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ORIGINAL ARTICLE

Investigation of the effect of dimethyl sulfoxide on growth and biofilm formation of *Pseudomonas aeruginosa*

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ABSTRACT

Background and Objectives: The antimicrobial resistance of *Pseudomonas aeruginosa* bacteria limits the spectrum of effective antibiotics. Considerable focus has been placed on the identification of more contemporary and cost-effective antimicrobial drugs. In this study, the antibacterial properties of a commonly used solvent, dimethyl sulfoxide (DMSO), against *P. aeruginosa* were investigated.

Materials and Methods: The microtiter broth dilution technique was employed to establish the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of DMSO. The solvent's impact on bacterial growth, biofilm formation and eradication was assessed. A quantitative polymerase chain reaction (qPCR) was carried out to assess the effect of varying DMSO concentrations ranging from 1% to 8% (v/v) on quorum sensing gene expression.

Results: All *P. aeruginosa* strains exhibited a DMSO MIC of 25% v/v and MBC of 50% v/v. DMSO caused significant growth inhibition and suppression of biofilm formation in all *P. aeruginosa* strains at sub-inhibitory concentrations, i.e. 1%-8% v/v. At these concentrations, the samples showed a reduction in biomass and reduced metabolic activity. These effects were concentration-dependent. A DMSO strength of 8% v/v was associated with a statistically significant downregulation of most of the quorum sensing genes; at a DMSO titer of 1% v/v, this effect was modest with only a few genes being significantly affected.

Conclusion: DMSO is a potential therapeutic agent against *P. aeruginosa* as it has been demonstrated that it exhibits antimicrobial characteristics. Moreover, the impact of DMSO on bacterial growth and biofilm formation complicates its use as a solvent in biologic and clinical research.

Keywords: Cystic fibrosis (CF); *Pseudomonas aeruginosa*; Dimethyl sulfoxide (DMSO); Anti-bacterial agents; Bacterial growth; Biofilms

INTRODUCTION

Pseudomonas aeruginosa (PA) can lead to chronic infections, and in combination with the associated inflammatory process in lung tissue, forms the principal etiology responsible for mortality in individuals with cystic fibrosis (CF) (1). Growth of PA occurs in both multicellular and sessile formations, which are referred to as biofilms. These are composed of bacteria which are self-amassing or surface-adherent

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within an extracellular matrix, and which are responsible for numerous long-term and invasive infections in humans, e.g. those related to clinical devices, chronic pulmonary infection in CF and chronic infections in wounds (2).

The virulence and biofilm forming capacity of PA is dependent on the bacteria's intercellular quorum-sensing (QS) communication (3), which comprises three separate systems, i.e. the Las, Rhl and Pseudomonas quinolone signal (PQS) systems. A further system which has been identified is the integrated quorum sensing (IQS) system (4). PA's QS system has been well-delineated. The modulator at the core of the QA pyramid is the Las system. Controlling Las-Rhl cascades has the ability to induce the expression of genes that encode the majority of exoenzymes and virulence factors (5). Elimination of PA using antimicrobial agents is hindered by the extreme degree of antibiotic resistance seen in this bacteria. This results from the generation of biofilm, a phenomenon which is particularly common in individuals with CF (3). This issue has inspired scientists to seek antimicrobials from de novo origins in order to find agents that demonstrate efficacy against these bacteria.

Dimethyl sulfoxide (DMSO) is a frequently utilized solvent used in in vivo and in vitro studies to create a solution of pharmaceutical agents or any test material which are insoluble in water (6). A number of reviews are available in the literature regarding the activity of DMSO as anti-inflammatory and bacteriostatic agent as well as a local analgesic, tranquilizer, and diuretic (6-8).

Some studies have suggested that the activity of certain pharmaceutical agents can be augmented by DMSO through the enhancement of substance penetration of physiologic membranes, although this observation remains the subject of debate (9). For the current study, a hypothesis is proposed which states that DMSO is able to suppress the formation of biofilm and inhibit PA growth. Studies relating to the ability of solvents, e.g. DMSO, to impede the growth of PA isolates from individuals with CF are scarce. Thus, the aim of the present research is to assess the degree of efficacy demonstrated by DMSO with respect to specific reference and clinically isolated PA strains. This work will offer useful empirical data in relation to the mechanisms underlying the function of DMSO as both an antibiotic and a solvent.

Although dimethyl sulfoxide (DMSO) is widely used as a solvent, it possesses intrinsic biological activity that can influence microbial membranes, quorum sensing, and stress responses, which may confer antimicrobial or antibiofilm effects (10).

Initial pharmacology studies indicated that the growth of *Escherichia coli, Pseudomonas aeruginosa*, and *Bacillus megaterium* was inhibited as the percentage of DMSO increased, with nearly complete suppression observed at approximately 15% and significant inhibition of *P. aeruginosa* noted above around 8% (11). A systematic evaluation of *P. aeruginosa* and *Streptococcus pneumoniae* revealed that 0.03-25% DMSO significantly inhibited biofilm formation at various levels; however, it intriguingly promoted biofilm formation at approximately 6%, indicating a non-linear (biphasic) response. Synergistic effects with standard antibiotics were noted at <1% DMSO, while the impact on *S. pneumoniae* was negligible within the same context. This study highlighted the prevalent under-reporting of solvent controls in antibiofilm assays (12). Distinct mechanistic scenarios exist in which DMSO acts as a respiratory electron acceptor, thereby stimulating biofilm formation. In *Shewanella algae*, the addition of DMSO or nitrate resulted in strain-specific biofilm formation, a phenomenon that was negated in DMSO/nitrate-reductase mutants. This suggests the involvement of respiration-linked signaling mechanisms, such as c-di-GMP, rather than solvent toxicity (13).

