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Article

Investigating Synergistic Effects of Different Antibiotics on Biofilm of *Pseudomonas aeruginosa*

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Abstract: Background: *Pseudomonas aeruginosa* is an opportunistic pathogen involved in number of hospital acquired infections such as catheter-associated urinary tract infections, bacteremia, septicemia, skin infections and ventilator-associated pneumoniae. Biofilm formation is an important trait implicated in chronic infections, such as cystic fibrosis and chromic pulmonary obstruction. **Method:** A total of 266 isolates were collected from the Armed Forces Institute of Pathology (AFIP). Antibiotic susceptibility was assessed by double disk synergy testing. ESBL and Modified Hodge testing was performed for phenotypic confirmation of carbapenemases. Molecular screening of the genes was done by PCR. Micro-dilution broth method was used to determine minimum inhibitory concentrations of antibiotics. Biofilm formation was done by micro-titer plate assay. **Results:** Overall, 20% of the *P. aeruginosa* isolates were extensively drug resistant (XDR-PA) and 25% were multi-drug-resistant (MDR-PA). Likewise, 43% of the isolates were ESBL producers and carbapenemase production was detected in 40% of the isolates. Molecular analysis confirmed occurrence of different resistant factors in ESBL positive isolates; 67% carried *bla-TEM,* 62% *bla-CTXM-15,* 41% *bla-SHV,* 34% *bla-CTXM-14* and 33% *bla-OXA-1*. In addition, 68% of carbapenem-resistant isolates were positive for *bla-NDM-1,* 25% for *bla-OXA-48* and 22% for *bla-KPC-2*. Biofilm formation was tested for 234 isolates, out of which 28% were strong biofilm formers. Moderate and weak biofilm formers were 46% and 23% respectively. Overall, ciprofloxacin, levofloxacin and cefepime showed inhibitory effects on *P. aeruginosa* biofilms. Antibiotics in combination showed strong synergistic effects (ciprofloxacin & cefepime); while gentamicin and cefepime resulted into complete eradication of *P. aeruginosa* biofilm. **Conclusion:** We confirm strong synergistic effects of gentamicin and cefepime that completely eradicated *P. aeruginosa* biofilm. We further confirm inhibitory effects of ciprofloxacin, levofloxacin and cefepime on *P. aeruginosa* biofilms.

Keywords: synergistic effects; gentamicin; cefepime; biofilm; *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa, is an opportunistic pathogen associated with different noscomial infections [1]. This bacterium is classified as "critical pathogen" by WHO and is one of the "ESKAPE" organisms. *P. aeruginosa* causes variety of infections including respiratory tract infections (RTIs), urinary tract infections (UTIs), wound infections, dermatitis, skin/soft tissue infections bone/joint infections, septicemia and bacteremia [2]. Higher mortality associated with *P. aeruginosa* infections (up to 30%) exceeds well beyond many other gram-negative bacteria, including ***Staphylococcus aureus [3]. In case of hospital-acquired pneumonia, multi-drug resistant*** strains of *P. aeruginosa* (MDR-PA) are an independent risk factor for mortality [4]. Quite recently, MDR-PA, resistant to levofloxacin, ciprofloxacin, imipenem-cilastatin, meropenem, aztreonam, cefepime, ceftazidime and piperacillin-tazobactam are suggested to be labeled as difficult to treat (DTR) infections [5,6]. In the current AMR scenario, combination therapy has been recommended to treat *P. aeruginosa* bloodstream infections [7,8]. Combining any conventional or novel β-lactam with aminoglycosides or a fluoroquinolones is highly sought [9,10]. Yet, for the use of empiric combination

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versus monotherapy, no consensus has been reached among scientific community [11]. Bacterial biofilms are a formidable barrier against the efficacy of antibiotics that makes biofilm associated infections notoriously difficult to treat. A three-dimensional structure of the biofilm results into anaerobic environment, hence some antibiotic such as fluoroquinolones, β-lactams, and aminoglycosides may lose efficacy in such environment [12]. On the other hand, exposure to sub-lethal or sub-minimal inhibitory concentrations of certain antibiotics expedite biofilm formation [13,14]. However, not much is known about the efficacy of antibiotic therapy in combination against infections involving pseudomonal biofilms. In the current study, we evaluated effects of ciprofloxacin (CIP), levofloxacin (LEV) and cefepime (CEF) on biofilm formation of *Pseudomonas aeruginosa*. It is one of the very few studies focusing on synergistic effects of multiple antibiotics on the biofilm of *Pseudomonas aeruginosa*.

