

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

Akinduti P.A^{1*}; Oluwadun A¹, Iwalokun B², Onagbesan O.M³, Ejilude O.¹

1. Department of Medical Microbiology & Parasitology, Olabisi Onabanjo University, Sagamu Campus, Ogun State, Nigeria.

2. Biotechnology Centre, University of Agriculture, Abeokuta, Ogun State, Nigeria.

3. Department of Biochemistry, Nigerian Institute of Medical Research, Lagos, Nigeria

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: 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, *bla*CTX-M.

*Corresponding author: Phone number: +234 7038450956 E-mail: niyiakinduti@gmail.com

1.0 Introduction

CTX-M β -lactamases are mutant, plasmid-mediated β -lactamase enzyme with potent hydrolytic activity against cefotaxime which 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 β -lactamases, may express altered antibiotic resistance phenotype [4]. Thus, organisms producing

CTX-M-type β -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 setting for implementing epidemiological measures to curtail and control this spread. Nevertheless, the increasing rate of 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 diarrhoeal and other enteric diseases raises speculation that oxyimino cephalosporins available in many patent 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 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 subjects 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). Then, incubated at 37°C for 18-24 hours. Each isolate obtained was identified using API enterobacteriaceae kit for their biotyping and interpreted according to World Health Organization (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 bacteria isolate against the commonly used antibiotics of 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 isolate 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]. 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 Muller-Hinton agar plate and ceftazidime (30 µg), ceftazidime + clavulanic acid (30/10 µg), cefotaxime + clavulanic acid (30/10 µg) and cefotaxime (30 µg) discs were placed 2 cm apart on the plate in a straight

line. A difference of 5mm or more between the inhibition zones of disc containing ceftazidime (30µg) and clavulanic acid (10µg) and ceftazidime (30µg) disc alone, was regarded as ESBL producer. *Escherichia coli* ATCC 25922 was used as control isolate.

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°C, containing 1:10 ratio of donor to recipient. It was incubated for 3 hours and then sub-cultured on MacConkey Agar plates supplemented with 6mg/ml ceftazidime (Oxoid). Transconjugants growing on selection plates were subjected to DDST to confirm transfer of β -lactamase genes and to examine the 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 *Escherichia coli* ATCC 25922 in Luria-Bertani broth supplemented with 2M Calcium chloride, then incubated at 37°C [11]. The mixture was later sub-cultured on selection plate of Muller Hinton Agar supplemented with 6mg/L ceftazidime and incubated at 37°C for 18-24 hours. Transformants growing on selection plates were subjected to DDST.

2.7 Plasmid profiling: Extra-chromosomal plasmid DNA was extracted from each isolate by Alkaline lysis method [10], [12]. 1000µL bacteria cell suspension was lysed in 1% SDS containing 0.2M NaOH (pH 8.0) and 150µL ice-cold 3.0M 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 14µl/g Ethidium bromide. The DNA was then 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 0.12-23.1Kbp (Roche Diagnostic GmbH).

2.8 PCR Amplification of *bla*CTX-M gene: Chromosomal DNA template was prepared by boiling 1ml suspension of pure colonies of ESBL isolate in DNase and RNase-free water at 55°C for 10 minutes. 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 2.5µl 10X PCR buffer, 1.5µl 25mM MgCl₂, 0.5µl 10mM dNTPs (dATP, dGTP, dCTP, dTTP), 0.2µl *bla*CTX-M F (2500 pmol), 0.2µl *bla*CTX-M R (2500 pmol), 0.2µl Taq polymerase enzyme (*Thermus aquaticus*), 17.9µl distilled water and 2.0µl of each extracted template DNA to make a final reaction volume of 25.0µl in a thermal cycler block (NYX Technik Inc; Model ATC401, USA). The PCR mixture was subjected to a 5 minutes initialization at 94°C, followed by 30 cycles of 45 s at 94°C, 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 100V electrophoresis in 2% agarose gel containing 0.5µg/ml ethidium bromide for 30 minutes, 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 at p value <0.05 at confidence interval of 95%. Significance of the high MIC and *bla*CTX-M gene was determined by Pearson test while the correlation coefficient was calculated by the method of least squares. Significance of high level 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 producer only 7 isolates transconjugated and 8 successfully transformed susceptible isolates respectively. Also, only 6(2.8%) harboured *bla*CTX-M genes among the identified ESBL producers.

