

Four Main Virotypes among Extended-Spectrum- β -Lactamase-Producing Isolates of *Escherichia coli* O25b:H4-B2-ST131: Bacterial, Epidemiological, and Clinical Characteristics

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A total of 1,021 extended-spectrum- β -lactamase-producing *Escherichia coli* (ESBLEC) isolates obtained in 2006 during a Spanish national survey conducted in 44 hospitals were analyzed for the presence of the O25b:H4-B2-ST131 (sequence type 131) clonal group. Overall, 195 (19%) O25b-ST131 isolates were detected, with prevalence rates ranging from 0% to 52% per hospital. Molecular characterization of 130 representative O25b-ST131 isolates showed that 96 (74%) were positive for CTX-M-15, 15 (12%) for CTX-M-14, 9 (7%) for SHV-12, 6 (5%) for CTX-M-9, 5 (4%) for CTX-M-32, and 1 (0.7%) each for CTX-M-3 and the new ESBL enzyme CTX-M-103. The 130 O25b-ST131 isolates exhibited relatively high virulence scores (mean, 14.4 virulence genes). Although the virulence profiles of the O25b-ST131 isolates were fairly homogeneous, they could be classified into four main virotypes based on the presence or absence of four distinctive virulence genes: virotype A (22%) (*afa* FM955459 positive, *iroN* negative, *ibeA* negative, *sat* positive or negative), B (31%) (*afa* FM955459 negative, *iroN* positive, *ibeA* negative, *sat* positive or negative), C (32%) (*afa* FM955459 negative, *iroN* negative, *ibeA* negative, *sat* positive), and D (13%) (*afa* FM955459 negative, *iroN* positive or negative, *ibeA* positive, *sat* positive or negative). The four virotypes were also identified in other countries, with virotype C being overrepresented internationally. Correspondingly, an analysis of XbaI macrorestriction profiles revealed four major clusters, which were largely virotype specific. Certain epidemiological and clinical features corresponded with the virotype. Statistically significant virotype-specific associations included, for virotype B, older age and a lower frequency of infection (versus colonization), for virotype C, a higher frequency of infection, and for virotype D, younger age and community-acquired infections. In isolates of the O25b:H4-B2-ST131 clonal group, these findings uniquely define four main virotypes, which are internationally distributed, correspond with pulsed-field gel electrophoresis (PFGE) profiles, and exhibit distinctive clinical-epidemiological associations.

In recent years, extended-spectrum β -lactamase (ESBL) production in *Enterobacteriaceae*, particularly *Escherichia coli*, has significantly increased in many countries, including Spain (1). In 2000, the first nationwide study of ESBL-producing *E. coli* (ESBLEC) in Spain was developed (GEIH-BLEE-2000) (2). The overall prevalence of ESBL production was 0.5%, with CTX-M-9, SHV-12, and CTX-M-14 predominating. No CTX-M-15-producing *E. coli* isolates were detected. In contrast, in 2006, a similarly designed nationwide study (GEIH-BLEE-2006) showed that in just 6 years, the prevalence of ESBLEC in Spain had increased 8-fold, to 4% (3, 4). In the 2006 study, it was found that CTX-M-15 had joined CTX-M-14, SHV-12, and CTX-M-9 as a prevalent ESBL type. Thus, the predominant ESBL type had changed quickly, primarily due to the introduction and dissemination of a single clonal group characterized by CTX-M-15, serotype O25b:H4, phylogenetic group B2, and sequence type 131 (ST131), i.e., the international O25b:H4-B2-ST131 clonal group (5–9).

Unlike most other antimicrobial-resistant *E. coli* isolates, most of which derive from non-B2 phylogenetic groups (i.e., A, B1, and D) but are similar to other group B2 clonal groups, O25b:H4-B2-

ST131 *E. coli* isolates typically exhibit multiple virulence factors, including adhesins, toxins, siderophores, and group 2 capsules. Thus, this clonal group combines both resistance and virulence genes, which in classical extraintestinal pathogenic *E. coli* (ExPEC) isolates have been mutually exclusive (6, 10–14).

In the report of the GEIH-BLEE-2006 study isolates (4), only the 37 CTX-M-15-positive isolates were screened for O25b-ST131 status, and all but five corresponded to this clonal group; the vir-

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ulence genotypes of the O25b-ST131 isolates were not assessed. Here, we screened all 1,021 ESBL-EC isolates from the GEIH-BLEE-2006 study for O25b-ST131 status and investigated a subset of the detected O25b-ST131 isolates for virulence genotypes and pulsed-field gel electrophoresis (PFGE) profiles. The main objective was to characterize the virulence profile diversity of the ESBL-producing *E. coli* human O25b-ST131 isolates from Spain, thereby identifying prominent virotypes. Secondarily, we screened for these virotypes among ST131 isolates from other countries and sought to find associations between individual virotypes and epidemiological and clinical features.

MATERIALS AND METHODS

Bacterial isolates. Forty-four hospitals representing all Spanish regions participated in the GEIH-BLEE-2006 project. During the study period (1 February to 30 March 2006), 1,021 ESBL isolates were obtained from clinical samples (4). Species identification was performed with the API 20E system (bioMérieux, Marcy l'Etoile, France). ESBL production was confirmed by broth microdilution according to the CLSI guidelines (15).

