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Original Article

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Comparative analysis of biofilm formation in carbapenem-resistant and carbapenem-susceptible *Klebsiella pneumoniae* clinical isolates from a tertiary hospital in Nigeria

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Abstract:

Background: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections pose a major challenge due to limited therapeutic options. Biofilm formation has been proposed as a potential contributor to antimicrobial resistance; however, evidence regarding its association with carbapenem resistance in *K. pneumoniae* remains conflicting. Notably, data from Nigeria are scarce. The objective of this study is to compare biofilm formation between carbapenem-resistant and carbapenem susceptible *K. pneumoniae* (CRKP and CSKP) isolated from blood samples.

Methodology: This comparative laboratory-based study analysed 46 stored blood culture isolates of *K. pneumoniae* from a tertiary hospital in Nigeria. Re-identification was done using VITEK® 2 automated system and carbapenem susceptibility testing was carried out using Kirby-Bauer disc diffusion method. Two methods of biofilm formation assessment; Congo Red Agar (CRA) and microtiter plate assay, were used. Statistical analysis was done using Fisher Exact test, with Odds ratio and 95% confidence interval, and $p < 0.05$ considered statistical significance. For statistical analysis of biofilm strength determined by the microtiter plate assay, isolates were dichotomised into strong and non-strong biofilm producers.

Results: A total of 46 *K. pneumoniae* blood isolates were studied, of which 30 (65.2%) were CSKP and 16 (34.8%) were CRKP. Using the CRA method, 16 isolates (34.8%) were biofilm-positive, while 30 (65.2%) were negative; 6 of 16 (37.5%) CRKP and 10 of 30 (33.3%) CSKP isolates were biofilm formers, but the difference was not statistically significant (OR=1.200; 95% CI=0.3385-4.254; $p=1.000$). Similarly, the microtiter plate assay identified 42 isolates (91.3%) as strong biofilm producers and 4 (8.7%) as non-strong biofilm producers; 14 of 16 (87.5%) CRKP and 28 of 30 (93.3%) CSKP isolates were strong biofilm producers, but the difference was also not statistically significant (OR=0.500; 95% CI=0.06355-3.934; $p=0.6019$).

Conclusion: Our study found no significant association between carbapenem resistance and biofilm formation, suggesting that biofilm formation may act as an independent bacterial survival strategy rather than a direct contributor to carbapenem resistance.

Keywords: *Klebsiella pneumoniae*, Biofilm formation, Carbapenem resistance, Congo Red Agar, Microtiter plate assay

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Analyse comparative de la formation de biofilm chez des isolats cliniques de *Klebsiella pneumoniae* résistants et sensibles aux carbapénèmes provenant d'un hôpital universitaire du Nigéria

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Résumé:

Contexte: Les infections à *Klebsiella pneumoniae* résistante aux carbapénèmes (CRKP) constituent un défi majeur en raison du nombre limité d'options thérapeutiques. La formation de biofilm a été proposée comme facteur potentiel de résistance aux antimicrobiens; cependant, les données concernant son association avec la résistance aux carbapénèmes chez *K. pneumoniae* restent contradictoires. Notamment, les données nigérianes sont rares. L'objectif de cette étude est de comparer la formation de biofilm entre les souches de *K. pneumoniae* résistantes et sensibles aux carbapénèmes (CRKP et CSKP) isolées à partir d'hémocultures.

Méthodologie: Cette étude comparative en laboratoire a analysé 46 isolats de *K. pneumoniae* provenant d'hémocultures conservées d'un hôpital de référence au Nigéria. La réidentification a été effectuée à l'aide du système automatisé VITEK® 2 et les tests de sensibilité aux carbapénèmes ont été réalisés par la méthode de diffusion sur disque de Kirby-Bauer. Deux méthodes d'évaluation de la formation de biofilm ont été utilisées: la gélose au rouge Congo (CRA) et le test sur microplaque. L'analyse statistique a été réalisée à l'aide du test exact de Fisher, avec calcul du rapport de cotes et de l'intervalle de confiance à 95%, et un seuil de signification statistique fixé à $p < 0,05$. Pour l'analyse statistique de la force du biofilm déterminée par le test sur microplaque, les isolats ont été classés en deux groupes: producteurs forts et faibles de biofilm.

