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A Study On Impact Of Biofilm Formation On Multidrug Resistance Among Gram Negative Isolates

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Abstract

Background: Biofilm has a remarkable impact in the field of infectious diseases. They confer advantages to the biofilm forming bacteria, such as protection from antimicrobial agents. A crucial factor in the persistence and treatment failure of bacterial infections is biofilm formation. Biofilm formation is recognized not only as a virulence factor but also as a significant contributor to multidrug resistance

Objectives: To assess the impact of biofilm formation on multidrug resistance among Gram-negative clinical isolates. Also to correlate biofilm-forming capacity with antimicrobial resistance profiles among them.

Materials and Methods: The present prospective study was conducted over a period of one year. All the Gram-negative isolates recovered from various clinical specimens were included. The antimicrobial susceptibility performed by Kirby Bauer disc diffusion method. The isolates were subjected for ESBL and MBL detection by standard tests and biofilm production by tube adherence method and Congo red agar method.

Results: A total of 450 non-repetitive gram-negative bacterial isolates recovered from various clinical specimens. Escherichia coli constituted 40% (180) followed by *Klebsiella spp.* 22% (99), *Acinetobacter spp.* 18% (81) and *Pseudomonas spp.*12% (54). *Citrobacter* species exhibited less resistance compared to other GNBs. A total of 280 (62.22%) isolates were biofilm producers and highest rate of 77.77% and 73.73% noted among *Pseudomonas spp*. and *Klebsiella spp*. respectively.

Conclusion: Biofilm producing isolates show higher antimicrobial resistance as compared to biofilm non producers. Early biofilm formation detection by routine incorporation of detection methods will be helpful in assisting the clinicians to initiate appropriate empirical antimicrobials for the treatment.

Keywords: Antimicrobial resistance, Biofilm, ESBL, MBL, Tube adherence method

Introduction

For the past few years, microbial biofilms have been emerging as a significant threat to the modern healthcare system, and their prevalence and antibiotic resistance threat gradually increase daily among the human population. [1] The biofilm has a remarkable impact in the field of infectious diseases.[1] These are an assembly of microbial cells formed by single or a mixture of bacterial species that are irreversibly associated with a surface and enclosed in a matrix of polysaccharide materials that allow the growth and survival in hostile environments. Biofilms confer advantages to the biofilm forming bacteria, such as

protection from antimicrobial agents, exchange of nutrients and metabolites, and/or genetic exchange between organisms. [2,3]

Biofilm impact all facets of human life, from public health to industrial concerns, and impact the economy.[3] Many human diseases and colonization of medical devices of are linked to microorganisms growing in biofilms and these microorganisms are very resistant to antimicrobial treatments.[3]

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Multidrug-resistant (MDR) organisms, including *Acinetobacter baumannii, methicillin-resistant Staphylococcus aureus* (MRSA), and extended spectrum beta-lactamase (ESBL) producing Gram-negative bacteria, are frequently implicated as the causative agents of acute and chronic infections contributing significantly to patient morbidity and mortality, as well as increased health care costs associated with treatment.[4] Adaptation to surface attached growth within a biofilm is accompanied by significant changes in gene and protein expression, as well as metabolic activity which confers resistance to antimicrobial therapy and host mechanisms of clearance.[4] The biofilm formation and beta-lactamases production synergistically contribute for extensive dissemination of multi-drug resistant strains of Gram-negative bacilli.[5] A crucial factor in the persistence and treatment failure of bacterial infections is biofilm formation.[1] Biofilms are implicated in up to 65–80% of chronic and device-associated infections.[2] Biofilms present a diagnostic and therapeutic challenge. These bacteria can display up to 100–1000-fold higher resistance to antibiotics compared to their counterparts, due to reduced drug penetration, slowed growth rates, and enhanced tolerance to antimicrobial agents.[2] Biofilm formation is recognized not only as a virulence factor but also as a significant contributor to multidrug resistance (MDR).[1,2]

Clinically important Gram-positive bacteria, including *Staphylococcus aureus* and *Enterococcus* species, are capable of robust biofilm formation. Gram-negative bacteria, including *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *Acinetobacter baumannii*, are also notorious for their ability to form resilient biofilms.[6]

The clinical implications of biofilm-associated multidrug resistance are profound. Biofilm-related infections tend to be persistent, recurrent, and refractory to conventional antibiotic therapy.

Despite growing recognition, comparative studies examining the impact of biofilm formation on multidrug resistance among both Gram-positive and Gram-negative clinical isolates remain limited.

