Original Research Article

ESBL Mediated Antimicrobial Nonsusceptibility of Uropathogenic *Escherichia coli* and *Klebsiella pneumoniae* Isolates from Pregnant Women in Nnewi, Nigeria.

Abstract

Background and Objective: Extended Spectrum β-lactamase (ESBL) producing UTI is an important public health issue due to lack of therapeutic antibiotic options and the danger it portends to the pregnant woman. This study was carried out to determine the prevalence and response to antimicrobials of ESBL-producing uropathogenic *E. coli* and *K. pneumoniae*, among pregnant women on ante natal care.

Study Design/Materials and Methods: Two hundred and fifteen pregnant women across three different hospitals in Nnewi North L.G.A of Anambra State were screened for these uropathogens. Modified Double Disc Synergy test (MDDST) was carried out on the isolates to phenotypically determine the presence of ESBL. Plasmid profiling as well as plasmid curing studies were undertaken. Molecular characterization of the phenotypically confirmed ESBL positive isolate via PCR was carried out using three ESBL primers (*bla*-TEM, *bla*-SHV, *bla*-CTX-M).

Results: 192 isolates were obtained of which 75(39.1%) were *E. coli* and 117(60.9%) were *K. pneumoniae*. A total of 130 (67.7%) of the pregnant women had ESBL-mediated UTI, the highest rate reported in recent times in Nigeria. Molecular characterization of the ESBL types revealed a predominance of *bla*-TEM (91.9%), followed by *bla*-SHV (73.3%) and *bla*-CTX-M (56.8%).

Conclusion: Majority of the isolates harbored multiple ESBL genes. Curing studies were largely ineffectual as most of the isolates retained their resistance determinants regardless of the concentration of the curing agent (acridine orange).

Key Words: Extended Spectrum Beta Lactamases, plasmid profiling, plasmid curing, polymerase chain reaction (PCR), Modified Double Disc Synergy test (MDDST), urinary tract infection.

Introduction

Extended spectrum ß–lactamases (ESBLs) are plasmid or chromosomally-mediated enzymes capable of hydrolyzing diverse spectra of ß–lactams such as penicillin, third generation cephalosporins (3GCs) and aztreonam but are inhibited by clavulanic acid [1,2]. Various phenotypic methods have been recommended for the routine detection of ESBL in Gram negative bacteria. These usually employ clavulanate (*ß*-lactamase inhibitor) in combination with a 3GC such as ceftriaxone, ceftazidime or cefotaxime. However, this inhibitor-based approach is most effective in isolates that do not co-produce an inhibitor-resistant ß -lactamase like AmpC (which mediates ampicillin resistance). The co-production of AmpC and ESBL makes ESBL detection problematic for microbiologists as ESBL detection can be camouflaged by high-level production of AmpC [3,4]. Cefepime (a fourth-generation cephalosporin) is a more suitable agent for detecting ESBLs even in the presence of AmpC ß-lactamases because it is minutely affected by production of high-level AmpC [5].

ESBL-producing urinary tract infections have emerged as an important cause of resistance among Gram negative bacteria. There have been worldwide reports [6,7,8,9] which are steadily increasing in community settings in Nigeria [10,11]. Among the ESBLs, CTX-M enzymes have replaced TEM and SHV mutants as the most prevalent ESBLs worldwide, with *E. coli* as the major host [12]. The resistance rate of ESBL-producing pathogens to 3GCs is a broad marker of the prevalence of ESBLs.

The prevalence of ESBL-producing uropathogens in pregnant women is an alarming trend as in addition to hydrolyzing most ß-lactams, bacteria harboring these ESBLs exhibit resistance to other unrelated antimicrobial agents, leading to recurrent UTI infection (as a result of antibiotic treatment failure). This is particularly dangerous in pregnant women where up to 50% of those with asymptomatic bacteriuria eventually develop pyelonephritis, thereby experiencing higher incidences of intrauterine growth restriction and low birth weight infants [13]. The occurrence of UTI has been reported to promote the risk of preterm labor, preterm birth, abortion, caesarean deliveries, pregnancy induced hypertension, preeclampsia, amnionitis and anemia [14].

At a wider geographic scale, the incidence of ESBL-producing organisms is quite tricky to monitor due to difficulties in detecting ESBL production (as a result of AmpC co-production) [15], inconsistencies in reporting clinical findings, and a dearth of epidemiological surveillance on the prevalence of ESBL among uropathogens. It is hoped that this research study will represent a true epidemiological picture on the prevalence of these ESBL producing organisms among the study population.

