

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

8 **ABSTRACT**

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

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

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

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

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

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

26

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

28

29 1.0 Introduction

30 CTX-M β -lactamases are mutants~~s of~~, plasmid-mediated β -lactamases with potent
31 hydrolytic activity against cefotaxime ~~and have been~~~~is now~~ commonly
32 encountered among numerous enteric bacteria isolates [1, 2]. In recent past,
33 increasing prevalence of bacterial pathogens producing CTX-M β -lactamases has
34 trigger multiple resistance to most commonly used 3rd Generation cephalosporin
35 [3]. However, some CTX-M-type ESBLs may actually hydrolyze ceftazidime and
36 confer resistance to this cephalosporin. It was noted that the same organism
37 harbouring both CTX-M-type and SHV-type ESBLs or CTX-M-type ESBLs and
38 AmpC-type β -lactamases, may express altered antibiotic resistance phenotype
39 [4]. Thus, organisms producing CTX-M-type β -lactamases typically are now
40 know~~n~~ to have high minimum inhibitory concentration (MIC) range against
41 cefotaxime, while ceftazidime MIC is usually in the apparently susceptible range
42 [5].

43 Moreover, faecal colonisation with ESBL-producing isolates is fast becoming
44 prevalent in our society while it is a common practice in the hospital setting for
45 implementing epidemiological measures to curtail and control this spread.
46 Nevertheless, the increasing rate of inpatients, outpatients and healthy residents
47 colonised with cephalosporin resistant strains are now potential risk ~~factor~~ for
48 acquisition and transmission of resistant CTX-M β -lactamases in many
49 households [6]. The relationship between antibiotic consumption and occurrence
50 of CTX-M β -lactamases has not been well studied in this locality. Although, the
51 prevalence of resistant strains causing community-acquired diarrhoea and other

52 enteric diseases raises speculation that oxyimino cephalosporins available in
53 many patent stores were taken without regulated prescription. Therefore, the
54 emergence and spread of antibiotic resistance bacteria with CTX-M β -lactamases
55 in this community which has become a threat to effective treatment of enteric
56 infection were studied.

57

58 2.0 Materials and methods

59 2.1 Community ~~S~~sample collection: Fresh faecal samples of two hundred and fifty
60 one (251) were randomly collected from the community residents in Abeokuta,
61 who consented to participate in the study and who have not used any
62 antimicrobial agents in the preceding two weeks either as therapy for gastro-
63 intestinal complication or prophylaxis between August 2010 and March 2013.
64 Their use of antimicrobial agents, feeding habit and hygiene in the last three
65 months was exclusion criteria used for the subjects selection. Informed consent
66 of all individual involved in this study was obtained while they were assured of
67 confidentiality in view of the intricacy and sensitivity of the study.

68

69 2.2 Isolation and identification: All the faecal samples collected were cultured on
70 Salmonella-Shigella agar (Oxoid CM 123, UK) and MacConkey agar without salt
71 (Oxoid CM 516, UK); and incubated at 37°C for 18-24 hours. Each isolate obtained
72 was identified using API enterobactericaea kit for the biotyping and were
73 interpreted according to World Health Organisation (WHO) manual for laboratory
74 investigation of acute enteric infections [7].

75

76 **2.3 Antibiotic susceptibility tests:** Standard broth micro-dilution method was used
77 to determine the MIC of each identified isolates against the commonly used
78 antibiotics with dilution ranges; Ampicillin (0.5–64 $\mu\text{g/mL}$),
79 Amoxicilli/Clavulanate (0.5–32 $\mu\text{g/mL}$), Gentamycin (0.5–64 $\mu\text{g/mL}$),
80 Ciprofloxacin (0.5–64 $\mu\text{g/mL}$), Cefuroxime (1–64 $\mu\text{g/mL}$), Ceftazidime (0.25–128
81 $\mu\text{g/mL}$), Tetracycline (0.25–6 $\mu\text{g/mL}$), Cefotaxime (0.25–64 $\mu\text{g/mL}$),
82 Azithromycin (0.5–64 $\mu\text{g/mL}$) and Imipenem (0.25–16 $\mu\text{g/mL}$). The reference
83 strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923
84 were used to check the quality and precision of the testing procedures. MIC of
85 each isolates was determined according to NCCLS recommended guidelines [8].

