

Identification and Broad Dissemination of the CTX-M-14 β -Lactamase in Different *Escherichia coli* Strains in the Northwest Area of Spain

German Bou,* Monica Cartelle, Maria Tomas, Delia Canle, Francisca Molina, Rita Moure, Jose Maria Eiros, and Antonio Guerrero†

Servicio de Microbiología, Complejo Hospitalario Juan Canalejo, 15006 La Coruña, Spain

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During the course of a molecular epidemiology study of mechanisms of antibiotic resistance in the area served by our hospital (516,000 inhabitants), we isolated the gene encoding CTX-M-14 β -lactamase. Thirty clinical strains (27 *Escherichia coli* and 3 *Klebsiella pneumoniae* isolates) with a phenotype of extended-spectrum β -lactamase were collected from January to October 2001 and studied for the presence of the CTX-M-14 β -lactamase gene. By isoelectric point determination, PCR, and nucleotide sequencing, we detected the presence of this gene in 17 *E. coli* strains belonging to 15 different genotypes (REP-PCR) causing infections in 17 different patients. Epidemiological studies based on medical records did not suggest any relationship between the patients infected with these *E. coli* strains and, interestingly, 7 of 30 patients harboring strains with extended-spectrum β -lactamases never had contact with the hospital environment before the clinical *E. coli* isolation. Conjugation experiments revealed that this gene was plasmid mediated in the 17 *E. coli* strains, and plasmid restriction fragment length polymorphisms showed 9 different patterns in the 17 *E. coli* strains. By PCR, the sequence of the *tnpA* transposase gene of the insert sequence ISEcp-1 was detected in all the plasmids harboring the CTX-M-14 gene. These results strongly suggest that plasmid dissemination between different *E. coli* strains in addition to a mobile element (transposon) around the β -lactamase gene may be involved in the spreading of the CTX-M-14 gene. This study reinforces the hypothesis that the epidemiology of the prevalence of the β -lactamase genes is changing and should alert the medical community to the increase in the emergence of the CTX-M β -lactamases worldwide.

The emergence of plasmid-mediated extended-spectrum β -lactamases (ESBLs) in members of the family *Enterobacteriaceae* has become a worldwide problem (6, 12, 21). ESBL molecular class A possesses an extended hydrolysis spectrum toward oxyimino- β -lactams and aztreonam but remains susceptible to cefoxitin and β -lactamase inhibitors (7). Most ESBLs are derivatives of TEM-1, TEM-2, or SHV-1 enzymes; however, reports describing the emergence of β -lactamases belonging to other families, such as CTX-M and/or OXA derivatives, are increasing worldwide (5, 8, 10, 11, 16).

CTX-M-related enzymes have been found in Europe, South America, and Mediterranean countries (3, 13, 15, 16, 18, 21). Unlike microorganisms harboring TEM and SHV β -lactamases, reports of outbreaks caused by organisms harboring non-TEM or non-SHV ESBL are very scarce. CTX-M-14 β -lactamase has recently been described in clinical isolates in Korea, China, and France (8, 11, 16, 19). In this communication we not only report for the first time the presence of the CTX-M-14 gene in Spain but we also show clear evidence of the dissemination of the gene encoding CTX-M-14 among 17 different patients, who had no apparent contact, living in the northwest part of Spain. This CTX-M-14 gene was detected in 15 different genotypes of *Escherichia coli* strains causing urinary tract infection in 17 patients in an area of 516,000 inhab-

itants. Plasmid dissemination and the presence of a putative transposon around the CTX-M-14 gene may account for the spreading of this gene. This finding emphasizes the increasing role of the CTX-M β -lactamases in antibiotic resistance worldwide and leads to consideration of empirical treatment for infections caused by *E. coli* strains, especially in patients compromised by underlying disease or immunological status.

MATERIALS AND METHODS

Selection of clinical isolates and patients. Between January and October 2001, 27 *E. coli* and 3 *Klebsiella pneumoniae* clinical strains with an ESBL phenotype were collected in the Department of Microbiology, Juan Canalejo Hospital, a 1,200-bed hospital serving a population of 516,000 in the northwest area of Spain. These isolates were identified by DadeMicroScan and the API 20E system (bioMérieux, Marcy l'Etoile, France).

Susceptibility tests and confirmation of ESBL production. The MICs of the β -lactams were determined by using E-test strips (AB Biodisk, Dalvågen, Sweden). The ESBL phenotype was detected by using ESBL detection E-test strips (AB Biodisk) as specified by the manufacturer. The double disk diffusion test was concomitantly used to confirm the E-test results.

