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RESEARCH ARTICLE OPEN ACCESS

Impact of Quorum Sensing System on Virulence Factors Production in *Pseudomonas aeruginosa*

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Abstract

***Pseudomonas aeruginosa* is an important pathogen that is frequently associated with nosocomial infections. The goal of this work was to determine the relationship between the quorum sensing system (QS) and the production of virulence factors in *P. aeruginosa*. A number of 100 *P. aeruginosa* isolates were collected from various clinical sources from different Mansoura university hospitals in the period from April 2018 till April 2019. PCR screening of QS genes in the isolates was carried out including *lasI, lasR, rhlI* and *rhlR*. Thereafter, assay of the production of different virulence factors in the isolates was established including biofilm formation, pyocyanin production, protease production, lipase production, hemolysin production as well as swimming motility. Finally, statistical analysis of the data was performed to confirm the relationship between the QS and the production of virulence factors. Out of the 100 *P. aeruginosa* isolates, 27 clinical isolates were QS deficient. PCR analysis revealed that 8 isolates lacked *lasR* gene, 15 isolates lacked *lasR* and *rhlR* genes, 1 isolate lacked *lasR* and *lasI* genes, 2 isolates lacked *lasR*, *lasI* and *rhlR* genes and 1 isolate lacked *rhlR*, *rhlI* and *lasR* genes. There was a significant decrease observed in the production of pyocyanin, protease, lipase, hemolysin and biofilm formation as well as swimming motility in *P. aeruginosa* QS deficient isolates in comparison to non-QS deficient ones. There was a clear association between QS and virulence factors production in *P. aeruginosa*. This could open the door for novel promising targets for developing new therapeutic strategies against infections caused by this pathogen.**

Keywords: *Pseudomonas aeruginosa*, Quorum sensing, Regulation, Virulence factors

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium, well known by its characteristic features as production of pyocyanin, oxidase positivity, grape-like odour and motility.[1-3] *P. aeruginosa* is an opportunistic bacterium and it harbours a variety of virulence factors that are responsible for its danger.[4] In hospitals, *P. aeruginosa* plays a pivotal role in nosocomial infections and is considered as a mutual cause of wound infections, especially for thermal burns.[5]

Community interaction between bacteria takes place via a well-developed framework called the quorum sensing system (QS).[6,7] QS is a signalling process, which is used by different bacterial species to organize gene expression of the population according to changes in the cell density.[1,8,9] QS is responsible for bacterial social behaviours, and this cell-to-cell communication or intercellular signalling takes place in Gram-negative bacteria via small N- acylated homoserine lactone molecules called autoinducers (AIs). These AIs coordinate the common actions associated with the succession of bacterial infection, which depends on the expression of virulence factors and invasion abilities.[1,8,10,11]

P. aeruginosa QS network is structured in a multi-layered hierarchy manner composed of at least four linked signalling systems, which are Las, Rhl, PQS and IQS.[3,8,10,12] Among these systems, the two well-defined interconnected QS systems, Las and Rhl, are well defined to control virulence factors production.[1,8,10,12] Other functions have been correlated with QS systems, which are involved in cell metabolism, stress responses, etc.[8] There is clear evidence that the QS regulatory network not only reacts to changes in the bacterial population, but can also react to environmental changes.[13,14] Thus, it is well established that during the discovery and development of anti-QS therapeutics, this plasticity should be taken into account.[9]

Clinical studies have shown that QS systems in infected tissues are fully functional, especially in patients with cystic fibrosis (CF) lungs who have been chronically infected with *P. aeruginosa*.[15-19] The sputum analysis of *P. aeruginosa* infection in CF patients revealed the existence of transcripts for both *lasI* and *lasR*.[15,18] Accumulation of these transcripts was associated with cumulation of transcripts for the *lasB, lasA* and *toxA* QS-controlled genes.[15,18] In addition, the presence of AIs within the sputum of CF patients was confirmed in other studies.[20,21] Moreover, the biological activation of these AIs was proved in other reports.[15]

