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Research Article

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Investigation of the Effect of Antibiotic Resistance Selected via Disk Diffusion and Cross-Resistance in *Klebsiella pneumoniae* on Biofilm Formation

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Abstract

Objective: Antibiotic resistance has become a global health issue, making it difficult to manage treatable infections. This study aimed to investigate the development of resistance in *Klebsiella pneumoniae* exposure to antibiotic diffusion gradients generated by disk diffusion. The emergence of cross-resistance and the impact of this resistance on biofilm formation are further evaluated.

Materials and Methods: *K. pneumoniae* was exposed to antibiotic diffusion gradients using Oxoid disks (36 µg) to select resistant mutants of piperacillin-tazobactam (TZP), leading to the development of a resistant strain (P_3). Comparative analyses of antibiotic susceptibility (TZP, amoxicillin/clavulanic acid [AMC], cefixime [CFM], and trimethoprim-sulfamethoxazole [SXT]) and biofilm formation were conducted between the parental (P_0) and resistant (P_3) strains. Biofilm production was evaluated under varying temperatures (37°C and 45°C) and glucose concentrations (0%-2.5%).

Results: Resistance to TZP increased significantly in P_3, with moderate cross-resistance observed against AMC and SXT ($p<0.05$). Although the strain was susceptible to most antibiotics at baseline (P_0), it exhibited primary resistance to CFM in both P_0 and P_3 stages. Biofilm formation was higher at 37°C than at 45°C under the tested conditions. Increasing the glucose concentration from 0 % to 2.5% reduced biofilm production by nearly 40%. However, no direct correlation was found between antibiotic resistance development and biofilm-forming capacity.

Conclusion: Selection through antibiotic gradients can induce resistance and cross-resistance in *K. pneumoniae*, but does not necessarily enhance biofilm production. Environmental parameters, particularly temperature and nutrient availability, and particularly glucose concentration, are more influential in modulating biofilm formation. Future molecular characterisation of β-lactamase and biofilm-associated genes are essential for elucidating the underlying mechanisms.

Keywords *Klebsiella pneumoniae* · Antibiotic resistance · Resistant mutant selection · Cross-antibiotic resistance · Biofilm

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INTRODUCTION

Klebsiella pneumoniae was isolated from the respiratory tract of a patient with pneumonia by the German microbiologist Edwin Klebs in 1875.[1] This bacterium belongs to the Enterobacteriaceae family, reproduces facultatively anaerobically, and is a Gram-negative bacterium. Morphologically, this bacterium is encapsulated and rod-shaped. However, it is nonmotile due to the absence of flagella and is classified as a nonspore-forming bacterium.[2] *K. pneumoniae* is typically found in healthy individuals' intestines and faeces. It is harmless in the intestines, but when it spreads to other parts of the body, it can cause serious infections. Among the infections caused by this bacterium is pneumonia, which is a severe lung infection. This bacterium is transmitted from person to person or through contaminated hospital equipment.[3] Thus pneumonia can be categorised into two groups: community-acquired pneumonia and hospital-acquired pneumonia.[4]

The global rise of antibiotic resistance has positioned *K. pneumoniae* as one of the most dangerous bacterial pathogens to human health. It is a critical threat because of its ability to easily colonise mucosal surfaces, including the gastrointestinal tract and the oropharynx, evade immune system components, and resist conventional antimicrobial treatments. Instead of actively growing and suppressing many immune system components, its ability to evade and survive makes it one of the most dangerous bacteria for living organisms.[4] Owing to the dangerous nature of *K. pneumoniae* infections, prolonged use of certain antibiotics can increase the chances of recovery. Although it has developed resistance to many antibiotics, some antibiotics, such as cephalosporins and carbapenems, are still effective in treating infections caused by this bacterium.[5,6]

Bacteria such as *K. pneumoniae* produce an enzyme known as carbapenemase, which renders carbapenem antibiotics ineffective in killing bacteria and treating infections. Unfortunately, carbapenem antibiotics are often considered the last line of defence against gram-negative infections that are resistant to other antibiotics. Treating infections caused by carbapenemase-producing bacteria is challenging because of the limited number of effective antibiotics.[7] Drug modification is a significant mechanism of resistance in *K. pneumoniae*. This bacterium produces β-lactamases, enzymes that hydrolyse the β-lactam ring, providing resistance to penicillins, cephalosporins, and carbapenems.[8] Antibiotic resistance is a significant global health issue affecting our ability to effectively combat infectious diseases. The ability of bacteria to form biofilms is one of the contributing factors to the development of resistance. A major contributing factor to the escalating antibiotic resistance crisis and a significant challenge in *K. pneumoniae* infections is the remarkable ability of the bacterium to form biofilms. Biofilms are complex structures composed of bacterial cells embedded in a protective matrix that have osmotic barrier properties and play a crucial role in antimicrobial resistance. Dense biofilms are formed to protect against antibiotics and increase protection by limiting and inactivating the penetration of antibiotics and inactivating them.[9] Thus biofilms increase the survival and persistence of resistant strains in clinical environments.[10] Infections caused by biofilm-forming bacteria often require prolonged or combined antibiotic therapies.[4] Clinical strategies for preventing biofilm formation are essential for treating infections. Disrupting or inhibiting biofilm formation can enhance the effectiveness of antibiotics and help limit the spread of antibiotic-resistant bacteria.[7]

