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DIABETIC FOOT ULCERS AND BIOFILM FORMATION

B.V.S Lakshmi[a]*, Hima Bindhu[b], M. Sudhakar[a], G. Apoorva[a], J. Uhasri[a], M. Pradeep[a], D. Sahaja[a]

[a]Department of Pharmacy Practice, Malla Reddy College of Pharmacy, Dhulapally, Secunderabad, Telangana-500100 (Affiliated to Osmania University).

[b]Department of General Surgery, Associate Professor, Malla Reddy Institute of Medical Sciences, Suraram, Hyderabad-500065.

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***Corresponding Author**
B.V.S Lakshmi
Department of Pharmacy Practice, Malla Reddy College of Pharmacy, Dhulapally, Secunderabad, Telangana-500100 (Affiliated to Osmania University).

ABSTRACT

Objectives: Diabetic foot infections are predominantly polymicrobial, most are multidrug resistant. Delayed wound healing appears to be largely related to the presence of micro-organism growing in a biofilm. This study aimed to identify the spectrum of multidrug-resistant bacteria associated with these infections, their antibiotic sensitivity pattern, and to detect the biofilm formation. **Methods:** A prospective study at a tertiary care hospital. 100 patients over the age of 18, having chronic diabetic foot ulcer, and attending the surgery outpatient department were included. Samples of pus were collected from deep wounds and processed using standard techniques for culture and sensitivity. Biofilm detection was done. Results were compiled and statistically analyzed. **Results:** 28(35%) Gram positive organisms and 52(65%) gram negative organisms were isolated. No polymicrobial infections were noted. *Psuedomonas aueroginosa1-8(22.5%)* *Staphylococcus aureus-14(17.5%)* was the most commonly isolated organism. Biofilm formation was seen in 34(42.5%). Gram negative- 23(67.64%) Gram positive-11(32.35%) microbes have predominant Biofilm production like *E.coli* (26.4%), *Klebsiella pneumonia* (23.1%), *Staphylococcus aureus (17.64%) Pseudomonas aueroginosa (14.7%), MSSA (8.82%), Betahemolytic streptococci (5.88%), Citrobacter(1%).* **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, additional

screening of multidrug-resistant organisms as well as non-resistant organisms like E.coli and Klebsiella pneumonia often associated with biofilms should be considered. Detection of biofilm formation is an easy and cost-effective test that can be performed routinely in the laboratoryand 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.

KEYWORDS: Biofilm; Diabetic Foot; Resistance; Amputation.

INTRODUCTION

Diabetes mellitus is one of the most common co morbid conditions among patients hospitalized for acute bacterial skin infections[1].

According to seventh edition of International Diabetes Federation (IDF)- Diabetes Atlas, there are 415 million diabetics in the World. A study in India has shown that 30.4% of the diabetics have infections, mainly wound infections[2]. India is ranked second (92.6 million) after China (109.6 million) for being home to largest number of adults with DM[3]. Approximately 25% of diabetics have a cumulative lifetime risk for foot ulcers with increased vulnerability for infections in 40-80 % of the cases[4]

Aetiology of the infection is variable and may ranges from gram-positive organisms such as *Staphylococcus aureus, Streptocooci, Enterocoocus* to gram-negative bacteria such as *Pseudomonas aeruginosa, Escherichia coli, Klebsiella* species, Proteus species, sometimes these organisms shows polymicrobial infection according to it the treatment also varies[5]

Bacteriologic investigation of bacterial specimens is an essential tool for active surveillance of antimicrobial drug resistance. Knowledge of causative bacterial species and their resistance profile enables targeted antimicrobial therapy, limits ineffective antimicrobial therapyPoly microbial infection, repeated interventions, antibiotic resistance leads to the chronicity of the wound and adversely affect the healing process. These poor treatment outcomes result in high healthcare costs, decreased life quality, increasedmortality[6-7]. These outcomes are facilitated by several factors, including biofilm formation[8].

