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Phenotypic and molecular characterization of extended spectrum β-lactamase producing *Pseudomonas aeruginosa* in Nigeria

Martina C. Agbo[1]*, Ifeoma M. Ezeonu[2], Maurice N. Odo[1], Chukwuebuka M. Ononugbo[2], Beatrice O. Onodagu[3], Chinelo C. Eze[1], Ezinwanne N. Ezeibe[1] and Chizoba A. Ozioko[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.
[3]Microbiology Laboratory Unit, University of Nigeria Teaching Hospital, Enugu, Nigeria.

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This present study was undertaken for detection of extended spectrum β-lactamases (ESBLS) enzyme genes among clinical isolates of *Pseudomonas aeruginosa* using phenotypic and molecular techniques. Thirty-four *P. aeruginosa* isolates from different hospitals in Nsukka and University of Nigeria Teaching Hospital (UNTH), Enugu were screened for the presence of ESBL-encoding genes. Phenotypic screening for ESBL producers was carried out using double disk synergy test and combined disk test. Genomic DNA was extracted from the isolates by modified boiling method. Extracted DNA was amplified by polymerase chain reaction (PCR) using ESBL specific primers namely *Bla GES, PER, OXA-50, SHV, CTX-M* and *TEM.* The results revealed that a total of 15 isolates of *P. aeruginosa* were identified as ESBL producer by phenotypic approaches which exhibited varying degrees of resistance to an array of antibiotics tested. While, the PCR screening revealed that 53.33% (n=8) of the isolates that were phenotypically ESBL positive harboured *bla OXA*-50 gene. However, the genes that encode *PER, GES, SHV, TEM* and *CTX-M* were not found in any of the *P. aeruginosa* isolates. This study highlights the need to establish antimicrobial resistance surveillance network to determine the appropriate empirical treatment regimen for *Pseudomonas* infections.

Key words: *Pseudomonas aeruginosa*, antibiotic resistance extended spectrum β-lactamase (ESBL), polymerase chain reaction (PCR), Nsukka.

INTRODUCTION

Pseudomonas aeruginosa is widely known as an opportunistic pathogen, frequently involved in infections of immunosuppressed patients and also causes outbreak of hospital-acquired infections (Wirth et al., 2009). According to the USA nosocomial infection surveillance system, *P. aeruginosa* is the third most common pathogen associated with hospital acquired infections, accounting for 10.1% of all nosocomial infections and is associated with high mortality rate (Moreaus-Marquis et al., 2008). This infection may cause septicaemia,

*Corresponding author. E-mail: martina.agbo@unn.edu.ng. Tel: +234 803 970 5442.

pneumonia, meningitis, wound, urinary tract, surgical wound, burn, and ear infections (Todar, 2008). It can tolerate diverse environmental conditions and resistant to many antimicrobial agents (Todar, 2014). This resistance in *P. aeruginosa* may be mediated via several mechanisms such as the production of β-lactamases, efflux pumps and target site or outer membrane modifications (Tam et al., 2010).

Generally, extended spectrum β -lactamases (ESBLS) are a group of β-lactamases that hydrolyze penicillin and cephalosporin, including oxyamino-β-lactamase (third and fourth generation of cephalosporin) and aztreonam. These ESBL enzymes are known to be inhibited by β-lactamase inhibitors, such as clavulanic acids, sulbactam and tazobactam (Peterson and Bonomo, 2005). These enzymes are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* and have been recently detected in *P. aeruginosa* at low frequency (Lee et al., 2005). These enzymes are encoded by different genes located on chromosomes or plasmids and transposon and are easily disseminated by conjugation. Based on Ambler classification these enzymes are grouped into four groups A to D (Livermore, 2002; Shen and Fang, 2015).

