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Nationwide Study of *Escherichia coli* and *Klebsiella pneumoniae* Producing Extended-Spectrum β-Lactamases in Spain

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Clonal dissemination of extended-spectrum β-lactamases (ESBL) in 170 *Escherichia coli* isolates and 70 *Klebsiella pneumoniae* isolates from a nationwide study of 40 Spanish centers in 2000 was not observed in most centers. The most prevalent ESBL were CTX-M-9 (27.3%), SHV-12 (23.9%), and CTX-M-14 (20.5%) for *E. coli* and TEM-3 (16.7%) and TEM-4 (25%) for *K. pneumoniae*. A new ESBL, TEM-133, with mutations L21F, E104K, and R164S, was identified.

The production of β-lactamases is the most relevant resistance mechanism against β-lactam antimicrobials in gram-negative organisms. Extended-spectrum β-lactamases (ESBL) of the TEM-, SHV-, OXA-, and, more recently, CTX-M-type enzymes have been described in many countries, including Spain (5, 6, 16).

The first ESBL-producing strain described in Spain was isolated in 1988, with several local ESBL outbreaks reported in Madrid (1, 5, 6) and Barcelona (14) since then. A nationwide epidemiological study was conducted in our country in 2000, revealing that the prevalences of ESBL-producing *Klebsiella pneumoniae* and *Escherichia coli* strains were 2.7% and 0.5%, respectively (8). Now we describe the clonal relationship and the susceptibility to antimicrobial agents of ESBL-producing *E. coli* and *K. pneumoniae* strains isolated in this study and also describe the molecular characterization of ESBL produced by these strains.

Bacterial isolates. Two hundred and forty clinical isolates (170 *E. coli* isolates and 70 *K. pneumoniae* isolates) corresponding to the GEIH-BLEE 2000 Project were included in the study (8). In this project, the prevalences of ESBL-producing *E. coli* and *K. pneumoniae* strains were evaluated over a period of 4 months. A significant number of *E. coli* isolates (51%) were derived from nonhospitalized patients. The organisms were identified to the species level by using the API 20E system (bioMérieux, Marcy-l'Étoile, France). ESBL production was confirmed by broth microdilution according to NCCLS guidelines (12).

Antimicrobial susceptibility testing. Broth microdilution assays were conducted with Mueller-Hinton broth according to NCCLS guidelines (12). The following antimicrobial agents were obtained from Sigma-Aldrich (Madrid, Spain): amoxicillin, cefotaxime, ceftazidime, cefoxitin, piperacillin, amikacin, gentamicin, tobramycin, ciprofloxacin, and co-trimoxazole. The following antimicrobial agents were obtained from their respective manufacturers: cefepime and cefpodoxime (Aventis Pharma, Madrid, Spain), aztreonam (Bristol-Myers-Squibb, Madrid, Spain), cefotetan and meropenem (AstraZeneca, Madrid, Spain), imipenem (Merck, Sharp & Dohme, Madrid, Spain), tazobactam (Wyeth-Lederle, Madrid, Spain), clavulanic acid (GSK, Madrid, Spain), and ticarcillin (GSK). *E. coli* ATCC 25922 and ATCC 35218, *K. pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 were used as control strains.

Conjugation experiments. Conjugation experiments were carried out by a broth mating method. *E. coli* BM21 and *E. coli* J53-AzR were used as recipients for mating experiments with ESBL-producing strains susceptible to rifampin and azide, respectively. Plates containing ceftazidime (1 μg/ml) or cefotaxime (2 μg/ml) and rifampin (100 μg/ml), when using the *E. coli* BM21 recipient, or azide (200 μg/ml), when using the *E. coli* J53-AzR recipient, were used to select transconjugants (5).

