Control of quorum sensing and virulence factors of *Pseudomonas aeruginosa* using phenylalanine arginyl β-naphthylamide

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The spread of multidrug-resistant *Pseudomonas aeruginosa* isolates constitutes a serious clinical challenge. Bacterial efflux machinery is a crucial mechanism of resistance among *P. aeruginosa*. Efflux inhibitors such as phenylalanine arginyl β-naphthylamide (PAβN) promote the bacterial susceptibility to antimicrobial agents. The pathogenesis of *P. aeruginosa* is coordinated via quorum sensing (QS). This study aims to find out the impact of efflux pump inhibitor, PAβN, on QS and virulence attributes in clinical isolates of *P. aeruginosa*. *P. aeruginosa* isolates were purified from urine and wound samples, and the antimicrobial susceptibility was carried out by disc diffusion method. The multidrug-resistant and the virulent isolates U16, U21, W19 and W23 were selected. PAβN enhanced their susceptibility to most antimicrobial agents. PAβN reduced QS signalling molecules N-3-oxo-dodecanoyl-L-homoserine lactone and N-butryl-L-homoserine lactone without affecting bacterial viability. Moreover, PAβN eliminated their virulence factors such as elastase, protease, pyocyanin and bacterial motility. At the transcription level, PAβN significantly (*P<0.01*) diminished the relative expression of QS cascade (lasI,lasR,rhlI,rhlR,pqsA and pqsR) and QS regulated-type II secretory genes lasB (elastase) and toxA (exotoxin A) compared to the control untreated isolates U16 and U21. In addition, PAβN eliminated the relative expression of pelA (exopolysaccharides) in U16 and U21 isolates. Hence, *P. aeruginosa*-tested isolates became hypo-virulent upon using PAβN. PAβN significantly blocked the QS circuit and inhibited the virulence factors expressed by clinical isolates of *P. aeruginosa*. PAβN could be a prime substrate for development of QS inhibitors and prevention of *P. aeruginosa* pathogenicity.

INTRODUCTION

*Pseudomonas aeruginosa* causes severe and persistent infection of immunocompromised and elderly patients. *P. aeruginosa* also causes opportunistic infections in many other conditions such as corneal infections, chronic otitis, implant infections, cystic fibrosis, urinary tract infection and burn infection (Stover *et al*., 2000). During infection, *P. aeruginosa* develops various pathogenicity factors and enzymes such as pyocyanin, protease, elastase, rhamnolipids and hydrogen cyanide which facilitate microbial dissemination (Jimenez *et al*., 2012). Moreover, it exhibits several cell-associated factors such as flagella, pili and LPSs which assist host colonization and chronic infection (Wagner *et al*., 2004). Quorum sensing (QS) allows microbial communication that coordinates the sense and the response of a single cell to its surrounding population via the release of the signal molecules, acyl homoserine lactones (AHLs), termed autoinducers (Jimenez *et al*., 2012).

In *P. aeruginosa*, the principal QS circuits are las, rhl and pqs which coordinate signal production and virulence expression. The three major signalling molecules of *P. aeruginosa* involve N-3-oxo-dodecanoyl-L-homoserine lactone (C12-HSL), N-butyryl-L-homoserine lactone (C4-HSL) and four-quinolone signal. Once those signals reach the significant levels, they activate their regulatory genes and enhance the transcription of virulence factors (Toder *et al*., 1994). C12-HSL is the basic molecule of las system; it triggers the expression of rhl and pqs circuits in a hierarchical manner (Pearson *et al*., 1994).

QS operates the release of *P. aeruginosa* virulence factors and the synthesis of biofilm during infection (Tang & Zhang, 2014). Therefore, QS inhibition is an advanced strategy to control microbial pathogenesis. Pure natural compounds

**Abbreviations:** AHL, acyl homoserine lactone; CLSI, Clinical Laboratory Standards Institute; C4-HSL, N-butryl-L-homoserine lactone; C12-HSL, N-3-oxo-dodecanoyl-L-homoserine lactone; PAβN, phenylalanine arginyl β-naphthylamide; QS, quorum sensing; RND, resistance nodulation division.
such as xanthones (Mohamed et al., 2014), ellagic acid (Sarabhai et al., 2013), eugenol (Zhou et al., 2013), ginseng (Schneper et al., 2012), patulin and penicillinic acid (Rasmussen et al., 2005), ajoene (Jakobsen et al., 2012) and various plant extracts (Adonizio et al., 2008; Koh et al., 2013) prevent the expression of QS-associated virulence. Recently, 1H-pyrrole-2-carboxylic acid from Streptomyces coelicoflavus soil isolate eliminates QS and its associated virulence factors in P. aeruginosa PAO1 (Hassan et al., 2016). Also, AHL analogues and halogenated furanones inhibit QS (Wang & Ma, 2014). Also, signal-degrading enzymes oxidase, lactonase and paraoxonase (Chun et al., 2004; Lin et al., 2003; Liu et al., 2008) have been identified. Moreover, vaccines that antagonize QS signalling molecules have been developed such as C12-HSL-BSA and C12-HSL-PrCrV (Golpasha et al., 2015; Miyairi et al., 2006).

