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Molecular Characterization of a Novel Plasmid-Encoded Cefotaximase (CTX-M-12) Found in Clinical *Klebsiella pneumoniae* Isolates from Kenya

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Nine *Klebsiella pneumoniae* isolates, six from blood and three from cerebrospinal fluid of newborn babies at Kenyatta National Hospital, Nairobi, Kenya, were analyzed for the mechanism of cephalosporin resistance. By using pulsed-field gel electrophoresis of *Xba*I-digested chromosomal DNA, all the nine isolates were found to be clonal. PCR and direct sequencing revealed a novel extended-spectrum β-lactamase, which we designated CTX-M-12. It has a more potent hydrolytic activity against cefotaxime than against ceftazidime and a pI of 9.0 and is encoded on a large self-transferable ca. 160-kbp plasmid.

Resistance to extended-spectrum cephalosporins in the family *Enterobacteriaceae* has commonly been associated with the expression of extended-spectrum TEM and SHV β-lactamases (ESBLs) (10, 14). However, since 1992, novel plasmid-mediated extended-spectrum β-lactamases—the cefotaximases, derived neither from these genes nor from AmpC cephalosporinases but with greater homology to the chromosomally encoded β-lactamases of *Klebsiella oxytoca* E23004—have been described (2, 4). The cefotaximases have more potent hydrolytic activity against cefotaxime than against ceftazidime and belong to Ambler's class A (1) plasmid-mediated enzymes and also within group 2be of the Bush classification (7). To date 10 variants of the CTX-M-type β-lactamases have been described in various enterobacterial species, including *Escherichia coli, Salmonella enterica* serovar Typhimurium, *Citrobacter* spp., and *Enterobacter* spp. (Table 1). A further six CTX-M-type sequences are registered in international databanks (EMBL and GenBank). In the present study we report a new CTX-M-type cefotataxime-hydrolyzing β-lactamase, which we have designated CTX-M-12.

Patients were babies aged 1 to 7 days with suspected sepsis admitted to the NewBorn Unit (NBU) of Kenyatta National Hospital, Nairobi, Kenya, between July 1999 and February 2000. A total of nine isolates of *Klebsiella pneumoniae* were obtained from blood (6) and cerebrospinal fluid (3), and their identity as *K. pneumoniae* was confirmed by biochemical tests using API 20E strips (bioMérieux, Basingstoke, United Kingdom). Isolates were frozen at −70°C on protect beads (TCS, Wirral, United Kingdom) until analyzed.

Disk susceptibility to ampicillin (10 μg), coamoxyclav (20:10 μg), cephradine (30 μg), cefuroxime (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), aztreonam (30 μg), carbenicillin (100 μg), cotrimoxazole (1:25 μg), gentamicin (10 μg), chloramphenicol (30 μg), streptomycin (10 μg), tetracycline (30 μg), and nalidixic acid (30 μg) (Oxoid Ltd., Basingstoke, United Kingdom) was measured using a controlled disk diffusion technique (23) on Iso-Sensitest agar (Oxoid). In addition, testing of susceptibility to cefotaxime or to ceftazidime alone (E test MIC strips; AB BioDisk, Solna, Sweden) and in combination with clavulanic acid (E test extended-spectrum-TEM-and-SHV-β-lactamase strips; AB BioDisk) was performed on donors and *E. coli* K-12 transconjugants. Screening to detect phenotypic production of AmpC β-lactamases was done as described by Coudron et al. (9) by employing both direct susceptibility testing with a cefoxitin 30-μg disk and their previously described three-dimensional extract test.

Chromosomal DNA from *K. pneumoniae* isolates was prepared in agarose plugs as described previously (15). DNA in agarose plugs was digested using 25 U of *Xba*I (Gibco Life Technologies, Paisley, United Kingdom). Pulsed-field gel electrophoresis (PFGE) was performed with a CHEF DR 11 system (Bio-Rad Laboratories, Richmond, Calif.) on a horizontal 1% agarose gel for 24 h at 120 V, with a pulse time of 1 to 40 s at 14°C. A lambda DNA digest consisting of a ladder (ca. 22 fragments) of increasing size from 48.5 to approximately 1,000 kb was included as a DNA size standard. The restriction endonuclease digest patterns were compared by the method of Tenover et al. (20).

