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Antibiotic Sensitivity Pattern and Plasmid Profile of Bacteria Isolated from Diabetic Ulcers in Mbano Metropolis, Imo State, Southeastern Nigeria

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Abstract
The study was undertaken to evaluate the bacteriology and antibiogram of isolates from diabetic patients with chronic foot ulcers in Nigeria. A total of 150 pus samples were collected and processed according to standard aerobic and anaerobic microbiological methods. Antibiogram was done using Kirby-Bauer method. Biofilm tests, ESBL & AmpC production was conducted using Congo red agar, Double disc synergy test and Cefoxitin disc test respectively. Total number of isolates obtained was 210. The Plasmid profiles of some of the Multi-Drug Resistance (MDR) isolates were carried out using the alkaline lysis method for plasmid extraction and electrophoresis on agarose gel with standard markers. The most frequently isolated aerobic organism in the study was *Escherichia coli* (32.1%) while the least occurring was *Enterobacter* spp (1.57%). For the anaerobes, *Peptostreptococcus* spp (40%) was the highest isolated bacterium. Percentage of Extended Spectrum β-lactamase (ESBL) producers among *E. coli* isolates was 44%. Percentages of biofilm formation potential among the isolates were: *E. coli* (36.8%), *S. aureus* (23.1%) and *Proteus vulgaris* (4.2%). *Escherichia coli* and *S. aureus* showed considerable levels of resistance to some common antibiotics. No methicilin resistant *S. aureus* was encountered. AmpC producers encountered were *Klebsiella pneumonia* (10%) and *E. coli* (8.1%). Post-curring antibiogram tests revealed that nine isolates carried plasmids, suggesting that the mode of resistance may be plasmid mediated.
Keywords: Diabetic ulcers, Bacteria, Antibiogram, Plasmid profile.

INTRODUCTION

Diabetic ulcer is a serious clinico-pathologic outcome of diabetes with recent studies revealing that life time risk of developing a foot ulcer in diabetic patients could be as high as 25% which leads to proximate and non-traumatic causes of leg amputation (Lipsky, 2004; Ahmed *et al.*, 2006). Presently there is increasing empirical evidence suggesting that diabetes is a risk factor for antibiotic-resistant *Streptococcus pneumonia*, Methicillin resistant *Staphylococcus aureus* (MRSA) with basal sensitivity to vancomycin and vancomycin-resistant enterococci as well as ESBL producing Gram negative bacteria and carbapenem-resistant *Pseudomonas* spp (Lyndmila *et al.*, 2013).

The prevalence of foot infections in persons with diabetes ranges from a lifetime risk of up to 25% in persons with the disease, to 4% yearly in patients treated in a diabetic foot center (Singh *et al.*, 2005). In diabetic foot ulcers, various organisms inhabit the wound and in most patients one or more species of organisms multiply in the wound which may lead inexorably to tissue damage. Utilizing a murine chronic wound model, some researchers found that DNA protected *P. aeruginosa* in the wounds of insulin-treated diabetic mice from antibiotic treatment (Watters *et al.*, 2014). Due to common or recurrent infections, diabetic patients have more antibiotic treatments compared with other subjects which can increase the antibiotic resistance rates in the bacteria (Watters *et al.*, 2014). In a study in Malaysia, *S. aureus* was found to be predominant followed by *Klebsiella pneumonia* and *Pseudominas aeruginosa* (Mohanasoundram, 2012). The increased relationship of multi-drug resistant (MDR) pathogens with diabetic ulcers, further compounds the problem faced by physicians or surgeons in treating diabetic ulcers without resorting to amputation (Yoga *et al.*, 2006). Infection with MDR pathogens incurs a lot of expenses and leads to delayed hospital stay, and sometimes increases the chances of mortality in diabetic patients.

Careful selection of antibiotics based on the susceptibility pattern of the isolates from the lesions is most reliable for the proper management of these infections.

