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Spread of Extended-Spectrum β-Lactamase CTX-M-Producing *Escherichia coli* Clinical Isolates in Community and Nosocomial Environments in Portugal[▽]

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Of the 181 unduplicated *Escherichia coli* strains isolated in nine different hospitals in three Portuguese regions, 119 were extended-spectrum β-lactamase (ESBL)-CTX-M producers and were selected for phenotype and genotype characterization. CTX-M producer strains were prevalent among community-acquired infections (56%), urinary tract infections (76%), and patients ≥60 years old (76%). In MIC tests, all strains were resistant to cefotaxime, 92% were resistant to ceftazidime, 93% were resistant to quinolones, 89% were resistant to aminoglycoside, and 26% were resistant to trimethoprim-sulfamethoxazole; all strains were sensitive to carbapenems, and 92% of the strains had a multidrug resistance phenotype. Molecular methods identified 109 isolates harboring a $bla_{CTX-M-15}$ gene, 1 harboring the $bla_{CTX-M-32}$ gene (first identification in the country), and 9 harboring the $bla_{CTX-M-14}$ gene. All isolates presented the IS*Ecp1* element upstream from the bla_{CTX-M} genes; one presented the IS*903* element (downstream of $bla_{CTX-M-14}$ gene), and none had the IS*26* element; 85% carried bla_{TEM-1B}, and 84% also carried a bla_{OXA-30}. Genetic relatedness analysis based on pulsed-field gel electrophoresis defined five clusters and indicated that 76% of all isolates (from cluster IV) corresponded to a single epidemic strain. Of the 47 strains from one hospital, 41 belonged to cluster IV and were disseminated in three main wards. CTX-M-producing *E. coli* strains are currently a problem in Portugal, with CTX-M-15 particularly common. This study suggests that the horizontal transfer of bla_{CTX-M} genes, mediated by plasmids and/or mobile elements, contributes to the dissemination of CTX-M enzymes to community and hospital environments. The use of extended-spectrum cephalosporins, quinolones, and aminoglycosides is compromised, leaving carbapenems as the therapeutic option for severe infections caused by ESBL producers.

Production of extended-spectrum β-lactamases (ESBLs) is a major mechanism of resistance to β-lactam antibiotics. First detected in 1986 (27), CTX-M (cefotaximases) is an ESBL family including more than 60 different enzymes in five phylogenetic groups (http://www.lahey.org/studies/webt.htm). CTX-M β-lactamases of group 2be (5), unlike other ESBL, are more active against cefotaxime than against ceftazidime (3). The rapid emergence and worldwide spread of this plasmid-mediated family is associated with mobile elements and particularly insertion sequences (1, 4, 13, 21, 31, 40, 49). This high mobility associated with inefficient antibiotic policies has led to community-acquired and nosocomial infections (24, 51).

Many strains that express CTX-M β-lactamases are multidrug resistant (16, 21, 23, 51). Genes conferring resistance to aminoglycosides and tetracycline and other *bla* genes have been found on the same plasmids as the bla_{CTX-M} genes (4). Genes conferring plasmid-mediated quinolone resistance have also been associated with bla_{CTX-M} genes (20).

CTX-M-15 appears to have the best dissemination capacity of all of the CTX-M family, probably due to successful genetic rearrangements (48). The corresponding gene is normally associated with an upstream IS*Ecp1* element. The gene has been detected in Europe (1, 11, 14, 24), Africa (30, 33, 43, 47), North and South America (4, 38), and Asia (21, 22) and has been associated with many community and hospital outbreaks. To our knowledge, only dispersed cases have been reported in Portugal (11, 12, 25, 26, 28).

We investigate the dissemination of CTX-M enzymes among clinical isolates of *Escherichia coli* recovered in various Portuguese hospitals from cases of community-acquired and nosocomial infections. We describe the phenotypes and genetic characteristics of these isolates.

MATERIALS AND METHODS

Bacterial strains. A total of 181 unduplicated *E. coli* strains isolated from patients were collected in nine hospitals in three different regions of Portugal between March 2004 and March 2006 and sent to the Antibiotic Resistance Unit at the National Institute of Health (NIH) Dr. Ricardo Jorge in Lisbon. The bacteriology laboratories of these hospitals collaborate with the NIH as contributors to the Antibiotic Resistance Surveillance Program in Portugal (ARSIP); they sent all *E. coli* strains identified as ESBL producers by different systems (ATB G-5, VITEK 1, VITEK 2, and Phoenix). Of the 181 strains received, 119 were detected as ESBL-(CTX-M) producers by phenotypic and genotypic characterization at NIH, as stated below. The NIH conducted the main study using 119 of 126 CTX-M producer strains because only the first bacterial isolate from the three patients with multiple isolates was considered (among a total of 181 ESBL producers) (Table 1). The other ESBL producers were not investigated in the present study. Of the 119 strains, 47 were nosocomial and 66 were community acquired, according to Centers for Disease Control and Prevention criteria (18), and 6 were of unknown origin (Table 1). Strains were isolated from urine (n = 90), wounds (n = 13), blood (n = 4), ascitic fluid (n = 3), sputum (n = 3), bronchoalveolar lavage (n = 2) and secretions (n = 3), and gastric fluid (n = 1). *E. coli* INSRA99 (IRT-2, pI 5.2), *E. coli* RP4 (TEM-2, pI 5.6), and *E. coli* SolRl

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TABLE 1. General characterization of the 119 CTX-M-producing *E. coli* strains studied (one per patient), characterized by hospitals, regions, and origin of isolates