For comparison, 52 international O25b:H4-B2-ST131 isolates (50 of human and 2 of avian origin), representing eight countries and three continents and taken from the reference collections of Nicolas-Chanoine et al. (6) and Johnson et al. (16), were analyzed to detect globally spread clonal variants in Spain. These isolates included the most prevalent ST131-associated XbaI pulsotypes (800, 812, 905, and 968) and others (699, 788, 797, 806, 903, 904, 909, 910, 911, 913, 915, 916, 917, 919, 979, 981, 1160, 1201, and 1202), as previously described by Johnson et al. (16).

Identification of O25b:H4-B2-ST131 isolates: serotyping, phylogenetic grouping, and multilocus sequence typing. The 1,021 Spanish study isolates were screened using a triplex PCR that was previously developed for the presumptive identification of O25b-ST131 isolates (5). Presumptive O25b-ST131 isolates were confirmed as O25:H4 by serotyping, using type-specific O and H antisera. The major *E. coli* phylogenetic group was determined by triplex PCR (17). Multilocus sequencing typing (MLST) relied on a sequence analysis of seven housekeeping genes (*adhA*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) according to the protocol and primers that are specified on the *E. coli* MLST website (<http://mlst.ucc.ie/mlst/dbs/Ecoli>) (18).

Antibiotic susceptibility testing and molecular characterization of resistance mechanisms. MICs were determined by the MicroScan Walk-Away automated system (Siemens, Spain), used according to the manufacturer's instructions, and were interpreted as specified by the CLSI (15). ESBL production was screened for by using cephalosporin resistance and the double-disk synergy test. ESBL genotype was determined by PCR using published TEM-, SHV-, CTX-M-1-, and CTX-M-9-group-specific primers (19). The resistance genes *bla*_{OXA-1} and *aac*(6')-Ib-cr were detected by PCR screening and bidirectional sequencing of amplicons (10). The genetic environment of *bla*_{CTX-M-15} was investigated by a specific PCR for upstream insertion elements (*ISEcp1* and *IS26*) in isolates of clone O25b-ST131 (20).

Virulence genotyping. The detection of 40 ExPEC-associated virulence genes was done by multiplex PCR (21, 22). The genes we sought to detect included *fimH*, *fimA*_{MT78}, F10 *papA*, *papA*, *papC*, *papEF* (positive isolates were tested for the *papG I*, *papG II*, and *papG III* alleles), *sfa/focDE* (positive isolates were tested for *sfaS* and *focG*), *afa/draBC*, *afa* operon FM955459, *iha*, *bmaE*, *gafD*, *sat*, *cdtB*, *cnf1*, *hlyA*, *iucD*, *iutA*, *iroN*, *fyuA*, *chuA*, *kpsM II* (with its *neuC-K1*, *K2*, and *K5* variants), *kpsMT III*, *cvaC*, *iss*, *traT*, *ibeA*, *malX*, *usp*, *tsh*, and *ompT* (Table 1). Isolates were classified as ExPEC if they carried ≥ 2 of *papEF* (P fimbriae), *sfa/focDE* (S/F1C fimbriae), *afa/draBC* (Afa/Dr adhesins), *iutA* (aerobactin receptor), and *kpsM II* (group 2 capsule synthesis) (23). The virulence score was the number of virulence genes that were detected in an isolate. PCR screening of the *fimB* insertion sequence (ISL3-like transposase) was performed as described elsewhere (24). Investigation of the *fim* operon sequence in the

E. coli EC958 ST131 strain revealed a 1,895-bp insertion in the *fimB* gene, which encodes the FimB recombinase that switches on the expression of type 1 fimbriae (24).

***fimH* subtyping.** The *fimH*_{TR} allele was identified based on sequence variation in the *E. coli* type-1 fimbrial adhesion gene (positions 64 to 552). Amplification and sequencing were performed as previously described (25).

Pulsed-field gel electrophoresis. XbaI PFGE analysis was performed as previously described (21). Profiles were analyzed with the BioNumerics fingerprinting software (Applied Maths, St-Martens-Latem, Belgium). Cluster analysis of the Dice similarity indices based on the unweighted-pair group method using average linkages (UPGMA) was done to generate a dendrogram describing the relationship among the PFGE profiles.

Epidemiological and clinical features. Epidemiological and clinical features were collected by using a structured questionnaire based on the following data: age, sex, health care environment, underlying conditions, invasive procedures performed during the preceding week, antimicrobial use during the preceding month, whether the isolate represented colonization or infection (and, if infection, the type of infection), and outcome. Isolates were classified as nosocomially acquired (NA) if obtained 48 h after hospital admission, as health care-associated (HCA) if the patient had been admitted to an acute or long-term care center or had received hemodialysis, specialized home care, or day hospital care during the preceding 3 months, and as community-acquired (CA) if none of these applied. The ethics committee of each participating center approved the study.

Statistical analysis. Comparisons of proportions and scores were tested using Fisher's exact test, chi-square test, and the Mann-Whitney U test, as appropriate. The criterion for statistical significance was set at a P value of <0.05.

Nucleotide sequence accession number. The sequence for the CTX-M-103 gene was deposited in EMBL under accession number [HG423149](https://www.ebi.ac.uk/EMBL/nuccore/HG423149).

RESULTS

Prevalence and distribution of the O25b:H4-B2-ST131 clonal group. According to PCR-based detection, the *E. coli* O25b-ST131 clonal group accounted for 195 (19%) of the 1,021 ESBL-EC isolates from the Spanish GEIH-BLEE-2006 project. The O25b-ST131 isolates were widely distributed across Spain and were recovered from 30 of the 44 participating hospitals, including in 13 of the 17 autonomous communities. By hospital, the prevalence of ST131 among the local ESBL study isolates ranged from 0% to 52% (see Table S1 in the supplemental material), with the highest values observed in Madrid and the Canary Islands (Fig. 1).

