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From Vasodilator to Virulence Disarmer: Glyceryl Trinitrate Modulates Quorum Sensing in *Pseudomonas Aeruginosa* Biofilms

Almujtaba Maytham Fadhil Tuama¹, Harith Jabbar Fahad Al-Mathkhury^{*2}

^{1,2}Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq, 10071

Email ID: almujtaba.maitham1702a@sc.uobaghdad.edu.iq

Email ID: harith.fahad@sc.uobaghdad.edu.iq

<https://orcid.org/0000-0002-5414-9834>

*Corresponding author: Harith Jabbar Fahad Al-Mathkhury

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq, 10071

Email ID: harith.fahad@sc.uobaghdad.edu.iq

ABSTRACT: Antimicrobial resistance, biofilm formation and quorum sensing (QS) are all involved in the persistence and therapeutic challenge of *Pseudomonas aeruginosa*, an opportunistic pathogen. This study aimed to determine the effect of glyceryl trinitrate (GTN) on pre-established biofilms and QS regulation in clinical isolates of *P. aeruginosa*. Conventional and automated methods were used to identify clinical isolates and their antimicrobial susceptibility was assessed as well as their ability to form biofilms. The biomass of the biofilms was measured by OD₆₃₀ of crystal violet staining and the antibacterial activity of GTN was measured by resazurin based broth micro dilution assay. The influence of GTN on existing biofilms was phenotypically tested and then analysed in detail by field-emission scanning electron microscopy (FE-SEM). *lasR*, *rhlR* and *mvfR* expression was studied by quantitative real-time PCR. The 83 clinical isolates differed substantially in their ability to form a biofilm ($F(82,166) = 15.01$, $p < 0.0001$) and there was only a weak positive non-significant correlation between biofilm biomass and the number of resisted antibiotics (Spearman's $\rho = 0.222$, $p = 0.0538$). GTN could not completely inhibit growth even at the highest concentration of 2 mg/mL. However, GTN decreased the amount of established biofilm biomass in 20/28 isolates (71.4%), of which 6 isolates showed significant decrease in biomass, while the remaining eight isolates showed an increase in the biomass. The corresponding isolate-dependent changes in the architecture of the biofilm were similarly found using FE-SEM. QS transcription was also a mixture with downregulation of *mvfR* and *rhlR* in 5 out of 6 isolates, and *lasR* in varying isolates. Interestingly, all three QS regulators were found to be up-regulated at the same time in PA1 with biofilm biomass being increased. These results show that GTN can affect the existing *P. aeruginosa* biofilms and the QS-related regulatory responses without the ability to detectably inhibit growth, although its impact is highly isolate dependent. GTN should be further investigated as an anti-virulence therapeutic agent, not as a conventional antibiotic.

INTRODUCTION

Pseudomonas aeruginosa is a potent opportunistic pathogen that causes recalcitrant and severe infections, mainly as a result of

its ability to form strong biofilms (Elbehiry et al., 2026, Shariati et al., 2024). Biofilm-encased bacteria are embedded in their own extracellular polymeric substance (EPS) matrix and display a coordinated phenotypic behaviour that helps them to persist in tissues, to exhibit increased virulence and to produce resistance to traditional antimicrobial therapy (Chen et al., 2024, Prieto-Rodriguez et al., 2026). Cell-to-cell communication through a density-dependent regulation of gene expression is known as quorum sensing (QS), and is at the core of orchestrating these collective behaviours (Nwobodo et al., 2026). The functions of major virulence determinants and the maturation of biofilms are regulated by a complex hierarchical network of three principals, interconnected signalling circuits: Las, Rhl, and Pqs systems in *P. aeruginosa* (Kapadia et al., 2022, Vieira et al., 2022).

LasR is the transcriptional regulator at the top of this hierarchy and has an effect on downstream signalling via the Rhl (RhIR) and Pqs (MvIR/PqsR) circuits (Bernabè et al., 2025). The expression of critical elastases, rhamnolipids and biofilm-maintenance traits are under control of rhIR, while the expression of the pqs cascade is controlled by mvIR in the presence of 2-alkyl-4-quinolones (Chadha et al., 2024). These regulatory nodes are well interconnected, meaning that the targeted disruption of QS signalling will attenuate multiple pathogenic characteristics without any direct selection pressure on the bacteria's viability, therefore decreasing the chance of selection for the emergence of traditional resistance pathway (Sudan et al., 2026, Sanchez-Mateos and Flores-Felix, 2026). As such, quorum sensing inhibition (QSI) is a promising anti-virulence strategy for the inhibition of *P. aeruginosa* (Sanchez-Mateos and Flores-Felix, 2026).

In order to speed up clinical translation of QSI strategies, drug repurposing has become a major approach by taking advantage of compounds that are already known to be safe and have established pharmacokinetic profiles (Al Khzem and Wali, 2025). Glyceryl trinitrate (GTN), a cardiovascular vasodilator and nitric oxide (NO) donor that is widely used, has come into focus as a novel anti-virulence candidate (Nazeih et al., 2023). Low concentrations of NO and NO-donating compounds have been found to affect bacterial signalling, the dispersion of bacterial biofilms, and the activity of some bacterial phenotypes regulated by QS, such as pyocyanin, total protease, and early biofilm formation in *P. aeruginosa* (Cai et al., 2021). These initial results indicate that GTN acts on the lasR- and rhIR-mediated pathways, either directly or indirectly, to inhibit virulence.

While these promising phenotypic findings, however, show that the *P. aeruginosa* QS network generates a response to GTN, the exact transcriptomic and molecular response to GTN under structured biofilm communities still remains incompletely described. Previous research has focused on the phenotypes, secreted virulence factors or planktonic growth models. The transcript levels of central nodes (*lasR*, *rhIR* and *mvIR*) in planktonic culture were preliminary noted to be down-regulated after exposure to GTN (Nazeih et al., 2023), however, the transcriptional responses of these central nodes specifically in mature biofilm architectures are less well understood. This is key because the physiological state of the cells in a biofilm is vastly different from the planktonic cells, including very well-established metabolic gradients and different regulatory scenarios that may alter the susceptibility to anti-virulence interventions (Booth et al., 2011).

Thus, the present study was aimed to evaluate the potential of GTN as a reused anti QS agent on the biofilm-associated *P. aeruginosa* and focus on its effect on the expression of the central QS regulatory genes *lasR*, *rhIR* and *mvIR*. This study combines phenotypic anti-biofilm assessments with quantitative transcriptomic analysis of these key nodes to uncover the molecular mechanism that prevents the growth of quorum sensing, but not degrades it, and lays the foundation for its potential clinical use in the control of problematic biofilm infections.

