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Characterisation of Extended Spectrum Beta-Lactamase Producing *E. coli* from Secondary and Tertiary Hospitals in South Eastern Nigeria

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Abstract: Clinical isolates of *Escherichia coli* (No. = 109) were collected from Microbiology Laboratory unit of a tertiary hospital (Ebonyi State University teaching hospital, Abakaliki, EBSUTH) and a secondary hospital (Federal Medical Center, Abakaliki, FMC) from four different clinical specimens (urine, stool, blood and sputum) between February to November 2006. Sixty-three clinical isolates of *E. coli* were isolated from EBSUTH while forty-six were from FMC Abakaliki. These organisms were characterized and identified to species level using standard identification technique. Sensitivity studies were carried out on the test organisms using disc diffusion method and later the organisms were characterized phenotypically for ESBL production using the Double Disc Synergy Test (DDST). A preliminary molecular characterization of the ESBL producing isolates were further carried out based on the evaluation of their plasmid profile via agarose gel electrophoresis. The overall result of the study revealed that the prevalence of ESBL producing organisms was high 18 (16.5%) in our environment. The rate of occurrence varied within the two hospitals with 11 (23.9%) from FMC (urine 2 (18.2%), blood 5 (35.7%), wound 3 (30%) semen 1 (33.3%) and non was isolated from sputum while 7(11.1%) were from EBSUTH (urine 2(9.5%), blood 3 (21.4%), wound 2 (18.2%) respectively and non was isolated from sputum and semen. The plasmid profile studies revealed the presence of low molecular weight plasmid DNA within the ranges of 21.3-29.4 kb.

Key words: *Escherichia coli*, ESBL, antibiotic susceptibility, tertiary, secondary hospital

INTRODUCTION

The continuous use of expanded-spectrum cephalosporins in various hospitals for life threatening infections have resulted to the outbreak of resistant organisms that were previously known to be sensitive to these agents (Wong-Beringer *et al.*, 2001). This resistance has spread to *E. coli* and some other gram negative organisms such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Citrobacter* spp., *Serratia* spp., *Salmonella* spp. and *Enterobacter* spp. (Hsueh *et al.*, 2002). This often occurs as a result of mutation that took place in the serine active site of the old forms of β -Lactamase enzyme where there is an addition of 4-6 new amino acids that gave rise to derivatives that possess an extended substrate profile compared to that of parental enzymes, hence the name extended spectrum β -lactamase enzyme (Patterson, 2001). These plasmid mediated enzymes confers resistance to cefotaxime, ceftazidime, ceftriaxone and monobactam such as aztreonam (Nathisuwan *et al.*, 2001). The first isolate, resistant to expanded spectrum cephalosporin was found in Germany in 1983 and

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was found to produce a sulphurhydryl variable (SHV)-type β -Lactamase (Sanders *et al.*, 1988). Subsequently, many ESBLs, predominantly SHV and TEM variants that are plasmid or chromosomally mediated have been reported in clinical isolates of *E. coli* (Bradford, 2001).

The aim of the present study was to detect the prevalence of ESBL producing organisms in clinical isolates of *E. coli* from a secondary and tertiary hospital in Ebonyi State area of South Eastern Nigeria.

MATERIALS AND METHODS

Bacterial Isolates

The 109 clinical isolates of *Escherichia coli* were isolated between February and November 2006 from EBSUTH (n = 63) and FMC (n = 46) Abakaliki, Microbiology laboratory unit, in Ebonyi, South Eastern Nigeria. The strains were isolated from various clinical specimen types, mostly from urine (n = 32 isolates), blood (n = 28 isolates), wound (n = 21 isolates), sputum (n = 17 isolates) and semen (n = 11 isolates). The isolates were identified and characterized using standard technique (Cowan *et al.*, 1983).

Antimicrobial Susceptibility Studies

The susceptibility to antibiotics was carried out on Mueller-Hinton (MH) agar by preparing two hundred and fifty sterile Petri-dishes each containing 20 mL of the prepared MH agar. A 0.1 mL of over-night cultures of *E. coli* diluted to 0.5 Macfarland equivalent standard was seeded into each of the Petri-dishes containing MH agar and was allowed to stand for 1 h to gel. The following antibiotic discs were placed on the surface of the agar plates (ofloxacin 5 μ g, gentamicin 10 μ g, streptomycin 5 μ g, ampicillin 25 μ g, augmentin 30 μ g, cefotaxime 30 μ g ceftazidime 30 μ g, ceftriaxone 30 μ g cefoxitin and imipenem 30 μ g (Oxoid UK) and was incubated for 18-24 h at 37°C, after which the zones of inhibition were taken. *Escherichia coli* ATCC 25723, obtained from the Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, Nigeria, was used as a control strain.

