Original Article

Synergistic Effect of Colistin with Selected Antibiotic on Colistin Resistant *Pseudomonas aeruginosa* Strains Isolated from Clinical Samples

Zahra Keramati[1], Leila Asadpour[1]*, Hadi Habibollahi[1]

[1]Department of Biology, Rasht Branch, Islamic Azad University, Rasht, Iran

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Abstract

Background: The spread of antibiotic resistance and the emergence of MDR strains, especially in gram-negative bacteria, has led to combining antibiotics to treat infections caused by these bacteria. The study aimed to evaluate some antibiotics' synergistic effect on multidrug-resistant Pseudomonas aeruginosa isolates isolated from clinical samples between thyroid dysfunction and asymptomatic bacteriuria in women of reproductive and postmenopausal age.

Materials and Methods: *P. aeruginosa* was isolated from Rasht city hospitals (North of Iran) clinical specimens with multiple antibiotic resistance. The antibiotic resistance of isolates was determined by the disk diffusion method. The screening checklist evaluated the synergistic effects of ciprofloxacin, colistin, meropenem, and gentamicin.

Results: The combination of meropenem and colistin in four isolates was additive, and in two isolates was indifferent. The combined effect of ciprofloxacin and colistin was also incremental in 4 isolates and indifferent in 2 isolates. The combined effect of gentamicin and colistin was indifferent in all selected strains.

Conclusion: Results of this study showed that combinations of colistin with ciprofloxacin and colistin with meropenem were successful against resistant isolates, while a combination of gentamicin and colistin showed no synergistic effect. Since the characteristics of each clinical isolate differ from the other isolates, more study with more isolates is recommended to make any precise and definite recommendation.

Keywords: *Pseudomonas aeruginosa*, Antibacterial Resistance, Colistin, Synergism

***Corresponding Author:** Leila Asadpour, Department of Biology, Rasht Branch, Islamic Azad University, Rasht, Iran. Email: l.asadpour@yahoo.com

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Introduction

Pseudomonas aeruginosa is a Glucose non-fermentative gram-negative bacillus. It is a ubiquitous opportunistic pathogen that causes nosocomial or hospital-acquired infections, especially in intensive care unit patients[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, bacteriaemia, urinary tract infection[4-6] and pneumonia, acute infections in burn victims or wounds[7].

These nosocomial infections caused by multi-drug-resistant *Pseudomonas aeruginosa* are difficult to eradicate, causing high mortality and high morbidity[8].

Carbapenem is the only available drug for treating GNFB infections in many cases. However, the recent increase of carbapenem resistance in GNFB has become a serious problem worldwide[9]. Most carbapenem-resistant *P. aeruginosa* strains identified are also extensively drug-resistant (XDR)[10].

Polymyxins are an old lipopeptide antibiotic Class that is increasingly used as a last-line therapy for life-threatening infections caused by XDR *P. aeruginosa*[11,12]. Polymyxins are natural products from *Paenibacillus polymyxa*, and polymyxin B (PMB) and colistin (i.e., polymyxin E) are the only two clinically available polymyxins, differing by only an amino acid at position 6[13,14].

Colistin sulfate (polymyxin E) is one of the cyclic polypeptide antibiotics known to interact primarily with LPS and phospholipids present at the outer membrane of Gram-negative bacteria with also the ability to disturb the permeability of the cytoplasmic membrane. It may allow the entry of hydrophobic and/or large molecules, ultimately leading to cellular content leakage[15]. Although polymyxin B and colistin remain effective against these Gram-negative pathogens, recent harmacokinetic/pharmacodynamics (PK/PD) studies suggested that polymyxin monotherapy is associated with increased polymyxin resistance[16]. Worryingly, there have been increasing reports of infections caused by polymyxin-resistant XDR *P. aeruginosa*, which are essentially untreatable byconventional antibiotic monotherapy[17].

Polymyxins have nephrotoxicity dose-limiting adverse effects; simply increasing the dose is not feasible to preserve the efficacy of polymyxins while minimizing the emergence of resistance; novel approaches are urgently needed[18-20]. Combination therapy has been proposed as a novel strategy to maximize the antimicrobial efficacy against XDR pathogens and suppress the spread of resistance[21–23]. Therefore, colistin can be combined with other antibiotics to obtain a synergistic effect.

The consensus is that combination therapy is probably more effective than monotherapy for infections with *P.aeruginosa*, primarily among patients with bacteriaemia and neutropenia[24]. Certain combinations of β-lactam antibiotics exhibit synergistic antibacterial effects against various gram-negative bacilli[25]. Synergism testing may improve antibiotic choice and clinical outcomes in patients with ventilator-associated pneumonia due to *Pseudomonas sp*[26]. Hence, the objective of the present study is to determine the antibiotic susceptibility pattern of *Pseudomonas aeruginosa by the* occurrence of the interaction between Colistin, Ciprofloxacin, Gentamicin, and Imipenem using the checkerboard method.