IEF assay. β -Lactamases were characterized by isoelectric focusing (IEF) of ultrasonicated bacterial extracts. Bacteria growing exponentially at 37°C in Luria-Bertani (LB) medium were harvested, and cell-free lysates were prepared by sonication. β -Lactamases were analyzed by isoelectric focusing of cell extracts on polyacrylamide gels containing ampholytes with a pH range of 3 to 9 (PhastGel; Amersham Pharmacia Biotech AB) in a PhastSystem apparatus (Amersham Pharmacia Biotech AB). The focused β -lactamases were detected by overlaying the gel with nitrocefin (0.5 mg/ml) in 100 mM phosphate buffer (pH 7.0). pIs were determined by comparison with those of β -lactamases with a known pI: TEM-1 (pI 5.4), SHV-1 (pI 7.6), IMP-2 (pI 8.2), and AmpC from *A. baumannii* (pI 9.4).

DNA extraction. Bacterial chromosomal DNA was obtained with the High Pure PCR template preparation kit (Roche Diagnostics Corp., Indianapolis, Ind.). The chromosomal DNA was checked by electrophoresis in agarose gels,

* Corresponding author. Mailing address: Servicio de Microbiología, Hospital Materno Infantil, Complejo Hospitalario Juan Canalejo, C/Xubias de Arriba 84, 15006 La Coruña, Spain. Phone: 34-981-178000, ext. 21171. Fax: 34-981-178216. E-mail: germanbou@canalejo.org.

† Present address: Hospital de la Ribera, Alzira, Valencia, Spain.

and the concentrations of the different extracts were standardized by spectrophotometric measurements.

Conjugation experiments, plasmid purification, cloning experiments, and DNA sequencing. Transfer of resistance by conjugation was attempted using *E. coli* BM21 and *E. coli* XL-10-Gold Kan cells (Stratagene, La Jolla, Calif.) as recipients. Overnight mating experiments were performed at 37°C, and the transconjugants were selected on LB agar plates supplemented with ampicillin (50 μ g/ml) and nalidixic acid (50 μ g/ml) for *E. coli* BM21 and ampicillin (50 μ g/ml) and kanamycin (25 μ g/ml) for *E. coli* XL-10-Gold Kan cells. Afterward, transconjugants were selected for the cefotaxime resistance phenotype.

Plasmids from different *E. coli* strains were purified by the alkaline-lysis method with the High Pure plasmid isolation kit (Roche Diagnostics Corp.). Cloning procedures were performed as described by Sambrook et al. (20). Restriction enzymes were purchased from Boehringer (Mannheim, Germany) and were used as specified by the manufacturer.

For cloning the CTX-M-14 gene, plasmid DNA from transconjugant *E. coli* clinical strain 3 (see Table 2) was digested with *Bam*HI. The resulting fragments were ligated into pBGS18 and digested with *Bam*HI, and the mixture was transformed into *E. coli* TG1 made competent by the calcium chloride method (20). After transformation, a few clones grew on LB agar plates supplemented with kanamycin (10 μ g/ml) and ampicillin (50 μ g/ml). They harbored an identical plasmid (pC) with an insert of about 6 kb. These plasmids were used as templates to determine the nucleotide sequence. Templates were sequenced on both strands by the method of Sanger et al. Sequencing was carried out with the *Taq* DyeDeoxyTerminator cycle-sequencing kit using primers specific to the coding sequence, and the sequence was analyzed in an automatic DNA sequencer (377 ABI Prism; Perkin-Elmer).

Detection of the CTX-M-14 gene. Plasmid DNA from different *E. coli* strains was used as the template in a PCR amplification. Amplification reactions were performed in a final volume of 50 μ l. Mg^{2+} -free PCR buffer was purchased as a 10 \times concentrate consisting of 500 mM KCl, 100 mM Tris-HCl (pH 9.0), and 1% Triton X-100 (Perkin-Elmer, Roche Molecular Systems, Inc., Nutley, N.J.) with 200 μ M (each) dATP, dCTP, dGTP, and dTTP (Perkin-Elmer, Roche Molecular Systems, Inc.). The Mg^{2+} concentration was 2.5 mM, and the primers were used at 0.5 μ M each. The primer pair CTX-M-1 (5'-AACACGGATTGACCGTAT TG-3') and CTX-M-2 (5'-TTACAGCCCTTCGGCGAT-3') was used to amplify the CTX-M-14 gene in the plasmid DNA. Amplification reactions were carried out in an Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany), with an initial denaturation (10 min at 94°C) followed by 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C), and extension (2 min at 72°C), with a single final extension of 10 min at 72°C. Aliquots (15 μ l) of each sample were subjected to electrophoresis in 1.0% agarose gels. Amplified products were detected after staining with ethidium bromide (50 μ g/ml) and photographed with Polaroid type 665 film.

