Occurrence and Emergence of New-Delhi Metallo Beta-lactamase-1 Positive *Pseudomonas aeruginosa* from Urine samples in Ebonyi State, Nigeria

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Abstract: This study was intended to find the extent of occurrence and prevalence of carbapenem resistance *Pseudomonas aeruginosa* from urine samples from both clinical and non-clinical settings in Ebonyi state. A cross sectional study was carried out from April-December 2021 in Alex-Ekwueme Federal University Microbiology Laboratory Ebonyi State amongst individuals using a pre-tested, self-administered questionnaire. A total of 185 urine samples were isolated from three (3) health institutions and four communities all in Ebonyi state and isolation of microorganisms and proper identification were done using standard microbial techniques. The sensitivity of the isolated organisms to commonly used antibiotics (Oxoid, UK) was determined by Kirby-Bauer diffusion method. Results for ESBL production shows that of the 185 isolates under study, ESBL production was seen in 35.7% (66/185) *Pseudomonas aeruginosa* by MDDST, only Cefepime and no other third generational Cephalosporins showed synergism with amoxicillin-clavulanate to five isolates of *P. aeruginosa*. Out of 185 *Pseudomonas aeruginosa* isolates, 32(17.3%) were carbapenem resistant, while 12(6.5%) of the isolates were confirmed as carbapenemases producers. DNA extraction of New-Delhi Metallo beta-lactamase genes (NDM-1) using Agarose gel electrophoresis shows that New-Delhi Metallo beta-lactamase genes were present at Lane 6 of NDM-1 gene band (950bp) and Lane A represents the 100bp molecular ladder of 1500bp. This increase in resistance and the presence of NDM-1 genes can be attributed to abuse of antibiotics in humans and animal settings. Hence formulation of good policy, usage of antibiotics by government agency is advocated and it's monitoring and enforcement encouraged

Keywords: Antibiotics, Resistance, Kirby-Bauer, Carbapenem, Modified double disc synergy test (MDDST). New-Delhi Metallo beta-lactamase (NDM), Occurrence.

INTRODUCTION

For over seventy years the antimicrobial era has been marked by successive discoveries of a wide range of antibiotics and the subsequent emergence of antibiotic resistance (Ajuba *et al.,* 2020). Enterobacteriaceae are rod-shaped, gram-negative bacteria that are normal inhabitants of the intestinal flora and among the most common human pathogens, causing infections (Poirel *et al.,* 2012). They have been renowned for the production of extended-spectrum beta-lactamases (ESBL) and have in recent time also shown remarkable production of the carbapenemases leading to limited antibiotic treatment option (Abdullahi *et al.* 2017). Very few studies have reported the occurrence and detection of carbapenemase in Nigeria and this limitation poses a lot of problem with finding in Ebonyi State Nigeria (Oduyebo *et al.,* 2015).

The objectives of this research work is to isolate and identify *P. aeruginosa* from both clinical and non-clinical urine samples, to determine their antimicrobial resistance profile and also determine the presence of New-Delhi metallo beta-lactamase (NDM) genes in multi resistant clinical and community isolates of *P. aeruginosa.*

MATERIALS AND METHODS

Study area

This study was conducted in Abakaliki, the capital of Ebonyi State, Nigeria. Ebonyi State located in the South-Eastern Nigeria was created in 1996. It has thirteen (13) Local Government Areas and occupies a land mass of 5933km^2 space and shares boundaries with Enugu and Cross River states in the East, Abia in the South-East and Benue in the North.

It has an estimated population of 2.3m, with 75% of these living in the rural area and farming as their major occupation.