Résultats: Au total, 46 isolats sanguins de *K. pneumoniae* ont été étudiés, dont 30 (65,2%) étaient des CSKP et 16 (34,8%) des CRKP. Par la méthode CRA, 16 isolats (34,8%) étaient positifs pour la formation de biofilm, tandis que 30 (65,2%) étaient négatifs; 6 des 16 isolats CRKP (37,5%) et 10 des 30 isolats CSKP (30,0 %) formaient un biofilm, mais la différence n'était pas statistiquement significative (OR=1,200; IC à 95%=0,3385-4,254; $p=1,000$). De même, le test sur microplaque a identifié 42 isolats (91,3%) comme producteurs forts de biofilm et 4 (8,7%) comme producteurs faibles de biofilm. Quatorze des seize isolats de CRKP (87,5%) et 28 des 30 isolats de CSKP (93,3%) étaient de forts producteurs de biofilm, mais la différence n'était pas statistiquement significative (OR=0,500; IC à 95%=0,06355-3,934; $p=0,6019$).

Conclusion: Notre étude n'a pas mis en évidence d'association significative entre la résistance aux carbapénèmes et la formation de biofilm, suggérant que cette dernière pourrait constituer une stratégie de survie bactérienne indépendante plutôt qu'un facteur direct de résistance aux carbapénèmes.

Mots-clés: *Klebsiella pneumoniae*, formation de biofilm, résistance aux carbapénèmes, gélose au rouge Congo, test sur microplaque

Introduction:

Infections caused by *Klebsiella pneumoniae* are becoming progressively more difficult to treat as antimicrobial resistance continues to expand at an alarming rate, especially resistance to carbapenems, which are often regarded as the last resort antibiotics for treatment of infections caused by multidrug-resistant (MDR) pathogens (1,2). Several international studies have documented a substantial occurrence in carbapenem-resistant *K. pneumoniae* (CRKP) over the years (3-5). Similarly, two systematic reviews have reported a rising trend of CRKP in Nigerian hospitals, with prevalence rates ranging from 20.2% to 26.3% (6,7).

This increasing trend is linked to increased death rates, extended hospital admissions and limited access to effective therapeutic options (8,9). Despite this, active surveillance of CRKP remains challenging in resource-limited settings due to financial constraints, which contributes to the under-recognition of the actual disease burden (10,11). In response to this growing threat, the World Health Organization (WHO) has identified CRKP as a priority for the development of novel therapeutic agents (12, 13).

Resistance to carbapenems largely arises from the acquisition of carbapenem-hydrolysing enzymes, notably KPC (*Klebsiella pneumoniae* carbapenemase), NDM (New Delhi metallo- β -lactamase) and OXA-48-type β -lactamases

(14-16). These enzymes hydrolyse carbapenems and render them ineffective, often resulting in treatment failures and prolonged hospital stays (17).

Beyond enzyme-linked resistance, the persistence and virulence of *K. pneumoniae* infections are strongly influenced by the ability of the organism to develop biofilms, which are complex microbial assemblies encased within a self-generated extracellular matrix that attaches to both living tissues and inert surfaces (18, 19). This protective structure limits the effectiveness of antimicrobial therapy and impairs host immune clearance, thereby complicating eradication of infection (19). According to Makhrmash et al., *fimH*, *mrkA*, and *mrkD* genes, which encode fimbrial adhesion proteins that help the bacteria attach to surfaces and host cells, are commonly found in *K. pneumoniae* and are strongly linked to biofilm-producing isolates (20).