At elevated concentrations, DMSO can decrease biofilm biomass and viability while modifying extracellular polymeric substances (EPS) (14). DMSO can protect bacteria from rapid killing by various antibiotic classes, such as aminoglycosides, likely by quenching reactive intermediates or altering uptake. This suggests that DMSO may interfere with standard susceptibility tests when included in assay mixtures.

Testing its activity is scientifically valuable, as it offers mechanistic insights into microbial physiology and identifies novel targets for biofilm disruption (15). DMSO's significant penetration capability renders it an effective drug delivery vehicle. It is crucial to comprehend its independent antimicrobial effects to differentiate between solvent influences and those of co-administered agents. Systemic use may be restricted due to toxicity and cost, whereas localized or controlled applications—such as catheter lock solutions or wound dressings—are still feasible (16). Research on DMSO establishes a standard for evalu-

ating safer derivatives or analogs, with findings that contribute to regulatory risk–benefit assessments and inform the creation of enhanced formulations (17). Therefore, despite practical limitations, a thorough evaluation of DMSO's antimicrobial and biofilm-modulatory effects is necessary.

MATERIALS AND METHODS

Bacterial isolates and their identification. The reference strain, American Type Culture Collection (ATCC) PA 27853, together with six clinical isolates, i.e. PA1, PA2, PA3, PA4, PA5 and PA6, were included in this research. PA ATCC is from the international PA panel and its whole genome sequence can be accessed. The clinical isolates were provided by the microbiology laboratory at the microbiology laboratory at Jordan University. These specimens were obtained from sputum samples given by individuals with CF. The following features were deemed indicative of PA: Gram staining; green pigment evident on nutrient agar; growth on both MacConkey and cetrimide agars; oxidase testing; and growth at a temperature of 42°C. The VITEK2 computer automatic bacteria identification system (Bio Merière, Lyon, France) was used to verify the presence of PA.

Culture conditions. Pseudomonas cetrimide agar (Oxoid™) was the selective medium used for PA isolation. Brain-heart infusion (BHI) medium (Oxoid™) was applied to the clinical sputum samples in order to enrich them. A streak and pour plate technique was employed to culture these specimens on the cetrimide agar. For each individual isolate, subcultures were setup on cetrimide agar plates from a one single colony; and incubated at a temperature of 37°C. Fresh cultures from each PA strain were prepared in a concentration of 0.5 McFarland Scale (1.5×10^8 CFU/mL), and were used in the assessment experiments.

Minimal inhibitory concentration assays. The minimal inhibitory concentration (MIC) was established with the use of a microtiter broth dilution technique (18). Admixtures of different DMSO and Mueller Hinton (MH) Broth volumes were added to the individual wells of assay plates containing 96 tissue culture-treated polystyrene wells (Costar Corning, NY). A 50% stock solution was prepared by mixing 190 µL of broth with 190 µL of DMSO; subsequent two-fold dilutions were performed to get concentrations of 25%, 12.5%, 6.25%, and 3.75%. An overnight growth of the test PA strains was used to make a 0.5 McFarland standard bacterial suspension and 20-µL of bacteria at a density of 5×10^5 CFU/mL was added. Each well contained a maximum volume of 400 µL. The MIC endpoint was defined as a lack of observed growth within the tubes, i.e. when DMSO was present in its most dilute titer.

MBC determination. Once the DMSO MIC had been established, 50 µL quantities of inoculated material were pipetted from all the tubes containing no obvious bacterial growth onto the BHI agar plates; these were then cultured for a 24-hour period at a temperature of 37°C. The minimum bactericidal concentration (MBC) could then be identified by inspection of the agar plates before and after incubation for evidence of bacteria. The MBC was defined as the most dilute titer of the antibiotic which eradicated 99.9% of the bacterial colony.

Microtiter-plate test for biofilm assay. The influence of a sub-inhibitory titer of DMSO on the PA strains growth and biofilm formation was investigated using Microtiter-plate test (19). A spectrum of MH broth and DMSO volumes were admixed within the individual wells of 96-well plates; the solvent dilution varied between 1% to 8% v/v. The reference bacteria suspension from the individual PA strains, which had undergone overnight incubation, was admixed with Tryptone Soya Broth to make up 20 µL volumes, the tests were performed in triplicate. Wells designated as positive and negative controls contained PA with broth but no DMSO, and broth alone, respectively.

A cover was applied to the plates which then underwent aerobic incubation for a 24-hour period at a temperature of 37°C. Then, aspiration of the admixture from the individual wells, 250 µL sterile physiological saline was used to rinse the wells thrice. The plates were then agitated to extract any free floating bacteria. 200 µL 99% methanol was pipetted into each well to retrieve the residual adherent bacteria. After 15 minutes, the contents of the plates were decanted, and the plates were allowed to dry before adding 0.2 mL/well. 2% Hucker's Crystal Violet (CV) was then applied for five minutes. The plates were cleaned with tap water to get rid of extra staining solution. Next, the plates were allowed to air dry. To resolubilize the dye associated to the adherent cells, 160 µL of glacial

acetic acid at a concentration of 33% (v/v) was applied to each well.

The optical density (OD) for every well was calculated using an automated reader (Infinite® 200 PRO NanoQuant, TECAN). Three measurements were taken: 1) at the beginning of the incubation phase (OD 600 nm), 2) at the end of the incubation phase (OD 600 nm), and 3) when the biofilm assay was established (OD, 570 nm). The biofilm computation was normalized against the bacterial growth data using a ratio of 570: 600. An OD value of 0 indicated a negative value. Every test was run three times in duplicate.

