

## Effect of azithromycin and phenylalanine-arginine beta-naphthylamide on quorum sensing and virulence factors in clinical isolates of *Pseudomonas aeruginosa*

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### ABSTRACT

**Background and Objectives:** *Pseudomonas aeruginosa* is a problematic opportunistic pathogen causing several types of nosocomial infections with a high resistance rate to antibiotics. Production of many virulence factors in *P. aeruginosa* is regulated by quorum sensing (QS), a cell-to-cell communication mechanism. In this study, we aimed to assess and compare the inhibitory effect of azithromycin (AZM) and EPI- PA $\beta$ N (efflux pump inhibitor- Phenylalanine-Arginine Beta-Naphthylamide) on QS system and QS-dependent virulence factors in *P. aeruginosa* clinical isolates.

**Materials and Methods:** A total of 50 *P. aeruginosa* isolates were obtained from different types of clinical specimens. Isolates were investigated for detection of QS system molecules by AHL cross-feeding bioassay and QS-dependent virulence factors; this was also confirmed by detection of QS genes (*lasR*, *lasI*, *rhlR*, and *rhlI*) using PCR assay. The inhibitory effect of sub-MIC AZM and EPI PA $\beta$ N on these virulence factors was assessed.

**Results:** All the *P. aeruginosa*, producing QS signals C<sub>4</sub>HSL, failed to produce C<sub>4</sub>HSL in the presence of sub-MIC AZM. In the presence of EPI PA $\beta$ N (20  $\mu$ g/ml) only 14 isolates were affected, there was a significant reduction in QS-dependent virulence factors production (protease, biofilm, rhamnolipid and pyocyanin) in the presence of either 20  $\mu$ g/ml EPI or sub-MIC of AZM with the inhibitory effect of AZM was more observed than PA $\beta$ N.

**Conclusion:** Anti-QS agents like AZM and EPI (PA $\beta$ N) are useful therapeutic options for *P. aeruginosa* due to its inhibitory effect on QS-dependent virulence factors production without selective pressure on bacteria growth, so resistance to these agents is less likely to develop.

**Keywords:** *Pseudomonas aeruginosa*; Virulence factors; Quorum sensing; Azithromycin; Phenylalanine-arginine  $\beta$ -naphthylamide

### INTRODUCTION

*Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the major opportunistic pathogens that can trigger a wide range of life-threatening nosocomial infections especially in immunocompromised patients (1). Quorum sensing (QS) is an intercellular signaling mechanism whereby an individual bacterium pro-

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duces small diffusible molecules called autoinducers (AIs) that can be detected by the surrounding microbial community. The QS signal-based communication system enables bacteria to modify their pattern of gene expression giving the bacteria a selective advantage over host defenses and this is important for the pathogenesis of the organism (2, 3). In *P. aeruginosa* and most Gram negative bacteria, these autoinducers are acyl-homoserine lactones (AHLs). Only when the concentration of AHLs in the environment increases, potentially in response to increasing bacterial cell density, the intracellular levels of AHLs reaching their threshold concentration to induce the activation of a cascade of signaling events, leading to the altering the expression of hundreds of genes underlying various biological processes related to bacterial virulence factors production. As a consequence, QS inhibition is an ideal target for anti-virulence strategies (3-5).

*P. aeruginosa* possesses four principal QS systems, namely the LasI/LasR and the RhII/RhIR systems the PqsABCDE/PqsR system, and the AmbB-CDE/IqsR system which are regulated by AHLs and each system has its own AI (autoinducer) (2). The two major QS systems are *las* and *rhl*; the *las* system is composed of the autoinducer synthase gene *lasI* responsible for the synthesis of N-(3-oxododecanoyl) homoserine lactone (3OH-C12-HSL) and the *lasR* gene that codes for its transcriptional activator protein. Similarly, the *rhl* system consists of *rhlI* autoinducer synthase (N-butyryl homoserine lactone (C4-HSL)), and the *rhlR* gene encoding its transcriptional activator protein. These QS systems are hierarchically connected (6, 7).

Control of *P. aeruginosa* infections is always complicated. This problem arises from the fact that *P. aeruginosa* is a multidrug-resistant bacteria. It is characterized by a high level of intrinsic resistance to a variety of antimicrobial agents; in addition, *P. aeruginosa* has acquired different mechanisms of resistance against all available antipseudomonal agents such as the active efflux that represent an important multidrug resistance mechanism (8). The most studied efflux pump in *P. aeruginosa* is the MexAB-OprM that is constitutively expressed in *P. aeruginosa* with broad substrate specificity. It exports diverse antimicrobial agents outside the bacterial cell causing resistance to broad-spectrum antimicrobials. Moreover, this efflux machinery participates in the selective transport of AHLs outside the cells, which

stimulates adjacent cells and completes the cell-to-cell cascade (9, 10).

Resistance mechanisms are regulated and coregulated to make *P. aeruginosa* one of the greatest therapeutic challenges; therefore it is becoming urgent to discover new therapeutic options to combat this resistance issue. Alternative mechanisms for targeting *P. aeruginosa*, other than traditional antibiotics, have been the focus of much research (10, 11). Therapeutics that target and inhibit QS in *P. aeruginosa* holds a promising strategy for combating this pathogen. QS inhibitors are agents that disrupt QS systems in bacterial cells leading to attenuation of virulence factors production without selective pressure on bacterial growth that leads to the development of resistance and therefore, no or low resistance is anticipated to arise against these agents. A great diversity of QS interfering agents has been described. These compounds can be either of natural or synthetic origin and can target different steps of the QS process (12, 13). The ability of different compounds to inhibit violacein production in the sensor strain *Chromobacterium violaceum* (*C. violaceum*) is a common assay used to evaluate anti-QS activity. In *C. violaceum*, synthesis of the violet pigment violacein is regulated by QS in response to the concentrations of the autoinducers; therefore *C. violaceum* is widely used as biosensor strain for screening anti-QS molecules (14).

