Quorum sensing inhibitory activity of sub-inhibitory concentrations of β-lactams

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Abstract

Introduction: The virulence factors of *Pseudomonas aeruginosa* are under the control of quorum sensing (QS) signals. Hence, interference with QS prevents its pathogenesis.

Objective: The aim of the present research is to assess the influence of some β-lactam antibiotics on cell communication and the release of different virulence factors.

Methods: The minimal inhibitory concentrations of ceftazidime, cefepime and imipenem were evaluated by microbroth dilution method. The effect of sub-inhibitory concentration of the tested antibiotics on QS signals was investigated using reporter strain assay. In addition, different virulence factors (elastase, protease, pyocyanin and hemolysin) were estimated in the presence of their sub-inhibitory concentrations.

Results: Low concentrations of ceftazidime, cefepime and imipenem caused significant elimination of the QS signals 3OH-C12-HSL and C4-HSL up to 1/20 MIC. Furthermore, low concentrations of the tested antimicrobials suppressed virulence factors elastase and hemolysin. Moreover, 1/20 of their MICs reduced elastase, protease, pyocyanin and hemolysin.

Conclusion: Utilization of β-lactam antibiotics at low concentrations could be an effective approach for prevention and treatment of *P. aeruginosa* infection.

Keywords: Quorum sensing inhibition, β-lactams, Pseudomonas aeruginosa

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen with remarkable metabolic versatility. Infections with *P. aeruginosa* are common in compromised patients suffering from cystic fibrosis, severe burns, deep wounds, in addition to patients having urinary tract infections. *P. aeruginosa* produces various virulence attributes, including biofilm, toxins and enzymes such as pyocyanin, protease, elastase, and rhamnolipids[1].

P. aeruginosa exhibits its virulence behavior via quorum sensing (QS)[2]. The common QS systems in *P. aeruginosa* have been assigned las and rhl. QS circuits in *P. aeruginosa* are connected by signaling molecules called autoinducers. The las system is composed of the synthase gene lasI and its transcriptional regulatory protein LasR. Its autoinducer is called N-(3-oxododecanoyl) homoserine lactone (3OH-C12-HSL). Similarly, the rhl system consists of rhlI synthase, and its transcriptional regulatory protein LasR. Also, it possesses autoinducer molecule N-butyryl homoserine lactone (C4-HSL)[2,3]. When bacterial growth reaches a specific threshold, the signals acyl homoserine lactones (AHL) are released and stimulates the expression of virulence genes[4]. Both the las and rhl systems are coregulated, and las system is dominant over the rhl pathway. Hence, inhibition of these signaling molecules hinders the pathogenicity of *P. aeruginosa*, and could be used for prevention or treatment of its infections[5].

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Several plants have QS inhibiting activities. Medicinal plants[6] and edible plants[7] exhibited QSI effects. Also, *Streptomyces coelicoflavus* isolated from soil microbiota produced 1 H-pyrrole-2-carboxylic acid with QSI effect[8]. Synthetic molecules and peptides exhibited QSI activity[9]. Previous studies focused on the effect of some antimicrobials such as aminoglycosides and quinolones on QS of *P. aeruginosa*. Azithromycin showed significant elimination of QS and virulence factors of *P. aeruginosa*[10]. The present study focused on the effect of sub-inhibitory concentrations of some β-lactam antibiotics on QS of *P. aeruginosa*. Furthermore, virulence factors of *P. aeruginosa* were assessed in the presence of sub-inhibitory concentrations of the tested antibiotics.

Materials and methods
Bacterial strains, growth media and conditions
The wild strain *P. aeruginosa* PAO1 was used for the assay of QSI effects of the tested antibiotics. Two reporter strains; *P. aeruginosa* pME3846 (rhlI-lacZ; Tc^r^) and *E. coli* MG4/pKDT17 (lasB::lacZplac-lasR Ap^r^)2,3 were used for the assessment of rhlI and lasI/R, respectively in the presence and absence of the tested antibacterials. The QS deficient *P. aeruginosa* isolate PAO-JP2 double mutant (Δ lasI::Tn10,Tcr;Δ rhlI::Tn5012, Hgr) was included as a negative control[11]. All bacterial cultures were grown in Luria Bertani medium (LB broth; tryptone 1%, yeast extract 0.5%, and NaCI 1%) at 37° C.

