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RESEARCH ARTICLE

Biofilm Production in Clinical Isolates and Their Antimicrobial Susceptibility Pattern in the Intensive Care Unit of a Tertiary Care Hospital in Rajasthan

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ABSTRACT

Aims and objectives: This study was carried out to know about the production of biofilm by the microorganisms in various clinical isolates and to compare the antimicrobial sensitivity pattern of biofilm- and nonbiofilm-producing organisms.

Materials and methods: One hundred and fifty samples collected from intensive care units for a period of 1 year were taken for the study. Samples included blood, urine, sputum, endotracheal tips, suction tips, pus/swabs, stents/valves, body fluids, etc. Samples were processed and identification of microorganisms and antibiotic sensitivity was tested by methods according to Clinical and Laboratory Standards Institute guidelines.

Biofilm production identification was done by tissue culture plate (TCP) method, tube method (TM), and Congo red agar (CRA) plate method.

Results: Out of 150 samples, 108 (72%) samples showed growth of Gram-negative bacilli, 16 (11%) samples showed growth of Gram-positive cocci, and Candida species were seen in remaining 26 (17%) samples. Among the total organisms isolated, 124 organisms (82.66%) showed production of biofilm, while 26 organisms (17.33%) did not produce biofilm. Antibiotic resistance was seen more in biofilm-producing organisms as compared with nonbiofilm-producing organisms.

Conclusion: Most of the biofilm-related infections are characterized particularly by high resistance to antibiotics and persistent infections, in turn leading to a very high morbidity and mortality. Therefore, detection of biofilm production is of high relevance to the clinician for appropriate approach to the treatment.

Keywords: Antibiotic susceptibility, Biofilms, Device-associated infections, Quorum sensing, Resistance.

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INTRODUCTION

Biofilm is a complex aggregate of microorganisms wherein cells adhere to each other (microcolony). These adherent cells are embedded in a self-produced matrix that consists of extracellular polymeric substances/slime, which are made up of polysaccharides and proteins.

Biofilm-associated microorganisms behave differently from planktonic (freely suspended) organisms with respect to growth rate and ability to resist antimicrobial treatments and therefore, pose a major health problem.[1] Chronic infections caused due to biofilm remain a major challenge to treat and are of great economic relevance because traditional antibiotic therapies are not sufficient to eradicate such infections.

Certain extracellular proteins, surface proteins, capsular polysaccharides, adhesins (PS/A), and autolysin (encoded by at/E gene) regulate biofilm production. [1]

Gram-positive and Gram-negative bacteria as well as yeasts can be associated with biofilm formation. Bacteria commonly involved include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. Candida species are also involved in biofilm formation.[2,3]

Biofilms have been found to be involved in a variety of microbial infections in the body. Infections associated with biofilm include common problems, such as urinary tract infections (UTIs), catheter infections, middle ear infections, dental plague, gingivitis, coating contact lenses, and some lethal processes, such as endocarditis infections in cystic fibrosis and infection of indwelling medical devices, such as central venous catheters, needleless connectors, endotracheal tubes, intrauterine devices, pacemakers, heart valves, peritoneal dialysis catheters, prosthetic joints, urinary catheters, etc.[4]

Pathogenic Mechanisms

Different pathogenic mechanisms of the biofilms have been proposed. These include[5]:

- Biofilm allow attachment to the various surfaces
- Host defenses, such as phagocytosis are evaded
- Obtaining a high density of microorganisms
- Gene exchange takes place resulting in more virulent strains of microorganisms
- Production of a large concentration of toxins
- Protection from antimicrobial agents
- Microbial aggregates get detached and are transmitted to other sites.

