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Biofilm Formation and its Association with Gram Negative Sepsis Pathogenicity

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Biofilm formation is an important virulence factor that protects an organism from antimicrobial agents as well as host immune effectors, thus allowing organisms to invade, survive, and cause persistent-reoccurring infection in host cells. The aim of this study was to investigate the ability of sepsis-causing gram-negative bacteria to form biofilms, evaluate the association between antibiotic resistance pattern and biofilm formation, determine the role and influence of biofilm formation on pathogenicity and clinical outcome of sepsis. A prospective study conducted from October 2020 to August 2021, non-replicated gram-negative bacteria isolates were recovered from blood samples of patients with suspected bacteremia, sepsis, and sepsis shock and identified using biochemical procedures. Antimicrobial susceptibility patterns of GNB isolates were determined using the Kirby-Bauer disc diffusion method and interpreted using CLSI guidelines. The ability of GNB isolates to form biofilm was assessed using Congo red agar and the tissue culture plate method. Of the 160 Gram-negative bacteria tested, biofilm formation was seen in 73 (45.63%) isolates. Isolates are Klebsiella pneumoniae (39.73%), Acinetobacter spp. (34.25%), Escherichia coli (23.29%), Pseudomonas aeruginosa (1.37%), and other non-fermenters (1.37%). Isolates were highly resistant to cephalosporins, fluoroquinolones, and the penicillin group of antibiotics. No statistical relationship was found between resistance pattern, clinical outcome, and biofilm formation. In the current study, we found that 45.63% of gram-negative bacteria causing sepsis were biofilm producers. Klebsiella pneumonia isolates exhibited the highest levels of biofilm formation and antimicrobial resistance. Based on the strength of biofilm formation, most isolates were weak biofilm producers, and there was no statistical correlation between the formation of biofilms and antimicrobial resistance, indicating that the formation of biofilms was not a determining factor for resistance.

Keywords: Antibacterial agent; Bacteria; Biofilm; Persistence infections; Virulence.

Biofilms are microbial-derived surface-associated cells that are enclosed in an extracellular polymeric substance matrix (EPS) and attached to a substratum or to each other in an irreversible way.[1,2] They are important virulence factors that are produced through a multi-step process that begins with a single species of bacteria with fimbriae, pilli, or flagella attaching to conditioning film and progresses to micro-colonies after longer exposures, eventually forming a three-dimensional structure that detaches after maturation. [2,3]

Biofilm serves as a protected mode of growth and an efficient barrier in hostile environments, allowing cells to survive while also dispersing to colonise new niches.[4,5] Research shows that 80% of chronic persistent bacterial infections

are linked to biofilms, which are usually formed at the primary focus of infection, such as meningitis, UTI, cystic fibrosis, or infective endocarditis, and then disseminated into the bloodstream via the penetration of injured tissues.[3,6,7] The presence of an organism in the bloodstream, particularly planktonic bacteria, triggers an immune response that typically destroys pathogens. However, a dysregulated response by host immune cells to infection results in sepsis, which further leads to organ dysfunction, septic shock, or death if left untreated.[8]

Biofilm formation is a serious clinical issue that promotes antimicrobial resistance by slowing antimicrobial diffusion and facilitating plasmid exchange, which requires aggressive treatment.[9,10] Several studies show that biofilm formation is associated with infection severity, persistence, and relapse. [2,10,11,12,13.] However, only a few studies looked at the biofilm-forming ability of gram-negative bacteria that cause sepsis and its correlation to antimicrobial resistance and sepsis outcome. Studies on biofilm formation in sepsis-causing gram-negative bacteria are very important as they will play a key role in understanding the virulence of GNB causing sepsis, provide information on the role of biofilm in resistance, as well as give a deeper insight into treatment strategies, which might help reduce the mortality and morbidity rate associated with sepsis. The aim of this study was to investigate the ability of sepsis-causing gram-negative bacteria to form biofilms, evaluate the association between antibiotic resistance pattern and biofilm formation, determine the role and influence of biofilm formation on pathogenicity and clinical outcome of sepsis.

