JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Correlation of quorum sensing and virulence factors in *Pseudomonas aeruginosa* isolates in Egypt

Hamida M Aboushleib[1], Hoda M Omar[1], Rania Abozahra[2], Amel Elsheredy[3], Kholoud Baraka[2]

[1] *Pharmaceutical Microbiology Department, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt*
[2] *Pharmaceutical Microbiology Department, Faculty of Pharmacy, Damanhour University, Damanhour, Egypt*
[3] *Microbiology department, Medical Research Institute, Alexandria University, Alexandria, Egypt*

Abstract

Introduction: *Pseudomonas aeruginosa* is one of the most virulent nosocomial pathogens worldwide. Quorum sensing (QS) regulates the production of pathogenic virulence factors and biofilm formation in *P. aeruginosa*. The four genes *lasR, lasI, rhlR*,and *rhlI* were found to regulate this QS system. In this study, we aimed to assess the correlation between these four genes and QS-dependent virulence factors and to detect the inhibitory effect of clove oil on QS.

Methodology: Fifty *P. aeruginosa* clinical isolates were collected. Susceptibility to different antibiotics was tested. Virulence factors including biofilm formation, pyocyanin production, and twitching motility were phenotypically detected. QS genes were amplified by polymerase chain reaction (PCR), and one strain subsequently underwent sequencing. The inhibitory effect of clove oil on virulence factors was also tested.

Results: A positive correlation was found between biofilm formation and the presence of *lasR* and *rhlI* genes. Twitching motility was positively correlated with the presence of *lasR*, *lasI*, and *rhlI* genes. On the other hand, no correlation was found between pyocyanin production and any of the studied genes. Only one isolate amplified all the tested QS gene primers, but it did not express any of the tested virulence factors phenotypically. Sequence analyses of this isolate showed that the four genes had point mutations.

Conclusions: Results emphasize the importance of QS in *P. aeruginosa* virulence; however, QS-deficient clinical isolates occur and are still capable of causing clinical infections in humans. Also, clove oil has an obvious inhibitory effect on QS, which should be clinically exploited.

Key words: quorum sensing; virulence; *Pseudomonas aeruginosa*;genes; biofilm.

J Infect Dev Ctries 2015; 9(10):1091-1099. doi:10.3855/jidc.6492

(Received 27 December 2014 – Accepted 06 April 2015)

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium found in almost every ecological niche, including soil, water, and plants. It is also an important opportunistic pathogen of humans, primarily infecting immunocompromised patients such as severely burned patients, patients with cystic fibrosis, and cancer patients undergoing chemotherapy [1].

The severity of *P. aeruginosa* infections is due to the production of different extracellular and cell-associated virulence factors including pyocyanin, flagella, and pili [2,3,4]. Another factor contributing to the pathogenesis of *P. aeruginosa* is its tendency to form organized communities, known as biofilms, when it attaches to biotic and abiotic surfaces [1].

Biofilm formation is important in the establishment of *P. aeruginosa* infections on different host tissues. The biofilms of *P. aeruginosa* may also be formed on medical devices such as urinary catheters. In these settings, the antibiotic resistance engendered by biofilms presents a serious challenge to the treatment of chronic *P. aeruginosa* infections [2]

Another virulence factor produced by *P. aeruginosa* is the blue redox-active exoproduct pyocyanin, the predominant phenazine pigment produced by this organism that can easily penetrate biological membranes. It is readily recovered in large quantities from cystic fibrosis patients infected by *P. aeruginosa* and from ear secretions of *P. aeruginosa*-mediated chronic otitis media. It was found to induce neutrophil apoptosis and impair neutrophil-mediated host defenses *in vivo* [5,6].

Among the cell-associated virulence factors are the cell surface structures, the type IV pili. These polar fimbriae are presumably the principal adhesins, mediating the adherence to eukaryotic cell surfaces and probably to abiotic surfaces as well. They are also responsible for the flagellum-independent mode of translocation called twitching motility. Type IV pilus-based twitching motility has been shown to be

required for the initial attachment and development of a biofilm by *P. aeruginosa* [7].

Recently, it has been discovered that the production of many virulence factors (including pyocyanin production and twitching motility) and the formation of biofilm by *P. aeruginosa* is regulated by a cell-to-cell communication mechanism known as quorum sensing (QS) [1,8,9]. This intercellular signalling is a cell-density dependent mechanism through which bacteria coordinate different activities including bioluminescence, plasmid conjugation, and the production of different virulence factors [9].

