Original Article

Simultaneous Application Of Non-Antibiotics With Antibiotics For Enhanced Activity Against Multidrug Resistant Pseudomonas Aeruginosa

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Abstract

The treatment of multi drug resistance (MDR) Pseudomonas aeruginosa has raised a major health concern worldwide. However the combine use of antibiotics and non-antibiotic could be beneficial in the treatment and prevention of MDR P. aeruginosa. The current study is therefore an attempt to evaluate the synergistic effect of antibiotics and non-antibiotic against MDR P. aeruginosa. Currently, a total of 62 P. aeruginosa clinical isolates were investigated. Kirby Bauer Disk diffusion method was used to determine the antibiotic susceptibility of the clinical isolates following the Clinical and Laboratory Standards Institute (CLSI) guidelines. The minimum inhibitory concentrations of the antibiotics including polymyxin B, ciprofloxacin and cefotaxime and non-antibiotics i-e., sertraline, loperamide, metformin and diclofenac sodium were determined using broth micro dilution technique. The synergistic effects of these antibiotics and non-antibiotics were further evaluated using checkerboard assay and fractional inhibitory concentration (FIC). Of the 62 clinical isolates, 53 (85.48%) were confirmed MDR. The individual MICs of cefotaxime, polymyxin B and ciprofloxacin were 32 µg/mL, 16 µg/mL and 64 µg/mL respectively. While the individual MIC of sertraline, loperamide, metformin and diclofenac sodium were 64 µg/mL, 32 µg/mL, 16 µg/mL and 64 µg/mL respectively. . Sertraline and loperamide when combined with polymyxin B showed synergistic antibacterial activity with an FIC value of 0.37 and 0.5 respectively. Similarly diclofenac sodium with cefotaxime also showed synergism with an FIC value of 0.25. However the combination of metformin and ciprofloxacin showed no synergistic activity (FIC=1). It was therefore concluded that the tested combinations had significantly lowered MICs values compared to antibiotics and non-antibiotics individually.

Keywords: Pseudomonas aeruginosa**,** Multi drug resistance, Minimum inhibitory concentration, Fractional inhibitory concentration.

INTRODUCTION

Known for many years to be a cause of serious wound and surgical infections, but often regarded as a secondary or opportunistic invader rather than a cause of primary infection in healthy tissues, Pseudomonas aeruginosa has now clearly emerged as a major nosocomial pathogen in immunocompromised and debilitated patients, as well as in cystic fibrosis patients [1]. Pseudomonas aeruginosa is the third most prevalent bacterium causing around 9-10% of the nosocomial infections [2]. In healthy people the prevalence of its colonization is typically low, but after hospitalization greater rate of colonization is experienced, particularly among people treated with broad spectrum antibiotics [3]. P. aeruginosa causes urinary tract infections, kidney infections, cystic fibrosis, surgical site infection and sepsis [4]. Colonization of the lungs of cystic fibrosis (CF) patients by the opportunistic bacterial pathogen Pseudomonas aeruginosa is the principal cause of mortality in CF populations [5]. In the past infections of P. aeruginosa have been treated by a combination of β-Lactams (i.e., penicillin) a group of antibiotics classified by its four-membered ring [6]. Pseudomonas aeruginosa displays resistance to a variety of antibiotics, including aminoglycosides, quinolones and β-lactams [7]. Generally, the major mechanisms of P. aeruginosa used to counter antibiotic attack involve Efflux pumps, β-lactamases, lower membrane permeability [8], biofilms [9], horizontal gene transfer and mutational changes [10]. Multi drug resistant P. aeruginosa is a gradually emerging concern in clinical practice [11]. Conventional antibiotic therapies against P. aeruginosa infections have become increasingly ineffective due to the rise of multidrug-resistant strains [12]. There are many significant reasons why the spread and emergence of MDR P. aeruginosa has recently become a public health issue (general opinion). First, it causes serious infections, especially in immunocompromised patients and health care settings [13]. Secondly it has an excellent potential for disseminating antimicrobial resistance [10].Third, the successful global dissemination of high risk clones of multi drug resistant P. aeruginosa poses a major public health problem which needs to be managed urgently and determinedly [14]. Carbapenems are widely used as first-line drugs to treat nosocomial infections and are effective against multidrug-resistant P. aeruginosa and other bacterial infections producing the cephalosporinase AmpC or extended-spectrum β-lactamases [15]. Nevertheless, carbapenem-resistant P. aeruginosa (CRPA) isolates are increasingly observed, probably due to the global clinical use of carbapenem [16]. The World Health Organization (WHO) has recently listed carbapenem-resistant P. aeruginosa as one of three bacterial species in which there is a critical need for the development of new antibiotics to treat infections (17). Therefore, an alternative strategy should be adopted to deal with the MDR Pseudomonas aeruginosa. Recently, drug repurposing and synergistic drug screens have provided alternative approaches to combat infections caused by MDR pathogens and emerging viral outbreaks [18]. The strategy has many advantages such as reducing the time, cost, and risk associated with the development of new antimicrobials [19]. The synergistic effect of various non-antibiotics with antibiotics might be of critical values [20, 21]. The synergistic effect of antibiotic and Non-antibiotics against MDR bacteria have been reported before. The combination of Fluconazole (antibiotic) with non-antibiotics like fluoxetine and propranolol have been found effective against C. albicans [22]. In addition, the antibacterial activity of combination of polymyxin B and wide variety of non-antibiotics has been reported against Pseudomonas aeruginosa, Acinetobacter baumannii, Escherichia coli and Klebsiella pneumonia [23]. Considering the worldwide MDR of P. aeruginosa and the efficacy of combination of antibiotic with non-antibiotic, the current study was aimed at the evaluation of synergistic effect of the selected antibiotic and non-antibiotics against MDR of P. aeruginosa. For this purpose checkerboard assay method was used to determine the MICs of the selected antibiotic and non-antibiotics in combination as well as alone.