In numerous *P. aeruginosa* infected animal models, the importance of QS in regulating virulence has been described involving the thermally injured mouse model as well as mouse models of acute and chronic lung infections.[22-26] Mutants of *P. aeruginosa* with deletions in the QS genes were compared to their parent strains according to their virulence. These studies showed that mice infected with QS mutants and thermally injured have lower mortality rate than mice infected with the parent strain.[23,24] The spreading of the mutant strains decreased locally in the skin of thermally damaged mice and decreased systemically in the infected thermally injured mice bodies.[23,24] Using the *P. aeruginosa* acute pulmonary infection mouse model, it was demonstrated that the mortality rate and the lung damage decreased with *P. aeruginosa* strains that carry deletions within the QS genes.[22] In addition, some studies reported that flat biofilms are produced by *lasI* deficient strains in which this produced biofilm is different from the normal wild types.[27] Another report indicated that QS deficiency takes place naturally and Las QS has important role in corneal infection development.[28] Mice models that were infected by mutants deficient in the *lasI* gene exhibited virulence reduction, and by implantation of functional *lasI* gene, *lasI* mutant virulence was fully restored.[29] From this background, in this study, we were motivated to screen the presence of QS genes in *P. aeruginosa* clinical isolates collected from different sources in Mansoura university hospitals, Egypt, and to reveal the impact of QS on virulence factors production in this pathogen.

MATERIALS AND METHODS

Bacterial isolates

This work was established after the agreement of the research ethics committee of the Faculty of Pharmacy, Tanta University, Egypt (Research Ethics Committee Code: TP / RE /12-21-M-002). A total of 100 *P. aeruginosa* clinical isolates were collected from different

Table 1. Specific amplification primer sets for the tested quorum sensing genes among *P. aeruginosa* isolates

Gene name	Type	Sequence	Amplicon size (bp)	Annealing temperature	References
lasR	F	ATGGCCTTGGTTGACGGTT	725	50°C	32, 33
	R	GCAAGATCAGAGAGTAATAAGACCCA			
lasI	F	ATGATCGTACAAATTGGTCGGC	605	50°C	32, 33
	R	GTCATGAAACCGCCAGTCG			
rhlR	F	CAATGAGGAATGACGGAGGC	730	50°C	32, 33
	R	GCTTCAGATGAGGCCCAGC			
rhlI	F	CTTGGTCATGATCGAATTGCTC	625	50°C	32, 33
	R	ACGGCTGACGACCTCACAC			

F: Forward; R: Reverse.

clinical sources including 35 from urine (U), 17 from wounds (W), 15 from burns (B), 14 from sputum (S), 10 from ear (E) and 9 from eye (EY). These isolates were collected from patients in different Mansoura hospitals under medical attention with strict aseptic precautions in the period from April 2018 till April 2019. According to the standard microbiological techniques,[30] the isolates were biochemically identified as *P. aeruginosa*. Moreover, identity of the isolates was further confirmed by PCR amplification of their 16s *rRNA* as previously described.[31] All cultures were grown at 37°C in Luria Bertani medium (LB broth; tryptone 1% w/v, yeast extract 0.5% w/v, and NaCl 1.0% w/v), otherwise specified, and stored in 80% glycerol/LB broth at -80°C.

Polymerase chain reaction

Chromosomal DNA was extracted from *P. aeruginosa* isolates by using QIA amp® DNA miniprep kit (Qiagen, Germany). All *P. aeruginosa* clinical isolates were screened for the presence of QS genes by PCR using the primers (*lasR, lasI, rhlR* and *rhlI*) shown in (Table 1) as previously reported.[32,33] The volume of PCR reactions was 25 µL, and each reaction composed of 12.5 µL Dream Taq PCR master mix 2x (Fermentas, USA), 1 µL forward primer (10 µM), 1 µL of reverse primer (10 µM) and 3 µL of template DNA, then the reaction was adjusted to total volume of 25 µL with nuclease free water. Negative control tubes were also performed without template DNA. The cycling conditions performed were; initial denaturing at 95°C for 5 min, then 35 cycles of (denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 2 min), and final extension at 72°C for 10 min. Visualization of the amplified genes by electrophoresis was done using 1% agarose gel stained with ethidium bromide (MP biomedicals, France), and compared with a 100 base pair plus DNA ladder (Thermo Scientific, UK). The presence of the tested gene was indicated by the appearance of a single sharp band with the specific amplicon size for each gene (Table 1).

