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Spread of Escherichia coli strains with high-level cefotaxime and ceftazidime resistance between the community, long-term care facilities, and hospital institutions.
Oteo J, Navarro C, Cercenado E, Delgado-Iribarren A, Wilhelmi I, Orden B, García C, Miguelañez S, Pérez-Vázquez M, García-Cobos S, Aracil B, Bautista V, Campos J.
J Clin Microbiol. 2006 Jul;44(7):2359-66.

which has been published in final form at https://doi.org/10.1128/JCM.00447-06

Spread of high-level of cefotaxime and ceftazidime resistance in *Escherichia coli* between the community, long-term care facilities, and hospital institutions

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Running title: Spread of cefotaxime and ceftazidime resistant *E. coli*

Word count: Abstract **250**; Text **4,150**

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Abstract

One hundred fifty-one *Escherichia coli* strains resistant to cefotaxime and ceftazidime were isolated during a prospective surveillance study. These strains were characterized by clinical, microbiological, and molecular analyses and were distributed into four clusters of 103, 11, 6 and 5 isolates, and 25 unrelated strains. The principal cluster was isolated from urine, wound, blood, and other samples in three hospitals, eight nursing homes, and a community healthcare center. This cluster was associated with both nosocomial (65%) and community acquired infections (35%). Most strains were resistant to ciprofloxacin, gentamicin, tobramycin, cefepime, amoxicillin/ clavulanic acid, and trimethoprim/ sulfamethoxazole, but susceptible to imipenem. All isolates from the four clusters expressed the extended-spectrum β-lactamase (ESBL) CTX-M-15. This enzyme was also present in 8 (30.8%) of the 26 unrelated isolates. The other ESBLs, CTX-M-14 and CTX-M-32, were detected in 5 and 7 cases, respectively, but they were detected in individual *E. coli* isolates only. In three clusters, $bla_{CTX\text{-}M\text{-}15}$ alleles were linked to an IS*Ecp1*-like element (implicated in their mobilization), while in eight strains of cluster II an IS*26* element preceded the $bla_{CTX\text{-}M\text{-}15}$ allele. An additional pool of resistance genes included *tet*A, *drf*A14 or *dfr*A17, *sul*1 or *su*l2, *aac* (6´)-1b, and *aac* (3)-IIb. All except one of the 27 isolates tested for genetic virulence markers harbored the same three virulence genes: *iut*A and *fyu*A (siderophores), and *tra*T (serum survival factor). Epidemic or occasional isolates of cefotaxime and ceftazidime resistant *E. coli* can spread between distinct health facilities including hospitals, community health centers, and long-term care centers.

Introduction

The production of extended spectrum β-lactamases (ESBLs) is one of the major sources of resistance to extended spectrum cephalosporins in *Enterobacteriaceae* (16). Classically, plasmid-mediated ESBL enzymes have been of the TEM and SHV types, but in recent years, CTX-M-type ESBLs have been increasingly found throughout the world (1). CTX-M enzymes predominantly hydrolyze cefotaxime but are weakly active against ceftazidime. However, some ESBLs of the CTX-M family display increased hydrolytic activities against ceftazidime, as is the case for CTX-M-15 (24) and CTX-M-32 (4).

In recent years, ESBL production in *Enterobacteriaceae*, particularly *Escherichia coli*, has significantly increased in several countries, including Spain (6,21,30). This increase is primarily due to the spread of CTX-M-type ESBLs (25). ESBL dissemination in *E. coli* is usually due to plasmid transmission between unrelated strains (9), while clonal spreading is more frequent in other *Enterobacteriaceae*, such as *Klebsiella pneumoniae* (23). However, outbreaks of ESBL-producing *E. coli* clones have recently been described (2,15,18,19,32). In Spain the proportion of CTX-M-*E. coli* producers is rapidly increasing in both nosocomial and community acquired infections (21,25).

We have observed an apparent and alarming increase of cefotaxime- and ceftazidime-resistant strains amongst the *E. coli* isolates submitted to our laboratory. These events prompted the present study in which we describe the emergence and spread of high-level cefotaxime and ceftazidime resistant *E. coli* isolates, their complex molecular epidemiology, and the characterization of several clusters of multi-drug resistant CTX-M-15-producing *E. coli* isolates. Epidemic strains were detected in

patients admitted to hospitals and nursing homes for the elderly, as well as in patients attending an outpatient community healthcare center.

