Extended Spectrum Beta- Lactamase (ESBL) Resistant Genes in *Escherichia coli* from women with Urinary Tract Infections (UTIs)

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Abstract: Complications in UTIs have increased among women due to the increased ability of *E coli* to produce ESBL, which makes treatment difficult due to multidrug resistance. This research was undertaken to detect ESBL genes in *E .coli* from women with UTI. Bacteria were isolated from urine samples using Eosin Methylene Blue Agar (EMB) and identified using biochemical tests. Antimicrobial resistance was determined using the disk diffusion method with different classes of antibiotics. Isolates were screened for ESBL production using the CLSI protocol and suspected ESBL producers were confirmed using the double disc synergy method. Detection and characterization of ESBL genes were done by single Polymerase chain reaction method. Results showed that 95 (34.17%) women tested positive for UTI, with 51(53.68) being *E .coli* positive and 44 (46. 31%) being other bacteria. Women with fistula had the highest prevalence of *E. coli* [30 (58.8%)] while women with diabetes had the least[2 (3.9%)]. Infection with *E. coli* was statistically significant among women with Fistula, pregnancy, HIV and diabetes (P = 0.0001, 0.002, 0.003 and 0.001 respectively). All the strains of *E. coli* showed high resistance to Beta lactams and other commonly used classes of antibiotics including the Carbapenems. Prevalence of ESBLs was 23.52%, while ESBL genes detected include TEM 8 (66.66%), TEM & CTX 2 (16 .66%). The SHV gene was not detected while 2 of the isolates could not be recovered for the test.

Key words: ESBL, Resistance Genes, *E. coli*, Women, Nigeria

INTRODUCTION

Beta lactam antibiotic which constitutes 60 % of the worldwide antibiotics in use are among the most effective and commonly used agents in the treatment of infectious diseases (Livermore and Wood ford 2006). Extended Spectrum Beta Lactamases (ESBL) are enzymes primarily produced by the enterobacteriaceae and are more predominant in *Escherichia coli, Klebsiella pneumoniae* and *Proteus spp. (*Falagas and Karageogopoulos, 2009). The presence of these enzymes gives limited therapeutic options for treatment, because the plasmids responsible for ESBL production can simultaneously carry multiple resistant genes to other classes of antimicrobials other than the Beta lactams, giving rise to the development of multidrug resistance (MDR) (Nathisuwan *et al.,*2001).

Urinary Tract Infection is one of the most prevalent infections with diverse etiological agents annually affecting 250 million and causes death of 150 million people worldwide (Stamm and Norrby, 2001). About 40-60% of women are said to have at least one UTI during their life time (Micali *et al.,* 2014), 80-90% of which are caused by *E.coli* (Flores-Mirelas *e*t *al*., 2015), making it the number I cause of urinary tract infections worldwide, with so much challenge in treatment due to ESBL production. Therefore, data on specific identification of ESBL will be needed for deciding local therapy options and control strategies, since this may vary according to regions. Such data will serve as a starting point for further studies concerning UTI caused by ESBL producing *E.* coli.

MATERIALS AND METHODS

Study area and Population

The study was carried out at Bingham University Teaching Hospital in Jos north, Plateau State of North-Central Nigeria. Jos is situated on the latitude 9.5^0N and longitude 8.5^0E, and is about 400ft above sea level. The state experience two types of seasons (dry and rainy season) which are modified as a result of its higher altitude. The annual temperature ranges from 50-99^0f, while the annual rainfall also ranges from 40-70 inches.

Bingham University Teaching Hospital is a 250 bed capacity facility located at No. 23 Zaria Bye- pass in Jos north Local Government area. It is a Mission private hospital which was established as ECWA Evangel Hospital in 1959 to provide health care services to missionaries in Nigeria, and also meet the health care needs of the indigenous populace. its mission is to render quality health care, as well as provide training and research. The hospital was later upgraded to a teaching Hospital for the Medical college of Bingham University and offers a range of qualified medical services in the following fields: Surgery, Obstetrics and Gynecology, Pediatrics, clinical and care services for up to 6000 HIV/AIDS cases, and is a centre for Vesico Vaginal Fistula care in the North central zone of Nigeria.