86

87 **2.4 Phenotypic detection of beta-lactamase and ESBL:** Isolates showing MIC >8
88 $\mu\text{g/mL}$ to Cefuroxime, Ceftazidime and Cefotaxime were tested for beta-
89 lactamase production by a starch-iodide paper acidometric method described by
90 Odugbemi *et al.* [in](#), 1977 [9]. ESBL Double Disc Synergy Test (DDST) was
91 performed according to recommended method and criteria of CLSI [10]. 0.5
92 McFarland standard of bacterial suspension was evenly spread onto the surface
93 of the Mueller–Hinton agar plate and Ceftazidime (30 μg), Ceftazidime + clavulante
94 (30/10 μg) Cefotaxime + clavulante (30/10 μg) and cefotaxime (30 μg) discs were
95 placed 2 cm apart on the plate in a straight line. A difference of 5 mm or more
96 between the inhibition zones of the ceftazidime (30 μg) plus clavulanic acid (10

97 | μg) and ceftazidime (30 μg) disc alone, was taken to indicate an ESBL producer.
98 | *Escherichia coli* ATCC 25922 was used as controls.

99

100 | **2.5 Mating activity:** The confirmed ESBL isolates were mated with susceptible *E.*
101 | *coli* recipient strains, ATCC 25567 (penicillin-resistant and lactose fermentation-
102 | positive strain). Overnight cultures of donor cells of identified ESBL isolates and
103 | recipient strains (*E. coli* ATCC 25677) were grown in 0.5% sucrose enriched Luria-
104 | Bertani broth at 37°C, after mixing together at a 1 :10 ratio (donor to recipient) and
105 | incubated for 3 hours and then sub-cultured on MacConkey Agar plates
106 | supplemented with 6 mg/ml ceftazime (Oxoid). Transconjugants growing in the
107 | selection plates were subjected to DDST to confirm transfer of β -lactamase
108 | genes and also examine for the co-transfer of other antibiotic resistance
109 | determinants present in the donor isolates.

110

111 | **2.6 Plasmid DNA transformation:** DNA plasmid extracted by an alkaline lysis
112 | method [10] of 20 μl was added to 5 ml of overnight enriched Luria-Bertani broth
113 | culture of cephalosporin susceptible *Escherichia coli* ATCC 25922 ~~isolates~~ in the
114 | presence of 2 M ~~C~~calcium chloride and the mixture was incubated at 37 °C for 6
115 | hours according to Smith et al., ~~(1981)~~ [11]. The mixture was later sub-cultured on
116 | selection plate of Muller Hinton Agar supplemented with 6 ~~m~~ $\mu\text{g/mL}$ ceftazidime
117 | and incubated at 37°C for 18-24 hours. Transformants growing in the selection
118 | plates were subjected to DDST.

119

120 2.7 Plasmid profiling: Extra-chromosomal plasmid DNA was extracted from each
121 isolates according to the alkaline lysis method [10], ~~[12]~~ using 1000 ~~μ~~^uL of the
122 bacteria cell suspension lysed in 1% SDS, 0.2_M NaOH (pH 8.0) and precipitated in
123 150 ~~μ~~^uL of ice-cold 3.0 M Potassium Acetate, pH 5.5 [12]. The obtained plasmids
124 DNAs ~~obtained~~ were then electrophoresed on 0.8% ~~A~~agarose gel, stained with 14
125 ~~μ~~^l/g Ethidium bromide. The DNA was then viewed using UV trans-illumination and
126 photographed with Polaroid camera ~~and viewed using UV trans-illumination~~. The
127 molecular weights were determined according to Meyers *et al.* [12] using
128 standard DNA molecular weight marker of 0.12-23.1_kbp (Roche Diagnostic
129 GmbH).

Comment [D1]: Please verify the concentration.
Normally, 0.5 μg/ml is used.