Materials and methods

Study design

A prospective surveillance study of infections caused by ESBL-producing *E. coli* is currently ongoing in the Autonomous Community of Madrid, Spain. Three hospitals located in three separate geographic areas participated in the study and included Hospital Gregorio Marañón (estimated catchment population of 650,000), Hospital Fundación de Alcorcón (estimated catchment population of 250,000, including eight associated nursing homes), Hospital Severo Ochoa de Leganés (estimated catchment population of 380,000), and a community healthcare center, Centro de Especialidades Argüelles. All collected the inital *E. coli* isolates that displayed ESBL production and sent them to the Centro Nacional de Microbiología (CNM), a public-health reference institution, to confirm ESBL production and to study the molecular epidemiology of the isolates and the ESBL type. Here we describe the analysis of all ESBL-producing *E. coli*, isolated between January 2004 and August 2005, which exhibited resistance to cefotaxime (>32 μg/mL) and ceftazidime (≥16 μg/mL).

Infections and patient characteristics

The participating institutions collected the following information: personal patient data (code, age, sex, clinical diagnosis, and risk factors), hospital and departmental data, and isolate data (clinical sample and antimicrobial susceptibility). This information was entered into the Whonet software program, a free microbiological database (WHO Collaborating Center for the Surveillance of Antibiotic Resistance).

Patients with acquired community infections were the outpatients and those who had a positive culture at the time of or within 48 hours of hospitalization, in both cases they have not been previously in a healthcare setting for 1 month.

Antimicrobial susceptibility testing and ESBL production detection

E. coli isolates were identified and antibiotic susceptibility was tested according to standard microbiological procedures performed in each clinical laboratory. At the CNM, isolates were subcultured in both Columbia blood agar and McConkey agar to ensure viability and purity. The identification of all isolates was confirmed according to standard microbiological methods; susceptibility testing was initially carried out by microdilution (BD Phoenix Automated Microbiology System; Becton-Dickinson Diagnosis, Sparks, MD, USA) and the disk-diffusion reference method (5). The antimicrobial agents tested were as follows: ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefoxitin, cefotaxime, ceftazidime, cefepime, amikacin, gentamicin, tobramicin, ciprofloxacin, imipenem, and trimetoprim/sulfametoxazole. ESBL production was confirmed as described by the Clinical Laboratory Standards Institute (CLSI) (5); inhibition zones obtained using disks that contained cefotaxime (30 μg) and ceftazidime (30 μg) were compared with those containing cefotaxime/clavulanic acid (30/10 μg) and ceftazidime/clavulanic acid (30/10 μg) (Oxoid, Madrid, Spain), respectively. In addition, the minimal inhibitory concentrations (MICs) to cefotaxime and cefotaxime/clavulanic acid, as well as to ceftazidime and ceftazidime/clavulanic acid, were determined by the E-test method (AB-Biodisk, Solna, Sweden). Antimicrobial susceptibility results were interpreted according to the breakpoints recommended by the CLSI (5). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

Molecular epidemiology

The molecular epidemiology of *E. coli* isolates that were resistant to cefotaxime and ceftazidime was determined by pulsed-field gel electrophoresis (PFGE). Total bacterial DNA was digested with *Xba*I (MBI Fermentas, Vilnius, Lithuania), and DNA fragments were separated on a 1% agarose gel in 0.5 X TBE buffer using the CHEF Mapper apparatus (BioRad, Madrid, Spain). The conditions were as follows: 14°C, 6 V/cm, pulse 2-54 s, and 24 h. Gels were stained with ethidium bromide and photographed under ultraviolet (UV) light. Genetic similarity was calculated by the unweighted pair group method using arithmetic averages (UPGMA) and presented in a dendrogram. Similarity was calculated by Dice's coefficients with a tolerance of 1.8% (Fingerprinting II Software, BioRad). Well-resolved bands that corresponded to fragments exceeding 48.5 kb were included in the computer analysis.

