

Virulence Factors of *Pseudomonas aeruginosa* after Postantibiotic Effects Induced by Different Antimicrobial Agents

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Abstract. Postantibiotic effects were induced in *Pseudomonas aeruginosa* clinical isolates after a short time (1 hr) treatment with 2X- and 4X-MICs of different antimicrobial agents (fluoroquinolones, amikacin, aztreonam, and β -lactam antibiotics). The postantibiotic effect of fluoroquinolones on bacterial regrowth and virulence factor activity were more than that of the amikacin, and the highest postantibiotic effect was observed with norfloxacin. The postantibiotic effect of fluoroquinolones at concentrations of 2X MICs induced suppression in the *Pseudomonas aeruginosa* elastase, protease, and phospholipase C activity to the ranges of 31-37%, 62-69.8%, and 62.4-68.8% of the control, respectively. While at higher concentrations (4X MICs), they reduce the enzymatic activity to the ranges of 17-20%, 30.6-36%, and 47.6-57% of the control, respectively. In the case of amikacin, the induced postantibiotic effects at concentrations of 2X and 4X MIC caused suppression in the *Pseudomonas aeruginosa* enzymatic activity to the ranges of 54-73.8% and 27-59.2% of the control, respectively. The postantibiotic effect of aztreonam and β -lactam antibiotics induced weak effect on the re-growth and virulence factors activity of the tested isolates. According to the obtained results, the postantibiotic effect of fluoroquinolones and amikacin induced significant suppression in the *Pseudomonas aeruginosa* elastase, protease, and phospholipase activity.

Keywords: Antibiotics, Postantibiotic effects, *Pseudomonas aeruginosa*, Virulence factors, Elastase, Protease, and Phospholipase C.

Introduction

The term postantibiotic effect (PAE) refers to a period of time after complete removal of an antibiotic during which there is no growth of the target organism^[1]. The PAE appears to be a feature of most antimicrobial

agents, and it has been documented with a variety of common bacterial pathogens. Several factors influence the presence or duration of the PAE including the type of organism; type and concentration of antimicrobial agents; duration of antimicrobial exposure, and antimicrobial combinations. The major clinical significance of the PAE is its potential influence on the antibiotic-dosing regimens^[2,3]. The knowledge of pharmacodynamic parameters such as bactericidal activity, postantibiotic effect, and activity on virulence factors (*e.g.* bacterial adhesion), allows the refinement of the choice of the antibiotic and the optimization of its posology^[4-8].

The antibiotic therapy is usually adopted on the assumption that the drug concentrations at the site of the infection reach the minimum bactericidal concentration, thus eliminating the virulence of the microorganisms by killing them. In contrast, although the microorganisms do not necessarily die, MICs can inhibit the growth of bacteria, and in most cases, significantly reduce their virulence^[9,10].

Pseudomonas aeruginosa is a particularly problematic pathogen frequently responsible for severe nosocomial infections, mostly urinary tract infections, septice-mias, wound infections, and chronic lung infections. *P. aeruginosa* is naturally resistant to many structurally unrelated antibiotics. This innate resistance is mainly associated with low-outer membrane permeability and with the production of β -lactamases^[11]. The effectiveness of these pathogenic organisms is due to an arsenal of well-regulated virulence factors^[12]. Some bacterial functions and virulence factors can be affected by the PAEs induced by different antibiotics^[11].

In the present study, the PAEs of fluoroquinolones (troflaxacin, levofloxacin and norfloxacin), amikacin, aztreonam and β -lactams (cefamandole, ceftazidime, ceftriaxone, and cefepime) on the re-growth of *P. aeruginosa* clinical isolates, plus on some of their virulence factors were studied.

Materials and Methods

Microorganisms

The clinical isolates of *P. aeruginosa* used in the present study, were obtained from Microbiology Laboratory at King Abdulaziz University Hospital. Identification of each isolate was confirmed by API

system (API Analytical Products, Division of Sherwood Medical, New York, USA).

The obtained isolates were tested for their virulence factors; elastase, protease, and phospholipase C activity for *P. aeruginosa* isolates by the methods described later.