Determination of minimal inhibitory concentration
Minimal inhibitory concentrations (MICs) of the studied β-lactams: cefepime (Cfp), ceftazidime (Cft), and imipenem (Imp), were estimated by broth microdilution method (CLSI, 2014). Two-fold serial dilutions of each antibiotic were prepared and inoculated with 0.1 ml of PAO1 inoculum contained 5×10^6 CFU/ ml and incubated at 37 °C for 24 h. Values of MIC were recorded as the lowest concentration of the antibiotic at which there was no visible growth of the organism[12].

Determination of the viable count of *P. aeruginosa*-PAO1
The viability of *P. aeruginosa* PAO1 wild type was examined in the presence of sub-inhibitory concentrations (1/4 MIC) of the tested β-lactams using pour plate counting method and cell proliferation was checked before supernatant collection[13]. Similarly, the viable count

of the untreated cells was performed and compared to the treated cultures.

Preparation of the supernatant
P. aeruginosa PAO1 was propagated in LB broth containing 1/4, 1/8 and 1/20 MIC of each antibiotic. *P. aeruginosa* PAO1 was also grown without antimicrobial agents as the positive control and PAO-JP2 was propagated under the same conditions as the negative control[14]. The supernatants of the treated and untreated *Pseudomonas* cultures were separated by centrifugation at 8.000 rpm for 10 min at 4°C. Cell-free supernatants were then stored at -20°C to be used for estimation of AHLs and assay of various virulence factors[15].

Effect of β-lactams on QS signal molecules
The QS signals 3OH-C12-HSL and C4-HSL were detected in treated and untreated cultures of PAO1, respectively. The overnight growth of the reporter strains *E. coli* MG4 (pKDT17) and *P. aeruginosa* (pME3846) were diluted up to OD600 of 0.1. The previously prepared cell-free supernatant (1 ml) was mixed with 0.5 ml of *E. coli* MG4 and 1 ml *P. aeruginosa* pME3846. Cells were propagated till they reached 0.3-0.4 at OD600 then pelleted. β-galactosidase was estimated according to Miller assay method[16].

Effect on virulence factors
The effect of the tested β-lactams on the virulence determinants of *P. aeruginosa* PAO1 was estimated using the prepared supernatants both in the presence and absence of tested β-lactams.

Evaluation of LasB elastase
The supernatant of *P. aeruginosa* was mixed with 10 mg of elastin Congo red (ECR, Sigma Chemicals, St. Louis, USA) in ECR buffer (100 mM Tris buffer, pH 7.2). The insoluble substrate was centrifuged and the absorbance of the supernatant was estimated at 495 nm[17].

Total protease production
Total proteolytic effects of the treated and untreated cells were detected using modified skim milk method[18]. Cultures supernatants of PAO1 were mixed with skim milk (1.25%) at 1:2 ratio and reaction was incubated at 37°C for 30 min. The turbidity of the solution was determined at OD 600nm. The results were calculated as relative protease production to the untreated PAO1 strain.

Determination of hemolysin production

The hemolysin test was performed by incubating 700 µl of 2% erythrocytes suspension with 0.5 ml of PAO1 supernatant for 2 h at 37°C. The suspension was centrifuged and the produced hemoglobin was estimated by measuring the OD at 540nm. The negative control; RBCs in LB broth was evaluated under the same conditions. The total RBCs lysed were detected using 0.1% SDS[19].

Pyocyanin assay

King A media (peptone 2%, K2SO4 1%, and MgCl2 0.14%) supplied with sub-MICs of β-lactams was inoculated and propagated at 37 °C for 48 h in triplicates. Media without antibiotics was also inoculated with PAO1 and propagated as a positive control. The culture was centrifuged and pyocyanin was extracted using chloroform in acidic pH and the red layer was separated. Its absorbance was measured at 520 nm and pyocyanin concentration

was expressed as µg/ml by multiplying the OD520 by 17.072[20].

Statistical analysis

The mean of three independent experiments and the standard deviation of the mean were calculated by Excel data package. *P. aeruginosa* treated with β-lactams were compared to the untreated control using GraphPad Instate software (version 3.05) and Tukey-Kramer multiple-comparison test. Significant difference between treated and untreated culture was assigned when the probability value was $P < 0.05$ or $P < 0.01$.