Control of P. aeruginosa infections is always complicated. This problem arises from the fact that P. aeruginosa is a multidrug-resistant bacteria. It exhibits different mechanisms of resistance where the active efflux is an important multidrug resistance mechanism. The resistance-nodulation-division (RND) family is the main efflux machinery among P. aeruginosa. RND is a tripartite efflux pump which consists of an inner protein and an outer membrane protein connected through a periplasmic membrane. The most studied tripartite drug efflux pump in P. aeruginosa is the MexAB-OprM (Du et al., 2013; Tomás et al., 2010). The efflux pump MexAB-OprM is constitutively expressed in P. aeruginosa with a broad substrate specificity. It exports diverse antimicrobial agents such as fluoroquinolones, tetracyclines, chloramphenicol, β-lactams, dyes and organic solvents outside the bacterial cell causing intrinsic resistance to broad-spectrum antimicrobials (Moore & Flaws, 2011). Moreover, efflux machinery participates in the selective transport of AHLs outside the cells, which stimulates adjacent cells and completes cell-to-cell cascade (Minagawa et al., 2012). Hence, P. aeruginosa QS and accompanied virulence characteristics are controlled by the activity of MexAB-OprM efflux (Blair & Piddock, 2009).

Phenylalanine arginyl β-naphthylamide (PAβN) is one of the main efflux inhibitors (Mahamoud et al., 2006). It enhances the susceptibility of P. aeruginosa to antimicrobial agents and decreases the dissemination of Pseudomonas infection (Hirakata et al., 2009). The influence of PAβN on QS and bacterial signalling has been assessed. In this study, the impact of PAβN on the level of QS signals and related virulence factors was evaluated among clinical isolates of P. aeruginosa. Also, the influence of PAβN on the expression of QS genes and exotoxin A was estimated.

**METHODS**

**Bacterial strains and media composition**

P. aeruginosa isolates were purified from patients with infected urinary tract and from wound samples. These isolates were identified as P. aeruginosa according to laboratory biochemical standards (Elmer et al., 2006). The samples were handled according to the ethical guidelines of the research ethics committee (permit no. 2016-19).

*Escherichia coli* MG4/pKDT17 (lac reporter; lasR::lacZ, lacZ, lasR Ap') and *E. coli* DH5α/pECP61.5 (rhl reporter; rhlR::lacZ, lasR, Ap') reporter strains were utilized for quantitation of the signals C12-HSL and C4-HSL, respectively (Pearson et al., 1997). P. aeruginosa PAO-JP2 (∆rhl::Tn10, Tc') is a QS double-mutant strain; it was used as a negative control. All isolates were cultivated using Luria–Bertani (LB) growth medium (+) at 37°C.

**Determination of antimicrobial susceptibility of P. aeruginosa isolates**

The antimicrobial susceptibility of the purified P. aeruginosa isolates was assessed by disc diffusion technique according to the Clinical Laboratory Standard Institute (CLSI, 2014). Mueller–Hinton plates were streaked with the pure cultures (1.5×10⁸ c.f.u. ml⁻¹). Subsequently, antimicrobial discs (cefazidime 30 µg, imipenem 10 µg, gentamicin 10 µg, amikacin 30 µg, ciprofloxacin 5 µg and levofloxacin 5 µg; Oxoid) were placed on the plates. The obtained filtrates were kept at 4°C. The obtained filtrates were kept at 4°C. The influence of PAβN on the growth of P. aeruginosa isolates was detected from the respective interpretation charts according to the CLSI guidelines (CLSI, 2014). The multidrug-resistant isolates and the most virulent isolates U16, U21, W19 and W23 were selected for further studies.

