Molecular Characterization of Extended-Spectrum Beta-Lactamase (ESBLs) genes in *Pseudomonas aeruginosa* from Pregnant Women Attending a Tertiary Health Care Centre in Makurdi, Central Nigeria

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Abstract- *Pseudomonas aeruginosa* is among the most common pathogen causing nosocomial infections. The objective of this study was to investigate the multidrug resistance pattern of *P. aeruginosa*isolatesfrom pregnant women and to determine the presence of extended-spectrum-beta-lactamase (ESBLs) genes. A total of 28 isolates of *P. aeruginosa* were isolated from pregnant women attending antenatal clinic in a tertiary health care Centre in Makurdi, Central Nigeria. Susceptibility to fifteen antimicrobial agents was performed by disk diffusion method. ESBL-genotypic detection of the presence of the genes encoding for bla(TEM) and bla(CTX-M), was studied by a multiplex polymerase chain reaction. The prevalence of ESBLs gene was 10.7% as the presence of genes encoding ESBLs was confirmed in three isolates. *P. aeruginosa*demonstrated the highest resistance rate to clindamycin (96.4%), ceftriaxone (89.3%) and clarithromycin (89.3%). A 100% of the isolates were sensitive to ofloxacin, perfloxacin, ciprofloxacin, gentamycin and streptomycin. Also, all of the isolates were (Multiple antibiotic drug resistance) MDR (resistant to three or more antibiotics). Proper infection control practices and barriers are essential to prevent spreading and outbreaks of ESBL-producing and MDR *P. aeruginosa* in our environment and consequently in pregnant women.

Index Terms- Pseudomonas aeruginosa, extended-spectrum-beta-lactamase, antimicrobial agents.

I. Introduction

Pseudomonas aeruginosa is a ubiquitous pathogen prevalent in hospital environments. It can cause severe nosocomial infections, particularly among immunocompromised patients. People with respiratory, gastro intestinal, urinary tract, and wound infections as well as burn victims, individuals with cancer, and patients hospitalized in intensive care units are affected by *P. aeruginosa* mostly due to nosocomial spread and cross contamination (Wolska*et al.,* 2012). *P. aeruginosa* accounts for 10% of all hospital acquired infections, a site specific prevalence which may vary from one unit to another (Jones *et al.,* 2000). Various possible sources of *P. aeruginosa* infection in hospitals have been identified, *i.e.* tap water, disinfectants, food, sinks, mops, medical equipments, hospital personnel and others (Deplano*et al.,* 2005; Morrison and Wentzel, 1984).

Pseudomonas aeruginosa can develop resistance to antibiotics either through the acquisition of resistance genes on mobile genetic elements (i.e., plasmids) or through mutational processes that alter the expression and/or function of chromosomally encoded mechanisms. Both strategies for developing drug resistance can severely limit the therapeutic options for treatment of serious infections. (Bonten*et al.,* 1999).

Extended-spectrum beta-lactamases (ESBL) are enzymes that confer resistance to most beta-lactam antibiotics, including penicillins, cephalosporins, and the monobactamaztreonam. Infections with ESBL-producing organisms have been associated with poor outcomes. Community and hospital-acquired ESBL-producing Enterobacteriaceae are prevalent worldwide (Bush *et al.,* 2010). The CTX-M enzymes are replacing SHV and TEM enzymes as the prevalent type of ESBLs in urinary tract infections, bloodstream and intra-abdominal infections (Falagas and Karageorgopoulos, 2009). They have been found in the Enterobacteriaceae and other Gram-negative bacilli. Identifying ESBL-producing organisms is a major challenge for the clinical microbiology laboratory. Multiple factors contribute to this, including production of multiple different β-lactamase types by a single bacterial isolate and the production of ESBLs by organisms that constitutively produce the AmpC β-lactamases, varying substrate affinities and the inoculum effect. ESBLs are often encoded by genes located on large plasmids, and these also carry genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol. (Yezli*et al.,* 2015). Recent studies have demonstrated fluoroquinolone resistance mediated by co-transfer of the *qnr* determinant on ESBL-producing plasmids.(Yetkin*et al.,* 2006). Thus, very broad antibiotic resistance extending to multiple antibiotic classes is now a frequent characteristic of ESBL-producing enterobacterial isolates. As a result, ESBL-producing organisms pose a major problem for clinical therapeutics. In *P. aeruginosa* other enzymes that were identified like PER (mostly in clinical isolates from Turkey), VEB (from South-East Asia, France and Bulgaria), GES/IBC (France, Greece and South Africa) and BEL types. The annual Hajj to Makkah in Saudi Arabia is considered one of the largest annually recurring religious mass gatherings in the world. Saudi Arabia could be a hot spot for the collection of multidrug resistant strains such as *P. aeruginosa* (Yezli*et al*., 2014). Previous data from Saudi Arabia suggest that VEB and OXA-10-like enzymes are also frequent ESBLs in *P. aeruginosa* from Saudi Arabia, in

