

Comparative Study of Biofilm and Non-Biofilm Producing *Klebsiella pneumoniae* with Special Reference to Metallo-Beta-Lactamase Production

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Abstract

Klebsiella pneumoniae is one of the most common bacteria among all biofilm-producing as well as the beta-lactamase producing strains, which is responsible for multi-drug resistance. For better therapeutic applications, it is important to detect the biofilm production by *Klebsiella pneumoniae* and their antibiogram along with the ability to produce ESBL, MBL, and AmpC β -lactamases. The aim of the study was to determine the prevalence of biofilm formation and ESBL, MBL, AmpC β -lactamase phenotypes in *K. pneumoniae* as well as the antibiogram of all (biofilm and MBL-producing and non-producing) isolates of *K. pneumoniae*. Isolates of *K. pneumoniae* were tested for biofilm formation by the Congo-red agar method. ESBL, MBL, and AmpC β -lactamase detection were done by both screening and confirmatory tests as per CLSI guidelines. The antibiogram was obtained by the Kirby-Bauer disc diffusion method. Among the total 100 isolates of *K. pneumoniae*, 40% were biofilm-producing. Most of them were from urine specimens. Out of biofilm-producing isolates, ESBL-28%, MBL- 47% and AmpC β -lactamase- 25.8% producers were observed. *K. pneumoniae* isolates were seen to have maximum resistance to ceftazidime and maximum sensitivity to nitrofurantoin. Study findings suggest the importance of assessment of biofilm formation for better treatment. The scenario further worsens if such biofilm-producing isolates are also MBL-positive leading to limited therapeutic options.

Keywords: Biofilm Formation, Extended Spectrum Beta-lactamases, Metallo-Beta-Lactamases, Ampicillinase Cephalosporinase

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INTRODUCTION

A biofilm is an organized collection of microorganisms which live embedded within a self-generated matrix of extracellular polymeric substances (EPS). This biofilm is usually attached to a biotic or abiotic surface.¹ The biofilm protects microorganisms from adverse effects of extreme environmental conditions. Studies have shown that biofilms can make microorganisms resistance to increase against UV radiation,² extreme pH and temperature, high salinity, poor nutrients, and importantly to various antibiotics.³ Among Gram-negative pathogens *Klebsiella pneumoniae* is one of the leading bacteria responsible for biofilm formation.

Klebsiella pneumoniae possesses virulence attributes, encompassing capsule polysaccharide, lipopolysaccharide, outer membrane proteins, type 1 and type 3 fimbriae as well as factors that govern iron acquisition and nitrogen source utilization.⁴ These virulence elements play a critical role in enabling the pathogen to evade the host's immune system and in facilitating the formation of robust biofilms.⁵ Pili along with lipopolysaccharides of biofilm-forming isolates increases the bacterial pathogenicity.⁶ *K. pneumoniae* are also known to cause biofilm-mediated infections in most hospitalized patients.⁴ Biofilms formed by *K. pneumoniae*, are bacterial communities enclosed within an extracellular matrix composed of proteins, DNA, exopolysaccharides, and lipopeptides.⁷ This biofilm holds the bacterial attachment to living or non-living surfaces. Also, biofilm protects the aggregate of *K. pneumoniae* in the biofilm from antimicrobial penetration and reduces its effects.⁸

K. pneumoniae has demonstrated a heightened ability to acquire resistance to antimicrobial agents compared to many other bacteria, primarily through the production of enzymes like Extended Spectrum β -Lactamase (ESBLs) and Carbapenemases.⁹ Furthermore, it can rapidly transfer this resistance mechanism to other bacteria. The relation between biofilm-forming and beta-lactamase-producing strains is going the extensive range to the dissemination of multi-drug resistant mechanism.¹⁰

Antimicrobial resistance (AMR) has emerged as a critical worldwide health challenge.

Limited information is available regarding the relationship between biofilm formation and antimicrobial resistance in clinical isolates of *K. pneumoniae*. The increase in the rate of antimicrobial resistance in *Enterobacterales* has become a serious issue.¹¹ Drug resistance is responsible for narrowed therapeutic options because the pathogens have developed resistance mechanisms against the commonly used antimicrobial groups like extended-spectrum beta-lactams, aminoglycosides, fluoroquinolones, and carbapenems, etc.¹² ESBL-producing bacteria are not only resistant to penicillin group but also other antibiotics classes like cephalosporins, aztreonam, aminoglycosides, and fluoroquinolones.¹³ Carbapenems are the drug of choice in the various clinical isolates.