MATERIALS AND METHODS

Isolation and identification of *Pseudomonas aeruginosa*

During the period from November 2025 to January 2026 150 clinical samples were taken from patients referred to Medical City Hospitals, Baghdad, Iraq. Specimens included wound swabs, burn swabs, midstream urine, and sputum. The isolates obtained were initially identified by their colony morphology, pigmentation, odour, haemolysis, and appearance under the microscope after Gram staining. Presumptive *P. aeruginosa* isolates were also confirmed with conventional biochemical tests such as growth on cetrimide agar and oxidase test (Tille, 2025). Final identification was done by the VITEK® 2 Compact system as usual microbiological procedures.

Antimicrobial susceptibility test

The Clinical and Laboratory Standards Institute's recommendations were followed for conducting the antimicrobial susceptibility testing (AST) (Clinical and Laboratory Standards Institute (CLSI), 2026). A standardised discs diffusion method was used for AST, Kirby-Bauer method. UPEC isolates were grown on Mueller Hinton agar to make a uniform bacterial lawn and then of

cefepime, ceftazidime, ciprofloxacin, imipenem, and tobramycin impregnated disc were placed on the surface of the agar. Plates were incubated 16-18 hours at 35-37°C and the diameter of each zone of inhibition was measured. Susceptibility was defined according to the CLSI breakpoints, with isolates either being susceptible or intermediate or resistant. The resistant isolates were subjected to molecular and phenotypic characterization to identify those that were multi-drug resistant (MDR) (resistant to ≥ 3 classes of antibiotics). Because of this formula a/b (a = number of antibiotics the isolate was resistant to, b = number of antibiotics to which the isolate was exposed) the multiple antibiotic resistance (MAR) index was estimated for all isolates. Any bacterial isolate developed an MAR index ≥ 0.2 would have originated from a high-risk source of contamination where multiple antibiotics are used (Duah et al., 2026).

Determination of GTN minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of GTN was investigated using a resazurin-based broth microdilution assay (Clinical and Laboratory Standards Institute (CLSI), 2026). Two-fold serial dilutions of GTN were made in Mueller–Hinton broth to a final concentration of 0.003–2 mg/mL in sterile 96-well microtiter plates. Bacterial suspensions were prepared using fresh cultures and adjusted to an approximately 0.5 McFarland standard ($\approx 1.5 \times 10^8$ CFU/mL) and then diluted appropriately to give a final inoculum of approximately 5×10^5 CFU/mL/well. For each plate there was an untreated growth control with the inoculum of bacteria but without GTN and a sterility control with uninoculated broth. A vehicle control, if available, was also provided. After 24 h of incubation at 37°C under aerobic conditions, 0.01% resazurin solution was added to all of the wells (30 μ L) and incubated for another 4 h at 37°C. Viability of bacteria was determined by reduction of a blue resazurin to a pink resorufin, which is a sign of metabolically active bacterial cells (Calvo et al., 2026). The MIC value was determined as the lowest concentration of GTN which did not record any metabolic activity when compared to the untreated growth control. The assays were carried out in independent biological replicates, and replicate wells for each concentration.

Biofilm assays

Determination of biofilm forming capacity

A modified crystal violet staining protocol (Ghafil et al., 2024) was used for the assessment of biofilm formation. Brain heart infusion broth cultures ($OD_{600} = 1$) containing 1% glucose. Aliquots (200 μ L) were added to sterile 96-well polystyrene microtiter plates and were then cultured aerobically for 24 hours at 37°C. After the incubation, the supernatant was removed and gently washed twice with sterile distilled water. The adherent cells were fixed by methanol and stained with 0.5% crystal violet which was removed by washing. Crystall violet was air dried and then dissolved in 33% glacial acetic acid. The biofilm growth was then determined by measuring the optical density (OD) at 630 nm thereafter. A negative control value of mean $OD_{nc} + 3 \times SD$ was used as the cut-off value (COV). The isolates were categorized based on their OD_{630} value as follows: non-biofilm-producing ($OD < COV$), weak biofilm-producing ($COV \leq OD < 2 \times COV$), moderate biofilm-producing ($2 \times COV \leq OD < 4 \times COV$), and strong biofilm-producing ($OD \geq 4 \times COV$).

In vitro biofilm inhibition test

Isolates with high biofilm-forming capacity and MDR phenotypes were initially selected for GTN testing. Eight isolates susceptible to all tested antibiotics were subsequently included for comparative evaluation. A modified crystal violet staining protocol (Bonacorsi et al., 2024) was used for the assessment of biofilm formation. Brain heart infusion broth cultures ($OD_{600} = 1$) containing 1% glucose. Aliquots (200 μ L) were added to sterile 96-well polystyrene microtiter plates and were then cultured aerobically for 24 hours at 37°C. After the incubation, the supernatant was removed and replaced with 200 μ L of fresh medium with or without GTN (2 mg/L). This was then incubated for another 24 h at 37°C and subsequently gently washed twice with sterile distilled water. The adherent cells were fixed by methanol and stained with 0.5% crystal violet which was removed by washing. Crystall violet was air dried and then dissolved in 33% glacial acetic acid. The biofilm growth was then determined by measuring the optical density (OD) at 630 nm thereafter.

Quantitative reverse transcription PCR (qRT-PCR)

Biofilm cells were harvested from microtiter plates by scraping after incubation with or without GTN. Total RNA was extracted with TRIzol reagent (Promega, USA) according to the manufacturer's instructions and genomic DNA contamination was removed using DNase treatment. Spectrophotometric quantification and purity of RNA was determined. 1 μ g of RNA was used in a reverse transcription kit to synthesize complementary DNA (cDNA). The gene *lasR*, *rhIR*, *myrR* and the housekeeping gene

fbp were quantified using gene-specific primers (Table 1). Reactions were assembled in Promega qPCR master mix and optimized for each gene including annealing temperatures of 60°C and melting curve analysis performed to validate amplification specificity. The level of gene expression was determined relative by $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) and compared the treated samples with the control samples. A fold change of ≥ 2 was defined as being biologically significant (Rasigade et al., 2011). Sequences were designed with the help of Geneious Prime 2026.1.2 (Geneious, Auckland, New Zealand) based on the sequences of the corresponding target gene.

