

Characterization of a Large Outbreak by CTX-M-1-Producing *Klebsiella pneumoniae* and Mechanisms Leading to In Vivo Carbapenem Resistance Development

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All extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae* isolates from patients admitted to an adult intensive care unit were prospectively documented from 2002 to 2005, when a large outbreak (51 patients affected) of multiresistant ESBL-producing *Klebsiella pneumoniae* infection was detected. The involvement of a single *K. pneumoniae* clone was demonstrated by pulsed-field gel electrophoresis. In addition to the ESBL-mediated resistance, the epidemic strain uniformly showed cross-resistance to ciprofloxacin, gentamicin, tobramycin, trimethoprim-sulfamethoxazole, and tetracycline, whereas resistance to the β -lactam- β -lactamase inhibitor combinations was variable. The ESBL involved was CTX-M-1, as demonstrated by isoelectric focusing, PCR amplification, and sequencing. CTX-M-1 as well as the aminoglycoside resistance determinants were encoded in a 50-kb plasmid that could be transferred to *Escherichia coli* only by transformation. In two of the infected patients, carbapenem resistance development (MICs of 8 to 12, 16, and >32 μ g/ml for imipenem, meropenem, and ertapenem, respectively) was documented, both in clinical samples and in intestinal colonization studies. The analysis of the outer membrane proteins of the carbapenem-susceptible and -resistant isolates revealed that the former expressed only one of the two major porins, OmpK36, whereas the latter did not express either of them. In one of the cases, the lack of expression of OmpK36 was demonstrated to be mediated by the interruption of the coding sequence by the insertion sequence IS26. This is the first report of a large outbreak of CTX-M-1-producing *Enterobacteriaceae* and, curiously, the first documented description in the literature of CTX-M-1 in *K. pneumoniae*, despite the fact that this enzyme has been found in multiple species. Furthermore, we document and characterize for the first time carbapenem resistance development in CTX-M-1-producing *Enterobacteriaceae*.

Since plasmid-mediated extended-spectrum β -lactamases (ESBLs) were first detected in a *Klebsiella pneumoniae* isolate in 1983 in Germany (22), they have been increasingly reported worldwide (5). The classical ESBLs are those derived from the broad-spectrum enzymes TEM-1, TEM-2, and SHV-1 by the acquisition of specific point mutations which expand their spectrum of hydrolysis to oximino-cephalosporins and aztreonam (7). Nevertheless, the most widespread plasmid-mediated ESBLs nowadays are the CTX-M enzymes, which are directly derived from the chromosomal β -lactamases of several species of the genus *Kluyvera* (3). Five different groups of CTX-Ms containing a total of over 30 different variants have been described so far, with CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 being the first reported representatives. CTX-M-1 (also known as MEN-1) was the first reported variant, detected from an *Escherichia coli* strain in 1989 in Germany (2), but CTX-M-2, CTX-M-3, CTX-M-14, and CTX-M-15 are the most widespread enzymes (3).

The production of ESBLs in *Enterobacteriaceae* confers resistance to all penicillins and cephalosporins (with the exception of cephamycins), with the organism generally remaining susceptible only to β -lactam- β -lactamase inhibitor combinations, such as amoxicillin-clavulanate, and the carbapenems, which are frequently the only therapeutic options available for treatment of hospital-acquired severe infections caused by these microorganisms (25). Coresistance to non- β -lactam antibiotics is also frequent, either by the cotransfer of the resistance determinants in the same genetic elements (such as aminoglycoside resistance) or simply by the coselection of both resistance mechanisms, as occurs with fluoroquinolones (32).

In this work we characterized a large outbreak caused by a CTX-M-1-producing multiresistant *K. pneumoniae* strain in a Spanish intensive care unit (ICU). Although CTX-M-1 was the first CTX-M described, over 15 years ago, and has been detected in various countries, including at least Germany and France (14), Italy (31), and recently, in a single *E. coli* isolate from cattle milk, Spain (6), and in several species, including *E. coli*, *Proteus mirabilis*, *Morganella morganii*, and *Citrobacter amalonaticus* (28), reports of CTX-M-1-producing *K. pneumoniae* strains have not yet been published, and this is the first report of a large outbreak of CTX-M-1-producing *Enterobac-*

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teriacae infection. Furthermore, in this work we document and characterize for the first time in vivo resistance development to carbapenems in CTX-M-1-producing *Enterobacteriaceae* due to the selection of mutations leading to the lack of expression of porins.

MATERIALS AND METHODS

Clinical strains and antibiotic susceptibility testing. All ESBL-producing *Enterobacteriaceae* isolates from patients admitted to the adult ICU (30 beds divided in three contiguous units) from Hospital Son Dureta (900 beds; reference public hospital from the Island of Mallorca, Spain) have been prospectively documented from 2002 to 2005, when a large outbreak caused by multiresistant ESBL-producing *K. pneumoniae* was detected. Bacterial identification and initial susceptibility testing were performed with the WIDER semiautomatic system (Francisco Soria Melguizo, Madrid, Spain) (8). Double-disk synergy testing (DDST) for the detection of ESBL production was performed using amoxicillin-clavulanate, cefotaxime, ceftazidime, cefepime, and aztreonam disks that were applied 30 and/or 20 mm apart (20). Additionally, the MICs of several antibiotics were determined for selected isolates by using Etest strips (AB Biodisk, Solna, Sweden), following the manufacturer's recommendations. For the screening of intestinal colonization by the epidemic *K. pneumoniae* ESBL-producing strain, from May to December 2005, rectal swabs, collected weekly, were plated on MacConkey agar supplemented with 2 µg/ml of cefotaxime and 10 µg/ml of gentamicin. The screening for intestinal colonization was carried out for all patients with positive clinical samples as well as those patients present in the same unit at the time of isolation. For those patients, weekly rectal swabs were obtained until discharge or until three consecutive negative colonization results.