Results

Growth inhibitory concentrations for *P. aeruginosa* PAO1 Table (1) showed the MIC values of tested β-lactams and the mean value for their sub-inhibitory concentrations used for testing QSI effect and virulence factors.

Table (1): Minimal inhibitory concentration (MIC) values of the tested β-lactam and their sub-inhibitory concentrations

Compounds	MIC value	1/4 MIC	1/8 MIC	1/20 MIC
Cefepime (Cfp)	200 (µg/ml)	50	25	10
Ceftazidime (Cft)	1.0 (µg/ml)	0.2	0.1	0.05
Imipenem(Imp)	1.0 (µg/ml)	0.2	0.1	0.04

Effect of sub-inhibitory concentrations of the tested β-lactams on the growth of *P. aeruginosa* **PAO1**

Viable counts of *P. aeruginosa* PAO1 treated with sub-inhibitory concentrations of cefepime, ceftazidime, and imipenem was performed using pour plate method. The tested concentrations (1/4 MIC) did not alter bacterial growth compared to the control untreated cultures and the same bacterial count of untreated PAO1 ($161\pm21\times10^7$ CFU/ml).

Inhibition of quorum sensing signal molecules
Effect on 3OH-C12-HSL

Untreated *P. aeruginosa* PAO1 produced the highest β-galactosidase activity (3125 Miller units). Double mutant strain devoid of QS genes (PAO-JP2) showed the lowest level of β-galactosidase (39 Miller units). The level of 3OH-C12-HSL was significantly reduced by all sub-MICs of cefepime (66%-76% inhibition) and imipenem (63%-78%). However, ceftazidime exhibited less significant effect ranged from 54% at 1/4 MIC to 47% at 1/8 MIC, while ceftazidime (1/20 MIC) did not affect 3OH-C12-HSL yield (Fig. 1) as compared to the untreated PAO1 strain.
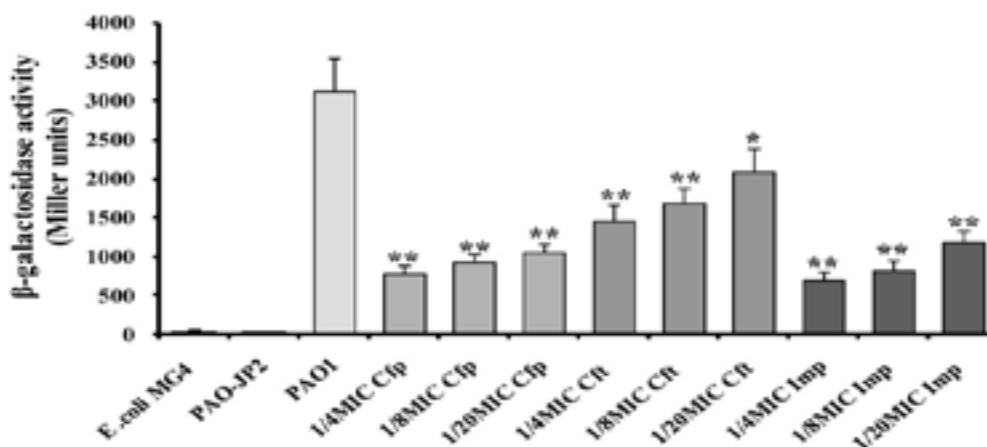
Figure (1): β-galactosidase assay of 3OH-C$_{12}$-HSL. Effect of sub-MICs of cefepime (Cfp), ceftazidime (Cft), and imipenem (Imp) on 3OH-C$_{12}$-HSL production (**, highly significant _P_<0.01 and *, significant _P_<0.05).

