Community-acquired CTX-M βeta-lactamase enteric isolates in Abeokuta, Nigeria.

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ABSTRACT

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

Study design: To determine the prevalence rate of CTX-M β-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% strains were ESBL and 2.8% harbour *bla*CTX-M genes. High resistance rate of 91.0% by *Eschericia 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, blaCTX-M.

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1.0 Introduction

CTX-M β-lactamases are mutant, plasmid-mediated β-lactamases with potent hydrolytic activity against cefotaxime is now commonly encountered among numerous enteric bacteria isolates [1, 2]. In recent past, increasing prevalence of bacterial pathogens producing CTX-M β-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 ß-lactamases, may express altered antibiotic resistance phenotype [4]. Thus, organisms producing CTX-M-type ß-lactamases typically are now

know to have high minimum inhibitory concentration (MIC) range against cefotaxime, while ceftazidime MIC is usually in the apparently susceptible range [5].
Moreover, faecal colonisation with ESBL-producing isolates is fast becoming prevalent in our society while it is a common practice in the hospital setting for implementing epidemiological measures to curtail and control this spread. Nevertheless, the increasing rate of inpatients, outpatients and healthy residents colonised with cephalosporin resistant strains are now potential risk factor for acquisition and transmission of resistant CTX-M β-lactamases in many households [6]. The relationship between antibiotic consumption and occurrence of CTX-M β-lactamases has not been well studied in this locality. Although, the prevalence of resistant strains causing community-acquired diarrhoea and other enteric diseases raises speculation that oxyimino cephalosporins available in many patent stores were taken without regulated prescription. Therefore, the emergence and spread of antibiotic resistance bacteria with CTX-M β-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 faecal samples of two hundred and fifty one (251) 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 the faecal samples collected were cultured on Salmonella-Shigella agar (Oxoid CM 123, UK) and MacConkey agar without salt (Oxoid CM 516, UK); and incubated at 37°C for 18-24hours. Each isolate obtained was identified using API enterobactericaea kit for the biotyping and were 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 with dilution ranges; Ampicillin (0.5–64ug/mL), Amoxicilli/Clavulanate (0.5-32ug/mL), Gentamycin (0.5–64ug/mL), Ciprofloxacin (0.5–64ug/mL), Cefuroxime (1–64ug/mL), Ceftazidime (0.25–128 ug/mL), Tetracycline (0.25–6ug/mL), Cefotaxime (0.25–64ug/mL), Azithromycin (0.5-64 ug/mL) and Imipenem (0.25–16ug/mL). 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-lactamase and ESBL: Isolates showing MIC >8ug/mL to Cefuroxime, Ceftazidime and Cefotaxime were tested for beta-lactamase production by a starch-iodide paper acidometric method described by Odugbemi *et al*, 1977 [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 onto the surface of the Mueller–Hinton agar plate and Ceftazidime (30µg), Ceftazidime + clavulante (30/10µg) Cefotaxime + clavulante (30/10µg) and cefotaxime (30µg) discs were placed 2cm apart on the plate in a straight line. A difference of 5mm or more between the inhibition zones of the ceftazidime (30µg)

plus clavulanic acid(10ug) and ceftazidime (30µg) disc alone, was taken to indicate an ESBL producer. *Eschericia coli* ATCC 25922 was used as controls.

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

2.6 Plasmid DNA transformation: DNA plasmid extracted by an alkaline lysis method [10] of 20ul was added to 5ml overnight enriched Luria-Bertani broth culture of cephalosporin susceptible *Escherichia coli* ATCC 25922 isolates in the presence of 2M Calcium chloride at 37^0C for 6hours according to Smith *et al*, (1981) [11]. The mixture was later sub-cultured on selection plate of Muller Hinton Agar supplemented with 6mg/L ceftazidime and incubated at 37^OC for 18-24hours. Transformants growing in the selection plates were subjected to DDST.