Bacteria exhibit high antibiotic resistance inside biofilms, making treating infections more challenging. Many strains of *K. pneumoniae* produce biofilms, further worsening the resistance issue. Biofilms formed by *K. pneumoniae* reduce the susceptibility of bacteria to antibiotics, such as gentamicin, ampicillin, ciprofloxacin, and cefotaxime.[8] Mechanisms of antibiotic resistance related to biofilms include the physical barrier of the biofilm matrix, the presence of dormant cells, the regulation of efflux pumps, and genetic factors such as horizontal gene transfer.[11] Despite the well-established role of biofilms in resistance, the precise environmental factors that modulate *K. pneumoniae* biofilm formation in clinically relevant contexts and how these factors might interact with antibiotic resistance profiles are not fully understood. Biofilm formation is a mechanism by which bacteria develop antibiotic resistance. Even under harsh conditions, such as high temperatures (45°C) and increased glucose concentration (2.5%), bacteria produce biofilms in small amounts. Therefore, in addition to antibiotics, discovering and using chemicals that inhibit biofilm formation could be a more successful strategy in combating microorganisms. Therefore, the current study addresses this critical gap by specifically investigating the impact of key environmental parameters, namely temperature and glucose concentration, on *K. pneumoniae* biofilm formation and how these effects relate to existing antibiotic resistance profiles. This study aimed to investigate the resistance development in *K. pneumoniae* associated with the selection of resistant mutants through disk diffusion gradients, observe the emerging cross-resistance due to this resistance, and examine the effect of this resistance change on biofilm-forming capacity. Additionally, this study aims to evaluate the impact

of various parameters, such as temperature (37°C and 45°C), glucose concentration (0%, 0.5%, 1%, 1.5%, 2%, and 2.5%), and resistance profiles, on the biofilm production capacity.

MATERIALS AND METHODS

Preparation of Microorganisms for Use

K. pneumoniae culture was taken from the main stock stored at -20°C and first transferred to nutrient broth (Merck, Germany) and then incubated at 37°C (Mipro, Türkiye) for 24 h. Subsequently, it was transferred to nutrient agar (Nutrient Broth: Merck, Germany; Agar: Conda, Spain) and incubated at 37°C for another 24 h.[12] This culture was labelled as passage zero (P_0).

Preparation of Bacterial Inoculum

Colonies were collected from nutrient agar using a sterile loop and suspended in a sterile 0.9% saline solution to prepare the inoculum. The suspension was then adjusted to a turbidity equivalent to 0.5 McFarland standard (~1.5 × 10^8 CFU/mL) (Biosan, Lithuania) to ensure a uniform bacterial concentration for subsequent experiments.[13]

Determination of Resistance Profile

The resistance profile of the *K. pneumoniae* strain was determined using the following antibiotics: piperacillin-tazobactam (TZP) (36 µg), trimethoprim-sulfamethoxazole (SXT) (25 µg), cefixime (CFM) (5 µg), amoxicillin/clavulanic acid (AMC) (30 µg), aztreonam (ATM) (30 µg), gentamicin (CN) (10 µg), cefepime (FEP) (30 µg), and ceftriaxone (CRO) (30 µg). The prepared inoculum was streaked onto solid media using a sterile swab. Eight antibiotic disks (four per Petri dish) were placed with adequate spacing using sterile forceps. The plates were then incubated at 37°C overnight. The inhibition zones around the antibiotic disks were measured using a ruler after incubation, and the results were recorded.[14]

Inducing Resistance in the Strain

During the determination of the resistance profile (distribution test), spontaneous mutant colonies that emerged within the inhibition zone of the TZP (36 µg) disk were selected for further investigation. The appearance of distinct colonies within a clear inhibition zone is a recognised phenotypic indicator of resistant mutants or subpopulations that possess higher resistance levels than the parent strain.[15,16] These colonies, which were exposed to the antibiotic diffusion gradient on the solid media, were collected in triplicate in antibiotic-free nutrient broth and incubated at 37°C for 24 h to ensure sufficient growth and phenotypic stability. This method of picking resistant derivatives from the inhibition zone gradient and sequentially passaging them in antibiotic-free media is an established protocol to confirm that the resistance is genetically stable and not a transient adaptive response.[16] The resulting culture was labelled as the first passage (P_1), stored as P_1 stock, and then transferred to nutrient agar for the next round of resistance profile determination. The process of selecting resistant colonies from the inhibition zones and subsequent subculturing in antibiotic-free nutrient broth was repeated sequentially. The resistance level was monitored at each stage until no inhibition zone was observed around the TZP disk, which was achieved at the third passage (P_3).

Comparison of Resistance Profiles Between P_0 and P_3

At the end of the study, the resistance profiles of P_0 and P_3 were compared for TZP, SXT, CFM, AMC, ATM, CN, FEP, and CRO.

Biofilm Experiments

In the biofilm experiments, P_0 and P_3 were tested for their biofilm-forming capacities. Additionally, the effects of temperature (37°C and 45°C) and glucose concentration (0%, 0.5%, 1%, 1.5%, 2%, and 2.5%) on biofilm formation were compared. As previously described, biofilm formation under these conditions was assessed using the crystal violet method in 96-well plates.[17] The retained crystal violet from biofilms was measured at 595 nm using a BioTek plate reader.

Culture media containing different glucose concentrations were used as negative controls (NC).

