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Faecal carriage of extended-spectrum beta-lactamase (ESBL)-producing commensal *Klebsiella pneumoniae* and *Escherichia coli* from hospital out-patients in Southern Nigeria

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Normal intestinal microflora is the major source from which common hospital- and community-acquired infections originate. This study aimed to determine faecal carriage of extended-spectrum beta-lactam (ESBL) resistant genes from commensals of out-patients in Nigeria. Non-duplicate *Klebsiella pneumoniae* and *Escherichia coli* from different hospitals were investigated for susceptibility to a panel of antibiotics, carriage of plasmid mediated β-lactam resistance, and analysis of plasmids present, including replicon typing. The minimum inhibitory concentrations (MICs) for β-lactam antibiotics showed MIC_{90} of ≥256 µg/ml for all antibiotics. *CTX-M* carriage was 36.8% for the 114 strains; 30 of these were *CTX-M-15* and 12 carried *CTX-M-2*. *TEM-1* genes were present in 102 isolates (89.5%), *SHV-1* genes in 24 (21.1%), *OXA-1* in 36 (31.6%) and 10 (8.8%) in *AmpC* genes detected. There was no significant difference in the proportion of ESBL genes detected in *E. coli* and *K. pneumoniae* (*t* test; $p = 0.64$; $p > 0.05$) and between hospitals ($\chi^2 = 0.35$; $p = 0.84$; $p > 0.05$). *IncF* was the common plasmid encoding β-lactamases. High faecal carriage of ESBL genes in commensals, importantly classical *CTX-M-15* in out-patients is a reflection of the prevalence from clinical specimens in diseased conditions in Nigeria.

Key words: Faeces, bacteria, plasmid, extended-spectrum beta-lactam (ESBL) genes, Nigeria.

INTRODUCTION

Production of extended-spectrum β-lactamases (ESBLs) is the most common mechanism of resistance to third-generation cephalosporins among *Enterobacteriaceae* including *Klebsiella pneumoniae* and *Escherichia coli* (Paterson and Bonomo, 2005; Pitout and Laupland, 2008). ESBL determinants have been detected not only in clinical isolates but also in commensal bacteria from humans and animals and in isolates from products of the food chain and sewage, revealing distribution and suggesting the presence of environmental reservoirs for these resistance determinants (Brinas et al., 2004).

Reducing the spread of plasmid-mediated resistance genes in hospitals requires the identification of the genes involved in order to control the movement of this resistance mechanism. Over the last 6 years, CTX-M (especially CTX-M-15) β-lactamase-producing *E. coli* have increased rapidly in number both within and outside the hospital environment and make up the vast majority of isolates (Woodford et al., 2004; White, 2008). CTX-M β-lactamases have been shown to be currently the most common cause of multidrug resistance in *E. coli* from other areas of the world; in particular, the Far East and South-East Asia, where rates can be as high as 50 to 70% (Hawkey, 2008), are a growing problem in some

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Table 1. Primers used to amplify genes for ESBLs.

Forward primer	Sequence (5´to3´)	Reverse primer	Sequence (5´to3´)	Annealing temp. (ºC)	Product size (bp)
CTXM	CGATGTGCAGTACCAGTAA	CTXM	TTAGTGACCAGAATAAGCGG	60	585
OXA	ATATCTCTACTGTTGCATCTCC	OXA	AAACCCTTCAAACCATCC	56	620
SHV1	AGGATTGACTGCCTTTTTG	SHV1	ATTTGCTGATTTCGCTCG	56	393
TEMH	CCCCGAAGAACGTTTTC	TEMC	ATCAGCAATAAACCAGC	51	517
CTXM1	GACGATGTCACTGGCTGAGC	CTXM1	AGCCGCCGACGCTAATACA	60	499
CTXM2	TGATACCACCACGCCGCTC	CTXM2	TATTGCATCAGAAACCGTGGG	60	341
CTXM8	CGCTTTGCCATGTGCAGCACC	CTXM8	GCTCAGTACGATCGAGCC	60	307
CTXM9	GCTGGAGAAAAGCAGCGGAG	CTXM9	GTAAGCTGACGCAACGTCTG	60	293
CTXM25	CGCTTTGCCATGTGCAGCACC	CTXM25	GCTCAGTACGATCGAGCC	60	307

parts of Europe (Hawser et al., 2011), Latin America (Pallecchi et al., 2007) and Senegal in Africa (Ruppe et al., 2009). To the best of our knowledge, no definitive studies have been done to determine faecal carriage of ESBLs-producing bacterial isolates in Nigeria in spite of report of high level of β-lactamases resistance genes in this environment including *TEM, SHV, OXA, CTX-M* and *AmpC* types (Ogbolu et al., 2011). This study was conducted to determine the carriage of ESBLs resistant genes in faecal isolates of commensal *E. coli and Klebsiella* species in out-patients from South Western hospitals in Nigeria.