P. aeruginosa possesses two QS systems, *las* and *rhl*, which are regulated by N-acyl-homoserinelactones (AHLs). The *las* system comprises *lasI* and *lasR*. The *rhl* system comprises both *rhlR* and *rhlI*. The *las* system positively regulates the expression of both *rhlR* and *rhlI* [10]

Multiple studies have demonstrated the contribution of QS to the pathogenesis of *P. aeruginosa*. The importance of QS in the virulence of *P. aeruginosa* has been demonstrated in different animal models, including mouse models of acute and chronic lung infections of *P. aeruginosa*. These studies compared the virulence of the *P. aeruginosa* mutants that carried deletions within QS genes with that of their parent strains. They showed that deletions within QS genes significantly reduced the lung damage caused by *P. aeruginosa*, as well as the mortality rate from the infection [9,10].

Since QS plays an important role in the virulence and survival of *P. aeruginosa* and other pathogenic bacteria, this signalling pathway became a novel and potential target for anti-infective agents [11].

Essential oils possessing anti-QS properties have been reported by many workers. Clove oil anti-QS activity has been demonstrated. It was found that clove oil extract inhibited QS-regulated phenotypes in *P. aeruginosa* PAO1 including swarming motility and pyocyanin production. These data suggested that clove extracts may be useful as an anti-infective drug. [12]

As QS inhibitors do not kill or inhibit bacterial growth, these agents have an advantage because they do not impose strong selective pressure for the development of resistance, as compared to antibiotics. However, a safe, broad-spectrum, and stable anti-QS compound with proved therapeutic uses is yet to be discovered and exploited [13].

In this study, we aimed to assess the role of the four previously mentioned genes in the presence of the QS-dependent virulence factors in *P. aeruginosa* clinical isolates. We also aimed to assess the inhibitory effect of clove oil on QS-dependent virulence factors.

Methodology

Bacterial strains, media, and growth conditions

In the present study, 50 *P. aeruginosa* clinical isolates were collected from the clinical microbiology laboratory of Damanhour General Hospital over the years 2012 and 2013. A total of 5 (10%) clinical isolates were collected from sputum, 2 (4%) from nasal discharge, 6 (12%) from ear swabs, 17 (34%) from wound swabs, and 20 (40%) from urine samples. The strains were identified by routine biochemical tests and confirmed by the VITEK 2 system (Bio-Merux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Susceptibility testing was performed for 20 antibiotics: ampicillin (AMP), amoxicillin (AML), amoxicillin-clavulanic acid (AMC), ampicillin sodium/sulbactam sodium (SAM), cefazolin (KZ), cefoxitine (FOX), cefadroxil (CFR), cefuroxime (CMX), ceftriaxone (CRO), cefoperazone (CFP), cefepime (FEP), meropenem (MEM), co-trimoxazole (SXT), tetracycline (TE), rifampicin (RD), chloramphenicol (C), polymyxin (PB), nalidixic acid (N), levofloxacin (LEV), and gentamycin (CN) (Oxoid, Basingstoke, United Kingdom) on Mueller-Hinton agar (Oxoid) according to Clinical and Laboratory Standards Institute (CLSI) guidelines [14].

Biofilm assay

The biofilm-forming ability of the isolates was tested using the polystyrene microtiter plate method described by O'Toole *et al*. [15] with slight modifications. Overnight broth cultures in nutrient broth were diluted 1:100 into fresh nutrient broth, and then 200 µL of the freshly inoculated medium was dispensed into wells of a 96-well polystyrene microtiter plate. The plate was incubated at 37ºC overnight without agitation. Biofilms were detected by staining the wells with 200 µL of crystal violet (0.1% [w/v] in H2O), after the stain was added the plate was incubated for another 15 minutes at room temperature and then washed thoroughly with distilled water to remove planktonic cells and residual dye.

Next, 200 µL ethanol (95%) was used to elute crystal violet from the biofilms and the absorbance of the solubilized dye was measured at 590 nm using a microtiter plate reader. Assays were performed in triplicate and the average was taken [15].

Pyocyanin assay

Pyocyanin pigment production by the *P. aeruginosa* clinicalisolates was detected using King A agar (P agar) (peptone 20 g/L ,magnesium chloride 1.40 g/L, potassium sulfate 10 g/L, and agar agar 15 g/L [Oxoid]). The final pH was adjusted to be 7.2 ± 0.2 at 25°C.

Slants of the medium were inoculated and incubated at 30–32°C for four to five days, and then the blue color was observed. Pyocyanin production was confirmed by adding 0.5–1 mL chloroform to the inoculated slants; these were shaken for a few minutes until the pyocyanin was diffused, which turned the solvent blue. After that, the chloroform was acidified with a few drops of HCl, which resulted in a rapid change in color from blue to red; this color change confirmed the presence of pyocyanin [16-18].

