PREVALENCE OF BIOFILM PRODUCING AEROBIC BACTERIAL ISOLATES IN BURN WOUND INFECTIONS AT A TERTIARY CARE HOSPITAL IN NORTHERN INDIA

PRÉVALENCE DES BACTÉRIES AÉROBIES PRODUCTRICES DE BIOFILM DANS LES INFECTIONS DE BRÛLURE AU SEIN D'UN HÔPITAL DE RÉFÉRENCE DU NORD DE L'INDE

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SUMMARY. Burn wounds frequently get infected due to a break in skin integrity and prolonged hospitalization. Microbial flora originating from the patient's own flora colonize and infect the burn wounds. Bacterial biofilms in particular are postulated as the culprit for the development of non-healing burn wounds by inducing chronic inflammation in these patients. In the present study, 190 wound isolates obtained from patients admitted to the burn ward at the Pt. B.D. Sharma PGIMS, Rohtak, were evaluated for biofilm formation along with Antimicrobial Susceptibility Testing (AST). Biofilm detection was done by modified Tissue Culture Plate method and AST was done by Kirby-Bauer disc diffusion method. A total of 190 isolates were studied, which included *Staphylococcus aureus*, Coagulase negative *Staphylococcus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella* spp., *Proteus* spp., *Citrobacter* spp., *Escherichia coli* and *Enterobacter* spp. Of these, 68.9% isolates showed biofilm formation. Biofilm formation was more common in *Pseudomonas aeruginosa* followed by *Klebsiella spp.* and *Staphylococcus aureus*. Biofilm producing isolates showed greater multidrug resistance than non-biofilm producers. In our study, a high rate of biofilm formation and antimicrobial drug resistance was seen.

Keywords: biofilm, burn injury, wound infection, modified tissue culture method

RÉSUMÉ. *Les zones brûlées sont fréquemment infectées, en raison de la lésion cutanée et de la longue durée d'hospitalisation. La colonisation et l'infection des zones brûlées sont le fait de la flore du patient. Le biofilm bactérien est suspecté responsable de l'absence de cicatrisation des brûlures, en raison de l'inflammation chronique qu'il entraîne. Dans cette étude, nous avons évalué, conjointement à leur antibiogramme, la capacité de formation de biofilm de 190 bactéries isolées chez des patients brûlés hospitalisés dans le service idoine de l'hôpital du Président Sharma PGIMS de Rothak. Les antibiogrammes étaient réalisées par méthode de diffusion en gélose, la recherche de formation de biofilm par culture tissulaire modifiée. Parmi les 190 bactéries isolées, on trouvait Staphylococcus aureus, SCN, Pseudomonas æruginosa, Acinetobacter baumannii, Klebsiella spp., Proteus spp., Citrobacter spp., Escherichia coli et Enterobacter spp. La production de biofilm, observée sur 68,9% des souches était plus fréquente par Pseudomonas æruginosa, suivi de Klebsiella spp. et de Staphylococcus aureus. Les bactéries productrices de biofilm s'avéraient plus résistantes que les autres. Dans cette étude, la formation de biofilm et une antibiorésistance sont fréquemment observées.*

Mots-clés: *biofilm, brûlure, infection cutanée, culture tissulaire modifiée*

Introduction

Burn injury is one of the most common types of trauma that requires urgent medical attention.[1] Normal protective defense mechanisms of the skin are lost after a burn injury, resulting in rapid colonization of the wound surface. Initially, gram-positive organisms derived from skin commensals colonize the wound bed, followed later by gram-negative organisms and yeasts.[2] *Staphylococcus* species and *Pseudomonas aeruginosa* are two of the most frequently isolated microorganisms from burn wounds around the world.[3] Wound sepsis is considered the most frequent cause of mortality and morbidity in such patients.[4]

A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material.[5] Biofilm producing microbial flora manifests an altered growth rate and transcribes genes that provide them with inherent resistance to antimicrobials and the host immune system. This allows them to survive in a relatively harsh environment. Thus, biofilms are reported to be a major factor contributing to many chronic inflammatory diseases.[6] As burn wounds are chronic, non-healing in nature and difficult to treat, this study was planned to evaluate the role of biofilm producing aerobic bacteria in causing burn wound infection.

