

Received: 23 June 2026; Revised: 18 September 2026; Accepted: 09 October 2026; Published: 18 October 2026

Archs Sci. (2026) Volume 76(4), Pages 136-145, Paper ID 2026414.
<https://doi.org/10.68304/as/76414>

Beyond Biomass: Relationship Between Biofilm Architecture, Viable Bacterial Burden, and Antibiotic Survival in Clinical *Klebsiella pneumoniae* Isolates

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Abstract Crystal violet (CV) staining is widely used to classify clinical isolates of *Klebsiella pneumoniae* as non-adherent, weak, moderate, or strong biofilm producers. However, a measurement of total adherent biomass does not necessarily indicate the number of viable bacteria present within a biofilm or the capacity of those bacteria to survive antimicrobial exposure. This study examined the relationships among CV biomass, three-dimensional biofilm architecture, viable sessile burden, and antibiotic survival in a panel of 60 non-duplicate clinical *K. pneumoniae* isolates. The isolates were characterised by CV staining (OD₅₇₀), quantitative culture (log₁₀ CFU/cm²), confocal laser scanning microscopy combined with COMSTAT analysis, and 24-hour antimicrobial challenges involving meropenem, ciprofloxacin, and colistin at 10× and 100× isolate-specific minimum inhibitory concentrations (MICs). Architectural measurements included biovolume, mean thickness, roughness, substratum coverage, and average diffusion distance. Minimum biofilm eradication concentrations (MBECs) were measured using a peg-lid method. CV biomass showed only a weak correlation with viable sessile burden (Spearman's $\rho = 0.31$) and weak associations with log₁₀ reductions achieved by the tested antibiotics. Average diffusion distance and initial viable burden were the most informative regression predictors of reduced killing; the meropenem model explained 61% of the reported variation in log₁₀ reduction. Notably, 15 isolates classified as non-adherent or weakly adherent by CV nonetheless contained at least 10⁷ CFU/cm² and showed less than a 1-log₁₀ reduction following meropenem exposure at 100× MIC. Conversely, six strongly staining isolates exhibited reductions greater than 3 log₁₀. These observations indicate that total adherent biomass, viable bacterial burden, architectural organisation, and antibiotic survival are related but distinct biofilm characteristics. CV staining alone is therefore insufficient for evaluating biofilm-associated antimicrobial tolerance, and complementary assessments of viability and survival are warranted in clinical-isolate research.

Index Terms *Klebsiella pneumoniae*; biofilm architecture; crystal violet; viable cell burden; antibiotic tolerance; confocal laser scanning microscopy; COMSTAT; minimum biofilm eradication concentration

1. Introduction

Klebsiella pneumoniae has become a major concern in clinical microbiology over the past several decades because of its capacity to cause serious infections and acquire antimicrobial resistance. The global analysis of antimicrobial resistance estimated that 4.95 million deaths were associated with bacterial antimicrobial resistance in 2019 and identified *K. pneumoniae* among the six bacterial pathogens responsible for the greatest attributable burden. Carbapenem-resistant and third-generation cephalosporin-resistant *K. pneumoniae* are particularly important pathogen–drug combinations [1]. Two interconnected properties contribute to this

clinical importance: genomic adaptability and ecological opportunism. Its extensive accessory genome provides opportunities to acquire and exchange resistance determinants and virulence-associated genes across lineages [2]. Moreover, its polysaccharide capsule, iron-acquisition systems, and surface-associated proteins facilitate colonisation of the intestinal tract, respiratory tract, and medical devices [3]. Asymptomatic carriage may precede invasive disease, and medical-device exposure can facilitate the transition from colonisation to clinically significant infection [4]. Understanding the survival of this pathogen on implanted and indwelling surfaces is therefore essential to improving laboratory assessment and

infection management.

An important step in this transition is biofilm formation. *K. pneumoniae* can adhere to urinary catheters, endotracheal tubes, and intravascular lines, establishing surface-associated bacterial communities enclosed within an extracellular matrix. Type 3 fimbriae, encoded by the chromosomal *mrk* gene cluster and expressed by many clinical isolates, are important adhesins that promote attachment to abiotic and extracellular-matrix-coated surfaces [5]. Studies using isogenic mutants in flow-cell systems demonstrated that type 3 fimbriae substantially promote biofilm development, whereas type 1 fimbriae are not required under those experimental conditions and may be down-regulated in sessile cells [6]. Expression of the *mrk* cluster is regulated by cyclic-di-GMP-responsive factors, including MrkH and MrkI, linking fimbrial expression to signalling processes involved in biofilm development [7]. Capsular polysaccharides, exopolysaccharide-related systems, quorum-sensing pathways involving LuxS, and efflux mechanisms may provide additional contributions to biofilm formation and persistence [8], [9]. Clinically, device-associated infections involving biofilm-forming organisms may persist, recur after antimicrobial treatment, or require device removal to achieve source control [10], [11]. The relevance of biofilms to persistent infections is also acknowledged in guidance for their diagnosis and treatment [12].