MATERIALS AND METHODS

Bacterial isolates

In total, 114 strains of *K. pneumoniae* (60) and *E. coli* (54) were obtained from 122 non-duplicate faecal specimens of out-patients submitted to Medical Microbiology and Parasitology laboratory, for routine medical examination between 2010 and 2011. It was assumed they would not have taken any antibiotics nor had any contact with hospitals or health care facilities prior to submission of their samples since they were not sick or have any obvious illness. Single isolate from specimens without positive results of bacterial intestinal pathogens were retained and without intestinal disorder or other diseases based on the information on the requisition form. Isolates were from three teaching hospitals in South-Western Nigeria, namely University College Hospital, Ibadan (33), Obafemi Awolowo University Teaching Hospital, Ile-Ife (51) and Ladoke Akintola University of Technology Teaching Hospital, Osogbo (30). All isolates were confirmed using API 20E strips (bioMérieux, Marcy l'Etoile, France).

Antibiotic susceptibility testing

Antimicrobial disc susceptibility tests were carried out on the isolates on freshly prepared Mueller-Hinton agar (Oxoid, England) and were standardized by the method of Clinical and Laboratory Standard Institute (CLSI, 2007). The antibiotics and the disc contents are shown in Table 1. All susceptibility testing runs included the control organisms *E. coli* NCTC 10418. Plates with antibiotic discs were incubated for 24 h at 37°C and sensitivity pattern was compared with that of the control strain.

Minimum inhibitory concentrations (MICs) of a panel of β-lactam antibiotics were determined and interpreted using the agar dilution method according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC). All susceptibility testing runs included the control organism *E. coli* NCTC 10418.

Detection of ESBL

ESBL production was confirmed by double disc synergy test (Jarlier et al., 1988). ESBL-positive *K. pneumoniae* ATCC 700603 and ESBL negative *E. coli* ATCC 25922 control strains were used in these experiments.

Detection of AmpC

AmpC β-lactamase production was detected using cefepime and cefpodoxime discs alone or in combination with clavulanic acid as previously described (Derbyshire et al., 2009). AmpC β-lactamases were detected using a difference of ≥14 mm between cefepime/ cloxacillin and cefpodoxime/cloxacillin discs. Difference of >5 mm between cefepime/clavulanate and cefepime or cefpodoxime/ clavulanate and cefpodoxime discs was used to detect ESBL in the presence of AmpC which would have been missed with double disc synergy test mentioned earlier.

Amplification of β-lactam genes

Polymerase chain reaction (PCR) was used to detect genes encoding resistance to β-lactams; *blaOXA, blaSHV, blaTEM, blaCTX-M* (Table 1) (Maynard et al., 2003) and *blaAmpC* (Table 2) (Tan et al., 2009) as previously described. All the amplimers resulting from these PCR reactions were sequenced to confirm the identity and specific variant of each gene identified and sequences were aligned to known reference sequences using ClustalW.

Conjugational transfer of antibiotic resistance

Mating was done for β-lactamases on selected *E. coli* or *K. pneumoniae* using *E. coli* DH5α with a chromosomal mutation conferring rifampicin resistance as recipient cells. All mating procedures were done on filters for 18 h at 37°C, 200 µl of mixed cultures were plated out onto selective plate containing rifampicin (100 µg/ml) and ampicillin (50 µg/ml). Transconjugants were confirmed by susceptibility and amplification of ESBL and *AmpC* genes.

Table 2. Primers used for amplification of *ampC* genes.