Hospital[a] code	Region	Origin and no. of isolates						Total no. of isolates
		Community acquired (n = 66)		Nosocomial (n = 47)		Unknown (n = 6)		
		PFGE profile (no. of strains)	Total	PFGE profile (no. of strains)	Total (ward)	PFGE profile (no. of strains)	Total	
A	North	0009 (1) 0010 (7)	8	0009 (1)	1 (internal medicine)			9
B	South	0009 (1) 0010 (5) 0012 (1) 0013 (1)	8	0010 (2)	2 (emergency, internal medicine)			10
C	North	0010 (27) 0003 (1)[c] 0005 (1)[c] 0011 (1) ND (1)[c]	31[b]	0010 (14) 0018 (1)[c] ND (1)[c]	16[b] (cardiology, surgery, internal medicine, nephrology,[c] pneumology,[c] ICU, urology)			47
D	Lisbon and Tagus Valley	0002 (1)[c] 0010 (7)	8[d]	0007 (1) 0010 (7) 0016 (1)	9[d] (cardiology, surgery, internal medicine, orthopedics, pneumology, gastroenterology, observation)	0014 (1)[c]	1	18
E	Lisbon and Tagus Valley	0001 (1)[c] 0006 (1) 0010 (6)	8	0004 (1) 0006 (1) 0008 (1) 0010 (4) 0017 (1) 0019 (1)	9[e] (emergency, internal medicine, surgery, nephrology, ICU, pediatric, neurology)	0006 (2) 0010 (1) 0019 (1)[f]	4	21
F	Lisbon and Tagus Valley			0010 (1)	1 (infectious diseases)			1
G	Lisbon and Tagus Valley			0010 (1)	1 (internal medicine)			1
H	Lisbon and Tagus Valley			0010 (5) 0015 (1)	6 (surgery, internal medicine)	0010 (1)	1	7
I	North	0003 (1)[c] 0010 (1) ND (1)	3	0010 (2)	2 (internal medicine)			5

[a] All hospitals were general hospitals.
[b] Two more strains were collected from the same patient: both were noted as community acquired, whereas the first was nosocomial (from the urology ward).
[c] CTX-M-14 producers.
[d] Three more strains were collected from the same patient (two from internal medicine and one from the community).
[e] Two more strains were collected from the same patient, all noted as nosocomial (from the pediatric ward).
[f] CTX-M-32 producers. All other isolates were CTX-M-15 producers excepting those from CTX-M-14 producers.

90 (AmpC, pI 9.2) were used only as control strains for isoelectric focusing (IEF). *E. coli* R111 (TEM-1, pI 5.4; bla_{TEM-1} plus *ampC*), *Salmonella enterica* serovar Typhimurium (OXA-1, pI 7.4; bla_{OXA-1}), *E. coli* C600 (SHV-1, pI 7.6; bla_{SHV-1}), and *E. coli* UA1526 (CTX-M-15, pI 8.9; $bla_{CTX-M-15}$) were used as control strains for both PCR and IEF. The control strains used for PCR with insertion sequence primers were INSRA5753 (IS*Ecp1*), INSRA5776 (IS*903*), and Kp125 (IS*26*).

Susceptibility testing and ESBL confirmation. The MICs of 23 antibiotics were determined by a broth microdilution method (MicroScan Panel Sólo 1S; Dade Behring, West Sacramento, CA) for all strains. The MICs of antibiotics were determined by the agar dilution method for all transformants obtained as previously (29). The results were interpreted by using CLSI (formerly National Committee for Clinical Laboratory Standards) criteria (10). Isolates were considered multidrug resistant if they had reduced susceptibility to three or more structurally unrelated antibiotics. ESBL production was confirmed by a broth microdilution method: strains with synergy between ceftazidime and ceftazidime-clavulanic acid were suspected of producing ESBL. To confirm ESBL producers, 52 of 119 strains were randomly chosen for further testing by the Etest ESBL method using strips with cefotaxime and ceftazidime both alone and associated with clavulanate (AB Biodisk, Solna, Sweden); findings were interpreted according to the manufacturer's instructions.

Transfer of resistance. We tested whether the ESBL phenotypes of strains producing CTX-M-14, CTX-M-15, and CTX-M-32 enzymes were transferable. Plasmid DNA was extracted from six producer strains, representative of the different CTX-M-type enzymes detected, and used to transform *E. coli* DH5α by electroporation. Transformants were selected on Luria broth medium containing 1 μg of cefotaxime/ml. PCR was used to test for the *bla* gene in transformants as described below for clinical isolates.

IEF. Cell extracts from all isolates were obtained by ultrasonic treatment, and IEF was used to characterize the pIs of ESBLs as previously described (6). The pIs of each β-lactamase were compared to those produced by control strains.

PCR amplification and gene sequencing. PCR amplification was used to test for the bla_{CTX-M} gene and IS*Ecp1*, IS*26* and IS*903* elements, and multiplex PCR was used to test for the bla_{TEM}, bla_{OXA}, bla_{SHV}, and *ampC* genes in all 119 ESBL producer strains as described previously (26, 29, 42). Specific primers were used for PCR and sequencing (Table 2). A particular specific primer (CTX15i) was used to determine the nucleotides of codon 288, which distinguish $bla_{CTX-M-15}$ from

TABLE 2. Primers used for PCR amplification and sequencing

Gene	Primer[a]	Primer sequence (5′→3′)	PCR product size (bp)	Source or reference
bla_{TEM}	P1	TACGATACGGGAGGGCTTAC	716	2
	P2	TTCCTGTTTTTGCTCACCCA		
	FIN	ATTCTTGAAGACGAAAGGGC	1,091	7
	DEB	ATGAGTAAACTTGGTCTGAC		
	P3[b]	TGGGTGAGCAAAAACAGGAA		7
	CLB[b]	AATGAAGCCATACCAAACGA		2
bla_{SHV}	SHVf1	TCAGCGAAAAACACCTTG	471	32
	SHVr2	TCCCGCAGATAAATCACCA		
bla_{OXA}	oxa1f	TATCTACAGCAGCGCCAGTG	199	17
	oxa1r	CGCATCAAATGCCATAAGTG		
	oxa1sf	ATGAAAAACACAATACATATC	816	9
	oxa1sr	AATTTAGTGTGTTTAGAATGG		
ampC	ampCf	CCCCGCTTATAGAGCAACAA	634	17
	ampCr	TCAATGGTCGACTTCACACC		
bla_{CTX-M}	CTXf[c]	TTTGCGATGTGCAGTACCAGTAA	543	15
	CTXr[d]	CGATATCGTTGGTGGTGCCATA		
$bla_{CTX-M-15}$	CTX15f	AGAATAAGGAATCCCATGGTT	875	This study
	CTX15r	ACCGTCGGTGACGATTTTAG		
	CTX15i[b]	GGAATCTGACGCTGGGTAAA		This study
$bla_{CTX-M-14}$	CTXg3f	CTGATGTAACACGGATTGACC	871	This study
	CTX14r	CGATTTATTCAACAAAACCAG		
IS*Ecp1*	ISEcp1	AAAAATGATTGAAAGGTGGT	Variable	14
IS*26*	IS26	AGCGGTAAATCGTGGAGTGA	Variable	14
IS*903*	IS903	CGGTTGTAATCTGTTGTCCA	Variable	14