accordance with reports from the Middle East, South East Asia, and parts of Europe (Omar *et al.,* 2015; Strateva and Yordanov, 2009).

II. MATERIALS AND METHODS

Sample Collection

This study was carried out in Makurdi, the capital city of Benue State-Nigeria between the months of September, 2014 and April, 2015. A purposive selection consisting of pregnant women attending the ante-natal clinic was taken. A total of 438 pregnant women attending the ante-natal clinic of the tertiary health institution Makurdi participated in this study. Information regarding each patient's medical and obstetric history was recorded on a predesigned proforma before collection of urine specimen. Information required on the proforma includes the age, parity, gestational age and symptoms relating to urinary tract infection. Written informed consent was obtained from the women for the collection of each specimen, in accordance with the ethical guidelines of the medical Institution.

Each of the women were instructed on how to collect a clean-catch midstream urine sample in a sterile container.

Processing and isolation of samples

The culture media used for isolation were Cystein-Lactose Electrolyte-Deficient (Difco Co, USA), Blood and chocolate agar plates. Each urine sample was inoculated and streaked with·; the aid of heat-flamed standard wire loop (delivering 0.001 ml urine)on to the agar plates. The plates were incubated aerobically at 37°C for 24hrs and then examined. Only plates with significant growth (i.e. at least 100cfu/ml) were considered significant and further analyzed. The cultural and morphological characteristics of distinct and isolated colonies were studied. This included size, elevation, opacity and colour. Distinct and isolated colonies from each significant growth were Gram stained. Those resembling *Pseudomonas* were inoculated onto Cystine Lactose Electrolyte Deficiency (CLED) and colonies that did not ferment Lactose were presumptively identified as *Pseudomonas aeruginosa*and confirmed by the oxidase slide and tube agglutination tests. *Pseudomonas aeruginosa* are usually Oxidase positive. Plates were then viewed under UV rays to observe a characteristic fluorescent. Gram-negative rods were identified as lactose or non-lactose fermenters using Eosin Methylene Blue (EMB and MacConkey agar. Plates were further sub cultured on a selective medium using *Pseudomonas* Chromagar and Centimide agar, this was incubated for 24hrs at 37°C. Plates were observed for green-mauve color on Pseudomonas chromagar and yellow green or yellow brown on centrimide agar, again plates were viewed under UV rays for fluorescent. Suspected organisms were inoculated on nutrient agar and incubated for 24hrs at 37°C. Colonies from the nutrient agar were presented for the Gram stain, Antibiotic Sensitivity tests, Biochemical tests which include; Citrate, Indole, Urease, Oxidase tests were carried out. Each isolate originating from a single colony of each patient's culture was identified as *P. aeruginosa* by analytical profile index test kit (API 20 E test; bioMérieux.)

Antimicrobial susceptibility test Discs

A total of 28 *Pseudomonas aeruginosa* strains from different women across different geographical regions within Makurdi metropolis were employed in this study. The disc diffusion method of Bauer *et al*(1966) was used in this study. Five colonies of each strain of the isolate were suspended in a sterile bijou bottle containing 5mls of peptone water (Lab M) and incubated overnight at 37°C. The overnight broth cultures were diluted to 10^6 colony-forming units per ml. A sterile cotton-tipped applicator was introduced into standardized inoculum and used to inoculate dried plate of sensitivity test agar (STA) for each isolate. Antimicrobial susceptibility test discs were obtained from Oxoid Ltd, UK. They comprise:Ampicillin (PN) 10 µg, Ciprofloxacin (CPX) 10µg, Gentamicin (CN) 10 µg. Erythromycin (E) 10µg, Nalidixic acid (NA) 30µg, Ceftriaxone (CRO) 30µg, Clindamycin (DA) 25µg, Clarithromycin (CLA) 10µg, Azithromycin (AZM) 25µg, Perfloxacin (PEF) 30µg, Ceporcx(CEP) 5µg, Septrin (SXT) 30µg, Augumentin(AU) 30µg, Ofloxacin (OFX) 5µg and Streptomycin(S)*30*µg. The plates were incubated at 37^{0}C for 18-24 hours. Zones of inhibition were measured and interpreted using the chart from Clinical Laboratory Standard Institute (CLSI), 2006.