Methods

The culture media used in the study were Nutrient agar, Cetrimide agar, and Muller Hinton media, all obtained from HiMedia. Antibiotic disks, including Colistin (5mcg/disk), Ciprofloxacin (10mcg/disk), Imipenem (10mcg/disk), and Gentamicin (10mcg/disk) that were purchased from Padtan teb (Tehran, Iran). *P. aeruginosa* was obtained from different clinical samples. This study was approved by the Ethics Committee of Islamic Azad University Rasht Branch (ID: IR.IAU.RASHT.REC.1401.029).

Isolation and maintenance of test organism: All received isolates from Rasht Poursina Hospital, Rasht, Iran, were subsequently confirmed by standard microbiology and biochemical tests. For this purpose, the single colony was streaked on a sterile Nutrient agar plate and incubated at 37oC for 24 hours. The colonies obtained were large, opaque, and irregular, with bluish-green pigmentation. The test microorganism was confirmed by Gram staining and subsequent culturing in selective media, Cetrimide agar, followed by the standard biochemical tests[27].

Inoculum preparation: The 24-hour culture of *P. aeruginosa* was inoculated into Muller Hinton broth and incubated at 37°C for 6 hrs. The product was used for the synergism test.

Antibiotic susceptibility test: Kirby-Bauer Disk diffusion test performed an antimicrobial susceptibility test of the isolates[28]. A 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 ciprofloxacin, gentamicin, and meropenem were placed on the plate's agar surface. Following incubation, a bacterial lawn appeared on the plate with inhibition zones around the antibiotic disks. Based on the highest zone of inhibition shown by the antibiotics, a combination of the two was used for the synergy testing using the

checkerboard method.

Preparation of antibiotic stock solution: Standard powder forms of ciprofloxacin, gentamicin, and meropenem 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 a concentration of 1000µg/ml in Muller Hinton broth.

Test for synergism: From the stock solutions, a twofold dilution of each antibiotic to at least double the MIC was distributed into each microfuge tube to obtain a varying concentration 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 and 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 microfuge tube 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 results (29). According to the Clinical Laboratory Standards Institute (2006) (30) guidelines for broth microdilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibited the organism's growth as detected with the naked eye. Synergy was more likely to be expressed when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was the 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 alone (31). The combination is considered synergistic when the fractional inhibitory concentration (ΣFIC) index is ≤0.5. Indifference was indicated by an FIC index >0.5 to ≤4, while antagonism was when the ΣFIC was>412.

Table 1: Synergistic assay of Ciprofloxacin and Colistin on MDR isolates of *P. aeruginosa*.

Antibiotic MIC for	P1	P2	P3	P4	P5	P6
Ciprofloxacin	31.2	15.6	15.6	15.6	500	125
Colistin	42.5	10.6	85	85	42.5	21.2
Ciprofloxacin in combination with colistin	15.6	7.8	3.9	7.8	62.5	62.5
Colistin in combination with Ciprofloxacin	10.6	5.3	42.5	21.2	42.5	21.2
FIC	0.75	1	0.75	0.75	1.12	1.5

Table 2: Synergistic assay of Meropenem and Colistin on MDR isolates of *P. aeruginosa*.

Antibiotic MIC for	P1	P2	P3	P4	P5	P6
Meropenem	39	1250	9.2	39	156	39
Colistin	42.5	10.6	85	85	42.5	21.2
Meropenem, in combination with colistin	19.5	312.5	4.6	39	39	9.7
Colistin, in combination with Meropenem	21.2	10.6	42.5	42.5	21.2	10.6
FIC	1	1.25	1	1.5	0.75	0.75

Results

In this study, 86 isolates of *P. aeruginosa* from clinical specimens were selected for further antimicrobial assays.

Our results showed that colistin was the most effective antibiotic, and 83 isolates (96.5%) were sensitive to this antibiotic. Resistance rates for gentamicin, meropenem,

Table 3: Synergistic assay of Gentamicin and Colistin on MDR isolates of *P. aeruginosa*.

Antibiotic MIC for	P1	P2	P3	P4	P5	P5
Gentamycin	125	31.2	125	125	156	125
Colistin	42.5	10.6	85	42.5	42.5	21.2
Gentamycin, in combination with colistin	125	15.6	62.5	125	39	62.5
Colistin, in combination with Gentamycin	42.5	10.6	42.5	42.5	42.5	21.2
FIC	2	1.5	2	2	1.25	1.5

and ciprofloxacin were 47 (55%), 41(48%), and 52 (60%), respectively. Based on our results, we studied *P. aeruginosa* isolates were multiple antibiotic resistance (MDR) strains.