REP-PCR. Amplification reactions were performed in a final volume of 50 μ l. Mg^{2+} -free PCR buffer was purchased as a 10 \times concentrate consisting of 500 mM KCl, 100 mM Tris-HCl (pH 9.0), and 1% Triton X-100 (Perkin-Elmer, Roche Molecular Systems, Inc.), with 200 μ M (each) dATP, dCTP, dGTP, and dTTP (Perkin-Elmer, Roche Molecular Systems, Inc.). The Mg^{2+} concentration was 3 mM, and the primers were used at 0.5 μ M each. The primer pair REP1 (5'-IIIGCGCCGICATCAGGC-3') and REP2 (5'-ACGTCTTATCAGGCCTAC-3') was used to amplify putative REP-like elements in the genomic bacterial DNA (23). A total of 500 ng of chromosomal DNA was added to the reaction mixture. Amplification reactions were carried out in an Eppendorf thermal cycler, with an initial denaturation (10 min at 94°C) followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 45°C), and extension (2 min at 72°C), with a single final extension of 16 min at 72°C. Aliquots (20 μ l) of each sample were subjected to electrophoresis in 1.0% agarose gels. Amplified products were detected after staining with ethidium bromide (50 μ g/ml) and photographed with Polaroid type 665 film. Strains belonging to the same DNA group showed identical or highly similar profiles (up to two bands different).

Plasmid restriction fragment length polymorphisms (RFLPs). Plasmid DNA from transconjugant *E. coli* strains 1 to 17 (see Table 2) was isolated as described above. The DNA was then digested with *Hind*III, and the resulting fragments were loaded on a 0.8% agarose gel. After electrophoresis, DNA fragments were detected by staining with ethidium bromide (50 μ g/ml) and photographed with Polaroid type 665 film.

Detection of the gene encoding the *tnpA* transposase. To detect the presence of the *tnpA* gene of the insert sequence ISEcp-1, we used the primer pair *tnpA*1 (5'-GAATTCATCAATTGTATT-3') and *tnpA*2 (5'-CAAGAAATACATACTT CAA-3'). About 10 ng of plasmid DNA was added to the reaction mixture. Amplification reactions were carried out in an Eppendorf thermal cycler, with an initial denaturation (10 min at 94°C), 30 cycles of denaturation (1 min at 94°C),

annealing (1 min at 48°C), and extension (2 min at 72°C), and a single final extension of 16 min at 72°C. Aliquots (20 μ l) of each sample were subjected to electrophoresis in 1.0% agarose gels. Amplified products were detected after staining with ethidium bromide (50 μ g/ml) and photographed with Polaroid type 665 film.

RESULTS

Selection of clinical isolates and patients. Between January and October 2001, 27 *E. coli* and 3 *K. pneumoniae* clinical strains with a phenotype suggestive of ESBL were selected. These strains were isolated from 30 different patients. One isolate per patient was selected (Table 1). The strains were associated with urinary tract infections (77%), wound infection (13.3%), catheter infection (3.3%), blood infection (3.3%), and conjunctival infection (3.3%). Most patients had underlying diseases. Moreover, although most of the patients were hospitalized before the *E. coli* and/or *K. pneumoniae* isolation, seven patients had no hospital admission noted in their medical records.

Determination of antibiotic susceptibility and ESBL production. MICs for the 30 clinical strains were determined by the DadeMicroScan. In addition, all MICs were confirmed by the E test (AB Biodisk) according to the NCCLS criteria (14). The overall results are shown in Table 2. For all strains, the amoxicillin MICs were higher than 256 μ g/ml; moreover, this concentration decreased drastically in the presence of clavulanic acid, suggesting the presence of a class A β -lactamase. Regarding broad-spectrum cephalosporins, strains 1 to 17 and 22 had a phenotype suggesting cefotaximase β -lactamase activity as shown by the cefotaxime MICs (the cefotaxime MICs were clearly higher than the ceftazidime MICs).

Interestingly, the *K. pneumoniae* isolates included in this study with an ESBL phenotype (strains 28 to 30) showed unique SHV-like β -lactamase activity band by IEF. This band probably corresponds to the SHV chromosomal β -lactamase that is constitutively present in *K. pneumoniae* strains; alternatively, it is an SHV ESBL indistinguishable from the chromosomal SHV-1. The presence of a second antibiotic resistance mechanism in these *K. pneumoniae* strains cannot be ruled out.

IEF assay. Protein extracts of the 30 clinical strains and their transconjugants obtained by sonication were resolved by IEF (3–9). Different pIs corresponding to different β -lactamases were obtained. Interestingly, strains 1 to 17 yielded an identical pI of 8.0; in addition, some of them yielded a pI of 5.4, suggestive of a TEM-1-like β -lactamase. Strains 18 to 30 showed different pIs such as 5.4, 5.9, 7.4, 7.6, 7.9, 8.5, and 9.0 (Table 2).