Mechanisms of the antimicrobial resistance of biofilms are:

Trapping of antibiotics: The slime causes a diffusion barrier by restricting the transport of antibiotic to the interior of the biofilm, or by chemically reacting with the molecules themselves. The concentration of the antibiotics gets diluted before they reach to the individual bacterial cells in the biofilm, thus making the antibiotics less effective in the treatment.[4,6]

Biofilm-producing bacteria escape the host immune system: Biofilm producers (BPs) escape the damaging effect of the antibodies produced by the host immune system.[7]

Quorum sensing and genotyping adaptations decrease the growth rate of bacteria: A cell-to-cell communication in bacterial biofilms is established through chemical signaling. Small, diffusible molecules of class of N-acylated homoserine lactones (AHLs) are liberated from the biofilm-producing bacteria into their surroundings. The amount of AHLs reaches a threshold level and induces the transcription of specific genes throughout the bacterial population. This regulation is known as quorum sensing.

We plan to observe the prevalence of biofilm-producing organism isolated from our ICU and also compare the antimicrobial susceptibility of biofilm-producing and nonbiofilm-producing bacteria.

MATERIALS AND METHODS

The study was carried out in the Department of Microbiology, Mahatma Gandhi Medical College & Hospital, Jaipur, Rajasthan, India from January 2013 to January 2014. The test group selected was the patients from the ICU of the hospital. One hundred and fifty samples were taken irrespective of all ages, sex, occupation, religion, and ethnicity.

Sample Collection

One hundred and fifty samples were collected from the ICUs for a period of 1 year. Variable samples were collected, which included blood, urine, sputum, endotracheal tips/secretions, suction tips, pus/swabs, stents/valves, body fluids, etc. All the samples were collected with due aseptic precautions and transported to laboratory as soon as possible under optimum transport conditions.

Laboratory Diagnosis

Direct microscopy

Sample Culture

Primary inoculation was done on blood agar and MacConkey agar which was incubated for 18 to 24 hours at 37°C. Samples were identified by standard techniques based on colony morphology, gram staining, and biochemical tests. Antimicrobial susceptibility test using modified Kirby Bauer disk diffusion method was done as per Clinical and Laboratory Standards Institute guidelines. Antibiotics discs used are mentioned in Table 1.

Methods for the Detection of Biofilm

Biofilm formation was assessed by the following methods. Following disks were placed.

Tissue Culture Plate Method

Optical density (OD) of stained adherent biofilm was obtained by using micro-enzyme-linked immunosorbent assay autoreader at a wavelength of 570 nm. Classification was done as shown in Table 2.

Table 1: Drugs used for antibiotic susceptibility testing

Gram-positive cocci	Gram-negative bacilli
AMP: Ampicillin (30 µg)	AMP: Ampicillin (30 µg)
AMC: Amoxyclav (30 µg)	AMC: Amoxyclav (30 µg)
COT: Cotrimoxazole (25 µg)	DO: Doxycycline (30 µg)
CD: Clindamycin (2 µg)	AK: Amikacin (30 µg)
GEN: Gentamycin (30 µg)	COT: Cotrimoxazole (25 µg)
CX: Cefoxitin (30 µg)	LE: Levofloxacin (5 µg)
E: Erythromycin (15 µg)	CTX: Cefotaxime (30 µg)
	CFM: Cefixime (5 µg)
LZ: Linezolid (30 µg)	CPM: Cefepime (30 µg)
VA: Vancomycin (30 µg)	PI: Piperacillin (100 µg)
LE: Levofloxacin (5 µg)	PIT: Piperacillin + Tazobactam (100/10 µg)
	IPM: Imipenem (10 µg)
	TGC: Tigecycline(15 µg)
	#TOB: Tobramycin (10 µg)
	CL: Colistin (10 µg)
	PB: Polymyxin B (300 units)
	#TI: Ticarcillin (75 µg)
	#TCC: Ticarcillin + Clavulanic acid (75/10 µg)
	*NIT: Nitrofurantoin (300 µg)
	*FO: Fosfomycin (200 µg)

*For urine samples only; #For *Pseudomonas* isolates only

Table 2: Classification of bacterial adherence by TCP method

Mean OD values	*Adherence*	*Biofilm formation*
<0.120	None	None/weak
0.120–0.240	Moderate	Moderate
≥0.240	Strong	High

Tube Method

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Tubes were examined and the amount of biofilm formation was scored as 1—absent/weak, 2—moderate, or 3—strong.