**Effect of phenylalanine arginyl β-naphthylamide on the sensitivity of P. aeruginosa**

The effect of various concentrations of phenylalanine arginyl β-naphthylamide (PAβN) (Sigma–Aldrich) on the growth of P. aeruginosa isolates U16, U21, W19 and W23 was detected (CLSI, 2014). Twofold serial dilutions of PAβN (100, 50, 25, 12.5, 6.25 and 3.125 µg ml⁻¹) were prepared in a microtitre plate. The dilutions were inoculated with 0.5×10⁶ c.f.u. ml⁻¹ of U16, U21, W19 and W23, and the plates were propagated for 18 h at 37°C. The MICs of cefazidime, imipenem, gentamicin, amikacin, ciprofloxacin and levofloxacin were determined using the broth micro-dilution assay (CLSI, 2014) in triplicates. Twofold or greater differences in MICs of antimicrobials in the presence of PAβN (50 µg ml⁻¹) compared to MICs of antimicrobials alone were assigned a significant efflux inhibition effect (Lamers et al., 2013).

**Effect of PAβN on the growth of P. aeruginosa clinical isolates**

Isolates U16, U21, W19 and W23 were cultivated in the presence of PAβN (50 µg ml⁻¹) at 37°C. The viability of the cultures either treated or untreated with PAβN was tested every hour by measuring the OD₆₀₀ of the suspension during the first 12 h (Nalca et al., 2006).

**Influence of PAβN on QS autoinducers**

**Preparation of P. aeruginosa cell-free filtrates**

Isolates U16, U21, W19 and W23 were inoculated into LB medium with and without PAβN (50 µg ml⁻¹) and incubated at 37°C for 18 h with shaking at 150 r.p.m. The bacterial suspension was centrifuged at 8000 g for 15 min at 4°C. Cell-free supernatant was separated and purified using syringe filters (0.45µm). The obtained filtrates were kept at −20°C and utilized in the assay of QS autoinducers and virulence determinants. Negative control, PAO-JP2, was prepared under the same conditions (Gupta et al., 2011).

**Assay of C12-HSL and C4-HSL**

Overnight cultures of the tested isolates were selected for further studies.
Each mixture was centrifuged at 8000 r.p.m. Elastin Congo red (20 mg ml⁻¹) was mixed with an equal volume of the previously prepared supernatants of the clinical isolates at 30 °C with shaking for 3–4 h. The β-galactosidase analysis was performed according to Miller (1972).

Assay of P. aeruginosa virulence factors

Assay of elastase. Elastase enzyme of both treated and untreated P. aeruginosa was quantified in triplicates according to elastin Congo red method. Elastin Congo red (20 mg ml⁻¹) in Tris/HCl buffer (700 µl) was mixed with an equal volume of the previously prepared P. aeruginosa supernatants, and the reaction was kept at 37 °C for 18 h. Each mixture was centrifuged at 8000 g for 10 min and soluble pigment formed was measured at A485 (Musthafa et al., 2011). The percent reduction in elastolytic activity in the presence of PAßN was estimated and compared to the control isolates propagated without PAßN.

Assay of total proteases. The assessment of P. aeruginosa proteolytic activity with and without PAßN was carried out using skimmed milk assay in triplicates (El-Mowafy et al., 2014a). The previously prepared P. aeruginosa supernatant (200 µl) was mixed with 1 ml of 1.25 % skimmed milk and incubated for 15 min at 37 °C, and the OD₆₀₀ was measured. Protease activity=OD₆₀₀ of skimmed milk–OD₆₀₀ of the sample.

Pyocyanin assessment. Pyocyanin assay was made using King’s A broth medium in triplicate (Essar et al., 1990). Overnight cultures of U16, U21, W19 and W23 were diluted at OD₆₀₀ to 0.1–0.15 into 5 ml of King’s A medium, which contained PAßN (50 µg ml⁻¹), and were incubated at 37 °C for 48 h with shaking at 150 r.p.m. Control cultures without PAßN and PAO-JP2 were simultaneously propagated. Cultures were centrifuged, and pyocyanin was extracted with chloroform (3 ml). The lower organic layer containing pyocyanin pigment was separated out PA and mixed with 1 ml of 0.2 N HCl, and the OD₅₂₀ was measured. The percent reduction in the pyocyanin level in the presence of PAßN was determined and compared to that of the control untreated cultures.

Motility assays. The influence of PAßN (50 µg ml⁻¹) on the swimming and twitching of P. aeruginosa was performed and compared to the control plates without PAßN in triplicates (Rashid & Kornberg, 2000). Pseudomonas swimming was assessed by surface inoculation of 0.5 % w/v LB agar plates with 2 µl of the diluted P. aeruginosa isolates U16, U21, W19 and W23 (1 x 10⁶ c.f.u. ml⁻¹). The swimming zone was measured post-incubation at 30 °C for 18 h. For twitching, diluted U16, U21, W19 and W23 cultures were stabbed into the twitching plates (1 % w/v tryptone, 0.5 % w/v NaCl and 0.5 % w/v Bacto agar). After incubation at 37 °C for 24 h, the agar was removed, and the bacteria attached to the plate surface were stained with crystal violet. The migration of P. aeruginosa on the interface of the twitch medium and bottom of the plate was measured in millimetre. Reduction in P. aeruginosa swimming and twitching diameter indicated the QS inhibitory effect of PAßN.