β-Lactamase characterization. β-Lactamases were characterized by isoelectric focusing (IEF) as previously described (3). Clonality was assessed by repetitive extragenic palindromic (REP)-PCR (3, 15). Isolates showing more than two different bands after electrophoresis of the PCR product and ethidium bromide staining were considered not clonally related. ESBL-encoding genes were characterized by PCR as described pre-

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TABLE 1. In vitro activity of several antimicrobial agents against ESBL-producing *E. coli* and *K. pneumoniae* isolates

Agent	*E. coli*				*K. pneumoniae*			
	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%S[a]	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%S
Cefotaxime	0.06–>64	>64	>64	0	≤0.03–>64	32	>64	0
Ceftazidime	0.5–>64	16	>64	0	2–>64	32	>64	0
Cefepime	≤0.03–>64	8	>64	0	≤0.03–>64	2	>64	0
Aztreonam	0.125–>64	8	>64	0	0.125–>64	8	>64	0
Cefpodoxime	0.5–512	128	256	0	≤0.25–512	64	256	0
Cefoxitin	1–256	4	16	76.5	1–256	4	8	94
Cefotetan	≤0.25–32	1	2	98	≤0.25–32	≤0.25	0.5	98.5
Amoxicillin-clavulanate (2:1)	2/1–128/64	8/4	32/16	69	4/2–128/64	16/8	32/16	40
Ticarcillin-clavulanate (2 μg/ml)	≤0.5–>1,024	128	>1,024	13	≤0.5–>1,024	256	>1,024	7
Piperacillin-tazobactam (4 μg/ml)	≤0.5–>1,024	2	128	85	≤0.5–>1,024	4	>1,024	74
Imipenem	≤0.06–1	0.125	0.25	100	≤0.06–0.5	0.125	0.25	100
Meropenem	≤0.06–0.25	≤0.06	≤0.06	100	≤0.06–0.125	≤0.06	≤0.06	100
Amikacin	0.5–128	2	16	93.5	0.5–64	1	16	91
Gentamicin	0.125–>128	1	128	66	0.5–>128	32	>128	33
Tobramycin	0.25–>128	1	64	65	0.25–128	8	32	38.5
Ciprofloxacin	≤0.06–>128	4	128	37.5	≤0.06–64	0.125	2	88.5
Co-trimoxazole	≤4.75/0.25–>608/32	304/16	>608/32	25	≥4.75/0.25–>608/32	19/1	608/32	40

[a] %S, percent susceptibility.

viously, using specific primers for TEM, SHV, CTX-M-1, and CTX-M-9 groups (6, 15). PCR products were purified with the Sephaglas BandPrep (Amersham Pharmacia Biotech, Uppsala, Sweden) purification kit for direct sequencing. ESBL gene sequences were developed with an ABI PRISM 377 sequencer (Applied Biosystems, Perkin-Elmer, Foster City, CA).

Nucleotide sequence accession number. The sequence of the novel β-lactamase, TEM-133, has been deposited in GenBank and assigned the accession number AY528425.

ESBL production was confirmed in 240 isolates (170 *E. coli* isolates and 70 *K. pneumoniae* isolates). As shown in Table 1, all isolates were susceptible to imipenem and meropenem. The most active β-lactam/β-lactamase inhibitor combination was piperacillin-tazobactam (85% and 74% susceptible *E. coli* and *K. pneumoniae* isolates, respectively).

One hundred thirty-seven and 26 different REP-PCR patterns were obtained for *E. coli* and *K. pneumoniae*, respectively. In 37 of 40 hospitals, ESBL-producing *E. coli* isolates were clonally unrelated. In three centers, more than one *E. coli* isolate (*n*, 2 to 14) presented the same REP-PCR pattern. For *K. pneumoniae*, the number of REP-PCR patterns per hospital ranged from one to four. Four hospitals had only one *K. pneumoniae* REP-PCR pattern with more than eight isolates each. The other patterns included only one or two isolates. Due to the diversity of REP-PCR patterns, all *E. coli* isolates were analyzed further. Based on REP-PCR and susceptibility patterns, 49 *K. pneumoniae* isolates were subsequently studied.

ESBL-encoding genes were transferable in 73.7% and 73.0% of *E. coli* and *K. pneumoniae* isolates, respectively. TEM-type β-lactamases (IEF bands ranging from pI 5.4 to 6.5) were detected in 132 *E. coli* isolates and 38 *K. pneumoniae* isolates; SHV-type enzymes were detected (IEF bands from pI 7.0 to 8.2) in 64 *E. coli* isolates and 51 *K. pneumoniae* isolates; and 85 *E. coli* isolates and 5 *K. pneumoniae* isolates had IEF bands with pIs of 8 to 8.1 (Table 2). These last isolates had a cefotaxime MIC greater than that of ceftazidime (consistent with a CTX-M-type β-lactamase). When two or more IEF bands were present, those with a pI of 5.4 were assumed to be TEM-1, and those with a pI of 7.6 in *K. pneumoniae* were assumed to be SHV-1.