METHODOLOGY

Sample collection and processing

A total of 100 wound pus samples were collected from patients visiting Hayatabad Medical Complex (HMC), Peshawar. Sterile cotton swabs were used for the collection of samples following CLSI (2020) guidelines [24]. The collected samples were processed following standard operating procedures (SOPs) laid-down by Pokharel et al. 2019 [25]. Nutrient and MacConkey agar was inoculated with swabs and the plates were incubated at 37°C for 24 hrs.

Characterization of P. aeruginosa

The identification of Pseudomonas aeruginosa isolates was done on the basis of colony morphology, gram staining and various biochemical tests according to Bergey's Manual [26].

Antibiotics susceptibility test

Antibiotic susceptibility testing (AST) of Pseudomonas aeruginosa was conducted using the Kirby Bauer disc diffusion method utilizing commercial antibiotic discs following compliance with CLSI 2020 guidelines [24]. Pseudomonas aeruginosa colonies were obtained from the surface of agar with the help of a sterile wire loop and inoculated in peptone water and incubated for 4 hours at 37 ºC and the broth turbidity was acclimated to the McFarland level by diluting the broth with normal saline. A clean swab was immersed in peptone water culture and twisted to suck out excess fluid, after which the swab was uniformly cultured over the cationic adjusted Muller Hinton Agar (CA-MHA) plate surface. This method was repeated several times, turning the plate about 60º to ensure equal inoculum distribution. The plate was permitted to dry for 5 minutes. Antibiotic discs were then mounted on medium's surface with the help of a sterile forcep. To ensure complete contact with the agar surface, the discs were gently squeezed. Just five antibiotic discs were put on a single pate at a time to avoid one mixing. When the discs were mounted on the plate, they were not removed because few of the antibiotics diffuses immediately. The plates were then incubated for 16-18 hrs.at 37 ºC, and the zone diameter was determined to the nearest mm with the aid of a ruler placed at the back of the inverted plate. The margin of zone was described as the region with no apparent, noticeable growth that could be identified with the unassisted eye. CLSI standards were used to assess the inhibition zone sizes [24]. The antibiotics evaluated were cefepime (30µg), gentamicin (10 µg), amikacin (30µg), ciprofloxacin (5 µg), ceftazidime (30µg), piperacillin/tazobactam (100/10 µg), amoxicillin/clavulanic acid (30 µg), chloramphenicol (30 µg), penicillin (10 µg), imipenem (30 µg), tetracycline (30 µg), sulphametaxazole/trimethoprim (100/10 µg), cefotaxime (30 µg), meropenem (30 µg), ceftriaxone (30 µg) and polymyxin B (300).