Molecular strain typing. The clonal relationship between the different isolates was studied by pulsed-field gel electrophoresis (PFGE). Agarose plugs containing total bacterial DNA were prepared as described elsewhere (21). Plugs were then digested with XbaI and loaded into a 1% Megabase agarose (Bio-Rad, La Jolla, Calif.) gel. DNA separation was performed in a CHEF-DR11 apparatus (Bio-Rad, La Jolla, Calif.) under the following conditions: 6 V/cm² for 20 h at 14°C, with initial and final pulse times of 2 s and 35 s, respectively. The results were interpreted following the criteria of Tenover et al. (37).

Characterization of β-lactamases and their genes. The pIs of the β-lactamases were determined by isoelectric focusing (IEF), applying the supernatants of crude sonic cell extracts to Phast gels (Pharmacia AB, Uppsala, Sweden) with a pH gradient of 3 to 9 in a Phast system (Pharmacia). β-Lactamases with known pI values (TEM-1, TEM-2, TEM-4, TEM-3, SHV-1, CTX-M-10, and CTX-M-1) were included as controls. Gels were stained with 500 µg/ml of nitrocefin (Oxoid) to identify the bands corresponding to β-lactamases. PCRs for genes encoding TEM, SHV, CTX-M-9, and CTX-M-10 β-lactamases were performed using primers and conditions described previously (10). Primers CTX-MF (5'-GACT ATTCATGTTGTTGTTATTC-3') and CTX-MR (5'-TTACAAACCGTTGG TGACG-3') were used for the amplification of a 923-bp DNA fragment (nucleotides -48 to the stop codon) from the genes encoding CTX-M-1 or closely related ESBLs. *Taq* Gold polymerase (PE-Applied Biosystems) was used for PCR amplification under the following conditions: 12 min at 94°C, 35 cycles of amplification (1 min at 94°C, 1 min at 58°C, and 1 min at 72°C), and a final extension step of 10 min at 72°C. Two independent PCR products were sequenced on both strands, using the BigDye terminator kit (PE-Applied Biosystems) for performing the sequencing reactions, which were analyzed with the ABI Prism 3100 DNA sequencer (PE-Applied Biosystems).

Conjugation, transformation, and plasmid analysis. Conjugation experiments were performed by filter mating using a rifampin-resistant mutant of *E. coli* strain HB101 as the recipient in 1:1 ratio. Alternatively, triparental crossings using the same recipient and *E. coli* HB101(pRK2013) as a mobilization helper (12) in a 1:1:1 ratio were attempted. Transconjugants were selected in Luria-Bertani (LB) agar plates containing 100 µg/ml of rifampin and 2 µg/ml of cefotaxime. For transformation experiments, plasmid DNAs from the ESBL-producing *K. pneumoniae* isolates obtained using the QIAGEN plasmid Midi kit (QIAGEN, Hilden, Germany) were transformed into the *E. coli* XL1-Blue strain made competent by CaCl₂ treatment. Transformants were selected in LB agar plates containing 50 µg/ml of ampicillin. Transconjugants or transformants were checked by DDST followed by IEF, PCR amplification of the appropriate ESBL-encoding gene, and Etest testing of susceptibility to all β-lactams and non-β-lactams (to determine the resistance determinants cotransferred with the ESBL) defined above. For the estimation of the plasmid size, plasmid DNA obtained from one of the transformants was digested with EcoRI, BamHI, and EcoRI plus

BamHI, and the restriction fragments were separated by electrophoresis in a 1% agarose gel. The sizes of the different fragments were estimated by plotting against the standard curve obtained with the Superladder-Mid1 100-bp ladder molecular weight markers (Gensura) and the lambda HindIII digest (New England Biolabs).

Isolation and analysis of OMP. Cell envelopes were isolated from *K. pneumoniae* strains grown in LB medium by centrifugation at 100,000 × g for 1 h at 4°C after French press cell lysis. Outer membrane proteins (OMP) were isolated as sodium lauryl sarcosinate-insoluble material (15). Electrophoretic analysis of OMP by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 11% acrylamide-0.35% bisacrylamide-0.1% SDS by using Laemmli's buffers (24) and Coomassie blue staining. Samples were boiled for 5 min in sample buffer before electrophoresis. Western blot analysis of SDS-PAGE-separated OMP was carried out with the buffers and conditions described by Towbin et al. (38). Immobilon P membranes (Millipore), rabbit antiserum raised against purified OmpK36 porin (1), and alkaline phosphatase-labeled anti-rabbit immunoglobulin G (Sigma). Enzyme was detected with 5-bromo-4-chloro-3-indolylphosphate toluidinium-nitroblue tetrazolium. *K. pneumoniae* LB1, which expresses only one porin, was used as a control (26).