ESBL enzymes produced by O25b:H4-B2-ST131 isolates. From the 195 total O25b-ST131 isolates, 130 (a maximum of 10 per hospital) were selected randomly for further characterization. Of these, 96 (74%) were positive for CTX-M-15, 15 (12%) for CTX-M-14, 9 (7%) for SHV-12, 6 (5%) for CTX-M-9, and 5 (4%) for CTX-M-32, with the remaining two isolates being positive, respectively, for CTX-M-3 and a new ESBL enzyme, CTX-M-103, which was first detected in this study. CTX-M-103 (EMBL accession no. HG423149) has a single amino acid change compared to CTX-M-15 and CTX-M-3, and it may be grouped in cluster CTX-M-1 (26). Three O25b-ST131 isolates from three different hospitals in the Galicia autonomous community exhibited both CTX-M-14 and CTX-M-15 (Fig. 1; see also Table S1 in the supplemental material).

Virulence genes and virotypes of O25b:H4-B2-ST131 isolates. Of the 40 studied ExPEC-associated virulence genes, 13 were detected by PCR in a majority of the 130 selected O25b-ST131 isolates (Table 1). These 13 genes included *fimH* (in 99%), F10 *papA* (77%), *iha* (75%), *sat* (76%), *iucD* (93%), *iutA* (94%), *fyuA*

TABLE 1 Comparison of virulence gene prevalences of 130 representatives of O25b:H4-B2-ST131 ESBLEC isolates in relation to their virotypes

Virulence gene	Isolates in virotype: ^a				O25b-ST131 isolates	<i>P</i> ^b
	A	B	C	D		
Total no.	29	40	41	17	130	
Adhesins (no. [%])						
<i>fimH</i>	29 (100)	40 (100)	40 (98)	17 (100)	129 (99)	
ISL3-like in <i>fimB</i>	29 (100)	40 (100)	41 (100)	0	110 (85)	<0.001
<i>fimA</i> _{MT78}	0	0	0	1 (6)	1 (1)	
F10 <i>papA</i>	27 (93)	31 (78)	41 (100)	0	100 (77)	<0.001
<i>papA</i>	0	0	0	12 (71)	12 (9)	<0.001
<i>papC</i>	0	0	2 (5)	9 (53)	11 (8)	<0.001
<i>papEF</i>	0	0	0	12 (71)	12 (9)	<0.001
<i>papG I</i>	0	0	0	0	0	
<i>papG II</i>	0	0	0	0	0	
<i>papG III</i>	0	0	0	12 (71)	12 (9)	<0.001
<i>sfa/focDE</i>	0	0	0	0	0	
<i>sfaS</i>	0	0	0	0	0	
<i>focG</i>	0	0	0	0	0	
<i>afal/draBC</i>	29 (100)	0	1 (2)	0	30 (23)	<0.001
<i>afa</i> FM955459	29 (100)	0	0	0	29 (22)	<0.001
<i>iha</i>	27 (93)	29 (73)	41 (100)	0	98 (75)	<0.001
<i>bmaE</i>	0	0	0	0	0	
<i>gafD</i>	0	0	0	0	0	
Toxins (no. [%])						
<i>sat</i>	28 (97)	30 (75)	41 (100)	0	99 (76)	<0.001
<i>cdtB</i>	0	0	0	5 (29)	5 (4)	<0.001
<i>cnf1</i>	0	0	0	10 (59)	10 (8)	<0.001
<i>hlyA</i>	0	0	0	10 (59)	10 (8)	<0.001
Siderophores (no. [%])						
<i>iucD</i>	27 (93)	39 (98)	41 (100)	13 (76)	121 (93)	
<i>iutA</i>	28 (97)	40 (100)	41 (100)	13 (76)	122 (94)	
<i>iroN</i>	0	40 (100)	0	13 (76)	53 (41)	<0.001
<i>fyuA</i>	29 (100)	40 (100)	41 (100)	17 (100)	130 (100)	
<i>chuA</i>	29 (100)	40 (100)	41 (100)	17 (100)	130 (100)	
Capsula (no. [%])						
<i>kpsM II</i>	29 (100)	31 (78)	28 (68)	17 (100)	106 (82)	
<i>kpsM II-K2</i>	29 (100)	0	0	0	29 (22)	<0.001
<i>kpsM II-K5</i>	0	31 (78)	28 (68)	15 (88)	75 (58)	<0.001
<i>neuC-K1</i>	0	0	0	2 (12)	2 (2)	
<i>kpsM III</i>	0	0	0	0	0	
Miscellaneous (no. [%])						
<i>cvaC</i>	0	1 (3)	0	9 (53)	10 (1)	<0.001
<i>iss</i>	0	39 (98)	0	16 (94)	55 (42)	<0.001
<i>traT</i>	21 (72)	21 (53)	36 (88)	16 (94)	96 (74)	
<i>ibeA</i>	0	0	0	17 (100)	17 (13)	<0.001
<i>malX</i> (PAI)	29 (100)	40 (100)	41 (100)	17 (100)	130 (100)	
<i>usp</i>	29 (100)	40 (100)	41 (100)	17 (100)	130 (100)	
<i>tsh</i>	0	0	0	0	0	
<i>ompT</i>	29 (100)	40 (100)	41 (100)	17 (100)	130 (100)	
ExPEC status (no. [%])	29 (100)	31 (78)	28 (68)	17 (100)	106 (82)	
Range of virulence genes (no.)	10–15	11–17	12–14	9–20	9–20	NA
Mean no. of virulence genes	14.4	14.5	13.6	16.8	14.4	NA

^a Significant differences are indicated in bold.^b *P* values (by Fisher's exact test) are shown where there was a *P* value of <0.05. NA, not applicable.