Determination of MICs

The minimum inhibitory concentration (MIC) of the selected antibiotics (cefotaxime, polymyxin B and ciprofloxacin) and non-antibiotics (sertraline, loperamide, metformin and diclofenac sodium) against a single MDR Pseudomonas aeruginosa was determined by broth micro dilution in Mueller-Hinton broth (MHB) using 96-well microtitre plates procedure [27]. The antibiotics and non-antibiotics dilutions tested in the micro broth dilution assay ranged from 1 µg/mL to 512 µg/mL at final concentration. The dilutions were formulated at twice the required final concentrations in Muller Hinton broth to enable a dilution of 1:2, when an equivalent amount of inoculum was added to it. Sterile test tubes were marked as follows: 512, 256, 128, 64, 32, 16, 8, 4, 2 and the last 1µg/mL. The inoculum was prepared with 2-3 h broth culture of each isolate, adjusted to a turbidity equivalent to 0.5 McFarland Standard and diluted in Mueller Hinton broth to give a final concentration of 5 × 105 cfu/mL. MIC was defined as the lowest concentration of antibiotic to completely inhibit visible growth.

Synergistic effect of selected non-antibiotics on antibiotics and FICs

Synergistic effects of selected non-antibiotics on antibiotics were determined by checker board assay [28]. Non-antibiotics and antibiotics combination evaluated in-vitro using checker board against Pseudomonas aeruginosa were Sertraline+ Polymyxin B, Diclofenac Sodium+ Cefotaxime, Loperamide+ Polymyxin B and Metformin+ Ciprofloxacin. The MIC of each antibiotic and non-antibiotic substance alone or in combination was determined by a broth micro dilution method in accordance with CLSI standards. The assay was performed in 96-well microtitre plates, a two-fold dilution of the antibiotic and was distributed into each well to obtain a varying concentration of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1ug/mL in the wells of the first column, while those of the non-antibiotics were similarly distributed among the first row (512 to 1 µg/mL). The antibiotic dilutions were started from the columns to the right and the non-antibiotic dilutions were started from the first row downwards. Thus, each of the wells held a unique combination of concentrations of AgNPs and the antibiotic. The broth micro dilution plates were inoculated with each test microorganism to yield the appropriate density (105 CFU/mL) in 100 _L Mueller–Hinton broth and incubated at the optimum temperature and time of growth conditions (37 ºC/24 h). The MIC was determined as the least dilution without any turbidity. The MICs of single antimicrobial A and B (MICA and MICB) and in combination were determined. A possible synergistic effect was indicated by the lack of growth below the MIC value of two substances. The fractional inhibitory concentration index (ΣFIC) was calculated as

ΣFIC= FIC A+FIC B

Where, FIC A=MIC of drug A in combination/ MIC of drug A alone.

FIC B= MIC of drug B in combination/ MIC of dug B alone.

From the above equation synergism is defined as: when the ΣFIC was less than or equal to 0.5, the combination was considered synergistic, indifferent when the value was greater than 0.5 and less than 2 and a value greater than 4 was classified as antagonistic [29].

RESULTS

Identification of Pseudomonas aeruginosa

Amongst 100 wounds pus samples, 62 were identified Pseudomonas aeruginosa. Pseudomonas aeruginosa culture on nutrient agar plates was opaque, slimy and irregular colonies with earthy odor. On MacConkey agar plates, it formed big, pale color, smooth egg-like colonies with flat margins. The results of biochemical tests performed for the identification of Pseudomonas aeruginosa are listed in the Table 1.

Table 1. Results of Biochemical tests for the identification of Pseudomonas aeruginosa.

