World Journal of Medical Sciences 11 (4): 497-503, 2014
ISSN 1817-3055

DOI: 10.5829/idosi.wjms.2014.11.4.82334

Prevalence of Extended Spectrum Beta-Lactamase-Producing Gram-Negatve Bacteria in a University Hospital in Ilisan Remo, Ogun State, Nigeria

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Abstract: This study was carried out to determine the prevalence of Extended-spectrum β-lactamase (ESBL) and their antibiotic resistance patterns from clinical isolates in a university hospital. A total of One hundred and seventy-two isolates of Gram-negative were identified using Standard Laboratory Methods from various clinical specimens. Antibiotics susceptibility test was performed using disc diffusion technique. Isolates obtained were subjected to the double disc synergy test (DDST) for extended-spectrum β-lactamases (ESBL) confirmation. Positive ESBL were further subjected to plasmid extraction and profiling. The highest percentage of occurrence was *Escherichia coli* (48.8%), followed by *Pseudomonas aeruginosa* (17.4%), while *Proteus spp* and *Klebsiella pneumonia* had the least value of 16.9% each. Extended spectrum β-lactamase production was detected in 17 isolates (9.9%), of which *E. coli* constitutes 11 (6.4%), *K. pneumonia* 4(2.3%) and *Proteus* spp. 2 (1.2%). Among the Gram-negative bacterial, the antibiotic resistances were more prevalent in amoxicillin (86.7-93.1%), cotrimoxazole (73.3-93.1%), augumentin (76.7-86.2%) and tetracycline (86.2-96.6%). A total number of six antimicrobial resistances to ESBL were also revealed in this study. Gentamicin, cotrimoxazole, ciprofloxacin, amoxicillin, ceftazidime and ofloxacin antibiotic showed the highest multiple resistances of 35.3%. Out of the ESBL producing isolates, the plasmid of the same molecular weight of 30.0kbp were shown for eight. The presence of plasmid might facilitate spread of antibiotic resistance among different members of Enterobacteria. Antimicrobial susceptibility testing and ESBL production monitoring are therefore recommended in patients.

Key words: Antibiotic · Gram-negative · Extended-Spectrum β-Lactamase · Resistance

INTRODUCTION

Among the wide array of antibiotics, β-lactams are the most varied and widely used agents accounting for over 50% of all systemic antibiotics in use [1]. Beta-lactam antibiotics such as penicillins, cephalosporins, monobactams and carbapenems are used in the treatment of bacterial infections due to the presence of β-lactam ring in their structure. Beta-lactam antibiotics are bactericidal by the mechanisms of combining to and inactivating penicillin-binding protein (PBPs). This inactivation of PBPs prevents cross-linking of peptidoglycan, weakening the cell wall, resulting in cell lysis [2].

Bacteria produce β-lactamase to confer resistance to β-lactam antibiotic [2] although; many of the second and third generation penicillins and cephalosporins were specifically designed to resist the hydrolytic action of major β-lactamases [3]. These enzymes are commonly produced by many members of *Enterobacteriaceae*, especially *Escherichia coli* and *Klebsiella. pneumoniae* which efficiently hydrolyse oxymino- cephalosporins and split the amide bond in the β-lactam ring conferring resistance to third generation cephalosporins such as cefotaxime, ceftazidime and ceftriaxone as well as to monobactam such as aztreonam [4, 5].

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Extended spectrum lactamases (ESBLs) are major problems in hospitalized patients worldwide and cause epidemic outbreaks in many institutions [6]. Typically, nosocomial outbreaks were associated with previous antibiotic therapy, especially ceftazidime monotheraphy. Hospital colonization by ESBL producing bacteria is usually a complex phenomenon involving many different mechanisms, dissemination of several epidemic strains and dissemination of plasmids and resistant genes [7]. Specific risk factors include; prolonged hospital stay, severity of illness, ICU urinary or arterial catheterization, intubation and mechanical ventilation. ESBLS commonly occur in surgical frequently from patients from extended care facilities [8].