Azithromycin (AZM) is a macrolide antibiotic that does not show significant bactericidal activity against *P. aeruginosa* at clinically therapeutic achievable concentrations and is therefore unlikely to select resistant clones. However, at sub-inhibitory concentrations (sub-MIC), AZM suppresses QS signal molecules and hence attenuates the virulence. It also possesses antibiofilm activity, and so combining it with an antipseudomonal antibiotic might be more effective than using antibiotics alone for the treatment of biofilm-associated infections (15, 16).

A large number of interesting molecules, acting on a series of efflux pumps in different bacteria, have been designed. These Efflux Pump Inhibitors (EPIs) could also affect bacterial virulence, in addition to enhancing antibiotic activity (17). The most widely used compounds as EPIs for *Pseudomonas* overexpressing Mex efflux pumps are the group of peptidomimetic molecules with phenylalanine arginyl  $\beta$ -naphthylamide (PA $\beta$ N) as a leading compound. The main drawbacks associated with these EPI com-

pounds are their toxic properties hindering their clinical applications (17, 18).

Hence the antivirulence activity of azithromycin is now well known and it has been used as adjuvant therapy in *P. aeruginosa* chronic lung infections (19), so we used it as a gold standard for comparing its inhibitory effect on QS-dependent virulence factors with that of EPI-PA $\beta$ N. The present study aimed to investigate the role of the QS signal production and QS-associated genes in the production of the QS-dependent virulence factors, we also aimed to assess and compare the inhibitory effect of AZM and EPI-PA $\beta$ N on QS system and QS-dependent virulence factors in *P. aeruginosa* isolates of different clinical sources.

## MATERIALS AND METHODS

**Bacterial isolates and their identification.** Fifty isolates of *P. aeruginosa* were included in this study. All isolates were collected from the clinical microbiology laboratory of the medical research institute, Alexandria University. The isolates were collected from different clinical specimens. The isolates were identified as *P. aeruginosa* using biochemical standard assays and growth at 42°C. The identity of isolates was confirmed by MALDI-TOF Mass Spectrometer (Bruker Daltonics).

Fresh colonies were selected for performing bacterial identification by MALDI-TOF MS. The identification was conducted directly from the bacterial colonies as follows; a bacterial colony was inoculated in the plate for mass spectrometry and 1  $\mu$ l of the organic matrix was added to the sample. Afterwards, the plate was positioned in the equipment for MALDI-TOF Mass Spectrometer analysis. The resulting spectra for each culture were analyzed and compared to a reference spectrum in the database by the MALDI-TOF software for identification of the best match from the database records (20).

For bacterial storage; one ml of a fresh bacterial culture grown on Luria Bertani (LB) broth was added to 1 ml of 20% sterile glycerol solution in screw-capped tubes, then stored at -80°C. These stock cultures were utilized for different assays to evade repeated subculturing of isolates. *P. aeruginosa* PAO1 was used as a positive control strain for phenotypic tests and QS genes detection by PCR. It was propagated under the same conditions as isolates.

**Antibiotic susceptibility testing.** Antibiotic susceptibility testing of the isolates was done using disk diffusion (Kirby-Bauer) method according to the Clinical Laboratory Standard Institute (CLSI) guidelines (21). The following antipseudomonal antibiotic discs were used: gentamicin (CN, 10  $\mu$ g), amikacin (AK, 30  $\mu$ g), imipenem (IPM, 10  $\mu$ g), ceftazidime (CAZ, 30  $\mu$ g), levofloxacin (LEV, 5  $\mu$ g), and ciprofloxacin (CIP, 5  $\mu$ g). All discs were provided by (Oxoid, UK).

**Determination of sub-inhibitory concentration (Sub-MIC) of AZM.** Checkerboard titration assay was performed to assess the MIC of AZM for the 50 clinical isolates to detect the sub-MIC that was used in the further phenotypic investigations.

Broth microdilution tests were used according to the CLSI guidelines (21) as follows; pure colonies from each isolate were suspended in nutrient broth and adjusted to 0.5 McFarland standard, then tested in a microtiter plate against AZM (in twofold serial dilutions in nutrient broth) at concentrations ranging from 2  $\mu$ g/ml to 256  $\mu$ g/ml. MIC was recorded as the lowest concentration of antibiotic inhibiting visible growth after overnight incubation at 37°C. The test was performed in duplicates to take the average MIC. Concentrations just below the MIC were considered as the sub-MIC.

**Autoinducer (AHLs) detection by cross-feeding bioassay.** Micro-AHL cross-feeding bioassay was used to detect AHL production. The assay relied on the induction of violacein pigment production by the biosensor indicator strain *Chromobacterium violaceum* CV026 (most sensitive to C<sub>4</sub>HSL) after the diffusion of the AHLs into the environment produced by the test organism. For this purpose, 200  $\mu$ l of LB agar was added into wells of microplates and left to solidify. *C. violaceum* CV026 strain was grown in LB agar for 18 h at 30°C and suspensions from two to three colonies were made in phosphate-buffered saline (PBS) and the wells of the microplate were inoculated with 25  $\mu$ l of *C. violaceum* CV026 suspensions. The plates were air-dried for 30 min and 20  $\mu$ l of the tested bacterial suspensions were pipetted into each well. The plates were covered and incubated at 37°C for 48 h. Positive results in the assay were determined by the purple pigmentation as judged by the unaided eye. Assays were performed in triplicate. *P. aeruginosa* PAO1 was used as a positive control strain for

AHLs production (22).

To analyze the ability of sub-MIC AZM and EPI-PA $\beta$ N to inhibit the quorum sensing-regulated violacein pigment production, the same procedure was carried out except for the tested bacterial suspensions which were incubated in LB broth with either sub-MIC AZM or 20  $\mu$ g/ml EPI-PA $\beta$ N, P4157 (Sigma Aldrich, Germany) for 24 hours prior to inoculation in the wells of the microplate.

