Susceptibility Pattern and Detection of Resistance Genes in *Escherichia coli* isolates in Clinical Specimens in Calabar, Cross Rivers State.

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ABSTRACT

Introduction: *Escherichia coli* is a facultatively anaerobic bacilli and microbiota of the gastro intestinal tract. It causes about 2 million deaths yearly through extra-intestinal and intestinal infections. Diagnosis and treatment of patients seem to be dependent on the detection of extended spectrum beta-lactamase (ESBLs) enzymes which hydrolyse antibiotics belonging to the cephalosporin and penicillin group rendering them ineffective on pathogens. This study aimed to detect ESBL encoding genes in *E. coli* isolated from various clinical specimens and to determine the relationship between the genes and their antibiotic susceptibility pattern.
Methods: Clinical isolates of *E. Coli* obtained from Laboratories in various clinics in Calabar, were re-identified and subjected to antibiotic susceptibility testing. Double disc synergy test was used for phenotypic detection of ESBL in isolates while conventional PCR method served the purpose for assessing ESBL encoding genes. **Results:** *Escherichia col*i solates phenotypicaly positive for ESBL production was (56.6%). Quinolones (54.7%) were the most effective antibiotics followed by fluoroquinolones (34.0%). Out of the three resistance genes encountered in the study (56.7%) were Cefotaximase (CTX-M) genes. The susceptibility range for the ESBL-producing isolates was from 3.3% to 60.0% while the Non-ESBL producer's susceptibility rates ranged from 8.7% to 47.8%. All the isolates were most susceptible to ofloxacin. Gene expression seem to determine antibiotic resistance ($p \leq 0.05$). The resistance rate to cephalosporins was (90.9%). **Conclusion:** The CTX-M gene was the most encoutered resistance encoding gene in our locality. Most phenotypic positive isolates possess the ESBL encoding genes. The genotypic method is better for detection of ESBL genes.

Keywords: Resistance, genes, *Escherichia coli*, Clinical isolates, Susceptibility

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INTRODUCTION

Escherichia coli is a Gram negative facultative anaerobic bacilli with optimum growth temperature of 37^0C and a commensal of the GIT of humans [1]. It is transmitted to humans through the orofecal route which includes: consumption of contaminated foods and faecal contamination of water [2].

The extra-intestinal and intestinal infections of *E. coli* causes about two million deaths yearly [3]. The ESBLs enzymes are plasmid mediated enzymes that hydrolyse antibiotics belonging to the cephalosporin and penicillin group rendering them ineffective. They are also defined as transmissible beta lactamases which are encoded by genes that can be exchanged between bacteria [4]. The success of EBLSs may be due to spread of bacterial clones and transfer of ESBLs encoding genes on mobile elements [5].

About 400 different ESBLs are known. They are grouped under Temoneira, Sulfhydryl variable and Cefotaximase (TEM, SHV and CTX-M) with several variants [6]. Molecular detection of ESBL genes in bacteria and the antibiotic susceptibility pattern of isolates may provide data on their spread [7]. Due to the ability to cause a wide range of infections and the increasing treatment failure, the detection of these resistant genes is paramount to chemotherapeutic success. It will also provide information on the prevalence of ESBL producing *Escherichia coli* in our locality.

The ESBLs are the result of point mutations [8]. The SHV gene has been reported from various places before the start of the 21st century. A newer type, called CTX-M has been reported in Asia and Europe [9-10]. Iroha *et al* [11] reported a high rate of CTX-M-1 cluster - ESBLs in clinical specimens in South-Eastern Nigeria. This confirms the spread of CTX-M ESBL in clinical isolates in Nigeria.The CTX-M has a greater activity against cefotaxime than ceftazidime. There is a need to determine the source and direction of spread of these resistance genes in pathogenic bacterial isolates in our locality.

This study aimed to detect ESBL encoding genes in *E. coli* isolated from various clinical specimens and to determine the relationship between the genes and their antibiotic susceptibility pattern.

MATERIALS AND METHODS

The study was carried out in the University of Calabar Teaching Hospital. The molecular studies was done in the Department of Medical Laboratory Science, Niger Delta University, Bayelsa State, Nigeria. This study was an experimental cross-sectional study. Ethical approval was not required because isolates were obtained from Laboratories in both public and private hospitals in Calabar metropolis. Clinical isolates already identified as *Escherichia coli* were obtained from various clinical specimens which include; urine, wound, blood, endocervical swab and stool. These specimens were obtained from University of Calabar Teaching Hospital, General Hospital and three private clinics into nutrient agar slants. The slants were incubated at 37^oC for 18-24 hours to allow the organism grow. The isolates were stored in the Lucky Goldstar (LG) refrigerator, South Korea at -20^oC until it was ready for processing.