Effect on C4-HSL

C4-HSL level was significantly reduced (P<0.01) by the assayed antibiotics at their sub-inhibitory concentrations with different degrees of signals inhibition (Fig. 2) as compared to the untreated _P. aeruginosa_ PAO1. C4-HSL levels were significantly lowered by sub-MICs of cefepime by 47-68% inhibition, ceftazidime by (40%-52%), and imipenem by (47-56%). Double mutant strain showed the lowest level of β-galactosidase activity (85 Miller units). All tested antibiotics produced no effect on both reporter strains.
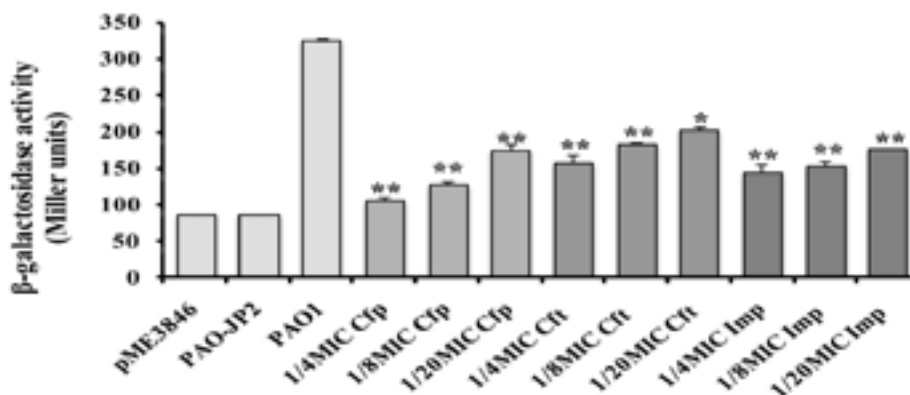

Figure (2): β-galactosidase assay of C$_4$-HSL. Effect of sub-MICs of cefepime (Cfp), ceftazidime (Cft), and imipenem (Imp) on the release of C$_4$-HSL (**, highly significant _P_<0.01 and *, significant _P_<0.05).

Effect of sub-inhibitory concentrations on virulence factors of _P. aeruginosa_ **PAO1**
Effect on elastase enzyme

Significant reduction in elastase production was detected when _P. aeruginosa_ PAO1 was grown in presence of sub-inhibitory concentrations of cefepime (61%-70% inhibition) and imipenem (52%-66%) with P<0.01. However, ceftazidime exhibited significant effect ranging from 54% at 1/4 MIC with P<0.01 to 42% at 1/8 MIC (P<0.05), while 1/20 MIC ceftazidime did not affect the elastase level (Fig. 3).
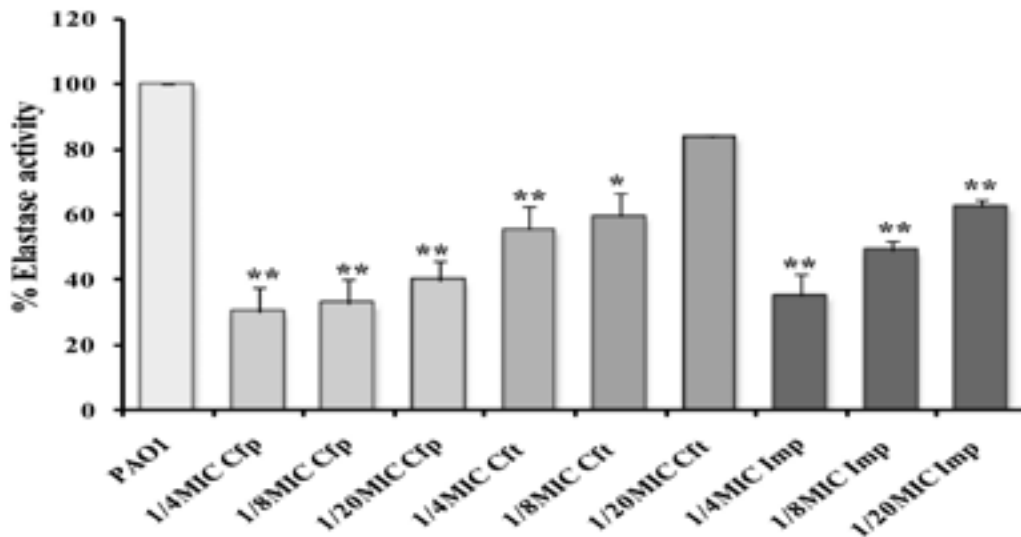
Figure (3): Relative production of elastase from *P. aeruginosa* PAO1 grown with sub-MICs of cefepime (Cfp), ceftazidime (Cft), and imipenem (Imp) (**, highly significant *P*<0.01 and *, significant *P*<0.05).

