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Extended-Spectrum β-Lactamase Enzymes in Clinical Isolates of *Enterobacter* Species from Lagos, Nigeria

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Over a 9-month period, 8 of 40 nonduplicate isolates of *Enterobacter* spp. producing extended-spectrum β-lactamase (ESBL) were detected for the first time from two hospitals in Lagos, Nigeria. Microbiologic and molecular analysis confirmed the presence of ESBL. Only four isolates transferred ESBL resistance as determined by the conjugation test, and pulsed-field gel electrophoresis showed genetically unrelated isolates.

In the preantibiotic era, species in the *Enterobacter* genus were not encountered in surveys of nosocomial infections (23). However, by the 1970s, they were becoming increasingly important nosocomial pathogens (13, 28), although they were much less commonly encountered than *Escherichia coli* and *Klebsiella* strains (23). They are reported in significant nosocomial infections, including urinary tract infections, respiratory tract infections, and bacteremia, particularly in elderly or debilitated patients (29). Recovery of antibiotic-resistant, especially to β-lactams, strains of *Enterobacter* is reported to be on the increase (3, 12), particularly during therapy with a β-lactam agent (7, 29). The molecular basis for the resistance in these *Enterobacter* isolates is a mutation to an *ampD* gene that normally prevents high-level expression of the chromosomal β-lactamase encoded by an *ampC* gene (28). Mutation to *ampD* permits constitutive production of the chromosomal Bush group 1 β-lactamase (4), producing an organism resistant to all β-lactam antibiotics, including inhibitor combinations based on clavulanic acid. Strains usually remain susceptible to carbapenems and cefepime. Such mutants have often been referred to as stably derepressed mutants (31). Recently, a different mechanism of resistance to expanded-spectrum cephalosporins has been recognized when *Enterobacter* species acquired a plasmid encoding Bush group 2be β-lactamase, the extended-spectrum β-lactamases (ESBLs). The occurrence of ESBLs in *Enterobacter* spp. possessing the inducible Bush group 1 chromosomal β-lactamase is increasingly reported worldwide (2, 6). These isolates are susceptible to clavulanic acid–β-lactam combinations and may have reduced susceptibility to some of the expanded-spectrum cephalosporins. There are no documented reports yet on the occurrence of ESBL enzymes in *Enterobacter* species from Nigeria, although such an enzyme is being recorded in strains of *Klebsiella* spp. and *E. coli* isolated in hospitals in Lagos, Nigeria (unpublished data). In the present study, we investigated ESBLs from clinical isolates of *Enterobacter* spp. recovered from specimens at 2 hospitals in Lagos, Nigeria.

A total of 40 isolates of *Enterobacter* species (25 *Enterobacter aerogenes* and 15 *Enterobacter cloacae*) were studied during a 9-month period from January to September 2001. They were recovered from 40 patients: 17 (42.5%) from intensive care units, 10 (25%) from surgical wards, 10 (25%) from medical wards, and 3 (7.5%) from pediatric wards. Specimens from which they were isolated included surgical wounds and wound ulcers of gunshot patients (45%) and urine (30%), blood (10%), and respiratory (15%) specimens. Species identification was carried out by using standard diagnostic procedures (14). Antibiotic susceptibilities were determined on Mueller-Hinton agar (Oxoid, Basingstoke, United Kingdom) by standard disk diffusion procedures (29) to the following antibiotics: ampicillin, amoxicillin, amoxicillin-clavulanic acid, aztreonam, cefoxitin, cefotaxime, ceftriaxone, ceftazidime, imipenem, gentamicin, amikacin, kanamycin, streptomycin, cefepime, piperacillin, ticarcillin, ticarcillin-clavulanic acid, ampicillin-sulbactam, cephalothin, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, nalidixic acid, ofloxacin, and ciprofloxacin (Oxoid). The following control strains were run simultaneously with the test organisms: *E. coli* ATCC 25922, *E. coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853. Results were interpreted with National Committee for Clinical Laboratory Standards criteria for disk diffusion (24). All the isolates were tested for the production of β-lactamase with the starch paper technique (19, 27) and nitrocefin test (19, 21). They were subjected to NCCLS guidelines for the detection of ESBL by the disk method. All the isolates were first tested for susceptibility to cefotaxime, ceftazidime, ceftriaxone, and aztreonam alone and then in combination with clavulanate. Double-disk synergy tests (DDST) (5, 8, 17, 29) were performed by placing disks of ceftazidime, cefotaxime, ceftriaxone, cefepime, and aztreonam (30 μg each) at a distance of 20 mm (center to center) (34) from a disk containing amoxicillin plus clavulanate (20 and 10 μg, respectively). Disk diffusion susceptibility tests showed all the strains to be resistant to ampicillin, amoxicillin, ticarcillin, cephalothin, and cefoxitin while there was 100% susceptibility to imipenem. Result of the susceptibility testing