**Effect of sub-MIC AZM and PA $\beta$ N on QS-dependent virulence factors: Pyocyanin production.**

Pyocyanin pigment was extracted from broth cultures of the tested *P. aeruginosa* clinical isolates grown in glycerol alanine minimal medium at 37°C for 48 h (23). The cells were removed by centrifugation and pyocyanin in the supernatant was extracted into chloroform (3 ml volume of chloroform to 5 ml of culture supernatant), then pyocyanin was re-extracted into 1 mL of 0.2 M HCl. PAO1 strain was propagated as a positive control, while negative control was prepared by applying the same procedure on an empty culture; without an isolate. For the quantitation of the pyocyanin, the absorbance was measured at OD 520. Assays were performed in triplicate where an average reading was then calculated in ( $\mu$ g/ml). To analyze the ability of sub-AZM and PA $\beta$ N to inhibit pyocyanin production, the same procedure was carried out except for the *P. aeruginosa* isolates were incubated in Glycerol alanine minimal media with sub-MIC AZM or 20  $\mu$ g/ml PA $\beta$ N for 24 hours prior to extraction of pyocyanin. The percent reduction in the pyocyanin level in the presence of AZM and PA $\beta$ N was determined and compared to that of the untreated cultures.

**Protease activity.** The isolates were assayed in triplicates for protease production by streaking on 2% skim milk agar and incubation at 37°C for 24 hours. Plates were checked for halo clearing zone around the colonies and streaks and the width of the clear zones was taken as a degree of protease production (24). The test was repeated in the presence of sub-MIC AZM or 20  $\mu$ g/ml PA $\beta$ N and the reduction in the degree of protease production was determined and compared to that of the untreated cultures.

**Rhamnolipid production: Drop collapse method.** Production of rhamnolipid was detected in triplicates by drop collapse method depending on destabilization of liquid droplets by biosurfactant (25). A

sterile microtiter plate was thinly coated with oil. The plate was left at room temperature for 24 hours and afterward, 5  $\mu$ l of culture supernatant from isolates was dropped to the surface of the oil in the center of each well. The drop was then left for 1 min on the oil surface and observed for its shape. Interpretation of results was according to the diameter of the drop; if the drop of the culture supernatant was collapsed it was considered as positive compared to un-inoculated media drop as a negative control. The test was repeated in the presence of sub-MIC AZM or 20  $\mu$ g/ml PA $\beta$ N to determine their effect on rhamnolipid production.

**Hemolysis assay.** Production of rhamnolipid was confirmed by hemolysis assay as rhamnolipid is considered a heat-stable hemolysin. *P. aeruginosa* cultures were grown for 16 to 20 h, cells were harvested by centrifugation, and the supernatants were filtered, this sterilized culture supernatant was autoclaved to destroy the enzymatic activity of the heat-labile hemolysin (phospholipase C) and then it was mixed with fresh blood at a ratio of 30:1 (supernatant to blood), observed after 20 min for hemolysis, and then left at room temperature to allow intact erythrocytes to sediment. Hemolytic activity was observed after 4 hours as a clearing of the blood solution indicated that rhamnolipid is present at a concentration above 50  $\mu$ g/ml, while a lack of hemolytic activity as characterized by intact erythrocytes precipitation indicated that little or no rhamnolipid was present in the supernatant (26). Hemolysis assay was done in the presence of sub-MIC AZM or 20  $\mu$ g/ml PA $\beta$ N to test for their inhibitory effect on hemolysis. The test was done in triplicates.

**Biofilm inhibition assay.** All *P. aeruginosa* isolates were assessed for their ability to produce biofilm using the microtiter plate method (27). Each *P. aeruginosa* isolate was incubated in LB broth (with and without sub-MIC AZM and 20  $\mu$ g/ml PA $\beta$ N to detect their biofilm inhibiting activity) in a glass tube overnight at 37°C. After incubation the turbidity of the culture was adjusted to an inoculum of  $1 \times 10^6$  CFU/ml, then an equal volume (200  $\mu$ l) was transferred to each isolate was tested in triplicate), then the plates were incubated 24 hours at 37°C. The contents of the wells were discarded and washed using PBS to remove the non-adherent bacteria. For biofilm fixation, 200  $\mu$ l of 2% sodium acetate solution was added

to each well and left for 5 minutes then washed using 200  $\mu$ l of sterile PBS. For biofilm staining, 200  $\mu$ l of 1% crystal violet solution was used. After washing 200  $\mu$ l of 95% ethanol was added to each well and left for 15 min to elute the crystal violet from the biofilm. The absorbance was read on a microtiter plate reader at 620 nm OD. The test was performed in triplicate and the mean OD was calculated. In each run, a negative control (LB broth without bacterial suspension) was included and absorbance was measured where ODC was calculated from the average OD of the negative control. The ability to form biofilm was scored as: Strong biofilm producer (isolate OD  $> 4 \times$  ODC), Moderate biofilm producer ( $2 \times$  ODC  $<$  isolate OD  $\leq 4 \times$  ODC), Weak biofilm producer (ODC  $<$  isolate OD  $\leq 2 \times$  ODC), and Non-biofilm producer (isolate OD  $\leq$  ODC).

**PCR for Detection of the QS genes.** Bacterial DNA was extracted from tested *P. aeruginosa* isolates and *P. aeruginosa* reference strain PAO1 by boiling method (28). Tested isolates were cultured overnight at 37°C on nutrient agar plates. Few colonies were emulsified in 200  $\mu$ l sterile distilled water to produce a heavy suspension. Bacterial suspensions were heated at 100°C for 15 minutes then cooled on ice for 5 minutes; afterward, they were centrifuged at 14,000 rpm for 5 minutes. The clear supernatant was used as the stock DNA extract and stored at -20°C.