Table 1 Primers utilized in this study

Target gene	Primer name	Sequence (5'-3')
<i>lasR</i>	F	GGCCTTGGTTGACGGTTTTC
	R	GGGTAGTTGCCGACGATGAA
<i>mvfR</i>	F	ATCTCCCGCTTCAACCAAGT
	R	CCTCGACGAAATAATGCGGC
<i>rhlR</i>	F	CCGAACAATTTGCTCAGCGT
	R	GGTTGGACATCAGCATCGGA
<i>fbp</i>	F	CCTACCTGTTGGTCTTCGACCCG
	R	GCTGATGTTGTCTGGGTGAGG

Scanning Electron Microscopy

All assays of the biofilm were performed as described above without staining procedures. Images were then obtained by the scanning electron microscope (SEM) imaging using the field emission electron microscope (FE-SEM) (Thermo Fisher Inspect F50, USA). In short, the samples were then washed 3 times for 5 minutes each in cacodylate buffer and then fixed in 1% (w/v) OsO₄ in cacodylate buffer for 1 hour. Fixed samples were rinsed three times in cacodylate buffer, then dehydrated with 25, 50, 70, 95 and 100% (v/v) ethanol for 10 minutes and critical spot dried with carbon dioxide. The dried surfaces of SEM were attached with tips and covered with evaporated carbon. Depending on the sample a voltage of 1-5 kV was applied (Korzekwa et al., 2026).

Statistical analysis

Each experiment was repeated three times and the normality was determined by Shapiro–Wilk test. The data were presented as mean $\pm$ SD for parametric data and median (IQR) for nonparametric data. Chi-square goodness of fit test was used to analyse categorical distributions, followed by appropriate post-hoc pairwise comparisons and multiplicity adjustment. The Cochran's Q test and exact McNemar tests with Holm correction were then used to compare antibiotic susceptibility patterns; the chi-square goodness-of-fit test and the Holm-adjusted exact binomial tests were then used to compare R/I/S frequencies for each antibiotic. Biofilm forming capacities among isolates were compared using ANOVA followed by Tukey's HSD post-hoc test. Changes following GTN exposure were analysed using paired t-tests or Wilcoxon signed-rank tests, as appropriate. The $2^{-\Delta\Delta C_t}$ method was used to calculate relative gene expression, and fold-change data were summarized using the geometric mean, since the fold-change data is multiplicative and exponential. Data was analysed by GraphPad Prism v10.6.1 and a P value of < 0.05 was taken as statistically significant.

RESULTS

Identification of *Pseudomonas aeruginosa*

In pure culture on nutrient agar, colonies were large, spreading, flat and irregular, greenish in colour and gave off a characteristic grape-like smell. Blood agar showed β -haemolysis and Gram-stained preparations showed Gram-negative, rod-shaped cells.

Colonies were pale/non-lactose fermenting colonies on MacConkey. On cetrinide agar all isolates were able to grow and gave a positive oxidase reaction. All 83 isolates (55.33%) were finally identified as *P. aeruginosa* with VITEK 2 system.

The distribution of the 83 isolates of *P. aeruginosa* was significantly different when the four types of specimens (Table 2) were compared [$\chi^2(3) = 34.93$, $P < 0.0001$]. A higher proportion of isolates were obtained from burn specimens (37/83; 44.58%) than from wound specimens (31/83; 37.35%) and fewer isolates were obtained from sputum (9/83; 10.84%) and mid-stream urine (6/83; 7.23%). There was no significant difference between burn or wound specimens and between sputum and urine specimens ($P > 0.05$) based on the post hoc exact binomial tests with Holm adjustment. Burn and wound specimens, however, had significantly higher numbers of isolates than did sputum or urine specimens (adjusted $P < 0.01$).

Table. 2 Frequency of *Pseudomonas aeruginosa* isolates across specimen type.

Specimen type	Number of isolates (%)
Burn	37 (44.58%) ^a
Mid-stream urine	6 (7.23%) ^b
Sputum	9 (10.84%) ^b
Wound	31 (37.35%) ^a
Total	83 (100%)

Values bearing different superscript lowercase letters are significantly different ($P < 0.05$), based on pairwise exact binomial tests with Holm adjustment following a significant chi-square goodness-of-fit test.

Antimicrobial susceptibility test

There was a great variation among the five antimicrobial agents for susceptibility of the 83 *P. aeruginosa* isolates (Table 3). The highest resistance frequency was observed for tobramycin (29/83; 34.94%), followed by Ceftazidime (25/83; 30.12%), cefepime (24/83; 28.92%), ciprofloxacin (16/83; 19.28%), and imipenem (9/83; 10.84%). Conversely, susceptibility was highest to imipenem (67/83; 80.72%), followed by ciprofloxacin (58/83; 69.88%), ceftazidime (50/83; 60.24%), cefepime (48/83; 57.83%), and tobramycin (38/83; 45.78%).

Table 3. Antibiotic susceptibility of study isolates

Susceptibility	Imipenem	Cefepime	Ceftazidime	Ciprofloxacin	Tobramycin
Resistant	9 ^{bc}	24 ^{abAB}	25 ^{ba}	16 ^{bBc}	29 ^{abAB}
Intermediate	7 ^{ba}	11 ^{ca}	8 ^{ca}	9 ^{ba}	16 ^{ba}
Sensitive	67 ^{aA}	48 ^{aBc}	50 ^{aBc}	58 ^{aAB}	38 ^{ac}
Total	83	83	83	83	83

Determination of GTN minimum inhibitory concentration

There was no direct observation of the MIC in the tested concentration range of the GTN (0.125– 2 mg/mL), and in the case of this study, metabolic activity of the bacteria was still detected at the highest concentration tested, 2 mg/mL, (Figure 1). Therefore, the MIC of GTN was recorded as > 2 mg/mL.

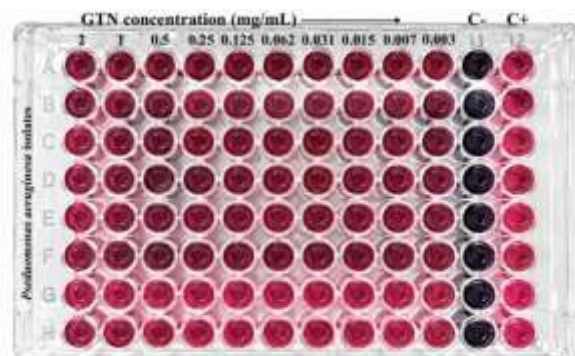


Figure 1. Resazurin-based broth microdilution assay illustrating the inhibitory activities of the glyceryl trinitrate (GTN). Rows A-H inoculated with *Pseudomonas aeruginosa* isolates. Columns 1–10 containing the 2-fold serially diluted GTN at the concentrations ranging from 0.003 to 2 mg/mL. Columns 11 and 12 represent the negative and positive controls, respectively.