Reported cases of diabetic infections which investigated the prevalence of microbes and their associated multi-drug resistance have been published in developed countries. Contrastingly, the bacteriology of diabetic ulcers in Nigeria has not been studied extensively especially in the south-eastern part of the country. This study was designed to identify the common bacterial agents found in foot ulcers of diabetic patients in Imo State and to determine their *in vitro* susceptibility to routinely used antibiotics.

MATERIALS AND METHODS

Study Location

The study was conducted in Ihitte Uboma town in Imo State. Samples were collected from Madonna Austrian Hospital Ihitte which serves as a referral hospital in the locality.

Study Design

This is a cross sectional surveillance study.

Study Population and study sample

The study population comprised of all diabetic patients that reported to Madonna Austrian Hospital Ihitte between June, 2018 and January, 2020. A total of 150 diabetic patients with diabetic foot ulcers were enrolled for the study, out of which 65 patients were on admission while 85 were out-patients. Some of the patients were referrals from other healthcare centres in the neighbouring towns. The collect foot ulcer samples were processed at the Microbiology laboratory of Michael Okpara University, Umudike and Oevent Research Laboratory Uzuakoli Road Umuahia respectively.

Sample collection from the diabetic foot ulcers patients

The surface of the wound was cleaned with physiological saline to avoid skin contaminants. With the aid of two sterile swab sticks, pus samples were collected from the foot ulcers of each diabetic patient. The sample collection was carried out before wound dressing.

Isolation of aerobic bacteria

The swab was inoculated on to blood agar, MacConkey agar and Mannitol Salt agar and incubated at 37°C for 24hrs (Turgeon, 2012).

Isolation of anaerobic bacteria

The second swab sample was inoculated in neomycin sulphate blood agar and cooked meat broth immediately. The blood agar plate was incubated anaerobically for 48hrs at 37^0C in an anaerobic jar with Gaspak Oxoid BROO38B (Gas Generating Kit). Saccharolytic reaction is shown by reddening of the meat with a rancid smell due to carbohydrate decomposition while proteolytic reaction is shown by blackening of the meat with a very unpleasant smell due to protein decomposition.

Identification of Organisms

The isolates were identified by standard techniques on the basis of their cultural morphology, motility, Gram staining reaction and biochemical properties according to CLSI (2015).

Antibiotic Sensitivity test

Antimicrobial susceptibility testing was performed using the disk diffusion method according to Clinical Laboratory Standard Institute (2015) on Mueller Hinton agar (Hardy Diagnostics, USA). Mueller Hinton culture plates were inoculated using a sterile cotton wool swab dipped into an overnight growth suspension of the test organism prepared to the density of 0.5 McFarland standard. The antibiotics tested were cotrimoxazole (25µg), levofloxacin (20µg), streptomycin (30µg), ciprofloxacin (10µg), amoxicillin (10µg), amoxicillin/clavulanate (30µg), gentamicin (10µg), perfloxacin (10µg), ofloxacin (10µg), ceftriaxone (30µg), ceftazidime (30µg), erythromycin (30µg). After overnight incubation, examination of the control and test plates were carried out to ensure the growth is semi- confluent. Using a ruler on the underside of the plate of each zone of inhibition was measured in mm. *Escherichia coli* ATCC 25922 was used as the control strain for this study. This organism was obtained from Aminu Kano Teaching Hospital (AKTH), Kano.

ESBL Screening and confirmation

The isolates were tested against third generation cephalosporins (Cefpodoxime, cefotaxime, and ceftrixone) using the WHO modified Kirby Bauer diffusion method (2003). Zone diameters were interpreted using the revised Clinical Laboratory Standard Institute document (2015). Isolates with reduced susceptibility to cefpodoxime (<17mm), cefotaxime (<27mm) and ceftriaxone (<25mm) were considered to be possible ESBL producers.