S. No	Biochemical tests	Results
1.	Catalase	Positive
2.	Oxidase	Positive
3.	Triple sugar iron (TSI)	Alkaline slant/Alkaline butt
4.	Citrate utilization test	Positive
5.	Indole production	Negative

Antibiotic Susceptibility Testing

The antibiotic susceptibility testing of Pseudomonas aeruginosa was performed according to Kirby Bauer Disc Diffusion method. Disc diffusion test results of all the 62 isolates of Pseudomonas aeruginosa indicated that 53 of isolates were MDR, which demonstrated in-vitro resistance to three or more than three different antibiotics and were termed as multi drug resistant. Significant level of resistance was observed among all the clinical isolates of Pseudomonas aeruginosa. Antibiogram of all the 62 isolates of Pseudomonas aeruginosa are given in Table 2.

Table 2. Antibiogram of Pseudomonas aeruginosa**.**

S. No	Antibiotics tested	Disc concentration	Sensitive	Intermediate	Resistant
			n (%)	**n (%)**	**n (%)**
1.	Ciprofloxacin	5 µg	10 (16.16)	----	52 (83.87)
2.	Penicillin	10 µg	3(4.83)	7 (11.29)	52 (83.87)
3.	Ceftriaxone	30 µg	5 (8.06)	4 (7.54)	53(85.48)
4.	Polymyxin B	300 µg	11 (17.74)	----	51 (82.25)
5.	Chloramphenicol	30 µg	5 (8.06)	4 (6.45)	53 (85.48)
6.	Sulphametaxazole/trime-thoprim	25 µg	7 (11.29)	-----	55 (88.70)
7.	Tetracycline	30 µg	8 (12.90)	-----	54 (87.09)
8.	Gentamicin	10 µg	7 (11.29)	2 (3.22)	53 (85.48)
9.	Imipenem	10 µg	5 (9.43)	3 (4.83)	54 (87.09)
10.	Meropenem	10 µg	5 (8.06)	-----	56(90.32)

11.	Cefepime	30 µg	8 (12.90)	-----	54 (87.09)
12.	Amikacin	30 µg	9 (14.51)	-----	53(85.48)
13.	Piperacillin/tazobactam	110 µg	5 (8.06)	1 (1.61)	56 (90.32)
14.	Ceftazidime	30 µg	6 (9.67)	-----	56 (90.32)
15.	Amoxicillin/clavulanic acid	30 µg	9 (14.51)	-----	53 (85.48)
16.	Ceftriaxone	30 µg	5(8.06)	2 (3.22)	55 (88.70)

Minimum Inhibitory Concentrations of antibiotics

MIC of antibiotics cefotaxime, polymyxin B and ciprofloxacin were performed for a single MDR Pseudomonas aeruginosa. The individual MIC of cefotaxime, polymyxin B and ciprofloxacin calculated were 32 µg/mL, 16 µg/mL and 64 µg/mL, respectively. The MIC50 and MIC90 values observed for these antibiotics were as follows; cefotaxime (MIC50=16 MIC90=29), polymyxin B (MIC50=8, MIC90=15) and ciprofloxacin (MIC50=32, MIC90=58). Results of MIC, MIC50 and MIC9 of selected antibiotics against a single MDR Pseudomonas aeruginosa are given in the Table 3.

Table 3. MIC of different antibiotics against for MDR Pseudomonas aeruginosa.

S. No	Antibiotics	MIC	MIC 50 µg/mL	MIC 90 µg/mL
1	Cefotaxime	32	16	29
2	Polymyxin B	16	8	15
3	Ciprofloxacin	64	32	58

Minimum Inhibitory Concentration of Non-Antibiotics

MIC of four non-antibiotics including sertraline, loperamide, metformin and diclofenac sodium were performed for a single MDR pseudomonas aeruginosa. The MIC sertraline, loperamide, metformin and diclofenac sodium were of 64 µg/mL, 32 µg/mL, 16 µg/mL and 64 µg/mL, respectively. The MIC50 values sertraline, loperamide, metformin and diclofenac sodium were 32, 16, 8 and 32 µg/mL respectively. In addition the MIC90 values observed were as follows; 58 µg/mL for Sertraline, 29 µg/mL for Loperamide, 15 µg/mL for Metformin and 58 µg/mL for Diclofenac sodium. Result of MIC, MIC50 and MIC90 values, of selected non-antibiotics are given in the Table 4.

