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EMERGENCE OF CARBAPENEM RESISTANCE AMONG EXTENDED SPECTRUM BETA-LACTAMASE ISOLATE OF *Escherichia coli* FROM CLINICAL SPECIMENS IN A TERTIARY HOSPITAL, NIGERIA

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Abstract- Extended-spectrum β-lactamases (ESβLs) are a rapidly evolving group of β-lactamases which share the ability to hydrolyze third-generation cephalosporins and aztreonam yet are inhibited by clavulanic acid. In this study, the aim was to determine the susceptibility of ESβL producing clinical isolates of *Escherichia coli* across various antimicrobial agents according to the Clinical Laboratory Standard Institute (CLSI) new breakpoints. Sixty five clinical isolates of *Escherichia coli* were collected from a Tertiary Hospital in Nigeria of which forty six ESβL -producing isolates emerged. Presence of ESβL was determined by double- disk synergy test, DNA extraction and amplification of ESβL genes- bla_{SHV}, bla_{TEM}, bla_{CTX-M} by Polymerase Chain Reaction. Susceptibility of the ESβL-producing isolates was determined by the CLSI agar diffusion method. Utilizing the CLSI new breakpoints, 76.09%, 73.91%, 73.91% and 63.04% were resistant to Cefotaxime, Ceftazidime, Aztreonam and Cefepime respectively. While, 19.6%, 15.2%, 21.7% and 40% were susceptible to Cefotaxime, Ceftazidime, Aztreonam and Cefepime respectively. Furthermore, though a significant number of ESβL-producing isolates were susceptible to carbapenem; ESβL isolates that harboured the bla_{SHV}, bla_{TEM}, bla_{CTX-M} genes showed resistance to carbapenem. Thus, carbapenem-resistant ESβL-producing isolates emerged from this study. In conclusion, Nigeria is a developing country affected by the spread of bacterial strains harbouring ESβL and with the emergence of carbapenem resistance; it is certain to create significant therapeutic problems as carbapenem is the drug of choice for serious infections caused by ESβL-producing *Escherichia coli.*

Keywords- Extended-spectrum β-lactamases (ESβLs), Carbapenem-resistance, Clinical specimens, *Escherichia coli* isolates

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Introduction

Beta lactamases (βLs) which are enzymes hydrolyzes β lactam antibiotics like penicillins, cephalosporins, carbapenems and monobactems [1]. Third-generation cephalosporins(3GCs) which are extended spectrum cephalosporins were thought to be resistant to hydrolysis by beta lactamases. But, in mid 1980s, it became evident that a new type of beta lactamase emerged which could also hydrolyze the extended spectrum cephalosporins. These are termed "extended spectrum beta lactamases" (ESβLs). These enzymes result from mutations of Temorina and Sulf hydryl variable (SHV) enzymes, usually plasmid mediated [2]. These and other newly detected β- lactamases (for example CTX-M) hydrolyze β-lactam antibiotics containing the oxymino side-chain. CTX-M preferentially hydrolyzes cefotaxime. Due to the changes in amino acids sequences they are divided into five groups. They are found mostly in the *Enterobacteriaceae* family [3]. Some derivatives of TEM and SHV are not inhibited by clavulanic acid thus are known as inhibitor resistant TEM (IRTs) [4].

ESβLs hydrolyzes penicillins, narrow-spectrum as well as third-generation cephalosporins, and monobactems. The ESβLs have hydrolysis rates for ceftazidime, cefotaxime, or aztreonam (aminothiazoleoxime β-lactam antibiotics). They are inhibited by clavulanic acid. In general, the fourth-generation cephalosporin, cefepime, is clinically less useful against ESβL-producing organisms [5].

The presence of ESβLs carries tremendous clinical significance [6]. The ESβLs are frequently plasmid encoded. Plasmids responsible for ESβL production frequently carry genes encoding resistance to other drug classes such as the aminoglycosides. Therefore, antibiotic options in the treatment of ESβL-producing organisms are extremely limited [7]. Carbapenems are the treatment of choice for serious infections due to ESβL-producing organisms, yet carbapenem-resistant isolates have recently been reported [8]. ESβL-producing organisms may appear susceptible to some extended-spectrum cephalosporins. Consequently, treatment with such antibiotics has resulted to high failure rates [9].