First-generation penicillin are used to treat streptococci infections, whereas extended-spectrum penicillin have a broader spectrum against many *Enterobacteriaceae*, while second-generation cephalosporins like cefuroxime are effective against a greater spectrum of Gram – negative bacterial which are often used to treat respiratory infections [2]. The third generation cephalosporins such as cefotaxime also shows greater activity to Gram- negative bacteria while the fourth – generation cephalosoporins like cefepime are active against Gram-negative bacilli expressing chromosomal beta-lactamases (AMPC) [2]. Essentially, most clinical diagnostic laboratory detects ESBL producers by phenotypic tests, which require a screening step followed by confirmation. Reference laboratory can test for genes encoding ESBLs by molecular analysis, using polymerase chain reaction amplification of specific nucleic acid sequences. This is usually reserved for epidemiological purposes as it identifies the particular genotype of ESBL [9].

Despite the widespread reports of the existence of ESBL, variable prevalence in different geographical areas [10-12] and its clinical significance in Europe, America and Asia, there is limited report of its existence in Sub-Saharan Africa, particularly in Nigeria [13]. Prevalence of ESBL from this study intends to provide the baseline data for eventual appreciation on the need for cautions approach and intervention measures. Therefore, the study aimed at investigating the prevalence of Extended-spectrum β-lactamases-producing *Enterobacteriaceae* in a University Hospital in Ilisan Remo, Ogun State.

MATERIALS AND METHODS

This study was conducted at the Medical Microbiology Laboratory Department of a University Teaching Hospital, Ilisan Remo, Ogun State, Nigeria with coordinates 6°52'N 3°43'E / ?6.867°N 3.717°E between July 2011 and January 2012.

Sample size was determined using the formula derived by Hsiesh *et al* [14].

$$\text{Sample Size (n)} = Z^2*(P)*(1-P)/C^2$$

Where: Z = 1.96 (for 95% confidence level), p = prevalence rate and, c = Confidence interval, 0.05 according to the procedure described by Iroha *et al.* [15].

One hundred and seventy-two with non-duplicated isolates of *Entreobacteriaceae* were identified from clinical specimens, which included wound swabs, Ear swab, urine, stool, High vaginal swabs and sputum. Isolation and identification of organism was carried out according to the method described by Cowan and Steel [16]. Samples were inoculated on blood agar and MacConkey agar. Urine samples were also cultured on Cysteine Lactose Electrolyte Deficient agar (CLED). Cultures were incubated aerobically at 37°C for 24 hours using Uniscope SM 9082 Laboratory Incubator (Surgifriend medicals, England). Method of bacteria identification by their colonial appearance such as size, shape, consistency, colour, elevation and its differential characteristics such as haemolysis on blood agar, lactose fermentation on MacConkey and Gram staining were done to identify the isolates. Biochemical characterization of extended spectrum beta lactamase isolates was done according to World Health Organization (WHO) Manual for laboratory investigation of Gram-negative organisms [17]. The antibiotics were tested against the isolates using the Kirby Bauer disc diffusion method on Mueller Hinton Agar according to Bauer *et al*. [18]. Bacterial culture suspended in saline equivalent to 0.5 MacFarland was spread on Mueller – Hinton Agar and allowed to dry at room temperature. Each antibiotic disc was placed at 20mm distance from each other on the inoculated agar and incubated at 37°C for 24 hours. The inhibition zones were measured using graduated metre rule to determine the diameter of the inhibition zones and interpreted as sensitive and resistant, according to CLSI guidelines [19].