Table 4. MIC of non-antibiotics.

S. No	Non-Antibiotics	MIC	MIC 50 µg/mL	MIC 90 µg/mL
1	Sertraline	64	32	58
2	Loperamide	32	16	29
3	Metformin	16	8	15
4	Diclofenac sodium	64	32	58

Synergistic effects of non-antibiotics on antibiotics

Non-antibiotics and antibiotics combination tested in-vitro against a single MDR Pseudomonas aeruginosa isolate were diclofenac sodium+cefotaxime, sertraline+polymyxin B, loperamide+polymyxin B and metformin+ciprofloxacin. Diclofenac sodium when combined with cefotaxime showed synergistic antibacterial activity with FIC value of 0.25. Similarly, the combination of loperamide with polymyxin B showed synergism with FIC values 0.5. The FIC values obtained for the combination of sertraline with polymyxin B was 0.37, while the FIC value observed for metformin when combined with ciprofloxacin was 1. Results of checkerboard assay are given in the Table 5.

Table 5. Results of combination of non-antibiotics and antibiotics against MDR Pseudomonas aeruginosa

Tested Non-antibiotic	**Antibiotic**	**MIC (µg/mL) of Non-antibiotic**		**MIC (µg/mL) of Antibiotic**		**FIC index**
		Alone	Combination	Alone	Combination	
Diclofenac sodium	Cefotaxime	64	8	32	4	0.25
Sertraline	Polymyxin B	64	8	16	4	0.37
Loperamide	Polymyxin B	32	8	16	4	0.5
Metformin	Ciprofloxacin	16	8	64	32	1

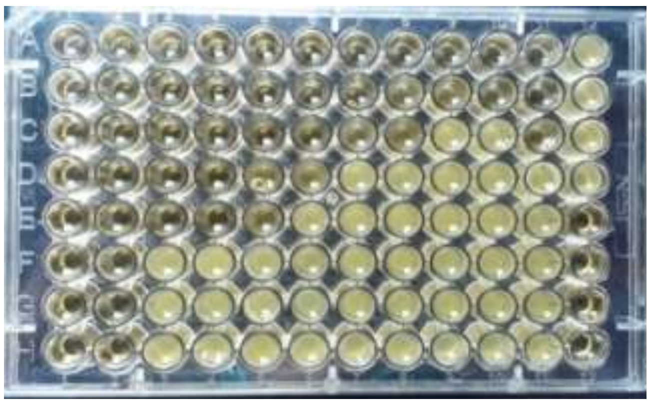

Fig 1. Showing Checkerboard assay for synergistic effect of selected antibiotics and non-antibiotics against MDR P. aeruginosa

