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Emergence and Outbreaks of CTX-M β-Lactamase-Producing *Escherichia coli* and *Klebsiella pneumoniae* Strains in a Tunisian Hospital[▽]

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Sixty-two isolates of *Enterobacteriaceae* (35 *Escherichia coli* and 27 *Klebsiella pneumoniae* isolates) producing CTX-M-type β-lactamases were collected between March 2000 and June 2003 in different wards of Charles Nicolle Hospital in Tunis (Tunisia). Sequencing identified the $bla_{CTX-M-15}$ determinant in 55 isolates and $bla_{CTX-M-16}$ in 7 isolates. The CTX-M-15-producing strains were isolated in several wards and consisted mainly of two successive clonal groups of *E. coli* and a major clonal group of *K. pneumoniae*. The second clonal group of *E. coli* belonged to phylogenetic group B2 and harbored more virulence factors than the first clonal group. Among the 22 transconjugants or electroporants obtained with selected *E. coli* and *K. pneumoniae* CTX-M-15-producing strains, a predominant plasmid restriction pattern was obtained with 17 isolates. The four CTX-M-16-producing strains of *E. coli* yielded the same pulsed-field gel electrophoresis (PFGE) pattern, while the three CTX-M-16-producing strains of *K. pneumoniae* yielded two different PFGE patterns. All of the CTX-M-16-producing isolates were recovered in the pediatric ward and had the same plasmid restriction pattern.

First reported in Argentina and France (3, 7), the CTX-M-type enzymes were subsequently found in several European countries as well as in Asia and North America (3). Recent studies have shown the presence of these enzymes in African countries (2, 13, 14, 24, 31, 37, 39). In Tunisia, the first identified CTX-M-producing strain (CTX-M-3), *Salmonella enterica* serovar Wien, was recovered in Tunis in 2001 (1). Later, a strain (*Salmonella enterica* serovar Livingstone) producing CTX-M-27 caused a nosocomial outbreak in a neonatal ward in Sousse in 2002 (4).

One of these enzymes, CTX-M-15, is now found worldwide, mainly in *Escherichia coli* isolates recovered in hospitals and in the community and responsible for outbreaks in France, the United Kingdom, Sweden, and Canada (11, 12, 25, 28, 29, 30, 33, 40). Molecular characterization of plasmids encoding CTX-M-15 from *E. coli* strains involved in outbreaks in different countries showed that they additionally carried other antibiotic resistance genes, such as bla_{OXA-1}, bla_{TEM-1}, *tetA*, *aac(6′)-Ib*, and *aac(3)-II*, and sometimes a class 1 integron (6, 23, 27).

Phylogenetic studies of *E. coli* isolates producing CTX-M enzymes indicate that most belong to phylogenetic group D, except that CTX-M-15 producers often belong to group B2 (8, 25, 28, 32).

Since the first isolation of an extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* strain at Charles Nicolle Hospital, Tunis, in 1984, a growing variety of *Enterobacteriaceae* and ESBL enzymes have been detected; 60% of the isolates were *Klebsiella* spp. and 12.5% were *E. coli* (5). In March 2000, an ESBL-producing clinical isolate of *E. coli* exhibiting an unusual resistance phenotype (a higher level of resistance to cefotaxime than to ceftazidime) was recovered. The aims of this retrospective study of all ESBL-producing *E. coli* and *K. pneumoniae* isolates recovered in Charles Nicolle Hospital from March 2000 to June 2003 were (i) to detect and identify CTX-M enzymes and associated resistance genes, (ii) to conduct an epidemiological investigation using chromosome and plasmid fingerprint analyses, and (iii) to determine the phylogenetic group and virulence factors of *E. coli* isolates.

MATERIALS AND METHODS

Bacterial strains. All ESBL-producing strains of *E. coli* and *K. pneumoniae* recovered at Charles Nicolle Hospital between March 2000 and June 2003 were collected and identified with API 20E systems (bioMérieux, Marcy l'Etoile, France). *E. coli* J53-2 (*pro met* Rif[r]) and *E. coli* DH10B (Invitrogen SARL, Cergy-Pontoise, France) were used for conjugation and electroporation, respectively.

The nosocomial character of the infections was defined when the first CTX-M isolate was recovered from clinical samples obtained 3 days or more after admission.

Study of antibiotic consumption. Broad-spectrum cephalosporin (cefotaxime, ceftazidime, and ceftriaxone) use data were collected from the pharmaceutical department of the Charles Nicolle Hospital between 2000 and 2003. Data were expressed in grams of active substance and then in the number of defined daily doses (DDD) according to the Anatomic Therapeutic Chemical classification from WHO Index 2006 (http://www.whocc.no/atcddd/). The number of hospital-

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TABLE 1. Primers for phylogenetic studies, virulence factors, and resistance genes used for PCR assays