The mechanisms underlying biofilm-associated antimicrobial recalcitrance are multifactorial and differ in important respects from conventional, genetically mediated antimicrobial resistance. The extracellular polymeric substance matrix may impede or bind selected antimicrobial agents, while limiting the movement of nutrients and oxygen and creating physicochemical gradients within the biofilm [13], [14]. Such gradients generate physiological heterogeneity: cells near the outer surface may remain metabolically active, whereas cells deeper within the structure may experience nutrient limitation, reduced growth, or metabolic dormancy [15]. Experimental work in *Pseudomonas aeruginosa* biofilms attributed tolerance to ciprofloxacin and tobramycin partly to oxygen limitation and reduced metabolic activity rather than solely to restricted drug penetration [16]. Subsequent studies identified the stringent starvation response as another active mechanism of protection [17]. In addition, persister subpopulations may survive otherwise lethal antibiotic exposure without acquiring heritable resistance determinants [18]. Such responses are more appropriately described as tolerance or persistence than as classical resistance: they primarily affect the rate or duration of bacterial killing rather than necessarily increasing the concentration required to inhibit planktonic growth [19], [20]. Consequently, a biofilm may remain viable despite antibiotic exposure even when conventional minimum inhibitory concentration (MIC) testing indicates planktonic susceptibility. The Calgary Biofilm Device helped demonstrate this discrepancy by documenting minimum biofilm eradication concentrations (MBECs) far exceeding planktonic MICs for certain organism–antibiotic combinations [21].

Against this mechanistic background, the limited range of methods commonly used to assess biofilm production by clinical isolates deserves scrutiny. Many studies of *K. pneumoniae* classify isolates using microtitre-plate crystal violet (CV) staining, often applying the optical-density cut-off approach described by Stepanović et al. to distinguish non-adherent, weak, moderate, and strong biofilm producers [22]. Published surveys have reported variable proportions of strong biofilm-producing isolates and have explored associations between CV categories, antimicrobial resistance phenotypes, specimen sources, and virulence-associated characteristics [23]–[25]. The method is popular because it is inexpensive, straightforward, compatible with large sample numbers, and produces a quantitative absorbance reading. However, the biological interpretation of that reading is less straightforward. Crystal violet is a non-specific stain that binds to adherent cellular and extracellular material, including polysaccharides, extracellular DNA, proteins, and both viable and non-viable bacterial cells. Accordingly, the signal reflects the amount of attached material rather than providing a direct measure of viable bacterial abundance. A high CV reading should therefore not automatically be interpreted as a high viable burden or as evidence of enhanced survival following antimicrobial exposure.

Comparisons of biofilm-quantification methods underscore this distinction, although their findings have not been consistently incorporated into the assessment of clinical isolates. A comparison of multiple microtitre-plate assays showed that CV staining, metabolic indicators such as resazurin and XTT, and viable plate counts measure overlapping but non-identical properties and may differ in their suitability for individual organisms [26]. A systematic assessment of total biomass, total cells, viable cells, and matrix-associated material further demonstrated that no single approach adequately characterises all aspects of biofilm status; complementary methods are needed to distinguish physical removal of biofilm from the killing of cells within it [27]. An interlaboratory investigation involving five laboratories similarly evaluated the reproducibility and responsiveness of CV, resazurin, and plate-count procedures, and documented differences in their performance after antimicrobial challenge [28]. Biomass may remain detectable after substantial loss of viability, while a relatively small amount of adherent material can contain clinically relevant viable organisms. CV staining therefore addresses the question, “How much material is attached?” By contrast, quantitative culture and antibiotic-challenge assays address distinct questions: “How many viable bacterial cells remain?” and “How readily are these cells killed?” These distinctions are fundamental when translating laboratory observations into clinically meaningful conclusions.

Biofilm architecture constitutes a third, partially independent dimension of biofilm phenotype. Confocal laser scanning microscopy permits the examination of hydrated, three-dimensional biofilm structures, while image-analysis software such as COMSTAT can quantify biovolume, mean and maximum thickness, roughness, surface coverage, and related spatial parameters [29]. These measures can be reproducible

when imaging, segmentation, and analytical procedures are standardised, although their interpretation remains dependent on image quality and sampling design [30]. Architectural measurements may also have mechanistic relevance for antibiotic action. For example, average diffusion distance estimates the distance separating biomass from an interface with surrounding void space and can serve as a geometric descriptor of the potential depth of local concentration and metabolic gradients. Roughness describes spatial heterogeneity in the distribution of biomass across the substratum. Two isolates may produce similar CV absorbance values while generating very different structures: one may form a relatively continuous, shallow layer, whereas another may form isolated, tall microcolonies separated by exposed surface. Their total adherent biomass can be similar, even though local physiological conditions and drug-exposure histories within the biofilms may differ markedly. Yet architecture is seldom assessed alongside both CV biomass and viable burden in routine clinical-isolate studies.

This distinction has practical consequences because CV-defined biofilm strength is sometimes proposed as a marker of clinical risk. Strong biofilm production has been investigated in relation to multidrug resistance, difficult-to-treat infections, and other clinical outcomes [8], [23], [25]. However, using a biomass category alone to guide decisions such as device removal, prolonged treatment, combination therapy, or trial enrolment would require evidence that the category reliably predicts viable sessile burden and antibiotic survival. If that assumption is incorrect, risk assessment may misclassify isolates in both directions. Weakly staining isolates with viable, highly tolerant microcolonies could be underestimated, whereas heavily staining isolates with relatively few viable cells could be overestimated. The need for antimicrobial-development endpoints that capture biofilm-specific activity rather than simply MIC has already been emphasised [32], but the corresponding need for more informative laboratory phenotyping remains important. Studies integrating biofilm assessment with the characteristics of urinary *K. pneumoniae* isolates have suggested that the magnitude of adherence and antimicrobial resistance are not necessarily interchangeable properties [33].

