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Research Article

Evolution of *Pseudomonas aeruginosa* resistance to antibiotics isolated from wound infections and its production of Biofilm

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A B S T R A C T

Introduction: Antimicrobial resistance in *Pseudomonas aeruginosa* represents a major challenge in the management of hospital-acquired and wound-related infections. The ability of this organism to form biofilms significantly contributes to treatment failure and persistence of infection. Novel anti-biofilm strategies are therefore required to address this growing public health concern.

Objective: To evaluate the antibiotic resistance patterns and biofilm-forming ability of *Pseudomonas aeruginosa* isolated from clinical specimens, and to assess the in-vitro anti-biofilm efficacy of biosynthesized gold nanoparticles against strong biofilm-producing isolates.

Materials and Methods: This laboratory-based cross-sectional study was conducted on 20 clinical isolates of *Pseudomonas aeruginosa* obtained from various clinical specimens at a tertiary care hospital. The isolates were identified using standard cultural, microscopic, and biochemical methods. Antibiotic susceptibility testing was performed using the Kirby–Bauer disc diffusion method.

Results: All isolates exhibited resistance to multiple antibiotics, with 100% resistance observed against cephalexin, clindamycin, ampicillin, cefixime, trimethoprim, and gentamicin. Resistance to meropenem was noted in 33.3% of isolates. Biofilm production was detected in 85% of isolates by the Congo red agar method and in 90% by the microtiter plate assay, with a substantial proportion identified as strong biofilm producers.

Conclusion: The study demonstrated a high burden of multidrug resistance and biofilm production among *Pseudomonas aeruginosa* clinical isolates. Biosynthesized gold nanoparticles showed effective anti-biofilm activity in vitro and may serve as a potential adjunctive strategy for controlling biofilm-associated infections caused by *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*; antimicrobial resistance; biofilm; gold nanoparticles; hospital-acquired infections; wound infections

Introduction

Antimicrobial resistance (AMR) has emerged as a major global public health concern and represents a serious threat to the effective control of communicable diseases. The increasing prevalence of multidrug-resistant (MDR) pathogens has significantly reduced the effectiveness of commonly used antibiotics, resulting in prolonged hospitalisation, increased healthcare costs, and higher morbidity and mortality.[1,2] Hospital-acquired infections caused by resistant organisms remain a critical challenge, particularly in developing countries.

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen frequently implicated in nosocomial infections such as wound infections, urinary tract infections, respiratory tract infections, and otitis media. Its clinical importance is largely attributed to its intrinsic resistance to multiple antimicrobial agents and its remarkable ability to acquire additional resistance through genetic mutations and horizontal gene transfer.[3,4] Consequently, infections caused by *P. aeruginosa* are often difficult to treat and are associated with poor therapeutic outcomes.

One of the most important virulence determinants of *P. aeruginosa* is its ability to form biofilms. Biofilms are organised microbial communities embedded within a self-produced extracellular polymeric substance matrix that enhances bacterial survival and persistence.[5] Biofilm-associated bacteria exhibit markedly increased tolerance to antibiotics and host immune responses, with resistance levels reported to be up to 10–100 times higher than those of planktonic cells.[6,7] The formation of biofilms therefore plays a crucial role in chronic and recurrent infections and contributes significantly to the global burden of antimicrobial resistance.

The limited effectiveness of conventional antibiotics against biofilm-associated infections has led to growing interest in alternative therapeutic strategies. Nanotechnology-based approaches, particularly the use of metal nanoparticles, have shown promising antimicrobial and anti-biofilm potential.[8] Gold nanoparticles (AuNPs) have attracted considerable attention due to their stability, biocompatibility, and ability to penetrate biofilm matrices and disrupt bacterial cell functions.[9] Biosynthesized AuNPs, produced using biological sources, offer additional advantages such as eco-friendly synthesis and enhanced biological activity.[10] Several studies have demonstrated the ability of gold nanoparticles to inhibit biofilm formation and reduce the virulence of *P. aeruginosa*.[11]

In view of the increasing clinical significance of multidrug-resistant *P. aeruginosa* and the urgent need for effective anti-biofilm strategies, the present study was undertaken to assess the antibiotic resistance patterns and biofilm-forming ability of *P. aeruginosa* isolates obtained from clinical infections. Additionally, the study evaluated the inhibitory effect of biosynthesized gold nanoparticles on biofilm production, highlighting their potential role as an adjunctive approach in the management of resistant *P. aeruginosa* infections.

