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

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ABSTRACT

Aim: To study the emergence of the community-acquired 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: β-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% β-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 now commonly encountered among numerous enteric bacteria isolates [1, 2]. In the recent past, increasing prevalence of bacterial pathogens producing CTX-M β-lactamases has triggered multiple resistance to most commonly used third 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 harboring both CTX-M-type and SHV-type ESBLs or CTX-M-type ESBLs and AmpC-type ß-lactamases, may express an altered antibiotic resistance phenotype [4]. Thus, organisms producing CTX-M-type ß-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, fecal colonization with ESBL-producing isolates is fast becoming prevalent in our society while it is a common practice in hospital settings for implementing epidemiological measures to curtail and control this spread. Nevertheless, the increasing rate of in-patients, out-patients and healthy residents colonized with cephalosporin resistant strains are now potential risk factors 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 diarrhea and other enteric diseases raises speculation that oxyimino cephalosporins available in many patient stores were consumed 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 fecal samples of two hundred and fifty one (251) individuals 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 complications or prophylaxis between August 2010 and March 2013. Their use of antimicrobial agents, feeding habits and hygiene in the last three months was exclusion criteria used for subject selection. Informed consent of all individuals 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 fecal 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 18-24 hours. Each isolate obtained was identified using API enterobactericaea kit for biotyping and was interpreted according to the World Health Organization (WHO) manual for laboratory investigation of acute enteric infections [7].

2.3 Antibiotic susceptibility tests: The standard broth micro-dilution method was used to determine the MIC of each identified isolate against the commonly used antibiotics with the following dilution ranges: Ampicillin (0.5–64 µg/mL), Amoxicillin/Clavulanate (0.5-32 µg/mL), Gentamycin (0.5–64 µg/mL), Ciprofloxacin (0.5–64 µg/mL), Cefuroxime (1–64 µg/mL), Ceftazidime (0.25–128 µg/mL), Tetracycline (0.25–6 µg/mL), Cefotaxime (0.25–64 µg/mL), Azithromycin (0.5-64 µg/mL) and Imipenem (0.25–16 µg/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 β-lactamase and ESBL: Isolates showing MIC >8 µg/mL to Cefuroxime, Ceftazidime and Cefotaxime were tested for β-lactamase production by a starch-iodide paper acidometric method described by Odugbemi *et al*, 1977 [9]. The ESBL Double Disc Synergy Test (DDST) was performed according to the recommended method and criteria of CLSI [10]. 0.5 McFarland standard of bacterial suspension was evenly spread onto the surface of 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 the Ceftazidime (30 µg) disc alone, was regarded as an indication for an ESBL producer. *Eschericia coli* ATCC 25922 was used as control.

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 at a 1:10 ratio (donor to recipient) and incubated for 3 hours 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 β-lactamase genes and also to examine the co-transfer of other antibiotic resistance determinants present in the donor isolates.

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

2.7 Plasmid profiling: Extra-chromosomal plasmid DNA was extracted from each isolate according to the alkaline lysis method [10], [12] using 1000 µL of bacterial cell suspension lysed in 1% SDS, 0.2 M NaOH (pH 8.0) and precipitated in 150 µL of ice-cold 3.0 M Potassium Acetate, pH 5.5. The plasmid DNA samples obtained were then electrophoresed on 0.8% agarose gel, stained with 14 µl/g ethidium bromide. The DNA was then visualized by UV trans-illumination and documented with a Polaroid camera. The molecular weights were determined according to Meyers *et al* [12] using standard DNA molecular weight markers of 0.12-23.1 Kbp (Roche Diagnostic GmbH).

2.8 PCR Amplification of *bla*CTX-M gene: A chromosomal DNA template was prepared by boiling picked colonies of ESBL isolates suspended thoroughly in 1 mL DNase and RNase–free water and incubated at 55^{0}C for 10 minutes. After centrifugation, the supernatant was used as template DNA. The template DNA was quantified by using a Thermoscientific Nanodrop Spectrophotometer at an absorbance of 260 nm. DNA was amplified for CTX-M gene by Polymerase Chain Reaction (PCR) using specific oligodeoxynucleotides for *blaCTX-M (F* 5` GCG ATG TGC AGG ACC AGT AA 3` and *R* 5` CCG CGA TAT CGT TGG TGG TG 3`) to amplify a 250 bp fragment (Norgen Biotek, Canada). The PCR was performed in 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 blaCTX-M F (2500 pmol), 0.2 µl blaCTX-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 minutes initialization at 94℃, followed by 30 cycles of 45 s at 94℃, denaturation at 94℃ for 60 seconds, annealing at 60℃ for 60 seconds, *extension at* 72℃ for 60 seconds. Amplified products were separated by 100V electrophoresis on a 2% agarose gel containing 0.5 µg/ml ethidium bromide for 30 minutes, after which they were viewed under ultraviolet light to determine the specific *bla* gene fragments.