For preservation of the obtained isolates, organisms were grown in Brain Heart infusion broth (Difco Laboratories, Detroit, MI USA) and aliquots of 1-2 ml in 15% glycerol were stored at -70°C .

Antimicrobial Agents

The used agents were: Trovafloxacin (Pfizer Laboratories, New York, NY, USA); levofloxacin (Aventis, Bridgewater, NJ, USA); cefamandole, ceftazidime, ceftriaxone, ciprofloxacin, norfloxacin, and amikacin (Sigma, Deisenhofen, Germany); aztreonam and cefepime (Bristol-Myer Squibb, Pharmaceutical Research Inst., NJ.). The stock solutions of the used agents were prepared, stored and diluted according to the manufacturer's instructions.

Determination of MICs

The minimum inhibitory concentrations (MICs) of the tested antimicrobial agents were determined by a standard broth microdilution method as described by the National Committee for Clinical Laboratory Standards^[13]. Serial dilutions of antimicrobial agents were prepared in 96 well microtiter plates. The range tested was 0.015 $\mu\text{g/ml}$ to 64 $\mu\text{g/ml}$. The bacterial suspensions in Mueller-Hinton broth (Oxoid) were standardized to yield a final inoculum size of 5×10^5 to 1×10^6 CFU/ml by turbidimetric assay and the counts verified by the colony forming units. The MIC was defined as the lowest concentration of the antimicrobial agent that prevented visible growth after 18 to 24 h of incubation at 37°C .

Determination of PAE

The postantibiotic phase was induced as described by Boswell *et al.*^[14]. Briefly; a logarithmic phase broth culture in Mueller Hinton (Approximately 10^7 cfu/ml) was exposed to 2 X or 4 X-MIC of the tested antimicrobial agents for 1 h at 37°C in a shaking water bath. To eliminate the antibiotic, the strains were washed three times for 5 min at

1400 Xg and diluted. Various dilutions (10^{-2} , 10^{-3} , 10^{-4}) of the control culture (without antibiotic) were made to obtain a control with an inoculum as close as possible to the inoculum of the treated culture. Then, all cultures were incubated (37°C) and the re-growth of bacteria were followed by measuring the absorbance (A_{540}) over a period of 24 h. Viable counts of the bacterial suspensions were determined before exposure to the tested antimicrobial agents, after induction of the postantibiotic phase and after dilution. The PAE was defined as $\text{PAE} = T - C$, where T was the time required for the count in the test culture to increase $1 \log_{10}$ above the count observed immediately after dilution, and C was the time required for the count in the untreated count to increase $1 \log_{10}$ above the count observed immediately after dilution^[8]. The results were expressed as mean of two separate assays.

The PAEs of the Antimicrobial Agents on the Virulence Factors of the Tested Bacterial Isolates

After re-growth, control and treated cultures were centrifuged at $10,000 \times g$ for 10 min at room temperature (Sigma-Aldrich Chemie GmbH, Munich, Germany), then filtered through $0.2 \mu\text{m}$ Acrodisc® PF Syringe Filter (Pall Corporation, USA). The culture supernatant was used to study the effect of the antimicrobial agents on the elastase, protease, and phospholipase C activity of *P. aeruginosa* as follows;

a. Elastase Assay

Elastase activity produced by *P. aeruginosa* isolates was measured by Elastine Congo-red (ECR) assay^[15]. One milliliter of culture supernatants filtrate was added to 1 ml of ECR buffer (0.1 M Tris-HCl/1mM CaCl_2 , pH 7.2) containing 20 mg of ECR (Sigma-Aldrich). The tubes were incubated for 3 hrs at 37°C with shaking at 250 rpm. Elastolytic activity results in the cleavage of ECR, which releases a soluble red pigment. After incubation, 0.2 ml of 0.12 M Na_2EDTA was added to stop the reaction. Insoluble ECR was separated by centrifugation at $3,500 \times g$ for 10 min, and the absorbance of the supernatant at 495 nm was measured. The A_{495} of samples incubated in the absence of culture filtrate was considered background activity, and this value was subtracted from all samples. The corresponding untreated bacterial culture supernatant was used as a control experiment. The values of elastase (mg/1) were determined from the standard curve prepared with elastase Type I (Sigma-Aldrich).