Hence, the present study was undertaken to assess the impact of biofilm formation on multidrug resistance among Gram-negative clinical isolates. By correlating biofilm-forming capacity with antimicrobial resistance profiles, this study elucidates the complex interplay between biofilm production and the development and persistence of MDR phenotypes.

Materials And Methods:

The present prospective study was conducted at the Department of Microbiology over a period of one year from Jan 2025 to December 2025. All the Gram negative isolates recovered from the various clinical specimens (blood, urine, pus, CSF, endotracheal tube, tracheal aspirate, fluids, genital swab, catheter tips, and sputum) submitted to the microbiology laboratory for routine culture and sensitivity testing were included. All specimens were inoculated on 5% sheep Blood agar and MacConkey agar as per the standard bacteriological procedures and incubated at 37 °C for 24–48 hours. The growth of bacteria identified on the basis of colony morphology, pigmentation, odour, and their unique biochemical tests by battery of reactions. [7]

The antimicrobial susceptibility test performed on Mueller Hinton agar by Kirby Bauer disc diffusion method. The representative antibiotics among all classes were used for sensitivity testing. Bacterial isolates demonstrating resistance to at least one agent each, in three antibiotic classes were considered as Multi Drug resistant (CDC MDRO/CDI, module, 2014). [8] The isolates were subjected for ESBL production and MBL detection by standard tests [7]. The results interpreted as per CLSI guidelines 2023. [9]

The biofilm production was detected by

1. Biofilm formation by tube adherence method: The growth of the isolate was inoculated onto trypticase soy broth and incubated for 24 h at 35 °C. After discarding the supernatant, the tube

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was washed with phosphate buffer saline(PBS) and was treated with 0.1% crystal violet(CV) for staining and then washed with water and air dried. The appearance of visible biofilm lining the bottom and wall of the tube considered positive.[10]

2. Congo red agar method: The isolates were inoculated on Congo red agar plate (Hi media laboratories, Mumbai) and incubated at 37 °C for 24 h. Biofilm producing bacteria exhibited black crystalline, dry, rough colonies, whereas non biofilm producers showed smooth, reddish coloured colonies. A 5point colour reference scale was applied for the quantification of biofilm production. Biofilm producers were regarded as BB Bright black or OB Opaque black. Anything between pink, red to Bordeaux was taken as non biofilm producer strains. [8]

Results

A total of 450 non-repetitive gram-negative bacterial isolates recovered from various clinical specimens received for aerobic bacterial culture and sensitivity were included in the study. Out of these, 40% (180) were Escherichia coli followed by *Klebsiella* spp. 22% (99), *Acinetobacter spp.* 18% (81) and *Pseudomonas spp*.12% (54). A small portion were of *Enterobacter spp.* 4% (18) and 2% (9) each of *Citrobacter spp*. and *Proteus species*. Among all the isolates, *Citrobacter* species exhibited less resistance compared to other GNBs. In the present study *Pseudomonas* species showed a resistance rate of 21.25% to Piperacillin–Tazobactam, however resistance to other tested antibiotics was between 30.58% to Imipenem & Ofloxacin, around 33. 84% to Ceftazidime.

Table 1: Distribution of biofilm formers and ESBL and MBL producers

Organisms	Total isolates (%)	Biofilm formers no. (%)	Biofilm ESBL producers no. (%)	Biofilm MBL producers no. (%)	Biofilm Both ESBL and MBL producers no. (%)	ESBL, MBL CRE and biofilm producers no. (%)
Escherichia coli	180(40)	102(56.66)	60(58.82)	14(13.72)	5(4.9)	4 (3.9)
Acinetobacter spp.	81(18)	42(51.85)	15 (35.71)	20(47.62)	2(4.76)	2(4.76)
Klebsiella spp.	99(22)	73(73.73)	20(27.4)	20(27.4)	4(5.48)	4(5.48)
Pseudomonas spp.	54(12)	42(77.77)	11(26.19)	15(35.71)	3(7.14)	3(7.14)
Enterobacter spp.	18(4)	12(66.66)	3(25)	4(33.33)	2(16.66)	1(8.33)
Citrobacter spp.	9(2)	5(56.66)	3(60)	0(00)	0(00)	0(00)
Proteus spp.	9(2)	4(44.44)	1 (25)	2(50)	0(00)	0(00)
Total	450(100)	280(62.22)	113(40.36)	75(26.78)	16(0.57)	14(0.50)

Among the Enterobacteriales, 14.22% of *Escherichia coli* were resistant to Amikacin, followed by 19.34% to Piperacillin–Tazobactam, and 20.49% to Imipenem. Whereas resistance to other antibiotics tested ranged between 50.24% to 61.23%. (Table No.3) Highest resistance was observed among *Klebsiella spp*. About 75.23% of isolates were resistant to Ceftazidime, 50.01% to Imipenem, 41.81% to Amikacin and 43.82% to Piperacillin–Tazobactam. Half of the isolates were noted to be multidrug resistant having resistance to more than three groups of antibiotics tested.

