

ANTIBIOTIC SYNERGY TEST: CHECKERBOARD METHOD ON MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA*

Spoorthi N. Jain¹, Vishwanatha T*¹, Reena V¹, Divyashree B.C¹, Aishwarya Sampath¹, Siddhalingeswara K.G¹,
Venugopal.N¹ and Ramesh I²

¹Dept of Microbiology, Maharani's Science College for Women, Bangalore 560 001 Karnataka, India

²Dept of Zoology, Maharani's Science College for Women, Bangalore 560 001 Karnataka, India

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*E-mail:lvphaget@rediffmail.com

ABSTRACT

Pseudomonas aeruginosa is a global emergence of multidrug resistant strains leading to a wide variety of nosocomial infections in humans. The clinical use of combination of antibiotic therapy for gram negative infections is probably more effective than monotherapy. Certain combinations of antibiotic exhibit synergistic antibacterial effects. Ampicillin and Kanamycin are the commonly used potent bactericidal antibiotics, active against gram negative bacteria and also act synergistically. Antibiotic susceptibility of *Pseudomonas aeruginosa* showed a high level of resistance with Optochin and Rifamycin. An intermediate effect was seen to Erythromycin and Chloramphenicol, Kanamycin and Ampicillin showed different minimum inhibitory concentration (MIC). Thus, the susceptible antibiotics were used in the checker board technique, which appears useful for determining antibiotic synergism against *Pseudomonas aeruginosa*. The Σ FIC results showed synergy for concentrations of 80 μ g/ml and 40 μ g/ml of antibiotic combinations with fractional inhibitory concentration (FIC) of 0.48 at 37°C over night incubation per ml. Thus, the combinations tested were bactericidal indicating the minimum inhibitory concentration and no growth illustrated the extensive activity of kanamycin which was enforced by the ampicillin resulting in an antibacterial effect.

Key words: Antibiotic resistance, FIC, *Pseudomonas aeruginosa* and Synergy Test

INTRODUCTION

Pseudomonas aeruginosa is a gram negative bacilli. It is a ubiquitous opportunistic pathogen, often causing nosocomial or hospital acquired infections^{1,2}. There is a global emergence of multi drug resistant strains of *Pseudomonas species*³ which is invasive, toxigenic, leading to a wide variety of infections in humans such as iatrogenic infections, bacteraemia, urinary tract infections^{4,5,6} and pneumonia, acute infections in burn victims or wounds⁷. These nosocomial infections caused by multi drug resistant *Pseudomonas aeruginosa* causing high mortality and high morbidity is difficult to eradicate⁸.

The clinical use of combination of antibiotic therapy for bacterial infections in general can be divided into two categories. In the first category, such therapy is used to improve clinical outcomes of infections with strains that are susceptible to one or more individual antibiotics. The primary rationale for combining two agents is to enhance the activity or either by achievement of a synergistic effect. This effect, synergy is defined as significantly greater activity provided by two agents combined than that provided by the sum of each agent alone.⁹

A secondary rationale is to allow lower doses of either antibiotic to reduce toxicity. Third, use of two antibiotics might prevent the emergence of resistance to either. The second category of antibiotic combination use has evolved during the last decade, during which certain clinical species have become resistant to all available antibacterial agents or to all except a single agent. The consensus is that combination therapy is probably more effective than monotherapy only for infections with *P.aeruginosa* and primarily among patients with bacteraemia and neutropenia¹⁰. Certain combinations of β -lactam antibiotics exhibit synergistic antibacterial effects against various gram-negative bacilli¹¹. Synergy testing may improve antibiotic choice and clinical outcome in patients with ventilator-associated pneumonia due to *Pseudomonas sp.*¹²

Ampicillin is a commonly used broad-spectrum aminopenicillin, which inhibits the final stage of bacterial cell wall synthesis and ultimately leads to cell lysis. Its usefulness is limited by its susceptibility to β -lactamase hydrolysis produced by the organism¹³. Kanamycin is an aminoglycoside which is a potent bactericidal antibiotic that acts by creating fissures in the outer membrane of the bacterial cell. They are particularly active against aerobic, gram-

negative bacteria and act synergistically. In terms of mechanism of action, kanamycin acts by binding to the bacterial 30S ribosomal subunit inhibiting the translocation of the peptidyl-tRNA from the A-site to the P-site and also causing misreading of mRNA, leaving the bacterium unable to synthesize proteins vital to its growth where as ampicillin acts by inhibiting cell wall synthesis or activation of enzymes that disrupts bacterial cell walls to cause loss of viability and often cell lysis¹⁴.