Assay of *P. aeruginosa* virulence factors

Pyocyanin assay

Pyocyanin was extracted as previously described.[34] King's A liquid medium (peptone 2% w/v, K_2SO_4 1.0% w/v, and $MgCl_2$ 0.14% w/v) was used to cultivate the isolates for 48 hours with shaking at 150 rpm. Cultures were then centrifuged and pyocyanin was recovered from the supernatant using 3 mL chloroform. Thereafter, 1 mL of 0.2 N HCl was added to the pyocyanin (bottom layer) after it was transferred to a new clean tube. The extracted acidic form of pyocyanin, which has a pink colour, was spectrophotometrically measured at OD_{520} nm. The optical density at 520 nm was multiplied by 17.072 to get the microgram amounts of pyocyanin.[35] All *P. aeruginosa* isolates were compared with the isolate with the highest amount of pyocyanin in percentage. In this experiment, King's A liquid medium was used as a negative control.

Hemolysin assay

P. aeruginosa clinical isolates were cultured overnight in LB and incubated at 37°C with shaking at 150 rpm for an appropriate time. Thereafter, cultures were centrifuged at 10,000 rpm for 10 min at 4°C. The obtained supernatants were filtered through 0.2 µM Millipore filter and subsequently used in the assay of hemolysin activity. The assay was performed as previously

described.[36] Prepared free supernatant of bacterial cultures (volume 600 µL) was mixed with equal volume of saline suspension of erythrocytes 2%, and incubated at 37°C for 2 hours. Then, centrifugation of the reaction mixtures was established at 10,000 rpm for 8 min. at 4°C, and the optical density at 540 nm was used to determine the degree of haemoglobin release. Control studies for spontaneous (negative control) and complete (positive control) lysis were conducted without hemolysin and with 0.2 % sodium dodecyl sulphate, respectively. The percentage (%) of cells lysed = [(X-B)/ (T-B)] × 100, where B (baseline) is a negative control and T is a positive control corresponding to the total lysis.

Protease assay

Assay of total proteases was established using skimmed milk assay as previously illustrated.[37] Volume of 200 µL of *P. aeruginosa* supernatant (prepared as described above in hemolysin assay) was mixed with 1 mL 1.25% skimmed milk and at 37°C incubation takes place for 15 min, and then, OD_{600} was measured. Comparison of OD_{600} of all *P. aeruginosa* isolates with OD_{600} of skimmed milk in percentage was established to detect the protease activity of each isolate. Protease activity = (OD_{600} of skimmed milk - OD_{600} of the sample) X 100. In this experiment, LB was used as a negative control.

Lipase assay

Lipase assay was established as formerly outlined.[38] Culture supernatants of *P. aeruginosa* isolates were prepared as described above in hemolysin assay. A stock solution of p-nitrophenyl palmitate (p-NPP) (Sigma, USA) was prepared in HPLC grade of isopropanol. The reaction mixture contained 75 µL of p-NPP stock solution, 5 µL bacterial supernatant, and completed to a final volume of 3 mL with 0.1 M Tris buffer (pH 8.5). To stop the reaction, the reaction mixture was incubated at 37°C for 10 min before being refrigerated at -20°C for 8 min. At OD_{410} nm, the optical density of emitted p-nitrophenol was measured. All *P. aeruginosa* isolates were compared with the isolate with the highest lipase activity in percentage. In this experiment, LB was used as a negative control.

Swimming motility assay

Swarming motility assay was performed as previously depicted.[39] Surface inoculation was used to measure the swimming ability of *P. aeruginosa* by using swimming agar plates (tryptone 1%, sodium chloride 0.5%, and agar 0.3%). The prepared plates were centrally stabbed with 5 µL of diluted overnight culture of *P. aeruginosa* isolates in tryptone broth. After 24 hours incubation period at 37°C, the swimming zones were measured. All *P. aeruginosa* isolates were compared with the isolate with the highest swimming activity in percentage. In this experiment, LB was used as a negative control.

Quantitative detection of biofilm using microtiter plate assay

By using the method described previously,[40,41] biofilm production was assayed. *P. aeruginosa* clinical isolates were cultured overnight in tryptic soy broth (TSB) (Oxoid, Thermo Fisher, UK) and diluted to produce a cell density of approximately 1×10^6 CFU/mL. Using 96-well flat-bottomed polystyrene microtiter plate, 100 µL aliquots of each bacterial suspension was inoculated into the wells of at least in triplicate. Then, plates were incubated at 37°C for 24 hours. The contents of each well were aspirated to eliminate all non-adherent cells. Each well was rinsed three times with phosphate buffer saline (PBS, pH 7.4). The remaining adherent bacteria were fixed with 100% methanol per well. The bacterial cells adhered to the well walls were stained with 1% crystal violet for 20 min. Thereafter, the dye bound to the adhering cells was resolubilized using 33% (v/v) glacial acetic acid. The absorbance of solubilized stain was measured at OD_{492} nm using Biotek spectrofluorimeter (Biotek, USA). All *P. aeruginosa* isolates were compared with the isolate with the highest biofilm formation in percentage. In this experiment, LB was used as a negative control.