Congo Red Agar Method

Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result.

Statistical Analysis

Statistical analysis was applied using Chi-square test to compare the antimicrobial susceptibility pattern of biofilm-forming and nonbiofilm-forming bacterial isolates. Chi-square test is applied when two or more set of variables are compared. The critical value of p indicating the probability of significant difference has been taken as $p < 0.05$.

RESULTS

A total of 150 samples were taken. Out of that, 108 samples showed growth of Gram-negative bacilli, 16 samples showed growth of Gram-positive cocci, and 26 samples had growth of Candida species. Out of 150 organisms isolated, 124 organisms were BP and 26 were nonbiofilm producer (NBP) (Table 3).

The various samples received were blood (14 samples), double J (DJ) stent (6), fluid (8), pus (22), urine (38), and respiratory secretions like endothelin, sputum (60). The maximum growth of Gram-negative bacilli was seen in respiratory secretions and least in DJ stent. While Gram-positive cocci were maximum isolated in pus samples, Candida were mostly isolated from urine samples.

The biofilm production was identified mainly by TCP method (82.66%) than by TM (36%), and by CRA method (9.33%) (Table 4).

Organism-wise Distribution of BP and NBP

In Gram-negative isolates, maximum number of isolates were *E. coli* (28), out of which 22 (78.5%) showed biofilm production, rest 6 (21.4%) were NBP. In *Pseudomonas* isolates (22), 18 (82%) were BP, rest 4 (18%) were NBP. Both *Acinetobacter* and *Citrobacter* isolates (20) showed 90% BP and 10% NBP (Table 5).

Gram-positive cocci and Candida species biofilm production in coagulase-negative *Staphylococcus* was 100%. Out of 12 isolates of coagulase-positive *Staphylococcus*, 7 (58.33%) were BPs, rest 5 (41.66%) were NBPs. Candida species showed 26 isolates out of which 21 (80.7%) were BPs and remaining 5 (19.2%) were NBPs (Table 6).

Comparison of Antimicrobial Resistance of Biofilm-forming and Nonbiofilm-forming Bacteria

Table 3: Distribution of total isolates obtained from the ICU

Isolates	*No. of isolates*	*Percentage*
Gram-negative bacilli	108	72
Gram-positive cocci	16	11
Candida species	26	17
Total samples	150	

Table 4: Percentage distribution of biofilm formation by different detection methods

	BP: Biofilm detected (%)	*NBP: No biofilms detected (%)*
CRA	9.33	90.66
TM	36	64
TCP	82.66	17.33

Table 5: Organism-wise distribution of BP and NBP in Gram-negative bacilli isolates (n = 108)

		BP	*NBP*
E. coli	28	22 (78.5%)	6 (21.4%)
Pseudomonas	22	18 (82%)	4 (18%)
Acinetobacter	20	18 (90%)	2 (10%)
Citrobacter	20	18 (90%)	2 (10%)
Enterobacter	8	8 (100%)	0 (0%)
Klebsiella	8	6 (75%))	2 (25%)
Proteus	2	2 (100%)	0 (0%)

Table 6: Organism-wise distribution of BP and NBP in Gram-positive organisms and Candida species (n = 42)

		BP	*NBP*
Coagulase-negative *Staphylococcus*	4	4 (100%)	0 (0%)
Coagulase-positive *Staphylococcus*	12	7 (58.33%)	5 (41.66%)
Candida species	26	21 (80.7%)	5 (19.2%)

DISCUSSION

Biofilm has long been considered as a virulence factor contributing to infections associated with various medical devices causing nosocomial infections. Different mechanisms by which biofilm-producing organisms cause disease are the following:

- Detachment of the cells from medical device biofilm causing bloodstream infections or UTIs
- Endotoxin formation
- Resistance to host immune system
- Generation of resistance through plasmid exchange[4]

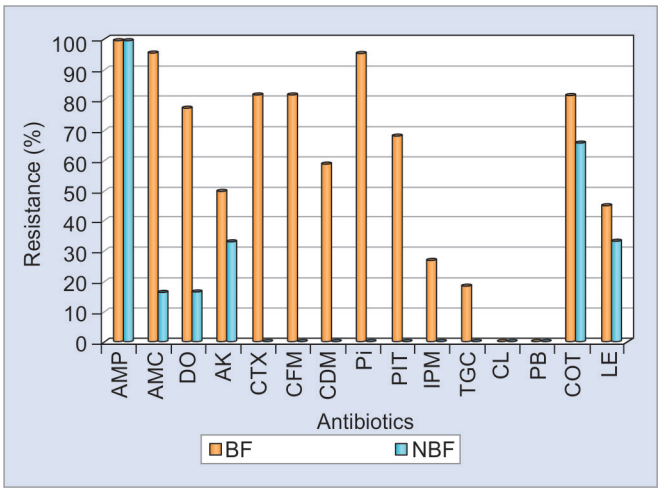
Comparison of Antimicrobial Resistance Pattern of Biofilm-forming and Nonbiofilm-forming Bacteria

Biofilm-forming bacteria generally show a greater resistance to antibiotics than nonbiofilm-forming bacteria because of the difficulty in penetration of drugs through the biofilm. In the current study, various organisms from variable samples in the intensive care setup were isolated. Results are depicted in Graphs 1 to 5. The BPs were more resistant to antibiotics as compared with the nonbiofilm-producing organisms.

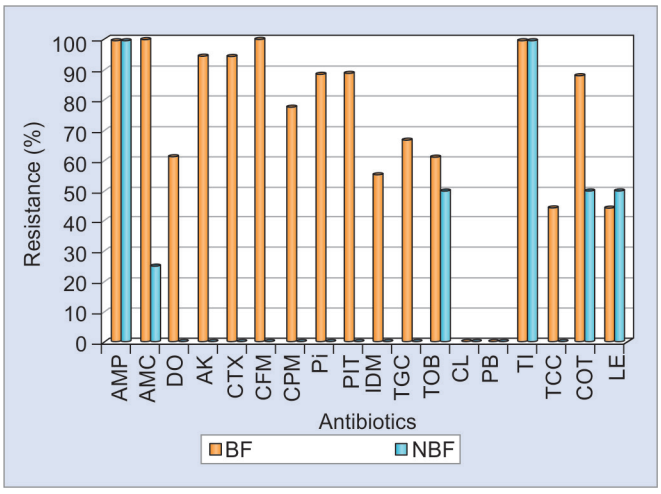
Many studies have been undertaken which reported high resistance among different biofilm-producing organisms. Most of the study results were similar to the present study but some differences in sensitivity to antibiotics were seen. Different authors have performed studies on different clinical samples and antibiotics susceptibility pattern vary with the geographical area and the hospital environment.

SUMMARY

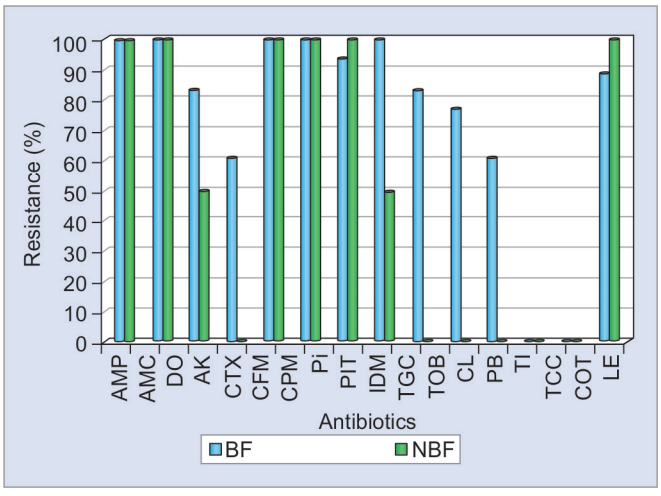
The study entitled "Biofilm Production in Clinical Isolates and their Antimicrobial Susceptibility Pattern in Critical Care Units" was conducted in the Department of Microbiology, Mahatma Gandhi Medical College & Hospital,



