Journal of Human Environment and Health Promotion
Print ISSN: 2476-5481 Online ISSN: 2476-549X

Plasmid Profile and Characterization of *Escherichia coli* Isolated from Diabetic Foot Ulcers in Imo State, South Eastern Nigeria

Emmanuel Onwubiko Nwankwo [a *] | Enyinnaya Nwagbara [b] | Ebubechi Uloma Okey-kalu [a]

a. Department of Microbiology, College Of Natural Sciences, Michael Okpara University of Agriculture, Umudike. P. M. B 7267, Umuahia, Abia State, Nigeria.
b. Department of Medical Laboratory Services, Savealife Mission Hospital, Onitsha, Anambra state, Nigeria.

***Corresponding author:** Department of Microbiology, College Of Natural Sciences, Michael Okpara University of Agriculture, Umudike. P. M. B 7267, Umuahia, Abia State, Nigeria.
E-mail address: emmaonwubiko@yahoo.com

ARTICLE INFO

Article type:
Original article

Article history:
Received: 25 June 2022
Revised: 24 July 2022
Accepted: 9 August 2022

© The Author(s)

DOI: 10.52547/jhehp.8.3.118

Keywords:

Diabetic ulcers
E. coli
antibiogram
plasmid profile
Isolates

ABSTRACT

Background: The study aimed at evaluating the plasmid profile and characterization of *E. coli* isolated from chronic diabetic foot ulcers among patients in Imo State, South East Nigeria.
Methods: A total of 150 wound swab samples were collected and analyzed. Standard microbiological methods processed samples. Antibiogram was done by Kirby-Bauer disc diffusion method. Tests were conducted to determine biofilm potential, ESBL and AmpC production. Plasmid profiling was done by the alkaline lysis method and Gel electrophoresis.
Results: Based on the results, out of the 150 swab samples, 61(40.6%) *E. coli* isolates were recovered. 57.8%, 32.8%, and 8.2% of the *E. coli* isolates were positive for biofilm formation, ESBL, and AmpC production, respectively. Further, the study revealed a pattern of multiple-drug resistance of *E. coli* to amoxicillin/clavulanic acid (96.7%), septrin (95.1%), and ampicillin (93.4%).
Conclusion: Analysis of the multiple antibiotic resistance (MAR) index showed that the organism had MAR index values above the low-risk region (≤0.2) for all the tested antibiotics. Post-curring antibiogram tests revealed that nine isolates carried plasmids, suggesting that the mode of resistance may be plasmid-mediated. Findings could act as a therapeutic guide for antibiotic prescriptions in this environment.

1. Introduction

Diabetes mellitus (DM) is a global health concern. It is a common metabolic disorder characterized by hyperglycaemia emerging from defects in insulin secretion and action [1]. Diabetes has a major clinicopathological consequence known as diabetic foot ulcers (DFU). In a person with DM, DFU is a partial or full-thickness wound that is not healing or is healing inadequately. The toes or the bottom of the foot are frequently affected areas [2]. In individuals with DM, non-healing infected DFU often leads to amputation of the affected limb [3]. Diabetes patients have a lifetime risk of up to 25% for foot infections; however, only 4% of patients treated in diabetic foot centers have this risk annually [4]. Numerous organisms live in diabetic foot ulcers. In most patients, one or more species of these organisms grow in the wound, which may inevitably cause tissue damage. *S. aureus, Staphylococcus epidermidis*, *P. aeruginosa, E. coli, Klebsiella* species, and *Proteus* species are the most common types of this bacteria [4], which impede healing and lead to chronic wounds that never heal. Due to common or recurrent infections, diabetic patients have more antibiotic treatments than other subjects, increasing antibiotic resistance rates in the bacteria [5]. The difficulty experienced by the doctor or

CC BY NC

How to cite: Nwankwo Emmanuel O, Nwagbara E, Okey-kalu Ebubechi U. Plasmid Profile and Characterization of Escherichia coli Isolated from Diabetic Foot Ulcers in Imo State, South Eastern Nigeria. *J Hum Environ Health Promot.* 2022; 8(3): 118-23.

surgeon in treating diabetic ulcers without resorting to amputation is made worse by the rising association of multi-drug resistance (MDR) bacteria with diabetic ulcers [6]. Controlling DFU infections has become more difficult due to widespread bacterial antibiotic resistance and increased infections from superbugs, including methicillin-resistant *Stapylococcus aureus* (MRSA), polymicrobic flora, MDR *E. coli*, and *Pseudomonas aeroginosa* [7]. Therefore, this study aimed to evaluate the incidence of biofilm formation, ESBL, AmpC production, and plasmid profile of MDR *E. coli* isolated from DFU infections within the study environment.