METHODOLOGY

Isolates collection

This was a prospective study conducted at SRM Medical College and Research Centre's Department of Medical Microbiology (October 2020–August 2021). 160 non-repetitive GNB were recovered from blood samples submitted by various outpatient and inpatient wards to the laboratory. All blood positive cultures identified as gram-negative bacteria between the study periods were included, while blood positive cultures identified as contaminants or gram-positive were excluded.

Colony characterization and bacterial identification

According to bacteriological guidelines, the specimens were cultured on blood, MacConkey, and chocolate agar, and incubated at 37°C for 24 hours. Following incubation, colony morphology was evaluated based on size, mucoid nature, pigment, odour, and lactose fermentation. Biochemical identification was done using unique tests such as Oxidase, Indole, Motility, Triple Sugar Iron, Citrate, Urease, Methyl Red Voges-proskauer, and Amino acid.

Susceptibility testing

Antimicrobial susceptibility testing was done using the Kirby Bauer disc diffusion technique and interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines.[14]

Detection of Biofilms

Biofilm detection was performed using Congo red agar and the tissue culture plate method as previously described by Hassan et al. and Dhanalakshmi et al. [1,10]

Congo Red Agar (CRA) Method

Congo red medium was prepared using brain heart infusion broth at 37gm/l, agar 10gm/l, sucrose 50gm/l, and Congo red stain 0.8gm/l. First, an aqueous concentrated solution of Congo red stain was prepared, autoclaved, and added to the other medium constituents at 55°C. Isolates were then cultured on CRA plates and incubated for 24 hours at 37°C. CRA was repeated in triplicates and the results were interpreted based on whether black colonies with a dry crystalline consistency were produced or red colonies. (Fig 1).[1,10]

Tissue culture plate method (TCP)

In brief, 200 µl of bacterial suspension was aliquoted into 96-well flat-bottom tissue culture plates and incubated for 24 hours at 37°C. Following incubation, wells were rinsed with phosphate buffer saline (pH 7.2). Adhering bacteria were fixed with 2% sodium acetate and stained with 0.1% crystal violet. An ELISA auto reader (Model 680) was used to quantify stained adhering biofilm at an optical density of 450 nm, and the results were interpreted according to Stepnovic *et al*. criteria. Each experiment was carried out in triplicate, with an uninoculated medium serving as a control. [1,10,15.]

Statistical Evaluation

Statistical calculations were performed using SPSS (IBM SPSS V23). The relationship

between resistance pattern and biofilm formation was assessed using Pearson correlation. The chi-square table was applied to compare variables (Table I). P value <0.05.

Ethics

The study was approved by our institution's ethical committee (2196/IEC/2020). Patient consent was not required for the study because isolates were collected directly from the laboratory.

RESULTS

Out of the one hundred and sixty (160) non-repetitive gram-negative bacteria isolates studied for biofilm formation, 73 were identified as biofilm producers via TCP method and 90 as biofilm producers via CRA method (Table II). A true biofilm producing organism is considered an organism that shows biofilm formation in both methods. The most common biofilm producing organism was *Klebsiella pneumoniae* 29 (39.73%), followed by *Escherichia coli* 17 (23.3%). Table II shows the percentages of biofilm and non-biofilm production identified by CRA and TCP.

Table III shows the diagnostic efficacy of Congo red agar method. Specificity, sensitivity, PPV, NPV, and accuracy as compared to tissue culture plate method, which is the standard method used for this study.