Detection of Extended-Spectrum Beta-lactamase Production Using Double- Disc Synergy Test: This test was performed as a disc diffusion test as recommended by CLSI [19] sterile swabs was dipped into standard bacteria suspensions (0.5 MacFarland turbidity) and these were spread on Mueller-Hinton sensitivity agar plates.

amoxicillin- clavulanic acid (20+10 µg) disc was placed at the centre of the plate. Ceftazidime and cefotaxime (3.0mg) disc were then placed 20mm (centre to centre) from the Amoxicillin – clavulanic acid disc and incubated at 37°C overnight. Enhancement of the zones of inhibition of the cephalosporin β-lactam antibiotic disc (cefotaxime and ceftazidime) caused by the synergy with the amoxicillin /clavulanic acid disc was taken as an evidence of ESBL production [20].

Plasmid Extraction and Profiling was performed using alkaline lysis method described by Birnboim and Doly [21]. Thousand µL of the bacteria cell suspension was placed into 2ml clean eppendorf tube and centrifuged at maximum speed of 2000 rpm for 20 seconds. The supernatant was withdrawn and discarded using a pipettor into a waste container, being careful not to disturb the cell pellet. Then 100 µL of Buffer 1 (containing 50 Mm Tris-HCL, 10 Mm EDTA, 100 µg/mL RNase A, Ph 8.0) was added to each tube and the cells were re-suspended by vortexing to make the cell suspension homogenous. 200 µL of Buffer 2 (containing 1% SDS, 0.2M NaOH) was the added and the cap was closed and the solutions were mixed by rapid inverting for some few times. It was not vortexed since the chromosomal DNA released from the broken cells could be sheared into small fragments and contaminates the plasmid preparation. The tubes were placed on ice for 5 minutes. 150 µL of ice-cold Buffer 3 (containing 3.0 M potassium acetate, pH 5.5) was added to the tube and the solution was mixed by rapidly inverting it for a few times to form a white precipitate. The tubes were placed on ice for another 5 minutes and later centrifuged at speed of 12,000 rpm for 5 minutes. The precipitate formed pellet along the side of the tube. The supernatant was carefully put into another clean 1.5 ml tubes to avoid not picking up any of the precipitate. The tubes containing the precipitate were discarded. To the tube of supernatant an equal volume of 400 µL of isopropanol was added to precipitate the nucleic acids, the caps were closed and mix vigorously. They were allowed to stand at room temperature for 2 minutes and later spun at 12,000rpm for 5 minutes. This was done to pellet the nucleic acids. The supernatant was carefully removed and discarded. The pellet is now visible. Absolute ethanol of 200 µL was added to each tube and mixed by inversion several times. The tubes were spun at 12,000 rpm in a centrifuge for 2-3 minutes. The supernatant was carefully removed and discarded, without dislodging the pellet which is mainly plasmid DNA. The tubes were placed in the fume hood with the caps open for 15-20 minutes to dry off the last traces of ethanol. TE buffer of 20 µL (containing 10 Mm Tris-HCL, 1 Mm EDTA, pH 8.0) was added to dissolve the pellet by repeated pipetting in and out, up the side of the tube to ensure that all of the plasmid DNA comes into contact with the TE buffer. Extracted plasmid DNA of 20 µL was pooled into a clean labelled tube and store in the freezer.

Electrophoresis of the DNA was carried out on a 0.8% agarose gel in a 0.5X concentration of Tris-Borate-EDTA (TBE) buffer. Agarose gel was prepared by boiling 0.8g of agarose powder in 100 ml of 0.5X TBE buffer. After boiling, the solution was allowed to cool and 10 µl of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes and the comb was removed. 20 µl of bromophenol blue was added. A DNA molecular weight marker was also loaded into one of the wells. The gel was thereafter electrophoresed in a horizontal tank at a constant voltage of 60V for about 1 hour 30 minutes. After electrophoresis, plasmid DNA bands were viewed by fluorescence of bound ethidium bromide under a short wave ultraviolet light transilluminator and the photograph were taken using a digital camera. Molecular weight of the DNA bands was calculated by comparing with Lambda DNA Hind III digest (Bangloregenei) as standard marker

RESULTS

The distribution of isolates obtained from clinical specimens. *E. coli* (48.8%) was the most common, followed by *P. aeruginosa* (17.4%), while *Proteus* spp. (16.9%) and *K. pneumoniae* (16.9%) had the least. ESBL were detected in 17(9.9%) of the isolates in which *E. coli*, 11(6.4%), *K. pnuenoniae* 4(2.3%) and *Proteus* spp. 2(1.2%) were ESBL producers.