Cloning of the CTX-M-14 gene. The cefotaxime MICs and pIs obtained by IEF suggested the presence of an identical or similar β -lactamase gene associated with resistance to cefotaxime in strains 1 to 17 (Table 2). We attempted to clone the β -lactamase gene responsible for this selective resistance to cefotaxime.

For this purpose, strain 3 (Table 2) was chosen for the cloning experiments. First, we tried to show whether the β -lactamase gene was plasmid or chromosomally mediated. A conjugation experiment was performed using *E. coli* XL-1-blue as a host, as described in Materials and Methods. Transconjugants were selected on LB agar plates supplemented with kanamycin and ampicillin. Several transconjugants were obtained, and IEF of the sonicated extracts of the clinical strain

TABLE 1. Patients from whom the *E. coli* and *K. pneumoniae* strains used in this study were isolated

Patient data (no., sex/age [yr])	Type of infection ^a	Underlying disease	Specimen	Hospitalization before the <i>E. coli</i> isolation
1. F/81	UTI	Diabetes	Urine	No
2. F/9	UTI	Acute pyelonephritis	Urine	Yes
3. M/66	UTI	Prostate tumor	Urine	Yes
4. F/1	UTI	Respiratory tract infection	Urine	Yes
5. F/Unknown	UTI	Unknown	Urine	Unknown
6. F/77	UTI	Blood infection, diabetes	Urine	Yes
7. F/80	UTI	No	Urine	No
8. F/Unknown	UTI	Unknown	Urine	Unknown
9. M/67	UTI	Colon cancer	Urine	Yes
10. M/58	UTI	Prostate tumor	Urine	Yes
11. F/73	UTI	HBP, ^b arthrosis	Urine	Yes
12. F/75	UTI	HPB, urinary incontinence	Urine	No
13. F/41	UTI	Unknown	Urine	No
14. F/57	WI	Stomach ulceration	Exudate	Yes
15. F/76	UTI	Type II diabetes, chronic hepatitis	Urine	Yes
16. M/86	WI	Unknown	Exudate	Unknown
17. F/37	UTI	No	Urine	No
18. F/73	UTI	HBP, subarachnoid hemorrhagia	Urine	Yes
19. F/32	UTI	Unknown	Urine	Yes
20. F/51	UTI	Unknown	Urine	Yes
21. F/73	UTI	HBP	Urine	Yes
22. M/25	WI	Unknown	Exudate	No
23. F/14	CI	Unknown	Catheter	Unknown
24. M/79	WI	Pneumonia	Exudate	Yes
25. F/66	UTI	Unknown	Urine	No
26. M/73	UTI	Prostate tumor	Urine	Yes
27. M/5	UTI	Several UTIs	Urine	Yes
28. M/53	UTI	Leukemia	Urine	Yes
29. F/1	BI	Hepatitis	Blood	Yes
30. F/1	CoI	Premature birth	Conjunctival swab	Yes

^a UTI, urinary tract infection; WI, wound infection; CI, catheter infection; BI, blood infection; CoI, conjunctival infection.

^b HBP, high blood pressure.

and the transconjugants showed a pI 8.0 activity band in all cases. Plasmid analysis of the clinical strain (strain 3) and one transconjugant (strain 3Tc) showed that the β -lactamase gene was associated with a plasmid of ca. 40 kb (data not shown). Moreover, MIC experiments performed with both parental (strain 3) and transconjugant (strain 3Tc) isolates showed almost identical antibiotic susceptibilities (Table 2), clearly suggesting that the β -lactamase gene was plasmid mediated.

Cloning of the CTX-M-14 gene was performed as described in Materials and Methods. The nucleotide sequence of the fragment revealed only one open reading frame with homology with β -lactamase genes. The CTX-M-14 open reading frame was 876 bp long and encoded a protein of 291 amino acid residues. This gene has previously been reported (GenBank accession numbers AJ416341 and AF252622). In the upstream region of the CTX-M-14 gene, the nucleotide sequence of the *trpA* gene, the transposase gene of insertion sequence ISEcp1, was also detected (24).

Spread of the CTX-M-14 gene in different *E. coli* strains. To elucidate whether the CTX-M-14 gene was plasmid mediated in all *E. coli* strains, conjugation experiments were performed. Regarding the kanamycin and/or nalidixic acid susceptibility of the *E. coli* clinical strains, two different *E. coli* isolates were used as hosts in the conjugation experiments, *E. coli* BM21 (resistant to nalidixic acid) when the *E. coli* clinical strains were susceptible to nalidixic acid and *E. coli* XL-10-Gold Kan (resistant to kanamycin) when the *E. coli* clinical strains were susceptible to kanamycin. Transconjugants were obtained in all

E. coli strains (isolates 1 to 17 in Table 2) that yielded a β -lactamase activity band of pI 8.0 with cefotaximase activity, strongly suggesting that in all cases the β -lactamase with cefotaximase activity was plasmid mediated.