PCR amplification of porin genes. The primers used to amplify the OmpK36 porin gene were OmpK36-0 (5'-AAGCTTGTGGATTATTCTGC-3') and OmpK36-end (5'-CAAGCTTAGAAGTGGTAAACC-3'). They anneal specifically to sequences of *ompK36* located 116 bp upstream and 1,084 bp downstream of the *ompK36* start codon (26), respectively. PCR amplifications were performed in a Thermoline Amplifitron 1 thermal cycler by using *Taq* polymerase (Pharmacia) with 30 cycles of amplification (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). Amplicons were visualized by agarose gel electrophoresis and ethidium bromide staining and sequenced as described above.

RESULTS

All ESBL-producing *Enterobacteriaceae* isolates from patients admitted to an adult ICU were prospectively documented from 2002 to 2005, when a large outbreak of multi-resistant ESBL-producing *K. pneumoniae* infection was detected. The incidence of ESBL-producing *Enterobacteriaceae* isolated during this period is shown in Fig. 1A. As can be observed, a dramatic increase in the number of patients infected by ESBL-producing *K. pneumoniae* was documented during 2005, with a total of 52 cases, in contrast to the 1 case documented in either 2003 or 2004 and the 0 cases documented in 2002. Figure 1B shows the distribution of new cases on a monthly basis during 2005, clearly showing that the epidemic started in February and reached its peak during May and June, with 13 and 12 new cases, respectively. From then on, the incidence of new cases on a monthly basis fluctuated from zero to six new cases per month. Since five new cases were detected in December, the outbreak still cannot be considered to have been controlled at the end of the year. In May 2005, in addition to measures for the contact isolation of infected patients, a protocol for routine detection and isolation of patients with intestinal colonization was established; rectal swabs were collected weekly and plated in selective media, and ESBL-producing *K. pneumoniae* was identified as described in Materials and Methods. The outbreak had a dramatic impact on the prevalence of ESBL-producing *K. pneumoniae* in the ICU, reaching 60.3% of patients infected (not including patients with only intestinal colonization) by *K. pneumoniae* during 2005, in contrast to 0%, 4.5%, and 3.8% for the years 2002, 2003, and 2004, respectively.

All but one of the *K. pneumoniae* isolates recovered from the 52 patients had the same pattern of multiresistance as initially documented with the WIDER microdilution-based semiautomatic system for bacterial identification and susceptibility testing (8), showing, in addition to the ESBL-mediated resistance

