ORIGINAL ARTICLE

GENOTYPING OF UROPATHOGENIC ESCHERICHIA COLI IN OFFA, KWARA STATE

Olatunji Matthew Kolawole[1]* & Kolawole Muftau Usman[2]

[1]Department of Microbiology, Infectious Diseases and Environmental Health Research Group, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria.

[2]Department of Science Laboratory Technology, Federal Polytechnic, Offa, Nigeria

ABSTRACT

Background: There has been an increase in the occurrence of infections due to Extended Spectrum Beta lactamases (ESBL) producing bacteria. ESBLs exhibit an enhanced capacity to hydrolyze the extended spectrum Beta-lactams, which has led to an increase in the antibiotic resistance capability of uropathogenic microorganisms. This study was aimed at determining the production of beta lactamase and extraction of beta lactamase genes from urinary tract infection due to *Escherichia coli*.

Methods: Plasmid curing was carried out using sub inhibitory concentration of 0.10 mg/ml of acridine orange to determine the location (plasmid-borne or chromosomal) of the drug resistance marker(s). Beta-lactamase test was performed using the Starch Paper Method, while DNA extraction, genomic gene analysis and polymerase chain reaction were done to determine the presence and analysis of beta lactamase genes.

Results: Ninety-eight (98) *Escherichia coli* isolates analyzed, thirty-one (31) were plasmid mediated and of this, Sixteen (16) was resistant to amoxillin, six (6) to augmentin, three (3) to nitrofurantoin and six (6) to tetracycline. Results further revealed that out of the *E. coli* isolates that were plasmid mediated only nine (9) were beta lactamase producers. None of the ESBL producing *E. coli* contained SHV beta-lactamase genes. However, three (3) and five (5) strains of ESBL producing *E. coli* contained TEM and CTX-M beta-lactamase genes respectively.

Conclusion: This study shows that the resistant of urinary tract infection (UTI) isolates to beta-lactams were due to production of TEM and CTX-M beta-lactamases. Identification of these genes provides for accurate treatment and further understanding of the mechanism of resistance.

KEY WORDS: Extended Spectrum Beta-lactamase, *Escherichia coli*, Antibiotics, Plasmid mediated.

INTRODUCTION

Urinary Tract Infections (UTIs) are regarded as one of the most common bacterial infections, which affect individuals across varying age groups[1].UTIs are associated with different clinical conditions, including; urethritis, cystitis, and acute pyelonephritis, with their infectious agents having the ability to invade tissues of the urinary tract [2].

Over the years, there has been an increase in the occurrence of infections due to Extended Spectrum Beta Lactamase producing bacteria[3]. This upsurge in antibiotic resistance especially to new generation cephalosporin has been largely associated to the acquisition of ESBL enzymes among Enterobacteriaceae[4,5]. Gholipour et al.2014 [6] referred to ESBLs as a group of enzymes with the ability to hydrolyze 3rd and 4th generation cephalosporin's and monobactams with the exception of carbapenems.

Extended spectrum β-lactamases (ESBLs) exhibit an enhanced capacity to hydrolyze the expanded-spectrum β-lactams. The rapid spread of ESBLs caused significant threat to the therapy for infections and usage of the expanded spectrum β-lactam. They are undergoing rapid and continuous mutation[4]thus, the task of scientists to recognize susceptibility pattern indicative of the presence of specific β-lactams, will become even more important, as the genus acquires additional antimicrobial resistance mechanisms[7].

Resistance to expanded spectrum cephalosporin

Corresponding Author: Olatunji Matthew Kolawole*

frequently materializes in Enterobacter species due to a mutation in an adenosine monophosphate deaminase (ampD) gene, which under normal circumstances would check high-level expression of this organism's chromosomal β-lactamase[8]. In addition, resistance to cephalosporins has arisen in Escherichia coli (*E. coli*) via the acquisition of plasmid containing the chromosomally encoded AmpC found in Enterobacter spp, Pseudomonas aeruginosa and Citrobacter spp.[9].

The purpose of this study is to determine the production of Extended spectrum β-lactamases (ESBLs) and to extract the beta lactamase genes from UTI Escherichia isolates, since there is little or no published data on it in Offa, Kwara State, Nigeria.

METHODS

Population of the study
Five hundred patients attending Offa Specialist Hospital's Medical Laboratory for urinalysis (not clinically diagnosed as having UTI) over an eight month period formed the population of this study.

Ethical Approval
Ethical approval was received from the Ethical Review Committee (ERC) of Offa Specialist Hospital, Offa, Kwara State, after all requirements had been met. An informed consent was also obtained from the subjects/guardian.

