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A study of biofilm production by gram-negative organisms isolated from diabetic foot ulcer patients

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Abstract
The present study was undertaken to study the difference in antibiotic resistance profile and minimum antibiotic concentration (MIC) of biofilm producing and non-biofilm producing gram-negative bacilli isolated from diabetic foot ulcer (DFU) patients in a tertiary care hospital in North India. Among the diabetic foot patients, 73.6% were males and 15% were females. 77.1% had T2DM whereas only 24.4% patients had T1DM. Poor glycemic control and poor HbA1c (>8) was observed in 68.7% and 70.1% patients respectively. Among the 57 patients, 97 gram-negative bacilli were isolated in which mixed bacterial infection was found in 67.8% and monomicrobial in 32.2% only. *Escherichia coli* was the most common (42.2%) isolate followed by *Pseudomonas aeruginosa* (23.7%)*, Klebsiella oxytoca* (11.3%)*, Klebsiella pneumonia* (9.2%)*, Proteus vulgaris* (5.1%)*, Acinetobacter* sp (5.1%), *Proteus mirabilis* (2%) and *Morganella morganii* (1.0%). 77.1% DFU patients had infection by biofilm producing organisms. BFP positive status was associated with the presence of neuropathy (O.R. 7.65), osteomyelitis (O.R. 3.14), duration of ulcer (O.R. 25.7), grade of ulcer (O.R. 9.12), necrotising ulcer (O.R. 14.4) and ulcer size >4cm^2 (O.R. 3.30) but not with patients characteristic, type of diabetes and type of diabetes, or duration of hospital stay. Poor glycemic control in 56.1% patients, amputation (24.5%), hospital stay (38.5%) and age distribution were independently associated with risk of biofilm producing infection in diabetic foot patients.

Keywords: Diabetic foot ulcer; bacterial profile; antibiotic resistance; biofilm production.

Introduction

Toole *et al.* (2005) who observed that, the bacteria are not free floating but grow upon submerged surfaces. The basic architecture of biofilms shows that the microcolony is actually the basic structural unit of the biofilm. The exhaustive structural analysis of hundreds of monospecies *in vitro* biofilms, and of dozens of multispecies natural biofilms, has shown that microcolonies are discrete matrix-enclosed communities of bacterial cells that may include cells of one or of many species. Depending on the species involved, the microcolony may be composed of 10–25% cells and 75–90% extracellular exopolysaccharide matrix (EEM). The matrix material often appears to be most dense in the area closest to the core of the microcolony, which is characterized by their lack of Brownian movement. Costerton *et al.* (1999) showed the arrangement of micro-colonies are in horizontal array in thin biofilms, but also form a vertical arrays in very thick sessile communities. Biofilm EEM, which is also referred to as *slime* (although not everything described as slime is a biofilm), is a polymeric conglomeration generally composed of extracellular DNA, proteins, polysaccharides, adhesins (PS/A) and autolysin (encoded by *atlE* gene) are involved in regulation of biofilm production present in various configurations. The *ica* gene codes for intracellular adhesion (ICA) and may also code for PS/A and, is required for biofilm production (Toole *et al*., 2005; Donlan *et al.,* 2002; Carol *et al.,* 2005).

Biofilm which forms on living or non-living surfaces establishes a protective environment of microbial life in natural, industrial and hospital settings (Stoodley *et al.,* 2004), which are, physiologically distinct from planktonic cells of the same organism, which, by contrast, are single-cells that may float or swim in a liquid medium (Karatan *et al*., 2009; Hoffman *et al*., 2005). When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in which large suites of genes are differentially regulated (An *et al*., 2007). Biofilms are also often the site for quorum sensing influence the availability of key nutrients for biofilm formation, chemotaxis towards surface, motility of bacteria, surface adhesion and presence of surfactants are certain factors which influence biofilm formation (Carol *et al*., 2005; Thomas *et al*., 2007). According to a recent public announcement from National Institute of Health (NIH), more than 60% of all infections are caused by biofilm (Kim *et al*., 2001). Moreover, these ulcers adversely influence the patients' quality of life, leading to decrease in

social, physical and physiological functions (Raiber *et al.*, 1998). Various factors including defects in host defense mechanisms (impaired leukocyte functions) are responsible for this increase in infection rates. Wound infection is known to impair wound healing in both acute and chronic DFUs (Robson *et al.,* 1997). That most of the infections in DFU are polymicrobial in nature have recently been documented in our studies also (Zubair *et al.,* 2010a,b). Although the numbers and type of bacteria in a wound are critical for infection to occur, recently a new concept of bacterial biofilms has emerged as a potential way to better understand how bacteria deter healing. Therefore, a better understanding of bacterial biofilms is needed, and this may ultimately result in development of novel therapeutics for the prevention and treatment of DFU infections. The biofilm producing organisms have an inherent resistance to antibiotics and in the long run they may be very damaging because of the development of immune complex diseases (Donlan *et al.,* 2002; Raad *et al.,* 1995; Souli *et al.,* 1998).

