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Research article

Biofilm Formation and Doripenem Susceptibility in Clinical *Pseudomonas aeruginosa*: Anti-Biofilm Effect of Sub-MIC Doripenem

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ABSTRACT

Pseudomonas aeruginosa is a major cause of burn, wound, and urinary tract infections. It poses a serious threat to global public health. Biofilm formation by this pathogen may contribute to its reduced susceptibility to carbapenems. *P. aeruginosa* was isolated from 390 clinical specimens and 50 healthy skin swabs. The 72 isolates were isolated and tested for susceptibility to six antibiotics by disk diffusion and for biofilm formation by the microtiter plate assay. The Pearson correlation coefficient between biofilm OD590 and doripenem inhibition zone diameter was calculated. Six doripenem-resistant, strong biofilm-producing isolates were exposed to doripenem from 1/2 to 1/2048 MIC. *P. aeruginosa* was isolated from 72 of 336 culture-positive clinical specimens (21.4%) and from none of the healthy controls. The present study showed that nineteen isolates (26.4%) were resistant to doripenem. Biofilm formation correlated negatively with doripenem zone diameter ($r = -0.328$, $P = 0.0049$). At 1/2 MIC, doripenem reduced biofilm in all six isolates by roughly 40–83%, and biofilm remained below the control at 1/32 MIC in all of them. Some isolates produced more biofilm at certain sub-MICs. Stronger biofilm formation was modestly associated with reduced doripenem susceptibility, and sub-MIC doripenem can limit biofilm formation in resistant isolates, although the effect varies between strains.

Keywords: Biofilm formation; carbapenem resistance; *Pseudomonas aeruginosa*; sub-inhibitory concentration.

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1. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen that causes burn and wound infections as well as complicated and catheter-associated urinary tract infections (UTIs) [1, 2]. Its clinical success rests on intrinsic resistance mechanisms, adaptability to hostile environments, the ability to form biofilms, and a high frequency of multidrug resistance [3,4]. Moreover, the ability of *P. aeruginosa* to cause disease is closely related to multiple virulence factors, such as extracellular proteases, elastases, rhamnolipids, lipopolysaccharides, and secretion systems, all of which facilitate damage to host tissue [5], the ability of *P. aeruginosa* to survive in the host and evade the host immune system, which happens because of the ability of this pathogen to form biofilms [6]. Carbapenems remain among the most important agents for treating serious *P. aeruginosa* infections, yet resistance to them is increasing. Resistance arises through reduced membrane permeability, efflux pump overexpression and enzymatic degradation, including carbapenemases such as New Delhi metallo-β-lactamase (NDM) [7]. Doripenem is a broad-spectrum carbapenem, but data on how resistance to it relates to other bacterial traits in clinical isolates are limited [8].

Biofilm formation is one trait that may be linked to reduced susceptibility. The extracellular polymeric matrix limits antibiotic penetration, and embedded cells show altered gene expression

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reduced metabolic activity, and greater stress tolerance [9]. Carbapenem-resistant isolates are often strong biofilm producers, which contributes to persistent infection and treatment failure [10]. Still, biofilm biomass and resistance do not always show a simple linear relationship [9], so the association needs to be tested for each drug and each isolate collection. Antibiotics at concentrations below the minimum inhibitory concentration (sub-MICs) can modulate bacterial physiology, including adhesion, quorum sensing and matrix production, without killing the cell [9, 11]. Whether sub-MIC doripenem reduces biofilm formation in doripenem-resistant *P. aeruginosa* has not been clearly shown.

The aims of this study were therefore to (i) isolate and identify *P. aeruginosa* from clinical specimens and healthy skin, (ii) determine the susceptibility of the isolates to six antibiotics, with emphasis on doripenem, (iii) test the relationship between biofilm formation and doripenem susceptibility, and (iv) assess the effect of sub-MIC doripenem on biofilm formation by strong biofilm-producing, doripenem-resistant isolates.