Amplification by Multiplex Polymerase Chain Reaction (PCR) for ESBL genes.

Two sets of primers (Forward and Reverse oligonucleotides) were mixed in the following manner: 75µl each of TEM and CTX-M forward primers, 75µl each of TEM and CTX-M Reverse primers, 600µl nuclease free water. These were mixed by gentle vortexing and primer mix for 100 reactions was prepared. Final concentration of primer is 0.3µM. Eppendorf Nexus Gradient Thermal Cycler was used for the DNA amplification following the modified protocol by Hijazi *et al.,* (2016) as follows: Water was used as no template control NTC, existing *Methicillin resistant Staphylococcus aureus (MRSA)* DNA was used as Negative Control (NC) and previously typed *P. aeruginosa* with *CTM9* was used as Positive Control (PC).PCR experiments were performed in a volume of 25µl with the following in a reaction tube: 5 µl of DNA template, 12.5µl 2x PCR Master Mix, 7.5µlprimer mix. PCR was performed with denaturation or an initial lysing step of 3 min at 94°C; 35 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final extension step of 3 min at 72°C. After PCR amplifications, 2.5 µl of each reaction was separated by electrophoresis in 1.5% agarose gel for 30 mins at 100 V in 0.5x TBE buffer. DNA was stained with ethidium bromide (1µg/ml) and the bands were detected using ultraviolet transilluminator. Photographs were then taken.

Primer selection for Extended Spectrum β-Lactamase (ESBLs) genes.

Enterobacteriaceae carrying extended-spectrum β-lactamases ESBLs have emerged as significant pathogens. Such strains are resistant to multiple antimicrobial agents, and can be challenging to treat, as their therapeutic options are few. Resistance to β-lactamases is primarily due to β-lactamases-mediated antibiotic hydrolysis; while an altered expression of efflux pumps and or porins play only a minor role. A multiplex PCR based on the TEM and CTX- M β-lactamases genes was designed to detect the presence of the genes.

Table 1: Primers for Extended Spectrum β-Lactamase (ESBLs) genes.

Primer Name	Sequence (5'-3')	Product Size (bp)
UMS125-F	AGT GCT GCC ATA ACC ATG AGT G	431 For TEM
UMA226R	CTG ACT CCC CGT CGT GTA GAT A	
UMS51-F	GGA TTA ACC GTA TTG GGA GTT T	164 For CTX-M
UMA427R	GAT ACC GCA GAT AAT ACG CAG G	

III. RESULTS

From 438 urine samples screened for bacteriuria, 277 were positive for significant bacteriuria (10^5cfu/ml) out of which a total of twenty-eight (28) were *Pseudomonas aeruginosa*isolates.Antibiotic sensitivity profiles of the 28*Pseudomonas aeruginosa* isolates from urine of pregnant women showed that most of the isolates were not susceptible to the inhibitory activities of clindamycin, ceftriaxone and clarithromycin amongst others. They were generallysensitive to ofloxacin, perfloxacin and ciprofloxacin generally classified as the quinolones by the clinical laboratory standard institute (CLSI, 2008). Table 2.

Table 2: Antibiotics Sensitivity Pattern of *Pseudomonas aeruginosa* Isolates from urine.

