

1 **Community-acquire CTX-M Beta-lactamase enteric isolates in Abeokuta, Nigeria.**

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ABSTRACT

Aim: To study the emergence of the community-acquire CTX-M beta-lactamase genes among the residents in Abeokuta, Nigeria.

Study design: To determine the prevalence rate of CTX-M beta-lactamase genes in enteric multi-resistant ESBL isolates found among the Abeokuta residents.

Place of study: Abeokuta, Nigeria between August 2010 and March 2013.

Methodology: Beta-lactamase and ESBL producing enteric bacteria were identified and their antimicrobial susceptibility was tested against commonly used antibiotics. Mating activity of the isolates was evaluated and their plasmid was profiled while a CTX-M chromosomal gene was assayed by PCR.

Result: Of the 27.4% beta-lactamase producers obtained, only 5.1% of the strains were ESBL and 2.8% harboured *bla*CTX-M genes. High resistance rate of 91.0% by *Escherichia coli* and *Klebsiella oxytoca* to commonly used antibiotics was observed.

Conclusion: There is urgent need to curb the community spread of undetected mobile genetic elements of CTX-M-encoded enteric bacteria among the residents in this community and if this persists, a possible outbreak of enteric infection caused by resistant organisms is eminent.

Key words: Community residents, ESBL, *bla*CTX-M.

1.0 Introduction

CTX-M beta-lactamases are mutant, plasmid-mediated enzymes with potent hydrolytic activity against cefotaxime which are now commonly encountered among numerous enteric bacteria isolates [1, 2]. In recent past, increasing prevalence of bacterial pathogens producing CTX-M beta-lactamases has trigger multiple resistance to most commonly used 3rd Generation cephalosporin [3]. However, some CTX-M-type ESBLs may actually hydrolyze ceftazidime and confer resistance to this cephalosporin. It was noted that the same organism harbouring both CTX-M-type and SHV-type ESBLs or CTX-M-type ESBLs and AmpC-type beta-lactamases, may express altered antibiotic resistance phenotype [4]. Thus, organisms producing CTX-M-type beta-lactamases typically are now known to have high minimum inhibitory concentration (MIC) range against cefotaxime, while ceftazidime MIC is usually in the apparently susceptible range [5].

Moreover, feecal colonisation with ESBL-producing isolates is fast becoming prevalent in our society while it is a common practice in hospital setting for implementing epidemiological measures to curtail and control this spread. Nevertheless, the increasing rate of patients and healthy residents colonised with cephalosporin resistant strains are now potential risk factor for acquisition and transmission of resistant CTX-M beta-lactamases in many households [6]. The relationship between antibiotic consumption and occurrence of CTX-M beta-lactamases has not been well studied in this locality. Although, the prevalence of resistant strains causing community-acquire diarrhoeal and other enteric diseases raises speculation that oxyimino cephalosporins available in many patent stores were administered without regulated prescription. Therefore, the emergence and spread of antibiotic resistance bacteria with CTX-M beta-lactamases in this community which has become a threat to effective treatment of enteric infection were studied.

2.0 Materials and methods

2.1 Community sample collection: Fresh feecal samples of two hundred and fifty one (251) individual were randomly collected from the community residents in Abeokuta, who consented to participate in the study and who have not used any antimicrobial agents in the preceding two weeks either as therapy for gastro-intestinal complication or prophylaxis between August 2010 and March 2013. Their use of antimicrobial agents, feeding habit and hygiene in the last three months was exclusion criteria used for the subjects' selection. Informed consent of all individual involved in this study was obtained while they were assured of confidentiality in view of the intricacy and sensitivity of the study.

2.2 Isolation and identification: All feecal samples collected were cultured on Salmonella-Shigella agar (Oxoid CM 123, UK) and MacConkey agar without salt (Oxoid CM 516, UK), and were incubated at 37 °C for 18-24 hours. Each isolate obtained was identified using API enterobactericaea kit for biotyping and was interpreted according to World Health Organisation (WHO) manual for laboratory investigation of acute enteric infections [7].