b. Protease assay

Protease activity produced by *P. aeruginosa* isolates was assayed using a method described by Zhang and Maddox^[16]. A 125- μ l aliquot of 2% azocasein solution in Tris buffer pH 7.8 was incubated with 75 μ l of bacterial supernatants at 37°C for 45 min. The reaction was stopped by adding 600 μ l of 10% trichloroacetic acid. After incubation for 10 min at room temperature, the mixture was centrifuged for 5 min at 12,500 rpm, and 600 μ l of the supernatant were transferred to a tube containing 500 μ l of 1 M NaOH. The absorbance was measured at 440 nm. The proteolytic activity (U/ml) was then determined from the calibration curve that was constructed by using protease type XVII-B (Sigma-Aldrich).

c. Phospholipase C assay

In such assay the tested organisms were grown in tryptone minimal medium, and the produced phospholipase was measured as described by Mizukane *et al.*^[17]. Aliquots of 10 μ l of the clear supernatant fluid were added to 90 μ l of NPPC reagent (250 mM tris; hydroxymethyl-aminomethane-hydrochloride buffer (pH 7.2); 60% glycerol (wt/wt); 1.0 μ M ZnCl₂, and 10 mM of NPPC [p-nitrophenylphosphorylcholine]) in microtiter plates. The plates were then incubated at 37°C for 1 h before the absorbance at 405 nm was measured spectrophotometrically. The values of the produced phospholipase were determined from the standard curve prepared with phospholipase C standard (Sigma-Aldrich).

Statistical Analysis

Statistical significance between means was tested by analysis of variance and “student’s” *t* test using InStat ANOVA software. The differences between means were considered statistically significant when the test yielded a value $p \leq 0.05$.

Results

Microorganisms

Seven clinical isolates *P. aeruginosa* were used for studying the PAE of the selected antimicrobial agents.

The results of evaluating the virulence factors (enzymatic activities) of selected isolates showed that five isolates of *P. aeruginosa*

have significant elastase, protease, and phospholipase C activity (with the range of 6.2-7.6 ug/ml, 28-142 U/ml, and 37-133 U/ml.). These isolates were used for studying the PAE of the selected antimicrobial agents on their enzymatic activity.

The MICs of the Tested Antimicrobial Agents

The MICs of the tested antimicrobial agents against the clinical isolates used in this study are summarized in Table 1. The MICs of the tested fluoroquinolones (trovafloxacin, levofloxacin and norfloxacin), amikacin, aztreonam, and β -lactam antibiotics (cefamandole, ceftazidime, ceftriaxone, and cefepime) against the selected isolates were in the ranges of 0.2-25, 1.6-12.5, 3.2-25, and 0.8-50.0 μ g/ml, respectively.

Table 1. The sensitivity of the tested antimicrobial agents against *P. aeruginosa* isolates.

Isolates	MIC Range of Antimicrobial Agents (μ g/ml)				
	TRO	LVX	NOR	AMK	AZM
<i>P. aeruginosa</i> (n = 7)	0.8-12.5	0.2-6.2	0.8-25.0	1.6-12.5	3.2-25.0
	CML	CFT	CFX	CFP	
	3.2-50.0	1.6-12.5	0.8-12.5	3.1-25.0	

Abbreviations: TRO = trovafloxacin, LVX = levofloxacin; NOR = norfloxacin; AMK = amikacin, AZM = aztreonam; CML = cefamandole; CFT = ceftazidime; CFX = ceftriaxone; CFP = cefepime; n = Number of Isolates

The PAEs of the Tested Antimicrobial Agents

According to the obtained results (Tables 2a & b), a variable PAEs among the used antimicrobial agents on the tested clinical isolates were observed. The longest PAEs were observed after treatment with the tested fluoroquinolones (trovafloxacin, levofloxacin and norfloxacin). The mean PAEs induced by fluoroquinolones at concentrations of 2X- and 4X-MIC were in the ranges of 1.2-3.7 h and 1.9-4.8 h, respectively. The PAEs of amikacin are slightly weaker than that of fluoroquinolones. The mean PAEs induced by amikacin at concentrations of 2X- and 4X-MIC were in the ranges of 1.0-2.1 h and 1.7-3.2 h, respectively. In the case of aztreonam and the tested β -lactam antibiotics (cefamandole, ceftazidime, ceftriaxone, and cefepime), the PAEs values were significantly shorter than that of fluoroquinolones and amikacin. Their mean PAEs at concentration of 2X- and 4X-MIC on the tested clinical isolates were in the ranges of 0.1-0.8 h and 0.2-0.9 h, respectively.