Antibiotics	**Disc Content (µg)**	**No. and % Resistant**	**No. and % sensitive**
Ofloxacin-OFX	5	0(0.00%)	28(100%)
Perfloxacin-PEF	30	0(0.00%)	28(100%)
Ciprofloxacin-CPX	10	0(0.00%)	28(100%)
Augumentin –AU	30	2(7.1%)	26(92.9%)
Gentamycin CN	10	0(0.0%)	28(100%)
Streptomycin S	30	0(0.0%)	28(100.0%)
Ceporcx-CEP	5	1(3.6%)	27(96.4%)
Nalidilic Acid-NA	30	3(10.7%)	25(89.3%)
Septrin-SXT	30	2(7.1%)	26(92.9%)
Ampicillin-PN	10	12(42.9%)	16(57.1%)
Erythromycin-E	10	22(78.6%)	6(21.4%)
Clarithromycin-CLA	10	25(89.3%)	3(10.7%)
Clindamycin-DA	25	27(96.4%)	1(3.6%)
Azithromycin-AZM	25	6(21.4%)	22(78.6%)

The Analytical profile index (API) test with kit was used to identify 28 *Pseudomonas aeruginosa* isolates. The frequency of distribution and their corresponding API Identification numbers are displayed on Table 3.

Table 3: API frequency distribution of 28 different isolates of *Pseudomonas aeruginosa*

Serial No	API ID No	Frequency	Prevalence in (%)
1	71424067621	1	3.6
2	71426067631	1	3.6
3	71772777671	1	3.6
4	73424067621	3	10.7
5	73466067631	1	3.6
6	73626067621	4	14.3
7	73636177721	2	7.1
8	75676567661	3	10.7
9	76776777761	1	3.6
10	77466067631	1	3.6
11	77636177721	3	10.7
12	77676577631	1	3.6
13	77772577671	2	7.1
14	77772777771	2	7.1
15	77772777671	2	7.1
Total		28	100

The (Multiple antibiotic drug resistance) MDR index of an isolate is defined as a/b where 'a' represent the number of antibiotics to which the isolates were resistant and 'b' represent the number of antibiotics to which the isolate was subjected to. The MDR indexes of the 28 *Pseudomonas aeruginosa* isolates were calculated and presented on Table 4. The isolates had MDR index of ≥0.2 and above being resistant to three or more types of antimicrobial agents.

Table 4: Multiple Antibiotic Resistance (MAR) index of *Pseudomonas aeruginosa* isolates

No. of isolate	MAR index
1	0.33
2	0.33
3	0.40
4	0.47
5	0.33
6	0.40
7	0.27
8	0.27
9	0.27
10	0.27
11	0.27
12	0.27
13	0.27
14	0.13
15	0.27
16	0.33
17	0.47
18	0.33
19	0.33
20	0.33
21	0.33
22	0.27
23	0.27
24	0.2
25	0.2
26	0.2
27	0.27

28	0.33

The results of the multiplex PCR amplifications for extended spectrum βeta- lactamase (ESBLs) gene testing is shown in figure 1. From the results it was observed that three, 3 (10.7%) tested positive for the presence of the ESBL gene.

Figure 1: Agar gel electrophoresis of PCR products amplified with a multiplex PCR method using the TEM and CTX-M genes coding for ESBL resistance in *Pseudomonas aeruginosa* isolates showing three (3) positive DNA amplicons for this gene.

IV. DISCUSSION

The findings in our study closely resembles those of Omar et al. 2015 who reported that about 28 (25.9%) of *P. aeruginosa* isolates were confirmed as ESBL producers.They further explained that ESBLs enzymes are primarily produced by the Enterobacteriaceae family in particular Klebsiella pneumonia and Escherichia coli and can be carried on chromosomes or plamids, (Falagas and Karageorgopoulos, 2009). They suggested also that they could also be produced by *P. aeruginosa* (Jacoby and MunozPrice, 2005).

