Journal of Antimicrobial Chemotherapy (2005) **56**, 1107–1110
doi:10.1093/jac/dki370
Advance Access publication 20 October 2005

JAC

Mechanisms of resistance to expanded-spectrum cephalosporins in *Escherichia coli* isolates recovered in a Spanish hospital

Laura Briñas[1], Marta Lantero[2], Isabel de Diego[2], María Alvarez[2], Myriam Zarazaga[1] and Carmen Torres[1]*

[1]*Area de Bioquímica y Biología Molecular, Universidad de La Rioja, Madre de Dios, 51, 26006 Logroño, Spain;* [2]*Servicio de Microbiología, Hospital Universitario Central de Asturias, Oviedo, Spain*

Received 4 April 2005; returned 20 August 2005; revised 5 September 2005; accepted 15 September 2005

***Objectives*: To characterize the β-lactamase genes of the expanded-spectrum cephalosporin-resistant *Escherichia coli* isolates recovered in a Spanish hospital during the March 2002–March 2003 period.**

***Methods*: Thirty-four of the 1700 *E. coli* isolates recovered from unrelated patients in a Spanish hospital showed expanded-spectrum cephalosporin resistance. The presence of genes encoding TEM, SHV, CTX-M, CMY-2-type or FOX β-lactamases as well as the existence of mutations in the regulatory region of the chromosomal *ampC* gene were studied by PCR and sequencing in these 34 *E. coli* isolates.**

***Results*: The following extended-spectrum β-lactamases (ESBLs) or plasmidic class C β-lactamase genes were detected (number of isolates): $bla_{CTX\text{-}M\text{-}14}$ (14), $bla_{CTX\text{-}M\text{-}9}$ (4), $bla_{CTX\text{-}M\text{-}32}$ (1), $bla_{TEM\text{-}52}$ (2), $bla_{SHV\text{-}12}$ (3) and $bla_{CMY\text{-}2}$ (2). The remaining eight isolates showed a mutation in the promoter/attenuator region of the *ampC* chromosomal gene at position –42, in combination with mutations at positions –18, –1 and +58. The $bla_{TEM\text{-}1}$ gene was also detected in 12 of the ESBL-producing isolates, in both CMY-2-producing isolates and in four of the eight isolates that showed a mutation at position –42 of the *ampC* promoter. Other mutations in the promoter/attenuator region were detected in association with ESBL or CMY-2 genes, such as the combination –18, –1 and +58, –28 and +58, or +22, +26, +27 and +32. No clonal relationship was found among the CTX-M-producing *E. coli* isolates by PFGE with *Xba*I enzyme.**

***Conclusions*: Approximately 1.5% of the *E. coli* isolates of our hospital harboured ESBL genes, those of the CTX-M-9 group being the most common ones.**

Keywords: *E. coli*, ESBLs, class C β-lactamases, Spain

Introduction

The production of extended-spectrum β-lactamases (ESBLs) is generally associated with resistance to expanded-spectrum cephalosporins in *Escherichia coli* and the dissemination of strains of this species harbouring ESBLs in clinical settings is rising.[1,2] Most ESBLs are derived from the classical TEM-1, TEM-2 and SHV-1 enzymes, by amino acid substitutions in their sequences, but CTX-M β-lactamases are increasingly being reported among human and animal *E. coli* strains.[3–7] This class of ESBLs is characterized typically by conferring more resistance to cefotaxime than to ceftazidime, and they were initially reported in 1989 in Germany, from an *E. coli* isolate, and in 1990 in Argentina, from a *Salmonella* isolate.[1] CTX-M β-lactamases are widely spread and to date have been reported in a wide variety of countries and continents.[1,2] Approximately 40 CTX-M enzymes have been described so far and they are classified into five different groups.[1]

Resistance to expanded-spectrum cephalosporins can also be associated in *E. coli* with the production of plasmidic class C β-lactamases, such as CMY enzymes,[8] or with the overproduction of the chromosomal AmpC β-lactamase.[7,9,10] The objective of this work was to characterize the β-lactam resistance mechanisms in all the clinical *E. coli* isolates with reduced susceptibility or resistance to expanded-spectrum cephalosporins, recovered during a 1 year period in a Spanish hospital, and to analyse their clonal relationship.