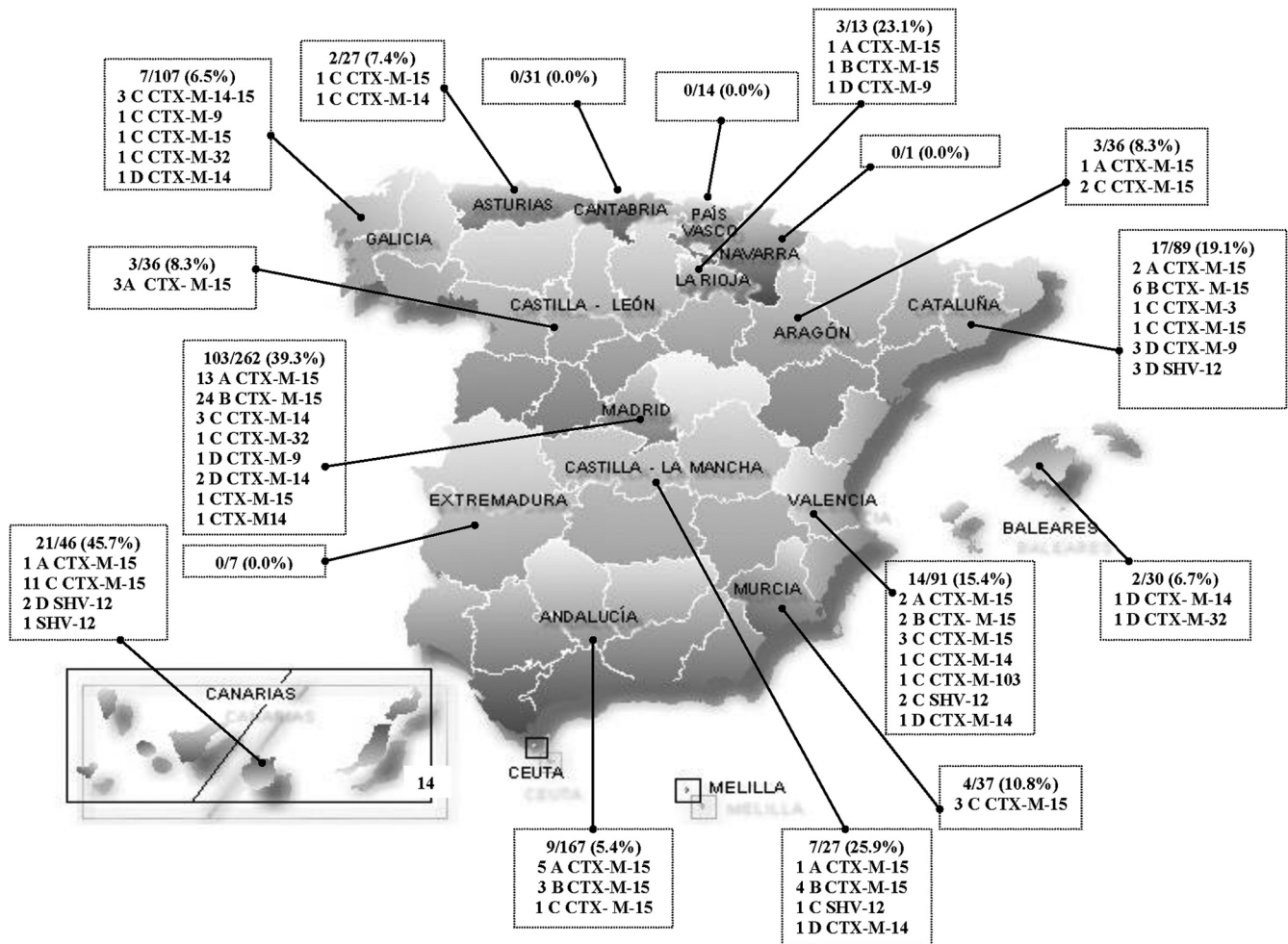


FIG 1 Distribution of O25b-ST131 isolates in Spain. The O25b-ST131 isolates were widely distributed across Spain, being recovered from 30 of the 44 participant hospitals, including 13 of the 17 autonomous communities of Spain.

(100%), *chuA* (100%), *kpsM II* (82%), *traT* (74%), *malX* (100%), *usp* (100%), and *ompT* (100%). In contrast, nine virulence genes were not detected in any O25b-ST131 isolate; these included *papG I*, *papG II*, *sfa/focDE*, *sfaS*, *focG*, *bmaE*, *gafD*, *kpsM III*, and *tsh*. Overall, the 130 O25b-ST131 isolates exhibited relatively high aggregate virulence scores (mean, 14.4; range, 9 to 20), and 106 (82%) satisfied the molecular criteria for ExPEC.