Twitching motility

The tested isolates were cultured by being stabbed into Luria Bertani (LB) agar plates (1.5% agar) and incubated overnight at 37°C. They were then left for one to two days at room temperature (< 25°C). Plates were then observed for the presence of flat, spreading, and irregularly shaped colonies and a hazy zone of cell growth within the agar substrate [19]. Another way to visualize the haze of growth at the interface between the agar and the petri dish is to remove the agar and stain the plate with crystal violet (0.1%) for 30 minutes at room temperature [7].

PCR for detection of the QS genes

The chromosomal DNA was extracted by boiling. Oligonucleotide primers synthesized by Eurofins MWG Operon were used. *lasR*1 (5'-ATGGCCTTGGTTGACGGTT-3') and *lasR*2 (5'-AGAGAGTAATAAGACCCA-3'), *lasI*1 (5'ATGATCGTACAAATTGGTCGGC-3') and *lasI*2 (5' GTCATGAAACCGCCAGTCG-3'), *rhlR*1 (5'-CAATGAGGAATGACGGAGGC-3') and *rhlR*2 (5'-GCTTCAGATGAGGCCCAGC-3'), *rhlI*1 (5'-CTTGGTCATGATCGAATTGCTC-3') and *rhlI*2 (5'-ACGGCTGACGACCTCACAC-3') were used to amplify *lasR, lasI, rhlR* and *rhlI* genes, respectively. [9].

PCR was performed in 25 µL of reaction mixture containing 5 µL of chromosomal DNA, 12.5 µL Maxima Hot Start Green PCR Master Mix (2X) (Thermo Scientific Fermentas, Vilnius, Lithuania), 0.3 µL (30 pmol) of forward and reverse primers, and H_2O. The thermal profile was as follows: initial denaturation at 94ºC for 3 minutes, followed by 35 cycles at 95ºC for 30 seconds, 50ºC for 30 seconds, 72ºC for 1 minute, and final extension at 72ºC for 10 minutes. DNA fragments were detected on 1% agarose gels with ethidium bromide staining [9].

Sequencing of lasR, lasI, rhlR, and rhlI genes

To search for the presence of mutations that may affect the QS genes, sequencing of *lasI*, *lasR*, *rhlR*, and *rhlI* genes was performed. The PCR products of the genotypically positive phenotypically negative case (p10) were purified using PureLink PCR purification kit (Invitrogen Life Technologies, Carlsbad, USA). Automated sequencing reactions were performed with the BigDye terminator cycle sequencing kit and an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster city, USA) using the same primers that were used in the amplification of the QS genes.

The derived sequences were aligned and compared with those of the published PAO1 strain of *P. aeruginosa* using the National Center for Biotechnology Information's BLAST server and BioEdit program for multiple sequences alignment.

Statistical analysis

The correlation between the presence of QS genes and the production of virulence factors was evaluated by using Statistical Package for Social Sciences (SPSS) software version 17 with Pearson's correlation test; $p < 0.05$ was considered statistically significant.

Influence of clove oil on certain QS-regulated functions

The minimum inhibitory concentration (MIC) of the clove oil (99%, Chemajet, Alexandria, Egypt) was determined against the 11 most virulent selected strains by the broth microdilution method (CLSI 2011). These 11 selected strains were all biofilm forming, pyocyanin producing, and twitching positive, and were positive for 1, 2, or 3 QS genes[20].

Biofilm inhibition assay

The effect of half the MIC of clove oil on biofilm formation was measured using the polystyrene microtiter plate method with the addition of 12.8% clove oil to the wells; clove-oil-free wells were used as controls. Then the assay was completed as previously described [11].

Table 1. Susceptibility of the 50 *P. aeruginosa*clinical isolates to 20 different antibiotics

Antibiotic	ResistantN (%)	SensitiveN (%)
Ampicillin (AMP)	49 (98)	1 (2)
Amoxicillin (AML)	50 (100)	0 (0)
Augmentin (AMC)	47 (94)	3 (3)
Unacyn (SAM)	49 (98)	1 (2)
Cefazolin (KZ)	49 (98)	1 (2)
Cefoxitine (FOX)	48 (96)	2 (4)
Cefadroxil (CFR)	49 (98)	1 (2)
Cefuroxime (CXM)	49 (98)	1 (2)
Ceftriaxone (CRO)	37 (74)	13 (26)
Cefoperazone (CFP)	37 (74)	13 (26)
Cefepime (FEP)	25 (50)	25 (50)
Meropenem (MEM)	15 (30)	35 (70)
Co-trimoxazole (SXT)	48 (96)	2 (4)
Tetracycline (TE)	29 (58)	21 (42)
Rifampicin (RD)	44 (88)	6 (12)
Chloramphenicol (C)	39 (78)	11 (22)
Polymyxin (PB)	3 (6)	47 (94)
Nalidixic acid (N)	17 (34)	33 (66)
Levofloxacin (LEV)	28 (56)	22 (44)
Gentamycin (CN)	27 (54)	23 (46)

