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Phenotypic Profiling of Biofilm Development and Efflux Pump Mechanisms in Multidrug-Resistant (MDR) *Klebsiella pneumoniae*

[1]Hadiza Misbahu Na`iya, [3]Abdulhamid Yusuf, [4]Zahra Misbahu Na`iya, [1]Dikwa K.B, [5]Shafa'atu Abubakar, [1]Bilkisu Isma'il, [1]Fadila Isyaku and [2]Adeleye Isaac Adewale

[1]Department of Biological Sciences, Nigerian Defence Academy, Kaduna, Nigeria
[2]Department of Microbiology, University of Lagos, Nigeria
[3]Department of Plant Science and Biotechnology, Federal University Dutsinma, Katsina, Nigeria
[4]Department of Microbiology, Umaru Musa Yar'adua University, Katsina, Nigeria
[5]Department of Biology, Usmanu Danfodiyo University, Sokoto, Nigeria
*Correspondence author: hmnaiya@nda.edu.ng

Abstract

Klebsiella pneumoniae causes diverse healthcare- and community-associated infections, frequently affecting patients with indwelling medical devices. Its ability to form biofilms and activate efflux pumps enhances antibiotic resistance. These two mechanisms work synergistically, accelerating the emergence of multidrug-resistant K. pneumoniae strains..This study investigated the antibiotic resistance patterns of Klebsiella pneumoniae isolates in relation to their potential for biofilm formation and efflux pump activity. A total of Sixteen (16) K. pneumoniae isolates were obtained from the Department of Microbiology, University of Lagos, Lagos State. Confirmation of K. pneumoniae was conducted through culture, microscopy, and biochemical assays. Antibiotic susceptibility testing was performed using six routinely prescribed antibiotics. Phenotypic assessment of efflux pump activity and biofilm formation was conducted using the ethidium bromide (EtBr) cartwheel assay and Congo red agar method, respectively. The results indicated that all isolates were multidrug-resistant, exhibiting high resistance to Ofloxacin (12, 75.00%), Cephalexin (9, 56.25%), and Ampicillin (8, 50.00%). Four 4(25%)isolates demonstrated biofilm-forming ability, while only two 2(12.5%) isolates exhibited efflux pump activity. The results highlight biofilm formation and efflux pump activity as key virulence mechanisms contributing to the pathogenicity and multidrug resistance of K. pneumoniae, highlighting the need for targeted therapeutic strategies to overcome these resistance mechanisms.

***Keywords:** Biofilm, Efflux pump, Multidrug-resistant (MDR), Klebsiella pneumoniae, Antibiotics.*

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative bacterium from the *Enterobacteriaceae* family and is considered an opportunistic pathogen. It frequently inhabits the mucosal linings of the human intestinal tract and oropharynx, but is also commonly found in environmental sources, such as water and soil (Barbosa & Lery, 2019). This bacterium is capable of causing both community-acquired and healthcare-associated infections, including pneumonia, bloodstream infections, meningitis, urinary tract infections, and liver abscesses. These infections are particularly dangerous for immunocompromised individuals, including those in neonatal care or intensive care units (Martin & Bachman, 2018).

Antibiotics are being used mainly to treat bacterial infections; they are either bacteriostatic or bactericidal. They allow the body's immune system to eliminate them. The use of antibiotics has led to numerous changes in medicine, enhancing health and the quality of human life. Based on their activity, the antibiotics have been classified. Some antibiotic groups include aminoglycosides, which block protein synthesis; quinolones, which interfere with nucleic acid synthesis; beta-lactams, which prevent cell wall formation; and sulphonamides, which possess antimetabolite properties (Kapoor *et al.*, 2017). Antibiotic resistance is defined as the phenomenon in which a drug loses its ability to inhibit bacterial growth effectively, allowing the bacteria to continue multiplying at therapeutic levels of antibiotics. The effectiveness of antibiotic chemotherapy is declining due to the emergence of antibiotic resistance, which usually occurs due to the misuse of antibiotics, inappropriate prescription, or self-medication. Multidrug

resistance (MDR) is a property whereby an organism withstands the effects of multiple common drugs or antibiotics used in the treatment of an infection caused by that organism. The multidrug resistance property may be due to the bacterium's biofilm-forming ability or the acquisition of an efflux pump mechanism. Multidrug-resistant *K. pneumoniae* is a major concern in public health, as its ability to cause infections and the difficulty encountered in treating these infections necessitate updating knowledge of its drug resistance. Additionally, the investigation of antibiotic susceptibility in this organism is essential for a proper understanding of the epidemiology of its multidrug resistance (Chakraborty *et al.*, 2016).

