Original Article

Phenotypic Profile and Antibiogram of Biofilm-Producing Bacteria Isolates from Diabetic Foot Ulcers in Zaria, Nigeria

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Abstract

Background: Diabetic foot ulcers (DFUs) present with high morbidity and reduce patient's quality of life. There is a gross paucity of data on biofilm-producing bacteria in DFU Infection in North-Western Nigeria. The study sought to determine the biofilm-forming ability of bacteria isolates from DFUs and determine their antimicrobial susceptibility pattern in Zaria, North-Western Nigeria. **Materials and Methods:** This hospital-based cross-sectional study of patients with DFUs was conducted from June 2018 to February 2020. Consecutive biopsies were aseptically collected. Bacteria were isolated and identified using a Microgen kit. Biofilm forming ability and antibiogram of isolates were determined using microtitre plate and disk diffusion methods, respectively. **Results:** Of the 225 participants enrolled, males constituted the majority, 144 (64.0%) with 88 (36.0%) females, the median age of participants was 54 (48–60) years, and the age range was 36–77 years. A total of 172 bacteria were isolated, and 123 (71.5%) were biofilm producers. *Staphylococcus aureus* (26.7%) was the highest biofilm producer, while *Citrobacter freundii* and *Stenotrophomonas maltophilia* were the least biofilm producers, 1 (0.6%) each. A disproportionate resistance pattern was demonstrated among the biofilm and non-biofilm producers against the cephalosporins tested, ceftazidime (68% vs. 18%), ceftriaxone (50% vs. 8.0%) and cefotaxime (21% vs. 0.0%). About 46% and 68% of the biofilm producers were resistant to gentamycin and ciprofloxacin, respectively. While only 2% of the non-biofilm producers were resistant to imipenem, 11% of the biofilm producers were resistant to it**. Conclusion:** These findings revealed a high proportion of biofilm-producing bacteria and were more resistant than non-biofilm producers.

Keywords: Antibiogram, bacteria, biofilms, diabetic foot ulcers, Nigeria, Zaria

Introduction

Diabetes is a global public health disease, and diabetic foot ulcers (DFUs) are the most common, costly, and devastating complication associated with reduced quality of life, lower-limbs amputation, hospitalization, high morbidity and mortality.[1] Greater than one-third of people with diabetes develop DFUs during their lifetime, with half of these becoming infected and causing diabetic foot infections (DFI). Fifteen per cent of patients with DFI require lower limb amputation to prevent the progression of the disease.[2,3] DFUs account for most non-traumatic amputations performed in most Nigerian tertiary hospitals, and DFI is the leading cause in almost 90%.[4] Research on wound bacteria has traditionally focused on planktonic cells. However, a recent report by the National Institutes of Health has estimated that approximately 65% and 80% of acute and chronic human infections, respectively, are biofilm associated.[5] Several studies have reported a broad spectrum of planktonic bacteria related to DFU in different parts of the globe,[6] with very little information on their ability to perform biofilm.[7,8] Currently, the medical team involved in DFI management relies on the isolation, identification of bacteria in planktonic form, and antimicrobial therapy instituted accordingly. This study aimed to determine the biofilm-forming ability of bacteria

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from DFU in Zaria-Nigeria and determine their antimicrobial sensitivity pattern.

Materials and Methods

Ethical considerations

Ethical approval was sought and approved on 20th December 2017 from the Health Research Ethics Committee (HREC) of the Ahmadu Bello University Teaching Hospital, Zaria, with reference number ABUTH/HREC/C08/2017. Also, for the Hajiya Gabo Sawaba General Hospital in Zaria study site, ethical clearance was sought and granted on 12th July 2018 by the HREC of Kaduna State Ministry of Health and Human Services (Independence way, P.M.B 2014, Kaduna, Kaduna State) with reference number MOH/ADM/744/VOL. 1/532 before the commencement of the study. We obtained informed consent from participants before enrolment into the study. All participants' rights were observed, and no financial burden was placed on them. Participants' data were treated with the utmost confidentiality.

Study area

The study was conducted in Zaria, with patients from Ahmadu Bello University Teaching Hospital (ABUTH) and Hajiya Gambo Sawaba General Hospital. Zaria is an ancient and a major city in Kaduna State in Northern Nigeria. It has 11°04'N 7°42'E as coordinates, a total land area of 300 km^2, and a population of 408,198, by the 2006 census.[9]

Study design and participants

The study was a hospital-based cross-sectional study. The study participants were diabetic patients with DFUs presenting to the surgical outpatient clinic and medical wards of the two study sites. Selection criteria were being 18 years of age or older with a diagnosis of diabetes and complication of DFUs. Other foot ulcers unrelated to diabetic were excluded.