Plasmid DNA extraction was performed using a Plasmid Mini Prep Kit (Qiagen Ltd., West Sussex, United Kingdom) according to manufacturer's instructions. Plasmids were separated by electrophoresis on horizontal 0.8% agarose gels at 100 V for 2 h. Plasmid sizes were determined by coelectrophoresis with plasmids of known sizes from *E. coli* strains V517 (NCTC 50193) (53.7, 7.2, 5.6, 3.9, 3.0, 2.7, and 2.1 kb) and 39R861 (NCTC 50192) (147, 63, 43.5, and 6.9 kb). DNA bands were visualized with an UV transilluminator (UVP Inc., San Gabriel, Calif.) after staining with 0.05% ethidium bromide. Mating experiments were performed in broth as described previ-

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TABLE 1. The evolution of the CTX-M-like enzymes (cefotaximases) isolated from various enterobacterial species from different parts of the world[a]

Enzyme	Isolate	pI	CTX MIC (μg/ml)	Source (year)	Reference
MEN-1	*E. coli*	8.4	28	France (1989)	4
CTX-M-1	*E. coli*	8.9	28	Germany (1990)	3
CTX-M-2	Serovar Typhimurium	7.9		Argentina (1990)	3
CTX-M-3	*Citrobacter freundii*	8.4	>512	Poland (1996)	13
	E. coli	8.4	>512		
CTX-M-4	Serovar Typhimurium	8.4	>512[b]	Russia (1998)	11
CTX-M-5	Serovar Typhimurium	8.8	128	Latvia (1998)	6
CTX-M-6	Serovar Typhimurium	8.4	>256[b]	Russia (1998)	12
CTX-M-7	Serovar Typhimurium	8.4	>256[b]	Russia (1998)	12
CTX-M-8	*Enterobacter cloacae*	7.6		Brazil (1997)	5
	Enterobacter aerogenes	7.6			
	Citrobacter amalonaticus	7.6	32		
CTX-M-9	*E. coli*	8.0	24	Spain (1996)	19
CTX-M-10	*E. coli*	8.1	8	Spain (2000)	17
CTX-M-11				Japan (2000)	GenBank
CTX-M-12	*K. pneumoniae*	9.0	24	Kenya (2000)	GenBank

[a] All data are as obtained from references listed.
[b] Expressed in *E. coli* transformants.

ously (22) using *E. coli* K-12 as recipient. Transconjugants were selected on MacConkey agar (Oxoid) supplemented with nalidixic acid (32 μg/ml) and cefotaxime (32 μg/ml).

As all nine *K. pneumoniae* isolates had similar antibiotic susceptibility patterns and were indistinguishable by PFGE, three representative isolates (two from blood and one from cerebrospinal fluid) were selected for further analysis. Extraction of β-lactamases was performed using the freeze-thaw method, and isoelectric focusing was performed as previously described (8) on polyacrylamide gels containing ampholines with pI's ranging from 3.5 to 9.5 (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). Native proteins were applied directly, following preincubation (15 min) with clavulanic acid (10 μg/ml). Gels were calibrated using an isoelectric focusing calibration kit (Amersham Pharmacia Biotech). Nitrocefin, a chromogenic cephalosporin, was used throughout the analysis for detection of production of β-lactamases. Detection of β-lactam hydrolysis by separated proteins postelectrophoresis was done by layering the ampholine gel with agar containing cefotaxime (0.4 μg/ml). After incubation at 37°C for 2 h, the agar was flooded with *E. coli* (NCTC 10418) and reincubated overnight. *E. coli* strains DP38 and DP42, encoding SHV-1 (pI 7.6) and TEM-1 (pI 5.4) β-lactamases, respectively, were used additionally as controls for isoelectric focusing.

Total DNA for PCR was extracted by suspending donors or transconjugants in 5% (wt/vol) Chelex-100 slurry (Bio-Rad) in injection-grade water followed by boiling for 10 min. PCR amplification of the entire coding sequence of the $bla_{\text{CTX-M}}$ gene (ca. 1-kb amplicon) was done by the method described by Gniadowski et al. (13), using primers P1C (5′-TCG TCT CTT CCA GA-3′) and P2D (5′-CAG CGC TTT TGC CGT CTA AG-3′). Sequence determination was performed using the PCR primers on both strands of the amplicons with a dideoxy-chain determination method using an automated DNA sequencer ABI PRISM 377 (Perkin-Elmer, Warrington, United Kingdom) and was analyzed using commercial software (Lasergene; DNAStar Inc., Madison, Wis.). The nucleotide sequence structure was compared to those of the $bla_{\text{CTX-M-1}}$ gene (EMBL accession no. X92506), $bla_{\text{CTX-M-3}}$ gene (EMBL accession no. Y10278), and $bla_{\text{SHV-1}}$ gene (GenBank accession no. X98100) in GenBank.

