

Molecular Characterization of Extended Spectrum Beta-lactamase from *Enterobacter cloacae*, *E. coli* and *Klebsiella pneumoniae* from Pregnant Women in South-south Nigeria

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Abstract: Extended spectrum beta-lactamase prevalence is still on the increase across the world and has been implicated in urinary tract infections in Nigeria. TEM, SHV and CTX-M are becoming more common with CTX-M-15 becoming more significant as it is associated with complicated urinary tract infections. This study was conducted to evaluate distribution of *bla*TEM, *bla*CTX-M-15 and *bla*SHV genes among *Enterobacter cloacae*, *E. coli* and *Klebsiella pneumoniae*. A total of 660 urine samples were collected from pregnant women in 3 general hospitals from Akwa Ibom state. *Enterobacter cloacae*, *E. coli* and *Klebsiella pneumoniae* were identified using Microbact 24E. The disc diffusion and combined discs methods were used for testing antimicrobial susceptibility. The presence of ESBL was detected using Double Disk Synergy Test (DDST) and CHROMagar ESBL, respectively. Plasmid extraction was carried out following the protocol of ZR Plasmid Miniprep-Classic extraction kit. The *bla*CTX-M-15, *bla*TEM and *bla*SHV was identified by PCR with specific primers in selected 50 ESBL producing isolates. A total of 252 clinical isolates were collected from three General Hospitals in South-South, Nigeria. ESBLs were found in 231 (92%) isolates. *bla*CTX-M-15 was the commonest genotype (84%), followed by *bla*SHV (60%) and *bla*TEM (60%). ESBL positive strains of *E. cloacae*, *E. coli* and *K. pneumoniae* are increasingly found in isolates from pregnant women. The current study demonstrated the predominance of gene encoding *bla*CTX-M-15 with a percentage frequency of (84%) commonly with plasmid in the setting.

Keywords: ESBL *blagene*, PCR, UTI

1. Introduction

ESBLs are enzymes that are most commonly found in *Escherichia coli* and *Klebsiella pneumoniae* and are becoming more preminent in Enterobacteriaceae [1]. These genes include temoneira (TEM β -lactamase), sulfhydryl variable (SHV β -lactamase) and cefotaximase (CTX-M β -lactamase) and are carried on plasmids [2]. Plasmids often carry these resistance genes to other antibiotic classes [3]. The most common genes within the ESBL family are temoneira

(*bla*TEM) and sulfhydryl variable (*bla*SHV) with increasing reports of cefotaximase (*bla*CTX-M) [4]. As a result of mutational changes, *bla*SHV and *bla*TEM subgroups arise resulting in subtypes. Examples include *bla*SHV which differs from *bla*SHV-1 due to a single mutation of an amino acid from glycine to serine at position 238 (G238S) [5] while the same mutation in the *bla*TEM gene is referred to as *bla*TEM-2 [6]. *bla*CTX-M is acquired on a plasmid from *Kluyvera* species, it is becoming more prevalent, over 125 different subtypes of the *bla*CTX-M gene have been described with *bla*CTX-M-15

most predominant around the world [7]. In clinical strains, CTX-M-encoding genes are commonly located on plasmids and has various sizes ranging from 7-200 kb [8]. The CTX-Ms is the largest group of ESBLs; it was first identified in Germany, [9] France [10] and South America [11]. The bla_{CTX-M-15} and bla_{CTX-M-14} have become globally disseminated, becoming the predominant genotypes. CTX-M ESBLs have increased in prevalence since 2000 [12] and this has become a major challenge to healthcare, with restricted options to treat infections caused by CTX-M-producing bacteria. The prevalence of CTX-M variants is a complex one globally, but it is clear that bla_{CTX-M-15} has increased over time in most countries, and is dominant in most regions exceptions are China, South-East Asia, South Korea, Japan and Spain, where group 9 variants (especially CTX-M-14) are dominant, and South America, where bla_{CTX-M-2} is still significant [13]. It is important to know the frequency of ESBL positive strains in hospitals to formulate effective policy of empirical therapy [14]. The aim of this study was to isolate and identify the types of extended spectrum beta-lactamases genes (ESBL) produced by *E. cloacae* and *E. coli* and *K. pneumoniae*.