2. Materials and Methods

2.1 Study Location

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

2.2 Study Population

150 diabetic patients were enrolled in the study. Samples were processed in the Microbiology laboratory of Michael Okpara University, Umudike, and Oevent Research Laboratory Uzuakoli Road, Umuahia.

2.3 Procedure for the Collection and Processing of Pus

The wound's surface was cleaned with physiological saline, and a sterile swab stick and a pus sample were collected from the diabetic ulcer. The swab was inoculated onto blood agar, MacConkey agar, and Eosine methylene blue agar by streak plate method and incubated at 37 °C for 24h [8].

2.4 Identification of E. coli

The *E. coli* isolates were identified by standard techniques based on their colonial morphology, Gram staining reaction, motility, and biochemical characteristics [9].

2.5 Antibiotic Sensitivity Test

Mueller Hinton agar was used for disk diffusion antimicrobial susceptibility testing (Hardy Diagnostics, USA). By inserting a sterile cotton wool swab into a suspension of the overnight growth of the organism that had been made to the density of an Mc Farland no 0.5 opacity standard, excess liquid from the swab was expressed before inoculation. The tested antibiotics were cotrimoxaxole (25mcg), levofloxacin (20mcg), streptomycin (30mcg), ciprofloxacin (10mcg), amoxicillin (10mcg), amoxicillin-clauvlanic acid (30mcg), gentamicin (10mcg), perfloxacin (10mcg), ofloxacin (10mcg), ceftriaxone (30mcg), ceftazidime (30mcg), and erythromycin (30 mcg). After overnight incubation, the researchers of the present study examined the control and test plates to ensure the growth was semi-confluent. The diameter of each zone of inhibition was measured in mm using a ruler on the plate's underside. The results were analyzed based on the Clinical and Laboratory Standards Institute (CLSI). The control strain was *Escherichia coli* ATCC 25922. This was interpreted according to [10].

2.5.1 Determination of Multiple Antibiotic Resistance (MAR) Index

MAR index was calculated by the method of [11], using the following formula: MAR = a/ (b.c). In this formula, (a) stands for the overall antibiotic resistanc e of all isolates, (b) for the total number of antibiotics, and (c) for the total number of isolates from the specimen location.

2.5.2 ESBL Screening and confirmation

The third-generation cephalosporins (Cefpodoxime, Cefotaxime, and Ceftriaxone) were tested on the isolates using a modified version of [12] disc diffusion technique developed by the WHO. We interpreted zone diameters using the CLSI guidelines [10]. Cefpodoxime (17mm), cefotaxime (27mm), and ceftriaxone (25mm) decreased susceptibility isolates were thought to be potential ESBL producers. The Double Disc Synergy test (DDST) was used for the phenotypic confirmation test. Amoxicillin-clavulanic acid discs with 10ug of the latter substance mounted precisely in the middle are set 15mm apart (edge to edge) and include the typical 10ug of cefpodoxime and 30ug of ceftazidine/ceftriaxone. Any enlargement of the zone of inhibition between beta-lactam and beta-lactamase inhibitor disks indicates ESBL after 16-20 h of incubation at 35°C [13].

2.5.3 Test for AmpC

A cefoxitin disk (30 g) was placed on Mueller-Hinton agar to screen AmpC –lactamase forming. On the screening, isolates with an inhibition zone diameter of less than 18 mm were considered positive [10].

2.5.3.1 Phenotypic confirmatory test

McFarland bacterial suspension (0.5) was produced from a blood agar plate left overnight. The conventional disk diffusion approach used the suspension to inoculate the Muller Hinton Agar surface. A disk containing 30 g of cefoxitin was applied to the MHA's infected surface. The disk was exposed to 20 l of 15 g/ml phenylboronic acid using sterile tips and absorbed it. At 35 °C, the plate was incubated overnight with the opened lid. The zone diameter around the antibiotic disk with additional boronic acid was compared to the antibiotic-containing disk alone during overnight incubation.The organism was identified as an AmpC produce r when it showed a distinct increase (5 mm) in the zone of in hibition surrounding the antibiotic disk with additional boro nic acid [14].