Isoelectric focusing

Isoelectric focusing was carried out on a total of 73 *E. coli* isolates representing the major clones identified by PFGE. Bacteria, exponentially growing at 37°C in Luria-Bertani medium, were harvested, and cell-free lysates were prepared by sonication. Isoelectric focusing was performed by applying this crude extract to Phast gels (pH = 3-9) in a Phastsystem apparatus (Pharmacia AB, Uppsala, Sweden) (10). β-lactamases with known pIs of 5.9, 5.4, 7.6, and 8.1 were used in parallel as controls. Gels were stained with 500 μg nitrocefin/mL (Oxoid) to identify β-lactamase bands.

Molecular analysis of ESBLs

Based on both antibiotic resistance profiles and pI values, molecular identification of β-lactamases was carried out by PCR amplification and DNA sequencing. All β-lactamases detected by isoelectric focusing were characterized by molecular methods. Genomic DNA from wild-type isolates was used as a template for PCR. ESBL amplification was performed with the appropriate primers and cycling

conditions for the TEM, SHV, CTX-M, OXA-1, OXA-2, and OXA-10 β-lactamase groups, as described elsewhere (3,22,28). The following primers were designed to amplify and sequence the *bla*$_{\text{CTX-M-15}}$ gene under standard conditions (CTX-M-15-F: 5´-ATG GTT AAA AAA TCA CTG CG- 3´ and CTX-M-15-R: 5´- TTA CAA ACC GTT GGT GAC G- 3´). PCR products were separated on 0.8% agarose gels, stained with ethidium bromide, visualized under UV light, further purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced using an ABI Prism 377 automated sequencer (Perkin-Elmer, Norwalk, Conn).

Linkage of *bla*$_{\text{CTX-M-15}}$ alleles with IS*Ecp1*-like elements, previously implicated in their expression and mobilization (13), was confirmed with primers PROM+ and PRECTX-M-3B as described previously (24,32). In addition, specific primers were used to amplify a 400-bp fragment spanning the link between IS*26* (inserted between IS*Ecp1* and *bla*$_{\text{CTX-M-15}}$) and *bla*$_{\text{CTX-M-15}}$; this fragment is believed to be characteristic of the epidemic *E. coli* strain A, a CTX-M-15 producer from the United Kingdom (32).

Identification of additional resistance genes

Based on antibiotic resistance profiles, additional mechanisms of antibiotic resistance were studied in 10 multiresistant CTX-M-15-producing isolates. Molecular identification of *sul*1, *sul*2, *tet*A, *aac6*-1´b, *aac3*-IIb, *dfr*A14, and *dfr*A17 genes was carried out by PCR amplification and DNA sequencing using primers previously described (8,14,15,20). In addition, the *acrR* gene of the AcrAB efflux system was amplified, including the promoter-operator region, as described elsewhere (31).

Virulence factors

Twenty seven isolates from different clinical samples belonging to cluster I (12 from urine, 9 from blood, 5 from wound and 1 from respiratory tract) and from different geographical sources (15 from three hospitals, 8 from the community, and 4 from four

long-term care facilities) were evaluated for the presence of ten genes encoding putative virulence factors characteristic of extraintestinal pathogenic *E. coli* using PCR as described previously (11). These genes included those encoding S fimbriae (*sfa*S), F1C fimbriae (*foc*G), M blood group antigen-specific M fimbriae (*bma*), glucosaminyl-specific G fimbriae (*gaf*), Dr family adhesins (*afa/dra*), toxin associated with extraintestinal pathogenic *E. coli* (*cnf*1), siderophores as aerobactin (*iut*A), yersiniabactin (*fyu*A), the serum survival gene (*tra*T), and the invasion of brain endothelium gene (*ibe*A).

Statistical analyses

Differences in the prevalence of antibiotic resistance between different groups were assessed by Fisher's exact test. Association was determined by calculation of the odds ratio (OR) with 95% confidence intervals (CI). The null hypothesis was rejected for values of $P < 0.05$. Statistical analyses were performed using GraphPad Prism version 3.02 software (GraphPad Software, Inc., San Diego, USA).

Results

Clinical isolates and population study

From January 2004 to August 2005, a total of 525 unduplicated *E. coli* ESBL producers were collected. Of these isolates, 151 (28.8%) were simultaneously resistant to cefotaxime and ceftazidime. Sixty-five (43%) were from males, 84 (55.6%) were from females, and two were from patients whose the gender was not reported. Fifteen (9.9%) were from children ≤ 14 years of age, 21 (13.9%) were from patients of 15 to 64 years of age, and 101 (66.9%) were from patients ≥ 65 years; in 14 cases, the patient's age was unknown.