A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharides, protein and DNA. Biofilm may have a pathogenic role across a spectrum of Diabetic foot ulcer (DFU) presentation. Delayed wound healing appears to be

largely related to the presence of micro-organism growing in a biofilm.[9] Bacterial biofilm causes chronic infections because they show increased tolerance to antibiotics and disinfectants as well as resisting phagocytosis and other components of the body's tolerance system[10-11] Biofilm-forming bacteria are refractory to host response and medical treatment[12-13]. Biofilm-forming bacterial colonies are 10 to 1000 times more resistant to antimicrobials in comparison with plank tonic (free-floating) bacteria[14] So, Biofilms are difficult to eradicate using conventional antibiotics, hence the identification of biofilm producers among microbial isolates may lead to better management of wound infections in diabetics who, in spite of repeated antibiotic treatment fail.

Hence, in this study, we aimed to evaluate factors affecting biofilm formation and biofilm formation in pathogenic bacteria.

These biofilms pave way for the re-emergence of multi-drug resistant strains and result in treatment failure[15] 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.

METHODS

This prospective study was conducted at the Department of Microbiology, in a tertiary care research and referral hospital attached to a medical college. One hundred 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 over 18 years of age having chronic diabetic foot ulcers where ulcer duration is greater than three months were included in the study. These patients had received antibiotics earlier. Children (<18 years), pregnant women, and patients with other comorbid conditions like HIV infection, chronic venous insufficiency, and osteomyelitis were excluded.

The patients were assessed through detailed history and clinical examination. Surgeons assessed the ulcers, and after debridement material for culture was collected with a cotton-tipped sterile swab from the deeper parts of the foot ulcer. The ulcers were not demarcated as per the Wegner classification of ulcers. The swabs were transported immediately to the

Department of Microbiology 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. Antibiotic sensitivity was done using Kirby Bauer's disc diffusion technique method as described in the Clinical Laboratory Standard Institute (CLSI) guidelines 2012. Multidrug-resistant organisms for gram-positive and gram-negative bacteria are resistant to three or more antimicrobial classes as per the guidelines. No concomitant blood cultures were collected.

The biofilm formation was detected by Congo Red method as described by Freeman et al. 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°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 24–48 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 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.[16]

Stepanovic et al. described the tissue culture plate method in plastic microtitre plates. 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°C. 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 one per cent 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. Theoptical density (O.D.) of each well was measured at 490nm using an ELISA auto reader.

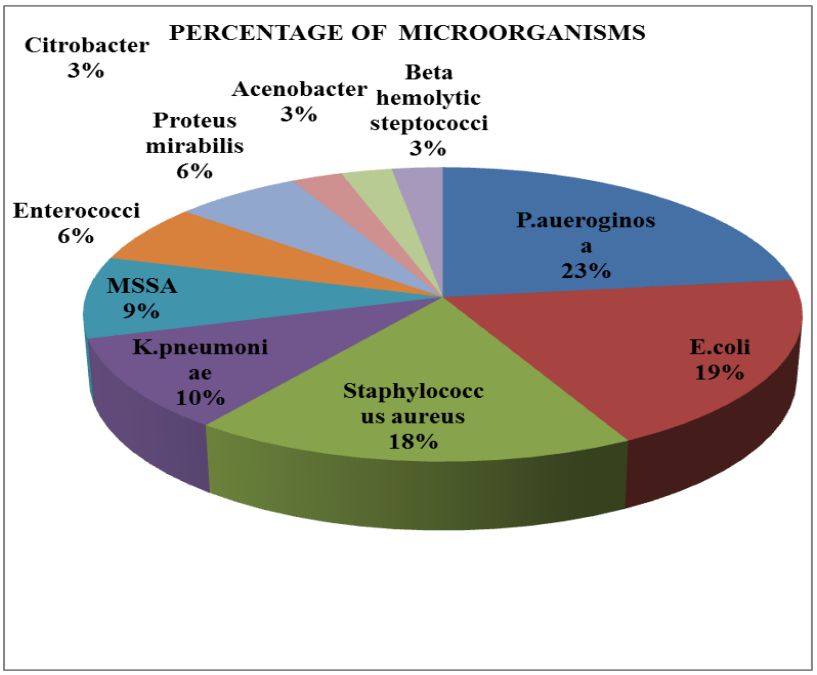
The tests were carried out in triplicate and the results were averaged. The cut-off O.D (O.D.c) was determined as three standard deviations above the mean O.D. of the negative control. Strains were classified as biofilm producer and no biofilm producer. Data was compiled and

