ABSTRACT

**Background/Purpose:** Based on fact there is high urinary tract infection and increasing treatment failure among pregnant women and this has led to increased mortality and morbidity among pregnant women, and increased stay in the hospital. This study was conducted to evaluate the prevalence of antimicrobial resistance and distribution of blaTEM, blaCTX-M-15 and blaSHV genes among *A. baumannii*, *P. mirabilis* and *E. cloaceae* strains isolated from urine samples from pregnant women attending antenatal at three secondary health care facilities south-south Nigeria.

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Keywords: ESBL blagene; PCR; UTI.

1. INTRODUCTION

Currently, the challenge of gradually increasing resistance to antibiotics has affected the entire world. The hydrolysis and inactivation of beta-lactam antibiotics, through the production of beta-lactamase has been one of the main resistance mechanisms of many bacterial species, especially in the family Enterobacteriaceae [1]. Gram-negative pathogens is increasingly associated with ESBLs, hence resulting in the resistance to beta-lactam antibiotics [2].

ESBL positive enterobacterial species are widely disseminating throughout the world [3]. The main reason for development of resistance is mainly the selection and preferential growth of resistant bacteria, together with inhibition of susceptible strains from prolonged use of antibiotic. Extended-spectrum -lactamases (ESBLs) were first described in the 1980s and they have been detected in Gram-negative bacilli [4,5,6].

A typical mechanism of AMR is the production of extended-spectrum beta-lactamase. (ESBL) enzymes, which confer resistance to penicillins, cephalosporins, and monobactams, but not to cephamycins and carbapenems [7,8].

Presently there is an increase in the emergence of ESBL producing bacteria. The increasing resistant to beta-lactam antibiotics used in treating urinary tract infections (UTIs) has made the treatment very challenging and frequently resistant to many of the antimicrobial agents recommended for the treatment of such infections [9]. Most ESBLs belong to the CTX-M, SHV (Sulfhydryl variable) and TEM (Temoniera) families. Due to the production of multiple enzymes such as the inhibitor-resistant ESBL variants and plasmid-borne AmpC, ESBL phenotypes have become more complex [10]. Commercial available chromogenic media such as CHROMagar (Paris, France) have been used to detect ESBL production. Chromogenic culture media is a rapid culture based methods used for detection of ESBL and presumptive organism identification. The media has a chromogenic enzyme substrate as a detection system. Chromogenic substrates consist of chromophor which is linked to an enzyme-recognizing part such as carbohydrate, amino acids or phosphate. Specific enzymes produced by the target microorganism will cleave to the chromogenic substrate liberating the chromophor which highlight the micro organism by coloration of the grown colony [11]. The aim of this study was to isolate and identify the types of extended spectrum beta-lactamases genes (ESBL) produced by A.baumannii and Enterobacter cloaeae and Proteus mirabilis.

2. MATERIALS AND METHODS

2.1 Sample Collection

The study was cried out within a period of six months. A total of 660 urine samples were collected from pregnant women attending antenatal at the three secondary health care facilities between July to December, 2018. All pregnant women who were not on any antibiotics
and willing to participate were included in the studies, while those on any antibiotic therapy were excluded from the studies.

Mid stream clean-catch urine samples were collected and inoculated on MacConkey and CHROMagar ESBL and incubated at 37°C for 24 hours. They were examined for growth and colony counts yielding bacterial growth of $10^5$/ml of urine were taken to be significant. Samples were Gram stained and also subjected to Microbact 24E identification.

2.2 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined by the disk diffusion method on Mueller-Hinton agar (Oxoid, UK) according to Clinical and Laboratory Standard Institute (CLSI) guidelines. ESBL-producing isolates were screened using double-disk synergy test in accordance with CLSI guidelines [12]. According to CLSI’s guidelines isolates showing inhibition zone size of ≤ 22 mm with Ceftazidime (30 µg), ≤ 25 mm with Cefotaxime (30 µg), ≤ 7mm with Azetronam (30 µg) and ≤ 22 mm with Cefodoxime (10 µg) was identified as potential ESBL producers and shortlisted for confirmation of ESBL production (CLSI, 2010). E. coli ATCC 25922 and S. aureus 6571 were used as quality control strains.

2.3 Double Disk Synergy Test

Double disk synergy test as described by Jarlier et al. [13] was used to confirm ESBL production. Test isolate was swabbed on the surface of Mueller Hinton agar, then placement of a ceftazidime disk close (20 or 30 mm) to an amoxicillin-clavulanate disk on a plate inoculated with the test organism. A clear extension of the zone of inhibition around the disk towards the amoxicillin-clavulanate disk that is centrally placed indicates the production of ESBL. This extension occurred due to the fact that the clavulanic acid present in the augmentin disc inactivated the ESBL produced by the organism.