Among the techniques employed in the evaluation of the combination of two antimicrobials potentially exhibiting synergism is the checkerboard technique. The checkerboard or fractional inhibitory concentration (FIC) technique employs a methodology similar to that utilized for the determination of the minimum inhibitory concentration (MIC). The combination is said to have synergistic effect if there is a 4-fold reduction in the MIC of each antimicrobial agent tested alone¹⁴. Hence, the objective of the present study is to determine the antibiotic susceptibility pattern of *Pseudomonas aeruginosa* by occurrence of the interaction between ampicillin and kanamycin, using the checkerboard method.

MATERIALS AND METHOD

Materials

The culture media used in the study were, Nutrient agar, Cetrinide agar and Muller Hinton media all obtained from HiMedia. Antibiotic disks- Ampicillin (10mcg/disc), Kanamycin (30mcg/disc), Chloramphenicol (30mcg/disc), Optochin (30mcg/disk), Erythromycin (15mcg/disc) and Rifamycin (30mcg/disk) from HiMedia. *Pseudomonas aeruginosa* was obtained from the clinical sample.

Isolation and maintenance of test organism

The inoculum was streaked on sterile Nutrient agar plate and incubated at 37°C for 24hrs. The colonies obtained were large, opaque, and irregular with bluish green pigmentation. The test microorganism was confirmed by Gram staining and by subsequent culturing in selective media i.e, Cetrinide agar, followed by the standard biochemical tests¹⁵.

Inoculum preparation: The 24 hours old culture of *Pseudomonas aeruginosa* was inoculated in to Muller Hinton broth, incubated at 37 °C for 6 hrs. This was used for synergy test.

Antibiotic susceptibility test

Antimicrobial susceptibility test of the isolates was performed by Kirby-Bauer Disk diffusion test¹⁶. Disk diffusion test was done, in which the test isolate was swabbed uniformly onto the surface of Muller Hinton agar plates. Antibiotic sterile disks such as Ampicillin, Kanamycin Chloramphenicol, Optochin, Erythromycin and Rifamycin were then placed on to the agar surface of the plate. Following incubation, a bacterial lawn appeared on the plate with zones of inhibition around the antibiotic disks. Based on the highest zone of inhibition shown by the antibiotics, a combination of the two was made use for the synergy testing using checkerboard method.

Preparation of antibiotic stock solution

Standard powder forms of ampicillin and kanamycin were stored at 4°C until use. The stock solution of each antibiotic was prepared by weighing and subsequently dissolving appropriate quantities of the antibiotics obtaining concentration of 1000µg/ml in Muller Hinton broth.

Test for synergism

From the stock solutions a twofold dilutions of each antibiotic to at least double the MIC were distributed into each microfuge tubes to obtain a varying concentrations of 2.5, 5.0, 10, 20, 40, 80, 160, 250 and 500µg/ml of each antibiotic was used for checkerboard method. A total volume of 2ml was made in each tube by distributing Muller Hinton broth along with 100µl of the inoculum. The microfuge tubes with one antibiotic of the combination were placed in rows in ascending concentrations starting at zero MIC and ending at two times the MIC. The other antibiotic was similarly distributed among the columns. Thus, each of the microfuge tubes was held in a unique combination of concentrations of the two antibiotics. The tubes were incubated overnight at 37°C and MIC was read as the least dilution without any turbidity. A fractional inhibitory concentration index (FICI) was used to interpret the results¹⁷. According to the Clinical Laboratory Standards Institute (2006)¹⁸ guidelines for broth microdilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected with the naked eye. Synergy is more likely to be expressed when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was same for all components of the mixture.

The ΣFICs were calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone¹⁹. The combination is considered synergistic when the fractional inhibitory concentration (ΣFIC) index is ≤0.5. Indifference was indicated by a FIC index >0.5 to ≤4 while antagonism when the ΣFIC is >4¹².