Primer	DNA sequence (5´ to 3´)	Target(s)	Amplicon size (bp)
MOXMF MOXMR	GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG C	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	520
CITMF CITMR	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	462
DHAMF DHAMR	AAC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC	DHA-1, DHA-2	405
ACCMF ACCMR	AAC AGC CTC AGC AGC CGG TTA TTC GCC GCA ATC ATC CCT AGC	ACC	346
EBCMF EBCMR	TCG GTA AAG CCG ATG TTG CGG CTT CCA CTG CGG CTG CCA GTT	MIR-IT, ACT-1	302
FOXMF FOXMR	AAC ATG GGG TAT CAG GGA GAT G CAA AGC GCG TAA CCG GAT TGG	FOX-1 to FOX5b	190

Table 3. Antimicrobial disc susceptibility pattern of 114 bacterial strains.

Antibiotic (µg/ml)	Sensitive strains (%)	Resistant strains (%)	Intermediate strains (%)
Ceftazidime (30)	78 (68.4)	21 (18.4)	15 (13.2)
Amoxyclav (30)	45 (39.5)	30 (26.3)	39 (34.2)
Cefotaxime (10)	6 (5.3)	66 (57.9)	42 (36.8)
Cefpodoxime (10)	27 (23.7)	81 (71.1)	6 (5.3)
Gentamicin (10)	45 (39.5)	66 (57.9)	3 (2.6)
Ciprofloxacin (5)	54 (47.4)	42 (36.8)	18 (15.8)
Pefloxacin (5)	60 (52.6)	42 (36.8)	12 (10.5)
Amoxicillin (25)	22 (19.3)	83 (72.8)	9 (7.9)
Tetracycline (30)	6 (5.3)	106 (93.0)	2 (1.8)

Digestion of plasmid DNA with restriction enzymes

Plasmids from transconjugants were digested with Eco RV (Promega MADISON, WI U.S.A.) to determine their physical characteristics. Reactions were carried out in samples that contained 8 µl of plasmid DNA, 2 µl of 10X buffers, 2 µl of EcoRV, and made up to 20 µl with sterile nuclease free water. They were incubated at 37°C for 3 h; 20 µl of each digestion mixture was subjected to electrophoresis on 0.7% agarose gel. They were visualised under ultra violet (UV) light on a 'Gene Genius' image analyser (SYNGENE, Cambridge, U.K.). The restriction digestion patterns were compared to each other.

Estimation of plasmid size

Plasmid size was estimated as previously described (Wang et al., 2003).

Identification of plasmids by PCR-based replicon typing

Incompatibility/replicon PCR-based typing was used in order to trace plasmids conferring drug resistance representing the major plasmid incompatibility groups circulating among the *K. pneumoniae* and *E. coli* as was previously described (Carattoli et al., 2005). Total DNA was generated for PCR using the Wizard genomic DNA purification System (Promega, Madison, WI).

Statistics analysis

Data were analysed using the statistical package within Microsoft Excel. Student *t*-test was done to determine the significant difference between distribution of ESBL genes in *E. coli* and *K. pneumoniae*, while Chi square was used to determine the association between distribution of ESBL genes and hospitals. In both cases, *p* value less than 0.05 was considered to be significant.

RESULTS

Table 3 shows the disc susceptibility of 114 bacterial isolates. Ceftazidime and pefloxacin had the highest susceptibility of 68.4 and 52.6%, respectively, while least

Table 4. MIC of bacterial strains Panel I: MIC of 114 bacterial strains.

Organism (no. of strains)	Panel I: MIC by strains			
	Antimicrobial agent	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Range (µg/ml)
	Ceftazidime	0.5	256	0.5 – 512
E. coli (54)	Cefpodoxime	1	256	0.5 - 512
	Cefotaxime	1	512	0.5 – 512
	Ceftazidime	0.5	256	0.5 – 512
K. pneumoniae (60)	Cefpodoxime	1	512	0.5 - 512
	Cefotaxime	1	512	0.5 – 512
Hospitals (no. of strains)				
	Ceftazidime	0.5	2	0.5 - 512
E. coli (10)	Cefpodoxime	0.5	4	0.5 - 512
	Cefotaxime	0.5	4	0.5 - 512
	Ceftazidime	0.25	1	0.5 - 512
K. pneumoniae (12)	Cefpodoxime	0.5	1	0.5 - 512
	Cefotaxime	0.5	2	0.5 - 512

MIC: Minimum inhibitory concentration.

Table 5. Faecal carriage of ESBL genes.