2. MATERIALS AND METHODS

2.1. Specimens, isolation and identification

A total of 390 clinical specimens were collected from patients with urinary tract infections (n = 143), burn wounds (n = 180) and other clinical conditions (n = 67). Skin swabs from 50 healthy individuals served as the control group. The Ethics Committee of the University of Baghdad, Baghdad, Iraq (CSEC/1025/0122; October 12, 2025), approved this study, which was a retrospective analysis of routinely collected clinical data; individual patient consent was waived in accordance with national ethical guidelines and the Declaration of Helsinki. All specimens were cultured aerobically on MacConkey agar and cetrimide agar at 37 °C for 24–48 h. *P. aeruginosa* was identified by colony morphology and phenotypic features: greenish pigmentation, smooth flat colonies, and a grape-like odor. The VITEK 2 system (bioMérieux, Marcy-l'Étoile, France) with the ID-GNB card was used for identification. Purified isolates were stored at −20°C in nutrient broth supplemented with 20% glycerol. The authors followed good laboratory practices when handling the biohazardous materials used in the current study [12, 13].

2.2. Antibiotic susceptibility testing

Susceptibility of 72 *P. aeruginosa* isolates was tested using the Kirby-Bauer disk diffusion method according to CLSI guidelines. The discs were doripenem (DOR, 10 µg). Inhibition zone diameters were measured in mm and interpreted as susceptible (S), intermediate (I), or resistant (R) using CLSI breakpoints [CLSI edition to be specified]. A 6 mm diameter corresponds to the disc itself, indicating no inhibition [14].

2.3. Biofilm formation assay

Biofilm production was determined using the microtiter plate crystal violet method in flat-bottom polystyrene 96-well plates. In short, 100 µL of Tryptic Soy Broth (TSB; 0.25 g/L glucose, HiMedia, India) along with 5 µL of a bacterial suspension at optical density (OD600) of 0.5 was incubated at 37°C for 24 h. After incubation, the wells were washed with phosphate buffer solution and air-dried at 65°C for an hour. Then, the wells were stained with 0.4% crystal violet for 15 minutes, and again the wells were washed with anhydrous ethanol and destained. OD was recorded at 590 nm using micro-plate reader (BioTek 800 TS; Winooski, USA). All the experiments were performed in triplicate and biofilm classification was done using the cut-off OD590 values [12]. The association between biofilm formation (OD590) and inhibition zone diameter (mm) was tested with Pearson's correlation coefficient (r). $P < 0.05$ was considered significant.

2.4. Minimum inhibitory concentration of DOR

The minimum inhibitory concentrations of DOR against *P. aeruginosa* isolates were evaluated using a round-bottom polystyrene microtiter plate and a spectrophotometric method to measure crystal violet. Briefly, a stock solution of DOR (1 mg/mL, PharmaCompass, Italy) was prepared in sterile Mueller-Hinton broth (MHB, HiMedia, India). A two-fold dilution series was prepared. Five microliters of a standard bacterial inoculum, adjusted to an optical density (OD600 nm) corresponding to 1 x 10^8 CFU/mL, was added. MHB with a bacterial isolate was considered the first control; MHB only and wells containing different antibiotic two-fold dilutions were considered the second and third controls. Plates were then incubated at 37 °C for 18 h, and growth was assessed visually. The lowest concentration of each agent that completely inhibited visible growth was considered the MIC [12]. MIC assays were performed in three independent experiments, and the modal MIC across the three runs is reported.

2.5. Effect of sub-MIC DOR on biofilm formation

Six doripenem-resistant isolates that were strong biofilm producers (Pa35R, Pa40R, Pa12R, Pa111, Pa25R and Pa37R) were selected. Each was grown on flat-bottom polystyrene plates (Bioland™, China) in the presence of eleven two-fold sub-MIC concentrations of doripenem, ranging from 1/2 MIC to 1/2048 MIC, alongside an untreated control. Biofilm biomass was quantified by crystal violet staining (OD590; section 2.3), and results are presented as mean ± SD.