There are only scarce reports on biofilm formation by clinical isolates from DFU especially in North India. Keeping this in mind, the present study was undertaken to study the difference in their antibiotic resistance profile and minimum antibiotic concentration of biofilm producing and non-biofilm producing gram-negative bacilli isolated from diabetic foot ulcer in a tertiary care hospital in North India.

Materials and Methods

Study Design

The study was carried out prospectively at the Diabetic and Endocrinology ward, J.N. Medical College, Aligarh Muslim University. Aligarh, India, from June 2009 to February 2010. Subjects studied were all in-patients of the male and female ward who had ulcer/infection in their foot with gram-negative bacterial infection.

Clinical Examination

A detailed clinical history and physical examination was carried out for every subject, which include a record of age, sex, anthropometric measurements, duration of ulcer, duration of diabetes and glycemic control. Foot ulcers were categorized into six grades (0-5) based on Meggit Wagner Classification System (Wagner *et al*, 1981). Neuropathy was quantified in each patient assessing vibration sensation using a 128 Hz tuning fork and a 10g monofilament (absence of perception of the Semmes Weinstein monofilament at 2 of 10 standardized plantar sites on either foot).

Ulcers were assessed for signs of infection (swelling, exudates, surrounding, cellulitis, odor, tissue necrosis and crepitation) and size was determined by multiplying the longest and widest diameters expressed in cm^2. Each patient was included only once in the study. All cases were monitored until discharged from the hospital. All the subjects gave informed consent and clearance was obtained from the hospital ethics committee.

Microbiological Methods

The microbiological methods described by Gadepalli *et al.* (2006) as adopted in our previous studies (Zubair *et al.,* 2010b, c) were used. Total transfer time to the laboratory was not more than 30 minutes. Direct microscopic examination of ulcer sample was performed and all the bacterial isolates were identified to the species level using standard identification techniques (Collee *et al.*, 1996).

Susceptibility Testing

Antimicrobial susceptibility testing was performed as described by the CLSI and adopted by us elsewhere (Zubair *et al.,* 2010b,c). Antimicrobial discs used were Aztreonam (30µg), Imipenem (10µg), Amoxyclav (30µg), Cefpodoxime (10µg), Cefepime (30µg), Cefoperazone (75µg), Cefoperazone/sulbactam (75/10µg), Cefixime (5µg), Piperacillin (100µg), Ceftazidime (30µg), Piperacillin/tazobactam (100/10µg), Ceftazidime/clavulanic acid (30/10µg), Amoxycillin (20µg), Cephotaxime (30µg), Cephotaxime/clavulanic acid (30/10µg), Ceftriaxone (30µg), Cephoxitin (30µg), Amikacin (30µg), Chloramphenicol (30µg), Gentamicin (10µg), Gatifloxacin (5µg), Ofloxacin (5µg), Levofloxacin (5µg). All discs were obtained from Hi-Media Laboratory, Mumbai, India. Interpretative criteria for each antimicrobial tested were those recommended by manufacturer's guidelines (Hi-Media Labs, Mumbai, India).

Biofilm Assay - Tissue Culture Plate (TCP) method

The biofilm assay described by Mathur *et al*. (2006) was adopted. Stated briefly, 10 ml of trypticase soy broth (TSB) with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar. The TSB broth was incubated at 37^{o}C for 24 hours. The culture was further diluted 1:100 with fresh medium and flat bottom tissue culture plates (96 wells) were filled with 200µl of diluted cultures individually. Uninoculated

sterile broth served as blank. Similarly, control organisms were also diluted and incubated. The culture plates were incubated at 37^{o}C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 200 µl of phosphate buffer saline (pH 7.2) four times to remove free-floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly. Optical densities (OD) of stained adherent biofilm were obtained with a micro ELISA auto-reader at wavelength of 570 nm. Experiments were performed in duplicate and the average of OD values of sterile medium were calculated and subtracted from all test values.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined in doubling dilutions from 512 µg/ml to 0.05 µg/ml (CLSI). Antibiotic powders were obtained from Hi-Media Labs, Mumbai, India, except potassium clavulanate (clavulanic acid) which was procured from the Center for Diabetes and Endocrinology, A.M.U., Aligarh.

Antibiotic Treatment

Antibiotics were selected according to published recommendation (Hartemann-Heurtier *et al*., 2009). In mild infections amoxicillin clavulanic acid was given empirically by the oral route. However, in moderate infections intravenous route was preferred taking into consideration the likelihood of osteomyelitis. Considering that the causative agent was polymicrobial, we initiated ampicillin-sulbactam plus an aminoglycoside/quinolone or piperacillin-tazobactam or ceftriaxone plus metronidazole/clindamycin. In the presence of severe infections, surgical debridement and amputation were performed immediately after admission. Metronidazole (500 mg I.V. every 8 hours) was added to the drug regimen if cellulitis or gangrene was also present. The treatment was later modified in accordance with the culture results. The duration of the treatment was at least 4-6 weeks and prolonged in cases of osteomyelitis. All patients also received an intensive insulin treatment.

