Original Article

Dissemination of IncF plasmids carrying beta-lactamase genes in Gram-negative bacteria from Nigerian hospitals

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Abstract

Introduction: Production of beta-lactamases is the predominant cause of resistance to beta-lactam antibiotics in Gram-negative bacteria. We investigated the diversity of plasmid-borne beta-lactamase genes and replicon type of the plasmids carrying the respective genes in Gram-negative bacteria recovered from clinical infection in Nigerian hospitals.

Methodology: A total of 134 Gram-negative bacteria of 13 species were analyzed for antimicrobial susceptibility, phenotypic and genotypic detection of various beta-lactamases, and plasmid analysis, including replicon typing.

Results: Of the 134 isolates, 111 (82.8%) contained beta-lactamases, while 28 (20.9%) carried extended-spectrum beta-lactamases. PCR and sequencing identified TEM-1 in 109 isolates (81.3%), SHV-1 in 33 isolates (24.6%), OXA-1 in 15 isolates (11.2%) and CTX-M enzymes (24 CTX-M-15 and 1 CTX-M-3) in 25 isolates (18.7%). Multiplex PCR showed that 6 isolates carried plasmidic AmpCs (ACT-1, DHA-1 and CMY-2); these enzymes were detected only in isolates possessing CTX-M beta-lactamases. Of 13 (76.9%) representative plasmids investigated in detail, 9 (69.2%) were self-transferable when selected by a beta-lactam and the plasmids once transferred coded for beta-lactam resistance. Replicon typing indicated IncF as the common vector encoding for beta-lactamases.

Conclusions: The study showed a diversity of beta-lactamase genes disseminated by conjugative IncF plasmids in Gram-negative bacteria; TEM-1, SHV-1, OXA-1, CTX-M-15, CTX-M-3and plasmidic AmpC enzymes are in common circulation in Nigeria.

Key words: ESBL; plasmid; resistant genes; bacteria; Nigeria

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Introduction

The presence of multidrug-resistant bacterial strains harbouring several expanded-spectrum beta-lactamases simultaneously (*e.g.*, extended-spectrum beta-lactamases (ESBLs), metallo-beta-lactamases) that are also resistant to fluoroquinolones and aminoglycosides, has been reported worldwide but there is scarcity of information in Nigeria. Indeed, only a few studies have reported isolation and frequency of beta-lactamases from Nigerian hospitals [1-3]. The CTX-M beta-lactamases are the most widespread ESBL enzymes globally [4]. They were initially reported in the second half of the 1980s, and their rate of dissemination among bacteria and in most parts of the world has increased dramatically since 1995 [5].

Enterobacteriaceae and *Pseudomonadaceae* are the most important Gram-negative causes of nosocomial and community acquired infections, and they are a major cause of nosocomial infections associated with high mortality. In Nigeria, extended-spectrum cephalosporins, particularly third-generation and fluoroquinolones, remain the drugs of choice to treat infections caused by these microorganisms. Carbapenems have recently been introduced in Nigeria and are rarely used by physicians. ESBLs have been described in Africa [6,7] and have considerable implications for the developing world, where use of antibacterial agents is largely indiscriminate and uncontrolled.

Recent studies have demonstrated the clonal expansion of certain enterobacterial clones that are

able to acquire plasmids harbouring different ESBLs and plasmidic AmpCs [8]. The high prevalence of bla_{ESBL} genes in different parts of the world is caused by the horizontal transfer of plasmids among unrelated clones and also within local or international epidemic clones. Plasmid transmission has played a significant role in the global spread of beta-lactamases. IncF plasmids have been associated with the abrupt worldwide emergence of clinically relevant ESBLs, such as CTX-M-15 [9-11], but also with the spread of plasmid-mediated *AmpC* genes (bla_{CMY} and bla_{DHA}) [12,13]. Characterization of plasmids encoding $bla_{CTX\text{-}M\text{-}15}$ from Canada, France, Spain, and the United Kingdom has classified most of them as members of incompatibility group FII [14,15]. Similarly, Coque *et al*. reported IncF plasmids harbouring multiple antimicrobial drug resistance determinants, including the *aac(6')-Ib-cr* gene, in several countries from America, Asia, Middle East and Europe [9]. The present study investigated the diversity of plasmid-borne beta-lactamases and replicon type of the plasmids carrying the respective genes in Gram-negative bacteria isolated from clinical specimens of patients attending tertiary care hospitals in Nigeria.