Although the virulence profiles were fairly homogeneous overall, four discrete virotypes (labeled arbitrarily as A, B, C, and D) were resolved based on the presence or absence of four distinctive virulence genes, including *afa* FM955459 (specific for an ST131 clone encoding an Afa/Dr adhesin), *iroN* (catechol siderophore receptor), *ibeA* (invasion of brain endothelium), and *sat* (secreted autotransporter toxin). The patterns were as follows: virotype A (*afa* FM955459 positive, *iroN* negative, *ibeA* negative, *sat* positive or negative), virotype B (*afa* FM955459 negative, *iroN* positive, *ibeA* negative, *sat* positive or negative), virotype C (*afa* FM955459 negative, *iroN* negative, *ibeA* negative, *sat* positive), and virotype D (*afa* FM955459 negative, *iroN* positive or negative, *ibeA* positive, *sat* positive or negative). The 130 isolates were distributed among the four virotypes as follows: A, 29 (22%), B, 40 (31%), C, 41 (32%), and D, 17 (13%), with three isolates remaining unclassified as to virotype since they lacked all four virotype-defining genes.

Interestingly, the 17 virotype D isolates exhibited significantly higher virulence scores than did those of other virotypes (mean, 16.8 for D versus 14.4 for A, 14.5 for B, and 13.6 for C) (Table 1).

All four virotypes identified for the Spanish O25b:H4-B2-ST131 isolates were also represented in 51 of the 52 studied international O25b:H4-B2-ST131 isolates. Virotype C was by far the most prevalent (65%), followed by virotypes A (17%), B (10%), and D (6%).

Insertion of ISL3-like transposase gene in *fimB* in relation to virotype. The 130 selected Spanish O25b-ST131 isolates were also analyzed for an insertion in *fimB*, similar to the ISL3 transposase gene observed in *E. coli* strain Nissle 1917 (GenBank accession no. AF188737). The *fimB* insertion was detected in all 113 isolates of virotypes A, B, and C but in none of the 17 virotype D isolates ($P < 0.001$ for all comparisons versus virotype D) (Table 1).

***fimH* alleles and O25b:H4-B2-ST131 virotypes.** *fimH* sequence analysis was done for 40 of the Spanish O25b-ST131 isolates, including 10 each for the four virotypes, which were selected to include a representation of all virulence gene profiles and PFGE groups encountered within a particular virotype. Two different *fimH* alleles were identified among these 40 isolates. All representatives of virotypes A, B, and C contained *fimH30*, whereas all

TABLE 2 ESBL enzymes, genes associated with CTX-M-15 plasmids, and antimicrobial resistance of 130 representatives of O25b:H4-B2-ST131 ESBLECs in relation to their virotypes

Characteristic	No. (%) of isolates by virotype ^a :				No. (%) of O25b-ST131 isolates
	A	B	C	D	
Total no.	29	40	41	17	130
ESBL enzymes					
CTX-M-3	0	0	1 (2)	0	1 (0.8)
CTX-M-9	0	0	1 (2)	5 (29)	6 (5)
CTX-M-14	0	0	8 (20)	6 (35)	15 (12)
CTX-M-15	29 (100)	40 (100)	26 (63)	0	96 (74)
CTX-M-32	0	0	4 (10)	1 (6)	5 (4)
CTX-M-103	0	0	1 (2)	0	1(0.8)
SHV-12	0	0	3 (7)	5 (29)	9 (7)
Genes associated with CTX-M-15					
<i>bla</i> _{OXA-1}	20 (69)	35 (88)	21 (51)	0	85 (65)
<i>aac(6′)-Ib-cr</i>	29 (100)	35 (88)	21 (51)	0	85 (65)
IS26 element	29 (100)	0	0	0	30 (23)
Antimicrobial resistance					
Ciprofloxacin	29 (100)	40 (100)	40 (98)	5 (29)	117 (90)
Gentamicin	0	27 (68)	16 (39)	1 (6)	46(35)
Tobramycin	29 (100)	33 (83)	28 (68)	3 (18)	95 (73)
Trimethoprim-sulfamethoxazole	29 (100)	31 (78)	20 (50)	10 (59)	92 (71)
Amoxicillin-clavulanic acid	10 (34)	6 (15)	9 (22)	0	26 (20)
Fosfomycin	5 (17)	3 (8)	2 (5)	0	11 (9)

^a Significant differences are indicated in bold. *P* values are shown in Results.

representatives of virotype D contained *fimH22* ($P < 0.001$ for all comparisons versus virotype D).

Virotype versus ESBL variants, CTX-M-15-associated genes, and antimicrobial resistance. Among the 130 selected O25b:H4-B2-ST131 isolates, all 69 isolates from virotypes A and B and 63% (26/41) of the virotype C isolates produced CTX-M-15; however, no virotype D isolates produced CTX-M-15 ($P < 0.001$ for all comparisons versus virotype D). In contrast, virotype D isolates produced CTX-M-9 (5 isolates), CTX-M-14 (6 isolates), SHV-12 (5 isolates), and CTX-M-32 (1 isolate) (Table 2).

As expected, the *bla*_{OXA-1} and *aac(6′)-Ib-cr* genes were associated with CTX-M-15-producing isolates. These genes were detected in 69% and 100% of virotype A isolates, respectively, 88% (both genes) of virotype B isolates, and 51% (both genes) of virotype C isolates but in 0% of virotype D isolates ($P < 0.001$ for all comparisons versus virotype D) (Table 2).

Only the 29 virotype A isolates showed an IS26 element within the terminal inverted repeat of the *ISEcp1*-like element upstream of *bla*_{CTX-M-15}, separating the *bla*_{CTX-M-15} allele from its usual promoter, as found in the epidemic ST131 “strain A” that is prevalent in the United Kingdom (5, 20) ($P < 0.001$ for comparisons with virotypes B, C, and D) (Table 2).