Statistical Analysis

In the first step of the data analysis, six replicates from the study were examined for the presence of outliers. Box plots were generated using all replicates, and interquartile range (IQR) analysis for outlier detection was conducted in R Studio. Prior to further analysis, identified outliers were replaced with the mean value for their respective group.[18,19] This approach ensured that extreme biological noise did not disproportionately skew the final results while allowing for a robust statistical test application. Data were evaluated for normal distribution and variance homogeneity to determine the appropriate statistical methods. The Shapiro-Wilk test was used to assess normal distribution, and the Bartlett test was chosen to analyse variance homogeneity, with a significance level set at $p<0.05$. The analysis of variance (ANOVA) test was applied to compare data that met the normal distribution and homogeneity of variance conditions, while the Kruskal-Wallis test was used to conduct non-parametric comparisons if the data did not meet the normal distribution or homogeneity of variance conditions. In addition, a Pearson correlation test

was applied to observe correlations. R Studio 2024.04.2 was used for all analyses.

RESULTS

Antibiotic Resistance Profiles between P_0 and P_3 Strains

The antibiotics were selected according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Clinical Breakpoint Table.[20] In the first passage (P_0), TZP was initially observed at the fully susceptible breakpoint. However, TZP resistance development in *K. pneumoniae* was later observed (P_3). In fact, in the current study, antibiotic susceptibility testing revealed significant differences between the parental (P_0) and the resistant (P_3) *K. pneumoniae* strains.

At baseline (P_0), the strain was susceptible to most tested β-lactam and non-β-lactam antibiotics, with inhibition zones ranging from 1.3 to 4.2 cm, except for cefixime (CFM), to which it showed primary resistance. However, the strain exhibited primary resistance to cefixime (CFM), showing no inhibition zone (0.0 cm) at the initial stage (P_0). Following the selection of resistant mutants from TZP inhibition zones (exposure to diffusion-based antibiotic gradients), the (P_3) strain demonstrated a complete loss of previously observable inhibition zones for TZP, AMC, and SXT. Combined with the baseline resistance already present for CFM, these results indicate that the P_3 strain attained full resistance to both β-lactam/β-lactamase inhibitor combinations and non-β-lactam agents. Resistance selection was achieved via the formation of diffusion-based antibiotic gradients around Oxoid TZP disks (36 µg).

In addition, partial decreases in zone diameters for CRO, ATM, and FEP were observed, which show cross-resistance tendencies within the β-lactam antibiotic class (Table 1).

These results confirm that TZP exposure to an antibiotic gradient not only induced direct resistance to TZP but also contributed to reduced susceptibility across structurally related β-lactam antibiotics and unrelated agents, such as SXT.

In the current study, the effects of different temperatures (37°C and 45°C), glucose concentrations (0%, 0.5%, 1%, 1.5%, 2%, and 2.5%), and resistance status on biofilm formation on the same *K. pneumoniae* strain (P_0 and P_3) were investigated. The initial phase of the analysis involved thoroughly examining the negative control groups. Specifically, the absorbance data obtained from negative control samples designated as P_0 and P_3, incubated at both 37°C and 45°C across all tested glucose concentrations, were assessed for their statistical properties. The results from these preliminary tests were favourable; for both incubation temperatures (37°C and 45°C) and both strains (P_0 and P_3), the data corresponding to all glucose concentrations adhered to a normal distribution (Shapiro-Wilk test, $p>0.05$) and exhibited homogeneity of variances (Bartlett's test, $p>0.05$). Since the critical assumptions for parametric testing were met ($p>0.05$ for both tests), ANOVA was deemed the appropriate statistical method and was subsequently performed. The objective of applying ANOVA specifically to these negative controls was to

Table 1. Comparison of antibiotic resistance profiles between P_0 and P_3 strains.

Antibiotic	Class	Zone diameter (cm, P_0)	Zone diameter (cm, P_3)	β-lactam ring	Resistance	Antibiotic Trend
CRO (Ceftriaxone)	Cephalosporin	3.2	2.4	(+)	Partial Decrease	CRO
ATM (Aztreonam)	Monobactam	3.2	2.4	(–)	Partial Decrease	ATM
CN (Gentamicin)	Aminoglycoside	1.3	1.0	(–)	Slight Decrease	CN
FEP (Cefepime)	Cephalosporin	4.2	2.3	(+)	Significant Decrease	FEP
CFM (Cefixime)	Cephalosporin	0.0	0.0	(–)	Resistant (No Zone)	CFM
TZP (Piperacillin-Tazobactam)	β-lactam/β-lactamase inhibitor	2.0	0.0	(+)	Resistant (No Zone)	TZP
AMC (Amoxicillin/Clavulanic acid)	β-lactam/β-lactamase inhibitor	2.4	0.0	(+)	Resistant (No Zone)	AMC
SXT (Trimethoprim–Sulfamethoxazole)	Sulphonamide	1.4	0.0	(–)	Resistant (No Zone)	SXT

Note: Zone diameters were measured after incubation for 24 h at 37°C. Values represent the mean of triplicate assays. A zone diameter of 0.0 cm indicates full resistance.

determine whether the varying glucose concentrations had any inherent effect on the measured absorbance values in the absence of the primary experimental treatment. Interestingly, the ANOVA results indicated a statistically significant effect ($p<0.05$). This finding demonstrates that altering the glucose concentration directly influenced the resulting absorbance measurements, even under negative control conditions. Consequently, the absorbance readings from these negative controls were subtracted from the absorbance readings of the corresponding experimental samples to ensure that this baseline effect of glucose concentration did not confound the results.