2.5.4 Test for Biofilm Formation Potential

The Congo Red Agar Method was used to test biofilm formation potential. Congo red and sugar were added as supplements to the blood agar base. The tested isolates were plated and cultured aerobically for 24 h at 37 °C. Black colonies with a dry crystalline consistency were a sign of success. Typically, non-slime producers stayed pink [15].

2.6 Plasmid Extraction

Plasmids were analyzed using the alkaline lysis method and Gel electrophoresis [16].

2.6.1 Plasmid Curing

A set of 20 test tubes received 10 ml of each bacterial culture that had been injected into peptone water and incubated for 24 h. The test tubes were then filled with ethidium bromide in the appropriate concentrations of 0, 20, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600,650, 700, 750, and 800 µl/ml, and were incubated at 37 °C for 24 h to determine the sub-lethal concentrations. Colonies were chosen and put onto freshly made Muller Hinton agar plates after a 1 ml aliquot from each test tube was cultured on nutrient agar plates for 24 h. The plates were then aseptically filled with antibiotic discs previously resistant to them, ensuring they made proper contact with the agar surface. After a 24-hour period of incubation at 37 °C, we checked the plates for cured colonies [17].

3. Results and Discussion

Table 1 shows the age and sex distribution of diabetic ulcer patients. The male-to-female ratio is 1:5. Their ages ranged from 41 to >90, and the highest number of patients (28%) were in the age group of 61-70, followed by 20.6% for the age group of 81-90 and 9.3% for two age groups respectively, (41-50) and (>90). Tables 2 and 3 present the prevalence of *E. coli* isolates in diabetic foot ulcer infections. Out of the 150 swabs analyzed samples, 61(40.6%) *E. coli* isolates were recovered. 57.8% (35), 32.8% (20), and 8.2% (5) of the *E. coli* isolates were positive for biofilm formation, ESBL, and AmpC production, respectively. Table 4 also explains the antibiogram and MAR index of the isolates. The study revealed a pattern of intense MDR of *E. coli* to amoxicillin-clavulanic acid (96.7%), septrin (95.1%), and ampicillin (93.4%). The MAR index analysis showed that the organism had MAR index values above the low-risk region (≤0.2) for all the tested antibiotics. Table 5 and Figure 1 indicate the distribution of plasmids among resistant isolates. Post-curring antibiogram tests revealed that nine isolates had plasmids, suggesting that the mode of resistance may be plasmid-mediated, as shown in Table 6. Male participants had 60% prevalence of diabetic ulcers compared to females (40%). This might be because men engage in more outdoor activities than women do. The finding is in line with [18, 19, 20] which showed that men are more likely than women to have diabetic foot ulcers. Since most patients are in the 61–70 age range, age likely plays a role in infections because the elderly are prone to these infections due to weak immune systems. The study results conform with the findings of [21]. Out of 150 studied samples, 61(29.05%) *E. coli* organisms were isolated. This finding is not consistent with the findings of [22, 23], who reported a lower *E. coli* preponderance of 17(17.89%) and 25(14.79%) in their studies on the bacterial associated with DFU infections in Pakistan and South India, respectively. Analysis of the sensitivity pattern revealed high-level resistance to ampicillin, septrin, amoxicillin-clauvlanic acid, streptin, and ceftazidim. There was demonstrable sensitivity of *E coli* to ciprofloxacin. Specifically, *E coli* recorded the highest sensitivity rate with Ciprofloxacin at 57.4% and Gentamicin at 41.0%. This is consistent with the findings of [24], where most gram-negative organisms were sensitive to Ciprofloxacin and Gentamicin, respectively. Furthermore, the multiple antibiotic resistance index analysis showed that all the isolates had MAR index values above the low-risk region (MAR index ≤0.2). Recent research has revealed that bacteria linked with biofilms might be up to 1000 times more antibiotic-resistant than freely floating planktonic germs. In the current research, 57.8% of *E. coli* were biofilm formers. A study by [25] recorded the highest biofilm-forming organism as *S. aureus* at 38.8%, followed *P aeruginosa* at 26.5%, *Citrobacter* spp, and *E coli* at 10.5% each. This deviation in the distribution of organisms may be associated with debridement procedures and the duration of some ulcers. The unusual number of biofilm-forming *E coli* may be due to the study′ s high prevalence rate of E coli. 32.8% of the *E. coli* organisms isolated were ESB L producers. This finding is consistent with the work of [26, 27], which recorded 53.66% and 44.7% of *E coli* , respectively. Contrastingly, this disagrees with the findings of [28], reported *Proteus* spp (13%) as the highest ESBL producer as against *E coli* (7%). This variance could be associated with the different sample sizes, as [28] used 75 patients for their study. The increasing prevalence of ESBL-producing organisms is alarming because of the problems associated with an antibiotic prescription. The chromosomes of several members of Enterobacteriaceae family, such as *Enterobacter* spp, *Shigella* spp, *Providencia* spp, *Citrobacter freundii, Morganella morganii, Serratia marcescens,* and *E. coli* [29] frequently contain genes for AmpC B-lactamases. Plasmid-mediated AmpC B-lactamases have increased through chromosomal genes transfer for the inducible AmpC B-lactamases onto plasmids. As a result of this transfer, isolates of *E. coli*, *Klebsiella pneumoniae*, *Salmonella* species, *Citrobacter freundii*, *Enterobacter aerogenes*, and Proteus mirabilis have plasmid-mediated AmpC B-lactamases [30]. In the study, 8.2% of the *E. coli* isolates that produced AmpC tested positive for the enzyme. The outcome contrasts with the study of [31], which reported *P aeruginosa* (13%), and is consistent with the findings of [32], who reported 3.3% E coli and 2.2% *Klebsiella pneumonia*. Extra- chromosomal elements with the capacity for independent replication are called plasmids. These DNA molecules are found in bacteria and are distinct from chromosomal DNA [17]. Agarose gel electrophoresis of plasmid DNA showed that nine out of 15 samples contained plasmids.