Inoculation was also done on CHROMagar ESBL, a completely new and innovative chromogenic medium designed specifically for the Screening of Extended Spectrum β-Lactamase-producing Enterobacteria (ESBL) [14]. Incubation was done for 18-24 hrs. Escherichia coli produced pink to burgundy colouration of β-glucuronidase-producing colonies Klebsiella, Enterobacter, Serratia, Citrobacter (KESC): green/blue to brown/yellow colouration of β-glucosidase-producing colonies Proteae (Proteus, Providencia, Moraganella) produced dark to light brown colouration.

2.4 Statistical Analysis

The SPSS statistical package version (20.0) was used for statistical analysis. A p-value <0.05 was considered as statistically significant.

2.5 Plasmid DNA Analysis

Plasmid extraction was carried out using ZR Plasmid Miniprep-Classic extraction kit according to the manufacturers instruction.

2.6 Detection of ESBL Genes Types by PCR

ESBL producing isolates were amplified using bla TEM/SHVCTX-15, specific primers listed in Table 1. The reaction was performed in Gene Amp PCR system 9700 thermocycler (Thermo Electron Corporation, USA) under the following conditions: Initial denaturation at 94°C for 5 minutes followed by 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 58°C, 60 seconds extension at 72°C and a final extension at 72°C for 7 minutes. Polymerase chain reaction (PCR) products was separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide. A molecular marker (DNA laddah size range: 10 kb) was used to assess PCR product size.

### Table 1. Primer sequences used to amplify and β-lactamase genes by the PCR technique

<table>
<thead>
<tr>
<th>Gene</th>
<th>Target</th>
<th>Primer</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| bla TEM   | β-lactam| F: AATAAATTTCTTGAAGAGCGAAA  
R: GACGTTACCAATTGCTAATC | 1080              | Sharma et al. 2010 |
| bla SHV   | β-lactam| F: CACTCAAGGATGTATTGTG  
R: TTAGCGTTGCCCAGTGCTCG | 928               | Sharma et al. 2010 |
| bla CTX-M-15 | β-lactam| F: CGCTTTGCGATGTGCGAG  
R: ACCGCGATATCGTTGCTT | 550               | Sharma et al. 2010 |
3. RESULTS AND DISCUSSION

During a six-month period, a total of 252 uropathogens from pregnant women attending antenatal at Government general hospital, Eket, Ikot Ekpene and Oron were identified. 231 isolates were confirmed as potentially ESBL producers using DDST and CHROMagar ESBL. Occurrence of ESBL isolates was as follows: Enterobacter cloacae (57%), A. baumanii (13.5%), Proteus mirabilis (32%), (Table 2).

Fifty ESBL producing isolates were selected for plasmid DNA extraction and bla gene amplification: P. mirabilis n=12, A. baumanii n=20, Enterobacter cloacae n=18. Plasmid DNA of size 10kb was extracted from the 50 isolates (Fig. 4). CTX-M-15 type ESBL gene was found in 17% of P. mirabilis, 25% of A. baumanii and 17% of E. cloacae, (Fig. 3) bla TEM ESBL resistant gene was found in 8.3% of P. mirabilis, 35% of A. baumanii, none was found in E. cloacae (Fig. 1) bla SHV resistant gene was found in 35% of A. baumanii, none was found in E. cloacae (Fig. 3). In this study, antimicrobial susceptibility testing of A. baumannii, E. cloacae, and P. mirabilis isolates originally showed highly significant resistance to different types of antibiotics. This resistance can be due to the presence of specific genes of ESBL such as blaTEM, blaCTX-M-15 and blaSHV. Knowing the types and frequency of these genes helps us to make a good decision for the treatment process of patients effectively.

High level of multi-drug resistance including Cefotaxime (CTX), Ceftazidime (CAZ), Amoxicillin/clavulanic acid (AMC), Cefoxacin (OFX) and Amikacin (Ak) was observed among the isolates under the study. Various factors, such as the abuse of antibiotics, the spread of clonal resistant microorganisms, can cause the release of highly resistant pathogens. Previous studies showed that the prevalence of A. baumannii MDR isolates ranged from 32.7% to 100% [15,16,17,18,19]. Previous researches has reported that the prevalence of ESBL producing E. cloacae isolates ranged from 18% to 75% [20,21] while our studies reports a prevalence of 57%. In the present study 32% of P. mirabilis was ESBL producer was consistent with previous studies by Habibu and Orhue [22,23].