Microtiter biofilm eradication assay. A technique to assess the consequences of DMSO application to pre-generated biofilms (Mature biofilm) was designed which involved using CV staining together with a metabolically activated tetrazolium dye (20). 200 µL bacterial suspension (0.5 McFarland) in the TSB was pipetted into the inside wells of a 96-well microtiter plate so as to create biofilms. Two microtitre plates in parallel were prepared, one was stained with CV and the other was treated with a metabolic dye, triphenyl tetrazolium chloride (TTC).

The two plates were placed in a temperature of 37°C for overnight incubation to enable adherence and growth of the biofilm. The next day, planktonic and unattached cells were aspirated from each well; the adhering biofilm was then washed three times with 150 µL of fresh sterile media using a multi-channel pipette.

Any surplus medium was aspirated and further aliquots of sterile media were then added to the individual wells, together with DMSO aliquots of varying concentrations, i.e. 1%-8%. In static conditions, the plates underwent overnight incubation at 37°C. Documentation of the OD600 was carried out the next day, which provided a measure of the accrued bacterial growth which comprised attached biofilm and planktonic cells. The latter, together with the used medium, were discarded, and the residual biomass was again washed thrice using distilled water. CV staining was applied to the plates for quantification of biofilm biomass, as detailed previously.

Metabolic parameters in relation to the second plate were then evaluated using TTC as a marker for viable bacteria (20). TTC is converted into a colored derivative of formazan in the presence of notable cellular metabolic activity. Quantification of the formazan derivative reflects the number of PA viable cells present.

The process followed was equivalent to that for the CV assay. The plates were aggressively stirred to remove any non-adherent organisms after an overnight incubation at 37°C. All of the wells had their media removed after the incubation times. A single rinse in 200 µL of phosphate-buffered saline (PBS) was performed on the adhering biofilm. To the wells containing biofilm and a suspension of biofilm created by vigorous pipetting, an additional 100 µL PBS was added. After that, this was moved to a fresh 96-well plate. To each well, 50 µl of 0.1% TTC (Sigma, USA) was applied. The experiment was run for 4-5 hours at 37°C, and the OD450 was measured at that time. Any decrease in bacterial growth might be calculated by calculating the colorimetric absorbance at 450 nm, as the presence of live bacteria causes a reduction in TCC and the subsequent formation of red formazan.

Gene expression tests associated with the construction of biofilm. The effect of varying DMSO titers on relative QS-regulatory gene expression was computed utilizing a Real-Time Quantitative Reverse Transcription Polymerase chain reaction (qRT-PCR) to establish the molecular constituents of the QS-regulatory genes. Extraction of the entire bacterial RNA from PA was performed at the central point of the log phase, i.e. equivalent to OD600 of 0.5-0.6, in the presence of low concentration (1% v/v) and a relatively high concentration (8% v/v) DMSO solutions in combination with a MH medium. Controls lacking DMSO underwent an identical procedure. During the exponential biofilm accumulation phase, i.e. following 24-48 hours' contact, a careful collection of the biofilm was carried out; this was then rinsed in 10 mM sodium chloride in order to eradicate any free cells. The biofilm RNA was extracted with the use of a RNeasy Mini Kit (Qiagen, Germany). The concentration of RNA was then quantified (ng/µL) using a Nanodrop ND-1000 instrument (Nanodrop Products Inc., Wilmington, NE). The latter also facilitated confirmation of the purity of the RNA at 260 nm and 280 nm absorbance.

A qRT-PCR, conducted at a temperature of 42°C and using random primers, RNaseOUT, dNTPs and SuperScript II reverse transcriptase (EasyScript, transgenbiotech, China), was utilized to manufacture cDNA. A BIO-RAD thermal cycler was used to carry out qRT-PCR. Amplification was achieved in a 20 µL reaction volume comprising an admixture of 2 µL template DNA, 0.5 µL forward and 0.5 µL reverse

primers,10 µL Luna Universal qPCR Master Mix and 7 µL nuclease-free water.

All PCR primers (Table 1) were subject to a gradient PCR reaction. The conditions for cycling were as follows: pre-denaturation cycle, 95°C, 3 minutes; 34 denaturation cycles, 94°C, 30 seconds; annealing, 50-63°C, 30 seconds; elongation, 72°C, 60 seconds; extension, 72°C, 5 minutes. Normalization of the relative QS-regulatory gene expression values was carried out in relation to *rpoD*, the housekeeping gene (21). The precise degree of PCR amplification was estimated using agarose gel electrophoresis. Analysis of the relative gene expression in cultures both with and without DMSO were conducted, following the 2-ΔΔCt procedure (21).

Statistical analysis. The test data results were obtained from a minimum of three replicate experiments, and presented as mean ± standard error. One-way analysis of variance (ANOVA) was used to evaluate differences between samples and controls where appropriate. Tukey's test was used to provide a comparison of the OD values obtained from the microtiter plate studies. The significance value was set at < 0.05. GraphPad InStat 6.0 software was used for data analysis.