Table 1: Age and Sex Distribution of Diabetic Ulcer Patients

Age	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		90	60	

Table 2: Prevalence of *E. coli* in Diabetic Foot Ulcers

Category	Number	Percentage prevalence
Samples analyzed	150	-
Total number of bacteria isolated	210	-
No. of *E coli* isolated	61	29.05

Table 3: Characterization of *E. coli* isolates

Category	No tested	No. positive	Percentage positive
Biofilm	61	35	57.4
ESBL	61	20	32.8
AmpC	61	5	8.2

Table 4: Antibiogram and multiple antibiotic resistance (MAR) index of *E. coli* isolates

Antibiotic	No. tested	No. sensitive (%)	No. resistant (%)	MARI
PEF	61	13(21.3)	48(78.7)	0.79
CPX	61	35(57.4)	26(42.6)	0.43
CRO	61	10(16.4)	51(83.6)	0.84
S	61	9(14.8)	52(85.2)	0.85
CAZ	61	3(4.9)	58(95.1)	0.95
AUG	61	2(3.3)	59(96.7)	0.97
SXT	61	3(4.9)	58(95.1)	0.95
OFX	61	20(32.8)	41(67.2)	0.67
GN	61	25(41.0)	36(59.0)	0.59
AMP	61	4(6.6)	57(93.4)	0.93

Table 5: Distribution of Plasmids among Resistant Isolates

SN	Sample Code	Base Pairs (bp)	No of Plasmids
1	E1		
2	E2		
3	E3	23130	1
4	E4	23130	1
5	E5	23130	1
6	E6	23130	1
7	E7	23130	1
8	E8	23130	1
9	E9	23130	1
10	E10	23130	1
11	E11	23130	1
12	E12		
13	E13		
14	E14		
15	E15		