Materials and Methods

Sample collection, Isolation, Identification and Susceptibility tests

Obioma Specialist Hospital, Nnamdi Azikiwe University Teaching Hospital and Nnewi Diocesan hospital were chosen for this study because of their strategic location and patient population. Two hundred and fifteen urine samples were obtained from pregnant women on ante natal care. Appropriate microbiological protocols were followed in sample collection and culturing. Isolates were identified as *Escherichia coli* and *Klebsiella pneumoniae* by standard biochemical tests and evaluated for antibiotic susceptibility by the Kirby-Bauer method on 4 mm thick Mueller-Hinton agar medium according to the Clinical and Laboratory Standards Institute (CLSI) [16].

Pre-screening for potential ESBL producers

Isolates exhibiting zones of inhibition ≤25mm for Ceftriaxone, and ≤22mm for Ceftazidime on antibiotic susceptibility plates were considered potential ESBL producers as recommended by the CLSI and further tested by confirmatory methods [16].

Modified Double Disc Synergy Test (MDDST)

All the potential ESBL producers were subjected to the confirmatory procedure of MDDST on Mueller-Hinton-agar plates [15] using a disc of amoxicillin-clavulanate (20/10μg) together with four cephalosporins; 3GC- cefotaxime, ceftriaxone, ceftazidime and 4GC-cefepime (Oxoid, UK). A lawn culture of the test isolate was made on a Mueller-Hinton agar plate using sterile swab sticks as recommended by CLSI, [16]. The disc containing amoxicillin-clavulanate (20/10 μg) was placed in the centre of the plate while the 3GC and 4GC discs were placed 15 mm and 20 mm apart respectively, centre to centre to that of the amoxicillin-clavulanate disc. Increase in the zone of the 3GCs and 4GCs towards the amoxicillin-clavulanate disc was considered positive for ESBL production. This method also served for detection of AmpC phenotype. A potentiation greater than 5 mm of the cefepime disc towards the amoxicillin-clavulanate disc signified AmpC production.

Plasmid Profiling

Selected confirmed ESBL producers were analysed for the presence of plasmid DNA using the alkaline lysis method 'TENS' (Tris 25 mM, EDTA 10 mM, 0.1 N NaOH and 0.5% SDS) of [17] and [18]. The extracted plasmid DNA was separated on 0.8% w/v agarose gel in a 20-40μl of TE (Tris-EDTA) buffer with a 100 bp ladder as standard. The electrophoretic products were viewed with ultraviolet trans-illuminator.

Plasmid Curing

Plasmid curing was carried out using acridine orange concentrations of 0.15, 0.35, 0.55, 0.75 and 0.95 µg/ml in sterile Mueller-Hinton broth. The different dilutions were added to different 24h broth cultures and incubated at 37^0C overnight [19]. Resultant cells were tested for loss of ESBLs by MDDST.

Genomic DNA extraction

The genomic DNA was isolated by the boiling method [17]. Briefly, seventy- two hour-broth cultures of the organisms were centrifuged at 12000 rpm for 5 minutes at 4^oC. Harvested cell pellets were washed and about 50µl of nuclease-free water was added to the residual pellet. The resulting solution was subjected to heat shock treatment in a water bath at 99^oC for 10 minutes and then to ice shock treatment for 30 minutes. Subsequently, the solution was centrifuged at 12000 rpms for 10 minutes at 4^oC after which 50 µl of the supernatant was transferred to another Eppendorf tube and stored at 4^oC until further use.

Detection of ESBL Genes Using Polymerase Chain Reaction (PCR)

The plasmid and genomic DNA of all the phenotypically confirmed ESBL positive isolates were analyzed for ESBL genes. To detect the existence of these genes, three primer pairs for screening the *bla*TEM, *bla*SHV, and *bla*CTX-M genes were used in the PCR reaction. The primer sequences are shown in Table 1.

Table 1. Primer sequence of the blaTEM, blaSHV, and blaCTX-M genes [30]

GENE	**PRIMERS (5' – 3')**	**AMPLICON SIZE (bp)**
SHV	F: 5'-GTCAGCGAAAAACACCTTGCC-3'	
	R: 5'-GTCTTATCGGCGATAAACCAG -3'	383bp
TEM	F: 5'-GAGACAATAACCCTGGTAAAT-3'	
	R: 5'-AGAAGTAAGTTGGCAGCAGTG-3'	459bp
CTX-M	F: 5'-GAAGGTCATCAAGAAGGTGCG-3'	
	R: 5'-GCATTGCCACGCTTTTCATAG-3'	560bp