2.6. Statistical and Data analysis

Data analysis was performed using Origin 8.6 (OriginLab, Northampton, USA). Biofilm formation was reported as optical density at 590 nm (OD590). The outcome of the study is presented as mean ± SD. Correlation was calculated using Pearson's correlation coefficient, which estimates the strength and direction of the association between the outcome and biofilm (OD590), as well as the size of the inhibition zone by doripenem (in mm). Pearson's correlation was calculated for the relationship between biofilm formation (OD590) and the inhibition zone by doripenem among 72 *P. aeruginosa* isolates, and the r^2 value was provided to represent the degree of association. One-way ANOVA was performed to compare biofilm biomass under sub-MIC conditions with that under control conditions for the same isolate. All studies were carried out independently three times, and a P value less than 0.05 was considered significant.

3. RESULTS

3.1. Isolation *and identification of P. aeruginosa*

Of the 390 clinical specimens, 336 (86.2%) yielded bacterial growth, and *P. aeruginosa* was isolated from 72 of them. This is 18.5% of all clinical specimens and 21.4% of the culture-positive specimens. Only 5 of the 50 skin swabs from healthy individuals grew bacteria, and none grew *P. aeruginosa* (Table 1). The proportion of culture-positive specimens that yielded *P. aeruginosa* was lowest in UTI (16.5%) and highest in burn wounds (23.1%) and other clinical samples (25.9%).

Table 1. Recovery of *P. aeruginosa* from clinical specimens and healthy skin.

Source	Specimens	Bacterial growth	*P. aeruginosa*	% of specimens	% of culture-positive
UTI	143	109	18	12.6	16.5
Burn wounds	180	169	39	21.7	23.1
Other clinical samples	67	58	15	22.4	25.9
All clinical specimens	390	336	72	18.5	21.4
Healthy cohort	50	5	0	0	0

3.2. Antibiotic susceptibility of the isolates

Among the six antibiotics tested on the 72 isolates, imipenem was the most active, with most isolates susceptible. Meropenem and doripenem also showed considerable activity. Resistance was more frequent for amikacin, levofloxacin and ertapenem. Isolates split almost evenly between susceptible and resistant for levofloxacin, and ertapenem was the least effective drug, with only a small share of susceptible isolates. For doripenem, inhibition zones ranged from 6 mm (no inhibition) to 41 mm (Fig. 1A). Nine isolates showed no inhibition at all (Pa69R, Pa37R, Pa2P, Pa8, Pa25R, Pa48R, Pa83R, Pa40R and Pa39N). When classified as in Figure 2, 47 isolates (65.3%) were susceptible, 6 (8.3%) intermediate and 19 (26.4%) resistants to doripenem.

3.3. Biofilm formation and DOR susceptibility

Biofilm formation varied widely among the isolates, from an OD590 of about 0.11 (Pa39N) to about 1.10 (Pa39R) (Fig. 1B). The three strongest producers were Pa39R and Pa2P (both about 1.1) and Pa37R (about 0.84). Pa37R also showed no inhibition zone for doripenem.