For ESβL-producing bacteria, there is a drastic rise of MIC for extended-spectrum cephalosporins as the inoculum is increased beyond that used in routine susceptibility testing. Same isolates may test susceptible at the standard inoculum and resistant at a higher

inoclum [10]. Therefore, false-negative results may possibly occur with both screening and confirmatory tests when lower inocula are used [11].

At present, however, organizations such as the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) provide guidelines for the detection of ESβLs in *Klebsiellae* and *Escherichia coli* (*E. coli*). In common to all ESβL detection methods is the general principle that the activity of extended-spectrum cephalosporins against ESβL-producing organisms will be enhanced by the presence of clavulanic acid [12]. ESβLs represent an impressive example of the ability of gram-negative bacteria to develop new antibiotic resistance mechanisms in the face of the introduction of new antimicrobial agents [13]. There are no documented reports yet on the emergence of carbapenem resistance among extended spectrum beta-lactamase clinical isolate of *E. coli* from Nigeria. Therefore, this work provides a first report on the emergence of carbapenem resistance among extended spectrum beta-lactamase clinical isolate of *E. coli* from Nigeria.

Materials and Methods

Study Area

The study area was a tertiary hospital which is a referral centre for many hospitals and clinics in neighbouring states - Imo, Abia and Delta State.

Collection of Isolates

Sixty five (65) properly identified *E. coli* isolates from five hundred clinical specimens (Urine, Semen, Wound Swab, High Vaginal Swab, Ear Swab and Sputum) in the routine bacteriology laboratory of Nnamdi Azikiwe University Teaching Hospital, Nnewi were collected for this work.

Identification of Isolates

Isolates were identified using standard conventional microbiological techniques (microscopy, biochemical testing and culturing). Pure cultures of the isolates were stored in nutrient agar slant at 4^0C for further analysis.

Determination of Extended Spectrum Beta- Lactamase

The presence of Extended Spectrum β-lactamase (ESβL) was determined by the Double Disk Synergy Test (DDST) for all isolates. A suspension of the test organism was prepared to turbidity equivalent to 0.5McFarland Standards and then an aliquot was inoculated on Muller Hinton agar plate using sterile swab stick. A disk containing Amoxicillin plus Clavulanic acid (moxclav 20/10µg) disc was placed centrally on the Muller- Hinton agar plate. Discs containing Ceftazidime (30µg) was placed 15mm out from the edge of moxclav disc, so that its inner edge was 15mm from it. The same was performed with Cefotaxime (30µg), 15mm from moxclav disc. Plates prepared were incubated at 35^0C, aerobically for 18-24hours; zone diameters and zone-enhancement toward moxclav disc was recorded for all the cephalosporins, as per Clinical Laboratory Standard Institute (CLSI) guidelines. Plates with negative result were further incubated at 37°C, aerobically for 18-24 hours. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive control.

Antimicrobial Susceptibility Testing

The antimicrobial agents used were: Cefotaxime (30µg), Ceftazidime (30µg), Augmentin (30 µg) (Amoxicillin 20 µg/ clavulanic acid combination 10µg), Ciprofloxacin (5µg), (From Abtek Biological Ltd, Liverpool, UK), Cefepime (30µg), Imipenem (10µg), Meropenem (10µg), Fosfomycin (50µg) and Aztreonam (30µg) (From Oxoid, UK). A suspension of the test organism was prepared to turbidity equivalent to 0.5 Mc Farland standards and an aliquot was inoculated on Muller Hinton agar plate using sterile swap stick. All plates were incubated for 18 - 24 hours at 37^0C in air. Antimicrobial susceptibility test was performed on each isolate by disk diffusion method and diameter of zones of inhibition were interpreted as; Susceptible (S), Intermediate (I) and Resistant (R) as recommended by Clinical Laboratory Standard Institute, CLSI [14]. *E. coli* ATCC 25922 was used as control.

DNA Extraction and Amplification of Extended Spectrum β-Lactamase Genes in the Isolates using Polymerase Chain Reaction (PCR)

Isolates producing ESβLs were subjected to polymerase chain reaction (PCR) targeting bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$ genes. Genomic DNA was extracted according to the published method of Johnson and Woodford [15].

