

MOLECULAR CHARACTERIZATION OF ESBLs PRODUCING ORGANISM CAUSING ANTIBACTERIAL DRUG RESISTANCE IN SOUTH-SOUTH NIGERIA

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ABSTRACT

Some members of the enterobacteriaceae were investigated for their ability to produce extended spectrum beta lactamases (ESBLs) implicated in inactivating the cephalosporins. Clinical specimens of urine, stool, sputum, blood, high vaginal, wound, pus and urinogenital swabs were obtained from health resource centers in Akwa Ibom, Cross River, Delta, Edo and Rivers State, all in South- South geopolitical zone of Nigeria. Double Disk Synergy Test (DDST) was used to detect ESBL production. Out of the 12 isolates of *E. coli* strains studied, isolate A, N, R and T had bands in TEM and SHV; M had bands SHV and CTX-15; S had weak bands in TEM and SHV but two strong bands in CTX-15; U had weak bands in SHV and CTX, while isolate J, K, O, Q and V had no band. For the second set of *E. coli* isolates; A₅, A₆, A₈ had band in TEM; X had bands in SHV and CTX- 15; A₁₁ and A₁₂ had bands in SHV and CTX-15; A₁₁ and A₁₂ had bands in CTX-15 and SHV respectively, while A₇, A₁₃ and A₁₄ had no bands. The PCR results of the phenotypically negative strains revealed that isolates B had CTX-M-15; E had TEM, SHV, CTX-M; F had SHV, CTX-M, CTX-M-15; G had amplification in TEM and a weak band in CTX-M-9; H had CTX-M-15; P had TEM, SHV while isolate C, D and I had no bands. For Bla genes recovered in this study, a total of 12(28.57%) TEM; 14(33.33%) SHV; 5(11.90%) CTX-M; 2(4.76%) CTX-M-9 and 9(21.43%) CTX-M-15 were obtained. Bla OXA and CTX-M-2 were not found among the strains, while 11 strains had no amplification with the primer sets used in this study. However, this study has shown that the genotypic method of characterizing ESBLs producing organisms is a more sensitive and dependable method compared to the phenotypic methods. Furthermore, the study has revealed the presence of several extended spectrum beta lactamases genes in the study area. It is believed to be responsible among other factors for the problem of antibiotic resistance experienced in this part of the world. The impact of this serious public health threat cannot be glossed over. Therefore, concerted efforts must be made by researchers to carry out further research with a view to completely understand the mode of action of these enzymes and how they can be effectively controlled.

Introduction

There has been increasing report of antibiotic resistance among isolates of enterobacteriaceae as well as other gram negative bacilli in many parts of the world and this is seen as a major threat to successful therapy of infections in many hospital (Andy *et al.*, 2019). The emergence of these enzymes initially appeared to the insignificant and of no consequence. However, it has proved to be one of the major resistance problems worldwide and is eroding the efficacy of the most commonly used group of antibiotics (Chidimma *et al.*, 2018).

Among the wide many of antibiotics, beta lactams are the most varied and widely used accounting for over 50% of all systematic antibiotics in use. The most common cause of all bacterial resistance to beta lactams antibiotics is the production of beta lactamases (Iroha *et al.*, 2017). Many of the second and third generation cephalosporins were specifically designed to resist the hydrolytic action of major beta lactamases, however, new beta lactamases emerged against new class of beta lactam antibiotic introduced and caused resistance, with the latest in their arsenal being the evolution of ESBLs (Raji *et al.*, 2013).