PCR Amplification Reactions and Conditions

The PCR amplification reactions were carried out in a 25 µl volume containing 12.5 µl of the master mix (comprising of 20 mM Tris-HCl at a pH of 8.9, 1.8 mM MgCl2, 22 mM NH_4Cl, 22mMKCl, 0.2 mM dNTPs, 5% glycerol, 0.06% IGEPAL™ CA-630, 0.05% Tween™ 20 and 25

units/ml *Taq* DNA polymerase), 0.5 µl each of the forward and reverse primer under evaluation, 6.5 µl of the nuclease free water and 5 µl of the template DNA; in a DNA thermal cycler. For all three of the genes, initial denaturation was set at 94^{o}C for 3 minutes of 35 cycles while denaturation was set at 94^{o}C for 45 seconds of 35 cycles. For the bla-SHV and bla-CTX-M genes, annealing was set at 60^{o}C for 30 seconds of 35 cycles, while for bla-TEM, annealing was set at 55^{o}C for 30 seconds of 35 cycles. Subsequently, in all of the three genes, extension was set at 72^{o}C for 3 minutes of 35 cycles, while final extension was set at 72^{o}C for 2 minutes of 35 cycles. Positive and negative controls were utilized in the study. The negative controls contained the master mix, forward and reverse primers, and nuclease water without template DNA while the positive controls contained all of the contents of the negative controls in addition to a standard multi-locus sequenced typed ESBL positive isolate.

Detection of Amplified DNA bands

After PCR amplification, 2.5 µl of each reaction was separated by electrophoresis in 1.5% agarose gel for 80 minutes at 90V in 0.5x TBE buffer. The DNA was stained with 5 µl ethidium bromide. Detection was in an ultraviolet transilluminator.

Results

Frequency of Occurrence of E. coli and K. pneumoniae from the Urine of Pregnant Women

Seventy-five (39.1%) *E. coli* isolates and 117(60.9%) *K. pneumoniae* isolates were isolated from the urine of the one hundred and seventy-five pregnant women subjected to the study. Results showed predominance of *K. pneumoniae* (60.9%) over *E. coli* (39.1%) in the urinary tract of pregnant women.

Phenotypic confirmation of ESBL and AmpC production by the Modified Double Disc Synergy Test (MDDST)

All the *E. coli* (n = 75) and *K. pneumoniae* isolates (n = 117) were found to be potential ESBL producers by the preliminary screening test. However, 78.1% (n = 64) of *E. coli* and 76.2% (n = 105) of *K. pneumoniae* were phenotypically confirmed as ESBL producers by MDDST (table 2). A substantial number (80) of ESBL producers were observed to harbor AmpC gene together with ESBL (tables 3 and 4).

Table 2. Distribution of ESBL among *E. coli* and *K. pneumoniae* isolates

S/N	Isolates	No of Isolates Screened	No of Positive ESBLS Producers	% Prevalence
1.	*E. coli*	64	50	78.1
2.	*K. pneumoniae*	105	80	76.2
	Total	**169**	**130**	**76.9**

Table 3. ESBL Distribution among *E. coli* and *K. pneumoniae* isolates

S/N	Isolates	No of Positive Isolates Screened	No of Isolates harboring only ESBLS	% Prevalence
1.	*E. coli*	64	17	26.6
2.	*K. pneumoniae*	105	33	31.4
	Total	**169**	**50**	**29.6**

Table 4. ESBL and AmpC Distribution among *E. coli* and *K. pneumoniae* isolates

S/N	Isolates	No of Positive Isolates Screened	No of Isolates harboring ESBLS +AmpC	% Prevalence
1.	*E. coli*	64	33	51.6
2.	*K. pneumoniae*	105	47	44.8
	Total	**169**	**80**	**47.3**

Refer to figures 1 – 3.