Extraction of mRNA and synthesis of cDNA. The impact of PAßN (50 µg ml⁻¹) on the relative expression of QS genes lasI, lasR, rhlI, rhlR, pqsA and pqsR in the isolates U16 and U21 was assessed using real-time polymerase chain reaction (RT-PCR). Moreover, the level of expression of other virulent genes including lasB, pelA and toxA was also evaluated in the presence of PAßN. P. aeruginosa isolates U16 and U21 were grown until the middle of exponential phase (OD₆₀₀, 0.5–0.6) with and without PAßN. Cultures were pelleted, and mRNA was extracted with Trizol reagent (Sigma-Aldrich). P. aeruginosa PAO-JP2 strain was similarly handled. The prepared mRNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen). The levels of gene expression were assessed using RT-PCR.

RT-PCR of virulence factor and QS regulatory genes. The RT-PCR was carried out via the thermocycler Rotor-Gene Q (Qiagen). The

Table 1. Specific amplification primer sets for P. aeruginosa isolates

<table>
<thead>
<tr>
<th>Gene type</th>
<th>Gene symbol</th>
<th>Type of primer</th>
<th>Primer sequence</th>
<th>Melting temp.</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference gene</td>
<td>rpoD</td>
<td>Fw 5'-CGAACTGCTTGCCGACTT-3'</td>
<td>56 °C</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 5'-GCCGAGGCTCCTCAAGGATAC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QS genes</td>
<td>lasI</td>
<td>Fw 5'-CGGCCATCGGGGAGTG-3'</td>
<td>56 °C</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 5'-CGGCCAAGGAAGGATAG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>lasR</td>
<td>Fw 5'-CTGCGGAGGTTTGCAG-3'</td>
<td>56 °C</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 5'-CTGCGGAGGTTTGCAG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rhlI</td>
<td>Fw 5'-CGGCCAAGGAAGGATAG-3'</td>
<td>58 °C</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 5'-CGGCCAAGGAAGGATAG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rhlR</td>
<td>Fw 5'-CTGCGGAGGTTTGCAG-3'</td>
<td>58 °C</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 5'-CTGCGGAGGTTTGCAG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pqsA</td>
<td>Fw 5'-CGGCCAAGGAAGGATAG-3'</td>
<td>58 °C</td>
<td>142</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Virulence genes</td>
<td>lasB</td>
<td>Fw 5'-CGGCCAAGGAAGGATAG-3'</td>
<td>56 °C</td>
<td>165</td>
<td></td>
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<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>pelA</td>
<td>Fw 5'-CGGCCAAGGAAGGATAG-3'</td>
<td>58 °C</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 5'-CGGCCAAGGAAGGATAG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>toxA</td>
<td>Fw 5'-CGGCCAAGGAAGGATAG-3'</td>
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<td>85</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Rev 5'-CGGCCAAGGAAGGATAG-3'</td>
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<td></td>
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</table>

temp., temperature; Fw, forward; Rev, reverse.
reaction was set up using FIREPol EvaGreenqPCR Mix (Solis Bio-Dyne) and primers shown in Table 1. The level of gene expression was relatively normalized to the expression of the housekeeping gene rpoD. The expression of genes in P. aeruginosa isolates U16 and U21 cultivated with PAβN was compared to their expression in the control cultures without PAβN. Gene expression of PAO-JP2 was also measured.

Statistical analysis

Each experiment was constructed in triplicates. Data was represented as mean±SD. The Student’s t-test (Tukey–Kramer test) was performed for statistical analysis using GraphPad Prism software (version 5.01) where \(P<0.05\) or \(P<0.01\) was considered statistically significant.

RESULTS

Antimicrobial susceptibility and virulence of P. aeruginosa isolates

The antimicrobial susceptibility patterns of the purified P. aeruginosa isolates were estimated. Eight P. aeruginosa isolates were multidrug resistant to the evaluated antimicrobial agents (Table S1, available in the online Supplementary Material). The QS signals C12-HSL and C4-HSL of the multidrug-resistant P. aeruginosa isolates were assessed (Table S2). Also, the virulence factors (elastase, protease and pyocyanin) of the multidrug-resistant isolates were estimated. The four isolates U16, U21, W19 and W23 revealed the highest level of C12-HSL. Moreover, the four isolates possessed high virulence levels (Table S2).