Collection of sample
Wide mounted plastic containers with screw cap tops (universal containers) were used to collect the mid-stream urine samples. The samples were analyzed and identified by conventional bacteriological tests[10].

Plasmid Curing
Plasmid curing was done to ascertain the location (plasmid-borne or chromosomal) of the drug resistance marker(s). Plasmid curing of the *E. coli* isolates was carried out using sub-inhibitory concentration of 0.10 mg/ml of acridine orange as described by Sheikh et al.[11, 12] with slight modification. Resistant isolates from antimicrobial susceptibility test were grown for 24 hours at 37°C in nutrient broth containing 0.10 mg/ml acridine orange. The content of the broth was homogenized after 24 hours through agitation with a loopful taken and sub-cultured on Mueller Hinton Agar (MHA) plates and antibiotic sensitivity testing was carried out. Plasmid mediated resistance was indicated when there was a zone of inhibition on the MHA plates while absence of any zone of inhibition on the MHA plates was suggestive of chromosome mediated resistance.

Test for Beta-Lactamase Production
Beta-lactamase test was carried out using the Starch Paper Method (SPM) described by Odugbemi et al.[13] Strips of starch paper about 4 – 6cm was cut and sterilized using 70% ethanol, after which the strips were soaked for about 10 min in phenoxymethyl penicillin tablet dissolved in phosphate buffer. The cut strips were spread evenly on Petri dishes and about 18 – 24 hrs old cultures grown on Nutrient Agar were inoculated on the surface of the test starch paper and spread over an area of 2 -3mm. The Petri dishes were incubated at 37°C for 30 minutes after which the plates were flooded and drained off immediately using Gram's iodine. The starch paper turned uniformly black within 30seconds of application. Colonies with decolorized zones were positive for beta-lactamase but colonies with black background showed beta-lactamase negativity.

Extraction of DNA from ESBL producing *E. coli* isolates
DNA was extracted from β-lactamase producing isolates of *Escherichia coli* by a standard Cetyltrimethyllammonium Bromide (CTAB) genomic DNA isolation method[14] as follows: 1ml of 24 hour broth culture was transferred into 1.5ml Eppendorf tube and spun at 14,000rpm for 30 mins (to harvest the cell). 400µl of a pre-warmed CTAB buffer (at 60°C) containing Proteinase k and β-mercapto ethanol was added. Then 75µl of 10% SDS (Sodium Deodycyl Sulphate) was added and heated in water bath at 65°C for 30 mins. 500µl chloroform was added and mixed for 15minutes (to purify the DNA) spun at 10,000 rpm for 10mins. The supernatant was collected in eppendorf tube to which 500µl isopropanol and 1µl (100mg/ml) RNase were added and incubated for 30 min at 37°C. The resultant mixture was kept at -20°C for 24 hours, spun at 10,000rpm for 10 mins. The supernatant was gently decanted and the pellet was washed with 200µl of 70% ethanol, gently mixed and spun at 10,000rpm for 5mins. The extracted DNA was air dried for 30 mins to 1 hour (to eliminate all traces of alcohol) and finally re-suspended in 200µl of sterile distilled water.

Genomic DNA Analysis
The DNA products were analyzed by agarose gel electrophoresis through 1% agarose gel. Accurately weighed 1g of agarose powder dissolved in 100ml of Tris-Acetate EDTA (TAE) melted in a microwave (AKAI model MW-MB 0930DP) and allowed to cool to 45°C. Then 20µl/5ml of ethidium bromide was carefully added inside the fume cupboard and gently poured into gel caster inside which chrome was already placed and allowed to solidify and placed inside electrophoresis machine in re-circulating TAE buffer. The DNA mixed with loading dye was loaded into gel wells along with Marker (A High Ranger 1kb DNA ladder) and negative control (NC) of water in place of template DNA, run was at 250V, current 300mA for 1 hour and visualized by UV trans-illumination.