The present investigation was designed to test the assumption that total adherent biomass adequately represents the clinically relevant features of biofilm phenotype. Sixty non-duplicate clinical *K. pneumoniae* isolates were evaluated using four complementary approaches: crystal violet staining to quantify adherent biomass; quantitative culture of disrupted biofilms to determine viable sessile burden; confocal microscopy combined with COMSTAT to describe three-dimensional architecture; and antibiotic-challenge assays to determine log₁₀ reductions in viable counts. Parallel MBEC measurements were also obtained. Three specific research questions guided the study. First, how closely is the CV biomass measurement associated with the number of viable bacteria present within a biofilm? Second, which of the measured characteristics—biomass, viable burden, or architectural

descriptors—are most strongly associated with survival following antibiotic exposure? Third, how frequently would reliance on biomass alone classify isolates in a manner inconsistent with their viable burden and observed antibiotic survival? Addressing these questions may help clarify the interpretation of biofilm categories used in clinical research and support more discriminating approaches to biofilm phenotyping.

II. Materials and Methods

A. Study design, setting and isolates

This cross-sectional laboratory study examined 60 consecutive, non-duplicate clinical *Klebsiella pneumoniae* isolates recovered from hospitalised patients in a 700-bed tertiary-care hospital over a 12-month collection period. Only one isolate per patient and infection episode was retained, and repeat recovery of the same organism from the same patient within 30 days was excluded unless the isolate differed meaningfully in antibiogram or specimen source. Isolates collected from surveillance or screening specimens, as well as those regarded by the treating clinical team as contaminants, were excluded. The final collection comprised 24 urinary isolates, 16 respiratory isolates obtained from specimens such as endotracheal aspirates or bronchoalveolar lavage, 12 bloodstream isolates, and eight isolates from wounds or drainage specimens. Twenty-eight isolates were associated with indwelling devices at the time of sampling. These selection criteria were intended to reduce duplicate representation while capturing isolates from multiple clinically important infection sites. The institutional ethics committee approved the study, and the authors report that individual informed consent was not required because anonymised data and residual diagnostic isolates were used. The hospital identity, study period, and ethics approval identifier should be confirmed by the authors before publication.

Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry using the Bruker Biotyper platform was employed for species-level identification, with a reported identification-score threshold of ≥ 2.0 . Identification was additionally confirmed by amplification of the *khe* gene specific to *K. pneumoniae*. Isolates were subcultured twice on MacConkey agar to verify purity and then stored at -80°C in tryptic soy broth containing 20% (v/v) glycerol. The number of passages from frozen stock was restricted before experimental testing, and all phenotypic assays for an individual isolate were conducted using the same working subculture to minimise passage-dependent variation in biofilm characteristics. The exact maximum permitted passage number is not specified in the original records supplied for editing and requires confirmation by the authors. *K. pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference or inter-assay control strains, while *Escherichia coli* ATCC 25922 served as a reference strain for antimicrobial susceptibility testing. These control procedures were used to support consistency across identification, culture, and antimicrobial testing workflows.

B. Antimicrobial susceptibility testing and MIC determination

Antimicrobial susceptibility was assessed by broth microdilution in cation-adjusted Mueller–Hinton broth following Clinical and Laboratory Standards Institute (CLSI) procedures, and results were interpreted using the susceptibility criteria specified by the cited CLSI standard [34]. The antibiotic panel included meropenem, imipenem, ciprofloxacin, levofloxacin, ceftazidime, cefotaxime, piperacillin–tazobactam, gentamicin, amikacin, and trimethoprim–sulfamethoxazole. Colistin MICs were determined exclusively by broth microdilution in polystyrene plates without added surfactant. Multidrug resistance (MDR) was defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories. Extensive drug resistance (XDR) was defined using the corresponding international criteria, namely non-susceptibility to at least one agent in all but two or fewer antimicrobial categories [35]. Extended-spectrum β -lactamase activity was assessed phenotypically by combination-disc testing, and carbapenemase activity was investigated using a modified carbapenem inactivation method. For biofilm challenges, concentrations of meropenem, ciprofloxacin, and colistin were expressed as multiples of each isolate's measured planktonic MIC rather than as fixed absolute drug concentrations. This approach allowed relative exposure to be standardised across isolates with differing planktonic susceptibility profiles, although it did not reproduce clinical pharmacokinetic conditions.

C. Biofilm biomass by crystal violet staining

Total adherent biofilm biomass was measured in flat-bottomed polystyrene 96-well microtitre plates using a crystal violet staining procedure adapted from the standardised approach described by Stepanović et al. [22]. Overnight cultures were adjusted to a 0.5 McFarland standard and then diluted 1:100 in tryptone soya broth supplemented with 1% (w/v) glucose. Each test well received 200 μ L of the diluted suspension, and the plates were incubated under static conditions at 37 °C for 24 hours. Planktonic cells were removed by aspiration, and the wells were washed three times with 250 μ L of phosphate-buffered saline. Plates were allowed to air-dry, fixed with methanol for 15 minutes, and stained with 0.1% (w/v) crystal violet for a further 15 minutes. The excess stain was removed by repeated rinsing until the wash solution was clear, after which bound stain was solubilised using 33% (v/v) acetic acid. Absorbance was determined at 570 nm and recorded as OD₅₇₀. For each isolate, eight technical wells were assessed in each of three independent biological experiments, providing 24 well-level measurements. The cut-off optical density (OD_c) was calculated as the mean absorbance of sterile-medium controls plus three standard deviations. Isolates were categorised as non-adherent (OD $\leq$ OD_c), weakly adherent (OD_c < OD $\leq$ 2 $\times$ OD_c), moderately adherent (2 $\times$ OD_c < OD $\leq$ 4 $\times$ OD_c), or strongly adherent (OD > 4 $\times$ OD_c). These categories describe staining intensity and do not themselves quantify cell viability.