Target	Sequence (5′–3′)	Primer name	Reference
chuA	GACGAACCAACGGTCAGGAT	chuA.1	9
	TGCCGCCAGTACCAAAGACA	chuA.2	
yjaA	TGAAGTGTCAGGAGACGCTG	yjaA.1	9
	ATGGAGAATGCGTTCCTCAAC	yjaA.2	
TspE4.C2	GAGTAATGTCGGGGCATTCA	TspE4C2.1	9
	CGCGCCAACAAAGTATTACG	TspE4C2.2	
papG allele II	GGGATGAGCGGGCCTTTGAT	AlleleII-f	18
	CGGGCCCCCAAGTAACTCG	AlleleII-r	
papG allele III	GGCCTGCAATGGATTTACCTGG	AlleleIII-f	18
	CCACCAAATGACCATGCCAGAC	AlleleIII-r	
sfa/*foc*	CTCCGGAGAACTGGGTGCATCTTAC	sfa1	18
	CGGAGGAGTAATTACAAACCTGGCA	sfa2	
afa/*draBC*	GGCAGAGGGCCGGCAACAGGC	Afa f	18
	CCCGTAACGCGCCAGCATCTC	Afa r	
fimH	TGCAGAACGGATAAGCCGTGG	FimH f	18
	GCAGTCACCTGCCCTCCGGTA	FimH r	
hlyA	AACAAGGATAAGCACTGTTCTGGCT	hly f	18
	ACCATATAAGCGGTCATTCCCGTCA	hly r	
cnf1	AAGATGGAGTTTCCTATGCAGGAG	cnf1	18
	CATTCAGAGTCCTGCCCTCATTATT	cnf2	
fyuA	TGATTAACCCCGCGACGGGAA	FyuA f′	18
	CGCAGTAGGCACGATGTTGTA	FyuA r	
iutA	GGCTGGACATCATGGGAACTGG	AerJ f	18
	CGTCGGGAACGGGTAGAATCG	AerJ r	
kpsMT II	GCGCATTTGCTGATACTGTTG	kpsII f	18
	CATCCAGACGATAAGCATGAGCA	kpsII r	
traT	GGTGTGGTGCGATGAGCACAG	TraT f	18
	CACGGTTCAGCCATCCCTGAG	TraT r	
sat	ACTGGCGGACTCATGCTGT	Sat 1	35
	AACCCTGTAAGAAGACTGAGC	Sat 2	
iha	CTGGCGGAGGCTCTGAGATCA	IHA f	19
	TCCTTAAGCTCCCGCGGCTGA	IHA r	
iroN	AAGTCAAAGCAGGGGTTGCCCG	IRONEC-F	19
	GACGCCGACATTAAGACGCAG	IRONEC-R	
tetA	GTTTCGGGTTCGGGATGGTC	tetA up	This study
	GCAGGCAGAGCAAGTAGAGG	tetA low	
$bla_{\text{OXA-1}}$	TATCAACTTCGCTATTTTTTA	OXA-1 up	This study
	TTTAGTGTGTTTAGAATGGTGA	OXA-1 low	
aac(6′)-Ib	ATGACTGAGCATGACCTT	AAC6′-Ib up	28
	GAAGGGTTAGGCATCACT	AAC6′-Ib low	
aac(3)-II	CAATAACGGAGGCAATTCG	AAC3-II up	28
	GATTATCATTGTCGACGG	AAC3-II low	
sul1	CGGCGTGGGCTACCTGAACG	Sul 1-F	26
	GCCGATCGCGTGAAGTTCCG	Sul 1-B	
sul2	GCGCTCAAGGCAGATGGCATT	Sul 2-F	26
	GCGTTTGATACCGGCACCCGT	Sul 2-B	

ization days was used to calculate the penetration index (ratio number of DDD to 1,000 hospitalization days).

Antibiotic susceptibility testing. Antibiotic susceptibility was tested with the agar disk diffusion method according to CLSI (formerly NCCLS) guidelines (10). ESBLs were detected using a standard double-disk synergy test (17). The CTX-M phenotype of ESBL producers screened in this study was based on a similar or smaller inhibition zone with cefotaxime than with ceftazidime. The MICs of the following antibiotics were determined by a dilution method in Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France): ticarcillin, cefotaxime, and ceftazidime alone and combined with clavulanic acid (2 mg/liter), cefoxitin, and cefepime. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains.

Characterization of β-lactamases and associated resistance genes. All ESBL-producing strains with a CTX-M phenotype were subjected to CTX-M consensus PCR (groups M-1, M-2, and M-9) using primers MA1 and MA2 as previously described (Table 1) (36). All PCR-positive strains were subjected to genomic DNA extraction with a QIAGEN mini kit (QIAGEN). Genes encoding TEM and CTX-M-1-type, CTX-M-2-type, and CTX-M-9 β-lactamases were amplified by PCR and sequenced as previously described (11). The nucleotide sequences and deduced protein sequences were analyzed with the BLAST and Clustal W programs (multiple-sequence alignment, pair-wise comparisons of sequences, and dendrograms).

Other antibiotic resistance genes, often found associated with $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, *aac(3)-II*, *aac(6′)-Ib*, and *tetA* as well as *sul1* and *sul2* genes, were screened by PCR using the primers listed in Table 1.

Fingerprinting analysis. Repetitive extragenic palindromic sequence PCR was performed with primers rep-1R and rep-2T for all of the *E. coli* isolates as previously described (11). Enterobacterial repetitive intergenic consensus sequence PCR was performed with primer ERIC-2 for all of the *K. pneumoniae* isolates as previously described (11). If isolates had similar patterns, they were subjected to pulsed-field gel electrophoresis (PFGE). PFGE was performed using a GenePath system (Bio-Rad, Marnes-la-Coquette, France) with genomic DNA digested with XbaI (Ozyme, Saint Quentin en Yvelines, France) at 14°C and 6 V/cm for 20 h, with pulse times of 5.3 to 49.9 s. Clonal relationships based on PFGE patterns were interpreted using the criteria established by Tenover et al. (38).