2. Results

2.1. Susceptibility profile

Clinical samples collected from Armed Forces Institute of Pathology (AFIP), Islamabad, Pakistan revealed that the major source of *P. aeruginosa* was pus 102 (38%) that was followed by urine 49 (18%), ear/throat/wound swab 27(10%), tissue culture 18 (7%), sputum 14(5%), endobronchial secretion 10 (4%) and NBL 9 (3%). Highest percentage of the *P. aeruginosa* isolates showed resistance against ciprofloxacin, 114(43%) and levofloxacin 116(44%) that was followed by meropenem 99(37%), ceftazidime 97(36%), imipenem 94(35%), aztreonam 94(35%), gentamicin 87(32%), amikacin 72(27%), cefepime 69(26%) and piperacillin/tazobactam 45(17%). The most effective antibiotics were polymyxin B and colistin showing 98% sensitivity. Moreover, 25% of the isolates were MDR and 20% XDR. The distribution of MDR, XDR and non-MDR strains of *P. aeruginosa* is shown in Table 1.

2.2. Molecular identification of ESBL and carbapenemases genes

Out of 266 isolates, 43% of the isolates were confirmed as ESBL producers by double disc synergy testing. Molecular analysis revealed that 62% of the isolates carried *bla*$_{\text{CTXM-15}}$ gene, and 34% were positive for *bla*$_{\text{CTXM-14}}$. Other molecular factors including *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{OXA-1}}$ were detected in 41%, 67% and 33% of the isolates respectively. In addition, 40% of the isolates were carbapenemase producers. Overall, 68% of the isolates carried *bla*$_{\text{NDM-1}}$ gene and 25% were positive for *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC-2}}$ was detected in 22% of the *P. aeruginosa* isolates.

2.3. Biofilm formation assay

Pseudomonas aeruginosa was assessed for its biofilm forming ability using micro-titer plate method. Out of 234 tested isolates, 227(97%) were biofilm producers. Out of these 65 (28%) were strong biofilm producers, 108 (46%) produced biofilms at moderate level and 54 (23%) were identified as weak biofilm producers.

2.4. Minimum inhibitory concentration

Minimum inhibitory concentrations were measured against ciprofloxacin, levofloxacin and cefepime using broth micro-dilution method. Six isolates, simultaneously strong biofilm formers and sensitive to ciprofloxacin, levofloxacin and cefepime were selected for measuring, minimum inhibitory concentration of biofilm (MIC-b). It was observed that MIC-b values for all six tested isolates were 128-1000 times higher, revealing that the isolates became tolerant to antibiotics in their biofilm forms. Furthermore, MBEC values indicated a ~2000 folds increase when compared with respective MIC-p, which clearly showed that complete eradication of biofilms of *P. aeruginosa* was difficult to achieve even at much higher concentration of antibiotics (Table 2).

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2.5. Effects of sub-MICs of CIP, LEV and CEF on biofilm formation

All six *P. aeruginosa* isolates, simultaneously strong biofilm formers and sensitive to ciprofloxacin, levofloxacin and cefepime were used to determine sub-minimal inhibitory concentrations of ciprofloxacin, levofloxacin and cefepime at different time intervals i.e. 4 hours, 8 hours, 12 hours and 24 hours. It was observed biofilm formation was significantly (p<0.05) and consistently reduced upon treatment with antibiotic at sub-minimal level. Reduction was observed in a dose dependent manner. In case of ciprofloxacin maximum reduction of biofilm was shown at 0.5 µg/ml (Figure 1). While in case of levofloxacin, only two isolates showed significant reduction (p<0.05) in biofilm formation at two different concentrations (0.125 µg/ml and 0.5µg/ml) after 24 hours of incubation. Overall however, effect of levofloxacin on the biofilm forming ability of tested isolates was inhibitory (Figure 2). In case of cefepime inhibitory effects were not observed on all except one isolate that showed significant reduction at 0.125 µg/ml and 0.5 µg/ml as shown in Figure 3.

2.6. Evaluation of synergistic effects by checkerboard method

Strong biofilm former *P. aeruginosa* isolates simultaneously sensitive to ciprofloxacin, cefepime and gentamicin were used to evaluate effects of antibiotics on biofilm formation. Antibiotics were tested individually and in combination. When tested gentamicin alone MBIC values was 512 µg/ml and MBEC (1024 µg/ml). However, when tested gentamicin in combination with cefepime MBIC for both antibiotics was substantially reduced (16 µg/ml). Similarly in combination MBEC of both antibiotics gentamicin and cefepime was reduced (32 µg/ml). Likewise, MBIC and MBEC were significantly reduced for another tested combination ciprofloxacin and cefepime (Table 3). FIC values clearly indicate significant synergistic potential of tested combinations in this study (Table 4).

2.7. Effect of sub-MIC on synergism

To assess the effect of sub-MIC on synergistic potential, the antibiotics were used in combination at sub-MIC. The sub-MIC of each antibiotic was used in following concentrations: 0.5µg/ml, 0.25µg/ml, 0.125µg/ml, and 0.0625µg/ml. An effective inhibition in biofilm formation was observed after 4-6 hours of incubation with CEF+CN and CEF+LEV, particularly at 0.5µg/ml. While growth controls (in the absence of antibiotics) showed strong biofilm production **(Figure 4).**