DISCUSSION

Hospital-acquired infections caused by P. aeruginosa are generally life-threatening and pose a great challenging to treat. During the current day study a total of 62 pseudomonas aeruginosa isolates were inestigated in which 85.48 % (53) were MDR, which is certainly is a huge number. Resistance to several antimicrobials by Pseudomonas aeruginosa is a major challenge in managing its infections [30, 31]. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements [32]. Similarly, Mirsalehian et al. (2010) reported 87.05 % of the MDR isolates that support our finding [33]. The present findings are also strengthened by Rahimzadehet al. (2016) who reported similar results [34]. Golshaniet al. (2015) reported that more than 63 % of the cases were MDR which is close to our findings [35]. More than 10 % of the isolates of Pseudomonas aeruginosa around the world are multi-drug resistant [36]. The present study further revealed that More than 90 % of the isolates were resistant to meropenem, piperacillin/tazobactam and ceftazidime. While the highest sensitivity was shown to polymyxin B (17 %) followed by ciprofloxacin (16 %), amikacin (14 %) and amoxicillin/clavulanic acid (14 %). Polymyxins are amongst the most important antibiotics in modern medicine and are currently used as last line treatment against MDR P. aeruginosa infections [37]. Like the present study, Banaret al. (2016) reported almost similar variations in the antibiotic resistance pattern of Pseudomonas aeruginosa [38]. However, the resistance to ceftazidime (61.4 %) was lower than the present findings. Similarly, Khosraviet al. (2016) reported highest rate of resistance to ceftazidime (90.5 %) and gentamicin (88.5 %) [39]. Resistance to piperacillin/tazobactam was 90.32 %, which is higher than that reported previously by Abbas et al. 2015 [40] and Miceket al. 2015 [41]. Sensitivity to ciprofloxacin and ceftazidime was 16.16 % and 9.67 %, respectively. In contrast, Sarwaret al. (2013) reported 41.5 % sensitivity to ciprofloxacin and 22 % to ceftazidime [42. Similarly, Ahmed at al. (2016) revealed 75.9 % sensitivity to ciprofloxacin and 67.6 % to ceftazidime [43], which support the present findings. Carbapenems have been recommended in a number of previous studies for the treatment of MDR isolates. However, in the present study, 90 % of the isolates were resistant to meropenem and 87 % to imipenem. Similarly, multiple studies have shown resistance to meropenem such as 35 % by Rahimiet al. 2012 [44] and 62 % by Fazeliet al. 2013 [45]. Detection of carbapenems resistance and its comparison with the current results indicates an increase in resistance to imipenem and meropenem. Therefore, it is suggested that the use of imipenem and meropenem is no longer effective in burn wounds.During the current study, the strategy of repurposing drugs was adapted to treat multidrug resistance P. aeruginosa. Amongst the different tested combination of the selected antibiotics and non-antibiotics, the combination of Diclofenac sodium+cefotaxime was the most effective against MDR P. aeruginosa, followed by Sertraline+ Polymyxin B and Loperamide+ Polymyxin B. In case of the combination of Metformin+ Ciprofloxacin indifference was observed (FIC=1). Similar results were shown by Liu et al. 2020) with no synergistic antibacterial activity of metformin with ciprofloxacin [46]. The escalating levels of drug resistance render it indispensable to explore newer drugs with lesser degrees of toxicity and possibly fewer chances of developing resistance [47]. However the concept of reversal of resistance by means of non-antibiotics could be a promising solution for bringing back drug resistant micro-organisms to their original sensitivity to the classical antibiotics. The synergistic effect of antibiotic and Non-antibiotics against MDR bacteria have been reported before. Haderaet al. (2018) reported the synergistic effect of Fluconazole (antibiotic) with non antibiotics like fluoxetine and propranolol against C. albicans [22]. Otto et al. (2018) documented the antibacterial activity of combination polymyxin B and a wide variety of non-antibiotics against Pseudomonas aeruginosa,Acinetobacter baumannii, Escherichia coli and Klebsiella pneumonia [23]. In particular, little is known about the underlying mechanisms of most drug interactions [48]. Therefore, in vitro data are important to identify promising regimens and provide a better understanding of the mechanisms of synergistic interaction [49].

CONCLUSION

The unchecked and unnecessary use of antibiotics is leading to the emergence of multidrug resistance bacteria which makes it difficult to treat these bacteria. Unfortunately, new antibiotics are discovered and are released to markets rapidly, which further contributes to the problem of antibiotic resistance. This study was aimed to evaluate the antibacterial potential of already present drugs against MDR P. aeruginosa. During the present study it was observed that, all the tested combinations except of the selected antibiotics showed synergism against MDR P. aeruginosa. Our findings suggest that these tested combinations have promising potential to treat MDR strains P.aeruginosa, against which almost all the conventional antibiotics are ineffective. However, further steps, including pharmacokinetic and pharmacodynamic studies and in vivo efficacy evaluation in experimental models of infection, including the dosage and safety, are required to confirm the usefulness of pentamidine in the treatment of infection by MDR P. aeruginosa.

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Conflicts of interest/Competing interests

The authors declare that they have no competing interests

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