Phenotypic Confirmation Test was carried out using Double Disc Synergy test. Disc containing the standard 10µg of cefpodoxime and the 30µg of ceftazidime/ ceftriaxone was placed 15mm apart (edge to edge) with amoxicillin-clauvlanic acid disc containing 10µg of the latter compound mounted exactly at their center. After 16-20 hours of incubation at 35°C any enhancement of the zone of inhibition between a beta-lactam disk and that containing the β-lactamase inhibitor is indicative of the presence of an ESBL (Jacoby *et al.*, 2004).

Screening for AmpC β-lactamase

Screening for AmpC ß-lactamase production was done by placing a cefoxitin disk (30µg) on Mueller-Hinton agar. Isolates showing an inhibition zone diameter of < 18mm were considered positive on the screening.

Amp C Detection test

A 0.5 McFarland suspension of multidrug resistant bacteria was inoculated on the surface of a MHA plate. A 30µg cefoxitin disc (Oxoid, England) was placed on the inoculated surface of the agar. A sterile plain disc inoculated with several colonies of the test organism was placed beside the cefoxitin disc almost touching it, with the inoculated disk face in contact with the agar surface. The plates were incubated at 37^0C for 24 hours. After incubation, the plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (negative result) (CLSI, 2015).

Test for Methicillin Resistant *Staphylococcus*.

Susceptibility to cefoxitin was determined by the Disk Diffusion method on Mueller–Hinton agar plates using a bacterial suspension with the turbidity adjusted to a 0.5 McFarland standard. Plates were incubated at 35°C for 24 hrs. Results were interpreted according to CLSI (2013) guidelines. The interpretive criteria for cefoxitin were: *S. aureus*, sensitive ≥22mm, resistant ≤21 ug/ml; Coagulase Negative *Staphylococcus* (CoNS), sensitive ≥25mm, resistant ≤24mm.

Test for Biofilm Formation Potential

This was done by the Congo Red Agar Method. Blood agar base supplemented with sucrose and Congo red was used. Plates were inoculated with the tested isolates and incubated aerobically for 24hrs at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency. Non slime producers usually remained pink (Mathur *et al.*, 2006).

Plasmid Extraction

Plasmid extraction was done following the method described by Birnboim, *et al.*, (1979). This analysis was carried out using the alkaline lysis method and gel electrophoresis.

Plasmid Curing

Ten millilitres of each bacterial culture inoculated into peptone water and incubated for 24hrs was introduced into a set of 20 test tubes, respectively. Ethidium bromide in various concentrations of 0, 20, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600,650, 700, 750 and 800 µl/ml were then introduced accordingly into the test tubes and incubated at 37ºC for 24hrs to determine the sub-lethal concentrations of ethidium bromide. After 24hrs of incubation, 1ml aliquot from each test tube was inoculated onto nutrient agar plates and incubated, after which colonies were selected and inoculated onto freshly prepared Muller Hinton agar plates. Then, antibiotic discs of prior resistance were aseptically introduced into the plates, ensuring that the discs made appropriate contact with the surface of the agar. These were incubated for 24 hrs at 37ºC after which plates were examined for cured colonies (Raghada *et al.*, 2013).

RESULTS

Table 1 shows the age and sex distribution of Diabetic ulcer patients. Of the 150 patients enrolled for this study, 90 (60%) were male and 60 (40%) were female. Their ages ranged from 41 to >90 and the highest number of patients were found in the age group of 61-70 years (28%), followed by those the aged of 81-90 years (20.6%) then the age groups 41-50 (9.3%) and >90 (9.3%).

Table 2 shows the distribution of aerobic and anaerobic isolates. A total 210 isolates were obtained from this study out of which 137 (65.5%) were Gram negative, 53 (25.5%) were Gram positive and 20 (9.5%) were anaerobes. The most frequently isolated organism in this study was *Escherichia coli* (32.1%), and the least was *Enterobacter* spp (1.57%) for the aerobes. The anaerobes, *Peptococcus* spp (15%), *Peptostreptococcus* spp (40%), *Bacteroides* spp (30%) and *Fusobacterium* spp (15%).