Biofilm forming capacity of *Pseudomonas aeruginosa* isolates

The 83 clinical *P. aeruginosa* isolates exhibited a broad range in their ability to form a biofilm with mean OD₆₃₀ values from 0.090 ± 0.003 for PA34 to 1.132 ± 0.372 for PA82 (Table 4). Several isolates exhibited pronounced biofilm formation, including PA75 (1.107 ± 0.081), PA73 (1.036 ± 0.603), PA19 (0.959 ± 0.018), PA81 (0.944 ± 0.058), and PA80 (0.907 ± 0.029). PA34, PA36, PA3, PA43 and PA15 had some of the lowest OD₆₃₀ values, on the other hand. The difference in biofilm biomass between the isolates was highly significant (F (82, 166) = 15.01, p < 0.0001) and accounted for a large percentage of the variation (R² = 0.8812) in the data. The results of Tukey's HSD post-hoc analysis revealed a critical difference of 0.431 (α = 0.05), with significant pairwise differences between those isolates with a mean OD₆₃₀ falling outside this limit. Taken together, these data indicate that there is significant inter-isolate variability in the ability to form biofilm by clinical *P. aeruginosa*.

Findings grouped in Figure 2 highlighted that the distribution of biofilm production was significantly different among the four categories (χ² = 27.70, df = 3, P < 0.001). Strong biofilm production was the most frequently observed phenotype (39/83, 46.99%), followed by moderate (25/83, 30.12%), weak (13/83, 15.66%), and non-production (6/83, 7.23%).

Table 4. Biofilm-forming capacity of clinical *Pseudomonas aeruginosa* isolates.

Isolate code	OD ₆₃₀ (SD)	Isolate code	OD ₆₃₀ (SD)	Isolate code	OD ₆₃₀ (SD)
PA1	0.468 (0.234)	PA31	0.414 (0.019)	PA61	0.891 (0.506)
PA2	0.355 (0.166)	PA32	0.429 (0.024)	PA62	0.273 (0.041)
PA3	0.098 (0.001)	PA33	0.119 (0.015)	PA63	0.526 (0.046)
PA4	0.669 (0.079)	PA34	0.090 (0.003)	PA64	0.507 (0.082)
PA6	0.623 (0.028)	PA35	0.232 (0.020)	PA65	0.168 (0.006)
PA7	0.390 (0.234)	PA36	0.093 (0.006)	PA66	0.347 (0.059)
PA8	0.123 (0.014)	PA37	0.662 (0.027)	PA67	0.319 (0.036)
PA9	0.138 (0.006)	PA38	0.354 (0.029)	PA68	0.496 (0.181)
PA11	0.864 (0.071)	PA39	0.265 (0.024)	PA69	0.337 (0.009)
PA12	0.395 (0.024)	PA40	0.235 (0.071)	PA70	0.456 (0.053)
PA13	0.235 (0.040)	PA41	0.117 (0.008)	PA71	0.802 (0.053)
PA14	0.207 (0.010)	PA42	0.279 (0.030)	PA72	0.220 (0.012)
PA15	0.099 (0.010)	PA43	0.098 (0.035)	PA73	1.036 (0.603)

PA16	0.464 (0.060)	PA44	0.164 (0.010)	PA74	0.827 (0.132)
PA17	0.397 (0.109)	PA45	0.146 (0.043)	PA75	1.107 (0.081)
PA18	0.194 (0.020)	PA46	0.115 (0.018)	PA76	0.557 (0.052)
PA19	0.959 (0.018)	PA47	0.113 (0.010)	PA77	0.735 (0.057)
PA20	0.444 (0.010)	PA48	0.490 (0.262)	PA78	0.899 (0.045)
PA21	0.477 (0.053)	PA49	0.897 (0.056)	PA79	0.280 (0.030)
PA22	0.126 (0.027)	PA50	0.230 (0.031)	PA80	0.907 (0.029)
PA23	0.225 (0.036)	PA51	0.707 (0.123)	PA81	0.944 (0.058)
PA24	0.700 (0.196)	PA52	0.463 (0.089)	PA82	1.132 (0.372)
PA25	0.379 (0.047)	PA55	0.291 (0.004)	PA83	0.597 (0.049)
PA26	0.101 (0.003)	PA56	0.284 (0.038)	PA84	0.489 (0.065)
PA27	0.280 (0.198)	PA57	0.437 (0.046)	PA85	0.747 (0.064)
PA28	0.287 (0.099)	PA58	0.490 (0.032)	PA86	0.558 (0.070)
PA29	0.178 (0.015)	PA60	0.514 (0.020)	PA87	0.598 (0.025)
PA30	0.157 (0.009)				

ANOVA ($F(82, 166) = 15.01, p < 0.0001; R^2 = 0.8812$). Tukey's HSD post-hoc test yielded a critical difference of 0.431 at $\alpha = 0.05$.

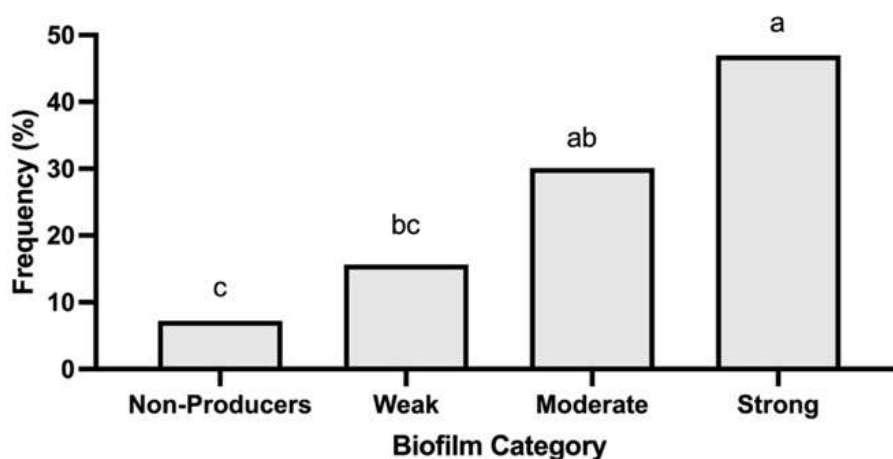


Figure 2. Distribution of *Pseudomonas aeruginosa* isolates across biofilm categories