Table 2. Production of virulence factors and occurrence of QS genes in the 50 *P. aeruginosa* isolates

Isolate no.	*lasI* gene	*lasR* gene	*rhlI* gene	*rhlR* gene	Biofilm*	Pyocyanin	Twitching motility
47, 59, 63	+	+	+	+	**S**	-	+
9, 32, 56	+	+	+	+	**M**	-	+
10	+	+	+	+	**N**	-	-
41	+	+	+	-	**M**	-	+
49	+	+	+	-	**S**	+	+
61, 71	+	+	+	-	**S**	-	+
43	+	-	+	+	**M**	+	+
50	+	-	+	+	**S**	+	+
154	+	-	+	+	**W**	+	+
104	+	-	+	+	**W**	-	+
12	+	+	-	-	**W**	-	+
77, 105	+	+	-	-	**S**	-	+
122, 124	+	+	-	-	**W**	+	+
74	-	+	+	-	**S**	-	+
92	-	-	+	+	**S**	-	+
149	-	-	+	+	**N**	+	+
99	+	-	+	-	**S**	+	+
128	-	+	-	+	**W**	-	+
129	-	-	-	+	**W**	-	+
131	-	-	-	+	**W**	+	+
106	-	-	-	+	**M**	-	+
109	-	-	-	+	**N**	-	-
18, 19	+	-	-	-	**M**	-	+
151	+	-	-	-	**W**	+	+
123	-	-	+	-	**W**	+	+
121	-	+	-	-	**M**	+	+
158	-	+	-	-	**M**	-	+
62	-	-	-	-	**M**	-	+
73, 100, 110, 112a, 113	-	-	-	-	**N**	-	-
157	-	-	-	-	**W**	+	+
81	-	-	-	-	**S**	-	+
112b, 125	-	-	-	-	**W**	-	+
118, 141, 150	-	-	-	-	**N**	+	-
13, 98	-	-	-	-	**S**	+	+

S: strong; M: moderate; W: weak; N: non-biofilm forming

Pyocyanin inhibition assay

Half the MIC of clove oil (12.8%) was added to the King A agar (P agar) during its preparation, and slants of this medium were inoculated by streaking. One slant was left untreated with clove oil to act as a control. Then the slants were incubated at 30–32°C for four to five days [11].

Twitching motility inhibition assay

The tested isolates were cultured by being stabbed into LB agar plates supplemented with 12.8% clove oil using sterile toothpicks, incubated overnight at 37°C degrees, then left for one to two days at room temperature (< 25°C) [11].

Results

Antimicrobial susceptibility testing

According to the antibiotic disk diffusion method, the highest resistance in the *P. aeruginosa* clinical isolates was exhibited towards amoxicillin (100%), followed by ampicillin, cefadroxil, cefazolin, cefuroxime, and ampicillin-sulbactam (98% for each), cefoxitine and co-trimoxazole (96% for each), and amoxicillin-clavulanic acid (94%). Only 6% of the isolates were resistant to polymixin (Table 1).

Phenotypic detection of QS-dependent virulence factors

Of the 50 studied *P. aeroginosa* isolates, 38 (76%) were found to be biofilm forming. They were distributed according to the strength of the formed biofilm into strong (30%), moderate (22%), and weak (24%) biofilm forming. Upon testing pyocyanin production, 19 isolates (38%) were found to be pyocyanin producers, and 44 isolates (88%) were found to be positive for twitching motility (Table 2).

PCR for detection of the QS genes

Upon amplifying the four tested QS genes using the conventional PCR technique, 24 isolates (48%) gave positive results for the *lasI* gene, 20 (40%) were positive for the *lasR* gene, 20 (40%) were positive for the *rhlI* gene, and 18 (36%) were positive for the *rhlR* gene (Figures 1 and 2).

Correlation between the presence of QS genes and the production of virulence factors

Positive correlation was found between biofilm formation and the presence of *lasR* and *rhlI* genes (p value for both = 0.01 [$p < 0.05$]). Twitching motility was positively correlated with the presence of *lasR* (p = 0.031 [$p<0.05$]), *lasI* (p = 0.006 [$p < 0.05$]), and *rhlI* genes (p = 0.031 [$p < 0.05$]). On the other hand, no correlation was found between pyocyanin production and any of the studied genes.