[a] For PCR amplification and sequencing (except those in used only for internal sequencing).
[b] Used only for internal sequencing.
[c] When used with primers ISEcp1, IS26, IS903, this also amplifies the downstream region of the bla_{CTX-M} gene. Various-sized PCR products are expected.
[d] When used with primer ISEcp1, IS26, or IS903, this also amplifies the upstream region of the bla_{CTX-M} gene. Various-sized PCR products are expected.

$bla_{CTX-M-28}$. PCR products were purified and further sequenced as previously described (29).

PFGE. Of the 119 isolates, 116 were analyzed by pulsed-field gel electrophoresis (PFGE). Seven other strains from three patients with multiple isolates in different hospitals were also subjected to PFGE analysis (Table 1). Plugs were prepared from bacterial suspensions (300 μl) with an optical density at 650 nm of 1.2 to 1.4 in phosphate buffer (50 mM). SeaPlaque GTG (Cambrex Bio Science Rockland, Rockland, ME) agarose was added (1.5% in 300 μl of TF buffer); the mixture was immediately taken up in a 1-ml syringe and placed for exactly 4 min at −20°C. The syringe was then incubated at room temperature for 10 min, and its end was cut off. The plugs were sliced into 1-mm-thick sections and incubated in EC buffer (6 mM Tris, 1 M NaCl, 0.1 M EDTA, 0.2% deoxycholic acid, 0.5% *N*-lauroylsarcosine, and 0.5% Brij 58) for 3 h at 37°C. The EC buffer was replaced with ES lysis buffer (0.5 M EDTA and 1% *N*-lauroylsarcosine) plus proteinase K (1 mg/strain), and the samples were incubated for 16 h at 55°C. The plugs were then washed with distilled water for 5 min and with TE buffer (100 mM Tris and 10 mM EDTA) four times for 30 min each time. The DNA in the plugs was digested with 30 U of XbaI for 16 h at 37°C. PFGE was performed on a CHEF MAPPER PFGE apparatus (Bio-Rad, Hercules, CA) with 1.2% Seakem Gold agarose (Cambrex) in 0.5× Tris-borate-EDTA at 11°C and 6 V/cm. The duration of the run was 24 h, with initial and final switch times of 0.1 and 36 s, respectively. The gels were stained with ethidium bromide and photographed with Gel Doc 2000 (Bio-Rad). Banding patterns were analyzed by using BioNumerics software (version 3.5; Applied Maths, Sint-Martens-Latem, Belgium). The unweighted-pair-group method was used to construct a dendrogram based on PFGE XbaI restriction patterns of the 123 *E. coli* isolates. The Dice band-based similarity coefficient, with a band position tolerance of 1.0% and an optimization of 1.8%, was used for clustering. A cutoff value of 80% similarity was determined by the cluster cutoff method according to Bionumerics software. Isolates with a Dice band-based similarity coefficient value of >80% were considered to belong to the same cluster.

RESULTS

Clinical ESBL producer strains and antibiotic susceptibility. We confirmed that the 119 isolates included in the study were ESBL producers. The CTX-M-32 producer strain was from a wound in a 51-year-old man, and 8 of the 9 CTX-M-14 producers were isolated from urine, and 1 was from a wound. Most of CTX-M-15 producers (92%) were from urine from men or women ≥60 years old (data not shown).

A total of 90% of the isolates were resistant to 11 of the 14 β-lactam antibiotics tested: more than 98% CTX-M group 1 enzyme producers were resistant to ceftazidime and cefotaxime, all CTX-M group 9 producers were resistant to cefotaxime, and 11% resistant to ceftazidime (Table 3). Only 26% CTX-M group 1 producers showed nonsusceptibility to amoxicillin-clavulanate and 12% showed nonsusceptibility to piperacillin-tazobactam. Imipenem and meropenem were the only antibiotics effective against all isolates producing either group 1 or group 9 enzymes (Table 3). A total of 92% of the isolates were multidrug resistant: 50% were isolated in the community, and 37% were isolated from hospitalized patients.

Transfer of antibiotic resistance. We tested whether bla_{CTX-M} genes in six selected isolates were transferable by transformation of *E. coli* strain DH5α. Only three transformants were obtained: two representative of CTX-M group 1 isolates (one carrying the $bla_{CTX-M-15}$ plus bla_{TEM-1B} genes and other carrying the $bla_{CTX-M-32}$ gene) and one representative of CTX-M group 9 isolates (carrying the $bla_{CTX-M-14}$ gene). Generally, the transformants had antibiotic resistance profiles similar to those of their parental clinical isolates (Table 4). All clinical strains were resistant to ciprofloxacin, but none of the transformants maintained that condition. Transformants of *E. coli* strains harboring CTX-M-32 or CTX-M-14 enzymes, whose clinical strains were resistant to gentamicin and trimethoprim-sulfamethoxazole, demonstrated susceptibility to those antibiotics.