Table 3 shows the distribution of organisms with biofilm formation, betalactamase producing potentials and AmpC producers. The percentage of biofilm forming organisms is as follows: *E coli* (36.8), *S aureus* (23.1%), *Klebsiella* spp (12.6%), *P. aeruginosa* (8.4%), *P. mirabilis*, *COANS* (7.3%) and *P. vulgaris* at (4.2%). It shows the

percentage of Extended Spectrum Beta-lactamse *(ESBL)* as follows: *E coli* (44%), *Klebsiella spp* and *P. mirabilis* (17%) each, *P. aeruginosa* (15%) and *P. vulgaris* (4%). Also, Table 3 the AmpC producers encountered as *Klebsiella* spp (28.5%) and *E. coli* (71.4%).

Table 4 shows the antibiogram for Gram negative and Gram positive isolates. *E coli* recorded the highest sensitivity rate with Ciprofloxacin (57.3%) and Gentamicin (40.9%) (Table 4). Similarly, *Klebsiella* spp recorded higher sensitivity rate of 70% to Ciprofloxacin and 55% to Perfloxacin and Ofloxacin (Table 4). The antibiogram for the Gram-positive organisms indicated that organisms like *Staphylococcus aureus* and Coagulase negative *Staphylococcus* spp (*COANS*) were sensitive to Ofloxacin at the rate of 63% and 100% respectively. *Enterococcus* spp was 100% sensitive to Streptomycin while COANS showed 100% sensitivity to Ofloxacin, Amoxil and Streptomycin (Table 4). *Citrobacter* spp was 100% sensitive to Perfloxacin, Ciprofloxacin, Ofloxacin and Gentamicin while *Enterobacter* spp was 100% sensitive to Ofloxacin and Pefloxacin (Table 4).

Table 5 shows the distribution of plasmids and their molecular weight. It also reveals the resistance pattern of isolates before and after curing.

Table 1: Age and Sex Distribution of Diabetic Ulcer Patients

Age (Years)	No. examined	Male	Female	Percentage (%)
41-50	14	7	7	9.3
51-60	21	11	10	14
61-70	42	32	10	28
71-80	28	18	10	18
81-90	31	16	15	20.6
>90	14	6	8	9.3
Total	150	90	60	

Table 2: Distribution of aerobic and anaerobic Isolates

Aerobic Isolates		
Organisms	**Number of Occurence**	**Percentage (%)**
Klebsiella spp	20	10.5
E coli	61	32.1
P. mirabilis	20	10.5
Enterobacter spp	3	1.57
COANS	10	5.2
S. aureus	30	15.7
P. aeruginosa	19	10
E. faecalis	8	4.2
Strept spp	5	2.6
Citrobacter spp	3	1.57
Total	190	
Anaerobic Organisms		
Peptococcus spp	3	15
Peptostreptococcus spp	8	40
Bacteroides spp	6	30
Fusobacterium spp	3	15
Total	20	

CONS = Coagulase Negative Staphylococci

Table 3: Distribution of Organisms with Biofilm Formation, Beta-lactamase producers and AmpC producers Number Positive (Percentage)

Organisms	Number Isolated	Biofilm forming potential	Beta-lactamase producers	AmpC producers
Klebsiella spp	20	12 (12.6%)	8 (17%)	2 (28.5%)
E. coli	61	35 (36.8%)	20 (44%)	5 (71.4%)
P.vulgaris.	11	4 (4.2%)	2 (4%)	Nil
P. mirabilis	20	7 (7.3%)	8 (17%)	Nil
Enterobacter spp.	3	Nil	Nil	Nil
CoNS	10	7 (7.3%)	Nil	Nil
S.aureus	30	22 (23.1%)	Nil	Nil
P. aeruginosa.	19	8 (8.4%)	7 (15%)	Nil
E. faecalis	8	Nil	Nil	Nil
Streptococcus spp.	5	Nil	Nil	Nil
Citrobacter spp.	3	Nil	Nil	Nil