Materials and Methods

Study Design and Setting

This laboratory-based cross-sectional study was conducted in the Department of Life Sciences, College of Science, Tikrit University, in collaboration with the Microbiology Laboratory of Tikrit Teaching Hospital. The study was carried out on clinical isolates of *Pseudomonas aeruginosa* obtained from hospitalised patients.

Bacterial Isolates

A total of 20 non-duplicate clinical isolates of *Pseudomonas aeruginosa* were collected from various clinical specimens, including wound swabs, urine samples, middle ear discharges, and respiratory specimens. All isolates were obtained from routine diagnostic samples processed at Tikrit Teaching Hospital.

Identification of Pseudomonas aeruginosa

The bacterial isolates were identified based on cultural characteristics, microscopic examination, and standard biochemical tests. The isolates were cultured on blood agar, MacConkey agar, and cetrimide agar and incubated aerobically at 37 °C for 24 hours. Colony morphology, pigment production, and hemolytic patterns were recorded.

Microscopic examination was performed using Gram staining. Biochemical identification included catalase and oxidase tests, which were performed according to standard microbiological procedures. Isolates showing characteristic phenotypic and biochemical profiles were confirmed as *Pseudomonas aeruginosa*.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar, following Clinical and Laboratory Standards Institute (CLSI) guidelines. Bacterial suspensions were prepared in sterile normal saline and adjusted to match 0.5 McFarland turbidity standard.The inoculated Mueller–Hinton agar plates were allowed to stand for 5 minutes before antibiotic discs were placed using sterile forceps. The plates were incubated at 37 °C for 24 hours. Zones of inhibition were measured in millimetres and interpreted as sensitive or resistant according to CLSI standards.The antibiotics tested included cephalexin, clindamycin, ampicillin, cefixime, meropenem, trimethoprim, and gentamicin.

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Detection of Biofilm Formation

Biofilm production by *Pseudomonas aeruginosa* isolates was assessed using **two different methods**.

Congo Red Agar (CRA) Method

Congo red agar medium was prepared using brain heart infusion agar supplemented with sucrose and Congo red dye. The medium was poured into sterile Petri dishes and allowed to solidify. Each isolate was inoculated onto the agar surface using a sterile loop and incubated aerobically at 37 °C for 24 hours.Biofilm production was determined based on colony morphology. Black, dry, crystalline colonies were interpreted as strong biofilm producers, while red or pink colonies were considered weak or non-biofilm producers.

Microtiter Plate Assay

The quantitative assessment of biofilm formation was performed using a **96-well microtiter plate assay**. Each isolate was inoculated into brain heart infusion broth and incubated at 37 °C for 24 hours. A volume of 200 µL of bacterial suspension was dispensed into each well in triplicate. Sterile broth without bacteria served as the negative control. After incubation, the wells were gently washed twice with phosphate-buffered saline to remove planktonic cells and allowed to dry. Crystal violet solution (1%) was added to each well and incubated for 45 minutes. Excess stain was removed by washing with sterile distilled water, and the plates were air-dried. Bound dye was solubilised using 99% ethanol. Optical density was measured at **630 nm** using an ELISA reader. Biofilm formation was categorised as weak, moderate, or strong based on optical density values relative to the control.

Gold Nanoparticles

Ready-made gold nanoparticles were obtained from the postgraduate laboratory, Department of Life Sciences, College of Science, Tikrit University. The nanoparticles were characterised before use to confirm their optical and structural properties.

Characterisation of Gold Nanoparticles

UV–Visible Spectroscopy

Ultraviolet–visible spectroscopy was performed using a Shimadzu UV-1601 spectrophotometer. Samples of gold nanoparticles were scanned within the wavelength range of **200–800 nm**. The absorption peak was recorded to confirm the formation and stability of the nanoparticles.

Evaluation of Anti-Biofilm Activity of Gold Nanoparticles

The effect of gold nanoparticles on biofilm formation by *Pseudomonas aeruginosa* was evaluated using **two methods**.