There are mixed findings on whether carbapenem resistance is linked to biofilm formation in *K. pneumoniae*. Some studies reported a significant association, suggesting that isolates resistant to carbapenems tend to form stronger biofilms (21,22). In contrast, Fang et al., (23) found that the regulatory gene *mrkH*, which plays a key role in biofilm development, is often absent in carbapenem-resistant strains, leading these isolates to produce weaker biofilms. Sabenca et al., (24) further showed that the presence of KPC enzymes does not necess-

arily correspond with increased biofilm-forming ability. Similarly, Cepas et al., (25) observed that multidrug-resistant strains were not more likely to form higher-level biofilms than non-multidrug-resistant strains (25). These studies indicate that although biofilm formation and carbapenem resistance may interact, the exact nature of this relationship is still unclear.

Several studies from Nigeria have reported the prevalence and antimicrobial resistance patterns of *K. pneumoniae*, with a primary focus on phenotypic resistance profiles and molecular characterisation (26-29). No study from Nigeria has specifically examined the association between carbapenem-resistant *K. pneumoniae* (CRKP) and carbapenem-susceptible *K. pneumoniae* (CSKP). Since enhanced biofilm formation can increase bacterial persistence and reduce treatment efficacy, this study aims to compare biofilm formation between CRKP and CSKP isolated from blood samples in a tertiary facility in Lagos and to determine if there is any relationship between biofilm formation and carbapenemase resistance.

Materials and method:

Study setting, design and ethical approval:

This was a comparative laboratory-based study carried out in the Department of Medical Microbiology and Parasitology Laboratory, Lagos University Teaching Hospital (LUTH). Ethical approval was obtained from the hospital's ethical committee with Health Research Committee Assigned Number: ADM/DSCST/HREC/APP/7759.

Sample size determination and collection of isolates:

Sample size was determined using a 5.2% prevalence of carbapenem-resistant *Klebsiella pneumoniae* at LUTH (30), with a 2:1 carbapenem-susceptible to carbapenem-resistant ratio to enhance statistical power. Based on this ratio, an estimated sample size of 30-45 isolates was calculated. Ultimately, 46 archived blood isolates (16 carbapenem-resistant and 30 carbapenem-susceptible) collected between January and August 2025 were included. These isolates were recovered from routinely stored glycerol stocks at -80°C in the Department of Medical Microbiology and Parasitology Laboratory, Lagos University Teaching Hospital (LUTH).

Re-identification of *K. pneumoniae*:

Stored isolates were first cultured on MacConkey agar and Blood agar and incubated at 37°C for 18–24 hours. On MacConkey agar, colonies were large, pink, and mucoid, reflecting

lactose fermentation. On blood agar, colonies appeared large, greyish-white, smooth, and non-haemolytic. Colonies showing these characteristic features were further analysed and confirmed using the VITEK®2 automated system for species identification.

Carbapenem susceptibility testing:

Carbapenem susceptibility testing was done using the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar, in accordance with 2025 Clinical and Laboratory Standards Institute (CLSI) guidelines. Carbapenem discs used were meropenem (10µg) and imipenem (10µg). Plates were incubated at 37°C for 18–24 hours. Inhibition zone diameters were measured and interpreted according to CLSI. Isolates with diameters ≥23mm were classified as CSKP, those between 20–22mm as intermediate, and ≤19mm as CRKP.

Biofilm detection:

Biofilm formation in the CRKP and CSKP isolates was assessed using two complementary methods; qualitative Congo Red Agar (CRA) assay and quantitative 96-well Microtiter Plate assay.

CRA assay:

The CRA method, described by Harika et al., (31) and Wilson et al., (32) was used as an initial qualitative screening technique. The assay is based on the principle that biofilm-producing bacteria synthesize extracellular polysaccharides capable of binding Congo red dye. When the dye interacts with the slime layer produced by the organism, it alters colony morphology. Biofilm-forming isolates typically appear as black colonies with a dry crystalline consistency, whereas non-producers appear red or pink. All CRA plates were independently interpreted by two observers to reduce subjectivity, and discrepancies were resolved by repeat testing. Duplicate testing was also performed to enhance result reliability.