D. Viable sessile burden

Viable sessile burden was quantified without fixation or staining by culturing biofilms grown in parallel wells on the same substratum and under the same incubation conditions as those used for CV analysis. After 24 hours of incubation and three phosphate-buffered saline washes, 200 μ L of sterile PBS was added to each well. Adherent cells were dispersed using a two-stage procedure consisting of bath sonication for 10 minutes at 40 kHz and 50 W, followed by vigorous scraping and 30 seconds of vortex mixing. Preliminary experiments involving five isolates assessed recovery under these conditions and indicated that exposure to the sonication procedure changed viable counts by less than 0.2 log₁₀ in matched planktonic controls. The recovered suspensions were diluted in ten-fold steps using PBS, and 20 μ L aliquots of the appropriate dilutions were drop-plated in triplicate onto tryptone soya agar. Colony counts were obtained following incubation for 18–24 hours at 37 °C. Counts were normalised to the reported wetted well area of 0.32 cm² and expressed as log₁₀ colony-forming units per square centimetre (CFU/cm²). The reported limit of detection was 2.5 log₁₀ CFU/cm². For each isolate, the assay was performed in six wells across three independent experimental runs. This measure characterises recoverable, culturable biofilm-associated cells rather than total adherent material.

E. Confocal laser scanning microscopy and architectural analysis

For architectural imaging, biofilms were grown for 24 hours at 37 °C on sterile borosilicate glass slides placed in 12-well plates, using inoculum preparation and medium conditions comparable to those of the microtitre-plate experiments. Slides were rinsed twice with phosphate-buffered saline and stained in the dark for 20 minutes using the LIVE/DEAD BacLight system containing SYTO 9 at 6 μ M and propidium iodide at 30 μ M. Imaging was conducted with an inverted confocal laser scanning microscope fitted with a 63 $\times$ /1.4 numerical-aperture oil-immersion objective. The excitation wavelengths were 488 and 561 nm, with emission windows of 500–540 and 600–650 nm, respectively. Five fields were selected from each of two separate slides for every isolate, yielding ten image stacks per isolate. Optical sections were acquired at 0.5 μ m intervals from the substratum to the first plane in which fluorescence was no longer detectable. This sampling procedure was intended to capture the spatial heterogeneity of each isolate's mature biofilm rather than relying upon a single representative field.

The confocal image stacks were analysed using COMSTAT 2 within the ImageJ environment; COMSTAT was originally developed for quantitative analysis of biofilm structure and was subsequently implemented as an ImageJ-compatible tool [29], [31]. Image segmentation employed an automated Otsu threshold applied consistently across the stacks. Parameters extracted from the image data included biovolume per unit substratum area (μ m³/ μ m²), mean biofilm thickness (μ m),

maximum thickness (μm), the dimensionless roughness coefficient, percentage substratum coverage, and average diffusion distance (μm). The latter parameter characterises the spatial distance from biomass voxels to surrounding void regions and was interpreted as a geometric descriptor relevant to the development of local gradients, rather than as a direct experimental measurement of antibiotic diffusivity. The green-to-red fluorescence-voxel ratio was recorded as an index of staining patterns, but was not treated as a quantitative estimate of viable-cell proportion because fluorescence exclusion and membrane integrity are imperfect indicators of culturability. Reproducibility between independent slides was assessed by analysis of variance applied to a reference strain, consistent with the general principles of replicate biofilm-image comparison described previously [30].

F. Antibiotic challenge of mature biofilms

Biofilms grown for 24 hours in microtitre plates were washed three times with PBS to remove unattached cells, then exposed for an additional 24 hours at 37°C to meropenem, ciprofloxacin, or colistin in fresh cation-adjusted Mueller–Hinton broth at either $10\times$ or $100\times$ the isolate-specific planktonic MIC. Untreated biofilms incubated in parallel served as growth controls. Following exposure, each well was washed three times to reduce residual antimicrobial carryover, and the surviving biofilm-associated bacterial burden was quantified using the dispersal and viable-culture procedure described in Section II-D. Antibiotic activity was expressed as a $\log_{10}$ reduction in recoverable viable cells, calculated by subtracting the $\log_{10}$ CFU/cm² measured in the treated well from the corresponding untreated control count obtained within the same experimental run. MBECs were assessed in parallel using a 96-peg-lid device and the Calgary Biofilm Device approach [21]. Briefly, biofilms grown on the pegs for 24 hours were challenged for another 24 hours over a two-fold antibiotic dilution series, rinsed, dispersed into recovery medium by sonication, and scored for regrowth after 24 hours. The MBEC was defined as the lowest tested concentration producing no detectable subsequent growth under those conditions. Antibiotic challenges were conducted in quadruplicate wells in three independent runs. Because exposure was based on fixed multiples of planktonic MIC rather than simulated patient dosing, these assays measured in-vitro tolerance under controlled conditions and should not be interpreted as direct estimates of clinical treatment efficacy.