PCR was carried out using the following primers (Table 1) to amplify *lasI*, *lasR*, *rhlI*, and *rhlR* genes. Each 25  $\mu$ l reaction mixture contained 12.5  $\mu$ l master mix (MyTaq™ Red Mix, Biorline), 5  $\mu$ l of the DNA extract, and 10 pmol of each primer. Amplification was performed according to the following parameters; initial denaturation at 95°C for 4 min followed by 35 cycles of denaturation at 95°C, annealing at

52°C and extension at 72°C with a final extension step at 72°C for 10 min. Negative control was prepared by the addition of the same contents with water instead of the DNA extract. Detection of the amplified products was done on 1.5% agarose gel electrophoresis with ethidium bromide staining.

#### Sequencing of *lasI*, *lasR*, *rhlI*, and *rhlR* genes.

For the six isolates that were positive for the 4 amplified QS genes and phenotypically negative for the tested virulence factors, sequencing of *lasI*, *lasR*, *rhlR*, and *rhlI* genes was performed to search for any possible mutations affecting and causing malfunctioning of these QS genes. The PCR products were purified using Thermo Scientific® GeneJET Gel Extraction Kit. Automated sequencing was performed using BigDye terminator cycle sequencing kit on ABI Prism 310 Genetic analyzer (Applied Biosystems) with the same primers used in the amplification. The obtained gene sequences were aligned and compared with the sequence of standard *P. aeruginosa* strain (ATCC27853) retrieved from the National Center for Biotechnology Information (NCBI) using BioEdit sequences alignment software.

**Statistical analysis.** Statistical analysis was done using SPSS software version 26.0. Chi-square test was used to study any significant association between qualitative variables. Fischer exact and Monte Carlo tests were used if more than 20% of the total expected cell counts  $< 5$  at 0.05 level of significance. McNemar test was done to detect any significant difference in the proportion of positive and negative different virulence factors before and after addition of AZM. Cochran's Q test and marginal test of homogeneity were done to study any statistically significant differences in the virulence factors before and after the addition

**Table 1.** Primers used for the detection of QS genes

Gene	Primer	SIZE	Reference
<i>las I</i>	F: 5'ATGATCGTACAAATTGGTCGGC-3' R: 5' GTCATGAAACCGCCAGTCG-3'	605 bp	
<i>las R</i>	F: 5'-ATGGCCTTGGTTGACGGTT-3' R: 5'-GCAAGATCAGAGAGTAATAAGACCCA-3'	725 bp	(29)
<i>rhl I</i>	F: 5'-CTTGGTCATGATCGAATTGCTC-3' R: 5'-ACGGCTGACGACCTCACAC-3'	625 bp	
<i>rhl R</i>	F: 5'-CAATGAGGAATGACGGAGGC-3' R: 5'-GCTTCAGATGAGGCCAGC-3'	730 bp	

F: Forward, R: Reverse

of AZM and EPI (PAβN). All statistical tests were judged at the 0.05 significance level.

**RESULTS**

**Detection of C<sub>4</sub>HSL molecules using cross feeding bioassay.** The 50 *P. aeruginosa* isolates were collected from different clinical sources; 21 urine samples, 23 wound swabs, 4 sputum samples, one endotracheal tube sample, and one bronchoalveolar lavage sample. Thirty six (72%) of isolates gave positive signals, while 14 (28%) isolates were defective in the production of the autoinducers C<sub>4</sub>HSL using a cross-feeding bioassay.

**Production of AHLs dependent virulence factors.** To determine the contribution of QS systems to the pathogenesis of *P. aeruginosa* infections, the 50 isolates were screened for the production of AHLs dependent virulence factors; 40 (80%), 32 (64%), 41 (82%) *P. aeruginosa* isolates were positive for the production of pyocyanin, rhamnolipid and protease respectively. Regarding the biofilm formation, 37 (74%) of *P. aeruginosa* isolates produced strong biofilm whereas 8 (16%) of the isolates produced moderate biofilm and 5 (10%) produced no biofilm.

In correlating between QS Signals production and the detected virulence factors in the isolates (Table 2), it was found that; there was a statistically significant difference in the production of virulence factors between positive and negative isolates for autoinducer production as among the 36 isolates with QS autoinducer; 88.9%, 83.3%, and 100% were positive for pyocyanin, rhamnolipid and protease production respectively compared to 57.1%, 14.3% and 35.7%

for the 14 isolates defective for the autoinducer production. Concerning biofilm formation, about 5.5% of isolates positive for autoinducer produced weak biofilm, meanwhile, 21.4% of isolates with negative QS signals formed weak biofilm. However, this difference was not statistically significant.