Congo Red Agar Method

Strong biofilm-producing isolates were treated with gold nanoparticles at concentrations of **12.5%, 25%, and 50%**. Treated bacterial suspensions were inoculated onto Congo red agar plates and incubated at 37 °C for 24 hours. Changes in colony morphology were assessed to determine the inhibition of biofilm production.

Microtiter Plate Method

Strong biofilm-producing isolates were exposed to gold nanoparticles at concentrations of 12.5%, 25%, and 50% in a 96-well microtiter plate. After incubation at 37 °C for 16 hours, biofilm formation was quantified using the crystal violet staining method. Optical density readings were obtained at 630 nm, and reductions in biofilm formation were recorded in comparison with untreated controls.

Ethical Considerations

Ethical approval for the study was obtained from the appropriate institutional ethics committee. All bacterial isolates were collected as part of routine diagnostic procedures, and no patient identifiers were used in the study.

Statistical Analysis

Data were recorded and analysed using descriptive statistical methods. Results were expressed as frequencies and percentages where appropriate.

Results

Identification of *Pseudomonas aeruginosa*

All 20 clinical isolates were identified as *Pseudomonas aeruginosa* based on characteristic cultural morphology on blood agar, MacConkey agar, and cetrimide agar, Gram-negative bacillary appearance on microscopy, and positive oxidase and catalase reactions.

Antibiotic Susceptibility Pattern

Antibiotic susceptibility testing revealed a **high level of resistance** among the *P. aeruginosa* isolates. All isolates (100%) were resistant to cephalexin, clindamycin, ampicillin, cefixime, trimethoprim, and gentamicin. Resistance to meropenem was observed in 7 isolates (33.3%), while the remaining isolates were sensitive. The detailed resistance pattern is presented in Table 1.

Biofilm Formation by Pseudomonas aeruginosa

Congo Red Agar Method

Using the Congo red agar method, 17 isolates (85%) demonstrated biofilm production. Among these, 8 isolates (40%) were classified as strong biofilm producers, 9 isolates (45%) were weak to moderate producers, and 3 isolates (15%) did not produce biofilm. The distribution of biofilm production is shown in Table 2.

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Microtiter Plate Assay

Quantitative assessment using the microtiter plate method showed that 18 isolates (90%) produced biofilms. Among these, 7 isolates (35%) were strong biofilm producers, 9 isolates (45%) were moderate producers, and 2 isolates (10%) were weak producers. Two isolates (10%) showed no biofilm formation. The results are summarised in Table 3.

Table 1.Antibiotic resistance pattern of *Pseudomonas aeruginosa* isolates

Antibiotic	Code	Resistant n (%)
Cephalexin	CL	20 (100)
Clindamycin	DA	20 (100)
Ampicillin	AM	20 (100)
Cefixime	CFM	20 (100)
Meropenem	MEM	7 (33.3)
Trimethoprim	TR	20 (100)
Gentamicin	CN	20 (100)

Table 2.Biofilm production by *P. aeruginosa* using Congo red agar method

(n = 20)

Biofilm production category	Number of isolates n (%)
Strong biofilm producers	8 (40.0)
Weak–moderate biofilm producers	9 (45.0)
Non-biofilm producers	3 (15.0)
Total	20 (100)

Table 3.Biofilm production by *P. aeruginosa* using microtiter plate assay

(n = 20)

Biofilm production category	Number of isolates n (%)
Strong biofilm producers	7 (35.0)
Moderate biofilm producers	9 (45.0)
Weak biofilm producers	2 (10.0)
Non-biofilm producers	2 (10.0)
Total	20 (100)

Effect of Gold Nanoparticles on Biofilm Production

Seven isolates identified as strong biofilm producers by the microtiter plate assay were further evaluated for the effect of gold nanoparticles. Following treatment with gold nanoparticles at concentrations of 12.5%, 25%, and 50%, a reduction in biofilm production was observed in all tested isolates. Four isolates showed a reduction from strong to moderate biofilm production, while three isolates showed a reduction from strong to weak biofilm production.