TABLE 3. MIC_{50}, MIC_{90}, range, percentage of resistant and nonsusceptible *E. coli* strains (n = 119) producing enzymes, from the CTX-M-1 and CTX-M-9 groups, collected in nine Portuguese hospitals

Antimicrobial agent	MIC (μg/ml) for strains producing:										CLSI breakpoints[b] (μg/ml)	
	CTX-M group 1[a] (n = 110)					CTX-M group 9 (n = 9)						
	MIC_{50}[c]	MIC_{90}[d]	Range	Susceptibility[e] R (%)	Susceptibility[e] IR (%)	MIC_{50}	MIC_{90}	Range	Susceptibility R (%)	Susceptibility IR (%)	S	R
Ampicillin	>16	>16	4–>16	110 (100)	110 (100)	>16	>16	4–>16	9 (100)	9 (100)	≤8	≥32
Ampicillin-sulbactam	>16/8	>16/8	8/4–>16/8	108 (98)	109 (99)	>16/8	>16/8	8/4–>16/8	9 (100)	9 (100)	≤8/4	≥32/16
Amoxicillin-clavulanic acid	8/4	16/8	4/2–16/8	3 (3)	28 (26)	≤4/2	8/4	≤4/2–16/8	0 (0)	0 (0)	≤8/4	≥32/16
Piperacillin	>64	>64	16–>64	110 (100)	110 (100)	>64	>64	16–>64	9 (100)	9 (100)	≤16	≥128
Piperacillin-tazobactam	≤16	32	≤16–64	7 (6)	13 (12)	≤16	≤16	≤16–64	0 (0)	0 (0)	≤16/4	≥128/4
Ticarcillin	>64	>64	8–>64	110 (100)	110 (100)	>64	>64	8–>64	9 (100)	9 (100)	≤16	≥128
Cephalothin	>16	>16	8–>16	110 (100)	110 (100)	>16	>16	8–>16	9 (100)	9 (100)	≤8	≥32
Cefazolin	>16	>16	1–>16	110 (100)	110 (100)	>16	>16	1–>16	9 (100)	9 (100)	≤8	≥32
Cefuroxime	>16	>16	2–>16	110 (100)	110 (100)	>16	>16	2–>16	9 (100)	9 (100)	≤8	≥32
Cefotaxime	>32	>32	0.5–>32	109 (99)	110 (100)	>32	>32	0.5–>32	9 (100)	9 (100)	≤8	≥64
Ceftriaxone	>32	>32	2–>32	110 (100)	110 (100)	>32	>32	2–>32	9 (100)	9 (100)	≤8	≥64
Aztreonam	>16	>16	1–>16	110 (100)	110 (100)	8	>16	1–>16	1 (11)	2 (22)	≤8	≥32
Ceftazidime	>16	>16	0.5–>16	108 (98)	109 (99)	2	>16	0.5–>16	1 (11)	1 (11)	≤8	≥32
Cefepime	>16	>16	0.5–>16	109 (99)	109 (99)	>16	>16	0.5–>16	7 (78)	9 (100)	≤8	≥32
Cefoxitin	≤4	8	≤4–16	1 (1)	5 (5)	≤4	16	≤4–16	0 (0)	1 (11)	≤8	≥32
Imipenem	≤0.5	≤0.5	≤0.5–8	0 (0)	0 (0)	≤0.5	≤0.5	≤0.5–8	0 (0)	0 (0)	≤4	≥16
Meropenem	≤1	≤1	≤1–8	0 (0)	0 (0)	≤1	≤1	≤1–8	0 (0)	0 (0)	≤4	≥16
Amikacin	≤8	16	≤8–32	4 (4)	8 (7)	≤8	16	≤8–32	0 (0)	0 (0)	≤16	≥64
Gentamicin	>8	>8	2–>8	87 (79)	87 (79)	≤2	>8	≤2–>8	2 (22)	2 (22)	≤4	≥16
Tobramycin	>8	>8	2–>8	103 (94)	104 (95)	≤2	>8	≤2–>8	2 (22)	2 (22)	≤4	≥16
Ciprofloxacin	>2	>2	0.5–>2	108 (98)	108 (98)	≤0.5	>2	≤0.5–>2	3 (33)	3 (33)	≤1	≥4
Norfloxacin	>8	>8	2–>8	108 (98)	108 (98)	≤2	>8	≤2–>8	3 (33)	3 (33)	≤4	≥16
Ofloxacin	>4	>4	0.5–>4	108 (98)	108 (98)	≤0.5	>4	≤0.5–>4	3 (33)	3 (33)	≤2	≥8
Trimethoprim-sulfamethoxazole	≤1/19	>2/38	≤1/19–>2/38	28 (26)	28 (26)	≤1/19	>2/38	≤1/19–>2/38	3 (33)	3 (33)	≤2/38	≥4/76

[a] There were a total of 99 CTX-M-15 producers and 1 CTX-M-32 producer, according to the phylogenetic group 1 defined by Bonnet (3).
[b] As scored according to CLSI guidelines: S, sensitive; R, resistant.
[c] MIC_{50}, MIC at which 50% of the isolates are inhibited.
[d] MIC_{90}, MIC at which 90% of the isolates tested are inhibited.
[e] R, number of resistant isolates; IR, number of nonsusceptible isolates.

IEF. As determined by IEF, 82% of the CTX-M group 1 strains (n = 110) contained enzymes with pIs 5.4, 7.4, and 8.9 (Table 5); 9% contained enzymes with pIs of 5.4 and 8.9; and 9% contained enzymes with pIs of 7.4 and 8.9. All CTX-M group 9 strains (n = 9) contained an enzyme of pI 8.1, and 44% contained an enzyme with a pI of 5.4.

Identification of *bla* genes and IS elements. The PCR tests used detected a $bla_{\text{CTX-M}}$ gene and the *ampC* gene in all isolates. A total of 91 of the 119 strains (76%) also contained bla_{TEM} and bla_{OXA} genes, 13 (11%) contained $bla_{\text{CTX-M}}$ plus bla_{TEM} but not the bla_{OXA} gene, and 10 (8%) had $bla_{\text{CTX-M}}$ and bla_{OXA} genes (Table 5). Sequencing identified the $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{CTX-M-32}}$ genes in 109 (92%), 9 (8%), and 1 (1%) strains, respectively; the $bla_{\text{TEM-1B}}$ gene was identified in 101 (85%) strains, $bla_{\text{TEM-1C}}$ was identified in 1 strain, and $bla_{\text{TEM-1A}}$ was identified in 1 strain (note that all of these bla_{TEM} genes had a P3 promoter region). One strain had more than one bla_{TEM} gene sequence. A total of 101 (85%) strains harbored a $bla_{\text{OXA-30}}$ gene. No strain carried the bla_{SHV} gene. In all strains, the IS*Ecp1* element was found upstream from the $bla_{\text{CTX-M}}$ genes. One strain also carried an IS*903* element downstream from its $bla_{\text{CTX-M-14}}$ gene (Table 5).