To assess the possibility of the spread of this CTX-M-14 gene in the 16 remaining *E. coli* strains, a PCR assay was performed. Plasmid DNA obtained from the 27 *E. coli* and 3 *K. pneumoniae* strains was used as a template in a PCR amplification with CTX-M-1 (5'-AACACGGATTGACCGTATTG-3') and CTX-M-2 (5'-TTACAGCCCTTCGGCGAT-3') primers, which annealed at the promoter and the 3' part of the CTX-M-14 gene, respectively. Interestingly, a positive PCR result, indicated by a band of 905 bp, was detected in 17 out of 30 clinical strains, including strain 3 (data not shown). The overall results showed that the CTX-M-14 gene was disseminated among 17 *E. coli* strains in the northwest area of Spain. Moreover, nucleotide sequence analysis of these 17 amplicons showed total identity to the CTX-M-14 nucleotide sequence.

It is worth mentioning that strain 18, although possessing a pI 8.5 band of β -lactamase activity, did not yield an amplification product with the CTX-M-1 and CTX-M-2 primers. Also, the antibiotic susceptibility pattern of this strain did not show the putative cefotaximase profile detected in the other 17 *E. coli* strains.

The next step was to try to determine whether these 17 *E. coli* strains were genetically related. This experiment was necessary to distinguish between two putative hypotheses: (i) one epidemic *E. coli* strain has been disseminated among all

TABLE 2. MICs for the clinical strains included in this study

Isolate no.	pI	MIC (μ g/ml) of ^a :										
		AMX	AMC	FOX	AT	CTX	CTX-CL	CAZ	CAZ-CL	IMP	MRP	CEP
1	5.4, 8.0	>256	4	8	4	24	0.094	1.5	0.25	0.25	0.016	2
2	8.0	>256	4	2	2	24	0.047	1.0	0.094	0.25	0.016	2
3	8.0	>256	6	4	3	16	0.064	1.0	0.25	0.25	0.016	2
3 (Tc) ^b	8.0	>256	4	4	3	32	0.047	1.0	0.125	0.125	0.032	4
4	5.4, 8.0	>256	4	2	0.75	16	0.023	0.5	<0.064	0.094	0.012	1.5
5	5.4, 8.0	>256	4	12	2	48	0.094	1.0	0.125	0.125	0.023	4
6	5.4, 8.0	>256	4	4	2	24	0.047	0.75	0.125	0.19	0.016	2
7	8.0	>256	4	2	2	24	0.032	1.0	0.064	0.19	0.012	1.5
8	8.0	>256	4	4	2	16	0.064	1.0	0.125	0.125	0.016	4
9	5.4, 8.0	>256	8	24	8	>128	>1.0	6.0	4.0	0.25	0.023	4
10	5.4, 8.0	>256	6	12	2	64	0.094	1.0	0.125	0.125	0.016	4
11	5.4, 8.0	>256	4	3	2	32	0.047	1.0	0.064	0.25	0.016	4
12	8.0	>256	4	4	2	64	0.047	1.0	0.094	0.19	0.016	4
13	8.0	>256	4	4	4	>128	0.047	1.0	0.094	0.125	0.012	2
14	8.0	>256	4	4	2	48	0.064	1.5	0.19	0.125	0.012	4
15	8.0	>256	4	3	1.5	>128	0.047	1.0	0.125	0.19	0.016	4
16	5.4, 8.0	>256	4	4	2	16	0.064	1.0	0.19	0.19	0.023	2
17	8.0	>256	4	4	2	24	0.064	1.0	0.125	0.125	0.012	2
18	5.4, 8.5	>256	4	2	8	24	>1	>32	>4	0.25	0.032	0.38
19	5.4	>256	16	16	24	0.5	0.19	>32	0.75	0.25	0.032	2
20	5.4	>256	2	1	0.5	<0.016	<0.016	12	0.064	0.19	0.023	0.032
21	5.4	>256	4	12	6	0.5	<0.016	>32	0.19	0.125	0.023	0.75
22	9.0	>256	4	2	4	>256	0.047	6	0.094	0.125	0.016	8
23	9.0	>256	12	>256	>256	>256	>1.0	>32	>4	0.125	0.064	>128
24	9.0	>256	12	>256	>256	>256	>1.0	>32	>4	0.125	0.047	128
25	5.9	>256	4	2	1	2	0.094	2	0.19	0.5	0.032	0.094
26	5.4, 5.9	>256	4	8	6	0.19	0.047	>32	0.125	0.125	0.016	0.5
27	7.6	>256	8	4	0.125	0.064	0.032	4	0.19	0.19	0.032	0.5
28 ^c	7.4	>256	4	1	6	24	<0.016	>32	0.19	0.19	0.032	3
29 ^c	7.9	>256	4	12	6	0.5	0.064	>32	0.19	0.125	0.023	0.75
30 ^c	7.4	>256	6	2	1	1	0.047	1.5	0.125	0.19	0.032	0.094
<i>E. coli</i> TG1		3	3	2	0.047	0.023	0.023	0.064	0.064	0.125	0.032	0.094
<i>E. coli</i> TG1 (pC) ^d	8.0	>256	6	2	48	>256	0.064	4	0.125	0.19	0.023	64