β-Lactam resistance transfer assays and plasmid fingerprint analysis. Conjugation was carried out in Trypticase soy broth (Bio-Rad), with *E. coli* J53-2 as the recipient. Mating broths were incubated at 37°C for 18 h. Transconjugants

were selected on Mueller-Hinton agar plates containing rifampin (250 mg/liter) and cefotaxime (2.5 mg/liter).

For transformation, plasmid DNA extracted from donors with a QIAGEN plasmid midi kit (QIAGEN, Courtaboeuf, France) were used to transform *E. coli* DH10B cells by electroporation following the manufacturer's instructions (Bio-Rad). Transformants were incubated for 1.5 h at 37°C and then mated on Drigalski agar (Bio-Rad) supplemented with 2.5 mg/liter cefotaxime.

For plasmid fingerprinting, plasmid DNA was extracted from the transconjugants and transformants with the QIAGEN plasmid midi kit and digested with EcoRI or HpaI. The resulting fragments were subjected to electrophoresis on a 0.8% agarose gel.

Phylotyping and virulence genotyping of *E. coli*. The phylogenetic group of the *E. coli* isolates was determined by the PCR method developed by Clermont et al. (9), using a combination of three DNA markers (*chuA*, *yjaA*, and TspE4.C2). All isolates were screened for 14 virulence factors often found in extraintestinal pathogenic *E. coli* (ExPEC), namely, *fimH*, *sfa*/*foc*, *papG* allele II and allele III, *afa*, *hlyA*, *cnf1*, *fyuA*, *iutA*, *kpsM* II, *traT*, *sat*, *iroN*, and *iha*, using single or multiplex PCR assays (18, 19, 35) and the primers listed in Table 1.

Three archetypal ExPEC strains, CFT073, ECOR66, and EC7372, producing various virulence factors were used as positive controls (16, 20, 22).

Statistical analysis. Factorial analysis of correspondence (FAC) was used to describe associations among clinical and bacterial data (15). FAC uses a covariance matrix based on χ^2 distances. The computation determines a plane defined by two principal axes of the analysis; the first axis, F1, accounts for most of the variance, and the second axis, F2, orthogonal to F1, accounts for the largest part of the variance not accounted for by F1. The F1/F2 plane allowed the positioning of the variables according to their coordinates on each of these factors. This positioning describes the relation between the variables. When two variables are closely related on the plane, they are strongly associated. On the contrary, when they are distantly related, they are not associated. FAC was conducted with SPAD.N software (Cisia, Saint Mandé, France) from two two-way tables. A first table was constructed for the *E. coli* strains and had 35 rows (one for each *E. coli* strain) and 31 columns corresponding to the following 31 variables: the 4 years of isolation (2000 to 2003), the four wards (surgery, urology, general medicine, and pediatrics), the three types of infection (urine, blood, and others), the three phylogenetic groups (A, B2, and D), the seven molecular profiles (E1 to E7), the CTX-M type (15 or 16), and the nine discriminating virulence factors (Table 1). A second table was constructed for the *K. pneumoniae* strains. It had 27 rows (one for each *K. pneumoniae* strain) and 18 columns corresponding to the following 18 variables: the five wards (surgery, general medicine, urology, pediatrics, and intensive care unit [ICU]), the three types of infection (urine, blood, and others), the nine molecular profiles (K1 to K9), and the CTX-M type (15 or 16). In each column, each strain was coded as a binary variable (present = 1, absent = 0).

RESULTS

Clinical isolates. The first isolate found to produce a CTX-M-type β-lactamase was an *E. coli* strain recovered from the urine of a surgical patient (8 March 2000). Two months later, other isolates harboring the CTX-M PCR consensus sequence were found in the general medicine ward and subsequently in other wards. By June 2003, 35 *E. coli* and 27 *K. pneumoniae* strains recovered from different patients and positive for CTX-M consensus PCR were detected. CTX-M-producing *E. coli* and *K. pneumoniae* strains represented 8% and 2.7% of ESBL producers, respectively, belonging to the same species in 2000, compared to 22% and 1.25% in 2001, 28.5% and 8% in 2002, and 27% and 30% in 2003 (January to June).

Their ward distribution was as follows: 28% general medicine, 28% surgery, 21% urology, 15% pediatrics, and 8% ICU. They were associated with urinary tract infections (52%), bacteremia (22%), wound infections (14%), lower respiratory tract infections (7%), and catheter colonization (5%) (Table 2). All of these infections were nosocomial.