Effect of PAβN on the susceptibility of P. aeruginosa virulent isolates

P. aeruginosa isolates U16, U21, W19 and W23 with the highest level of QS signal C12-HSL and high virulence levels were selected to test the effect of PAβN on their QS and virulence. The MICs of U16, U21, W19 and W23 were calculated in the presence and absence of PAβN (50 \(\mu\)g ml\(^{-1}\)) (Table 2). PAβN significantly reduced MICs of ceftazidime, imipenem and ciprofloxacin for the four isolates. The largest reduction in MIC was for isolates U16 and W23 treated with ceftazidime, where 50 \(\mu\)g ml\(^{-1}\) PAβN caused eightfold decrease in the MIC of ceftazidime. However, no effect of PAβN was observed on the MICs of gentamicin and amikacin for all isolates.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>MICs without PAβN ((\mu)g ml(^{-1}))</th>
<th>MICs PAβN ((\mu)g ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAZ</td>
<td>IPM</td>
</tr>
<tr>
<td>U16</td>
<td>1250</td>
<td>625</td>
</tr>
<tr>
<td>U21</td>
<td>2500</td>
<td>625</td>
</tr>
<tr>
<td>W19</td>
<td>39</td>
<td>18.5</td>
</tr>
</tbody>
</table>

CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin; CN, gentamicin; AK, amikacin.

Influence of PAβN on the viability of P. aeruginosa

Different concentrations of PAβN (3.126–100 \(\mu\)g ml\(^{-1}\)) did not change the visible growth of P. aeruginosa isolates U16, U21, W19 and W23. The effect of PAβN (50 \(\mu\)g ml\(^{-1}\)) on the growth of the isolates U16 and U21 was assessed by monitoring the OD of the cultures at 600 nm (Fig. 1). The viability of the isolates treated with PAβN was similar to the control untreated cultures through the first 12 h of the growth indicating that PAβN did not affect the growth of the isolates. Furthermore, the isolates with and without PAβN attained the stationary growth phase within 8 h, denoting that PAβN (50 \(\mu\)g ml\(^{-1}\)) did not impair bacterial growth along the exponential phase (Fig. 1a, b).

Effect of PAβN on QS signals

The two signalling molecules C12-HSL and C4-HSL were evaluated in the presence of PAβN using the reporter strain assay and compared to control cultures. The addition of PAβN (50 \(\mu\)g ml\(^{-1}\)) to the isolates U16, U21, W19 and W23 caused 36, 77, 22 and 54 % decrease in C12-HSL signal, respectively, compared to the untreated cultures (\(P<0.01\)). PAβN reduced the level of C4-HSL in U16 by 54 % (\(P<0.01\)) and in U21 by 65 % (\(P<0.01\)) compared to the control cultures without PAβN. However, PAβN did not affect the level of C4-HSL in wound isolates W19 and W23. PAO-JP2 strain was included as the QS double mutant (Fig. 2a, b).

Impact of PAβN on the release of virulence factors

PAβN inhibited the elastase enzyme activity in the tested isolates as estimated via elastin Congo red method (Fig. 3a). PAβN reduced elastase activity of U16, U21 and W23 by 33 % (\(P<0.05\)), 44 % (\(P<0.01\)) and 35 % (\(P<0.01\)), respectively.

Additionally, protease activities of P. aeruginosa with PAβN were evaluated using the skimmed milk assay. PAβN significantly reduced protease activity of U16 by 54 % (\(P<0.01\)) and of U21 by 37 % with \(P<0.05\). Also, PAβN significantly diminished protease activity of W19 and of W23 by 51 and 37 %, respectively, with \(P<0.01\) (Fig. 3b).
The effect of PAβN on pyocyanin production was quantified using King A media (Essar et al., 1990). It was noticed that PAβN decreased the level of pyocyanin in U16, U21, W19 and W23 by 43, 56, 27 and 43%, respectively, with P<0.01 (Fig. 3c).

**Inhibition of Pseudomonas motility by PAβN**

P. aeruginosa exhibits swimming motility in aqueous environments (low agar concentration) and twitching on dry environments (high agar concentration) (Rashid & Kornberg, 2000). PAβN-supplemented plates caused a significant decrease in the twitching by 52.3, 60, 36.4 and 55% in P. aeruginosa isolates U16, U21, W19 and W23, respectively (P<0.01) (Fig. 4a). Moreover, the swimming diameter of both isolates U16 and U21 was significantly reduced by 69 and 68.6%, respectively (P<0.01). Also, the swimming of the wound isolates W19 and U21 was decreased by 21 and 23%, respectively (Fig. 4b).