Prevalence of resistant enteric isolates in the studied community: β -lactamase producer show very high resistance rate of 98.8% to cefuroxime while 91.0% resistance to Ampicillin, cefotaxime and ceftazidime were respectively observed. It is worth noting that *Escherichia coli* and *Klebsiella oxytoca* show resistance rate of 5.2% and 7.8% respectively, to imipenem 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)	<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 spp</i>	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 aeruginosa</i>	39(18.3)	10(4.7)	1(2.4)	7.4	1	1	1(0.5)
<i>Proteus mirabilis</i>	31(14.6)	9(4.2)	1(3.2)	8.7	1	1	1(0.5)
<i>Shigella spp</i>	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)

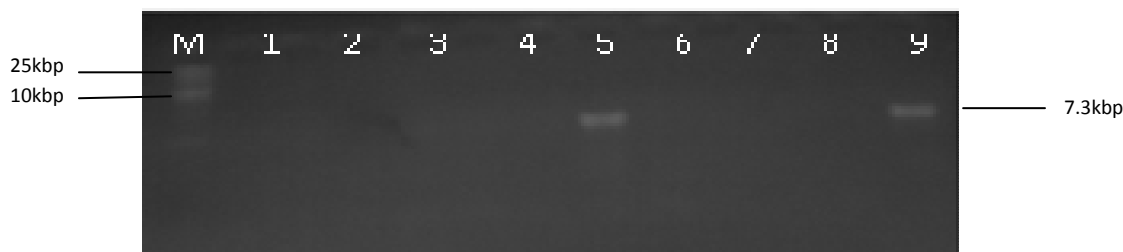


Figure 1 show the *bla*_{CTX-M} DNA amplicons recovered from the ESBL positive isolates on Agarose gel. Lane M is high molecular weight DNA markers (Supercoiled DNA Ladder, Gibco BRL, France); lane 5 and 9 are *bla*_{CTX-M} DNA bands.

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

Enteric isolates	Antimicrobial agents (MIC range 2 to 8ug/ml)									
	AMP	AMC	CFX	AZT	CPX	IMP	CTX	TET	CFZ	GN
<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 spp</i>	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)

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 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 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, 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 residents and this could be elicited by self-medication and use of self-prescribed antibiotics which is a common practice in this locality. Plasmid DNA isolated from enteric bacteria obtained from the 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 β -lactamase could decrease antibiotic susceptibility to commonly use antibiotics, thereby causing economic loss 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 bacterial 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 with poor standard of food hygiene. 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 cephalosporin, such as ceftazidime, cefuroxime and cefotaxime with MIC >16 μ g/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 use of antibiotic for food preservatives, animal consumption of antibiotics, and frequent patient contact with relatives during illness may be some major factors to consider.

However, some of the antibiotic resistance genes usually associated with mobile elements are also associated with chromosomal transmission of resistant genes which maintain 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 gene could encode virulence alleles which could be involved in colonization, infection, and persistence in humans [20, 21]. These factors simultaneously explain the spread and infection persistence of the 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 those 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 faecal collection was used.

Authors' contribution: 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 review and all the authors read and approved the final manuscript.