Beta lactamase of gram negative bacteria are the most important mechanisms of resistance against Beta lactam drugs which include first, second and third generation cephalosporins, penicillins, monobactams and aztreonam (Raji *et al.*, 2013). The extended spectrum Beta lactamases contain multi- resistance genes that may be easily transmitted among members of the enterobacteriaceae. They also confer resistance to other classes of antibiotics such as aminoglycosides and fluoroquinolones (Kiratisin *et al.*, 2009). The most noticeable feature of these enzymes is their ability to attack and hydrolyze extended spectrum or never cephalosporins such as ceftazidime, cefotaxime, ceftriazone, monobactams such as cephalothin (Paterson and Bonomo, 2005). Extended spectrum beta lactamases have been on the increase worldwide. Over the last twenty years, many new beta lactam antibiotics have been developed specifically to resist the hydrolytic action of ESBLs. However with each class of drugs, newer beta lactamases have emerged (Lee *et al.*, 2011). This is presumably due to selective pressure of use and over use of the new antibiotic. Though antibiotic resistance seems to be inevitably, there are measures that can be taken to retard it. Some of these include infection control, developing new antibiotics, quick identification and isolation of patients with drug resistant infections, improved sewage system and water purity in developing nations and establishment of national surveillance programs on antibiotics (Ejikellgwu, *et al.*, 2013). It is therefore, pertinent to carry out studies on these powerful enzymes which have clearly become a nightmare in successful antibacterial chemotherapy and by extension our existence. Hence, there is need to be vigilant, proactive and also, to understand the molecular nature of resistance posed by these organisms.

MATERIAL AND METHODS

Collection of isolates

Six hundred and forty-six isolates were obtained from various clinical specimens including urine, wound swab, stool, blood, high vaginal swab and sputum. The isolates were collected on nutrient agar slants and immediately transported to the laboratory in cool boxes.

Sources of samples

The clinical isolates were obtained from five of the six states of the south-south geo-political zone of Nigeria namely; Akwa-Ibom, Cross River, Delta, Edo and Rivers States. In each of these states, they were obtained from two major hospitals; Akwa Ibom (Unvieristy of Uyo Teaching Hospital and St. Luke's Hospital and General Hospital, Calabar), Delta (Federal Central Hospital Asaba and NNPC Clinic Warri); Edo (University of Benin Teaching Hospital and Central Specialist Hospital, Benin) and Rivers (University of Port Harcourt Teaching Hospital and Braitwaite Memorial Hospital, Port Hartcourt).

Preparation of cell lysate

Three to five pure colonies of isolates were sub-cultured onto nutrient agar and blood agar and incubated at 37⁰C overnight. The overnight cultures were harvested into 500µl of distilled water and was boiled on heating mantle for 10 minutes at 95⁰C. After boiling, they were centrifuged for 10 minutes in a bench top centrifuge at 8,000rpm. The supernatant was used for downstream procedures such as PCR.

ESBL typing using Polymerase chain reaction (PCR)

The genes coding for the following; beta lactamases (bla TEM, SHV, CTX, OXA, CT-Z, CT-9, CT- 15) were detected by PCR amplification of genomic DNA in the cell lysates. The oligonucleotide PCR primers specific for beta lactamase genes, melting temperature and PCR products length are as shown in Table 1.

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TABLE 1

Primers Guide used in the Study

Primers	Nucleotide Sequence	Tm	Product Length	Ref.
TEM	ATGAGTATTCAACATTTCCGTG TTACCAATGCTTAATCAGTGAG	55	840	Gracia <i>et al.</i> , 2008
SHV	ATTTGTCGTTCTTTACTCGC TTTATGGGCGTTACCTTTGACC	55	1051	Gracia <i>et al.</i> , 2008
CTX-M	TTTGCGATGTGCAGTACCATAA CGATATCGTTGGTGGTGCCATA	51	544	Eldestem <i>et al.</i> , 2003
OXA	TTTTCTGTTTGGGTTTT TTTCTGGCTTTTGTGTCTTG	52	427	Park <i>et al.</i> , 2006
CTX-2	AAATGTGCTGGCTCCTTTCGTGAGC AGGGTTCGTTGCAAGACAAGACTG	60	1122	Gracia <i>et al.</i> , 2008
CTX-9	GTGACAAAGAGAGTGCAACGC TAGATTCTCGCCCTAAGGCC	60	856	Sabata <i>et al.</i> , 2000
CTX-15	CACACGTGGAATTTAGGGACT GCCGTCTAAGGCGATAAACA	55	996	Muzaheed <i>et al.</i> , 2008

Source: Sidjabat *et al.*, 2009

The gel was viewed in a gel doc system (biorad Co ltd, USA) after electrophoresis. Standard kilobase ladder used to confirm band sizes as shown in Table 2.