*Corresponding author. Tel: +34-941299750; Fax: +34-941299721; E-mail: carmen.torres@daa.unirioja.es

Materials and methods

A total of 1700 *E. coli* isolates were recovered during a 1 year period (March 2002–March 2003) from unrelated patients of different wards of the Hospital Universitario Central de Asturias (Oviedo, Spain). Thirty-four of these *E. coli* isolates (2%) showed an MIC value of ceftazidime or cefotaxime of ≥4 mg/L (by the automatic Wider system) and were included in this study. The origin of these resistant isolates was as follows (number of isolates): urine (21), exudates and surgical wounds (7), blood (2), tracheobronchial aspirate (2), peritoneal fluid (1) and bile (1). The MIC values of 10 β-lactams were further analysed by the agar dilution method according to the NCCLS recommendations. Susceptibility to non-β-lactam antibiotics was studied by the disc diffusion method (NCCLS). *E. coli* ATCC 25922 was used as a quality control strain. ESBL production was screened by the double disc method (ceftazidime or cefotaxime combined with clavulanic acid).

The presence of genes encoding TEM (forward, 5′-ATTCTTGAAGACGAAAGGGC-3′; reverse, 5′-ACGCTCAGTGGAACGAAAAC-3′), SHV (forward, 5′-CACTCAAGGATGTATTGTG-3′; reverse, 5′-TTAGCGTTGCCAGTGCTCG-3′), CTX-M (forward, 5′-GTGACAAAGAGAGTGCAACGG-3′; reverse, 5′-ATGATTCTCGCCGCTGAAGCC-3′), CMY-2-type (forward, 5′-GATTCCTTGGACTCTTCAG–3′; reverse, 5′-TAAAACCAGGTTCCCAGATAGC-3′) and FOX (forward, 5′-CACCACGAGAATAACCAT-3′; reverse, 5′-ATGTGGACGCCTTGAACT-3′) β-lactamases was studied by specific PCRs[7] and their identification was verified by sequencing and comparison with those sequences included in the EMBL database. The promoter and attenuator region of the chromosomal *ampC* gene was also amplified by PCR (forward, 5′-AATGGGTTTTCTACGGTCTG-3′; reverse, 5′-GGGCAGCAAATGTGGAGCAA-3′), sequenced and compared with the same region of the *E. coli* K12 *ampC* gene,[10] in order to analyse the mutations in that region, associated with the overexpression of the *ampC* gene. Positive and negative controls were included in all PCR assays.

The clonal relationship among the strains was studied by PFGE, using *Xba*I as restriction enzyme. PFGE patterns were classified into four groups: indistinguishable (all the bands match), closely related (1–3 different bands), possibly related (4–6 different bands) and unrelated (>6 different bands).

Results and discussion

Thirty-four expanded-spectrum cephalosporin-resistant *E. coli* isolates (MIC of ceftazidime or cefotaxime of ≥4 mg/L) were recovered from unrelated patients in a Spanish hospital during a 1 year period, representing 2% of the total clinical *E. coli* isolates obtained. Table 1 shows the MIC values of different β-lactams as well as the resistance mechanisms detected in all these 34 isolates. The MIC ranges of some of the β-lactams tested were as follows (mg/L): ampicillin (64 to >256), amoxicillin/clavulanic acid (2–32), cefoxitin (1–256), ceftazidime (0.125–128), cefotaxime (0.25 to >256), imipenem (≤0.06–0.25) and aztreonam (1–256).

Mechanisms of resistance in the ESBL-positive strains

A positive ESBL screening test was demonstrated in 24 of the 34 studied isolates, which represents 1.4% of the 1700 *E. coli* isolates recovered in the 1 year period. A higher MIC value of cefotaxime (4 to >256 mg/L) than of ceftazidime (0.125–16 mg/L) was detected in 19 of them, and the MIC values of ceftazidime were higher than those of cefotaxime in three other isolates (2–8 and 32–128 mg/L, respectively). Similar MIC values of cefotaxime and ceftazidime were found in the last two isolates (32 mg/L). Specific PCR and sequencing allowed identification of five different ESBL genes among these 24 ESBL-positive isolates: $bla_{\text{CTX-M-14}}$ (14 isolates), $bla_{\text{CTX-M-9}}$ (4 isolates), $bla_{\text{CTX-M-32}}$ (1 isolate), $bla_{\text{SHV-12}}$ (3 isolates) and $bla_{\text{TEM-52c}}$ (2 isolates). A $bla_{\text{TEM-1}}$ gene (molecular variants $bla_{\text{TEM-1b}}$ or $bla_{\text{TEM-1c}}$) was also detected in 12 of them that harboured $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-9}}$ or $bla_{\text{SHV-12}}$ genes (Table 1).