Statistical analysis

Significance of difference was assessed in the results by repeating the experiments three independent times and comparing the data using Student's t-test. P values lower than 0.05 were regarded significantly different.

RESULTS

Detecting the presence of QS-genes in *P. aeruginosa* isolates

In this study there were 27 isolates out of the 100 *P. aeruginosa* clinical isolates were QS deficient isolates (Table 2). PCR analysis revealed

Table 2. Results of tested quorum sensing genes among *P. aeruginosa* isolates and their impact on different phenotypes

Isolate code	Isolate source	*lasR*	*lasI*	*rhlR*	*rhlI*	% of pyocyanin production to (B59)	% of hemolysin production	% of protease production	% of lipase production to (E90)	% of swimming motility to (W43)	% of biofilm formation to (E88)
U1	Urine	+	+	+	+	20.88	70.12	23	52.24	48.125	67.5
U2	Urine	+	+	+	+	20.34	73.57	30	59.45	60.625	63.48
U3	Urine	+	+	+	+	20	62.29	35	51.03	46.25	66.66
U4	Urine	+	+	+	+	18.9	79.57	26	68.19	44.6875	30.44
U5	Urine	+	+	+	+	25.97	83.13	43.3	76.93	38.125	46.24
U6	Urine	+	+	+	+	26.04	64.83	41.6	57.37	47.5	60.73
U7	Urine	+	+	+	+	24.63	78.048	38.33	86	43.75	81.37
U8	Urine	+	+	+	+	20.2	89.63	30.3	58.14	48.125	33.69
S9	Sputum	+	+	+	+	23.6	59.75	45.6	73.77	43.125	64.61
U10	Urine	+	+	+	+	19	67	21.3	52.67	48.125	87.81
U11	Urine	+	+	+	+	40.18	91.56	44.6	78.68	56.875	80.90
U12	Urine	+	+	+	+	20.53	96.44	57	49	48.125	80.31
U13	Urine	+	+	+	+	12.76	61.58	27	85.57	48.75	39.37
U14	Urine	+	+	+	+	13.77	78.45	31	69.94	46.25	28
S15	Sputum	+	+	+	+	15.6	73.67	24.3	58.57	38.125	82.87
EY16	Eye	+	+	+	+	32.11	66.56	46.6	78.14	65	50.75
EY17	Eye	+	+	+	+	17.35	62.093	36.6	54.42	59.375	35
EY18	Eye	+	+	+	+	25.2	62.60	32.6	76	46.875	40.57
EY19	Eye	+	+	+	+	14.16	97.45	53.3	54.64	40.625	30.99
EY20	Eye	+	+	+	+	12	93.90	44	62.73	46.25	32.45
EY21	Eye	+	+	+	+	69.8	82.11	35	55.73	46.25	33.40
EY22	Eye	+	+	+	+	29.7	71.95	33	72.67	71.25	43
U23	Urine	+	+	+	+	14.9	62.70	23.6	63.49	36.875	39.37
U24	Urine	+	+	+	+	18.63	58.73	39.6	53.66	57.5	48.84
U25	Urine	+	+	+	+	50.53	100	71	51.80	45.625	94.47
U26	Urine	+	+	+	+	10.93	83.63	35.67	76.93	70	66.55
U27	Urine	+	+	+	+	25.30	75.20	43	93.87	45.625	49.87
U28	Urine	+	+	+	+	60.15	64.12	74.6	84	80	41.74
U29	Urine	+	+	+	+	29.96	87.70	41.6	95.3	40	99.89
S30	Sputum	+	+	+	+	49.88	94.71	61	89	90.625	81.22
U31	Urine	+	+	+	+	40.70	99.49	35.3	93.22	71.25	78.9

Table 2. Cont...