130
131 2.8 PCR Amplification of *bla*CTX-M gene: Chromosomal DNA template was
132 prepared by boiling pure colonies of ESBL isolates which was suspended
133 thoroughly in 1_mL DNase_ and RNase-free water and boiled at 55_°C for 10
134 minutes. After centrifugation, supernatant was used as template DNA. The
135 template DNA was quantified by Thermoscientific Nanodrop Spectrophotometer
136 of absorbance of 260_nm filter and estimated. The extracted DNA template was
137 amplified for CTX-M gene by Polymerase Chain Reaction (PCR) using specific
138 oligodeoxynucleotides *bla*CTX-M F 5_^_GCG ATG TGC AGG ACC AGT AA_3_^_
139 and *bla*CTX-M R 5_^_CCG CGA TAT CGT TGG TGG TG_3_^_ to amplify minimum of
140 250_bp amplicon (Norgen Biotek, Canada). The PCR was performed in mix of 2.5
141 ~~pl of 10x~~ PCR buffer, 1.5 ~~pl of~~ 25 mM MgCl₂, 0.5 ~~pl of~~ 10 mM dNTPs (dATP, dGTP,
142 dCTP, dTTP), 0.2_ ~~pl of~~ *bla*CTX-M F of 2500_pmole, 0.2_ ~~pl of~~ *bla*CTX-M R of 2500

Formatted: Not Superscript/ Subscript

Comment [D2]: μl? Please check all the
paragraph.

Formatted: Subscript

143 | pmole, 0.2_μl of Taq polymerase enzymes (*Thermus aquaticus*), 17.9_μl of distilled
144 | water and 2.0_μl of each extracted template DNA to make final reaction volume of
145 | 25.0_μl in a thermal cycler block (NYX Technik Inc; Model ATC401, USA). The PCR
146 | mixture was subjected to a 5 minutes initialization at 94°C, followed by 30 cycles
147 | of 45 s at 94°C, denaturation at 94°C for 60 seconds, annealing at 60°C for 60
148 | seconds, extension at 72°C for 60 seconds. Amplified products were separated by
149 | ~~100V~~ electrophoresis at 100 V in a 2% agarose gel containing 0.5_μg/ml ethidium
150 | bromide for 30 minutes, after which they were viewed under ultraviolet light to
151 | determine the specific *bla* gene amplicons.

152

153 | **2.9 Statistical analysis:** Risk factors for ESBL prevalence among the studied
154 | community was assessed by using Chi square to determine the significance of
155 | ESBL isolates at p- value <0.05 at confidence interval of 95%. Significance of the
156 | high MIC to the occurrence of *bla*CTX-M gene was determined by Pearson test
157 | while the correlation coefficient was calculated by the method of least squares
158 | and high level of resistance among the isolates was determined at p<0.005.

159

160 | **3.0 Results**

161 | **Molecular characteristic of study strains:** Of 215 non-duplicated enteric isolates
162 | obtained from the community residents, 11_ (5.1%) strains met CLSI screening
163 | criteria as potential ESBL producer, with very high average molecular plasmid
164 | weight of 7.91_kbp. Among the 11 ESBL producers, only 7 isolates
165 | transconjugated and 8 successfully transformed susceptible isolates

166 | respectively. Also, only 6 (2.8%) harbour *bla*CTX-M genes among the identified
 167 | ESBL producers.

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

174

175 | 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</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 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</i> 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)

Formatted: Spanish (Mexico)

Formatted: Font: Not Italic, Spanish (Mexico)

Formatted: Font: Not Italic, Spanish (Mexico)

Formatted: Spanish (Mexico)

Formatted: Font: Not Italic

176

177

178

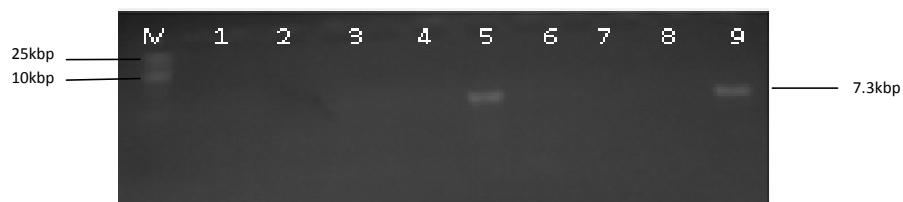


Figure 1. Photograph of electrophoresis showing the bla_{CTX-M} DNA amplicons recovered from the ESBLs positive isolates on Agarose gel. Lane M is high rang ladder DNA markers (Supercoiled DNA Ladder, Gibco BRL, France) while lanes 5 and 9 showing the bla_{CTX-M} DNA bands.