A cross-sectional study of patients with urinary tract infections (UTI) and individuals with history of Urinary tract infections from four (4) random communities which adopted semi-structured questions about patients in health institutions and individual from the communities. One hundred and eighty-five (185) non duplicate *P. aeruginosa* isolates were obtained consecutively from urine specimen of patients submitted to the Medical Microbiology Laboratory Unit of Alex-Ekwueme Federal University Teaching Hospital Abakaliki (AE-FUTHA). AE-FUTHA is a tertiary health institution in Ebonyi State and also a major referral centre serving individuals in South-Eastern Nigeria and the country Nigeria. This study carried out from April to December, 2021.

The urine samples were from patient admitted in Hospital with Urinary tract infection (UTI) and also from patients in Intensive Care Unit (ICU) and from health institutions within Abakaliki and its environs.

Ethical Approval

This study received approval from the research and ethics committees of AE-FUTH, Abakaliki, with reference number AE-FUTHA/REC/VOL 3/2021/232. Ethical consideration was based on the general ethical principles as applicable to human subjects. These are respect to person, beneficence, non malificience and justice. All participants in this study gave their verbal consent.

Sample size

The sample size was calculated using the structural formula based on the prevalence of 12.4% of patients and a confidence level of 95%. The Formula used to calculate sample size is

$$N = \frac{Z^2 \alpha}{d^2} pq$$

Where n= the desired sample size.

Z= the standard normal deviation usually set at 1.96 which corresponds to 95% confidence interval

P= prevalence of the disease

Q= 1-P

d= degree of accuracy (set at 0.05)

$$n = \frac{(1.96)^2 (0.124) (0.944)}{(0.05)^2} = 180$$

180+5 (10% ATTRITION RATE)

=185 for each group (minimal sample size)

Sample size = 185

Sample collection

Each urine sample was collected from April to September 2021 from seven different locations with a sterile wide-mouthed and screwed-capped container and transported immediately to the Microbiology Laboratory at AE-FUNAI for processing within 1 hour of collection. Information concerning the patient's age, gender and occupation were also obtained.

Bacterial isolation

Each Urine sample was centrifuged and sediments inoculated on Pseudomonas isolation agar (PIA) and MacConkey agar (Oxoid, UK). All plates were incubated aerobically at 37ºC for 24 hrs. Then growth was confirmed by visible colonies on the surface of the culture media.

Bacteria identification

The isolated organisms were gram-stained and subjected to standard biochemical tests for *P.aeruginosa* identification. These tests include methyl red test, Indole production, citrate utilization, Vogues Proskauer, catalase and oxidase. The suspected *P. aeruginosa* isolates were then confirmed as *P. aeruginosa* by the production of diffusible pigments. Some pseudomonads did not grow well at 35ºC. After 24 hours when there was no growth, the plate(s) were incubated at 25ºC for an additional 24 hours then sub-cultured on MacConkey Agar and incubated for 18-24h at 37ºC (Cheesbrough 2010).

Antimicrobial susceptibility testing

The sensitivity of the isolated organisms to commonly used antibiotics including Amikacin (AK) 30µg, Amoxicillin (AMC) 10µg, Aztreonam (ATM) 30µg, Cefepime(FEP) 30µg, Cefotaxime (CTX) 30µg, Ceftriaxone (CRO) 30µg, Ertapenem (ETP) 10µg, Imipenem (IPM) 10µg, Meropenem (MEM) 10µg and Gentamycin (CN) 30µg (Oxoid, UK) were determined by Kirby-Bauer diffusion method (Cheesbrough 2010). Incubation was performed at 37ºC for 18-24hours. After incubation, the inhibition zone was measured for each antibiotic produced by the *P. aeruginosa* isolates was measured in millimeters, and this was interpreted as either sensitive or resistant based on CLSI standard interpretive criteria.

Detection of ESBL production using modified double disc synergy test.