Table 1. Primers used in assess the expression of quorum sensing genes

Gene	Primer sequence
ropD	F 5′-CGAACTGCTTGCCGACTT-3′
	R 5′-GCGAGAGCCTCAAGGATAC-3
lasI	F 5′-CGCACATCTGGGAACTCA-3′
	R 5′-CGGCACGGATCATCATCT-3′
lasR	F 5′-CTGTGGATGCTCAAGGACTAC-3′
	R 5′-AACTGGTCTTGCCGATGG-3′
rhlI	F 5′-GTAGCGGGTTTGCGGATG-3′
	R 5′-CGGCATCAGGTCTTCATCG-3′
rhlR	F 5′-GCCAGCGTCTTGTTCGG-3′
	R 5′-CGGTCTGCCTGAGCCATC-3′
pqsA	F 5′-GACCGGCTGTATTCGATTC-3′
	R 5′-GCTGAACCAGGGAAAGAAC-3′
pqsR	F 5′-CTGATCTGCCGGTAATTGG-3′
	R 5′-ATCGACGAGGAACTGAAGA-3′
lasB	F 5′-GGAATGAACGAAGCGTTCTCCGAC-3′
	R 5′-TGGCGTCGACGAACACCTCG-3′

RESULTS

DMSO: MIC and MBC. In the test tubes containing DMSO in concentrations of 25% and 50%, there was an absence of turbidity following aerobic incubation over a 24-hour period at a temperature of 37°C, suggestive of PA inhibition. At DMSO titers ≤ 12.5%, the solution demonstrated some turbidity. For the 25% and 50% DMSO concentrations, the contents of these tubes were plated onto sterile agar using the streak plate technique to inoculate the bacteria under investigation. Incubation was carried out at 37°C over a 24-hour period. An MBC of 50% was determined, as evidenced by the presence of bacterial growth in the 25% but not in the 50% dilutions of DMSO (Fig. 1).

DMSO impact of PA growth and biofilm formation and eradication. All the PA strains were shown by the microtiter plate assay to be potent generators of biofilm. When the PA strains were cultured with 1%-8% v/v DMSO titers, PA growth was suppressed, although the effect was variable amongst the differing strains. ANOVA demonstrated significant inhibition at high DMSO concentrations ($F(4, 16.64) = (6.659)$, $p = 0.002$). Statistically significant growth inhibition was seen in strains PA1, PA2, PA3 and PA4 ($p < 0.05$) with DMSO strengths of 6% and 8% v/v. PA5 and PA6 showed growth suppression in DMSO dilutions of 4%, 6% and 8% v/v (Fig. 1).

Fig. 2 illustrates the impact of DMSO on the formation of the biofilm. Biofilm formation inhibition was dictated by the DMSO concentration. ANOVA revealed that DMSO titers of 6% and 8% v/v affected biofilm generation in ATCC, PA1, PA3 and PA6 ($F(9, 22.64) = (11.659)$, $p=0.002$); a DMSO concentration of 8% v/v led to significant biofilm formation inhibition of the other PA strains (Fig. 2).

The biofilm eradication assays, designed to study the effect of DMSO on 1-day old pre-generated biofilms, demonstrated that the PA biomass was significantly influenced by 6% and 8% v/v DMSO concentrations in strains ATCC and PA2; four strains were only affected by 8% v/v DMSO. The strain, PA4, was unaffected by any of the DMSO strengths to a significant level (Fig. 3).

The day-old PA biofilm demonstrated a fall in metabolic activity at DMSO titers which were less than those required to suppress the biomass. Concentra-

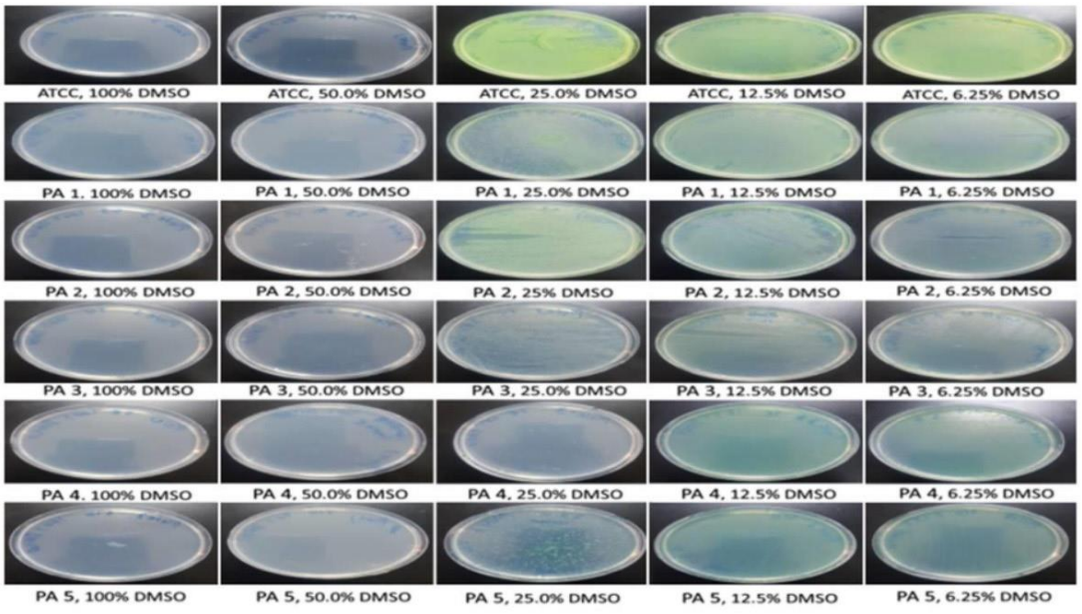

Fig. 1. Growth of the PA strains, ATCC and PA1-PA6 following the incubation with 1%-8% v/v DMSO over 24 hours of incubation. Y-axis: OD, 600nm; x-axis: DMSO concentration. ****<0.0001, ***0.0001, **<0.001, *<0.01.