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

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

193 The prevalence of CTX-M family of ESBLs is now becoming an emerging global
194 health threat due to its unfavourable outcome in the treatment of common enteric
195 infectious diseases in the community and among the hospital patients. It was
196 clearly observed from the study that out of 27.4% beta-lactamase producing
197 enteric flora obtained from the community residents, only 5.1% were ESBL
198 producers. This rate was lower to 11.4% recorded in Enugu, Enugu State, South-
199 East Nigeria [13]; and also 25% rate in Lagos, Nigeria [14]. Although there is no
200 established report of ESBLs prevalence in any community in Nigeria, but similar
201 study in Cambodia recorded 36.6% community-acquire ESBLs [15]. Our findings
202 suggest that there is undetected dissemination of community-acquired *bla*CTX-M
203 genes among this community residents and this could be elicited by self-
204 medication and use of unprescribed antibiotics which is a common practice in
205 this locality. Conjugative DNA plasmid obtained from the community resident
206 further indicate a potential reservoir of antibiotic resistance bacteria that could
207 contain a pool of mobile genetic elements, which could be readily disseminated
208 to other human pathogens. Therefore, transfers of naked resistant DNA plasmids
209 encoding β -lactamase mostly decrease antibiotic susceptibility to commonly use
210 antibiotics, thereby causing economic loss to the society and prolonged
211 treatment of infection. High transformation achieved by the ESBL isolates from
212 both the community and the hospital, suggest a very high frequency of resistant
213 gene transfer between the same bacterial species or other different species. This
214 transformation process could be promoted by constant release of naked DNA by
215 diedding bacteria which can be taken up by other isolates-bacteria through

216 unhygienic eating habit of most residents in poorly located food joints,
217 restaurants and street food vendors and sachet-water supplied for drinking that
218 usually contain this naked DNA_s genes [16, 17], which has high propensity for
219 transforming a susceptible isolates. This would definitely prone these residents
220 to live in grave danger of increasing antibiotic resistant resulting to poor
221 therapeutics, causing increasing morbidity.

222 Highly diverse antibiotics resistance rates against all cephalosporin, such as
223 ceftazidime, cefuroxime and cefotaxime with MIC >16 µg/mL₁ represent a very
224 critical situation as compared to investigations from other regions of the world
225 reporting resistance towards first, second and third generation of cephalosporin
226 [18,19]. The spread of antimicrobial resistance among bacterial pathogens to
227 most antibiotics in this locality is fast becoming an important fatal challenge for
228 the community and health institutions due to unguided use of over-the-counter
229 drugs mostly antibiotics. Unfortunately, data regarding bacterial resistance to
230 antimicrobial agents is limited in South-western, Nigeria. The cause of this
231 upsurge in resistant of community-acquired ESBL-producing organisms is not
232 yet clear, but associations with foodstuffs, animal consumption of antibiotics,
233 and frequent patient contact with relatives during illness may be a major factor to
234 consider.

235 However, some of the antibiotic resistance genes₁ usually associated with mobile
236 elements₁ were also associated with chromosomal transmission of resistant gene
237 which maintains the spread of CTX-M extended spectrum β-lactamases among
238 pathogenic bacteria populations, even if the subjects are not treated with

239 extended-spectrum cephalosporins, especially in countries where antibiotics
240 usage is unregulated. Interestingly, *bla*CTX-M clone could exhibit virulence traits
241 which could be involved in colonization, infection, and persistence in humans
242 [20, 21]. These factors simultaneously explain the spread and the persistence of
243 community spread of the CTX-M-encoding genes among enteric isolates in this
244 community.