Species	N	Phenotypic detection n (%)		PCR positive for ESBL genes n (%)					
		ESBL	AmpC	*TEM-1*	*SHV-1*	*OXA-1*	*CTX-M-15*	*CTX-M-2*	*AmpC*
E. coli	54	12 (22.2)	3 (5.6)	44 (81.5)	9 (16.7)	12 (22.2)	17 (31.5)	5 (9.3)	3 (5.6)
K. pneumonia	60	6 (10)	8 (13.3)	58 (96.7)	15 (25)	24 (40)	13 (21.7)	7 (11.7)	7 (11.7)
Total	114	18 (15.8)	11 (9.6)	102 (89.5)	24 (21.1)	36 (31.6)	30 (26.3)	12 (10.5)	10 (8.8)

susceptibility of 5.3% was found in cefotaxime and tetracycline. Appreciable level of intermediate was also recorded especially with cefotaxime, 36.8% and augmentin, 34.2%. The MICs for β-lactam antibiotics are shown in Table 4. The results showed a high degree of resistance, with MIC_{90} values (MIC for 90% of the organisms) of ≥256 µg/ml for all antibiotics. It is noteworthy, the low value of MIC_{50} in spite of the high MIC_{90}.

Of the 114 bacteria strains, 18 (15.8%) produced ESBL by double disc diffusion test, of which 12 of 54 (22.2%) *E. coli* and 6 of 60 (10%) *K. pneumoniae* are ESBL producers. Similarly, phenotypic detection of AmpC revealed that AmpC enzymes were found in 11 strains (9.6%), 3 (5.6%) in *E. coli* and 8 (13.3%) in *K. pneumoniae.*

All 114 strains were amplified by PCR; *CTX-M* carriage (Figure 1) was found in 42 strains (36.8%). Sequencing identified these as 30 *CTX-M-15* and 12 *CTX-M-2*. *OXA-1* genes (Figure 2) were present in 36 isolates (31.6%), *TEM-1* genes (Figure 3) in 102 (89.5%) and *SHV-1* (Figure 4) in 24 (21.1%). Multiplex PCR for detection of *AmpC* genes (Figure 5) confirmed 10 (8.8%) isolates carry non-chromosomal AmpC enzymes (Table 5), these genes were only found in isolates with *CTX-M* genes. Of the 10 *AmpC*-positive genes, 5 amplified using ACC group primers, 3 by DHA group primers and 2 by FOX primers. Sequencing identified these genes as *ACC*, *DHA-1* and *FOX-1*, respectively. The ESBL genes cut across the three hospitals in varying proportions (Table 6). There was no significant difference in the proportion of positive ESBL genes detected in *E. coli* and *K. pneumoniae* (*t* test; $P = 0.64$; $P > 0.05$). Furthermore, no association was found between hospital and distribution of ESBL genes in patients' faeces ($X^2 = 0.35$; $p = 0.84$; $p > 0.05$).

Transfer of the ESBL resistance phenotype was successful for all the selected strains. Restriction digestion products to further type plasmids showed several bands having similar patterns (Figures 6 and 7). The transconjugant plasmids sizes were estimated to be 108 kb. Replicon type of the common plasmids for all β-lactamases was found to be *IncF*. This confirmed presence of a common plasmid in these strains.

Table 6. Distribution of ESBL genes in hospitals.

Hospital	N	ESBL genes [n (%)]					
		TEM-1	***SHV-1***	***OXA-1***	***CTX-M-15***	***CTX-M-2***	***AmpC***
UCH	33	30 (90.9)	8 (24.2)	12 (36.4)	10 (30.3)	2 (6.1)	3 (9.0)
OAUTH	51	46 (90.2)	11 (21.6)	16 (31.4)	14 (27.5)	6 (11.8)	3 (5.9)
LTH	30	26 (86.7)	5 (16.7)	8 (26.7)	6 (20.0)	4 (13.3)	4 (13.3)

N: Frequency of strains in hospital; UCH: University College Hospital, Ibadan; OAUTH: Obafemi Awolowo University Teaching hospital; LTH: Ladoke Akintola University Teaching Hospital. Chi square= 0.35; p = 0.84, p > 0.05.