2.8. Figures and Tables

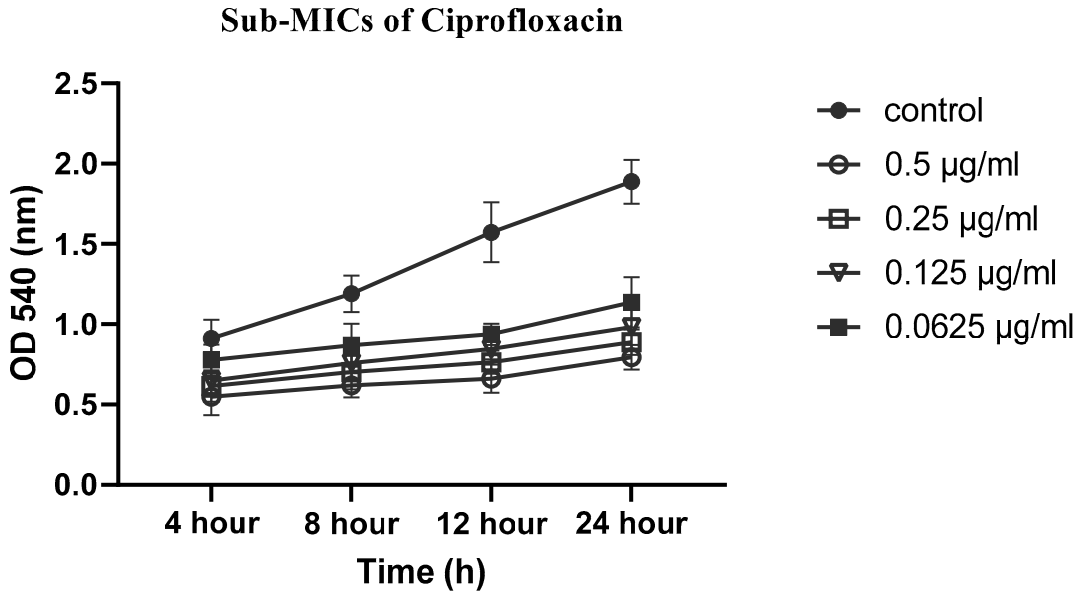

Figure 1. Sub-minimal inhibitory effect of Ciprofloxacin on selected isolates of *Pseudomonas aeruginosa.*

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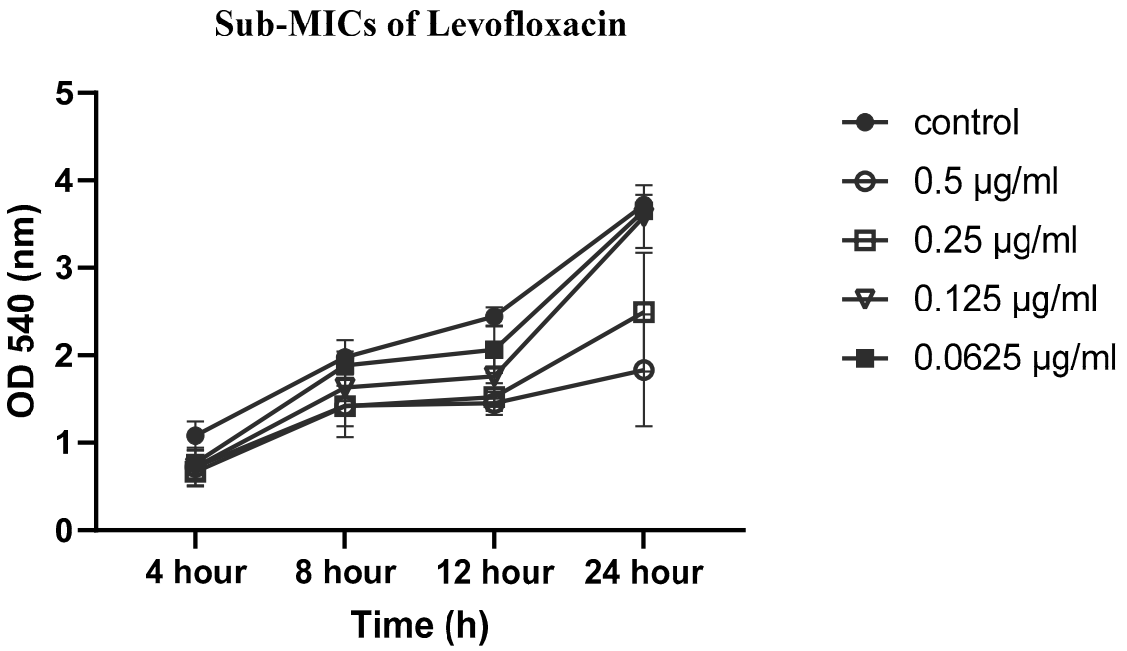

Figure 2. Sub-minimal inhibitory effect of Levofloxacin on selected isolates of *Pseudomonas aeruginosa.*