Of the 151 *E. coli* isolates, 76 (50.3%) were isolated from the urinary tract, 30 (19.9%) were from wounds, and 22 (14.6%) were from blood; the remaining 23 cases were isolated from other clinical samples (15.2%). Ninety-eight of the patients (64.9%) were admitted into different hospital departments: 33 (21.8 %) to internal medicine, 16 (10.6%) to the emergency room, 12 (7.9%) to pediatric units, 7 (4.6%) to surgery, 5 (3.3%) to the ICU, and 25 (16.5%) to other departments. Twenty-six patients (17.2%) came from long- term care facilities for elderly people, and 21 patients (13.9%) came from the community. In six cases, this information was not available.

In total, 92 isolates (60.9%) produced nosocomial infections (including those from nursing homes), 55 (36.4%) produced community-acquired infections, and in 4 isolates, this information is unknown.

The clinical diagnoses included urinary tract infections (76 cases or 50.3%), wound infection (30 cases or 19.9%), sepsis (22 cases or 14.6%), pneumonia (7 cases or 4.6%), peritonitis (3 cases or 2%), and abscess (2 cases or 1.3%).

Seventy-nine patients (52.3%) had predisposing underlying conditions with 21 cases being respiratory/cardiac diseases (13.9%). Also among these conditions were 16 cases of impaired immunity (10.6%) (diabetes: 6; tumoral pathology: 4; transplantation: 3; HIV infection: 2; rheumatic arthritis: 1), 11 cases of cognitive disorders (7.3%), 7 cases of urinary diseases (4.6%) (including 3 with a vesical catheter), 6 cases of premature birth (4%), and 4 cases of liver pathology (2.6%).

Figure 1 shows the monthly distribution of the 101 *E. coli* strains that were isolated during 2004 in two of the participating hospitals. In the period from January to June, 29 (28.7%) cases were detected, and from July to December 72 (71.3%) cases were detected ($P = 0.02$; OR: 0.57, 95% CI: 0.35-0.91).

Antimicrobial susceptibility

Data on antimicrobial susceptibility for all 151 *E. coli* isolates resistant to cefotaxime and ceftazidime are provided in Table 1. The prevalence of antimicrobial resistance, calculated according to the MICs, are as follows: 59.9% for amoxicillin/clavulanic acid, 38.8% for piperacillin/tazobactam, 98% for cefepime, 8% for cefoxitin, 89.3% for ciprofloxacin, 82% for cotrimoxazole, 70% for gentamicin, 82.7% for tobramycin, and 1.3% for amikacin (Table 1).

Compared to nosocomial infections, community acquired infections exhibited a lower resistance to gentamicin (56.4% *vs.* 71%; $P = 0.001$; OR: 0.38, 95% CI: 0.18-0.79), and tobramycin (72.2% *vs.* 87.6%; $P = 0.025$; OR: 0.36, 95% CI: 0.15-0.86). Most of the isolates were resistant to the majority of antibiotics tested. As shown in Table 2, 56 of the isolates (37.1%) exhibited multi-resistance pattern A, being susceptible to amikacin, cefoxitin, and imipenem. Eleven of the isolates (7.3%) were classified as having multi-resistance pattern B, being susceptible to amikacin and imipenem. Eleven of the isolates (7.3%) were susceptible to amikacin, cefoxitin, imipenem, and gentamicin (pattern C).

Molecular epidemiology

Cluster analyses of DNA fingerprinting performed on the 151 isolates is shown in Figure 2. *Xba*I did not digest the DNA of one isolate.

Using PFGE analysis, four clusters of isolates were detected which exhibited a genetic relatedness of 85–100% (Figure 2). One hundred three isolates made up cluster I, 11 isolates made up cluster II, 6 isolates made up cluster III, and 5 isolates made up cluster IV. The remaining 25 isolates had a genetic similarity of < 85% (Figure 2) and were considered unrelated. Cluster I was distributed among all participant centers and included nosocomial (65%) and community isolates (35%).

Clusters II and IV were distributed among three of the participant centers while the six isolates of cluster III belonged exclusively to one of the centers.