Table 1. Chi square table. P valve = 0.057

	Negative	Positive	Total
TCP	87	73	160
CRA	70	90	160
Total	157	163	320

Table 1 compares data obtained via the Congo red agar (CRA) method with the Tissue Culture Plate (TCP) method. P<0.05

DISCUSSION

The formation of biofilm is an efficient defence barrier used by microbes to invade hostile environments.[5] It is associated with antimicrobial resistance, persistence, and severity of chronic infections and is a major cause of sepsis relapse.[8,13] Sepsis is a serious health threat with over 30 million causes recorded annually. Bacterial sepsis has been identified as a major cause of mortality and morbidity, even though its pathophysiology is not yet fully understood. In this study, the ability

Table 2. Biofilm detection by two different phenotypic (CRA & TCP) methods

Methods	Biofilm producers No (%)				Non-biofilm producers No (%)	Total No (%)
Congo red agar method (CRA)	Strong 12(13.33) n=90	Moderate 6(6.67) n=90	Weak 72(80.00) n=90	Total 90(56.25) n=160	70(43.75) n=160	160(100)
Tissue culture plate (TCP)	0(0.00) n=73	8(10.96) n=73	65(89.04) n=73	73(45.63) n=160	87(54.38) n=160	160(100)

Table 2 shows the percentages of biofilm producers and non-biofilm producers detected using the Congo Red method and the Tissue Culture method.

Table 3. Diagnostic efficacy of Congo red agar method

Biofilm detection	Specificity (%)	Sensitivity (%)	*PPV (%)	**NPV (%)	Accuracy (%)
Congo red method (CRA)	92	76	92	76	83

*PPV- Positive predictive valve. ** NPV- Negative predictive valve.

Table III shows the diagnostic efficacy of the Congo red agar method. Specificity, sensitivity, PPV, NPV, and accuracy as compared to the tissue culture plate method, which is the standard method used for this study.

Table 4. shows the bacteriological profile and percentage of biofilm producing isolates

Blood Isolates	Biofilms producers Number (%)
Klebsiella pneumonia	29(39.73)
Escherichia coli	17(23.29)
Acinetobacter spp	25(34.25)
Pseudomonas aeroginosa	1(1.37)
Other -Non fermenting GNB	1(1.37)
Salmonella typhi	0(0.00)
Total	73(100)

Table 4 shows the distribution and percentages of gram-negative bacteria isolates producing biofilm in our study. *Klebsiella pneumonia* was found to be the highest biofilm producer, followed by *Escherichia coli. Salmonella typhi* was found to be a non-biofilm producer.

of gram-negative sepsis-causing bacteria recovered from the bloodstream to form biofilm was studied and correlated with the strength of biofilm formation, resistance pattern, and its influence on pathogenicity and clinical outcome.

Our finding indicates 73 (45.63%) of GNB organisms causing sepsis formed biofilm. This is nearly similar to Cepas *et al.*[17] which reported 49.3% of biofilm formation in isolates and is contrary to studies by Swarna *et al.* [18] and Zubair *et al*.[19] which receptively reported 91% and 80% of biofilm formation in their studies. The highest biofilm producing organism was *Klebsiella pneumonia*. Similar to our results, *Klebsiella pneumonia* was found to be the most common biofilm producing organism by Cepas *et al*.[17], Karmi *et al.*[20] while De *et al*.[21] and Dumaru *et al*.[13]