All the strains that showed a diameter of less than 27mm for cefotaxime and less than 25mm for ceftriaxone, were selected for ESBL production examination. The ESBL production was tested by the modified Double Disc Synergy Test (MDDST) by using a disc of am oxicillin-clavunate (20/10) with other four cephalosporins

3 generational cephalosporins (3GC): cefotaxime, ceftriaxone and cefazidime and 4 generational cephalosporins (4GC): cefepime (Manchanda *et al*., 2003). The increased prevalence of bacterial pathogens producing both ESBLs and AmpC β-lactamases creates a requirement for laboratory testing can accurately detect the presence of these enzymes in clinical isolates. (Aibinu *et al.,* 2004) (Thomson, 2010).

DNA extraction

DNA from *Pseudomonas aeruginosa* was extracted using boiling method of DNA extraction (Maria-Eleni., 2020). Five hundred microlitres of an overnight broth culture of bacterial isolated from *P. aeruginosa* in Luria Bertani medium were added into an Eppendorf tube. After which normal saline of 1000µl (i.e 1ml) was added and mixed by vortexing. After vortexing, the suspension was spine at 14,000 rpm for 5 mins. The supernatants were re-suspended in 1000µl of normal saline and mix properly using a vortex mixer. This process was done three times; the supernatant was once again discarded. The sediment was re-suspended again, this time in 500µl of DNA elution buffer and again vortexed. this mixture was heated on a heating block at 95ºC for 25mins and fast cooled on ice and spun again for 3 mins at 14,000 rpm, then the supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20ºC for further downstream reactions (Maria-Eleni., 2020).

Molecular detection of new-delhi metallo beta-lactamase

Polymerase Chain reaction (PCR) was used to amplify genes encoding NDM group of Carbapenemases. Polymerase chain reaction is a method widely used to rapidly make millions to billions of copies (complete copies or partial copies) of a specific DNA sample, allowing scientists to take a very small sample of DNA and amplify it to a large enough amount to study in detail (Castanheira *et al*., 2004).

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of was initialized with 2µl of sterile distilled water band blanked using Normal Saline. Two Microlitres of the extracted DNA was loaded onto the lower pedestal. The DNA concentration was measured by clicking on the measure button (Maria-Eleni., 2020).

16S rRNA amplification

The 16s rRNA region of the rRNA gene of the isolates was amplified using the 27F: 5'-AGAGTTTGATCTGGCTCAG-3'and
1492R: (Maria-Eleni., 2020; Fazeli., 2015).
5'-CGGTTACCTTGTTACGACTT-3'
Primers on a AB19700 Applied Biosystem thermal cycle at a final volume of 40 microlitres for 35 cycles. The PCR mix includes: The X_2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl).

The primers at a concentration of 0.5µM and the extracted DNA as template, the PCR conditions were as follows for NDM:
Initial denaturation, 95ºC for 5 minutes, denaturation was done for 95ºC for 30seconds, annealing at 52ºC for 30 seconds, extension at 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transillumination (Yong *et al.,* 2009).

Table 1: Primer sequence used in the study

Primers [a]	Sequence (5'-3')[c]	Gene	Expected size of PCR product	Reference
NDM (F)	5'-GGTTTGGCGATCTGGTTTTC-3'	bla_{NDM}	621	L. Pourel *et al.* (2011).
NDM (R)	5'-CGGAATGGCTCATCACGATC-3'	bla_{NDM}	264	

RESULTS

Antimicrobial susceptibility of 185 isolates of *Pseudomonas aeruginosa*

This work phenotypically evaluated urine samples from four (4) communities and three (3) health institutions. Bacterial isolation and biochemical characterization show that a total 130 (130/185) (68.4%) isolates of *Pseudomonas aeruginosa* species was isolated from 185 urine samples bacteriologically analyzed (Table 1).