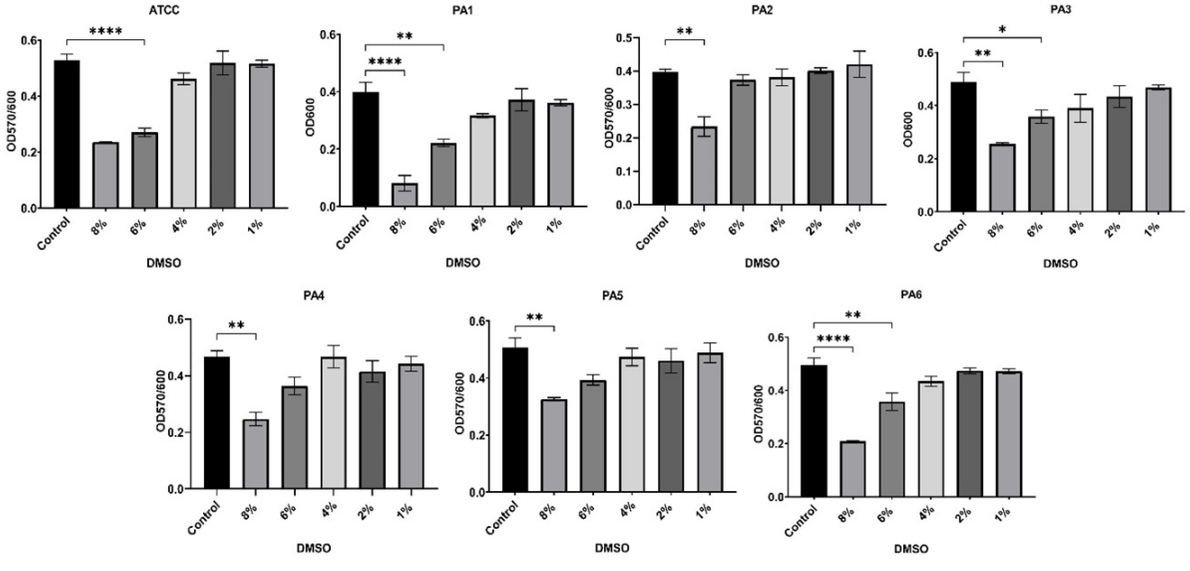

Fig. 2. Biofilm formation for PA strains, ATCC and PA1-PA6, following the incubation with 1%-8% v/v DMSO over 24 hours of incubation. Y-axis: OD, 570/600nm; x-axis: DMSO concentration. ****<0.0001, ***0.0001, **<0.001, *<0.01.

tions of DMSO of 2%-8%, 4% and 6% v/v caused a decline in metabolic activities of strains PA1 and PA5, ATTC and PA3 and PA2, PA4 and PA6, respectively (Fig. 4).

QS-regulated gene expression. The Ct values were used as a reference to determine the relative expression of a number of QS-regulatory genes, i.e. *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsR*, *LasB* and *pqsA*. Normalization of mean relative measures of the genes under investigation was carried out with respect to the mean relative quantity of the reference gene, *ropD*, within the identical sample.

A comparison of the relative degrees of expression for cultures both with and without DMSO administration was performed; the minutiae were assessed. Fig. 5

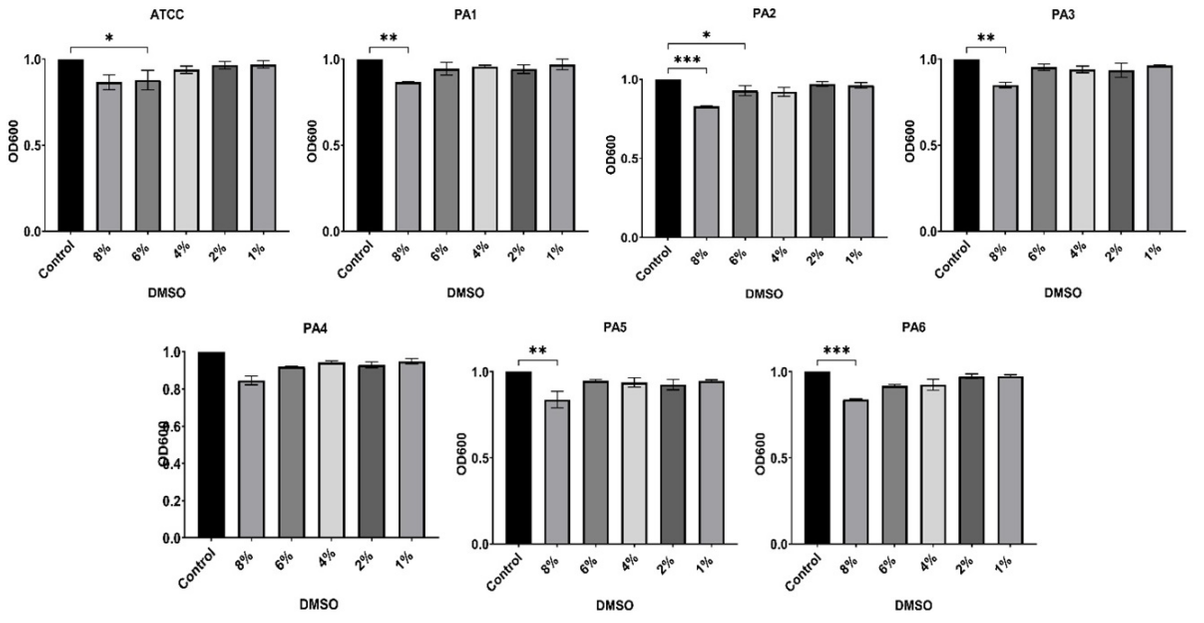

Fig. 3. The impact of DMSO on Mature biofilm biomass compared to control following the incubation of 1%-8% v/v DMSO over 24 hours of incubation using Crystal Violet staining. X-axis: PA strains, Y-axis represents the percentage of biofilm biomass compared to control (100%). ***0.0001, **<0.001, *<0.01, ns: not significant.