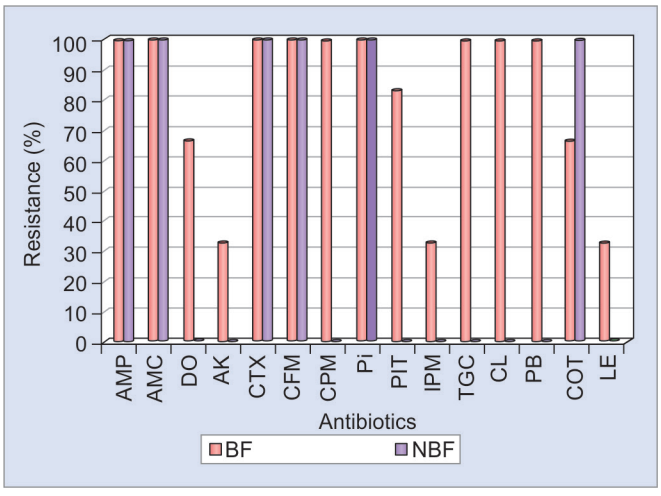
Graph 1: Antimicrobial resistance percentage of biofilm-producing and nonbiofilm-producing *E. coli* isolates



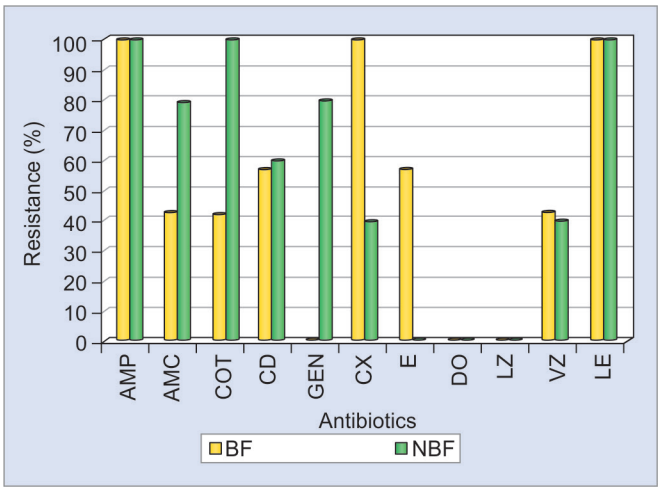
Graph 2: Antimicrobial resistance percentage of biofilm-forming and nonbiofilm-forming *Pseudomonas* isolates



Graph 3: Antimicrobial resistance percentage of biofilm-forming and nonbiofilm-forming *Acinetobacter* isolates



Graph 4: Antimicrobial resistance percentage of biofilm-forming and nonbiofilm-forming *Klebsiella* isolates



Graph 5: Antimicrobial resistance percentage of biofilm- and nonbiofilm-forming *S. aureus* isolation

Jaipur, India with the main objective of detecting biofilm formation in clinical isolates by different methods and comparing their results along with determination of antimicrobial susceptibility pattern in both biofilm-producing and nonbiofilm-producing isolates.