TABLE 2

Standard band sizes used to confirm enzyme bands in PCR

Kilo base	Base pairs
- 10	(10,000)
- 8	(8,000)
- 6	(6,000)
- 5	(5,000)
- 4	(4,000)
- 3	(3,000)
- 2	(2,000)
- 1.5	(1,500)
- 1.0	(1,000)
- 0.5	(500)

Cycling conditions for polymerase chain reaction (PCR) is shown on Table 3. The concentration of each reagent in the mixture was 100-500µg of total genomic DNA, 10mM tris HCL (pH 8.3), 2mm MgCl₂, 0.5pm of each primer pair (forward and reverse). 250Mm of each deoxynucleoside and triphosphate and 1µl of Tag DNA polymerase for all the genes, bla TEM, SHV, CTX, OXA, CTX-2, CTX-9, CTX-15. The PCR products were separated in a 1% gel stained with ethidium bromide. The gel was run in an electrophoresis chamber at a voltage of 80mA for 2 hours. A standard 1kbDNA ladder was used to size the bands.

TABLE 3
Steps of PCR process

Step	Procedure involved
1.	Initial denaturation at 95°C for 4 minutes
2.	Denaturation at 95°C for 30 seconds
3.	Primer annealing at 55°C for 30 seconds
4.	Primer extension at 72°C for 1 minute
5.	Final extension at 72°C for 10 minutes
6.	Holding temperature 4°C forever

Preparation of agarose gel

The agarose gel was used for electrophoresis after polymerase chain reaction. To prepare a 1% gel, 1g of agarose powder was weighed into 100ml of Tris- borate EDTA (TBE). This was placed in a microwave oven to melt and mix properly. Care was taken to ensure that the contents do not evaporate due to over-heating. It was allowed to cool to about 50°C. Ethidium bromide was then added. The importance of this step was to allow for visibility of the amplicon bands under ultra violet light. The mixture was allowed to cool further and then poured into gel tray with appropriate gel combs. The combs were used to create wells in the agarose gel where the PCR products will travel during electrophoresis. The gels were left to solidify (set) after which the combs were removed.

Gel electrophoresis

The PCR products were introduced into the gel. They were then placed in the electrophoretic tank to separate the bands. DNA is negatively charged thus movement is from the negative to the positive end of the electrophoretic chambers. The electrophoresis step lasted for 1 hours 30mins to 2hours. All PCR process were carried out in bowls containing ice cubes to prevent the delvoepment of primer dimmers.

Results

The result of the genotype typing of ESBL producing isolates by PCR is found in Fig. 1,2 and 3. Figure 1 shows the result of PCR for 12 isolates of *E. coli* strains. Isolate A, N, R and T had bands in TEM and SHV; M had bands SHV and CTX-15; S had weak bands in TEM and SHV but two strong bands in CTX-15; U had weak bands in SHV and CTX while isolates J, K, O, Q and V had no bands. Figure 2 shows the result for PCR test of these cored set of *E. coli* isolates. Isolates A₅, A₆, A₈ had bands in TEM; X had bands in SHV and CTX-15; A₁₁ and A₁₂ had bands in CTX-15 and SHV respectively. Isolates A₇, A₁₃ and A₁₄ had no bands. Fig. 3 shows the PCR result of the phenotypically negative strains. It revealed that isolate B had CTX-M-15; G had

amplification in TEM and a weak band in CXT-M-9; H had CTX-M-15; P had TEM and SHV. Only isolate C, D and I had no bands.