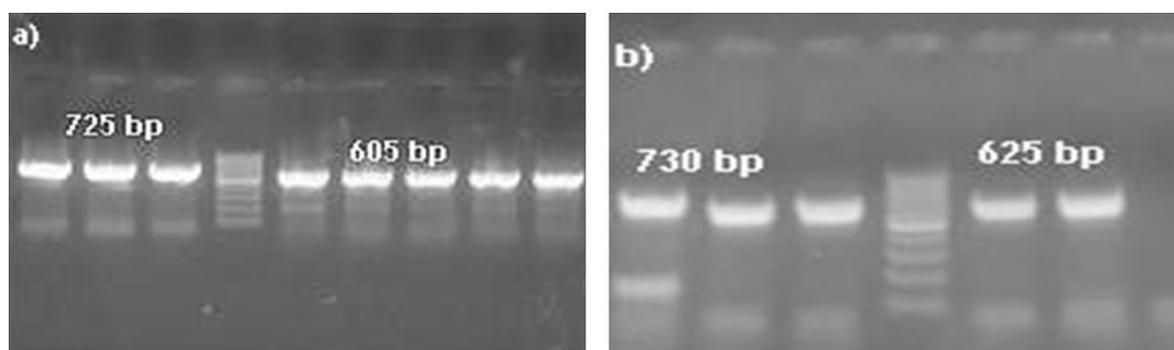
**Detection of QS genes (*lasI*, *lasR*, *RhlI*, and *rhlR*) by PCR.** PCR analysis of QS genes (Fig. 1) revealed that the four QS genes were present in 42 *P. aeruginosa* isolates. On the other hand, 8 isolates were negative for one, two, or three genes; *lasI* gene was the least absent gene (2%) among the 50 *P. aeruginosa* isolates whereas, *rhlI* gene was the most absent gene (10%). All 36 (72%) isolates producing autoinducer C<sub>4</sub>HSL had the 4 QS genes present. On the contrary, in the remaining 14 isolates with deficient QS signals 8 of them had absent one or more QS genes while 6 were positive for QS genes. There was a statistically significant difference between the proportion of positive QS autoinducer C<sub>4</sub>HSL and the presence of the four QS genes. (Fep <0.001). Since the Rhl system relays on the autoinducer C<sub>4</sub>HSL, the relation between the QS signals C<sub>4</sub>HSL and each of *rhlI* and *rhlR* of the QS system was statistically analyzed and there was a statistically significant association between the production of signals and each of the QS genes; *rhlI* and *rhlR*. (p=0.004 and p=0.001 respectively).

The relation between QS-dependent virulence factors and QS genes was analyzed, although most of the isolates with positive QS genes were phenotypically positive for QS-dependent virulence factors production, the association between the presence of QS genes and the production of QS-dependent virulence factors was statistically significant only for

**Table 2.** Relation between the C<sub>4</sub>HSL QS Signals production and the detected virulence factors in the 50 *P. aeruginosa* isolates

Virulence factors	Biofilm			Pyocyanin		Rhamnolipid		Protease	
	Strong n=37	Moderate n=8	Weak n=5	+ve n=40	-ve n=10	+ve n=32	-ve n=18	+ve n=41	-ve n=9
Positive (n=36)	28 (77.8%)	6 (16.7%)	2 (5.5%)	32 (88.9%)	4 (11.1%)	30 (83.3%)	6 (16.7%)	36 (100%)	0 (0%)
Negative (n=14)	9 (64.3%)	2 (14.3%)	3 (21.4%)	8 (57.1%)	6 (42.9%)	2 (14.3%)	12 (85.7%)	5 (35.7%)	9 (64.3%)
*Significance	**MCp=0.28			***Fep=0.02		****P<0.001		***Fep <0.001	
	x <sup>2</sup> =20.858								

\*Results ≤ 0.05 are significant. \*\* Monte Carlo Test. \*\*\* Fischer exact test. \*\*\*\* Chi-Square test.



**Fig. 1.** Detection of PCR amplicons of QS genes on the ethidium bromide-stained gel. a) Detection of *lasI* and *lasR*: lanes number 1, 2, 4, 5, and 6 are positive for *lasR* gene (show amplified bands at 725 bp.). Lanes number 8-13 are positive for *lasI* gene (show amplified bands at 605 bp.). b) Detection of *rhII* and *rhIR*: lanes number 1, 2, 3, 4 and 13 are positive for *rhIR* gene (show amplified bands at 730 bp.). Lanes number 5, 6, 8, 9 and 11 are positive for *rhII* gene (show amplified bands at 625 bp.). Lane number 7 shows DNA 100 bp. ladder.

protease and rhamnolipid production ( $p=0.005$ ).

**Sequence analysis of *lasI*, *lasR*, *rhII* and *rhIR* genes.** Out of the 14 *P. aeruginosa* isolates that were defective in the production of autoinducer  $C_4$ HSL, 8 isolates had one or more negative genes, whereas 6 isolates (isolates number; 8, 9, 10, 19, 23 and 54) had the four QS genes present and they were also defective in the production of the most of the virulence factors. Hence, these 6 isolates were further investigated for their probable mutational defects in their QS genes. Sequence analysis of the PCR products of these isolates showed that these six isolates appeared to carry various point mutations in one or more of the QS genes. Isolates 10 and 23 had mutational defects in the *lasI* and *rhII* genes while isolate 9 had mutations in the *rhII*, and *rhIR* genes. Isolate 8 had mutations in *lasI*, *rhII* and *rhIR* genes. Whereas each of the isolates number 19 and 54 had mutations in one gene; *lasI* and *rhII*, respectively.

**Antibiotics susceptibility pattern of the 50 *P. aeruginosa* isolates.** Among the 50 *P. aeruginosa* isolates, 32 (64%) isolates were multidrug-resistant (MDR) as defined by non-susceptibility to at least one agent in three or more antimicrobial categories. The isolates showed the highest resistance to fluoroquinolones; 74% of isolates were resistant to both levofloxacin and ciprofloxacin. On the other hand, isolates showed the highest susceptibility to ceftazidime (38%), followed by amikacin (34%), and then imipenem (30%).

Regarding the relation between the resistance to different classes of antibiotics and QS signals pro-

duction, isolates with negative QS signals were more resistant to fluoroquinolones (85.7%) followed by (78.6%) for each of amikacin, imipenem, and ceftazidime and (64.2%) for gentamycin. Nevertheless, there was no statistically significant association between *P. aeruginosa* resistance to antibiotics and QS signals ( $p > 0.05$ ).

**Determination of MIC of AZM for the 50 *P. aeruginosa* isolates.** The 50 *P. aeruginosa* isolates included in the study had MIC values for AZM ranged from 16-250  $\mu\text{g/ml}$  according to broth microdilution tests, with a median value of 62  $\mu\text{g/ml}$  and a mean value of 99.26  $\mu\text{g/ml}$ . Accordingly, the chosen sub-MIC of AZM was 8  $\mu\text{g/ml}$  and was used for all further phenotypic experiments.