Table 1: Antimicrobial susceptibility of 185 isolates of *P. aeruginosa*

Antibiotics (µg)	Susceptible N (%)	Resistant N (%)
Amikacin(10)	30(23.1)	100(76.9)
Ceftriaxone (30)	12(9.2)	118(90.8)
Meropenem(10)	3(2.3)	127(97.7)
Cefotaxime (30)	7(5.4)	123(94.6)
Gentamycin(10)	54(41.5)	76(58.5)
Aztreonam(30)	0(0)	130(100)
Cefepime(30)	0(0)	130(100)
Imipenem(10)	42(32.3)	88(67.7)
Amoxicillin(10)	4(3.1)	126(97.7)
Ertapenem	15(11.5)	115(88.5)

Key: %= percentage

ESBL production

Of the 185 isolates which were studied, ESBL production was seen in 35.7% (66/185) *Pseudomonas aeruginosa* by MDDST. Only Cefepime and no other third generational Cephalosporins showed synergism with amoxicillin-clavulanate to five isolates of *P. aeruginosa.*
Twenty-nine percent (54/185) of the ESBL producers were from in patients. While 10.3% of the ESBL producers were from Urinary Tract infected (UTI) patients. These ESBL isolates were obtained from 94 female and 91 male patients. They are distributed in the range of 6 to 76 years as seen below. Out of 185 *P. aeruginosa* isolates, 32(17.3%) were carbapenem resistant while 12 (6.5%) were detected in 2/185 (1.1%).

Table 2: ESBL production *Pseudomonas aeruginosa*

Organism	Total ESBL producers #
***P. aeruginosa* (n-185)**	**66 (37.5%)**

Antibiotics	*Pseudomonas aeruginosa* (n=66)	Total (n=66)
Cefotaxime	8	8(12%)
Ceftriaxone	5	5(23%)
Ceftazidime	4	4(6%)
Cefepime	12	12(18%)