The percentages of resistance to non-β-lactam antibiotics detected in the series of 24 *E. coli* isolates harbouring ESBLs were as follows: nalidixic acid, 75%; ciprofloxacin, 50%; tetracycline, 71%; trimethoprim/sulfamethoxazole, 63%; streptomycin, 67%; kanamycin, 25%; gentamicin, 13%; tobramycin, 8%; amikacin, 4%; and chloramphenicol, 17%.

A CTX-M-32-harbouring *E. coli* strain, recovered from one urine sample, has been detected in this study. This ESBL was first described by Cartelle *et al.*[4] from a human clinical *E. coli* strain and, very recently, has been also found in one animal *E. coli* strain,[7] in both cases in Spain. CTX-M-32 differs from CTX-M-1 through a single amino acid substitution (Asp-240→Gly), which confers by itself hydrolytic activity against ceftazidime.[4] As a matter of fact, our $bla_{\text{CTX-M-32}}$-containing *E. coli* strain showed a relatively high MIC value of ceftazidime (4 mg/L).

It is interesting to underline the extended dissemination of $bla_{\text{CTX-M}}$ genes, of different groups, among ESBL-containing *E. coli* strains of our hospital (19 of the 24 ESBL producers, 79%), $bla_{\text{CTX-M-14}}$ being the most frequent one (14 of the 19 isolates, 74%). CTX-M-9-group β-lactamases, such as CTX-M-14 or CTX-M-9, have been increasingly found in human clinical *E. coli* isolates in different countries[1–3,5,6] and they begin to be the most frequently found ESBLs in Spain either in clinical or faecal human and animal *E. coli* isolates.[3,6,7]

Mechanisms of resistance in the ESBL-negative strains

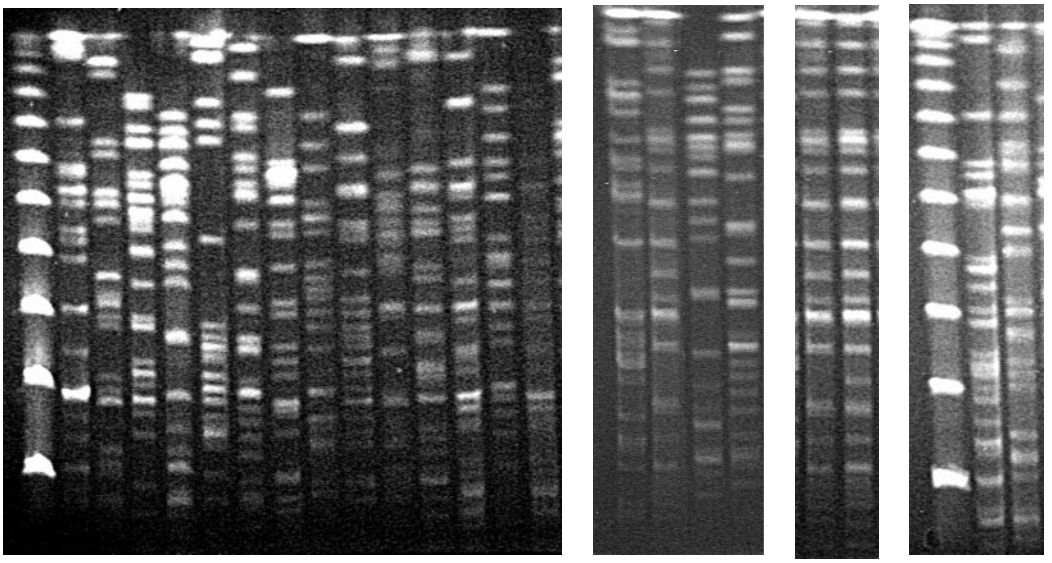
Ten of the 34 *E. coli* isolates of our series showed a negative result for the ESBL screen test, being resistant to amoxicillin/clavulanic acid (MIC 32 mg/L) and cefoxitin (16–256 mg/L). The $bla_{\text{CMY-2}}$ gene was found in two of these isolates, associated with either the $bla_{\text{TEM-1a}}$ or $bla_{\text{TEM-1b}}$ genes. No ESBLs or plasmidic cephalosporinases were detected in the remaining eight isolates. Nevertheless, mutations in the promoter and attenuator region of the *ampC* gene (at positions –42, –18, –1 and +58) were identified together in all these eight isolates (Table 1). It is known that specific mutations in the *ampC* promoter region render an increase in the MIC values of expanded-spectrum cephalosporins as well as of cephamycins.[9,10] In this sense, mutations at positions –42 (C→T) and at –18 (G→A) create new –35 and –10 boxes separated by 17 bp, the optimal distance to enhance the expression, resulting in the formation of a strong promoter.[9,10] Mutations at position –42 have not been detected in association with ESBLs either in this study or, to our knowledge, in others.