Statistical Analysis

The data was analyzed using SPSS version 17.0 for descriptive statistics. Quantitative variables were expressed as mean±sd while qualitative variables were expressed as percentage (%). Continuous variables were compared using 2 sample *t tests* for independent samples. Odds ratios and 95% confidence interval (CI) were reported for independent variables associated with the outcome variable: presence of anaerobic infection.

Results

Clinical

Males were predominant 42(73.6%) in the study subjects. Majority 44(77.1%) of subjects had T2DM. The mean age of the subjects was 49.1±12.4 years. The mean duration of diabetes was 12.6±6.4 years. Thirty-four patients (59.4%) had neuropathy, 35(61.4%) nephropathy, 32(56.1%) retinopathy, and 33(57.8%) were hypertensive. Osteomyelitis was present in 18(31.5%) subjects. Majority (77.0%) of the DFU patients were from Meggit Wagner grade II to grade IV. Grade I ulcer was found in 8.7%, Grade II in 14%, Grade III in 28%, Grade IV in 35%, and Grade V in 8.7% of patients. Majority of the subjects 31(54.3%) had lesions for >1 month before presentation at the hospital. The ulcer was necrotic in 25(43.8%) cases. Glycemic control was poor in 67(65.6%). HbA1c was <7% in 12 patients (21%), 7%-8% in 5(8.7%) and >8% in 40(70.1%) subjects. More than 38(66.6%) received surgical treatment, mainly in the form of debridement. 19(33.3%) patients were subject to amputation and 3(5.3%) died during the hospital stay (mean hospital stay 19.6±12.5) (Table 1). Majority of the ulcers were found on interdigits and the plantar surface (47.3% each), followed by heels (42.1%), margins (28%), malleoli (24.5%), and legs (8.7%) and on multiple (≥2 sites) 47.3%. Size of ulcer ≤4cm^2 was observed in 21% patients and ≥4cm^2 in 64.9% patients.

Microbiological Observations

A total of 97 gram-negative bacteria were isolated from 57 DFU patients, averaging 1.7 species per patient. Monomicrobial infection was observed in 32.2% patients whereas polymicrobial etiology was observed in 67.8% patients. In the direct microscopic examination of ulcer samples, 96% findings correspond with the culture growth on next day and in 4% patients, direct smear result differed with their culture growth. The frequency of bacterial isolates from DFU is shown in Table 2. *Escherichia coli* was the most common isolate, accounting for 41(42.2%), followed by *Pseudomonas aeruginosa* 23(23.7%), *Klebsiella oxytoca* 11(11.3%), *Klebsiella*

pneumoniae 9(9.2%), *Proteus vulgaris* 5(5.1%), *Acinetobacter* sp. 5(5.1%), *Proteus mirabilis* 2(2%) *and Morganella morganii* 1(1%).

Biofilm Assay

Among the 97 gram-negative bacterial isolates, 60(59.4%) were biofilm producers. A total of 80% *P. vulgaris* isolates were biofilm producers, followed by *K. pneumoniae* (77.7%), *E. coli* (63.4%), *K. oxytoca* (63.4%), *Acinetobacter* sp. (60%) and *P. aeruginosa* (52.1%). The lone isolate of *M. morganii* was a biofilm producer (Table 2).

Antibiotic Resistance Profile of BFP and BFN Isolates

The result of resistance studies are summarized in Fig. 1. High degree of antibiotic resistance was exhibited by all the BFP isolates compared with NBP. High degree of resistance by BFP isolates was observed against cefoparazone (79.6%) followed by piperacillin (68.4%), cephotaxime (67.3%), amoxyclav (64.3%), cefixime (64.3%), amoxycillin (63.3%), ofloxacin (63.3%), cefepime (59.2%), gatifloxacin (57.1%), levofloxacin (51.0%), cefpodoxime (49.0%), ceftriaxone (44.9%), ceftazidime (42.9%), amikacin and gentamicin (40.8% each), astreonam (39.8%), cephoxitin (36.7%), chloramphenicol (31.6%), imepenem (24.5%), piperacillin+tazobactum (21.4%), cefotaxime+clavulanic acid (12.2%), and Ceftazidime+clavulanic acid (9.2%).

Minimum Inhibitory Concentration (MIC)

The MIC values of the piperacillin (with/without tazobactam), cefoparazone (with/without sulbactam), ceftazidime (with/without clavulanic acid) and levofloxacin between the BFP and NBP were given in Table 3. Percentage of BFP isolates that had an MIC of ≥2µg/ml was 93.3% for cefoparazone followed 90% for piperacillin, 81.6% for ceftazidime, and 75% for levofloxacin. The isolates that had an MIC ≥2µg/ml antibiotics with inhibitor were 80% for piperacillin+tazobactum, followed by 73.3 % for cefoparazone+sulbactum and 48.3% for ceftazidime + clavulanic acid.