Analysis of the Results Obtained for P_0 and P_3 at 37°C and 45 °C

Regarding the data for the P_0 strain cultured at 37°C in culture media with varying glucose concentrations, while all datasets exhibited a normal distribution ($p>0.05$), the assumption of homogeneity of variances was not met ($p<0.05$). The effect of different glucose concentrations on the biofilm absorbance produced by the P_0 strain at 37°C was examined using the non-parametric Kruskal-Wallis test. This analysis revealed a statistically significant difference among at least some glucose concentration groups ($p=1.4x10^{-4}$). Dunn's test was employed as a post hoc analysis to determine which groups differed. The results of Dunn's test indicated that the effect of the 0% and 0.5% glucose concentrations on biofilm formation was statistically similar, and the two glucose concentrations were significantly different compared to the other glucose concentrations tested ($p<0.05$). The highest biofilm formation was observed in 0.5% glucose.

Figure 1A compares the biofilm formation data of the P_0 strain cultured at 37°C with different glucose concentrations with those of the negative controls. Although these data followed a normal distribution, they again failed the homogeneity of variance test. Therefore, the comparison between the experimental groups and the negative controls was performed using the Kruskal-Wallis test. Since this test indicated a statistically significant overall difference ($p<0.05$), Dunn's post hoc test was subsequently applied for pairwise comparisons. Based on post hoc results, only at 0% glucose concentration did the P_0 strain produce a significantly different amount of biofilm at 37°C compared to the negative control ($p=0.0282$). Analysis of the P_0 strain cultured at 45°C confirmed that the datasets for all glucose concentrations followed a normal distribution and showed homogeneity of variances. Therefore, an ANOVA was performed, which indicated a significant effect of glucose concentration on biofilm formation ($p<0.05$). The subsequent Tukey post hoc test indicated that while biofilm formation was similar among most groups, the biofilm absorbance was significantly lower at 2% glucose than at other concentrations ($p<0.05$).

Figure 1B compares the biofilm formation data of the P_0 strain cultured at 45°C with various glucose concentrations and the negative controls. Statistical analysis showed that the biofilms formed at 45°C for P_0 were not statistically different from the negative controls. Analysis of the data for the P_3 strain cultured at 37°C confirmed that the datasets for all glucose concentrations did not exhibit homogeneity of variances, although they followed a normal distribution. Therefore, the non-parametric Kruskal-Wallis test was performed, which indicated a significant overall effect of glucose concentration on biofilm formation ($p<0.05$). The subsequent Dunn's post hoc test indicated that although biofilm formation was similar among most groups, a statistically significant difference existed between the 0% and 2% glucose concentrations ($p<0.05$). Specifically, the 0% concentration yielded the highest biofilm absorbance, whereas the 2% concentration yielded the lowest.

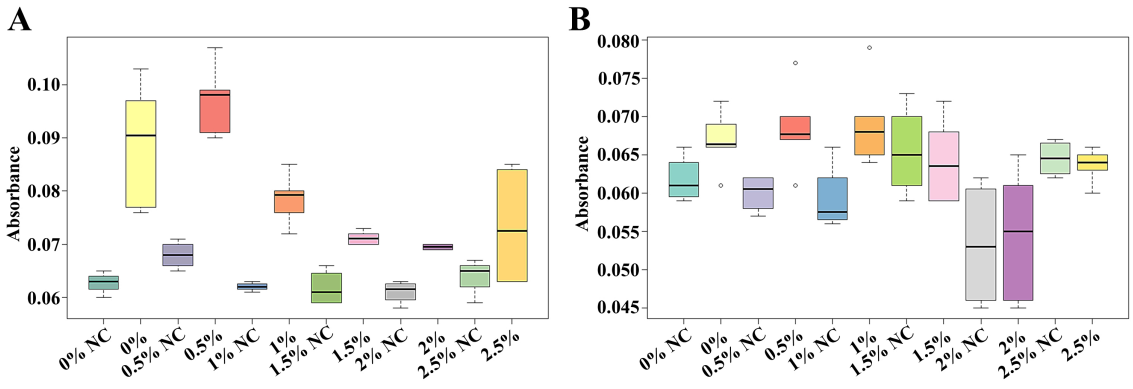

Figure 1. Biofilm formation of the *Klebsiella pneumoniae* P_0 strain at different glucose concentrations compared with negative controls (NCs) (A) Culture at 37°C; (B) culture at 45°C. NC represents wells containing culture medium with the corresponding glucose concentration but without bacterial inoculation.

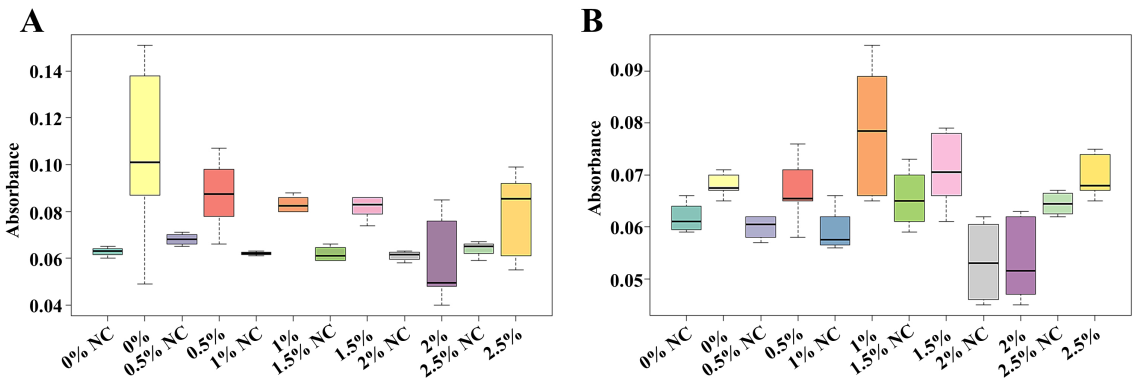

Figure 2. Biofilm formation of the *Klebsiella pneumoniae* P_3 strain at various glucose concentrations compared with negative controls (NC). (A) Culture at 37°C; (B) culture at 45°C. NC, negative control wells without bacterial inoculation.