It was also found that two (4%) of the fifty tested isolates gave negative results for gene detection, but phenotypically they expressed all the tested virulence factors. On the other hand, five (10%) isolates failed to amplify the four primers used and also failed phenotypically to express any of the tested virulence factors.

Another three genotypically negative isolates produced only pyocyanin pigment but could not form biofilm and did not show twitching motility. On the other hand, only one isolate (p10) amplified all the tested QS gene primers used, but phenotypically it did not express any of the tested virulence factors.

Sequencing of lasR, lasI, rhlR, and rhlI genes for the isolate (p10)

Sequence analysis of the PCR products showed that this genotypically positive and phenotypically negative isolate appeared to carry various mutations in

Figure 1. Detection of *lasI* and *rhlI* on 1% agarose gel. *LasI* (605 bp) is shown in lane 1, ladder in lane 2, and *rhlI* (625 bp) in lane 4.

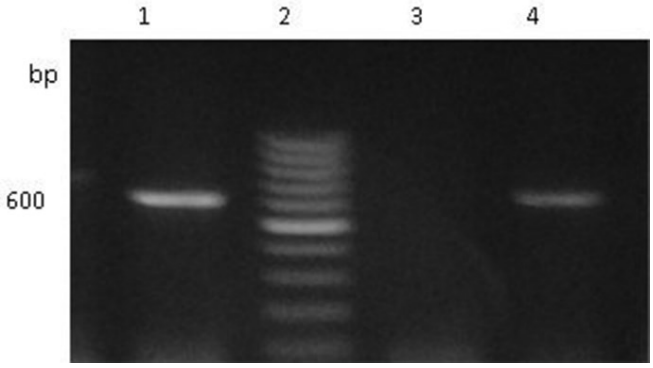
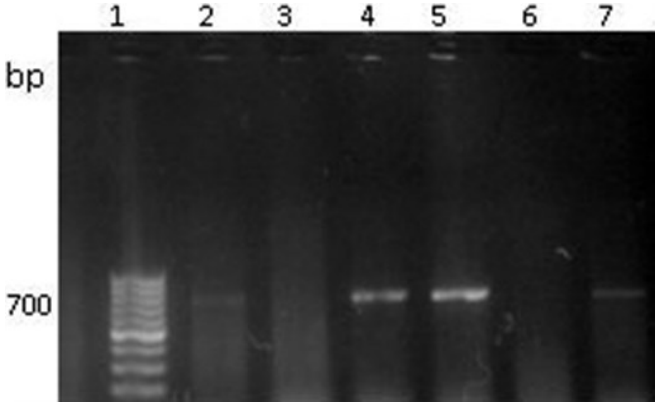


Figure 2. Detection of *lasR* and *rhlR* on 1% agarose gel. The ladder is shown in lane 1, *lasR* (725 bp) in lane 4, and *rhlR* (730 bp) in lanes 5 and 7.



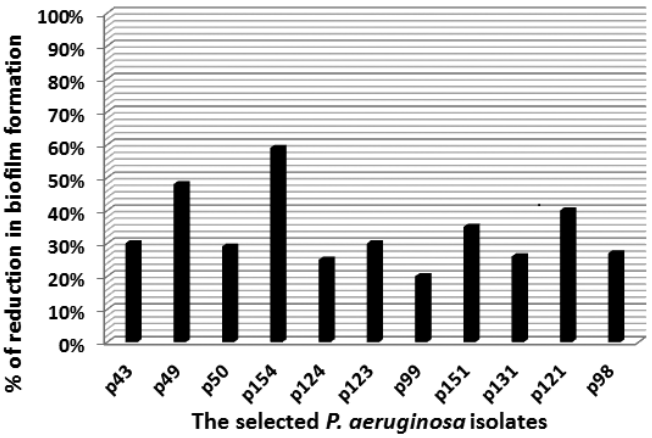
the QS genes. The results of alignment of nucleotide sequence of the tested isolate with the published PAO1 strain of *P. aeruginosa* showed 4 mismatches and 99.28% identity with *lasI* gene, 3 mismatches and 99.25% identity with *lasR* gene, 10 mismatches and 98.21% identity with *rhlI* gene, and 8 mismatches and 98.52% identity with *rhlR* gene. These mutations could probably explain its virulence factor negative phenotype.

Influence of clove oil on certain QS-regulated functions

When the MIC of the clove oil was determined against the 11 selected *P. aeruginosa* isolates by the broth microdilution method, it was found to be 25.6%.

When the inhibitory effect of half the MIC of clove oil (12.8%) was tested on the twitching motility, pyocyanin production, and biofilm formation of the selected 11 *P. aeruginosa* isolates, these QS-dependent virulence factors were inhibited in all the 11 (100%) tested isolates (Figure 3).