2.7 Plasmid profiling: Extra-chromosomal plasmid DNA was extracted from each isolates according to the alkaline lysis method [10], [12] using 1000 uL of the bacteria cell suspension lysed in 1% SDS, 0.2M NaOH (pH 8.0) and precipitated in 150 uL of ice-cold 3.0 M Potassium Acetate, pH 5.5. The plasmids DNA obtained were then electrophoresed on 0.8% Agarose gel, stained with 14µl/g Ethidium bromide. The DNA was then photographed with Polaroid camera and viewed using UV trans-illumination. The molecular weights were determined according to Meyers *et al* [12] using standard DNA molecular weight marker of 0.12-23.1kbp (Roche Diagnostic GmbH).

2.8 PCR Amplification of *bla*CTX-M gene: Chromosomal DNA template was prepared by boiling pure colonies of ESBL isolates which was suspended thoroughly in 1mL DNase and RNase–free water and boiled at 55^0C for 10 minutes. After centrifugation, supernatant was used as template DNA. The template DNA was quantified by Thermoscientific Nanodrop Spectrophotometer of absorbance of 260nm filter and estimated. The extracted DNA template was amplified for CTX-M gene by Polymerase Chain Reaction (PCR) using specific oligodeoxynucleotides *blaCTX-M F* 5` GCG ATG TGC AGG ACC AGT AA 3` and *blaCTX-M R* 5` CCG CGA TAT CGT TGG TGG TG 3` to amplify minimum of 250bp amplicon (Norgen Biotek, Canada). The PCR was performed in mix of 2.5ρl 10X PCR buffer, 1.5ρl 25mM MgCl2, 0.5ρl of 10mM dNTPs (dATP, dGTP, dCTP, dTTP), 0.2ρl blaCTX-M F of 2500pmole, 0.2ρl of blaCTX-M R of 2500pmole, 0.2ρl Taq polymerase enzymes *(Thermus aquaticus*), 17.9ρl distilled water and 2.0ρl of each extracted template DNA to make final reaction volume of 25.0ρl in a thermal cycler block (NYX Technik Inc; Model ATC401, USA). The PCR mixture was subjected to a 5 minutes initialization at 94℃, followed by 30 cycles of 45 s at 94℃, d *enaturation* at 94℃ for 60 seconds, *annealing* at 60℃ for 60 seconds, *extension at* 72℃ for 60 seconds. Amplified products were separated by 100V electrophoresis in a 2% agarose gel containing 0.5ug/ml ethidium bromide for 30 minutes, after which they were viewed under ultraviolet light to determine the specific *bla* gene amplicons.

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 at p- value <0.05 at confidence interval of 95%. Significance of the high MIC to the occurrence of *bla*CTX-M gene was

determined by Pearson test while the correlation coefficient was calculated by the method of least squares and 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%) strains met CLSI screening criteria as potential ESBL producer, with very high average molecular plasmid weight of 7.91kbp. Among the 11 ESBL producers, only 7 isolates transconjugated and 8 successfully transformed susceptible isolates respectively. Also, only 6(2.8%) harbour *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 by beta-lactamase producers while 91.0% resistance was observed among the isolates to Ampicillin, cefotaxime and ceftazidime respectively. It is worth noting that *Eschericia coli* and *Klebsiella oxytoca* show resistance rate of 5.2% and 7.8% to iminepem respectively 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 (±0.5kbp)	Conjugants (n=11)	Transformants (n=11)	blaCTX-M n(%)
Eschericial coli	62(29.1)	12(5.6)	5(8.1)	8.5	2	3	2(0.9)
Klebsiella oxytoca	37(17.4)	9(4.2)	1(2.7)	10.2	1	1	1(0.5)
Salmonella spp	14(6.6)	8(3.6)	1(7.1)	7.5	1	0	1(0.5)
Citrobacter freundii	8(3.8)	5(2.3)	1(12.5)	6.8	0	1	0(0.0)
Enterobacter cloacae	22(10.3)	6(2.8)	1(4.6)	6.3	1	1	0(0.0)
Pseudomonas aeruginosa	39(18.3)	10(4.7)	1(2.4)	7.4	1	1	1(0.5)
Proteus mirabilis	31(14.6)	9(4.2)	1(3.2)	8.7	1	1	1(0.5)
Shigella spp	2(0.9)	0(0.0)	0(0.0)	0.0	0.0	0.0	0(0.0)
Total	215(100.	59(27.4)	11(5.1)	7.91	7	8	6(2.8)