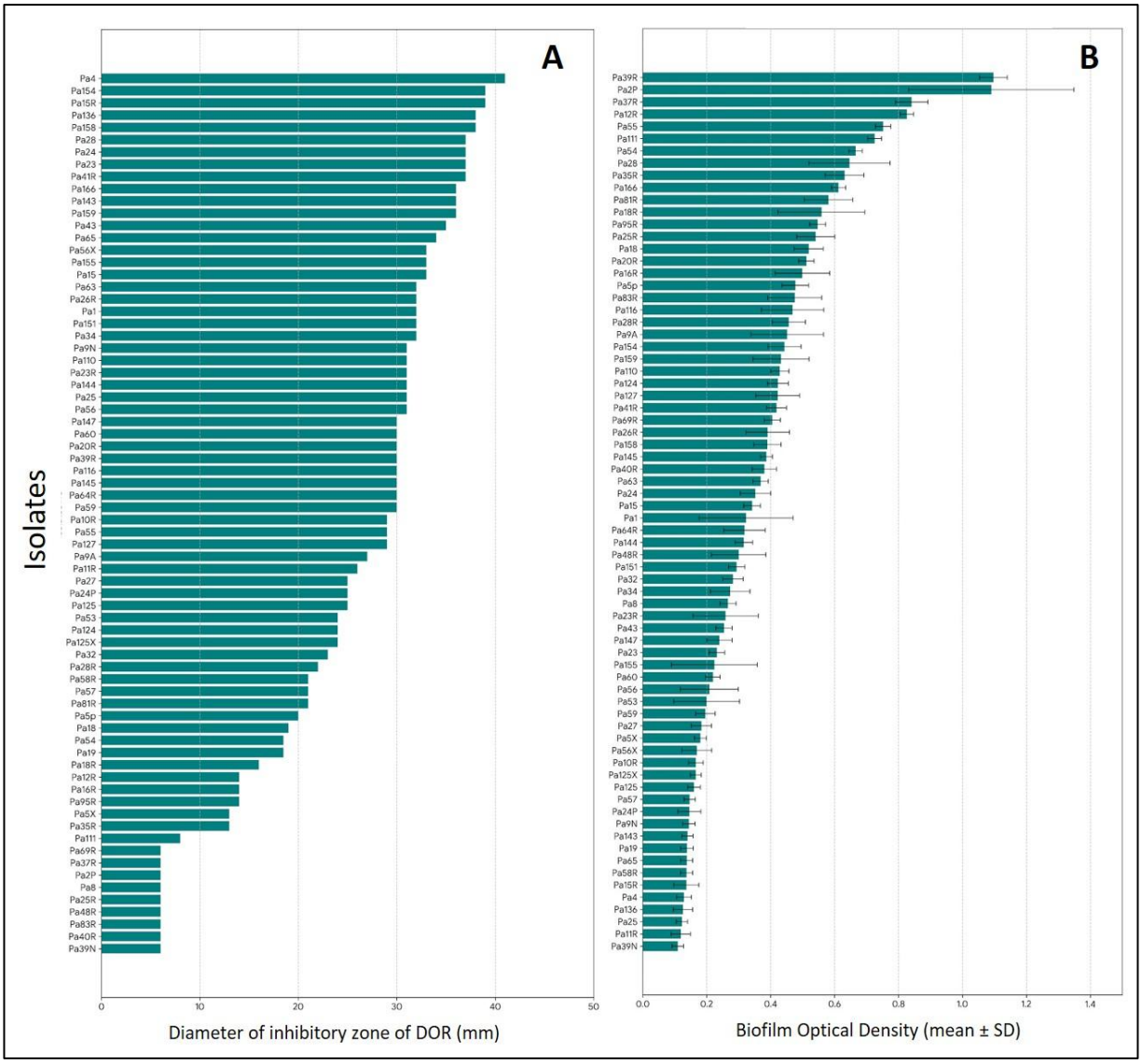

Fig 1. (A) Doripenem inhibition zone diameter and (B) biofilm formation (OD590, mean ± SD) of the 72 *P. aeruginosa* isolates.

When the isolates were ranked by biofilm OD590 and colored by doripenem category, resistant isolates were concentrated among the strong producers (Fig. 2). Eleven of the 19 resistant isolates (58%) were among the 18 strongest biofilm producers, which are the top quartile of the collection. Susceptible isolates were concentrated at the low end. There were clear exceptions: Pa39R was the strongest producer yet remained susceptible (30 mm), while Pa39N was resistant with the lowest biofilm OD.