Figure 2A compares the biofilm formation data of the P_3 strain cultured at 37°C with various glucose concentrations and the negative controls. No significant difference was observed between the absorbance readings of the experimental samples and the negative controls for the P_3 strain cultured at 37°C with various glucose concentrations. Significant variation in biofilm formation based on glucose concentration was detected for the P_3 strain cultured at 45°C using the Kruskal-Wallis test ($p<0.05$). This non-parametric approach was employed because, although the datasets for all glucose concentrations passed normality tests, they failed the homogeneity of variances test. Further analysis with Dunn's post-hoc test pinpointed the difference: the 2% glucose concentration yielded significantly lower biofilm absorbance than the other concentrations tested ($p<0.05$), whereas most other groups showed similar levels of biofilm formation. A graphical comparison of these P_3 strain biofilm results at 45°C against the negative controls is presented in Figure 2B. Comparison with the negative controls revealed that the obtained absorbance values were not significantly different for the P_3 strain cultured at 45°C with various glucose concentrations.

Comparison of Results Obtained for P_0 and P_3

A direct comparison between the biofilm absorbance of strains P_0 and P_3 at 37°C across the tested glucose concentrations is shown in Figure 3A. Figure 3B compares these two strains at 45°C under the same glucose conditions. Direct comparisons of biofilm absorbance between strains P_0 and P_3 were conducted at 37°C (Figure 3A) and 45°C (Figure 3B) across the tested glucose concentrations. Statistical analysis employing the Kruskal-Wallis test and Dunn's post-hoc test for pairwise comparisons revealed no statistically significant differences ($p>0.05$) in biofilm absorbance between the two strains when compared under the same specific temperature and glucose concentration. This shows that the differences in resistance profiles between strains P_0 and P_3 did not significantly impact the biofilm production under these experimental conditions.

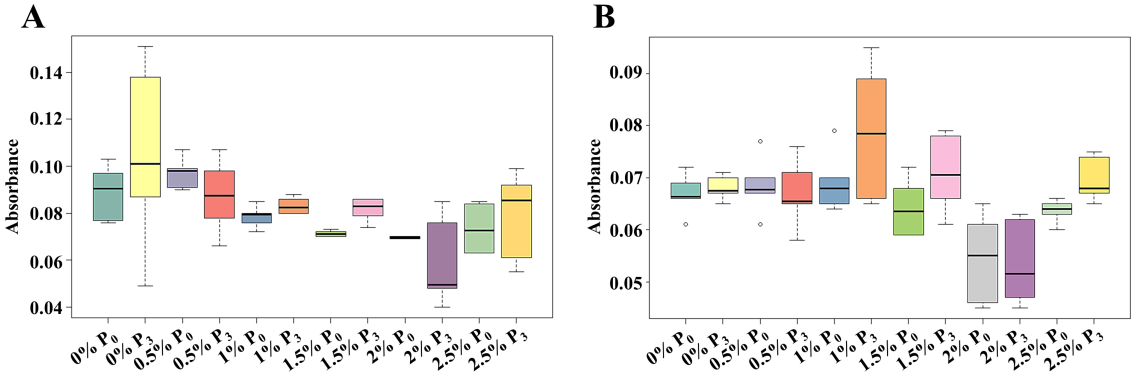

Figure 3. Comparison of biofilm formation between P_0 and P_3 strains of *Klebsiella pneumoniae* across different glucose concentrations (A) Culture at 37°C; (B) culture at 45°C.

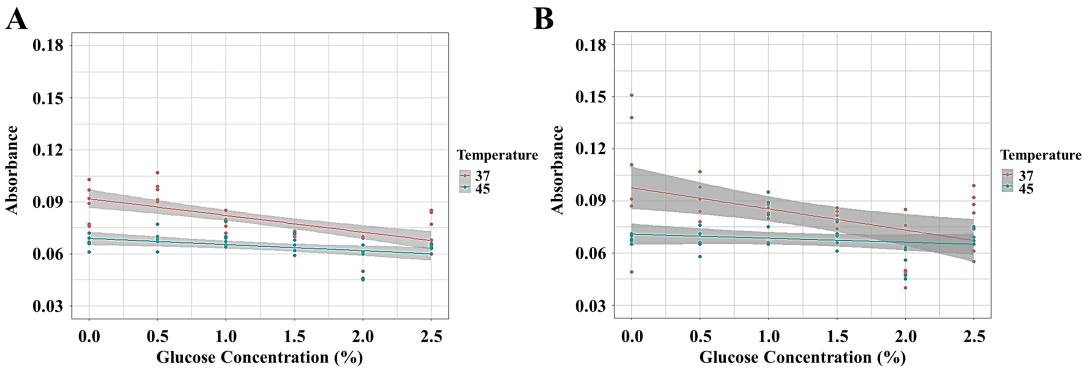

Figure 4. Effect of temperature on the biofilm absorbance of strains P_0 and P_3 at 37°C and 45°C: (A) P_0 and (B) P_3.