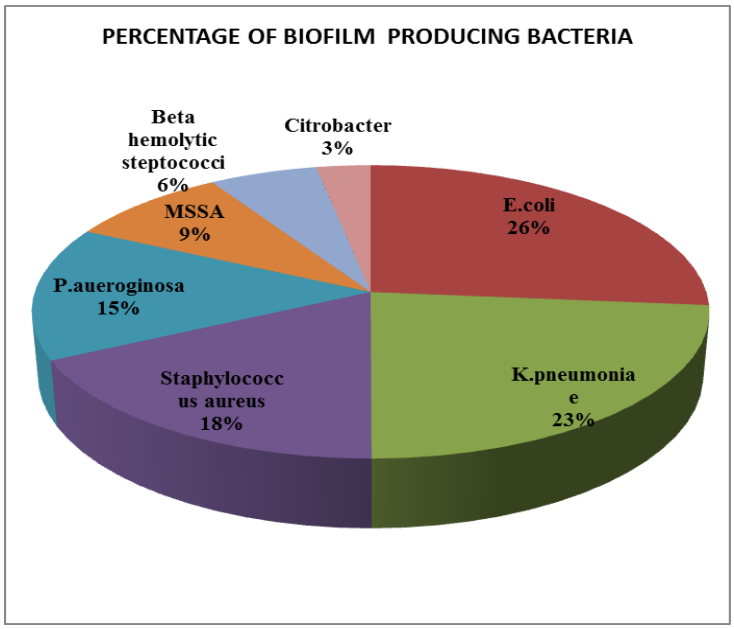
descriptive statistics were applied using Microsoft Excel 2010 Edition (Microsoft, Seattle, WA).

RESULTS

100 samples were collected from patients with chronic diabetic foot ulcer. The study group comprised 70 male patients and 30 female patients, whose ages ranged from 30-70 years. From these samples, 80 isolates were obtained. 28(35%) Gram positive organisms and 52(65%) gram negative organisms were isolated. No polymicrobial infections were noted. *Psuedomonas aueroginosa1 8(22.5%) Staphylococcus aureus 14(17.5%)* were the most commonly isolated organisms followed by *E.coli15(18.75%), Klebsiella pneumonia10(12.5%), Methicillin Sensitive Staphylococcus aureus7(8.75%), Enterococcus5(6.25%), Proteus mirabilis 5(6.25%), Citrobacter 2(2.5%), Acenobacter 2(2.5%).* 34(42.5%) Diabetic Ulcer patients had Biofilm Producing bacteria. From the figure it is clear that Gram negative 23(67.64%) Gram positive 11(32.35%) Microbes have predominant Biofilm production like *E.coli*(26.4%), *Klebsiella pneumoniae*(23.1%), *Staphylococcus aureus(17.64%) Pseudomonas aueroginosa(14.7%), MSSA(8.82%), Betahemolytic streptococci(5.88%), Citrobacter(1%).*

Out of 80 isolates 75(93.75%) were Multi Drug Resistant with 31(41.33%) of the MDR isolates also showing biofilm formation.





DISCUSSION

According to seventh edition of International Diabetes Federation (IDF)- there are 415 million diabetics in the World. A study in India has shown that 30.4% of the diabetics have infections, mainly wound infections[17]. India is ranked second (92.6 million) after China (109.6 million) for being home to largest number of adults with DM [18]. Approximately 25% of diabetics have a cumulative lifetime risk for foot ulcers with increased vulnerability for infections in 40-80 % of the cases. According to the NIH over 80% of chronic bacterial infections are associated with biofilms.