Table 2a. PAE induced by the tested fluoroquinolones, amikacin, and aztreonam on *P. aeruginosa* isolates.

Isolates	Conc. of Antimicrobial Agents	Mean PAE (h) ± SD (PAE rang)				
		TRO	LVX	NOR	AMK	AZM
<i>P. aeruginosa</i> (n = 7)	2X MIC	1.8 ± 0.54 (1.2-2.6)	1.9 ± 0.62 (1.3-2.8)	2.6 ± 0.69 (1.8-3.7)	1.6 ± 0.4 (1.0-2.1)	0.3 ± 0.18 (0.1-0.6)
	4X MIC	2.9 ± 0.60 (2.1-3.8)	2.7 ± 0.65 (1.9-3.6)	3.6 ± 0.96 (2.3-4.8)	2.2 ± 0.49 (1.7-3.2)	0.5 ± 0.23 (0.2-0.9)

Abbreviations: See footnote Table 1.

Table 2b. PAE induced by the tested β-lactam antibiotics on *P. aeruginosa* isolates.

Isolates	Conc. of Antimicrobial Agents	Mean PAE (h) ± SD (PAE rang)			
		CML	CFT	CFX	CFP
<i>P. aeruginosa</i> (n = 7)	2X MIC	0.4 ± 0.13 (0.2-0.6)	0.5 ± 0.17 (0.3-0.7)	0.5 ± 0.17 (0.3-0.8)	0.5 ± 0.10 (0.4-0.7)
	4X MIC	0.5 ± 0.13 (0.3-0.7)	0.7 ± 0.17 (0.4-0.9)	0.8 ± 0.18 (0.4-1.0)	0.8 ± 0.11 (0.6-0.9)

Abbreviations: See footnote Table 1.

The PAEs of the Tested Antimicrobial Agents on *P. aeruginosa* Virulence Factors Activities

The PAEs of the tested antimicrobial agents on *P. aeruginosa* elastase, protease, and phospholipase C activities were studied in the supernatant filtrates that were prepared after the re-growth of the treated isolates. The obtained results showed that the tested fluoroquinolones and amikacin at concentrations of 2X- and 4X-MIC significantly ($p < 0.05$) reduced elastase, protease, and phospholipase C activity and their effects were concentration dependent. The PAEs of the tested fluoroquinolones were stronger than that of amikacin. The PAEs of fluoroquinolones at concentrations of 2X MICs induced suppression in the *P. aeruginosa* elastase, protease, and phospholipase C activity to the ranges of 31-37%, 62-69.8%, and 62.4-68.8% of the control, respectively. While at higher concentrations (4X MICs), they reduce the enzymatic activity to the ranges of 17-20%, 30.6-36%, and 47.6-57% of the control, respectively. Among the tested fluoroquinolones, the highest PAE effects on the virulence factors of the tested *P. aeruginosa* isolates was observed by norfloxacin.

In case of amikacin, the induced PAEs at concentrations of 2X and 4X MIC caused suppression in the *P. aeruginosa* enzymatic activity to the ranges of 54-73.8% and 27-59.2% of the control, respectively. In the case of aztreonam and β-lactam antibiotics, their PAEs induced a

week effect on the tested enzymatic activities as compared to that of the control (Fig. 1a, b - 3a, b).

