Original Research Article

Study of Biofilm formation and Antibiogram of *Staphylococcus* species from Diabetic Foot ulcer patients

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Abstract

Background: India has the world's largest number of diabetics. Non- traumatic lower limb amputation is the most common devastating complication of diabetes, primarily due to diabetic foot ulcers (DFU) and diabetic foot infections (DFI). In India, the incidence of foot ulcers ranges from 8–17 per cent. DFIs are predominantly polymicrobial and multidrug- resistant (MDR) with the ability to form biofilm, which is an important virulence factor and results in treatment failure. **Material and Methods:** This is prospective and observational study conducted from September 2020 – August 2021 at a tertiary care teaching hospital. The clinical samples were collected for diagnostic purposes by the bacteriology laboratories of Hospital and were from wound secretions of DFU. Infected sites were aseptically cleaned using normal saline and sterile gauzes. Then a wound swab from each patient was collected using sterile cotton swabs. Antibiotic susceptibility tests were done to confirm the results from hospital. **Results:** *Staphylococcus aureus* were the most commonly isolated organisms (21.4 %), *Escherichia coli* (20.0%) and followed by *Pseudomonas aeruginosa* (18.3 %), *Citrobacter sp.* (18.3 %), *Klebsiella oxytoca* (15.4%), and *Proteus sp*. (6.6 %). **Conclusion:** This study showed that bacteria isolated from diabetic foot ulcers were biofilm producers and presented resistance to commonly used antibiotics. Knowledge on antibiotic sensitivity pattern and biofilm phenotype of the isolates will be helpful in determining the drugs for the treatment of diabetic ulcers.

Keywords: Biofilm, Diabetic foot, Antibiogram, Biofilm Formation.

Introduction

Diabetic foot ulcers (DFU) have a lifetime prevalence of 15–25%. Infection is the most common in diabetic foot ulcer which results in severe and costly DFU complication with high risk of mortality and morbidity associated with lower limb amputation. [1] The diagnosis of diabetic foot infection (DFI) is often difficult, leading to the inappropriate use of antibiotics. The bacterial organization in DFU and the involvement of multidrug-resistant (MDR) bacteria require new antimicrobial solutions. [2]

60 to 80% of chronic wounds harbor bacterial structures in a biofilm. For the clinician, the main difficulty is to distinguish between infecting and colonizing bacteria. Misclassification can lead to inappropriate antibiotic prescriptions that facilitate the emergence of MDR bacteria, a major DFU health issue. [3] Better understanding of the bacterial organization of biofilms in chronic wounds would allow development of tailored antimicrobial strategies and improving wound healing. In this context a large majority of current fundamental studies on DFUs focuses on bacterial cooperation and the impact of local microenvironment on microorganisms. Thus, the host-microorganism interface plays a major role in DFI development. [4]

In DFU, bacteria are classically organized in functionally equivalent path groups (FEP) where pathogenic and commensal bacteria co-aggregate symbiotically in a pathogenic biofilm to maintain a chronic infection. Polymicrobial biofilms have been observed both in pre-clinical studies using animal models and in clinical research on DFU. [5] They represent the main cause of healing delay. Recently, some approaches have targeted biofilm formation with the aim of controlling infections. Better understanding of the host-bacterial interactions is essential to develop new therapeutic solutions that take into account the biofilm to limit the diffusion of MDR bacteria. [6]

Biofilm formation is a multistep process where heterogeneous communities of microorganisms (bacteria and/or fungi) are embedded into an extracellular polymeric substance (EPS) matrix that contains proteins, deoxyribonucleic acid (DNA), glycoproteins and polysaccharides, and confers the ability to adhere to biotic or abiotic surfaces. [7] In DFU, the biofilm architectural structure differs among patients due to the variability of the involved bacterial genera and species. Conversely, the multistep formation process is similar. [8]

Biofilm formation is a major mechanism of adaptation that protects bacteria from antibiotics, due to several mechanisms. Biofilm structure provides a protective layer against antimicrobial compounds. Wound biofilms are polymicrobial, formed by complex and order combinations of microorganisms. [9] Hence, compounds produced by different bacterial strains might impair the contact between the bacterial cell wall and the antibiotic by changing the composition of the EPS. Finally, the production of degradative enzymes by different pathogens can act in synergy against antibiotics. These biofilm aspects are responsible for a reduced diffusion of the antibiotic within the biofilm matrix leading to an inefficient activity of the antibiotic treatment.