This research aimed to bridge the knowledge gap regarding the effect and correlation between biofilm formation in *K. pneumoniae* and antimicrobial resistance. Besides the prevalence of biofilm formation among *K. pneumoniae* clinical isolates, another objective was to study the antibiogram of Biofilm and MBL-producing and nonproducing *K. pneumoniae*.

MATERIALS AND METHODS

A total of 100 non-repetitive *K. pneumoniae* isolates from clinical specimens of patients of all ages and genders admitted to Krishna Hospital and Medical Research Centre in Karad, India, were included in the study. The specimen processing and identification was done on the basis of standard methodology.¹⁴ The project was approved by the Institutional Ethics Committee and the informed consent was taken from all enrolled patients before collecting the specimen.

Biofilm detection by Congo red agar method

Congo red agar media was prepared by mixing brain heart infusion broth, sucrose, Congo red dye, and agar in distilled water. Isolates were inoculated on Congo red agar media and incubated at 37°C for 18-24 hrs. After incubation, the black-coloured colonies with metallic dry consistency were considered as biofilm producers and red-coloured colonies as non-producer (Figure 1).¹⁵

Antibiotic sensitivity profile

After the isolation of pure culture, organisms were tested for antimicrobial susceptibility test, which was performed by the Kirby-Bauer disc diffusion method as per the CLSI guidelines.¹⁶ For this, antibiotic discs included were nitrofurantoin (300 µg), imipenem (10 µg), ceftazidime (30 µg), ceftaxitin (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), gentamicin (10 µg), amikacin (30 µg), piperacillin/tazobactam (100/10 µg), fosfomycin (200 µg), co-trimoxazole (1.25/23.75 µg), meropenem (10 µg), tigecycline (15 µg). The zone of inhibition was interpreted as per the CLSI (2021) standards.

Phenotypic detection of drug resistance

The isolates were further screened for phenotypic detection of enzymes like ESBL, MBL, and AmpC β-lactamase. For the screening of ESBL production ceftazidime and ceftazidime plus clavulanic acid discs were used and a difference of 5mm zone size between the two was considered significant.¹⁷ MBL production was detected with imipenem and imipenem EDTA discs with respect to 7 mm inhibition zone size¹⁸ difference and for the AmpC β-lactamase detection ≥4 mm inhibition zone difference was considered significant between ceftoxitin and ceftoxitin plus cloxacillin discs.¹⁹

RESULTS



Figure 1. Biofilm detection on Congo red agar

Among 100 isolates of *K. pneumoniae* maximum biofilm-producing isolates were from urine 12 (12%) followed by ETT 9 (9%), pus 7 (7%), blood 8 (8%), and least in sputum 3 (3%) and CSF 1 (1%) (Table 1).

Among the biofilm-producing strains of *K. pneumoniae*, 25% were AmpC β-lactamase-producing, 28% were ESBL-producing and 47% were MBL-producing isolates (Figure 2).

When the biofilm-producing strains of *Klebsiella pneumoniae* were tested against antimicrobial agents, they showed maximum sensitivity to nitrofurantoin 23 (57.5%), meropenem 22 (55%) and resistance to ceftazidime 36 (90%),

Table 1. Distribution of biofilm-producing and non-producing *K. pneumoniae* among various clinical specimens

Clinical Specimens	Biofilm producers		Biofilm non-producers	
	Number (n)	%	Number (n)	%
Urine	12	12	13	13
ETT	9	9	13	13
Pus	7	7	15	15
Blood	8	8	10	10
Sputum	3	3	5	5
CVP tip	0	0	2	2
Ascitic fluid	0	0	1	1
CSF	1	1	0	0
Pleural fluid	0	0	1	1
Total	40	40	60	60

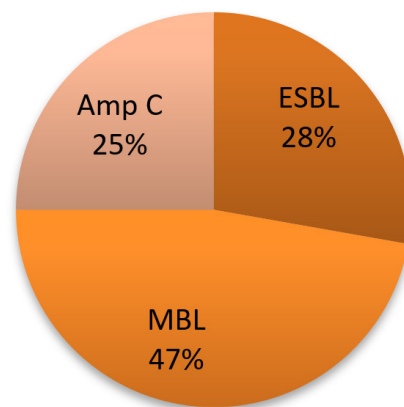


Figure 2. Distribution of ESBL, MBL, and AmpC producers among biofilm-producing isolates

ciprofloxacin, cefoxitin, tigecycline 31 (77.5%) each (Table 2).

Antimicrobial sensitivity pattern of Biofilm and MBL-producing *K. pneumoniae* isolates showed maximum sensitivity to nitrofurantoin 10 (58.82%), fosfomycin 9 (52.94%) and resistance to ceftazidime, 16 (94.11%) (Table 3).