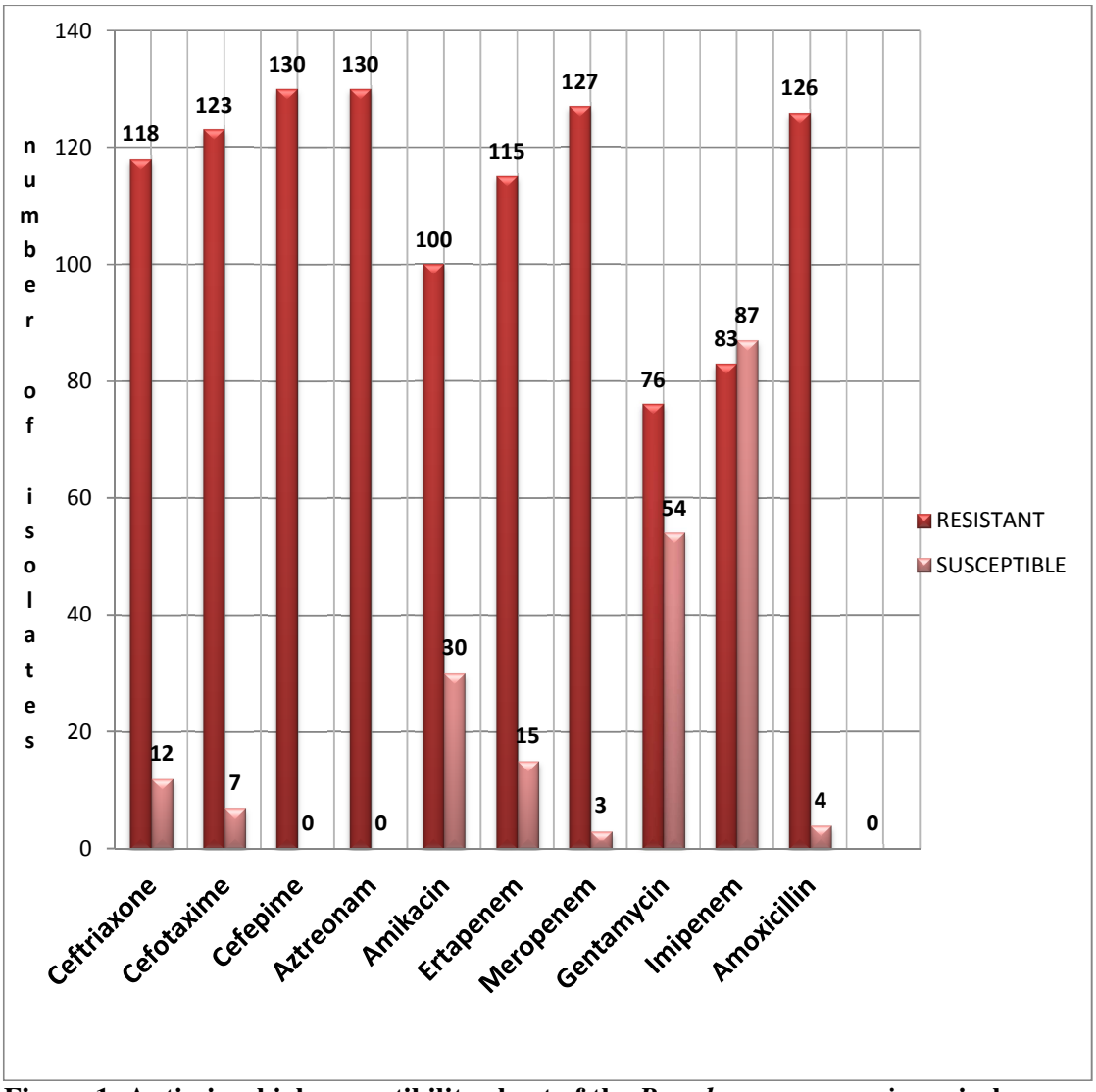

Figure 1: Antimicrobial susceptibility chart of the *Pseudomonas aeruginosa* isola

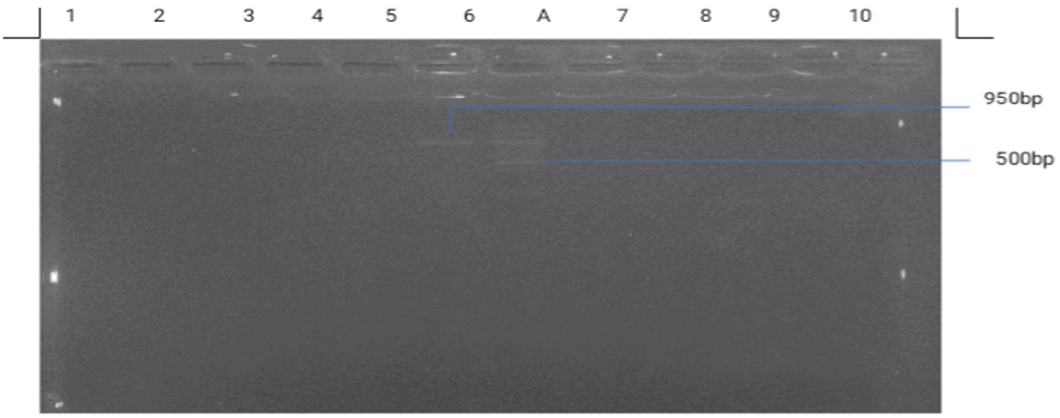

Plate: Agarose gel electrophoresis of NDM gene of some selected bacteria isolates. Lane 6 represents t he NDM gene band (950bp). Lane A represents the 100bp Molecular ladder of 1500bp.

Figure 2: Agarose gel electrophoresis showing the amplified New-Delhi Metallo beta-lactamase gene bands. Lane 6 represents the NDM gene band (950bp). Lane A represents the 100bp molecular ladder of 1500bp.

DISCUSSION

The increase in resistance of carbapenem in both hospital and community acquired infections all over the world over the last decade has raised concern among healthcare professionals and wider community. Also the emergence and dissemination of carbapenemases among gram-negative bacteria are considered a significant public health challenge. Information on the emergence and spread of carbapenemases-producing Enteriobactericeae in Ebonyi state, south-eastern Nigeria are limited.