Effect on total protease

There was significant lowering in total protease production in cultures supplied with sub-MICs of cefepime (51-61% decrease) and imipenem (50-62%) with P<0.01. However, ceftazidime exhibited variable reduction levels ranged from 62% to 54% at 1/4 and 1/8 MIC, respectively (P<0.01). However, it showed less effect at 1/20 MIC (39%, P<0.05) (Fig. 4).
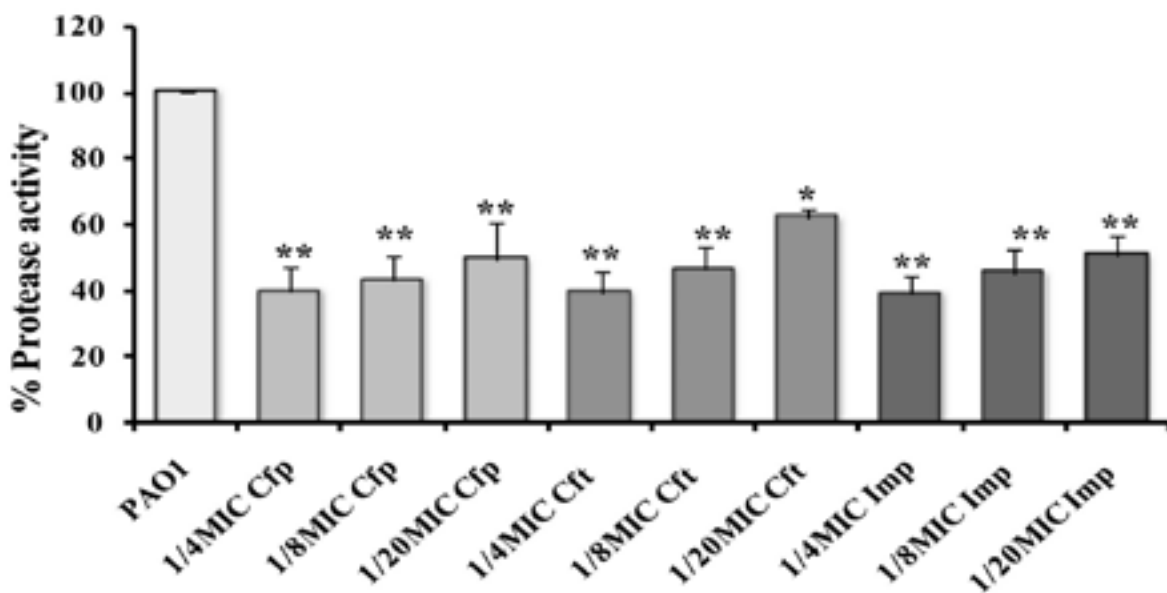

Figure (4): Effect on the production of total protease in PAO1 by sub-MICs of cefepime (Cfp), ceftazidime (Cft), and imipenem (Imp) (**, highly significant *P*<0.01 and *, significant *P*<0.05).

Effect on hemolysin production

The effect on hemolytic activity was expressed as percentage lysis of RBCs by PAO1culture supernatants treated with sub-inhibitory concentrations, as compared to lysis obtained by the untreated PAO1 supernatant[19]. Significant reduction in hemolysin production (P<0.01) was recorded with sub-MICs of cefepime (69-83%), ceftazidime (58-72%), and imipenem (55-69%) (Fig. 5).
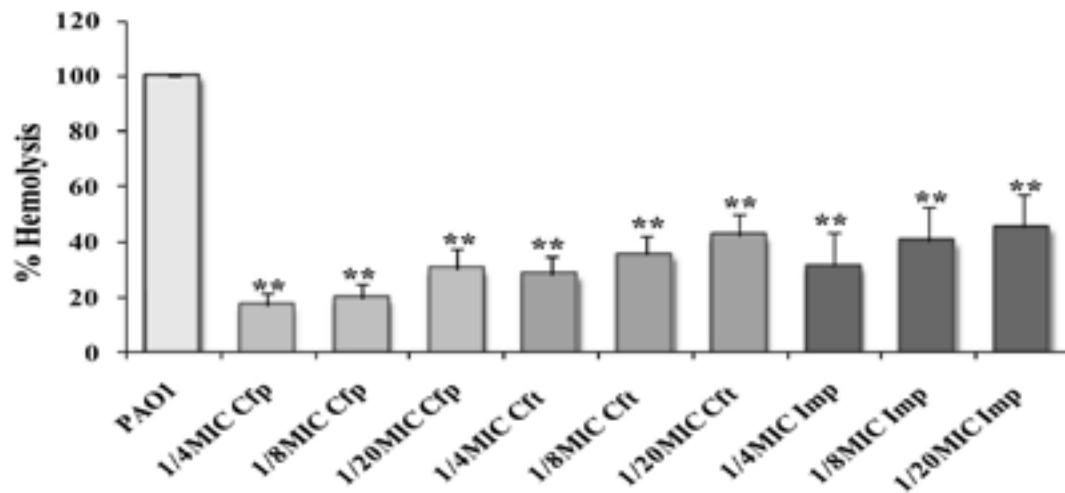
Figure (5): Inhibition of hemolytic activity in *P. aeruginosa* PAO1 using sub-inhibitory concentrations of cefepime (Cfp), ceftazidime (Cft), and imipenem (Imp) (**, highly significant *P*<0.01 and *, significant *P*<0.05).