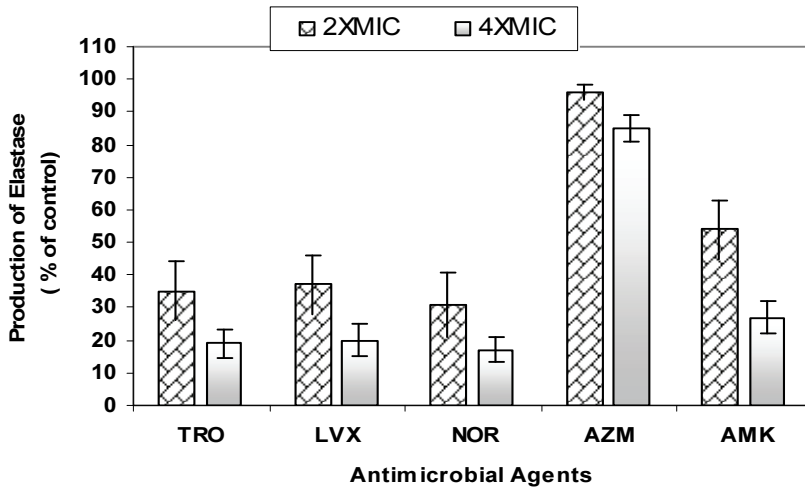


Fig. 1a. Relative elastase activity of five *P. aeruginosa* isolates after short time (1 h) treatment with trovafloxacin (TRO), levofloxacin (LVX), norfloxacin (NOR), Aztreonam (AZM), Amikacin (AMK) at concentrations of 2X- and 4X-MIC. SD = Standard deviation.

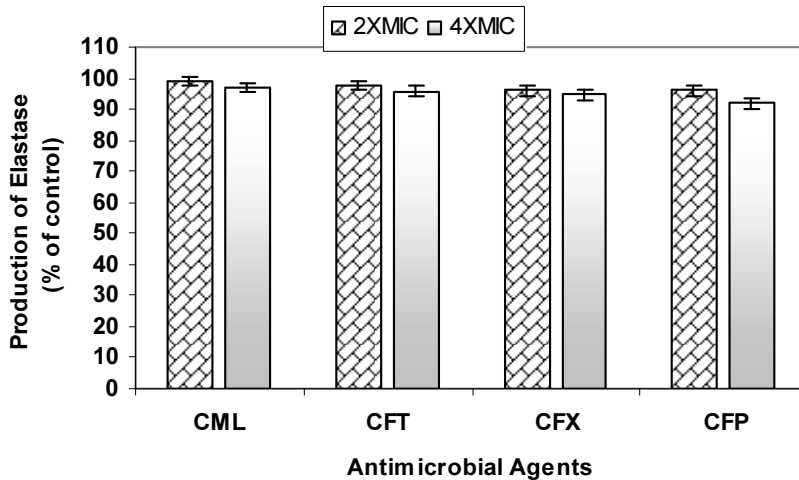


Fig. 1b. Relative elastase activity of five *P. aeruginosa* isolates after short time (1 h) treatment with cefamandole (CML), ceftazidime (CFT), ceftriaxone (CFX), cefepime (CFP) at concentrations of 2X- and 4X-MIC. SD = Standard deviation.

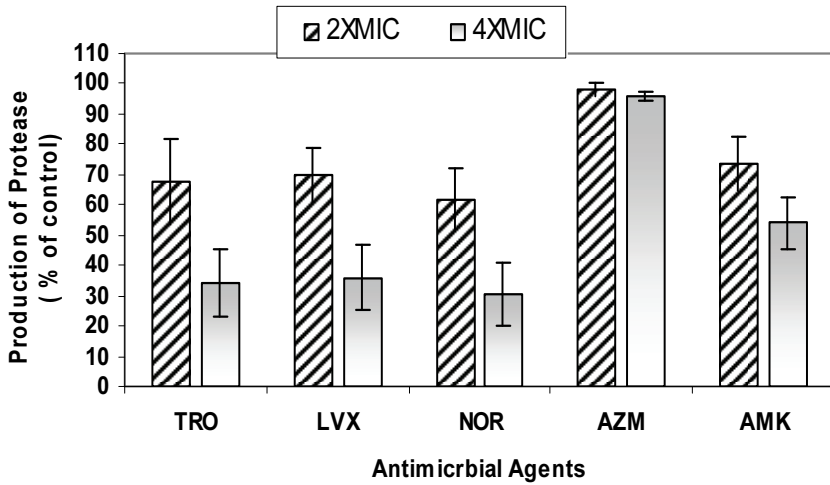


Fig. 2a. Relative protease activity of five *P. aeruginosa* isolates after short time (1 h) treatment with trovafloxacin (TRO), levofloxacin (LVX), norfloxacin (NOR), Aztreonam (AZM), Amikacin (AMK) at concentrations of 2X- and 4X-MIC. SD = Standard deviation.