During the same period, the total number of ESBL-producing *E. coli* and *K. pneumoniae* strains were, respectively, 50 and

TABLE 2. Epidemiological data, resistance determinants, and plasmid backbones of the different clones of *E. coli* and *K. pneumoniae* producing CTX-M-15 or CTX-M-16 β-lactamase at the Charles Nicolle Hospital in Tunis

Clone (*n*)	CTX-M allele	Period of isolation (mo/yr)	Ward(s)	No. of clones with indicated type of infection:				No. of clones with[a]:							Plasmid backbone(s)[b]
				Urine	Blood	Pus	Respiratory	Catheter	*bla*$_{TEM-1}$	*bla*$_{OXA-1}$	*aac(3)-II*	*aac(6′)-Ib*	*tetA*	*sulI*	*sul2*	Cip^r	
E1 (10)	15	03/00–01/02	Surgery, medicine 1, urology	9	0	1	0	0	10	8	9	10	10	0	10	10	P1 (2/2)
E2 (3)	16	07/01–12/01	Pediatric	3	0	0	0	0	0	0	0	0	0	0	0	0	P3 (3/3)
E3 (3)	15	09/01	Medicine 1	3	0	0	0	0	3	0	3	3	0	0	2	3	P1 (3/3)
E4 (1)	16	03/02	Pediatric	1	0	0	0	0	1	0	0	1	0	1	0	0	P3 (1/1)
E5 (1)	15	10/02	Surgery	0	1	0	0	0	1	1	1	1	0	0	1	1	P1 (1/1)
E6 (16)	15	12/02–06/03	Surgery, medicine 1 and 2, urology	4	5	3	2	2	15	15	15	15	0	14	0	16	P1 (2/2), P5 (1/1)
E7 (1)	15	12/02	Medicine 1	1	0	0	0	0	0	1	1	0	0	0	0	1	P1 (1/1)
K1 (2)	16	05/00–06/00	Pediatric	2	0	0	0	0	0	2	0	2	2	0	0	0	P3 (2/2)
K2 (1)	16	08/01	Pediatric	1	0	0	0	0	0	1	0	1	1	1	0	0	P3 (1/1)
K3 (1)	15	05/02	Medicine 3	1	0	0	0	0	1	0	1	1	0	1	0	0	P6 (1/1)
K4 (17)	15	10/02–06/03	Surgery, medicine 1, urology, ICU	4	8	4	2	1	14	17	17	17	0	1	17	17	P1 (5/5)
K5 (1)	15	12/02	Urology	1	0	0	0	0	1	1	1	1	1	1	1	1	P1 (1/1)
K6 (2)	15	12/02–03/03	Surgery, medicine 1	0	2	0	1	1	0	2	2	2	2	2	0	2	P1 (2/2)
K7 (1)	15	03/03	Pediatric	0	1	0	0	0	1	1	1	1	1	0	0	0	P7 (1/1)
K8 (1)	15	03/03	Surgery	1	0	0	0	0	1	1	1	1	0	0	0	1	P2 (1/1)
K9 (1)	15	05/03	ICU	0	0	0	1	0	1	1	1	1	0	0	0	0	P4 (1/1)

[a] These resistances were detected by PCR as described in Materials and Methods, except for the resistance to ciprofloxacin (Cip^r), which was detected only by the disk diffusion method.
[b] The plasmid backbone has been analyzed only in transconjugants or electroporants. Values in parentheses are numbers of plasmid analyses/numbers of transconjugants or electroporants.

73 in 2000, 54 and 81 in 2001, 35 and 113 in 2002, and 37 and 54 from January to June 2003.

Broad-spectrum cephalosporin consumption. The evolution of the consumption of broad-spectrum cephalosporins, evaluated by the penetration index (number of DDD per 1,000 hospitalization days), showed a global increase of 27% between 2000 and 2003 (from 34.5 to 43.9). The penetration index of cefotaxime increased from 29.8 to 34.9, and that of ceftazidime increased from 4.7 to 8.9. The consumption of ceftriaxone was very small (less than 1%).

β-Lactam susceptibility. All of the strains were highly resistant to ticarcillin (MIC > 1,024 mg/liter). The cefotaxime MICs ranged from 256 to >2,048 mg/liter (MIC_{90}, 1,024 mg/liter), and those of ceftazidime ranged from 64 to >2,048 mg/liter (MIC_{90}, 128 mg/liter). Thirty *E. coli* (83%) and seven *K. pneumoniae* (22%) isolates had a higher level of resistance to cefotaxime than to ceftazidime. However, 5 *E. coli* and 20 *K. pneumoniae* isolates showed similar levels of resistance to cefotaxime and ceftazidime (256 to >2,048 mg/liter). Clavulanic acid partially or completely restored the activities of cefotaxime (0.5 to 64 mg/liter) and ceftazidime (1 to 128 mg/liter). All of the isolates were resistant to cefepime and aztreonam (16 to >128 mg/liter) but remained susceptible to imipenem.

