



## Research Article

# Antibiogram and Plasmid Profile analysis of Bacterial Strains from Diabetic Skin Ulcers

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### ABSTRACT

A diabetic skin ulcer (DSU) is an injury found on the skin of diabetics which affects both the surface and deeper tissue of the skin leading to disintegration. Infections on diabetic skin ulcers are usually caused by an array of bacterial strains; therefore, appropriate management of the infections involves choosing the appropriate antibiotics to treat the wounds. Swabs samples from the wounds were collected using swab sticks. The isolates were cultured using standard microbiological techniques. Antibiotics susceptibility of the bacterial isolates was determined using the modified Kirby-Bauer disc diffusion method. The multidrug-resistant bacteria were subjected to plasmid profiling and curing to determine if the resistances displayed were plasmid or chromosomally mediated. The Gram-positive bacteria showed the highest susceptibility to gentamycin (71.33 %) and were least sensitive to amoxicillin (21.11 %); while the Gram-negative bacteria showed the highest susceptibility to ciprofloxacin (78.83 %) and were least susceptible to augmentin (26.08 %). *Staphylococcus aureus*, *Alcaligenes faecalis* and *Acinetobacter* sp. lost their antibiotic-resistant plasmid markers after acridine orange mediated curing while *E. coli* and *K. pneumoniae* retained their markers.

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## INTRODUCTION

Diabetes mellitus (DM) is a health condition which is on the increase and has become a major challenge, especially in developing countries like Nigeria (Raimi and Fasanmade, 2018). It is rising rapidly as a major global health issue which threatens to reach pandemic numbers by 2030. The total number of diabetics worldwide has been projected to escalate from 382 million in 2013 to 592 million by 2035 (Guariguata *et al.*, 2014). Diabetes is responsible for about 1.5 million deaths annually as well as its associated complications like heart attack, stroke, blindness, kidney failure and lower limb amputation (WHO, 2022).

Diabetic skin ulcers (DSU) are the most dreadful and difficult to treat complications of diabetes mellitus. About one-fifth of the total population of diabetics stands a risk of having a diabetic skin ulcer in their lifetime (Mathew and Suchithra, 2014). Generally, diabetics have a 0.25 probability of having diabetic skin ulcers in life; most of which could be infected (Spinchler *et al.*, 2015). In urban and rural Nigerian populations, the occurrence of diabetic skin ulcer is reported as 0.8 % to 11 % (Edo and Eregie, 2007). Worldwide, diabetic skin ulcers have become the major cause of non-traumatic extremity amputations. Increased incidence of infection in the wound further contributes to the complications. Reports show that diabetic skin ulcers are the cause of 85 % of amputation cases (Boulton *et al.*, 2005).

The colonization of wounds by bacterial pathogens is a great impediment to the management of DSU (Lipsky, 2004). The increased hyperglycemia associated with diabetes activates advanced glycation of lipids and proteins which accumulate in the blood capillaries, thus resulting in decreased flow of blood and oxygen perfusion to the wound site. This impaired circulation prevents the

phagocytes' penetration thus enhancing the development of infection (Mathew *et al.*, 2015). Hence, increased susceptibility to infection is found in diabetic patients, more than in the non-diabetic persons.

Since diabetic skin ulcers are caused by myriads of microorganisms, either alone or mixed, it is pertinent to employ the most effective antibiotics in the management of the wounds to enhance proper healing. Therefore, it requires identifying the causative bacteria of the wound infection. *Medical personnel should avoid unnecessary, excessively broad-spectrum or unduly prolonged antibiotic therapy, as this may result in the development of drug-related adverse effects, incur excessive finances and support the development of antimicrobial resistance* (Richard *et al.*, 2012).

It is common knowledge that mechanisms of antibacterial action differ from one antibiotic to another and bacteria develop resistance to some antibiotics; in which case if such antibiotics are used in treating the wounds it could lead to delayed wound healing and further complications. Resistant bacterial strains develop from the misuse of antibiotics (Zubair *et al.*, 2010). These resistance traits can be transferred genetically (plasmids and horizontal gene transfer) or by mutations among the bacterial species (Raghunath, 2008; Anderson and Hughes, 2010; Bengtsson-Palme *et al.*, 2017).