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

248

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

251

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

257

258 **Authors' contributions:** Akinduti P.A and Oluwadun A designed the study,
259 performed the statistical analysis, wrote the protocol, and wrote the first draft of
260 the manuscript. Iwalokun B.A and Onagbesan K.O managed the analyses of the

261 study. Ejilude O. managed the literature searches and all the authors read and
262 approved the final manuscript

263
264

265 Reference

- 266 1. Tzouveleakis LS, Tzelepi E, Tassios PT, Legakis NJ. CTX-M-type beta-lactamases: an
267 emerging group of extended-spectrum enzymes. *Int. J. Antimicrob. Agents.* 2000; 14:137-
268 142.
- 269 2. Yu WL, Pfaller MA, Winokur PL, Jones RN. Cefepime MIC as a predictor of the extended-
270 spectrum beta-lactamase type in *Klebsiella pneumoniae*, Taiwan. *Emerg. Infect.*
271 *Dis.*, 2002; 8:522-524.
- 272 3. Poirel L, Kampfer P, Nordmann P. Chromosome-encoded Ambler class A beta-lactamase
273 of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum
274 beta-lactamases. *Antimicrob. Agents Chemother.* 2002; 46:4038-4040.
- 275 4. Yan JJ, Ko WC, Tsai SH, Wu HM, Jin YT, Wu JJ. Dissemination of CTX-M-3 and CMY-2
276 beta-lactamases among clinical isolates of *Escherichia coli* in southern Taiwan. *J. Clin.*
277 *Microbiol.* 2000; 38:4320-4325.
- 278 5. Davis J. Inactivation of antibiotics and the dissemination of resistant genes. *Science.*
279 1994; 264: 375-382.
- 280 6. Valverde A, Grill F, Coque TM, Pintado V, Baquero F, Cantón R. High rate of intestinal
281 colonization with extended-spectrum-beta-lactamase-producing organisms in household
282 contacts of infected community patients. *J. Clin Microbiol.* 2008; 46(8):2796-2799.
- 283 7. World Health Organisation. Manual for laboratory investigation of acute enteric infections,
284 CDD/83.3 1983. WHO, 121 Geneva, 27-Switzerland.
- 285 8. National Committee for Clinical Laboratory Standards. *Performance Standards for*
286 *Antimicrobial Susceptibility Testing: Twelfth Informational Supplement M100-S12*, 2002
287 NCCLS, Villanova, PA, USA.
- 288 9. Odugbemi TO, Hafiz S, McEntegart MG. Penicillase producing *Neisseria gonorrhoea*:
289 Detection by Starch Iodide Paper Techniques. *Br. J. Med.* 1997; 11:500.
- 290 10. Birnboim HC and Doly JA. Rapid alkaline extraction procedure for screening recombinant
291 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. Bacteriol.* 1976; 1127: 1529-1537.
13. Iroha IR, Adikwu MU, Esimone CO, Aibinu I, Amadi ES. Extended spectrum Beta – Lactamase (EBSL) in *E. coli* isolated from a tertiary hospital in Enugu state, Nigeria. *Pakistan Journal of Medical Sciences*. 2009; 25:279-282.
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. Chin. Microbiol.* 2003; 41:2197-2200.
15. Ruppé E, Hem S, Lath S, Gautier V, Arieu F, Sarthou JL, Monchy D, Arlet G, 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. *Emerging Infectious Diseases*. 2009; 15:741-7485.
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 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. *Journal of Medical Microbiology*. 2003; 52:42-45.
19. Guillaume A. CTX-M beta-lactamase in *Escherichia coli* from community-acquired urinary tract infections, Cambodia. *Emerging Infectious Diseases*. 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. *Journal of Life Sciences*. 2011; 5:340-344.
21. Sirot D, Sirot J, Labia R, Morand A, Courvalin P, Darfeuille-Michaud A, Perroux R, Cluzel R. Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella*

Formatted: English (United States)

Formatted: Font: Not Italic

Comment [D3]: Please verify this reference. It seems the same with reference 15

324 *pneumoniae*: identification of CTX-1, a novel beta-lactamase. *J. Antimicrob. Chemother.*
325 | 1987;20:323-334.
326