

Diabetic foot ulcers and biofilm formation- The culprits

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Abstract

India leads the world with largest number of diabetic subjects earning the dubious distinction of being termed the “diabetes capital of the world”. DFIs are predominantly polymicrobial caused by a combination of Gram positives like *Staphylococcus aureus*, *Enterococcus*, Gram negatives like *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Proteus* species etc. and Anaerobes of which, most are multidrug resistant. A Biofilm is defined as a thin robust layer of mucilage adhering to a solid surface and containing a community of bacteria and other microorganisms. These biofilms pave way for the re-emergence of multi-drug resistant strains and result in treatment failure.

Therefore proper screening of multi drug resistant organisms that are often associated with biofilms, is essential. Detection of biofilm formation is an easy, cost effective test that must be and can be performed as a routine in the laboratory in all these cases.

Hence, liberal debridement in combination with appropriate antibiotics will help surgeons to effectively manage the situation and hereby decrease the incidence of emergence of multi drug resistant organisms thereby reducing mortality and the morbidity in patients.

Keywords: Biofilm; Diabetic Foot; Resistance; Amputation.

1. Introduction

Diabetes is a common disease in India with a prevalence rate of 12-17% in the urban population and 2.5% in the rural population.[1] India leads the world with largest number of diabetic subjects earning the dubious distinction of being termed the “diabetes capital of the world”.[2] According to the IDF Diabetes Atlas (6th Edition), India had a diabetic population of 7,60,429 in 2013.

In diabetic patients, the commonest devastating complication is non- traumatic lower limb amputation mostly due to diabetic foot ulcers (DFU) and infections (DFI).[1] The incidence of foot ulcers ranges from 8 to 17% in India.[3]

DFIs are predominantly polymicrobial caused by a combination of Gram positives like *Staphylococcus aureus*, *Enterococcus*, Gram negatives like *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Proteus* species etc. and Anaerobes of which, most are multidrug resistant.[4] The microorganism that colonizes the surface wound also provides an ideal niche for further invasion resulting in these infections. An interesting factor is that these different microorganisms can exist independently or combine together

to form micro-communities within a matrix of extracellular polymeric substances and are called as biofilms.

A Biofilm is defined as a thin robust layer of mucilage adhering to a solid surface and containing a community of bacteria and other microorganisms. Its formation begins with the attachment of free floating microorganisms to a surface. At first, these colonize through weak van der Waals forces. If left unseparated, they anchor themselves more firmly via cell adhesion structures such as pili. Hydrophobicity plays an important role in determining the ability of bacteria to form Biofilms, as those with increased hydrophobicity have reduced repulsion between the extracellular matrix and bacterium. The ability of a microorganism to form biofilm is an important virulence factor as it establishes a protective environment for the organisms to survive and evade antibiotics and such biofilms are the main cause of many chronic infections such as diabetic foot ulcers (DFU).

These biofilms pave way for the re-emergence of multi-drug resistant strains and result in treatment failure [1] and are therefore difficult to eradicate by conventional antibiotics. Hence identification of biofilm producers among

clinical isolates may help in better management of wound infections in diabetics who in spite of repeated antibiotic treatment fail to respond, as this is not being tested routinely.

The objectives of the study are:

1. To identify the spectrum of bacteria causing diabetic wound infection and antibiotic sensitivity pattern in our hospital.
2. To detect the biofilm formation among these bacteria.

2. Materials and methods

This study was conducted as a prospective study at the Department of Microbiology, in a tertiary care research and referral hospital attached to a Medical College and Research Institute. 100 patients attending the surgery outpatient department of the hospitals were included in the study. Institutional ethical clearance was taken and informed consent was obtained from the subjects in their own language.

All patients above 18 years of age having chronic diabetic foot ulcer where ulcer duration is greater than 3 months were included in the study.[5] Children (<18years), pregnant women and patients with other co morbid conditions like HIV infection, chronic venous insufficiency were excluded.

The patients were assessed through detailed history and clinical examination. The ulcers were assessed by the surgeons and after debridement, material for culture was collected with cotton tipped sterile swab from the deeper parts of the foot ulcer. This was transported immediately to the microbiology department for culture and sensitivity and biofilm formation. Swabs received were cultured on Blood agar and McConkey agar and the plates were incubated overnight at 37°C. Colonies obtained were identified by using standard techniques.[6] Antibiotic sensitivity was done using Kirby Bauer's disc diffusion technique method as described in clinical laboratory standard institute guidelines 2012 (CLSI).[7]

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 (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and congo red stain (0.8 gms/L) was used. Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. Biofilm formers produced black colonies with a dry crystalline consistency while weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. Indeterminate results were characterized 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.[9]

Stepanovic *et al* 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. 20 µl 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°C. The content of the wells were poured off and the wells were washed three times with 300 µl of sterile distilled water. The bacteria adhering to the wells were fixed with 250 µl of methanol for 15 minutes. Then the wells were stained with 250 µl of 1% solution of crystal violet for 5 minutes. Excess stain was removed by washing and the wells were air-dried. The dye bound to the wells was resolubilised with 250 µl of 33 % (v/v) glacial acetic acid. The optical density (O.D.) of each well was measured at 490nm using an ELISA autoreader.