Klebsiella pneumoniae strains can be categorized into three major groups based on their genetic makeup and observable traits: classical strains, hypervirulent variants, and multidrug-resistant types (Wang *et al.*, 2020). In 2019 alone, antimicrobial resistance was associated with an estimated 4.95 million deaths globally, with 1.27 million of those directly attributed to infections caused by resistant bacteria. *Klebsiella pneumoniae* was one of the primary organisms contributing to this burden (Murray *et al.*, 2022). The emergence of hypervirulent and multidrug-resistant strains poses a significant threat to public health (Lan *et al.*, 2021). Due to the rise in resistance, the World Health Organization (WHO) has identified carbapenem-resistant *K. pneumoniae* (CRKP) as a critical priority for the development of new antibiotics (Zhang *et al.*, 2021). As one of the ESKAPE pathogens (a group of bacteria known for their capacity to evade the effects of antibiotics), *K. pneumoniae* poses a serious clinical challenge. Its resistance to treatment is further strengthened by its ability to form biofilms, which protect the bacteria from antimicrobial agents and host immune responses (Saha *et al.*, 2023).

Biofilms are structured communities of microorganisms that adhere to both living tissues and non-living surfaces. They are commonly found on various parts of the human body, including the skin, mucous membranes, and teeth, as well as on medical implants such as central venous catheters and artificial joints (Varma *et al.*, 2023). In *Klebsiella pneumoniae*, biofilm development is considered a major factor contributing to its ability to cause disease (Shadkam *et al.*, 2021). During biofilm formation, microbes produce and utilize a matrix known as extracellular polymeric substances (EPS) to adhere to various surfaces, whether living or nonliving (Ashwath *et al.*, 2022). This EPS matrix is primarily composed of substances such as polysaccharides, proteins, lipids, nucleic acids, and extracellular DNA (Bertoglio *et al.*, 2018). The polysaccharides found in *K. pneumoniae* biofilms include sugars such as mannose and glucose, along with their derivatives and acetylated forms. Protein expression within the biofilm can vary, influenced by differences in the surrounding environment (Singh *et al.*, 2019). Although biofilm formation may occur at different rates depending on the bacterial strain, the developmental stages generally include initial attachment, formation of microcolonies, biofilm maturation, and eventual dispersion (Koo *et al.*, 2017).

Efflux pumps are active transport systems present in bacterial cells that expel toxic substances, including antibiotics, from the interior of the cell to the external environment. These transporters play a significant role in bacterial resistance by lowering the intracellular concentration of antimicrobial agents, thereby diminishing their effectiveness (Sharma *et al.*, 2019). In *Klebsiella pneumoniae, specific efflux pump systems, such as AcrAB-TolC and MdtK,* are crucial in mediating resistance to multiple drugs. AcrAB-TolC belongs to the resistance-nodulation-division (RND) family, while MdtK is part of the multidrug and toxic compound extrusion (MATE) family. Research has demonstrated that these systems contribute to decreased susceptibility to various antibiotics, including tetracycline, quinolones, and chloramphenicol, particularly in multidrug-resistant (MDR) strains of *K. pneumoniae* (Mohsin *et al.*, 2022).

Given the significant role of biofilm formation and efflux pump activity in enhancing the pathogenicity and antibiotic resistance of *Klebsiella pneumoniae,* particularly in multidrug-resistant strains, this study aims to phenotypically characterize these two key virulence mechanisms to better understand their contributions to treatment failure and persistence in clinical settings.

MATERIALS AND METHODS

Collection and Identification of *Klebsiella pneumoniae* Isolates

A total of sixteen (16) *K. Pneumoniae* isolates were obtained from the Department of Microbiology, University of Lagos, Lagos State.

Microbiological analysis was conducted to confirm *Klebsiella pneumoniae*. The isolates were subcultured onto prepared nutrient agar plates and incubated at 37°C for 24 hours to obtain pure cultures. Phenotypic identification was performed using Gram staining and a series of biochemical tests, including the Indole test, Methyl Red (MR) test, Voges-Proskauer (VP) test, Citrate utilization test, Oxidase test, and Catalase test, as described by Perla (2016). Confirmed *K. pneumoniae* colonies were then inoculated onto nutrient agar slants, incubated at 37°C for 24 hours, and subsequently stored at refrigeration temperature for preservation.