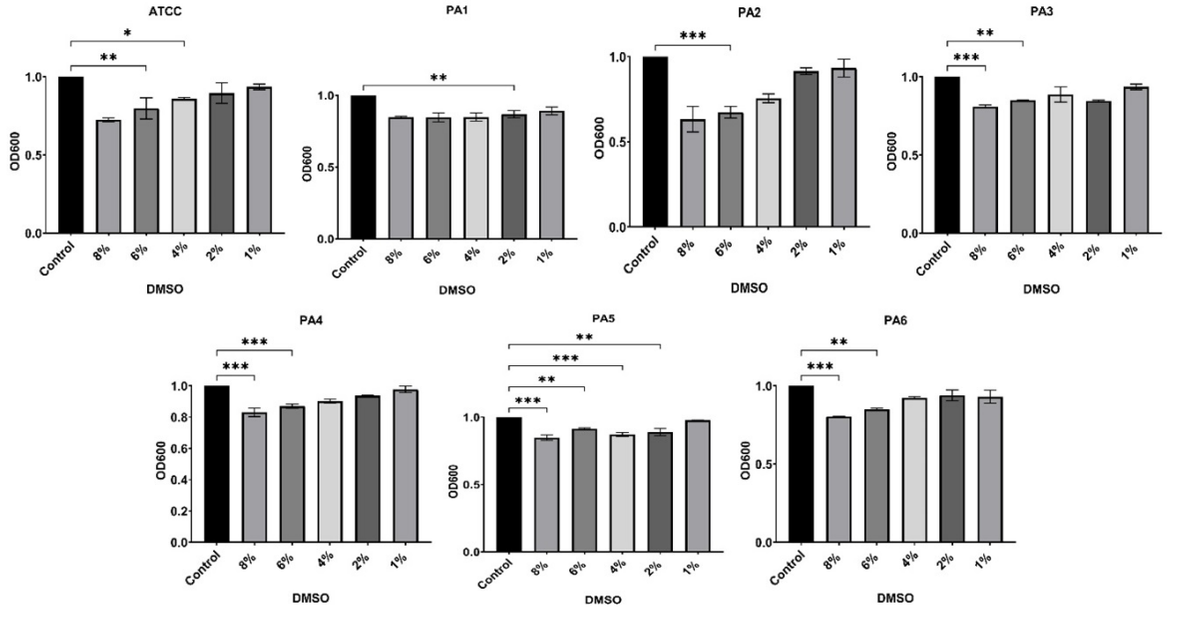

Fig. 4. The impact of DMSO on PA Mature biofilm metabolic activity compared to control following the incubation of 1%-8% v/v DMSO over 24 hours of incubation using triphenyl tetrazolium chloride. X-axis: PA strains, Y-axis represents the percentage of metabolic activity compared to control (100%). ***0.0001, **<0.001, *<0.01, ns: not significant.

indicates alterations in gene expression.

Following culturing with 8% v/v DMSO strength, each strain displayed a significant downregulation of expression of all the QS-regulatory genes (ANOVA test F (12, 42) = 5.228, P<0.0001). Las system was the most affected compared to other system especially LasI and LasR genes. When a DMSO titer of 1% v/v was applied, there was a trend towards expression decline but this did not reach statistical significance (Table 2).