- Out of the 150 isolates obtained, 72% were Gram-negative bacilli, 11% Gram-positive cocci, and 17% Candida species.
- Out of 150 isolate samples, 14 from blood, 60 from respiratory samples (endotracheal secretions/suction secretions/sputum), 38 from urinary samples, 22 from pus/swabs, 8 from body fluids, and 8 from DJ stents were taken.
- Biofilm formation was detected using three different methods:
  - Congo red agar method
  - Tube method
  - Tissue culture plate method
- Out of 150 isolates, biofilms formation was seen by TCP method in 82.66% isolates. Biofilm production by TM was found in 36% isolates. Biofilm production by CRA method was found in 9.33% isolates.
- On comparing the results by three different methods, TCP method was found to be more reliable than TM and CRA.
- The TCP method also detected different grades of biofilm production by the isolates (150), which were strong/high (79), moderate (49), and weak/none (26) biofilm production.
- The isolates which graded strong/high and moderate by TCP method were considered as biofilm-producing isolates for the study.
- Biofilm-producing isolates showed a high resistance to antibiotics as compared with nonbiofilm-producing isolates.
- The biofilm-producing Gram-negative isolates showed high resistance to ampicillin, amoxiclav, doxycycline, amikacin, cefepime, piperacillin, piperacillin + tazobactam, imipenem, cotrimoxazole, levofloxacin. Lesser resistance was shown by tigecycline. Colistin and polymyxin B were 100% effective drugs against both biofilm and nonbiofilm-producing isolates.

The biofilm-producing Staphylococci isolates showed a high resistance to ampicillin amoxiclav, cotrimoxazole, clindamycin, gentamicin, erythromycin, doxycycline, and levofloxacin; cefoxitin showed no resistance in the study. Vancomycin and linezolid were 100% sensitive.

CONCLUSION

Bacteria that adhere to the surface of indwelling medical devices or damaged tissue can become the cause of persistent infections. With the increasing use of catheters, artificial implants and antimicrobials especially in immunocompromised patients admitted in the ICU or critical care units are a major cause of concern over biofilm infections.

Most of the biofilm-related infections are characterized particularly by high resistance to antibiotics and formation of persistent foci that may complicate therapy and lead to chronic infections. Hence, biofilm detection holds a great relevance to the clinician for adopting appropriate approach to the treatment.

Reliable and sensitive methods for the detection of this pathogenicity factor in the clinically important organisms, suitable for use in routine microbiological laboratories, are needed for this purpose. Presently, a wide array of methods are available for the detection of biofilm, and each of these methods has limitations; hence, the best results can be achieved by combining different approaches.

Prophylaxis against Biofilm Formation

A local antibiotic prophylaxis should be given to inhibit the colonization of microorganisms on the devices and the contamination of the surrounding tissue.

Various forms of prophylaxis are:

- *Device coating*: Devices coated with antibiotics or the quorum sensing inhibitors.
- *Device immersion*: Dipping the device in the antimicrobial solution.
- Surgical site irrigation.
- *Antibiotic-loaded cements*: Antibiotic loaded bone cements (in joint arthroplasties) provide local delivery of antibiotics, stabilization of soft tissue, and better clinical outcome.

- *Antibiotic lock therapy*: Catheter lumen is filled with concentrated antibiotic solution and is then locked in place for an extended period when not in use.

There are many other experimental studies going on for prevention of biofilm formation. Biofilm formation is significant because of its association with chronic nature of the subsequent infections and with their inherent resistance to antibiotic chemotherapy.

Keeping in view the possibility of biofilm formation in patients admitted in the ICU who are immunocompromised having multiple indwelling medical devices, the antibiotic selection should be based on the relevant antibiogram.

We recommend the use of TCP method as a reliable method for biofilm detection. Colistin, polymyxin B, and tigecycline are the drugs recommended for all biofilm-forming Gram-negative organisms. Vancomycin and linezolid are the drug of choice to treat Staphylococcal biofilm production in the suspected patients.

In the end, it can be summarized that if effective control measures are devised to control the growth of biofilms, it can result in an enormous saving of finances, drugs, manpower, and finally life itself.

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