Table 4: Antibiogram result of the bacterial isolates

Organism	No. tested	No. sensitive									
Gram Negative		PEF	CPX	CRO	S	CAZ	AUG	SXT	OFX	GN	PN
E coli	61	13 (21.3)	35 (57.3)	10(16.3)	9 (14.7)	3 (4.9)	2 (3.2)	3 (4.9)	20(32.7)	25(40.9)	-
Klebsiella spp	20	11 (55)	14 (70)	5(25)	1(5)	0 (0)	2 (10)	0 (0)	11(55)	7(35)	6(30)
P. aeruginosa	19	6 (31)	9 (47)	0(0)	-	3 (15.7)	1 (5.2)	1 (5.2)	11(57)	8(42)	
P. vulgaris	11	7 (63)	3 (27.2)	1(9)	2 (18)	1(9)	1 (9)	0 (0)	9(81)	2(18)	3(27)
P.mirabilis	20	12 (60)	7 (35)	3 (15)	3 (15)	3 (15)	2 (10)	1 (5)	16(80)	4(20)	7(35)
Citrobacter spp.	3	3(100)	3(100)	0(0)	-	0 (0)	-	0 (0)	3(100)	3(100)	-
Enterobacter spp.	3	3(100)	2(66.7)	1(33.3)	1(33.3)	-	2 (66.7)	0 (0)	3(100)	2(66.7)	-