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[19,20] However,

some studies have shown lower than expected rates of polymicrobial infection.[21] The monomicrobial nature of infection is associated with the duration of the ulcer and antimicrobial treatment. Earlier on in the infection, the monomicrobial state prevails and as the infection progresses with time, a polymicrobial state arises. Also, ulcers that are shallower and that have a lesser degree of necrosis 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. In our study, it could also be attributed to the fact that all the patients were on antimicrobial treatment during sampling and only the multidrug-resistant organisms not responding to the treatment would have been cultured.

Of the isolates, 35 % were found to be grampositive while 65% were gram-negative. This corresponds with the findings of Bhansal et al., 14 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.[23] However, certain studies have established a higher proportion of gram-positive organisms. In this study, Pseudomonas aueroginosa (22.5%) and Escherichia coli (18.75%) were the most commonly isolated organisms followed by S.auereus(17.5%). These results were similar to those obtained by Bhansal et al. All of the organisms were sensitive to linezolid, vancomycin, and imipenem (100 per cent). This is similar to the study by Rani et al., where the gram-positive organisms showed complete sensitivity to vancomycin, linezolid, and teicoplanin.

The gram-negative organisms in our study showed a high level of resistance to amoxicillin+clavulanic acid (56.1 per cent), ceftazidime (53.66 per cent), ciprofloxacin (46.34 per cent), cefoxitin and cefuroxime (43.9 per cent each). The organisms were most sensitive to piperacillin+tazobactam (90.2 per cent) and Ceftriaxone (100 per cent). This corresponds with the findings of Rani et al. in which imepenam, cefaperazone+sulbactam, cefepime+tazobactam, and piperacilllin+tazobactam are reported as the most effective drugs against ESBL-producing gram-negative bacilli18 and those of Aasha et al. Studies have shown that biofilm-associated microorganisms can be up to 1,000 times more resistant to a antibiotics than free-floating planktonic bacteria.[24]

In the present study, 75 isolates (93.75%) were multidrug resistant with 31(41.3%) of the MDR isolates also showing biofilm formation. Swarna et al. reported that 80.4 per cent of the MDR organisms were biofilm formers,[25] and this is a significantly larger percentage in comparison to the present result. The mechanism of multidrug 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.[26] Organisms, which form biofilms, are also characterised by tolerance, which is a temporary, nonheritable characteristic. The mechanisms for tolerance are: (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. (2) Drug permeation is hindered the polysaccharide matrix of the biofilm. (3) Drug efficacy is altered in the microenvironment of the biofilm (pH and osmotic variations). In addition to their effect on antimicrobial agents, biofilms also block host defences. They have an antiphagocytic property, which inactivates leukocytes in the polysaccharide matrix. There is also an element within the matrix that disables both complement and host antibodies.In our study 42.5% of the isolates showed biofilm formation. This was lower compared to prior studies in which it ranged from 73–77.1%[25] A study by James et al. recorded a rate of 60 per cent in chronic wounds, and 6 per cent in acute wounds.10 Such a deviation from the norm could be due to effective debridement procedures or shorter duration of ulcer in the patients. E.coli was the predominant biofilm former, with 60% of the isolates testing positive for biofilm formation. This is an expected result, with existing literature supporting the biofilm forming nature E.coli.[27] E.coli is followed by Klebsiella pneumoniae with 80%.

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, additional screening of multidrug-resistant organisms as well as non-resistant organisms like E.coli and Klebsiella pneumonia often associated with biofilms should be considered. 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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CONFLICTS OF INTEREST

No conflicts of interest have been declared.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

Ethics Approval

All procedures performed in studies involving human participants were in accordance with the ethical Standards of the Institutional Ethics Committee and with the 1964 Helsinki declaration and its later amendments or Comparable ethical standards.

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