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Weak biofilm formation among carbapenem-resistant *Klebsiella pneumoniae*

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Abstract

Biofilm formation of multidrug and extensively drug resistant *Klebsiella pneumoniae* isolates is poorly understood. We investigated 139 diverse clinical *K. pneumoniae* isolates that possess various resistance patterns to evaluate the relationship between biofilm formation and resistance. Antimicrobial resistance was compared among a diverse collection of weak versus strong biofilm-forming *K. pneumoniae*, and predictors of strong biofilm formation were identified. Multi-drug resistant isolates were more common among weak (97.9%) versus strong biofilm formers (76%; p=0.002). Carbapenem-resistant *K. pneumoniae* were 91% less likely to form strong biofilm (odds ratio 0.09; 95% confidence interval 0.02-0.33). The statistically significant inverse relationship between biofilm formation and antibiotic resistance suggests that virulence may be a trade-off for survival.

Keywords: *Klebsiella pneumoniae;* biofilm; carbapenem resistance

INTRODUCTION

Klebsiella pneumoniae, the most common and most concerning carbapenem-resistant *Enterobacteriaceae* (CRE) (1), is associated with mortality rates up to 50% (2). Adding to this challenging infection is *K. pneumoniae*'s high propensity to form biofilms (3, 4). Biofilm-forming *K. pneumoniae* are associated with foreign indwelling device related infections (4), as well as urinary stones (5-7). The most common *K. pneumoniae* infections include urinary tract infections, pneumonia, as well as intra-abdominal infections, which are prone to biofilm formation(4, 8). Biofilm eradication requires high antimicrobial concentrations (9), which often cannot be physiologically achieved in the blood stream or at the site of infection, thus potentially leading to infection recurrence (4). Unfortunately, clinical microbiology labs cannot routinely test for biofilm formation; however, tested phenotypic characteristics may help clinicians predict biofilm potential.

Multidrug-resistant (MDR) organisms have been associated with biofilm formation when *Klebsiella pneumoniae* (10, 11), *Staphylococcus aureus* (11), *Acinetobacter spp.* (10, 11), *Pseudomonas aeruginosa* (10, 11), *Escherichia coli* (10, 11), coagulase-negative staphylococci (10), or *Enterococcus spp*. (10) are assessed together (10, 11). However, the relationship between biofilm-forming *K. pneumoniae* alone and antimicrobial resistance has not been fully elucidated (12-14). The study objective was to determine whether certain antimicrobial class resistance in *K. pneumoniae* was predictive of strong biofilm formation.

MATERIALS AND METHODS:

Our study included 139 unique *K. pneumoniae* clinical isolates obtained from the Centers for Disease Control and Prevention (CDC; n=66), Biodefense and Emerging Infections (BEI; n=36), American Type Culture Collection (ATCC; n=3), and Providence Veterans Affairs (VA) Medical Center and Rhode Island Hospital (n=34). These isolates were selected because they are known to be resistant to a range of antibiotic classes. Isolates from the Providence VA Medical Center

were collected per the VA approved Institutional Review Board (IRB), Research and Development (R&D), and safety committee protocols.

A previously described biofilm assay was performed to assess biofilm formation (15-19). This assay is considered the standard for evaluation of bacterial attachment and biofilm formation in vitro (20). Isolates were obtained from culture stocks, stored at -80°C. After streaking on tryptic soy agar and incubating for 18 to 24 hours, an inoculum of 6 $\log_{10}$ CFU/mL in tryptic soy broth with added 25 mg/L calcium, 12.5 mg/L magnesium, and 1.25% total dextrose (21). Media was selected and validated based on the greatest biofilm formation, which is consistent with previously published data for *Escherichia coli* (22) and *Klebsiella pneumoniae* (17, 18). Each isolate was incubated in a 96-well plate (Costar 3596) for 24 hours at 37°C and 120 rpm in octuplicate (17, 18). Wells were stained with 0.1% crystal violet (CV) and resolubilized with 33% glacial acetic acid (19). *K. pneumoniae* (ATCC 700603) served as the biofilm positive control and media alone was the negative control (12, 23-25).