Methodology

Bacterial strains

One hundred and thirty-four clinical Gram-negative bacterial isolates of 13 species were obtained from 585 non-duplicate clinical specimens including aspirates, ear swab, wound swab, throat swab, high vaginal swab, eye swab, sputum, urine, cerebrospinal fluid and blood culture for the period between 2005 and 2007. Single isolates from each specimen were retained. The isolates were from four teaching hospitals in south-western Nigeria: University College Hospital, Ibadan; Obafemi Awolowo University Teaching Hospital, Ile-Ife; Ladoke Akintola University of Technology Teaching Hospital; and Osogbo and Olabisi Onabanjo University Teaching Hospital, Sagamu. All isolates were speciated using API 20E strips (BioMerieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of beta-lactam antibiotics were determined. MIC testing was performed using the agar dilution method according to the recommendation of the British Society for Antimicrobial Chemotherapy (BSAC) (http://www.bsac.org.uk/susceptibility_testing/guide_to_antimicrobial_susceptibility_testing.cfm). All runs included the control organisms *Escherichia coli* (NCTC 10418) and *Pseudomonas aeruginosa* (NCTC 10662). A start and finish plate without antibiotic was also included as a growth control.

Detection of beta-lactamases by nitrocefin hydrolysis

Broth cultures of each test strain as well as an ampicillin-resistant strain (*E. coli* NCTC 10418, carrying pUC18) were incubated overnight in 5 ml of LB broth. Two hundred microlitres of the resulting overnight cultures were transferred into wells of a microlitre tray; sterile broth was included to serve as a negative control. Ten microlitres of nitrocefin solution prepared according to the manufacturer's instructions (Fisher Scientific, Loughborough, UK), were added to each well. Beta-lactamase production was inferred when the broth turned red within 30 minutes of nitrocefin addition, as directed by the manufacturer.

Detection of extended-spectrum beta-lactamases (ESBLs) by the double disc method

Suspensions of broth culture of each test strain were prepared in sterile water to give an inoculum equivalent to a 0.5 McFarland standard before being used to inoculate the surface of iso-sensitest agar plates [16]. ESBL positive (K. pneumoniae ATCC 700603) and negative (E. coli ATCC 25922) control strains were used in these experiments. The double disc method described by Jalier *et al*. was followed [17].

Detection of AmpC *by simple disc diffusion*

AmpC beta-lactamase production was detected for strains producing ESBL using cefepime and cefpodoxime disks in combination with clavulanate as previously described [18]. The plate and the inoculum were essentially the same as for ESBL detection described above. Following overnight incubation at 37ºC, AmpC beta-lactamases were detected using a difference of ≥14 mm between cefepime/cloxacillin and cefpodoxime/cloxacillin discs.

Amplification of beta-lactam genes

PCR was used to detect genes encoding resistance to beta-lactams (bla_{OXA}, bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$). Multiplex PCR was used for bla_{AmpC} genes [19]. 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 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Gene transfer

Transformation experiments were performed by electroporation (Gene pulser; Bio-rad, Hemel Hempstead, UK) of purified plasmid DNA into competent *Escherichia coli* α-select (Bioline, London, UK, efficiency of > 10^9). The protocol of the manufacturer was followed to determine the properties of plasmids. The mixtures (200 µl) containing transformants were plated onto selective agar (ampicillin, 16µg/ml), allowed to stand for a few minutes, and incubated at 37ºC overnight. To control for antibiotic activity, the wild type strain was also inoculated onto the selective plates. Minimum inhibitory concentrations of the transformants and recipient strain were measured. Plasmid DNA from the transformants were isolated and restricted with EcoRV and BamHI.