Table 6: Distribution of Cured Plasmids and Antibiotic Resistance

Organisms	Resistance before curing	Resistance after curing
E1	PEF,CPX,CAZ,CRO,S,GN	
E2	CPX,S,OFX,CRO,CAZ,AU	
E3	OFX,CPX,CAZ,CRO, PEF,GN	OFX, CPX, PEF
E4	OFX,CAZ,CRO,CPX, AU,S	CRO,OFX, CAZ
E5	CAZ,CRO,OFX,GN, AU,PEF	CRO,AU, OFX,
E6	CRO,CAZ,OFX,GN, AU,PEF	CAZ,CRO, PEF,OFX
E7	CPX,S,CRO,CAZ,PEF,GN	CRO,CAZ
E8	CRO,CAZ,CPX,PEF, AU,S	S, CPX, PEF
E9	OFX,CPX,CRO,CAZ, P EF,S	OFX,CPX, CRO,CAZ, PEF,S
E10	CAZ,CRO,OFX,PEF,AU,S	CRO,OFX,S
E11	PEF,AU,CPX,CAZ, CRO,GN	PEF,CRO, CPX
E12	CRO,CAZ,AU,PEF,CPX,S	
E13	CPX,PN,OFX,CRO, CAZ,	
E14	CRO.CPX,PN,GN,PEF,S	
E15	CAZ,CRO,OFX,CPX, AU,S.	

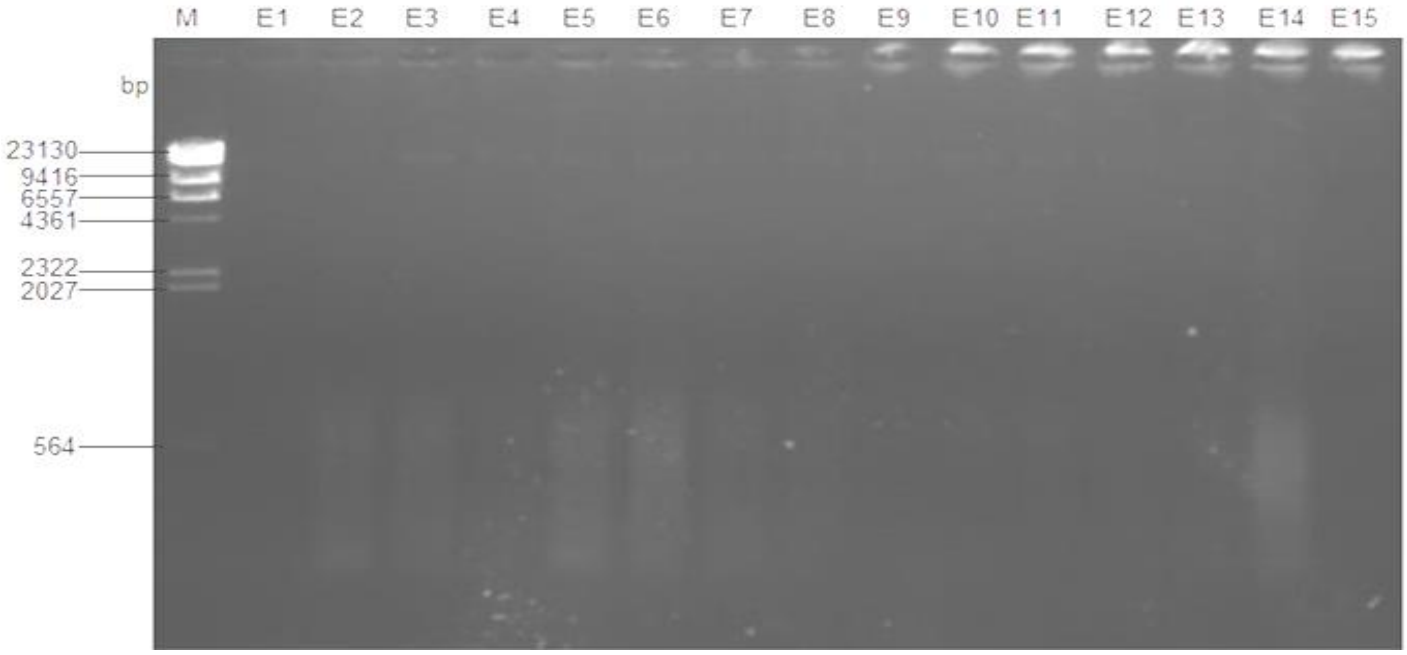

Figure 1: Plasmid profile of resistant isolates from the study

Hence, the resistance is plasmid-mediated because when the isolates that were cured of plasmids were re-exposed to the initially resistant antibiotics, it was discovered that they became sensitive. According to [33], curing agents like ethidium bromide operate on plasmids by either inhibiting DNA gyrase, which is in charge of plasmid DNA replication, or by inhibiting plasmid efflux pumps on the plasmid membrane. Additionally, they stated that this curing agent outperformed other physical and chemical agents like sodium dodecyl sulphate and acridine orange in terms of activity. Moreover, it has been noted that the effectiveness of the cure varies depending on the type of plasmid and the bacterial host harboring it [33]. According to [17], ethidium bromide's curative action against most of the isolates may cause their susceptibility to antimicrobial drugs to which they had previously been resistant.