The study participants include all consenting out and inpatient women from the age of 19 years and above, who visited the hospitals (BHUTH) from August 2019 to June 2021. The socio-demographic characteristics and other related risk factors were collected using a structured questionnaire.

Sample size

A total of 278 out and in-patients women who consent to the study from 19 years and above, attending Bingham university teaching hospital were enrolled for this study. The women were tested randomly for urinary tract infections.They were grouped according to the risk factors for UTI already established including fifty women with obstetric fistula,57 Pregnant women,57 HIV infected women, 57 Diabetic women and, 57 women with no risk factor.

Inclusion and Exclusion criteria

All consenting women ≥ 19 years were enrolled while those who were unwilling to volunteer/non-consenting, were excluded from the study

Ethical Approval:

This was applied for and obtained from the Health Research Ethics Committee of Bingham university teaching hospital Jos Plateau state with certificate number NHREC/21/05/2005/00641.

Sample Collection and processing

Ten (10) ml clean- catch mid- stream urine samples were collected from women, using sterile containers. The women were properly educated on how to collect the urine, after which they were given the containers for urine production. Urine samples were properly labelled with the patients details and recorded before processing.

Urine Macroscopy and Microscopy: The color of the urine samples were examined to check if they were clear, cloudy and/or bloody . **The** urine samples were examined microscopically as a wet preparation to detect significant pyuria, i.e. WBC in excess of 10 cells /µl of urine.

Urine Chemical tests using dipstick: dipstick test was carried out on each urine sample, by placing the strip of chemicals in the urine to detect protein, nitrite and leucocytes which may be signs for Urinary Tract Infections.

Bacterial Isolation and Identification

The urine specimens were inoculated on Eosin Methylene Blue (EMB) Medium, with a sterile wire loop, the inoculated plates were incubated overnight (24hrs) at 37^0c. After overnight incubation, the bacterial growths on each culture plate were examined. All positive urine cultures showing significant growth of bacteria with distinct metallic green sheen colonies were subjected to Gram's staining, motility test and biochemical tests including Indole, Methyl red, Citrate and Triple Sugar Iron to confirm *E. coli* as described by Cheesebrough (2006). All media were prepared according to manufacturer's instructions.

Antimicrobial Susceptibility Testing

This was done using the modified Kirby-Bauer disc diffusion method described by Bauer *et al*, (1966) using Muller-Hinton Agar.

A suspension was made from a 24hr growth of the organism in normal saline to match the 0.5 McFarland turbidity standard. Beta lactam antibiotics and representatives of other classes of antibiotics used include; PENICILINNE (e.g Ampicillin, Amoxicillin), CEPHALOSPORINES (Ceftriazone, Ceftazidime, Cefepime), CABAPENEMES (Imipenim, Meropenem), MACROLIDES (Erythromycin), QUINOLONES, (Ciprofloxacin), .NITROFURANTOIN (Nitrofurantoin), all of Oxoid company.

Screening for ESBL Genes

The guideline provided by the National Committee for clinical Laboratory Standard 2002 (NCCLS) was used. All *E.coli* isolates were subjected to ESBL screening tests using Ceftaxidime (30µg) and Ceftriaxone (30µg) discs. Those isolates with Ceftaxidime zone of inhibition ≤22mm and Ceftriaxone zone of inhibition ≤ 25mm were subjected to ESBL confirmatory tests.

ESBL confirmatory test (The double disc synergy method)

The double –disc synergy test method described by the US Clinical and Laboratory Standard Institute (2002), was used to confirm ESBL production. Ceftazidime (30µg) and Ceftriaxone (30µg) discs were placed on either sides of co-amoxiclave (20+10µg respectively) 15mm apart. ESBL positive strains showed an expansion of the zone of inhibition of either Cephalosporin toward the clavulanate giving a dumb bell shape. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as both negative and positive controls respectively.