Correlation of Biofilm Assay and Clinical Characteristics of DFU Patients

Table 1 also shows the result of univariate analysis of factors to be associated with the presence of biofilm producing organism infections. The age distribution [O.R. 1.23, P = 0.489], Type 2 diabetes [O.R. 2.16, P<0.207], duration of ulcer >1 month [O.R. 25.7, P < 0.001] was observed in 52.6% patients having biofilm producing infection. The size of ulcer more than 4 cm^2 [O.R. 3.30, P < 0.89] was found in 64.9% patients with biofilm positive infection and in 14.0% patients having ulcer size less than 4 cm^2. The neuropathy [O.R. 7.65, P < 0.003], osteomyelitis [O.R. 3.14, P <0.136], necrotising ulcer [O.R. 14.4, P< 0.002] and poor glycemic control (HbA1c : >8%)[O.R. 1.66, P<0.32] were significantly associated with biofilm producing bacterial infection. There was a significant relation between the biofilm producing bacterial growth with Wagner's grading. Majority of the biofilm positive patients were from grade 4 [O.R. 9.12, P<0.001] followed by grade 3 [O.R. 2.56, P< 0.23], grade 2 [O.R. 2.27, P< 0.40] and grade 5 [O.R. 1.5, P< 0.68]. (Fig. 5).

Discussion

This study presents a comprehensive clinical and microbiological profile of infected diabetic foot ulcers in hospitalized patients with special reference to the study of biofilm production in the gram-negative bacterial isolates.

With the rise in the prevalence of diabetes mellitus there is increasing problem of infections, especially foot infections. According to some studies, patients with diabetic foot infections account for 20% of hospital admissions (Shankar *et al.,* 2005). India is the home for the largest number of diabetic individuals. As higher resistance is a growing problem, effort was made to study the association of different study characteristics with the presence of resistant organisms. The prevalence of diabetic foot ulcers among male subjects was found to be 73.6% against 26.3% in female i.e. a ratio of 2.3:1 which may be due to higher level of outdoor activity among males compared to females (Zubair *et al.,* 2010b,c). With increasing duration of diabetes, there is increased risk of diabetes related complications especially chronic complications like sensory neuropathy. This study also reports a high prevalence of neuropathy (59.4%). There was a marked variation of sensory neuropathy from our earlier studies (Zubair *et al.,* 2010b,c), which showed a slightly higher percentage (66.6% & 78.5%) of neuropathy in North India. Ako *et al.* (2006) in a Nigerian study, showed the increase in neuropathy to 77.8% and 56.8% in a South Indian study (Shankar *et al.,* 2005). This marked variation in the prevalence may be due to difference in the methods used for the diagnosis of these conditions (10g monofilament or biothesiometer).

In Table 1, duration of infection >1month, prior antibiotic use and ulcer size >4cm^2 were independent predictors of

infection. Thus patients with a large ulcer, with a history of prior antibiotic use and duration of infection >1month were more likely to harbor BFP organisms. In the present study, mean duration of ulcer was found to be 41.5±47.6 days with 54.3% having ulcer for more than 1 month. About 78.9% presented with a large ulcer of approximate size of >4cm^2, thereby accounting for approximately 77.1% of the patients presenting with Wagner's grade II and IV. The reasons for presentation with advanced grade and stage of ulceration could be because of lack of structured health care delivery in the country, attempted self-medication and trust in traditional healers (Boulton *et al*., 2001; Zubair *et al*., 2010b,c). Diabetic foot infections are usually polymicrobial in nature and this has been well documented in the literature. In our study also, we found polymicrobial etiology in 67.8% and monomicrobial in 32.2% patients with the rate of isolation of about 1.7 bacteria per patient which is higher than the previous reports (Zubair *et al.,* 2010a,b,c) whereas Gerding *et al*., (1995) and Gadepalli *et al*. (2006) have reported higher isolation rate of 2.0%-5.8%. The present study also confirms the high resistance among the DFU isolates which was extremely common in hospitalized patients with diabetic foot ulcers. This is in accordance with the reports of Hartemann-Heurtier *et al*. (2009) and Zubair *et al*. (2010a,b,c).

This high degree of antibiotic resistance may be due to the fact that ours is a tertiary care hospital with widespread usage of broad spectrum antibiotics leading to selective survival advantage of pathogen. Our results of antimicrobial resistance pattern were similar to the recent studies done in India and outside (Shankar *et al,* 2005; Raja *et al.,* 2007). Gram-negative bacteria that are regarded as normal flora of the skin, like *P. aeruginosa*, may cause severe tissue damage in diabetics and should never be automatically disregarded as insignificant in diabetic foot ulcers (Zubair *et al.,* 2010b).

Another reason for this high antimicrobial resistance among the BFP appears to be due to the close cell-cell contact that permits bacteria to more effectively transfer plasmids to one another than in the planktonic state. These plasmids can encode for resistance to several different antimicrobial agents (Mah and Toole, 2001). Another factor contributing to resistance is quorum sensing, which through the processes described above can force bacteria into a slow-growing state when placed in an environment with adverse growth conditions; when in this state of intermission, bacteria are less susceptible to antimicrobial attack (Mertz, 2003). The biofilm also provides a physical protection to bacteria because antimicrobial agents are also ineffective at penetrating the biofilm, decreasing the concentration acting on the bacterial cells within the biofilm and as a consequence their efficacy (Mah and Toole, 2001). In addition to the resistance to antimicrobials, biofilms also appear to have an antiphagocytic property within the biofilm, which renders leukocytes present within the matrix ineffective (Leid, 2002). Additionally, there appears to be a component within the polysaccharide that inactivates and traps both complement and host antibodies. These factors lead to an accumulation of host immune factors that can lead to host tissue damage and eventually chronic inflammation (Percival and Bowler, 2004).