The tests were carried out in triplicate and the results were averaged. The cut-off O.D (O.D.c) was determined as three standard deviations above the mean O.D. of the negative control. Strains were classified as biofilm producer and no biofilm producer.

Data was compiled and descriptive statistics were applied using Microsoft Excel 2010 Edition.

3. Results

100 samples were collected from patients with chronic diabetic foot ulcer. The study group comprised 84 male patients and 16 female patients, whose ages ranged from 35-80 years. From these samples, 82 isolates were obtained. No polymicrobial infections were noted. *Staphylococcus aureus* and *E. coli* were the most commonly isolated organisms (24.40% each) followed by *Pseudomonas aeruginosa* (17.07%), *Citrobacter sp.* (12.1%), *Klebsiella oxytoca* (12.1%) and *Proteus sp.* (9.76%). This is depicted in Figure 1. Overall, 20 organisms (24.4%) were Gram positive and 62 organisms (75.6%) were Gram negative.

38 (46.34%) of the isolates showed biofilm formation. *Staphylococcus aureus* was the predominant biofilm former, with 14 (38.84%) of the isolates testing positive for biofilm formation. All 10 (100%) of the MRSA isolates were biofilm formers while only 4 (40%) of the MSSA isolates formed biofilm. The second highest biofilm formation was by *Pseudomonas aeruginosa* (26.52%) followed by *Citrobacter sp.* (10.53%), *E. coli* (10.53%), *Proteus sp.* (10.53%) and *Klebsiella oxytoca* (5.26%) This is represented in Figure 2.

The antimicrobial resistance pattern of Gram positive organisms is represented in figure 3 and resistance pattern of Gram negative organisms is represented in chart 4. 80 isolates (97.56%) were Multi Drug Resistant with 37(46.25%) of the MDR isolates also showing biofilm formation.

Figure 1: Isolate showing chronic diabetic foot ulcer

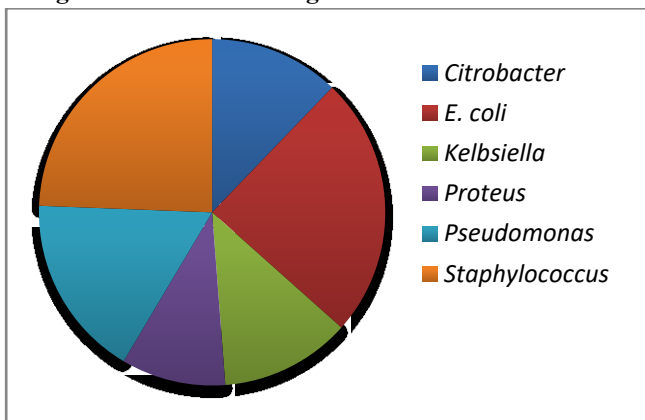


Figure 2: Isolate showing biofilm formation

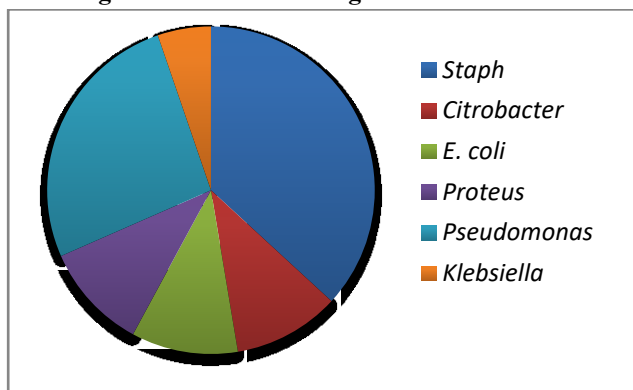
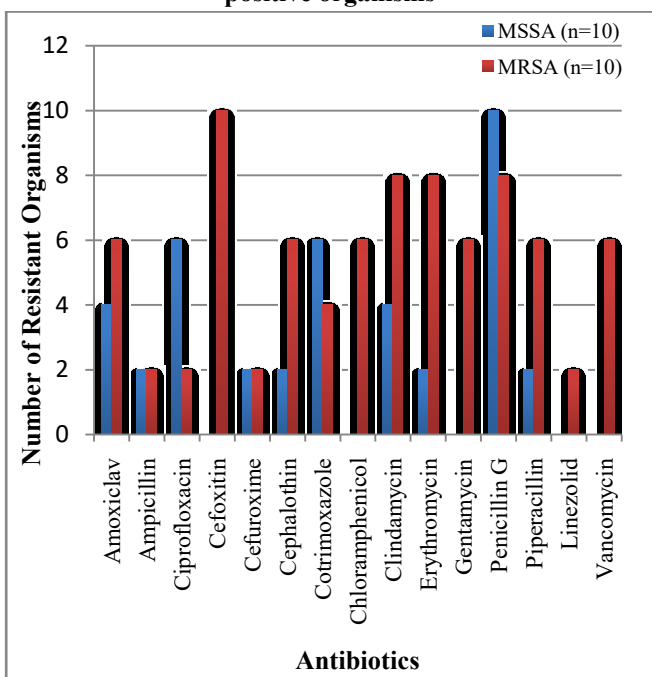