**Effect of sub-MIC AZM and PA $\beta$ N on  $C_4$ HSL QS signals production using cross-feeding bioassay.** On reassessment of these 36 positive isolates in QS signals molecules,  $C_4$ HSL production in the presence of sub-MIC of AZM (8  $\mu\text{g/ml}$ ); all the 36 isolates failed to produce the signals. On the other hand, in the presence of PA $\beta$ N (20  $\mu\text{g/ml}$ ); only 14 out of the 36 isolates producing QS signals were affected as follows; 6 isolates failed to produce the QS signals molecules  $C_4$ HSL and 8 isolates showed a reduction in the production of the QS signals in the form of a visual reduction in the color intensity of the bioassay. There was a significant difference ( $P < 0.01$ ) in the number of *P. aeruginosa* isolates producing QS signals in presence of sub-MIC AZM and PA $\beta$ N.

**Effect of sub-MIC AZM on the production of**

**QS-dependent virulence factors.** The effect of sub-MIC of AZM on the production of QS-dependent virulence factors among the 50 *P. aeruginosa* isolates was investigated. Table 3 shows a decreased number of isolates producing QS-dependent virulence factors in the presence of sub-MIC of AZM (8 µg/ml). For the rhamnolipid production; only 3 isolates produced rhamnolipid in the presence of sub-MIC of AZM. Regarding protease production; only 4 isolates produced protease, 9 isolates did not produce protease and 28 isolates showed reduced protease production; observed as reduced halo zone around separate colonies on skimmed milk agar. Concerning pyocyanin production; 28 isolates failed to produce pyocyanin and 12 isolates showed decreased pyocyanin production. The number of isolates producing biofilm was also reduced in the presence of sub-MIC of AZM, where 48% of isolates produced almost no biofilm compared to 5% in the absence of AZM. There was a statistically significant difference in the proportion of positive QS-dependent virulence factors between isolates in the absence and presence of sub-MIC of AZM (p<0.001).

**Effect of EPI PAβN on the production of QS-dependent virulence factors.** Since EPI PAβN affected the production of QS signals C<sub>4</sub>HSL in only 14 *P. aeruginosa* isolates producing signals, therefore, its effect on the production of QS-dependent virulence factors among these 14 isolates was investigated and compared to the effect of sub-MIC of AZM on these 14 isolates (Table 4).

Only 5 isolates produced rhamnolipid in the pres-

ence of 20 µg/ml EPI PAβN. Regarding protease production, only 6 isolates produced protease, 3 isolates did not produce protease and 5 isolates showed reduced protease production. All the 14 isolates failed to produce pyocyanin. The number of isolates producing biofilm was also reduced in the presence of 20 µg/ml EPI PAβN; as only 11 isolates produced biofilm in the presence of EPI PAβN. There was a statistical significant difference in the proportion of positive QS-dependent virulence factors between these 14 isolates in the absence and presence of either 20 µg/ml EPI PAβN or sub-MIC of AZM.

By comparing the results of the effect of sub-MIC of AZM and 20 µg/ml EPI PAβN on the QS-dependent virulence factors, the inhibitory effect of AZM was more observed but this was not statistically significant.

**DISCUSSION**

Alternative strategies aiming at the inhibition of bacterial virulence have been proposed to face the serious challenge of multidrug resistant bacteria that represent a major medical threat worldwide (30, 31). *P. aeruginosa* causes many different types of infections and it frequently develops resistance to available antimicrobial agents. Improved treatments and preventive measures are so urgently required for combating this pathogen. QS is a complex cell-to-cell signaling network that allows coordinated gene expression according to cell density and regulates many major virulence factors in *P. aeruginosa*. As a

**Table 3.** Number of isolates producing virulence factors in the presence and absence of sub-MIC AZM

Virulence factors Number of isolates (%) (producing virulence factors) In absence of sub-MIC AZM	Biofilm	Pyocyanin	Rhamnolipid	Protease
	-37 (74%) strong	40 (80%)	32 (64%)	41 (82%)
	-8 (16%) moderate			
	-5 (10%) weak			
	-0 (0%) no biofilm			
In presence of sub-MIC AZM	-11 (22%) strong	12 (24%)	3 (6%)	4 (8%)
	-15 (30%) moderate			
	-17 (34%) weak			
	-7 (14%) no biofilm			
*Significance	**p<0.001	***p<0.001	***p<0.001	***p<0.001

\* Results ≤ 0.05 are significant. \*\* Marginal test of homogeneity \*\*\* McNemar test p-value

**Table 4.** Effect of each EPI and AZM on the virulence factors production in the 14 *P. aeruginosa* isolates (with affected QS signals in presence of EPI)

Virulence factors		Number of isolates (n=14)			***Significance
		In absence of AZM & EPI	In presence of AZM	In presence of EPI	
Biofilm	Strong	10 (71.4%)	3 (21.4%)	3 (21.4%)	<sup>a</sup> p=0.132
	Moderate	3 (21.4%)	4 (28.6%)	8 (57.1%)	
	**Significance	-----	<sup>a</sup> p=0.004	<sup>a</sup> p=0.007	
Pyocyanin	Positive	12 (85.7%)	3 (21.4%)	0 (0%)	<sup>aa</sup> p=0.867
	**Significance	-----	<sup>aa</sup> p=0.004	<sup>aa</sup> p=0.001	
Rhamnolipid	Positive	12 (85.7%)	1 (7.1%)	5 (35.7%)	<sup>aa</sup> p=0.472
	**Significance	-----	<sup>aa</sup> p=0.001	<sup>aa</sup> p=0.004	
Protease	Positive	14 (100%)	1(7.1%)	6 (42.9%)	<sup>a</sup> p=0.225
	Decreased*	0	10 (71.4%)	5 (35.7%)	
	**Significance	-----	<sup>a</sup> p=0.317	<sup>a</sup> p=0.317	

\*Reduction in diameter of halo zone \*\*tests for presence and absence of AZM or EPI \*\*\*tests for presence AZM and EPI.