Hence, the present study was focused on the determination of antibiotic susceptibility of bacterial isolates from diabetic skin ulcers and plasmid profile analysis of the antibiotic-resistant isolates (before and after curing).

## MATERIALS AND METHODS

### Sample Collection

Pus samples were collected from the wounds of 100 diabetic skin ulcer patients and 50 wound swabs from non-diabetic patients with wounds

(control) in some health facilities in Benin City (Edo State) and Asaba (Delta State).

#### **Isolation and Characterization of Bacteria**

The bacteria were isolated by streaking the overnight peptone broth cultures containing the swab inocula onto already prepared Nutrient and MacConkey agar plates and incubating for 24 hr at 37 °C. The bacteria were isolated, characterized and identified using standard bacteriological procedures based on cultural, morphological and biochemical characteristics.

#### **Antibiotic Susceptibility Test**

Antibiotics susceptibility of the bacterial isolates was tested using the modified Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966; CLSI, 2010). The antibiogram analysis was conducted against the Gram negative and Gram-positive bacteria using a total of 14 commercially available and commonly used antibiotics namely: Gentamycin (10 µg), Pefloxacin (10 µg), Tarivid (30 µg), Streptomycin (30 µg), Septrin (30 µg), Sparfloxacin (10 µg), Chloramphenicol (30 µg), Ciprofloxacin (10 µg), Augmentin (30 µg), Amoxicillin (30 µg), Ampiclox (30 µg), Zinnacef (20 µg), Rocephin (25 µg) and Erythromycin (10 µg). The results were interpreted as Susceptible (S) or Resistant (R) using the standard published by Clinical and Laboratory Standards Institute (CLSI, 2010).

#### **Determination of Multiple Antibiotic Resistance Index**

Multiple antibiotic resistance index (MAR) was determined using the formula,  $MAR = x/y$ , where x was the number of antibiotics to which the test isolate displayed resistance and y was the total number of antibiotics to which the test isolate was evaluated for sensitivity.

#### **Plasmid Profiling and Agarose Gel Electrophoresis**

The multidrug resistant bacteria were subjected to plasmid profiling and curing to determine if the resistances displayed were plasmid or chromosomal mediated. Plasmid extraction was carried out using ZymoPURE Plasmid miniprep kit following the manufacturer's instructions.

Agarose gel electrophoresis was carried out by dissolving 1.6 g of agarose gel powder in 200 ml of TBE (Tris-Borate-EDTA) buffer. The mixture was homogenized by heating for 30 min and allowed to cool to 56 °C. Five microlitres (5 µl) of ethidium bromide was then added, after which the mixture was poured on the gel mould with an inserted comb and was left to solidify for 1 hr.

TBE buffer was poured into the gel electrophoresis machine and the solidified gel was placed on the machine. A molecular ladder containing DNA of known fragment sizes was placed in the first well. Two microlitres (2 µl) of the DNA loading dye was then added to 8 µl of the plasmid template while 10 µl of the loading dye-template of each isolate was transferred into individual wells on the gel. The DNA ladder was also placed into a separate well on the gel. The negative control was also placed into another well. The gel electrophoresis machine was run at 90 mA for 1 hr (Brown, 2000). After 1 hr, the gel was taken from the electrophoresis machine and was placed in a UV transilluminator where it was read by adjusting the camera to a clear resolution.

#### **Plasmid Curing**

The plasmids were cured by treatment with acridine orange according to the method of Brown (2000), by preparing nutrient broth and supplementing it with 0.1 mg/ml of acridine orange. From the overnight bacterial culture, 20 µl was taken and sub-cultured into 5 ml of

nutrient broth which contained acridine orange, after which the samples were placed in the incubator at 37 °C for 72 hr. After 72 hr incubation, the bacterial isolates were sub-cultured onto Mueller-Hinton agar and the susceptibility test and plasmid extraction was repeated on some of the organisms to verify if the plasmids were successfully cured.

#### **Ethical Clearance**

Approved Ethical clearance was obtained from the appropriate Institutional Ethical Committees as well as informed consent and permission from the subjects used in this study.