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In addition to this feature, the ability to form a biofilm is an effective strategy to enhance survival and persistence of microorganisms by increasing their antimicrobial resistance. The antimicrobial resistance in organisms producing biofilms acts by delayed penetration of the antimicrobial agents through the biofilm matrix, altered growth rate of biofilm organisms, and other physiological changes due to the biofilm mode of growth.

Material and Methods

This is a prospective and observational study conducted from September 2020 – August 2021 at a tertiary care teaching hospital.

The clinical samples were collected from the wound secretion of diabetic foot ulcer for diagnostic purposes at the bacteriology laboratories of Hospital and were from wound secretions of DFU. Infected sites were aseptically cleaned using normal saline and sterile gauzes. Then a wound swab from each patient was collected using sterile cotton swabs. Isolated bacteria on Trypticase soy agar medium were received in the Laboratory of

Antibiograms of each isolated Staphylococcus sp. strains using the Kirby Bauer method on Mueller Hinton Agar were realized with the following antibiotic disks: Erythromycin, Ciprofloxacin, Cefoxitin, Clindamycin, Penicillin G, Amikacin, Penicillin G, Amikacin, Co-trimoxazole, Ciprofloxacin, Linezolid Amoxy-clav and Gentamicin. Test for methicillin resistance was performed with diffusion method using Oxacillin (1 µg) on Mueller Hinton agar with 4% NaCl. Gram negative strains were tested against the following antibiotic disks: Gentamycin (10 µg), Amikacin (30 µg), Co-trimoxazole (25 µg) and Cefoxitin (30 µg). After incubation of plates at 37˚C for 24 hours, diameters of zone of inhibition were measured. Evaluation of the results was done according to the criteria of Clinical Laboratory Standards Institute (CLSI).

The biofilm formation was detected by Congo Red method as described by Freeman et al.8 A specially prepared medium composed of Brain Heart Infusion (BHI) broth (37gm/L), sucrose (50gm/L), agar no.1 (10gm/L) and Congo Red stain (0.8gm/L) was used. Congo Red was prepared as concentrated aqueous solution and autoclaved at 121℃ for 15 minutes, separately from other medium constituents and was added when the agar had cooled to 55℃. Plates were inoculated and incubated aerobically for 24–48 hours at 37℃. Biofilm formers produced black colonies with a dry crystalline consistency, while weak slime producers usually remained pink, though occasional darkening at the centres of colonies was observed. Indeterminate results were characterised by darkening of the colonies with the absence of a dry crystalline colonial morphology. The tests were carried out in triplicate and repeated three times.

Experimental and Pharmaceutical Microbiology for biofilm formation studies. Antibiotic susceptibility tests were done to confirm the results from hospital.

Wound swabs were inoculated into mannitol-salt and Mac Conkey agars and incubated at 37˚C for 24 hours. Staphylococcus sp. were identified by standard microbiological methods such as Gram staining, catalase tests. S. aureus suggestive colonies were confirmed by coagulase and DNase testing. Gram-negative bacilli were identified using micro-biological conventional methods including Gram staining, oxidase tests, indole and urease production, citrate utilization, hydrogen sulphide, gas production and fermentation of sugars, phenylalanine deaminase, lysine decarboxylase (L.D.C.), ornithine decarboxylase (O.D.C.), arginine di-hydrolase (A.D.H.) tests, and methyl red reaction. In our laboratory Gram negative bacilli were confirmed as Enterobacteriaceae species using the same tests. Pseudomonas aeruginosa were confirmed after 24 hours incubation time into Cetrimide agar.