Different patterns of associated antimicrobial resistances were detected in relation to virotype. Specifically, ciprofloxacin and tobramycin resistance were significantly associated with virotypes A, B, and C ($P < 0.001$ for all comparisons versus virotype D). In contrast, virotypes B and C were significantly associated with gentamicin resistance ($P < 0.05$ for all comparisons versus virotypes A and D), and virotype A was significantly associated with trimethoprim-sulfamethoxazole resistance ($P < 0.05$ for all comparisons versus virotypes B, C, and D) (Table 2).

PFGE profiles of O25b:H4-B2-ST131 isolates in relation to virotype. In the PFGE-based dendrogram, 116 of the 130 Spanish O25b-ST131 isolates were distributed among 4 large virotype-specific clusters, defined at similarity levels of approximately 72% (11 virotype D isolates), 76% (28 virotype A isolates), 77% (33 virotype C isolates), and 74% (39 virotype B isolates) (see Fig. S1 in the supplemental material).

A similar clustering of PFGE profiles by virotype was detected when the 130 Spanish study isolates were compared with the 52 international isolates (see Fig. S2 in the supplemental material). Four main clusters, each largely virotype specific, comprised 162 of the 182 total isolates. Each of these clusters, defined at similarity levels of approximately 80% (47 isolates, 40 of virotype B), 78% (60 isolates, 58 of virotype C), 82% (37 isolates, 36 of virotype A), and 67% (18 isolates, 16 of virotype D), included isolates from multiple countries.

In the present study, among 13 international isolates representing the top four pulsotypes described by Johnson et al. (16), we found that virotype A corresponded with pulsotype 812, virotype B with pulsotype 905, and virotype C with all four pulsotypes (968, 800, 905, and 812), whereas none of the virotype D isolates belonged to these top four pulsotypes.

Epidemiological and clinical associations of ESBLECs O25b-ST131. Associated clinical data were compared for O25b-ST131 and non-O25b-ST131 Spanish ESBLECs isolates (Table 3). In univariate analyses, compared with the non-O25b-ST131 isolates, the O25b-ST131 isolates were significantly associated with older age, nursing home residents, a health care-related origin, asymptomatic bacteriuria, and bacteremia. However, after adjustment for acquisition type and age by logistic regression analysis, the association of O25b-ST131 isolates with bacteremia was not statistically

TABLE 3 Epidemiological and clinical data of patients with colonization or infection due to ESBLEC O25b:H4-B2-ST131 compared to those with non-ST131 infection

Patient variable	Value for isolate ^a :		P ^c
	ST131	Non-ST131	
Total no.	190	818	
Male gender (no. [%])	70 (37)	328 (40)	
Median age (IQR) (yr)	75 (68–84)	69 (50–79)	0.01 ^b
No. (%) of pediatric patients (age ≤ 14 yr)	4 (2)	53 (6)	0.01 ^c
Acquisition type (no. [%])			
Nosocomial	66 (35)	238 (29)	
Health care related	47 (25)	115 (14)	<0.001
Community	77 (41)	465 (57)	<0.001
Nursing home resident	37 (19)	39 (5)	<0.001
Infection site (no. [%]) ^d			
Urinary tract	136 (72)	651 (80)	0.02
Soft tissue	102 (75)	485 (75)	
Digestive tract	16 (12)	71 (11)	
Other types	6 (4)	37 (6)	
Bacteremia (primary or secondary)	20 (15)	116 (18)	
Bacteremia (primary or secondary)	22 (16)	56 (9)	0.01
Crude 30-day mortality (no. [%]) ^d	5 (4)	12 (2)	

^a Data from 5 patients with ST131 and from 8 patients with non-ST131 isolates were unavailable. P values were calculated by chi-square test except where otherwise noted.

^b Calculated by Mann-Whitney U test.

^c Calculated by Fisher's exact test.

^d Only patients with infection were considered.

^e P values are shown where P < 0.05.

significant (odds ratio [OR], 1.58; 95% confidence interval [CI], 0.91 to 2.75) (P = 0.10).

Associations of the four virotypes with demographic data, acquisition type, and type of infection also were explored (Table 4). Virotype B was significantly associated with older patients and a lower likelihood of symptomatic infection, specifically urinary tract infection, but a higher likelihood of respiratory tract infection. In contrast, virotype C was significantly associated with a higher likelihood of symptomatic infection, whereas virotype D was significantly associated with younger patients and community acquisition. Additionally, virotypes A and B were more highly associated with nursing home residents (17/69 [25%]) than were virotypes C and D (4/61 [7%]) (P = 0.005).

Triplex PCR for virotyping. A triplex PCR based on the detection of *sat*, *iroN*, and *ibeA* was designed for virotyping of the O25b:H4-B2-ST131 isolates. When applied to the present 130 Spanish and 52 international O25b-ST131 isolates, this assay exhibited 100% specificity and sensitivity (not shown). This assay can be combined with a previously described triplex PCR (based on detection of the *afa* operon FM955459, the O25b *rfb* allele, and the 3' end of *bla*_{CTX-M-15}) to establish the virotypes of the O25b-ST131 isolates (Table 5; see also Fig. S3 in the supplemental material).