Data are presented as percentage of isolates (total number of isolates is 83). Differences in the distribution of isolates among biofilm-intensity categories were evaluated using the chi-square goodness-of-fit test followed by pairwise comparisons of proportions with Bonferroni adjustment ($\chi^2 = 27.70, df=3, P < 0.001$). Values bearing different lowercase superscript letters are significantly different, whereas values sharing at least one letter are not significantly different (adjusted $p < 0.05$). A weak positive correlation was found between biofilm-forming ability (OD_{630}) and the number of antibiotics to which the isolates are resistant (Spearman's $\rho = 0.222, 95\% \text{ CI: } -0.010 \text{ to } 0.432$). The association was not statistically significant, however ($p = 0.054$; Figure 3). Therefore, there was no significant association between increased antibiotic resistance (number of antibiotics) and increased biofilm biomass in the clinical *P. aeruginosa* isolates.

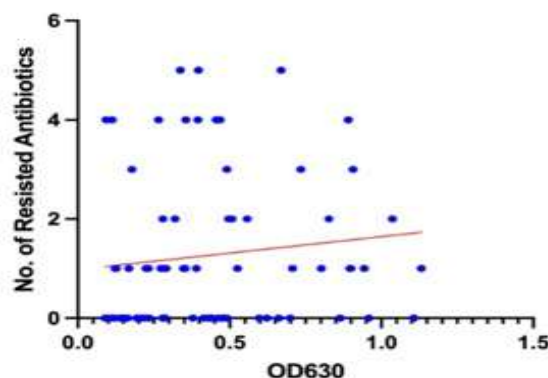


Figure 3. Plot of correlation between the number of antibiotic resistances and biofilm biomass (OD₆₃₀) for the isolates. Individual clinical isolates are represented as points. Spearman's rank correlation revealed a weak positive, but insignificant, correlation ($\rho = 0.222$, 95% CI: -0.010 to 0.432 , $p = 0.0538$).

Effect of GTN on Biofilm Biomass

Six isolates showed significant biofilm inhibition varying from 31.04 % to 67.20 % (Table 5). The greatest reduction occurred in PA43 (67.20%; $p = 0.0033$), followed by PA9 (59.11%; $p = 0.0023$), PA33 (57.64%; $p = 0.0035$), PA15 (45.41%; $p = 0.0019$), PA61 (36.17%; $p = 0.0038$), and PA77 (31.04%; $p = 0.0179$). Several of the other isolates showed levels below significance, but PA70 was close to the statistical significance level ($p = 0.0507$). In contrast, eight isolates had greater biofilm biomass with GTN and only PA1 had a significant increase (−73.98% inhibition; $p = 0.0161$). Overall, the response to GTN was mainly a reduction in biofilm biomass, but varied significantly among different *P. aeruginosa* isolates.

Table 5. Effect of Glyceryl Trinitrate on Biofilm Biomass of Clinical *Pseudomonas aeruginosa* Isolates

Isolate code	Without GTN		With GTN		P value	Inhibition %
	Mean OD ₆₃₀	SD	Mean OD ₆₃₀	SD		
PA1	0.515	0.261	0.896	0.104	0.0161	-73.9806
PA4	0.51	0.124	0.434	0.095	0.2666	14.90196
PA9	0.829	0.314	0.339	0.02	0.0023	59.10736
PA15	0.414	0.172	0.226	0.059	0.0019	45.41063
PA26	0.762	0.096	0.522	0.26	0.3209	31.49606
PA33	0.628	0.054	0.266	0.049	0.0035	57.64331
PA34	0.441	0.223	0.432	0.149	0.8536	2.040816
PA41	0.66	0.135	0.461	0.313	0.0386	30.15152
PA43	0.75	0.067	0.246	0.03	0.0033	67.2
PA46	0.308	0.105	0.185	0.036	0.1912	39.93506
PA49	0.639	0.117	0.422	0.03	0.1244	33.95931
PA51	1.533	0.094	1.786	0.774	0.6584	-16.5036
PA52	0.497	0.117	0.308	0.039	0.1644	38.02817
PA58	0.702	0.115	0.551	0.183	0.0796	21.50997
PA61	0.622	0.032	0.397	0.236	0.0038	36.17363

PA63	0.517	0.04	0.717	0.121	0.1367	-38.6847
PA64	0.479	0.028	0.463	0.058	0.6287	3.340292
PA68	0.621	0.157	0.372	0.246	0.361	40.09662
PA70	0.464	0.079	0.401	0.054	0.0507	13.57759
PA71	0.324	0.056	0.28	0.026	0.4292	13.58025
PA73	0.609	0.157	0.847	0.198	0.3278	-39.0805
PA74	0.661	0.072	0.525	0.182	0.4495	20.57489
PA76	0.479	0.135	0.483	0.179	0.9402	-0.83507
PA77	0.335	0.052	0.231	0.035	0.0179	31.044
PA78	0.649	0.059	0.731	0.05	0.207	-12.6348
PA80	0.624	0.213	0.349	0.159	0.325	44.07051
PA81	0.513	0.017	0.716	0.176	0.175	-39.5712
PA82	0.767	0.069	0.81	0.023	0.315	-5.60626

The FE-SEM analysis showed that there were clear differences in the architecture of the biofilm after the GTN treatment, consistent with the quantitative crystal-violet data. The untreated control showed a classic biofilm with significant surface colonization, cellular aggregation and heterogeneous surface-associated material. The surface in the PA1 isolate treated with GTN showed a higher amount of biofilm biomass, and the biofilm was more present, meaning that the surface was covered by a dense and interconnected bioarchitecture, where the cellular aggregates were closer together, and the underlying surface was less exposed. In contrast, the PA43 isolate that produced less biofilm biomass grew into a less compact and more discontinuous structure in the GTN-treated isolate with smaller and more spatially distributed cellular aggregates as well as increased exposure of the underlying surface (Figure 4). Therefore, FE-SEM was used to support the qualitative morphological data obtained from the crystal-violet assay for the divergent biofilm responses obtained under the separate conditions.