The idea of disrupting a biofilm that is already formed is attractive. This could be accomplished in a number of ways, including physical methods and/or application of topical substances. Among potential physical methods, debridement, electrical stimulation, or ultrasound could be used. Debridement may not only remove the bacteria and biofilm but also may aid in the removal of necrotic tissue for which the bacteria would thrive on. Electrical stimulation has been used over the years to assist penetration of various topical agents but have a limited application (e.g., electroporation and electrophoresis have been shown to enhance the penetration of a photosensitizer) (Johnson and Oseroff, 2002).

Changing the perspective about chronic infectious disease to include biofilm enables two important insights. First, it opens new methods for detection and treatment. Second, it provides a global reconceptualization of many chronic infectious diseases as resulting from a biofilm, allowing biofilm principles to be shared across disciplines. Recent studies have investigated new methods for detecting the components of a biofilm. Several investigations have used modern molecular methods, such as denaturing gradient gel electrophoresis and denaturing high performance liquid chromatography, along with imaging techniques including fluorescent in situ hybridization. Also, molecular methods such as polymerase chain reaction (PCR) and pyrosequencing in conjunction with conventional culture methods have been used to determine the bacterial species composition of chronic infections (Dowd *et al.*, 2008). Performing molecular tests as part of routine bacterial analysis is becoming a real option for clinical laboratories. These tests could include

methods such as PCR, reverse transcriptase–PCR, microarrays, antigen testing, and rapid sequencing. Only a few of these methods are being used to test for certain pathogens, but culture-free identification of all pathogens and their corresponding resistance markers may soon become routine (Espy *et al.*, 2006). A biofilm focus also provides new strategies for treatment of chronic infections. Biofilm-based treatments might block initial bacterial attachment to a surface, block or destroy EPS formation, interfere with cell-cell signalling pathways, and use bacteriostatic or bactericidal agents at the same time. Concomitant therapies that not only attempt to eradicate bacteria but also affect the biofilm's community structure and communications may prove more effective than a single or sequential strategy such as antibiotic therapy (Ehrlich *et al.*, 2005). This multimodality approach to therapy is commonly used in other areas of medicine, such as the treatment of human immunodeficiency virus for which combination antiretroviral therapy is used to achieve the best clinical outcome.

Conclusion

Diabetic foot infections are a significant burden on patients as well as a burden on the health care delivery system. It is important to quickly and effectively identify and treat these ulcers and prevent complications. Biofilm formation on these wounds may be responsible for the chronicity of these wounds and for their common infectious complications. The presence of biofilm also represents an important barrier to effective treatment. Although in vitro study of novel approaches to control or eradicate biofilm formation are being performed, *in vivo* testing is necessary because various factors (e.g., wound fluid, proteases, growth factors, and so forth) need to be taken into consideration to determine the true efficacy of these agents. Treating the DFU by shifting from the planktonic model of microbiology to the biofilm model makes available new methods for detection and treatment. Because of molecular methods, science now has the ability to detect biofilms and understand the implications of interspecies chaos that contribute to infections. With these new scientific approaches along with coordination of clinical and laboratory efforts, education, and research, it is possible to imagine overcoming much of biofilm disease.

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Table 1: Demographic presentation of DFU patients in response to biofilm assay positive and negative bacterial infections (mean±sd and n(%) of otherwise indicated).