Sample size determination

The minimum sample size was determined using single proportion formula below:

$N = Z2Pq/d2$.

where,

N = Minimum sample size

$Z2$ = Standard Normal deviate set at 1.96

P = Prevalence rate of 46.0% (0.46) was recorded according to Banu *et al*.[7]

d = acceptable error of 10% (0.1)

Thus, the minimum required sample size was calculated to be 95.

However, 225 participants were enrolled to increase the statistical power of the study. Participants were enrolled purposively. Consecutive non-duplicate participants were recruited from 1st June 2018 to 20th February 2020.

Sample collection

Following aseptic measures, a sterile surgical blade was used to collect biopsies from the participants' ulcers and placed into a falcon tube containing normal saline. The samples were immediately transported to the medical microbiology laboratory of the Ahmadu Bello University Teaching Hospital, Zaria, Nigeria, for laboratory analyses.

Microbiological isolation and identification of bacteria

The biopsy tissue was minced using a sterile surgical blade, centrifuged and the supernatant discarded. The deposit was inoculated onto blood, and MacConkey agar was incubated at 37°C for 24–48 h to cater for slow-growing bacteria. Standard microbiological technique was used in the identification of the isolates demonstrated by Carvalho *et al*.[10] The bacterial isolates were preserved at −70°C in Tryptone-Glycerol broth.

Phenotypic detection of biofilm production

The tissue culture plate method was employed in the determination of biofilm production.[11] A colony was picked from a new subculture, and a suspension was made using tryptone soy broth with one per cent glucose. The suspension was diluted to make a 1: 100 dilution. A sterile pipette was used to pick 200 µl of the suspension and dispences into 96 sterile microtitre wells. It was then incubated for good 24 h at 37°C. After that, the plates were washed thoroughly five times using Phosphate buffered saline. It was allowed to be fixed by air drying. 200 µl of crystal violet was applied to staining the plates and allowed for 15 min at room temperature. This was followed by washing using PHS; P.H. 7.2. Ethanol was finally applied for 30 min. The absorbance was read using Vitek Microplate Reader at a wavelength of 570 nm. The analysis was performed in triplicate, and average values were taken.). Optical density cut-off (ODc) was determined. O.D. of 0.133 was considered as non-biofilms. >/=0.113 = weak biofilms producer. 0.227–0.452 = Moderate and O. D. of >0.453 were considered as Strong biofilm.[11]

Antimicrobial susceptibility tests

The Kirby–Bauer disk diffusion method of antimicrobial susceptibility testing was performed by overnight culture on Mueller Hinton agar (Oxoid) plates.[12] The following antibiotic discs were used in this study: ciprofloxacin (CIP), penicillin (P), ceftazidime (CAZ), ceftriaxone (CTN), trimethoprim/sulfamethoxazole, gentamicin (G.N.), cefoxitin (CTN), imipenem (IPM), amoxicillin, (AMC), amoxicillin-clavulanic acid (AML) and erythromycin (E), and all were purchased from Oxoid (U.K.). After overnight incubation at 37°C, zones of inhibition were measured and reported using the CLSI guidelines.[12] A *Pseudomonas aeruginosa* standard strain was used as quality control (ATCC 27853).

Statistical analysis

Data were analysed using Prism-GraphPad version 8 (San Diego, CA, USA). Results were presented as frequencies,

percentages and confidence intervals (in some instances). Tabular presentations of bacterial isolates, biofilm production and antibacterial resistance were presented.

Results

Of the 225 participants enrolled, males constituted the majority, 144 (64.0%) with 88 (36.0%) females, median (IQR) age of participants was 54 (48–60) years, and the age range was 36–77 years. The sociodemographic features of the subjects are shown in Table 1. Of 152/225 (67.6%) participants, a total of 172 bacteria were isolated, with 151 causing mono-microbial infections and 21 being part of poly-microbial infections. The highest isolated organism was *Staphylococcus aureus* 46 (27%) followed by *P. aeruginosa* 31 (18.0%), with *Stenotrophomonas maltophilia* 1 (0.6%) as the least occurring bacteria [Table 2]. Out of the 172 isolates, 123 (71.5%) were biofilm producers. *S. aureus* (26.7%) was the highest biofilm procuring organism, followed by pseudomonas while *Citrobacter freundii* and *S. maltophilia* were the least biofilm producers with 1 (0.6%) each [Table 2].