Preservation of Isolates

Isolated *E. coli* strains were preserved by refrigerating at 4^0C in refrigerator using peptone water in sterile Bijou bottles.

Characterisation of ESBL genes

Plasmid DNA Isolation

Plasmids DNA were isolated using QIA amp DNA minikit (50) Cat. No 51304. Bacterial colonies from 24hrs culture plates were transferred to labeled tubes. Phosphate buffer saline (PBS) was added to each tube, while the tubes were vortexed and centrifuged at 8000 rpm for 30s at room temperature.The supernatant from each tube was then decanted and theprocesses repeated 3 times to wash off unwanted substances from the bacterial cells.

Twenty (20) µl of QIAGEN protease (protinase k) was put into the bottom of 1.5 ml micro centrifuge tubes, to which 200µl of the samples were added. This was followed by addition of 200µl buffer AL to the samples and the whole mixture was thoroughly mixed by pulse vortexing for 15 s. The tubes were then incubated at 56^0 c for 10min.

The microcentrifuge tubes were centrifuged briefly to remove drops from the inside of the lid, and the mixtures were applied to the QIA amp mini spin column in 2ml collection tubes and centrifuged at 8000 rpm for 1 min. The QIA amp mini spin column were then removed and placed in another 2ml clean collection tubes while the tubes containing the filtrate were discarded.

A 500µl buffer AWI was added to each tube and centrifuged at 8000 rpm for 1 min before the QIA amp mini spin columns were removed again and placed in clean 2ml collection tubes, and the tubes containing the filtrate discarded. The QIA amp mini spin column were then carefully opened and 500µl buffer AW2 was added in each tube, closed and centrifuged at full speed (14000 rpm) for 3 min.

The QIA amp mini spin column were again removed from the tubes, placed in clean 1.5ml micro centrifuge tubes and the collection tube containing the filtrate discarded. This was followed by adding 200µl buffer AE and incubated at room temperature for 1min and then centrifuged at 8000rpm for 1min to elute the DNA from the column.

PCR FOR 16Sr RNA

The Primer sequence for 16S rRNA gene is as follows;

F - 5' CCCCCTGGACGAAGACTGAC 3', R - 5' ACCGCTGGCAACAAAGGATA 3'

Procedures

Nuclease free water (8.5µl) was put in tubes and followed by the addition of 12.5µl of 2× green Taq (Takara product), 1.0µl 16s Forward primer, 1.0µl 16s Reversed primer, and 2.0µl of the extracted DNA samples making a total of 25µl mixture in each tube. The DNA amplification was done using single PCR method at the following temperatures for 30 cycles.

Table 1. Temperature conditions for 16S rRNA DNA amplification for 30 cycles

	Temperature (°C)	time
Initial Temperature	95	8min
Denaturation Temperature	95	30sec
Annealing Temperature	58	30sec
Elongation Temperature	72	30 sec
Proofreading Temperature	72	7min
Holding Temperature	4	

16 SrRNA GENE ELECTROPHORESIS

We dissolved 1.5 % agarose powder in 100ml of 1×TBE buffer. The electrophoresis was done at 100v for 35min, with Ethidium bromide used to stain the gene bands, which were viewed using UV, light. The expected band size for 16sr RNA gene is 401bp while 100bp DNA ladder was used for the process.

Table 2. Conditions for PCR Assay of β- lactamase genes

Target gene	Primer sequence	Product size
BLA SHV – R	5' GGTTAGCGTTGCCAGTGCT 3'	888 bp
BLA SHV - F	5'TGGTTATGCGTTATATTCGCC 3'	
BLA TEM -R	5' TTGGTCTGACAGTTACCAATGC 3'	972 bp
BLA TEM – F	5' TCCGTCATGAGACAATAACC 3'	
BLA CTX –M R	5' TGGGTRAARTARGTSACCAGA 3'	593 bp
BLA CTX –M F	5 'ATGTGCACCAGTAARGT 3'	

Procedure

Nuclease free water (5.5µl) was added into tubes, followed by 12.5µl of 2×mm green taq (Takara product). Then 1.0µl forward primers for SHV, TEM and CTX were added into their appropriate tubes, followed by 1.0µl Reverse primers in their corresponding tubes. Then 5.0µl of each DNA isolate was added in each tube, to give a total mixture of 25µl in each tube.