SHV-encoding genes were identified in 94% of *K. pneumoniae* isolates. ESBL-encoding genes were sequenced from 91 *E. coli* isolates and 26 *K. pneumoniae* isolates, based on pI values and antimicrobial susceptibility profiles. In selected *E. coli* isolates, TEM-type ESBL were identified as TEM-3, TEM-4, TEM-10, TEM-20, TEM-24, TEM-26, TEM-28, TEM-29, TEM-52, and TEM-116, and in *K. pneumoniae* strains, TEM-type ESBL were identified as TEM-3, TEM-4, and TEM-25. Three *E. coli* isolates and one *K. pneumoniae* isolate had only one pI 5.4 IEF band, subsequently identified as TEM-1. Twelve clonally related *K. pneumoniae* isolates recovered from the same hospital and with a pI 5.6 IEF band had the following mutations in their ESBL-encoding gene sequence based on TEM-1: L21F, E104K, and R164S. This new enzyme, found only in Tenerife (the Canary Islands), has been designated TEM-133 (Table 3). Sequencing of SHV-type ESBL-encoding genes in selected *E. coli* isolates yielded SHV-2 and SHV-12, and it yielded SHV-2, SHV-2a, SHV-5, and SHV-12 in *K. pneumoniae* isolates (Table 3). One *K. pneumoniae* isolate had a single pI 7.6 IEF band, subsequently identified as SHV-1. CTX-M-type ESBL-encoding genes sequenced in *E. coli* strains corresponded to CTX-M-9, CTX-M-10, and CTX-M-14. In *K. pneumoniae* isolates, CTX-M-10 was the only CTX-M-type ESBL produced (Table 3). The most prevalent ESBL

TABLE 2. IEF bands expressed by *E. coli* and *K. pneumoniae* isolates

pI	No. of indicated isolates (total) with indicated band	
	E. coli (170)	*K. pneumoniae* (49)
5.4	111	16
5.6	6	3
5.9	6	11
6.3	8	8
6.5	1	0
7.0	7	9
7.6	17	35
8.0	25	0
8.1	60	5
8.2	40	7

TABLE 3. TEM-, SHV-, and CTX-M-type ESBL detected in *E. coli* (n = 88) and *K. pneumoniae* (n = 24) isolates

ESBL type	ESBL (no. of isolates) in indicated strain	
	E. coli	*K. pneumoniae*
TEM	TEM-3 (3) TEM-4 (2) TEM-10 (2) TEM-20 (1) TEM-24 (1) TEM-26 (1) TEM-28 (2) TEM-29 (1) TEM-52 (2) TEM-116 (2)	TEM-3 (4) TEM-4 (6) TEM-25 (2) TEM-133 (2)
SHV	SHV-2 (4) SHV-12 (21)	SHV-2 (2) SHV-2a (1) SHV-5 (1) SHV-12 (3)
CTX-M	CTX-M-9 (24) CTX-M-10 (4) CTX-M-14 (18)	CTX-M-10 (3)

in selected *E. coli* isolates were CTX-M-9 (24 isolates; 27.3%), SHV-12 (21 isolates; 23.9%), and CTX-M-14 (18 isolates; 20.5%). The geographical distribution of ESBL in Spain is shown in Fig. 1. In the strains producing either a single TEM-1 or SHV-1 enzyme, the phenotype consistent with ESBL production could be caused by either an ESBL of another family with the same pI or other mechanisms as previously described (11, 18).

In the first nationwide study of clinical isolates of ESBL-producing *E. coli* and *K. pneumoniae* carried out in Spain, the prevalences of ESBL-producing *E. coli* and *K. pneumoniae* isolates in 40 Spanish hospitals were 0.5% and 2.7%, respectively (8). Other studies developed in Italy and France described similar results for *E. coli* isolates (1.2% and 0.2%, respectively) but higher values for *K. pneumoniae* isolates (20% and 9.4%, respectively) (7, 17).