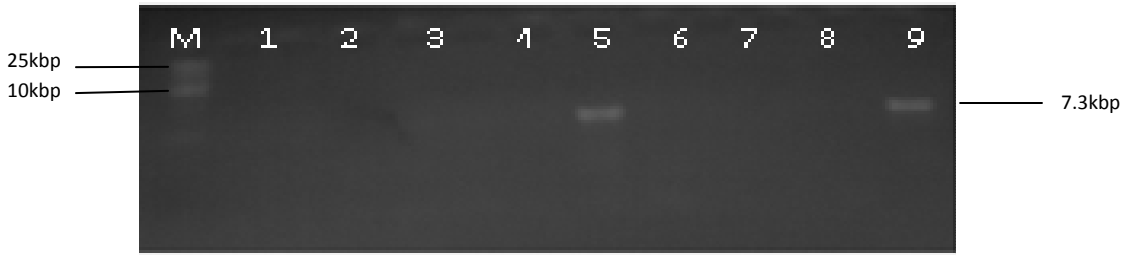

Figure 1 shows the $bla_{CTX\text{-}M}$ DNA amplicons recovered from the ESBLs positive isolates on Agarose gel. Lane M is high rang ladder DNA markers (Supercoiled DNA Ladder, Gibco BRL, France) while lane 5 and showing the $bla_{CTX\text{-}M}$ DNA bands.

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

	Antimicrobial agents (MIC range 2 to 8ug/ml)									
Enetric isolates	**AMP**	**AMC**	**CFX**	**AZT**	**CPX**	**IMP**	**CTX**	**TET**	**CFZ**	**GN**
					n(%)					
Eschericial coli	**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)**
Klebsiella oxytoca	**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)**
Salmonella 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)**
Citrobacter freundii	**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)**
Enterobacter cloacae	**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)**
Pseudomonas aeruginosa	**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)**
Proteus mirabilis	**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)**

Key: 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

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 to 11.4% recorded in Enugu, Enugu State, South-East Nigeria [13]; and also 25% rate in Lagos, 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-acquired *bla*CTX-M genes among this community residents and this could be elicited by self-medication and use of unprescribed antibiotics which is a common practice in this locality. Conjugative DNA plasmid obtained from the community resident 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, transfers of naked resistant DNA plasmids encoding β-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 the same bacteria specie or other species. This transformation process could be promoted by constant release of naked DNA by dying bacteria which can be taken up by other isolates through unhygienic eating habit of most residents in poorly located food joints, restaurants and street food vendors and sachet-water supplied for drinking that usually contain this naked DNA genes [16, 17], which has high propensity for transforming a 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 >16µg/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 [18,19]. 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 South-western, Nigeria. The cause of this upsurge in resistant of community-acquired ESBL-producing organisms is not yet clear, but associations with foodstuffs, animal consumption of antibiotics, and frequent patient contact with relatives during illness may be a major factor to consider.

However, some of the antibiotic resistance genes usually associated with mobile elements were also associated with chromosomal transmission of resistant gene which maintains the spread of CTX-M extended spectrum β-lactamases among pathogenic bacteria populations, even if the subjects are not treated with extended-spectrum cephalosporins, especially in countries where antibiotics usage is unregulated. Interestingly, *bla*CTX-M clone could exhibit virulence traits which could be involved in colonization, infection, and persistence in humans [20, 21]. These factors simultaneously explain the spread and the persistence of community spread of the CTX-M-encoding genes among enteric isolates in this community.

Conclusion: Adequate detection of new resistance mechanisms of the emerging $bla_{CTX\text{-}M}$ 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 individuals 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 done.

Authors' contributions: Akinduti P.A and Oluwadun A designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Iwalokun B.A and Onagbesan K.O managed the analyses of the study. Ejilude O. managed the literature searches and all the authors read and approved the final manuscript

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