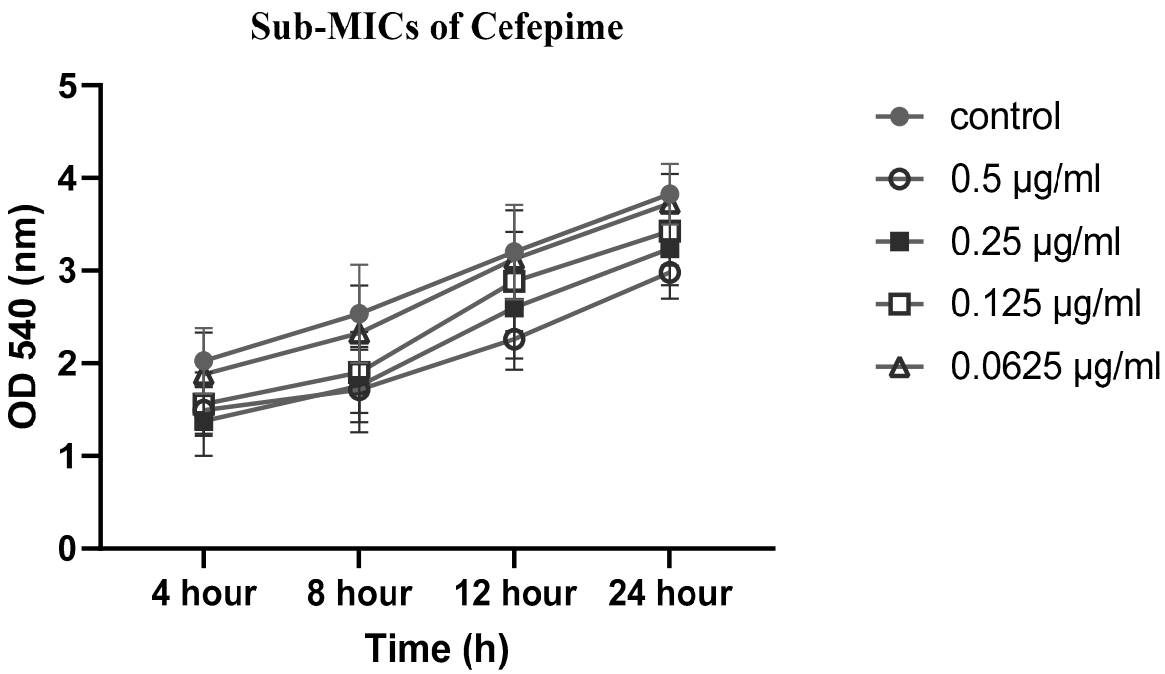

Figure 3. Sub-minimal inhibitory effect of Cefepime on selected isolates of *Pseudomonas aeruginosa.*

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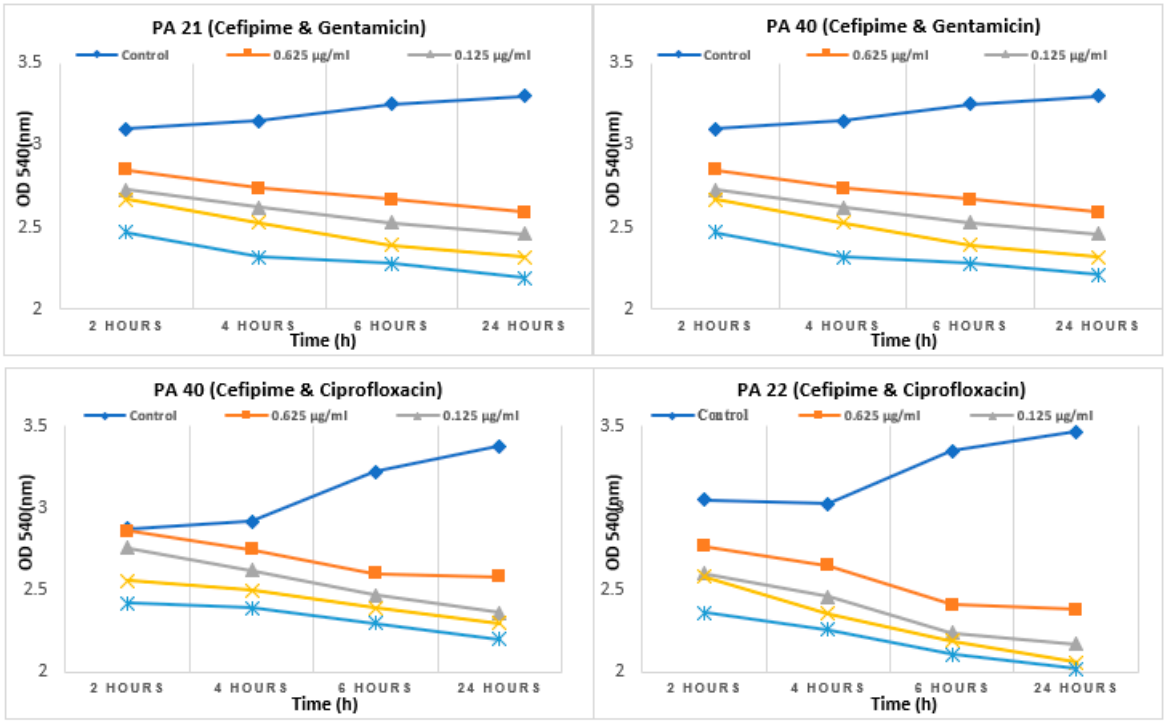

Figure 4. Sub-minimal inhibitory effect of Combination Therapy on selected isolates of *Pseudomonas aeruginosa.*

Table 1. Frequency of MDR and XDR *Pseudomonas aeruginosa* among clinical samples.