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Table 2: Detection of biofilm production

Organisms	Total isolates (%)	Biofilm formers no(%)	Tube adherence method	Congo red agar method
Escherichia coli	180(40)	102(56.66)	73(71.56)	29(28.44)
Acinetobacter spp.	81(18)	42(51.85)	34(80.95)	8(19.05)
Klebsiella spp.	99(22)	73(73.73)	61(83.56)	12(16.44)
Pseudomonas spp.	54(12)	42(77.77)	32(76.19)	10(23.81)
Enterobacter spp.	18(4)	12(66.66)	9(75)	3(25)
Citrobacter spp.	9(2)	5(56.66)	3(60)	2(40)
Proteus spp.	9(2)	4(44.44)	3(75)	1(25)
Total	450(100)	280(62.22)	211(75.35)	69(24.65)

Table 3: Antibiotic resistance among all GNB

Antibiotic tested	Escherichia coli	Acinetobacter spp.	Klebsiella spp.	Pseudomonas spp.	Enterobacter spp.	Citrobacter spp.	Proteus spp.
Amikacin	14.22%	40.44%	41.81%	32.21%	32.33%	12.52%	40.56%
Gentamicin	20.97%	50.40%	42.94%	34.24%	44.14%	23%	62%
Ofloxacin	61%	50.04%	51.14%	30.58%	37.53%	17.5%	61%
Levofloxacin	50.24%	49.38%	52.14%	31.46%	22.63%	25%	58%
Ceftazidime	61.23%	70.02%	75.23%	33.84%	50.85%	53%	41%
Cefepime	52 %	71.26%	53.18%	32.84%	41.44%	51%	39%
Imipenem	20.49%	32.68%	50.01%	30.58%	32.03%	13%	62%
Piperacillin	51.19%	51.56%	62.27%	33.58%	40.44%	62.5%	61%
Piperacillin – Tazobactam	19.34%	40 %	43.82%	21.25%	31.33%	25%	43%
Colistin	0%	0%	0%	0%	0%	0%	N/A
Polymyxin B				12.2%			
Tobramycin				30.5%			
Carbenicillin				33.9 %			

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Out of 450 isolates, 280 (62.22%) isolates were biofilm formers. More than 50% of all species were biofilm producers except *Proteus spp.* Highest rate of 77.77% and 73.73% was noted among *Pseudomonas spp*. and *Klebsiella spp*. respectively. However, 56.66% of *Escherichia coli* and 51.85% of *Acinetobacter spp*. too produced biofilms. (Table No.2). Amongst the two methods for biofilm detection, tube adherence method revealed a detection rate from 71.56% to 83.56%, where as Congo red agar method identified from 16.44% to 40%. (Table. No 2)

According to the number of antibiotic families to which the isolates were resistant, they were classified into multidrug resistant (MDR), those with resistant to three or more antibiotic families and extensively drug resistant (XDR); non-susceptible to at least one agent in all but two or fewer antimicrobial categories. [11] In the present study MDR was extensively noticed among biofilm producers.

Among 102 biofilm producing Escherichia coli, 60 (58.82%) were ESBL producers and 14 (13.72%) MBL producers. Those producing both ESBL & MBL were 5 (4.9%) and however 4 (3.9%) were ESBL, MBL & CRE producers.

However, among *Klebsiella spp*. the frequency of ESBL, MBL & CRE production was less compared to *Escherichia coli* as shown in Table no1. Among biofilm producing *Acinetobacter* spp. ESBL production was 35.71% and MBL was 47.62%. Whereas among *Pseudomonas spp*. it was 26.19% and 35.71% respectively.