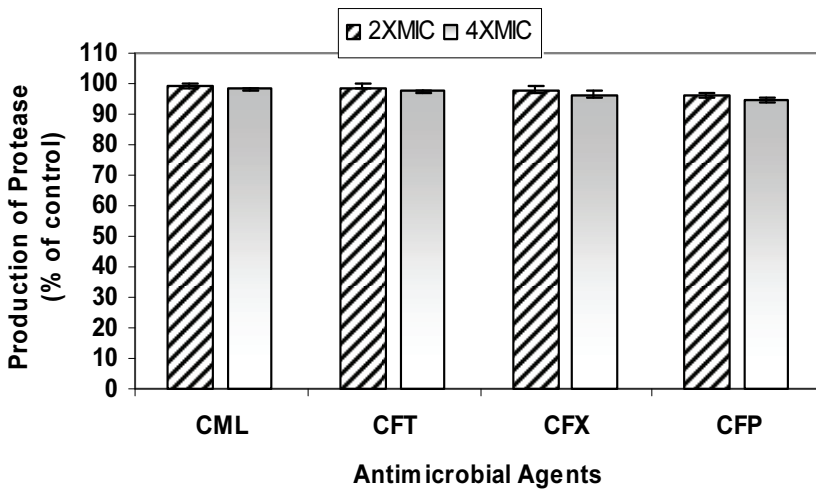


Fig. 2b. Relative protease activity of five *P. aeruginosa* isolates after short time (1 h) treatment with cefamandole (CML), ceftazidime (CFT), ceftriaxone (CFX), cefepime (CFP) at concentrations of 2X- and 4X-MIC. SD = Standard deviation.

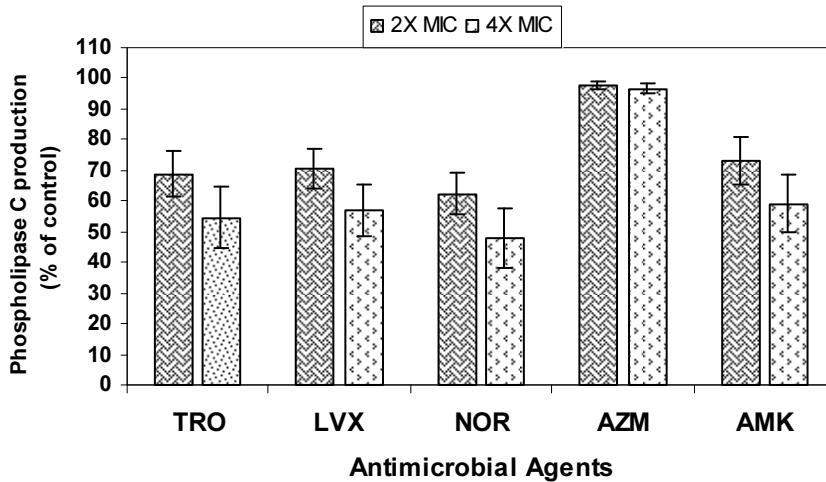


Fig. 3a. Relative phospholipase C activity of five *P. aeruginosa* isolates after short time (1 h) treatment with trovafloxacin (TRO), levofloxacin (LVX), norfloxacin (NOR), Aztreonam (AZM), Amikacin (AMK) at concentrations of 2X- and 4X-MIC. SD = Standard deviation.