2.3 Antibiotic susceptibility tests: Standard broth micro-dilution method was used to determine the MIC of each identified isolates against the commonly used antibiotics at the following dilution ranges (µg/mL): Ampicillin (0.5–64), Amoxicilli/Clavulanate (0.5-32), Gentamycin (0.5–64), Ciprofloxacin (0.5–64), Cefuroxime (1–64), Ceftazidime (0.25–128), Tetracycline (0.25–6), Cefotaxime (0.25–64), Azithromycin (0.5-64) and Imipenem (0.25–16). The reference strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used to check the quality and precision of the testing procedures. MIC of each isolates was determined according to NCCLS recommended guidelines [8].

2.4 Phenotypic detection of beta-lactamases and ESBL: Isolates showing MIC >8 µg/mL to Cefuroxime, Ceftazidime and Cefotaxime were tested for beta-lactamase production by a starch-iodide paper acidometric method described by Odugbemi *et al.* [9]. ESBL Double Disc Synergy Test (DDST) was performed according to recommended method and criteria of CLSI [10]. 0.5 McFarland standard of bacterial

suspension was evenly spread on Mueller–Hinton agar plates and Ceftazidime (30 µg), Ceftazidime +clavulante (30/10 µg) Cefotaxime +clavulante (30/10 µg) and Cefotaxime (30 µg) discs were placed 2 cm apart on the plate in a straight line. A difference of 5 mm or more between the inhibition zones of the Ceftazidime (30 µg) + Clavulanic acid (10 µg) and Ceftazidime (30 µg) disc alone, was regarded as ESBL producer. *E. coli* ATCC 25922 was used to control the test.

2.5 Mating activity: The confirmed ESBL isolates were mated with susceptible *E. coli* recipient strain, ATCC 25567 (penicillin-resistant and lactose fermentation-positive strain). Overnight cultures of donor cells of identified ESBL isolates and recipient strain (*E. coli* ATCC 25677) were grown in 0.5% sucrose enriched Luria-Bertani broth at 37 °C, after mixing at a 1:10 ratio (donor to recipient) and incubated for 3 h and then sub-cultured on MacConkey Agar plates supplemented with 6 mg/ml ceftazime (Oxoid). Transconjugants growing on the selection plates were subjected to DDST to confirm transfer of beta-lactamase genes and to examine co-transfer of other antibiotic resistance determinants present in the donor isolates.

2.6 Plasmid DNA transformation: Plasmid DNA of identified ESBL isolates were extracted by Alkaline lysis method [10] and was mixed with 0.5 MacFarland broth of cephalosporin susceptible *E. coli* ATCC 25922 in Luria-Bertani broth supplemented with 2 M Calcium chloride, then incubated at 37 °C [11]. The mixture was later sub-cultured on selection plate of Muller Hinton Agar supplemented with 6 mg/mL ceftazidime and incubated at 37 °C for 18-24 h. Transformants obtained were subjected to DDST.

2.7 Plasmid profiling: Extra-chromosomal plasmid DNA was extracted from each isolate by Alkaline lysis method [10, 12]. An aliquot of 1000 µL bacteria cell suspension was lysed in 1% SDS containing 0.2 M NaOH (pH 8.0) and 150 µL ice-cold 3.0 M Potassium Acetate (pH 5.5) was added to precipitate the cell suspension. Then rapidly inverted and centrifuged to obtain supernatant which was precipitated by adding equal volume of isopropanol. It was centrifuged to obtain clear supernatant which absolute ethanol was added and re-centrifuged to obtain plasmid DNA pellet. The plasmid DNA obtained was then electrophoresed on 0.8% agarose gel containing 0.5 µg/mL Ethidium bromide. The

DNA was visualized by ultraviolet trans-illuminator and documented with digital Polaroid camera. The molecular weights were determined according to Meyers *et al.* [12] using standard DNA molecular weight marker of 0.12-23.1 Kbp (Roche Diagnostic GmbH).