Type of Specimen	P. aeruginosa (n=266)	MDRs	XDRs	Non-MDRs	P value
Pus	102(38.34%)	16(15.68%)	25(24.5%)	61(50%)	0.44
Urine	49 (18.42%)	7(14.28%)	13(26.53%)	29(28.6%)	0.5670
Endobronchial sec.	10 (3.75%)	0	1(10.0%)	9(88.88%)	0.1815
Sputum	14 (5.26%)	1(7.14%)	1(7.14%)	12(75%)	0.2251
NBL	09 (3.38%)	4(44.9%)	2(22.25%)	3(14.3%)	0.0257
Tissue	18 (6.76%)	1(5.55%)	1(5.55%)	16(100%)	0.0774
Fluid	11 (4.13%)	1(9.09%)	1(9.09%)	9(50%)	0.4537
Ear, Throat and Wound swab	27 (10.15%)	4(14.81%)	3(11.11%)	20(100%)	0.3778
Blood	13 (4.88%)	0	7(53.84%)	6(66.66%)	0.0105
Pleural fluid	02 (0.75%)	1(50%)	0	1(50%)	0.3174
CVP tip	05 (1.87%)	1(20%)	2(40%)	2(40%)	0.4956
Peritoneal fluid	02 (0.75%)	1(50%)	0	1(50%)	0.3174
Tracheal tube tip	01 (0.37%)	0	1(100%)	0	0.1588

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Chest tube tip	01 (0.37%)	0	0	1(100%)	0.7567
Catheter tip	02 (0.75%)	1(50%)	0	1(50%)	0.3174

Note: Numbers (and percentage) of MDR and XDR strains obtained from corresponding samples. A significant *P*-value ($P<0.05$) indicates strong association of the sample type within the two population subgroups (MDR and XDR). MDR= Multidrug resistance, XDR= Extensive drug resistance.

Table 2. MIC-p, MIC-B and MBEC analysis on selected *Pseudomonas aeruginosa* isolates.

Sample ID	MIC-p (µg/ml)			MIC-b (µg/ml)			MBEC (µg/ml)		
	CIP	LEV	CEF	CIP	LEV	CEF	CIP	LEV	CEF
20	<0.25	2	0.5	128	64	128	>2048	>2048	>2048
44	1	0.5	2	256	16	128	>2048	>2048	>2048
92	1	2	1	128	64	256	>2048	>2048	>2048
147	0.25	0.5	1	128	64	256	>2048	>2048	>2048
172	0.25	0.5	0.5	256	64	128	>2048	>2048	>2048
188	1	0.5	1	256	64	256	>2048	>2048	>2048

MIC-p: Minimum Inhibitory Concentration for planktonic bacteria; MIC-b: Minimum Inhibitory concentration for biofilm; MBEC: Minimum Biofilm Eradication Concentration.

Table 3. Analysis of MBIC and MBEC of selected antibiotics (individual vs combination) against bacterial biofilm.

S. no.	Isolate no.	Gentamicin (GEN)		Cefepime (CEF)		GEN+CEF (Combination)	
		MBIC (µg/ml)	MBEC (µg/ml)	MBIC (µg/ml)	MBEC (µg/ml)	MBIC (µg/ml)	MBEC (µg/ml)
1.	PA21	512	1024	512	1024	32CEF+16GEN	32 CEF + 32GEN
2.	PA40	512	1024	256	512	32CEF+16GEN	32CEF+ 32GEN
S. no.	Isolate no.	Ciprofloxacin (CIP)		Cefepime (CEF)		CIP+CEF (Combination)	
		MBIC (µg/ml)	MBEC (µg/ml)	MBIC (µg/ml)	MBEC (µg/ml)	MBIC (µg/ml)	MBEC (µg/ml)
1.	PA21	512	1024	512	1024	64CIP+ 16CEF	64 CIP+ 32 CEF
2.	PA40	512	1024	256	512	128CIP+16CEF	128 CIP+ 32 CEF

MBIC/MRC: Minimum Biofilm Inhibitory Concentration, MBEC: Minimum Biofilm Eradication Concentration.

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Table 4. Antibiotic concentration having synergistic interaction in inhibition and eradication of biofilm.