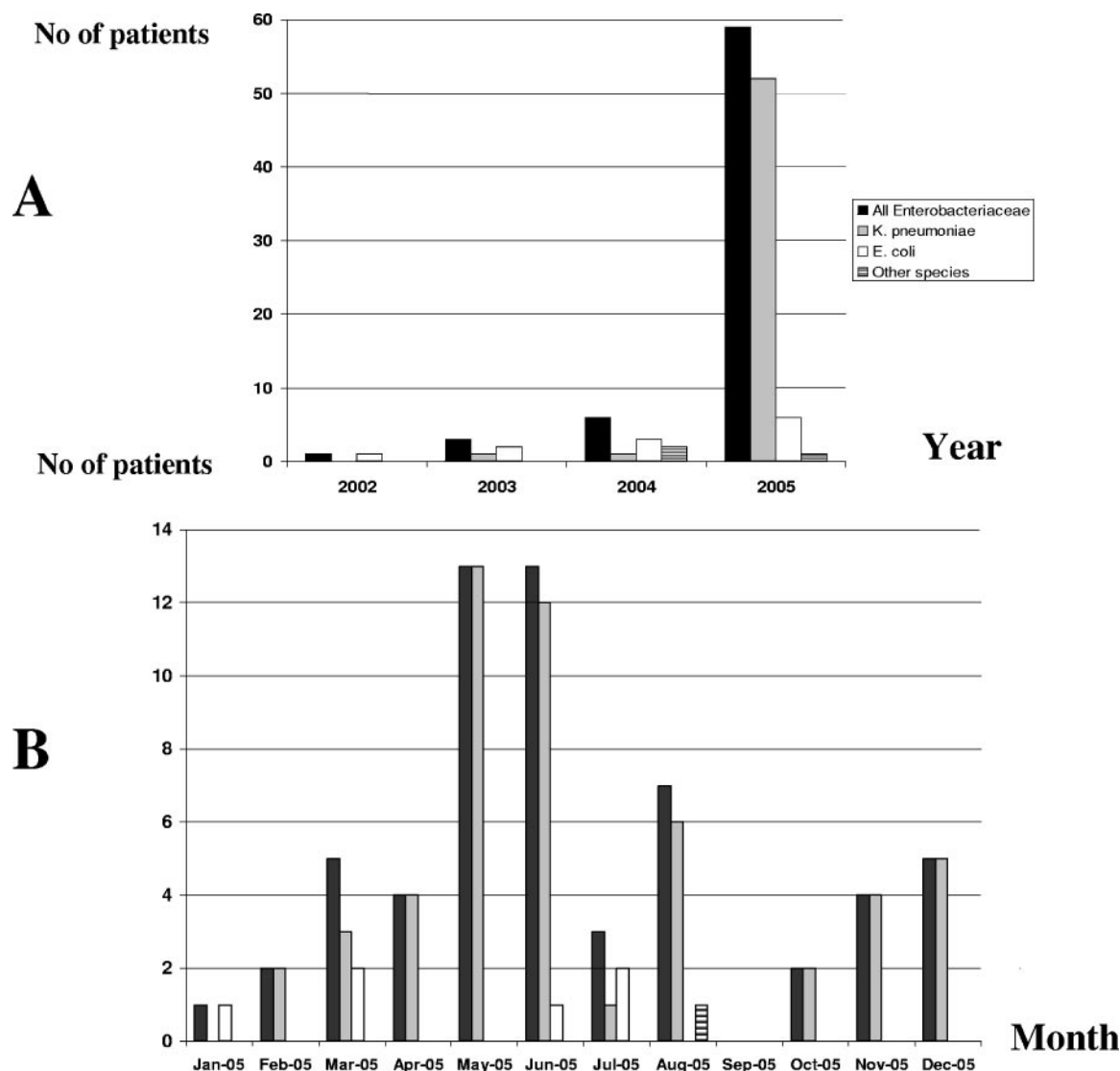


FIG. 1. A. Incidence of ESBL-producing *Enterobacteriaceae* (one isolate per patient) in the ICU during the period from 2002 to 2005. B. Distribution of new cases of isolation of ESBL-producing *Enterobacteriaceae* on a monthly basis during 2005.

to penicillins and cephalosporins, cross-resistance to ciprofloxacin, gentamicin, tobramycin, trimethoprim-sulfamethoxazole, and tetracycline. All isolates with this pattern of multi-resistance were uniformly susceptible, when first recovered (see below), only to amikacin and carbapenems. The single isolate with a different resistance pattern was ciprofloxacin susceptible and amikacin resistant.

All ESBL-producing *K. pneumoniae* isolates (one randomly selected isolate from each of the 52 patients) were confirmed to belong to a single epidemic, multiresistant clone, with the exception of that isolate with a different resistance pattern, by PFGE. Furthermore, the single isolate from 2003 was found to be nonrelated to the epidemic clone by PFGE, but the isolate from 2004 (detected in November, 3 months before the initiation of the outbreak) was found to be highly related to the epidemic clone, as shown in Fig. 2.

The sources of the ESBL-producing *K. pneumoniae* epidemic clone for the 51 ICU patients during the 2005 outbreak

are shown in Table 1. In 44 (86.3%) of the patients the epidemic clone was isolated from clinical samples, most frequently from the respiratory tract (30 patients; 68.2%), whereas in 7 (13.7%) of them it was detected only in intestinal colonization studies. Intestinal colonization studies were performed for 34 of the 44 infected patients (77.3%), with a positive result obtained for 32 (94.1%), thus showing that intestinal colonization occurs in most infected patients.

To characterize the ESBL produced by the epidemic *K. pneumoniae* clone, IEF was performed with three random isolates, revealing in all cases the presence of a β -lactamase band with a pI of ≥ 8.4 cofocusing with the CTX-M-1 control. PCR amplification was found to be positive only when using the primers for the gene encoding CTX-M-1 or related enzymes and sequencing of the PCR products confirmed the presence of *bla*_{CTX-M-1}. As could be expected, the single ESBL-producing *K. pneumoniae* isolate from 2004 (clonally related to the 2005 outbreak isolates) also produced CTX-M-1. On the other

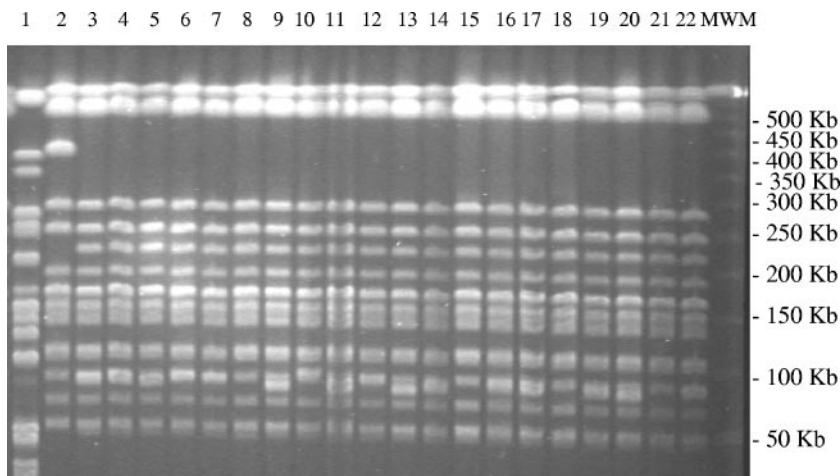


FIG. 2. XbaI PFGE patterns of ESKL-producing *K. pneumoniae* isolates from ICU patients. The single ESKL-producing *K. pneumoniae* isolates detected in 2003 and 2004 are represented in lanes 1 and 2, respectively, whereas lanes 3 to 22 contain representative isolates from the 2005 epidemic. MWM, molecular weight markers.

hand, the 2003 isolate produced an ESKL from the CTX-M-9 group.

Several attempts to transfer *bla*_{CTX-M-1} by conjugation to the *E. coli* recipient consistently failed. Nevertheless, the plasmid location of *bla*_{CTX-M-1} was demonstrated by transformation experiments. Plasmid DNA was transformed into *E. coli*, and three transformants growing on LB agar plates containing 50 µg/ml of ampicillin were first checked by DDST followed by IEF, which revealed the presence of a single β-lactamase band with a pI of ≥8.4, and then by PCR amplification with the specific primers. The size of the plasmid determined by the analysis of the EcoRI and BamHI restriction fragments was estimated to be approximately 50 kb.