#### **Statistical Analysis**

Frequencies and percentages were calculated for the study variables.

### **RESULTS AND DISCUSSION**

An array of bacterial strains was obtained from the diabetic skin ulcers (DSU) and non-diabetic wounds, which included *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter* spp., *Enterococcus faecalis*, *Proteus vulgaris*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Escherichia coli*, Coagulase-negative *Staphylococcus aureus*, *Klebsiella oxytoca*, *Alcaligenes faecalis* and *Citrobacter freundii* (Table 1). This finding on the bacteria that are implicated in DSU corroborates with that of Macdonald *et al.* (2021) in which *S. aureus* was the most common bacterial isolate. The colonization of the wounds by bacterial pathogens and in some cases the production of proteolytic enzymes which disrupt or cause a delay in the wound healing process (Okojie and Okotie, 2021) is a major challenge in the management of DSU. Appropriate antibiotic therapy becomes pertinent in this regard. The type, concentration of the antibiotic and the duration of the antibiotic treatment play significant roles in the healing process of the wound (Priyadarshini *et al.*, 2013). The antibiotics susceptibility results in this study revealed that the Gram-positive bacteria

(*Staphylococcus aureus*, *Enterococcus faecalis* and Coagulase-negative *Staphylococcus*) overall, showed the highest sensitivity to gentamycin (71.33 %) and were the least sensitive to amoxicillin (21.11 %). *Staphylococcus aureus* showed 68 % sensitivity to streptomycin while 56 %, 60 % and 52 % of this isolate were resistant to pefloxacin, amoxicillin and septrin respectively. The results also showed that 70 % of the *Enterococcus faecalis* strains were susceptible to gentamycin. Coagulase-negative *Staphylococcus* (CONS) showed 100 % sensitivity to streptomycin, 33.33 % to erythromycin and 0 % to gentamycin, meaning none of the Coagulase-negative *Staphylococcus* (CONS) strains was sensitive to gentamycin (Table 2). In the case of the Gram-negative bacteria, results showed that these bacteria exhibited the highest susceptibility to ciprofloxacin (78.83 %) and were least susceptible to augmentin (26.08 %). This can be compared to the study by Mathew and Suchithra (2014) in which the Gram-negative bacteria were highly sensitive to gentamycin (93.9 %) and poorly susceptible to amoxicillin (14.9 %); though in contrast to the present study, the highest sensitivity was shown by Gram-negative bacteria towards gentamycin while amoxicillin had the lowest sensitivity of 18.2 %.

Furthermore, the susceptibility pattern for Gram-negative bacteria from the diabetic skin ulcers revealed that streptomycin and ciprofloxacin were each active against 75 % of *Proteus mirabilis* strains while they also showed 75 % resistance to gentamycin and augmentin. Sensitivity to pefloxacin in the case of *Citrobacter freundii* was 100 %, 85.71 % to ciprofloxacin and 57.14 % to gentamycin, streptomycin, septrin, sparfloxacin and amoxicillin respectively. The results also revealed that 71.43 % of *Citrobacter freundii* strains were resistant to tarivid,

chloramphenicol and augmentin respectively. As for *E. coli*, 79.17 % of the strains were sensitive to tarivid, while 79.17 % showed resistance to augmentin and 90 % of *P. aeruginosa* were sensitive to sparfloxacin, while 70 % showed resistance to amoxicillin (Table 3).

Mechanisms of antibacterial action differ from one antibiotic to another. The most common mechanism is the cell wall synthesis inhibition and protein synthesis inhibition. Some fight the bacteria by damaging or altering cell membranes, inhibiting nucleic acid synthesis or through anti-metabolite activity. So, the antibiotic treatment has to be changed according to the microbes involved and their sensitivity to antibiotics (Mathew and Suchithra, 2014).

From the antibiogram analysis, the multiple antibiotic resistance indices of the bacterial isolates ranged from 0.80-1.00 as shown in Table 4. These results showed that the isolates were resistant to more than eight antibiotics. These multidrug resistant isolates were identified as *Escherichia coli*, *Staphylococcus aureus*, *Alcaligenes faecalis*, *Klebsiella pneumoniae* and *Acinetobacter* species. These isolates were subjected to plasmid profiling and curing using acridine orange and the results obtained before and after plasmid curing are shown in Table 4.