S.No./ Isolate no.	Well no.	Drug Concentration Drug A: Cefepime Drug B: Gentamicin	FIC index value Synergy ≤ 0.5	S.No./ Isolate no.	Well no.	Drug Concentration Drug A: Ciprofloxacin Drug B: Cefepime	FIC index value Synergy ≤ 0.5
1.	D2	128A, 16B	0.28	3.	D2	128A, 16B	0.28
	D3	128A, 32B	0.3		D3	128A, 32B	0.312
	D4	128A, 64B	0.37		D4	128A, 64B	0.375
	E2	64A, 16B	0.15		D5	128A, 128B	0.5
	E3	64A, 32B	0.18		E2	64A, 16B	0.15
	E4	64A, 64B	0.25		E3	64A, 32B	0.19
	E5	64A, 128B	0.37		E5	64A, 128B	0.375
	F2	32A, 16B	0.09	4.	D2	128A, 16B	0.31
	F3	32A, 32B	0.12		D3	128A, 32B	0.375
	F4	32A, 64B	0.18		D4	128A, 64B	0.5
	F5	32A, 128B	0.31				
2.	E2	64A, 16B	0.28				
	E3	64A, 32B	0.31				
	E4	64A, 64B	0.375				
	F2	32A, 16B	0.156				
	F3	32A, 32B	0.186				
	F4	32A, 64B	0.25				
	F5	32A, 128B	0.375				

3. Discussion

A critical ESKAPE pathogen, *P. aeruginosa* is associated with variety of infections including, skin infections, ventilator-associated pneumoniae, bacteremia and septicemia. Sever infections such as blood-stream infections (BSI) caused by this bacterium are linked to higher mortality rates up to 30%. In the current study, 266 clinical isolates of *P. aeruginosa* were collected from Armed Force Institute of Pathology (AFIP) located in Rawalpindi. We scrutinized antibiotic resistance profile and evaluated synergistic effects of different antibiotics on biofilm formation, inhibition and eradication of *P. aeruginosa*. Out of 266 isolates majority (38%) were isolated from pus and other 18% from the urine samples. Frequent isolation of *P. aeruginosa* from pus and urine was reported earlier from Pakistan [27,28]. In case of sever *P. aeruginosa* infections such as BSI several antibiotics are listed as the first line therapeutic options, including piperacillin/tazobactam. In addition, aminoglycoside in combination with piperacillin/tazobactam are recommended to treat noscomial pneumonia. Out of tested *P. aeruginosa* isolates 17% were resistant to piperacillin/tazobactam. Resistance to tested aminoglycosides ***gentamicin,*** and amikacin was 32% and 27% respectively. Among ***cephalosporins, cefepime is the most frequently used β-lactam class of antibiotic for*** *P. aeruginosa* infections, others being ceftazidime and cefoperazone. In this study 36% of the isolates were resistant to ceftazidime and 26% to cefepime. Likewise, fluoroquinolones are considered first-line therapeutic options to treat BSI caused by *P. aeruginosa*. Ciprofloxacin and levofloxacin are the only options which can be administered orally during bloodstream infections. Upon testing these two antibiotics 43% of the isolates showed resistance to ciprofloxacin and 44% to levofloxacin. Taken together resistance to above mentioned frontline antibiotics indicate significant constraints on available therapeutic options to treat *P. aeruginosa* infections in Pakistan. ***Meropenem is considered***

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as a single agent therapy for complicated skin infections caused by *P. aeruginosa*. For treating *P. aeruginosa* sepsis carbapenems are considered second line therapy; particularly use of meropenem is favored over imipenem because former is linked to the induction of resistance during the continual course of treatment [29]. Overall however, cephalosporins are preferred over carbapenems because of their better potency and narrower spectrum of activity to treat sepsis. In this study 37% of the isolates were resistant to meropenem and 35% to imipenem.

P. aeruginosa is notorious for using variety of mechanisms of antibiotic resistance including production of β-lactamase enzymes, aminoglycoside modifying enzymes, modification of target sites, modification of outer membrane protein (OprD), production of variety of carbapenemases and efflux pump gene (MexAB, MexXY) [11]. In this study, 43% of the isolates were ESBL producers and 40% produced carbapenemases. In three or more classes of antibiotics, resistance to at least one agent suffices MDR status, while resistance to at least one agent in all antibiotic classes, except two or fewer than two classes confers XDR status. In current study, 25% of the isolates were MDR while 20% were XDR.