Of the cluster I isolates, three (2.9%) were from children ≤ 14 years of age, 13 (12.6%) were from patients of 15 to 64 years of age, and 78 (75.7%) were from patients ≥ 65 years; in 9 cases, the patient's age was unknown (Figure 3A). These isolates caused urinary tract infections in 59 cases (57.3%), wound infections in 21 cases (20.4%), sepsis in 15 cases (14.6%), pneumonia in 6 cases (5.8%), and abscesses in 2 cases (1.9%) (Figure 3B). As shown in Figure 3A, the isolates of cluster I were most common in patients ≥ 65 years (75.7% *vs.* 47.9%; $P = 0.0014$; OR: 3.4, 95% CI: 1.6-6.9). They were also more frequently implicated in urinary tract infections (57.3% *vs.* 35.4%; $P = 0.0147$; OR: 2.4, 95% CI: 1.2-4.9) than other isolates that were resistant to cefotaxime and ceftazidime (Figure 3B).

Cluster I isolates were also significantly more resistant to ciprofloxacin, gentamicin, tobramycin, amoxicillin/clavulanic acid, and piperacillin/tazobactam than other *E. coli* isolates (Table 3). However, these isolates exhibited a lower resistance to cefoxitin than did other strains (Table 3). Multi-resistance pattern A was the most common pattern among the cluster I isolates (46.6%), followed by pattern D (9.7%) (Table 2).

Sixty-seven of the 103 cluster I strains (65%) were collected during 2004 by two of the participating hospitals. Sixteen (23.9%) of these strains were isolated between January and June, and 51 strains (76.1%) were isolated from July to December (Figure 1).

Isoelectric focusing

Seventy-three *E. coli* isolates, which included a representative sample of the different clusters identified by PFGE and of the unrelated strains, were tested by

isoelectric focusing. Fifty of 73 (68.5%) contained four β-lactamases with apparent pIs equal to 5.4, 6.8, 7.4, and 8.6. The 41 cluster I isolates tested had this profile while only 10 (31.2%) of the 32 isolates that belonged to other clusters had this profile ($P <$ 0.0001; OR: 177.9, 95% CI: 9.9-3180).

Molecular analysis of ESBLs

All 151 *E. coli* isolates resistant to cefotaxime and ceftazidime were screened with specific CTX-M-10-group primers; DNA from a total of 140 isolates was amplified, 133 isolates were identified as containing CTX-M-15, and 7 were identified as containing CTX-M-32. The remaining 11 strains were analyzed using universal CTX-M-type primers; 5 strains contained CTX-M-14, and 6 did not amplify.

All isolates of the four clusters as well as 8 (30.8%) of the 26 unrelated isolates expressed CTX-M-15. All of the *E. coli* isolates expressing CTX-M-14- or CTX-M-32- exhibited individual PGFE profiles.

Sequence analysis of PCR products obtained with bla_{TEM}- and bla_{OXA-1}-specific primers identified the β-lactamases TEM-1 (pI = 5.4) and OXA-30 (pI = 7.4). No PCR products were obtained using the bla_{SHV}, bla_{OXA-2}, and bla_{OXA-10} primers. The band corresponding to the pI of 6.8 did not amplify with any of the specific primers for the following β-lactamase groups: TEM, SHV, CTX-M, CTX-M-10, OXA-1, OXA-2, OXA-9, OXA-10, OXA-22, OXA-23, OXA-51, and GES-1 (http://www.lahey.org/studies/webt.asp).

Eighteen isolates from clusters I, II, III or IV, which expressed CTX-M- 15 were analyzed using PROM+ and PRECTX-M-3B primers, as described in Material and methods. Cluster I, III, and IV strains yielded PCR products of approximately 1000 bp. Further sequencing of this fragment revealed that $bla_{CTX-M-15}$ was directly linked to an upstream IS*Ecp1*-like element, previously implicated in expression and mobilization of

295 CTX-M-15 (13). These isolates failed to yield amplicons using specific primers
296 designed to amplify a 400-bp fragment spanning the IS*26* element and $bla_{\text{CTX-M-15}}$ – a
297 characteristic of the epidemic strain A from the United Kingdom (32). In contrast, 8 of
298 the 11 isolates belonging to the cluster II yielded amplicons of approximately 2000 bp
299 and contained the fragment spanning the IS*26* element and $bla_{\text{CTX-M-15}}$ characteristic of
300 strain A (32).