Elimination of pyocyanin production

The effect of the tested antibiotics on the production of pyocyanin pigment from PAO1 cells was assessed. Pyocyanin production was significantly reduced by sub-MICs of cefepime (63-73%), 1/4 and 1/8 MIC of imipenem (51-57%) with P<0.01, and 1/20 MIC of imipenem by 40% (P<0.05). However, ceftazidime exhibited significant reduction ranged from 57% at 1/4 MIC to 37% at 1/8 MIC, while 1/20 MIC did not affect pyocyanin yield (Fig. 6). On the other hand, PAO-JP2 showed the lowest pyocyanin level (0.6 µg/ml)
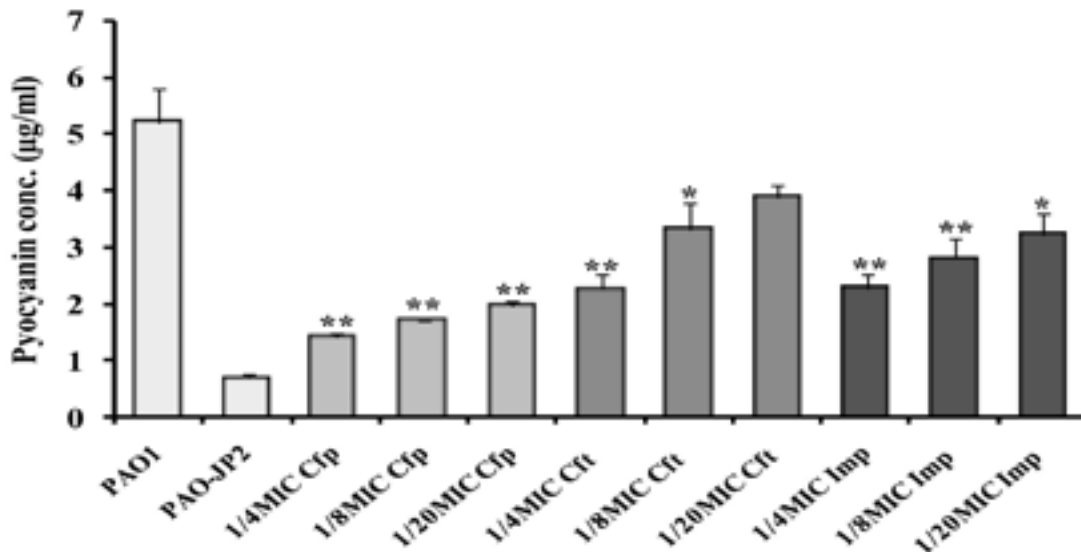

Figure (6): Pyocyanin production; cefepime (Cfp), ceftazidime (Cft), and imipenem (Imp) compared to untreated standard strain PAO1. (**, highly significant *P*<0.01 and *, significant *P*<0.05).