Biofilm forming capacity of *P. aeruginosa* facilitates chronic colonization of host tissues such as cystic fibrosis and persistence in implanted medical devices. These micro communities also enhance its resistance potential and protect it from the host defenses. In current study 28% of the isolates were strong, 46% moderate, 23% weak and 3% were non-biofilm formers. Among strong biofilm producers 25% and 20% of the isolates were MDR and XDR respectively. In terms of tolerance towards antibiotics, biofilm are crucial and may lead to persistent cell formation, replacement of sensitive cells with resistant phenotypes, impairment of antibiotic diffusion due to extensive exopolysaccharides presence in matrix. In present study, efficacy of the tested ciprofloxacin, cefepime and levofloxacin was much higher in planktonic state when compared with the biofilm mode. For example, overall in biofilm mode, MIC values were 128-1000 fold higher when compared with MIC values in planktonic state. We observed that at sub-MIC level fluoroquinolones inhibited biofilm at different time intervals. For *A. baumannii*, it was shown recently that sub-minimal concentration of antibiotics can significantly alter expression of genes involved in biofilm formation and antibiotic resistance [30]. It's fascinating that in biofilm matrix bacteria can sense minute concentrations of antibiotics and alter their behavior remarkably; however underlying molecular mechanisms for alteration in gene expression still remain obscure. Though for the treatment of severe *P. aeruginosa* infections, empirical combination therapy as an option remains inconclusive due to lack of robust prospective studies [11]. Yet due to AMR scenario and robust intrinsic resistance of *P. aeruginosa* to several different antibiotics, combination therapy is encouraged, particularly a β-lactam backbone combined with aminoglycoside or a fluoroquinolones are highly sought. It is pertinent to mention that according to the current guidelines of European Society of Clinical Microbiology and Infectious Diseases (ESCMID) treatment with ceftolozane-tazobactam is recommended for severe *P. aeruginosa* infection [31]. Though combination antibiotic therapy might be more advantages in case of biofilm mediated infections, yet not much is known about the synergistic effects of antibiotics on bacterial biofilms. In this study we tested strong biofilm former clinical *P. aeruginosa* strains which are simultaneously sensitive to all three antibiotics (ciprofloxacin, cefepime and gentamicin). In this study when tested single antibiotic, MIC-b values for all three tested antibiotics were 128-1000 fold higher and MBEC were even higher (~2000 fold). Ciprofloxacin was shown to defuse well in *K. pneumoniae* biofilms, while ampicillin was neutralized by β-lactamase enzymes [32]. Fourier transform infrared spectroscopy showed diffusion of fluoroquinolones in *P. aeruginosa* biofilms [33]. In this study, all the tested isolates were sensitive to all three tested antibiotics (ciprofloxacin, cefepime and gentamicin), we confirm that the observed increase in MIC-b (128-1000 fold) and MBEC (~2000 fold) is independent of any intrinsic or acquired mechanisms of antibiotic resistance; hence tolerance towards antibiotics is solely dependent on sessile growth of *P. aeruginosa*.

In order to avoid catheter associated infections antibiotic lock therapy (ALT) is highly recommended therapeutic intervention. Catheter is treated with higher concentration of antibiotic expecting to limit a biofilm associated infection. For a successful eradication, ALT is managed by parallel administration of systemic antibiotics to the patients as well. Given the increase in MIC-b and

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MBEC levels, 128-1000 fold and ~2000 fold respectively shown in this study, limited success can be expected for such eradication to avoid catheter associated biofilm infections with antibiotic lock therapy with single antibiotic. Here we evaluated efficacy of different antibiotics in inhibiting and eradicating biofilms of *P. aeruginosa* in unique combinations. We report a substantial reduction in MBIC of gentamicin when combined with cefepime (16µg/ml). Similarly, MBEC of both antibiotics in combination (gentamicin & cefepime) was reduced substantially (32µg/ml). We also confirm significant reduction in MBIC and MBEC of combination of ciprofloxacin and cefepime. Further, in this study we clearly observed strong synergistic effects of CEF+CN; and CEF+LEV, at sub-MIC concentration, 0.5µg/ml for inhibition of *P. aeruginosa* biofilm. This is one of the few studies, in which synergistic effects of antibiotics were evaluated on *P. aeruginosa* biofilm inhibition and eradication.

4. Materials and Methods

4.1. Sample collection and susceptibility profile

A total of 266 samples were collected from Armed Force Institute of Pathology (AFIP), Rawalpindi. Approval for this study was granted by the Ethical Board of National Institute of Health (NIH) Islamabad. The strains were isolated from different specimens such as pus, urine, sputum, non-directed bronchial lavage (NBL), pleural fluid, ear swab, tissue, CVP tip, tracheal tube tip, end bronchial secretion, blood, fluid, chest tube tip, catheter tip and peritoneal fluid. Antibiotic testing was performed by disc diffusion methods according to the CLSI guidelines [15].

4.2. Phenotypic and genotypic detection of ESBLs and carbapenemases

Phenotypic detection of ESBL and carbapenemase was performed by double disc synergy and Modified Hodge testing [16, 17]. Genotypic screening of ESBL genes, $bla_{CTXM-15}$, $bla_{CTXM-14}$, bla_{SHV}, bla_{TEM} and bla_{OXA-1} was done as described elsewhere [18]. Genes, bla_{NDM-1}, bla_{OXA-48}, and bla_{KPC-2} were screened by using PCR. Amplification conditions were as follows; denaturation at 95°C, for for 1 min, annealing at 55°C, 56°C and 57°C (for bla_{NDM-1}, bla_{OXA-48}, and bla_{KPC-2} respectively) for 1.5 min, extension at 72°C for 1 min and the final extension was done at 72°C for 10 min.

4.3. Minimum Inhibitory Concentration planktonic (MIC-p)

For ciprofloxacin, levofloxacin, and cefepime, MIC-p was determined by using broth micro-dilution method as per CLSI recommendation (CLSI 2021). Briefly, a 100µl of standardized inoculum of *P. aeruginosa* was added into tissue culture plate (TCP) containing 100µl of two-fold serial dilution of tested antibiotics. Each bacterial isolate was tested against a series of antibiotic dilutions; 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125µg/ml. The TCP were incubated overnight at 37 °C and MICs were noted as lowest concentration that inhibited the visible growth of the bacterium.