N=57	Total	Biofilm + (n=44)	Biofilm – (n=13)	P-value	OR(95%CI)
Sex					
Male	42(73.6)	32	10	0.532	0.8(0.18-3.4)
Female	15(26.3)	12	3		
Age distribution (years)	49.1±12.4	44.6±7.3	54.3±10.2		
<40	10(17.54	7(12.2)	3(5.2)		
41-60	33(57.8)	26(45.6)	7(12.2)	0.489	**1.23(0.35-4.3)**
>61	14(24.5)	11(19.2)	3(5.2)		
Type of Diabetes					
Type 1	14(24.4)	9(15.7)	5(8.7)		
Type 2	44 (77.1)	35(61.4)	9(15.7)	0.207	**2.16(0.57-8.0)**
Duration of Ulcer	41.5 ± 47.5	39.6±2.6	22.7±1.0		
< 1month	26 (45.6)	14(24.5)	12(21.0)		
>1 month	31(54.3)	30(52.6)	1(1.7)	**0.0001**	**25.7(3.0-217.7)**
Hospital stay(days)	19.6 ± 12.5	20.6±12.3	9.2±10.2		
≤20	19(33.3)	10(17.5)	9(15.70		
20-40	24(42.1)	22(38.5)	2(3.5)	0.46	**1.22(0.42-3.5)**
>40	14(24.5)	12(21.0)	2(3.5)		
Ulcer Grade (Wagner)					
grade 0	3(5.2)	0(0)	3(5.2)	-	-
grade 1	5(8.7)	0(0)	5(8.7)	-	-
grade 2	8(14)	7(12.2)	1(1.7)	0.40	**2.27(0.25-20.3)**
grade 3	16(28)	14(24.5)	2(3.5)	0.23	**2.56(0.5-13.1)**
grade 4	20(35)	19(33.3)	1(1.7)	**0.001**	**9.12(1.08-76.3)**
grade 5	5(8.7)	4(7.0)	1(1.7)	0.68	**1.2(0.12-11.7)**
Status					
discharge	54 (94.7)	41(71.9)	12(22.2)		
Dead	3 (5.3)	2(3.5)	1(1.7)	0.656	**0.878(0.08-9.2)**
Treatment					
conservative	38(66.6)	30(52.6)	8(14.0)		
amputation	19 (33.3)	14(24.5)	5(8.7)	0.447	**0.74(0.28-2.6)**
Diabetes duration(years)	12.6 ± 6.40	14.9±2.6	7.6±2.7		
Size of ulcer	20.14 ± 44.85	19.2±3.7	9.8±2.6		
≤4 cm^2	12 (21)	7(12.2)	5(8.7)		
>4 cm^2	45 (78.9)	37(64.9)	8(14.0)	0.89	**3.30(0.83-13.1)**
Complications					
neuropathy	38(66.6)	34(89.4)	4(10.5)	**0.003**	**7.65(1.9-30.1)**
nephropathy	35(61.4)	27(77.1)	8(22.8)	0.627	0.49(0.27-3.54)
retinopathy	32(56.1)	22(68.7)	10(31.2)	0.078	0.30(0.07-1.24)
hypertension	33(57.8)	24(72.7)	9(27.2)	0.269	0.53(0.14-1.99)
osteomyelitis	18(31.5)	16(88.8)	2(11.1)	0.136	**3.14(0.61-15.9)**
Nature of Ulcer					
necrotising	25(43.8)	24(96)	1(4)	**0.002**	**14.4(1.72-120)**
non-necrotising	32(56.1)	20(62.5)	12(37.5)		
Body Mass Index	20.59±4.41	20.3±2.1	18.6±1.8		
Plasma Glucose					
fasting	174.28±85.33	184.7±24.7	142.4±2.8		
postprandial	222.72±92.18	238.4±32.7	187.4±12.7		
HbA1c %	10.11±2.50	10.7±1.7	7.1±2.5		
<7 %(good control)	12(21.0)	9(15.7)	3(5.2)		
7-8 % (fair control)	5(8.7)	3(5.2)	2(3.5)		
>8 % (poor control)	40(70.1)	32(56.1)	8(14.0)	0.32	**1.66(0.45-6.11)**

Table 2: Gram-negative bacilli isolated from 57 diabetic foot ulcers (N=97).

	Name of DFU isolates	Biofilm assay		Total
		Positive	***Negative***	
1	*Escherichia coli*	26(63.4)	15(36.5)	**41(42.2)**
2	*Pseudomonas aeruginosa*	12(52.1)	13(47.9)	**23(23.7)**
3	*Klebsiella oxytoca*	7(63.6)	4(36.4)	**11(11.3)**
4	*Klebsiella pneumoniae*	7(77.7)	2(22.3)	**9(9.2)**
5	*Proteus vulgaris*	4(80)	1(20)	**5(5.1)**
6	*Proteus mirabilis*	-	2(100)	**2(2.0)**
7	*Acinetobacter sp*	3(60)	2(40)	**5(5.1)**
8	*Morganella morganii*	1(100)	-	**1(1.0)**
	Total	**60(59.4)**	**37(38.1)**	**97**

Fig. 1: Average resistance percentage of biofilm positive and negative gram-negative DFU isolates tested against various antibiotics.