Effect of Temperature on Biofilm Formation

The effect of temperature on the biofilm absorbance of strains P_0 and P_3 at 37°C and 45°C is shown in Figure 4. Figure 4 illustrates the relationship between glucose concentration and biofilm absorbance for strains P_0 (Figure 4A) and P_3 (Figure 4B) at 37°C and 45°C incubation temperatures. For both strains, a consistent visual trend was observed where biofilm absorbance was generally higher at 37°C than at 45°C across the tested range of glucose concentrations (0%–2.5%). Specifically, for strain P_0, increasing glucose concentration was associated with a slight decrease in biofilm absorbance at both temperatures. A similar, but potentially more pronounced, negative correlation between glucose concentration and absorbance was observed for strain P_3 at 37°C, particularlynoticeable when comparing 0% glucose to higher concentrations. Conversely, at 45°C, glucose concentration had a less pronounced effect on biofilm formation by strain P_3, with the line appearing relatively flat. Despite these visual trends suggesting a temperature effect, it is important to recall that previous statistical analysis (Dunn's test comparing 37°C vs. 45°C at each specific glucose concentration for each strain individually, as detailed in Figures 3A and Figure 3B) did not find these differences to be statistically significant ($p>0.05$). The shaded areas represent 95% confidence intervals (CI).

Figure 5 shows a direct comparison of biofilm absorbance between strains P_0 and P_3 as a function of glucose concentration (0%–2.5%). The data for incubations at 37°C and 45°C are presented separately (Figure 5). The lines present a general negative trend for both strains at both temperatures, indicating that higher glucose concentrations tend to be associated with lower biofilm absorbance under these conditions. At 37°C, the line for P_3 is positioned slightly above that of P_0 across the glucose concentration range, potentially having marginally higher biofilm formation by P_3. However, the 95% confidence intervals (shaded areas) for the two strains showed substantial overlap, particularly at higher

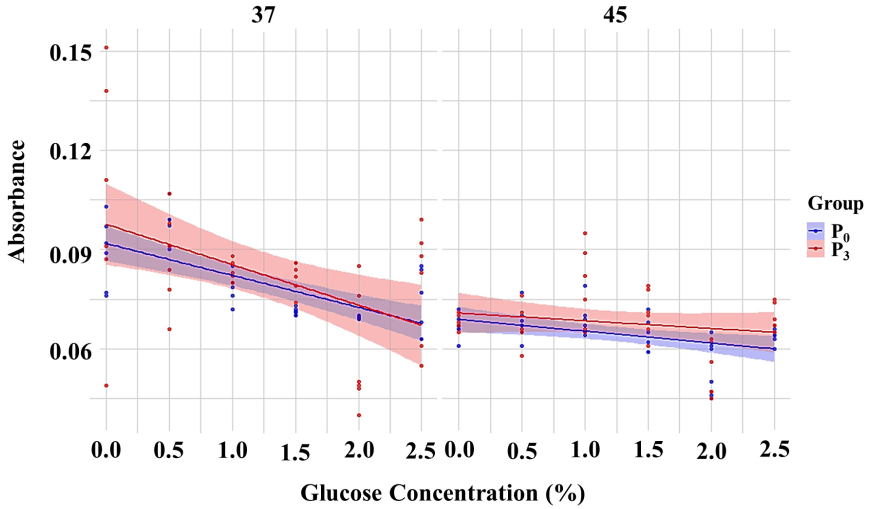

Figure 5. Biofilm absorbance between strains P_0 and P_3 as a function of glucose concentration (0%–2.5%) at 37°C and 45°C.

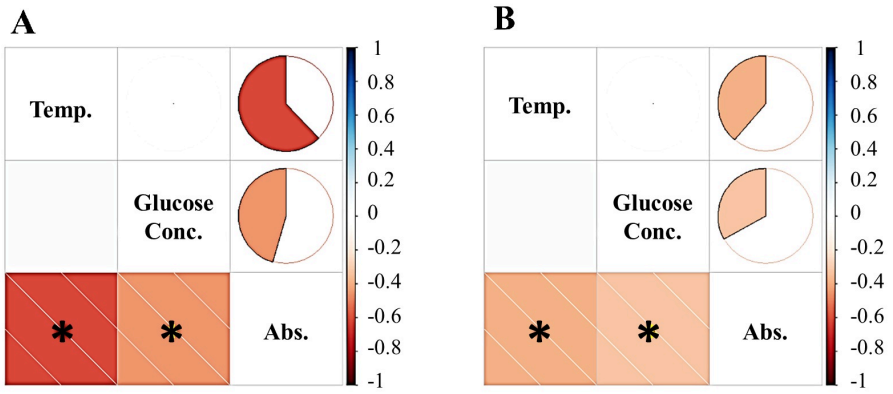

Figure 6. Pearson correlation plots for strains a) P_0 and b) P_3 (* denotes a statistically significant correlation ($p<0.05$)).

glucose concentrations. At 45°C, the regression lines for P_0 and P_3 are very close, with the CIs overlapping almost entirely.

Correlation Analysis

A Pearson correlation test was performed to investigate the relationship between temperature, glucose concentration, and biofilm absorbance in strains P_0 and P_3 (Figure 6). Pearson's correlation tests indicated significant correlations between biofilm absorbance and temperature and between biofilm absorbance and glucose concentration.

DISCUSSION

This study investigated the effects of different glucose concentrations, temperature conditions, and resistance profiles on biofilm formation and antibiotic resistance in *K. pneumoniae* strains. TZP resistance was the most pronounced, accompanied by moderate tolerance towards AMC and SXT. These findings are consistent with previous reports highlighting that β-lactam and sulphonamide classes can exert cross-selective pressure through overlapping metabolic and efflux-related pathways. The obtained results are consistent with those of the existing literature.