Figure 3. The inhibitory effect of ½ MIC of clove oil on biofilm formation on selected *P. aeruginosa* isolates



Discussion

P. aeruginosa is one of the leading nosocomial pathogens worldwide [21]. It infects vulnerable patients including those with postoperative immune suppression [22]. Nosocomial infections caused by this organism are often hard to treat since practically all known mechanisms of antimicrobial resistance can be seen in it. Worryingly, these mechanisms are often present simultaneously, thereby conferring multi-resistant phenotypes [21]. This is consistent with our results, where all the tested isolates showed high resistance to most of the antibiotics.

P. aeruginosa produces multiple virulence factors and causes different types of infections [9]. Depending on the infected tissue, some virulence factors play more important roles in the pathogenesis of *P. aeruginosa* infections than others [23].

The production of these multiple virulence factors in *P. aeruginosa* is controlled by a cell-to-cell communication system known as QS [9]. QS plays a crucial role in *P. aeruginosa* virulence [10]. 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, by employing QS-deficient strains [10].

In the present study, we investigated the role of the QS systems in the pathogenesis of *P. aeruginosa* by screening the presence of three QS-dependent virulence factors in 50 different clinical isolates obtained from sputum, nasal discharge, ear swabs, wound swabs, and urine and correlating them with the presence of the four previously mentioned genes which are regulated by N-acetylated homoserine lactone molecules.

It was found that 76% of the isolates produced biofilm, 38% produced pyocyanin pigment, and 88% were positive for twitching motility, indicating that these isolates were QS proficient. PCR analysis for the presence of QS genes in the same isolates revealed a positive correlation between biofilm formation and the presence of *lasR* and *rhlI* genes ($p < 0.05$). Also, the twitching motility was positively correlated to the presence of *lasR*, *lasI*, and *rhlI* genes ($p < 0.05$). On the other hand, no correlation was found between pyocyanin production and any of the studied genes.

This finding is in agreement with that of previous studies [8,9,24,25],which confirms the crucial role of QS in *P. aeruginosa* virulence.In these studies, inadequacy of QS-deficient strains to establish successful infection was proposed to be associated with reduced production of virulence factors.

However, among the 50 tested isolates in our study, we identified five isolates that were defective in the production of all virulence factors tested; they also failed to amplify our four primer pairs. The presence of these QS-deficient phenotypes could be due to the loss of QS genes. These isolates had lost most of their known virulence factors, but they could still cause infections in humans. These findings agree with those of Schaber *et al*. [9],who identified one QS-deficient clinical isolate that lost all its virulence factors, but still caused a wound infection.

Similar to our findings, Dénervaud *et al*. [26]identified three *P. aeruginosa* strains obtained from intubated patients. These strains were defective

in the production of virulence factors. The results of these two studies suggest that, besides the known virulence factors, there may be additional QS-independent virulence factors that could compensate for the loss of the QS-dependent ones, making QS-defective strains capable of producing infections [9,10,26].

Another possibility that may lead a QS-deficient strain to cause infection is the presence of multiple *P. aeruginosa* strains in the infection site. A single patient may be infected by both QS-proficient and QS-deficient strains of *P. aeruginosa*. QS-deficient strains could profit from the extracellular enzymes produced by QS-proficient partners. Production of signalling molecules and/or QS-regulated factors by QS-proficient strains may enable a QS-deficient strain to participate in an infection [10].

Two of our tested isolates gave negative results for the four QS genes, but phenotypically they expressed all the tested virulence factors. The lack of PCR amplification does not actually indicate the absence of the QS genes. There may be enough base pair mismatches in the region(s) of the used primer(s) to prevent amplification of the gene product.

On the other hand, three other genotypically negative isolates produced only pyocyanin pigment but could not form biofilm and did not show twitching motility; this was supported by our statistical analysis, in which we found that there was no correlation between any of the tested genes and pyocyanin production.

Only one isolate (p10) amplified all the tested QS genes primers that we used, but phenotypically it did not show any of the tested virulence factors. This may be explained by the presence of inactivating mutations, which caused the impaired QS-dependent phenotypes. Sequence analysis of the PCR products of this isolate showed that it carried various point mutations in the QS genes. These mutations could probably explain its virulence factor negative phenotype.

Similar to our findings, Senturk *et al*. [10] identified four isolates that were defective in the production of AHLs and all the virulence factors tested, but they contained all the QS genes. Sequence analyses of these isolates showed that these QS genes had point mutations. The combination of these mutations probably explains their AHL and virulence factor deficiencies. The results of the study suggest that QS-deficient clinical isolates exist and are still capable of causing clinical infections in humans.