Class B comprises metallo-β-lactamases, which use zinc for the facility reaction and can be inhibited by ethylenediaminetetracetic acid (EDTA), whereas classes A, C and D comprise serine β-lactamases, which can be resistant to many classes of cephalosporin and oxacillin. Class A is the most diverse class; it comprises enzymes that can inhibit β-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam. Class A-lactamases are encoded by many genes such as those that encode *VEB, PER, CTX, SHV* and *TEM* (Bush and Jacoby, 2010). Class C and D β-lactamases can be resistant to cephalosporin and oxacillin, respectively. The enzymes in these classes cannot be inhibited by β-lactamase inhibitors. There are many variants of the genes that encode class D β-lactamases, such as *OXA-1, OXA-2, OXA-10* and *OXA-50* (Poirel et al., 2010).

Most studies on molecular characterization of ESBL producing organisms have been carried out worldwide but only few data are available concerning the genetic characterisation of clinical isolates from Nigeria. Rapid and prompt detection of ESBL producing *P. aeruginosa* is of utmost importance to declare the appropriate antimicrobial therapy and also for preventing cross-transmission to other patients in the hospital. Several phenotypic methods have been proposed for ESBL detection on isolated *P. aeruginosa* strains such as double disk synergy test (DDST), combined disk tests (CDT) methods and the ESBL-E test, but their discrepancy in sensitivity has been reported (Drieux et al., 2008). Moreover, there are specific molecular test for ESBL detection in bacterial isolates which reduce the time of detection and increase the sensitivity and specificity (Sharma et al., 2013). The application of molecular biology technique has enhance the specificity and accuracy in diagnosis of *P. aeruginosa* strains and in the discovery of genotypic form of ESBL *P. aeruginosa* and how these genes disseminate into various isolates. However, there is little information on the molecular characterization of ESBL producing *P. aeruginosa* in Nigeria particularly in Southeastern Nigeria. Therefore, this study aimed to investigate the presence of ESBL production among the clinical isolates of *P. aeruginosa* using phenotypic and genotypic methods.

MATERIALS AND METHODS

The bacterial strains

Thirty four (34) strains of *P. aeruginosa* were collected from Microbiology Laboratories from various hospitals in Nsukka and UNTH, Enugu between May and August, 2018. Ethical approval and informed consent was not required by our Institution Ethics Committee because all bacterial isolates were collected, processed and stored as part of routine diagnosis by the hospitals. No patient information was associated with the data. The isolates obtained from the various laboratories were further characterized using 16S rRNA primer targeting *P. aeruginosa* consensus region (Inqaba biotechnical Company, South Africa).

Antimicrobial susceptibility test

The antimicrobial susceptibility testing of *P. aeruginosa* was performed using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI, 2014) guidelines. Commercially available antimicrobial disks of ceftazidime 30 µg (CAZ), cefepime 30 µg (FEP), ceftriaxone 30 µg (CRO), cefotaxime 30 µg (CTX), gentamicin 10 µg (CN), imipenem 10 µg (IMP), Aztreonam 30 µg (ATM), piperacillin/tazobactam 100/10 µg (TZP), ciprofloxacin 5 µg (CIP), meropenem 10 µg (MEM) and piperacillin 100 µg (PPL) (Oxoid, U.K.) were used on Mueller Hinton Agar (Oxiod, UK) to test susceptibility. Zone of inhibition was recorded as sensitive or resistant according to CLSI (2014) guidelines. *P. aeruginosa* ATCC 27853 was used as positive control.

Detection of ESBL by Double disk diffusion synergy test and combined disk method

ESBL production in all isolates were detected by double disk synergy test (DDST) and combined disk test as described by Jarlier et al. (1998) and CLSI (2014), respectively. 100 µl of the standardized inoculum equivalent to 0.5 McFarland turbidity standards was inoculated onto sterile Mueller Hinton agar. Amoxicillin-Clavulanic acid disk consisting of 20 µg Amoxicillin and 10 µg of clavulanic acid (AMC) (Hi-media) was placed in the centre of the plate and disks of third generation cephalosporin Ceftazidime (CAZ), ceftriaxone (CRO), cefotaxime (CTX) and Aztreonam (ATM) 30 µg each were placed at 20 mm distance (centre to centre) from Amoxicillin-Clavulanic acid disk prior to incubation. The plate was incubated at 37^0C for 24 h. Enhancement of the zone of inhibition of any one of the four drug disks toward Amoxicillin-Clavulanic acid suggested the presence of extended spectrum beta-lactamases.