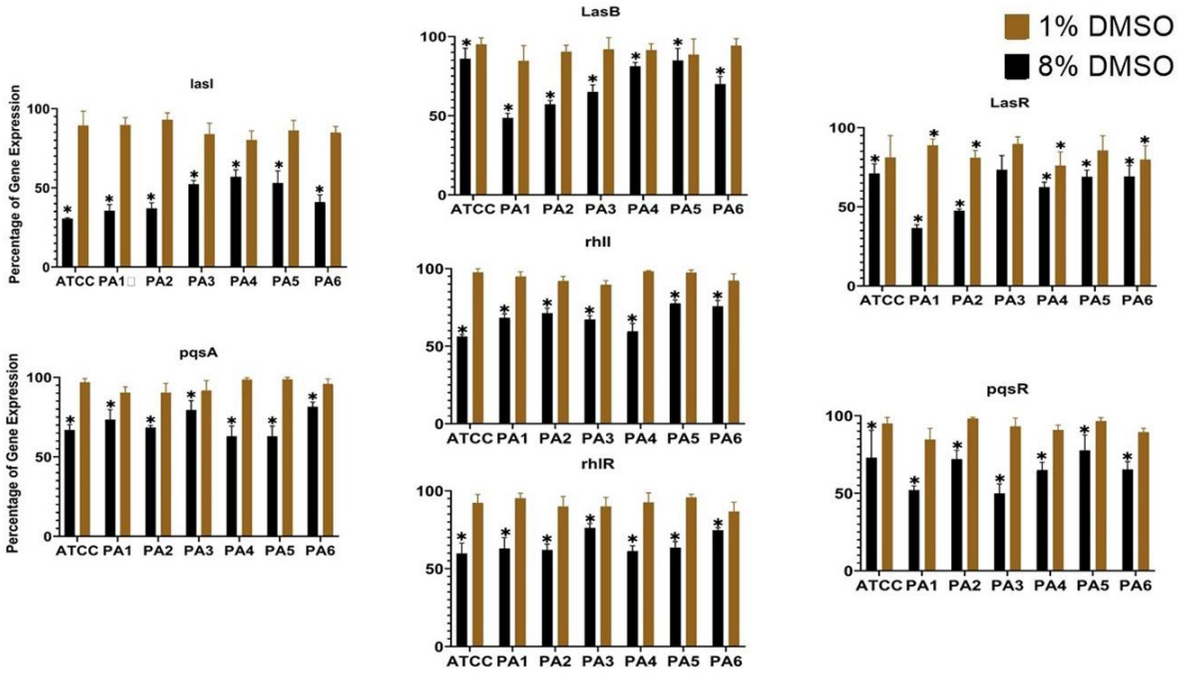

Fig. 5. Relative (% versus controls) quorum sensing-regulatory gene expression (y-axis) in PA strains following the incubation with 1%-8% v/v DMSO versus controls (no DMSO) over 24 hours of incubation. X-axis: PA strain. *<0.01.

Table 2. Percentage of QS-gene inhibition in PA strains following the incubation with 1%-8% v/v DMSO versus controls (no DMSO,100%) over 24 hours of incubation. X-axis: PA strain.

	LasR		LasB		LasI		pqsA		pqsR		rhlI		rhlR	
	8% DMSO	1% DMOSO	8% DMSO	1% DMOSO	8% DMSO	1% DMOSO	8% DMSO	1% DMOSO	8% DMSO	1% DMOSO	8% DMSO	1% DMOSO	8% DMSO	1% DMOSO
ATCC	28.9 %	18.8%	14.1%	4.9%	69.4%	10.6%	33.2%	2.9%	27.1%	5%	43.8%	2.4%	40.3%	7.6%
PA1	63.3%	11.2%	51.4%	15.3%	69.3%	10.3%	26.7%	9.6%	47.9%	20%	34.7%	5.1%	37%	4.8%
PA2	52.4%	24.3%	42.8%	11.9%	66.7%	7%	31.5%	9.6%	27.9%	1.9%	32.4%	8%	38%	10%
PA3	26.6%	10.3%	34.9%	8%	54.6%	16.1%	20.5%	8.4%	50.2%	6.8%	38%	15.2%	23.8%	10%
PA4	37.6%	24%	18.7%	10.3%	74.9%	19.9%	37%	1.3%	35%	12.5%	44.4%	1.7%	38.6%	7.4%
PA5	31%	14.5%	15.1%	11.5%	52%	13.7%	37%	1.1%	22.4%	3.5%	34.8%%	2.4%	36.6%	4.2%
PA6	30.8%	20.2%	30.1%%	5.7%	62.1%	15.1%	18.5%	4.1%	34.7%	16.1%	32.3	7.8%	25.2%	13.2%

DISCUSSION

DMSO is frequently employed as a solvent to dissolve conventional antimicrobial agents and additional anti-infective compounds which do not easily form an aqueous solution (22). It is advised for utilization in antimicrobial susceptibility examinations as a solvent for the dissolution of insoluble antimicrobial agents by the Clinical & Laboratory Standards Institute (23). However, for over half a century, DMSO itself has been recognized as having a number of pharmacological features, such as antibiotic properties (24).

Thus, it is essential to determine whether DMSO per se is a promising agent for use against infective organisms. Additionally, it has been noted that despite the excellent solubilizing characteristics of DMSO for traditional antimicrobials and new anti-infective compounds under evaluation, using it as a research tool may engender a risk of the DMSO causing bacterial growth inhibition rather than the compound being tested, which may lead to false positive outcomes for the latter (25).

Figs. 1 and 2 illustrate the impact of DMSO on the

growth and biofilm formation of PA, both of which are significantly affected by high titers of DMSO. At more dilute DMSO strengths, bacterial growth and biofilm generation were unaffected significantly. However, there was a generalized inhibition compared to the control.

The current findings are in keeping with earlier studies Kirkwood, Millar (26) in which researchers became aware that DMSO concentrations of between 5% and 10% had the capacity to inhibit the replication of a number of bacteria, such as *Mycobacterium tuberculosis.* The present data substantiate those published by Randhawa (27). In the latter study, an agar diffusion technique was used to investigate the response of dermophytes to a spectrum of DMSO titers, i.e. 0.125%-10%. At the highest concentration, there was no fungal growth observed, but at strengths of 1.25%-5%, the growth of the fungi was linearly correlated with the DMSO titer; this pattern was not seen in the control sample. The response to DMSO varied among different species (27). A further study by Wu, Wang (28) acquired similar data regarding *Escherichia coli.* Although only a modest decline in growth rate was observed over 6 hours in the presence of 5% DMSO, a significant decrease in viable bacteria was noted when 10% DMSO was utilized over the same duration. Mature PA biofilms demonstrated a diminished attached biomass and metabolic activity according to the DMSO strength applied. At titers of 8% v/v DMSO, there was a 20% loss of biomass seen on CV staining; at 5% v/v DMSO there was only a negligible impact when judged against controls with no exposure to DMSO. Where concentrations in excess of 6% v/v were applied, five strains of PA demonstrated significant inhibition of metabolic activity.

The bacterial planktonic cells were more vulnerable to the effects of DMSO than the cells within the biofilm, evidencing increased inhibition at equivalent or lesser DMSO titers and thus, potentially demonstrating the protective nature of the biofilm configuration. A number of studies have demonstrated that biofilms exhibit increased resilience to antibiotic therapy than planktonic cells, an observation that may additionally be relevant to contact with DMSO (29). When contrasted against planktonic cells, bacterial cells within a biofilm have different biological functions, metabolic pathways and stress reactions (30). Mature biofilms also exhibit greater resistance to toxins, such as antibiotics, than early stage biofilms and planktonic cells (31).