In two of the patients infected by the epidemic multiresistant *K. pneumoniae* clone, development of carbapenem (imipenem, meropenem, and ertapenem) resistance was documented. For one of them, the carbapenem-resistant isolate was recovered from respiratory samples after treatment with imipenem, and for the other the isolate was recovered from urine after treatment with meropenem. In both patients, the carbapenem-resistant isolates were additionally detected in the intestinal colonization studies. PFGE macrorestriction patterns of the two carbapenem-resistant isolates were found to be identical to

those of the carbapenem-susceptible isolates, and the acquisition of additional β-lactamases was ruled out by IEF.

Table 2 shows the MICs (determined by Etest) of several β-lactam and non-β-lactam antibiotics for five representative isolates of the epidemic clone recovered from different patients and during different periods and for the two carbapenem-resistant isolates. The MICs in both resistant isolates were slightly higher for ertapenem (MIC of >32 µg/ml), followed by meropenem (MIC of 16 µg/ml) and imipenem (MIC of 8 to 12 µg/ml). The MICs for the *E. coli* transformant harboring the *bla*_{CTX-M-1}-carrying plasmid (pAMR-1) are also shown in Table 2. As can be observed, in addition to the ESKL-mediated resistance pattern, aminoglycoside (gentamicin and tobramycin) resistance was cotransferred in the same plasmid. As for carbapenems, pAMR-1 significantly raised the MICs for *E. coli* XLI-Blue of ertapenem (from 0.012 to 0.125 µg/ml) and, to a lesser extent, of meropenem (from 0.016 to 0.047 µg/ml) but not of imipenem (0.38 µg/ml) (harboring or not pAMR-1). Nevertheless, the MICs of the three carbapenems remained far below the resistance breakpoints.

In order to investigate whether carbapenem resistance was caused by alterations of the outer membrane permeability, we analyzed the outer membrane proteins of susceptible and resistant pairs of *K. pneumoniae* clinical isolates from two patients. SDS-PAGE analysis of the OMP of *K. pneumoniae* grown in LB showed only two major proteins, of about 33 and 36 kDa, in the 31- to 45-kDa range for the carbapenem-susceptible isolates, corresponding to OmpA and OmpK36, respectively. On the other hand, the 36-kDa protein was absent in both carbapenem-resistant isolates (Fig. 3A). The porin nature of the 36-kDa protein present in the carbapenem-susceptible isolates and absent in the resistant ones was confirmed by Western blot analysis using specific antibodies (Fig. 3B). The specific antiserum reacted with the band present in the susceptible isolates and the control *K. pneumoniae* LB1 but failed to detect the protein in the resistant isolates.

To investigate the cause of the porin deficiency observed in the carbapenem-resistant isolates, we amplified the entire

TABLE 1. Sources of the ESKL-producing *K. pneumoniae* epidemic clone for the 51 ICU patients during the 2005 outbreak

Source	No. (%) of patients
Intestinal colonization only	7 (13.7)
Isolation from clinical samples	44 (86.3)
Respiratory tract	30 (68.2)
Urinary tract	7 (15.9)
Wound infection	11 (25.0)
Vascular catheter	8 (18.2)
Blood	4 (9.1)
Others	4 (9.1)
≥2 sources	15 (34.1)

TABLE 2. MICs (determined by Etest) of several β -lactam and non- β -lactam antibiotics for the epidemic CTX-M-1-producing multiresistant *K. pneumoniae* strain, the two carbapenem-resistant mutants, and the *E. coli* transformant harboring the CTX-M-1-encoding plasmid pAMR-1

Antibiotic	MIC (μ g/ml) for:				
	CTX-M-1-producing <i>K. pneumoniae</i> epidemic strain ^a	<i>K. pneumoniae</i> carbapenem-resistant mutants		<i>E. coli</i> XL1-Blue	<i>E. coli</i> XL1-Blue (pAMR-1)
		KpCR-1	KpCR-2		
Ampicillin	>256	>256	>256	4	>256
Amoxycillin + clavulanate	16–32	>256	32	4	16
Ticarcillin	>256	>256	>256	8	>256
Piperacillin	>256	>256	>256	1.5	>256
Piperacillin + tazobactam	32–>256	>256	>256	1.5	16
Cefoxitin	6–48	>256	128	2	8
Ceftazidime	16–128	>256	>256	0.5	24
Ceftazidime + clavulanate	1.5–4	>4	>4	0.5	2
Cefotaxime	>256	>256	>256	0.19	>256
Cefotaxime + clavulanate	0.38–1	>1	>1	<0.25	0.5
Cefepime	32–>256	>256	>256	0.125	128
Cefepime + clavulanate	0.094–0.5	>4	>4	0.094	0.19
Aztreonam	48–>256	>256	>256	0.125	>256
Imipenem	0.38–0.5	8	12	0.38	0.38
Meropenem	0.094–0.25	16	16	0.016	0.047
Ertapenem	0.094–1.5	>32	>32	0.012	0.125
Gentamicin	12–24	32	48	0.19	8
Tobramycin	16–32	48	96	0.125	16
Amikacin	1.5–3	3	6	0.75	0.75
Ciprofloxacin	>32	>32	>32	0.047	0.047
Trimethoprim + sulfamethoxazole	>32	>32	>32	0.012	0.032
Tetracyclines	>256	>256	>256	>256	>256