**Effect of PAβN on the expression of QS genes**

The influence of PAβN on genes involved in the control of Pseudomonas QS was performed using RT-PCR. PAβN showed more specificity for lasR gene than lasI (C12-HSL synthase gene), as it suppressed lasR gene by 78.2 and 87.76% and caused 69.2 and 34% inhibition of lasI expression in U16 and U21, respectively, compared to the untreated cultures (Fig. 5a).

Moreover, PAβN significantly decreased the rhlR expression in U16 and U21 by 93 and 91% (P<0.01), respectively, as well as the expression of rhlR gene which reduced by 92.3 and 85.1% (P<0.01) resulting in the termination of the rhl system (Fig. 5b).

In addition, PAβN significantly reduced the relative expression of pqsA in U16 and U21 by 84.5 and 81.1% and lowered pqsR relative gene expression in U16 and U21 by 75.4 and 88%, respectively, with P<0.01 (Fig. 5c).

**PAβN inhibited the expression of virulence genes**

The relative expression of the virulence genes was also inhibited by PAβN. The relative expression of the lasB gene in isolates U16 and U21 propagated with PAβN significantly decreased by 73 and 67% (P<0.01), respectively. The addition of PAβN to U16 and U21 isolates significantly lowered the expression level of pelA gene by 84 and 77% (P<0.01), respectively, which correlated to the control cultures. Moreover, PAβN caused a significant decline in the expression values of toxA by 83% in U16 isolate and by 63.2% in U21 isolate compared to untreated isolates (P<0.01) (Fig. 6).

**DISCUSSION**

P. aeruginosa pathogenesis is related to plenty of virulence attributes, biofilm formation, toxin secretion and antibiotic resistance to most conventional antimicrobials (Karatuna & Yagci, 2010). QS controls the expression of virulence characteristics among P. aeruginosa, biofilm formation and cellular functions including synthesis of polysaccharides and bacterial enzymes. P. aeruginosa inactivates antimicrobials and triggers multiple-drug resistance through antibiotic-degrading enzymes, target modification and bacterial efflux pumps which expel bactericidal drugs outside the bacterial cell (Srinivas et al., 2010). RND efflux family contributes to the intrinsic resistance among P. aeruginosa isolates; it removes various classes of antimicrobial agents outside bacterial membranes. The major RND pump in P. aeruginosa is MexAB-OprM, which is composed of three subunits: the inner membrane pumps (MexB), the membrane adapter protein (MexA) and the outer membrane protein (OprM). Each subunit plays a distinct role in the extrusion process (Nikaido & Pages, 2012). In addition, inhibition of RND with an efficient efflux pump inhibitor enhances the microbial susceptibility to a wide array of antimicrobial agents (Lomovskaya et al., 2001).

The expression of MexAB-OprM has been linked to the cell density and the quorum circuits (Whitehead et al., 2001). Targeting QS is a new anti-Pseudomonas approach to diminish its pathogenicity without induction of microbial resistance (Ben Haj Khalifa et al., 2011). Several QS inhibitors have been approved including plant extracts (Zaki et al., 2013), natural (Tang & Zhang, 2014) or synthetic compounds (Wang & Ma, 2014) and QS-degrading enzymes (Lin et al., 2003). Also, sub-MICs of azithromycin, ceftazidime and ciprofloxacin inhibit the QS activity of P. aeruginosa (Nalca et al., 2006; Skindersoe et al., 2008).

P. aeruginosa QS is composed of three interwoven circuits: las, rhl and pqs with interconnected signalling mechanisms. For microbial communication and response to cell density,
autoinducers need to be transferred extracellularly. In *P. aeruginosa*, C4-HSL freely diffuses across the bacterial membranes. However, C12-HSL and PQS autoinducers require membrane transporters to cross the outer membrane barrier (Pearson et al., 1999).

We selected four isolates U16, U21, W19 and W23 with multidrug resistance to different classes of antimicrobial agents. They also produced significant levels of QS signals and related virulence factors. The isolates revealed high resistance level against assessed antimicrobials according to

**Fig. 2.** The effect of PAβN on the QS signals among isolates U16, U21, W19 and W23. (a) PAβN showed a significant decrease in the levels of C12-HSL in isolates U16, U21, W19 and W23. (b) PAβN caused a significant decline in the levels of C4-HSL in isolates U16 and U21. PAO-JP2 was utilized as a negative control. Error bars indicate sd of three independent experiments (*significant $P<0.05$ and **highly significant $P<0.01$).