Isolate code	Isolate source	*lasR*	*lasI*	*rhlR*	*rhlI*	% of pyocyanin production to (B59)	% of hemolysin production	% of protease production	% of lipase production to (E90)	% of swimming motility to (W43)	% of biofilm formation to (E88)
U32	Urine	+	+	+	+	32.58	100	72.3	62.29	48.75	72.37
S33	Sputum	+	+	+	+	14.14	81.70	30.3	90.81	41.25	28.39
U34	Urine	+	+	+	+	31.39	75	32	54.20	43.125	32.12
U35	Urine	-	+	+	+	3.5	44.61	14	14.97	34.375	23.38
U36	Urine	+	+	+	+	22.26	82.62	34.3	49	77.5	33.18
U37	Urine	+	+	+	+	34.09	67.68	34.3	86.12	45.625	80.20
U38	Urine	+	+	+	+	9.89	71.74	51.6	72.67	45.625	32.19
U39	Urine	+	+	+	+	55.68	86.38	23.6	62.62	93.125	30
U40	Urine	-	+	+	+	3.85	44.7	13.6	15.62	29.375	22.64
W41	wounds	+	+	+	+	13.9	56.40	45.6	52.45	56.875	31.75
W42	wounds	+	+	+	+	10.86	61.788	28	57.15	84.375	36.99
W43	wounds	+	+	+	+	6.813	62.70	29.6	49.61	100	95.31
W44	wounds	+	+	+	+	16.21	72.76	22.3	49	93.125	43.61
W45	wounds	-	+	+	+	3.92	39.93	13.3	15.62	32.5	23.81
W46	wounds	+	+	+	+	24.43	100	23	50.81	55	32.12
W47	wounds	-	+	+	+	4.2	43.5	12.6	13.33	31.25	23.38
W48	wounds	-	+	+	+	3.6	39.73	12	14.97	30.625	24.44
W49	wounds	-	+	+	+	2.041	39.6	13.3	12.7	33.125	23.45
W50	wounds	+	+	+	+	5.57	86.48	33.3	52.89	40.625	58.28
W51	wounds	+	+	+	+	64.99	65.04	24.6	72.67	53.75	32.12
W52	wounds	+	+	+	+	22.51	91.66	33.3	66.33	61.25	35.12
W53	wounds	+	+	+	+	10.44	84.75	39.3	80.21	40	39.59
W54	wounds	+	+	+	+	40.21	59.55	29	58.79	45	30.58
W55	wounds	+	+	+	+	17.57	67.88	38.6	52	49.375	38.74
B56	Burns	+	+	+	+	84.2	100	46.3	65.35	87.5	69.48
B57	Burns	+	+	+	+	12.21	64.43	25	77	70	32.38
B58	Burns	+	+	+	+	31.81	73.78	51.3	80	56.25	60.88
B59	Burns	+	+	+	+	100	100	32.6	91.47	58.75	77.71
B60	Burns	+	+	+	+	20.39	55.79	40.6	47.54	40.625	31.72
B61	Burns	+	+	+	+	12.811	60.87	46.3	55.3	45.625	35
B62	Burns	+	+	+	+	12.04	63.414	28	74.53	40.625	52.50

Table 2. Cont...

Isolate code	Isolate source	*lasR*	*lasI*	*rhlR*	*rhlI*	% of pyocyanin production to (B59)	% of hemolysin production	% of protease production	% of lipase production to (E90)	% of swimming motility to (W43)	% of biofilm formation to (E88)
B63	Burns	+	+	+	+	26.01	74.69	29.6	74.53	54.375	70.25
B64	Burns	+	+	+	+	75.31	75.30	21	55.95	38.75	41.93
B65	Burns	-	-	+	+	0.57	31.7	4.6	8.41	20	15.18
S66	Sputum	+	+	+	+	34.06	63.92	29	55.62	58.125	65.20
S67	Sputum	+	+	+	+	17.72	86.38	27.3	54.31	50	33.15
S68	Sputum	+	+	+	+	9.8	71.036	26.6	68.08	93.125	32.30
S69	Sputum	+	+	+	+	28.23	65.85	31	49.18	80	68.31
S70	Sputum	+	+	+	+	64.60	83.84	24.6	54.20	89.375	63.59
S71	Sputum	+	+	+	+	14.735	56.30	30.3	69.39	42.5	63.11
S72	Sputum	+	+	+	+	71.21	58.73	21.3	44.37	46.25	94.25
B73	Burns	-	+	-	+	1.6	35	11.6	10.81	22.5	18.47
W74	Wounds	-	+	-	+	1.03	37	10.3	12	26.25	19
B75	Burns	-	+	-	+	1.75	35.6	12	8.9	25.625	17
U76	Urine	-	+	-	+	1.75	36.1	9	11.91	28.125	21.62
S77	Sputum	-	+	-	+	1.03	37	12.3	13.22	28.125	21.51
U78	Urine	-	+	-	+	1.85	33.8	9.6	12.02	26.25	17.34
S79	Sputum	-	+	-	+	1.48	36.99	10.3	11.25	26.875	17.52
U80	Urine	-	+	-	+	1.23	35.1	7.3	12.67	28.125	16.79
EY81	Eye	-	+	-	+	2.07	33.84	11	12.56	28.125	20.23
S82	Sputum	-	+	-	+	2.17	37.5	9.6	13.33	26.875	17.70
EY83	Eye	-	+	-	+	1.87	34.65	10	10.2	27.5	18.25
B84	Burns	-	+	-	-	0.28	30.5	4	6.77	20.625	19.1
U85	Urine	-	-	-	+	0.27	29.1	1.6	6.44	18.75	14.96
U86	Urine	-	-	-	+	0.098	26.72	1.3	6.1	18.125	14.12
E87	Ear	+	+	+	+	8.56	93.39	28.3	60.43	40.625	85.72
E88	Ear	+	+	+	+	33.25	65.54	23	81.20	51.25	100
E89	Ear	+	+	+	+	43.74	77.13	26.3	56.83	40	65.78
E90	Ear	+	+	+	+	88.86	100	33.6	100	58.75	66.70
E91	Ear	+	+	+	+	15.84	72.25	24.3	74.97	53.75	95.35
E92	Ear	+	+	+	+	18.78	85.67	49.3	87.21	66.875	80.75
E93	Ear	+	+	+	+	16.29	88.61	25	53.11	49.375	64.58