Antibiotic sensitivity testing

Aseptic procedures were followed to subculture pure *Klebsiella pneumoniae* colonies overnight on nutrient agar at 37°C. From these, a bacterial suspension was prepared and adjusted to the 0.5 McFarland standard. A sterile cotton swab dipped in the suspension was used to evenly spread the bacteria over Mueller-Hinton agar plates. Antibiotic discs were carefully placed onto the plates with sterile forceps and gently pressed into place. The plates were incubated at 37°C for 24 hours, and antibiotic susceptibility was assessed using the standard disk diffusion method (Kirby–Bauer) according to CLSI guidelines (CLSI, 2024). The antibiotic sensitivity was evaluated using discs of Ofloxacin (5 µg), Ciprofloxacin, amoxicillin-clavulanate (Augmentin), Gentamicin, cephalexin, and Ampicillin.

Assessment of biofilm development using the Congo red agar method

Congo red stain was prepared as a concentrated aqueous solution and sterilized separately by autoclaving. Following sterilization, it was incorporated into autoclaved brain heart infusion (BHI) agar supplemented with 5% sucrose, cooled to 55°C. The resulting Congo red agar was dispensed into Petri dishes, which were subsequently inoculated with the test organism and incubated aerobically at 37°C for 24 hours. All experiments were conducted in triplicate to ensure reproducibility, as described by Samia et al. (2016).

Detection of efflux pump mechanism

The ethidium bromide (EtBr)-agar cartwheel method, with slight modifications from the procedure of Martins *et al.* (2013), was employed to assess efflux pump activity in *Klebsiella pneumoniae* isolates. Initially, the isolates were cultured on nutrient agar at 37°C for 24 hours. Mueller-Hinton agar plates were then prepared with graded concentrations of EtBr (0.5 mg/L, 1 mg/L, 1.5 mg/L, and 2 mg/L). Each isolate was streaked onto the EtBr-supplemented plates in a cartwheel pattern. The plates were wrapped in aluminum foil to shield them from light and incubated overnight at 37°C. Post-incubation, they were examined under UV light. The lowest concentration of EtBr that induced visible fluorescence in the bacterial colonies was recorded. Plates were subsequently re-incubated for an additional 16 hours at 37°C and re-examined under a UV transilluminator.

RESULT

Data analysis

A total of sixteen (16) presumptive *Klebsiella pneumoniae isolates* (labeled K4 to K39) were recovered following primary culture on MacConkey agar at 37°C for 18–24 hours. All isolates produced pink, mucoid colonies indicative of lactose fermentation. Gram staining confirmed that the isolates were Gram-negative rods. Biochemical profiling revealed consistent results across all isolates: Indole-negative, Methyl Red-negative, Voges–Voges-Proskauer-positive, Citrate-positive, Oxidase-negative, and Catalase-positive. These findings support the identification of all isolates as *Klebsiella pneumoniae*.

Table 1. Biochemical Characterization of *Klebsiella pneumoniae* Isolates (n = 16)

Test	Result	Interpretation
Gram stain	Gram-negative	Rod-shaped bacilli
Lactose fermentation	Positive (pink colonies)	Lactose fermenter
Indole test	Negative	Does not produce indole
Methyl Red (MR) test	Negative	No mixed acid fermentation
Voges-Proskauer (VP) test	Positive	Acetoin production confirmed
Citrate utilization	Positive	Utilizes citrate as sole carbon source
Oxidase test	Negative	Lacks cytochrome *c* oxidase
Catalase test	Positive	Produces catalase enzyme

Antibiotic Susceptibility Profile of *Klebsiella pneumoniae* Isolates

The antimicrobial susceptibility testing of the sixteen *Klebsiella pneumoniae* isolates against six commonly used antibiotics revealed varying resistance and sensitivity patterns. A significant proportion of the isolates exhibited high resistance to Ofloxacin (75%, n = 12), followed by Cephalexin (56.25%, n = 9) and Ampicillin (50%, n = 8). Moderate resistance was observed for Gentamicin (43.75%, n = 7), while both Ciprofloxacin and Augmentin showed lower resistance rates (31.25%, n = 5 each).