**Fig. 3.** Impact of PAβN (50 $\mu$g ml$^{-1}$) on virulence factors of *P. aeruginosa* isolates U16, U21, W19 and W23 compared with the untreated cultures. PAβN eliminated (a) elastase, (b) protease activity and (c) pyocyanin. Error bars represent sd of three independent experiments. (*significant $P<0.05$ and **highly significant $P<0.01$).
CLSI (2014). Co-incubation of antimicrobials ceftazidime, imipenem and ciprofloxacin with the efflux inhibitor PAβN showed two- to eightfold decrease in their MICs indicating that the multidrug-resistant isolates U16, U21, W19 and W23 exhibited an active efflux pump (Table 2). PAβN is a substrate for RND pumps of *P. aeruginosa* (MexB, MexD and MexF). PAβN enhances the susceptibility of a MexAB-OprM-overproducing strain of *P. aeruginosa* to fluoroquinolone antibiotics such as levofloxacin and sparfloxacin (Lomovskaya et al., 2001). Molecular dynamics simulation study of Vargiu & Nikaido (2012) indicated that PAβN prevents the movement of MexAB-OprM substrates from the proximal to the distal binding sites. Additionally, a docking study of Vargiu et al. (2014) suggested that PAβN induces a conformational change in the extrusion channel which hinders the binding of the substrates to this site. Alternatively, PAβN demonstrates another mechanism of action by impairment of the outer membrane permeability of the Gram-negative bacteria (Lamers et al., 2013; Lomovskaya et al., 2001) associated with the increase in the influx of the

**Fig. 4.** Motility inhibition via PAβN (50 µg ml⁻¹). (a) PAβN significantly reduced the twitching and (b) swimming of isolates U16, U21, W19 and W23 compared to the control untreated cultures. Error bars demonstrate SD of three independent experiments (*significant* *P*<0.05 and **highly significant* *P*<0.01).

**Fig. 5.** Significant reduction of the QS cascade in *P. aeruginosa* isolates U16 and U21 treated with PAβN (50 µg ml⁻¹). PAβN reduced the expression of the QS genes (a) lasI and lasR, (b) rhlI and rhlR and (c) pqsA and pqsR compared with the untreated cells and PAO-JP2 double mutant. Error bars indicate SD of independent three experiments (*significant* *P*<0.05 and **highly significant* *P*<0.01).
The present research focused on the influence of PAβN on the quorum process and on the release of QS-mediated virulence factors among clinical isolates of *P. aeruginosa*. PAβN significantly reduced QS signal C12-HSL among the tested isolates U16, U21, W19 and W23 (Fig. 2a) and caused a significant elimination of C4-HSL signal in the isolates U16 and U21 (Fig. 2b). On the molecular level, PAβN reported a significant (*P*<0.01) decrease in the relative expression of *las* circuit and the interconnected cascades *rhl*/R and *pqsa*/R in both isolates U16 and U21 (Fig. 5). Other studies reported that the resistant nodulation efflux system MexAB-OprM affects the transport of QS signals and its precursors (Ben Haj Khalifa *et al.*, 2011). MexAB-OprM participates in the efflux of C12-HSL (Pearson *et al.*, 1999). Accordingly, inhibiting MexAB-OprM by PAβN in the present study was accompanied by low extracellular levels of the signal C12-HSL in all tested isolates. C12-HSL is the main signal that controls the expression of the subsequent QS cascade, so elimination of the C12-HSL efflux causes a significant interruption of bacterial communication accompanied by a further decrease in C4-HSL signals. Consequently, inhibition of *P. aeruginosa* C4-HSL and C12-HSL signals and associated QS circuit develops a marked decline in the QS-related virulence of *P. aeruginosa*. Similarly, sub-MICs of some antibiotics such as azithromycin and ciprofloxacin repress QS, via restraining cellular permeability and inhibiting the release of QS signal C12-HSL in the surrounding environment (Bala *et al.*, 2011; Malloy *et al.*, 2005). Pearson *et al.* (1999) showed high intracellular levels of C12-HSL among MexAB-OprM mutants. The intracellular accumulation of C12-HSL is not accompanied by irreversibly binding to the transcriptional activator LasR or RhlR protein.

Elastase and protease enzymes play an important role in the pathogenesis of *P. aeruginosa* through breaking down and disintegration of the connective tissue in human cells (Ben Haj Khalifa *et al.*, 2011). The release of both enzymes is managed by *las* and *rhl* systems. The release of pyocyanin pigment is also controlled by RhlR/R and PQS signals (Gupta *et al.*, 2011). In the present research, inhibition of QS signal release was followed by a significant reduction in total protease, elastase and pyocyanin (Fig. 3). Moreover, the viability of U16 and U21 isolates was not influenced by PAβN (50µg ml⁻¹) compared to the control cells without PAβN (Fig. 1a, b). Hence, reduction in quorum expression and decrease of the associated virulence factors were not related to the decrease in cell viability. Previous studies showed that elimination of QS signals is accompanied by inhibition of *P. aeruginosa* virulence artifacts involving total protease, elastase and pyocyanin as exhibited by furanone derivatives, natural products (Zaki *et al.*, 2013) or known drugs such as ascorbate (El-Mowafy *et al.*, 2014b).