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TABLE 1. Percent resistance of *Enterobacter* isolates to various antibiotics

Antibiotic	% Resistance of:	
	ESBL isolates	non-ESBL isolates
Ampicillin	100	100
Amoxicillin	100	100
Ticarcillin	100	100
Piperacillin	100	79
Ampicillin-sulbactam	87.5	53.9
Ticarcillin-clavulanic acid	87.5	53.9
Amoxicillin-clavulanic acid	100	82.5
Cefotaxime	62.5	10.3
Ceftazidime	100	20.5
Cefepime	37.5	10.3
Imipenem	0	0
Aztreonam	87.3	17.9
Kanamycin	87.5	51.3
Gentamicin	100	59
Amikacin	62.5	10.3
Nalidixic acid	75	12.8
Ofloxacin	75	15.4
Ciprofloxacin	75	23.1
Tetracycline	87.5	69
Chloramphenicol	82.1	61.5
Trimethoprim-sulfamethoxazole	100	82.1
Cephalothin	100	10
Cefoxitin	100	100
Ceftriaxone	73	40
Streptomycin	89	20

are summarized in Table 1. Using both the starch paper technique (19, 27) and nitrocefin test (19, 21), β-lactamase production was detected in 33 (82.5%) of the 40 *Enterobacter* isolates. Eight of these 40 isolates, six of which were *E. aerogenes* and two of which were *E. cloacae*, obtained from wound and blood cultures were found to produce an ESBL by the DDST. Resistance to beta-lactam or beta-lactamase inhibitors, the broad-spectrum cephalosporins, and the non-beta-lactam antibiotics is shown to be much higher in the ESBL-producing *Enterobacter* isolates than in the non-ESBL-producing isolates.

The isolates that were resistant or had decreased susceptibility to extended-spectrum cephalosporins and were positive by the DDST were mated with *E. coli* recipient strains, M2423 (nalidixic acid-resistant and lactose fermentation-negative strain) or M2424 (rifampin-resistant and lactose fermentation-positive strain), which were tested to be susceptible to the antibiotic resistance markers used in this study. Overnight cultures of recipient and donor strains, grown in Luria-Bertani broth at 37°C, were mixed together at a 1:10 ratio (donor to recipient) and incubated for 3 h. Samples (100 and 200 μl) of this mixture were spread onto the surfaces of MacConkey agar plates supplemented with 25 μg of nalidixic acid/ml or 100 μg of rifampin/ml and 100 μg of ampicillin/ml or 25 μg of tetracycline (Oxoid)/ml. Samples from the donor and recipient were used as controls. Transconjugants growing in the selection plates were subjected to DDST to confirm the presence of ESBL genes and were examined for the cotransfer of other antibiotic resistance determinants present in the donor clinical isolates. The DDST patterns of the transconjugant mimicked patterns of the donor. Only 4 of the 8 ESBL-producing *Enterobacter* isolates transferred resistance by conjugation. The most notable synergistic effect was seen with clavulanic acid and ceftazidime on the ESBL-producing *Enterobacter* and *E. coli* transconjugant with the disks placed 20 mm apart. Table 2 shows the sensitivity patterns of the donor, transconjugants, and *E. coli* recipient. Bacteria exponentially growing at 37°C in Luria-Bertani medium were harvested, and cell-free lysates were prepared by sonication (20). Isoelectric focusing (IEF) was performed by applying the crude sonic extract to polyacrylamide ampholyte gels with pHs ranging from 3.5 to 9.5 in a Multiphor II unit (LKB, Bromma, Sweden) (25). β-Lactamases with known pIs (TEM-1, pI 5.4; TEM-2, pI 5.6; SHV-2, pI 7.6; and SHV-5, pI 8.2) were focused in parallel as controls (25). Gels were stained with 200 mg of nitrocefin (Oxoid) per ml in 10 mM phosphate buffer (pH 7) to identify β-lactamase bands (20). IEF of the sonic extract of the ESBL-positive isolates showed that they possessed a class C Bush group inducible β-lactamase enzyme with an alkaline pI of >8.2, which was not sensitive to inhibition by clavulanic acid, and a Bush group 2be enzyme with a pI of 7.6. One of the ESBL isolates also had another additional enzyme with a pI of 5.6, and three others had a pI of 7.0. IEF of extracts from donors and *E. coli*