4.4. Biofilm formation assay

Biofilm formation assays were done by using micro-titer plate method as described elsewhere [19]. For the biofilm formation assays, *P. aeruginosa* strains were cultured on fresh media plates and incubated overnight. The overnight incubated cultures were then diluted 1:100 into fresh Mueller Hinton broth (MHB). A 200 µl of the culture dilution was added per well in a sterile micro-titer plate and incubated overnight at 37°C. Every isolate was tested in triplicate. After incubation, media was discarded and plate was washed thrice with phosphate buffer saline (PBS). After initial washing, 200µl methanol was added in each well for fixation of biofilm. Subsequently, biofilms were stained using 1% crystal violet (CV) stain. The wells were again rinsed with PBS three times and then allowed to air dry. Afterwards, acetic acid (33%) was added to each well for solubilization of crystal violet dye. The plate was incubated at room temperature for 20-30 min and absorbance was checked in plate reader (RT-6000) at 540 nm. *P. aeruginosa* ATCC 27853 was used as a quality control.

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4.5. Effect of sub-MICs of CIP, LEV, and CEF on biofilm formation

The effect of sub-MICs of different antibiotics was evaluated by micro-titer plate assay. Six *CIP, LEV and CEF* sensitive *P. aeruginosa* clinical strains were selected for this assay. Biofilm formation was analyzed in the presence and absence of ciprofloxacin, levofloxacin, and cefepime. All three antibiotics were tested at following sub-minimal concentrations; 0.5 µg/ml, 0.25µg/ml, 0.125 µg/ml, 0.0625 µg/ml for different time intervals i.e. 4 hours, 8 hours, 12 hours and 24 hours. *P. aeruginosa* ATCC 27853 was used as quality control. All assays were performed in triplicates.

4.6. Evaluation of synergistic effect of antibioticsby checkerboard method

Two different combinations of antibiotics: (i) ciprofloxacin with cefepime and (ii) gentamicin with cefepime were selected to determine minimum biofilm inhibitory combination (MBIC) and minimum biofilm eradication concentration (MBEC) by checkerboard assay as described elsewhere [20,21,22]. Briefly, three strong biofilm producing isolates of *P. aeruginosa* simultaneously sensitive to selected antibiotics were processed for the checkerboard assay. Serial two-fold dilutions of selected antibiotics were prepared in MHB ranging from 8µg/ml to 2048µg/ml in sterile test tubes and 100µl of each serially diluted antibiotic was added into the wells of micro-titer plate containing preformed biofilm of the selected isolates. After incubation at 37°C for 24 hours MBIC was visualized, antibiotic dilutions were then discarded, followed by washing of wells with PBS (phosphate saline buffer). After washing, 100µl recovery media (MHB) was added into each well and micro-titer plate was again incubated for 24 hours. Following the overnight incubation, the lowest antibiotic concentration in combination which inhibited the re-growth of the biofilm was considered as MBIC [23,24]. Experiment was performed in triplicates. Wells containing, only bacterial suspension and MHB with antibiotics were considered as positive and negative controls respectively. MBEC was determined the same way as MBIC, except that after incubation step (of recovery media), wells with no visible growth were thoroughly scrapped and suspended in 1ml PBS. The suspensions were then vortexed to disrupt the biofilm followed by plating on fresh tryptic soy agar (TSA). Plates were incubated overnight. MBEC was recorded as the minimum concentration showing no growth on TSA plates. In order to confirm the synergistic effects at which eradication occurred, fractional inhibitory concentrations were determined using formula: $FIC_A+FIC_B = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$

FIC value of ≤ 0.5 indicated synergy, FIC between 0.5 – 4 indicated indifference and FIC >4 indicated antagonism [25].

4.7. Evaluation of synergistic effect of antibiotics at sub-MIC level by checkerboard method

Two-fold serial dilutions of ciprofloxacin, cefepime and gentamicin were prepared at concentrations; 0.5µg/ml, 0.25µg/ml. 0.125µg/ml and 0.0625µg/ml in MHB. A 50µl of antibiotic solution was added to treat wells containing 100µl of standardized bacterial suspension making the final volume in the well 200µl. The TCP were incubated for 2 hours, 4 hours, 6 hours and 24 hours at 37°C [20,26]. Biofilm quantification was done at 540 nm following the crystal violet staining. All experiments were performed in triplicates. Wells containing, only bacterial suspension was considered positive control and MHB with antibiotics was taken as negative control.

Conclusion

Study highlights include prevalence of MDR and XDR *P. aeruginosa* isolates having notable resistance against frontline antibiotics used to treat sever *P. aeruginosa* infections. Conclusively, in this study antibiotics having two different mechanisms of actions showed strong synergistic affects on inhibition and eradication of *P. aeruginosa* biofilm at sub-MIC level.

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