Isolates were re-identified macroscop-ically, microscopically and biochemically before use. The following biochemical test were used to re-identify the isolates; Indole test, Citrate utilization, oxidase test and Kligler Iron Agar inoculation test. Kirby-Bauer dics diffusion antibiotic susceptibility testing was carried out. The

antibiotics tested were fluoroquinolones namely; levofloxacin (5µg), ciprofloxacin (5 µg), ofloxacin (5µg). Others include; cefuroxime (30µg), amoxicillin/clavulanic acid (30µg), ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), imipenem (10µg), gentamicin (10µg), and ampiclox (30µg) [12].

Extended spectrum Beta Lactamase detection was done using phenotypic and genotypic methods. Isolates with zone diameters for the following antibiotics; ceftazidime ≤ 22mm, ceftriaxone ≤ 25mm and cefotaxime ≤ 27mm were indicated as a potential ESBL producer [13]. Isolates with zone diameter as above were selected for the double disc synergy method of phenotypic testing.

Molecular Identification of ESBL Genes

DNA Extraction

A pure colony was emulsified in luria Bertani medium and incubated at 37^0C overnight. Two ml of the turbid suspension was put into a 2.0ml microcentrifuge tube, and then centrifuged at 11,000rpm for 1 minute. The supernatant was discarded and 2ml of sterile H_2o was used to resuspend the sediment and votexed. It was spun for a minute at 11,000rpm.One ml of sterile water was added to the sediment after discarding the supernatant. This preparation was heated on a heating block at 95^0C for 20 minutes and further cooled for 10 minutes at -20^0C thereafter it was centrifuged at 11,000rpm for I minute. Three hundred µl of the clear supernatant was transferred into 1.5 ml eppendorf tubes [14-15].

DNA Quantification

The Nanodrop 1000 spectrophotometer, Thermo Fisher Scientific, USA, was used for bacterial DNA quantification.

Amplification of ESBL Gene

Amplification of CTX-M, SHV and TEM genes was done using Eppendorf Master cycler (thermal cycler), California, USA, for a final volume of 25µl. The PCR tubes were set up in an ice rack and 12.5µl of the PCR master mix which contained a thermostable DNA polymerase, dNTPs, $MgCl_2$, and proprietary additives in a buffer optimized for PCR, 0.5µl of the forward and reverse primers (Table 1) obtained from Inquaba Biotech Industry, Pretoria, South Africa, 10.5 µl of nuclease water, Amresco Life Science, United Kingdom, was dispensed into each tube followed by addition of 1µl of the extracted bacterial DNA before loading into the thermal cycler. The cycling conditions for SHV amplification were set at 95^0C for 3 minutes initial denaturation accompanied by 35 cycles for 30 seconds denaturation at 95^0C and 40 seconds annealing at 56^0C, 30 seconds extension at 72^0C, followed by final extension of 3 minutes at 72^0C. For CTX-M; 95^0C for 3 minutes initial denaturation followed by 40 cycles of 30 seconds denaturation 95^0C, 40 seconds annealing at 52^0C, 30 seconds extension at 72^0C, followed by final extension of 3 minutes at 72^0C and for TEM 95^0C for 3 minutes initial denaturation followed by 30 cycles of 30 seconds denaturation 95^0C, 40 seconds annealing at 58^0C, 30 seconds extension at 72^0C and a 3 minutes final extension at 72^0C [16].

Agarose gel electrophoresis was carried out for the visualization of the base pairs.The PCR products were analyzed in 1% agarose gel containing 25 µg of ethidium bromide in tris-EDTA buffer. The gel was visualized on a UV transilluminator for band detection using gel documentation system (Bio-Rad, USA), with 100 bp DNA ladder included in each run [17]

Statistical Analysis

Data obtained in the study was analyzed with the Statistical Package for Social Science (SPSS) version 21. Frequencies were calculated. Chi square test was used to analyze the associations between categorical variables. Analysis of variance was performed to test whether group variance was significant. A p-value of ≤ 0.05 was considered to be statistically significant.