Determination of Beta-lactamase Genes

Table 1: Forward and Reverse Primers Used in the Determination of Beta Lactamase Genes

Primer	Forward	Reverse
SHV	5^1- CTT TAC TCG CTT TAT CG-3^1	5^1- TCC CGC AGA TAA ATC ACC A-3^1
TEM	5^1- CCA ATG CTT AAT CAG TGA GC- 3^1	5^1- ATG AGT ATT CAA CAT TTC CG-3^1
CTX	5^1- ATG TGC AGY ACC AGT AAR GT- 3^1	5^1- TGG GTR AAR TAR GTS ACC AGA-3^1

The Polymerase Chain Reaction (PCR) was performed with a final volume 10µl in 0.5ml Eppendorf tube. Each reaction contained 5µl 2x master mixture, 1µl primer F (forward), 1µl primer R (reverse), 1.5µl of germ-free distilled water and 1.5µl of DNA. The mixture was vortex (using vortex mixer SA3) and placed in PCR thermal cycler. The PCR program for Sulfhydryl-variable (SHV) primer consisted of an initiation step at 94°C for 5 mins followed by initial denaturation step at 94°C for 1 min, primer annealing at 39.5°C for 30s, elongation step at 72°C for 1 min. After the last cycle, a final elongation step at 72°C for 4 minutes. The PCR program for Triethylene Melamine (TEM) 42.9C primer consisted of an initiation step at 94°C for 5 mins followed by initial denaturation step at 94°C 1 min, primer annealing at 39.5°C 30s, elongation step at 72°C for 1 min. after the last cycle, a final elongation step at 72°C for 4 minutes and the PCR program for Cefotaxime (CTX-M) primer consisted of an initiation step at 94°C for 5 mins followed by initial denaturation step at 94°C for 1 min, primer annealing at 49°C for 30s, elongation step at 72°C for 1 min. after the last cycle, a final elongation step at 72°C for 4 minutes was added. 10µl aliquots of PCR products were analyzed by gel electrophoresis with 1% agarose. Gels were stained with 20µl/5ml of ethidium bromide and visualized by transillumination. A High Ranger 1kb DNA ladder was used as marker for CTX-M and SHV amplicons while PCR Sizer 100bk DNA Ladder was used as marker for TEM. Negative controls (NCs) were PCR mixtures with addition of DNAse free water in place of template DNA.

RESULTS

Production of β-lactamase by *E. coli* isolates

The plates showed that isolates; Ec_3, Ec_7, Ec_8, Ec_{55}, Ec_{56}, Ec_{83}, Ec_{84}, Ec_{92} and Ec_{93} demonstrated the production of ESBL by leaving clear zone on acridine orange impregnated starch paper strip following the addition of crystal dye while Ec_1, Ec_2, Ec_4, Ec_5, Ec_6, Ec_{10} and other isolates demonstrated no production of β-lactamase by having blue black background on acridine orange impregnated starch paper strip following the addition of crystal dye (Figure 1 and 2).

Extraction of DNA from β-lactamase producing E. coli isolates

The result of extraction of DNA from β-lactamase producing E. coli isolates in Figure 3 showed that the genomic DNA of *Escherichia coli* isolates: Ec_3 Ec_7 Ec_8 Ec_{55} Ec_{56} Ec_{83} Ec_{84} Ec_{92} and Ec_{93}, this confirmed the concentration of *E. coli* DNA in the isolates.

PCR Amplification of Isolates' DNA with β-lactamase Primers

Results showed that for the amplification of *E. coli* isolates DNA with SHV gene primer none of the tested isolates had SHV beta-lactamase gene. Figure 4 revealed that isolates; Ec55, Ec84 and Ec92 contain TEM beta-lactamase gene, while Figure 5 revealed that isolates; Ec_8, Ec_{55}, Ec_{83}, Ec_{84} and Ec_{92} have CTX-M beta-lactamase gene with about 500bp and concentration of 23ng/10µl

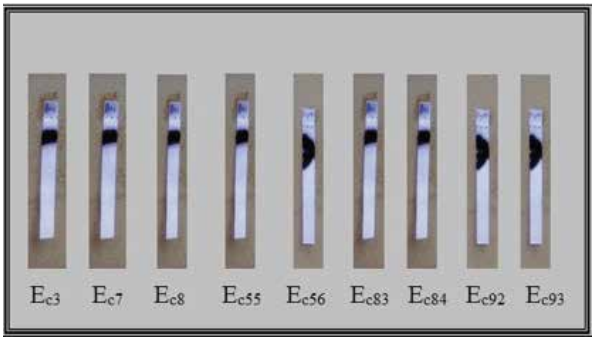

Figure 1: UTI *E. coli* isolates producing β-lactamase enzymes

Escherichia coli isolates showing colorless background on starch paper strip after the addition of crystal iodine.