<sup>a</sup> marginal test of homogeneity. results ≤ 0.05 are significant. <sup>aa</sup>Cochrane's Q test. results ≤ 0.05 are significant

consequence, QS is an ideal target for anti-virulence strategies (31).

The present study aimed to investigate the role of the QS signal production and QS-associated genes in the production of the QS-dependent virulence factors, also we aimed to assess and compare the inhibitory effect of AZM and EPI- PAβN on QS system and QS-dependent virulence factors in *P. aeruginosa* isolates of different clinical sources.

In this study, we measured the production of C<sub>4</sub> HSL and four QS-dependent virulence factors; 72% of the isolates were QS signals C<sub>4</sub>HSL producers, while 28% of them were defective in the production of the autoinducers. Production of virulence factors was significantly higher among the QS-proficient isolates than the QS-deficient isolates; 88.9%, 83.3%, and 100% were positive in proficient isolates compared to only 57.1%, 14.3%, and 35.7% in deficient ones for pyocyanin, rhamnolipid, and protease production, respectively. Regarding biofilm formation, there was a difference in the biofilm formation between the QS producers (94.5%) and the QS-deficient isolates (78.6%). This proves the correlation between the production of QS signals and the virulence in *P. aeruginosa*.

These results agree with previous observations of other studies which confirmed the crucial role of QS in *P. aeruginosa* virulence; Wang et al. observed that 80% of the isolates were found to exhibit the QS-dependent phenotypes; 81% of the isolates produced

protease, 78% produced pyocyanin, 82% of the isolates produced elastase and 89% produced biofilm with only 7% out of their isolates did not produce AHLs based on cross-feeding bioassay (32). Also, Senturk et al. found that 64.6% of the isolates produced rhamnolipid, 86.58% of the isolates produced elastase and 78% produced biofilm and all these isolates were QS-proficient (33). Another confirmation for the importance of QS to establish a successful infection has been shown in a number of different infection models such as mouse burn wound, pulmonary infection, and keratitis. In these studies, the inadequacy of QS-deficient strains to establish successful infection was proposed to be associated with reduced production of virulence factors (34, 35).

PCR analysis of QS genes (*lasI*, *lasR*, *rhlI*, and *rhlR*) revealed that the four QS genes were present in 42 *P. aeruginosa* isolates, while 8 isolates were negative for one, two, or three genes; these 8 isolates were also QS-deficient and failed to produce many of the QS-dependent virulence factors. The results of this study revealed a significant association between the proportion of positive QS autoinducer and the presence of the four QS genes; out of the 42 genotypic positive *P. aeruginosa* isolates, 36 isolates were QS signals producers while the remaining 6 isolates were deficient in the production QS signals and most of the studied QS-dependent virulence factors. The presence of a PCR product does not exclude the possibility that the QS genes may have had inacti-

vating mutations which could explain the impaired QS-dependent phenotypes observed in these 6 isolates in spite of the presence of the four QS genes. Therefore, these 6 isolates were further investigated for the probable mutational defects in their QS genes by sequence analysis revealing that; each had several point mutations in one or more of the four QS genes which may explain their virulence factor-negative phenotype, as well as their deficiency in AHL production.

These observations agree with previous studies. Senturk et al. found, among 82 *P. aeruginosa* isolates, 6 were defective in the production of all the virulence factors tested, as well as in the AHLs. PCR analysis of these isolates for the presence of QS genes revealed that two isolates were negative for one or more genes while four isolates contained genes that had several mutations (33). Also, Bosgelmez-Tinaz et al. reported that QS-deficient clinical isolates that were unable to produce both the C<sub>4</sub>-HSL signaling molecule and C<sub>4</sub>-HSL dependent virulence factors had mutations in the *rhlR* and/or *rhlI* genes (36).

In the present work, despite that the 14 isolates with deficient QS signals also showed deficiency in most of the QS-dependant virulence factors examined, they were capable of causing different types of infections; urinary tract infections (4 isolates), respiratory tract infections (2 isolates) and wound infections (8 isolates). This was also reported by previous studies where QS-deficient clinical isolates that lost all QS-dependent virulence factors tested were capable of causing clinical infections in humans (33, 36). These findings emphasize that; in addition to known QS-dependent virulence factors, there may be other factors that may not be stringently controlled by QS. In addition, the loss of any single virulence factor may be compensated by others during infection.

The susceptibility pattern of the 50 *P. aeruginosa* isolates for anti-pseudomonal antibiotics showed that the isolates had the highest resistance to the fluoroquinolones; 74% for levofloxacin and ciprofloxacin, followed by imipenem (70%). This susceptibility pattern was different from that of Wang et al. who found that resistance was most often observed to cefazolin (81.2%), followed by trimethoprim-sulfamethoxazole (73.5%), and ceftriaxone (62.4%) while resistance to levofloxacin and meropenem was 58.8% and 20%, respectively. Unfortunately, 64% of our isolates were MDR, this percentage was higher than that reported by Micek et al. found in an international multicenter

study of *P. aeruginosa* pneumonia infections that 30.5% of the strains were MDR (37).

In this study, the high resistance of the isolates to imipenem 70% is a striking observation because this class of antibiotics (carbapenem) is considered one of the few treatment options especially against MDR *P. aeruginosa* infections. Isolates with negative QS signals were more resistant to different anti-pseudomonal antibiotics. Nevertheless, there was no significant association between antibiotic resistance and QS signals production. This was also reported by other workers, where Wang et al. and Karatuna et al. found that QS-deficient *P. aeruginosa* strains tend to be less susceptible to antimicrobials (22, 32). This finding could be interpreted partly by that; efflux pumps which represent the main antibiotic resistance mechanism in *P. aeruginosa* may also expel autoinducer molecules, in which case the overexpression of this efflux pumps could lead to decreased intracellular concentrations of the autoinducers and hence diminished production of virulence factors.