2. Materials and Method

2.1. Specimen Collection

A total of 660 urine samples were collected from pregnant women attending antenatal at the three General Hospitals between July to December, 2018. All pregnant women who were not on any antibiotics and willing to participate were included in the research, while those on any antibiotic therapy were excluded from the research.

Mid-stream clean-catch urine samples were collected and inoculated on MacConkey and incubated at 37°C for 24 hours. The presence of Extended-Spectrum Beta-lactamase (ESBL) was also detected by ESBL Chromogenic Culture Medium (France, paris). A prepared suspension of the test organism to turbidity equivalent to 0.5 McFarland standards was inoculated on CHROMagar ESBL Culture agar plate. Inoculated plates were incubated at 37°C aerobically for 24 hours and 48 hours; change in color of colonies was observed and interpreted as per guidelines.

2.2. Study Area

This was a hospital-based study conducted at three general hospitals, in Akwa Ibom state, south-south Nigeria.

2.3. Antimicrobial Susceptibility Testing

Isolates prepared in suspension equivalent to 0.5

McFarland standards were used for antibiotic susceptibility testing with ceftazidime (30µg), cefotaxime (30 µg), azetronam (30 µg), and cefodoxime (10 µg) (Oxoid, United Kingdom). The test was conducted in accordance with Kirby-Bauer Disc Diffusion Method. Zones of Inhibition were interpreted as concurring to Clinical Laboratory Standard Institute (CLSI) [15]. *E. coli* ATCC 25922 and *S. aureus* 6571 were used as quality control strains.

2.4. Detection of Extended-Spectrum Beta-lactamase

Isolates showing inhibition zone size of <22mm with ceftazidime (30µg), <25mm with cefotaxime (30 µg), <27mm with azetronam (30 µg) and <22mm with cefodoxime (10 µg) were suspicious for producing ESBL and thus subjected to screening.

2.5. Double Disc Synergy Test (DDST)

Extended-Spectrum Beta-lactamase (ESBL) was detected by the Double Disc Synergy Test (DDST) [16, 17]. The prepared suspension of the isolates to turbidity equivalent to 0.5 McFarland standards was inoculated on Mueller-Hinton agar plate. Clavulanic-amoxicillin (30 µg) disc was placed at the center of the Mueller-Hinton agar plate. Ceftazidime (30 µg) and cefotaxime (30 µg) discs were placed 20 mm out from the edge of clavulanic-amoxicillin disc. Inoculated plates were incubated at 37°C aerobically for 24h. Observation of Cephalosporin/ clavulanate synergy was interpreted as positive for ESBL production [18, 19].

2.6. Ethical Consideration

Ethics committee of Akwa Ibom State Ministry of health provided ethical clearance for the study. Participants' privacy and confidentiality were assured and all data and results were handled and treated confidently.

2.7. Statistical Analysis

Chi square was used for statistical analysis. A p-value of <0.05 was considered as statistically significant.

2.8. Plasmid DNA Analysis

Plasmid extraction was carried out using ZR Plasmid Miniprep-Classic extraction kit. A modified alkaline lysis protocol together with Zymo-Spin technology to yield high quality plasmid DNA in minutes was followed, the buffers were color-coded (red, green, yellow) for easy visualization of complete cell lysis and neutralization. Extracted DNA was stored initially at -22°C for further use.

Table 1. Primer sequence for Beta-lactamase gene Detection.

Gene	Target	Primer	Product size (bp)	Reference
bla _{TEM}	β-lactam	F: ATAAAATTCTTGAAGACGAAA R: GACAGTTACCAATGCTTAATC	42	Sharma <i>et al.</i> , 2010
bla _{SHV}	β-lactam	F: CACTCAAGGATGTATTGTG R: TTAGCGTTGCCAGTGCTCG	36	Sharma <i>et al.</i> , 2010
bla _{CTX-M-15}	β-lactam	F: CGCTTTGCGATGTGCAG R: ACCGCGATATCGTTGGT	34	Sharma <i>et al.</i> , 2010

2.9. Detection of ESBL Genes Types by PCR

ESBL producing isolates were amplified using blaTEM/SHVCTX-15 specific primers listed in Table 1. The reaction was performed in Gene Amp PCR system Px2thermocycler (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 gel and stained with ethidium bromide. A molecular marker (DNA ladder size range: 1kb) was used to assess PCR product size.