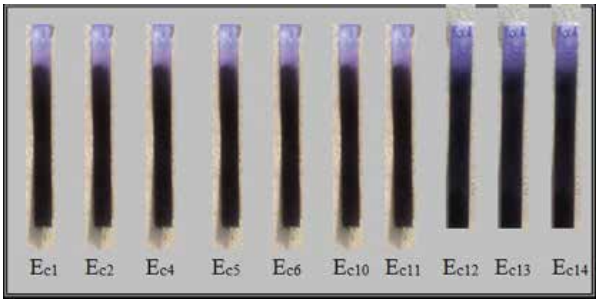

Figure 2: Samples of UTI *E. coli* Isolates not producing β-lactamase enzymes
Escherichia coli isolates showing blue black background on starch paper strip after the addition of crystal iodine.

Figure 3: Genomic DNA of B-lactamase producing *Escherichia coli* isolates.
Lane 1 represents (M) High ranger 1kb DNA Ladder, lanes 2- 10 are genomic DNA of *Escherichia coli* isolates and lane 11 represents negative control (NC).

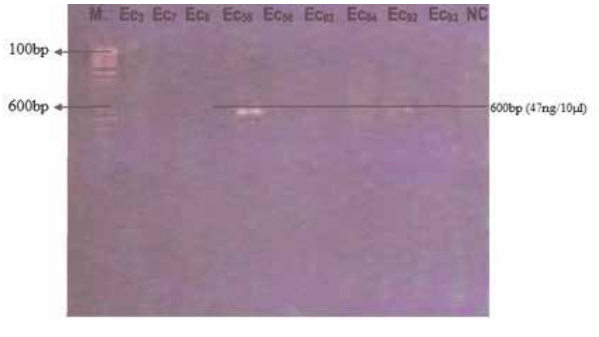

Figure 4: PCR Products' Analysis of *E. coli* DNA with TEM Primer
Lane 1 represents M PCR Sizer 100bp DNA Ladder, lanes 2- 10 are PCR products of DNA of *Escherichia coli* isolates and lane 11 represents negative control (NC). Right hand side: molecular size of amplicons of DNA with TEM genes for Ec_{55}, Ec_{84} and Ec_{93} isolates

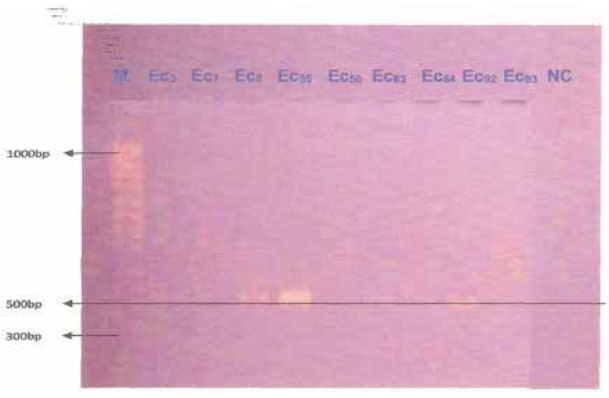

Figure 5: PCR Products' Analysis of E. coli DNA with CTX-M primer
Lane 1 (M) represents High Ranger 1kb DNA Ladder, lanes 2- 10 are PCR product of DNA of *Escherichia coli* isolates and lane 11 represents negative control (NC). Right hand side: size of amplicons of DNA with CTX-M gene for Ec_{8}, Ec_{55}, Ec_{83}, Ec_{84} and Ec_{92}.

DISCUSSION

Drug resistance is one of the natural endless processes by which the organisms develop tolerance to new environmental condition. It may be due to a pre-existing factor in organisms or result from the acquired factor(s) whereby the genes that confer this resistance (environmental resistome), transferred from non-disease causing bacteria to those that do cause disease, leading to clinically significant resistance[15]. Over the past decade, ESBL producing organisms have emerged as serious nosocomial pathogens throughout the world[16]. Outbreak due to this type of pathogen among the most critically ill patients in intensive care units has been reported by Jacoby and Archer[17]. One of the major contributors to the emergence of multidrug resistance in bacteria has been attributed to the inevitable genetic response to misappropriated exposures of microbial populations to antimicrobial therapy[11].

The locations of genes of resistant UTI *E. coli* isolates investigated revealed that 16 strains of *E. coli* (i.e. Ec_{3}, Ec_{4}, Ec_{10}, Ec_{12}, Ec_{18}, Ec_{19}, Ec_{26}, Ec_{37}, Ec_{38}, Ec_{39}, Ec_{44}, Ec_{45}, Ec_{47}, Ec_{92}, Ec_{97} and Ec_{98}), 6 strains of *E. coli* (i.e. Ec_{4}, Ec_{18}, Ec_{19}, Ec_{20}, Ec_{47} and Ec_{85}), 3 strains of *E. coli* (i.e. Ec^{15}, Ec^{23} and Ec^{41}) and 6 strains of *E. coli* (i.e. Ec^{8}, Ec^{17}, Ec^{18}, Ec^{44}, Ec^{59} and Ec^{63}) that were

unaffected by Amoxillin, Augmentin, Nitrofurantoin, and Tetracycline respectively, suddenly became vulnerable to these antibiotics following their treatment using acridine orange. These results confirmed that, the resistance of those strains to the antibiotics in question is plasmid mediated.