All nine *K. pneumoniae* isolates were uniformly resistant to ampicillin, cephradine, cefuroxime, cefotaxime, carbenicillin, imipenem, and tetracycline. However, they were susceptible to coamoxyclav, aztreonam, streptomycin, cotrimoxazole, gentamicin, and nalidixic acid. When the E test was used, the MICs of cefotaxime and ceftazidime were 24 and 1 μg/ml, respectively. The presence of clavulanic acid lowered the MIC of cefotaxime 750 times to 0.032 μg/ml, indicating that resistance was due to production of extended-spectrum β-lactamases. All nine isolates gave an identical PFGE pattern and contained plasmids with molecular sizes of ca. 160 kbp. All the isolates transferred resistance to ampicillin, cephradine, cefuroxime, cefotaxime, and tetracycline to *E. coli* K-12 on the 160-kbp plasmid. A summary of the evolution of CTX-M-type β-lactamases is shown in Table 1. The cefotaximase produced by the *K. pneumoniae* outbreak strains and their *E. coli* K-12 transconjugants had a pI of 9.0. SHV-type β-lactamases were not detected in either parent strains or *E. coli* K-12 transconjugants. When a PCR assay for CTX-M-type genes was used, amplicons were detected in the parents, transconjugants, and extracted plasmid DNA from the transconjugants. Our sequence data indicate an open reading frame of 879 bp, corresponding to 293 amino acid residues. The four amino acid changes previously reported between $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-3}}$ were detected (13). However, compared to $bla_{\text{CTX-M-3}}$, five silent point changes were found at positions Ala-52 (GCA to GCG), Phe-66 (TTT to TTC), Leu-102 (CTT to CTG), Ala-223 (GCT to GCA), and Ile-250 (ATC to ATT) and three amino acid substitutions were found at positions 12, threonine (ACC) to alanine (GCC); 89, asparagine (AAT) to serine (AGT); and 278, valine (GTA) to isoleucine (ATA). Table 2 shows amino acid changes in CTX-M-12 compared to $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-3}}$. The four consensus motifs ^{70}SXXK^{73}, ^{130}SDN^{132}, E-166, and ^{234}KTG^{236} typical of class A serine β-lactamases (1) were also found in the amino acid sequence of this new β-lactamase. As these substitutions are

TABLE 2. Amino acid differences between CTX-M-12 β-lactamase and the related cefotaxime-hydrolyzing enzymes CTX-M-1 (MEN-1) and CTX-M-3

β-Lactamase	Amino acid residue at position:						
	12	77	89	114	140	278	288
CTX-M-1	Thr	Val	Asn	Asp	Ser	Val	Asn
CTX-M-3	Thr	Ala	Asn	Asn	Ala	Val	Asp
CTX-M-12	Ala	Ala	Ser	Asn	Ala	Ile	Asp

not shared by other recorded CTX-M-type β-lactamases, the enzyme from Kenyan strains (pI 9.0) appears to be a novel extended-spectrum β-lactamase and has been designated CTX-M-12.

During the study period, morbidity due to bacterial sepsis in the NBU rose to 40% with 35% mortality, compared to a morbidity rate of 17% during 1997. This rise was attributed to a steep increase in admissions to the NBU, thus increasing congestion and nosocomial spread of *K. pneumoniae*. Clonal spread was detected by PFGE in all nine *K. pneumoniae* isolates.

The cefotaxime-hydrolyzing β-lactamase was encoded on a ca. 160-kbp self-transferable plasmid, which also conferred resistance to ampicillin, cephradine, cefuroxime, carbenicillin, imipenem, and tetracycline. These agents comprise most of the commonly available drugs in the hospital and therefore posed a major problem in the treatment and management of *K. pneumoniae* sepsis in the newborn babies. Although these outbreak strains were sensitive to ceftazidime, the cost of treatment is difficult for the majority of patients. Previous studies have also demonstrated that cefotaximases were found on a large self-transferable plasmid that also conferred resistance to other β-lactams as well as to aminoglycosides (6, 11, 21). Although the origin of these large plasmid-encoded β-lactamases is still unknown, they are closely related to the chromosomally mediated β-lactamase from *K. oxytoca* (18).

Previously CTX-M-type β-lactamases were detected in species of the *Enterobacteriaceae* from different parts of the world, including Europe and South America. However, to our knowledge, this is the first report from Africa of a CTX-M-type β-lactamase from a nosocomial *K. pneumoniae* outbreak. Although the Kenyatta National Hospital has a functioning Infection Control Committee, the introduction of more prudent infection control strategies in the NBU may be helpful in the control of nosocomial *K. pneumoniae* outbreaks, which have been a major problem in the hospital (16).

Nucleotide sequence accession number. The nucleotide sequence data reported appears in the GenBank nucleotide sequence database under accession no. AF305837.

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