Some agents like acridine (acridine orange) can hinder plasmid replication and so the multidrug-resistant isolates were screened to determine the location (plasmid-borne or chromosome) of the resistance. After the curing experiment was carried out, it was observed that some of the resistance markers were stably lost, this is in agreement with the study by Yah *et al.* (2007). Hence, *S. aureus*, *Alcaligenes faecalis* and *Acinetobacter* sp. which were initially resistant to some antibiotics became sensitive; while the other two isolates – *E. coli* and *K. pneumoniae* remained resistant. It can therefore be explained that the resistances displayed by three of the isolates - *S. aureus*, *Alcaligenes faecalis* and *Acinetobacter* species were plasmids-mediated meaning they were transferable; while the other two isolates – *E. coli* and *K. pneumoniae* were non-plasmid mediated and are probably chromosomally mediated.

Plate 1 shows the plasmid profile of the multidrug-resistant bacteria, analyzed with 0.8 % agarose gel electrophoresis stained with ethidium bromide. All three isolates subjected to plasmid profiling namely, *S. aureus*, *A. faecalis* and *Acinetobacter* sp. were positive for plasmid at the 20kb band.

**Table 1:** Frequency of occurrence of bacterial isolates in diabetic skin ulcers and non-diabetic wounds

<b>Bacteria</b>	<b>Diabetic Skin Ulcers n=138 (%)</b>	<b>Non-Diabetic Wounds n=18 (%)</b>
<b>GRAM POSITIVE</b>		
<i>Staphylococcus aureus</i>	25 (18.12)	4 (22.22)
<i>Enterococcus faecalis</i>	20 (14.49)	-
Coagulase negative staphylococcus (CONS)	3 (2.17)	-
<b>Gram Negative</b>		
<i>Escherichia coli</i>	24 (17.39)	6 (33.33)
<i>Klebsiella pneumoniae</i>	13 (9.42)	2 (11.11)
<i>Proteus mirabilis</i>	12 (8.70)	1 (5.56)
<i>Pseudomonas aeruginosa</i>	10 (7.25)	2 (11.11)
<i>Klebsiella oxytoca</i>	10 (7.25)	-
<i>Citrobacter freundii</i>	7 (5.07)	-
<i>Proteus vulgaris</i>	7 (5.07)	-
<i>Alcaligenes faecalis</i>	4 (2.90)	3 (16.67)
<i>Acinetobacter spp.</i>	3 (2.17)	-

**Table 2:** Antibiotics susceptibility pattern of Gram-positive bacteria from diabetic skin ulcers

Bacteria (n)	PEF	CN	APX	Z	AM	R	CPX	S	STX	E
<i>S. aureus</i> (25)	11 (44 %)	11 (44 %)	15 (60 %)	13 (52 %)	10 (40 %)	10 (40 %)	12 (48 %)	17 (68%)	12 (48%)	12 (4%)
<i>E. faecalis</i> (20)	13 (65 %)	14 (70%)	14 (70 %)	10 (50 %)	10 (50%)	12 (60 %)	15 (75 %)	12 (60 %)	9 (45%)	11 (55%)
CONS	1 (33.33%)	3 (100%)	1 (33.33%)	2 (66.67%)	1 (33.33 %)	2 (66.67%)	2 (66.67%)	0 (0%)	1 (33.33%)	1 (33.33%)

**Key:** PEF – Pefloxacin, CN – Gentamycin, APX – Ampiclox, Z – Zinacef, AM – Amoxicillin, R – Rocephin, CPX – Ciprofloxacin, S – Streptomycin, STX – Septrin, E – Erythromycin