Microtiter plate assay:

Although CRA provides a simple and rapid visual assessment, it is limited in its ability to distinguish between weak and moderate biofilm producers. Therefore, a standardized quantitative microtiter plate assay was employed for more accurate measurement of biofilm production strength. This method follows the principles described by Wilson et al., (32) and Stepanović et al., (33). Briefly, isolates were allowed to adhere and form biofilms in sterile 96-well microtiter plates for 24 hours. The resulting biofilm was stained with crystal violet, after which the

bound dye was solubilized with ethanol.

The optical density (OD) of each well was measured at 532–630 nm using a microplate reader. Four negative control wells containing only sterile broth were included. Their mean OD and standard deviation (SD) were calculated, and the optical density cut-off (ODc) was determined using the formula: $ODc = \text{mean OD of negative controls} + (3 \times SD)$. For each isolate, the average OD from its duplicate wells was calculated and the ODc was subtracted to obtain the final OD value used for classification. Biofilm production was categorized as follows; No biofilm: $OD \leq ODc$; Weak biofilm: $ODc < OD \leq 2 \times ODc$; Moderate biofilm: $2 \times ODc < OD \leq 4 \times ODc$; and Strong biofilm: $OD > 4 \times ODc$.

Although a standard positive control strain was not available during laboratory analysis, quality assurance was maintained through the use of negative control wells, strict adherence to established protocols and duplicate testing to ensure consistency and reproducibility of the results.

Statistical analysis:

The Fisher's Exact test was performed using IBM SPSS (34) to determine significant association between carbapenem resistance and biofilm formation, with Odds ratio and 95% confidence interval. Statistical significance was set at $p < 0.05$. For the purpose of inferential analysis, the strength of biofilm production determined by the microtiter plate assay was dichotomized into two categories; strong biofilm producers and non-strong biofilm producers, with the latter comprising weak, moderate, and non-biofilm-producing isolates for analysis using the Fisher's Exact test.

Results:

Identification and distribution of isolates:

A total of 46 stored *Klebsiella pneumoniae* isolates obtained from blood cultures were identified using the VITEK®2 system. Of these, 30 (65.2%) were carbapenem-susceptible (CSKP), while 16 (34.8%) were carbapenem-resistant (CRKP).

Results of biofilm detection by Congo Red Agar assay:

Using the Congo Red Agar method, 16 (34.8%) of the 46 *K. pneumoniae* isolates produced black, dry crystalline colonies indicative of biofilm formation, while 30 (65.2%) produced pink colonies and were classified as non-biofilm producers.

Association between biofilm formation and carbapenem resistance by CRA assay

Biofilm formation was observed in both carbapenem-resistant and carbapenem-susceptible isolates. Six of 16 (37.5%) CRKP and 10 of 30 (33.3%) CSKP isolates were biofilm formers. However, Fisher's exact test showed no statistically significant association between carbapenem resistance and biofilm formation ($OR = 1.200$; 95% CI = 0.3385 - 4.254; $p = 1.000$) (Table 1).

Biofilm quantification by Microtiter Plate assay:

The distribution of isolates into non-biofilm, weak, moderate and strong biofilm producers is summarised in Table 2. The result of the microtiter plate assay is shown in Fig 1.

Table 1: Frequency of biofilm formation by qualitative Congo Red agar assay among carbapenem-resistant and carbapenem-susceptible *Klebsiella pneumoniae* blood isolates

Isolates	Biofilm positive (%)	Biofilm negative (%)	Total	OR (95% CI)	p value
Carbapenem-Resistant	6 (37.5)	10 (62.5)	16	1.200 (0.3385-4.254)	1.000
Carbapenem-Susceptible	10 (33.3)	20 (66.7)	30		
Total	16 (34.8)	30 (65.2)	46		

OR=Odds ratio; CI=Confidence interval

Table 2: Classification of biofilm production by *Klebsiella pneumoniae* isolates on Microtiter plate assay

Biofilm production category	Number of isolates	Percentage (%)
No biofilm	0	0
Weak biofilm	2	4.3
Moderate biofilm	2	4.3
Strong biofilm	42	91.4
Total	46	100.0

