ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2005, p. 1262–1264 Vol. 49, No. 3
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.3.1262–1264.2005

Monitoring and Characterization of Extended-Spectrum β-Lactamases in *Escherichia coli* Strains from Healthy and Sick Animals in Spain in 2003

Laura Briñas,[1] Miguel Angel Moreno,[2] Tirushet Teshager,[2] Yolanda Sáenz,[1] María Concepción Porrero,[2] Lucas Domínguez,[2] and Carmen Torres[1]*

Área Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño,[1] and Departamento de Patología Animal I, Facultad de Veterinaria, Universidad Complutense, Madrid,[2] Spain

Received 4 June 2004/Returned for modification 25 August 2004/Accepted 3 November 2004

Genes encoding CTX-M-14, CTX-M-9, CTX-M-1, CTX-M-32, SHV-12, TEM-52, or CMY-2 β-lactamases were detected in 21 *Escherichia coli* strains recovered during 2003 from sick animals (11 of 459 [2.4%] strains) and healthy animals (10 of 158 [6.3%] strains) in Spain. Twelve of these strains harbored $bla_{\text{CTX-M}}$ genes and showed unrelated pulsed-field gel electrophoresis patterns.

Escherichia coli strains harboring extended-spectrum β-lactamases (ESBLs) or plasmidic class C β-lactamases have been arising as detected in humans (3, 5, 19) but have been rarely reported for animals (6, 7, 13, 22, 24–26). A new group of class A ESBLs, CTX-M enzymes, are increasingly being detected among human clinical *E. coli* strains in the last several years (3).

Since 1996, there has been a Veterinary-Antimicrobial-Resistance-Surveillance (VAV) Network in Spain focused on monitoring antibiotic resistance in bacteria of animal origin (16). The characterization of ESBLs and plasmidic class C β-lactamases in *E. coli* strains of sick and healthy animals recovered through the VAV Network during the periods 1997 to 2001 and 2000 to 2001, respectively, had been previously reported by our group (6, 7). The objective of the present work was to follow their evolution during the whole year of 2003. A total of 459 *E. coli* isolates from sick animals were submitted during 2003 to the VAV Network from eight public and private laboratories of animal health from all over Spain. No previous selection of the *E. coli* isolates according to their antimicrobial susceptibility was performed. Of the 459 *E. coli* isolates, 13 (2.8%) showed resistance or diminished susceptibility (drug MIC $\geq$ 2 μg/ml) to ceftazidime (CAZ) or cefotaxime (CTX), which were included in the study (Table 1). In addition, 160 fecal samples of healthy chickens (1 per farm) from nine Spanish slaughterhouses were obtained via the VAV Network during 2003. Fecal samples were seeded into non-antibiotic-supplemented MacConkey agar plates, and one *E. coli* isolate per sample was studied. A total of 158 *E. coli* isolates were recovered, and 13 of them (8.2%) showed resistance or diminished susceptibility to CAZ or CTX, which were also included in this study (Table 2).

Determination of the MICs of 10 β-lactams was carried out by the agar dilution method. Disks of CAZ and CTX supplemented or not supplemented with clavulanic acid were used to screen the ESBL production (17). The detection and characterization of genes encoding TEM (2), SHV (20), FOX (1), CTX-M (12, 18), and CMY (23) β-lactamases was carried out by PCR and sequencing. The regulatory region of the chromosomal *ampC* gene was amplified by PCR (8), sequenced, and compared with the same region in the *E. coli* K12 *ampC* gene (9, 15).

Characterization of β-lactamase genes in *E. coli* strains from sick animals. The characteristics of the 13 *E. coli* strains from sick animals with resistance or diminished susceptibility to broad-spectrum cephalosporins are shown in Table 1. ESBL production was detected by the screening method for 10 of these strains.