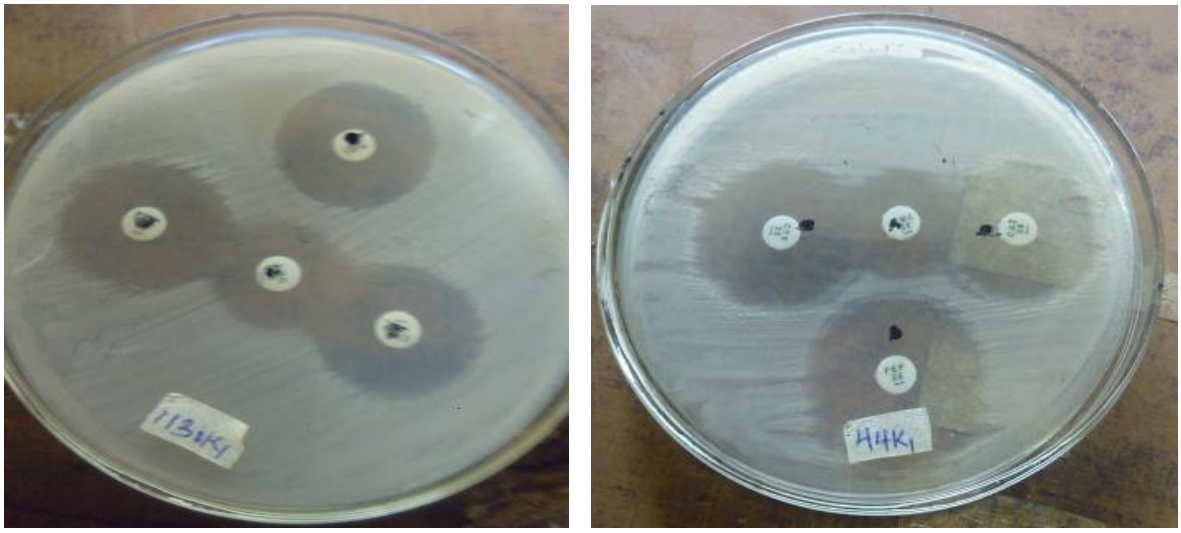


Fig. 1. Plates showing only ESBL (+) organisms

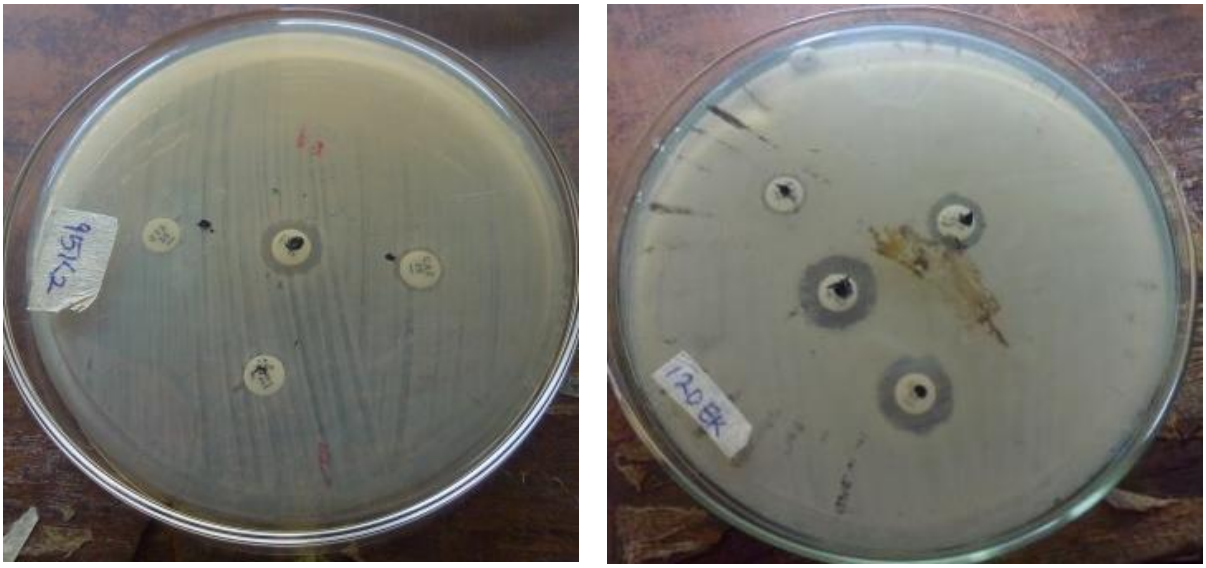
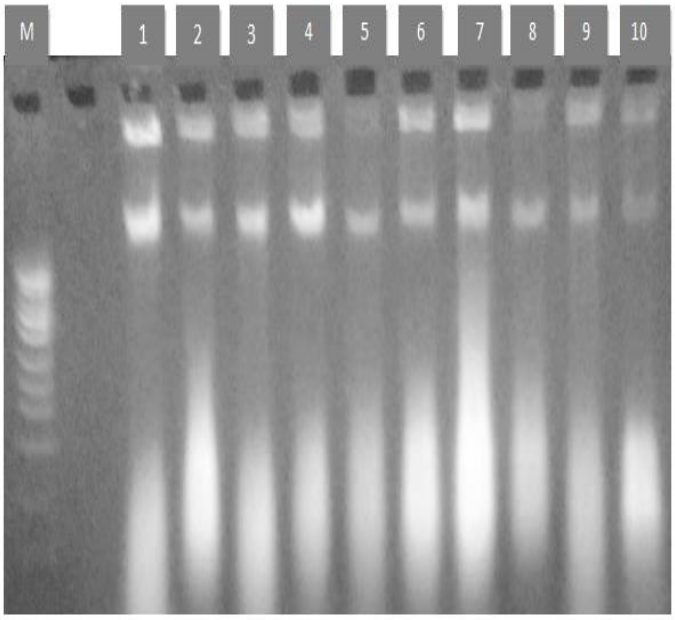


