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Distribution Profile of Extended Spectrum Beta Lactamase (ESBL) Producing *Escherichia coli* Isolates from Asa River (Nigeria)

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ABSTRACT: *Escherichia coli* are known pathogenic organism that has caused diseases which has led to severe morbidity and increased death rate. The occurrence of extended spectrum beta Lactamase (*bla*) producing *Escherichia coli* has been on the rise. Water samples were investigated as a potential reservoir for the Extended Spectrum Beta-Lactamase (ESBL) - producing *E. coli* using phenotypic (culture-based) and molecular methods. Double disc synergy test was determined between a disc of amoxicillin-clavulanate (20µg/10µg) (augmentin) and a 30-µg disc of each third-generation cephalosporin antibiotic placed at a distance of 20 mm from centre to centre on a Mueller-Hinton Agar plate streaked with the isolate. An isolate was considered to be ESBL negative if there was no enhancement between any of the cephalosporin and the clavulanate-containing discs and were then subjected to specific Polymerase Chain Reaction (PCR). Eighty-four environmental *E. coli* was isolated. 58(69.04%) showed positivity for ESBL production. *E. coli* isolates positive for ESBL-production selected and subjected to plasmid curing were all plasmid mediated. 16 isolates subjected to PCR to identify the presence of *bla*SHV (Sulphydryl Variable), *bla*TEM (Temoneira) and *bla*CTX-M (Cefotaximase) genes revealed that 11(68.7%) of these had at least one ESBL gene (either *bla*CTX-M or *bla*TEM, or both), 5(31.3%) isolates do not have any of the three ESBL genes, and *bla*SHV was not detected in any of the isolates. The results of this study indicate the widespread prevalence of ESBLs in *E. coli*. Therefore, beta-lactam antibiotics and beta-lactamase inhibitors should be prescribed based on an antibacterial susceptibility test. © JASEM

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The spread of genes coding for extended-spectrum beta-lactamases (ESBLs) among Enterobacteriaceae is alarming globally and is of significant public health concern (NORM, 2010). It is thought that over one billion people are colonized with ESBL-producing Enterobacteriaceae intestinally. The major agents of the propagation of these strains are misuse and over usage of antibiotics in humans and animals and circulation of antibiotic residues and ESBL in the environment (Zhang *et al*., 2015; Canton *et al*., 2012; Tenaillon *et al*., 2010). Patients with infections caused by ESBL are known to suffer from increased incidence of diseases and death rate compared to patients with infections caused by non-ESBL-producing Enterobacteriaceae (Rottier *et al*., 2012).

Extended-spectrum beta-lactamase-producing Enterobacteriaceae can be disseminated in humans through contaminated food or water (Zhang *et al*., 2015) and capable of inactivating extended-spectrum cephalosporin. They can be inhibited by extended spectrum β-lactamase inhibitors, such as clavulanic acid via plasmid-encoded or chromosomonally-encoded β-lactamases. Water environments are considered as one of the major reservoirs for resistance genes (Zhang *et al*., 2015), and possibly play a significant role in transfer of drug-resistant genes between bacteria (Malakoff, 2002; Kummerer, 2004).

Escherichia coli is the most widespread pathogenic organism among Enterobacteriaceae in human diseases and causes serious and common infections such as septicemia and urinary tract infection (UTI) (NORM, 2010). It is also the most commonly occurring ESBL-producing enterobacteriaceae, which are inhabitants of gastrointestinal tract and important pathogens in nosocomial and in the environment (Song *et al*., 2011; Tenaillon *et al*., 2010).

To date in Nigeria, there are numerous studies on ESBL-producing Enterobacteriaceae from clinical isolates. However, information on ESBL-producing Enterobacteriaceae isolated from waters from rivers is very limited. The global occurrence of antibiotic resistance of *E. coli* in water is of increasing concern; this informed the need to assess the impact of pollution on Asa river segments and evaluate the effectiveness of augmentation of clavulanate with Cefotaxime, Ceftazidime, Aztreonam and Ceftriaxone in detecting ESBL production in the *E. coli* isolates. This study aimed at investigating the prevalence of genes coding for ESBL-production in *E. coli* isolated from Asa River in Ilorin, Nigeria.