Discussion

P. aeruginosa is one of the main causes of the persistent urinary tract, respiratory tract, burn and wound infections[21]. Pathogenesis of *P. aeruginosa* is attributed to secreted virulence factors such as elastase, proteases, and pyocyanin. Its pathogenesis is also disseminated through bacterial adhesion and biofilm formation[5]. QS is the key regulator for Pseudomonas virulence and biofilm formation. Hence, reduction in bacterial communication by QS inhibitors results in reduced levels of virulence factors[22]. Therefore, targeting QS cascade developed a new therapeutic approach for control of *Pseudomonas* infections. This study showed that the sub-inhibitory concentrations of the three tested β-lactams exhibited significant

elimination of QS signals. Quantitative assessment of both QS signals has clearly indicated that anti-QS activity of the tested drugs is concentration dependent. Regarding the autoinducers 3OH-C12-HSL and C4-HSL, they significantly decreased (P<0.01) by sub-inhibitory concentrations of cefepime, ceftazidime and imipenem compared to the untreated PAO1 strain (Figs. 1 and 2). In line with these findings, certain macrolide antibiotics (azithromycin) are capable of repressing the synthesis of *P. aeruginosa* AHLs signal molecules when applied at sub-MICs[23]. Furthermore, sub-inhibitory concentrations of tobramycin, ciprofloxacin, and ceftazidime were effective in lowering the AHL levels in *P. aeruginosa*[24].The QSI of some antimicrobials is attributed to the alteration in membrane permeability, and subsequent elimination of the flux of N-3-oxo-dodecanoyl-l-homoserine lactone[25]. Also, efflux inhibitor phenylalanine arginyl β-naphthyl-amide caused significant elimination of QS signals and related virulence factors among multidrug resistant *P. aeruginosa* clinical isolates[26].

To confirm that the elimination of QS signals and virulence was not related to the antimicrobial activities of the tested β-lactams, viable counts of the treated cells were performed and our data have shown no change in the cell growth. Bacterial growth in the presence of Sub-MICs of tested β-lactams produced viable bacterial count similar to the positive control (untreated *P. aeruginosa* PAO1).

The effect of sub-MIC concentrations on *P. aeruginosa*-virulence has been verified. One of the main pathogenic factors in *P. aeruginosa* is LasB elastase. LasB elastase and proteases interfere with host defense mechanisms and destroy tissue components[27]. Most of sub-MICs of the tested compounds resulted in a significant decrease in LasB elastase and proteases enzymes (P<0.01 and P<0.05) (Figs. 3-4). The inability of PAO1 strain to produce elastase and protease has been linked with their reduced level of associated QS molecules released under the influence of the assayed antibiotics. Much lower concentration (1/20 MIC) of some of these antibiotics did not affect bacterial QS signaling network and hence, showed no remarkable effect on elastase and protease activities. QS-deficient strains were non-virulent and lose the ability to produce elastase and protease[28].

Hemolysin is another aspect of *P. aeruginosa* virulence which enhances its pathogenicity and helps in the sur-vival of pathogen by inhibiting the host defense factors. Significantly reduced levels (P<0.01) of hemolysin were observed after treating *P. aeruginosa* PAO1 with sub-inhibitory concentrations of cefepime, ceftazidime, and imipenem (Fig. 5). Similarly, a low hemolytic activity of *P. aeruginosa* was reported with sub-inhibitory concentrations of erythromycin[29], eugenol[30] and the 7-fluoroindole[17].

On the same instance, QS system coordinates the release of secondary metabolites such as pyocyanin[15]. Direct antagonists of QS in *P. aeruginosa* have a great impact on the production of pyocyanin[31]. In this study, the level of pyocyanin was significantly reduced (P<0.01) with sub-MICs of cefepime, ceftazidime and imipenem (Fig. 6).

These results indicated that controlling the production of QS molecules hinders most of the associated virulence agents. Also, inhibition of AHL signaling in Pseudomonas caused a reduction in the related virulence factors and attenuation of this pathogen when tested on animal models of pneumonia. On the same instance, synergistic effect has been achieved upon treating mice with both the QSI furanone C-30 and tobramycin, with increase in the clearance of *P. aeruginosa* in a foreign-body infection model[32].

Conclusion
The fact that these antibiotics are already approved drugs for human use is a significant benefit in the future application and development of anti-pathogenic drugs. Hence, further investigations are necessary to detect anti-quorum sensing potential of antimicrobial combinations. Also, further in-vivo studies are required to confirm their possible utilization as anti-pathogenic agents for competing with *P. aeruginosa* infections.

Conflict of interest
The authors have no conflict of interest to declare.

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