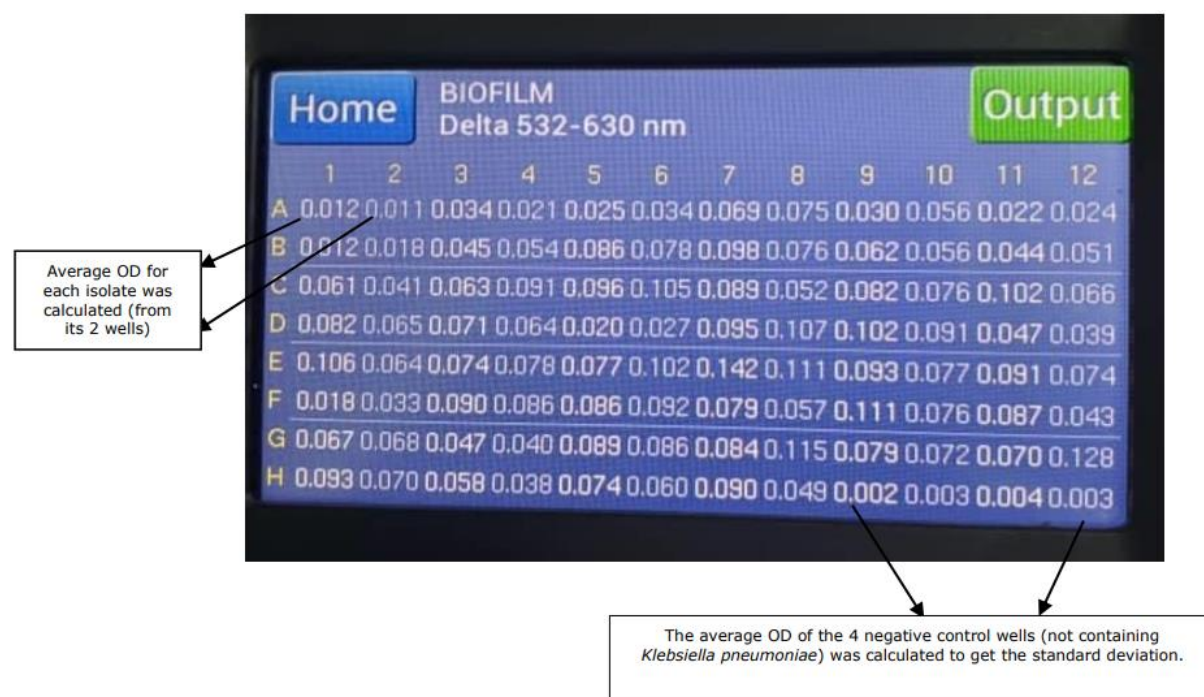


Fig 1: Result of Microtitre Plate assay for biofilm detection

Table 3: Frequency of biofilm formation by quantitative Microtitre Plate assay among carbapenem-resistance and carbapenem-susceptible *Klebsiella pneumoniae* blood isolates

Isolates	Strong biofilm (%)	Non-strong biofilm (%)	Total	OR (95% CI)	p value
Carbapenem-Resistant	14 (87.5)	2 (12.5)	16	0.500 (0.0636-3.934)	0.6019
Carbapenem-Susceptible	28 (93.3)	2 (6.7)	30		
Total	42 (91.3)	4 (8.7)	46		

OR=Odds ratio; CI=Confidence interval

Association between biofilm strength and carbapenem resistance by Microtiter Plate assay:

When biofilm strength was analysed, majority of the carbapenem-resistant and carbapenem-susceptible isolates (91.3%) were classified as strong biofilm producers; 14 of 16 (87.5%) CRKP and 28 of 30 (93.3%) CSKP isolates were strong biofilm producers. Fisher's Exact test demonstrated no statistically significant association between carbapenem resistance and biofilm strength (OR=0.500; 95% CI=0.06355-3.934; $p=0.6019$) (Table 3).