MATERIALS AND METHODS

Study Area and Water Sampling: Water sampling was carried out at weekly intervals for a period of 8 weeks on Asa River in Ilorin, Nigeria. The study was conducted on 5 different Asa River segments; Amilegbe road, Station road, Unity road, Offa-garage road and Asa dam. The samples were collected into pre-sterilized bottles kept on ice and transported

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immediately to the laboratory for analyses within 2 hours.

Isolation and Identification: Isolates were recovered from the water samples after incubation on Eosin Methylene Blue Agar and MacConkey Agar, for 18–24 hours at 37°C. Presumptive characteristic *E. coli* isolates were identified and confirmed using IMViC tests while potential ESBL producers were identified based on manufacturers' instructions (Oxoid Ltd, England).

Double Disc Synergy Test / DDST: The double disc detection test was done by determining the synergy between a disc of amoxicillin-clavulanate (AMC30) and a 30-µg disc of each third-generation cephalosporin test antibiotic placed at a distance of 20 mm from centre to centre on a Mueller-Hinton Agar (MHA) plate inoculated with the test isolate. Clear extension of the edge of the inhibition zone of cephalosporin toward the amoxicillin-clavulanate disc was interpreted as positive for ESBL production (Bradford *et al*., 1997) while no enhancement between any of the cephalosporins and the clavunate-containing discs was regarded as negative.

Plasmid Curing: The ESBL positive isolates were subjected to acridine orange (Merck) mediated plasmid elimination using the method of Stanisich and were also re-tested for ESBL production using DDST (Iroha *et al*., 2010).

Isolation of genomic DNA of ESBL E. Coli and Detection of resistance gene using PCR method: Pure cultures were grown overnight in Nutrient broth and genomic DNA was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction procedure (Devi *et al.,* 2013).

In order to detect genes encoding Ambler class A beta-lactamases (*bla*), standard PCR was performed with genomic DNA as a template. For amplification of genes encoding SHV beta-lactamases (bla_{SHV}), primers SHV-F (5'GACAGTTACCAATGCTTAATCA3') and SHV-R (5'TCGGGCCGCGTAGGCATGAT 3') were used (Mojtaba and Behnaz, 2012). Reaction conditions used were 94°C for 300 s, followed by 36 cycles of denaturation (94°C; 60 s), annealing (39.5°C; 30 s) and extension (72°C; 120 s), and a final extension at 72°C for 240 s.

For amplification of genes encoding TEM beta-lactamases (bla_{TEM}), primers TEM-F (5'ATAAAATTCTTGAAGACGAAA 3') and TEM-R (5'GACAGTTACCAATGCTTAATCA3') were used with thermal cycling conditions of 94°C for 300 s, followed by 36 cycles of denaturation (94°C; 60 s), annealing (42.9°C; 30s), extension (72°C; 120 s), and a final extension at 72°C for 240 s. In order to detect genes for CTX-M beta-lactamases (bla_{CTX-M}), primers CTX-MU1 (5'TTAATGATGACTCAGAGCATTC 3') and CTX-MU2 (5'GATACCTCGCTCCATTTATTG3') were used and the thermal cycling condition as described above except annealing (49.2°C; 30 s).

The PCR product stained with ethidium bromide was electrophorese in a 1.7% (w/v) agarose gel for 1hr at 140V together with High Ranger 1kb and PCR Sizer 100bp DNA ladder (NORGEN) and visualized under UV illumination.

RESULTS AND DISCUSSION

Eighty- four isolated *E. coli* were selected for phenotypic testing while 58(69.04%) showed positivity for ESBL. Table 1 shows The Percentage Resistance Pattern of the Isolates on the Various Beta Lactam Antibiotic Discs.
The results of the plasmid curing with acridine orange revealed that all the ESBL positive *E. coli* could not express the ESBL enzyme after their incubation with 0.1 mg/ml acridine orange.

Figure 1 shows 16 of the 58 ESBL positive isolates that were subjected to PCR for the presence of *bla*SHV, bla_{TEM} and *bla*CTX-M genes. 11(68.7%) of these had at least one ESBL gene (either *bla*CTX-M or *bla*TEM, or both), 5(31.3%) isolates didn't have any of the three ESBL genes, and *bla*SHV was not detected in any of the isolates (Figures 2-4).