Clonality of CTX-M producer strains. PFGE analysis was used to establish the genetic relatedness of the 116 CTX-M producer strains. We identified 19 PFGE profile types: 14 included a single clone genetically unrelated to other isolates in the study (types 0001, 0002, 0004, 0005, 0007, 0008, and 0011 to 0018). The five other types, defined as clusters I to V, included numerous related (>80% similarity) or indistinguishable (100% homology) isolates (Fig. 1). Of the 102 (88%) strains in these five clusters, 91 (89%) had profile type 0010 (cluster IV). These 91 isolates included clones from all hospitals (isolated from 53 outpatients, 36 inpatients, and 2 with no information) (Tables 1 and 5 and Fig. 1).

Hospital C provided more isolates than any other hospital, including four single-isolate profile types (0005, 0011, and 0018) and two profile types including numerous isolates (0003 and 0010) (Fig. 1). All isolates from the internal medicine, attending, intensive care unit (ICU), and urology services of hospital C were profile type 0010. Only the nephrology, pediatric, and emergency services of hospital C presented clones belonging to other different profile types (Table 6).

Isolates of profile types 0006 to 0013, 0015 to 0017, and 0019 expressed a CTX-M enzyme of group 1 and were multidrug resistant. Isolates of profile types 0001 and 0005 were also multidrug resistant and expressed CTX-M enzyme of group 9. Isolates with profile types 0002, 0003, 0014, and 0018 also produced the CTX-M group 9 enzyme but were not multidrug resistant (Table 5).

Three patients (from three different hospitals, C, D and E)

TABLE 4. MICs of antibiotics for clinical isolates and *E. coli* transformants and recipients[a]

Antimicrobial agent[b]	MIC (μg/ml) of antibiotic for *E. coli* strain:						
	DH5α	INSRA5776 (CTX-M-14 + TEM-1B)	DH5α-URA5776 (CTX-M-14)	INSRA5905 (CTX-M-15 + TEM-1B)	DH5α-URA5905 (CTX-M-15 + TEM-1B)	INSRA5924 (CTX-M-32 + TEM-1B)	DH5α-URA5924 (CTX-M-32)
Amoxicillin	8	>4,096	>4,096	>4,096	>4,096	>4,096	>4,096
Amoxicillin-clavulanic acid*	8	32	32	64	32	128	8
Ticarcillin	4	>4,096	>4,096	>4,096	>4,096	>4,096	>4,096
Piperacillin	2	256	256	>512	512	>512	512
Piperacillin-tazobactam†	1	8	2	2	2	4	2
Mecillinam	0.125	1	0.5	2	2	2	0.5
Cephalothin	8	>1,024	1,024	>1,024	>1,024	>1,024	>1,024
Cefuroxime	4	>256	>256	>256	>256	>256	>256
Cefoperazone	≤0.25	256	16	>512	256	256	64
Ceftriaxone	0.03	32	32	>512	256	256	64
Ceftriaxone-clavulanic acid*	0.06	0.25	0.06	≤0.016	≤0.016	0.125	0.06
Cefotaxime	0.06	32	32	512	512	256	128
Cefotaxime-clavulanic acid*	0.06	0.5	0.06	0.25	0.03	0.125	0.06
Ceftazidime	0.25	2	1	128	64	16	16
Ceftazidime-clavulanic acid*	0.125	0.5	0.25	0.25	0.25	0.5	0.25
Aztreonam	0.06	8	8	128	64	64	64
Aztreonam-clavulanic acid*	0.06	0.5	0.125	0.06	0.06	0.25	0.125
Cefepime	0.03	8	2	32	8	16	4
Cefoxitin	4	32	4	16	4	16	8
Imipenem	0.25	≤0.06	0.5	0.25	0.25	0.125	0.125
Ciprofloxacin	≤0.125	256	≤0.125	1,024	≤0.125	8	≤0.125
Gentamicin	≤0.125	32	0.5	1	0.25	16	≤0.125
Trimethoprim	≤0.125	>64	≤0.125	0.25	≤0.125	64	≤0.125

[a] *E. coli* DH5α-URA5776 (harboring CTX-M-14 enzymes), *E. coli* DH5α-URA5905 (harboring TEM-1B and CTX-M-15 enzymes), and *E. coli* DH5α-URA5924 (harboring CTX-M-32 enzymes) were transformants of *E. coli* INSRA5776 (harboring TEM-1B and CTX-M-14 enzymes), *E. coli* INSRA5905 (harboring TEM-1B and CTX-M-15 enzymes), and *E. coli* INSRA5924 (harboring TEM-1B and CTX-M-32 enzymes), respectively; *E. coli* DH5α was the recipient.

[b] *, Clavulanic acid, 2 μg/ml; †, tazobactam, 4 μg/ml.

gave multiple isolates; all provided isolates with PFGE profile type 0010, although the patient from hospital D also gave one isolate of PFGE profile type 0007 (Table 1).

DISCUSSION

The prevalence of CTX-M-type β-lactamases has increased substantially since 1992 (3): in Portugal, they have been found in isolates from animals, healthy humans, and patients (12, 26). Here, we investigated the dissemination of these enzymes among clinical strains responsible for community and hospital-acquired infections. In a survey conduced in 1999, no ESBL enzymes of the CTX-M family were detected in clinical *E. coli* isolates in Portugal (M. Caniça, unpublished data).

We included only strains resistant to extended-spectrum cephalosporins and monobactam-producing CTX-M β-lactamases collected in three different regions of Portugal between March 2004 and March 2006: 119 (66%) CTX-M producer strains out of 181 ESBL producers. The majority of the strains had CTX-M enzymes of group 1, CTX-M-15 (n = 109) and CTX-M-32 (n = 1), and 9 strains had CTX-M-14, an enzyme of group 9. Most of CTX-M-15 producers were isolated in urine from men or women ≥60 years old as in Spain (35).