The resistance percentage of the isolated Gram-negative bacteria *among* clinical samples shown in Table 2 revealed that among the *Enterobactriaceae* isolates, high antibiotic resistance observed ranged in amoxicillin (86.7-93.1%), cotrimoxazole (73.3-93.1%), augumentin (76.7-86.2%) and tetracycline (86.2-96.6%).

ESBL isolates showed eight multi-drug resistance patterns (Table, 3). Gentamycin, cotrimoxazole, ciprofloxacin, amoxicillin, ceftazidime and ofloxacin showed the highest multiple resistance patterns at 35.3%.

Table 1: Distribution of ESBL among isolates obtained from clinical specimens

	Number of isolates		ESBL	
Isolates	(n)	(%)	(n)	(%)
Proteus spp.	29	16.9	2	1.2
P. aeruginosa	30	17.4	0	0
E. coli	84	48.8	11	6.4
K. pneumoniae	29	16.9	4	2.3
Total	172	100	17	9.9

Table 2: Antimicrobial disc resistance pattern of isolates obtained from clinical specimens.

Antibiotics	µg/disc	*E. coli* n=84	*K. pneumoniae* n = 29	*Proteus* spp. n = 29	*P. aeruginosa* n = 30
Amoxicillin (AMX)	20	92.8	93.1	93.1	86.7
Cotrimozazole (COT)	10	88.1	93.1	89.7	73.3
Ciprofloxacin (CF)	5	27.4	31	37.9	26.7
Gentamicin (GEN)	10	47.6	65.5	55.2	76.7
Perfloxacin (PEF)	5	27.4	27.5	31	33.3
Ofloxacin (OFL)	5	33.3	31	37.9	20
Augumentin (AUG)	20-Oct	80.9	86.2	82.8	76.7
Tetracycline (TET)	30	91.7	96.6	86.2	93
Cefotaxime (CTX)	30	67.9	58.6	48.2	66.7
Ceftazidime (CFZ)	30	45.2	51.7	51.7	60

Table 3: Multi drug resistance pattern of ESBL obtained from clinical specimens

Antimicrobial agents	Number of Resistant isolates (n)	Percentage resistance
AMX,COT,CF,GEN,OFL,AUG,TET,CTX,CAZ	1	5.9
AMX,CF,COT,GEN,PEF,OFL,AUG,TET,CTX	3	17.6
AMX,COT,CF,GEN,PEF,OFL,AUG,TET	0	0
COT,AMX,CF,GEN,PEF,OFL,AUG	5	29.4
GEN,COT,CF,AMX,CAZ,OFL	6	35.3
AMX,COT,CF,GEN,CTX	1	5.9
CAZ,COT,CF,GEN	0	0
CF,COT,AMX	1	5.9
Total	17	100

AMX- Amoxicillin	COT- Cotrimozazole	CF- Ciprofloxacin	GEN- Gentamycin
PER- Perfloxacin	OFL- Ofloxacin	AUG- Augumentin	TET- Tetracycline
CTX- Cefotaxime	CFZ- Ceftazidime		

Table 4: Occurrence of resistance plasmid in ESBLs producing Gram-negative isolates found in clinical specimens

Isolates	ESBL producer (n=17)	Percentage
Proteus spp.2	25	
P. aeruginosa	0	0
E. coli	5	62.5
K. pneumoniae	1	12.5
Total	8	100

The plasmid profile of the ESBL producing Gram-negative isolates from clinical samples is shown in Table 4. It shows that eight out of the seventeen ESBL were found to have the same plasmid size of 30 kbp. The highest number of plasmids was produced by *E. coli* (62.5%).