Discussion

Antimicrobial resistance among *Pseudomonas aeruginosa* has emerged as a significant challenge in the management of hospital-acquired and wound-related infections. In the present study, all clinical isolates of *P. aeruginosa* demonstrated high levels of resistance to commonly used antibiotics, including cephalexin, clindamycin, ampicillin, cefixime, trimethoprim, and gentamicin. Similar high resistance rates have been reported in earlier studies from different regions, highlighting the global nature of antimicrobial resistance in *P. aeruginosa.*[4,12] The resistance observed in the present study may be attributed to intrinsic resistance mechanisms, reduced outer membrane permeability, efflux pump activity, and the acquisition of resistance genes through horizontal gene transfer.[13] In contrast, resistance to meropenem was comparatively lower in the present study (33.3%). Comparable findings have been reported by Roulová et al., who observed lower resistance rates to carbapenems compared to other antibiotic classes.[14] Carbapenems remain one of the few effective therapeutic options against multidrug-resistant *P. aeruginosa*; however, the emergence of carbapenem resistance, as observed in a subset of isolates in the present study, is a cause for concern and underscores the need for

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antimicrobial stewardship and continuous surveillance. Biofilm formation was found to be highly prevalent among the *P. aeruginosa* isolates in this study. Using the Congo red agar method, 85% of isolates were identified as biofilm producers, while the microtiter plate assay revealed biofilm production in 90% of isolates. These findings are consistent with previous studies reporting biofilm formation rates ranging from 90% to 100% among clinical isolates of *P. aeruginosa*.[15,16] The high prevalence of biofilm-producing isolates may explain the extensive antibiotic resistance observed, as biofilms provide a protective environment that limits antibiotic penetration and enhances bacterial survival.[17]

The microtiter plate assay detected a higher proportion of biofilm producers compared to the Congo red agar method, supporting earlier reports that quantitative methods are more sensitive and reliable for biofilm detection.[18] The presence of strong biofilm producers in more than one-third of the isolates further emphasises the clinical relevance of biofilm-associated infections, which are often persistent and difficult to eradicate. The inhibitory effect of gold nanoparticles on biofilm production observed in the present study aligns with findings from several previous investigations. Treatment with biosynthesized gold nanoparticles resulted in a marked reduction in biofilm formation among all strong biofilm-producing isolates. Similar anti-biofilm effects of gold nanoparticles against *P. aeruginosa* have been reported by Salam et al. and Kang et al., who demonstrated significant disruption of biofilm architecture and suppression of virulence properties following nanoparticle exposure.[10,11] The reduction in biofilm production from strong to moderate or weak levels observed in the current study further supports the potential role of gold nanoparticles as effective anti-biofilm agents.The anti-biofilm activity of gold nanoparticles may be attributed to their ability to penetrate the extracellular polymeric substance matrix, interact with bacterial cell membranes, and disrupt metabolic and quorum-sensing pathways essential for biofilm maintenance.[8] Biosynthesized nanoparticles, in particular, have been shown to possess enhanced biological activity due to the presence of bioactive functional groups on their surface.[9] Overall, the findings of the present study reinforce the growing body of evidence indicating that biofilm formation plays a critical role in antimicrobial resistance among *P. aeruginosa*. The observed anti-biofilm efficacy of gold nanoparticles highlights their potential as an adjunctive therapeutic strategy in the management of biofilm-associated *P. aeruginosa* infections, especially in settings where conventional antibiotics are increasingly ineffective.

Conclusion

This study demonstrated a high prevalence of multidrug resistance among *Pseudomonas aeruginosa* isolates obtained from clinical infections. A substantial proportion of isolates exhibited strong biofilm-forming ability, which likely contributed to the observed antibiotic resistance. The microtiter plate assay proved to be a sensitive method for detecting biofilm production. Treatment with biosynthesized gold nanoparticles effectively reduced biofilm formation in all strong biofilm-producing isolates. These findings highlighted the potential of gold nanoparticles as promising anti-biofilm agents. The study underscored the need for alternative strategies to combat biofilm-associated antimicrobial resistance in *P. aeruginosa* infections.

Limitations

The study was limited by a relatively small sample size, which may restrict the generalisability of the findings. Isolates were obtained from a single healthcare centre, limiting geographic representation. Molecular characterisation of resistance genes and biofilm-related genes was not performed. The anti-biofilm activity of gold nanoparticles was evaluated only in vitro, without in vivo validation. Long-term toxicity and clinical safety of gold nanoparticles were not assessed. Further multicentric and molecular studies are required to confirm these findings.

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Conflict of Interest: None

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