Furthermore, the biofilm-producing bacteria were classified as weak, moderate or strong biofilm producers based on the concentration of optical density in the microtitre wells. Eighteen (14.6%), 40 (42.5) and 65 (52.8) were found to be weakly, moderately and strongly form biofilm, respectively. Table 3 shows the distribution of the different biofilm-producing bacteria according to the degree of biofilm formation. Figure 1 shows the antibiogram of biofilm and non-biofilm bacterial isolates, respectively. High level antimicrobial resistance to penicillin, amoxicillin and amoxicillin-clavulanate was recorded (>80%). Conversely, a disproportionate resistance pattern against ceftazidime was found among the biofilm and non-biofilm producers (68% vs. 18%). About 46% and 68% of the biofilm producers were resistant to gentamycin and ciprofloxacin, respectively. While only 2% of the non-biofilm producers were resistant to imipenem, 11% of the biofilm producers were resistant to it.

Table 1: Sociodemographic characteristics of study participants

Characteristic	Frequency (n=225), n (%)	95% CI
Sex		
Male	144 (64.0)	57.4-70.2
Female	81 (36.0)	29.7-42.6
Age group		
36-45	30 (13.3)	9.2-18.5
46-55	98 (43.6)	37.0-50.3
56-65	70 (31.1)	25.1-37.6
66-75	21 (9.3)	5.8-13.9
≥76	6 (2.7)	1.0-5.7
Occupation		
Business	61 (27.1)	21.4-33.4
Civil servants	89 (39.6)	33.1-46.3
Farmers	49 (21.8)	16.6-27.7
Others	26 (11.6)	7.7-16.5
Residence		
Urban	146 (64.9)	58.3-71.1
Rural	79 (35.1)	28.9-41.7
Tribe		
Hausa	99 (44.0)	37.4-50.6
Fulani	27 (12.0)	8.1-17.0
Igbo	3 (1.3)	0.3-3.8
Yoruba	23 (10.2)	6.6-14.9
Others	73 (32.4)	26.4-39.0
Level of education		
None	7 (3.1)	1.3-6.3
Primary	24 (10.7)	7.0-15.5
Secondary	81 (36.0)	29.7-42.6
Tertiary	89 (40.0)	33.1-46.3
Qur'anic	24 (10.7)	7.0-15.5

Univariate analysis with 95% CI. CI: Confidence interval

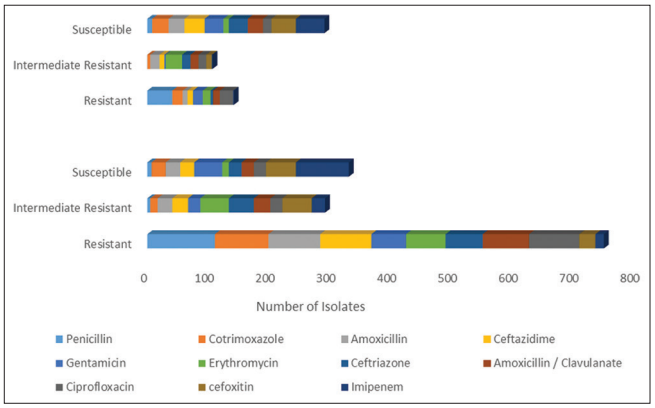

Figure 1: Antibiogram profile of bacterial isolates from participants with diabetic foot ulcers. NB: The first three composite bars are for biofilm-producing isolates while the last three for non-biofilm producers

Discussion

Bacterial biofilm is a major factor that impaired in wound healing, and high levels of biofilm production have been repeatedly described in multidrug-resistant bacteria.[13]

Among patients with DFUs, the present study found more males than the female counterpart. In our study, males were 64.0% which is similar to the findings of Jeffcoate *et al.* and Malepati *et al*., who also reported a higher incidence of DFI in males with 67% and 71.7%, respectively.[14,15] The male population was more exposed to harder work than females, which posed a greater risk of trauma in their means of searching for livelihood.[14] In this study, a total of 152 patients out of the 225 enrolled participants had culture-positive results (67.6%). Of which 151 as monomicrobial infection and 21 yielded polymicrobial isolates. This reveals the role of bacteria in the pathogenesis of DFUs. The relatively high bacterial load identified from this study is consistent with the many other previous reports.[16-19] This observation could be because DFUs are mainly exposed to skin commensal bacteria that can colonize the wound as multi-layered microbial communities surrounded by a self-produced protective extracellular biofilm.[20] However, this finding is lower than the prevalence

Table 2: Biofilm and non-biofilm producing bacterial isolates from diabetic foot ulcers participants