In this study, of 56 clinical MDR isolates, three isolates, which were resistant to gentamicin, meropenem, colistin, and ciprofloxacin in disk diffusion and MIC assay, plus three isolates from previously available isolates in our laboratory were selected for evaluation of the synergistic effect of these antibiotics. The MIC results of the synergism effect in these isolates are shown in Tables 1-3. Based on the calculated FIC, the results were interpreted. The combination of meropenem and colistin was effective on four isolates but not on two isolates.

The combined effect of ciprofloxacin and colistin was also incremental in 4 isolates and indifferent in 2 isolates. The combined effect of gentamicin and colistin was indifferent in all selected strains.

Discussion

Antibiotic resistance is one of the major problems in treating infectious diseases worldwide. *P. aeruginosa*, as a nosocomial and opportunistic pathogen, is the cause of a wide range of infections associated with high drug resistance. The drug resistance mechanisms of this bacterium are divided into intrinsic and acquired groups. These bacteria are inherently resistant to several antibiotics due to the low permeability of the outer membrane, the constant expression of several efflux pumps, and the production of a variety of antibiotic-inactivating enzymes (such as cephalosporinases), and readily transmit genes of resistance to many antibiotics. They are more comfortable in the multiplication and spreading of the multiple resistances. Increased MDR strains of these bacteria have been reported as a global health problem in different parts of the world, leading to reduced treatment options for infections caused by these bacteria[32,33].

The present study investigated *P. aeruginosa* strains from different clinical samples from Rasht, Iran, for antibiotic synergism. The most effective combination was the combination of colistin and ciprofloxacin antibiotics. The combined effect of these two antibiotics was additive in all studied MDR isolates. In addition, the combination of meropenem and colistin was additive in two isolates and had no effect in one isolate. In the study of Marie et al., The combination of colistin with meropenem by checkerboard method had a synergistic effect[34], consistent with our study. In the study of Song et al., The combination of colistin with meropenem by time-kill method had a synergistic effect[35]. Some studies have reported the synergistic effect of ciprofloxacin with the antibiotic beta-lactam against Enterobacteriaceae. In one study, ciprofloxacin and imipenem had a synergistic effect in 25% of cases[36]. In a study, the combined effect of vancomycin with tigecycline, levofloxacin, colistin, and meropenem and the combination of colistin with meropenem and rifampin against enterococci were indifferent and was not more significant than the action of these drugs alone[37].

Regarding the combination of antimicrobial agents, combining the two bactericidal antibiotics may have a synergistic or additive effect. Combining two bacteriostatic antibiotics is usually incremental, and combining a bacteriocidal antibiotic with a bacteriostatic antibiotic may have an antagonistic or indifferent effect[38]. Meropenem is a beta-lactam antibiotic and an inhibitor of cell wall synthesis. This drug is broad-spectrum and has bactericidal properties. Colistin is a polypeptide antibiotic with limited use in treating superficial and local infections and systemic infections caused by *P. auroginosa*. It affects bacterial

and human cytoplasmic membranes, so it should not be administered arbitrarily. One of its main side effects is severe nephrotoxicity. Colistin, in combination with gentamicin, a bactericidal antibiotic, did not show synergistic effects and is not recommended. Gentamicin is an aminoglycoside antibiotic that inhibits protein synthesis. The combination of gentamicin and colistin in vitro in the present study did not show a synergistic effect. Ciprofloxacin is a fluoroquinolone antibiotic that binds to the bacterial DNA gyrase to prevent replication and transcription. This antibiotic has bactericidal properties; it is quickly absorbed orally. The combination of ciprofloxacin with colistin, a bactericidal antibiotic, showed an excellent synergistic effect in vitro on the isolates of the present study. Gentamicin is effective on the 30s subunit of the ribosome and inhibits protein synthesis.

Synergistic tests are performed using different methods, and consequently, the results of different methods differ[39]. The checkerboard and time-kill methods are the best tests used[40]. This study showed that combining colistin with meropenem is suitable (66.7%) in treating infection caused by MDR bacteria. Reasons for using an antibiotic combination in the treatment of infections caused by resistant isolates of *P. aeruginosa* providing a wide range of antibiotics to treat infections in critically ill patients, reducing resistant strains, reducing dose-dependent toxicity of antibiotics, and increase the inhibitory power and lethal effect of microbes[37].

Conclusion

Considering the major health problem rising by MDR isolates of *P. aeruginosa*, this study showed that combinations of colistin with ciprofloxacin and colistin with meropenem were successful against resistant isolates. In contrast, a combination of gentamicin and colistin showed no synergistic effect. Since the characteristics of each clinical isolate are different from the other isolates, more study with more isolates is suggested to make any precise and definite recommendation.

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Conflict of interest

The authors further declare that they have no conflict of interest.

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