2.8 PCR Amplification of *bla*CTX-M gene: Chromosomal DNA template was prepared by boiling 1 ml of suspension of pure colonies of ESBL isolate in DNase and RNase-free water at 55 °C for 10 min. After centrifugation, its supernatant was used as template DNA. The template DNA was quantified by Thermoscientific Nanodrop Spectrophotometer at absorbance of 260 nm. The template DNA was amplified for CTX-M gene by Polymerase Chain Reaction (PCR) using specific oligodeoxynucleotides *bla*CTX-M Forward 5'-GCG ATG TGC AGG ACC AGT AA-3' and Reverse 5'-CCG CGA TAT CGT TGG TGG TG-3' (Norgen Biotek, Canada). The PCR was performed with 2.5 µl 10X PCR buffer, 1.5 µl 25 mM MgCl₂, 0.5 µl 10 mM dNTPs (dATP, dGTP, dCTP, dTTP), 0.2 µl *bla*CTX-M F (2500 pmol), 0.2 µl *bla*CTX-M R (2500 pmol), 0.2 µl Taq polymerase enzyme (*Thermus aquaticus*), 17.9 µl distilled water and 2.0 µl of each extracted template DNA to make a final reaction volume of 25.0 µl in a thermal cycler block (NYX Technik Inc; Model ATC401, USA). The PCR mixture was subjected to a 5 min initialization at 94 °C, followed by 30 cycles of denaturation at 94 °C for 60 seconds, annealing at 60 °C for 60 seconds, extension at 72 °C for 60 seconds. Amplified products were separated by electrophoresis in 2% agarose gel containing 0.5 µg/ml ethidium bromide for 30 minutes at 100 V, after which they were viewed under ultraviolet light to determine the specific *bla* gene bands.

2.9 Statistical analysis: Risk factors for ESBL prevalence among the studied community was assessed by using Chi square to determine the significance of ESBL isolates (p value <0.05) at confidence interval of 95%. Significance of the high MIC among the organism harbouring *bla*CTX-M gene was determined by Pearson test while the correlation coefficient was calculated by the method of least squares. High level of resistance among the isolates was determined at p<0.005.

3.0 Results

Molecular characteristic of study strains: Of 215 non-duplicated enteric isolates obtained from the community residents, 11 (5.1%) met CLSI screening criteria as potential ESBL producer with very high molecular weight (average 7.91 kbp) plasmid. Among the 11 ESBL producers only 7 were successfully transformed the susceptible strain. Also, only 6 (2.8%) harboured *bla*CTX-M genes among the identified ESBL producers.

Prevalence of resistant enteric isolates in the studied community: Very high resistance rate of 98.8% to cefuroxime was shown for beta-lactamase producers while 91.0% resistance was observed among the isolates to Ampicillin, cefotaxime and ceftazidime respectively. It is worth noting that *E. coli* and *Klebsiella oxytoca* show resistance rate of 5.2% and 7.8% respectively to iminepem and a high resistance of 31.6% to most antimicrobial agents.

Table 1; Molecular characteristics of enteric isolates obtained from the Community residents

Enteric isolates	Total isolates obtained n (%)	beta-lactamase n (%)	ESBL isolates n (%)	Plasmid size ($\pm$ 0.5kbp)	Conjugants (n=11)	Transformants (n=11)	<i>bla</i> CTX-M n (%)
<i>Escherichia coli</i>	62(29.1)	12(5.6)	5(8.1)	8.5	2	3	2(0.9)
<i>Klebsiella oxytoca</i>	37(17.4)	9(4.2)	1(2.7)	10.2	1	1	1(0.5)
<i>Salmonella</i> spp.	14(6.6)	8(3.6)	1(7.1)	7.5	1	0	1(0.5)
<i>Citrobacter freundii</i>	8(3.8)	5(2.3)	1(12.5)	6.8	0	1	0(0.0)
<i>Enterobacter cloacae</i>	22(10.3)	6(2.8)	1(4.6)	6.3	1	1	0(0.0)
<i>Pseudomonas</i>	39(18.3)	10(4.7)	1(2.4)	7.4	1	1	1(0.5)
<i>aeruginosa</i> <i>Proteus mirabilis</i>	31(14.6)	9(4.2)	1(3.2)	8.7	1	1	1(0.5)
<i>Shigella</i> spp.	2(0.9)	0(0.0)	0(0.0)	0.0	0.0	0.0	0(0.0)
Total	215(100.0)	59(27.4)	11(5.1)	7.91	7	8	6(2.8)