PCR conditions were as follows: reactions were carried out in MWG thermo cycler in 25µl mixtures containing 12.5µl PCR Master Kit (From Qiagen, Hilden, Germany), 9.5µl sterile *deionized* water, 200µM of dNTP, 1µl template DNA and 1µl of each oligonucleotide primer [16]. Initial denaturation at 95°C for 4min followed by 30cycles of denaturation at 95°C for 1min, annealing for 1min and at 48°C for TEM, and 60°C for SHV, CTX-M, extension at 72°C for 1min. The final extension step was extended to 10min at 72°C for all genes [17]. The amplified genes were seen in an electrophoretic gel. *Escherichia coli* 6681 containing bla_{SHV}, $bla_{CTX\text{-}M}$ and bla_{TEM} gene were used as controls.

Statistical Analysis

Standard percentage occurrence was used for the calculations of data received. Parametric method (Two factor ANOVA using Microsoft Excel) were used for statistical analysis.

Results

From our study, *Escherichia coli* had highest prevalence (65 isolates) among other gram negative organisms isolated from the clinical specimens. Following detection of ESβL isolates of *Escherichia coli* (46 isolates), prevalence of the enzyme across various clinical specimens showed that the ESβL-producing isolates recovered from Sputum, Ear Swab, Semen and Endo Cervical Swab were 100% respectively [Table-1]. The susceptibility profile showed 93.5% susceptibility of the ESβL -producing isolates to Meropenem, 80.4% susceptibility to Imipenem, 76% susceptibility to Fosfomycin. The resistance patterns across the antimicrobial agents are: Meropenem (2.17%), Imipenem (6.52%), Fosfomycin (15.22%), Co-amoxiclav (32.61%), Cefepime (63.04%), Ciprofloxacin (65.22%), Aztreonam (73.91%), Ceftazidime (73.91%) and Cefotaxime (76.09%). [Table-2] showed the results of percentage of intermediate pattern across the antibiotics in ESBL positive isolates. A total of 35(76%) ESβL producing isolates showed resistance to one or more of the third generation cephalosporins and aztreonam. The resistance zone of inhibition for Cefotaxime ranged from ≤ 22mm, for Ceftazidime ranged from ≤ 17mm, for Aztreonam ranged from ≤ 16mm, for Cefepime ranged from ≤14mm. PCR amplification was performed using the primers listed in [Table-3]. It was observed that there was a relationship between ESβL genes- bla_{SHV}, bla_{TEM}, bla_{CTX}

-M genes and resistance to carbapenem with *bla*CTX-M having the highest percentage (43.48%) [Table-4].

Table 1- Prevalence of Escherichia coli ESβL among the clinical specimens

Clinical Specimen	Number of *E. coli* isolate	Number/Percentage of ESβL *E. coli* isolates (%)
Urine	30	22(73)
High Vaginal Swab (HVS)	16	10(62.5)
Wound Swab	7	2(28.6)
Sputum	5	5(100)
Ear Swab	3	3(100)
Semen	3	3(100)
Endo Cervical Swab (ECS)	1	1(100)
Total	65	46

Table 2- Percentage of Susceptibility, Intermediate and Resistance pattern among the ESβL isolates across the antimicrobial agents

Antimicrobial agents	Susceptible (%)	Intermediate (%)	Resistant (%)
Meropenem (10µg)	43(93.5)	2(4.35)	1(2.17)
Imipenem (10µg)	37(80.4)	6(13.04)	3(6.52)
Fosfomycin (50µg)	35(76)	4(8.70)	7(15.22)
Co-amoxiclav (30µg)	10(21.7)	21(45.65)	15(32.61)
Cefepime (30µg)	17(40)	0(0)	29(63.04)
Ciprofloxacin (5µg)	13(28.3)	3(6.52)	30(65.22)
Ceftazidime (30µg)	7(15.2)	5(10.87)	34(73.91)
Aztreonam (30µg)	10(21.7)	2(4.35)	34(73.91)
Cefotaxime (30µg)	9(19.6)	2(4.35)	35(76.09)

Table 3- Primers used for amplification

Primer Name	Primer Sequence(5¹ to 3¹)	Size(bp)	Gene	Reference
SWSHV-A	AAG ATC CAC TAT CGC CAG CAG	200	blaSHV	[15]
SWSHV-B	ATT CAG TTC CGT TTC CCA GCG G			
TEM-A	GAG TAT TCA ACA TTT CCG TGT C	800	blaTEM	[15]
TEM-B	TAA TCA GAG GCA CCT ATC TC			
CTX-M A	CGC TTT GCG ATG TGA AG	550	blaCTX-M	[15]
CTX-M B	ACC GCG ATA TCG TTG GT			