301 **Identification of additional resistance genes**

302 The *tet*A gene was detected in 10 *E. coli* isolates resistant to tetracycline.
303 Resistance to trimethoprim and to sulphamides was associated with a combination of
304 *sul*2 and *dfr*A14 (8 isolates) or a combination of *sul*1 and *dfr*A17 (2 isolates). In
305 relation to the aminoglycosidae resistance genes, 7 isolates resistant to both gentamicin
306 and tobramycin had *aac (6´)*-1b and *aac (3)*-IIb genes. The remaining 3 isolates, which
307 were resistant to tobramycin but susceptible to gentamicin, contained only *aac (6´)*-1b.

308 The *acr*R gene amplified in all 10 isolates examined. Six of these isolates
309 exhibited no gene modifications. In contrast, 2 exhibited a single amino acid
310 substitution (Gly28Val), and 2 exhibited a single nucleotide deletion (Gua85 and
311 Cyt249).

312 **Virulence factors**

313 All of the 27 isolates examined harbored the same three virulence genes with
314 one exception. Two genes encoded siderophores (*iut*A and *fyu*A) and one encoded a
315 serum survival factor (*tra*T). One isolate had only the *tra*T gene. None of the genes
316 encoding S, G, M, and F1C fimbriae were detected in these 27 isolates.
317

Discussion

We have previously described significant increases in ESBL production, by both nosocomial and community acquired *E. coli*, in recent years in Spain (21). Other worldwide studies have documented similar findings (6,30). This increase has been attributed to the rising prevalence of the CTX-M family of ESBLs that has emerged as an important and rapidly developing problem worldwide (1,9,25). The first CTX-M-type enzyme detected in Spain was CTX-M-9, reported in 1996 (26). In 2003, the first report documenting the isolation of a CTX-M-like ESBL in the United States was published (9 strains from 5 US States) (27).

At present, the more prevalent CTX-M-types isolated from clinical samples in Spain have been CTX-M-9 and the genetically related CTX-M-14, followed by CTX-M-10 (9,17,25). No clonal dissemination of ESBL-producing *E. coli* was observed in a nationwide study of 40 Spanish centers in 2000 (9). CTX-M-15 was not detected in any of the three clinical Spanish studies mentioned (9,17,25). Also, CTX-M-15 was not detected in one study of fecal carriers performed in Madrid in 2003 (29). All ESBLs found were of the CTX-M-9, CTX-M-10, and CTX-M-14 types (29). However, in another fecal carrier study from Barcelona (2001-2002), CTX-M-15 was detected in five cases, as were both CTX-M-9 and CTX-M-14 (17).

Data reported here indicate that dissemination of high-level cefotaxime- and ceftazidime-resistant *E. coli* may be attributable to the following. First, the majority of resistant isolates were clonally associated and may have caused epidemics in several clinical settings. Second, ESBL CTX-M-15 demonstrates the ability to spread among different clusters of *E. coli*. Third, the simultaneous presence of other ESBLs of the CTX-M type like CTX-M-32 and CTX-M-14 may produce the same 3rd generation cephalosporin resistance profile.

The main epidemic *E. coli* strain was detected in 2004–2005 in isolates from patients of very distinct origins, which included three hospitals with a joint catchment population of approximately 1,300,000 persons (total population of the Madrid Autonomous region is approximately 5.5 million) covering several distinct administrative health areas, eight different nursing homes, and one community healthcare center. Infection-control measures were reinforced afterwards.

ESBL CTX-M-15 was first described in New Delhi in 1999 and is carried on large plasmids in *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes* (13). Since then, outbreaks of CTX-M-15 *E. coli* producers have been described in France, the United Kingdom, and Canada (2,15,32). In some studies, the simultaneous presence of OXA-30 was also reported (7).