The occurrence of multidrug-resistant *P. aeruginosa* strains is increasing worldwide and limiting therapeutic options for clinicians (Bonomo and Szabo, 2006). Our study reported the highest resistance rate to clindamycin, (96.4%) clarithromycin, (89.3%), ceftriaxone, (89.3%) erythromycin, (78.6%) respectively. In our study, gentamicin was very effective, as 100% of the isolates exhibited sensitivity contrary to the findings of Zahra *et al*. (2011) and Zhanel*et al*. (2010) who reported that gentamycin had the highest resistance value in their work. In this study, ofloxacin, perfloxacin and ciprofloxacin (generally classified as the quinolones by the Clinical Laboratory Standard Institute, CLSI) all exhibited an excellent susceptibility pattern (100%) thoughZhanel*et al*. (2010) reported that 19.0 to 24.1% isolates were resistant to quinolones. Variations in this findings may be due to differences in physiological and genetical differences of the subjects under study, as we know that pregnancy is a unique state with anatomic and physiologic urinary tract changes. Cephalosporins, are known anti-pseudomonal drugs, especially the third-generation ceftriaxone, however, in our study it has demonstrated a high resistance pattern (89.3%) of*P. aeruginosa* isolates, contrary to reports of Zahra *et al*. (2011) who reported that about 61% of their isolates were susceptible to the action of ceftriaxone. They further reported the use of cefotaxime and ceftriaxone as the two cephalosporin drug tested, with susceptibility value of 14 and 60.5% respectively comparable with the report from Malaysia of 40 and 31% (Jombo*et al*., 2008). In our study, prevalence of MDR *P. aeruginosa* isolates, was 100% and theirs was 32.5% which was lower than values reported in other studies in Iran (Mirsalehian*et al*., 2010), Malaysia (Lim *et al*., 2009) and Pakistan (Ullah*et al.,* 2009) and (Moniri*et al*., 2005). A very low values was by reported by Tam *et al*. (2010) who found 14.0% of *P. aeruginosa* isolates as MDR. The multidrug resistant phenotype in *P. aeruginosa* could be mediated by several mechanisms including multidrug efflux systems, enzyme production, outer membrane protein (porin) loss and target mutations. Our study reported 10.1% ESBL gene among *P. aeruginosa* isolates. The studies conducted by others depicted lower rates, 3.7% (Woodford *et al*., 2008), 4.2% (Lim *et al*., 2009), 7.7% (Jacoby, 1997) respectively, of ESBL production in *P. aeruginosa*. The results of various studies, showed high rate 20.3% (Aggarwal *et al*., 2008), 39.41% (Mirsalehian*et al*., 2010) and 35.85% (Ullah*et al*., 2009) of ESBLs in the samples of *P. aeruginosa* isolates examined. As shown by the multiplex PCR, three of the *P. aeruginosa* isolates showed the presence of some

genes (CTX-M and TEM) that code for ESBL production, some of the isolates did not show amplicons, indicating that the resistant genes primers used for this study are not contained in these isolates or the amount of gene was too small. It could also be due to the fact that the gene is not expressed at the molecular level of the primers used or these isolates might contain other resistant genes, which are also expressed at molecular level. The report of Charlene *et al.,* (2004) tends to support these assertions. In their study they did not observe bands similar to those genes tested and concluded that additional resistance genes may exist but not expressed at molecular level. The findings in this study that some isolates may contain more than one ESBL gene are in agreement with the report of Nadine *et al.,* (2012) that of the twenty-seven CTX-M carriers were additionally PCR-positive for blaTEM genes. Thirty-four isolates were CTX-M-1 producers, eight expressed additional TEM-1 and one isolate--from a pig-- additionally expressed a TEM-type enzyme, six isolates carried CTX-M-14 with TEM-1 and five isolates specified CTX-M-15, one of which producing additional TEM-1. One isolate from a calf produced TEM-1 in combination with CTX-M-117 a novel CTX-M group 1 ESBL with an amino acid sequence never found before (nucleotide sequence accession number JN227085). Currently, therapy for strains of Enterobacteriaceae that express ESBLs is limited to such broad-spectrum agents as imipenem. However, there have already been reports of therapeutic failures of this drug with strains that produce multiple β-lactamases (Ahmad *et al.,* 1999). There are limited therapeutic options left for some of these organisms. Strains expressing extended-spectrum β-lactamases will present a host of challenges for clinical microbiologists and clinicians alike as we head into the next century.

V. CONCLUSION

In our study Multiplex PCR showed that some of the test *P. aeruginosa* isolates harbour genes that encode ESBL as well as genes that code for resistance to β-lactam antibiotics. The detection of TEM and CTX-M encoding genes for resistance to ESBLs shows that these resistance determinants are found in our environment. These resistant traits call for surveillance in clinical settings because ESBLs gene is a world growing trait for resistance but its epidemiological effect and evaluation is still under estimated with low awareness therefore, early detection of these β-lactamase producing isolates in a routine laboratory could help to avoid treatment failure.

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