Combined disk test (CDT) as recommended by the CLSI, for detecting ESBLS in *K. pneumoniae* and *E. coli*, were also performed in all presumed to be ESBL producer by placing disks of ceftazidime (CAZ), cefotaxime (CTX) (30 µg each), ceftazidime-

Table 1. Primer sequences and PCR conditions used to detect ESBL genes.

Gene	Primer	Sequence (51 – 31)	PCR Conditions	Amplicon size (bp)	Reference
Bla GES	GES IF GES IR	ATGCGCTTCATTCACGCAC CTATTTGTCCGTGCTCAGG	Initial denaturation of 94°C for 4 min; 35 cycles of denaturation of 94°C for 1 min; annealing at 50°C for 1 min; extension at 72°C for 1min and final extension at 72°C for 5 min	860	Poirel et al. (2001)
BLa PER	PER-lF PER 1R	AATTTGGGCTTAGGGCAGAA ATGAATGTCATTATAAAAGC		933	Z21957
OXA-50	OXA-F OXA-R	GAAAGGCACCTTCGTCCTCTAC CAGAAAGTGGGTCTGTTCCATC	Initial denaturation of 95°C for 5 min; 35 cycles of denaturation of 95°C for 1 min; annealing at 54°C for 1 min; extension at 72°C for 45S and final extension at 72°C for 10 min	400 -	Accession No.: Am117128 -
TEM	F R	GAGACAATAACCCTGGTAAAT AGAAGTAAGTTGGCAGCAGTC	Initial denaturation of 94°C for 5min; 35 cycles of denaturation of 94°C for 45S; annealing at 55°C for 30S; extension at 72°C for 3 min and final extension at 72°C for 5 min	459 -	Parajuli et al. (2016) -
SHV	F R	GTCAGCGAAAAACACCTTGCC GTCTTATCGGCGATAAACCAG	Initial denaturation of 94°C for 5 min; 35 cycles of denaturation of 94°C for 45 S; annealing at 60°C for 30 S; extension at 72°C for 3 min and final extension at 72°C for 5 min	383	Parajuli et al. (2016)
CTX-M	F R	GAAGGTCATCAAGAAGGTGCG GCATTGCCACGCTTTTCATAG		560 -	Parajuli et al. (2016)

clavulanic acid (CAZ/CAC), and cefotaxime-clavulnaic acid (CTX/CEC), (30/10 µg each) (Hi-Media) on Mueller-Hinton agar plates which was inoculated with test strain at a distance of 20 mm from each other and then incubated at 37°C for 18 to 24 h. Isolate that showed increase of ≥5 mm in the zone of inhibition of the combination disks in comparison to that of the ceftazidime or cefotaxime disk alone was considered as ESBL producer.

DNA extraction and PCR for detection of gene encoding ESBLS

The genomic DNA extraction was performed using the modified boiling method (Katvoravutthichai et al., 2016). First, *P. aeruginosa* isolates were inoculated into a sterile brain heart infusion broth (Oxoid, U.K.) and incubated at 37°C for 72 h. One millilitre of the incubated broth was centrifuged at 12,000×g for 5 min and harvested cells were washed and re-suspended in 50 µl of Nuclease-free water (Norgen, Biotek Corop, Canada). The cells suspension were boiled at 95 to 100°C for 10 min; subjected to cold shock treatment on ice for 10 min and then centrifuged at 12000×g for 10 min.

Fifty microliters of the supernatant was transferred to a new micro centrifuge tube. To the supernatant, 100 µl of ice-cold absolute ethanol was added; mixed to precipitate out the DNA and kept on ice for 30 min. This was centrifuged at 12000×g for 10 min to pellet the DNA. The pelleted DNA was washed using 100 µl of 70% ice-cold ethanol and centrifuged at 12000×g for 2 min. The pellet was washed three times; air dried and dissolved in 50 µl of 1X TE buffer, pH 8.0.