^a Range of MICs for five randomly selected isolates, each recovered from a different patient at different periods of the outbreak.

ompK36 gene from the two pairs of susceptible and resistant isolates by using specific primers. PCR amplification of the genomic DNA from the susceptible isolates yielded an amplicon with the expected size, 1,200 bp (data not shown). On the other hand, PCR amplification of the *ompK36* gene in the resistant isolate KPCR1 consistently failed, and the amplicon obtained with the genomic DNA from the resistant strain

KPCR2 was 800 bp larger than expected (data not shown). Sequencing of this amplicon revealed that the size increase was due the presence of the insertion sequence IS26 in the *ompK36* coding region.

DISCUSSION

The dissemination of ESBL-producing *Enterobacteriaceae* in the hospital setting is a problem with major therapeutic and epidemiological consequences, particularly when it affects wards caring for critically ill patients such as the ICU. Multiple outbreaks of ESBL-producing *Enterobacteriaceae* have indeed been reported over the past two decades, and certainly *K. pneumoniae* has been the most frequently involved organism (33, 35, 36). Nevertheless, to the best of our knowledge, this is the first report of a large outbreak by CTX-M-1-producing *Enterobacteriaceae* and, curiously, the first documented description in the literature of CTX-M-1 in *K. pneumoniae*, despite the fact that this enzyme has been found in multiple species, including *E. coli*, *P. mirabilis*, *M. morganii*, and *C. amalonaticus*. A hospital outbreak due to a *K. pneumoniae* strain producing an ESBL related to CTX-M-1 was described in Japan (23), but since the gene encoding the ESBL was not sequenced and CTX-M-1 has not been reported in the Far East, it is more likely that the enzyme involved was actually one of its close relatives, either CTX-M-3 or CTX-M-15, both of which were already found in Japanese hospitals (3).

CTX-M-2, CTX-M-3, CTX-M-14, and CTX-M-15 are the most widespread CTX-M enzymes (3). As for Spanish hospitals, most CTX-M ESBLs found in a nationwide study were

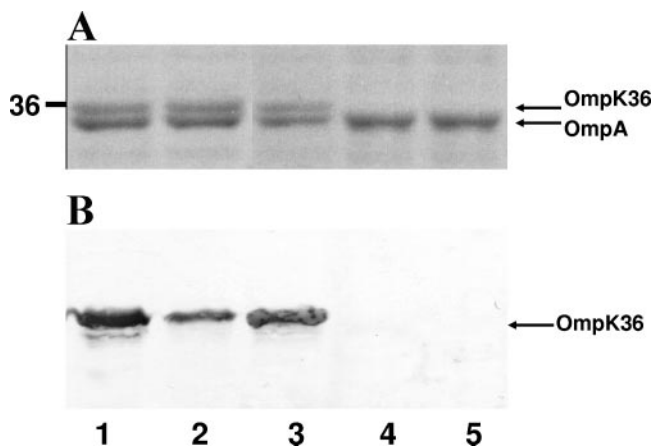


FIG. 3. SDS-PAGE (A) and Western blotting with anti-OmpK36 serum (B) of OMP from *K. pneumoniae* LB1 (lane 1), the carbapenem-susceptible *K. pneumoniae* clinical isolates (lanes 2 and 3), and their respective in vivo-selected carbapenem mutants (lanes 4 and 5). The size of the molecular mass marker (in kilodaltons) is indicated on the left of panel A. Only the relevant parts of the gel and Western blot are shown.

either CTX-M-14 or its close relative CTX-M-9 (16), whereas CTX-M-10, which is closely related to CTX-M-1, was highly disseminated among multiple *Enterobacteriaceae* species in single hospital from Madrid (10, 30). At this stage, the only published documentation of CTX-M-1-producing *Enterobacteriaceae* in Spain is the report of a single *E. coli* isolate detected in cattle milk (6).

Carbapenems are frequently the only therapeutic options available for treatment of hospital-acquired severe infections caused by multiresistant ESBL-producing *Enterobacteriaceae* such as the strain described in this work. Nevertheless, universal susceptibility to these last-line antimicrobials in *Enterobacteriaceae* is no longer guaranteed. Indeed, several carbapenemases have been described as occurring in *Enterobacteriaceae*, including representatives from classes A, such as the highly disseminated KPC enzymes; B, such as IMP or VIM metallo- β -lactamases; or D, such as OXA-48 (29, 34, 39). Additionally, carbapenem resistance development in strains producing ESBLs or AmpC β -lactamases due to the selection of mutants with reduced permeability to these antimicrobials is being increasingly reported (4, 9). Previous work has shown that the expression of both major *K. pneumoniae* porins, OmpK35 and OmpK36, plays a role in the susceptibility to carbapenem antibiotics and that mutants lacking expression of both outer membrane proteins show reduced susceptibility to these antimicrobials, especially when combined with AmpC enzymes and some class A ESBLs (11, 13, 19, 27). Nevertheless, in this work we document and characterize for the first time the development of in vivo resistance to carbapenems in CTX-M-1-producing *Enterobacteriaceae* due to the selection of mutations leading to the lack of porin expression. As frequently occurs in clinical ESBL-producing *K. pneumoniae* isolates (18), the epidemic strain described in this work did not express OmpK35, favoring carbapenem resistance development through the inactivation of OmpK36. Inactivation of OmpK36 in one of the strains was demonstrated to be mediated by the interruption of the coding sequence by an insertion sequence, as has been previously reported for other clinical strains (17). As for the second carbapenem-resistant isolate, the failure of *ompK36* amplification could suggest that the mechanism for inactivation was either a deletion of part or the complete coding sequence or its interruption by an insertion sequence too large to be amplified by conventional PCR.