4. Conclusion

The rate of resistance of some of the *E. coli* organisms explains their clinical significance in diabetic foot ulcer infections. This is reinforced by the fact that ESBL production was reported alongside AmpC production and Biofilm formation.

Authors' Contributions

Emmanuel Onwubiko Nwankwo: Supervision; Methodology; Data curation; Investigation; Writing-Review; Editing. Enyinnaya Nwagbara: Conceptualization; Data curation; Funding acquisition; Formal analysis; Resources. Ebubechi Uloma Okey-Kalu: Investigation; Writing-original draft; Writing-Review; Editing.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

We extend our gratitude to Madonna Austrian Hospital Ihitte, Imo State, Nigeria, for approving this study and providing the used samples. This research was approved by the Health Research Ethical Committee of Madonna Austrian Hospital. (project number REF/MAH/18/216).

References

1. Gabriel UP, Amadi AN, Ebirim CI. Type 2 Diabetes Mellitus in Ambulatory Adult Nigerians: Prevalence and Associated Family Biosocial Factors in a Primary Care in Eastern Nigeria: a Cross Sectional Study. *British Journal of Medicine and Medical Research.* 2015; 9(3):1-12.

2. Sanders LJ, Robbins JM, Edmonds ME. History of the Team Approach to Amputation Prevention: Pioneers and Milestones. *Journal of Vascular* . 2010; 52(3): 3S-16S.

3. Sweitzers SM, Fann SA, Borg TK. What is the Future of Diabetic Wound Care? . *The Diabetes Educator.* 2006; 32(2): 197-210.

4. Singh N, Armstrong DG, Lipsky BA. Preventing Foot Ulcers in Patients with Diabetes. *Journal of American Medical Association (JAMA).* 2005; 293: 217–28.

5. Watters C, Everett A, Haley A. Insulin Treatment Modulates the Host Immune System to Enhance Pseudomonas Aeruginosa Wound Infections. 2014; 82(1): 92-100.

6. Yoga R, Khairul A, Sunita K, Suresh C. Bacteriology of Diabetic Foot Lesions. *Medical Journal of Malaysia.* 2006; 61: 14-6.

7. Akinjogunla OJ, Adegoke AA, Mboto C, Chukwudebelu IC, Udokang IP. Bacteriology of Automobile Accident Wounds Infection. *International Journal of Medicine and Medical Sciences.* 2009; 2: 23-7.

8. Mary LT. Linne and Ringsrud's Clinical Laboratory Science. 2012: 53-4.

9. Cheesebrough M. Medically Laboratory Manual for Tropical Countries. Butterworth Heinemann Ltd, Oxford United Kingdom. 2006: 200-34.

10. Clinical and Laboratory Standards Institute CLSI. Performance Standards for Antimicrobial Disk Susceptibility Tests. Approved Standard, 11th Edition. West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA. 2013.

11. Riaz S, Faisal M, Hasnain S. Antibiotic Susceptibility Pattern and Multiple Antibiotic Resistances (MAR) Calculation of Extended Spectrum ß-Lactamase (ESBL) Producing Escherichia Coli and Klebsiella Species in Pakistan. *African Journal of Biotechnology.* 2011; 10(33): 6325-31.

12. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic Susceptibility Testing by a Standardized Single Disc Method. *American Journal of Clinical Pathology.* 1996; 45: 493-6.

13. Jacoby GA, Mederios AA. More Extended Spectrum B-Lactamases the/e CTX-M Enzymes. *Antimicrobial Agents and Chemotherapy.* 2004; 48: 1-14.

14. Livermore DM, Woodford N. The Betalactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter. *Trends in Microbiology.* 2006; 14: 413–20.

15. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of Biofilm Formation Among the Clinical Isolates of Staphylococci: An Evaluation of Three Different Screening Methods. *Indian Journal of Medical Microbiology.* 2006; 24(1): 25-9.

16. Appuhamy S, Low JC, Coote JG, Parton R. PCR Methods and Plasmid Profile Analysis for Characterization of Histophilus Ovis Strains. *Journal of Medical Microbiology.* 1998; 47: 987-92.