A summary of the Bla genes recovered in our study is shown in figure 4. A total of 12 (28.57%) TEM; 14(33.33%) SHV; 5(11.90%) CTX-M; 2(4.76%) CTX-M-9; and 9(21.43%) CTX-M-15 were obtained. Bla OXA and CTX-M-2 were not found among the strains while 11 strains had no amplification with the primer sets used in this study.

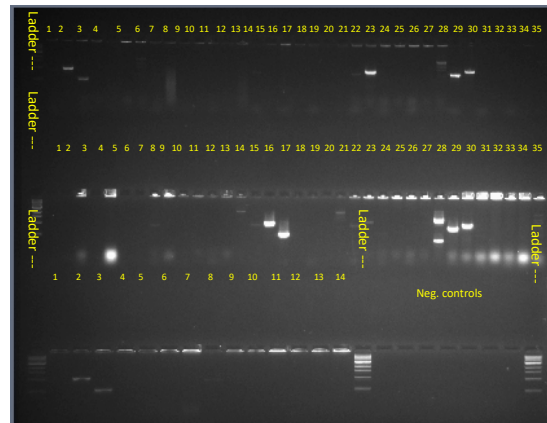


Fig. 1: ESBL typing for 12 *E. coli* isolates (A-U) using primers: TEM = Lane1, SHV = Lane 2, CTX-M = Lane 3, OXA = Lane 4, CTX-M2 = Lane 5, CTX-M9 = Lane 6 and CTX- M15 = Lane 7

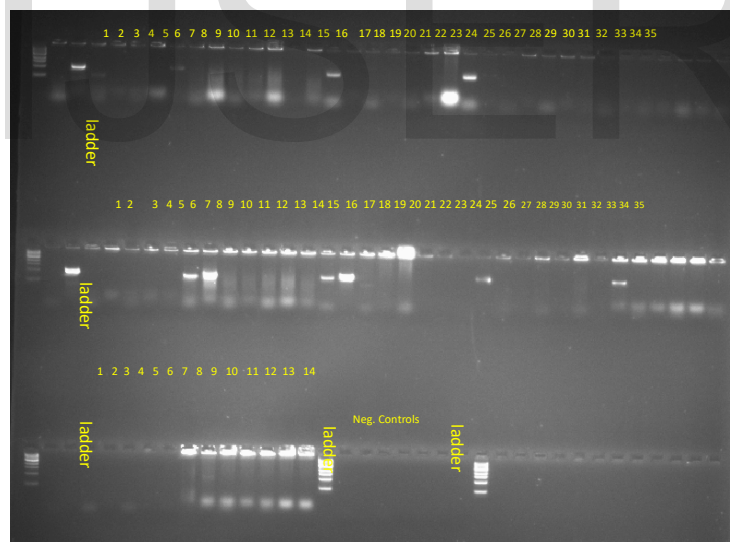


Fig. 2: ESBL typing for 12 *E. coli* isolates (X - A14) using primers: TEM = Lane1, SHV = Lane 2, CTX-M = Lane 3, OXA = Lane 4, CTX-M2 = Lane 5, CTX-M9 = Lane 6 and CTX-M15 = Lane 7