^a AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; AT, aztreonam; CTX, cefotaxime; CTX-CL, cefotaxime-clavulanic acid; CAZ, ceftazidime; CAZ-CL, ceftazidime-clavulanic acid; IMP, imipenem; MRP, meropenem; CEP, cefepime.

^b This isolate corresponds to the transconjugant of clinical strain 3.

^c This isolate corresponds to *K. pneumoniae*.

^d *E. coli* transformed with plasmid pC, which carries the CTX-M-14 gene.

the patients, and so one ancestor strain may be responsible for the spreading; it implies a link between all the patients, and epidemiological studies must confirm this hypothesis; and (ii) the CTX-M-14 gene has disseminated among different *E. coli* strains. Therefore, the possibility that a plasmid harboring the CTX-M-14 gene or a mobile genetic element carrying this gene had been disseminated in different *E. coli* strains cannot be ruled out. A REP-PCR assay was performed as described in Materials and Methods. This assay showed that most of the *E. coli* strains were genetically unrelated (Fig. 1). Fifteen different genotypes may be assigned to the 17 *E. coli* clinical strains (isolates 3 and 4 and isolates 13 and 15 yielded the same band patterns). This result therefore eliminated the possibility of clonal dissemination of one epidemic *E. coli* strain.

Plasmid RFLP. Purified plasmids from transconjugants of strains 1 to 17 (Table 2) were digested with *Hind*III, and the resulting fragments were separated on 0.8% agarose gels (Fig. 2). The overall results showed a similarity between plasmids in strains 1, 4, 5, 7, 9, 10, 12, 13, and 14 (RFLP pattern I in Table 3) obtained from the 17 *E. coli* strains, thus suggesting identity in the plasmids harboring CTX-M-14 on these strains. However, strains 2, 3, 6, 8, 11, 15, 16, and 17 did not show a

similarity in their RFLP patterns, and their patterns were different from RFLP pattern I. Therefore, the CTX-M-14 gene was harbored in different plasmids on these strains, and so the possibility that a mobile genetic element might be involved in the dissemination of the CTX-M-14 gene in these plasmids cannot be ruled out.

Detection of the *tnpA* transposase gene. Plasmid DNAs from the conjugants of the 17 *E. coli* clinical strains (strains 1 to 17 in Table 2) were subjected to PCR amplification with primers which annealed to the *tnpA* gene, as described in Materials and Methods. A positive band of 405 bp was obtained in all the plasmid purifications (data not shown), suggesting that a putative functional transposon was present in all *E. coli* strains and that this transposon may be involved in the mobilization of the CTX-M-14 gene.

DISCUSSION

In 1992 (1, 2) a novel type of ESBL that conferred a high level of resistance to cefotaxime but not to ceftazidime was identified in some members of *Enterobacteriaceae*. This new family of plasmid-mediated ESBLs of Ambler class A was