G. Statistical analysis

Continuous variables were summarised using medians and interquartile ranges (IQRs). Comparisons between two independent groups employed the Mann–Whitney U test, whereas comparisons involving more than two groups used the Kruskal–Wallis test followed, where applicable, by Dunn’s post hoc pairwise comparisons with Bonferroni adjustment. Relationships among CV biomass, viable sessile burden, architectural descriptors, and antibiotic-associated $\log_{10}$ reduc-

tions were examined using Spearman’s rank correlation coefficient (ρ). Corresponding 95% confidence intervals were estimated using bias-corrected bootstrap procedures with 2,000 resamples. Multivariable linear regression models for each antibiotic were developed to examine the association of $\log_{10}$ reduction with viable burden, CV OD₅₇₀, mean thickness, the roughness coefficient, and average diffusion distance. Multicollinearity was assessed using variance inflation factors (VIFs), with a threshold of five specified for identifying potentially problematic predictor overlap. Agreement between CV-defined biofilm categories and categories based on tertiles of viable burden was assessed using a weighted Cohen’s κ statistic. Statistical tests were two-sided, with $p < 0.05$ considered statistically significant. All analyses were performed using R software, version 4.3.1. Because the manuscript reports observational associations from a laboratory study, statistical significance was interpreted as evidence of an association rather than a demonstration of causal mechanisms.

III. Results

Crystal violet biomass was only weakly correlated with viable sessile burden and was not strongly associated with antibiotic-associated reductions in viable counts. In contrast, both viable burden and selected architectural measurements showed more substantial associations with biofilm survival. The characteristics of the 60 clinical isolates are summarised in Table 1. Four isolates were classified as non-adherent by the CV cut-off procedure, 17 as weakly adherent, 22 as moderately adherent, and 17 as strongly adherent. These categories refer specifically to the CV signal, and do not imply that isolates in the non-adherent category lacked recoverable viable cells in the biofilm culture assay. The isolate collection also included both susceptible/non-MDR and resistant phenotypes, providing an opportunity to examine several aspects of biofilm phenotype within the same clinical panel.

Table 1: Characteristics of the 60 clinical *K. pneumoniae* isolates

Characteristic	Category	n (%)
Specimen	Urine	24 (40.0)
	Respiratory	16 (26.7)
	Blood	12 (20.0)
	Wound/drain	8 (13.3)
Device-associated	Yes	28 (46.7)
Resistance phenotype	Susceptible/non-MDR	14 (23.3)
	MDR	31 (51.7)
	XDR	15 (25.0)
	β -lactamase	22 (36.7)
CV biofilm category	ESBL only	19 (31.7)
	Carbapenemase	4 (6.7)
	Non-adherent	17 (28.3)
	Weak	22 (36.7)
	Moderate	17 (28.3)
	Strong	17 (28.3)

The CV-based categories differentiated adherent biomass clearly but provided only limited discrimination of the viable sessile bacterial burden, as shown in Table 2. Across the categories, the median viable count increased by approximately $1.4 \log_{10}$ CFU/cm², from 5.9 in the non-adherent

category to 7.3 in the strong category. Nevertheless, the interquartile ranges of adjacent groups overlapped substantially, indicating pronounced within-category variation. Agreement between CV categories and viable-burden groupings was low: the reported weighted Cohen's κ was 0.21 (95% confidence interval, 0.04–0.38), consistent with only slight-to-fair agreement between these alternative classification approaches. In contrast, architectural descriptors such as mean thickness, substratum coverage, and average diffusion distance increased markedly across CV groups, demonstrating that the staining categories were more directly aligned with total structural development than with recoverable viable-cell abundance.

Table 2: Viable burden, architecture, and antibiotic-associated reductions across crystal violet categories. Values are medians (interquartile ranges)

Parameter	Non-adherent (n=4)	Weak (n=17)	Moderate (n=22)	Strong (n=17)	p
CV OD ₅₇₀	0.09 (0.07–0.11)	0.21 (0.18–0.27)	0.44 (0.37–0.52)	0.91 (0.78–1.16)	<0.001
Viable burden (log ₁₀ CFU/cm ²)	5.9 (5.4–6.3)	6.4 (5.8–7.4)	6.9 (6.1–7.6)	7.3 (6.6–7.9)	0.04
Biovolume (μm ³ /μm ²)	2.1 (1.6–2.9)	6.8 (4.2–9.9)	14.3 (10.1–19.4)	27.6 (21.8–34.2)	<0.001
Mean thickness (μm)	3.4 (2.8–4.1)	8.1 (5.9–11.2)	16.7 (12.4–21.0)	29.8 (24.1–36.5)	<0.001
Roughness coefficient	1.42 (1.30–1.55)	1.11 (0.84–1.39)	0.73 (0.55–0.98)	0.49 (0.36–0.67)	<0.001
Substratum coverage (%)	18 (14–24)	39 (28–52)	64 (51–77)	83 (74–91)	<0.001
Average diffusion distance (μm)	1.9 (1.5–2.4)	4.6 (2.7–7.8)	7.2 (4.9–10.3)	9.4 (6.8–12.7)	<0.001
Meropenem log ₁₀ reduction (100× MIC)	2.9 (2.4–3.3)	1.8 (0.7–3.0)	1.6 (0.8–2.7)	1.4 (0.6–2.5)	0.21

Spearman correlation analysis, presented in Table 3, further distinguished overall adherent biomass from biologically relevant survival characteristics. CV absorbance correlated positively but weakly with viable burden ($\rho = 0.31$), and its correlations with the log₁₀ reductions achieved by meropenem, ciprofloxacin, and colistin were small and negative ($\rho = -0.14$, -0.09 , and -0.18 , respectively). By comparison, baseline viable burden showed more pronounced inverse correlations with antibiotic-associated killing across all three agents ($\rho = -0.52$, -0.44 , and -0.47 , respectively). Average diffusion distance exhibited the strongest inverse correlations among the reported predictors ($\rho = -0.61$, -0.55 , and -0.58 , respectively). Higher values of these two descriptors were therefore associated with smaller reductions in recoverable viable bacterial counts under the specified in-vitro challenge conditions. Biovolume, mean thickness, and roughness exhibited additional associations of differing magnitudes; however, correlation alone does not establish whether these parameters independently determine antibiotic survival.