Conjugation experiments were performed by broth and filter mating assay using *E. coli* DH5α with a chromosomal mutation conferring rifampicin resistance as recipient cells. Suspensions of 200 µl were plated out onto selective plates containing rifampicin (100 µg/ml) and ampicillin (16 µg/ml). For controls, test strains were plated on rifampicin plates, while recipient cells were plated on separate plates of rifampicin and ampicillin.

Estimation of plasmid size

Plasmid size was estimated as previously described [20].

Identification of plasmids by PCR-based replicon typing

Incompatibility/replicon PCR-based typing was used to trace plasmids conferring drug resistance. In this method, 18 pairs of primers were designed for five multiplex- and three simplex-PCRs, recognizing the FIA, FIB, FIC, HI1, HI2, I1-Iy, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons, representing the major plasmid incompatibility groups circulating among the *Enterobacteriaceae* [21]. Because of the high level of homology between the K and B/O replicons, the same forward primer was used in both these simplex PCRs. Plasmid DNA of transformants and transconjugants were generated as a DNA template for PCR [22].

Results

Identification of bacterial species

A total of 13 Gram-negative enteric bacteria were identified and they consisted of *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Morganella morganii*, *Enterobacter cloacae*, *Aeromonas hydrophilia*, *Serratia adorifera*, *Stenotrophomonas maltophilia*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Pseudomonas oryzihabitans*, *Pseudomonas luteola*, *Burkholderia cepacia*. The strains were obtained from diverse clinical sources to capture as much diversity as possible.

Susceptibility testing

Determination of precise MIC values confirmed the numbers of isolates resistant to clinical breakpoint concentrations for all antibiotics using BSAC guidelines. The MIC results also showed that the level of resistance to beta-lactam antibiotics was extremely high, MIC_{50} greater than clinical breakpoints. The mean percentage of strains resistant to each of the beta-lactam drugs was 85% (Table 1).

Phenotypic detection of beta-lactamase

Beta-lactamase production was inferred when diluted broth turned red within 30 minutes of adding nitrocefin. Of the 134 isolates, 111 (82.8%) were found to be beta-lactamase positive by this method: 85.7% of *K. pneumoniae*s (54 out of 63), 71.4% of *E. coli* (20 out of 28), 69.2% of *P. aeruginosa* (9 out of 13), and 81.8% of *P. mirabilis* (9 out of 11). The rest of the strains were uniformly positive in the nitrocefin reactions.

Phenotypic detection of ESBLs

ESBL production was inferred when the zone of inhibition around the ceftazidime and cefotaxime discs was expanded by the clavulate by ≥ 5 mm. Among the 134 isolates, 28 (20.9%) were found to be ESBL positive by the combined disc test and all 20.9% ESBL-positive isolates were also beta-lactamase positive by nitrocefin.

Phenotypic detection of AmpC

Phenotypic detection of AmpC by disc diffusion using cefepime and cefpodoxime revealed that AmpC enzymes were present in 29 of 44 ESBL (ESBL by DDDT and *CTX-M* by PCR) producing isolates (65.9%). Twenty-five AmpC positive isolates were detected and interpreted as positive using an increase of ≥ 14 mm in zone diameter around the antibiotic disc with added inhibitor compound between cefepime and cefpodoxime.

Table 1. MICs of selected strains to beta-lactam antibiotics

Organisms (no of strains)	Antimicrobial Agents	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Range (µg/ml)
Escherichia coli n = 28	Ceftazidime	32	256	0.25-256
	Ceftriazone	8	256	0.25-256
	Amoxyclav	256	256	0.25-256
	Amoxycillin	256	256	0.25-256
K. pneumoniae ssp *pneumoniae* n = 63	Ceftazidime	256	256	0.25-256
	Ceftriazone	256	256	0.25-256
	Amoxyclav	256	256	0.25-256
	Amoxycillin	256	256	0.25-256
Proteus mirabilis n = 11	Ceftazidime	128	256	0.25-256
	Ceftriazone	256	256	0.25-256
	Amoxyclav	256	256	0.25-256
	Amoxycillin	256	256	0.25-256