DISCUSSION

The prevalence and epidemiology of ESBLEC are changing rapidly. In recent years, ESBL production in *E. coli* has increased significantly,

TABLE 4 Association of virotype with epidemiological and clinical data among 130 O25b:H4-B2-ST131 ESBLEC isolates

Patient variable	Isolates by virotype ^a :			
	A	B	C	D
Total no.	29	40	41	17
Male gender (no. [%])	10 (35)	16 (40)	22 (54)	7 (41)
Age ≤ 14 yr (no. [%])	1 (3)	0	1 (2)	2 (12)
Median age (IQR) (yr)	77 (63–82)	75 (64–83) ^a	64 (56–79)	60 (34–78) ^b
Acquisition type (no. [%])				
Nosocomial	9 (31)	18 (45)	18 (44)	4 (24)
Health care related	9 (31)	9 (23)	7 (17)	1 (6)
Community	11 (38)	13 (32)	16 (39)	12 (71) ^c
Nursing home resident	7 (24)	10 (25)	3 (7)	1 (6)
Infection type	22 (85)	21 (57) ^d	36 (92) ^e	13 (77)
Urinary tract	20 (69)	13 (33) ^f	25 (61)	13 (77)
Respiratory tract	0	4 (10) ^g	1 (2)	0
Digestive tract	0	0	2 (5)	0
Primary bacteremia	0	2 (5)	3 (7)	0
Other types	2 (7)	2 (5)	5 (12)	0
Bacteremia (primary or secondary)	5 (17)	8 (20)	8 (15)	0
Crude 30-day mortality (no. [%])	0	6 (15)	4 (10)	1 (6)
Infection-related deaths (no. [%])	0	3 (8)	1 (2)	0

^a P = 0.05 (calculated by Mann-Whitney U test). All comparisons other than those indicated by a footnote have a P value of >0.05.

^b P = 0.03 (calculated by Mann-Whitney U test).

^c P = 0.03 (calculated by Fisher's exact test).

^d P = 0.009.

^e P = 0.01.

^f P = 0.0001.

^g P = 0.03 (calculated by Fisher's exact test).

due primarily to the spread of CTX-M types. In fact, the prevalence of ESBLEC strains in Spain increased 8-fold between 2000 and 2006, from 0.5% to 4% (4). The emergence and dissemination of ESBLEC have two possible explanations, that it occurs through dissemination of mobile genetic elements between non-clonally related strains or through clonal spread. The two mechanisms might occur simultaneously, thereby contributing to the rapid dissemination of ESBLEC strains. Until a few years ago, most ESBLEC strains were clonally unrelated; however, recently, the O25b:H4-B2-ST131 intercontinental *E. coli* clonal group that produces CTX-M-15 with high virulence potential has been reported worldwide, representing a major public health problem (6, 10).

In the present study, 195 (19%) of the 1,021 total ESBLEC study isolates were of the O25b-ST131 group. The O25b-ST131 isolates were widely distributed across Spain, as they were at 30 of the 44 participating centers and accounted for up to 52% of ESBLEC strains per hospital. This contrasts with the 9% prevalence of ST131 among the 92 analyzed ESBLEC isolates from a similar study from 2004 done in 11 Spanish hospitals (7 of which were included in the present study) (8). More recent studies found even higher prevalences of O25b-ST131 among ESBLECs in various Spanish cities, including Lugo in 2008 (23%) (5) and in 2012 (61%) (27), Barcelona (32% in 2008) (28), Seville (13% in 2010) (29), and Madrid (21% in 2008) (30). Other countries have also had high reported prevalences of ST131 among the ESBLEC strains, including Denmark (38%) (31), Japan (41%) (32), the United States (47%) (33), and Canada (78%) (34).

The Spanish O25b-ST131 isolates in this study carried not only CTX-M-15 but also CTX-M-14, SHV-12, CTX-M-9, CTX-M-32,

TABLE 5 Primers included in the two triplex PCR assays used for specific identification of clonal group O25b:H4-B2-ST131 and virotyping

Gene	Primer	Oligonucleotide sequence (5' to 3')	Fragment size (bp)	Annealing temp (°C)	Reference
<i>afa</i> FM955459 ^a	AFA-O25F	GAGTCACGGCAGTCGCGGCGG	207	55	5
	AFA-O25R	TTCACCGGCGACCAGCCATCTCC			
<i>rfbO25b</i> ^a	rfb.1bis	ATACCGACGACGCCGATCTG	300	55	45
	rfbO25b.r	TGCTATTCATTATGCGCAGC			
<i>bla</i> _{CTX-M-15} ^a	CTX-M-F1	ATAAAACCGGCAGCGGTG	483	55	19
	CTX-M-F2	GAATTTTGACGATCGGGG			
<i>iroN</i> ^b	IRON-F	AAGTCAAAGCAGGGGTTGCCCG	667	60	46
	IRON-R	GACGCCGACATTAAGACGCAG			
<i>sat</i> ^b	SAT-F	ACTGGCGGACTCATGCTGT	387	60	47
	SAT-R	AACCCTGTAAGAAGACTGAGC			
<i>ibeA</i> ^b	IBEA 10F	AGGCAGGTGTGCGCCGCGTAC	170	60	22
	IBEA 10R	TGGTGCTCCGGCAAACCATGC			

^a Triplex PCR used for specific identification of O25b-ST131 isolates (5).

^b Triplex PCR used for virotyping of O25b-ST131 isolates (developed in this study).

CTX-M-3, and the new ESBL enzyme CTX-M-103 first described here. Although in most countries, ST131 is associated mainly with CTX-M-15 (6, 33–35), exceptions do occur, such as in Japan, where O25b-ST131 is frequently associated with CTX-M-14 (36), or in Ireland, where it is associated with CTX-M-3 (37). In the present study, we showed that association between *E. coli* O25b-ST131 and CTX-M types other than CTX-M-15 was mainly identified in the isolates with virotype D.