Biofilm formation was measured as an optical density (OD_{570}). We further categorized isolates as either weak, moderate, or strong biofilm formers based on tertiles of OD_{570}. In order to assess differences between the highest and lowest OD_{570}, we removed isolates in the moderate range (19, 26).

Organism susceptibility was obtained from the site of collection (eg. CDC, BEI). When results were unavailable testing was performed by E-test or Kirby-Bauer disc diffusion on Mueller-Hinton agar, and were interpreted according to 2017 CLSI susceptibility breakpoints (27, 28). The FDA package insert for tigecycline (E-test MIC ≤2) (29) and EUCAST breakpoints for colistin (E-test MIC ≤2) and fosfomycin (E-test MIC ≤32 and disc diffusion ≥24; both contained glucose-6-phosphate) (30) were used as CLSI breakpoints were not available. Isolates were categorized as multi-drug resistant (MDR), extensively drug-resistant (XDR), or resistant to specific

antimicrobial classes/agents, according to CDC and European CDC expert consensus definitions for *Enterobacteriaceae* (31). MDR isolates demonstrated non-susceptibility to at least one agent in three or more antimicrobial categories out of 16 antimicrobial categories and XDR isolates demonstrated susceptibility to at least one agent in less than or equal to two out of 16 antimicrobial categories (31).

To assess the relationship between biofilm formation and resistance to specific antimicrobial classes/agents, we grouped the 16 antimicrobial categories into 12 categories based on mechanism of action (Table 1). Piperacillin/tazobactam and penicillin/β-lactamase inhibitors were grouped as penicillins plus β-lactamase inhibitors, and non-extended spectrum cephalosporins, extended-spectrum cephalosporins, cephamycins, and ceftaroline were grouped as cephalosporins. This allowed us to avoid collinearity in our statistical models due to overlap in resistance between antimicrobial categories.

Differences in antimicrobial resistance among the weak and strong biofilm formation groups were assessed with chi-square, Fisher's exact, or t-test as appropriate. Predictors of strong biofilm formation were identified from a logistic regression model. A p-value of 0.1 was used for initial inclusion in the model (Table 1) and stepwise backward elimination was used to identify statistically significant predictors of strong biofilm formation (all p-values <0.05). We assessed multicollinearity between potential predictors in the initial model from variance inflation factors, and confirmed the absence of collinearity. A sensitivity analysis was conducted to identify predictors of biofilm formation as a continuous measure (OD_{570}) using linear regression.

RESULTS

Optical density (OD_{570}) for all 139 isolates were divided using tertiles as follows: weak (n=47; $OD_{570} \leq 0.16$), moderate (n=46; $0.16 < OD_{570} < 0.59$), and strong biofilm formers (n=46; $OD_{570} \geq 0.59$) (26). This method was internally validated as the positive control was consistently

categorized as a strong biofilm former (OD_{570} ≥ 0.59) (26). Moderate isolates were removed for a total cohort of 93 isolates (Table 1) to best predict biofilm formation extremes (26).

MDR isolates (n=81) were more common among weak biofilm formers (n=46, 97.9%) versus strong biofilm formers (n=35, 76.1%; p=0.002), and XDR (n=25) isolates were similar between the groups (n=12, 25.5% vs. n=13, 28.3% p=0.77). Number of resistant antimicrobial categories and OD_{570} are shown in Figure 1. Resistance to all classes of beta-lactams (i.e. penicillins plus β-lactamase inhibitors, cephalosporins, monobactams, and carbapenems), aminoglycosides, chloramphenicol, and fluoroquinolones were more common among weak biofilm formers ($p<0.05$). In the multivariate model, the only predictor of biofilm formation was carbapenem resistance, which was inversely associated with strong biofilm formation (odds ratio, OR 0.09; 95% confidence interval, CI 0.02-0.33). Therefore, carbapenem-resistant *K. pneumoniae* were 91% less likely to form strong biofilm.