Table 1 Primer sequences and base sizes of SHV, CTX-M and TEM genes

Genes	Primers	Primers sequence (5'-3')	Base size
SHV	SHV-F	CGCCTGTGTATTATCTCCCT	281
	SHV-R	CGAGTAGTCCACCAGATCCT	
CTX-M	CTX-M-F	CGCTTTGCGATGTGCAG	500
	CTX-M-R	ACCGCGATATCGTTGGT	
TEM	TEM-F	TTTCGTGTCGCCCTTATTCC	950
	TEM-R	ATCGTTGTCAGAAGTAAGTTGG	

RESULTS

Plate 1shows the phenotypic confirmation using double disc synergy for ESBL production.

Out of 53 isolates, 30(56.6%) were ESBL phenotypic positive while 23(43.4%) were ESBL phenotypic negative (Fig. 1).

Table 2 shows the frequency of SHV, CTX-M and TEM encoding genes in phenotypic positive ESBL *E. coli*. The CTX-M gene 17(56.7%) was the most prevalent gene followed by TEM 7(23.3%) and SHV 6(20.0%). Statistically significant difference was observed ($\chi^2 = 7.400$, $p \leq 0.05$) in the distributions of the genes among the phenotypic ESBL producing isolates.

The Non-ESBL producer's susceptibility rates ranged from 8.7% to 47.8% while the ESBL-producers were less susceptible with a range of 3.3% to 60.0% (Table 3).

Table 4 shows the association between antibiotic resistance and gene expression in ESBL producing isolates. There was positive relationship between antibiotic resistance and gene expression. All the isolates that produced SHV genes were resistant to Ceftazidime and Ampicloxacillin. Isolates with CTX-M gene were more resistant to third generation Cephalosporins (96.1%). All isolates with TEM genes were resistant to Gentamicin and Ceftazidime. Resistance rate of (90.9%) was observed in third and fourth generation cephalosporins.

Plate 2-4 The agarose gel electrophoresis showing the amplified SHV gene at 281bp, CTX-M gene at 500bp and TEM gene at 950bp. The different Lanes represent the gene bands while Lane L represents the 100bp DNA ladder.

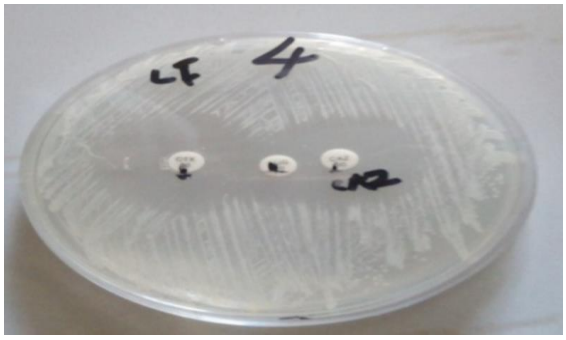

PLATE 1: Positive Double Disc Synergy Test

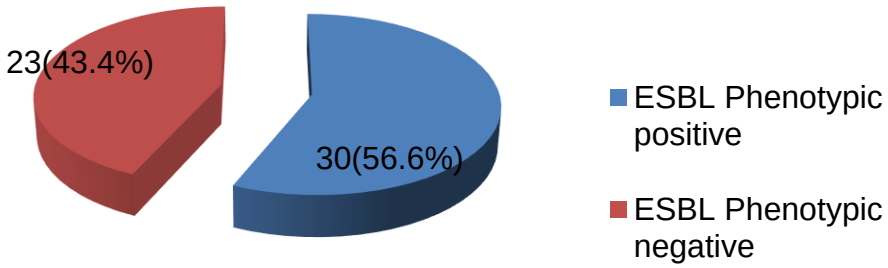

Fig. 1 Distribution of ESBL phenotypic positive *Escherichia coli* isolates

Table 2 Prevalence of TEM, SHV and CTX-M genes in ESBL producing *Escherichia coli*

Gene types	Phenotypic ESBL producer No (%) of isolates	Statistics
SHV	6 (20.3)	$\chi^2 = 7.400$
CTX-M	17(56.7)	p =0.025
TEM	7(23.3)	
Total	30(100)	