Amplification: The amplifications for the genes SHV, TEM and CTM were done as follows:

Both Initial and Denaturation temperature was 95^0 c at 30sec. The Annealing temperatures were 56^0c and 58^0c at 1 min for TEM /CTX and SHV respectively, while the Elongation and proofreading temperatures were 72^0c at 1 min.& 10min respectively. Holding temperature was 4^0 c. All at 30 cycles.

Gel Electrohporesis

1.5 % agarose powder was dissolved in 100ml of 1×TBE buffer, and the the electrophoresis was done at 100v for 35min while Ethidium bromide was used to stain the gene bands, which was viewed using UV light.

STATISTICAL ANALYSIS

Data generated was analysed using SPSS (version 25).The statistical significance of variables was estimated using Chi-squretest, p- values equal to or less than 0.05 was considered significant.

RESULTS

Table 3 shows the prevalence of UTI among the study population**.** Out of the 278 women screened for UTI, 95 (34.2 %) tested positive. Out of the 95 UTI positive isolates,51 (53.7%) were *E* .coli while 44 (46.3%) were other bacterial species including *Proteus spp, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus saprophyticus, klebsiella strainand Enterococcus faecalis.*

Table 4 shows the prevalence of *E. coli* according to age group of study population. The bacteria was mostly isolated among women of age groups 19 -28 [39 (65%)] with the least isolation of 3 (20), in women of 39-48yrs. *E. coli* was not isolated from age group 59 and above of the study population.

The prevalence of *E. coli* according to risk factors among the study population is presented on Table 5. Out of the five categories of women studied, women with obstetric fistula had the highest prevalence of infection with *E.coli* [30 (58.8%)], while women with diabetes had the least[2 (3.9%)]. Infection with *E.coli* was statistically significant among women with Fistula, pregnancy, HIV and diabetes (P = 0.0001, 0.002, 0.003 and 0.001 respectively), as shown on the table.

The antimicrobial resistance of *E. coli* isolates from women with UTI is shown on figure 1. Isolates were mostly resistant to Ampicillin (98%), while the least resistance was towards Imipenem (22%). Resistance generally varied from 22 % - 98%, with the isolates showing higher resistance to the Penicillins and Macrolides, followed by the Fluoroquinolones and Nitrofurantoin. The Cephalosporins and Carbapenems groups were only moderately resisted by the isolates as shown in the figure.

Table 3. Prevalence of UTI among women attending BHUTH Jos

Bacteria	No. possitive	Prevalence (%)	N=278
E. coli	51	18.4	
Others	44	15.8	
Total	95	34.2	

No.= Number

Table 4. Occurrence of *E. coli* according to age group of women with UTI

Age Group	No. positive for UTI	No. (%) of *E. coli*
19 -28	60	39 (65)
29-38	16	9 (56)
39-48	15	3 (20)
49-58	2	0(0)
59 and above	2	0(0)
Total	95	51 (53.68)

No. = Number, *P*= 0.49

Table 5. Prevalence of *E. coli* in Relation to Risk Factors among Women attending Bingham University Teaching Hospital Jos

Risk Factors	*No. of Women examined*	*No. (%) +ve*	*P-val.*
Fistula	50	30 (31.57)	0.0001
Pregnancy	57	8 (8.42)	0.002
HIV	57	5 (5.26)	0.003
Diabetes	57	2 (2.10)	0.008
Others	57	6 (6.31)	0.001
Total	**278**	**51 (18.35)**	**< 0.05**