Table 5. Showing the resistance pattern of gram-negative bacteria isolates

Antibiotics	Resistance in percentage (%) *Escherichia coli*	*Klebsiella pneumonia*	*Acinetobacter* spp	*Pseudomonas aeruginosa*	*Citrobacter*	*Proteus*
Aminoglycosides						
Amikacin	4.00	37.50	42.22	8.33	75.00	50.00
Gentamicin	38.00	41.67	53.33	8.33	-	50.00
Cephalosporins						
		Cefazolin	71.00	79.17	25.00	50.00
Cefepime	58.00	66.67	26.67	9.09	25.00	50.00
Cefotaxime	67.00	70.83	37.78	-	33.33	
Ceftriaxone	67.00	66.67	35.56	-	25.00	50.00
Ceftazidime	60.00	68.75	35.56	8.33	25.00	50.00
Cefoxitin	20.00	64.58	-	-	25.00	
Cefuroxime	69.00	72.92	-	-	25.00	50.00
Carbapenem						
Imipenem	4.00	45.83	42.22	-	-	-
Meropenem	4.00	47.92	33.33	8.33	-	-
Ertapenem	5.00	47.92	-	-	-	-
Fluroquinolones						
Ciprofloxacin	73.00	68.75	28.89	-	-	50.00
	Levofloxacin		-	-	-	9.09
-	-					
Pencillins						
Ampicillin	91.00	100	-	-	-	-
B lactam combination						
Amoxacillin- clavulanate	69.00	85.42	81.82	81.82	25.55	25.00
Piperacillin tazobactam	11.00	50.00	31.11	0.00	0.00	0.00
Ceftazidime clavanic acid	11.00	52.08		0.00	0.00	0.00
Tetracyclines						
Tetracycline	58.00	36.17	35.56	0.00	0.00	100.00

NA-Non applicable

Table 5 shows the resistance pattern of gram-negative bacteria isolates causing sepsis.

identified *Escherichia coli* as the most common Gram-negative biofilm producing organism.

For biofilm detection, two (2) phenotypic methods were used; they are Congo red agar (CRA) and the Tissue Culture Plate method (TCP). Although there are many other biofilm detection methods, there is no standard procedure for the detection of biofilm formation. TCP was used as the gold standard for this study.[10] Sensitivity and specificity of 75% and 92% were observed in the Congo red method. The percentage observed in sensitivity is similar to studies by Mathur *et al.* [22] (90.02%), Bose *et al*. [23] (96.23), and Chandana *et al*.[2] (86.2%), while the sensitivity observed was lower in their study. The specificity percentage observed in this study is consistent with findings by Dhanalaskshmi *et al*.[10] and Tayal *et al.*[24] which had 80% and 94.59%, respectively.

On account of antimicrobial resistance, our findings revealed maximum percentage resistance to penicillin, cephalosporins, fluoroquinolones**,** and B-lactam combination agents (Table 5). *Klebsiella pneumoniae* was 100% resistant to Ampicillin, 83.64% to Amoxicillin-clavulanate, 80% to Cefazolin, 70.91% to Cefuroxime, and 63.64% to Ciprofloxacin. These findings are in line with those of Karimi *et al.*[20] and Chandana *et al*.[2], who found that *Klebsiella pneumonia* isolates had the highest resistance to cefotaxime, ampicillin, and ciprofloxacin in their studies. No significant statistical relationship was found between the resistance pattern and the strength of biofilm formation (Table VI), although it is important to note that in this study, no strong biofilm formation was observed by the TCP method, and the majority of strains were weak biofilm-formers.

Table 6. Relationship between Biofilm formation and Resistance pattern P(<0.05)

Antibiotics	*Escherichia coli*	*Klebsiella pneumoniae*	*Acintobacter* spp	*Psedomonas aeroginosa*
Aminoglycosides				
Amikacin	0.886	0.834	0.962	0.753
Gentamicin	0.292	0.806	0.961	0.753
Cephalosporins				
Cefazolin	0.436	0.321	0.685	
Cefepime	0.529	0.822	0.418	0.753
Cefotaxime	0.642	0.442	0.390	-
Ceftriaxone	0.762	0.822	0.488	-
Ceftazidime	0.377	0.822	0.461	-
Cefoxitin	0.572	0.179	0.642	-
Cefuroxime	0.642	0.265	0.239	-
Carbapenem				
Imipenem	0.886	0.533	0.853	-
Meropenem	0.886	0.156	0.524	0.753
Ertapenem	0.886	0.533	0.560	-
Fluroquinolones				
Ciprofloxacin	0.790	0.293	0.423	-
Levofloxacin	-	-	-	0.753
Pencillins				
Ampicillin	0.277	-	0.520	-
B lactam combination				
Amoxacillin- clavulanate	0.528	0.231	0.991	0.546
Piperacillin tazobactam	0.445	0.657	0.418	-
Ceftazidime clavanic acid	0.917	0.744	0.303	0.7563
Tetracyclines				
Tetracycline	0.622	0.661	0.580	-