As the proportion of XDR isolates did not vary between weak and strong biofilm formers, we conducted a post-hoc sensitivity subgroup analysis excluding XDR isolates (n=68) (Table 2). Predictors of strong biofilm formation were again identified from a stepwise backward elimination logistic regression model, with a p-value of 0.1 used for initial inclusion in the model (Table 2). The only predictor of strong biofilm formation was the number of resistant categories, with an odds ratio of 0.70 (95% CI 0.56-0.86), where the odds of strong biofilm formation decreased by 30% with each increase in the number of resistant categories.

In the sensitivity analysis of the continuous measure of biofilm formation, only fluoroquinolone resistance was predictive of the OD_{570}, with a parameter estimate of -0.44 and an intercept of 0.86 ($p<0.001$). According to this model, fluoroquinolone susceptibility had an OD_{570} of 0.86, while fluoroquinolone resistance had an OD_{570} of 0.42. In other words, fluoroquinolone susceptible

isolates were predicted to be strong biofilm formers, and fluoroquinolone resistant isolates were predicted to be moderate biofilm formers.

DISCUSSION

This is the first study to our knowledge, to identify a statistically significant inverse relationship between *K. pneumoniae* antimicrobial resistance and biofilm formation, where carbapenem-resistant *K. pneumoniae* isolates were 91% less likely to be strong biofilm formers. Several published studies have described higher biofilm formation in resistant *K. pneumoniae* isolates, however, these descriptive studies did not assess whether resistance was predictive of biofilm formation in multivariate analyses (10-14). This is also the first study to assess resistance and biofilm formation of a diverse collection of *K. pneumoniae* isolates from multiple sources and centers. Inclusion of isolates only from a single-center introduces potential bias if patients are infected with the same organism, especially if isolates are from an outbreak (14). Additional rationale for the difference in findings requires further research.

Potential limitations of our study include the overall resistance patterns of our isolates. The majority of our isolates (n=81, 87.1%) were MDR, with the most isolates (n=64, 68.8%) resistant to at least 12 out of 16 antimicrobial classes, but only 26.9% (n=25) were XDR. Inclusion of more susceptible or XDR isolates may have resulted in different predictors of biofilm formation. As a post-hoc sensitivity analysis we excluded XDR isolates, which support the findings from the weak versus strong analysis, where strong biofilm formation was 30% less likely with each increase in the number of resistant categories.

Findings from previous studies may also be limited by misclassification of biofilm formation, which may affect conclusions that biofilm formation is more common among resistant isolates. The definition of biofilm formation varies across studies but the most common definition was originally

described for *Staphylococcus spp.* (15, 16). This method utilizes an OD cut-off (ODc), defined as three standard deviations above the average OD of the negative control, to determine biofilm formation. Isolates are either non-adherent (OD ≤ ODc), weakly adherent (ODc < OD ≤ 2xODc), moderately adherent (2xODc < OD ≤ 4xODc), or strongly adherent (4x ODc < OD). Categorization by this method however, is limited when the negative control has a negligible OD reading, which was the case for our study and previously described literature (12, 13). Applying this method to our cohort, zero isolates were non-adherent, two weak, 12 moderate, and 125 strong biofilm formers. Therefore, we divided biofilm formation into tertiles (26), to overcome potential bias of overestimating strong biofilm formation (12). Previous utilization of tertile biofilm categorization was also utilized for *S. aureus,* however categorization should not be affected by organism as our biofilm quantification method utilized was adapted for *K. pneumoniae.* We also assessed biofilm formation as a continuous variable since there is no standard categorization. However, interpretation of resulting odds ratios is challenging when compared to a dichotomous variable of resistance versus susceptible, and is less clinically meaningful.

Optical density remains an indirect measurement of biofilm formation and standardized methods for both quantification and categorization of *K. pneumoniae* biofilm formation are needed. Varied definitions of biofilm formation are utilized, making direct comparisons across studies difficult. It is imperative to standardize biofilm quantification to allow for accurate assessment of predictors of biofilm across settings and to quantify the relationship between biofilm forming isolates and clinical outcomes.

In our study, strong biofilm formation was 91% less likely with carbapenem-resistant *K. pneumoniae*, which allows clinicians to better predict *K. pneumoniae*'s ability to produce biofilm by phenotypic resistance. This inverse relationship between biofilm formation and antibiotic resistance suggests that virulence may be a trade-off for bacterial survival.