The isolates *pseudomonas aeruginosa* showed high resistance to Aztreonam (100%), Cefepime (100%). Amoxicillin (97.7%) and Meropenem (97.7%) Resistance was also high for Cefotaxime (94.6%), Ceftriaxone (90.8%), Ertapenem (88.5%) While moderately resistance were recorded for Amikacin (76.9%), Imipenem (67.7%) and with lowest resistance to Gentamycin (58.5%) Above results showed reduced susceptibility to the Carbapenem, Imipenem (67.7%), high for meropenem (97.7%) and 88.5% for Ertapenem. *Pseudomonas aeruginosa* were very resistance to the Cephalosporins used in the study which include Ceftriaxone (90.8%), Cefotaxime (94.6%), Cefepime (100%). Amikacin (76.9%) and Gentamycin (58.5%) are both Aminoglycosides. Aibinu *et al* (2003) in his work shown that in western part of Nigeria, the recent prevalence of this Carbapenem resistance is as a result of indiscriminate use of broad-spectrum antibiotics.

CONCLUSION

The identification of rare NDM-1 genes in *P. aeruginosa* isolated from patients with Urinary tract Infection portends a very high risk to public health. This detection shows that this virulence and antibiotic resistance genes which are plasmid mediated shows a great possibility of gene transfer between and within related species and even unrelated ones around our hospitals and communities and is of a great concern. Massive spread of this gene will hamper the effectiveness of antibiotic treatment.

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REFERENCES

Abdullahi S.A., Arzai A.H., Yusuf I., Adamu S., & Koki Y.A. (2017). Molecular Detection of New-Delhi metallo-Beta-Lactamase-1(NDM-1) Producing Bacteria isolates in Kano North Western Nigeria *Annu. Rev. Biol 14*: 1-6

Aibinu I, Fashae K, Ogunsola F, Odumgbemi T., & Mee B.J. (2004). Extended spectrum Beta-lactamase (ESBL) in Klebsiella pneumonia isolates from septicaemic children in Ibadan, Nigeria. Nigeria *J health Biomed Sci 3*(2):79-84.

Ajuba I.M., Akujiobi C.N., & Aghanya I.N. (2020). Emergence of New-Delhi metallo-betalactamase-1 and oxacillinase-48 positive *Escherichia coli* in South-Eastern Nigeria. *Nigeria Journal of Medicine 4*:701-706.

Akingbade, O., Balogun, S., Ojo, D., Akinduti, P., Okeretugba, P. O, Akinjogunla, O. J., & Enabulele, I. O. (2010). Virulence plasmid profile analysis of multidrug resistant Escherichia coli isolated from patients with acute otitis media. *J Am Sci. 6* (2): 1022-1033

Brusselaers N., Vogelaers D. & Blot S. (2011). The rising problem of antimicrobial resistance in the intensive care unit. *Ann Intensive Care* 1(1):47.

Castagnola E., Tatarelli P., Mesini A., Baldelli I., La Masa D., & Biassoni R. (2019). Epidemiology of carbapenemase-Producing Enterobacteriaceae in a Pediatrics hospital in a country with high endemicity *J.infect Public health 12*: 270-274.

Castanheira M, Toleman M.A, Jones R.N, Schmidt F.J, & Walsh T.R. (2004). Molecular characterization of a beta-lactamase gene, blaGIM-1, encoding a new subclass of metallo-beta-lactamase *Antimicrob Agents Chemother 48*:4654-61.

Cheesbrough M (2010) Biochemical test too identify bacteria in:District Laboratory practice in tropical countries (Part 2). *Cambridge University Press, Cambridge,* UK. PP: 77-76

Cuzon G, Naas T, Lesenne A, Benhamou M. & Nordmann P. (2010) Plasmid-mediated carbapenem Microbiol Infect 17: 552–556.