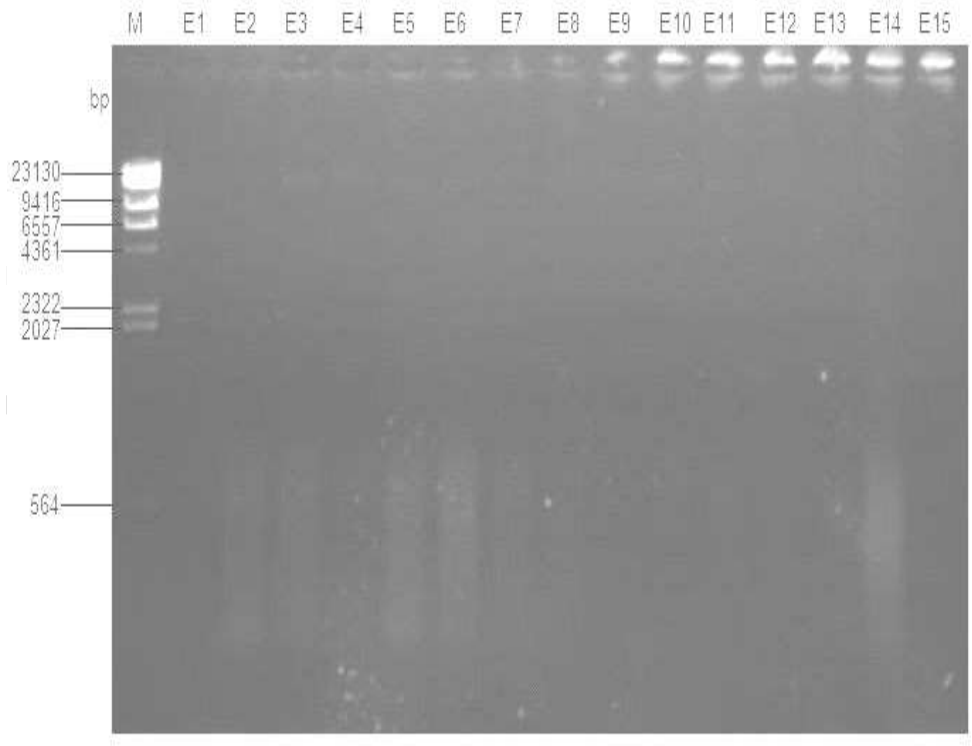
Table 4 continue

Gram positive	No. tested	No. sensitive										
		PEF	LEV	CPX	OFX	ERY	GN	APX	AUG	AM	S	R
S. aureus	30	10 (33.3)	18 (60)	15 (50)	19 (63)	15 (50)	21(70)	0(0)	9 (30)	0 (0)	5 (16)	5(16)
Streptococcus spp	5	0 (0)	0 (0)	1 (20)	3 (60)	3 (60)	1(20)	1(20)	0(0)	1(20)	0 (0)	2(40)
CoaNS	10	0 (0)	0 (0)	7 (70)	10 (100)	9 (90)	5 (50)	0(0)	0(0)	10 (100)	10 (100)	8(80)
Enterococcus spp	8	0 (0)	0 (0)	1 (12.5)	3 (37.5)	1 (12.5)	1 (12.5)	1(12.5)	1(12.5)	0 (0)	8 (100)	1(12.5)

KEY*: PEF= Perfloxacin, CPX=Ciprofloxacin, CRO = Ceftriaxone, S = Streptomycin, CAZ = Ceftazidime, AUG=Amoxicillin-clavulanate, OFX=Ofloxacin, LEV=Levofloxacin, ERY= Erythromycin, APX= Ampicillin, AM=Amoxicillin, SXT= Septrin, GN=Gentamicin, PN=Ampicillin, *CoNS= Coagulase Negative Staphylococci*, *Strept. Spp* = *Streptococcus* spp, AM= Amoxicillin, R=Rifampicin, APX= Ampiclox

Table 5: Distribution of Plasmids among Resistant Isolates, Cured Plasmids and Antibiotic Resistance

Organisms	Base pair (bp)	Resistance before curing	Resistance after curing
E1		PEF,CPX,CAZ,CRO,S,GN	
E2		CPX,S,OFX,CRO,CAZ,AU	
E3	23130	OFX,CPX,CAZ,CRO,PEF,GN	OFX, CPX, PEF
E4	23130	OFX,CAZ,CRO,CPX,AU,S	CRO,OFX,CAZ
E5	23130	CAZ,CRO,OFX,GN,AU,PEF	CRO,AU,OFX,
E6	23130	CRO,CAZ,OFX,GN,AU,PEF	CAZ,CRO,PEF,OFX
E7	23130	CPX,S,CRO,CAZ,PEF,GN	CRO,CAZ
E8	23130	CRO,CAZ,CPX,PEF,AU,S	S, CPX, PEF
E9	23130	OFX,CPX,CRO,CAZ,PEF,S	OFX,CPX,CRO,
E10	23130	CAZ,CRO,OFX,PEF,AU,S	CAZ, PEF, S
E11	23130	PEF,AU,CPX,CAZ,CRO,GN	CRO,OFX,S
E12		CRO,CAZ,AU,PEF,CPX,S	PEF,CRO, CPX
E13		CPX,PN,OFX,CRO,CAZ,	
E14		CRO.CPX,PN,GN,PEF,S	
E15		CAZ,CRO,OFX,CPX,AU,S.	



DISCUSSION

The prevalence of diabetic ulcers among male subjects was found to be 60% and 40% in females. This may be due to higher level of outdoor activity among males compared to females. This corresponds with the findings of some researchers as they indicated the preponderance of diabetes among males compared to females (Lilian *et al.*, 2015; Mohanasoundram, 2012; Nwachukwu *et al.*, 2009). Majority of the patients were in the age group of 61-70, suggesting that age could be a risk factor as the elderly are naturally predisposed to infections. This agrees with a report in South India by Viswanathan *et al.* (2002).