The presence of genes encoding ESBLs or plasmidic class C β-lactamases of the type TEM, SHV, CTX-M, or CMY in 11 of the 13 *E. coli* strains was demonstrated. The following combination of genes were found: $bla_{\text{CTX-M-14}}$ + $bla_{\text{TEM-1b}}$ (two strains), $bla_{\text{CTX-M-14}}$ + $bla_{\text{TEM-1c}}$ (one strain), $bla_{\text{CTX-M-14}}$ + $bla_{\text{TEM-1e}}$ (one strain), $bla_{\text{CTX-M-1}}$ + $bla_{\text{TEM-1b}}$ (one strain), $bla_{\text{CTX-M-9}}$ (one strain), $bla_{\text{CTX-M-32}}$ (one strain), $bla_{\text{SHV-12}}$ + $bla_{\text{TEM-1b}}$ (one strain), $bla_{\text{SHV-12}}$ (one strain), $bla_{\text{TEM-52b}}$ (one strain), $bla_{\text{CMY-2}}$ + $bla_{\text{TEM-1b}}$ (one strain), and none (two strains).

As mentioned above, one of our ESBL-producing strains (BAD159), recovered from a lung sample of a sick pig, harbored the $bla_{\text{CTX-M-32}}$ gene, whose sequence was very recently found in one human clinical *E. coli* strain in Spain (10). The CTX-M-32 β-lactamase shows an Asp240-Gly amino acid substitution with respect to CTX-M-1 that seems to be associated with the increase in the level of resistance to CAZ (10). As a matter of fact, the results obtained with our $bla_{\text{CTX-M-32}}$-containing *E. coli* strain showed a high CAZ MIC (32 μg/ml) as well as high CTX and ceftriaxone (both $\geq$256 μg/ml) and aztreonam (>64 μg/ml) MICs.

The specific C→T mutation at position −42 of the promoter-attenuator region of *ampC* chromosomal gene was only detected in the two strains in which no plasmidic β-lactamase genes were found (both strains showed resistance to amoxicillin-clavulanic acid and cefoxitin) (Table 1). Mutations at positions −18, −1, and +58 were also detected in these two strains

* Corresponding author. Mailing address: Área Bioquímica y Biología Molecular, Universidad de La Rioja, Madre de Dios 51, 26006 Logroño, Spain. Phone: 34 941 299750. Fax: 34 941 299721. E-mail: carmen.torres@daa.unirioja.es.

TABLE 1. MIC values of different β-lactams and β-lactamase genes detected in the 13 *E. coli* isolates obtained from sick animals with diminished susceptibility or resistance to broad-spectrum cephalosporins

E. coli isolate	Animal (sample)	MIC (μg/ml)[a]										ESBL test result[b]	Plasmidic β-lactamase genes detected	Mutations in the promoter-attenuator of the *ampC* gene
		AMP	TIC	AMC	CFZ	FOX	CAZ	CTX	CRO	IMP	ATM			
TER008	Pig (gut)	>256	>128	4	>128	8	1	32	128	0.125	4	+	bla_{TEM-1c} + $bla_{CTX-M-14}$	−18, −1, +58
BAR037	Pig (liver)	>256	>128	4	>128	4	1	32	64	0.125	2	+	bla_{TEM-1b} + $bla_{CTX-M-14}$	No mutations
TER007	Pig (gut)	>256	>128	8	>128	8	1	128	>256	0.125	4	+	bla_{TEM-1b} + $bla_{CTX-M-14}$	−18, −1, +58
AND014	Poultry (lung)	>256	>128	8	>128	8	1	128	256	≤0.06	4	+	bla_{TEM-1e} + $bla_{CTX-M-14}$	−18, −1, +58
MAD156	Poultry (yolk)	>256	>128	8	>128	4	≤0.5	8	32	0.125	2	+	$bla_{CTX-M-9}$	−18, −1, +58
BAD159	Pig (lung)	>256	>128	4	>128	8	32	256	>256	0.125	>64	+	$bla_{CTX-M-32}$	No mutations
BAR045	Cattle (milk)	>256	>128	8	>128	8	2	128	>256	0.125	16	+	bla_{TEM-1b} + $bla_{CTX-M-I}$	No mutations
BUR048	Rabbit (lung)	>256	>128	8	32	8	4	32	32	≤0.06	4	+	$bla_{TEM-52b}$	−18, −1, +58
COR029	Pig (liver)	>256	>128	8	32	8	16	2	2	0.125	16	+	bla_{TEM-1b} + bla_{SHV-12}	−18, −1, +58
A32/03/Hg	Poultry (liver)	>256	>128	2	32	4	16	4	2	≤0.06	32	+	bla_{SHV-12}	−18, −1, +58
TAR004	Partridge (gut)	>256	>128	64	>128	128	64	64	64	0.06	16	−	bla_{TEM-1b} + bla_{CMY-2}	−18, −1, +58
LUG699	Cattle (feces)	>256	>128	64	32	32	4	2	1	0.125	4	−	No *bla* genes detected	−42, −18, −1, +58
LUG737	Pig (gut)	128	64	32	128	64	4	4	4	0.06	4	−	No *bla* genes detected	−42, −18, −1, +58