TABLE 2. Resistance patterns of recipient *E. coli,* donors, and transconjugants to antibiotic resistance markers and extended-spectrum cephalosporins

Organism[b]	pI transferred	Result[a] for:											
		STR	CM	GEN	KAN	SXT	RIF	NAL	ATM	CTX	CAZ	CRO	TET
E. coli M2423		S	S	S	S	S	S	S	R	S	S	S	S
E. coli M2424		S	S	S	S	S	S	R	S	S	S	S	S
D1 *E. aerogenes*		R	R	R	R	R	R	S	R	S	R	R	R
TS1	7.6	S	R	S	R	S	R	S	R	S	S	R	S
D2 *E. aerogenes*		R	R	R	R	S	R	S	R	R	R	R	S
TS2	7.6	S	R	S	R	S	R	S	R	S	S	R	S
D3 *E. aerogenes*		R	R	R	R	S	R	S	S	R	S	R	R
TS3	7.6	S	R	S	R	S	R	R	S	S	S	R	S
D4 *E. cloacae*		R	R	R	R	R	R	R	R	R	R	R	R
TS4	7	S	R	R	S	S	R	S	R	S	S	R	S

[a] R, resistant; S, sensitive; STR, streptomycin; AMP, ampicillin; CM, chloramphenicol; GEN, Gentamicin; KAN, kanamycin; SXT, trimethoprim-sulfamethoxazole; RIF, rifampin; NAL, nalidixic acid; ATM, aztreonam; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; TET, tetracycline.

[b] *E. coli* M2423 is nalidixic, acid resistant. *E. coli* M2424 is rifampin resistant. D1, D2, D3, and D4 are *Enterobacter* donors 1 to 4. D1, D2, and D4 were mated with *E. coli* M2423 Nal^r. D3 was mated with *E. coli* M2424 Rif^r. TS1, TS2, TS3, and TS4 are transconjugants 1 to 4.

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TABLE 3. Result of isolates positive by DDST and conjugation test

Organism	Source	β-Lactamase pIs	pI of enzyme transferred	Resistance marker(s) transferred[a]
E. aerogenes	Wound	>8.2, 7.6, 5.6	7.6	Caz, Gen, Sxt, Amp
E. aerogenes[b]	Wound	>8.2, 7.6, 7.0	7.6	Caz, Gen, Sxt, Amp
E. aerogenes[b]	Wound	>8.2, 7.6, 7.0	7.6	Caz, Gen, Sxt, Amp
E. aerogenes	Wound	>8.2, 7.6	None	None
E. aerogenes	Blood	>8.2, 7.6	None	None
E. aerogenes	Blood	>8.2, 7.6	None	None
E. cloacae	Blood	>8.2, 7.6, 7.0	7	Caz, Amp, Tet, Cm
E. cloacae	Wound	>8.2, 7.6	None	None

[a] Caz, ceftazidime: Gen, gentamicin; Amp, ampicillin; Tet, tetracycline; Cm, chloramphenicol; Sxt, trimethoprime-sulfamethoxazole.