Polymerase chain reaction for detection of ESBL genes

The PCR for detection of ESBL genes was carried out using the new England Bio labs one Taq 2X master mix with standard buffer. Amplification was carried out in a 25 µl total volume of PCR mixture containing 12.5 µl of 1X master mix (England Bio Lab) with standard buffer, 20 µM Tris-Hcl, 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 22 mM KCL, 0.2 mM dNTPs, 5% glycerol, 0.06% GEPAL CA-630, 0.05% Tween 20, 25 units/ml Taq DNA polymerase; 0.5 µl of 10 µM each of primers (Inqaba, Biotech, South Africa) (Table 1); 5 µl of the extracted DNA and 6.5 µl of sterile Nuclease free water (Norgen, Biotek Corop, Canada).

The PCR amplification program for the primers used is shown in Table 1. The PCR was performed in a thermal cycler machine (BIBBY) - Scientific Ltd., UK. The PCR products were separated on 1.5% agarose gel stained with ethidium bromide (5 µg/ml) and electrophoresis was carried out at 70 V for 90 min. The gel was visualized under UV transilluminator (UP Land, USA). A 100 bp DNA Ladder (New England Bio labs) was used as DNA molecular weight marker.

Table 2. Antimicrobial susceptibility profiles of *P. aeruginosa* isolates.

Group of antibiotics	Antibiotic	Sensitive (%)	Resistant (%)
Cephalosporin	Cefepime	16 (47.06)	18 (52.94)
	Ceftriaxone	3 (8.82)	31 (91.18)
	Ceftazidime	16 (47.06)	18 (52.94)
	Cefotaxime	-	34 (100)
Monobactam, Fluoroquinlones, Aminoglycosides, Lipopetide, Carbapenems	Aztreonam	6 (17.65)	28 (82.35)
	Ciprofloxacin	18 (52.94)	16 (47.06)
	Gentamicin	20 (58.82)	14 (41.18)
	Polymyxin B	27 (79.41)	7 (20.59)
	Imipenem	31 (91.18)	3 (8.82)
	Meropenem	29 (85.29)	4 (11.76)
Penicillin, B-lactam inhibitors	Piperacillin	5 (14.71)	28 (82.35)
	Piperacillin/Tazobactam	18 (52.94)	16 (47.06)

Table 3. Resistance patterns of *P. aeruginosa* isolates.

Resistance patterns	No. of isolate
MEM^R, CIP^R, PB^R, CN^R, CEF^R, CRO^R, CAZ^R, CTX^R, ATM^R, PRL^R, TZP^R	-
MEM^R,CIP^R, CN^R, CEF^R, CRO^R, CAZ^R, CTX^R, ATM^R,PRL^R, TZP^R	4
IMP^R, CIP^R, CN^R, CEF^R, CAZ^R, CTX^R, ATM^R ,TZP^R	2
CRO^R, CAZ^R,CTX^R, ATM^R	24
MEM^R ,CIP^R, PB^R, CEF^R, CRO^R, CAZ^R, CTX^R, ATM^R, PRL^R	2
CRO^R, CTX^R, ATM^R	2
Total	34

CIP^R = Ciprofloxacin resistant; CRO^R =Ceftriaxone Resistant; PB^R = polymyxin-B resistant; CAZ^R = Ceftazidime resistant; CN^R = Gentamicin resistant; CTX^R = Cefotaxime resistant; CEF^R = Cefepime resistant; MEM^R = Meropenem resistant; IMP^R = Impenem resistant ; ATM^R = Aztreonam resistant.