ExPEC isolates have specialized virulence factors enabling them to colonize host surfaces, injure host tissues, and avoid host defense systems. Thirteen genes (*fimH*, F10 *papA*, *iha*, *sat*, *iucD*, *iutA*, *fyuA*, *chuA*, *kpsM II*, *traT*, *malX*, *usp*, and *ompT*) were detected in most of the 130 Spanish O25b-ST131 ESBLEEC isolates in this study. In two recent studies, Coelho et al. (28) in Spain and Johnson et al. (38) in the United States found that these virulence genes were significantly more prevalent among ST131 than non-ST131 isolates. Therefore, they might be important in the worldwide dissemination of *E. coli* O25b-ST131. Moreover, the present 130 Spanish O25b-ST131 isolates exhibited high virulence scores, and most qualified molecularly as being ExPEC (23).

After analyzing all the virulence gene profiles together with the PFGE pulsotypes, we observed that the virotypes we established corresponded well with the PFGE clusters. Although ST131 isolates share a large set of putative virulence factors, we selected the four virulence genes (*afa* FM955459, *iroN*, *ibeA*, and *sat*) that clearly define the four virotypes within the clonal group O25b-ST131. Notably, the four virotypes, most prominently virotype C, were also present in other countries (France, Portugal, Switzerland, United States, Canada, Korea, and Lebanon). The XbaI PFGE dendrogram revealed four major clusters that corresponded closely with the virotypes, suggesting a clonal basis for the virotypes. Isolates of virotype D exhibited significantly higher virulence scores, which might explain the association of this virotype with younger patients and community-acquired infections.

Like Banerjee et al. (39), we found that patients with O25b-ST131 isolates were older and more frequently had health care-associated acquisition (particularly through nursing home resi-

dency) than those with non-O25b-ST131 ESBLEEC isolates. Although the O25b-ST131 isolates were associated with bacteremia in univariate analyses, this association was not significant when age and acquisition type were considered in multivariate analysis. Chung et al. (40) found that patients with clone ST131 were more likely to have secondary bacteremia than those with non-ST131 isolates. As has been found in other studies (39–41), we did not find that O25b-ST131 isolates were associated with increased mortality. Further studies are needed to more fully assess the clinical implications of the comparatively high virulence scores and the group B2 background of the O25b-ST131 ESBLEEC isolates. Of note, virotype D was linked with community-acquired infections and virotypes A and B with nursing home residents.

In a recent PFGE analysis of 579 ST131 isolates from diverse years, hosts, and locales, Johnson et al. (16) found that the four most prevalent pulsotypes (among 170 total) accounted for 46% of the population and tended to occur in more recent years, which is consistent with recent emergence and expansion and implies greater fitness of these pulsotypes. In the present study, among 13 international isolates representing the top four pulsotypes of Johnson et al. (16), we found virotypes A, B, and C.

Seven distinct *fimH*-based putative clonal lineages (H15, H22, H27, H30, H35, H41, and H49) were found among 352 historical and recent ST131 isolates obtained primarily from the United States (42). The H22 subclone was dominant (73%) among the historical isolates (those from 1967 to 1999), whereas the H30 subclone was the most prevalent (75%) among the current clinical isolates (those from 2000 to 2011) and accounted for nearly all fluoroquinolone-resistant isolates. Interestingly, in the present study, the H30 subclone accounted for all analyzed Spanish O25b-ST131 isolates of virotypes A, B, and C, whereas the H22 subclone accounted for all analyzed virotype D isolates. Consistent with the association of the H30 ST131 subclone with fluoroquinolone resistance, we found the prevalence of ciprofloxacin resistance to be significantly higher among the (H30-derived) virotype A, B, and C isolates (100%, 100%, and 98%, respectively) than among the (non-H30) virotype D isolates (29%) ($P < 0.001$).

Diverse explanations have been proposed for the rapid and successful dissemination of *E. coli* O25b-ST131 among humans, including possible transmission through animal contact and food consumption (43). Mora et al. (44) reported an association of *E. coli* O25b-ST131 with clinical disease in poultry and identified this clonal group in retail chicken meat in Spain. In that study, the 19 avian O25b-ST131 isolates (of which 7 produced CTX-M-9) were of virotype D, defined according to the criteria specified here. However, in the present study, only 2 of the 17 Spanish human isolates of virotype D (isolates 16.30 and 16.31; *fimH*, *iucD*, *iroN*, *kpsMIII-K1*, *iss*, *traT*, *ibeA*, *malX*, *usp*, and SHV-12) and the 2 avian virotype D isolates from the United States (isolates CD285 and CD287; *fimH*, *iucD*, *iroN*, *kpsMIII-K1*, *iss*, *traT*, *ibeA*, *malX*, *usp*, and *tsh*) showed virulence gene profiles that were highly similar to those of the Spanish avian isolates (44).

In conclusion, we found a high prevalence of *E. coli* O25b:H4-B2-ST131 among (mostly CTX-M-15-producing) ESBL-EC isolates from hospitals across Spain in 2006. We newly defined four main virulence gene profile variants within ST131, which we termed virotypes A to D, and showed these to be prevalent also among isolates from other countries. The four ST131 virotypes, which appeared to represent clonal variants within ST131, exhibited distinctive distributions and clinical and epidemiological associations. Future studies of the conventional and molecular epidemiology of ST131 should address relevant within-ST divisions, including *fimH*-based subclones and virotypes. Such studies should improve our understanding of the basis for the worldwide dissemination and emergence of the *E. coli* ST131 clonal group.

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