Bacteria	Biofilm producers, *n* (%)	Non-biofilm producers, *n* (%)	Total, *n* (%)
Staphylococcus aureus	35 (20.3)	11 (6.4)	46 (26.7)
Pseudomonas aeruginosa	25 (14.5)	6 (3.5)	31 (18.0)
Escherichia coli	20 (11.6)	5 (2.9)	25 (14.5)
Acinetobacter baumannii	10 (5.8)	4 (2.3)	14 (8.1)
Proteus mirabilis	9 (5.2)	2 (1.2)	11 (6.4)
Klebsiella oxytoca	7 (4.1)	3 (1.7)	10 (5.8)
Morganella morganii	7 (4.1)	1 (0.6)	8 (4.7)
Klebsiella pneumoniae	3 (1.7)	5 (2.9)	8 (4.7)
Enterococcus faecalis	3 (1.7)	4 (2.3)	7 (4.1)
Citrobacter freundii	1 (0.6)	5 (2.9)	6 (3.5)
Proteus vulgaris	2 (1.2)	3 (1.7)	5 (2.9)
Stenotrophomonas maltophilia	1 (0.6)	0	1 (0.6)
Total	123 (71.5)	49 (28.5)	172 (100)

Table 3: Degree of biofilm formation by bacterial isolates from diabetic foot ulcers participants

Bacteria	Weak, *n* (%)	Moderate, *n* (%)	Strong, *n* (%)	Total, *n* (%)
Acinetobacter baumannii	0	1 (0.8)	9 (7.3)	10 (8.1)
Citrobacter freundii	0	1 (0.8)	0	1 (0.8)
Escherichia coli	5 (4.1)	5 (4.1)	10 (8.1)	20 (16.3)
Enterococcus faecalis	1 (0.8)	0	2 (1.6)	3 (2.4)
Klebsiella oxytoca	2 (1.6)	3 (2.4)	2 (1.6)	7 (5.7)
Klebsiella pneumoniae	0	1 (0.8)	2 (1.6)	3 (2.4)
Morganella morganii	0	2 (1.6)	5 (4.1)	7 (5.7)
Proteus mirabilis	0	2 (1.6)	7 (5.7)	9 (7.3)
Proteus vulgaris	0	1 (0.8)	1 (0.8)	2 (1.6)
Pseudomonas aeruginosa	3 (2.4)	9 (7.3)	13 (10.6)	25 (20.3)
Staphylococcus aureus	7 (5.7)	14 (11.4)	14 (11.4)	35 (28.5)
Stenotrophomonas maltophilia	0	1 (0.8)	0	1 (0.8)
Total	18 (14.6)	40 (32.5)	65 (52.8)	123 (100)

reported from previous studies with a range between 74% and 88%.[21,22] The differences in bacterial isolation proportions from one place to another or within the same location might be due to different factors including geographic variation, inadequate awareness for personal sanitary measures, specimen collection and transportation methods, bacteriological media used for bacteria isolation, differences in study period, and case inclusion criteria. Furthermore, it could be that most of our participants presented late to the hospital, as this may explain the relatively high bacterial colonization of most DFUs patients.

From our findings, monomicrobial cultures accounted for a greater percentage than those with polymicrobial cultures. This is similar to a previous report by Amaefule *et al*. and Tiwari *et al*.[18,23] However, this is contrary to the reports that DFI is known to be predominantly polymicrobial, especially in severe/late cases in South-Eastern Nigeria.[24] Improved culture techniques and the use of nucleic acid-based techniques for isolating organisms is another plausible reason. The polymicrobial nature of DFIs has been observed in various studies within and outside the country.[23,25] The monomicrobial feature of the DFI could be associated with antimicrobial treatment and a longer duration of the DFU. At first, the infection starts as monomicrobial, but later on, it progresses to encompasses multiple microbes. In addition, ulcers that are shallower and that have a lesser degree of necrosis tend to be monomicrobial.[7] It is worth mentioning that reports have it that total eradication of polymicrobial microbes is not a prerequisite for a significant prognosis in DFU healing. As observed in this study, antimicrobial treatment during sampling might have accounted for the polymicrobial state of the DFI.