Characterization of β-lactamase-encoding genes and other resistance genes. The results of PCR and sequence analysis are summarized in Table 2. CTX-M-encoding genes were detected in all of the isolates and in their transconjugants/electroporants. The deduced amino acid sequences corresponded to CTX-M-15 in 55 isolates (31 *E. coli* and 24 *K. pneumoniae* isolates) and CTX-M-16 in 7 isolates (4 *E. coli* and 3 *K. pneumoniae* isolates). The bla_{TEM-1} gene was identified in 30 *E. coli* and 19 *K. pneumoniae* isolates; all but one (an *E. coli* isolate, Ec7) of the seven isolates carrying $bla_{CTX-M-16}$ were negative for bla_{TEM}. The bla_{OXA-1} gene was detected in 52 isolates (25 *E. coli* and 27 *K. pneumoniae* isolates). The aminoglycoside resistance genes *aac(3)-II* and *aac(6′)-Ib* were found in 55 and 58 isolates, respectively. *tetA* was found in 11 *E. coli* isolates and 7 *K. pneumoniae* isolates. *sul2* was detected in 13 *E. coli* isolates and 18 *K. pneumoniae* isolates, whereas *sul1* was detected in 15 *E. coli* and 6 *K. pneumoniae* isolates. Only two *K. pneumoniae* isolates produced both *sul1* and *sul2*. Thirty-one *E. coli* isolates and 21 *K. pneumoniae* isolates were resistant to ciprofloxacin. None of the pediatric isolates was resistant to ciprofloxacin.

Epidemiological results. The 35 *E. coli* isolates yielded seven distinct repetitive extragenic palindromic sequence PCR patterns, and the 27 *K. pneumoniae* isolates yielded nine different enterobacterial repetitive intergenic consensus sequence PCR patterns (Table 2 and data not shown). Isolates with similar patterns were subjected to PFGE and were classified as clonally related (Fig. 1) (38). Two major clones producing CTX-M-15 were observed among *E. coli* isolates and were designated clone E1 (10 isolates, 28.6%) and clone E6 (16 isolates, 45.7%). Two minor clones were observed (E2 and E3), each comprising three isolates; one of them, E3, which produced CTX-M-16, was recovered only in the pediatric ward. Clone E1 predominated in 2000 to 2001, and clone E6 predominated in 2002 to 2003 (Fig. 1). Seventeen *K. pneumoniae* strains producing CTX-M-15 had the same profile, designated K4 (63%); all were isolated in 2002 to 2003 (Table 2 and Fig. 2). The three *K. pneumoniae* strains producing CTX-M-16 (clones K1 and K2) were recovered, all in the pediatric ward (Table 2). *tetA* was associated with the *E. coli* clonal strain E1. *sul2* was also detected in clonal strain E1 and in all *K. pneumoniae* isolates belonging to clone K4, whereas *sul1* was detected only in *E. coli* clonal group E6 (Table 2).

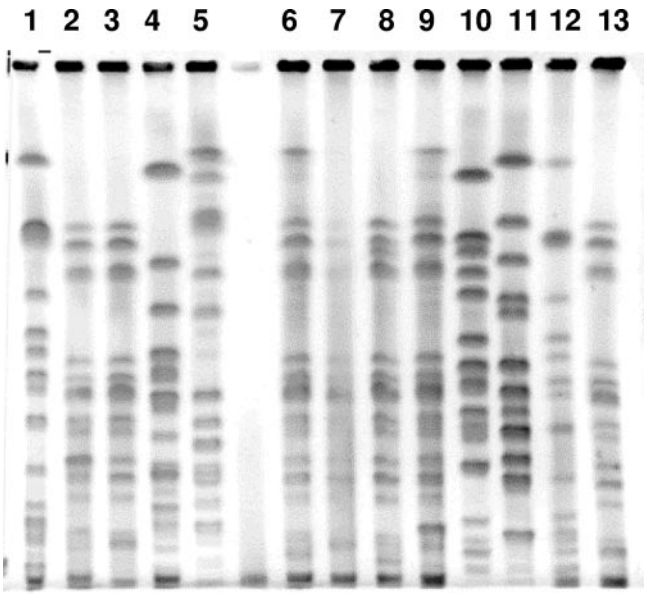

FIG. 1. PFGE patterns of *K. pneumoniae* strains carrying bla_{CTX-M} genes. Lanes 1 to 13, Kp19, Kp20, Kp65, Kp18, Kp15, Kp61, Kp67, Kp62, Kp69, Kp13, Kp14, Kp56, and Kp30 corresponding to molecular types K1, K4, K4, K9, K6, K4, K4, K4, K4, K3, K7, K1, and K4, respectively.

Transferability of CTX-M determinants and plasmid fingerprint analysis. Fourteen *E. coli* and 15 *K. pneumoniae* isolates were selected according to their bla_{CTX-M} gene, their fingerprint, and their antimicrobial resistance pattern. Four and eight *E. coli* isolates with the E1 and E6 fingerprints, respectively, were selected. All 29 strains were tested for conjugal transfer of cefotaxime resistance, and 19 (9 *E. coli* and 10 *K. pneumoniae* isolates) were positive. Electroporation of plasmid DNA from the other 10 strains into *E. coli* DH10B successfully transferred cefotaxime resistance. Large plasmids were found in *K. pneumoniae* and *E. coli* transconjugants or electroporants. EcoRI restriction of plasmids from transconjugants and electroporants of the 22 strains producing CTX-M-15-type enzymes yielded six different patterns, with a major plasmid restriction pattern (P1) in 17 strains (data not shown). This P1 plasmid was found in all *E. coli* clones and in three *K. pneumoniae* clones, including epidemic clone K4. Plasmids isolated from the six electroporants and one transconjugant of the CTX-M-16-producing strains yielded similar restriction patterns, named P3, after digestion with HpaI (Fig. 3).