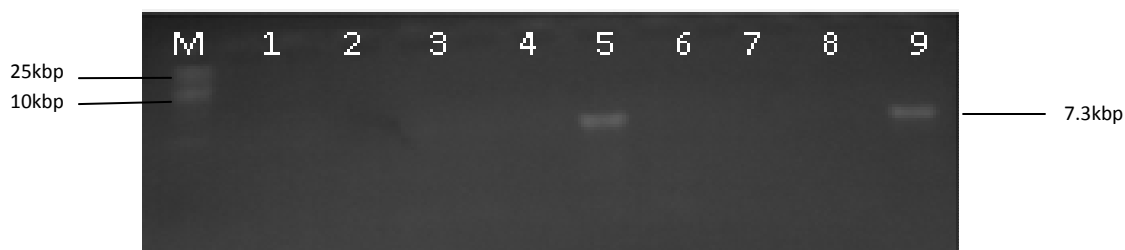


Figure 1. Photograph showing the *bla*_{CTX-M} amplicons obtained from ESBL positive isolates on agarose gel after electrophoresis. Lane M is Supercoiled DNA Ladder (Gibco BRL, France); lane 5 and 9 are *bla*_{CTX-M} specific DNA bands.

Table 2; Percentage resistance of ESBL-positive strains to commonly used drug

Enteric isolates	Antimicrobial agents (MIC range 2 to 8 µg/ml)									
	AMP	AMC	CFX	AZT	CPX	IMP	CTX	TET	CFZ	GN
	n (%)									
<i>Escherichia coli</i>	12(31.6)	5(13.0)	12(31.6)	3(7.8)	2(5.2)	2(5.2)	12(31.6)	2(5.2)	11(28.6)	2(5.2)
<i>Klebsiella oxytoca</i>	12(31.6)	3(7.8)	12(31.6)	8(20.8)	7(18.2)	3(7.8)	12(31.6)	6(15.6)	12(31.6)	6(15.6)
<i>Salmonella</i> spp.	2(5.2)	1(2.6)	2(5.2)	0(0.0)	2(5.2)	0(0.0)	2(5.2)	2(5.2)	2(5.2)	0(0.0)
<i>Citrobacter freundii</i>	3(7.8)	1(2.6)	4(10.4)	2(5.2)	2(5.2)	0(0.0)	3(7.8)	0(0.0)	3(7.8)	0(0.0)
<i>Enterobacter cloacae</i>	1(2.6)	0(0.0)	2(5.2)	0(0.0)	1(2.6)	0(0.0)	2(5.2)	0(0.0)	2(5.2)	1(2.6)
<i>Pseudomonas aeruginosa</i>	3(7.8)	1(2.6)	3(7.8)	1(2.6)	1(2.6)	0(0.0)	2(5.2)	1(2.6)	3(7.8)	1(2.6)
<i>Proteus mirabilis</i>	2(5.2)	1(2.6)	3(7.8)	1(2.6)	2(5.2)	1(2.6)	2(5.2)	2(5.2)	2(5.2)	1(2.6)
Total	35(91.0)	12(31.6)	38(98.8)	15(39.0)	17(44.2)	6(15.6)	35(91.0)	13(33.8)	35(91.0)	11(28.6)

Abbreviations: AMP-Ampicillin, AMC-Amoxicilli/Clavulanate, CFX-Cefuroxime, AZT-Azithromycin, CPX-Ciprofloxacin, IMP-Iminepem, CTX-Cefotaxime, TET-Tetracycline, CFZ-Ceftazidime, GN-Gentamycin, n=total number of resistant isolate, %= percentage resistance