Table 2. Beta-lactamase production by the isolates

Species	n	No. (%) of		No. (%) of PCR positive			
		beta-lactam[a] Positive	ESBL[b] Positive	TEM	SHV	OXA	CTX-M
K. pneumoniae	63	54 (85.7)	8 (12.7)	47 (74.6)	9 (14.3)	1 (1.6)	4 (6.3)
E. coli	28	20 (71.4)	7 (25)	24 (85.7)	10 (35.7)	5 (17.9)	9 (32.1)
P. aeruginosa	13	9 (69.2)	1 (7.7)	12 (92.3)	4 (30.8)	2 (15.4)	3 (23.1)
P. mirabilis	11	9 (81.8)	3 (27.3)	10 (90.9)	3 (27.3)	3 (27.3)	3 (27.3)
P. oryzyhabitans	6	6 (100)	2 (33.3)	6 (100)	3 (50)	0 (0)	1 (16.7)
B. cepacia	2	2 (100)	1 (50)	2 (100)	1 (50)	0 (0)	1 (50)
A. hydrophilia	1	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (100)
E. cloacae	2	2 (100)	2 (100)	0 (0)	0 (0)	0 (0)	0 (100)
M. morganii	3	3 (100)	2 (66.7)	2 (66.7)	1 (33.3)	1 (33.3)	1 (33.3)
P. luteola	1	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
S. adorifera	1	1 (100)	1 (100)	1 (100)	0 (0)	1 (1000	1 (100)
S. maltophilia	2	2 (100)	0 (0)	2 (100)	1 (50)	1 (50)	1 (50)
C. freundii	1	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)

n: frequency,
a: positive by nitrocefin test
b: positive for ESBL production by DDDT
( ): number in parenthesis is %

Genotypic detection of ESBLs

The data revealed that TEM-1 was present in 109 of 134 isolates (81.3%), SHV-1 in 33 isolates (24.6%), OXA-1 in 15 isolates (11.2%), and CTX-M in 25 isolates (18.7%), while all beta-lactamases were present in 6 (4.5%) isolates (data not shown). Of the 63 *K. pneumoniae,* TEM-1 was present in 47 (74.6%); SHV-1 in 9 (14.3%); OXA-1 in 1 (1.6%); and CTX-M-15 in 4 (6.3%) isolates. Of the *Escherichia coli,* 24 (85.7%) carried TEM-1; 10 (35.7%) SHV-1; 5 (17.9%) OXA-1; 8 (28.6%) CTX-M-15*;* and 1 (3.6%) isolate CTX-M-3. Of *Pseudomonas aeruginosa,* 12 (92.3%) were found to have TEM-1; 4 (30.8%) SHV-1; 2 (15.4%) OXA-1*;* and 3 (23.1%) CTX-M-15 (Table 2). PCR identified that a larger number of strains carried ESBLs than were predicted by the disc diffusion assays. In 25 CTX-M-positive strains, only 8 were phenotypically ESBL producers. Only one of the CTX-M-positive strains was TEM-negative; 14 and 11 were SHV and OXA negative, respectively.

Genotypic detection of plasmidic AmpC enzymes

Multiplex PCR was used to screen for isolates with plasmidic AmpCs. The results showed that six isolates were positive by PCR for *AmpC* genes and these genes were only found in isolates carrying *CTX-M* genes. The AmpC-positive strains cut across most of the clinical sites of the specimens, ranging from wound, ear, aspirate, blood culture, and catheter tip. Out of the six AmpC-positive genes, one was amplified using EBC primers, four by DHA group primers and one by CIT primers. Sequencing identified these genes as *ACT-1*, *DHA-1* and *CMY-2*, respectively**.** DHA-like enzymes were present predominantly in the strains. No enzymes belonging to the ACC, FOX, or MOX family were detected. Only four of the six AmpC-positive strains produced an inducible AmpC. AmpC beta-lactamase-positive strains appeared to have low-level resistance to the third-generation cephalosporins with an MIC range of 0.25 to 256 µg/ml comparatively. Resistance to quinolones among AmpC-positive strains was high with MICs ranging between 8 µg/ml and 256 µg/ml (Table 3).

Plasmid DNA analysis

The strains were found to harbour plasmids ranging from < 20 to > 200 kb as observed by plasmid extraction and electrophoresis. A number of plasmids were common to multiple isolates. Restriction digestion to further type plasmids used EcoRV endonuclease to select representative restriction patterns from the diverse profiles for further studies. Digestion products were found to yield several bands with sizes ranging from 6kb to > 200 kb (data not shown).