The inhibition of QS-regulated virulence factors, including biofilm formation, is a recognized anti-pathogenic drug target. The search for safe and effective anti-QS agents is expected to be useful to combat diseases caused by multidrug-resistant bacteria [27]. Compared with traditional antibiotics, potential QS inhibitors (QSIs) inhibit the QS mechanism and attenuate virulence without influencing bacterial growth [28].

Several reports have indicated the potential of various plant extracts and phtyocompounds in inhibiting one or more QS-controlled traits in *P. aeruginosa*. Although the mechanism of action has not been studied yet, it is possible that plant essential oils exhibiting anti-QS activity might influence bacterial QS-controlled phenotypes directly through inhibiting AHL synthesis or through some indirect mechanisms [11,27-30].

In the present study, attempts were made to determine the effect of sub-MICs of clove oil on *P. aeruginosa*'s QS system and its efficacy against bacterial virulence. When the inhibitory effect of half the MIC (12.8%) of clove oil was tested on the twitching motility, pyocyanin production, and biofilm formation of the selected 11 *P. aeruginosa* strains, these three QS-dependent virulence factors were inhibited in all the tested isolates.

These findings are in agreement with those reported by Husain *et al*. [11],who found that clove oil at sub-MICs significantly reduced different QS-regulated virulence factors and biofilm formation in *P. aeruginosa* clinical isolates.

Our findings are also in agreement with those of Krishnan *et al*. [12]and Khan *et al*.[13], who demonstrated that clove extract inhibited QS-regulated phenotypes in *P. aeruginosa* PAO1, such as swarming motility and pyocyanin production.

The presence of active compounds exhibiting anti-QS activity in the clove extracts maybe useful for the development of anti-infective drugs that differ from traditional antimicrobials that are bactericidal and/or bacteriostatic, as they are not expected to disrupt beneficial flora critical for health, nor lead to selective resistance.

Conclusions

The results of this study confirmed that the QS systems play an important role in the pathogenicity of *P. aeruginosa* infections. Consequently, compounds that attenuate QS may offer significant promise as potential therapeutic agents. These compounds provide alternative medicine for treating emerging bacterial infections without leading to antibiotic resistance as they do not pause selection pressure. Our study also

revealed the anti-QS and biofilm inhibitory activity of clove oil against *P.aeruginosa* isolates. This property could be exploited in developing the oil as an antipathogenic agent alone or in combination with antibiotics against drug-resistant pathogenic bacteria. The results of these new therapeutic strategies should be confirmed in animal models and future prospective clinical studies.

Our results also indicated that *P. aeruginosa* is capable of causing clinical infections in humans despite having an impaired QS system. These findings do not contradict the theory that QS plays a major role in *P. aeruginosa* pathogenicity, but emphasize that, in addition to known virulence factors, there may be other virulence factors contributing to infection and which are not controlled by QS.

References

1. Wagner VE, Filiatrault MJ, Picardo KF, Iglewski BH (2008) *Pseudomonas aeruginosa* virulence and pathogenesis issues. In Cornelis P, editor. *Pseudomonas* genomics and molecular biology, 1st edition. Norfolk: Caister Academic Press. 129-158.
2. Schaber AJ, Hammond A, Carty LN, Williams CS, Colmer-Hamood AJ, Burrowes HB Dhevan V, Griswold JA, Hamood AN (2007) Diversity of biofilms produced by quorum-sensing deficient clinical isolates of *Pseudomonas aeruginosa.* J Med Microbiol 56: 738-748.
3. Lyczak JB, Cannon CL, Pier GB (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect 2: 1051-1060.
4. Wiener-Kronish JP, Frank D, Sawa T (2001) Mechanisms of lung epithelial cell injury by *Pseudomonas aeruginosa*. In Wong HR, Shanley TP, editors. Molecular biology of acute lung injury, 1st edition. Norwell: Kluwer Academic. 149-161.
5. Lau WG, Hassett JD, Ran H, Kong F (2004) The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends Mol Med 10: 599-606.
6. Allen L, Dockrell HD, Pattery T, Lee GD, Cornelis P, Hellewell GP, Whyte MK (2005) Pyocyanin production by *Pseudomonas aeruginosa* induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. J Immunol 174: 3643-3649.
7. DeZiel E, Comeau Y, Villemur RA (2001) Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. J Bacteriol 183: 1195-1204.
8. Karatuna O, Yagci A (2010) Analysis of quorum sensing-dependent virulence factor production and its relationship with antimicrobial susceptibility in *Pseudomonas aeruginosa* respiratory isolates. Clin Microbiol Infect 16: 1770-1775.
9. Schaber AJ, Carty LN, McDonald AN, Graham DE, Cheluvappa R, Griswold AJ, Hamood AN (2004) Analysis of quorum sensing-deficient clinical isolates of *Pseudomonas aeruginosa*. J Med Microbiol 53: 841-853.
10. Senturk S, Ulusoy S, Bosgelmez-Tinaz G, Yagci A (2012) Quorum sensing and virulence of *Pseudomonas aeruginosa* during urinary tract infections. J Infect Dev Ctries 6: 501-507. doi:10.3855/jidc.2543.
11. Husain MF, Ahmad I, Asif M, Tahseen Q (2013) Influence of clove oil on certain quorum-sensing-regulated functions and biofilm of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. J Biosci 38: 835-844.
12. Krishnan T, Yin WF, Chan KG (2012) Inhibition of quorum sensing controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by Ayurveda spice clove (*Syzygiumaromaticum*) bud extract. Sensors (Basel) 12: 4016-4030.
13. Khan MSA, Zahin M, Hasan S, Husain FM, Ahmad I (2009) Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil. Lett Appl Microbiol 49: 354-360.
14. Clinical and Laboratory Standards Institute (2011) Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement. M100-S21. Wayne, PA: CLSI.