Detection of ESBL Producers

All the isolates that are resistant to ceftazidime, cefotaxime, ceftriaxone and cefoxitin (\leq 14 mm for 30 μ g disc) were tested for the production of ESBL using the Double Disc Synergy Test method (DDST). Thirty eight Petri-dishes of Mueller Hinton agar were prepared with a central amoxicillin-clavulanic acid (20:10 μ g) disc (Oxoid UK) and a disc of third generation cephalosporins (ceftazidime and cefotaxime 30 μ g) placed 15 mm center to center from each other. The test was considered to be positive for ESBL production when the bacteria had a champagne cork appearance and for each stain the test was repeated in triplicate.

Molecular Studies

Extraction of Plasmid DNA

The ESBL-producing isolates of *E. coli* were grown in a 3 mL of double strength Mueller Hinton broth for 72 h at 37°C. The overnight cultures were centrifuged in a micro centrifuge for 10 min at 10,000 rpm to obtain pellets. The supernatant was gently decanted and the cell pellets were vortexed for 5 min. Then 300 mL of Tris EDTA (TE) buffer and 150 mL of 3.0 M sodium aqueous acetate was added at pH 5.2 and was vortexed for 5-10 min to lyse the bacteria cell pellet. The samples were centrifuged again for 5 min in a micro centrifuge (Biofuge Biotra Heareus Sepatech Co. Ltd. USA) and the supernatant was transferred to a fresh tube, mixed well with 0.9 mL of 70% ethanol that was pre-cooled to 20°C so as to precipitate the bacteria DNA. It was centrifuged again for 5 min and the supernatant discarded. The pellet was rinsed twice with 1 mL of 70 % ethanol and dried under vacuum for 2-3 min, after which it was resuspended in 20-40 μ L of TE buffer for further use (O'Neal, 1999).

Preparation of Gel

A 0.4 quantity of agarose was melted in 50 mL of Tris Borate EDTA buffer (TBE) to form 0.8% gel. The agarose solution was allowed to cool to temperature of about 40°C before it was poured into a gel tray. This was allowed for 25-30 min to solidify and the comb was carefully removed from the gel. The plastic gel carrier was removed from the pouring tray and placed in the gel electrophoresis box. A 250-300 mL of TBE was used to fill the electrophoresis box until the gel was submerged, then 6 mL sample of each plasmid DNA and 3 µL of loading dye were added together and this was carefully mixed together by pipetting the solutions up and down (O'Neal, 1999).

Electrophoresis of DNA Samples

Each sample was loaded carefully into the gel wells, one sample per well and was placed on the gel box. A 90 volts current was applied and DNA migration was observed so as to be sure that it migrates towards the positive electrode. The set up was allowed to run for 90 min, after which it was removed from the electrophoresis box and stained with a solution of 0.5 mg mL⁻¹ of ethidium bromide (EtBr) for 20-30 min. The DNA was then visualized under UV light with a UV certified safety glasses (O'Neal, 1999).

RESULTS AND DISCUSSION

Table 1 shows the prevalence rate of ESBL producing clinical isolates of *E. coli* from EBSUTH Abakaliki. A total of 7 clinical isolates of *E. coli* (11.1%) out of 63 were positive for DDST indicating that these organisms are ESBL producers. Out of the 11.1% that produced ESBL, 2 isolates (9.5%) were isolates from urine, 3 (21.4) from blood, 2 (18.2) from wound while no ESBL producing isolates were isolated from sputum and semen. Figure 1 shows the confirmatory result of ESBL production by *E. coli* from FMC and EBSUTH.

Table 2 shows the prevalence rate of ESBL producing clinical isolates of *E. coli* from FMC Abakaliki. A total of 10 clinical isolates of *E. coli* (21.7 %) out of 46 were positive for DDST, out of the 21.7% that produced ESBL, 2 isolates (18.2%) were isolates from urine, 5 (35.7%) from blood, 3 (30%) from wound, 1 (33.3) from semen while no ESBL producing isolates were isolated from sputum. The result of plasmid profile studies reveals the presence of low molecular weight plasmid DNA (23.1-29.4 kb) which is known to harbour ESBL enzymes (Fig. 2, 3).