Overall, CTX-M-1-producing *K. pneumoniae* isolates from 2 of the 44 infected patients (4.5%) developed carbapenem resistance in clinical samples. Nevertheless, both patients had intestinal colonization by the carbapenem-resistant isolate when it was detected in the clinical samples, which could suggest that resistance development may have taken place in the intestines of the colonized patients. Since the development of carbapenem resistance was not systematically explored in the intestinal colonization studies, we may indeed be underestimating the magnitude of the potential problem of carbapenem resistance development in colonized patients as a source of carbapenem-resistant isolates.

In summary, we have described a large outbreak due to a multiresistant CTX-M-1-producing and OmpK35-deficient *K. pneumoniae* strain in an ICU and have characterized the mechanisms leading to in vivo carbapenem resistance development

through the inactivation of OmpK36 in two of the infected patients.

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REFERENCES

- Alberti, S., F. Rodríguez-Quinones, T. Schirmer, G. Rummel, J. M. Tomás, J. P. Rosenbusch, and V. J. Benedí. 1995. A porin from *Klebsiella pneumoniae*: sequence homology, three-dimensional structure, and complement binding. *Infect. Immun.* **63**:903–910.
- Bauernfeind, A., H. Grimm, and S. Schweighart. 1990. A new plasmidic cefotaxime in a clinical isolate of *Escherichia coli*. *Infection* **18**:294–298.
- Bonnet, R. 2004. Growing group of extended-spectrum β -lactamases: the CTX-M enzymes. *Antimicrob. Agents Chemother.* **48**:1–14.
- Bornet, C., A. Davin-Regli, C. Bosi, J. M. Pages, and C. Bollet. 2000. Imipenem resistance of *Enterobacter aerogenes* mediated by outer membrane permeability. *J. Clin. Microbiol.* **38**:1048–1052.
- Bradford, P. A. 2001. Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistant threat. *Clin. Microbiol. Rev.* **14**:933–951.
- Brinas, L., M. A. Moreno, T. Teshager, Y. Sáenz, M. C. Porrero, L. Domínguez, and C. Torres. 2005. Monitoring and characterization of extended-spectrum β -lactamases in *Escherichia coli* strains from healthy and sick animals in Spain in 2003. *Antimicrob. Agents Chemother.* **49**:1262–1264.
- Bush, K., G. A. Jacoby, and A. A. Medeiros. 1995. A functional classification scheme for β -lactamases and its correlation with molecular structure. *Antimicrob. Agents Chemother.* **39**:1211–1233.
- Canton, R., M. Perez-Vazquez, A. Oliver, B. Sanchez Del Saz, M. O. Gutiérrez, M. Martínez-Ferrer, and F. Baquero. 2000. Evaluation of the WIDER system, a new computer-assisted image-processing device for bacterial identification and susceptibility testing. *J. Clin. Microbiol.* **38**:1339–1346.
- Cao, V. T. B., G. Arlet, B. M. Ericsson, A. Tammelin, P. Courvalin, and T. Lambert. 2000. Emergence of imipenem resistance in *Klebsiella pneumoniae* owing to combination of plasmid-mediated CMY-4 and permeability alteration. *J. Antimicrob. Chemother.* **46**:895–900.
- Coque, T. M., A. Oliver, J. C. Perez-Diaz, 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.
- Crowley, B., V. J. Benedí, and A. Domenech-Sanchez. 2002. Expression of SHV-2 β -lactamase and of reduced amounts of OmpK36 porin in *Klebsiella pneumoniae* results in increased resistance to cephalosporins and carbapenems. *Antimicrob. Agents Chemother.* **46**:3679–3682.
- Davison, M. S., and A. O. Summers. 1983. Wide-host-range plasmids in the genus *Thiobacillus*. *Appl. Environ. Microbiol.* **46**:565–572.
- Domenech-Sánchez, A., L. Martínez-Martínez, S. Hernández-Allés, M. C. Conejo, A. Pascual, J. M. Tomás, S. Alberti, and V. J. Benedí. 2003. Role of *Klebsiella pneumoniae* OmpK35 porin in antimicrobial resistance. *Antimicrob. Agents Chemother.* **47**:3332–3335.
- Doutour, C., R. Bonnet, H. Marchandin, M. Boyer, C. Chanal, D. Sirot, and J. Sirot. 2002. CTX-M-1, CTX-M-3, and CTX-M-14 β -lactamases from *Enterobacteriaceae* isolated in France. *Antimicrob. Agents Chemother.* **46**:534–537.
- Filip, C., G. Fletcher, J. L. Wulf, and C. F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. *J. Bacteriol.* **115**:717–722.
- Hernández, J. R., L. Martínez-Martínez, R. Cantón, T. M. Coque, A. Pascual, and the Spanish Group for Nosocomial Infections. 2005. Nationwide study of *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β -lactamases in Spain. *Antimicrob. Agents Chemother.* **49**:2122–2125.
- Hernández-Allés, S., V. J. Benedí, L. Martínez-Martínez, A. Pascual, A. Aguilar, J. M. Tomas, and S. Alberti. 1999. Development of resistance during antimicrobial therapy caused by insertion sequence interruption of porin genes. *Antimicrob. Agents Chemother.* **43**:937–939.
- Hernández-Allés, S., M. Conejo, A. Pascual, J. M. Tomás, V. J. Benedí, and L. Martínez-Martínez. 2000. Relationship between outer membrane alterations and susceptibility to antimicrobial agents in isogenic strains of *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* **46**:273–277.
- Jacoby, G. A., D. M. Mills, and N. Chow. 2004. Role of β -lactamases and