Despite the resistance trends, Ciprofloxacin demonstrated the highest susceptibility rate among the antibiotics tested, with 56.25% (n = 9) of isolates showing sensitivity. Gentamicin and Augmentin also showed moderate susceptibility at 43.75% (n = 7) and 37.5% (n = 6) respectively. Cephalexin, Ampicillin, and Ofloxacin displayed comparatively lower sensitivity profiles, with a notable percentage of isolates falling into the intermediate resistance category. The susceptibility patterns were categorized as Resistant (R), Intermediate (I), and Susceptible (S), and are presented in Table 2. Percentages of each response pattern per antibiotic are detailed in Table 3 and visually represented in Figure 1.

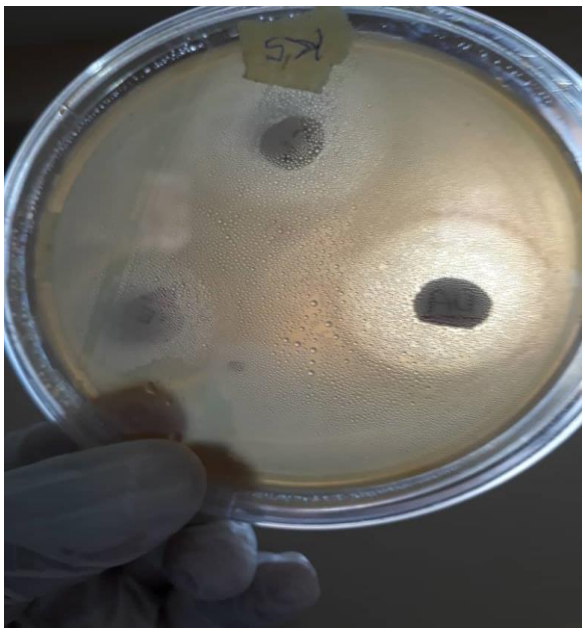

Figure 1: Plate Showing Antibiotic Sensitivity Testing

The table shows how different *Klebsiella pneumoniae* isolates (labeled K4 to K39) responded to six antibiotics by measuring the diameter of the zone of inhibition (in millimeters). This zone indicates how well an antibiotic stops bacterial growth: Larger zones mean the bacteria are more sensitive to the antibiotic; smaller or no zones mean the bacteria are resistant. Each number in the table shows the size of the inhibition zone (in mm), followed by a letter in brackets showing the result: S = Susceptible (the antibiotic worked), I = Intermediate (moderate effect), R = Resistant (the antibiotic didn't work). The findings as indicated from the table shows that; Ampicillin (AMP) had poor performance overall, with most isolates showing resistance, Cefalexin (CEF) also had a high rate of resistance, Ofloxacin (OFL) and Ciprofloxacin (CPR) had mixed results — some isolates were susceptible, while others were resistant or intermediate, Gentamicin (GEN) and Augmentin (AUG) showed relatively better activity, with several isolates being susceptible.

Table 2. Diameter of Zone of Inhibition (mm) for *Klebsiella pneumoniae* Isolates (K4-K39) Against Selected Antibiotics

	Diameter of Zones of Inhibition (mm)					
Isolate	**OFL**	**CEF**	**AMP**	**GEN**	**AUG**	**CPR**
K4	23 (R)	0 (R)	12 (R)	13 (R)	13 (R)	21 (S)
K5	13 (S)	25 (S)	0 (R)	28 (S)	29 (S)	28 (S)
K6	34 (R)	0 (R)	12 (R)	31 (S)	30 (S)	31 (S)
K9	25 (R)	12 (R)	19 (S)	20 (S)	22 (S)	25 (S)
K10	27 (I)	16 (S)	12 (R)	21 (S)	15 (I)	15 (R)
K11	26 (I)	0 (R)	11 (R)	20 (S)	26 (S)	28 (S)
K12	11 (R)	18 (S)	12 (R)	20 (S)	13 (R)	22 (S)
K13	28 (I)	20 (S)	23 (R)	13 (R)	0 (R)	27 (R)
K15	19 (R)	21 (S)	18 (S)	12 (R)	15 (I)	14 (R)
K17	11 (R)	15 (R)	10 (R)	18 (I)	17 (I)	19 (I)
K19	0 (R)	21 (S)	23 (S)	0 (R)	0 (R)	0 (R)
K21	25 (R)	14 (R)	16 (S)	20 (S)	16 (I)	13 (R)
K22	19 (R)	0 (R)	16 (S)	14 (R)	0 (R)	0 (R)
K24	14 (R)	22 (S)	23 (S)	21 (S)	20 (S)	25 (S)
K28	21 (R)	11 (R)	27 (S)	0 (R)	18 (S)	22 (S)
K39	12 (R)	0 (R)	0 (R)	0 (R)	19 (S)	18 (I)