Strains producing CTX-M of group 1 were more resistant to ceftazidime and aztreonam (100%) than those producing CTX-M of group 9 (11%). This is consistent with previous reports (3). CTX-M-14 differs from its parental enzyme CTX-M-9 by a single substitution (Ala231→Val), which does not confer an extension of resistance (37, 45). CTX-M-32 differs from its parental enzyme CTX-M-1 by a single substitution (Asp240→Gly), which confers high-level resistance to ceftazidime (8). The same substitution is found in CTX-M-15 relative to its parental enzyme CTX-M-3, similarly conferring high-level resistance to ceftazidime (41).

A total of 98% of strains producing CTX-M of group 1 were resistant to quinolones and 95% were resistant to aminoglycosides; only 33 and 22% of isolates producing CTX-M of group 9 were resistant, respectively. Resistance to quinolones (93%) was considerably more prevalent among our isolates than the 21% reported by Edelstein (15); 89% of the isolates in both studies were resistant to aminoglycosides. Resistance to trimethoprim-sulfamethoxazole was more prevalent in isolates carrying CTX-M of group 9 (33%) than CTX-M of group 1 (26%).

Combined production of CTX-M and OXA enzymes by *E. coli* improved resistance to β-lactamase inhibitors. Less than 24% of the isolates were nonsusceptible to clavulanate and tazobactam, and more than 98% were resistant to sulbactam (Table 3). The usefulness of β-lactams plus a β-lactamase inhibitor is uncertain for patients with treatment failure (24). Ninety-two percent of our strains harboring the $bla_{CTX-M-15}$ gene (Table 5) also possessed a bla_{OXA-30} gene, presumably explaining the high percentage of nonsusceptibility to ampicillin-sulbactam. Strains carrying the bla_{CTX-M} of group I enzymes were the only strains not susceptible to amoxicillin-clavulanate and to piperacillin-tazobactam, which may imply a low expression of β-lactamase OXA-30 or even another mechanism of resistance (34).

The genes $bla_{CTX-M-15}$ plus bla_{OXA-1} (bla_{OXA-30}) have been

TABLE 5. Phenotypic and genotypic characteristics of 119 *E. coli* CTX-M producer strains

Antimicrobial resistance pattern[a]	pI(s)[b]	Genotype by PCR (gene type by sequencing)[c]:						PFGE profile[d] (no. of strains)	Hospital code	Total no. of strains
		bla_{TEM}	bla_{CTX-M}	bla_{OXA}	IS*Ecp1*	IS*26*	IS*903*			
AM A/S AT CF CT FX TX TZ XM KZ PM PI P/T TI CI NX OF AK GM TO	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0010	A	1
AM A/S A/C AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF GM TO	7.4; 8.9	−	+ (15)	+ (30)	+	−	−	0010	D	2
AM A/S A/C AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF AK TO	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0006	E	1
AM A/S AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF AK GM TO	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0010	C	1
AM A/S AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF GM TO T/S	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0010	C	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF AK GM TO T/S	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0010	B	1
AM A/S AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF GM TO	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0010	C	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF GM TO T/S	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0009 (1), 0010 (13), 0012 (1), 0013 (1), 0015 (1), 0017 (1), ND (1)	A, B, C, D, E, F, H	19
	5.4; 7.4; 8.9	+ (1)[e]	+ (15)	+ (30)	+	−	−	0010	C	1
	5.4; 8.9	+ (1b)	+ (32)	−	+	−	−	0019	E	1
	5.4; 8.9	+ (1b)	+ (15)	−	+	−	−	0019	E	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF GM TO	8.1	−	+ (14)	−	+	−	−	0005	C	1
	7.4; 8.9	−	+ (15)	+ (30)	+	−	−	0007 (1), 0010 (3)	B, D, H	4
	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0016 (1), 0009 (2), 0010 (48), 0011 (1)	A, B, C, D, E, G, H, I	52
	5.4; 7.4; 8.9	+ (1a)	+ (15)	+ (30)	+	−	−	0010	H	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF TO T/S	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0010	I	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF GM	5.4; 8.9[f]	+ (1b)	+ (15)	+ (30)	+	−	−	0010	A	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF TO	7.4; 8.9	−	+ (15)	+ (30)	+	−	−	0010	D, E	3
	5.4; 8.9	+ (1b)	+ (15)	−	+	−	−	0010	D	1
	5.4; 7.4; 8.9	+ (1b)	+ (15)	+ (30)	+	−	−	0006 (2), 0008 (1), 0010 (7)	C, D, E, I	10
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF T/S	5.4; 8.9	+ (1b)	+ (15)	−	+	−	−	0010	A	2
AM AT CF CT TX TZ XM KZ PM PI TI CI NX OF TO	7.4; 8.9	−	+ (15)	+ (30)	+	−	−	0010	E	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF[g]	5.4; 8.9	+ (1b)	+ (15)	−	+	−	−	0006(1), 0010 (1)	E	2
AM A/S CF CT TX XM KZ PI TI CI NX OF GM TO T/S	5.4; 8.1	+ (1b)	+ (14)	−	+	−	+	0001	E	1
AM A/S CF CT TX XM KZ PI TI CI NX OF T/S	5.4; 8.1	+ (1b)	+ (14)	−	+	−	−	ND	C	1
AM A/S AT CF CT TX XM KZ PM PI TI T/S[g]	5.4; 8.9	+ (1b)	+ (15)	−	+	−	−	0004	E	1
AM A/S CF CT TX XM KZ PM PI TI T/S[g]	5.4; 8.1	+ (1b)	+ (14)	−	+	−	−	0018	C	1
AM A/S CF CT TX XM KZ PM PI TI[g]	8.1	−	+ (14)	−	+	−	−	0002 (1), 0003 (2), ND (1)	C, D, I	4
	5.4; 8.1	+ (1c)	+ (14)	−	+	−	−	0014	D	1
AM AT CF TX XM KZ PI TI[g]	5.4; 8.9	+ (1b)	+ (15)	−	+	−	−	0010	E	1

[a] AM, ampicillin; A/S, ampicillin-sulbactam; A/C, amoxicillin-clavulanic acid; AT, aztreonam; CF, cephalothin; FX, cefoxitin; CT, cefotaxime; TX, ceftriaxone; TZ, ceftazidime; XM, cefuroxime; KZ, cefazolin; PM, cefepime; PI, piperacillin; P/T, piperacillin-tazobactam; TI, ticarcillin; CI, ciprofloxacin; NX, norfloxacin; OF, ofloxacin; AK, amikacin; GM, gentamicin; TO, tobramycin; T/S, trimethoprim-sulfamethoxazole.