4.0 Discussion

The prevalence of CTX-M family of ESBLs is now becoming an emerging global health threat due to its unfavourable outcome in the treatment of common enteric infectious diseases in the community and among the hospital patients. It was clearly observed

from the study that out of 27.4% beta-lactamase producing enteric flora obtained from the community residents, only 5.1% were ESBL producers. This rate was lower than the values recorded in Enugu (11.4%), Enugu State at South-East Nigeria [13] and in Lagos (25%) of Nigeria [14]. Although there is no establish report of ESBLs prevalence in any community in Nigeria, but similar study in Cambodia recorded 36.6% community-acquire ESBLs [15]. Our findings suggest that there is undetected dissemination of community-acquire *bla*CTX-M genes among the residents and this could be elicited by self-medication and use of non-prescribed antibiotics which is a common practice in this locality. Plasmid DNA from bacteria isolates obtained from community residents further indicate a potential reservoir of antibiotic resistance bacteria that could contain a pool of mobile genetic elements, which could be readily disseminated to other human pathogens. Therefore, transfer of naked resistant DNA plasmids encoding beta-lactamase mostly decrease antibiotic susceptibility to commonly use antibiotics, thereby causing economic loss to the society and prolonged treatment of infection. High transformation achieved by the ESBL isolates from both the community and the hospital, suggest a very high frequency of resistant gene transfer between bacteria of the same specie or other species. This transformation process may be enhanced by constant release of naked DNA from dying bacteria which can be taken up by other isolates through unhygienic eating habit of residents at dirty food joints, restaurants and street food vendors. Moreover, sachet-water meant for drinking could contain resistant bacteria DNA [16, 17] which can easily transform susceptible isolates. This would definitely prone these residents to live in grave danger of increasing antibiotic resistant resulting to poor therapeutics, causing increasing morbidity.

Highly diverse antibiotics resistance rates against all cephalosporin, such as ceftazidime, cefuroxime and cefotaxime with MIC >16 µg/ml represent a very critical situation as compared to investigations from other regions of the world reporting resistance towards first, second and third generation of cephalosporin [15, 18]. The spread of antimicrobial resistance among bacterial pathogens to most antibiotics in this locality is fast becoming an important fatal challenge for the community and health institutions due to unguided use of over-the-counter drugs mostly antibiotics.

Unfortunately, data regarding bacterial resistance to antimicrobial agents is limited in the South-western Nigeria. The cause of this upsurge in resistant of community-acquire ESBL-producing organisms is not yet clear, but contamination of foodstuffs, animal consumption of antibiotics, and frequent patient contact with relatives during illness may be factors to consider.

However, some of the antibiotic resistance genes usually associated with mobile elements was also associated with chromosomal transmission of resistant genes which maintain the spread of the CTX-M extended spectrum of beta-lactamases among pathogenic bacteria populations, even if the subjects are not treated with extended-spectrum cephalosporins, especially in countries where antibiotics usage is poorly prescribed. Interestingly, *bla*CTX-M gene could exhibit virulence traits which could be involved in colonization, infection, and persistence in humans [19, 20]. These factors simultaneously explain the spread and infection persistence of CTX-M-encoding genes among enteric isolates in this community.

Conclusion: Adequate detection of new resistance mechanisms of the emerging *bla*_{CTX-M} isolates is necessary for infection control in the community and prevention of possible outbreaks of resistant organisms.

Competing interest: Neither financial nor personal relationships with other people or organizations inappropriately influence our work in any way.

Consent: The Authors declared that informed consent was obtained personally from all individual subjects involved in this study and any interested subject that met the criteria of the study. They were informed of the benefit of the study and freedom to withdraw if they wish as non-invasive method of fecal collection was used.

Authors' Contributions: Akinduti PA and Oluwadun A designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Iwalokun BA and Onagbesan KO managed the analyses of the study. Ejilude O managed the literature and the authors read and approved the final manuscript.

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