Table 1: Prevalence of ESBL isolates from EBSUTH hospital Abakaliki

Hospitals	Total No. of Isolates	Sources of collection	No. of organisms collected from each source	No. of ESBL from each source (%)
EBSUTH	63	Urine	21	02 (9.5)
		Blood	14	03 (21.4)
		Wound	11	02 (18.2)
		Sputum	09	-
		Semen	08	-

EBSUTH: Ebonyi State University Teaching Hospital

Table 2: Prevalence of ESBL isolates from FMC hospital Abakaliki

Hospitals	Total No. of Isolates	Sources of collection	No. of organisms collected from each source	No. of ESBL from each source (%)
FMC	46	Urine	11	02 (18.2)
		Blood	14	05 (35.7)
		Wound	10	03 (30)
		Sputum	08	-
		Semen	03	01 (33.3)

FMC: Federal Medical Center Abakaliki Ebonyi State

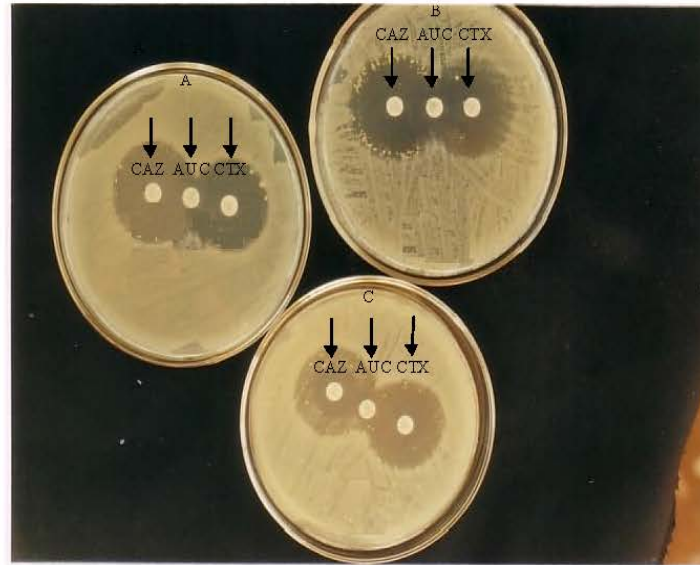


Fig. 1: The confirmatory result of ESBL production by *E. coli* (A-B from FMC and C from EBSUTH. Antibiotic disc contain cefotaxime (CAZ), ceftazidime (CTX) and augumentin (AUG) (combination disc (amoxicillin and clavulanic acid)

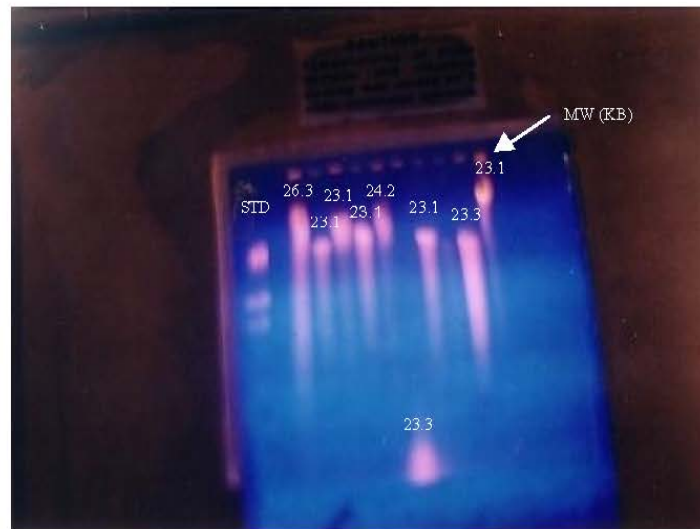


Fig. 2: Plasmid profile studies of ESBL producing *E. coli* from FMC Abakaliki, MW: Molecular weight, KB: Kilobase, The figures attached to the bands are the molecular weight of each isolates of ESBL, STD: Standard *E. coli* strain use as a control

The prevalence rate of ESBL producing organisms varies from country to country. A high prevalence was reported in USA from different isolates of the *Enterobacteriaceae* (Coudron *et al.*, 2000), while less than 1% of *E. coli* and *K. pneumoniae* strains possess ESBL in Europe and Netherlands but in France as many as 40% of *K. pneumoniae* were identified as ESBL producers (De Champs *et al.*, 2002). The 16.5% prevalence recorded in this study is thus noteworthy, especially