Delcour A.H. (2009). Outer membrane permeability and antibiotic resistance. *Biochem Biophys Acta* 1794(5):808–16.

Du D., Van-Veen H.W., Murakami S., Pos K.M. & Luisi B.F. (2015). Structure, mechanism and cooperation of bacterial multidrug transporters. *Curr Opin Struct Biol* 33:76–91.

Ejikeugwu C, Edeh C., Iroha I, Orji J. & Eluu S. (2016). Antibiogram and detection of metallo-Beta-Lactamase (MBL) positive *Escherichia coli*

isolates from abattoir. *Nature and science 14* (11): 65-69.

Iroha, I. R., Amadi, E. S., Oji, A. E., Nwuzo, A., & Ejike-Ugwu, P. C. (2010).Detection of Plasmid Borne Extended Spectrum Beta Lactamase Enzymes from Blood and Urine Isolates of Gram-Negative Bacteria from a University Teaching Hospital in Nigeria. *Current Research in Bacteriology. 3***:** 77-83.

Manchanda V. & Singh N.P. (2003). Occurrence and detection of AmpC β-lactamases among gram negative clinical isolates using a modified three-dimentional test at Guru Tegh Bhadur Hospital, Delhi, India. J Antimicrob Chemother. 51:415-18.

Maria-Eleni D, Venia S., CXhry Soula K., & Apostolos V. (2020). Boiling extraction Method vs Commercial kits for bacterial DNA isolation from food samples. *J. food Sci. Nutr. Res 3*(4): 311-319.

Motayo B.O., Akinduti P.A., Adeyakinu F.A., Okerentugba P.O., Nwanze J.C ., Onoh C.C., Innocent-Adiele H.C., & Okonkwo I.O. (2013). Antibiogram and Plasmid profiling of carpenease and extended spectrum Beta-lactamase (ESBL) producing Escherichia col, Klebsiella Pneumoniae in Abeokuta, South Western Nigeria. *Afr. Health.Sci 134*(4): 1091-1097.

Neyestanaki D.K., Mirsalehian A., Rezagholiazadeh F., Jabalameli F., Yaheri Kalani M. & Emaneini M.(2014) Determination of extended spectrum beta-lactamases and AmpC-beta-lactamases among Carbapnem resistant Pseudomonas aeruginosa isolated from burn patients. *burns* 40 (8): 1556-61

Nordamann P., Naas T., & Poirel L. (2011). Global spread of carbapenemse-producing enterobacteriaceae. *Emerg infect Dis* 1791-1798.

Oduyebo O.O, Falayai O.M, Oshun P., & Ettu A.O., (2015). Phenotypic Determination of carbapenemase producing Enterbacteriaceae isolates from Clinical Specimens at a tertiary hospital in Lagos Nigeria, *Niger postgrad Med J.22*:223-227.

Okoche D., Asumwe B.B., Katabazi F. A., Kato L., & Najjuka C.F. (2015). Prevalence and characterization of Carbapenem-Resistant Enterobacteriaceae Isolated from Mulago Nationasl Refferal Hospital, Uganda. PLoS one 10:e0135745.

Poirel L, Potron A & Nordmann P.(2012) OXA-48-like carbapenemases: the phantom menace. *J Antimicrob Chemother* 67: 1597–1606.

Queenan A.M., & Bush K. (2007) Carbapenemases: the versatile beta-lactamases. *Clin Microbiol Rev* 20:440-458.

Thomson K.S., (2010). Extended-spectrum-beta-lactamase, AmpC, and Carbapenemase issues. *J Clin Microbiol*; *48*:1019-25.

Yong D., Toleman M.A., & Giske C.G. (2009). Characterization of a new metallo-Beta-lactamase gene bla (NDM-1) and a novel erythromycin Esterase gene carried on a unique genetic structure in Klebsiella pneumonia Sequence type 4 from India. *Antimicrob Agent Chemother 53*:5046-5054.