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 a confidence interval of 95%. Significance of the high MIC for the presence of the *bla*CTX-M gene

was determined by the 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 characteristics 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 producers with very high average molecular plasmid weight of 7.91 Kbp. Among the 11 ESBL producers only 7 isolates transconjugated and 8 successfully transformed susceptible isolates, respectively. Also, only 6 (2.8%) harbored *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 β-lactamase producers while 91.0% resistance was observed among isolates to Ampicillin, Cefotaxime and Ceftazidime, respectively. It is worth noting that *E. coli* and *Klebsiella oxytoca* show resistance rates 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 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)

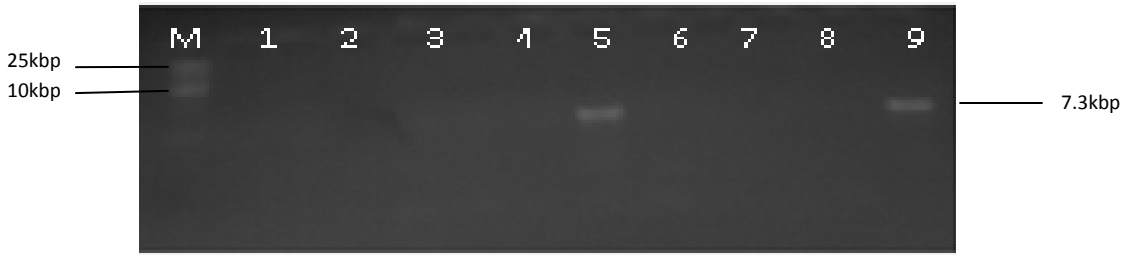

Figure 1. *bla*$_{CTX-M}$ DNA fragments recovered from the ESBL positive isolates subjected to agarose gel electrophoresis. Lane M: high molecular weight DNA markers (Supercoiled DNA Ladder, Gibco BRL, France); lane 5 and 9: *bla*$_{CTX-M}$ specific DNA bands.

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 the CTX-M family of ESBLs is now becoming an emerging global health threat due to its unfavorable outcome in the treatment of common enteric infectious diseases in the community and among hospital patients. It was clearly observed from the study that out of 27.4% β-lactamase producing enteric flora obtained from the community residents, only 5.1% were ESBL producers. This rate was lower than the 11.4% recorded in Enugu, Enugu State, South-East Nigeria [13] and also the 25% rate in Lagos, Nigeria [14]. Although there is no established report of ESBLs prevalence in any community in Nigeria, a similar study in Cambodia recorded 36.6%

community-acquired ESBLs [15]. Our findings suggest that there is undetected dissemination of community-acquired *bla*CTX-M genes among its 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 isolated from community residents further indicated 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 β-lactamase mostly decrease antibiotic susceptibility to commonly used antibiotics, thereby causing economic losses to the society and prolonged treatment of infections. 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 species or other species. This transformation process might be enhanced by constant release of naked DNA from dying bacteria, which can be taken up by other isolates through unhygienic eating habits of residents at food joints, restaurants and street food vendors of poor standards. Moreover, sachet-water supplies of drinking contain bacterial DNA [16, 17], which is highly susceptible to transformation of 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 Cephalosporins, 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 generations 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 association with foodstuff, animal consumption of antibiotics, and frequent patient contact with relatives during illness may be major factors to consider.

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

Conclusion: Adequate detection of new resistance mechanisms of the emerging $bla_{\text{CTX-M}}$ 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 individual that met the criteria of the study. They were informed of the benefit of the study and freedom to withdraw if they wished as a non-invasive method of fecal collection was applied.

Author 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 search and all authors read and approved the final manuscript

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