CTX = Cefotaximase, TEM = Temoneira, SHV = Sulfhydryl variable

Table 3Antibiotic susceptibility pattern of *E. coli*

Antibiotics	Non-ESBL producers (n=23) No. (%) susceptibility	ESBL producers (n=30)	Chi square	P value
AUG	5(21.7)	5(16.7)	0.641	0.423
CTX	5(21.7)	4(13.3)	2.314	0.128
CAZ	2(8.7)	1(3.3)	3.000	0.083
CRO	9(39.1)	7(23.3)	4.129	0.042*
CXM	8(34.8)	5(16.6)	6.231	0.013*
ACL	4(17.4)	4(13.3)	0.533	0.465
CN	8(34.8)	9(30.0)	0.533	0.465
OFX	11(47.8)	18(60.0)	1.333	0.248
CIP	9(39.1)	9(30.0)	1.174	0.279
LEV	7(30.4)	7(23.3)	0.925	0.336
IMI	6(26.1)	9(30.0)	0.286	0.593

Key : CXM –Cefuroxime, AUG – Amoxicillin+ Clavulanic acid, CAZ-Ceftazidime, CRO – Ceftriaxone, CTX-Cefotaxime, ACL – Ampicolxacillin , CN – Gentamicin, OFX-Ofloxacin , CIP – Ciprofloxacin , LEV – Levofloxacin , IMI - Imipenem

Table 4 Relationship between gene expression and antibiotic resistance of ESBL producing isolates

	Gene Types expressed No.(%) of isolates resistant		
Antibiotic	**SHV (n = 12)**	**CTX-M (n = 26)**	**TEM (n = 11)**
AUG	10(83.3)	24(92.3) *	11(100)
CTX	11(91.9)	25(96.1) *	10(90.9)
CAZ	12(100)	25(96.1) *	11(100)
CRO	8(66	25(96.1) *	10(90.9)
CXM	9(75.0)	25(96.1) *	10(90.9)
ACL	12(100)	23(88.5)	10(90.9)
CN	9(75.0)	23(88.5) *	11(100)
OFX	7(58.3)	17(65.3)	6(54.5)
CIP	8(66.7)	25(96.1) *	9(81.8)
LEV	8(66.7)	24(92.3) *	8(72.7)
IMI	8(66.7)	22(84.6) *	10(90.9)

KEY: *Denotes Significant differences (P≤0.05)
KEY : CXM –Cefuroxime, AUG – Amoxicillin+ Clavulanic acid, CAZ-Ceftazidime, CRO – Ceftriaxone, CTX-Cefotaxime, ACL – Ampicolxacillin , CN – Gentamicin, OFX-Ofloxacin , CIP – Ciprofloxacin , LEV – Levofloxacin , IMI – Imipenem.

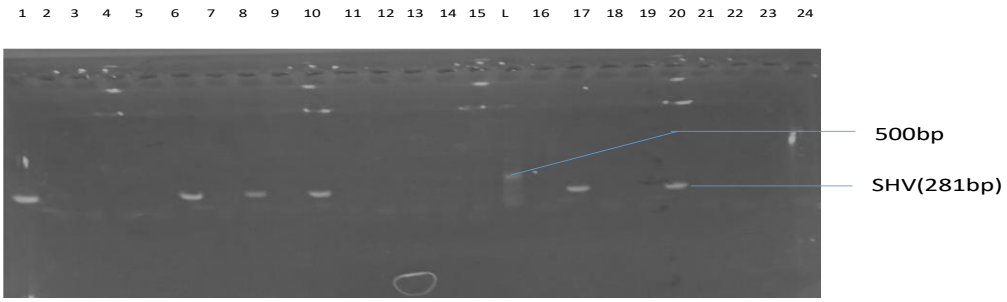

Plate 2: Agarose gel electrophoresis showing the amplified SHV bands at 281bp. Lane 1, 8, 10, 17 and 20 showed SHV gene band. Lane L represents the 100bp DNA ladder

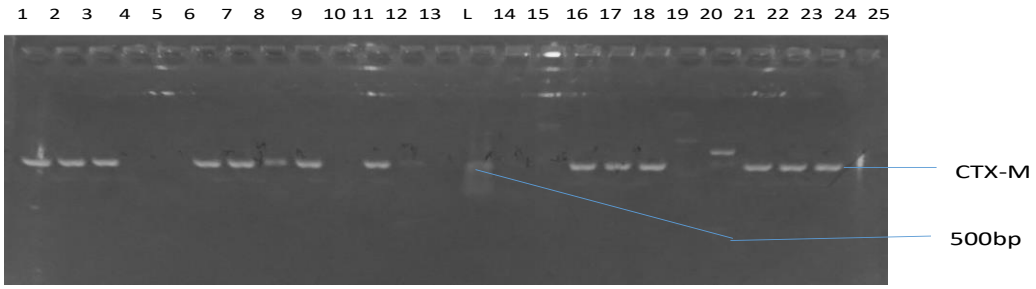

Plate 3: Agarose gel electrophoresis showing the amplified CTX-M bands at 500bp. Lane 1, 2, 3, 6, 7, 8, 9, 11,12, 16, 17, 18, 21,22 and 23 showed CTX-M gene band Lane L represents the 100bp DNA ladder