Like many human pathogens, *K. pneumoniae* is classified as a mesophile, with optimal growth typically observed around 37°C, corresponding to human body temperature. This temperature is ideal for its metabolic and physiological processes, including biofilm formation. Biofilm formation is a multifaceted process influenced by bacterial growth rate, gene expression, and temperature. *K. pneumoniae* achieves near-optimal growth rates and metabolic activity at 37°C. This temperature supports efficient ATP production and the synthesis of extracellular polymeric substances (EPS), which form the structural matrix of biofilms. Studies have shown that metabolic processes at this temperature are energetically favourable for the maturation of biofilms and the development of microcolonies. Enzymes involved in EPS biosynthesis exhibit optimal activity within this range.[21] Esener et al.[21] also proposed that growth rates and metabolic activity started to decrease after 39°C, with a sharp decrease observed around 43-44°C. Consistent with these findings, we observed a significant decrease in biofilm formation at 45°C compared with that at 37°C. This reduction aligns with Esener et al.'s[21] observations, reinforcing the negative impact of supra-optimal temperatures on the physiological processes of *K. pneumoniae* vital for biofilm development. A recent study discussed temperature-dependent changes in biofilm formation and related virulence factors in *K. pneumoniae*.[22] The current study shows that temperatures above the optimal (around 37°C) impact the biofilm formation capacity, physiology, and expression of virulence genes in *K. pneumoniae* strains. For example, increasing the temperature beyond 37°C leads to changes in mucoviscosity, plasmid copy number, and biofilm-related gene expression that influence biofilm development and pathogen behaviour.[22] Several other studies corroborate this, demonstrating that temperatures deviating significantly from 37°C, particularly elevated temperatures, impair metabolic activity and hinder biofilm maturation in various bacterial species, including *K. pneumoniae*.[23-25] This reduction is likely attributable to heat-induced protein denaturation and enzymatic inefficiencies at supra-optimal temperatures, thereby limiting EPS synthesis and cellular adhesion.[22]

High glucose concentrations inhibit cyclic AMP (cAMP) production, a critical second messenger in bacteria. cAMP forms a complex with the CRP, which regulates the transcription of genes involved in biofilm formation, such as those encoding type 3 fimbriae.[26] This can be attributed to glucose-mediated repression of the mrkHI operon via decreased cAMP-CRP activity, as noted by Panjaitan et al. (2023). The reduced expression of type 3 fimbriae (mrkA, mrkB, and mrkC) limits the adhesion and maturation stages of biofilm formation. Reduced cAMP levels impair the CRP-cAMP

complex, thereby decreasing the expression of genes, such as mrkHI, essential for biofilm development. This results in the diminished production of type 3 fimbriae, crucial for biofilm adhesion and maturation.[26] Our findings, demonstrating reduced biofilm formation under high glucose conditions, are in strong agreement with this mechanism. Specifically, the observed inhibition is consistent with the literature that excess glucose negatively impacts fimbrial expression, a key initial step in biofilm formation.[27]

Increased glucose availability promotes a metabolic shift that favours planktonic (free-floating) growth over biofilm formation. Nutrient-rich environments, such as those with high glucose concentrations, encourage rapid bacterial growth in the planktonic state rather than the resource-conserving biofilm state. This phenomenon has been observed in *K. pneumoniae* and other bacteria, such as *Escherichia coli*.[26,28] Our results further support this metabolic trade-off, where abundant glucose diverts bacterial resources towards rapid proliferation in a planktonic state, rather than investing in the more energy-intensive process of biofilm construction. This common adaptive strategy is observed across various pathogenic bacteria, where nutrient availability dictates growth morphology.[29,30] High glucose concentrations can reduce free water availability, leading to osmotic stress and physiological changes that negatively impact the ability of *K. pneumoniae* to form biofilms. This effect is likely mediated through a combination of impaired EPS production, altered gene regulation, and changes in cellular priorities under stress conditions.[26,28] Our results showed that glucose concentration influenced biofilm formation, which could be related to the osmatic stress due to glucose concentration. CPS are key components of the EPS matrix in biofilms. High glucose concentrations can disrupt CPS production by altering regulatory pathways involving CRP and other metabolic signals. Reduced CPS levels weaken the biofilm matrix's structural integrity, hindering its formation.[26,31] Since high glucose concentrations are known to disrupt CPS production, this alteration could led to changes in biofilm formations.

Finally, quorum sensing (QS), a bacterial communication system that regulates biofilm maturation, can also be affected by high glucose levels. Changes in environmental conditions, including sugar concentrations, may interfere with QS signalling molecules or their detection, disrupting coordinated biofilm development.[26,28] Moreover, the decrease in biofilm formation observed in our study under high glucose conditions is multifactorial, aligning with the combined impact of osmotic stress, impaired EPS/CPS production, and disruption of QS reported in the literature.[26,28,31] The consistency of our findings with these diverse mechanisms strengthens the understanding of how nutritional stress influences the biofilm dynamics of *K. pneumoniae*.