Phylogenetic analysis and virulence genotyping of *E. coli* isolates. The results of phylogenetic studies and virulence factor (Vf) determination are reported in Table 3. The two largest clones, E1 and E6, belong to phylogenetic groups A and B2, respectively. The two minor clones, E2 and E3, belong to groups B2 and D, respectively. Clone E2 (group B2) expressed virulence factors often encountered in ExPEC strains (*fyuA*,

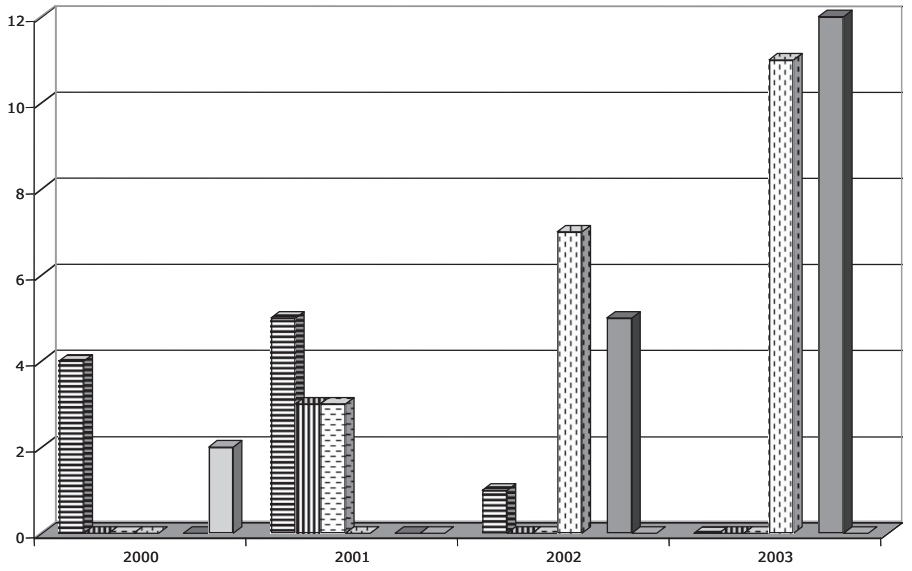

FIG. 2. Number of patients infected by clonal strains of *E. coli* and *K. pneumoniae* producing CTX-M-type β-lactamases and isolated at Charles Nicolle Hospital between March 2000 and June 2003. Bar with horizontal hatching, *E. coli* clone E1 (CTX-M-15); bar with vertical hatching, *E. coli* clone E2 (CTX-M-16); bar with horizontal dashes, *E. coli* clone E3 (CTX-M-15); bar with vertical dashes, *E. coli* clone E6 (CTX-M-15); light-gray bar, *K. pneumoniae* clone K1 (CTX-M-16); dark-gray bar, *K. pneumoniae* clone K4 (CTX-M-15).

papG allele III, *hlyA*, *cnf1*, *kpsMT* II, *iha*, and *sat*), whereas clone E6, which belongs to the same phylogenetic group, expressed fewer Vfs and did not produce cytotoxin or hemolysin. Curiously, major clone E6 exhibited the same Vf profile as minor clone E3 belonging to group D.

Statistical analysis. To explore the associations among bacterial characteristics and epidemiological characteristics, FACs were done. A first FAC was conducted on the *E. coli* data. The F1/F2 plane accounted for 55% of the total variance (Fig. 4). The planes obtained from the other factors of the FAC accounted for lower percentages of the variance than F1 and F2 and did not significantly improve the data interpretation. The projections of the variables on the F1/F2 plane distinguished three groups of variables: (i) CTX-M-16 type, pediatrics, Vfs *cnf1*, *hlyA*, and *papG* allele III, and clone E2, which were projected on the positive values of the second factor, F2, are closely related on the plane, and thus are strongly associated; (ii) phylogenetic group A, clones E1, E5, and E7, urology, and urinary tract infection, which were distinguished by the negative values of F1 and are associated; and (iii) phylogenetic group B2, the Vfs *fyuA*, *fimH*, *KpsMT* II, *iha*, *iutA*, and *sat*, the type of infection (blood and other), the surgery and medicine wards, and clones E3 and E6, which were distinguished by the positive values of F1 and are associated. Moreover, the projections of the variable "year of isolation" followed the increasing values of F1 from 2000 and 2001, projected on its negative values, to 2002 and 2003, projected on its positive values.

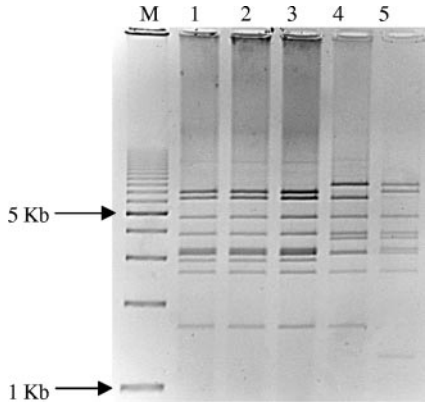

FIG. 3. HpaI-digested plasmid profiles of transconjugants or electroporants producing CTX-M-16. Lanes 1 to 4, electroporants of Ec3, Ec38, Kp12, and Kp19, respectively; lane 5, transconjugant Ec7; lane M, molecular weight marker, 1-kb DNA ladder (Bio-Rad).