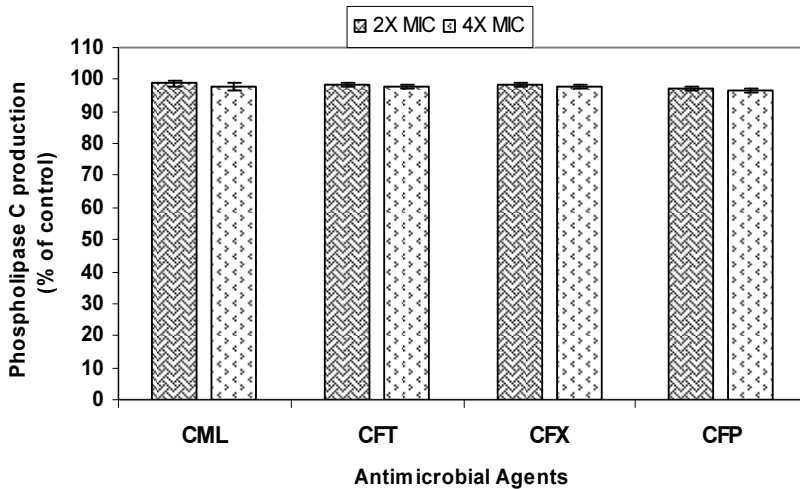


Fig. 3b. Relative phospholipase C activity of five *P. aeruginosa* isolates after short time (1 h) treatment with cefamandole (CML), ceftazidime (CFT), ceftriaxone (CFX), cefepime (CFP) at concentrations of 2X- and 4X-MIC. SD = Standard deviation.

Discussion

In the present study, variable PAEs among the used antimicrobial agents on the tested clinical isolates were observed. The longest PAEs were observed after treatment with the tested fluoroquinolones (trovafloxacin, levofloxacin and norfloxacin). The mean PAEs induced by fluoroquinolones at concentrations of 2X- and 4X-MIC were in the range of 1.2-3.7 and 1.9-4.8h, respectively. The PAEs were concentration dependent, since the higher concentrations (at 4X-MIC) induced longer lasting PAEs as compared to that obtained at a lower one. As compared to fluoroquinolones, weaker PAEs were observed after treatment with amikacin. The mean PAEs induced by amikacin at concentrations of 2X- and 4X-MIC were in the ranges of 1.0-2.1 and 1.7-3.2 h, respectively.

The obtained PAE results of fluoroquinolones (trovafloxacin, levofloxacin, and norfloxacin) and aminoglycosides (amikacin) were in agreement with that reported previously by other investigators^[7,18,19].

Pankuch *et al.*^[18] reported that treatment of *P. aeruginosa* strains with 10-MICs of the trovafloxacin for 1 h induced PAEs ranged from 2.4 h to 4.4 h. Norfloxacin at suprainhibitory concentrations induced PAEs in the range of 6 - 11.4 h (2X-MIC) and of 10.1 - >13.4 h (4X-MIC) on three *P. aeruginosa* strains^[20]. Other fluoroquinolones (levofloxacin, gemifloxacin, and ibafloxacin) induced PAEs on different types of gram-negative bacteria ranged from 0.7 h to 2.13 h and 0.2 h to > 6 h, respectively^[21,22]. Treatment of *P. aeruginosa* with 2X- and 4X-MIC of gentamicin or amikacin for 30-60 min induced PAE values ranged from 0.6 h to 1.2 h and of 3.0 h to 4.6 h, respectively^[11,23].

In case of β -lactam antibiotics and aztreonam, their weak PAEs, observed in the present study, were reported previously by other investigators^[7,24-27]. Odenholt *et al.*^[25] reported that β -lactam antibiotics exhibited no postantibiotic effect against the gram-negative strains. However, the weak PAEs of ceftriaxone on *P. aeruginosa* were observed by Buxbaum and Georgopoulos^[28]. The superior PAE of gentamicin as compared to that of cefotaxime and ceftriaxone was reported by Karlowsky *et al.*^[29]. The variability in the PAEs may result from the difference in the microorganisms, time of contacts, and the type and concentration of antimicrobial agents.

The major clinical significance of the PAE is its potential influence on the antibiotic-dosing regimens^[2,3,8]. The knowledge of pharmacodynamic parameters such as bactericidal activity, postantibiotic effect, activity on virulence factors (e.g. bacterial adhesion), allows the refinement of the choice of the antibiotic and the optimization of its posology^[5,7,30].