Gene transfer

Transformation experiments revealed that the plasmids once transferred coded for beta-lactam resistance. Beta-lactamase transformants showed significantly higher resistance to amoxicillin and ceftazidime (Table 4). The transformant strains carried a common plasmid approximately of 108-kb.

Replicon type of plasmids

To identify the replicon type of the common 108-kb plasmid observed, 18 specific primer pairs were used to amplify replicon specific sequences, on the basis of the multiple comparative analysis of nucleotide sequence deposited in the Gene Databank. A positive result was seen for IncF, indicating this is the replicon type of the common plasmids encoding beta-lactamases.

Discussion

This study indicates that *bla* genes were located on epidemic plasmids encoding different resistance phenotypes from strains from different hospitals with an estimated size of 108-kb. This result implies the spread of a common plasmid among different strains and essentially in different parts of Nigeria. Some of these plasmids encoded more than one type of ESBLs or AmpC. Plasmids with the ability to spread efficiently, or epidemic plasmids, are believed to be responsible for disseminating CTX-M ESBLs [23] by conjugative plasmids rather than by clonal expansion of a bacterial host strain [24]. The replicon type of the common plasmids for the beta-lactamases was IncF; this suggests the circulation of a prevalent IncF plasmid carrying different *bla* genes. To our knowledge, this study is the first report of plasmid identification by incompatibility group based on PCR in Nigeria. This observation suggests interesting clues on the evolution of related plasmids that can gain additional resistance determinants and/or lose the transferability gene-associated functions, evolving in different plasmid variants. Our study corroborates the current worldwide spread of clinically relevant *bla* genes such as *CTX-M-15* [9-11], also with the spread of plasmid-mediated *AmpC* genes (bla_{CMY} and bla_{DHA}) [12,13].

IncF plasmids have been reported to be of low copy number with a size of > 100 kb having more than one replicon promoting the initiation of replication.

Table 3. The relationship of AmpC-positive strains with other beta-lactamases and antibiotic susceptibility

AmpC	Strain	Specimen	Source	MICs (µg/ml)							Nitrocefin	AmpC	Target	CTX-M	TEM	SHV	OXA
				NAL	CIP	OFX	PEF	SPX	CAZ	CRO	Positive	phenotype	Site				
U2	*P. mirabilis*	Ear	In	>256	256	256	256	256	256	8	YES	YES	DHA-1	NO	YES	NO	NO
U7	*K. pneumoniae*	Aspirate	In	>256	256	64	256	256	256	0.5	YES	YES	DHA-1	1 (15)	YES	YES	YES
U13	*B. cepacia*	B/culture	In	>256	256	256	256	256	256	2	YES	NO	DHA-1	NO	YES	NO	NO
U73	*M. morganii*	C/tip	Out	>256	256	128	256	256	32	256	YES	YES	DHA-1	1 (15)	YES	NO	YES
Uo	*K. pneumoniae*	Wound	Out	>256	256	8	256	256	8	0.25	YES	NO	ACT-1	1 (15)	YES	NO	NO
Ut	*C. freundii*	B/culture	Out	>256	8	32	32	32	0.25	0.25	YES	YES	CMY-2	1 (15)	YES	YES	YES

MICs: minimum inhibitory concentrations, In: inpatient, Out: outpatient
NAL: nalidixic acid, CIP: ciprofloxacin, OFL: ofloxacin, PEF: pefloxacin, SPX: sparfloxacin, CAZ: ceftazidime, CRO: ceftriazone