The widespread resistance to antibiotics combined with the shortage of novel antimicrobial compounds results in an urgent need for new strategies to combat bacterial infections. Hence, antivirulence therapy has become an attractive anti-infective approach. Target-based rational design and screening of chemical libraries allowed the identification of a variety of virulence inhibitors (38, 39). The unpredicted antivirulence activity observed among macrolide antibiotics prompted revisiting of laboratory and clinical data to assess the potential of these compounds as antivirulence drugs (40).

In this study, the effect of AZM on the production of C<sub>4</sub>HSL and the four QS-dependent virulence factors was studied to investigate its role as antivirulence drugs. Also, the effect of EPI PAβN on the QS system and was investigated the results were compared to that of AZM.

AZM is neither bactericidal nor bacteriostatic against *P. aeruginosa* at achievable tissue concentrations and there are no published breakpoints for this species. Nevertheless, sub-inhibitory concentrations of macrolides cause substantial inhibition of virulence factors in bacteria (40). Several previous studies reported that; the antivirulence potential of sub-MIC AZM in *P. aeruginosa* through inhibition of QS signal molecules. Several mechanisms have been suggested for the inhibitory effect of AZM on QS including; interfering with biofilm formation,

interaction with ribosomes inhibiting translation, or increase in the outer membrane permeability. It was reported by many previous studies that, sub-MIC AZM reduced the expression of several genes in *P. aeruginosa* including *lasI/lasR* and *rhlI/rhlR* (40-42).

In the present study, the effect of sub-MIC of AZM (8 µg/ml) on the production of the QS signal C<sub>4</sub>HSL and QS-dependent virulence factors were evaluated; all the 36 clinical isolates (100%) producing QS signals, failed to produce the C<sub>4</sub>HSL autoinducer, indicating that the AZM interfered with the production of QS signals in *P. aeruginosa*. This result was also reported by several workers although there was variation in the value of sub-MIC. Other workers used another macrolide; erythromycin, as in Sofer et al. study that showed that its sub-MIC also reduced the production of QS signal molecules (43).

In the present work, there was a significant decrease in the number of isolates producing QS-dependent virulence factors in the presence of sub-MIC of AZM; 6%, 8%, and 24% only produced rhamnolipid, protease, and pyocyanin, respectively compared to 64%, 82%, and 80% in absence of AZM. Furthermore, 48% of the isolates produced almost no biofilm compared to only 10% in absence of AZM. The inhibitory effect of AZM on the production of virulence factors was reported by many studies; Hoffmann et al. and Wagner et al. reported that different concentrations of AZM suppressed the production of many QS-regulated virulence factors (42, 44). A similar finding was reported by Skindersoe et al. who found that rhamnolipid and protease production were reduced in the presence of AZM compared with the untreated cultures (26).

Bacterial efflux pumps export not only antibiotics but also host-derived antimicrobial agents and virulence determinants, such as adhesins, toxins, or other proteins that are important for colonization and infection, thus contributing to bacterial pathogenicity. Role of efflux pumps in exporting virulence determinants that allow *P. aeruginosa* to be invasive and cause infection was reported. Hirakata et al. and Join-Lambert et al. proposed that overexpression of Mex efflux pumps might trigger the efflux of some virulence determinants (45, 46). Furthermore, in *P. aeruginosa* AHLs also are substrates of the efflux system. Hence, the blocking of the efflux pump function by EPIs is one of the different strategies to overcome this pathogen.

PAβN is a dipeptide amide compound; it has been

identified as EPI with the ability to broadly inhibit several known multidrug efflux pumps in *P. aeruginosa*. The mechanisms involved in inhibition of efflux systems are not clearly understood but it has been proposed that the inhibitor binds directly to the pump, and blocks it in competitive or non-competitive pathways (47-49). The results of the present work revealed that PAβN reduced the production of both the autoinducer C<sub>4</sub>HSL and the studied QS-dependent virulence determinants as follows; in the presence of EPI (20 µg/ml), 14 (38.9%) *P. aeruginosa* isolates producing QS signals were affected; 6 (16.7%) of the isolates failed to produce C<sub>4</sub>HSL and 8 (22.2%) showed a reduction in its production.

Since EPI PAβN affected the production of QS signals in only 14 *P. aeruginosa* isolates, therefore, its effect on the production of virulence factors among these 14 isolates was investigated and also compared to the effect of sub-MIC of AZM on these isolates. In the presence of 20 µg/ml EPI PAβN, there was a reduction in the number of the tested isolates producing QS-dependent virulence factors. The percentage of reduction was 50%, 85.7%, 21.5%, and 14.3% for rhamnolipid, pyocyanin, proteases, and biofilm production, respectively. It worth mentioning that the biofilm, rhamnolipid, and pyocyanin production showed statistically significant difference between the absence and presence of PAβN. This could be attributed to the inhibition of the efflux pump exporting the virulence determinants by PAβN.

By comparing the effect of 20 µg/ml EPI PAβN and sub-MIC of AZM on the QS signals production our results revealed that; in presence of sub-MIC of AZM all the 36 *P. aeruginosa* isolates failed to produce the autoinducer C<sub>4</sub>HSL, meanwhile, in presence of PAβN only 14 isolates were affected. On the other hand, by comparing their effect on the QS-dependent virulence factors, each of PAβN and AZM has a significant effect on biofilm formation, rhamnolipid, and pyocyanin production. Although the inhibitory effect of AZM was more observed, this was not statistically significant.

## CONCLUSION

In conclusion, the development of antibiotic resistance by *P. aeruginosa* makes it indispensable to search for new therapeutic options for this challenging pathogen. Anti-quorum sensing agents like AZM

and EPI (PAβN) are useful agents in this approach due to their inhibitory effect on QS-dependent virulence factors production with no selective pressure on bacteria growth, so the resistance to these agents is less likely to develop.

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