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Table 1. *Klebsiella pneumoniae*: antimicrobial resistance and biofilm formation

Variable	Total Cohort (n=93)	Weak Biofilm Formation (n=47)	Strong Biofilm Formation (n=46)	p-value
Number of Resistant Categories (n=16), Median, (IQR)	13 (11-14)	13 (12-14)	11.5 (3-14)	**0.01**
Multidrug-resistant (MDR), n (%)*	81 (87.1)	46 (97.9)	35 (76.1)	**0.002**
Extensively drug-resistant (XDR), n (%)**	25 (26.9)	12 (25.5)	13 (28.3)	0.77
Penicillins + β-lactamase inhibitors, n (%)‡	79 (84.9)	46 (97.9)	33 (71.7)	**0.0004**
Cephalosporins, n (%)	82 (88.2)	46 (97.9)	36 (78.3)	**0.003**
Monobactam, n (%)	73 (78.5)	45 (95.7)	28 (60.9)	**<0.0001**
Carbapenems, n (%)‡ ‡	70 (75.3)	44 (93.6)	26 (56.5)	**<0.0001**
Aminoglycosides, n (%)	72 (77.4)	43 (91.5)	29 (63.0)	**0.001**
Chloramphenicol, n (%)	65 (69.9)	38 (80.9)	27 (58.7)	**0.02**
Fluoroquinolones, n (%)	73 (78.5)	45 (95.7)	28 (60.9)	**<0.0001**
Tigecycline, n (%)	13 (14.0)	6 (12.8)	7 (15.2)	0.73
Tetracyclines, n (%)	44 (47.3)	23 (48.9)	21 (45.7)	0.75
Folate pathway inhibitor, n (%)	66 (71.0)	37 (78.7)	29 (63.0)	0.09
Fosfomycin, n (%)	61 (65.6)	29 (61.7)	32 (69.6)	0.42
Colistin, n (%)	11 (11.8)	8 (17.0)	3 (6.5)	0.12

Bolded p-values indicate potential predictors of strong biofilm formation included in the initial logistic regression model

*MDR isolates demonstrated non-susceptibility to at least one agent in three or more antimicrobial categories out of 16 antimicrobial categories

**XDR isolates demonstrated susceptibility to at least one agent in less than or equal to two out of 16 antimicrobial categories

‡penicillin + β-lactamase inhibitors category includes piperacillin/tazobactam and penicillin/ β-lactamase inhibitors

‡ ‡cephalosporins category includes non-extended spectrum cephalosporins, extended-spectrum cephalosporins, cephamycins, and ceftaroline

Table 2. Sensitivity analysis of non-XDR *Klebsiella pneumoniae*: antimicrobial resistance and biofilm formation

Variable	Total Cohort (n=68)	Weak Biofilm Formation (n=35)	Strong Biofilm Formation (n=33)	p-value
Number of Resistant Categories (n=16), Median, (IQR)	12 (8-13)	13 (12-13)	9 (1-12)	<0.0001
Multidrug-resistant (MDR), n (%)*	56 (82.4)	34 (97.1)	22 (66.7)	**0.001**
Extensively drug-resistant (XDR), n (%)**	0	0	0	---
Penicillins + β-lactamase inhibitors, n (%)	54 (79.4)	34 (97.1)	20 (60.6)	**0.0002**
Cephalosporins, n (%)	57 (83.8)	34 (97.1)	23 (69.7)	**0.002**
Monobactam, n (%)	48 (70.6)	33 (94.3)	15 (45.5)	**<0.0001**
Carbapenems, n (%)	45 (66.2)	32 (91.4)	13 (39.4)	**<0.0001**
Aminoglycosides, n (%)	48 (70.6)	32 (91.4)	16 (48.5)	**0.001**
Chloramphenicol, n (%)	40 (58.8)	26 (74.3)	14 (42.4)	**0.008**
Fluoroquinolones, n (%)	48 (70.6)	33 (94.3)	15 (45.5)	**<0.001**
Tigecycline, n (%)	6 (8.8)	4 (11.4)	2 (6.1)	0.67
Tetracyclines, n (%)	20 (29.4)	12 (34.3)	8 (24.2)	0.36
Folate pathway inhibitor, n (%)	41 (60.3)	25 (71.4)	16 (48.5)	0.053
Fosfomycin, n (%)	41 (60.3)	19 (54.3)	22 (66.7)	0.30
Colistin, n (%)	6 (8.8)	4 (11.4)	2 (6.1)	0.44