Table 3: Spearman correlation coefficients (ρ) for viable burden and log₁₀ reductions at 100× MIC. Bold values indicate $p < 0.01$

Predictor	vs viable burden	Meropenem	Ciprofloxacin	Colistin
CV OD ₅₇₀	0.31	−0.14	−0.09	−0.18
Viable burden (log ₁₀ CFU/cm ²)	—	−0.52	−0.44	−0.47
Biovolume	0.36	−0.21	−0.16	−0.24
Mean thickness	0.34	−0.29	−0.22	−0.31
Roughness coefficient	−0.27	0.33	0.26	0.35
Average diffusion distance	0.48	−0.61	−0.55	−0.58

Multivariable regression findings are summarised in Table 4. Average diffusion distance and initial viable burden showed consistent inverse associations with log₁₀ reductions for all three tested antibiotics. For meropenem, their reported regression coefficients were -0.19 and -0.41 , respectively, with a model R^2 of 0.61. The corresponding models explained

49% and 54% of the observed variation in the log₁₀ reductions associated with ciprofloxacin and colistin, respectively. The additional CV OD₅₇₀ term displayed for meropenem was not statistically significant ($p = 0.67$); its coefficient and confidence interval are retained in the table rather than omitted. Variance inflation factors were reported to remain below 2.8, suggesting that substantial predictor collinearity was not detected under the stated criterion. These findings support the interpretation that viable burden and spatial structure were more informative predictors than total CV biomass in this collection, although the relative importance of the predictors cannot be independently recalculated without the original isolate-level data.

Table 4: Multivariable linear regression models for log₁₀ reduction at 100× MIC

Agent	Predictor	β (95% CI)	p	Model R ²
Meropenem	Average diffusion distance (μm)	−0.19 (−0.26 to −0.12)	<0.001	0.61
	Viable burden (log ₁₀ CFU/cm ²)	−0.41 (−0.63 to −0.19)	<0.001	
Ciprofloxacin	CV OD ₅₇₀	0.12 (−0.44 to 0.68)	0.67	0.49
	Average diffusion distance (μm)	−0.16 (−0.23 to −0.09)	<0.001	
Colistin	Viable burden (log ₁₀ CFU/cm ²)	−0.35 (−0.58 to −0.12)	0.004	0.54
	Average diffusion distance (μm)	−0.18 (−0.25 to −0.11)	<0.001	
	Viable burden (log ₁₀ CFU/cm ²)	−0.38 (−0.60 to −0.16)	0.001	

Fifteen isolates (25.0% of the full panel) displayed a discordant low-biomass, high-burden phenotype: they fell into the non-adherent or weakly adherent CV categories but yielded at least 7.0 log₁₀ CFU/cm² and showed less than 1 log₁₀ reduction after meropenem exposure at 100× the isolate-specific MIC. As detailed in Table 5, the low-biomass, high-burden group had a median viable burden of 7.4 log₁₀ CFU/cm², a median maximum thickness of 31.2 μm, and a median meropenem-associated reduction of 0.6 log₁₀. Although the overall staining signal was low, these isolates formed spatially heterogeneous structures with prominent localised microcolonies. Conversely, six isolates assigned to the strong CV category exhibited reductions greater than 3 log₁₀ following the same challenge. The reported MBEC-to-planktonic-MIC ratios had medians of 256 (IQR, 64–1,024) for meropenem, 128 (32–512) for ciprofloxacin, and 64 (16–256) for colistin. No statistically significant variation in these fold differences was reported across CV categories ($p = 0.38$). The disparity between biomass classification and survival was therefore evident in both directions, rather than being restricted to low-biomass isolates.

IV. Discussion

The principal finding of this study is that crystal violet biomass, although widely used to classify *K. pneumoniae* isolates as weak, moderate, or strong biofilm producers, was an inadequate standalone surrogate for viable sessile burden and survival during antibiotic challenge. CV staining was associated with major features of biofilm architecture, yet it correlated only weakly with the number of recoverable cells and showed little association with the amount of bacterial killing achieved by the three antibiotics. Agreement between CV-based and viable-burden classifications was similarly limited, with marked discordance in 15 of the 60 isolates (25%): these

Table 5: Discordant biofilm phenotypes: comparison of biomass category, viable burden, and antibiotic survival

Group	n	CV OD ₅₇₀	Viable burden (log ₁₀ CFU/cm ²)	Max thickness (μm)	Roughness	Meropenem log ₁₀ reduction
Low biomass, high burden, tolerant	15	0.19 (0.14–0.25)	7.4 (7.1–7.8)	31.2 (24.6–38.9)	1.28 (1.07–1.44)	0.6 (0.3–0.9)
High biomass, susceptible	6	0.84 (0.76–0.98)	6.2 (5.9–6.6)	26.8 (22.1–30.4)	0.44 (0.37–0.58)	3.4 (3.1–3.9)
Concordant remainder	39	0.47 (0.29–0.72)	6.8 (6.2–7.3)	28.1 (19.7–35.2)	0.81 (0.58–1.09)	2.0 (1.3–2.7)

isolates produced relatively little CV staining despite carrying a high viable burden and showing low meropenem-associated killing. Conversely, six strongly staining isolates were comparatively susceptible to killing in the specified experimental assay. In the regression analysis, average diffusion distance and viable burden were consistently associated with smaller log₁₀ reductions, and the meropenem model explained 61% of the reported variation. These results distinguish biomass, architecture, viability, and antibiotic survival as related but non-equivalent dimensions of biofilm phenotype. They do not, by themselves, establish that measuring architectural features will improve patient outcomes, but they do question the assumption that CV categories can reliably represent antibiotic tolerance.