17. Raghada SM, Munera CI, Ayad MAF. Detection of Plasmid DNA Profile in Bacterial and Fungal Isolates from Catheterizeds Patients and its Relation with Antibiotic Susceptibility. *Journal of Biotechnology Research Center.* 2013; 7: 51-60.

18. Lilian A, Fokunang NC, Elias NN, Emmanuel KT, Armel HN. Bacteriology of Diabetic Foot Ulcers with Reference to Multidrug Resistance Strains at the Yaounde Central Hospital. *Journal of Diseases and Medicinal Plants.* 2015; 1(4): 53-8.

19. Mohanasoundram KM. The Microbiological Profile of Diabetic Foot Infections. *Journal of Clinical and Diagnostic Research.* 2012; 6(3): 409-11.

20. Nwachukwu NC, Orji FA, Udora EC. Bacteriological Evaluation of Diabetic Ulcers in Nigeria. *African Journal of Diabetes Medicine.* 2009: 19-21.

21. Viswanathan V, Jasmine JJ, Snehalatha C, Ramachandran A. Prevalence of Pathogens in Diabetic Foot Infection in South Indian Type 2 Diabetic Patients. *Journal of Association of Physicians India.* 2002; 50: 1013-6.

22. Ayesha N. The Most Prevalent Organism in Diabetic Foot Ulcers and Its Drug Sensitivity and Resistance to Different Standard Antibiotics. *Journal of the College of Physicians and Surgeons Pakistan.* 2016; 26 (4): 293-6.

23. Ranjini CN, Vidhya RR. A Clinicomicrobial Study of Diabetic Foot Ulcer Infections in South India. *International Journal of Medicine and Public Health.* 2015; 5(3). 236-41.

24. Ruth CB, Richard KD, Ben AE, John A. Bacterial Profile of Diabetic Ulcers of Patients Visiting a Specialist Diabetic Clinic at Komfo Anokye Teaching Hospital Kumasi Ghana. *British Journal of Medicine and Medical Research.* 2014; 4(27): 4501-10.

25. Asima B, Mir Mohammad NH, Janani R, Sathyabheemarao S. Spectrum of Bacteria Associated with Diabetic Foot Ulcer and Biofilm Formation: A Prospective Study. *Australian Medical Journal.* 2015; 8(9): 280–5.

26. Shashikala V, Farman A, Narendranath L, Joseph M. Diabetic Foot Ulcer and Biofilm Formation. *International Journal of Biomedical and Advance Research.* 2016; 7(9): 428-33.

27. Bansal E, Garg A, Bhatia S, Attri AK, Chander J. Spectrum of Microbial Flora in Diabetic Foot Ulcers. *Indian Journal of Pathololgy and MIcrobiology.* 2008; 51(2): 204-8.

28. Samir P, Lovely B, Ashraf JJ, Ashraful H. A Bacteriological Study of Diabetic Foot Infection in an Urban Tertiary Care Hospital of Dhaka City. *Ibrahim Medical College Journal.* 2009; 3(2): 50-4.

29. Mohamudha PR, Harish BN, Parija SC. Ampc Beta Lactamases Among Gram Negative Clinical Isolates From A Tertiary Hospital, South India. *Brazilian Journal of Microbiology.* 2010; 41: 596-602.

30. Medeiros AA. Evolution and Dissemination of B-Lactamases, Accelerated by Generations of B-Lactam Antibiotics. *Clinical Infectious Disease.* 2010; 24 (1): 19-45.

31. Suranjana A, Manjusri B. AmpC Lactamase Producing Bacterial Isolates from Kolkata Hospital. *Indian Journal of Medical Research.* 2005; 122: 224233.

32. Subha A, Renuka DV, Ananthan S. AmpC B-Lactamase Producing Multi-Drug Resistant Strains of *Klebsiella* Spp & *Escherichia coli* Isolated from Children Under Five in Chennai. *Indian Journal of Medical Research.* 2003; 117: 13-8.

33. Zaman MA, Pasha MH, Akhter MZ. Plasmid Curing of E. Coli Cells with Ethidium Bromide, Sodium Dodecyl Sulphate and Acridine Orange. *Bangladesh Journal of Microbiology.* 2010; 27: 28-31.