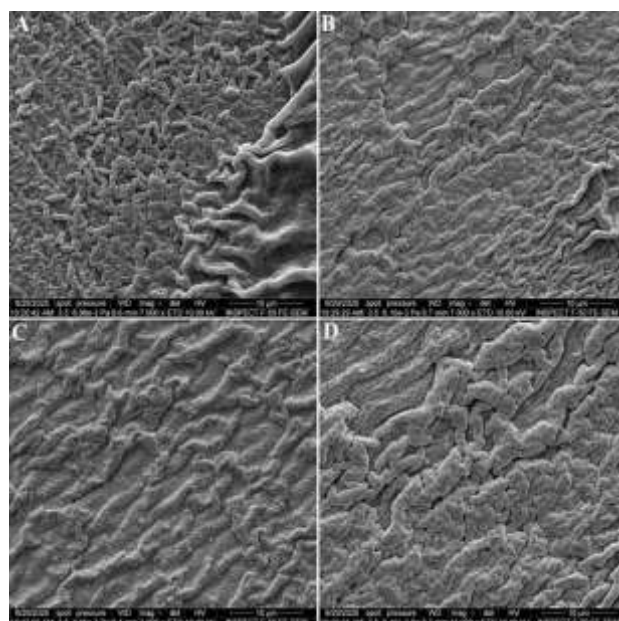


Figure 4. FE-SEM analysis of isolate-dependent changes in established *Pseudomonas aeruginosa* biofilms following GTN treatment.

(A) Untreated control showing an established biofilm corresponding to PA43 isolate. (B) GTN-treated biofilm of PA43 isolate exhibiting decreased biofilm biomass, showing denser and more extensive surface-associated architecture with closely aggregated cells. (C) Untreated control showing an established biofilm corresponding to PA1 isolate. (D) GTN-treated biofilm corresponding to PA1 isolate exhibiting increased biofilm biomass, showing reduced architectural compactness, smaller and more dispersed cellular aggregates, and greater exposure of the underlying surface. Representative FE-SEM micrographs were acquired at 7,000× magnification; scale bar = 10 µm. GTN, glyceryl trinitrate; FE-SEM, field-emission scanning electron microscopy.

Effect of GTN on Gene Expression of Quorum-Sensing-related genes

Relative expression analysis revealed that the response of the isolates to GTN treatment was isolate dependent (Table 6). The geometric mean fold changes for *lasR*, *mvfR*, and *rhlR* were 1.098, 0.584, and 0.887, respectively, and were near 1. *mvfR* and *rhlR* were found to have been downregulated in 5/6 isolates; while *lasR* showed mixed effect with 3 isolates being downregulated and 3 isolates were upregulated. Interestingly, all 3 QS regulators were upregulated on PA1.

Table 6. Effect of GTN on the Gene expression (fold change) of *lasR*, *mvfR*, and *rhlR*

Gene	Geometric mean fold change	Isolates downregulated
<i>lasR</i>	1.098	3/6 (50.0%)
<i>mvfR</i>	0.584	5/6 (83.3%)
<i>rhlR</i>	0.887	5/6 (83.3%)

DISCUSSION

Pseudomonas aeruginosa is an opportunistic pathogen that can cause a plethora of life-threatening, acute and chronic infections especially in those with compromised immune defence. Previous studies revealed that the antibiotic resistance of bacteria when they are in a biofilm state is responsible for the persistence of the bacteria and creates an issue in eliminating infection completely (Letizia et al., 2025). Sulaiman and Abdulhasan (2020) reported that 50 (67.6%) *P. aeruginosa* isolates were isolated from different clinical specimens and identified as *P. aeruginosa*; whereas AL-Ameen and Ghareeb (2022) isolated *P. aeruginosa* in 46 (17.35%) isolates.

The antimicrobial susceptibility profile showed great variation of the different agents tested against *P. aeruginosa* isolates from clinical samples. Imipenem demonstrated the highest susceptibility rates with 80.72% of isolates being susceptible and 10.84% being resistant followed by tobramycin, with 34.94% resistance and ceftazidime with 30.12% resistance, followed by Cefepime with 28.92% resistance. There were considerable differences between the antibiotics suggesting that resistance was not evenly spread throughout the antibiotics that were tested.

The relative resistance to imipenem is a positive development in the context of the role that carbapenems play in the treatment of severe *P. aeruginosa* infections, but the presence of resistant isolates is of clinical relevance. In addition to the loss or modification of OprD, increased efflux, overexpression of AmpC, and the presence of acquired carbapenemases, resistance to antipseudomonal cephalosporins also stems from changes in permeability (Zhao et al., 2024, Meletis and Karastergiou, 2025). This increased resistance to imipenem compared to ceftazidime may therefore be due to resistance mechanism differences between the individual β -lactam classes, and not due to a common resistance phenotype. In the same way, resistance mechanisms to tobramycin include aminoglycoside modifying enzymes, reduced permeability and target modification and multidrug efflux. Together, these mechanisms demonstrate genomic and phenotypic adaptability of *P. aeruginosa* and highlight the need for isolate-specific susceptibility testing.

Although the relatively low imipenem resistance level seen here should be viewed in the local context, resistance prevalence differs from one geographical region to another, clinical setting, specimen source, antimicrobial exposure and circulating clones. This is even more critical as carbapenem-resistant *P. aeruginosa* is considered a high priority resistant organism in the 2024 WHO bacterial priority pathogens list and should continue to be monitored locally (Meletis and Karastergiou, 2025).

The results presented here reveal that there was a marked variation in the ability of clinical isolates of *P. aeruginosa* to form biofilms, with more than tenfold difference in OD₆₃₀ values. The overall difference between the isolates was highly significant and accounted for a large proportion of the variance ($R^2 = 0.881$) suggesting that biofilm production was a characteristic of the isolates and not a uniform property of the clinical population. The level of phenotypic diversity within *P. aeruginosa* has been observed and

clinical isolates can vary significantly in ability to attach to surfaces, form a mature biofilm architecture and produce extracellular matrix.

This variability is biologically plausible because the development of biofilms of *P. aeruginosa* is regulated by an interconnected regulatory network of QS, cyclic-di-GMP signalling, motility, adhesins and extracellular matrix components, including Pel, Psl and alginate. Variations in the expression and/or regulation of these systems can produce very different biofilm phenotypes between even isolates of the same species. In clinical populations there may be further phenotypic variation due to additional adaptation to different host environments, exposure to antimicrobials and infection niches.