The great majority of isolates belonging to our epidemic cluster I was from urinary tract infections that affect older patients, in accordance with other studies (15,19). However, our data may indicate a complex underlying epidemiology of these CTX-M-15 producers. The high spreading capacity of CTX-M-15 includes two possible scenarios. One is the spread of an epidemic clone with some selective advantages (e.g., multiple antibiotic resistance and enhanced virulence) between different hospitals, long-term care facilities, and the community; the other is the horizontal transfer of plasmids or genes that carry $bla_{\text{CTX-M-15}}$ alleles. Here we demonstrate the presence of $bla_{\text{CTX-M-15}}$ within two different genetic environments in the same geographical region. The $bla_{\text{CTX-M-15}}$ gene was directly linked to an upstream insertion sequence IS*Ecp1*-like element (clusters I, III and IV of this study), as described in Canada, Cameroon, and India (2,7,13). In other isolates (8 strains of cluster II of this study), an IS*26* element was inserted within ISEcp1-like element

preceding the *bla*$_{\text{CTX-M-15}}$ gene, as described in the epidemic strain A of United Kingdom (32).

In addition to β-lactamase resistance mechanisms, CTX-M-15 *E. coli* producers carried an important pool of mobile resistance genes including *tet*A, *dfr*A14 or *dfr*A17, *sul*1 or *sul*2, *aac (6´)*-1b, and *aac (3)*-IIb. Resistance to gentamicin and tobramycin was associated with a combination of the *aac (6´)*-1b and *aac (3)*-IIb genes, while resistance to tobramycin was linked to *aac (6´)*-1b.

We also examined possible mutations in *acr*R, the regulator of the *acrAB* gene encoding a multi-drug efflux pump that has been associated with antimicrobial resistance – principally fluoroquinolone resistance (31). Four of the seven multi-resistant *E. coli* isolates tested demonstrated different amino acid substitutions or deletions in the *acr*R system; two of them had an amino acid substitution in a position (Gly28) previously described to be connected to norfloxacin, chloramphenicol and tetracycline resistance (31). Acquisition of antibiotic resistance may be associated to decreased expression of virulence determinants (12). This has also been observed in ESBL *E. coli* producers isolated from urinary tract infections (15). We did not detect F1C, S, G and M fimbriae genes by PCR in our strains (that came from non-invasive infections in the 86% of the cases); however, the strains isolated from blood also had the same virulence genetic profile.

In this study, 35% of the cluster I isolates were implicated in community-acquired infections. With probable origins of hospitals or nursing homes, these findings are demonstrative of the disruption of hospital-community barriers. In addition, the high and increasing use of fluoroquinolones in Spain and other countries (21) may be associated with a co-selection phenomenon, which facilitate the persistence and spread of this epidemic strain in fecal flora of healthy carriers. Long-term care centers may

represent a significant reservoir for multi-resistant ESBL-producing *E. coli* isolates, and infection control efforts must be addressed in these settings.

In summary, this study reports the spread of epidemic or occasional resistant *E. coli* isolates between the community, long-term care facilities and hospital settings. It also documents the first outbreak of a CTX-M-15 *E. coli* producer in Spain, affecting patients admitted to hospitals, nursing homes, and community outpatient centers. The CTX-M-15 extended spectrum β-lactamase was found to be widespread in a main epidemic *E. coli* strain and also in the majority of other isolates resistant to cefotaxime and ceftazidime. The same 3rd generation cephalosporin resistance profile was also due to isolates expressing other ESBLs like CTX-M-32 and CTX-M-14. The majority of these isolates were found to be susceptible to carbapenems but resistant to the vast majority of antibiotics tested.

Dissemination of these strains type could lead to important changes in the epidemiology of *E. coli* ESBL producers and generate important therapeutic problems.

Acknowledgements

This study was supported by research grants from Instituto de Salud Carlos III, *Programa Intramural* (reference 03/ESP32); Fondo de Investigaciones Sanitarias, Ministry of Health, Spain (FIS PI040837, and the *Red Española de Investigación en Patología Infecciosa, REIPI*).

J. Oteo was supported by the Dirección General de Salud Pública, Ministry of Health, Spain (reference SBVI1284/02-13).

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534 TABLE 1. Antimicrobial susceptibility of 151 *Escherichia coli* isolates resistant to

535 cefotaxime and ceftazidime.