A second FAC was conducted on the *K. pneumoniae* data. The projections of the variables on plane F1/F2, which accounted for 32.86% of the total variance, distinguished three groups of variables: (i) CTX-M-16 type, clones K1 and K2, and pediatrics, which were projected on the negative values of the first factor, F1; (ii) clones K3, K5, and K8 and the origin of infection (urine), which were projected on the negative values of F1 and F2; and (iii) clone K4, the origin of infection (blood), and the ward (ICU), which were distinguished by the positive values of the two factors (Fig. 5).

DISCUSSION

At the Charles Nicolle Hospital in Tunis, 62 enterobacterial strains producing CTX-M β-lactamase were collected between March 2000 and June 2003. The isolation rate increased during this period, from 6 isolates in 2000 to 13 in 2001, 18 in 2002, and 26 between January and June 2003. All of our isolates produce CTX-M-15 or CTX-M-16, both of which harbored the substitution of Asp-240→Gly which increases the activity against ceftazidime (5, 6, 7, 14, 34). During this period, the increase of consumption of cefotaxime and ceftazidime could

TABLE 3. Phylogenetic groups and virulence factors of *E. coli* producing CTX-M β-lactamase at the Charles Nicolle Hospital in Tunis[a]

Clone (*n*)	CTX-M allele	Phylogenetic group	Detection of indicated virulence factor:								
			fimH	*fyuA*	*iutA*	*papG* allele	*hlyA*	*cnf1*	*KpsMT* II	*iha*	*sat*
E1 (10)	15	A	−	−	−	−	−	−	−	−	−
E2 (3)	16	B2	+	+	−	III	+	+	+	−	−
E3 (3)	15	D	+	+	+	−	−	−	+	+	+
E4 (1)	16	D	+	+	−	−	−	−	+	−	−
E5 (1)	15	A	−	+	−	−	−	−	−	−	−
E6 (16)	15	B2	+	+	+	−	−	−	+	+	+
E7 (1)	15	A	+	−	−	−	−	−	−	−	−

[a] All strains were negative for *papG* allele II, *sfa*/*foc*, *iroN*, and *afa* genes, and all strains were positive for the *tra* gene.

have contributed to the emergence of ESBLs and particularly to these CTX-M-type enzymes. This is the first report of CTX-M-15-type β-lactamases in Tunisia and the first report of CTX-M-16-producing *Enterobacteriaceae* in an African country. All of these strains were multiresistant, producing other β-lactamases (e.g., TEM-1 and OXA-1) and aminoglycoside-modifying enzymes. They were resistant to ciprofloxacin, except for the strains recovered from the pediatric ward. Multiresistance has often been described for ESBL (and particularly CTX-M)-producing clinical isolates (3, 6, 7, 11, 23, 27, 28).

K. pneumoniae and *E. coli* isolates producing CTX-M-16 were isolated only in the pediatric ward. Three of the four *E. coli* (clone E2) and two of the three K. *pneumoniae* (clone K1) isolates producing CTX-M-16 were epidemiologically related, suggesting probable clonal spread. This clonal grouping was illustrated on the two FACs (Fig. 4 and 5). For *E. coli* isolates, clone E2 was closely related to three particular Vfs (*cnf1*, *hlyA*, and *papG* allele III) and to the pediatric ward by their projections on the positive values of the second factor, F2 (Fig. 4). For *K. pneumoniae* isolates, clones K1 and K2 and pediatric origin were grouped according to the negative values of the first factor, F1 (Fig. 5). Furthermore, all of the CTX-M-16-producing strains were found to carry very similar HpaI restriction P3 plasmid patterns, pointing to the existence of one common backbone for the CTX-M-16-encoding plasmids. Epidemiological studies showed that the dissemination of CTX-M-16 could be the consequence of both strain spreading and plasmid diffusion.

CTX-M-15-producing *E. coli* and *K. pneumonaie* isolates were recovered in all of the other wards. Twenty-six of the 31 CTX-M-15-producing *E. coli* isolates belong to two major clones. The first clone, E1 (phylogenetic group A), was predominant in the years 2000 to 2001 and was essentially recovered from urine (9/10). The second clone, E6 (phylogenetic group B2), appeared in December 2002, and 7 of the 16 isolates were recovered from blood and the lower respiratory tract (Table 2). In the FAC performed on *E. coli* data, the first axis opposed the variables (phylogenetic group A, clone E1, urology ward, and year of isolation [2000]) projected on its negative values with the variables (phylogenetic group B2, clone E6, blood and other sites of infection, several Vfs [*iha*, *iutA*, *fuyA*, *kpsM* II, and *fimH*], and year of isolation [2003]) projected on its positive values (Fig. 4). This well-established opposition between two levels of intrinsic virulence among ExPEC strains (8) illustrated the fact that CTX-M-15 resistance had been transferred from a less virulent *E. coli* clone (E1) to a more virulent *E. coli* clone (E6). In the same period (October 2002), we observed the emergence of the predomi-