The generation of biofilm and its maintenance is facilitated by QS in PA; this is also necessary for the bacteria to become attached (32). In PA, initial studies that determined the role of QS in biofilm formation observed that the produced biofilm was less thick in the *lasl* mutant, and had increased vulnerability to being destroyed by detergents (33). We hypothesized that DMSO exerts a quorum quenching effect and so qRT-PCR was utilized to quantify relative QS-regulatory gene expression; these genes govern the generation of biofilm in PA. The results indicated downregulation of the expression of the QS-regulatory genes, *lasI, lasR, rhlI, rhlR, pqsR* and *pqsA*, in the presence of 8% v/v DMSO. The currently noted biofilm formation decrease and the data obtained following the RT-PCR was in keeping with results published by Yahya, Alias (12). These researchers noted that DMSO acted as a key polar aprotic solvent to alter the expression of numerous genes associated with the QS in a non-inhibitory titer, i.e. 2% v/v, for an alternative PA strain (PAO1). They surmised that there was a notable negative influence of DMSO on the synthesis of the PA virulence factor mediated by QS with effects on the establishment of the biofilm, together with elastase, LasA protease, rhamnolipid and pyocyanin manufacture, all of which are under the governance of the rhl QS system (12).

DMSO has been reported to be an organic solvent with a relatively low toxicity level in an in vivo study, with LD50 (a lethal dose to 50% of a large number of test animals) values obtained of 6.2 mL/kg in mice, and 9.9 mL/kg in rats (34). Maximal DMSO strengths of 40% have been used safely for oral and topical routes of administration for the therapy of human pathologies (35); the United States Food and Drug Administration have sanctioned topical indications to a maximum strength of 50% (22). A number of characteristics support to the potential of DMSO to be used as an antibiofilm agent, e.g. the low incidence of systemic adverse effects, its efficacious metabolism in humans (36), and our finding indicating its antimicrobial and anti-biofilm actions. Thus, DMSO could forms a possible approach to counter pathologies related to the presence of biofilms.

In addition, given that DMSO is frequently employed as a solvent when antibiotics or alternative antimicrobial agents are being evaluated, the current data indicate that during such experiments, an appropriate control should be utilized so that any possible

bacterial inhibitory effects of DMSO are recognized. It has also been recommended that the maximum DMSO strength used in such studies should be no greater than a 1: 10 dilutions, and that a neat solution should be avoided when assessing certain bacteria.

A further issue is the potential for the interaction of DMSO with other antibiotics. One study observed that a number of reactive oxygen species (ROS)-dependent antibiotics are inhibited by DMSO when used against *E. coli* (37). The performance of six antibiotics in the presence of DMSO against PA was ascertained by Guo, Wu (38). In contrast to the previously described study, the sensitivity of PA was enhanced with respect to ciprofloxacin at DMSO titers of 1% or 2%, with a fall in MIC measured from 0.4 g/mL to 0.2 g/mL. A modest reduction in PA sensitivity was noted in relation to ceftazidime and chloramphenicol, with a doubling of the MIC. The efficacy of tetracycline, carbenicillin and meropenem were unchanged (38). Therefore, DMSO's effect on antimicrobial agents may be specific to both the antibiotic and to the species of bacterium, a fact which should be taken into account when prescribed simultaneously and need further studies.

Another consideration is that any solvents involved in biological investigations should not only offer an optimum degree of solvation, but be compatible with the system under investigation, e.g. non-aqueous solvents may be toxic to the tested bacterium. Toxicity assessments, which should incorporate a control arm, should therefore be carried out before a formal study is commenced in order to determine the highest solvent strength that can be applied safely. It should additionally be noted that solvents may have a selective action, and that for similar concentrations, differing bacteria may exhibit varying susceptibilities. Thus, the optimal method should only encompass use of organic solvents in assays at the maximum feasible dilution.

The way in which DMSO affects bacterial growth has not been investigated in the current study. However, there is increasing data to indicate that the MIC cannot be evaluated in the presence of DMSO owing to its capacity to scavenge radicals (24). Reactive oxygen species are major actors in the ability of antimicrobials to kill bacteria (39). Furthermore, organic solvent biochemical activity is key with respect to their transfer and location within bacteria, and thus their contact with enzyme systems, which may engender conformational abnormalities and inhibition of active sites, and therefore lower the immunity of the cell in stressful circumstances (40). DMSO may also alter phospholipid system fluidity within the membrane non-polar core. DMSO, at a strength of 0.5%, was shown to cause a 25% rise in phospholipid system rigidity, which could act as a barrier to key nutrients required for growth entering the cells. It remains uncertain whether this is a major component of the growth inhibitory properties of DMSO but it may apply in this instance (40).

CONCLUSION

The aim of this study is to draw attention to the appropriate selection of solvents for difficult-to-solubilize anti-infective compounds, and to ensure that such solvents do not adversely affect the assay or system under investigation. Such precautions are of vital importance, as any possible antimicrobial or anti-inflammatory properties exhibited by the solvent carrier may have an adverse effect on experiments and on the effectiveness of de novo pharmacological agents, concealing the true properties of the drug being tested. The current data are therefore of interest to academics in this sphere, as they offer a foundation for future studies, and facilitate the circumvention of study design hazards that could be expensive in terms of cost and time when poorly soluble novel antimicrobial molecules are appraised.

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