The study findings indicated that Gram negative microbes were the predominant pathogens isolated with 65% Gram negative, 25% Gram positive and 9.5% anaerobes. A similar result was documented in Malaysia revealing more Gram-negative bacteria (52%) than Gram positive bacteria (45%) (Raja, 2007). In addition, this agrees with the report of Lilian *et al.* (2015) but contradicts with that of Mohanasoundram (2012) whose study showed *S. aureus* as the most prevalent followed by *E coli*, *Klebsiella* spp, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and Non Fermenting Gram Negative Bacteria (NFGNB).

The Gram-negative organisms isolated from this study were sensitive to perfloxacin ciprofloxacin, ofloxacin and gentamycin. The Gram-positive organisms were susceptible to ofloxacin, erythromycin and gentamicin. This is consistent with the findings in Kumasi, Ghana where most of the Gram-negative organisms were sensitive to ciprofloxacin and gentamicin (Brenyah *et al.*, 2014). Similarly, Gram positive organisms like *S. aureus* and *CONS* were observed to be highly sensitive to ofloxacin. This sensitivity pattern agrees with the findings of Lilian *et al.* (2015).

Five out of seven Gram negative organisms isolated from this study were observed to be ESBL producers. Among the Gram negative, *E. coli* (at 44%) had the highest number of ESBL producers while, *Proteus vulgaris* had the least at4%. This is in accordance with the study of Bansal *et al.* (2008) and Shashikala *et al.* (2016) who reported a high level of ESBL production among *Ecoli* isolates. However, this contradicts with the work of Samir *et al.* (2009) who reported *Proteus* spp as having the highest ESBL producing potential from their study. This variance could be associated with the difference in sample size as Samir *et al.* (2009) used 75 patients for their study. The increasing prevalence of ESBL producing organisms is

disturbing because of the problems associated with antibiotic prescription.

Recent studies have shown that biofilm associated microorganisms can be up to 1000 times more resistant to antibiotics than free floating planktonic bacteria. In the present study, seven organisms were biofilm formers. *E. coli* had the highest biofilm forming potential followed by *S. aureus, Klebsiella* spp, *P. aeruginosa*, *P. mirabilis, CONS* and *P. vulgaris*. This result agrees with that of Gordon *et al*. (2008) on the biofilm forming nature of *Staphylococci*. This result disagrees with the study of Asima *et al*. (2015) who reported *S .aureus* as having the highest potential for biofilm formation followed by *P. aeruginosa*, *Citrobacter* spp and *Ecoli*. The unusual number of biofilm forming *E.coli* may be as a result of the high prevalence rate of *E. coli* in this study.

This study reports a high level of AmpC production in *K. pneumonia* and *Ecoli*. This is at variance with the report from researches at Kolkata which recorded a high level of AmpC production in *P. aeruginosa* (Subha *et al.,* 2003; Suranjana *et al.,* 2005). Agarose gel electrophoresis of plasmid DNA in this study showed that nine out of fifteen samples contained plasmids. Hence, the resistance is plasmid mediated because upon re-exposure of the isolates to antibiotics after plasmid curing, they were observed to be sensitive.

It is recommended that ESBL producers be subjected to further molecular procedures to define their various types and transformation studies are encouraged to better understand the dynamics of multidrug resistance.

CONCLUSION

This study established the presence of ESBL and AMPC production among biofilm forming bacterial isolates obtained from samples of diabetic patients with foot ulcers. This is of great public health concern as this could contribute to the resistant of these bacterial pathogens to antibiotic like carbapenems.

RECOMMENDATIONS

Regular studies of the antibiotic susceptibility pattern of diabetic ulcer isolates commonly observed in Mbano will guide clinical judgment and sustain veritable antibiotic prescriptions. Discoveries from such surveys will illuminate the current knowledge on multi drug resistant isolates and proffer solutions about the right choice of antibiotics. The detection of ESBL producers, AmpC producers, Biofilm forming organisms and plasmids are vital.

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