This dissociation is plausible when the chemistry and biological scope of CV staining are considered. Crystal violet is a basic triarylmethane dye that binds to charged cellular and extracellular materials. The extracellular polymeric matrix contains polysaccharides, extracellular DNA, and proteins that contribute to the total adherent signal [13]. Consequently, dead cells, cellular debris, and matrix material may all contribute to absorbance without reflecting the number of live, recoverable organisms. The relative contributions of these components are not quantified by the CV assay itself. Several discordant isolates in the present panel appeared to produce sparse but tall biofilm structures, consisting of localised towers separated by extensive uncovered substratum. Such morphology can yield comparatively low integrated biomass while still generating substantial local diffusion distances and potentially steep oxygen or nutrient gradients within particular microcolonies. Those gradients are among the conditions known to promote physiological heterogeneity and antimicrobial tolerance in biofilms [14]. Thus, the measured differences are consistent with the possibility that spatial organisation, rather than total matrix quantity alone, is related to survival following antibiotic exposure.

Existing mechanistic studies provide a framework for interpreting these findings. Biofilm-associated tolerance has long been understood as an emergent property of multicellular communities, influenced by chemical gradients, physiological heterogeneity, and stress responses rather than by a single resistance mechanism [10], [11], [36]. In *P. aeruginosa*, experimentally measured responses to ciprofloxacin and tobramycin showed that oxygen availability and the reduction in metabolic activity could contribute to survival even when inadequate antibiotic penetration was not the sole explanation [16]. The stringent starvation response has likewise been implicated in protection of nutrient-limited biofilm populations [17], while persister cells provide another potential mechanism of survival without heritable resistance [18], [37]. Physiological

differences between rapidly growing cells near the biofilm exterior and slower-growing cells deeper within the community have been demonstrated in several experimental systems [15], [38]. Oxidative stress may also contribute to genetic or phenotypic diversification within biofilms [39]. Collectively, these mechanisms illustrate why total adherent mass need not determine the response to an antimicrobial agent. In the present study, average diffusion distance was the strongest architectural correlate of reduced killing, although it remained a geometric indicator and was not a direct measurement of antibiotic concentration gradients inside the biofilm.

The results also offer a possible explanation for apparently inconsistent associations between biofilm production and antimicrobial resistance reported in the *K. pneumoniae* literature. Studies using CV classifications have found that strongly staining isolates may be associated with multidrug resistance in some collections, while other investigations have reported weaker, absent, or differently directed relationships between the two phenotypes [23]–[25]. Reviews have emphasised the complexity of biofilm development and antimicrobial susceptibility in this organism [8], [9], [33]. These discrepancies may arise partly because CV measures adherent material whereas susceptibility testing and biofilm-challenge assays assess different biological processes. Matrix accumulation can vary independently of genetic resistance determinants, the number of recoverable sessile cells, and the physiological state that contributes to tolerance. For example, the cited study by Karimi et al. reported strong biofilm formation in approximately one-fifth of its clinical isolates and found an association with antimicrobial resistance [23]. Such results should not be generalised automatically across assay systems or interpreted as proving that every strongly staining isolate survives antibiotic challenge. The present study adds an explicitly multicomponent comparison and therefore supports a more cautious interpretation of biomass-based categories.

Published methodological comparisons have raised similar concerns for several years. Direct assessments of microtitre-plate procedures demonstrate that CV staining, metabolic readouts, and viable-cell counts can produce non-identical estimates because they quantify distinct biological properties [26]. A broader comparison of total biomass, total cell abundance, viable counts, and biofilm matrix showed that combining approaches is necessary when the purpose is to distinguish biofilm removal from microbial killing [27]. An interlaboratory study conducted in five laboratories further demonstrated that CV, resazurin, and plate counts can achieve reproducible results when appropriate protocols and analyses are used, but their responsiveness differs after an antimicrobial challenge [28]. In particular, the presence of persistent adherent biomass does not necessarily imply that viable organisms

remain at the same concentration after treatment. These observations support the practical value of measuring both attached biomass and recoverable bacterial burden in the same isolate collection, rather than treating the results of one assay as a substitute for those of another.

Two practical implications deserve emphasis. First, CV staining alone should not be used to infer the viable bacterial burden or to rank isolates by their in-vitro survival following antibiotic exposure. In this panel, such an approach would have underestimated the substantial viable burden and meropenem tolerance of 15 low-biomass isolates, while potentially overestimating tolerance in six strongly staining isolates that showed comparatively large $\log_{10}$ reductions. However, these laboratory findings cannot yet justify changing patient management, including device-removal decisions, treatment duration, or antibiotic selection, without prospective clinical validation. Second, the observed MBEC-to-MIC ratios substantially exceeded unity, including a median 256-fold difference for meropenem, and did not vary significantly across CV categories. This result is consistent with the general distinction between planktonic susceptibility and biofilm eradication described using the Calgary Biofilm Device [21]. It also reinforces the case for studying viable-cell-based and killing-kinetic endpoints during the development of anti-biofilm interventions, rather than relying solely on a reduction in the amount of stainable biomass [32]. Existing guidance for biofilm infections already recognises the difficulty of predicting biofilm-associated treatment outcomes from standard susceptibility measurements alone [12]. The present findings indicate that CV staining, by itself, does not resolve that diagnostic limitation.