KEY: OFL: Ofloxacin; CEF: Cefalexin; AMP: Ampicillin; GEN: Gentamicin; AUG: Augmentin; CPR: Ciprofloxacin, R: Resistant; I: Intermediate; S: Susceptible. (EUCAST, 2019).

Table 3 summarizes the antibiotic sensitivity pattern of 16 different *Klebsiella pneumoniae* isolates. The bacteria were classified based on their sensitivity to each antibiotic as follows: Resistant – the antibiotic did not work against the bacteria; Intermediate – the antibiotic had a moderate or limited effect; and Susceptible –

the antibiotic was effective at stopping bacterial growth. Each row shows the number and percentage of isolates that fell into each category for a particular antibiotic.Ciprofloxacin (CPR) was the most effective antibiotic overall, with a 56.25% susceptibility rate. Ofloxacin (OFL) had the highest resistance rate (75%), while the other antibiotics showed mixed effectiveness, with no single drug being universally effective against all isolates.

Table 3: Antibiotic Sensitivity Pattern of *Klebsiella pneumoniae* Isolates

Antibiotic	Resistant n (%)	Intermediate n (%)	Susceptible n (%)
OFL (Ofloxacin)	12 (75.00%)	3 (18.75%)	3 (18.75%)
CEF (Cefuroxime)	9 (56.25%)	0 (0.00%)	7 (43.75%)
AMP (Ampicillin)	8 (50.00%)	0 (0.00%)	8 (50.00%)
GEN (Gentamicin)	7 (43.75%)	1 (6.25%)	8 (50.00%)
AUG (Amoxicillin-Clavulanate)	5 (31.25%)	4 (25.00%)	7 (43.75%)
CPR (Ciprofloxacin)	5 (31.25%)	2 (12.50%)	9 (56.25%)

Detection of biofilm formation

The sixteen (16) *Klebsiella pnuemoniae* isolates were examined for biofilm formation using the Congo Red Agar method. Biofilm-forming ability was indicated by the development of black, dry, and crystalline colonies with a metallic sheen, whereas non-biofilm producers exhibited red to dark red, smooth colonies without crystalline structure. Out of the 16 isolates examined, four isolates (25%)—specifically K11, K15, K21, and K22—demonstrated positive biofilm formation. These isolates developed characteristic black colonies, suggesting the presence of exopolysaccharide production associated with biofilm matrix development. The remaining twelve isolates (75%) did not exhibit significant colony blackening or crystalline morphology, indicating a lack of detectable biofilm production under the given conditions.

Figure 2: Plate showing biofilm development

Phenotypic profiling of efflux pump activity

Efflux pump activity was evaluated among multidrug-resistant (MDR) *Klebsiella pneumoniae* isolates using a phenotypic fluorescence-based screening approach. The ability of isolates to actively expel ethidium bromide (EtBr), a DNA-intercalating fluorescent dye, served as an indicator of efflux system function. Out of the tested MDR isolates, only two (12.5%)—specifically K5 and K15—demonstrated detectable efflux activity, evidenced by the absence of fluorescence at lower dye concentrations under UV light

Figure 3: Plates viewed under UV light to assess efflux pump activity.

DISCUSSION

The findings from this study reveal important insights into the antibiotic resistance patterns, biofilm formation, and efflux pump activity among *Klebsiella pneumoniae* isolates. The high level of resistance observed across multiple antibiotics, especially to Ofloxacin (75%) and Cefalexin (56.25%), underscores the growing concern of antimicrobial resistance in *K. pneumoniae*, a pathogen often implicated in nosocomial infections. Ciprofloxacin showed the highest susceptibility (56.25%), indicating it may still retain therapeutic value against certain isolates, although resistance is evidently emerging. These results align with recent

findings by Paczosa and Mecsas (2016), who noted increasing multidrug resistance in clinical *K. pneumoniae* strains due to the accumulation of resistance determinants.