Biofilms provide a protective environment for bacteria, making them more resistant to antibiotics than their planktonic counterparts. This is due to several factors, including reduced penetration of antibiotics into the biofilm matrix and the presence of efflux pumps that actively expel antibiotics from bacterial cells.[11, 32] Some studies have shown a positive correlation between antibiotic resistance profiles and biofilm-forming ability in *K. pneumoniae*.[32] However, the relationship is not always straightforward, as some carbapenem-resistant strains have formed weaker biofilms than carbapenem-sensitive strains.[11] The current study observed less biofilm formation in the P_3 strain than in the P_0 strain. This finding adds to the complex and sometimes contradictory literature on the interplay between antibiotic resistance and biofilm formation. Some studies indicate that resistant strains often form robust biofilms as a survival mechanism.[33,34] Our result for the P_3 strain aligns with the observations of other researchers[11], who reported that certain carbapenem-resistant strains of *K. pneumoniae* formed weaker biofilms than their sensitive counterparts. Several factors could explain this apparent discrepancy. Genetic mutations or physiological adaptations conferring high-level antibiotic resistance, such as the overexpression of efflux pumps or modifications in membrane permeability, might sometimes impose a fitness cost that inadvertently compromises biofilm production or integrity.[35,36] For example, Singh et al.[37] showed that certain resistance mechanisms could alter metabolic pathways, thereby affecting EPS synthesis. Therefore, the reduced biofilm in our P_3 strain might not indicate a general lack of biofilm-forming capacity in all resistant strains, but rather a strain-specific consequence of its particular resistance mechanism or genetic background. This highlights the importance of characterising individual isolates and calls for further research to fully understand the specific mechanisms by which resistance, particularly in strains like P_3, influences biofilm formation in *K. pneumoniae*. In addition, the observed decrease in biofilm productivity in the resistant P_3 strain may reflect a metabolic trade-off, where energy allocated to resistance maintenance (e.g., efflux systems, membrane modifications) reduces biofilm synthesis, a classic fitness cost scenario. Further investigation, including genetic analysis of the P_3 strain's resistance determinants and their impact on biofilm regulatory genes, is needed to fully understand the interplay between resistance and biofilm formation in this pathogen. Although the current study did not include molecular characterisation, future work should focus on β-lactamase genes (blaKPC, blaTEM, blaSHV) and

biofilm-associated loci (mrkABC, fimH), which could provide mechanistic insights linking phenotypic resistance to gene expression.

In contrast to our initial hypothesis and some reports in the literature suggesting that the acquisition of antibiotic resistance enhances biofilm formation, our study found no statistically significant difference in biofilm production between the P_0 and P_3 strains ($p>0.05$). This observation shows that the development of high-level resistance to TZP did not synergistically increase the biofilm-forming capacity of *K. pneumoniae* under the tested conditions. It is possible to explain this phenomenon by the "fitness cost" theory. The physiological adaptations and genetic mutations required to achieve full resistance to β-lactam/β-lactamase inhibitor combinations often impose a significant metabolic burden on the bacteria. Energy resources that would normally be allocated to EPS synthesis and biofilm matrix maturation may be redirected towards resistance maintenance mechanisms, such as the overexpression of efflux pumps or structural modifications of the cell wall. Consequently, the resistant P_3 strain may prioritise survival over the energy-intensive biofilm construction process. Furthermore, our findings align with studies by Vuotto et al. (2017)[33] and Hjort et al. (2022)[35], which reported that certain antibiotic-resistant strains, particularly those with high-level resistance, might actually exhibit reduced or unchanged biofilm integrity compared to their susceptible counterparts due to these metabolic trade-offs.

CONCLUSION

The current study comprehensively investigated the effects of different glucose concentrations and temperature conditions on biofilm formation and its complex relationship with antibiotic resistance in *K. pneumoniae* strains. In addition, this study revealed that *K. pneumoniae* adapts to antibiotic stress in diffusion gradients by developing moderate resistance. Although the present study did not include molecular characterisation of β-lactamase genes (e.g., *blaKPC*, *blaTEM*, *blaSHV*), particularly within β-lactam and sulphonamide classes. However, these adaptations did not correspond to an increase in biofilm formation. Our findings clearly demonstrated that the optimal physiological temperature (37°C) strongly promotes the biofilm development of *K. pneumoniae*. In contrast, a notable decrease in biofilm formation was observed at supra-optimal temperatures (45°C), underscoring the critical role of temperature in modulating this pathogenic process. Furthermore, elevated glucose concentrations resulted in significantly reduced biofilm production. This presents that high glucose environments, such as diabetic wounds or catheter-associated infections, can paradoxically inhibit biofilm formation in *K. pneumoniae* through various metabolic and regulatory pathways. Therefore, environmental factors, such as temperature and glucose concentration, exerted a far greater influence on biofilm development than resistance acquisition. A direct and uniform correlation between antibiotic resistance and biofilm production was not established. While some literature suggests a positive link, our study, exemplified by observations in the P_3 strain, indicates a more complex and strain-dependent interplay where certain resistant strains may exhibit weaker biofilm-forming capabilities. This highlights that the costs associated with resistance mechanisms can sometimes negatively impact biofilm integrity. In conclusion, these results emphasise the crucial impact of environmental cues, specifically temperature and glucose availability, on the ability of *K. pneumoniae* to form biofilms, a key virulence factor. Understanding these modulatory effects is vital for developing targeted strategies to prevent and treat *K. pneumoniae* infections, especially when these environmental factors fluctuate. Future research should focus on elucidating the precise molecular mechanisms underlying glucose-mediated inhibition and the specific genetic determinants that influence the observed biofilm formation variations among antibiotic-resistant strains. Furthermore, future investigations should integrate molecular analyses of β-lactamase and biofilm-related genes to elucidate the mechanisms connecting resistance and virulence in *K. pneumoniae*.

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