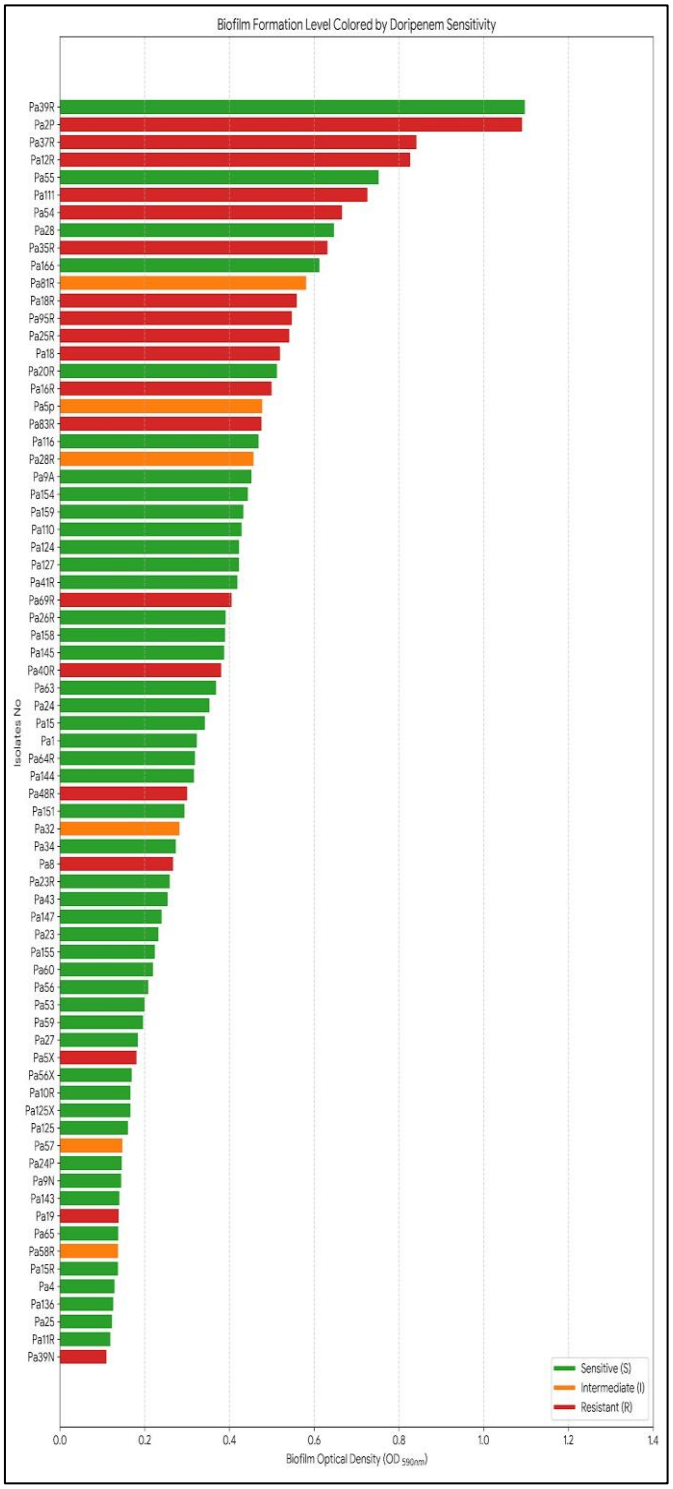

Fig 2. Biofilm formation of the 72 isolates in descending order, colored by doripenem category (green susceptible, orange intermediate, red resistant).

Biofilm OD590 and doripenem inhibition zone diameter were negatively correlated across the 72 isolates (Pearson r = −0.328, P = 0.0049; Fig. 3). Isolates producing more biofilm therefore tended to show smaller inhibition zones. The coefficient of determination ($r^2 \approx 0.11$) shows that biofilm biomass accounts for only about 11% of the variation in doripenem zone size.