RESULTS

The disk diffusion study for various antibiotics tested, following incubation showed zones of inhibition around the antibiotic disks against *Pseudomonas aeruginosa* indicating the susceptibility based on the size of the zone obtained. The antibiotic susceptibility pattern of *Pseudomonas aeruginosa* showed that the isolate was resistant to kanamycin and moderately to ampicillin, and sensitive to erythromycin and chloramphenicol. High level of resistance was observed with optochin and rifamycin. In the Checkerboard technique, the interaction between combination of ampicillin and kanamycin against *Pseudomonas aeruginosa* were predominantly synergistic, although there were few variations. Thus no growth or turbidity clearly illustrated the extensive activity of aminoglycoside which was enforced by the second drug, ampicillin resulting in an antibacterial effect as shown in Fig 1.

The ΣFIC results for the checker board method showed synergy for 80µg/ml and 40µg/ml antibiotic combinations with ΣFIC of 0.48; whereas rest were (indifferent)additive effect indicating the activity of two antibiotics in combination being greater to the sum of their independent activity.

DISCUSSION

Pseudomonas aeruginosa is a frequently recovered species from clinical specimens, with infections commonly occurring at any site where moisture tends to accumulate. Initial localized infections can often lead to invasion of the bloodstream resulting in bacteraemia⁸. Despite advances in sanitation facilities and the introduction of a wide variety of antimicrobial agents with antipseudomonal activities, life threatening infections caused by *Pseudomonas aeruginosa* continue to persist in hospital infections. A critical factor in the survival of *Pseudomonas aeruginosa* in an unfavorable environment and its ability to transform from a mobile "swarmer" cell to a glycocalyx enclosed microcolony which serves to protect the organisms against the active phagocytes, surfactants, enzymes and high levels of specific antibodies³. Infections due to this pathogen are especially prevalent among patients with cystic fibrosis, acute leukaemia, organ transplants and intravenous drug abuse¹². Urinary tract infections caused by *Pseudomonas sps* can be severely detrimental and often fatal in immune-compromised hosts⁵. Risk factors found to occur more frequently in patients with presence of intravascular catheters, ventilatory support, and prolonged stay in hospital¹².

In our study, antibiotic susceptibility pattern of *Pseudomonas aeruginosa* by the disk diffusion method showed that the isolate, *Pseudomonas aeruginosa* was resistant to the antibiotic kanamycin and ampicillin. Thus, these antibiotics were used in the checker board technique, which appears useful for determining antibiotic synergism against the isolated *Pseudomonas aeruginosa*. Since various concentrations of antibiotic combinations can be studied by broth two fold dilutions, the synergistic effect can be detected by turbidity of the test broth which can be readily and easily visualized²⁰.

Synergy is defined as a decrease in the viable organism as a result of the combination when compared with the most effective antibiotic when tested alone. Concentrations of 40µg of ampicillin with 80µg of kanamycin per ml and of 80µg of ampicillin and 40µg of kanamycin per ml resulted in synergy at 37°C over night incubation. Thus the combinations tested were bactericidal indicating the minimum inhibitory concentration whereas, the few of varying concentrations of antibiotic combinations showed no presence of turbidity but an additive effect. Rest of the combinations of antibiotic showed turbidity and thus no synergism.

Hence our study indicates that combinations of the beta lactam antibiotic ampicillin, which differs from penicillin only by the presence of an amino group that helps the antibiotic penetrate the outer membrane of gram-negative bacteria by cell wall synthesis and there by increases the permeability of the bacterium to the aminoglycoside kanamycin binding to the 30S ribosome inhibiting the protein synthesis, thus leading to a synergistic effect in the *in vitro* studies. This indicates that the synergistic effect of the MIC of antibiotic combination is more effective than any of the single antibiotic tested.

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KANAMYCIN ($\mu\text{g/ml}$)	500									
	250	■	■							
	160	■	■	■						
	80	■	■	■	■					
	40	■	■	■	■	■				
	20	■	■	■	■	■	■			
	10	■	■	■	■	■	■	■		
	5	■	■	■	■	■	■	■	■	
	2.5	■	■	■	■	■	■	■	■	■
	0	■	■	■	■	■	■	■	■	■
		00	2.5	05	10	20	40	80	160	250
	AMPICILLIN ($\mu\text{g/ml}$)									

Fig 1: Checkerboard method showing the synergy effect on *Pseudomonas aeruginosa*.

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