When biofilm and MBL-producing strains were analyzed, their antimicrobial pattern showed maximum resistance to ceftazidime (94.11%) and imipenem (94.11%). The biofilm-

producing non-MBL isolates showed resistance to ceftazidime (86.95%) and imipenem (60.86%). The MBL-positive isolates were comparatively more resistant than MBL-negative isolates among the biofilm-producing *Klebisella pneumoniae* isolates. (Figure 3).

DISCUSSION

K. pneumoniae isolation in patients can raise serious concern because globally it is one of

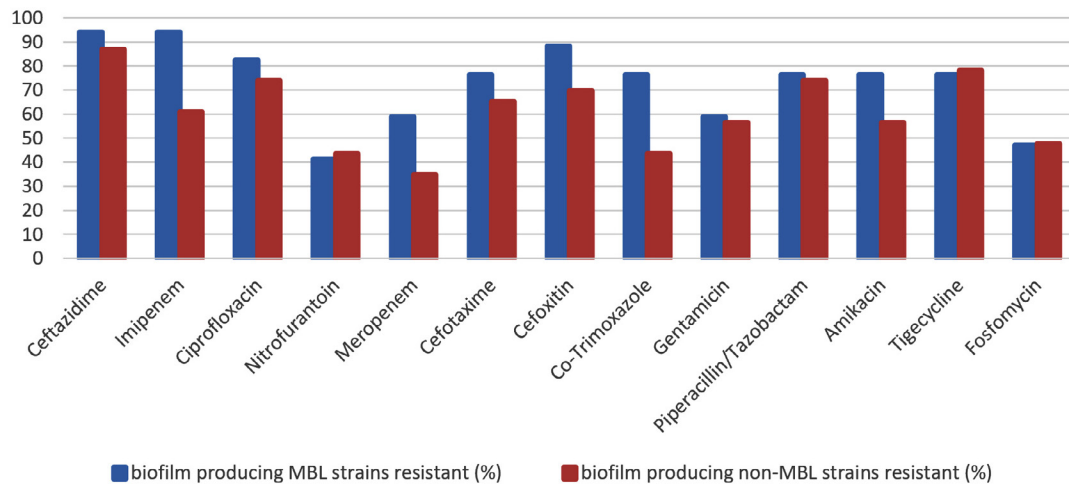


Figure 3. Resistogram of biofilm and MBL producing versus biofilm and MBL non-producing isolates

Table 2. Antimicrobial profile of biofilm producing strains of *K. pneumoniae*

Antibiotics	Sensitive	Sensitive %	Resistant	Resistant %
Ceftazidime	4	10	36	90
Imipenem	10	25	30	75
Ciprofloxacin	9	22.5	31	77.5
Nitrofurantoin	23	57.5	17	42.5
Meropenem	22	55	18	45
Cefotaxime	12	3	28	70
Cefoxitin	9	22.5	31	77.5
Co-trimoxazole	17	42.5	23	57.5
Gentamicin	17	42.5	23	57.5
Piperacillin/Tazobactam	10	25	30	75
Amikacin	14	35	26	65
Tigecycline	9	22.5	31	77.5
Fosfomycin	21	52.5	19	47.5

Table 3. Antimicrobial profile of biofilm and MBL-producing strains

Antibiotics	Sensitive	Sensitive %	Resistant	Resistant %
Ceftazidime	1	5.88	16	94.11
Ciprofloxacin	3	17.64	14	82.35
Nitrofurantoin	10	58.82	7	41.17
Cefotaxime	4	23.52	13	76.47
Cefoxitin	2	11.76	15	88.23
Co-Trimoxazole	4	23.52	13	76.47
Gentamicin	7	41.17	10	58.82
Piperacillin/Tazobactam	4	23.52	13	76.47
Amikacin	4	23.52	13	76.47
Tigecycline	4	23.52	13	76.47
Fosfomycin	9	52.94	8	47.05

the most important causes of MDR infections.¹⁹ Also in the case of HAIs outbreaks or longer stays in the hospitals due to MDR; it results in inflated healthcare costs.²⁰