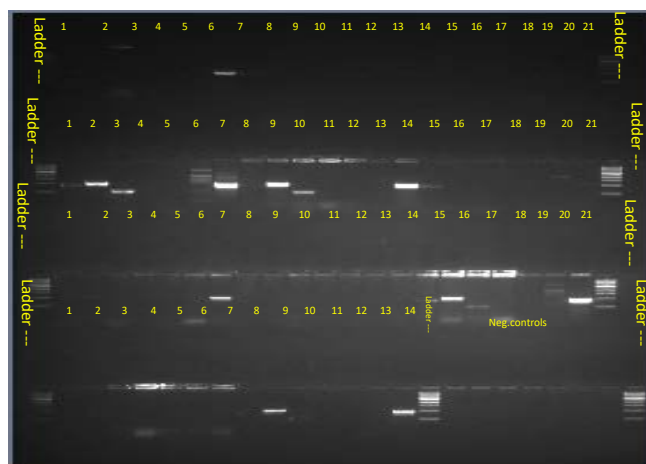


Fig. 3: ESBL typing for phenotypically negative isolates using primers: TEM = Lane1, SHV = Lane 2, CTX-M = Lane 3, OXA = Lane 4, TX-M2 = Lane 5, CTX-M9 = Lane 6 and CTX-M15 = Lane 7

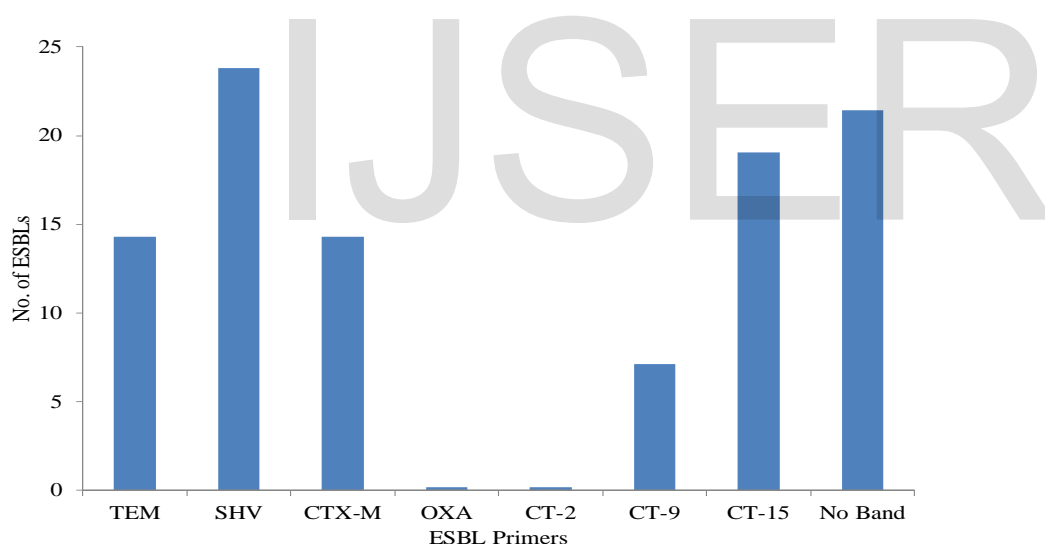


Fig.4: Bla genes recovered from study states

Discussion

The PCR experiment is designed to detect ESBL producers genotypically. Several isolates in this study gave amplification. Some isolates had more than one band which compounded with various band sizes by standard markers. The most amplification was observed in CTX-M. This is an indication of the ESBL type prevalent in the study area and this finding agrees with earlier reports, which stated that CTX-M enzymes are the most common of the ESBL types. The work of Kiratisin *et al.*, (2008) and Sidjabat *et al.*, (2009) are among the various reports which confirmed that the CTX-M enzymes causes the widespread of the ESBLs. Interestingly, it was observed in this study that some isolates that were positive phenotypically did not give any amplification in the PCR analysis, in which case they may not be ESBL producers. Nine (9) *E. coli* and one (1) *K. pneumonia* isolate did not give any amplification. This means that some organisms actually harbored the

ESBL even though it was not detected through phenotypic tests. Out of the twelve (12) phenotypically negative strains, six (6) isolates of *K. pneumonia* gave various bands while four (4) did not yield any amplification. However, two (2) isolates of *E. coli* also yielded amplifications.

Conclusion

The study has shown that the genotypic method of analysis is more sensitive than the phenotypic method. Although the genotypic method of characterization of ESBL producing organism is more expensive, it has however, proven to be the most dependable and best option.

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