The result in figure 1 shows that ESBL production is indicated by the presence of synergy between cefotaxime-amoxicillin/clavulanic acid-ceftazidime. The Non-ESBL producing isolate without zone of inhibition (No synergy) was observed between CTX -AUG -CAZ. Thus, indicating non-ESBL production (Figure 2). The results in figure 3 show that the plasmid sizes of all the ESBL producers are the same (30.0kbp).

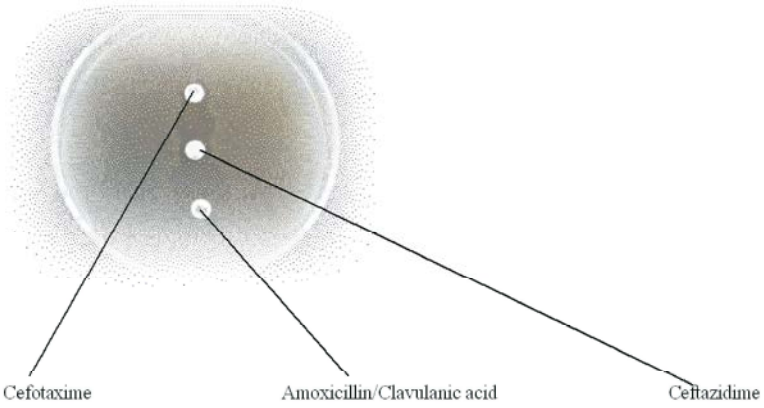

Fig. 1: Organism showing enhanced zone of inhibition between ceftazidime and cefotazime and amoxicillin/clavulanic acid containing disc indicating ESBL production.

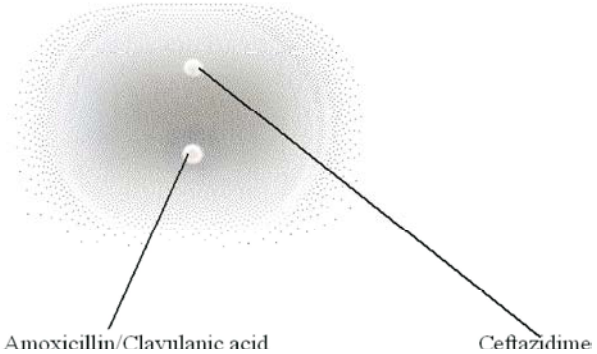

Fig. 2: Organism showing no synergistic action between ceftazidime and amoxicillin/clavulanic acid containing disc indicating no ESBL production.

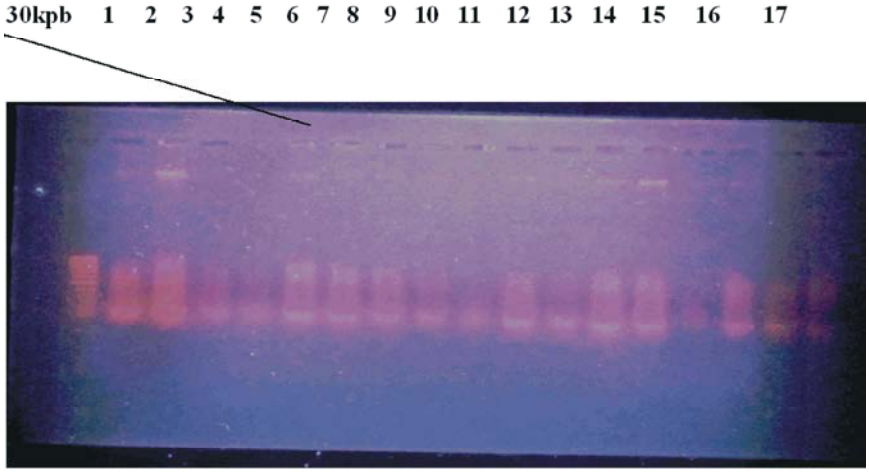

Fig. 3: Plasmid profile of ESBL isolates
Lanes 2, 5, 6, 7, 8, 10, 12 and 13 are positive and 1, 3, 4, 9, 11, 14, 15, 16, 17 are negative
Negative bacterial spp