[a] AMP, ampicillin; TIC, ticarcillin; AMC, amoxicillin-clavulanic acid; CFZ, cefazolin; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; IMP, imipenem; ATM, aztreonam.
[b] +, positive; −, negative.

and in other eight of this series. According to previous reports (9), the mutations at position −42 or −32 are associated with overexpression of the *ampC* gene in *E. coli*. Nevertheless, other mutations such as those at positions −18, −1 (attenuator region), and +58 are frequently found in β-lactam-susceptible *E. coli* strains (8).

It is interesting to underline that 8 of the 10 ESBL-harboring *E. coli* strains were recovered from pigs or poultry. In addition, 11 of the 13 strains, especially those of the CTX-M type (7 of these strains), presented an ESBL or a plasmidic class C β-lactamase as the mechanism of resistance. In our previous work carried out with *E. coli* strains from sick animals recovered during the period 1997 to 2001, ESBL or plasmidic class C β-lactamases were detected in a small proportion of strains and mutations in the promoter of *ampC* gene were the main mechanism of broad-spectrum cephalosporin resistance (6).

Characterization of β-lactamase genes in *E. coli* strains of healthy animals. The characteristics of the 13 *E. coli* strains from healthy animals with diminished susceptibility or resistance to broad-spectrum cephalosporins are shown in Table 2. ESBL production in eight of these strains was detected by the screening method.

ESBL or plasmidic class C β-lactamase genes were detected in 10 of these 13 *E. coli* strains and in 5 of them were combined with the bla_{TEM-1b} gene (Table 2). The genes found were the following: $bla_{CTX-M-14}$ (four strains, in one case associated with bla_{TEM-1b} gene), bla_{SHV-12} (three strains, in two cases associated with bla_{TEM-1b} gene), $bla_{CTX-M-9}$ (one strain, associated with bla_{TEM-1b} gene) and bla_{CMY-2} (two strains, in one case associated with bla_{TEM-1b} gene).

The remaining three *E. coli* strains showed CTX and CAZ MICs in the range of 1 to 2 μg/ml and harbored the specific mutation (C→T) at position −42 in the promoter region of the *ampC* gene related to its overexpression (in one of the cases associated with the bla_{TEM-1b} gene) (Table 2).