536

Antibiotic	S (%)	I (%)	R (%)	MIC_{50}*	MIC_{90}*	Range*
Ampicillin	0	0	151 (100)	>16	>16	>16
Amoxicillin/Clavulanic acid	13 (8.7)	48 (32)	90 (59.3)	>16	>16	4->16
Piperacillin/Tazobactam	41 (27)	52 (34.2)	58 (38.8)	64	>128	4->128
Cefotaxime	0	0	151 (100)	>32	>32	>32
Ceftazidime	0	15 (10)	136 (90)	>16	>16	16->16
Cefepime	2 (1.3)	1 (0.7)	148 (98)	>16	>16	4->16
Cefoxitin	128 (84.7)	11 (7.3)	12 (8)	8	16	≤ 4->16
Ciprofloxacin	16 (10.7)	0	135 (89.3)	>2	>2	≤ 0.12->2
Gentamicin	45 (30)	0	106 (70)	>8	>8	≤2->8
Tobramycin	25 (16.6)	1 (0.7)	125 (82.7)	>8	>8	≤2->8
Amikacin	143 (94.7)	6 (4)	2 (1.3)	≤8	16	≤8->32
Trimethoprim/Sulfamethoxazole	27 (18)	0	124 (82)	>2	>2	≤0.5->2
Imipenem	151 (100)	0	0	≤ 1	≤ 1	≤ 1

537 S: Susceptible; I: Intermediate; R: Resistant.

538 * μg/mL.

539 TABLE 2. Most common multi-resistance patterns found in *Escherichia coli* isolates
540 belonging to cluster I and other isolates resistant to cefotaxime and ceftazidime.
541

Multi-resistance patterns	Cluster I isolates (%)	Other isolates (%)
A: Amp-A/Clav-Ctx-Caz-Cef-Pip/Taz-Gen-Tob-Cip-Sxt	48 (46.6)	8 (16.7)
B: Amp-A/Clav-Ctx-Caz-Cef-Pip/Taz-Gen-Tob-Cip-Sxt-Fox	6 (5.8)	5 (10.4)
C: Amp-A/Clav-Ctx-Caz-Cef-Pip/Taz-Tob-Cip-Sxt	6 (5.8)	5 (10.4)
D: Amp-A/Clav-Ctx-Caz-Cef-Pip/Taz-Gen-Tob-Cip	10 (9.7)	0
E: Amp-A/Clav-Ctx-Caz-Cef-Gen-Tob-Cip-Sxt	8 (7.8)	0

542

543 Amp: ampicillin, A/Clav: amoxicillin/clavulanic acid, Ctx: cefotaxime, Caz:
544 ceftazidime, Cef: cefepime, Pip/Taz: piperacillin/tazobactam, Gen: gentamicin, Tob:
545 tobramycin, Cip: ciprofloxacin, Sxt: trimethoprim/sulfametoxazole, Fox: cefoxitin.

546

547

548 TABLE 3. Comparison of antimicrobial resistance data between *Escherichia coli*
549 isolates belonging to cluster I (n = 103) and other isolates (n = 48) resistant to
550 cefotaxime and ceftazidime.
551

Antibiotics	Cluster I N (%)	Other strains N (%)	OR	95% CI	*P*
Amoxicillin/Clavulanic acid*	99 (96.1)	39 (81.2%)	5.7	1.7-19.6	0.004
Piperacillin/Tazobactam*	84 (81.6)	28 (58.3)	3.2	1.5-6.7	0.005
Cefoxitin	2 (1.9)	10 (20.8)	0.07	0.02-0.4	0.0002
Ciprofloxacin	99 (96.1)	36 (75)	5.7	1.7-19.6	0.004
Gentamicin	85 (82.5)	22 (45.8)	5.6	2.6-12	<0.0001
Tobramycin	93 (90.3)	32 (66.7)	4.6	1.9-11.3	0.0008

552
553 * Intermediate and resistant isolates included.
554

Figure legends:

FIGURE 1. Monthly distribution of total cases of *Escherichia coli* isolates belonging to cluster I and other isolates resistant to cefotaxime and ceftazidime in two participating hospitals during 2004.

FIGURE 2. Dendrogram that illustrates the genetic relationship of 150 ESBL-producing *Escherichia coli* isolates resistant to cefotaxime and ceftazidime.

*Isolates identification number

FIGURE 3. Comparison of patient's age (A) and clinical diagnostics (B) between *Escherichia coli* isolates belonging to cluster I and other isolates resistant to cefotaxime and ceftazidime.

1: $P = 0.001$

2: $P = 0.01$