Fig. 2. Plates showing ESBL (-) organisms

Fig. 3. Plates showing ESBL (+) and AmpC (+) organisms

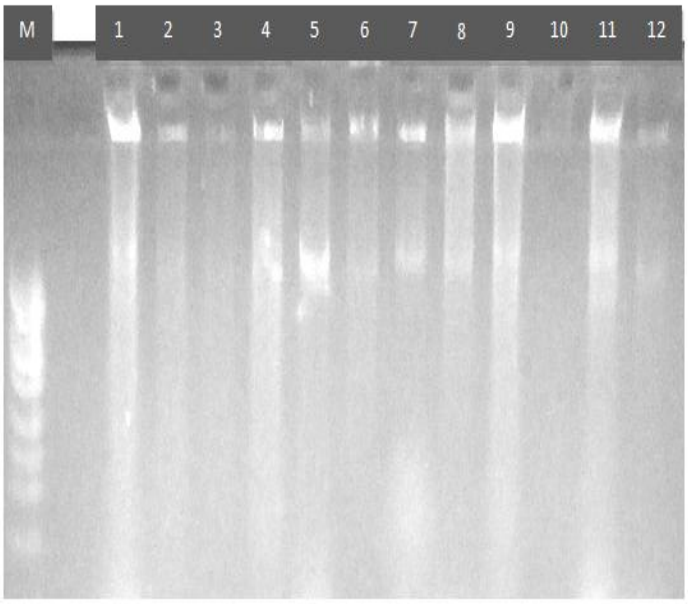
Plasmid profile of ESBL+ Escherichia coli and Klebsiella pneumoniae isolates

The profiles of the plasmid DNA obtained from ESBL-producing *E. coli* and *K. pneumoniae* isolates were analyzed. It was observed that almost all the isolates harbored varying number of plasmids of diverse molecular weights, some considerably higher than the molecular weight standard. This is seen in Figures 4 and 5.



Lanes	Isolate	Mol.Wt (kb)
Standard (M)	1kb DNA marker	1.5-24
1	*K. pneumoniae* 101	25 17
2	*K. pneumoniae* 162	26 19
3	*K. pneumoniae* 63	25 21
4	*K. pneumoniae* 161	25 18
5	*K. pneumoniae* 105	18
6	*K. pneumoniae* 113	25 18
7	*K. pneumoniae* 108	25 18
8	*K. pneumoniae* 171	18
9	*K. pneumoniae* 47	25 19
10	*K. pneumoniae*127	26 19

Fig. 4. Plasmid profile of ESBL + *K. pneumoniae* isolates



Lanes	Isolate	Mol. Wt. (kb)
Standard (M)	1kb DNA marker	1.5-24
1	*E. coli* 128	35
2	*E. coli* 83	34
3	*E. coli* 58	-
4	*E. coli* 76	32
5	*E. coli* 85	34 14
6	*E. coli* 148	33
7	*E. coli* 159	33 14
8	*E. coli* 164	35 15
9	*E. coli* 93	33
10	*E. coli* 123	-
11	*E. coli* 93	33 15
12	*E. coli* 79	35 13

Fig. 5 Plasmid profile of ESBL + *E. coli* isolates

Table 5 below shows the plasmid curing studies that were done on selected ESBL + isolates

Table 5. Plasmid curing studies on ESBL+ isolates

Bacteria Isolates	Resistance Properties (Before Treatment)	Resistance Properties (After Treatment with varying concentrations of Acridine Orange)				
		0.15	0.35	0.55	0.75	0.95
E. coli 53	ESBL +	ESBL +	ESBL +	ESBL +	ESBL -	ESBL -
K. pneumoniae 108	ESBL +	ESBL +	ESBL +	ESBL +	ESBL +	ESBL +
E. coli 83	ESBL +	ESBL +	ESBL +	ESBL +	ESBL -	ESBL -
K. pneumoniae 66	ESBL +	ESBL +	ESBL +	ESBL +	ESBL +	ESBL +

β-Lactamase Genes among the Phenotypically Confirmed ESBL Producers

The amplification of the plasmid DNA did not yield any result as no band could be seen when viewed using the ultraviolet trans-illuminator (figure 6) suggesting that the genes were not located on the plasmids. However, the genomic DNA amplification revealed clear bands with corresponding amplicon sizes to the positive control strain (Figures 7 – 9).