**Table 3:** Antimicrobial susceptibility pattern for Gram negative bacteria from diabetic skin ulcers

Bacteria (n)	CN	PEF	OFX	S	STX	CH	SP	CPX	AM	AU
<i>E. coli</i> (24)	10 (41.67%)	13 (54.1%)	19 (79.17%)	17 (70.83%)	10 (41.67%)	11 (45.83%)	10 (41.67%)	14 (58.33%)	7 (29.17%)	5 (20.83%)
<i>K. pneumoniae</i> (13)	3 (23.08%)	9 (69.23%)	9 (69.23%)	5 (38.46%)	2 (15.38%)	2 (15.38%)	10 (78.92%)	12 (92.3%)	8 (61.54%)	5 (38.46%)
<i>P. mirabilis</i> (12)	3 (25%)	7 (58.33%)	6 (50%)	9 (75%)	5 (41.67%)	3 (25%)	8 (66.67%)	9 (75%)	4 (33.33%)	3 (25%)
<i>P. aeruginosa</i> (10)	6 (60%)	7 (70%)	7 (70%)	4 (40%)	4 (40%)	6 (60%)	9 (90%)	8 (80%)	3 (30%)	1 (10%)
<i>K. oxytoca</i> (10)	7 (70%)	7 (70%)	7 (70%)	6 (60%)	3 (30%)	3 (30%)	6 (60%)	8 (80%)	5 (50%)	5 (50%)
<i>C. freundii</i> (7)	4 (57.14%)	7 (100%)	2 (28.57%)	4 (57.14%)	4 (57.14%)	2 (28.57%)	4 (57.14%)	6 (85.71%)	4 (57.14%)	2 (28.57%)
<i>P. vulgaris</i> (7)	2 (28.57%)	5 (71.43%)	2 (28.57%)	4 (57.14%)	6 (85.71%)	4 (57.14%)	4 (57.14%)	5 (71.43%)	3 (42.86%)	2 (28.57%)
<i>A. faecalis</i> (4)	1 (25%)	2 (50%)	2 (50%)	1 (25%)	2 (50%)	1 (25%)	2 (50%)	4 (100%)	0 (0%)	0 (0%)
<i>Acinetobacter spp.</i> (3)	2 (66.67%)	1 (33.33%)	1 (33.33%)	1 (33.33%)	2 (66.67%)	1 (33.33%)	2 (66.67%)	2 (66.67%)	0 (0%)	1 (33.33%)

**Key:** CN – Gentamycin, PEF – Pefloxacin, OFX – Tarivid, S - Streptomycin, STX – Septrin, CH – Chloramphenicol, SP–Sparfloxacin, CPX – Ciprofloxacin, AM – Amoxicillin, AU - Augmentin

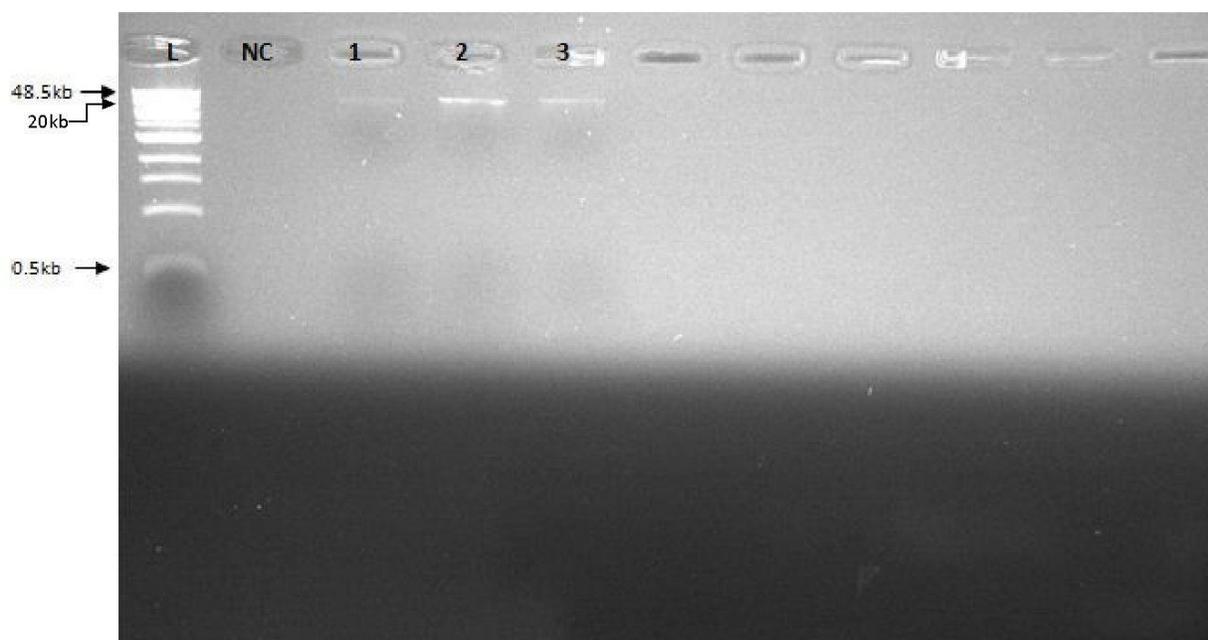
**Table 4:** Multiple Antibiotic Resistance Pattern of bacteria before and after plasmid curing