Figure 3: The antimicrobial resistance pattern of Gram positive organisms



4. Discussion

The prevalence of foot ulcers ranges from 4% to 10% in patients with diabetes mellitus, with the annual population-based incidence is estimated to be 1.0% to 4.1%. The lifetime rate extends to around 25%. A common

complication of these ulcers is infection, which if left untreated, results in the need for distal limb amputation.[11] According to the NIH over 80% of chronic bacterial infections are associated with biofilms.[12]

In the present study all the samples yielded monomicrobial isolates. This is significantly different from the results of the majority of studies, in which DFUs are polymicrobial in nature.[13-22] However, some studies have shown lower than expected rates of polymicrobial infection.[1,20,23] Monomicrobial nature of infection is associated with the duration of the ulcer. Earlier on in the infection, monomicrobial state prevails and as the infection progresses with time, a polymicrobial state arises. Also, ulcers that are shallower and which have lesser degree of necrosis (<grade 2 as per Wegner classification) tend to be monomicrobial. It is necessary to note that studies have shown that in polymicrobial infections not all isolates have to be eradicated to ensure an improvement in the ulcer’s healing process.[24]

75.6% of the isolates were Gram positive while 24.4% were Gram negative in our study. This corresponds with the findings of Bhansal *et al*[21] in which 76% of the microbes were Gram negatives and 24% were Gram positive. The predominance of Gram negative organisms has been noted in several studies.[4,25] However, certain studies ^(1, 26, 27)have established a higher proportion of Gram positive organisms.

In our study, *Staphylococcus aureus* and *Escherichia coli* were the most commonly isolated organisms (24.40% each) followed by *Pseudomonas aeruginosa* (17.07%). These results were similar to those obtained by Bhansal *et al*[21]. However, a study in Malaysia reported *Proteus sp.* to be the predominant Gram negative organism.[20]

Amongst the Gram positive organisms MRSA (12.2%) and MSSA (12.2%) were obtained. MSSA exhibited high-level resistance (100%) to penicillin G, cotrimoxazole (60%) and ciprofloxacin (60%). There was a lower level of resistance to the other antibiotics that were tested against, and the MSSA were completely susceptible to chloramphenicol, linezolid and vancomycin. The prevalence of MRSA in our study was lower as compared to prior studies in which it ranged from 40%-69.8%.[21,25,26,28] However a similar prevalence of 12% was seen in a Malaysian study.[20] The MRSA displayed high level of resistance to clindamycin (80%), erythromycin (80%) and penicillin G (80%). Most of the organisms were sensitive to linezolid (80%). However, a serious concern is the large number of isolates showing vancomycin resistance (60%). According to previous studies, vancomycin resistance is rare, and isolation of community-acquired VRSA is an important issue as it identifies cases, which are highly refractory to treatment.[29] In the study by Rani *et al*, the Gram positive organisms showed complete sensitivity to vancomycin, linezolid, teicoplanin, more than 95% sensitivity to cefaperazone + sulbactam, cefepime +

tazobactam, piperacillin + tazobactam, amikacin & gentamycin, 80% to fluoroquinolones, 65% to cephalosporins and only 30% to ceftioxin.[26] Bhansal *et al* noted that nearly all Gram positive strains were sensitive to ceftriaxone and imipenem, while a good level of sensitivity existed for amikacin and ciprofloxacin.[21]

With reference to the Gram negative organisms, 53.66% of the organisms were ESBL producers, with the highest production by *E. coli*. This is in parallel with pre-existing studies in which 44.7% - 57.4% are ESBL positive [21,26], but significantly lower than the 80% ESBL formers found by Mamdouh *et al*[30]. The Gram negative organisms in our study showed a high level of resistance to amoxicillin+clavulanic acid (56.1%), ceftazidime (53.66%), ciprofloxacin(46.34%), ceftioxin, cephalothin and cefuroxime (each 43.9%). The organisms were most sensitive to piperacillin+tazobactam (90.24%) and imipenem (85.36%).