Mutations at positions +22, +26, +27 and +32 of the *ampC* attenuator region were found together in one isolate which harboured the $bla_{\text{CMY-2}}$ gene and mutations at positions –18, –1 and +58 were found in the other CMY-2-producing isolate (Table 1). The high MIC of cefoxitin for these two isolates could be explained by the expression of the $bla_{\text{CMY-2}}$ gene, although any effect due to the mutations in the promoter (position –18) or attenuator (positions +22, +26, +27 and +32) *ampC* region cannot be excluded.[9]

Table 1. Resistance mechanism detected and MIC values for the 34 expanded-spectrum cephalosporin-resistant *E. coli* isolates of this study

No. of *E. coli* strains (n = 34)	MIC ranges (mg/L)										Plasmidic β-lactamase gene detected	Mutations in *ampC* promoter/ attenuator at positions[a]
	AMP	TIC	AMC	CFZ	FOX	CAZ	CTX	CRO	IPM	ATM		
5	64 to >256	>256	2–4	128 to >256	1–4	0.5–16	32–128	32 to >128	≤0.06–0.125	2–256	$bla_{\text{CTX-M-14}}$	ND
2	64 to >256	>256	8	>256	4–16	0.5–8	16–256	128 to >256	0.125	2–32	$bla_{\text{CTX-M-14}}$	no mutations
2	>256	>256	8	>256	16	2	32–256	16–128	0.125	8	$bla_{\text{CTX-M-14}}$	–28, +58
4	>256	>256	4	>256	4–8	0.5–1	32 to >256	32–256	≤0.06–0.25	2–8	$bla_{\text{CTX-M-14}}$ + $bla_{\text{TEM-1b}}$	ND
1	>256	>256	4	>256	8	1	64	128	0.125	4	$bla_{\text{CTX-M-14}}$ + $bla_{\text{TEM-1c}}$	ND
4	>256	>256	4	>256	2–4	0.125–2	4–64	16–256	0.125	1–32	$bla_{\text{CTX-M-9}}$ + $bla_{\text{TEM-1b}}$	ND
1	>256	>256	2	>256	4	4	256	256	0.125	32	$bla_{\text{CTX-M-32}}$	–28, +58
1	>256	>256	4	32	4	128	8	4	0.06	128	$bla_{\text{SHV-12}}$ + $bla_{\text{TEM-1c}}$	ND
1	>256	>256	4	128	8	64	8	16	≤0.06	128	$bla_{\text{SHV-12}}$ + $bla_{\text{TEM-1b}}$	no mutations
1	>256	>256	4	32	4	32	2	8	0.125	32	$bla_{\text{SHV-12}}$ + $bla_{\text{TEM-1b}}$	–18, –1, +58
2	>256	>256	4	64–128	16	32	32	64–128	≤0.06	8–16	$bla_{\text{TEM-52c}}$	–18, –1, +58
1	>256	>256	32	>256	256	128	16	64	0.125	64	$bla_{\text{CMY-2}}$ + $bla_{\text{TEM-1a}}$	–18, –1, +58
1	>256	>256	32	256	64	16	8	4	0.125	2	$bla_{\text{CMY-2}}$ + $bla_{\text{TEM-1b}}$	+22, +26, +27, +32
4	128 to >256	16 to >256	32	16–128	32–64	4–8	1–2	0.25–1	0.125–0.25	2–8	no *bla* genes detected	–42, –18, –1, +58
3	>256	>256	32	16–64	16–32	4–16	0.25–4	0.25	0.125	1–4	$bla_{\text{TEM-1b}}$	–42, –18, –1, +58
1	>256	>256	32	128	64	8	2	0.5	0.06	4	$bla_{\text{TEM-1a}}$	–42, –18, –1, +58

AMP, ampicillin; TIC, ticarcillin; AMC, amoxicillin/clavulanic acid; CFZ, cefazolin; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; IPM, imipenem; ATM, aztreonam; ND, not determined.
[a]Mutations detected: –42 (C→T), –28 (G→A), –18 (G→A), –1 (C→T), +22 (C→T), +26 (T→G), +27 (A→T), +32 (G→A) and +58 (C→T).