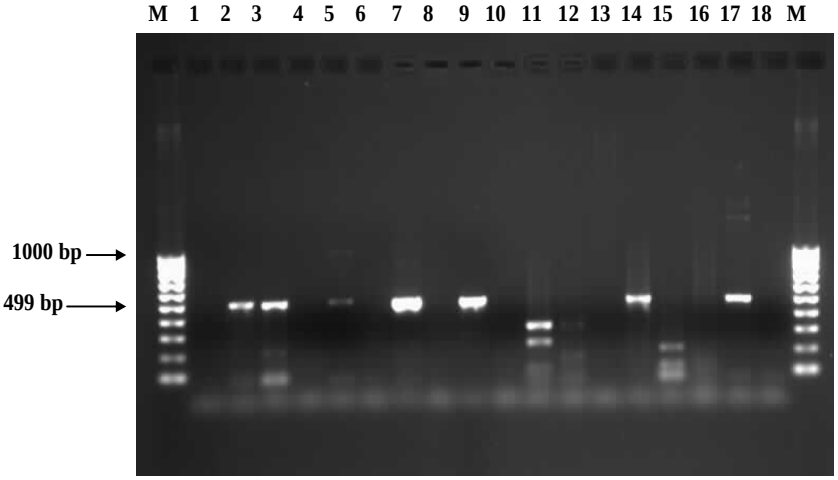

Figure 1. PCR amplification of CTX-M group 1. Lane m: Hyperladder IV; 9 Bands from 100-1000bp, lane 25: positive control, lane 26: negative control, lanes 1 to 24: tests. Product size is 499 bp.

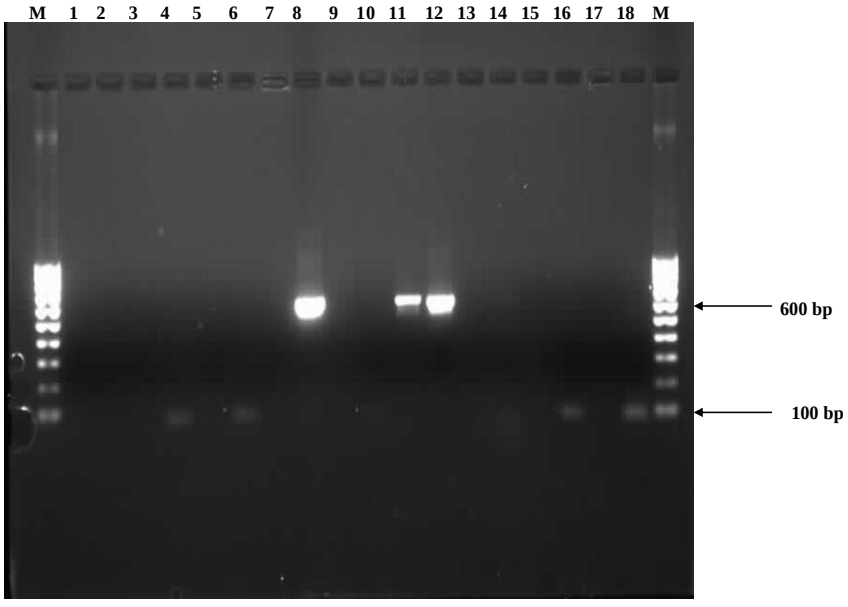

Figure 2. PCR amplification of OXA gene type for ESBL. Lane M: Hyperladder IV; 9 Bands from 100-1000bp, lane 18: Negative control, lanes 1 to 17: tests. Product size is 620 bp.