Stepanovic et al. the biofilm formation also detected by enzyme linked immunosorbent assay (ELISA) described the tissue culture plate method in plastic microtitre plates. [10] On a sterile 96 well flat-bottomed polystyrene microtitre plate, 230µl of Trypticase Soya Broth (TSB) was added. Also, 20µl of overnight bacterial culture was added to the corresponding well (each strain in three successive wells). The negative control wells contained broth only. The plates were incubated aerobically for 24 hours at 35℃. The content of the wells was poured off and the wells were washed three times with 300µl of sterile distilled water. The bacteria adhering to the wells were fixed with 250µl of methanol for 15 minutes. Then the wells were stained with 250µl of 1% solution of crystal violet for five minutes. Excess stain was removed by washing and the wells were air-dried. The dye bound to the wells was solubilised with 250µl of 33 per cent (v/v) glacial acetic acid. The optical density (O.D.) of each well was measured at 490nm using an ELISA auto reader.

Results

Sixty samples were collected from patients with chronic diabetic foot ulcers. The study group comprised 41 male patients and 19 female patients, whose ages ranged from 31–70 years in table 1.

Table 1: Gender wise frequency distribution of the population under study

Gender	No. of individuals	Percentage
Male	41	68.4
Female	19	31.6
Total	60	100.0

Table 2: Frequency of Age distribution among the study subjects

Age	No. of individuals	Percentage
31-40 years	11	18.3
41-50 Years	13	21.6
51-60 Years	19	31.8
61-70 Years	17	28.3
Total	60	100.0

In table 2, majority of subjects belonged to 51-60 years (31.8%) followed by 61-70 years (28.3%) of age range.

Table 3: Frequency of gram-positive and gram-negative among the study subjects

Bacteria	No.	Percentage
Gram-Negative	42	70
Gram-positive	18	30
Total	60	100.0

In table 3, Overall, 18 organisms (30%) were gram-positive and 42 organisms (70%) were gram-negative.

Table 4: Comparison of biofilm-forming organisms

organisms	No.	Percentage
Staphylococcus aureus	13	21.4
Escherichia coli	12	20.0
Pseudomonas aeruginosa	11	18.3
Citrobacter sp	11	18.3
Klebsiella oxytoca	9	15.4
Proteus sp.	4	6.6
Total	**60**	**100**

In table 4, *Staphylococcus aureus* were the most commonly isolated organisms (21.4 %) followed by *Escherichia coli* (20.0%), *Pseudomonas aeruginosa* (18.3 %), *Citrobacter sp*. (18.3 %), *Klebsiella oxytoca* (15.4 %) and *Proteus sp*. (6.6 %). With reference to the gram-negative organisms, 53.6 per cent of the organisms were extended spectrum beta lactamase (ESBL) producers, with the highest production by *E. coli*.

Table 5: Distribution of MRSA and MSSA isolates among *Staphylococcus aureus*

Organisms	No. of isolates	Percentage
MRSA	7	53.8%
MSSA	6	46.2%

Table 6: Antibiotic susceptibility profile of Staphylococcus isolates

Antibiotic drugs	Sensitive (%)	Resistant (%)
Cefoxitin	6 (46.2%)	7 (53.8%)
Erythromycin	3 (23.1%)	10 (76.9%)
Clindamycin	5 (38.4%)	8 (61.6%)
Penicillin G	3 (23.0%)	10 (76.9%)
Amikacin	13 (100%)	0 (0%)
Co-trimoxazole	0 (0%)	13 (100%)
Ciprofloxacin	4 (30.7%)	9 (69.3%)
Linezolid	13 (100%)	0 (0%)
Gentamicin	3 (23.0%)	10 (76.9%)
Amoxy-clav	6 (46.2%)	7 (53.8%)

Table 7: Biofilm formation by Gram positive and gram-negative isolates

Biofilm Formation	No.	Percentage
Staphylococcus aureus	10	34.5%
Escherichia coli	5	17.2%
Pseudomonas aeruginosa	7	24.1%
Citrobacter sp	4	13.8%
Klebsiella oxytoca	1	3.5%
Proteus sp.	2	6.9%
Total	29	100

In table 7, Twenty-nine (48.33%) of the isolates showed biofilm formation. *Staphylococcus aureus* was the predominant biofilm former, with 10 (34.5%) of the isolates testing positive for biofilm formation. The second highest biofilm formation was by *Pseudomonas aeruginosa* was 7 (24.1%) followed by *Citrobacter sp.* was 4 (13.8%), *E. coli* was 5 (17.2 %), *Proteus sp.* (6.9 %), and *Klebsiella oxytoca* (3.5%).