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Material and methods

A prospective study was conducted from March to August 2015 on 133 pus samples from burn patients received in the Department of Microbiology, Pt. B.D. Sharma PGIMS, Rohtak, Haryana, India. Gram stain was done, followed by inoculation of the sample on MacConkey agar and blood agar plates. The inoculated media were then incubated overnight at 37°C aerobically. Identification of the organisms was done by colony morphology, and gram staining and biochemical reactions as per standard microbiological protocol.[7-9] Antimicrobial Susceptibility Testing was done by Kirby-Bauer disc diffusion method in accordance with CLSI guidelines 2015.[10] The antimicrobial drugs tested for gram-positive bacteria included erythromycin (15µg), penicillin (10units), cefoxitin (30µg), cephalexin (30µg), trimethoprim-sulfamethoxazole (1.25/23.75µg), linezolid (30µg), doxycycline (30µg), clindamycin (2µg), vancomycin (30µg) and amoxicillin/clavulanic acid (20µg/10µg). For gram-negative bacteria they were gentamicin (10µg), amikacin (30µg), amoxicillin/clavulanic acid (20µg/10µg), piperacillin/tazobactam (100µg/10µg), cefepime (30µg), ciprofloxacin (5µg), imipenem (10µg), meropenem (10µg), trimethoprim/sulfamethoxazole (1.25µg/23.75µg), aztreonam (30µg), ceftazidime (30µg), netilimicin (30µg) and doxycycline (30µg). An isolate was considered as multi-drug resistant (MDR) if it was resistant to at least three classes of antimicrobial agents.[11]

Biofilm production was detected by Modified Tissue Culture Plate (MTCP) method.

Modified Tissue Culture Plate (MTCP) method[12]

Isolates from fresh agar plates were inoculated in brain heart infusion (BHI) broth supplemented with 2% sucrose dispensed in 3ml in test tubes and incubated overnight at 37°C aerobically. This cultured broth was diluted in the ratio of 1:100 with fresh medium. 200µl of this diluted cultured broth was then added to 96-well flat bottom, non-adherent, non-treated polystyrene tissue culture plates. These tissue culture plates were further incubated for 24 hours at 37°C. After incubation, the content of the wells was removed by gently tapping the plates and was washed four times with 0.2ml of phosphate buffered saline (PBS). Lastly, the wells were fixed with 2% sodium acetate for 30 minutes and stained with crystal violet (0.1% w/v) for 30 minutes. Excess stain was rinsed off with distilled water. After drying, the wells were then treated with 160µL of 33% glacial acetic acid for 15 min at room temperature to solubilize the dried crystal violet stain which was adherent to any biofilm. Optical densities (OD) were then determined by an automated micro ELISA reader at a wavelength of 570nm. These OD values were considered as an index of bacterial adhesion and biofilm formation. Biofilm formation was considered as weak/no biofilm formation if OD value was less than 2.63, moderate if OD value was between 2.66-5.32 and strong when OD value was greater than 5.32.

Results

A total of 190 bacterial isolates were obtained from 133 pus samples. Of these, 82 (61.6%) showed monomicrobial infection while multimicrobial infection was seen in only 51 (38.4%) samples. Rate of isolation of gram-negative bacteria was found to be 76% while gram-positive microorganisms were only 24%. *P. aeruginsa* (32.1%) was the most commonly isolated bacteria followed by *Klebsiella* spp. (16.3%) and *Staphylococcus aureus* (14.7%). The bacteriological profile has been depicted in *Table I*.

Table I - Aerobic bacteriological profile of burn wound isolates

Organisms	N	%
S. aureus	28	14.7
CONS	18	9.5
P. aeruginosa	61	32.1
Acinetobacter spp.	10	5.3
Klebsiella spp.	31	16.3
E. coli	7	3.7
Citrobacter spp.	14	7.4
Proteus spp.	16	8.4
Enterobacter spp.	5	2.6
Total	190	100

Table II - Antibiotic resistance pattern of gram-positive isolates

Antibiotics	*S. aureus* (n=28)		CONS (n=18)	
	N	%	N	%
Erythromycin	14	50	07	38.9
Penicillin	26	92.9	17	94.5
Cefoxitin	20	71.4	11	61.1
Co-trimoxazole	16	57.2	10	55.6
Clindamycin	10	35.7	05	27.8
Cephalexin	18	64.3	10	55.6
Amoxicillin- clavulanate	16	57.2	06	33.3
Doxycycline	15	53.6	05	27.8
Linezolid	03	10.7	02	11.1
Vancomycin	00	00	00	00