Among the mechanisms that create resistance to drugs, ESBLs play an important role in resistance to commonly used antibiotics such as penicillin and cephalosporins. ESBL genes, due to the widespread diffusion of pathogens in the community through plasmids and integrons, can further lead to an increase in resistance to drugs including MDR isolates [24].

Safari et al. [24] reported that SHV (58%) and TEM (20%) were the highest numbers of ESBL genes in their study [14]. Azhar et al. [25] based on a study that conducted in Iraq, reported that SHV (25%) was the most frequently detected ESBL gene [26]. Reza et al. [27] reported that...
TEM (52.1) was the most frequently detected ESBL gene. Khalizadegan and colleagues [18] identified that CTX-M and TEM have most ESBL genes [28] while in our studies blaCTX-M-15 (58.3) was the most frequently detected gene.

The reason to the observed differences in resistance patterns and the prevalence of A. baumannii, E. clocae and P. mirabilis in various investigations include the following: abuse and misuse of antibiotics, differences in the type of antibiotics used, long-term hospitalization, type of samples taken, differences in diagnostic methods used to identify genes, geographical conditions, gender and etc [29,30].
Fig. 4. Agarose gel Electrophoresis of plasmids recovered from ESBL producing bacterial isolates. Lane M: 10kb DNA ladder, lanes 1=P. mirabilis, Lanes 5, 6=A. baumanii (Upper gel)
Lanes 4,5,6 =E. clocae

Table 2. Frequency of ESBL producing isolates across the three study area

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumanii</td>
<td>31</td>
<td>13.5</td>
</tr>
<tr>
<td>Acinetobacter haemolyticus</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>A. iwoffii</td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>E. coli</td>
<td>10</td>
<td>4.3</td>
</tr>
<tr>
<td>Citrobacter youngae</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Citrobacter diversus</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Hafnia alvei</td>
<td>17</td>
<td>7.4</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>17</td>
<td>7.4</td>
</tr>
<tr>
<td>Enterobacter cloace</td>
<td>57</td>
<td>24.8</td>
</tr>
<tr>
<td>S. maltophilia</td>
<td>12</td>
<td>5.2</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>32</td>
<td>13.9</td>
</tr>
<tr>
<td>Salmonella subspecies</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>P. stuarti</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>2</td>
<td>0.7</td>
</tr>
<tr>
<td>Enterobacter hormaechei</td>
<td>4</td>
<td>1.7</td>
</tr>
<tr>
<td>Enterobacter gresoviae</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Seratia luquefaciens</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Morganella morganii</td>
<td>6</td>
<td>2.6</td>
</tr>
<tr>
<td>Citrobacter sakazaki</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>231</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>
4. CONCLUSION

In the present study bla$_{CTX-M-15}$ had the highest frequency of 58.3% obtained from pregnant women attending antenatal at the three study areas. The biological characteristics of ESBL isolates suggest that the predominant bla$_{CTX-M-15}$ is carried by plasmids. Antibiotic use, poverty, hygiene failures have enhance the high increment in ESBL producing Gram negative organism disseminating in African continent [31].


Ethics committee of Akwa Ibom State Ministry of health, provided ethical clearance for the study. Participants’ privacy and confidentiality have been assured (no names have been used, only serial numbers were used) and all data and results have been handled and treated confidentially. Ref:MH/PRS/99/VOL.IV/200.

ETHICAL APPROVAL

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


3. Timko J. Changes of antimicrobial resistance and extended spectrum beta-


Table 3. Detection of bla$_{ESBL}$ genes of SHV, TEM and CTX-M-15 in ESBL producing Proteus mirabilis, Acinetobacter baumanii and Enterobacter cloacae

<table>
<thead>
<tr>
<th>Strain identification</th>
<th>No. of isolates tested</th>
<th>$bla_{SHV}$</th>
<th>$bla_{TEM}$</th>
<th>$bla_{CTX-M-15}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteus mirabilis</td>
<td>12</td>
<td>1(8.3)</td>
<td>1(8.3)</td>
<td>2(16.7)</td>
</tr>
<tr>
<td>Acinetobacter baumanii</td>
<td>20</td>
<td>7(35)</td>
<td>7(35)</td>
<td>5(25)</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>3(16.6)</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>8(16)</td>
<td>8(16)</td>
<td>10(20)</td>
</tr>
</tbody>
</table>

No. (%) positive isolates


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