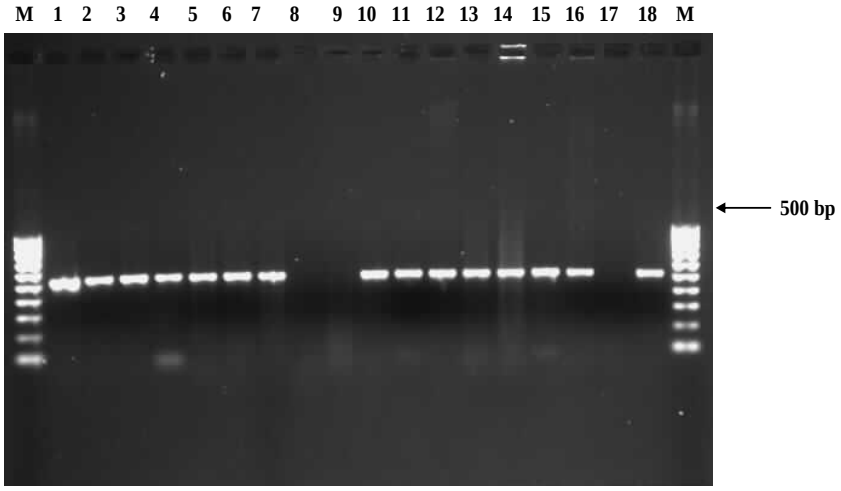

Figure 3. PCR amplification of TEM gene type for ESBL. Lane M: Hyperladder IV; 9 Bands from 100-1000 bp, lanes 1 to 18: tests. Product size is 517 bp.

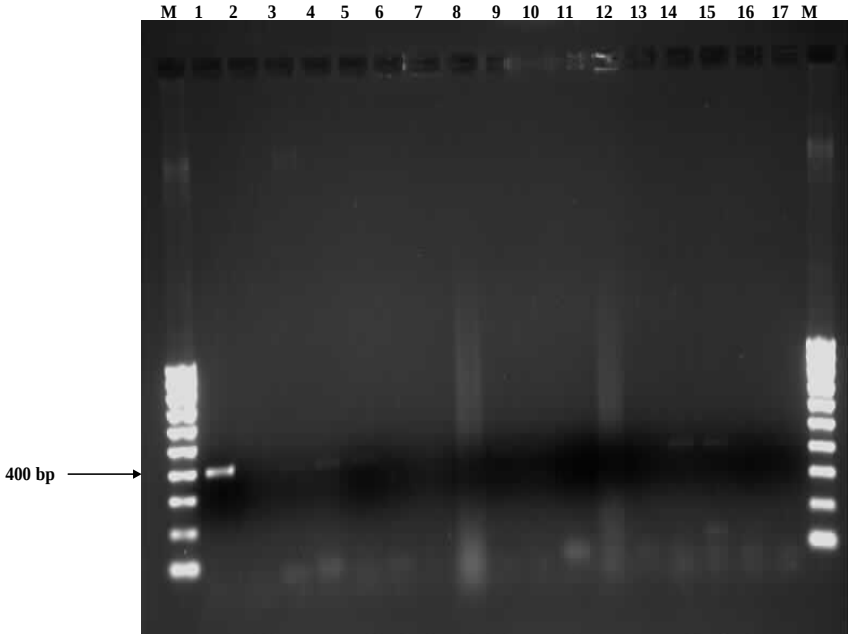

Figure 4. PCR amplification of *SHV* gene type for ESBL. Lane M: Hyperladder IV; 9 Bands from 100-1000 bp, lanes 1 to 17: tests. Product size is 393 bp.

DISCUSSION

The faecal strains demonstrated high level of resistance to all the drugs tested, including the cephalosporins and fluoroquinolones. These faecal strains can serve as reservoir to pathogens in the spread of resistance. *E. coli* and *K. pneumoniae* are known to be the leading cause of primary and opportunistic infections in human. Hence, they can be incriminated in virtually any type of infectious disease. ESBL phenotype is lower, 18 (15.8%) than previous reports from Nigeria, where a previous study that found 8 (20%) of 40 *Enterobacter* isolates to produce an ESBL by the double disc diffusion test (DDDT) (Aibinu et al., 2003) and 28 (28.9%) of 134 Gram negative bacteria carried an ESBL (Ogbolu et al., 2011). This lower rate may not be unconnected with the fact that the strains are commensals in view of their origin, though they may cause various types of infection in other sites. Similarly, 8.8% occurrence of AmpC producers was obtained, this was predominantly found in *K. pneumoniae*. High level faecal carriage ESBL genes was found in our environment, previous studies from other countries showed Senegal, 10% (Ruppe et al., 2009) and Europe, 21.6% travellers with resistant *E. coli* post-travel