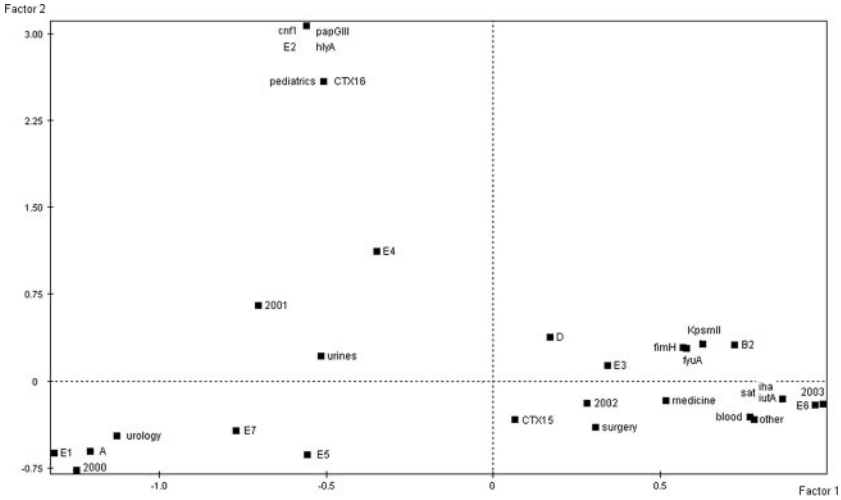

FIG. 4. Projections of the bacterial and clinical variables of the 35 *E. coli* strains on F1/F2 planes computed by factorial analysis of correspondence. A, B2, and D, phylogenetic groups A, B2, and D; E1 to E7, *E. coli* molecular types 1 to 7; CTX15 and CTX16, β-lactamase types CTX-M-15 and CTX-M-16; urine, blood, and other, urinary tract infection, bacteremia, and other infections; 2000 to 2003, years of isolation. The nine VFs are named as in Table 1.

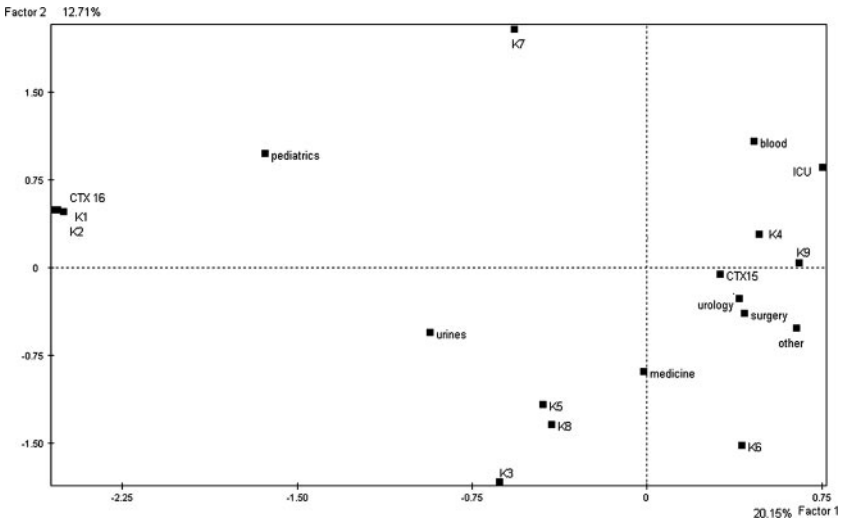

FIG. 5. Projections of the bacterial and clinical variables of the 27 *K. pneumoniae* strains on F1/F2 planes computed by factorial analysis of correspondence. K1 to K9, *K. pneumoniae* molecular types 1 to 9; CTX15 and CTX16, β-lactamase types CTX-M-15 and CTX-M-16; urine, blood, and other, urinary tract infection, bacteremia, and miscellaneous infections; 2000 to 2003, years of isolation.

nant and multiresistant clone K4 of *K. pneumoniae*. Similarly to that of *E. coli*, the FAC indicated the close relatedness between *K. pneumoniae* clone K4 and the variables blood and ICU (Fig. 5), illustrating the association between clinical virulence and antibiotic resistance.

As suggested by plasmid fingerprinting, the same plasmid (same backbone) encoding CTX-M-15 could have been transferred first from clone E1 to the *K. pneumoniae* clone K4 and then from clone K4 to clone E6 (which emerged 2 months later). This shift (from clone E1 to clone E6) could be explained by the virulence genotype. Clone E1 is a typical commensal strain with fewer than two virulence factors, unlike clone E6 (18–20). Interestingly, clone E6 showed lower intrinsic virulence than archetypal ExPEC strains and particularly the absence of toxins (18–20). But the virulence traits of this clone could be involved in colonization, infection, and persistence in humans (nonspecific adhesin, siderophore, and resistance to the serum and to phagocytosis) (18, 19). These factors could simultaneously explain the spread and the persistence of this "successful" *E. coli* clone. This recalls the results of two French studies that investigated nosocomial outbreaks in long-term-care facilities due to *E. coli* isolates producing CTX-M-15 β-lactamase and similar virulence traits (25, 28). Previous studies on the relation between antibiotic resistance and virulence in human isolates of *E. coli* suggested that antibiotic-resistant clones (except those resistant to fluoroquinolones) were less virulent than susceptible strains (21). In our study, we observed the success of a multiresistant and virulent clone of *E. coli*.

In summary, clonal spread of strains, multiresistance, several virulence factors, plasmid transfer, and broad-spectrum cephalosporin consumption have contributed to the nosocomial dissemination of the CTX-M-encoding genes among *E. coli* and *K. pneumoniae* strains in our hospital.

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