3. Results

During a six-month period, a total of 252 uropathogens from pregnant women attending antenatal at government general hospitals, Eket, Ikot Ekpene and Oron were identified. Two hundred and thirty one isolates were confirmed as potentially ESBL producers using DDST and CHROMagar ESBL. Occurrence of ESBL isolates was as follows: *E. cloacae* (25%),

E. coli (19%), *K. pneumoniae* (15%) (Table 2).

Table 2. Frequency of ESBL producing Isolates.

Bacterial Isolates	Total No.	%
<i>E. cloacae</i>	58	25
<i>E. coli</i>	44	19
<i>K. pneumoniae</i>	34	15
<i>H. alvei</i>	17	7.4
<i>S. aureus</i>	17	7.4
<i>A. haemolyticus</i>	6	2.5
<i>A. iwoffii</i>	3	1.3
<i>S. subspecies</i>	7	3.0
<i>M. morgani</i>	5	2.2
<i>E. hormaechei</i>	4	1.7
<i>E. agglomerans</i>	11	4.7
<i>Citrobacter sakazaki</i>	1	0.4
<i>S. luquefaciens</i>	1	0.4
<i>E. gresoviae</i>	1	0.4
<i>Serratia marcescens</i>	7	3.0
<i>S. maltophilia</i>	12	5.2
<i>Citrobacter youngae</i>	1	0.4
<i>Citrobacter diversus</i>	1	0.4
<i>Citrobacter freundii</i>	1	0.4
Total	231	100

Table 3. Detection of bla_{ESBL} genes of SHV, TEM and CTX-M-15 in ESBL producing *Enterobacter cloacae*, *E. coli* and *K. pneumoniae*.

Strain identification	No. of isolates tested	bla _{SHV}	bla _{TEM}	bla _{CTX-M-15}
<i>E. coli</i>	12	3 (25)	3 (25)	5 (42)
<i>K. pneumoniae</i>	20	7 (35)	7 (35)	5 (25)
<i>Enterobacter cloacae</i>	18	0	0	3 (17)

No. (%) positive isolates.

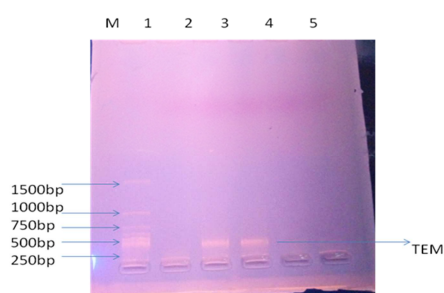


Figure 1. Agarose gel electrophoresis of PCR products for specifying blaTEM broad spectrum beta-lactamases genes. M: DNA ladder (1kb), No 1: positive control for blaTEM, No. 2, 3 isolates with blaTEM gene.

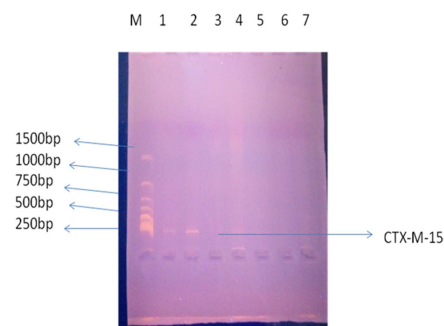


Figure 2. Agarose gel Electrophoresis of PCR products for specifying blaCTX-M-15 broad spectrum beta-lactamases genes. M: DNA ladder (1kb), No 1: positive control for blaCTX-M-15, No. 2, 4 isolates with blaCTX-M-15 gene.

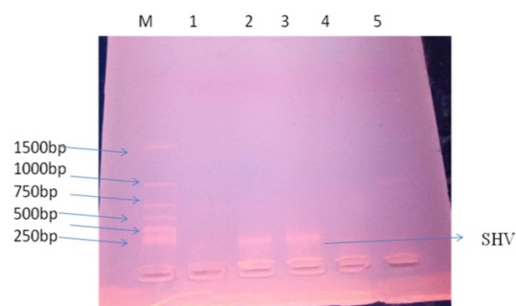


Figure 3. Agarose gel electrophoresis of PCR products for specifying blaSHV broad spectrum beta-lactamases genes. M: DNA ladder (1kb), No 1: positive control for blaSHV, No. 2, 3 isolates with blaSHV gene.