Table III - Antibiotic resistance pattern of gram-negative isolates (non-fermenters)

Antibiotics	*Pseudomonas aeruginosa* (n=61)		*Acinetobacter* spp. (n=10)	
	N	%	N	%
Ceftazidime	43	70.5	06	60
Gentamicin	36	59	06	60
Piperacillin-tazobactam	80	13.1	05	50
Amikacin	22	36.1	04	40
Aztreonam	23	37.7	05	50
Cefepime	24	39.4	NA	NA
Ciprofloxacin	30	49.2	08	80
Meropenem	70	11.5	04	40
Imipenem	20	03.3	02	20
Netilmicin	33	54.1	NA	NA
Doxycycline	NA	NA	07	70
Co-trimoxazole	NA	NA	08	80

The gram-positive bacterial isolates showed 93.5% resistance to penicillin, 67.4% to cefoxitin, 60.9% to cephalexin, 56.5% to co-trimoxazole, 47.8% to amoxicillin-clavulanate, 45.7% to erythromycin, 43.5% to doxycycline, 32.6% to clindamycin and 10.9% resistance to linezolid. No resistance was observed against vancomycin. This has been depicted in *Table II*.

P. aeruginosa isolates showed 70.5% resistance to ceftazidime, 59% to gentamicin, 54.1% to netilmicin, 49.2% to ciprofloxacin, 39.4% to cefepime, 37.7% to aztreonam, 36.1%

to amikacin, 13.1% to piperacillin-tazobactam, 11.5% to meropenem and 03.3% to imipenem. Antibiogram of *Acinetobacter* spp. isolates showed 80% resistance to ciprofloxacin and co-trimoxazole, 70% to doxycycline, 60% to ceftazidime and gentamicin, 50% to piperacillin-tazobactam and aztreonam, 40% to meropenem and amikacin, and 20% resistance to imipenem. The antibiogram of non-fermenters has been depicted in *Table III*.

Table IV - Antibiotic resistance pattern of gram-negative isolates other than non-fermenters

Antibiotics	N	%
Gentamicin	51	69.9
Amikacin	27	37
Amoxycillin-clavulinic acid	40	54.8
Piperacillin-tazobactam	24	32.9
Ciprofloxacin	34	46.6
Meropenem	20	27.4
Imipenem	03	04.1
Co-trimoxazole	50	68.5
Ceftazidime	58	79.5
Doxycycline	54	74

Table V - Rate of biofilm formation by MTCP method

Organism	Number	Number of biofilm forming isolates	Percentage (%)
S. aureus	28	20	71.4
CONS	18	12	66.7
P. aeruginosa	61	45	73.8
Acinetobacter spp.	10	04	40
Klebsiella spp.	31	22	71
E. coli	7	04	57.1
Citrobacter spp.	14	9	64.3
Proteus spp.	16	11	68.7
Enterobacter spp.	5	04	80
Total	190	131	68.9

Table VI - Grading of biofilm formation in aerobic bacterial isolates by MTCP method

Biofilm formation	Optical density	MTCP	
		Number	Percentage
Strong	>5.32	45	23.7
Moderate	2.66-5.32	86	45.3
Weak/None	<2.66	59	31
Total		190	100

Antibiogram of members of the *Enterobacteriaceae* family showed 79.5% resistance to ceftazidime, 74% resistance to doxycycline, 69.9% to gentamicin, 68.5% to co-trimoxazole, 54.8% to amoxicillin-clavulanate, 46.6% to ciprofloxacin, 37% to amikacin, 32.9% to piperacillin-tazobactam, 27.4% to meropenem and 04.1% to imipenem. This is shown in *Table IV*.

In the current study, 68.9% of isolates showed biofilm production by modified tissue culture plate method. Maximum biofilm production was shown by *P. aeruginosa* (73.8%) followed by *S. aureus* (71.4%). Rate of biofilm production by individual bacterial isolates is shown in *Table V*. Strong biofilm production was seen in 23.7% isolates while 45.3% were moderate biofilm producers. The rest were weak/non biofilm producers. Grading of biofilm formation is shown in *Table VI*.