[b] The two identical strains isolated from the intensive care unit and surgical ward.

transconjugants showed that 3 *E. aerogenes* isolates (from wound swabs) transferred the pI 7.6 enzyme to their transconjugants. The transfer of resistance to gentamicin, trimethoprim-sulfamethoxazole, ampicillin, and ceftazidime accompanied the transfer of the β-lactamase enzyme with a pI of 7.6. An *E. cloacae* blood isolate transferred the β-lactamase enzyme with a pI of 7.0. The enzyme with a pI of 5.6 was not transferred. The remaining 4 *Enterobacter* isolates positive by DDST but found not to transfer their resistance through conjugation experiment all have pIs of >8.2 and 7.6 and were comprised of 3 *E. aerogenes* isolates (2 from blood samples and 1 from a wound sample) and 1 *E. cloacae* isolate (from a wound sample). The ESBL gene in these isolates may not have been transferable because these β-lactamase genes may be carried on the chromosome (which is usually not transferable) or on a transfer-deficient plasmid (16). For PCR amplification, DNA was obtained from the ESBL wild-type isolates and corresponding transconjugants by heating a suspension of a colony in 50 μl of water to 95°C for 10 min. The DNA-containing supernatant was then used as a template in specific PCR for the detection of the bla_{TEM} and bla_{SHV} genes. PCR amplification was performed by using the following primers: for TEM β-lactamase genes, TEM A (5′ ATA AAA TTC TTG AAG AC 3′) and TEM B (5′ TTA CCA ATG CTT AAT CA 3′) (22); for SHV β-lactamase genes, OS 5 (5′ TTA TCT CCC TGT TAG CCA CC 3′) and OS 6 (5′ GAT TTG CTG ATT TCG CTC GG 3′) (1). Cycling conditions were as follows: initial denaturation at 96°C for 5 min; 35 cycles of 96°C for 60 s, 60°C for 60 s (SHV) or 42°C for 60 s (TEM), and 72°C for 60 s; and a final period of extension at 72°C for 10 min. The PCR products were separated on 0.8% agarose gels. Bands were visualized under UV after staining with ethidium bromide and photographed. The TEM primers produced a 1,076-kb fragment, and the SHV primers produced a 790-kb DNA fragment. Amplification by PCR showed that enzymes with pIs of 7.6 were SHV-derived β-lactamases while the enzyme with a pI of 5.6 is a TEM-type β-lactamase. The enzyme with a pI of 7.0 could not be amplified with the SHV and TEM primers (Table 3). Bacterial DNA was prepared, and pulsed-field gel electrophoresis (PFGE) of chromosomal DNA was performed according to published procedures (25) with the restriction enzyme *Xba*I (Promega, Madison, Wis.). Plugs were loaded onto a 0.8% agarose gel (molecular biology certified agarose; Bio-Rad, Hemel Hempstead, United Kingdom). DNA separation was performed in a CHEF-DRIII apparatus with the following conditions: 200 V for 20.2 h with pulse times of 5.5 to 52.0 s at 14°C. A lambda ladder (Bio-Rad) was included for a molecular size marker. PFGE showed clearly different patterns for most of the isolates except for 2 strains found to be indistinguishable, suggesting they were related. These 2 strains were obtained from different wards of the same hospital (the intensive care unit and the surgical ward) and from different patients. The patient from the intensive care unit was moved to the surgical ward. The first strain was isolated in the intensive care unit, and the other strain was subsequently isolated in the surgical ward. This indicates a possible spread of an ESBL-producing strain in the ward. Nosocomial bacterial infections constitute a substantial cause of morbidity and mortality in developing countries such as Nigeria. In recent years, *Enterobacter* spp. have emerged as major nosocomial pathogens in the Lagos clinical environment and significant antibiotic resistance has emerged among these pathogens (18). The rate of beta-lactamase production in Lagos among enterobacteria has been reported to be very high (26). This is attributed to the indiscriminate and widespread use of antibiotics, particularly the beta-lactam antibiotics, which are easily bought over the counter without a prescription from a doctor. This misuse and abuse of antibiotics in this country has adversely contributed to the rate of antibiotic resistance in the country and might also be the cause of the emergence of ESBL enzymes compromising the efficacy of the extended-spectrum cephalosporins in our environment. In the past few years, ESBL production in *Enterobacter* spp. has been described in France (9, 10), Spain (5), and Mexico (32). Several other outbreaks have also been reported (29, 34). Reports exist on the resistance of *Enterobacter* spp. in Lagos to extended-spectrum cephalosporins (18), but there has been no documented investigation into the resistance mechanisms occurring in this environment. To our knowledge, this is the first report on the occurrence of ESBL resistance in *Enterobacter* spp. in Lagos, Nigeria. Eight of the 40 *Enterobacter* isolates (20%) investigated in this study were found to be ESBL producers. This report is similar to that of the prevalence of ESBL-producing *K. pneumoniae* isolates (20.8%) but higher than that of *E. coli* isolates (14.7%) in our environment (unpublished data). The rate is considered rather high when compared with the prevalence of ESBL production in *Enterobacter* isolates from other countries (5, 9).