No. = Number

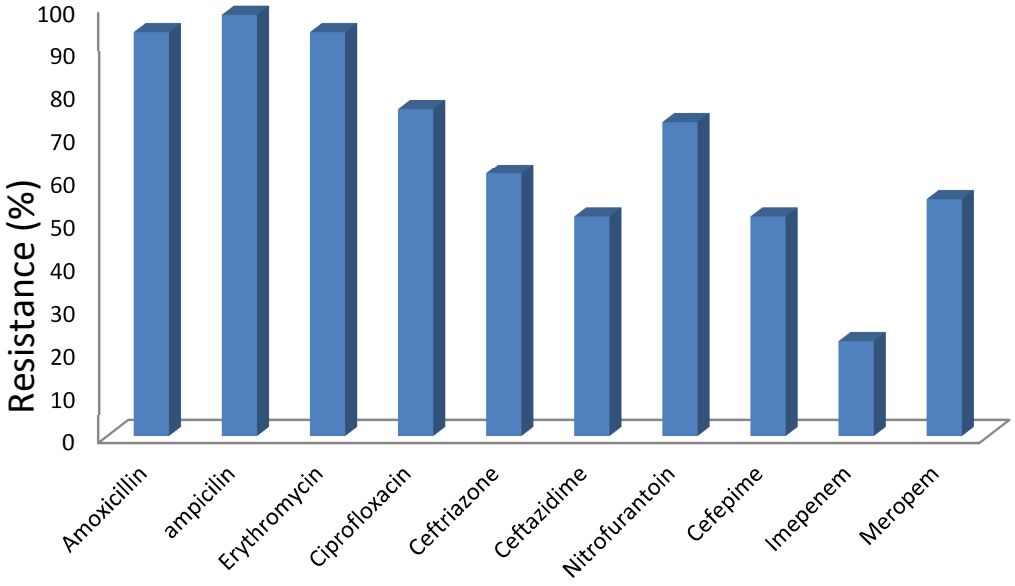

Figure 1: Antimicrobial resistance profile of *E .coli* from women with UTI

Out of the 51 (53.6%) isolates of *E. coli* screened, 21(41.17%) were ESBL producers while 30 (58.82%) were non ESBL producers, but the confirmatory test gave an ESBL prevalence of 23.53% (12/51) among the population ((Table 6). Women with obstetric fistula had the highest [9 (42.8%)] prevalence of ESBL while pregnant women had the least [1 (4.8%)]. No ESBL producing *E. coli* was detected among women with diabetes and HIV.

The most prevalent ESBL gene in the study is the bla-TEM which was detected in 8 (66.6%) isolates,and while 2 of the isolates carried both bla-TEM and bla-CTX genes, the bla-SHV gene was not detected in our study (table 7). Also 2 of the ESBL phenotypically +ve isolates tested negative for the genes.

Table 6. ESBL Production by *E. coli* based on UTI Risk Factor among study population

UTI risk factor	No. of E.coli tested	ESBLs by Screening	ESBLs by confirmation
Fistula women	30	14 (27.4)	9(42.8)
Pregnant women	8	3 (5.9)	1(4.8)
HIV	5	1(2.0)	0(0)
Diabetes	2	0 (0.0)	0(0.0)
Others	6	3(5.9)	2 (9.5)
Total	51	21 (41.2)	12(57.1)

Table 7. Occurrence of ESBL resistant genes on *E. coli* isolated from UTI positive women attending BhUTH

ESBL Resistant genes	Occurrences (%)
TEM	8(66.70)
TEM + CTX	2(16.7)
SHV	0(0)
NO GENE DETECTED	2(16.7)
TOTAL	12

DISCUSSION, CONCLUSION AND RECOMMENDATION

Although the prevalence of UTI was high in our study (34.2%), most of the women have asymptomatic bacteuria as also reported by Bigotte *et al.* (2018), who also stated that the bodies' normal defense mechanisms prevent symptomatic infections in most cases. Our study showed a higher (53 .6 %) prevalence of *E. coli* compared to that of other bacteria (46.3%). This agrees with the study of Okonko *et al.* (2010), who reported *E. coli* as the most common isolated bacteria from urine. This has been attributed to its presence in fecal matters which can easily get to the urinary tract since the urethra is close to the anus.