Escherichia coli is a known pathogenic organism that has caused severe nosocomial, urinary tract infection, blood borne disease and gastroenteritis which has led to severe morbidity and increased death rate. Following the wide consumption and constant abuse of the extended spectrum beta lactam agent, the outbreaks of infection caused by extended spectrum β-lactamase producing *E. coli* have been widely reported throughout the world (Branger *et al.,*1998). The production of ESBLs is a major threat to the use of new generation of cephalosporins (Putman *et al*., 2000; Mendes *et al*., 2004). Long hospitalization, diabetes, age over 60 and antibiotic treatment have been reported previously as the risk factors to acquire infections with ESBL strains (Silva *et al*., 2006).

The present results showed that, out of the 84 *E. coli isolates* screened for β- lactamase production using third-generation Cephalosporins (Cefotaxime, Ceftriaxone and Ceftazidime) and monobactam (Aztreonam); 58(69.04%) showed positivity for ESBL production. Previous studies reported that,

primary antimicrobial susceptibility test of *E. coli isolates* observed high resistance to third-generation Cephalosporins such as Cefotaxime, Ceftriaxone and Ceftazidime. Some studies also show resistance to β-lactam antibiotics (especially third-generation Cephalosporins) and non-β- lactam antibiotics, amongst which clinical isolates of gram-negative bacteria are increasing worldwide (Goossens, 2000; Andrew, 2001) and this correlates with the present research.

In this study, Ceftriaxone detected 28.6% of the ESBL producers at a distance of 20 mm. This was followed by Cefotaxime at 26.2%, Ceftazidime at 23.8% and Aztreonam at 21.4% in the presence of Amoxicillin- clavulanic acid. It was observed that this study is in contrast to the work of Babypadmini and Appalara, (2004).

The effect of plasmid curing studies in this study shows that the plasmid resistant maker of positive ESBL isolates was eliminated which means that the ESBL genes of these isolates (*E. coli*) are plasmid-mediated. The percentage of isolates expressing ESBL production is variable in relation to previous reports of ESBL occurrence from Nigeria with respect to the chromosomal and extra-chromosomal location of the resistance determinants. On the other hand, Enabulele and Uraih (2009) reported that, the resistance by gram negative wound isolates was plasmid-mediated. Hence this study correlates with the study of Enabulele and Uraih (2009).

However, in a later study, Yah *et al.,* (2007) and Iroha *et al.,* (2009) reported their findings that the production of ESBL can be chromosomal or plasmid in origin. When these are considered in relation to this present finding they seem to be in contrary. The ESBLs can either be carried on chromosomes or plasmids and plasmid-mediated ESBLs can carry genes that have the ability to transfer a replica of themselves to other bacteria. The transferable plasmids also code for resistant determinants to other antimicrobial agents, hence, multidrug resistance is anticipated to be commonest among ESBL-producing organisms. ESBLs that are chromosomal in origin cannot easily be transferred from organism to organism (Iroha *et al.,* 2009).
The most ESBLs in this study were CTX-type and TEM-type enzymes while failed to detect SHV-type in isolated *E. coli*. 68.8% of these had at least one ESBL gene (either *bla*CTX-M or *bla*TEM, or both), 5(31.3%) isolates didn't have any of the three ESBL genes. The results in this study showed prevalence of CTX-type and SHV-type to be 68.7% and 25.0% respectively.

In a similar report, the most abundant ESBL gene was $bla_{\text{CTX-M}}$, the second-most prevalent gene was bla_{TEM} and the bla_{SHV} gene was not found (Su-Ying *et al.,* 2010). It has also been reported by Iroha *et al*., (2009) that, *bla*SHV gene could not be found in any of their isolates. The above studies correlate with the present finding while Mojtaba and Behnaz, (2011) failed to detect CTX-M type ESBL in the isolated strains which is in contrary to this research. The variation in this study results compared with others about prevalence rate of ESBLs may be due to different reasons such as difference in type and volume of consumption of antibiotics and difference in time which the isolates were collected (Al-Agamy *et al*., 2009).

In epidemiological studies, the $bla_{\text{CTX-M}}$ gene has been widespread in clinical settings globally (Coque *et al.,* 2008), but it has seldom been identified in environmental isolates. Recently, Reinthaler *et al.* reported that the $bla_{\text{CTX-M}}$ gene was dominant in ESBL-producing bacteria isolated from Austrian sewage sludge, with the bla_{TEM} gene as the second-most prevalent type (Reinthaler *et al*., 2010). The present research was in concordance with the above study, but the latter data indicated that the CTX-M-type ESBL- gene had already been prevalent in a river sediment environment.