In this study, we investigated the biofilm-producing capabilities of *Klebsiella pneumoniae* isolated from a range of clinical specimens. Our findings show some of the *K. pneumoniae* isolates were biofilm-producing strains. The formation of biofilms by nosocomial opportunistic pathogens like *K. pneumoniae* on host-tissue surfaces represents a pivotal step in the progression of infection. In this study, 40% of isolates were biofilm producers. Similar findings are reported by Madhavi S. Hullur *et al.*²¹ stated that 54% *K. pneumoniae* isolates were identified as biofilm producers. In another study, Rakesh Prasad Sah *et al.*²² reported that 57.4% *K. pneumoniae* strains were biofilm producers. Among biofilm-producing strains of *K. pneumoniae* the higher reported findings were 77.55% by Rabina Dumar *et al.*¹⁰ The variation in biofilm-forming ability among each isolate can be attributed to a multitude of influencing factors, including the physiochemical properties of *K. pneumoniae*, the nature of the surface to which the biofilm adheres as well as the environmental factors like temperature and pH.²³ The findings in other studies were 60% by Gedif Meseret Abebe *et al.*,²⁴ 65.46% by Roshani Nhuchhen Pradhan *et al.*²⁵ etc. The occurrence of the lowest biofilm-producing strains of *K. pneumoniae* was reported as 24.3% by Sabina Fatima *et al.*²⁶

Because of the ability to produce β -lactamase enzymes and biofilms, *Klebsiella* spp. is resistant to a wide range of antibiotics,²⁷ which is also observed in our study. Among the 40 biofilm-producing *K. pneumoniae* isolates 28% were ESBL producers, 47% MBL producers and 25% AmpC β -lactamases producers. These findings are slightly comparable with Susmita Kuinkel *et al.*²⁷ where they observed 17.9% ESBL producers, 33.3% MBL producers, and 20.5% AmpC β -lactamases producers among biofilm-positive strains. In the study of Rabina Dumar *et al.*¹⁰ 30.61% were ESBL and 26.53% were MBL producers,¹⁰ respectively among biofilm producers.

Comparatively slightly higher prevalence of ESBL, MBL and AmpC β -lactamases production in biofilm-producing *K. pneumoniae* in present study is justifiable as half (49.05%) of these biofilm producers were from various ICUs where patients usually have an invasive equipment for bacteria to form biofilm on.

Biofilm-producing strains have the tendency of ESBL, MBL, and AmpC β -lactamases production which is responsible for multi-drug resistance factors worldwide.²⁷

Overall, 40% of isolates were detected as biofilm producers in our study. When these biofilm-producing strains were further got tested with antimicrobials, they showed multiple antibiotic resistance patterns. In the present study, maximum isolates were resistant to cephalosporin-ceftazidime (90%), carbapenem - imipenem (75%), and fluoroquinolone-ciprofloxacin (77.5%). On the

other hand, Hera Nirwati *et al.*⁸ found in their study that most of the biofilm-producer isolates were resistant to cefazolin (65.29%), cefuroxime (58.74%) and ceftriaxone (56.64%) respectively. *Klebsiella* with resistance of ceftazidime (66.5%), cefepime (59.39%), piperacillin/Tazobactam (57.87%), and ofloxacin (57.87%) were observed in the study of Rabina Dumar *et al.*¹⁰

Biofilm producing MBL-positive isolates showed more resistance to aminoglycoside, cephalosporins, quinolones, and carbapenem group of antimicrobials compared to MBL negative biofilm producing isolates.

Biofilms are the bacterial cells that have ability to self-produce a polymeric matrix which is capable to adhering a layer on living or non-living surface. The ability of microorganisms to produce biofilm leads to high prevalence of multidrug resistance which in turn leads to treatment failure. Hence, it is crucial to emphasize both antimicrobial therapy and anti-biofilm strategies, as they collectively offer the most effective treatment options. The resistance pattern in biofilm producing isolates is further aggravated if the isolates are Metallo-Beta-Lactamase producing strains.

CONCLUSION

In the present study, we observed high prevalence (40%) of biofilm formation among *Klebsiella pneumoniae* isolates. Most of the biofilm producers were from urine and catheter tips. Among these biofilm producing strains prevalence of ESBL, MBL and AmpC β -lactamases. β -lactamases was seen in 28%, 47%, and 25% of the isolates, respectively. *K. pneumoniae* as well as biofilm producing *K. pneumoniae* strains had higher resistance to ceftazidime and higher sensitivity to nitrofurantoin antibiotic.

Significant proportions of the study isolates (40%) were positive for biofilm formation suggesting importance of biofilm assessment routinely. The scenario further worsens if such biofilm producing isolates are also MBL-positive making limited therapeutic options. The study suggests that it is very important to screen biofilm production and phenotype detection in urinary and catheter related *Klebsiella pneumoniae* infections.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

The study was approved by the Institutional Ethics Committee, Krishna Institute of Medical Sciences, KVV DU, Karad, with Protocol number 051/2021-2022.

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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