Table 4. Resistance profiles of donor strains and transformants

Strains	**MICs (µg/ml)**			
	CAZ	CRO	AMC	AMX
Clinical strains (Donors)				
2	256	8	256	256
5	256	256	256	256
10	256	256	256	256
21	4	0.5	256	256
22	32	256	256	256
53	256	256	256	256
72	16	256	256	256
73	32	256	256	256
80	256	256	256	256
I5	4	1	256	256
I7	256	256	256	256
I9	256	256	256	256
I16	256	256	256	256
Recipient *E. coli* K12 DH5α	0.03	0.03	0.125	0.125
Beta lactamase selected				
2	>0.25	0.5	32	64
5	>0.25	1	32	64
10	>0.25	0.5	64	64
21	>0.25	0.5	64	64
22	>0.25	0.5	64	64
53	0.5	1	64	64
72	>0.25	1	64	64
73	>0.25	0.5	64	64
80	>0.25	1	64	64
I5	>0.25	0.5	64	64
I7	>0.25	1	64	64
I9	>0.25	2	64	128
I16	>0.25	1	64	64

MICs: minimum inhibitory concentrations
CAZ: ceftazidime, CRO: ceftriazone, AMC: amoxy/clav, AMX: amoxycillin

The multi-replicon status has been described to be one means by which plasmids with a narrow host range can accomplish broad host range replication [11], which explains why they are widely diffused in clinically relevant *Enterobacteriaceae,* representing one of the most frequently encountered plasmid types. The IncF plasmids carried various ESBLs including *AmpC*-type *DHA-1* and *CMY-2* genes. IncF plasmids harbouring multiple antimicrobial drug-resistance determinants including *aac(6')-Ib-cr* gene have been described [9,25]. The phenotypic detection of beta-lactamases indicated a high prevalence in clinical isolates in this country; these strains prove a clinical treatment challenge and are likely to be associated with high levels of mortality and morbidity. TEM- and SHV-derived ESBLs are most often found in *E. coli* and *K. pneumoniae* [26,27]. TEM-1 was the most commonly identified beta-lactamase in this study. In previous African studies, this enzyme has previously been reported only from South Africa, where it was identified initially in ESBL-producing *Klebsiella* species, *Proteus* species, *Enterobacter* species, and *E. coli* strains [28,29] and in strains of *Salmonella* [30].
There has been an increase in the isolation of CTX-M-producing bacteria globally, with reports from Europe, North and South America, Africa and Asia [4]. In

Africa, clonal CTX-M-12-producing *K. pneumoniae* strains were isolated in Kenya [31]. There are few reports of *bla* genes, especially bla_{CTX-M}, in Nigeria. Twenty-five isolates were CTX-M-positive (18.7%), although among 28 ESBL-producing isolates, only 8 were CTX-M-positive by PCR. Plasmids coding for ESBLs that express a low level of resistance to beta-lactams [32] or contain multiple silenced antibiotic resistance genes [33] are of particular concern, as they may serve as reservoirs of antibiotic resistance determinants in bacteria that cannot be detected phenotypically. A previous report of CTX-M in Nigeria identified CTX-M-15 [2]. In this study, sequencing identified most of the CTX-M enzymes to be CTX-M-15 and only one was CTX-M-3. CTX-M-14, CTX-M-15, CTX-M-9, CTX-M-3 and CTX-M-2 are the most commonly isolated enzymes worldwide.

The prevalence of plasmidic AmpC-mediated resistance in Nigeria is not known due largely to the few surveillance studies seeking clinical strains producing AmpC beta-lactamases and to the technical difficulties for laboratories to detect this resistance mechanism. Plasmid-mediated AmpC was detected in 6 isolates by PCR and these genes were found in isolates co-producing other ESBLs such as CTX-M-15, TEM-1, SHV-1and OXA-1. The AmpC-positive strains cut across most of the clinical sites of the specimens, ranging from wound, ear, aspirate, blood culture and catheter tip. The majority of these patients had actually been treated with beta-lactam antibiotics. Sequencing identified these genes as *ACT-1, DHA-1* and *CMY-2*. Only four of the six AmpC-positive strains produced an inducible AmpC with the disc diffusion method, while two of the AmpC positive strains did not express inducible AmpC by the phenotypic method.

Conclusion

In conclusion, we describe the dissemination of IncF plasmids in *K. pneumoniae*, *E. coli*, *P. mirabilis*, and *P. aeruginosa* carrying various types of ESBLs including CTX-M-15 and CTX-M-3 as well as AmpCs including DHA-1, CMY-2 and ACT-1 in hospitals in Nigeria.

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