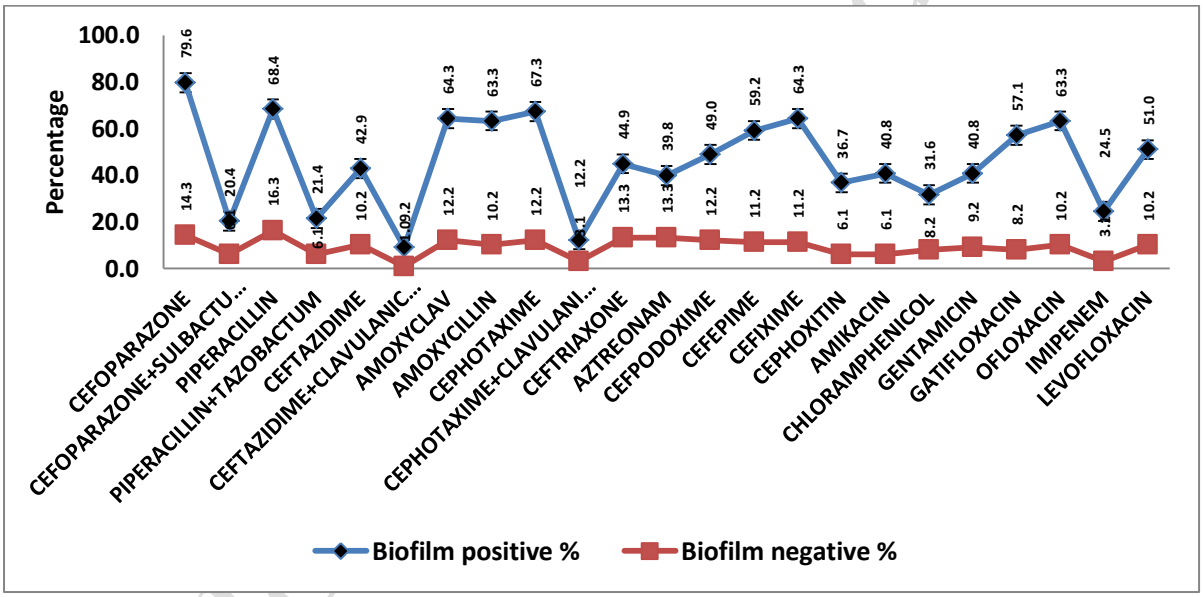


Table 3: MIC of gram-negative bacilli (GNB) isolated from 57 DFU patients (N=97).

MIC	Biofilm producers	Non-biofilm producers
	≥2µg/ml	**≥2µg/ml**
Piperacillin	54(90)	6(10)
Piperacillin+Tazobactum	48(80)	12(20)
Cefoparazone	56(93.3)	1(6.7)
Cefoparazone+Sulbactum	44(73.3)	16(27)
Ceftazidime	49(81.6)	11(18.4)
Ceftazidime+Clavulanic acid	29(48.3)	31(51.6)
Levofloxacin	45(75)	15(25)

Fig. 2: Fasting and postprandial blood glucose level among DFU patients having infection with the biofilm producing and non-producing gram-negative bacterial infections at the time of admission and discharge from the hospital.

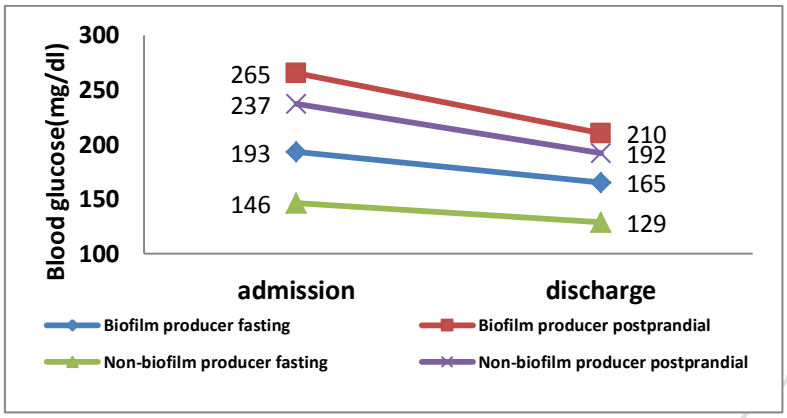


Fig. 3: HbA1c values among the DFU patients having infection with the biofilm producing and non-producing gram-negative bacterial infections at the time of admission and discharge from the hospital.

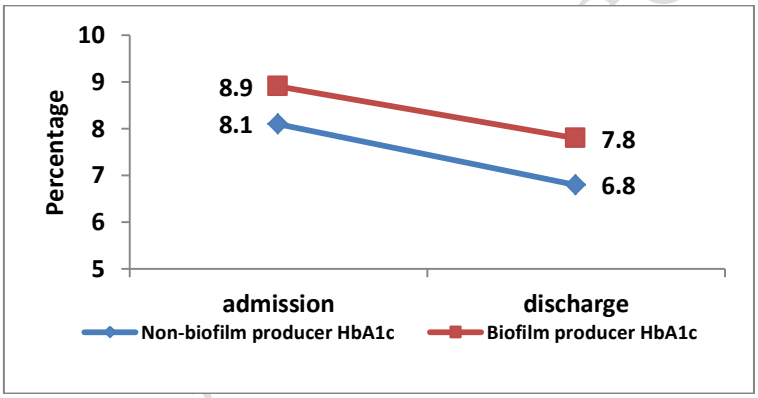


Fig. 4: Tissue culture plate showing the result of biofilm assay, A1 and B1 were blank.