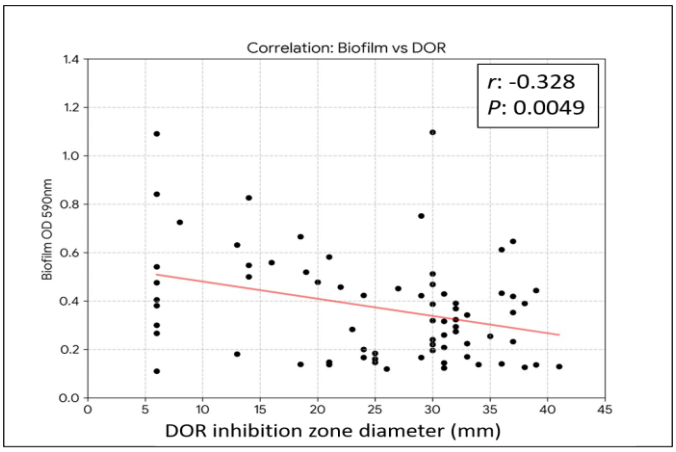

Fig 3. Correlation between biofilm formation (OD590) and doripenem inhibition zone diameter (mm) for 72 isolates; r = −0.328, P = 0.0049.

3.4. Effect of sub-MIC DOR on biofilm formation

Fig. 4 portrays the effect of sub-MIC concentrations of doripenem (1/2 to 1/2048 MIC) on biofilm formation by six doripenem-resistant and strong biofilm-producing isolates of *P. aeruginosa* (Pa35R, Pa2R, Pa12R, Pa111, Pa54R and Pa37R) on polystyrene microtiter plates. Untreated controls displayed various OD590 values of 0.67 for Pa35R and Pa54R and 1.14 for Pa2R (Table 2).

Doripenem reduced biofilm formation among all six isolates, and the effect was greatest at the highest sub-MICs. At 1/2 MIC, the OD590 values for Pa35R and Pa37R were approximately 0.14 and 0.20, respectively; for Pa12R and Pa54R, 0.21 and 0.25, respectively, and for Pa111 and Pa2R, 0.25 and 0.21, respectively. These corresponded to reductions of approximately 70–84% when compared to their controls. Furthermore, all of these values were statistically significant. The reduction remained significant in all six isolates, albeit slightly less, at 1/4 MIC, with OD590 values between 0.15 and 0.46.

Biofilm formation gradually increased in most isolates as doripenem concentration decreased, and OD590 values approached those of the controls at the lowest concentrations. As an illustration of this, the OD590 of Pa37R increased from about 0.14 at 1/2 MIC to about 0.54 at 1/512 MIC, while the control value for Pa37R was approximately 0.86. Significant differences were observed in Pa37R, Pa111, Pa54R, and Pa35R despite these varying concentrations in all of these isolates, with the differences from the control values being non-significant at the lowest concentrations, particularly for Pa35R from 1/128 MIC and for Pa111 at 1/1024 and 1/2048 MIC.

Notably, the response for Pa12R showed some variations, with several of its intermediate and low concentrations not showing any differences from the control. The OD590 value at the concentration of 1/256 MIC was approximately 0.89, and the control OD590 value was only a bit less, at 0.85. All resistant isolates displayed a concentration-dependent reduction in biofilm formation, with doripenem inhibiting their formation at the

strongest sub-MIC concentrations, particularly at 1/2 and 1/4 MIC concentrations, and a significant reduction was still observed in all of the isolates even at the lowest concentration of 1/32 MIC.

Table 2. Biofilm formation (OD590) of six doripenem-resistant *P. aeruginosa* isolates in the untreated control and after treatment with ½ MIC of doripenem (DOR), and the percentage reduction relative to the control. Values are approximate means read from Figure 4, and all reductions were significant compared with the control ($P < 0.05$).

Isolate	Control (OD590)	½ MIC DOR (OD590)	Reduction (%)
Pa2R	1.14	0.24	79
Pa35R	0.67	0.14	79
Pa12R	0.85	0.20	76
Pa111	0.79	0.21	73
Pa54R	0.67	0.20	70
Pa37R	0.86	0.14	84

Reduction (%) = (control − treated) / control × 100.

4. DISCUSSION

P. aeruginosa was recovered from about one in five culture-positive clinical specimens and from none of the healthy skin swabs. This supports its role as an opportunistic pathogen of damaged or compromised tissue rather than a member of the normal skin flora [4]. The highest proportions were in burn wounds and other clinical samples, in line with reports that identify *P. aeruginosa* as a leading pathogen of burn and wound infections because of its virulence, environmental adaptability, and resistance [1, 3]. Its recovery from UTIs agrees with its recognized role in complicated and catheter-associated urinary infections, where biofilm formation and adaptive resistance favor persistence [2].