Table 2. Cont...

Isolate code	Isolate source	*lasR*	*lasI*	*rhlR*	*rhlI*	% of pyocyanin production to (B59)	% of hemolysin production	% of protease production	% of lipase production to (E90)	% of swimming motility to (W43)	% of biofilm formation to (E88)
E94	Ear	+	+	+	+	41.15	81.80	28.6	71.36	79.375	72.63
B95	Burns	-	+	+	+	3.85	40.4	12.3	14.97	33.125	22.17
W96	Wounds	-	+	-	+	2.44	38	10.3	11.7	28.75	18
E97	Ear	-	+	+	+	4.8	41.56	12	16.61	31.25	25.17
U98	Urine	-	+	-	+	2.36	34.6	11.3	12.15	28.75	19.46
E99	Ear	-	+	-	+	1.65	36.58	11.6	11.03	28.75	20
B100	Burns	-	+	-	+	2.024	38.41	12.6	11.91	26.875	21.84

B: burns; EY: eye; W: wound; E: Ear swabs; S: sputum; U: urine.

that 8 isolates lacked *lasR* gene, 15 isolates lacked *lasR* and *rhlR* genes, 1 isolate lacked *lasR* and *lasI* genes, 2 isolates lacked *lasR*, *lasI* and *rhlR* genes and 1 isolate lacked *rhlR, rhlI* and *lasR* genes. Among the QS deficient *P. aeruginosa* isolates, 8 were collected from urine, 6 isolates from wounds, 6 isolates from burns, 3 isolates from sputum, 2 isolates from ear and 2 isolates from eye.

Assay of *P. aeruginosa* virulence factors

Pyocyanin assay

As shown in (Table 2), percentages of pyocyanin produced by *P. aeruginosa* QS deficient isolates according to the sample (B59), which produced the highest amount of pyocyanin, were in between 4.8% and 0.098%. Sample W50 had the lowest pyocyanin production percentage of 5.57% in all non-QS deficient isolates. The amount of pyocyanin production that was produced by *P. aeruginosa* QS deficient isolates was less than the isolates that possess all QS genes (Fig. 1A).

Hemolysin assay

As illustrated in (Table 2), a number of 73 non-QS deficient isolates produced more than 50% hemolysis including 6 isolates that produced complete (100%) hemolysis. On the other hand, the 27 QS deficient isolates produced less than 50% hemolysis (Fig. 1B). This gives indication that QS deficient isolates produce hemolysin less than non-QS deficient isolates.

Protease assay

As depicted in (Table 2), the activity of QS deficient isolates was in between 14% and 1.3%, while isolate B64 had the lowest protease activity (21%) in all non-QS deficient isolates. Skimmed milk assay indicates that QS deficient isolates have lower protease activity in comparison to other non-QS deficient isolates (Fig. 1C).