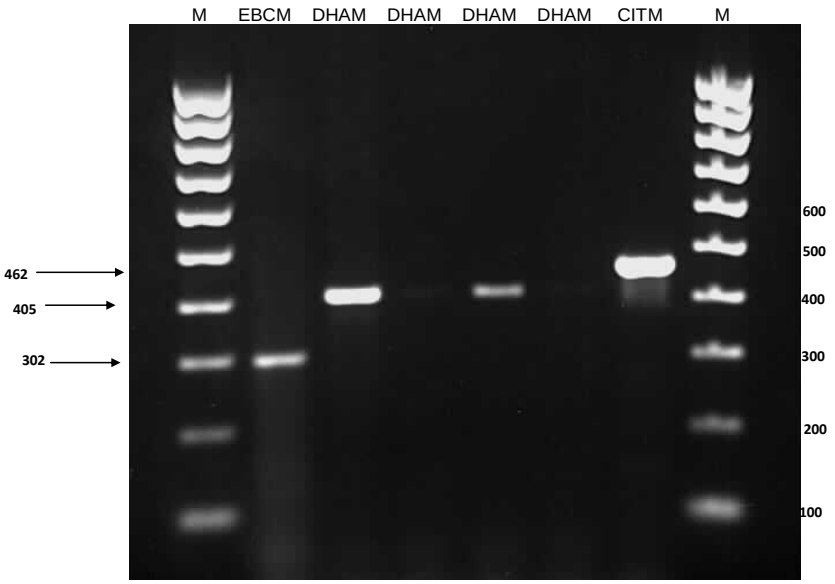

Figure 5. Positive ampC multiplex PCR products. Lanes are labeled with the ampC primers used. Lane M: Hyperladder IV. The amplified product size is indicated on the left. EBCM and CITM are positive controls, while DHAMs are tests

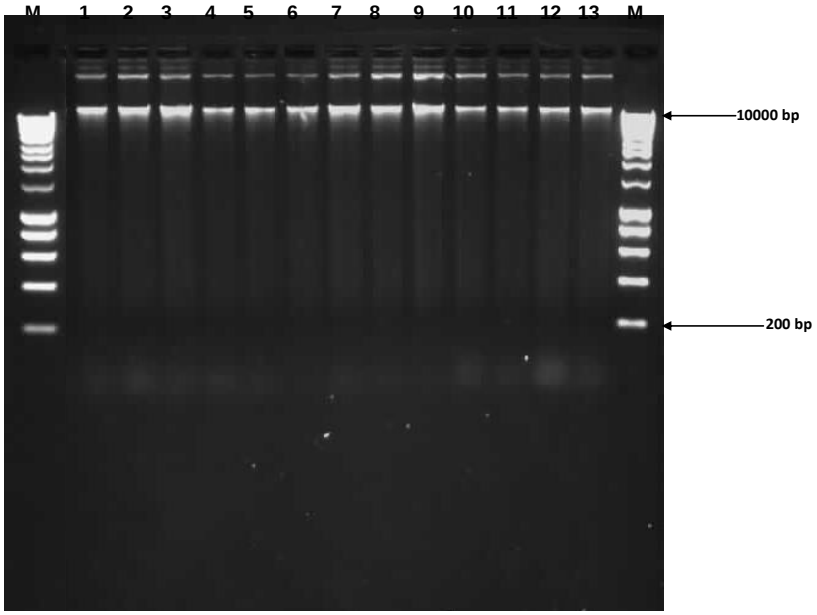

Figure 6. Transformants plasmid DNA. Lane M Hyperladder I; 14 bands from 200-10,000 bp. Lane 1 to 13 are transformants.

(Kennedy and Collignon, 2010). Extremely high rates from 50 to 70% have been found in the Far East and South-East Asia (Hawkey, 2008); particularly India, 100% (Muzaheed et al., 2009). There has been an increase in the isolation of CTX-M-producing bacteria in the last 20 years globally and these genes are spread worldwide. Classical *CTXM-15* has been described in Nigeria from clinical specimens (Ogbolu et al., 2011) and its presence confirms the rapid spread and global phenomenon (Ruppe et al., 2009; Oteo et al., 2010). It is however surprising that *CTX-M-3* that was first reported in Nigeria (Ogbolu et al., 2011) in the same environment was not found, instead *CTX-M-2* was detected, a new variant to Nigeria strains. DHA-1 was the only AmpC in this study