[b] pIs were determined by IEF: 5.4 corresponds to TEM-1A, TEM-1B, or TEM-1C enzyme production; 7.4 corresponds to OXA-30 production; 8.1 corresponds to CTX-M-14; and 8.9 corresponds to CTX-M-15 or CTX-M-32 enzyme production.

[c] bla_{TEM} genes amplified by PCR were identified by sequencing as bla_{TEM-1A}, bla_{TEM-1B}, or bla_{TEM-1C}; bla_{CTX-M} genes were identified by sequencing as $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, or $bla_{CTX-M-32}$; and bla_{OXA} genes were identified by sequencing as bla_{OXA-30}. +, Amplification by PCR; −, no amplification by PCR.

[d] PFGE profile and number of strains with each profile, deduced from the dendrogram presented on Fig. 1. ND, not determined.

[e] More than one bla_{TEM-1} was present.

[f] β-Lactamase with pI 7.4, corresponding to OXA-30 enzyme production, was not detected by IEF.

[g] Non-multidrug-resistant pattern.

Dice (Opt:1.80%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]

PFGE

strain code	PFGE profile types	hospital codes	β-lactamases	Clusters
5776	0001	E	CTX-M-14	
5862	0002	D	CTX-M-14	
5944	0003	C	CTX-M-14	I
5993	0003	I	CTX-M-14	I
5986	0004	E	CTX-M-15	
5975	0005	C	CTX-M-14	
5842	0006	E	CTX-M-15	II
5852	0006	E	CTX-M-15	II
5847	0006	E	CTX-M-15	II
5840	0006	E	CTX-M-15	II
5861	0007	D	CTX-M-15	
5756	0008	E	CTX-M-15	
6241	0009	A	CTX-M-15	III
6242	0009	A	CTX-M-15	III
6381	0009	B	CTX-M-15	III
	0010		CTX-M-15	IV
5977	0011	C	CTX-M-15	
6372	0012	B	CTX-M-15	
6379	0013	B	CTX-M-15	
5785	0014	D	CTX-M-14	
5913	0015	H	CTX-M-15	
5958	0016	D	CTX-M-15	
5848	0017	E	CTX-M-15	
5820	0018	C	CTX-M-14	
5924	0019	E	CTX-M-32	V
5984	0019	E	CTX-M-15	V

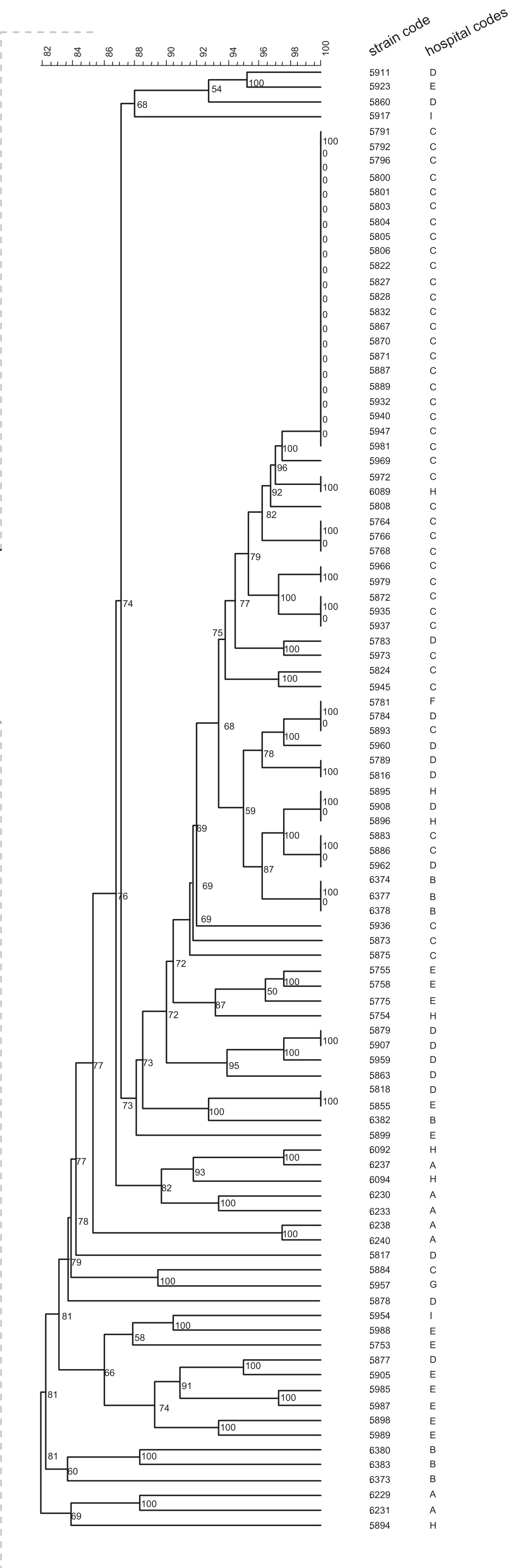

TABLE 6. Distribution of 47 strains from hospital C with corresponding PFGE profile types, by service, between May 2004 and May 2005

Service	Profile type (no. of strains)[a]												
	2004								2005				
	May	June	July	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Cardiology													0010 (1)
Attending	0010 (1)			0010 (2)			0010 (3)	0010 (1)	0010 (1)				
Emergency room							0010 (1)		0010 (1)	0010 (2)	0011 (1)	0010 (1)	
Gastrology	0010 (1)												
ICU										0010 (1)			
Internal medicine	0010 (1)	0010 (2)	0010 (1)	0010 (3)	0010 (1)	0010 (4)	0010 (2)	0010 (1)	0010 (1)				
Nephrology						0018 (1)				ND			
Pediatric									0003 (1)		0005 (1)		
Pneumology			0010 (1)						0010 (ND)				
Surgery												0010 (1)	
Unknown									0010 (1)				
Urology							0010 (2)	0010 (3)					