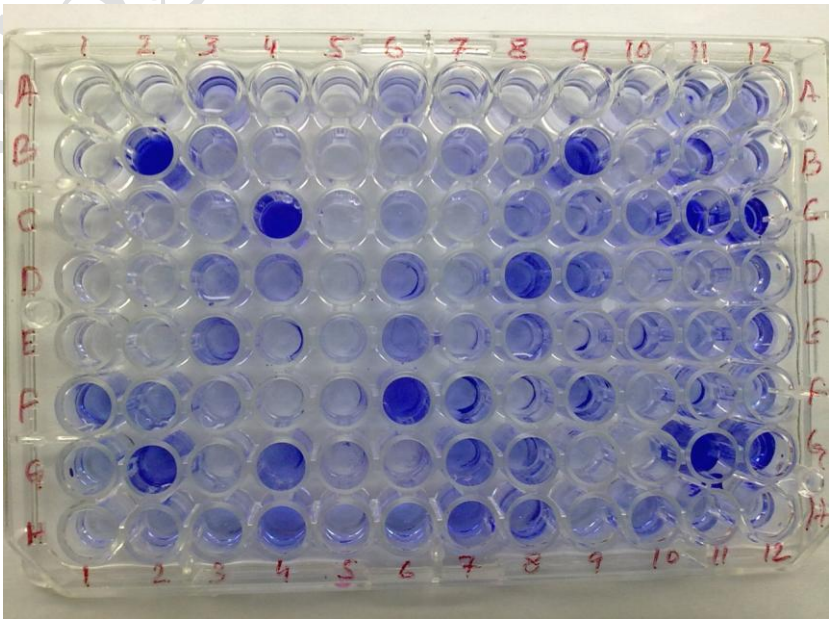
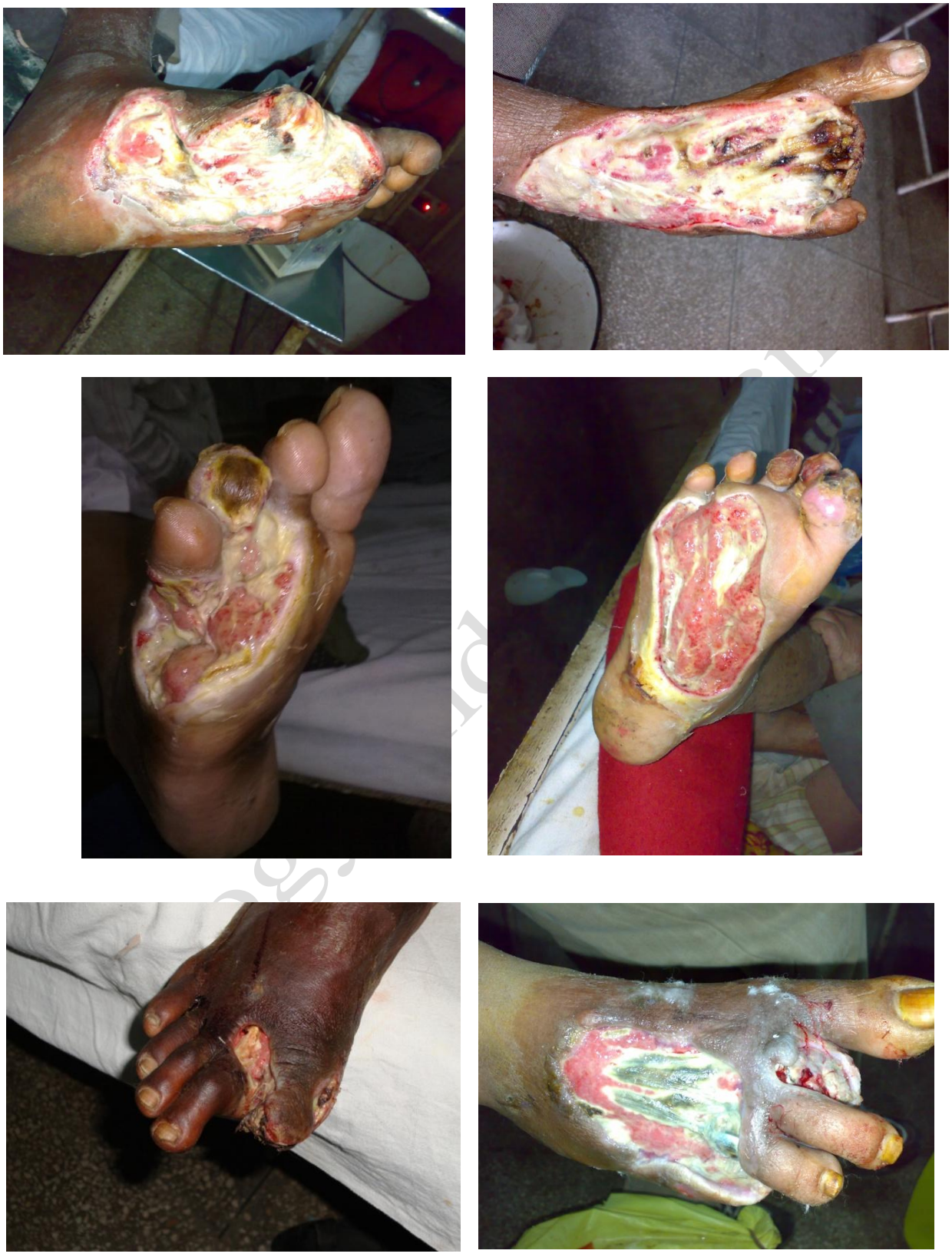


Fig. 5: Images of Diabetic Foot Ulcer.



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