Table 6 shows the statistical relationship between antibiotic resistance and biofilm formation. The P value is <0.05

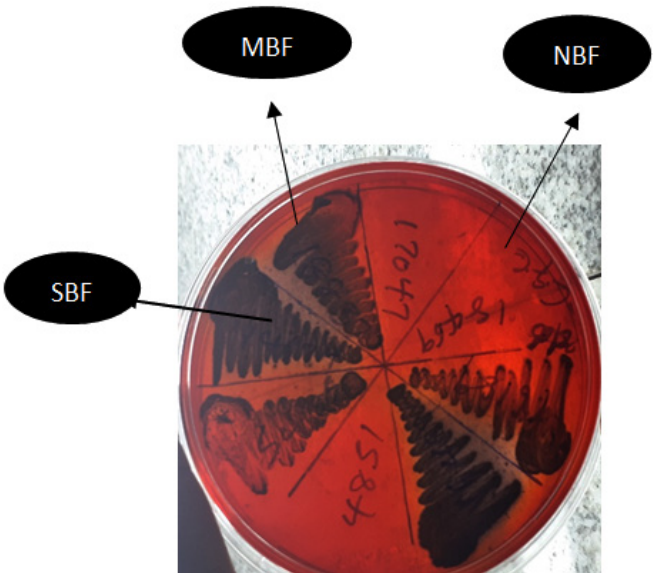

Fig. 1. Congo red method

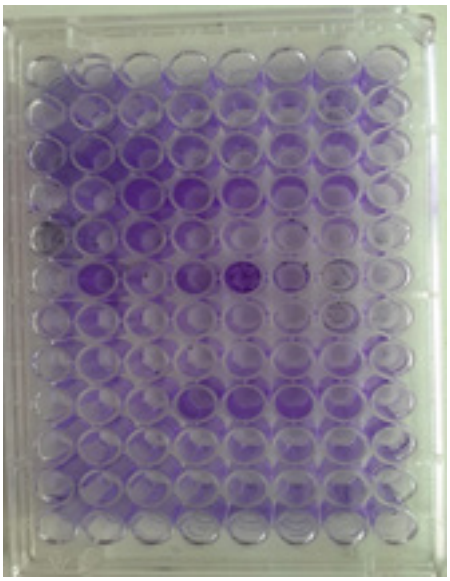

Fig. 2. Tissue culture plate method

In most articles where a strong correlation was found between biofilm formation, pathogenicity, and resistance patterns, the biofilm formed was either strong or moderate. suggesting that biofilm formation strength may play an essential role in resistance. [16,20,25,26.] Devanga Rugupathi *et al.*[25] discovered a stronger correlation between strong biofilm formation and carbapenem resistance than between moderate and weak biofilm formation. This might imply that the susceptibility pattern of an organism is dependent on the strength of the biofilm formed by that organism. Several scientific studies have hypothesised that the formation of biofilm prevents the efficient diffusion of antibiotics, resulting in a significant decrease in bacteria's exposure to antimicrobial agents and antibiotic activity.

CONCLUSION

In this study, resistance, pathogenicity, and clinical outcome of patients were found to be independently associated with weak biofilm formation. *Klebsiella pneumoniae* was the most resistant organism and had the highest biofilm production. No statistical relationship was found between biofilm formation and antibiotic resistance, and this could be because most isolates were weak biofilm producers. Further molecular investigation into biofilm associated genes and their role in sepsis severity is very important as findings will help in accurate treatment development, thus reducing mortality and morbidity associated with sepsis-associated Gram-negative bacteria.

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Conflict of interest

The author(s) declare that there is no conflict of interest.

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