Several limitations must be acknowledged. First, the investigation was conducted at a single centre and included 60 clinical isolates. Although the panel allowed descriptive and correlational comparisons, it was not designed to support definitive subgroup conclusions by specimen type, resistance mechanism, or clinical outcome. Second, the biofilms were established for 24 hours under static conditions on polystyrene or borosilicate surfaces in glucose-supplemented medium. Biofilms formed on silicone catheters, within body fluids, under flow conditions, and in the presence of host immune responses may differ considerably in architecture, cell physiology, and antibiotic tolerance. Third, antibiotic exposure was performed for a fixed 24-hour period at $10\times$ and $100\times$ isolate-specific planktonic MIC, rather than under conditions simulating human pharmacokinetics or clinically achievable concentrations. Accordingly, the observed $\log_{10}$ reductions are laboratory indices of survival and should not be read as predictions of patient-level therapeutic response. Fourth, the isolates were not genotyped; possible associations of the discordant phenotype with sequence type, capsular background, the *mrk* locus, or cyclic-di-GMP regulation therefore remain unexamined [5], [7]. Finally, although green-to-red voxel ratios were recorded from LIVE/DEAD staining, these values were intentionally not treated as quantitative viability estimates. Propidium iodide uptake may occur in certain vi-

able cells, and dye distribution within complex matrices can be heterogeneous, limiting the interpretation of membrane staining as an exact estimate of colony-forming viability [40], [41]. Together, these limitations argue for replication in larger collections, preferably with external validation and clinically relevant biofilm conditions.

Future investigations should determine whether the architectural information provided by average diffusion distance can be approximated using simpler, more scalable assays than confocal microscopy coupled with COMSTAT. Although detailed three-dimensional imaging is valuable for mechanistic research, it may not be practical for routine assessment of every clinical isolate. Potential alternatives include depth-resolved oxygen measurements, electrical or impedance-based descriptors of surface-attached structure, and combined interpretation of CV biomass with quantitative viable-cell recovery. The latter approach is comparatively inexpensive because it adds culture-based counts after physical disruption of the biofilm to the widely available CV assay. Nevertheless, the clinical utility, reproducibility, cost-effectiveness, and predictive value of any simplified procedure would require independent evaluation. Future work should also investigate whether the discordant phenotypes are associated with specific genotypes and whether biofilm parameters predict persistent infection, device-associated recurrence, or responses to antimicrobial treatment. Until such validation is available, investigators should report distinct measurements of biomass, viable-cell burden, architectural organisation, and antibiotic survival wherever feasible, rather than using a single adjective such as “strong” to imply all of these properties.

V. Conclusion

In this study of 60 clinical *Klebsiella pneumoniae* isolates, crystal violet-stained biomass was a poor standalone predictor of viable biofilm burden and in-vitro antibiotic-associated killing. CV biomass showed only a weak positive correlation with viable sessile burden and small correlations with $\log_{10}$ reductions after exposure to meropenem, ciprofloxacin, or colistin. By contrast, average diffusion distance and initial viable burden were consistently associated with lower levels of killing across all three agents. Fifteen isolates—25% of the collection—had low CV biomass despite harbouring at least $7.0 \log_{10}$ CFU/cm² and showing less than 1 $\log_{10}$ reduction with meropenem at $100\times$ their own planktonic MIC. Conversely, six isolates with high CV biomass exhibited reductions greater than 3 $\log_{10}$. The median MBEC-to-MIC ratio reached 256 for meropenem and did not differ significantly by CV category. These findings underscore the distinction between the amount of stainable material on a surface, the number of recoverable viable bacteria within it, and the degree to which those organisms survive antibiotic challenge.

For clinical research, biofilm phenotype should therefore be described by several complementary measurements rather than a single ordinal classification. At a minimum, CV staining can be paired with culture-based enumeration of viable bacteria recovered from physically disrupted biofilms; such

a combination is more informative than biomass measurement alone and can be undertaken without advanced imaging equipment. For the assessment of experimental anti-biofilm treatments, viable-cell burden, $\log_{10}$ reductions, and well-defined eradication endpoints may be more meaningful than changes in stainable biomass alone. Architectural measures can provide further mechanistic insight, especially when spatial structure appears to be associated with survival despite low total biomass. Importantly, however, prospective clinical validation is needed before these laboratory measures can be used to stratify patients or guide treatment. Describing an isolate as a strong biofilm producer communicates the quantity of adherent material detected under a specified assay; it does not, by itself, establish that the isolate contains more viable cells or that its biofilm will be harder to eradicate with antibiotics. These outcomes should be evaluated and reported separately.

Author Contributions

All authors contributed equally to the conception, development, analysis, and preparation of this manuscript. All authors have carefully read and approved the final version of the manuscript for publication.

Competing Interests

The authors declare no competing interests.

Funding

The authors received no specific financial support, research grant, or external funding for the research, authorship, or publication of this manuscript.

Data Availability

Data are available from the corresponding author upon request.

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