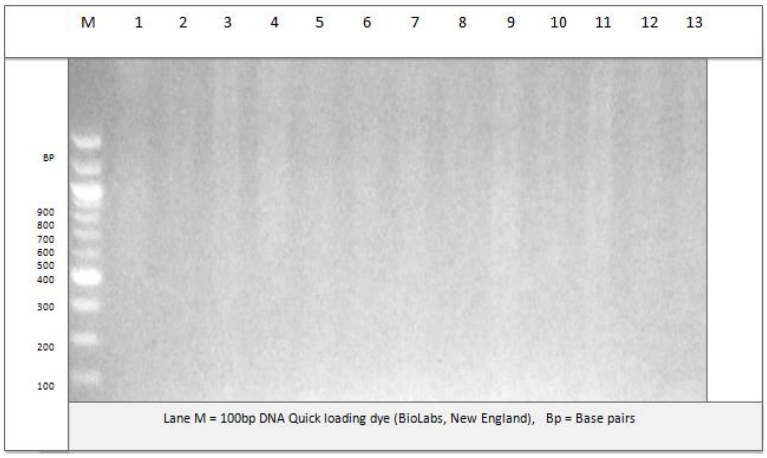


Fig. 6. Agarose gel electrophoresis of plasmid DNA from selected ESBL + Isolates

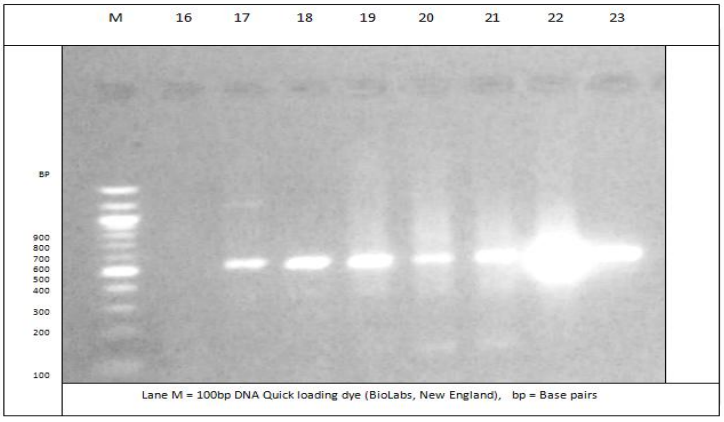


Fig 7. Agarose gel electrophoresis with bands of bla-CTX-M from ESBL+ *Klebsiella pneumoniae* Isolates

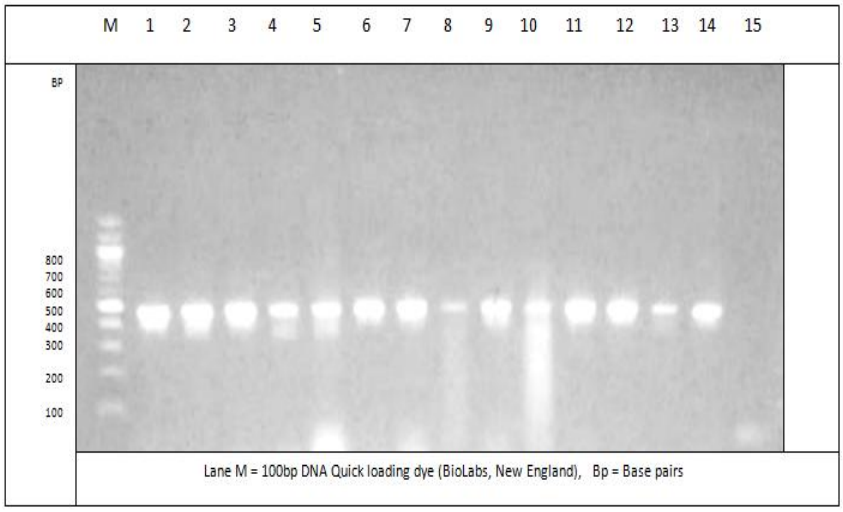


Fig. 8. Agarose gel electrophoresis with bands of bla-TEM from ESBL + *Escherichia coli* isolates