The ability to form biofilms was detected in 25% of the isolates, as indicated by dark crystalline colonies on Congo Red Agar, a key virulence factor for *K. pneumoniae* that contributes to persistent infections, resistance to antimicrobial agents, and survival on medical surfaces and devices. The Congo Red Agar method, although qualitative, remains a rapid and cost-effective screening tool for initial biofilm assessment in clinical isolates (Mosa *et al.*, 2021; Khosravi *et al.*, 2020). Biofilm formation enhances bacterial survival by providing protection from antibiotics and host immune responses (Vuotto *et al.*, 2017). Notably, three of the biofilm-forming isolates (K15, K11, K21) also demonstrated resistance to multiple antibiotics, suggesting a correlation between biofilm production and multidrug resistance. This supports prior studies indicating that biofilm-producing *K. pneumoniae* are more likely to be antibiotic-resistant due to impaired drug penetration and metabolic dormancy of cells within the biofilm matrix (Anderl *et al.*, 2018).

Phenotypic characterization of efflux pump activity using the Ethidium Bromide Cartwheel method revealed that only two isolates (K5 and K15) exhibited active efflux mechanisms. Interestingly, K15 was both a biofilm former and exhibited efflux pump activity, suggesting a potentially synergistic mechanism of resistance. These findings suggest that while efflux pump-mediated resistance may not be widespread among all the MDR *K. pneumoniae* isolates in this study, it remains a contributing resistance mechanism in select strains. The presence of functional efflux systems in isolates K5 and K15 suggests their potential role in reducing intracellular antibiotic accumulation and enhancing survival under antimicrobial stress. This aligns with existing literature highlighting the role of efflux pumps, particularly those from the RND and MATE families, in mediating resistance to multiple drug classes, including fluoroquinolones, tetracyclines, and chloramphenicol (Ghai *et al.*, 2023; Godoy *et al.*, 2022). Efflux pumps play a crucial role in conferring resistance by expelling a broad range of antibiotics, and their co-occurrence with biofilm formation in the same isolate may explain its enhanced resistance profile (Roy *et al.*, 2021). However, the low prevalence of efflux activity suggests that resistance in these isolates may primarily result from other mechanisms such as ß-lactamase production or porin loss.

Overall, the findings highlight the multifactorial nature of antibiotic resistance in *K. pneumoniae*, with biofilm formation and efflux pumps contributing variably among isolates. These results underscore the need for continuous surveillance and the development of therapeutic strategies targeting both planktonic and biofilm-associated cells.

CONCLUSION

This study demonstrates the significant antimicrobial resistance exhibited by *Klebsiella pneumoniae* isolates, with high resistance rates observed particularly against Ofloxacin, Cefalexin, and Ampicillin. The detection of biofilm-forming ability in 25% of the isolates suggests that biofilm production may be contributing to the persistence and resistance of certain strains. Moreover, the identification of efflux pump activity in two multidrug-resistant isolates, including one biofilm-forming isolate, indicates that multiple resistance mechanisms may coexist within individual strains, thereby compounding treatment challenges.

These findings highlight the multifactorial nature of resistance in *K. pneumoniae*, where both structural adaptations, such as biofilm formation, and active processes, like efflux, contribute to antibiotic evasion. The observed susceptibility to Ciprofloxacin and Gentamicin in a subset of isolates suggests these agents may retain some therapeutic utility; however, their effectiveness may be limited by emerging resistance mechanisms.

Given the public health implications of rising multidrug resistance in *K. pneumoniae*, this study underscores the importance of continuous antimicrobial surveillance, rational antibiotic use, and the development of novel treatment strategies that target biofilm and efflux-mediated resistance. Antibiofilm compounds that could inhibit bacterial adhesion, modulate quorum sensing, and disrupt mature biofilms are being developed. Despite the growing volume of research on this, none of the agents have been licensed for clinical use. The treatment of biofilm-related infections still remains an unsolved problem because clinical and investigative observations have shown that antibiotic therapy is insufficient to eradicate biofilm infections. It is therefore an urgent concern that alternative strategies for the treatment of biofilm be discovered

Future studies incorporating molecular methods are recommended to further elucidate the genetic basis of these phenotypic traits and to guide more effective infection control and therapeutic interventions.

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