum(n=12)	75%	-	-	50%
Pus(n=5)	-	-	100%	50%

DISCUSSION

The synergy testing methods aids in accessing the effective antibiotic combination in treating the infections especially due to multidrug resistant bacteria. In a scientific report published recently, the experimental results theoretically indicate that synergistic combination of antibiotics with closer MSWs had a great potency to prevent resistance according to the hypotheses of MSW and MPC (*Zhao, X et al.,2002 and Drlica, K et al.,2007*)[13,14] and deduced that in vivo synergistic validity of a combination was likely a key to prevent resistance (*Xu, X et al.,2018*)[15].

In a study done by *Sopirala et al.,2010* who compared E test and checkerboard assay with time kill assay to assess synergy of antibiotic combination against pan resistant *Acinetobacter* isolates, synergy was detected with the three-drug combination of imipenem, tigecycline, and amikacin by both CB and time-kill methods among several tested clones. These findings indicate the utility of checkerboard assay which is used to compare any combination of antibiotics[7].

In our study we evaluated the antibiotic combinations colistin and amikacin, ceftazidime and amikacin since most of the isolates were resistant to these antibiotics. The most effective synergistic combination was colistin+amikacin with synergy being shown in 70% of all isolates except *Pseudomonas*. This indicates the effectiveness of this combination in majority of isolates from various sites of infection. In a recent study done by *Ontong JC et al.,2021* where MDR isolates are tested against the above combination showed synergy of 72.7% which is similar to our study[16].This emphasizes the universality of effectiveness of this combination for clinicians to consider it as an empirical antibiotic combination in treating serious MDR infections.

Karki et al.,2021 conducted a study on XDR isolates and reported synergy, indifference and antagonism of 28.7% ,42.86% and 28.57% of klebsiella isolates when antibiotic combination amikacin+ colistin was tested.They also reported 0% synergy and 14.29% antagonism in the isolates of E.coli with the same antibiotic combination. This is in contrast to our study which showed 63.6% synergy and 0% antagonism of klebsiella and 75% synergy and 0% antagonism among E.coli isolates to the above antibiotic combination[17].A similar scenario is seen with Acinetobacter isolates also with synergy being only 1% in the above study as compared to synergy of 72.7% synergy in our study.This may be due to the extensive drug resistant nature of the isolates in the above study.This indicates that combination therapy in XDR infections is not as effective ascompared to MDR infections.But itcan be considered as an initiative in preventing emergence of XDR isolates where we are combating various resistance mechanisms at the same time by this strategy.

Only 55% of Acinetobacter isolates has shown synergy when amikacin is tested in combination with ampicillin sulbactam which was seen in a study done by *Fatemeh S et al., 2021*[18]. A study conducted by *Saida meliani et al.,2020* showed synergy among 42.86% of Acinetobacter isolates when a combination of amikacin and colistin is tested[19]. Our study showed a synergy among 75% of Acinetobacter isolates for the same combination. In therapeutic practice the combination of colistin with amikacin is not recommended because of the toxicity of both antibacterial families (*Martis N et al.,2014*)[20].But studies show that their combination is considered in meningitis caused by MDR Acinetobacter (*Fulnecky EJ et al.,2005*)[21].

In a study conducted by *Ontong JC et al.,2021,*72.72% of klebsiella isolates showed synergy to colistin amikacin combination which is in concordance to our study[16].The study also shown synergy of colistin in combination with other classes of antibiotics like fluoroquinolones, carbapenems etc but less as compared to colistin amikacin combination.

Antibiotic combinations amikacin and ceftazidime showed synergy in 58.3% of pseudomonas isolates in a study conducted by Douka E et al., 2015 which is in contrast to our study in which 80% of isolates were synergistic to the above combination[22].

The use of combination antibiotic susceptibility testing to guide clinical decisionsis generally limited to multidrug-resistant organisms in thecystic fibrosis population (*Tamma PD et al.,2012*) [23]. But a retrospective observational study conducted by *Bodey et al.1985* in patients with malignancies over a 10-year period observed that patients who received an antipseudomonal betalactam antibiotic and aminoglycoside had a significantly higher cure rate (defined as eradication of all signs and symptoms of pseudomonal infections) than patients who received only an aminoglycoside[24].This evidence is supported by our study which has shown synergy of 70.6% when a combination of ceftazidime and amikacin is tested against pseudomonas isolates.

*Gribble et al., 1983*observed that the emergence of resistant organisms during therapy was more frequent among patients receiving piperacillin alone (42%) than among patients receiving combination therapy (17%) [25]. Even though the clinical outcome is not measured in our study, the invitro results itself supported the idea of improving clinical outcome

when combination therapy is used andalso the possibility of reduction in resistance mechanisms.

With the global implementation of antibiotic stewardship program, creation of own antibiogram for isolates in a particular health centre has gained importance since it is aiding the clinicians in deciding the treatment regimen. Similarly devising a combination antibiogram imparts greater advantage than simple antibiogram for treating patients in intensive care unit. A recent study in which combination antibiogram has been devised against pseudomonas isolates has shown considerable increase in susceptibility to few combinations (Ai, Ming-Ying, et al.,2022)[26]. This in the near future becomes essential due to the emergence of XDR organisms. Combination therapy is hence the future of affective therapy in infections caused by MDR and XDR gram negative organisms.

CONCLUSION

Combination therapy has gained attention due to increased efficacy and scope for decreasing the toxicity and development of resistance especially against drug-resistant strains.Our study results supports the concept of determining antibiotic combination synergy by using checker board assay. This can be implemented to test MDR pathogens especially in critically ill patients in the tertiary hospital setting.Combination antibiograms assist physicians to choose the most effective combination therapy in the treatment of critically ill patients.

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