Carbapenems retained good activity against most isolates, as compared with other antibiotic groups. Doripenem was active against about two-thirds of isolates, yet about a quarter were resistant. This is consistent with carbapenem resistance in *P. aeruginosa* arising from reduced membrane permeability, efflux overexpression and enzymatic degradation [7].

Biofilm production correlated negatively with doripenem zone diameter ($r = -0.328$, $P = 0.0049$), and resistant isolates clustered among the strongest producers. A protective matrix that limits drug penetration, together with altered gene expression, lower metabolic activity and greater stress tolerance of embedded cells, is a plausible explanation [9]. The result also agrees with reports that carbapenem-resistant isolates are often strong biofilm formers, which contributes to persistence and treatment failure in hospitals [10].

The association is modest, however. Biofilm biomass explained only about 11% of the variation in zone diameter, and clear exceptions existed, such as the susceptible but strongly biofilm-forming Pa39R and the resistant but weak producer Pa39N. Resistance in *P. aeruginosa* is multifactorial and involves efflux, porin changes and enzymatic mechanisms that act independently of biofilm biomass [7, 9, 15]. Moreover, a disk-diffusion zone measures the susceptibility of planktonic growth, whereas the biofilm assay was run without antibiotic, so the two readouts reflect different phenomena [16]. The correlation should therefore be read as an association, not as evidence that biofilm causes doripenem resistance. Sub-MIC doripenem lowered biofilm biomass in all six resistant, strong-biofilm isolates at 1/2 MIC and still did so at 1/32 MIC, a concentration