Table 4- Relationship between Antimicrobial resistance pattern and ESβL genes among ESβL-producing isolates

Antimicrobial agents	%Resistant	% blaTEM	% blaCTX-M	% blaSHV
Meropenem (10µg)	2.17	0	2.17	0
Imipenem (10µg)	6.52	2.17	2.17	2.17
Fosfomycin (50µg)	15.22	4.35	8.7	2.17
Co-amoxiclav (30µg)	32.61	10.87	10.87	10.87
Cefepime (30µg)	63.04	13.04	32.61	17.39
Ciprofloxacin (5µg)	65.22	21.74	21.74	21.74
Ceftazidime (30µg)	73.91	30.43	10.87	32.61
Aztreonam (30µg)	73.91	21.74	39.13	13.04
Cefotaxime (30µg)	76.09	19.57	43.48	13.04

Discussion

In this study, we found that ESβL-producing isolates of *Escherichia coli* showed different patterns of resistance to various antimicrobial agents when using the new CLSI Susceptibility breakpoints. This may reflect the different abilities of ESβLs to hydrolyze different antimicrobial agents especially cephalosporins and aztreonam.

All ESβL-producing isolates of *Escherichia coli* showed resistance to Cefotaxime. The susceptibility of ESβL-producing isolates of *Escherichia coli* to Ceftazidime, Cefepime and Aztreonam varied. Resistance to Cefotaxime: maybe a better marker for the presence of ESβL than resistance to Ceftazidime. Surprisingly, 2.17% - 6.52% of ESβL-producing isolates of *Escherichia coli* appeared to be resistant to Meropenem and Imipenem which are carbapenems-the treatment of choice for serious infections due to ESβL-producing organisms [18]. Though, a significant number of ESβL-producing isolates were susceptible to carbapenems and fosfomycin. The variety profiles of susceptibility to carbapenem might be due to the combination of porin loss and β-lactamase production which results to carbapenem resistance [19]. This may explain the presence of *bla*SHV, *bla*TEM, *bla*CTX-M genes of ESβL harboured by the isolates.

We also observed that Imipenem resistant ESβL -producing isolates emerged from urine and semen with more from urine. While, Meropenem resistant ESβL-producing isolate emerged from High Vaginal Swab (HVS). All ESβL isolates were susceptible to Meropenem except isolates from High Vaginal Swab (HVS). ESβL isolates from urine showed resistance to all the antimicrobial agents except Meropenem. While, ESβL isolates from High Vaginal Swab (HVS) showed resistance to all the antimicrobial agents except Imipenem. ESβL isolates from wound swab showed the lowest resistance to the antimicrobial agents followed by ESβL isolates from Sputum.

From our study, Fosfomycin and Co-amoxiclav may serve as suitable therapeutic option for uncomplicated infections by ESβL-producing *E. coli*. Imipenem and Meropenem could serve as suitable therapeutic options for serious infections ranging from nosocomial urinary infection to bacteremia. Although, Carbapenem, Fosfomycin and Co-amoxiclav resistant isolates emerged, resistance to these antimicrobial agents was minimal.

Further well designed clinical studies are needed to determine the efficacy of Carbapenem (Imipenem and Meropenem), Fosfomycin and Cefepime in the treatment of infections caused by ESβL-producing strains of *Escherichia coli* most especially in treating uncomplicated to serious infections such as non-bacteremic urinary infection to nosocomial infections.

Conclusion

Escherichia coli are known pathogenic organism that has caused clinically important infections which has led to sever morbidity and mortality. The presence of ESβLs plus carbapenem resistance will surely create significant therapeutic problems in the future. Prolong and extensive use of carbapanem in treatment of infection caused by ESβL isolates as led to carbapenem resistant strains. Thus, heavy antibiotic use poses a risk factor for the acquisition of an ESβL-producing organism [20]. Continual alteration of antibiotic susceptibility breakpoints may become necessary but need to be carefully considered in combination with clinical data.

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Conflict of Interest- There are no conflict of interest between authors.

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