The molecular study of the selected strains in this study revealed a highly diverse population structure with a low clonal relationship among ESBL-producing *E. coli* strains (170 strains/137 clones), even in those isolated within the same institution. Of the 70 *K. pneumoniae* isolates, 26 different REP-PCR patterns were obtained. In some hospitals, all the isolates were clonally related, indicating that in Spain, as has been described in other countries, ESBL-producing *K. pneumoniae* isolates are frequently involved in outbreaks (1, 4, 9, 14).

The most active antimicrobial agents against ESBL-producing *E. coli* and *K. pneumoniae* isolates were carbapenems (100% susceptible) and amikacin. Among the β-lactam/β-lactamase inhibitor combinations, piperacillin-tazobactam was the most active agent against these microorganisms. The concurrence of ciprofloxacin resistance with ESBL production, particularly in isolates of *K. pneumoniae*, was also observed in this study. The actual causes of this association are not well known but may be related not only to target mutations in DNA gyrase or topoisomerase IV but also to other mechanisms,

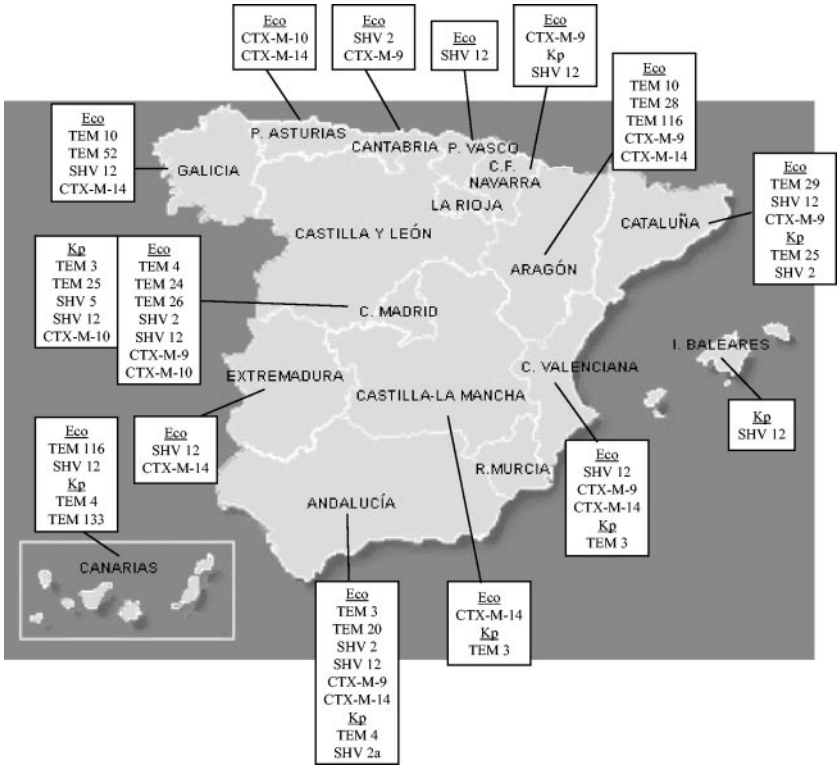

FIG. 1. Distribution of ESBL described in this study. Eco, *Escherichia coli*; Kp, *Klebsiella pneumoniae*.

including porin loss, active efflux, and target protection (2, 10, 13).

Among ESBL-producing *E. coli* isolates, there was a great variety of TEM-type ESBL, with TEM-3 being the most prevalent. In addition to TEM-3 and TEM-25 being detected in some *K. pneumoniae* isolates, this is the first report of TEM-10, TEM-20, TEM-26, TEM-28, TEM-52, and TEM-116 in Spain. SHV-12 was the most prevalent SHV-type ESBL expressed by *E. coli* isolates (16 of 33 hospitals). CTX-M-type ESBL, an emerging group of Ambler class A plasmidic β-lactamases, were the most prevalent *E. coli* ESBL isolated in Spain (52.3% of total sequenced ESBL; 23 of 40 hospitals), with 91% belonging to the CTX-M-9 group (52% CTX-M-9 and 39% CTX-M-14). CTX-M-9 and CTX-M-14 were encountered in several regions of Spain, whereas CTX-M-10 was found mostly in the central region of Spain.

In conclusion, the great diversity of ESBL and the prevalences of clinical isolates of *E. coli* and *K. pneumoniae* producing these enzymes indicate that this is an important problem in Spain. Microbiology laboratories need to be alert to the correct identification and control of infections caused by such microorganisms.

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