This corresponds with the findings of Rani *et al* in which imipenem, cefepime+sulbactam, cefepime+Tazobactam and piperacillin+tazobactam are reported as the most effective drugs against ESBL producing Gram negative bacilli.[26] However, another Indian study conducted on Gram negative biofilm formers reported high degree of resistance of Gram negative isolates against ceftazidime (79.6%) followed by piperacillin (68.4%), ceftioxin(67.3%) amoxiclav (64.3%), ceftioxin (64.3%) amoxicillin (63.3%), ofloxacin (63.3%) cefepime (59.2%), gatifloxacin(57.1%) levofloxacin (51.0%), ceftioxin(49%) ceftriaxone (44.9%), ceftazidime amikacin and gentamicin (40.8% each), aztreonam (39.8%), ceftioxin (36.7%), chloramphenicol (31.6%), imipenem (24.5%), piperacillin+tazobactam (21.4%), ceftioxin+clavulanic acid (12.2%), and Ceftazidime+clavulanic acid (9.2%).[31]

This high degree of drug resistance could be due to the excessive usage of broad-spectrum antibiotics, which cause organisms to acquire a survival advantage. This is often seen in tertiary care centers.[31]

Studies have shown that biofilm associated microorganisms can be up to 1000 times more resistant to antibiotics than free floating planktonic bacteria.[12]

In the present study, 80 isolates (97.56%) were Multi Drug Resistant with 37(46.25%) of the MDR isolates also showing biofilm formation. Swarna *et al* reported that 80.39% of the MDR organisms were biofilm formers [1], and this is a significantly larger number as compared to the present result.

The mechanism of multi-drug resistance in Biofilm forming organisms is believed to be a direct result of close cell-cell contact in the biofilm, which allows for easy transfer of plasmids containing MDR genes amongst one another.[35] Organisms, which form biofilms, are also characterized by tolerance, which is a temporary, nonheritable characteristic. The mechanisms for tolerance are as follows: (1) Antibiotics

whose mechanism of action depends on the division of cells are inactive against microbes in a biofilm, which are in a slow-growing, dormant state.[36] (2) Drug permeation is hindered by the polysaccharide matrix of the biofilm.[35] (3) Drug efficacy is altered in the microenvironment of the biofilm (pH and osmotic variations).[34] In addition to their effect on antimicrobial agents, biofilms also block host defenses. They have an antiphagocytic property, which inactivates leukocytes in the polysaccharide matrix.[37] There is also an element within the matrix that disables both complement and host antibodies.[38]

In our study 46.34% of the isolates showed biofilm formation. This was unusual, as compared to prior studies in which it ranged from 73%-77.1%. (1,31) A study by James *et al* recorded a rate of 60% in chronic wounds, and 6% in acute wounds.[10] Such a deviation from the norm could be due to effective debridement procedures [31] or shorter duration of ulcer in the patients.

Staphylococcus aureus was the predominant biofilm former, with 38.84% of the isolates testing positive for biofilm formation. This is the expected result, with prior literature supporting the biofilm forming nature of *Staphylococci*. [32] *S. aureus* is followed by *Pseudomonas aeruginosa* with 26%. Studies have reported *P. aeruginosa* to form biofilms more readily in the diabetic wound environment.[12]

The limitation of this study was the inability to isolate anaerobes. Numerous studies report anaerobes as comprising a majority of the isolated organisms.[33]

5. Conclusion

Difficulty in eradicating a chronic diabetic foot infection associated with biofilm formation has been reported and biofilm producing bacteria have been shown to resist higher antibiotic and disinfectant concentrations than non biofilm producing bacteria.

Therefore proper screening of multi drug resistant organisms that are often associated with biofilms, is essential. Detection of biofilm formation is an easy, cost effective test that must be and can be performed as a routine in the laboratory in all these cases.

In the present study all the samples yielded monomicrobial isolates. This is significantly different from the results of the majority of studies, in which DFUs are polymicrobial in nature. In our study, *Staphylococcus aureus* and *Escherichia coli* were the most commonly isolated organisms followed by *Pseudomonas aeruginosa*.

The organisms were most sensitive to piperacillin+tazobactam and imipenem . The Gram negative organisms in our study showed a high level of resistance to amoxicillin+clavulanic acid, ceftazidime, ciprofloxacin, ceftioxin, cephalothin and cefuroxime.

In our study, a significant decrease in the number of isolates forming biofilms was observed, which just about

46.34% was. This was unusual, as compared to prior studies in which it ranged from 73%-77.1%.[10] Such a deviation from the norm could be due to effective debridement procedures or shorter duration of ulcer in the patients.

Therefore, liberal debridement in combination with appropriate antibiotics will help surgeons to effectively manage the situation and hereby decrease the incidence of emergence of multi drug resistant organisms thereby reducing mortality and the morbidity in patients.

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