RESULTS

Out of 34 clinical isolates of *P. aeruginosa* 15 (44.12%) were found to be potential ESBL producers by preliminary screening. Antimicrobial susceptibility pattern revealed that most of *P. aeruginosa* isolates were resistant to cefotaxime (100%), ceftriaxone (91.18%) and aztreonam (82.35%) while the isolates were generally sensitive to carbapenems group (imipenen 91.18% and meropenem 85.29%), followed by polymyxin B (79.41%) and gentamicin (58.82%) (Table 2). Six different resistance patterns were identified among the *P. aeruginosa* isolates. Twenty four (24) out of thirty-four (34) *P. aeruginosa* isolates were resistant to four antibiotics ceftazidime, cefotaxime, ceftriaxone and Aztreonam shown in Table 3. The information describing the detection ESBL- positive *P. aeruginosa* isolates by different phenotypic tests in relation to the resistance patterns is shown in Table 4. The first group, 24 out of 34 *P. aeruginosa* isolates, were resistant to ceftazidime, cefotaxime, ceftriaxone and Aztreonam while the second group, 2 out of 34 isolates consisted of isolates that were resistant to ceftriaxone, cefotaxime, and aztreonam and at the same time sensitive to ceftazidime.

The highest percentage of ESBL-positive strains among these two groups was detected using the combined disk test (CDT) with emphases on cefotaxime (CTX) alone and cefotaxime-clavulanic acid (CTX/CEC) combination (Figure 1). The PCR method was used to investigate β-lactamase genes in the 15 isolates of ESBL producing *P. aeruginosa* for six genes, namely *bla PER, GES, CTX-M, TEM, SHV* and *OXA-50*. Out of fifteen (15) ESBL producing *P. aeruginosa* isolates that were screened for six ESBL genes. Only *bla OXA*-50 genes was able to show positive amplification in eight (8) isolates (53.33%) (Figure 2).

However, none of the isolates was positive for the *bla-PER, GES, CTX-M, SHV,* and *TEM* genes.

Table 4. Detection of ESBL producing *P. auriginosa* isolates by phenotypic test among the isolates that were resistant to ceftazidime, ceftriaxone, cefotaxime and aztreonam.

Resistant patterns of isolate	No. of positive isolates		
	DDST-AMC	CAZ/CAC	CTX/CEC
CRO, CAZ CTX ATM (24)	3	-	13
CRO, CTX, ATM (2)	-	-	2
Total	3	-	15

CRO^R = Ceftriaxone Resistant; CAZ^R = Ceftazidime resistant; CTX^R = Cefotaxime resistant; ATM^R = Aztreonam resistant.

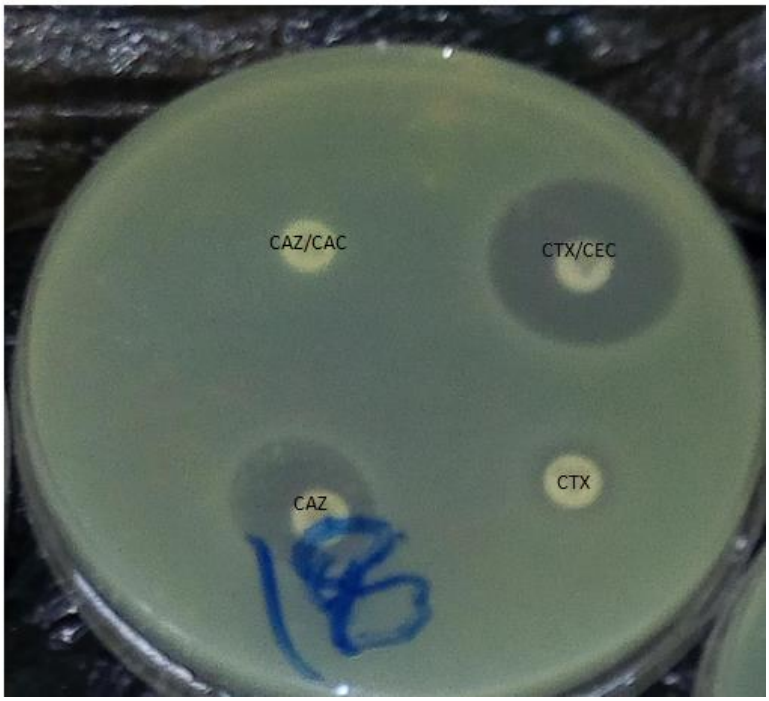

Figure 1. Combined disk test using CAZ/CAC and CTX/CEC.