Genes encoding CMY-2, CTX-M-14, and SHV-12 β-lactamases were previously found by our group in fecal samples obtained during the period 2000 to 2001 from healthy chickens (7), and the presence of ESBL or plasmidic class C β-lactamase genes was demonstrated in 2.5% of those strains at that

TABLE 2. MIC values of different β-lactams and β-lactamase genes detected in the 13 *E. coli* strains obtained from fecal samples of healthy chickens with diminished susceptibility or resistance to broad-spectrum cephalosporins

E. coli isolate	MIC (μg/ml)										ESBL test result[b]	Plasmidic β-lactamase gene(s) detected	Mutations in the promoter-attenuator of the *ampC* gene
	AMP	TIC	AMC	CFZ	FOX	CAZ	CTX	CRO	IMP	ATM			
A3SAEC 269–271	>256	>128	4	>128	8	1	64	128	0.125	2	+	bla_{TEM-1b} + $bla_{CTX-M-14}$	No mutations
A3SZEC 377–379	>256	>128	4	>128	4	1	64	128	0.125	2	+	$bla_{CTX-M-14}$	No mutations
A3SZEC 443–445	>256	>128	8	>128	4	≤0.5	32	128	≤0.06	2	+	$bla_{CTX-M-14}$	No mutations
A3YEEC 209–211	>256	>128	4	>128	4	1	64	128	≤0.06	4	+	$bla_{CTX-M-14}$	−18, −1, +58
A3SZEC 431–433	>256	>128	8	>128	4	≤0.5	8	16	0.125	2	+	bla_{TEM-1b} + $bla_{CTX-M-9}$	−28, +58
A3AGEC 425–427	>256	>128	8	32	4	16	4	4	0.125	32	+	bla_{TEM-1b} + bla_{SHV-12}	No mutations
A3SZEC 368–370	>256	>128	8	32	8	16	4	4	0.125	32	+	bla_{TEM-1b} + bla_{SHV-12}	+22, +26, +27, +32
A3LEEC 064–066	>256	>128	4	32	8	32	8	8	0.125	32	+	bla_{SHV-12}	No mutations
A3YEEC 296–298	>256	>128	32	>128	64	8	8	16	0.125	8	−	bla_{TEM-1b} + bla_{CMY-2}	No mutations
A3AAEC 031–033	256	64	64	>128	64	2	8	16	0.125	4	−	bla_{CMY-2}	No mutations
A3SAEC 239–241	>256	>128	32	32	16	2	1	0.5	0.125	2	−	bla_{TEM-1b}	−42, −18, −1, +58
A3AVEC 404–406	256	32	64	32	16	2	2	0.5	0.125	2	−	No *bla* genes detected	−42, −18, −1, +58
A3COEC 551–553	128	32	32	16	16	2	1	0.5	0.125	2	−	No *bla* genes detected	−42, −18, −1, +58

[a] AMP: ampicillin; TIC: ticarcillin; AMC: amoxicillin-clavulanic acid; CFZ: cefazolin; FOX: cefoxitin; CAZ: ceftazidime; CTX: cefotaxime; CRO: ceftriaxone; IMP: imipenem; ATM: aztreonam.
[b] +, positive; −, negative.

time. In the present work, the ratio of this type of genes was higher (6.3%) and the diversity of β-lactamase genes, including the $bla_{CTX\text{-}M\text{-}9}$ gene, was also wider. This ratio is especially high when we consider that no antibiotic-supplemented medium was used for the analysis of the animal fecal samples.

Clonal diversity of *E. coli strains*. All eight *E. coli* strains from sick and healthy animals which carried the $bla_{CTX\text{-}M\text{-}14}$ gene showed unrelated pulsed-field gel electrophoresis patterns with the XbaI enzyme (14). Similarly, no clonal relationship was detected among strains containing $bla_{CTX\text{-}M\text{-}9}$ (two strains), $bla_{SHV\text{-}12}$ (four strains), or $bla_{CMY\text{-}2}$ (three strains) genes.

It is interesting to underline the broad dissemination of $bla_{CTX\text{-}M}$ genes of different groups among *E. coli* strains of sick and healthy animals, with $bla_{CTX\text{-}M\text{-}14}$ being the most frequently detected. This specific β-lactamase gene has been increasingly found in human clinical *E. coli* isolates in different countries, including Spain, in the last several years (3, 4, 11, 21). The factors that could have contributed to the dissemination of $bla_{CTX\text{-}M}$ genes among animal *E. coli* strains are unknown but could be associated with a potential coselection with other antibiotics due to the possible association of the resistance determinants in the same genetic mobile elements.