Bacteria in the postantibiotic phase are damaged, they are physiologically altered. During and after PAEs induced by different antibiotics, some bacterial activities (mainly changes in protein and/or DNA synthesis, in the production of some enzymes and/or virulence factors) were affected^[10,31-34]. *P. aeruginosa* proteases, elastase, and phospholipase C are considered important virulence factors, which damage host tissues and interfere with host bacterial defense mechanism^[11,35-38].

In the present study, the induced PAEs of the tested antimicrobial agents caused variable effects on *P. aeruginosa* (protease, elastase, and phospholipase C) virulence factors. All the tested enzymatic activities were suppressed after short-time (1h) treatment with suprainhibitory concentrations (2X- & 4X-MICs) of trovafloxacin, levofloxacin, norfloxacin, and amikacin. These effects were concentration dependents. The induced PAEs of the tested fluoroquinolones on the tested enzymatic activities were stronger than that of amikacin, and the highest PAE effects was produced by norfloxacin. However, weak effects on the enzymatic activities were induced by the PAEs of the tested β -lactam antibiotics and aztreonam.

The PAEs of different antimicrobial agents on the virulence factors of different gram-negative bacteria were studied previously by other investigators. *P. aeruginosa* elastase and protease activities were suppressed after a short-time (30 min) treatment with suprainhibitory concentrations (2X- and 4X-MICs) of pefloxacin, ciprofloxacin, amikacin and tobramycin^[11,39,40]. A significant reduction in some of the *P. aeruginosa* and *E. coli* virulence factors was observed during the postantibiotic periods that induced by levofloxacin, ciprofloxacin, and gentamicin^[41]. The induced PAEs of ciprofloxacin, pefloxacin, netilmicin, tobramycin, and gentamicin significantly suppressed the production of phospholipase C in a *P. aeruginosa*^[17,23,42].

According to the obtained results, the presence of long lasting PAEs and virulence factors suppression after a short-time treatment with trovafloxacin, levofloxacin, norfloxacin, and amikacin (2X- and 4X-MIC) allow these agents to be dosed infrequently during antimicrobial therapy. Whereas, the very short PAE of β -lactam antibiotics and aztreonam without any effect on the bacterial virulence factors activity suggests frequent or continuous dosing. The implications of these findings for clinical use of these drugs need further evaluation.

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عوامل الحدة للسودوموناس ايريوجينوزا بعد التأثير الحيوي لأنواع مختلفة من مضادات الميكروبات

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المستخلص. في هذا البحث تم معالجة عازلات إكلينيكية من نوع السودوموناس ايريوجينوزا لمدة ساعة واحدة بأنواع مختلفة من مضادات الميكروبات (مركبات فلوروكينولون، وأميكيسين وأز تريونام، ومركبات البيبتالاكتام) لدراسة تأثيرها بعد إزالتها من الوسط، على نمو ونشاط عوامل الحدة للعازلات المختبرة. وقد أظهرت النتائج أن مركبات الفلوروكينولون أقوى من الأميكيسين تأثيراً على نمو ونشاط عوامل الحدة للعازلات المختبرة، وأقوى المركبات تأثيراً هو النورفلوكساسين. إن وجود مركبات الفلوروكينولون بتركيز اربعة أضعاف التركيز المثبط للنمو تخفض من نشاط عوامل الحدة (إنزيم الإلاستيز وإنزيم البروتيز وإنزيم الفوسفوليباز) المنتجة من العازلات المختبرة إلى ١٧ - ٢٠٪ و ٣٠,٦ - ٣٦٪ و ٦,٤٧ - ٥٧٪ على التوالي، مقارنة بالعازلات الغير معالجة. وفي حالة استخدام مركب الأميكيسين بتركيزات أربعة أضعاف التركيز المثبط للنمو ينخفض نشاط عوامل الحدة إلى ٢٧ - ٥٩,٢٪ مقارنة بالعازلات الغير معالجة. أما بالنسبة لمركبات البيبتالاكتام والأز تريونام فليس لها أي تأثير على نشاط عوامل الحدة الخاصة بالعازلات المختبرة. من النتائج السابقة يتضح أن مركبات الفلوروكينولون وأميكيسين بعد إزالتها من الوسط تؤخر من نمو عازلات لسودوموناس ايريوجينوزا، وتخفض من نشاط عوامل الحدة لهذه العازلات.