DISCUSSION

The *P. aeruginosa* is one of the most important nosocomial pathogens, being responsible for various types of infections with more and more limited therapeutic options. One of the most alarming characteristic of *P. aeruginosa* is its resistance antibiotic susceptibility. This situation is due to expression of various resistance mechanisms such as drug inactivation through the production of β-lactamases, alteration of target site (e.g. alteration of PBP-the target site of penicillin), alteration of metabolic pathway and reduced drug accumulation by decreasing drug permeability or increasing active efflux (pumping out) of the drug across the cell surface.

Extended-spectrum Beta-lactamase-producing bacteria are one of the fastest emerging resistance problems worldwide. Increased global prevalence and dissemination of ESBL genes among pathogenic microorganisms are a serious peril for medical fraternity. The rates of ESBL-positive *P. aeruginosa* (44.12%) found in our study were in accordance with similar studies conducted in Bangladesh, North West of Pakistan and South West of Iran (Ullah et al., 2009; Begum et al., 2013; Mohammadi et al., 2015), although low detection rates of 3.7 to 8.1% were noted in studies conducted by others (Woodford et al., 2008; Lim et al., 2009; Tavajjohi et al., 2011).

The relatively high prevalence of ESBLS recorded in this study might be due to the extreme empirical use of third-generation cephalosporins in clinical settings. The differences in the ESBL rates may be attributable to the geographic difference, antimicrobial stewardship programme and infection control practices. ESBLS producing organisms pose unique challenges to clinical microbiologists, clinicians, and infection control agents

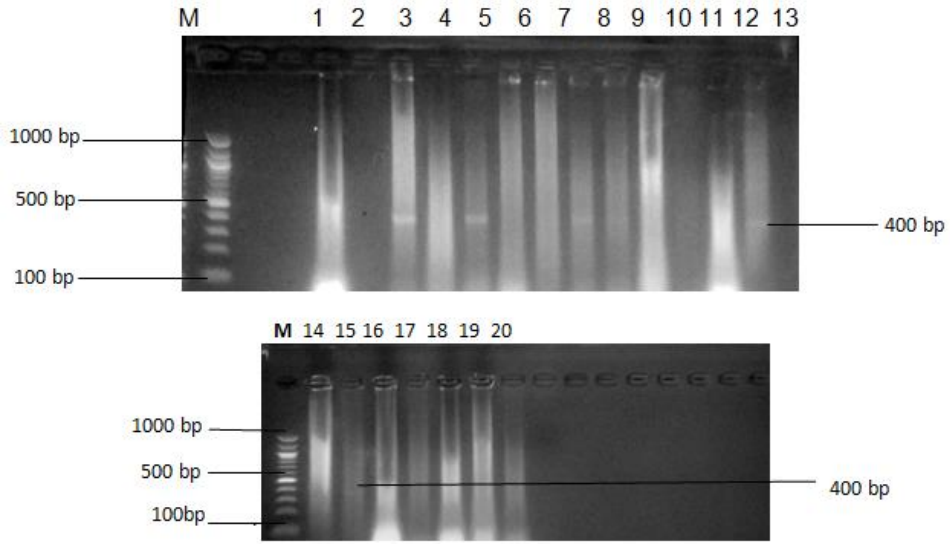

Figure 2. PCR detection of 400 bp amplicons of OXA-50 gene for identification of ESBL. Lane M shows bands for 100 bp molecular weight standard. Lanes 1, 3, 5, 8, 9, 12, 13 and 15 show positive amplification bands indicating the presence of OXA 50 gene in *P. aeruginosa* isolates analyzed. Other lanes show negative amplification and produced no visible band.