An increase in the percentage and variety of ESBL-producing *E. coli* strains in healthy and sick animals in this study (year 2003) compared to the results of previous studies (before 2002) carried out by our group has been observed. Monitoring ESBL-producing *E. coli* strains, including both clinical and commensal strains, should be continued in the human and animal fields to investigate their evolution and to analyze the factors that contribute to their selection and spread.

This work has been supported in part by grants FIS01/973 and AGL2002/02637. The VAV work was supported by the Ministery of Agriculture, Fish and Food of Spain.

We acknowledge Fernanda Ruiz-Larrea and Myriam Zarazaga for reviewing the manuscript.

REFERENCES

1. **Bauernfeind, A., S. Wagner, R. Jungwirth, I. Schneider, and D. Meyer.** 1997. A novel class C β-lactamase (FOX-2) in *Escherichia coli* conferring resistance to cephamycins. Antimicrob. Agents Chemother. **41:**2041–2046.
2. **Belaaouaj, A., C. Lapoumeroulie, M. M. Caniça, G. Vedel, P. Névot, R. Krishnamoorthy, and G. Paul.** 1994. Nucleotide sequences of the genes coding for the TEM-like β-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). FEMS Microbiol. Lett. **120:**75–80.
3. **Bonnet, R.** 2004. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. **48:**1–14.
4. **Bou, G., M. Carelle, M. Tomas, D. Canle, F. Molina, R. Moure, J. M. Eiros, and A. Guerrero.** 2002. 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. **40:**4030–4036.
5. **Bradford, P. A.** 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance treat. Clin. Microbiol. Rev. **14:**933–951.
6. **Briñas, L. M. A. Moreno, T. Teshager, M. Zarazaga, Y. Sáenz, C. Porrero, L. Domínguez, and C. Torres.** 2003. β-lactamase characterization in *Escherichia coli* isolates with diminished susceptibility or resistance to extended-spectrum cephalosporins recovered from sick animals in Spain. Microb. Drug Resist. **9:**201–208.
7. **Briñas, L. M. A. Moreno, M. Zarazaga, C. Porrero, Y. Saénz, M. García, L. Domínguez, and C. Torres.** 2003. Detection of CMY-2, CTX-M-14, and SHV-12 β-lactamases in *Escherichia coli* fecal-sample isolates from healthy chickens. Antimicrob. Agents Chemother. **47:**2056–2058.
8. **Caroff, N., E. Espaze, I. Bérard, H. Richet, and A. Reynaud.** 1999. Mutations in the *ampC* promoter of *Escherichia coli* isolates resistant to oxyiminocephalosporins without extended spectrum β-lactamase production. FEMS Microbiol. Lett. **173:**459–465.
9. **Caroff, N., E. Espaze, D. Gautreau, H. Richet, and A. Reynaud.** 2000. Analysis of the effects of −42 and −32 *ampC* promoter in clinical isolates of *Escherichia coli* hyperproducing AmpC. J. Antimicrob. Chemother. **45:**783–788.
10. **Cartelle, M., M. M. Mar, F. Molina, R. Moure, R. Villanueva, and G. Bou.** 2004. High-level resistance to ceftazidime conferred by a unique enzyme, CTX-M-32, derived from CTX-M-1 through a single Asp240-Gly substitution. Antimicrob. Agents Chemother. **48:**2308–2313.
11. **Chanawong, A., F. H. M'Zali, J. Heritage, J.-H. Xiong, and P. M. Hawkey.** 2002. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. Antimicrob. Agents Chemother. **46:**630–637.