Of the 190 isolates, 105 (55.6%) showed multi drug resistance (MDR). The rate of MDR isolates in biofilm positive isolates was 66.4% while only 30.5% non-biofilm producing isolates showed MDR. This difference in resistance pattern was found to be statistically significant ($p< 0.05$) (*Table VII*).

Discussion

The extent of thermal injury along with the types of microorganism colonizing the burn wound influence the future outcome of the wound. In the present study, 61.6% burn wounds had monomicrobial etiology while 38.4% were due to multimicrobial infection. Similar findings were obtained by Al-saad et al., who studied 100 pus swabs obtained from burn patients. They found 76.4% were due to single species infection and 23.6% were due to mixed species infection.[13] In another study, Alharbi et al. found single species infection in 78.9% of samples while mixed species infection was seen in 21.1% of cases.[14]

In the current study, gram-negative bacterial isolates were predominant, accounting for 76% as compared to 24% gram-positive bacterial isolates. Similar findings were observed by Alharbi et al., where 65.7% of the isolates were gram-negative and 34.3% were gram-positive cocci.[14] Similar results revealing gram-negative predominance have been observed by various researchers.[15-18]

In the current study, *P.aeruginosa* was the most frequently isolated bacteria, followed by *Klebsiella* spp. and *St. aureus.* Agnihotri et al. observed that *P. aeruginosa* was the most common bacterial isolate obtained from burn wounds, followed by *St. aureus*.[16] Similar findings were also obtained by Alharbi et al.[13] and Singh *et al.*[17] However, two separate studies conducted by Mehta et al. and Ramakrishnan et al. showed *Acinetobacter*

Table VII - Comparison of MDR among biofilm forming (BF) and non-biofilm forming (NBF) isolates

Organism	No. of BF isolates	BF MDR		No. of NBF isolates	NBF MDR		'p' value
		N	%		N	%	
S. aureus	20	12	60	8	3	37.5	<0.05
CONS	12	7	58.3	6	2	33.3	<0.05
P. aeruginosa	45	38	84.4	16	5	31.2	<0.05
Acinetobacter spp.	4	1	25	6	1	16.6	<0.05
Klebsiella spp.	21	13	61.9	10	4	40	<0.05
Escherichia coli	4	2	50	3	1	33.3	<0.05
Citrobacter spp.	10	6	60	4	1	25	<0.05
Proteus spp.	11	6	54.5	5	1	20	<0.05
Enterobacter spp.	4	2	50	1	0	00	NA
Total	131	87	66.4	59	18	30.5	<0.05

spp. to be the second most common bacterial isolate after *Pseudomonas* species.[15,18] This may be due to the emergence of acinetobacter as a nosocomial pathogen. Since burn patients are admitted to hospital for long periods, they are at increased risk of being infected by this organism.

In our study, biofilm producing bacterial isolates exhibited a high level of resistance to all drugs that are commonly prescribed, like fluoroquinolones, cephalosporins, aminoglyosides and tetracyclins. Vancomycin and carbepenems were found to be the most effective antimicrobial drugs against gram-positive and gram-negative bacterial isolates respectively. Mehta et al. also observed high resistance to aminoglycosides, fluroquinolones and cephalosporins by gram-negative bacterial isolates, and netilmicin and erythromycin by gram-positive bacteria.[15] In the study by Alharbi et al, amoxycillin, ampilcillin and cefaclor showed low activity. The authors attributed this to the extensive use of these drugs in their centre.[14] A similar observation of high antimicrobial resistance by biofilm producing bacterial isolates has been made in other studies.[16-18]

In our study, biofilm formation was seen in 68.9% isolates while 31.1% bacterial isolates showed no biofilm production. In the study by Al-saad et al., the rate of biofilm production was 54.2% while the rest were non/weak biofilm producers.[13] Ramakrishnan et al. observed a slightly low rate of biofilm production, where 43% of isolates were biofilm producers and 57% were weak/non biofilm producers.[18]

Conclusion

We observed a high rate of antimicrobial resistance and biofilm production in bacterial isolates obtained from burn patients. It was also observed that the majority of MDR pathogens were also biofilm producers. Thus, early identification of infection caused by biofilm producing strains might help to modify treatment and outcome in burn patients.

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Conflict of interest. The authors declare that they have no conflict of interest related to this article.