Another aspect of *Pseudomonas* virulence is the motility behaviour which assists microbial invasion and bacterial attachment. *P. aeruginosa* motility is coordinated via the QS system; it is relayed on *las* and *rhl* systems. Therefore, *P. aeruginosa* PAO-JP2 double mutant is completely devoid of the bacterial motility (Köhler *et al.*, 2000). In this study,
cultivation of *P. aeruginosa* on media supplied with PAβN inhibited the motility function of *P. aeruginosa* (Fig. 4). Similarly, ellagic acid derivatives eliminate motility of *P. aeruginosa* (Sarabhai et al., 2013).

Moreover, this study revealed a marked decline in the relative expression of *pelA*, in *P. aeruginosa* treated with PAβN (Fig. 6). The *pel* operon is fundamental for the formation of exopolysaccharide matrix (Vasseur et al., 2005) whose expression is regulated by *las* system and its signal C12-HSL (De Kievit, 2008). Furthermore, the relative expression of *lasB* and *toxA* representing elastase and exotoxin A, respectively, were reduced at the transcriptional level (Fig. 6). Both genes (*lasB* and *toxA*) are linked to Xcp type II secretion pathway and upregulated via *QS* (Gambello et al., 1993), and *lasl* transcript is required for the expression of *lasB* and *toxA* (Storey et al., 1998). Deletion of *mexAB-oprM* from *P. aeruginosa* PAO1 eliminates its capacity to invade Madin–Darby canine kidney cells. Efflux pump inhibitor (PAβN) reduces the invasiveness of *P. aeruginosa* (Hirakata et al., 2009). Hence, *P. aeruginosa mexAB-oprM* deficient mutants display low traffic of C12-HSL signals to the extracellular environment and so hinder cell communication and eliminate the pathogenesis of *P. aeruginosa*. Likewise, some efflux pump inhibitors impair efflux activities and abolish biofilm assembly in *Klebsiella* and *E. coli* (Kvist et al., 2008).

QS controls the expression of virulence characteristics according to population density. Sensing of the cell density is attained via the extracellular autoinducer molecules AHLs and PQS. Once the concentration of signalling molecules accomplishes a certain threshold level, they trigger a signal to the bacterial cells which respond by altering the expression of several genes in a consistent manner. In the study of Amaral & Molnar, (2012), trifluoromethyl ketones inhibit AHL signals as detected by the reporter strain *Chromobacterium violaceum* CV026. On the same instance, phenothiazines and trifluoromethyl ketones as proton motive force inhibitors eliminate efflux pump systems and restrict QS systems and accompanied swarming and swimming motilities (Varga et al., 2012).

Previous studies tested the influence of efflux inhibitors on *P. aeruginosa* standard strains and derived mutants. In the study of Pearson et al. (1999), *P. aeruginosa* standard strain PA01 treated with the proton motive force inhibitors such as azide and carbonyl cyanide 3-chlorophenylhydrazone has less extracellular levels of C12-HSL compared to the untreated cells. Also, the extracellular concentration of C12-HSL has been reduced in *P. aeruginosa* mutants lacking efflux pump MexAB-OprM by eightfold of the intercellular levels. Moreover, the AHL-dependent response of *P. aeruginosa* was affected by inhibition of MexB transport activity in *P. aeruginosa mexB* mutant (Minagawa et al., 2012). PAβN increases the potency of the externally added C12-HSL to the pump-active QS double-mutant *P. aeruginosa* PAO-JP2 (Moore et al., 2014). The present work explored the influence of the efflux pump inhibitor PAβN among clinical isolates; the tested isolates showed significant decrease in the level of QS signals on both phenotypic and genotypic levels.

Here, inhibition of efflux pumps by PAβN reduced the extracellular accumulation of QS signals and hence inhibited cell–cell signalling (quorum phenomena) in *P. aeruginosa* clinical isolates. This is followed by a reduction in the expression of virulence machinery on both translation and transcription levels and bacteria lose its virulence attributes. Therefore, efflux inhibition could be an imperative approach in the management of *P. aeruginosa* virulence and pathogenesis. Moreover, impairment of drug efflux by PAβN will enable this molecule to be used as antimicrobial adjuvant that can lower the effective doses of the current antimicrobials.

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