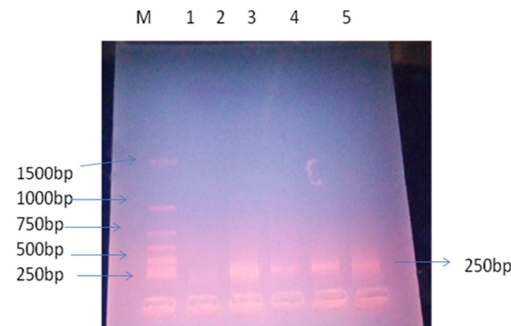


Figure 4. Agarose gel electrophoresis of plasmids recovered from ESBL producing bacterial isolates. Lane M: 1kb DNA ladder, lanes 2, 3-*E. coli*, Lanes 4-*K. pneumoniae*, Lanes 5=*E. cloacae*.

4. Discussion

The 660 urine samples from different pregnant women yielded 252 clinical isolates. The isolates were screened for ESBL production and 92% (231) were found to be ESBL producers including *E. cloacae* (25%), *E. coli* (19%), *K. pneumoniae* (15%), *H. alvei* (7.4%), *A. haemolyticus* (2.5%), as shown in table 1.

Fifty ESBL producing isolates were selected for plasmid DNA extraction and *bla* gene amplification as follows; *E. coli* n=12, *K. pneumoniae* n=20, *E. cloacae* n=18 (Table 2). Plasmid DNA of size 1kb was extracted from the 50 isolates (Figure 4). *bla*CTX-M-15 type ESBL gene was found in 42% of *E. coli*, 25% of *K. pneumoniae* and 17% of *E. cloacae*. *bla*TEM ESBL resistant gene was found in 25% of *E. coli*, 35% of *K. pneumoniae*, none was found in *E. cloacae*. *bla*SHV resistant gene was found in 35% of *K. pneumoniae*, 25% of *E. coli*, while none was found in *E. cloacae* (Table 2).

The agarose gel electrophoresis of the *bla* genes after PCR amplification is shown in figures 1, 2 and 3. The agarose gel electrophoresis of plasmids recovered from the ESBL isolates is shown in figure 4.

In the present study, the prevalence of ESBL-producing bacteria was 92%, which is almost similar to that found in Pakistan 95%, south western Uganda 89% and Saudi Arabia 84% [20, 21, 22]. However, our result was higher than 72% reported at a tertiary care hospital in Riyadh capital, Saudi Arabia [23]. High prevalence of ESBL-producing bacteria has been observed in many countries, such as 47.6% in Algeria [24], 60% in Pakistan [25], 42.3% in India [26], 37.8% in Bangladesh and 65% in Nigeria [34]. By contrast, the lowest prevalence of ESBL-producing Enterobacteriaceae has been reported in Europe, such as below 1% in Sweden [27] and 5% in Netherlands [28]. These reports together with the current research indicate the global dissemination of β -lactamase-producing microorganisms, most especially in developing countries including Nigeria. This could be caused by abuse of antibiotics, poor hygiene conditions in developing countries [29].

In the present study, a frequency of 25% *E. cloacae*, 19% *E. coli* and 15% for *K. pneumoniae* species respectively were noted. Although, *E. cloacae* was found to be the commonest ESBL producing isolate, other researchers from Iran has reported a significantly higher prevalence of 78.2% for *E. cloacae* [30]. This shows the important role of this species in human infections, which was in agreement with other, reports [31-34]. ESBL production among *E. cloacae* isolates is becoming a major clinical concern because of its ability to develop resistance to several classes of antimicrobial agents and has high potential for transmission of resistance to other bacterial species [35]. In the current study, the frequency of ESBL producing *E. cloacae* (25%), *E. coli* (19%) and *K. pneumoniae* (15%) was different from other results in Saudi Arabia and south-south Nigeria in which a frequency of 3.5% *E. cloacae*, 9.5% *E. coli*, 16.5%, *K. pneumoniae* and 6% *E. cloacae*, 38%, *E. coli*, 56% *K. pneumoniae* was recorded

respectively [22, 36].