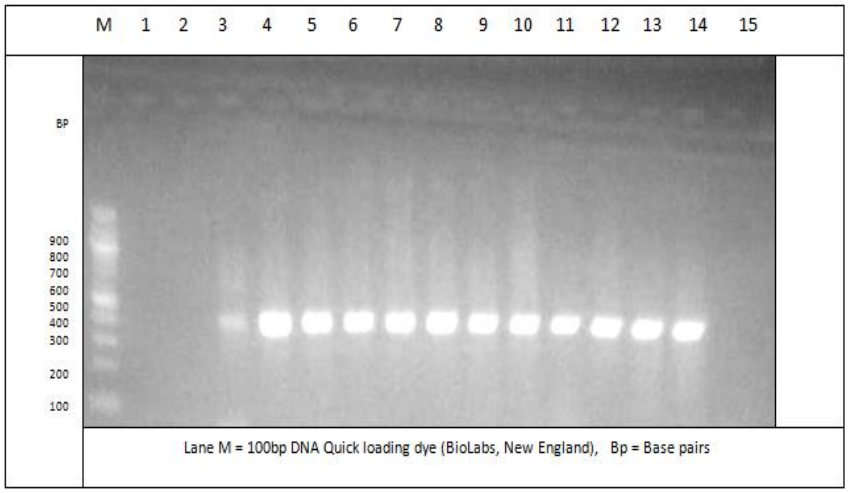


Fig. 9. Agarose gel electrophoresis with bands of bla-SHV from ESBL + *Klebsiella pneumoniae* Isolates

Distribution of ESBL Genes Among Selected Phenotypically Confirmed ESBL Positive Isolates

Among the ESBL genes under study (bla-CTX-M, bla-TEM, and bla-SHV), prevalence of bla-TEM (91.9%) was highest, followed by bla-SHV (73.0%) and bla-CTX-M (56.8%) (Table 6). Multiple occurrences of genes were found in some of the isolates. The coexistence of all three genes was seen in 17 (45.9%) of the isolates while bla-TEM and bla-SHV coexisted in 24 (64.9%) of the isolates, bla-TEM and bla-CTX-M coexisted in 20 (54.1%) of the isolates and bla-SHV and bla-CTX-M coexisted in 18 (48.6%) of the isolates.

It was also observed (Table 6) that among the selected nineteen phenotypically confirmed ESBL positive *Escherichia coli* isolates, subjected to molecular analysis for the presence of bla-CTX-M, bla-TEM, and bla-SHV genes, all three genes were present in 4 (21.1%) of the isolates, bla-

TEM and bla-CTX-M coexisted in 7 (36.8%) of the *E. coli* isolates, bla-TEM and bla-SHV coexisted in 9 (47.4%) of the *E. coli* isolates and the coexistence of bla-SHV and bla-CTX-M was found in 4 (21.1%) of the *E. coli* isolates. The prevalence of bla-TEM, bla-CTX-M, and bla-SHV for *E. coli* was 100%, 58.3% and 47.4% respectively.

Table 6. Distribution of ESBL genes among the bacterial isolates

	ESBL Genes													
Isolates	**TEM**		**SHV**		**CTX-M**		**TEM + SHV**		**TEM + CTX-M**		**CTX-M + SHV**		**TEM + SHV + CTX-M**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Escherichia coli (n = 19)	19	100	9	47.4	7	58.3	9	47.4	7	36.8	4	21.1	4	21.1
Klebsiella pneumoniae (n = 18)	15	83.3	18	100	14	77.8	15	83.3	13	72.2	14	77.8	13	72.2
Total (n = 37)	**34**	**91.9**	**27**	**73.0**	**21**	**56.8**	**24**	**64.9**	**20**	**54.1**	**18**	**48.6**	**17**	**45.9**

Among the selected eighteen phenotypically confirmed ESBL positive *Klebsiella pneumoniae* isolates subjected to molecular analysis for the presence of bla-CTX-M, bla-TEM, and bla-SHV genes, all three genes were present in 13 (72.2%) of the isolates, bla-SHV and bla-CTX-M coexisted in 14 (77.8%) of the *E. coli* isolates, bla-TEM and bla-SHV coexisted in 15 (83.3%) of the *E. coli* isolates. Coexistence of bla-TEM and bla-CTX-M was found in 13 (72.2%) of the *E. coli* isolates. The prevalence of bla-SHV, bla-TEM, and bla CTX-M for *E. coli* was 100%, 83.3% and 77.8% respectively.