ESBL producing bacteria are frequently resistant to many classes of antibiotics resulting in difficult to treat infections. The ESBL producing *P. aeruginosa* isolates exhibited high level of resistance against most of the antibiotics tested (Table 2). Currently carbapenems are regarded as the drugs of choice for treatment of infections caused by ESBL producing organisms. In this study, 91.18 and 85.29% of all the *P. aeruginosa* isolates were sensitive to imipenem and meropenem, respectively. The introduction of carbapenems into clinical practice represented a great advance for the treatment of serious bacterial infection caused by β-lactam resistant bacteria. Due to their broad spectrum of activity and stability to hydrolysis by most beta lactamases, carbapenems have been the drug of choice for treatment of infections caused by penicillin or cephalosporin-resistant Gram-negative bacilli especially ESBL producing strains (Mandiratta et al., 2005). Unfortunately, use of carbapenems has been associated with the emergence of carbapenem-resistant *P. aeruginosa* as observed in this study, 8.82 and 11.76% of isolates were resistant to imipenem and meropenem, respectively. This finding is consistent with the work of Tripathi et al. (2011) and Jayanthi and Jeya (2014) who reported that 5.88 and 9.77% of *P. aeruginosa* isolates were resistant to imipenem. Similar findings have been documented in other countries such as Egypt and India (Senthamarai et al., 2014; Raafat et al., 2016). So, proper infection control practices and antimicrobial susceptibility testing before treatment are essential to prevent the spread and outbreaks of ESBL-producing bacteria. Since *P. aeruginosa,* is the recipient of various genes due to its genetics nature such as plasmids and transposon, therefore it can quickly be resistant to various antibiotics.

With the recent detection of *GES, PES, CTX-M, SHV* and *TEM* producing strains in several countries (Tavajjohi et al., 2011; Ahmed and Asghar, 2017; Laudy et al., 2017), the appearance of ESBL producing clinical isolates of *P. aeruginosa* can be anticipated in Nsukka. Our results suggest the lack of these genes on the *P. aeruginosa* isolates tested. Extended spectrum β lactamase in the bla *GES, PES, CTX-M, SHV* and *TEM* negative isolates is most likely due to other mechanisms such as active drug efflux pumps and cell membrane mechanism and gene mutation. Further studies are necessary to conclude that these genes are not present in the *P. aeruginosa* isolates circulating in this area of the country.

The present study showed that 15 strains of *P. aeruginosa* were identified as ESBL by phenotypic method. Of these 15 phenotypic ESBL isolates, 8 (53.33%) expressed the bla *OXA-50* gene. This gene was identified for first time in *P. aeruginosa* isolates from Turkey and France (Aktas et al., 2005; Peterson and Bonomo, 2005) and in Romania (Crăciunas et al., 2010). In Taiwan, *bla oxa-17* and *bla oxa-10* genes have been detected in *P. aeruginosa* (Du et al., 2010). This finding is in agreement with the work conducted by Porjafari et al., (2013) who reported similar occurrence of *OXA-50* gene among the *P. aeruginosa* strains collected in their hospitals. In Bangkok, Thailand *Oxa-10* was the predominant clone of *P. aeruginosa* clinical isolates (Katvoravutthichai et al., 2016). This raises concern about oxacillinases among *P. aeruginosa* clinical isolates.

The *OXA-50* gene detected in this study belonged to group D β-lactamases. Selective antibiotic pressure that develops in response to over use of β-lactam antibiotics particularly in hospitals can be responsible for the expression and dissemination of these enzymes. The threat of treatment failure is amplified by the evolution of *P. aeruginosa* strains expressing extended spectrum oxacilinase activity.

In conclusion, to the best of our knowledge, this study is the first to report the presence of *bla OXA* gene among clinical isolates of *P. aeruginosa* in Nsukka, Southeast Nigeria. The emergence of extended spectrum group D β-lactamases among *P. aeruginosa* isolates must be taken seriously. There is a need for a comprehensive review in antibiotic prescription and usage to prevent the spread of these pathogens.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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