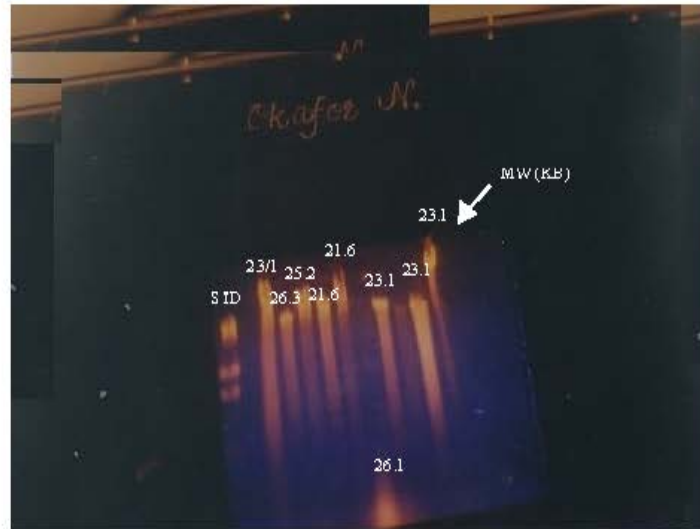


Fig. 3: Plasmid profile studies of ESBL producing *E. coli* from EBSUTH Abakaliki, MW: Molecular weight, KB: Kilobase, The figures attached to the bands are the molecular weight of each isolates of ESBL, STD: Standard *E. coli* strain use as a control

in our environment were studies on ESBL is still scanty and their medical implication yet to be appreciated. Aibinu *et al.* (2003) have shown that in Western part of Nigeria, the recent prevalence of ESBL is as a result of indiscriminate use of broad spectrum antibiotics and the only way to circumvent this is to strictly adopt a functional antibiotic policies such as restricting the use of a particular agents, limiting the introduction of antibiotic resistant organisms to the hospital environment, limiting the indiscriminate use of antibiotics and cycling of antibiotics.

The ESBL-producing isolates were predominantly isolated from blood samples followed by wound, urine and sputum. The prevalence rate of ESBL producers in FMC (a secondary hospital) was higher than in EBSUTH (a tertiary hospital). Previous studies show that ESBL producers are more prevalent in a tertiary hospital than in secondary hospitals (Aibinu *et al.*, 2003). This is because it is thought that 2nd and 3rd generation cephalosporins are used more often in tertiary hospitals for life threatening infections than in secondary hospitals.

The result of plasmid profile studies shows similarities in molecular weight, these could be that the same organism is responsible for the dissemination of the ESBL enzymes. ESBL genes are known to be plasmid or chromosomally-mediated, SHV-1 TEM-1 and TEM-2 β -lactamases arise by mutations that alter the amino-acid configuration around the active site of these enzymes so as to expand their spectrum of activity (Hawkey *et al.*, 2004). TEM-1 and TEM-2 β -lactamases are determined by plasmids belonging to many different incompatibility (Inc) groups in enteric organisms and are often carried on transposons (Mathew *et al.*, 1989). SHV-1 is also encoded by several plasmid types of β -lactamases. The small molecular weight of plasmid is a typical distribution of TEM-1 type beta-lactamase in nosocomial isolates of *E. coli* in which TEM is most often carried along with only a few other resistant genes by plasmids. The Inc specificity of the plasmid is challenging to investigate because their multi-resistance often makes suitable test plasmid without overlapping markers difficult to find (Aibinu *et al.*, 2003). Also considering the variety of plasmid types encoding TEM-1, TEM-2 and SHV-1, distribution of extended spectrum β -lactamase seems limited and raises question of whether certain plasmid types are more successful in nosocomial environment or have properties that promote resistance gene evolution. This study has shown that ESBL producing organisms of *E. coli*

were highly present in our environment and it is a very serious problem in our medical institution. This is because they are multi-drug resistance organisms that appear to be sensitive to expanded-spectrum cephalosporins *in vitro* but are not *in vivo* (Aibinu *et al.*, 2003). Such result could be misleading thus leading to failure during treatment because patients having infection caused by ESBL producing organisms are at an increased risk of not responding to treatment with an expanded spectrum beta-lactam antibiotic. Therefore it is suggested that organisms confirmed to be ESBL producers based on NCCLS criteria should be reported as resistant to all expanded spectrum cephalosporins regardless of their *in vitro* susceptibility test results.

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