The result from this study reported a high occurrence of *bla*CTX-M-15 gene, although CTX-M-15 gene now occurs in a higher percentage across the globe. A study by Abrar *et al.*, from Pakistan indicated 72% of isolates had *bla*CTX-M-15 gene which was similar to the prevalence of *bla*CTX-M-15 gene found in this study [37]. Several studies from other parts of world have shown different prevalence of *bla*CTX-M-15 gene among isolates including 84.7% (Chile), 98.8% (China) and 13.6% (Tanzania) [38-40]. Several studies from Europe and Asia have also reported that CTX-M gene is now replacing TEM and SHV genes as the commonest ESBL type in that part of the world [41, 42]. We observed *bla*TEM and *bla*SHV genes had 60% prevalence respectively in our study. Report by Sid *et al.*, from Qatar stated that CTX-M group has evolved through mutations in *bla*TEM and *bla*SHV genes and is recent endemic [43].

Our study reported that 3 (17%) of ESBL producing *E. cloacae* tested had *bla*CTX-M-15 gene. The spread of *bla*CTX-M-15 *E. cloacae* with a frequency of 11.8% was reported from Iran [43]. In our study, there was no *bla*SHV and *bla*TEM detected among ESBL strains of *E. cloacae*, the result reported was 0%. This showed that other factors such as presence of gene other than TEM, SHV and CTX-M-15 were effective in producing resistance to beta-lactam antibiotics.

In our study, 35% of ESBL producing *K. pneumoniae* were harbouring *bla*TEM and *bla*SHV respectively. A study from northern India and southern India revealed a prevalence of 72% and 77.58% for *bla*SHV production [44, 45]. Our study, revealed a frequency of 25% for *bla*CTX-M-15 gene in *K. pneumoniae*. Kotekani and Kotigadde reported a significantly high occurrence of 75.51% of CTX-M-15 genes among *K. pneumoniae* from India. Many other researchers have reported similar result [46, 47]. The gene of *bla*CTX-M-15 has been reported in *Klebsiella pneumoniae* isolated from various hospitals in Iran [48]. To further buttress the findings from this study, Ahmad and Khali reported a prevalence of 64.7% for *bla*TEM gene in *K. pneumoniae* from Iraq which is significantly higher than what we reported [49].

E. coli isolates carried *bla*TEM, *bla*SHV and *bla*CTX-M-15 type ESBL gene. In our study, CTX-M-15 type ESBLs was confirmed in 42% of the isolates. This result emphasized that this enzyme is now one of the commonest CTX-M beta-lactamase in Nigeria. Among 1168 ESBL-*E. coli* isolates obtained from various clinical specimens in China, 18.2% of the isolates were detected to harbor *bla*CTX-M-15 type ESBL gene [50]. A study by Siddaramappa *et al.*, observed the preponderance of *bla*CTX-M-15 *E. coli* (50%) in India [51].

K. pneumoniae, *E. coli*, and most Gram-negative bacteria possess the ability to acquire plasmid encoding for ESBL genes such as *bla*-TEM, *bla*SHV and *bla*-CTX-M and become highly resistant to different antibiotics and wide-spectrum of 3rd generation cephalosporins in hospitals and in community [52].

5. Conclusion

In this study, we observed that the major groups of ESBL genes, TEM, SHV and CTX-M-15 were present in some strains of *E. coli* and *K. pneumoniae* while only CTX-M-15 ESBL gene was found in *E. cloacae*. CTX-M-15 ESBL gene was the highest occurring gene. Therefore, there is likely a “strong selection pressure” for the maintenance and dispersal of the bla_{CTX-M-15} genotype among uropathogenic *E. coli*, *K. pneumoniae* and *E. cloacae* in Nigeria. In view of this, diagnostic laboratories should routinely test clinical isolates of *E. coli*, *E. cloacae* and *K. pneumoniae* for bla_{CTX-M-15}, and hospitals should develop and implement an antibiotic stewardship program to reverse the trend. It is also recommended that further studies should be carried out with a larger population size.

Conflict of Interest

There is no conflict of interest.

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