1-*K.pneumomiae*	*3-K. pneumonia*	*4-K.pneumoniae*
9-E.coli	*11-E.coli*	*14- E.coli*
15- E.coli	*16- E.coli*	*17- E.coli*

DISCUSSION

The prevalence of extended spectrum Beta-lactamase (ESBL) producing Gram-negative bacterial is fast becoming an emerging global health threat due to its unfavourable outcome in the treatment of common infectious diseases among the hospital patients. The Gram-negative isolates recovered included, *E. coli*, *P. aeruginosa*, *K. pneumonia* and *Proteus* spp. This finding agreed with work done by Mathur *et al.* [22] where, *Klebsiella* spp. was reported as the most common Gram-negative produced ESBL. Seventeen isolates confirmed to produce ESBL indicating overall prevalence of 12% among gram negative bacteria. In the present study, the ESBL prevalence may be considered high, due to unreported cases from previous data on ESBL in the study area. However, the prevalence may be considered moderate when compared with similar study in Enugu, South East, Nigeria [15]. ESBLs rates among *E. coli, K. pneumoniae* and *Proteus* spp. were 6.4, 2.3 and 1.2% respectively. In this study, ESBL isolates were predominantly present among *E. coli* than other Gram-negative isolates. This conforms with the findings of Ananthakrishnan *et al.* [23] who reported a high prevalence of ESBLs among *E. coli*, because *E. coli* is a common human pathogen that causes opportunistic or hospital-acquired infections. None of the isolates of *Pseudomonas spp*. were positive for Esbl production by the method we used; whether they were actually non-producers, or whether some of them did produce β-lactamases that were not inhibited by clavulanate needs to be investigated In the present study, resistance was observed to commonly used antibiotics such as amoxicillin, augumentin, cotrimoxazole and tetracycline due to the fact that antibiotics can be bought without prescription and some practitioners and pharmacist frequently prescribe/sell unnecessary antibiotics for their petty gain. In addition, antibiotics are prescribed without drug sensitivity testing due to lack of laboratory facilities in most of the health care centres. Even where the facilities are available, medical practitioners do not routinely recommend the test because of negligence or patients' economic status. All these factors might have contributed to high prevalence of ESBL in this study [24]. A total number of eight different multidrug resistance patterns to ESBL isolates show the highest multiple resistances due to the fact that gene encoding for resistance for both ESBL and other classes of antibiotics are often found associated on the same mobile DNA (plasmid). The propagation of this plasmid during conjugation leads to development of multidrug resistance in previously sensitive organisms. (Bush *et al*., 1993) Beta-lactamase production could be chromosomal or plasmid mediated. Genes coding for ESBL enzymes are plasmid mediated and are often acquired through transfer of genetic information among many strains of Gram-negative bacteria [4; 25]. Eight out of the eighteen ESBL-producing isolates found to harbor a single resistance plasmid of molecular weight 30.Kbp agrees with the report of Jyoti *et al*. [26], stating that ESBL producers express their Bet lactamase genes from plasmids. The emergence of the resistance plasmid might facilitate the spread of antibiotic resistance among different members of Gram-negative bacterial.

CONCLUSION AND RECOMMENDATION

In conclusion, a low prevalence rate of ESBL was obtained in this study compared to other studies. The findings from this study also demonstrated an increase in resistance to amoxicillin, augumentin, cotrimoxazole and tetracycline. Eight Multidrug resistance patterns observed showed the highest multiple resistance patterns.

In view of the emerging drug resistance amongst bacteria, therapy should only be advocated as far as possible after culture and sensitivity has been performed hence suggesting that routine diagnosis of ESBL producing strains should be done as this would not only help in the proper treatment of the patient but also prevent further development of bacterial drug resistance.

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