Women in age group 19 – 28 recorded the highest prevalence of UTI in this study and prevalence decreases with age, but no statistically significant relationship was established between UTI and age group in this study. This is expected due to their high sexual activity and the fact that most of the women with obstetric fistula in this study belong to this age group. Our study found a highly significant relationship between UTI and obstetric fistula. This has been attributed to frequent bladder infections, incontinence of urine and stool which further predisposes them to health related problems like UTI (Hilton, 2003).

The high resistance of *E. coli* in our study agrees with the report by Ali *et al.* (2020), but differ from that of Adenipekun *et al.* (2016) and Aworh *et al.* (2019), who reported lower resistance to 3rd and 4th generation cephalosporins in the South-Western and North-Central part of Nigeria, respectively. The high resistance to Ampicillin recorded in our study is significant owing to the fact that Ampicillin (AMP), a semi-synthetic β-lactam antibiotic, is widely used to treat human and livestock *E. coli* infections, but recently its resistance rate has increased (Li *et al*., 2019). This calls for concerted effort toward modifying antibiotic treatment policies with respect to infections caused by *E.coli* in regions where this has been widely reported.

Similarly, the 22% and 55% resistance to imipenem and Meropenems recorded in our study is worthy of note as the Carbapenems are currently the drug of choice for the treatment of MDR bacteria.

The increasing frequency of ESBL production by *E.coli* has led to Carbapenems usage which may have led to the production and spread of Carbapenemases among Enterobacteriaceae.This signals danger in the effort to combat MDR as a threat to public health, and calls for more studies from different regions to make available data that can inform appropriate decisions.

The prevalence of 23.53% (12/51) of ESBL +ve isolates recorded in our study is similar to the 5-44 % prevalences previously reported from several studies by Olowe and Aboderin (2010), Akujobi and Ewuru, (2010), Mohammed *et al.* (2016) and Ogefere *et al.* (2015) in Ogun, Nnewi, Maiduguri and Zaria respectively. Persistent exposure of bacterial strains to a multitude of β-lactams may have induced a selective pressure in favour of the resistant strains having eliminated the sensitive strains in the process. This could expand the activity of the resistant strains, even against newly developed β-lactam antibiotics.

Our study corroborated the report by Ali *et al.* (2020) and Mohammed *et al.* (2016), who reported that ESBL genes were prevalent in Jos and Maiduguri respectively. The predominance of the TEM gene over the CTX gene earlier reported by them was also confirmed in our study, just as both studies could not detect the SHV gene among the populations examined. The high occurrence of TEM gene, and absence of SHV gene in our study is contrary to reports from other parts of the globe where CTX-M gene have high prevalence. 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 (Šeputienė*et al* 2010, Livermore *et al* 2007). It is possible that the changing pattern of CTX-M gene observed in other parts of the world is yet to catch up with us in Nigeria.

The absence of ESBL genes in 2 of the positive isolates suggests that other factors such as presence of genes other than TEM, SHV and CTX-M could be responsible for the resistance. Our study also observed that there were multiple occurrences of genes in some of the isolates. This finding is similar to a study by Goyal *et al.* (2009) where majority of the ESBL strains harboured 2 or more ESBL genes. The clinical significance of this finding is that patients having organisms possessing this multiple genes are more likely to have multi drug resistance and more likely to have the tendency for widespread transmission.

We recommend futher studies to look at other parts of the state and region for these and other ESBL resistant genes in other to confirm and update existing data. Also, further work to detect Carbapenemeses-producing strains should be done so as to predict and forestall possible emergence of public health disaster as resistance to the Carbapenamases was demonstrated by some of the *E. coli* strains in our study.

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