- porins in resistance to ertapenem and other β -lactams in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **48**:3203–3206.
20. Jarlier, V., M. Nicolas, G. Fournier, and A. Philippon. 1988. Extended broad-spectrum β -lactamases conferring transferable resistance to newer β -lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. **10**:867–878.
 21. Kaufmann, M. E. 1998. Pulsed-field gel electrophoresis. Methods Mol. Med. **15**:17–31.
 22. Kliebe, C., B. A. Nies, J. F. Meyer, R. M. Toixdorf-Neutzing, and B. Wiedemann. 1985. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob. Agents Chemother. **28**:302–307.
 23. Komatsu, M., N. Ikeda, M. Aihara, Y. Nakamachi, S. Kinoshita, K. Yamasaki, and K. Shimakawa. 2000. Hospital outbreak of MEN-1-derived extended spectrum beta-lactamase-producing *Klebsiella pneumoniae*. J. Infect. Chemother. **7**:94–101.
 24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
 25. Livermore, D. M. 1997. β -Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8**:557–584.
 26. Martínez-Martínez, L., S. Hernández-Allés, S. Albertí, J. M. Tomás, V. J. Benedí, and G. A. Jacoby. 1996. In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. **40**:342–348.
 27. Martínez-Martínez, L., A. Pascual, S. Hernández-Allés, D. Álvarez-Díaz, A. I. Suárez, J. Tran, V. J. Benedí, and G. A. Jacoby. 1999. Roles of β -lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **43**:1669–1673.
 28. Mugnaioli, C., F. Luzzaro, F. De Luca, G. Brigante, G. Amicosante, and G. M. Rossolini. 2005. Dissemination of CTX-M-type extended-spectrum β -lactamase genes to unusual hosts. J. Clin. Microbiol. **43**:4183–4185.
 29. Nordmann, P., and L. Poirel. 2002. Emerging carbapenemases in gram-negative aerobes. Clin. Microbiol. Infect. **8**:321–331.
 30. Oliver, A., T. M. Coque, D. Alonso, A. Valverde, F. Baquero, and R. Cantón. 2005. CTX-M-10 linked to a phage-related element is widely disseminated among Enterobacteriaceae in a Spanish hospital. Antimicrob. Agents Chemother. **49**:1567–1571.
 31. Pagani, L., E. Dell'Amico, R. Migliavacca, M. M. D'Andrea, E. Giacobone, G. Amicosante, E. Romero, and G. M. Rossolini. 2003. CTX-M-type extended-spectrum β -lactamases in nosocomial isolates of Enterobacteriaceae from a hospital in northern Italy. J. Clin. Microbiol. **41**:4264–4269.
 32. Paterson, D. L., L. Mulazimoglu, J. M. Casellas, W. C. Ko, H. Goossens, A. Von Gotteberg, S. Mohapatra, G. M. Trenholme, K. P. Kugman, J. G. McCormack, and V. L. Yu. 2000. Epidemiology of ciprofloxacin resistance and its relationship to extended-spectrum beta-lactamase production in *Klebsiella pneumoniae* isolates causing bacteremia. Clin. Infect. Dis. **30**:473–478.
 33. Peña, C., M. Pujol, C. Ardanuy, A. Ricart, R. Pallares, J. Liñares, J. Ariza, and F. Gudiol. 1998. Epidemiology and successful control of a large outbreak due to *Klebsiella pneumoniae* producing extended-spectrum beta-lactamases. Antimicrob. Agents Chemother. **42**:53–58.
 34. Poirel, L., C. Héritier, V. Tolün, and P. Nordmann. 2004. Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **48**:15–22.
 35. Quale, J. M., D. Landman, P. A. Bradford, M. Visalli, J. Ravishankar, C. Flores, D. Mayorga, K. Vangala, and A. Adedeji. 2002. Molecular epidemiology of a citywide outbreak of extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* infection. Clin. Infect. Dis. **35**:834–841.
 36. Rebuck, J. A., K. M. Olsen, P. D. Fey, A. N. Langnas, and M. E. Rupp. 2000. Characterization of an outbreak due to extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* in a pediatric intensive care unit transplant population. Clin. Infect. Dis. **31**:1368–1372.
 37. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33**:2233–2239.
 38. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
 39. Yigit, H., A. M. Queenan, G. J. Anderson, A. Domenech-Sanchez, J. W. Biddle, C. D. Steward, S. Alberti, K. Bush, and F. C. Tenover. 2001. Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **45**:1151–1161.