Discussion

In the past two decades, *E. coli* and *K. pneumoniae* have become important pathogens associated with both hospital and community acquired ESBL-producing UTIs. In pregnant women, the incidence of ESBL-producing infection is more dangerous as approximately 90% of pregnant women develop ureteral dilation (hydronephrosis of pregnancy) which persists until delivery. About 30% go on to develop pyelonephiritis and cystitis leading to increased mortality and morbidity for mother and child in addition to a host of other syndromes [20].

The prevalence of ESBL producing *Enterobacteriaceae* varies among countries probably due to antimicrobial stewardship programs and infection control practices. In Nigeria, several studies have been carried out on the prevalence of these organisms [10,2,21]. In this study, of the 192 isolates of *E. coli* and *K. pneumoniae,* 130 (67.7%) were phenotypically confirmed as ESBL

producers. This is a high prevalence considering previous works that had been done in other parts of Nigeria. [10] and [21] reported a 26.1% prevalence of ESBLs in South Western Nigeria; [2] reported 13.8% prevalence for ESBLs in South Eastern Nigeria.

Findings from this work are in tandem with global scale reports on the prevalence rates of ESBLs. Independent studies carried out in Southern India by [22,23,24] reported 66.78%, 63.6% and 75% prevalence of ESBLs respectively. In Peru, [25] reported a 40.85% prevalence of community acquired ESBL-producing UTIs.

The high prevalence recorded in our study may be attributable to uncontrolled distribution and usage of antibiotics within the study area [2].

In our study, the incidence of ESBL in *E. coli* was a bit higher (78.1%) compared to *K. pneumoniae* (76.2%). This correlates with studies carried out by [26]. [27] reported a 91.7% prevalence of ESBL-producing *E. coli* over *K. pneumoniae* (8.3%) while [28] reported an 11.2% prevalence of ESBL-producing *K. pneumoniae* over *E. coli* (7.72%). The predominance of *E. coli* could be attributed to its commensal nature in the intestinal tract and its propensity to be easily transferred via faecal carriage to the urinary tract as a result of poor personal hygiene [29].

Results showed that the isolates were not cured of ESBL genes when subjected to varying concentrations of the curing agent (Acridine orange) except two isolates of *E. coli* which became ESBL negative after curing and subjection to the Modified Double Disc Synergy Test (MDDST). These findings corroborate the findings of [2] who reported non-transference of ESBL resistance determinants during conjugations studies. Plasmid curing had no effect on the isolates except *P. aeruginosa* which became susceptible to Ceftazidime following exposure to acridine orange.

We conducted molecular screening studies on the ESBL genes (bla-CTX-M, bla-SHV, and bla-TEM) using polymerase chain reaction (PCR) and it was established that the location of the ESBL resistance determinants were on the chromosomes. Examination of plasmid DNA on agarose gel electrophoresis (AGE) after PCR amplification with the ESBL primers, did not reveal any band. When the chromosomal DNA was examined after PCR amplification with the ESBL primers, the bands were clearly visible.

Majority of the phenotypic ESBL positive strains carried multiple *bla* genes, with TEM-type as the most predominant (91.9%) *bla* gene followed by SHV-type (73.3%) and CTX-M type (56.8%). Majority of *K. pneumoniae* (83.3%) and *E. coli* (100%) harbored TEM type, 100 percent of *K. pneumoniae* and 47.4% of *E. coli* harbored the SHV type while 77.8% of *K.*

pneumoniae and 58.m3% of *E. coli* harbored the CTX-M type. This finding is in sharp contrast with the findings of [30] and [31], who reported *bla*-CTX-M as the most prevalent ESBL type with dominant percentage prevalence of 91.4% and 95.8% respectively. In another study, [32] reported *bla*-TEM as the dominant ESBL type with 72.2% occurrence in *E. coli* and 75% occurrence in *K. pneumoniae.*

Additionally, there was no variation in the number of ESBL-producing organisms detected in either of the phenotypic and genotypic methods employed, thus emphasizing the reliability of the MDDST for clinical detection of ESBLs.

The alarming increase of these ESBL-producing strains (especially among pregnant women) is an ugly trend that urgently needs to be checkmated. A great deal of epidemiological studies is recommended to fully understand the degree of spread of these ESBLs, the causes and major risk factors so as to formulate base line strategies for effectively combating these superbugs.

Conclusion

A high prevalence of ESBL-producing UTI among pregnant women was observed. There are socio and economic burden associated with ESBL-producing urinary tract infections. This calls for health education and personal hygiene among pregnant women. Also necessary is routine screening of their urine to check for these resistance determinants during antenatal clinics whether or not they present with clinical symptoms.

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