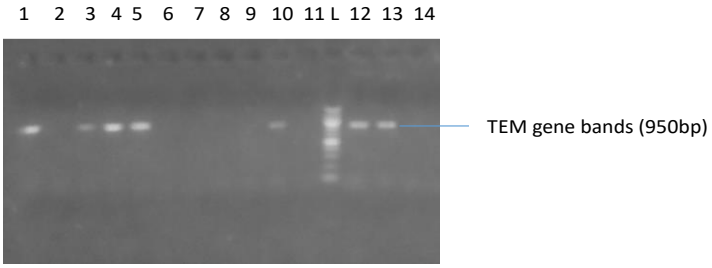

Plate 4: Agarose gel electrophoresis showing the amplified TEM gene. Lanes 1, 3, 4, 5, 10, 12, 13 showing the TEM gene bands while lane L represents the 100bp molecular ladder.

DISCUSSION

All isolates were most susceptible to ofloxacin. There was preponderance of high resistance to aminoglycoside, penicillins and third-generation cephalosporins in both groups. The high prevalence (56.6%) of ESBL producing *E. coli* in this study may be attributed to high antibiotic abuse in the locality. Kaur *et al* [18] attributed the variation in ESBLs prevalence to the complex epidemiology of ESBLs and methods used for ESBL detection.

In this study, CTX-M gene was the most prevalent (53.3%) among the isolates followed by SHV (24.5%) and TEM (22.5%) gene. This report is in agreement with the work of Sid Ahmed *et* al [19] who reported CTX-M as the most prevalent gene (66.1%), followed by SHV gene (53%). The variation in the gene expressions of the ESBL *E coli* isolates may be due to geographical variation in the types of antibiotics mostly abused.

Gene detection by PCR does not necessarily indicate its expression on the isolate. In this study 30(56.6%) *E. coli* were phenotypically positive. The genes that code for antibiotic resistance: CTX-M, SHV and TEM were detected in all the isolates. However, some of the non-ESBL producing isolates also expressed these resistant genes. It is possible that some ESBL negative isolates in this study produced insufficient enzymes and could not be detected by the phenotypic method used, or were masked by the expression of other chromosomal or plasmid-mediated beta-lactamases [20-21].

In this study isolates susceptibility peaked at (54.7%) for ofloxacin followed by (34.0%) for other fluoroquinolones but

showed low susceptibility to the carbapeneme tested (IMI) (28.3%). This is an indication that the carbapenemes which were used as second line therapy ESBL producing *E. coli* are no longer reliable. Most of the isolates were resistant to cephalosporins and ampicloxacillin (84.9%).
The ESBL-producers (56.6.0%) and non-ESBL producers (43.4%) were most susceptible to ofloxacin. This shows that ofloxacin could be used for the treatment of infections caused by ESBL producing *E coli*. Nwakaeze *et al* [22] also reported ofloxacin as an effective antibiotic against ESBL producing *E coli*. On the other hand non-ESBL producers were more susceptible to cephalosporins compared to the ESBL-producers. However, Imipenem a carbapenemase which is used for the treatment of multidrug resistant *E. coli* isolates was less effective against the ESBL-producing organism than some of the quinolones. This shows that the isolates may have developed or acquired resistance to imipenem. In this study, there was significant relationship between antibiotic resistance and gene expression in isolates with ESBL genes ($p \leq 0.05$).

Conclusion: The most common resistance gene in our locality was CTX-M gene. Most phenotypic positive isolates possess the ESBL encoding genes. The genotypic method is the best method for detection of ESBL genes.

Declaration: This research was presented at the Microbiology Society Conference at Northern Ireland in April, 2019 and posted online in their book of abstract.

Conflicts of interest
All authors have disclosed no conflicts of interest.

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Ethics approval
Ethical approval was waived sinced the research did not involve animal or human subjects.

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