15. O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R (1999) Genetic approaches to study biofilms. Methods Enzymol 310: 91-109.
16. King EO, Ward YM, Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescein. J Lab Clin Med 44: 301-307.
17. Lennette EH, Spaulding WE, Trouant PJ (1974) Manual of Clinical Microbiology. 2nd Ed ASM. Washington DC: 407-422p.
18. United States Pharmacopia (2008) Microbial Limit Tests. US Pharmacopeial Convention Inc.31st edition. Rockville: 19: 2412-2745 p.
19. O'Toole AG, Kolter R (1998) Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol 30: 295-304.
20. Luber P, Bartelt E, Genschow E, Wagner J, Hahn H (2003) Comparison of broth microdilution, E Test, and agar dilution methods for antibiotic susceptibility testing of *Campylobacter jejuni* and *Campylobacter coli*. J Clin Microbiol 41: 1062-1068.
21. Strateva T, Yordanov D (2009) *Pseudomonas aeruginosa* - a phenomenon of bacterial resistance. J Med Microbiol 58: 1133-1148. doi: 10.1099/jmm.0.009142-0.
22. .Driscoll JA, Brody SL, Kollef MH (2007) The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs 67: 351-368.
23. Wang H, Tu F, Gui Z, Lu X, Chu W (2013) Antibiotic resistance profiles and quorum sensing-dependent virulence factors in clinical isolates of *Pseudomonas aeruginosa.* Indian J Microbiol 53: 163-167.
24. Le Berre R, Nguyen S, Nowak E, Kipnis E, Pierre M, Ader F, Courcol R, Guery BP, Faure K; Pyopneumagen Group (2008) Quorum-sensing activity and related virulence factor expression in clinically pathogenic isolates of *Pseudomonas aeruginosa*. Clin Microbiol Infect 14: 337-343.
25. Kumar R, Chhibber S, Harjai K (2009) Quorum sensing is necessary for the virulence of *Pseudomonas aeruginosa* during urinary tract infection. Kidney Int 76: 286-292.
26. Dénervaud V, TuQuoc P, Blanc D, Favre-Bonte S, Krishnapillai V, Reimmann C, Haas D, van Delden C (2004) Characterization of cell-to-cell signalling deficient *Pseudomonas aeruginosa* strains colonizing intubated patients. J Clin Microbiol 42: 554-562.
27. Husain FM, Ahmad I (2013) Doxycycline interferes with quorum sensing-mediated virulence factors and biofilm formation in Gram negative bacteria. World J Microbiol Biotechnol 29: 949-957.
28. Norizan SNM, Yin WF, Chan KG (2013) Caffeine as a potential quorum sensing inhibitor. Sensors (Basel) 13: 5117-5129.
29. Zhou L, Zheng H, Tang Y, Yu W, Gong Q (2013) Eugenol inhibits quorum sensing at sub-inhibitory concentrations. Biotechnol Lett 35: 631-637.
30. Koh CL, Sam CK, Yin WF, Tan LY, Krishnan T, Chong YM, Chan KG (2013) Plant-derived natural products as sources of anti-quorum sensing compounds. Sensors (Basel) 13: 6217-6228.

Corresponding author

Rania Abozahra
Faculty of Pharmacy, Damanhour University
Elgomhoreya Street, Damanhour, Egypt
Phone: + 002 01284458834
Fax: + 002 0453334596
Email: rania_abozahra@yahoo.com

Conflict of interests: No conflict of interests is declared.