Of 31 *E. coli* isolates whose resistant were plasmid mediated, only nine isolates produced beta-lactamase. This might be due to the possibility of plasmid coding for resistance to other antibiotics different from beta-lactam antibiotics. As the isolates without ESBL were very much susceptible to the different antibiotics used, this could be attributed to the lack of mutation that has occurred in the active serine site of ESBL producing organism[18]. Results from this study further showed that all the nine (9) ESBL producing *E. coli* in this study were comparable to reports from other parts of the world, which also revealed multiple drug resistance among gram negative rods[18,19]. These findings support the hypothesis that ESBL producing strains of *E. coli* are more probable to have diminished susceptibility to non β-lactam antibiotics compared with *E. coli* that are not producing ESBL. The finding is also similar to that of Procop et al.[20]. This study also made known a decreased susceptibility of ESBL producing Escherichia coli to the tested antibiotics which may also be as a result of the presence of multidrug resistance gene in plasmids that they are habouring[21]. Therefore, it is important to use the antimicrobial susceptibility profile of the individual isolates to guide treatment.

The global incidence rate of AmpC-mediated resistance is unknown due to the fact that a limited number of studies are focusing on AmpC β-lactamases as well as accurately identifying this resistance mechanism[22]. Thus, reducing the global spread of plasmid-mediated AmpC resistance would largely depend on identification of these genes and arresting their movement among human population.

The SSR (single sequence repeat) PCR technique described in this report is an important tool for the detection of transferable (i.e., plasmid-mediated) AmpC β-lactamase genes in gram-negative bacteria.

The result of this study revealed that none of the selected beta-lactamase producing *E.coli* contained SHV beta-lactamase enzyme. Thus their beta-lactamase gene is different from SHV beta-lactamase since there are several genes coding for beta-lactamase among beta-lactamase producing microorganism. However, isolates; Ec_{55}, Ec_{84} and Ec_{92} confirmed availability of TEM beta-lactamase; a class A member of broad spectrum beta-lactamase in them which conferred on them resistance to many antibiotics except for Ofloxacin and Gentamicin that inhibited the growth of most test bacteria. This finding somewhat agrees with the finding of Albinu[23] in Lagos Nigeria, who reported that ESBL producing microorganisms are multi-drug resistant and the organisms were unaffected by all the antibiotics except Gatifloxacin which was active against them. Nevertheless, ESBL isolates; Ec_3, Ec_7, Ec_8, Ec_{56}, Ec_{84} and Ec_{93} did not display the existence of TEM beta-lactamase. The absence of TEM beta-lactamase suggests the presence of other form of broad spectrum beta-lactamase such as OXA, PER, VEB, GES and IBC beta-lactamases which could as well confer resistance to microorganisms possessing them[24]. This study confirmed possession of CTX-M beta-lactamase enzyme in some of the ESBL producing isolates, such as Ec_8, Ec_{55}, Ec_{83}, and Ec_{92}. It was also established by this study that a microorganism can carry two or more genes coding for resistance against two or more antibiotics as seen in the case of isolates; Ec_{55} and Ec_{84} carrying gene for enzymes TEM and CTX-M at the same time.

Data on the sensitivity of ESBL producing strains showed that these strains are not only resistant to beta lactams but also to other classes of antimicrobials including Gentamicin. The most frequent encountered mechanisms of resistance to beta-lactams found in this study were the productions of TEM and CTX-M beta-lactamases. These results conform to reports of previous studies elsewhere in which most of ESBL producing Enterobacteriaceae reported was *E. coli* and most of them expressed CTX-M enzymes[25, 26]. The occurrence of ESBL producing microorganism in the environment could be to some extent traced to lack of surveillance studies seeking clinical strains producing β-lactamases and the difficulty that laboratories are inaccurately detecting this resistance mechanism.

Reducing the spread of ESBL resistance would largely depend on establishing and using molecular techniques in the isolation and identification of these resistant genes.

CONCLUSION

This study shows the presence of TEM and CTX-M beta-lactamases in *E. coli* isolated from patients with urinary tract infections which could be responsible for their resistance against antibiotics. Thus, identification of these genes among the study population will help to provide more information for better treatment for patients with persistent urinary tract infections.

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