Lipase assay

As demonstrated in (Table 2), percentage of lipase activity produced by *P. aeruginosa* isolates according to the sample (E90), which had the highest lipase activity, was in the range between 16.61% and 6.1%. Sample S72 had the lowest lipase activity percentage (44.37%) in all non-QS deficient isolates. The amount of lipase produced by QS deficient isolates is less in comparison to the other isolates, which possess all QS genes that were examined in this study (Fig. 1D).

Swimming motility assay

As depicted in (Table 2), percentages

of swimming zone diameters produced by *P. aeruginosa* QS deficient isolates according to the sample (W43), which had the highest swimming zone diameter, were in the range between 34.375% and 18.125%. Sample U23 had the lowest swimming zone diameter percentage (36.875%) in all non-QS deficient isolates in this study (Fig. 1E). This gives an indication that the swimming motility of QS deficient isolates is less than the non-QS deficient ones.

Quantitative assay of biofilm formation

As shown in (Table 2), 24 QS deficient isolates were characterized by weak biofilm formation. Moreover, three isolates that lacked Las system (*lasI* and *lasR* genes were absent) were non-adherent. Percentage of biofilm formation produced by QS deficient isolates according to sample (E88), which had the highest capability of biofilm formation, was in the range between 25.17% and 14.12%. Sample U14 was weakly adherent and had the lowest biofilm formation percentage (28%) in all non-QS deficient isolates. The amount of biofilm formation, which produced by QS deficient isolates is less than the other of non-QS deficient isolates (Fig. 1F).

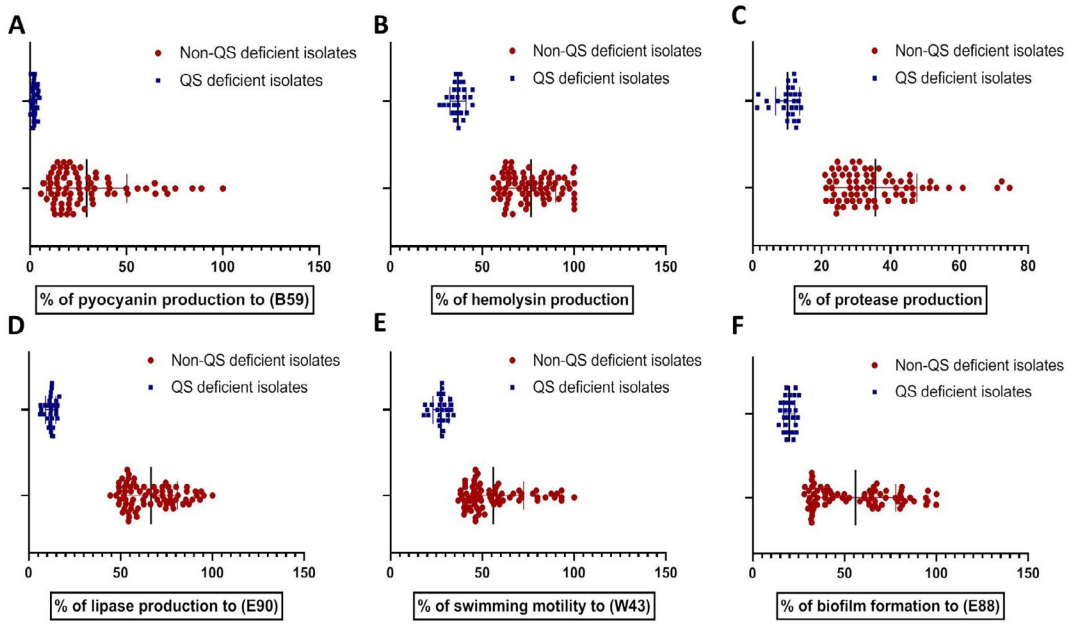

Fig. 1. Virulence factors production and biofilm formation in *P. aeruginosa* non-QS deficient and QS deficient isolates. (A) Pyocyanin production. (B) Hemolysin production. (C) Protease production. (D) Lipase production. (E) Swimming motility. (F) Biofilm formation.

DISCUSSION

P. aeruginosa virulence is multifactorial and has been accredited to cell-associated factors like alginate, lipopolysaccharide, flagellum, pilus and non-pilus adhesins as well as exoenzymes or secretory virulence factors like protease, elastase, pyocyanin, exotoxin A, exoenzyme S, hemolysins (rhamnolipids and phospholipase) and siderophores.[42] Pathogenesis of *P. aeruginosa* takes place by these factors inducing infections like respiratory tract infections, burn wound infections and keratitis.[4] *P. aeruginosa* produces a number of extracellular components that once colonised can cause significant tissue damage, bloodstream invasion and spread.[5] In our study we investigated the relationship between *P. aeruginosa* QS system and its virulence factors, which have an important role in the pathogenicity of this pathogen.