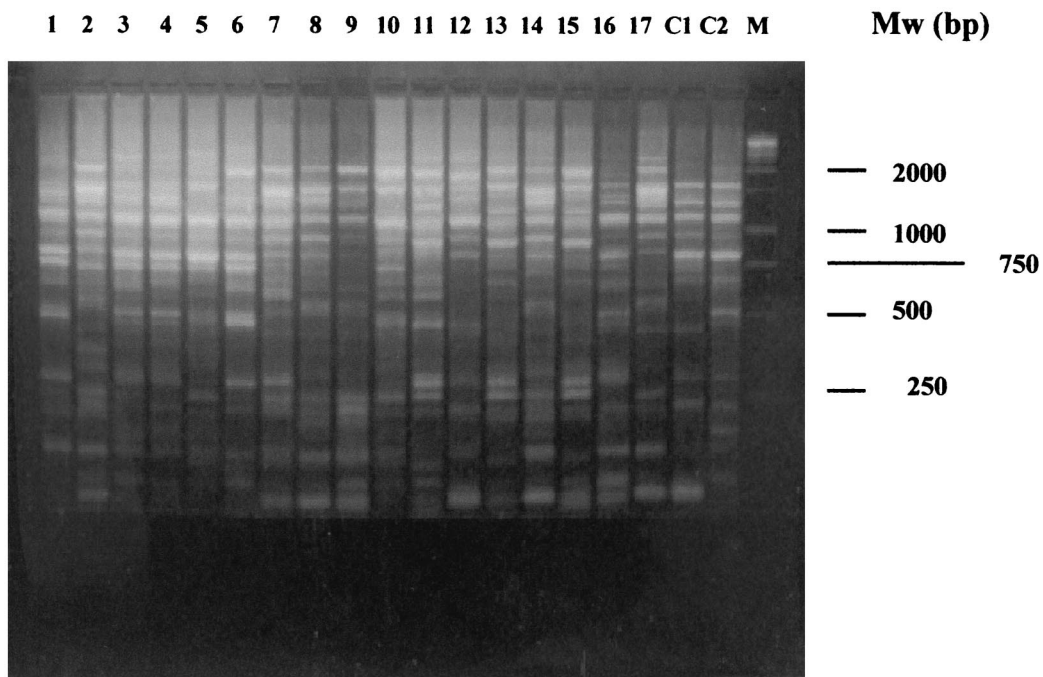


FIG. 1. REP-PCR of the 17 *E. coli* strains and 2 genetically unrelated *E. coli* controls (C1 and C2). The strain numbers are shown above the gel. Mw (molecular size markers) corresponds to the 1-kb DNA ladder (Promega, Madison, Wis.).

assigned to the cefotaximase (CTX-M) family. Cefotaximases (CTX-M) have been detected mainly in South America, Eastern Europe, Japan, Spain, Kenya, and France (3, 13, 15, 17, 22).

The family of the CTX-M-type ESBLs comprises several members belonging to four major phylogenetic trees on the basis of their amino acid sequence similarities (4): the CTX-

M-1 group (CTX-M-1, CTX-M-3, and CTX-M-10), the CTX-M-2 group (CTX-M-2, Toho-1, CTX-M-4, CTX-M-5, and CTX-M-6), the CTX-M-9 group (CTX-M-9 and Toho-2), and the CTX-M-8 group. The CTX-M-14 β -lactamase is an Ala231-Val mutant of CTX-M-9; therefore, it may be assigned to the CTX-M-9 group.

We have detected the CTX-M-14 β -lactamase for the first

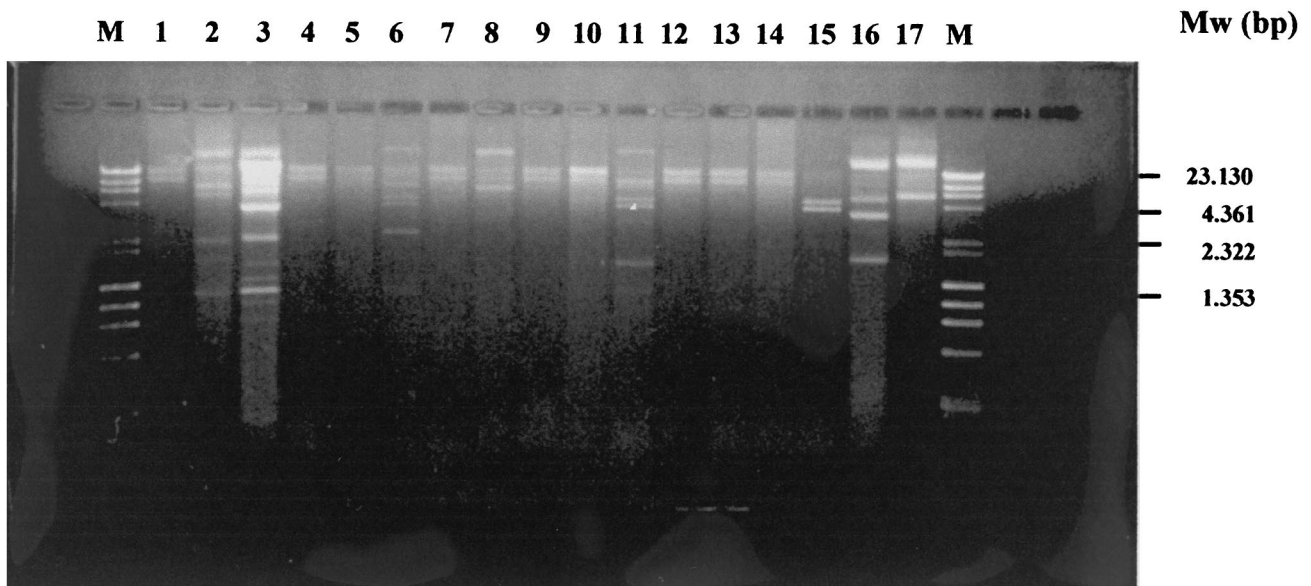


FIG. 2. Plasmid RFLP obtained by *Hind*III digestion with plasmid purifications of the transconjugants of the 17 *E. coli* strains. The strain numbers are shown above the gel. Mw (molecular size markers) corresponds to the lambda DNA *Hind*III digest and bacteriophage Φ 174 *Hae*III digest (Fynnzymes OY).