Isolates that are especially capable of forming biofilms are also clinically significant because the biofilm growth phenomenon can render bacteria more persistent thereby limiting penetration of antimicrobials and changing the metabolic state of cells and the induction of phenotypic tolerance to antimicrobial and host-mediated stresses. However, a high biofilm biomass should not be considered synonymous with antimicrobial resistance as this is a related but different phenomenon. AL-Fridawy et al. (2020) reported significant variations in the phenotype of the isolates studied as 3 (13.0%) were weak biofilm producers, 7 (30.4%) were moderate biofilm producers, 11 (47.8%) were strong biofilm producers and only 2 (8.6%) were non-biofilm producers

There was a weak positive correlation between biofilm biomass and number of antibiotics resisted by the *P. aeruginosa* population ($\rho = 0.222$, $p = 0.0538$), and this correlation was not statistically significant, which indicates that there was no strong association between biofilm-forming ability and conventional antimicrobial resistance observed in the clinical *P. aeruginosa* population analysed. The confidence interval, which just touched zero (-0.010 to 0.432), also suggests some uncertainty about the existence and strength of the association. The present data therefore do not allow for a statistically significant relationship between increased resistance burden and increased biofilm biomass to be established, although there was a slightly higher resistance burden in isolates with higher biofilm biomass.

There is biological plausibility in this apparent independence because the processes of antimicrobial tolerance in biofilms and those of inherited antimicrobial resistance are different. Conventional resistance is primarily attributed to several resistance mechanisms including enzymes that inactivate antimicrobials, modification of the target, decreased permeability and active efflux, whereas the matrix-mediated diffusion effects, altered metabolic state and physiological heterogeneity or persister-cell formation are associated with biofilm growth and survival during exposure to antimicrobials. Therefore, the MDR isolates do not always show higher biofilm biomass nor do strong biofilm producers necessarily be resistant to a higher number of antibiotics.

This discovery also provided an experimental approach to the subsequent GTN assays where MDR phenotype and high biofilm forming capacity were used independently as selection criteria. The lack of significant correlation suggests that the MDR status would not be a reliable predictor of the best biofilm producer isolates. Taking both features into account enabled the GTN experiments to be directed to isolates carrying a clinically relevant degree of antimicrobial resistance and high biofilm production.

The extreme inter-isolate variation examined in the present study also served as a good foundation for the following assessment of GTN. High biofilm forming isolates that had MDR phenotypes were given special attention and the effect of GTN was studied against clinically relevant and well-established biofilms. The following observation that GTN resulted in opposing outcomes (significant decrease in biofilm for some isolates and increase in biofilm for others) further highlights the biological heterogeneity which is already apparent in the baseline biofilm assay. Therefore, the response to GTN should be considered within an intrinsically heterogeneous background of biofilm-forming capacity, and not as a species-level response.

In addition to the more classical antimicrobial susceptibilities, one of the most interesting results emerging from the study of the effects of GTN was the lack of complete growth inhibition over the range of concentrations tested. After reaching a concentration level of 2 mg/mL no endpoint was obtained, hence the MIC was taken as being >2 mg/mL. The antibacterial activity of GTN has been found to vary in previous studies. This showed no significant activity against methicillin-resistant *Staphylococcus aureus* (MRSA) or methicillin-resistant *S. epidermidis* (MRSE) at 0.04% while (Abbas et al., 2019) reported activity against *S. aureus* at 0.5 mg/mL. Nazeih et al. (2023) reported the inhibition of *P. aeruginosa* and *Serratia marcescens* at concentrations higher than 1 mg/mL while *P. aeruginosa* was found metabolically active at 2 mg/mL in the present study.

Such differences could be biological and/or methodological. *P. aeruginosa* isolates from clinical patients have various outer membrane permeabilities and efflux activities, which may affect the susceptibility to nitrosative stress, as well as their metabolic state and ability to handle stress. In addition, bioactivation of GTN may produce NO or other reactive nitrogen species, and thus the composition of the media, pH, the amount of oxygen, bacterial density and the conditions of the incubation could affect its

biological activity. Exposure may also be affected by the GTN formulation and excipients. There are also methodological differences. This study employed a broth microdilution assay system that measures cellular metabolic activity by the reduction of the dye resazurin. There could be populations of metabolically active cells present even if proliferation is severely suppressed; endpoint may differ from those based on turbidity, OD or viable counts.

While not totally inhibiting, GTN had a primarily inhibitory, but highly isolate-dependent, effect on existing *P. aeruginosa* biofilms. For 20/28 isolates (71.4%), the biomass of the biofilm was reduced, and in six isolates the biofilm was inhibited, ranging from 31.04% inhibition in PA77 to 67.20% inhibition in PA43 ($p > 0.05$). This is in line with earlier studies that showed that GTN inhibits the formation of *P. aeruginosa* biofilm and disrupts pre-existing biofilms. Nazeih et al. (2023) also reported reduced biofilm formation along with down-regulation of *lasI*, *lasR*, *rhII*, *rhIR*, *pqsA* and *pqsR*, which suggests that GTN may exert its effect on biofilm-regulatory pathways as well as through growth inhibition.

The quantitative biofilm results were corroborated by the FE-SEM results showing different architecture responses to exposure to GTN. The untreated control had a well-developed biofilm with extensive areas of surface coverage and cell aggregation, while the isolate treated with GTN had lower biomass with less cell aggregation, forming less continuous aggregates and exposing more of the surface. The isolate that increased biofilm biomass when grown with GTN, however, had denser and more extensive surface-associated architecture. Both these morphological patterns are in agreement with crystal violet results and suggest that the impact of GTN on mature *P. aeruginosa* biofilms is isolate-dependent and not generally inhibitory.

The FE-SEM observations also allow for complementary information to the crystal-violet assay. FE-SEM can be used to qualitatively assess surface colonisation, cellular aggregation and overall biofilm organisation, while crystal violet quantifies the total attached biomass. These observations are consistent with the study of reduced crystal-violet biomass, which is associated with decreased compactness of the structures after GTN treatment, and further support the evidence that GTN disrupts established biofilm organisation. The opposite is true for the isolate with elevated crystal-violet biomass, however, which has a denser architecture in support of the paradoxical improvement seen in a subset of isolates. However, FE-SEM cannot differentiate between viability and non-viability of cells and cannot determine if cell surface reduced due to active dispersal, detachment, reduction in matrix production or loss of viability. Similarly, the material seen on the surface using FE-SEM should not be conclusively interpreted as being extracellular polymeric substances unless accompanied by a supporting compositional analysis.