The $bla_{\text{CTX-M}}$ group is unique among ESBL genes. CTX-M was initially identified in 1989 and its pandemic (CTX-M) has changed the prevalence of ESBLs, and it has rapidly become the dominant ESBL worldwide (Canton and Coque, 2006). Previous reports suggest that the CTX-Ms had several different environmental origins, in contrast to TEM and SHV, with single ancestors (Barlow *et al.,* 2008). CTX-M not only is associated with nosocomial outbreaks but is also mostly found in community-acquired infections. The high degree of diversity of $bla_{\text{CTX-M}}$ genes in this present study, in the river could have two potential origins: the hospital effluent or naturally occurring strains. This result indicates that novel $bla_{\text{CTX-M}}$ genes could be identified from the environment and that the river environment could be a potential reservoir of novel ESBL genes that may pose a potential risk to public health.

Table 1: The Percentage Resistance Pattern of the Isolates on the Various Beta Lactam Antibiotic Discs

Antibiotic disc	Inhibition zone	No of isolates (%)
Ceftriaxone	≤ 25mm	24 (28.60)
Cefotaxime	≤ 27mm	22 (26.20)
Ceftazidime	≤ 22mm	20 (23.80)
Aztreonam	≤ 27mm	18 (21.40)

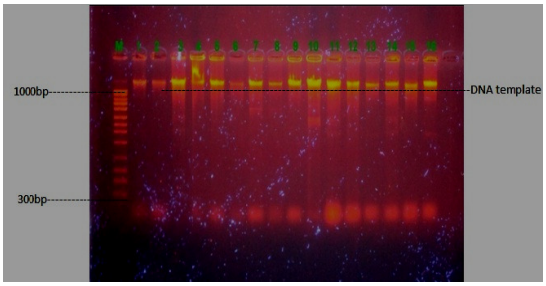

Fig 1: *E.coli* genomic DNA.M:1kb DNA marker; NC: negative control; Lanes 1-16: *E. coli* isolates genomic DNA template.

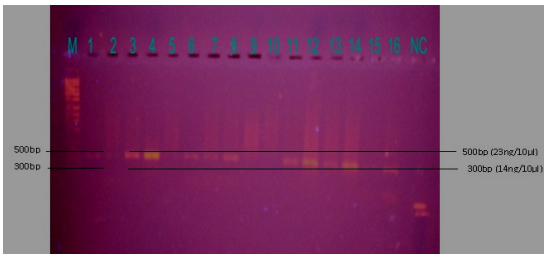

Fig 2: *bla*CTX-M gene PCR amplicon. M: 1kb DNA marker; NC: negative control; lanes 1-16: *E. coli* isolates positive for the *bla*CTX-M gene.

Fig 3: *blaSHV* gene PCR amplicon. M: 100 bp DNA marker; NC: negative control; lanes 1-16: *E. coli* isolates negative for the *blaSHV*gene.

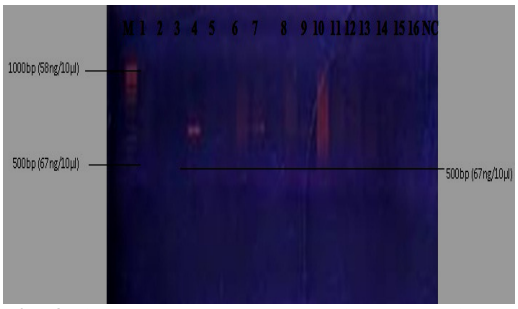

Fig 4: *bla*TEM gene PCR amplicon. M: 1kb DNA marker;
NC: negative control; lanes 1-16: *E. coli* isolates positive for the *blaTEM* gene**.**

*Conclusion***:** There is apparently high prevalence of ESBL producing strains of *E. coli* in present study environment and the enzyme genes are plasmid mediated. The high prevalence of *E. coli* that showed resistance to Beta lactam antibiotics in the studied river in Kwara State call for a serious concern as this river serve as a source of livelihood for the people living in the environment. The outcome of this study has demonstrated the palpable presence of ESBL producing bacteria strains from community within Ilorin metropolis to the best of our knowledge. This kind of study remains relevant towards providing adequate baseline for the future projection and effective management of infectious diseases caused by the ESBL producing bacteria strains.

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