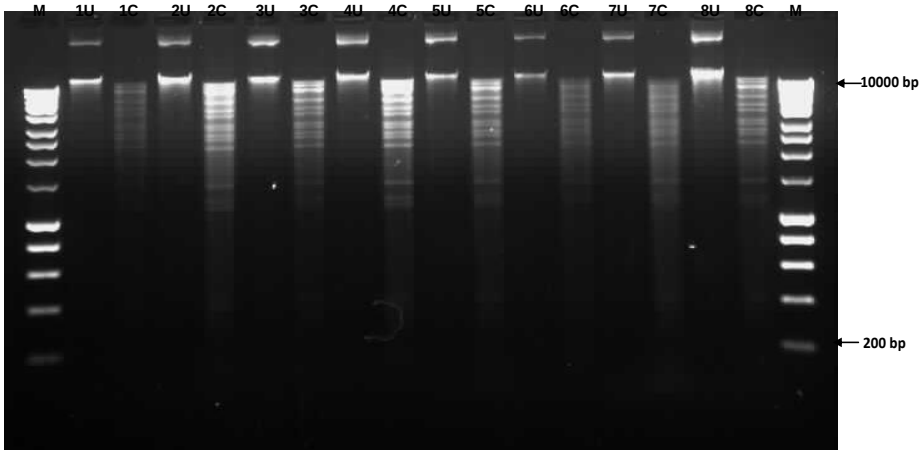

Figure 7. Restriction enzyme EcoRV digest of transformants plasmid DNA. Each transformant has two lanes, the first uncut and second cut DNA. Lane M: Hyperladder I; 14 bands from 200-10,000 bp. U-Uncut; C-Cut.

that was previously identified in our environment (Ogbolu et al., 2011) and was the most common. In accordance with our previous study, *AmpC* genes were found in isolates co-producing other ESBL genes such as *CTX-M-15, CTX-M-2, TEM-1, SHV-1* and *OXA-1* genes. This is different from the study of Muzaheed et al. (2009) where *TEM* and *SHV* were not detected in *K. pneumoniae* faecal strains of patients with acute gastroenteritis in India.

There was no significant difference in the proportion of positive ESBL genes detected in *E. coli* and *K. pneumoniae* and between hospitals. This may be due to the fact that these hospitals are within the South-West region of Nigeria about 100 km to each other and obviously there are contacts among these inhabitants within this region, including inter-hospital patients transfer. Also, orally administered antimicrobials including fluoroquinolones are available over-the-counter and frequently used for the purpose of self-medication in Nigeria. There is a common plasmid conferring different antimicrobial resistance in *bla* genes in the region evident by restriction digestion and *IncF* obtained from PCR-based replicon typing.

The discordance found between ESBL phenotype and PCR method could be that bacteria also reduce the fitness cost of resistance by silencing the genes when not required. There is little documented evidence for gene silencing in bacteria in general or for silencing of resistance genes in particular (Yarmolinski, 2000). In clinical settings, carriage of antibiotic resistance genes is generally assumed on the basis of phenotype, and in most genotypic investigations, only resistant isolates are screened for the presence of particular genes conferring antibiotic resistance. Accordingly, if silent genes were present, most surveys of resistant bacteria would fail to detect them. Expression of intact antibiotic resistance gene systems can be switched off in bacteria, that is, resistance genes can be silenced (Enne et al., 2006), and this process is reversible.

The existence of faecal carriage of *CTX-M* genes has clinical implications, as intestinal tract colonization is prerequisite for infection by ESBLs-producing organisms. What is less clear is the route by which community infections arise. On one hand, many patients with 'community' infections with CTX-M-β-lactamase-producing *E. coli* have a history of recent hospitalization (Soge et al., 2006), where they may have been colonized. Not all colonized individuals have a history of hospitalization and it may be that low-level gut colonization occurs in the community, via the food chain. This study showed that β-lactam determinants were located in transferable plasmids. The transferable nature of these resistance genes is particularly worrisome, and treatment options for infections caused by these organisms are very limited, and this may account in part for the association between fluoroquinolones resistance and expanded-spectrum cephalosporins. The selection of such highly resistant isolates in countries such as Nigeria may act as a reservoir of resistant strains that can be transferred to other countries in the era of global travel.

Conclusion

There is high faecal carriage of ESBL genes in commensal isolates, importantly classical *CTX-M-15* in out-patients in Nigeria. Screening populations for faecal or rectal carriage would be the obvious way to resolve these issues, but has not yet been undertaken on any wide scale. This calls for enhanced infection control and a better understanding of resistance mechanisms, molecular epidemiology and the means by which spread occurs.

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