Conversely, studies have suggested that the communication of organisms within biofilms might results into the expression of virulence factors, such as enzymes and short-chain fatty acids that cause inflammation, halts wound healing process, and contribute to the persistent of the infection.[26] So, due to the formation of extracelular matrix of biofilms, penetration of antimicrobial agents is impeded into the infected site. Therefore, the presence of multiple species can have important clinical implications that should not be overlooked. In this study, Gram-positive bacteria were the predominant pathogens, with *S. aureus* being the commonest aerobic isolate. Similarly, the predominance of *S. aureus* has been demonstrated in many

studies within and outside the country.[19,25,27-29] In addition, this agrees with the results of Perim *et al*., Nageen, and Pradeep *et al*.[30-32]

Out of the 172 isolates, 71.5% were biofilm producers. This is consistent with prior studies in which range from 73% to 78.2%.[33-35] Other studies by James *et al.*[36] Banu *et al*.[7] and Lakshmi *et al.,*[8] Biofilm producing bacteria isolates were 60% and 46.3% 42.5% in DFUs. These differences could be a result of proper removal of dead tissues of the DFU (debridement) or lesser duration of ulcers in the subjects. *S. aureus* was the most prevalent biofilm producer. This is an expected result, with existing literature supporting the biofilm-forming nature of Staphylococci.[36,37] *S. aureus* is followed by *P. aeruginosa*. Studies have reported *P. aeruginosa* to form biofilms more readily in the diabetic wound environment.[38] In addition to *S. aureus* being the most recovered bacteria, it was also the highest biofilm procuring organism. A similar observation was reported by Ibrahim *et al*. in North-Eastern Nigeria.[19] Biofilm formation is a heterogeneous property amongst clinical strains and is associated with bacterial species and certain clonal types. *S. aureus* biofilm is a multilayered biofilm embedded within a glycocalyx or slime layer of the glycocalyx as primarily composed of teichoic acids and Staphylococcal host proteins.[39] The result of this study has a serious implication on the patients and health care system because biofilm-producing bacteria have been shown to be resistant to most antimicrobial agents, antiseptics, biocides, and host immunity. This worsens prognosis and increases morbidity and mortality of infected patients.

Bacteria isolates were found to be multidrug-resistant among DFU cases in this work, and this might be as a result of antimicrobial abuse in which there is a high rate of unrestricted access to antimicrobials in developing countries, including Nigeria. This was also observed in some studies conducted in other developing nations.[40] In contrast, there are findings of many studies from developed countries, including France, which reported a low prevalence of bacteria that were multidrug-resistant among DFI subjects.[41] A significant proportion of aerobic bacteria isolated in this study was found to be multidrug-resistant when compared to reports from Asia and sub-Saharan Africa with soaring MDR rates particularly *P. aeruginosa*, *S. aureus* and *Escherichia coli*.[31,42-44] There was a huge resistance rate of most of the bacterial isolates to antibiotics, particularly β-lactamases which give resistance to cephalosporins and penicillin, especially in the biofilm-producing *Enterobacteriaceae* and included *E. coli, Klebsiella*, and *Citrobacter* species. The burden of ESBL producing gram-negative bacteria is colossal among patients with DFI, especially in resourced constrained countries, with a reported prevalence rates ranging from 23% to 49% across Asian and African continents.[40,45-48] Antimicrobial resistance to ciprofloxacin appeared to be the most common after all beta-lactam antibiotics, 68% and 45% in biofilm and non-biofilm producers, respectively. A similar finding was reported by Pontes *et al*.[49] Although these percentages refer to bacterial resistance demonstrated *in vitro*, these data point to the need to adapt the empirical antibiotic therapy initially used for the treatment of patients with the infected diabetic foot at the studied hospitals.

The close proximity of bacterial cells in biofilms has been reported to be one of the mechanisms of resistance to antimicrobials. This allows for easy transfer of mobile genetic elements like plasmids containing multidrug resistance genes from one bacteria to another, delayed penetration of antimicrobial agents through the biofilm matrix, an altered growth rate of biofilm-forming bacteria, long-term persistence of bacteria in various environments surfaces, decreased bacterial growth rate in a biofilm, and restricted penetration of antibiotics into the biofilm.[50-52] Unfortunately, antibiotic resistance in biofilm-producing bacteria still remains a major public health burden among DFU infections, as it makes treatment outcomes poor and worsens patients' prognosis.

The few limitations of this study were that genotyping of key resistant, antimicrobial, anaerobic culture, and visualization of biofilms were not performed due to logistical issues.

Conclusion

This study revealed a high proportion of biofilm-producing bacteria colonizing DFUs and were more resistant compared to their planktonic counterpart. Furthermore, the high prevalence of Gram-negative bacilli in DFI, resistance to beta-lactams, imipenem, and fluoroquinolone emphasizes the need for continuous monitoring of antibiotics resistance patterns of the frequently found isolates in chronic wound ulcers and should be taken into consideration when choosing empiric antibiotic therapy. Therefore, additional screening of multidrug-resistant organisms often associated with biofilms should be considered. Detection of biofilm formation by bacteria will ultimatetly assist the medical team to properly managing these infections with appropriate antibiotics that will significantly decrease morbidity and mortality.

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Conflicts of interest

There are no conflicts of interest.

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