12. **Coque, T. M., A. Oliver, J. C. Pérez-Díaz, F. Baquero, and R. Cantón.** 2002. Genes encoding TEM-4, SHV-2 and CTX-M-10 extended-spectrum β-lactamases are carried by multiple *Klebsiella pneumoniae* clones in a single hospital (Madrid, 1989 to 2000). Antimicrob. Agents Chemother. **46:**500–510.
13. **Costa, D., P. Poeta, L. Brinas, Y. Saenz, J. Rodrigues, and C. Torres.** 2004. Detection of CTX-M-1 and TEM-52 β-lactamases in *Escherichia coli* strains from healthy pets in Portugal. J. Antimicrob. Chemother. **54:**960–961.
14. **Gautom, R. K.** 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. J. Clin. Microbiol. **35:**281–294.
15. **Jaurin, B., T. Grundström, T. Edlund, and S. Normak.** 1981. The *E. coli* β-lactamase attenuator mediates growth-dependent regulation. Nature **290:**221–225.
16. **Moreno, M. A., L. Dominguez, T. Teshager, I. A. Herrero, and M. C. Porrero.** 2000. Antibiotic resistance monitoring: the Spanish programme. The VAV Network. Red de Vigilancia de Resistencias Antibióticas en Bacterias de Origen Veterinario. Int. J. Antimicrob. Agents **14:**285–290.
17. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. NCCLS document M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
18. **Pagani, L., E. Dell'Amico, R. Migliavacca, M. M. D'Andrea, E. Giacobone, G Amicosante, E. Romero, and G. M. Rossolini.** 2003. Multiple CTX-M-type extended-spectrum β-lactamases in nosocomial isolates of *Enterobacteriaceae* from a hospital in northern Italy. J. Clin. Microbiol. **41:**4264–4269.
19. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46:**1–11.
20. **Pitout, J. D., K. S. Thomson, N. D. Hanson, A. F. Ehrhardt, E. S. Moland, and C. C. Sanders.** 1998. β-lactamases responsible for resistance to expanded-spectrum cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* isolates recovered in South Africa. Antimicrob. Agents Chemother. **42:**1350–1354.
21. **Saladin, M., V. T. B. Cao, T. Lambert, J. L. Donay, J. L. Herrmann, Z. Ould-Hocine, C. Verdet, F. Delisle, A. Philippon, and G. Arlet.** 2002. Diversity of CTX-M β-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS Microb. Lett. **209:**161–168.
22. **Shiraki, Y., N. Shibata, Y. Doi, and Y. Arakawa.** 2004. *Escherichia coli* producing CTX-M-2 β-lactamase in cattle, Japan. Emerg. Infect. Dis. **10:**69–75.
23. **Stapleton, P. D., K. P. Shannon, and G. L. French.** 1999. Carbapenem resistance in *Escherichia coli* associated with plasmid-determined CMY-4 β-lactamase production and loss of an outer membrane protein. Antimicrob. Agents Chemother. **43:**1206–1210.
24. **Teshager, T., L. Dominguez, M. A. Moreno, Y. Saénz, M. Zarazaga, C. Torres, and S. Cardeñosa.** 2000. Isolation of an SHV-12 β-lactamase-producing *Escherichia coli* strain from a dog with recurrent urinary tract infections. Antimicrob. Agents Chemother. **44:**3483–3484.
25. **Winokur, P. L., D. L. Vonstein, L. J. Hoffman, E. K. Uhlenhopp, and G. V. Doern.** 2001. Evidence for transfer of CMY-2 AmpC β-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. **45:**2716–2722.
26. **Zhao, S., D. G. White, P. F. McDermott, S. Friedman, L. English, S. Ayers, J. Meng, J. J. Maurer, R. Holland, and R. D. Walker.** 2001. Identification and expression of cephamycinase bla_{CMY} genes in *Escherichia coli* and *Salmonella* isolates from food, animals, and ground meat. Antimicrob. Agents Chemother. **45:**3647–3650.