Reference

1. Tzouveleakis LS, Tzelepi E, Tassios PT, Legakis NJ. CTX-M-type beta-lactamases: an emerging group of extended-spectrum enzymes. *Int. J. Antimicrob. Agents*. 2000; 14:137-142.
2. Yu WL, Pfaller MA, Winokur PL, Jones RN. Cefepime MIC as a predictor of the extended-spectrum beta-lactamase type in *Klebsiella pneumoniae*, Taiwan. *Emerg. Infect. Dis*. 2002; 8:522-524.
3. Poirel L, Kampfer P, Nordmann P. Chromosome-encoded Ambler class A beta-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother*. 2002; 46:4038-4040.
4. Yan JJ, Ko WC, Tsai SH, Wu HM, Jin YT, Wu JJ. Dissemination of CTX-M-3 and CMY-2 beta-lactamases among clinical isolates of *Escherichia coli* in southern Taiwan. *J. Clin. Microbiol*. 2000; 38:4320-4325.
5. Davis J. Inactivation of antibiotics and the dissemination of resistant genes. *Science*. 1994; 264:375-382.
6. Valverde A, Grill F, Coque TM, Pintado V, Baquero F, Cantón R. High rate of intestinal colonization with extended-spectrum-beta-lactamase-producing organisms in household contacts of infected community patients. *J. Clin Microbiol*. 2008; 46:2796-9.
7. World Health Organisation. Manual for laboratory investigation of acute enteric infections, CDD/83.3 1983. WHO, 121 Geneva, 27-Switzerland.
8. National Committee for Clinical Laboratory Standards. *Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement M100-S12*, 2002 NCCLS, Villanova, PA, USA.
9. Odugbemi TO, Hafiz S, McEntegart MG. Penicillase producing *Neisseria gonorrhoea*: Detection by Starch Iodide Paper Techniques. *Br. Med. J*. 1997; 11:500.

10. Birnboim HC and Doly JA. Rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 1979; 7:1513-1523.
11. Smith CJ, Markowitz SM, Macrina FL, 1981. Transferable tetracycline resistance in *Clostridium difficile*. *Antimicrob. Agents Chemother.* 1981; 19:997–1003.
12. Meyers, J.A., Sanchez, D., Elwell, L.P. and Falkows. Simple agarose gel electrophoretic method for the identification and characterization of plasmids deoxyribonuclease acid. *J. Bacterial.* 1976; 1127:1529-1537.
13. Iroha IR, Adikwu MU, Esimone CO, Aibinu I, Amadi ES. Extended spectrum Beta – Lactamase (ESBL) in *E.coli* isolated from a tertiary hospital in Enugu state, Nigeria. *Pakistan J. Med. Sci.* 2009; 25:2.
14. Aibinu IE, Olaegbulam VC, Adenipakan EO, Ogunsola FT, Odugbemi JO, Mee BJ. Extended spectrum beta-lactamase enzymes in clinical isolates of Enterobacteria species from Lagos. *Nig. J. Clin. Microbiol.* 2003; 41:2197-2200.
15. Etienne R, Sopheak H, Sovannarith L, Valerie G, Frederic A, Jean-Louis S, Didier M, Guillaume A. CTX-M beta-lactamase in *Escherichia coli* from community-acquired urinary tract infections, Cambodia. *Emerg. Infect. Dis.* 2009; 15:5.
16. Stobberingh, EE, Arends J, Kostanje JA, Goessens WHF, Visser MR, Buiting AGM, Ossenkopp YJ, Van Ketel RJ, Vanogtrop ML, Sabbe LJ, Voorn GP, Winter HL, Van Zeijl JH. Occurrence of extended spectrum β -lactamases in Dutch hospitals. *Infection* 2000; 27:348-354.
17. Xiong, Z, Zhu D, Zhang Y, Wang F. Extended spectrum β -lactamases in *K. pneumonia* and *E. coli* isolates. *Zhonghua Yi Xue Za Zhi*; 2002; 82:1476-1479.
18. Jain A, Roy I, Gupta MK, Kumar M, Agarwal SK, 2003. Prevalence of extended spectrum β -lactamase producing Gram-negative bacteria in septicemia neonates in a tertiary care hospital. *J. Med. Microbiol.* 2003; 52:42-45.
19. Guillaume A. CTX-M beta-lactamase in *Escherichia coli* from community-acquired urinary tract infections, Cambodia. *Emerg. Infect. Dis.* 2009;15: 5.
20. Akinduti PA, Akinbo JA, Adenuga WF, Ejilude O, Umahoin KO, Ogunbileje JO. Occurrence of Extended-Spectrum β -lactamase Producing Enterobacteriaceae (ESBLPE) among Primary School Pupil in Obafemi-Owode, Nigeria. *J. Life Sci.* 2011; 5:340-344.

293 **21. Sirot D, Sirot J, Labia R, Morand A, Courvalin P, Darfeuille-Michaud A, Perroux R, Cluzel R.**
294 **Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella***
295 ***pneumoniae*: identification of CTX-1, a novel beta-lactamase. *J. Antimicrob. Chemother.***
296 **1987; 20:323-334.**
297