[a] ND, not determined.

found to be associated in the same strain in India (21), the United Kingdom (24), and Canada (4). Note that $bla_{\mathrm{OXA\text{-}1}}$ and $bla_{\mathrm{OXA\text{-}30}}$ have the same sequence and thus code for the same enzyme, called OXA-1 or OXA-30 (36, 46). The combination of $bla_{\mathrm{CTX\text{-}M\text{-}15}}$ plus $bla_{\mathrm{OXA\text{-}30}}$ (or $bla_{\mathrm{OXA\text{-}1}}$) plus $bla_{\mathrm{TEM\text{-}1}}$ has been reported in seven strains from Korea (20), in two strains from Senegal (50), and also in Spain (35). An association between $bla_{\mathrm{CTX\text{-}M\text{-}14}}$ and $bla_{\mathrm{TEM\text{-}1B}}$ has been described in Korea (22). However, our detection of $bla_{\mathrm{CTX\text{-}M\text{-}32}}$ in the same strain as $bla_{\mathrm{TEM\text{-}1B}}$ is, to our knowledge, a first.

There was an IS*Ecp1* element upstream from all $bla_{\mathrm{CTX\text{-}M}}$ genes detected. Thus, we add the $bla_{\mathrm{CTX\text{-}M\text{-}32}}$ gene to the list of $bla_{\mathrm{CTX\text{-}M}}$ genes associated with the IS*Ecp1* element (1, 4, 13, 21, 31, 40, 49), which contributes to their mobilization (40). The IS*903* element was detected downstream from only the $bla_{\mathrm{CTX\text{-}M\text{-}14}}$ gene, a finding in agreement with other studies (13, 26). The IS*26* element, characteristic of the epidemic strain A from the United Kingdom, was not detected among our strains but was recently detected in clinical isolates from Spain that were collected in the same period as the strains of our study (35, 51).

We tested for gene transfer from six isolates and genes were successfully transferred by electroporation to another *E. coli* strain from only three. This may suggest a major horizontal transfer by mobile elements. We obtained transformants containing CTX-14 alone, CTX-M-32 alone, and CTX-M-15 plus TEM-1B. The clinical strain containing CTX-M-14 enzyme was the only one not susceptible to cefoxitin; however, the transformant was susceptible, indicating another mechanism of resistance. None of the transformants presented the same resistance to quinolone, aminoglycosides, and trimethoprim as the clinical strains. Nevertheless, plasmid-determined resistance to quinolones and aminoglycosides, recently described in Portugal (25), involving a variant of aminoglycoside acetyltransferase AAC(6′)-Ib (44), was not possible to identify in the three studied strains.

Resistance to quinolones (93%), aminoglycosides (89%), and trimethoprim-sulfamethoxazole (26%) explains why most CTX-M producers were multidrug resistant. Another study reported lower prevalences of nonsusceptibility to quinolones (55%) and aminoglycosides (37%) but more frequent resistance to trimethoprim-sulfamethoxazole (34%) (16), and yet another describes very different percentages of resistance for trimethoprim-sulfamethoxazole (78% resistance) and aminoglycosides (43%) (30). Eckert et al. (14) reported that 58% of isolates were resistant to trimethoprim-sulfamethoxazole and 74% were resistant to aminoglycosides and that 100% of CTX-M enzymes producers were multidrug resistant. In our study, the value was 92% for multidrug resistance, with a higher percentage of isolates from the community (50%) than from hospital environments (37%), which was consistent with the predominance of *E. coli* expression of the CTX-M enzyme in the community described by Pitout et al. (39). Indeed, the high-level consumption of antibiotics in outpatients in Portugal may be responsible for this resistance (19). A wide diversity of resistance genes of numerous families coding for various antibiotic resistance mechanisms have now been described in CTX-M-15 producer strains.

We used PFGE to classify the strains. Three quarters of the strains clustered together (>80% similarity) in cluster IV, indicating countrywide dissemination of this multidrug-resistant clone. Various factors may have contributed to this dissemination: the small size of the country, the proximity of population areas, and/or an inadequate antibiotic use. Since all strains were susceptible to carbapenems, the use of these or other appropriate drugs could help reduce the prevalence of strains from cluster IV. Note that similar values for susceptibility to carbapenems were reported by others (23, 47, 50). The spread of a single clone in hospital C suggested nosocomial dissemination, especially in the internal medicine service. Nevertheless, the genetic similarity of clones in community services, in particular the attending and emergency rooms, also suggest

FIG. 1. Genetic relatedness among 123 *E. coli* strains by PFGE. PFGE profile types and clusters are shown from left to right. A total of 116 strains were the first isolate from each patient; seven additional isolates were collected from three of the patients. Strains INSRA5966, INSRA5979, INSRA5876, INSRA5879, INSRA5907, INSRA5863, INSRA5758, and INSRA5775 are multiple isolates from three patients. Strains with PFGE profile types 0003, 0006, 0009, 0010, and 0019 were defined as forming clusters I to V, respectively (indicated by vertical bands on the right).

dissemination within the community. The hospital-community and community-hospital dissemination of the $bla_{CTX-M-15}$ enzyme, mainly in hospital C (Tables 1 and 6), suggest the presence of an epidemic strain. The analysis of the three patients with multiple isolates shows that nosocomial infections due to *E. coli* CTX-M-15 producer strains are persistent (hospitals D and E), that nosocomial infection is easily transferred among services (hospital D), and that nosocomial infection can become a community infection (hospitals C and D) (data not shown).

Our study suggests that *E. coli* CTX-M producers are widespread in at least three regions of Portugal, possibly a consequence of the dissemination of major clones between hospitals and community and between regions and of the horizontal transfer of plasmids or mobile elements. Our findings also illustrate the potential dangers that the misuse of antibiotic can cause and the importance of measures to control infection. Nevertheless, rational use of other drugs may improve the situation. As previously noted (24), the use of extended-spectrum cephalosporins, quinolones, and aminoglycosides could be replaced with the use of carbapenems for treating infections in which ESBL-producing strains are likely to emerge.

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