Pyocyanin synthesis by *P. aeruginosa* reduces the acute inflammatory response by speeding up neutrophil apoptosis and lowering local inflammation, which is beneficial for bacterial survival.[3] In this study, the production of pyocyanin in QS deficient isolates was less than

non-QS deficient ones. The Las system regulates the production of LasA, whereas the Rhl system regulates the production of pyocyanin. Pyocyanin production should, however, be regulated by the Las system due to the hierarchical nature of the QS systems and the relevance of the Las system in the hierarchy.[29,43] Las and Rhl are two interconnected QS systems that control production of pyocyanin.[44,45] A link was discovered between *P. aeruginosa* QS deficient isolates that were gained from sputum or endotracheal tubes and some virulence factors such as elastase and pyocyanin.[46] Moreover, there was another study reported that *P. aeruginosa* QS deficient strains and strains that produce no or low amount of AIs as N-(3-oxododecanoyl) homoserine lactone and N-butyryl homoserine lactone, produce lower amount of pyocyanin.[33]

Elastase and protease, which are produced by *P. aeruginosa*, are extracellular enzymes that aid in colonization of the host.[3] Las and Rhl QS systems control the production of alkaline protease and LasA protease in *P. aeruginosa*.[29,43,44] It was observed that major protease and elastase were reduced by deleting the *lasR* open reading frame of some strains.[47] In other studies, *P. aeruginosa* QS deficient strains were characterized by low protease activity.[28,33] Our study has similar outcome, where, *P. aeruginosa* QS deficient isolates produce lower levels of protease in comparison to the non-QS deficient isolates.

Swimming motility takes place by flagella and type IV pili, where bacteria can incorporate itself in the epithelial cells, and initiate the first step of its colonization on the epithelium.[48] Las and Rhl systems in *P. aeruginosa* take important role in controlling the swimming motility.[10,44] Therefore, in our study, the swimming motility of *P. aeruginosa* QS deficient isolates were lower than that non-QS deficient ones. It is well reported that pyocyanin synthesis, protease production and swimming motility in *P. aeruginosa* decreased by using febuxostat,[12] phenylalanine arginyl b-naphthylamide,[11] sodium ascorbate[49] and aspirin,[50] which all act as QS inhibitors.

Biofilms are formed by adherent cells on cellular or inert substrata and are tangled in 60% of all infections that are characterized by symptoms of moderate severity, chronic development and antimicrobial resistance.[51] Previous reports depicted that biofilm formation is controlled by QS.[3,52] *P. aeruginosa* QS is used to initiate growth and produce biofilms.[27] A decrease in biofilm formation takes place by sodium ascorbate[49], aspirin[50] and febuxostat,[12] which act as QS inhibitors. Another study indicated that the amount of biofilm formation that was produced by *P. aeruginosa* QS deficient strains was lower than that of the non-QS deficient strains,[33] and this is in accordance with the findings of our study.

Lipase enzyme plays important role in the colonization of *P. aeruginosa* on human respiratory tract and skin.[1,3] While hemolysin, which is a hydrolytic enzyme enhances the spread of bacteria inside the tissues of the host and resistance to the host defence.[12] Lipase production is controlled by Rhl system.[44] Las and Rhl systems plays important role in lipase and hemolysin production.[10] Hemolysins are inhibited by some QS inhibitors as sodium ascorbate,[49] febuxostat[12] and aspirin.[50] According to our study, lipases and hemolysin levels in *P. aeruginosa* QS deficient isolates were lower than that non-QS deficient ones.

CONCLUSION

The QS system in *P. aeruginosa* is important for controlling the production of virulence factors and connected to numerous pathogenic phenotypes including bacterial capacity to attach to tissues and build biofilms. Discovery and development of anti-QS therapeutics are very important to stop or decrease the severity of *P. aeruginosa* virulence and to overcome the problem of drug resistance; especially *P. aeruginosa* is one of the most abundant causes of nosocomial infections in developing countries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All datasets analysed in the study are included in the manuscript and presented as tables and figures.

ETHICS STATEMENT

This study was approved by the Research Ethics Committee of Tanta University, Faculty of Pharmacy, Egypt. Protocol approval code TP/RE /12-21-M-002.

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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