A potential mechanism by which the inhibitory phenotype occurs is through the NO-responsive c-di-GMP signalling. Bioactivation by GTN can produce NO related signalling molecules, whereas a low NO level has been linked to *P. aeruginosa* biofilm dispersal, which occurs due to an increase in phosphodiesterase activity and a decrease in the levels of c-di-GMP within the cell (Andersen et al., 2021, Tan et al., 2024). Given that c-di-GMP is linked to matrix production and sessility, and that its depletion is associated with motility and altered biofilm architecture after GTN exposure, modulation of this signalling pathway could be linked to the reduced biomass and altered architecture that was seen after exposure to GTN, (Cordery et al., 2024). The response is, however, not universal, and the contrasting FE-SEM phenotype in the isolate showing increased biofilm biomass suggests the dispersal mechanism to be GTN is not uniform. Instead, differences in the QS activity and c-di-GMP homeostasis, metabolic state and stress responses of strains may define the direction and magnitude of the response.

This interpretation is especially compelling when viewed in conjunction with the transcriptional studies. The much more complex architecture would correlate with the simultaneous upregulation of *lasR*, *myfR* and *rhIR* (2.445-, 2.949- and 3.294-fold respectively) in the much thicker biofilm, while significant decreases in biofilm biomass were correlated with the dominant suppression of the examined QS regulators. An isolate-dependent response to GTN would, therefore, be supported by complementary phenotypic (biomass measurements) and molecular (FE-SEM morphology and QS transcription levels) evidence. However, these correlations are not causal and direct measurements of c-di-GMP, QS signalling molecules and viable biofilm cells would be needed to pinpoint the mechanism.

The response was not universal, there was an increase in biofilm biomass after GTN exposure in eight isolates, including a significant increase in PA1. This divergent response is biologically plausible as clinical *P. aeruginosa* isolates exhibit significant differences in the architecture of their biofilm, composition of their matrix, stress response, metabolism, and QS activity, as well as regulation of c-di-GMP. However, this regulatory divergence could lead to either biofilm decrease or biomass increase after applying GTN to individual isolates. Importantly, the crystal violet is a measure of biomass on the surface as a whole, not viability or a particular matrix component. Therefore, reduced OD₆₃₀ can be used for biofilm disruption/dispersal, but is not able to

differentiate between biofilm disruption/dispersal or reduced matrix production or cellular detachment or changes in viable cell abundance. Determination of these mechanisms would require complementary viable-count, microscopic and matrix analyses.

At the transcript level, the isolate-dependent biofilm response was even further observed. *mvfR* had the highest overall fold change described as a geometric mean (0.584) followed by *rhIR* (0.887) and *lasR* (1.098). Downregulation of both *mvfR* and *rhIR* was observed in 5 (83.3%) out of 6 isolates whereas *lasR* was equally distributed between up and downregulation. The overall suppression of *rhIR* was modest, but directionally consistent, while *mvfR* was frequently and more strongly suppressed.

This is very similar to the molecular-docking analysis results reported by Nazeih et al. (2023) regarding anti-QS activity in which they noted downregulation of various Las, Rhl and Pqs components after exposure to GTN. Accordingly, they suggested interactions between GTN and LasR, RhlR and PqsR, but further experiments are needed to validate these computational suggestions. This dramatic decrease in the expression of *mvfR* is especially interesting since PqsR/MvfR is a key regulator of the PQS system and is an important factor in virulence and biofilm formation. The relatively low uniformity of *lasR* response could be a consequence of the flexibility of regulation of clinical *P. aeruginosa*, as variations in QS phenotypes and *lasR* integrity can occur significantly between isolates (Yang et al., 2024).

We found PA1 to be particularly informative because it showed simultaneous upregulation of *lasR*, *mvfR* and *rhIR* (2.445-, 2.949- and 3.294-fold, respectively) together with a large increase in the levels of biofilm biomass. Isolates, however, like PA9, PA43 and PA77 resulted in substantial reductions in biofilm formation, with mostly decreased expression of the studied QS regulators. These pheno-transcriptional correlations provide evidence of a correlation between QS modulation and GTN response and PA1 is an internal counterexample that the effect is not universal and suppressive. However, these concurrent responses are not causal since the development of biofilms is regulated by many other regulatory and metabolic pathways, including ones not studied here that were not included in this analysis.

Generally, the consistent repression of *rhIR* and the biofilm reduction that are seen in the majority of isolates suggest a biofilm regulation effect by GTN which is isolate-dependent. The effects could not be attributed to a classical MIC level antibacterial effect, given the lack of full growth inhibition up to 2 mg/mL. But partial effects on the growth, metabolism or fitness of the bacteria can't be ruled out. However, the results suggest, but do not definitively demonstrate, that GTN has an anti-virulence effect. The application of growth kinetics, viable cell counts and direct measurement of NO and c-di-GMP in future studies would facilitate the separation of the effects on bacterial fitness from those of specific modulation of QS and biofilm regulatory pathways.

CONCLUSION

The present study revealed a significant difference in the antimicrobial resistance and in the ability to form biofilm among the clinical isolates of *P. aeruginosa* and also showed that there is not a close linkage between these two phenotypes. While GTN did not produce complete growth inhibition up to 2 mg/mL, it significantly disrupted already formed biofilms, in most of the tested isolates decreasing the biomass, and in a smaller number of isolates increasing the biomass. These isolate-dependent effects on biofilm architecture were confirmed by morphological changes that were observed using FE-SEM. In addition, GTN influenced the expression of central QS regulators *lasR*, *rhIR*, and *mvfR*, mostly downregulating the expression of *rhIR* and *mvfR*, but heterogeneously affecting the expression of *lasR*. The fact that an increase in biofilm biomass coincided with an increase in QS-gene expression was particularly notable for PA1, as this same pattern was not observed in other species. All these results suggest that GTN may be a biofilm- and virulence-modulating compound and not a typical growth-inhibiting antimicrobial agent. However, the variability in response to the isolates suggests that its activity should not be extrapolated to other populations of *P. aeruginosa*. Additional work using direct measurements of nitric oxide and c-di-GMP, functional QS-controlled virulence phenotypes and wider collections of isolates are needed to pinpoint the mechanisms of GTN responsiveness and to assess its potential for anti-virulence applications.

Ethics approval and consent to participate

The Research Ethics Committee of the College of Science, University of Baghdad approved this work (Ref. No. CSEC/1025/0126). All patients freely consented to provide their specimens for research purposes.

Patient consent for publication

Not applicable

Author declaration

The authors declare that they have no conflicts of interest.

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