TABLE 3. REP-PCR pattern, detection of the CTX-M-14 gene and RFLP of the plasmids of the transconjugants of the clinical strains used in this study

Isolate no.	pI	REP-PCR pattern	PCR CTX-M-14	Transconjugant plasmid RFLP pattern
1	5.4, 8.0	1	+	I
2	8.0	2	+	II
3	8.0	3	+	III
4	5.4, 8.0	3	+	I
5	5.4, 8.0	4	+	I
6	5.4, 8.0	5	+	IV
7	8.0	6	+	I
8	8.0	7	+	V
9	5.4, 8.0	8	+	I
10	5.4, 8.0	9	+	I
11	5.4, 8.0	10	+	VI
12	8.0	11	+	I
13	8.0	12	+	I
14	8.0	13	+	I
15	8.0	12	+	VII
16	5.4, 8.0	14	+	VIII
17	8.0	15	+	IX
18	5.4, 8.5	ND ^a	–	ND
19	5.4	ND	–	ND
20	5.4	ND	–	ND
21	5.4	ND	–	ND
22	9.0	ND	–	ND
23	9.0	ND	–	ND
24	9.0	ND	–	ND
25	5.9	ND	–	ND
26	5.4, 5.9	ND	–	ND
27	7.6	ND	–	ND
28	7.4	ND	–	ND
29	7.9	ND	–	ND
30	7.4	ND	–	ND

^a ND, not done.

time in Spain. This β -lactamase has previously been described in China, Korea, and France (8, 11, 16, 19).

As described above, the CTX-M-14 gene is plasmid mediated; indeed, we detected it in the plasmids of the 15 different genotypes of *E. coli* strains. The plasmid fingerprints yielded 9 different patterns; therefore, 9 of the 17 profiles were identical. This result suggests that the spreading of this plasmid among different *E. coli* strains was important in the dissemination of the CTX-M-14 gene. However, in the other eight *E. coli* strains, different plasmids were detected, as shown by restriction analysis. This result implies some kind of mobilization of the CTX-M-14 gene in the different *E. coli* strains in addition to the horizontal transfer. The analysis of the CTX-M-14 upstream region showed the presence of the transposase gene (*tnpA*) of the insertion sequence named ISEcp-1 in all plasmids detected in the *E. coli* strains. It has previously been suggested that the ISEcp-1 element could be associated with the mobility of the CTX-M genes (11). Therefore, the possibility that the ISEcp-1 insertion sequence can be associated with the spreading of the CTX-M-14 genes in our *E. coli* strains cannot be ruled out. In fact, this is the only genetic element we have found associated with the high dissemination of the CTX-M-14 gene.

These 17 *E. coli* strains (15 genotypes) caused infections in 17 patients. In 15 patients the *E. coli* strain harboring CTX-M-14 caused urinary tract infections whereas in the other two they caused wound infections. Furthermore, most patients had

underlying diseases and, interestingly, no hospital admission was noted in the medical records of 7 patients. Thus, on the basis of these data, we may assume the possible existence of outpatients with bacterial strains causing infections harboring antibiotic resistance mechanisms. Therefore, the data presented here raise the hypothesis that microorganisms with antibiotic resistance mechanisms can be present in patients in the community. Another interesting issue is that no apparent contacts between the 17 patients occurred. Indeed, most inpatients were admitted to different hospital wards of the hospital complex. The outpatients live in different areas far away from each other. With the current data, how can we explain the presence of up to 62% of the ESBL-producing strains as harboring the CTX-M-14 gene? Several reports have been published describing the detection of CTX-M-type enzymes in different areas worldwide. Here we report a practical study showing that CTX-M-type β -lactamases are highly disseminated; indeed, they are replacing the TEM and SHV derivative β -lactamases (9). The reasons why this is happening remain to be elucidated.

In a hospital environment, spreading between patients can be easy when a bacterium with an antibiotic resistance mechanism has been established, especially if this mechanism is associated with mobile genetic elements. However, we report here that seven patients from whom bacterial strains harboring ESBL were isolated never had contact with the hospital environment before the clinical *E. coli* isolation. Further epidemiological studies need to be performed to explain these data.

In summary, we have found a high prevalence of the CTX-M-14 β -lactamase in the northwest area of Spain. On the basis of these data, the empirical administration of cefotaxime in serious infections should be avoided in order to prevent clinical complications. In addition, we alert the medical community of the increase of these β -lactamases worldwide.

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