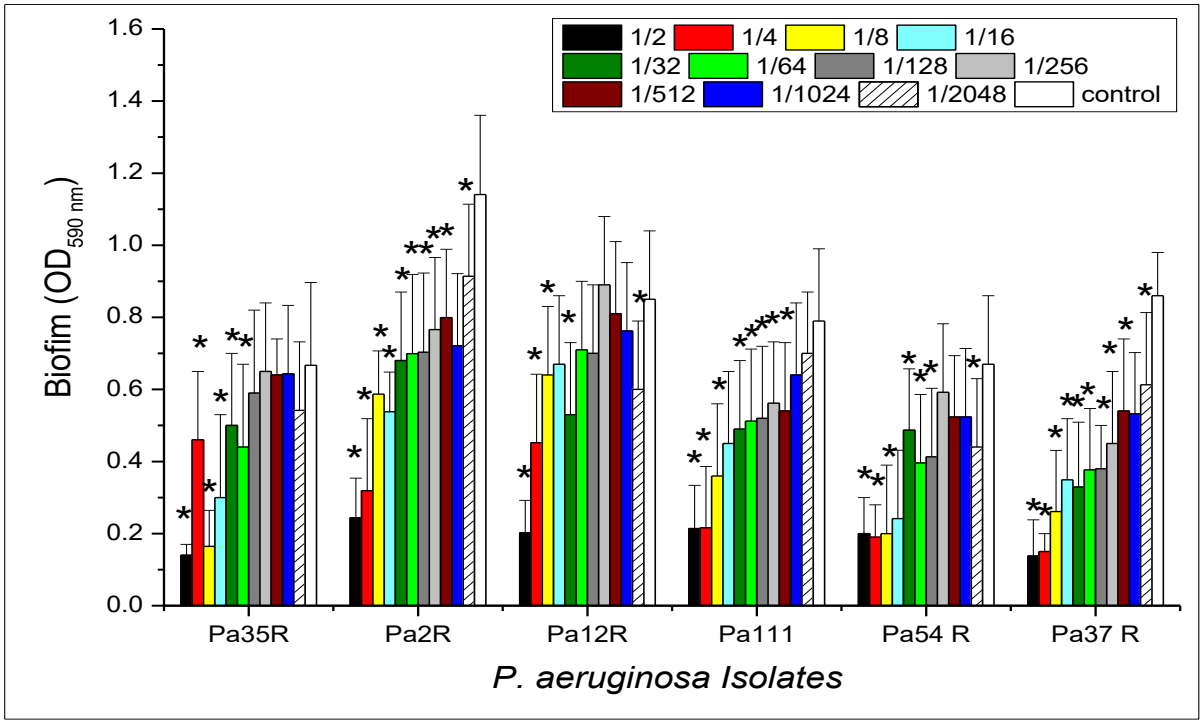

Fig 4. Impact of sub-MIC levels of doripenem (DOR) on biofilm production from doripenem-resistant **P. aeruginosa** isolates (Pa35R, Pa2R, Pa12R, Pa111, Pa54R and Pa37R) using microtiter plates made of polystyrene. Each isolate was exposed to DOR concentrations at ½, ¼, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, and 1/2048 MIC, and the resulting biofilm was determined by crystal violet staining and measured as optical density at 590 nm (OD590). Results are displayed as mean ± SD. '*' denotes significant difference ($P < 0.05$) from the non-exposed control group.

far below that needed to inhibit growth. Pa37R, the isolate with the highest resistance and a high biofilm optical density (OD), showed the clearest graded response. Similar effects of sub-inhibitory antibiotics on biofilm have been reported, and they have been linked to changes in adhesion, quorum sensing and production of extracellular polymeric substances [4, 9]. These mechanisms were not examined here and remain hypotheses for doripenem.

The response was not uniform. Pa35R and Pa12R produced more biofilm than the untreated control at some sub-MICs (1/4 to 1/16 MIC and 1/256 to 1/512 MIC, respectively). This strain-dependent variation shows that sub-MIC exposure cannot be assumed to be beneficial in every isolate, and it should be explored in a larger set of isolates before any therapeutic use is considered.

This study has several limitations. Isolates were identified by phenotypic methods only, and the healthy cohort was small (n = 50) and limited to skin swabs. The MIC was reported for Pa37R only, and the sub-MIC experiment used six isolates in a static polystyrene model. Crystal violet measures total biomass and was not normalized to bacterial growth, so part of the reduction at the higher sub-MICs may reflect growth inhibition. The absolute doripenem concentrations at ½ MIC of a resistant isolate (for Pa37R, 125 µg/mL) are probably well above what can be reached in tissue. Finally, statistical comparison of treated and control biofilms is not reported here. Gene expression, adhesion and matrix assays, and *in vivo* models would be needed to show how sub-MIC doripenem affects biofilm.

5. CONCLUSION

P. aeruginosa was recovered from 21.4% of culture-positive clinical specimens, most often from burn wounds and other clinical samples, and from none of the healthy skin swabs. Stronger biofilm formation was modestly but significantly associated with reduced susceptibility to doripenem ($r = -0.328$, $P = 0.0049$), although biofilm biomass explains only a small part of the variation. Sub-MIC doripenem reduced biofilm formation in six doripenem-resistant, strong-biofilm isolates at 1/2 MIC and down to 1/32 MIC, but some isolates produced more biofilm at certain sub-MICs. Doripenem at sub-inhibitory concentrations may therefore limit biofilm development in resistant *P. aeruginosa*, but the effect is strain-dependent and needs confirmation with growth-normalized assays, statistical testing, mechanistic studies, and *in vivo* models.

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Author contributions

Jaber NA. Conceptualization; data curation; formal analysis; investigation; methodology; and writing – original draft; and Writing – review & editing.

Ghafil JA. Funding acquisition; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing – original draft.

All authors reviewed and approved the final manuscript and agreed to be accountable for all aspects of the work.

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Conflict of interest

The authors declare no conflicts of interest.

Ethical Approval

The Ethics Committee of the University of Baghdad, Baghdad, Iraq (CSEC/1025/0122; October 12, 2025), approved this study

AI Declaration

During the preparation of this work, the authors used Claude AI and Gemini Pro to refine the academic tone and improve the readability of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

Data availability

Data will be made available on request.

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