(a) (b) (c) (d)

Figure 1. PFGE patterns of *Xba*I-digested total DNAs of *E. coli* isolates harbouring ESBL- or plasmidic cephalosporinase-encoding genes. (a) Lane 1, ladder marker; lanes 2–15, $bla_{\text{CTX-M-14}}$-containing isolates. (b) Lanes 1–4, $bla_{\text{CTX-M-9}}$-containing isolates. (c) Lanes 1 and 2, $bla_{\text{TEM-52}}$-containing isolates. (d) Lane 1, ladder marker; lanes 2 and 3, $bla_{\text{CMY-2}}$-containing isolates.

Mutations in the ampC promoter/attenuator region in the ESBL-producing strains

Mutations at positions –18, –1 and +58 were found together in three of our isolates that harboured ESBL genes ($bla_{\text{SHV-12}}$, one isolate; $bla_{\text{TEM-52}}$, two isolates). These isolates showed cefoxitin MIC values of 4–16 mg/L, lower than those presented by isolates harbouring a mutation at position –42 (16–64 mg/L). Caroff *et al.*[10] indicate that mutation at position –18, by creating a new –10 box, plays an important role in *ampC* expression. Our results suggest that further studies are necessary to determine the real role of this mutation.

Besides, mutations at positions –28 (G→A) and +58 (C→T) were also detected together in three isolates harbouring $bla_{\text{CTX-M-14}}$ or $bla_{\text{CTX-M-32}}$ genes (Table 1), which showed cefoxitin MIC values of 4–16 mg/L. Any association between a mutation at position –28 and overexpression of the *ampC* gene has not been previously demonstrated. Nevertheless, the nucleotide at this position could interact with RNA polymerase, due to its localization in the spacer, as suggested by Mulvey *et al.*[9]

Clonal relationship of ESBL-producing strains

No clonal relationship was observed among the 19 CTX-M-producing or the two CMY-producing *E. coli* strains when their PFGE patterns were compared after *Xba*I digestion. Nevertheless, an indistinguishable PFGE pattern was obtained for the two TEM-52-producing *E. coli* strains, both of them recovered from urine samples (Figure 1). These results suggest the existence of horizontal transfer of $bla_{\text{CTX-M}}$ genes rather than the dissemination of specific clones, as was also suggested by other authors.[1,3,6]

In summary, ∼1.5% of the *E. coli* isolates of our hospital harboured ESBLs, those of the CTX-M-9-group being the most common ones. Other resistance mechanisms to expanded-spectrum cephalosporins have been less frequently found, such as *ampC* overexpression or the presence of $bla_{\text{CMY-2}}$ genes. The evolution of these mechanisms of resistance should by monitored in the future.

Transparency declarations

No declarations were made by the authors of this paper.

References

1. Bonnet R. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. *Antimicrob Agents Chemother* 2004; **48**: 1–14.

2. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance treat. *Clin Microbiol Rev* 2001; **14**: 933–51.

3. Bou G, Cartelle M, Tomas M *et al.* Identification and broad dissemination of the CTX-M-14 β-lactamase in different *Escherichia coli* strains in the northwest area of Spain. *J Clin Microbiol* 2002; **40**: 4030–6.

4. Cartelle M, Tomás MM, Molina F *et al.* High-level resistance to ceftazidime conferred by a novel enzyme, CTX-M-32, derived from CTX-M-1 through a single Asp240-Gly substitution. *Antimicrob Agents Chemother* 2004; **48**: 2308–13.

5. Lartigue MF, Poirel L, Nordmann P. Diversity of genetic environment of $bla_{\text{CTX-M}}$ genes. *FEMS Microbiol Lett* 2004; **234**: 201–7.

6. Valverde A, Coque MT, Sánchez-Moreno MP *et al.* Dramatic increase in fecal carriage of extended-spectrum β-lactamase-producing *Enterobacteriaceae* during nonoutbreak situations in Spain. *J Clin Microbiol* 2004; **42**: 4769–75.

7. Briñas L, Moreno MA, Teshager T *et al.* Monitoring and characterization of extended-spectrum β-lactamases in *Escherichia coli* strains from healthy and sick animals in Spain in the year 2003. *Antimicrob Agents Chemother* 2005; **49**: 1262–4.

8. Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type β-lactamases. *Antimicrob Agents Chemother* 2002; **46**: 1–11.

9. Mulvey MR, Bryce E, Boyd DA *et al.* Molecular characterization of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. *Antimicrob Agents Chemother* 2005; **49**: 358–65.

10. Caroff N, Espaze E, Gautreau D *et al.* Analysis of the effects of –42 and –32 *ampC* promoter mutations in clinical isolates of *Escherichia coli* hyperproducing AmpC. *J Antimicrob Chemother* 2000; **45**: 783–8.