The result of this study showed a high frequency of ESBLs in the isolates of *E. aerogenes* (25%; 6 of 24) compared with those in isolates of *E. cloacae* (12.5%; 2 of 16) examined. This finding agrees with previous reports on ESBL production in *E. cloacae*, which is documented to be generally low (8, 11, 30). Among isolates of *Enterobacter* strains studied, a high level of resistance to ampicillin-sulbactam, ticarcillin-clavulanic acid, amoxicillin-clavulanic acid, gentamicin, and trimethoprim-sulfamethoxazole was observed while there was reduced susceptibility to the extended-spectrum cephalosporins, the fluoroquinolones, and amikacin (Table 1). This resistance pattern among *Enterobacter* isolates in our study agrees with reports from other parts of the world (15, 17, 33) where resistance to these antibiotics is most prevalent among *Enterobacter* spp.

This study has shown that *Enterobacter* isolates in Lagos, Nigeria possess in addition to the chromosome-borne Bush group 1 β-lactamase, ESBL enzymes with pIs of 7.6 (SHV-2 type) or 5.6 (TEM-2 type) or an enzyme with a pI of 7.0, which

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could not be amplified by the bla_{SHV} and bla_{TEM} genes by using PCR. The most frequently encountered of these enzymes was the ESBL enzyme with a pI of 7.6. This enzyme was also widely disseminated among clinical strains of *Klebsiella-pneumoniae* and *E. coli* isolated in some Lagos hospitals (unpublished data). It is noteworthy in this study that the ESBL-producing isolates of *Enterobacter* strains with β-lactamase enzymes of pI 7.6 transferred this enzyme together with resistance to gentamicin, ampicillin, and trimethoprim-sulfamethoxazole to an *E. coli* recipient strain, suggesting that the genes responsible for the ESBL are carried on the same plasmid as those encoding resistance to these antimicrobial agents and confirming the study previously carried out by Sanders and Sanders (31). Further epidemiological study needs to be carried out in the hospital where PFGE showed 2 identical strains to ascertain the presence of an epidemic strain so that preventive measures may be introduced to prevent further dissemination of this strain in this hospital, but the clearly different pattern of the other isolates suggests that the spread of ESBL-producing *Enterobacter* spp. in this environment is mainly by plasmid dissemination. In conclusion, this study highlights the need to establish an antimicrobial resistance surveillance network for members of the family *Enterobacteriaceae* to monitor the trends and new types of resistance mechanisms in Nigeria. Factors responsible for the selection and dissemination of ESBL-producing strains need to be identified, controlled, and where possible, prevented to avoid major outbreaks of such strains in the country.

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