<b>Gram Positive Isolates</b>											
	PEF	CN	APX	Z	AM	R	CPX	S	SXT	E	(MAR)
<i>Staphylococcus aureus</i>											
<b>Before curing</b>	R	R	R	R	R	R	R	R	R	R	<b>1.00</b>
<b>After curing</b>	S	R	R	R	R	R	R	R	R	R	
<b>Gram Negative Isolates</b>											
	CN	PEF	OFX	S	SXT	CH	SP	CPX	AM	AU	MAR
<i>Escherichia coli</i>											
<b>Before curing</b>	R	R	R	R	R	R	R	R	R	R	<b>1.00</b>
<b>After curing</b>	R	R	R	R	R	R	R	R	R	R	
<i>Alcaligenes faecalis</i>											
<b>Before curing</b>	R	R	R	R	R	R	R	S	R	R	<b>0.90</b>
<b>After curing</b>	R	R	R	R	R	R	S	S	R	R	
<i>Acinetobacter sp.</i>											
<b>Before curing</b>	R	R	R	R	R	R	R	R	R	R	<b>1.00</b>
<b>After curing</b>	R	S	S	R	R	R	R	R	R	R	
<i>Klebsiella pneumoniae</i>											
<b>Before curing</b>	S	S	R	R	R	R	R	R	R	R	<b>0.80</b>
<b>After curing</b>	S	S	R	R	R	R	R	R	R	R	

**Key:** R – Resistant; S - Susceptible

PEF – Pefloxacin, CN – Gentamycin, APX – Ampiclox, Z – Zinacef, AM – Amoxicillin, R – Rocephin, CPX – Ciprofloxacin, S – Streptomycin, STX – Septrin, E – Erythromycin, OFX – Tarivid, CH – Chloramphenicol, SP – Sparfloxacin, AU - Augmentin

MAR- Multiple Antibiotic Resistance index



**Plate 1:** Plasmid profile of multiple drug resistance bacterial isolates, analyzed with 0.8 % agarose gel electrophoresis stained with ethidium bromide. L is 0.5kb-48.5kb DNA ladder (molecular marker). Samples 1, 2 and 3 are positive for plasmid genes with band at 20kb. NC is a no plasmid DNA template control.

### Key

- 1 – *Staphylococcus aureus*
- 2- *Alcaligenes faecalis*
- 3- *Acinetobacter* spp.

### CONCLUSION

Diabetic skin infections are mostly polymicrobial caused by a mixture of different bacterial strains and most of which are Gram-negative, which play significant roles in the healing process of the wounds. Overall, gentamycin and ciprofloxacin had the highest antibacterial action on the Gram-positive and Gram-negative bacteria respectively. Therefore, appropriate management of the infections involves selecting the appropriate antibiotics. Empirical treatments may be used, but a therapy directed at the specific causative

organism may give better results. So, the antibiotic treatment has to be changed according to the microbes involved and their sensitivity to antibiotics. Some of the bacterial isolates which displayed antibiotic resistance markers were plasmid-borne and others were not.

### CONFLICT OF INTEREST:

The authors declare no financial or conflicts of interest in the design and interpretation of the study results.

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