Discussion:

This study compared biofilm formation using both qualitative and quantitative phenotypic methods. The consistently higher detection of biofilm production by the microtiter plate (tissue culture plate) shows the greater sensitivity of quantitative approaches in capturing biofilm-forming capacity, irrespective of carba-

penem susceptibility. In contrast, the Congo Red Agar method identified fewer biofilm-producing isolates, reflecting its reliance on subjective colour interpretation and limited ability to detect weaker or intermediate biofilm phenotypes. Similar discrepancies between CRA and microtiter-based assays have been reported elsewhere, reinforcing the view that CRA is best suited for preliminary screening rather than definitive assessment of biofilm formation (35-37).

The central finding of this study is the absence of an association between biofilm-forming capacity and carbapenem resistance in *K. pneumoniae*. This suggests that biofilm formation and carbapenem resistance may represent distinct adaptive strategies rather than interdependent phenotypes. Similar observations have been reported by Sabença et al., (24) and Bandeira et al., (38) who demonstrated that strong biofilm formation does not necessarily coincide with carbapenemase production or

high-level antimicrobial resistance. Collectively, these findings support the notion that carbapenem resistance in *K. pneumoniae* is more strongly driven by specific resistance mechanisms, such as carbapenemase production, porin alterations and efflux pump activity than by biofilm-forming ability alone (39).

Comparing these results with other studies shows a mixed pattern. For instance, Rahdar et al., (21) while correlating biofilm formation with carbapenem resistance, found a strong positive association between these two factors, with the majority of resistant isolates being strong biofilm producers. The researchers suggested that the biofilm formation may function as an adaptive mechanism increasing antibiotic resistance and, therefore, making such strains harder to treat. Jaisal et al., (22) also found an association between carbapenemase-producing *K. pneumoniae* and biofilm formation, showing that most of the isolates were moderate to strong biofilm producers. However, it did not compare biofilm formation between resistant and susceptible isolates. Contrarily, Fang et al., (23) identified that carbapenem-susceptible isolates were more likely to carry the *mrkH* gene, thus helping biofilm formation, and showed stronger biofilm formation compared with the resistant ones. The conflicting outcomes across studies might be related to differences in sample size, detection methods, and the local pattern of antimicrobial resistance.

From an infection-control perspective, the high prevalence of biofilm formation among both carbapenem-resistant and susceptible isolates has important clinical implications. Biofilm-producing strains can persist on medical devices, hospital surfaces, and indwelling catheters, facilitating recurrent or chronic infections even in the absence of overt antimicrobial resistance. Consequently, infection-prevention strategies should incorporate measures that directly target biofilm disruption, rather than focusing solely on antimicrobial susceptibility profiles (40). Moreover, biofilm-associated persistence may promote the maintenance and dissemination of resistance determinants, highlighting the need to address biofilm formation as an independent risk factor in healthcare-associated infections (41,42).

Conclusion:

This study found no evidence of an association between biofilm-forming capacity and carbapenem resistance in *K. pneumoniae*, indicating that biofilm formation may represent an independent bacterial survival strategy rather than a direct determinant of carbapenem

resistance. The ability of both carbapenem-resistant and carbapenem-susceptible isolates to form strong biofilms underscores the clinical relevance of biofilm-mediated persistence in healthcare settings, irrespective of antimicrobial susceptibility profiles.

The findings should be interpreted in light of certain limitations, including the relatively small sample size, the absence of a standard positive control strain, and the exclusive reliance on phenotypic methods for biofilm detection. While these approaches are practical and widely used, they do not capture the molecular complexity underlying biofilm regulation and resistance mechanisms. Future studies incorporating larger isolate collections and molecular characterization of both carbapenem resistance determinants and biofilm-associated genes are warranted to better elucidate the interplay between resistance and virulence in *K. pneumoniae*.

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Contributions of authors:

The study was a collaborative effort, with all authors making substantive contributions to the manuscript. OBM led the experimental design and conducted the data analysis. AV provided overall supervision of the study, while ORO contributed through critical review and intellectual refinement of the manuscript.

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Conflict of interest:

Authors declare no conflicts of interest.

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