Bolded p-values indicate potential predictors of strong biofilm formation included in the initial logistic regression model

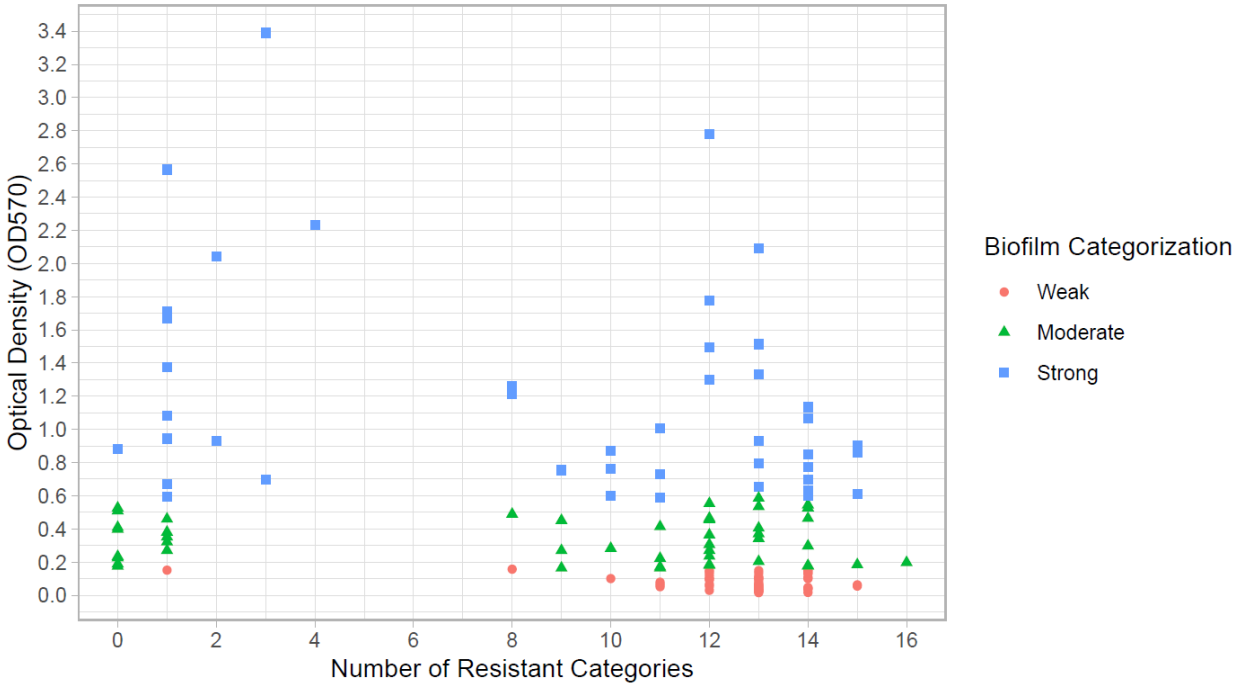
*MDR isolates demonstrated non-susceptibility to at least one agent in three or more antimicrobial categories out of 16 antimicrobial categories

**XDR isolates demonstrated susceptibility to at least one agent in less than or equal to two out of 16 antimicrobial categories

‡penicillin + β-lactamase inhibitors category includes piperacillin/tazobactam and penicillin/ β-lactamase inhibitors

‡ ‡cephalosporins category includes non-extended spectrum cephalosporins, extended-spectrum cephalosporins, cephamycins, and ceftaroline

Figure 1. *Klebsiella pneumoniae* biofilm formation and resistance (n=139)



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