Discussion:

Infections caused by Gram-negative microorganisms are a significant cause for both community and nosocomial settings. The emergence of microorganisms resistant to multiple antibiotics used in the treatment of infections has become an important health problem worldwide. [11]

In the present study, *Escherichia coli* was the predominant isolate accounting for 40% (180/450) followed by Klebsiella spp of 22% (99/450) which is in concordance with a study by Dumaru et al & Fatima et al. [8,10, 12]

Overall biofilm production rate among all GNB in the present study is 62.22% (280/450) which is in *concordance* to 61.4% reported by Sanchez et al. [4] A study at Telangana reported a similar biofilm rate of 54% among MDR isolates in nearing to present study.[8]

However, biofilm rate of 47% was noted among urinary isolates, predominance in *Pseudomonas aeruginosa of 51.7% & only 44.32% in Escherichia coli*. [2] A study in Indonesia found 85.63% of Klebsiella pneumoniae to be biofilm producers, which is almost similar to this study. [13]

Various studies have with different methods for detection of biofilm production have noted varying rates from 11.62% to 63.6% by tissue culture methods to and 39.3 % to 49% by tube adherence methods [12], however in the present study it was 75.35% by tube adherence methods and 24.65% by congo red agar method. This could be because of varying sensitivity rate of different methods in their detection and methodology. A study at Dharan, have reported 62.73% biofilm production, similar to present study but noted only 26.75% of ESBL and 16.24% of MBL production among them in contract to present study. However, E.*coli* was the most prevalent isolate of 38% identical to present study. [14]

Reported biofilm production of 19.57% to 24.29% by different methods and 32.72% *Klebsiella pneumoniae and 28.44% of Escherichia coli.* [12]

However, detection of biofilm-forming genes may be a good tool for the evaluation of biofilm production, which will help in prompt and better management.[15]

Although the molecular method is more sensitive and specific, phenotypic methods are comparatively less expensive and easier to perform in resource developing nations.

A study at Jodhpur reported maximum isolates to be resistance to ampicillin (93.27%) followed by amoxiclav (87.46%), ceftazidime (74%) and Ciprofloxacin (71.25%) and were sensitive to meropenem (70.34%), piperacillin-tazobactam (68.20%) and cefepime (55.05%), which is similar to the present study. [12]

Acinetobacter baumannii and *Pseudomonas aeruginosa* are commonly causing hospital acquired infection and were many a times found to be multidrug resistant.

Present study noted 40.36 % ESBL, 26.78% MBL rates among biofilm producers but Agarwal et al. reported 12.66% and 8.44%, they also noted highest

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among *Klebsiella pneumoniae* of 19.26% which is in contrast to present study where 56.66% of *Escherichia coli* were biofilm producers. This could be because of different geographical area and population types. [12] High rates of biofilm-producing K.*pneumoniae* have been reported in MDR strains, mainly Extended Spectrum Betalactamases producers harbouring blaCTX-M genes.[11]

No resistance was observed against colistin in the present study, which is similar to many other studies. It's the last viable option for MDR strains whether being biofilm non producers or producers of ESBL, MBL and biofilm.[14]

However, a study highlights variability in biofilm formation among Carbapenem Resistant clinical isolates and underscores the differences between the bacteria found as carriage versus infection. They also concluded that bacterial species and environmental factors variably influence biofilm formation.[16]

A higher proportion of antibiotic resistance in biofilm producers in comparison to non-producers has also been documented in many studies. The multicellular bacterial biofilm is a key player for the development of antibiotic resistance. These biofilms' multi-layered resistance against antibiotics is made possible by their sluggish growth rate, decreased penetration, and persisted cell presence. For many years conventional antibiotic resistance mechanisms among bacteria are because of excessive overuse, abuse, and disproportionate underuse of antibiotics. There has been a significant shift in the field of antibiotics since the discovery.[17]

The acquisition of specific antimicrobial resistance among these Gram negative bacteria can compromise or enhance biofilm formation in several species.

The notable occurrence of biofilm among MDR organisms provides a glimpse of upcoming threat. Biofilm-related infections are subject to develop recurrences and MDR may increases mortality. Biofilm production may play a crucial role in the infection severity and persistence. [16] Detection of biofilm is crucial for the prevention of treatment failure.

Conclusion:

Antimicrobial resistance is an issue of global concern, and biofilm producing isolates show higher antimicrobial resistance as compared to biofilm non producers, as is evident in the present study. Early detection about the biofilm formation by the infecting pathogen, by routine incorporation of detection methods in the laboratories, will be helpful in assisting the clinicians in initiation of the appropriate empirical antimicrobial for the treatment.[4]

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