Discussion

In the present study, all the samples yielded monomicrobial isolates. This is significantly different from most study results in which DFUs are polymicrobial in nature. However, some studies have shown lower than expected rates of polymicrobial infection. [11]

In our study isolates, 30% were found to be gram- positive while 70 % were gram-negative. This corresponds with the findings of Bhansal et al., in which 76 per cent of the microbes were gram-negative and 24 per cent were gram-positive. The predominance of gram- negative organisms has been noted in several studies. [12]

However, certain studies have established a higher proportion of gram-positive organisms. In this study, *Staphylococcus aureus* were the most commonly isolated organisms (21.4 %) followed by *Escherichia coli* (20.0%), *Pseudomonas aeruginosa* (18.3 %). These results were similar to those obtained by Bhansal et al. [12]

Amongst the *Staphylococcus aureus*, MRSA (53.8%) and MSSA (46.2%) were obtained. In our study, Antibiotic drugs exhibited resistance 100% to Co-trimoxazole and 76.9% Erythromycin and Gentamicin. All of the organisms were sensitive to Amikacin (100 %). This is similar to the study by Rani et al., where the gram-positive organisms showed complete sensitivity to vancomycin. [13]

In our study 48.33% of the isolates showed biofilm formation. This was more compared to prior studies in which it ranged from 73–77 %. [14] A study by James et al. recorded a rate of 60% in chronic wounds, and 6 per cent in acute wounds. [15] Such a deviation from the norm could be due to effective debridement procedures or shorter duration of ulcer in the patients. *Staphylococcus aureus* was the predominant biofilm former, with 38.8 per cent of the isolates testing positive for biofilm formation. This is an expected result, with existing literature supporting the biofilm forming nature of *Staphylococci*. [16]

Biofilm formation is a multistep process whereby heterogeneous communities of microorganisms (bacteria and/or fungi) are embedded into an extracellular polymeric substance (EPS) matrix that contains proteins, deoxyribonucleic acid (DNA), glycoproteins and polysaccharides, and confers the ability to adhere to biotic or abiotic surfaces. [17] In DFU, the biofilm architectural structure differs among patients due to the variability of the involved bacterial genera and species. [18]

Conversely, the multistep formation process is similar. Biofilm formation is a major mechanism of adaptation that protects bacteria from antibiotics, due to several characteristics. Biofilm structure provides a protective layer against antimicrobial compounds. Wounds biofilms are polymicrobial, formed by complex and order combinations of microorganisms. Hence, compounds produced by different bacterial strains might impair the contact between the bacterial cell wall and the antibiotic by changing the composition of the EPS. [19] Finally, the production of degradative enzymes by different pathogens can act in synergy against antibiotics. These biofilm aspects are responsible for a reduced diffusion of the antibiotic within the biofilm matrix leading to an inefficient activity of the antibiotic treatment.

In addition to this feature, the ability to form a biofilm is an effective strategy to enhance survival and persistence of microorganisms by increasing their antimicrobial resistance. The antimicrobial resistance in organisms producing biofilms acts by delayed penetration of the antimicrobial agents through the biofilm matrix, altered growth rate of biofilm organisms, and other physiological changes due to the biofilm mode of growth.

Conclusion

Detection of biofilm formation is an easy and cost-effective test that can be performed routinely in the laboratory. Detection of biofilm will help surgeons to effectively manage these infections by providing more aggressive source control and appropriate antibiotics resulting in decrease mortality and the morbidity in patients.

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