Prospective Multicenter Study of Carbapenemase-Producing Enterobacteriaceae from 83 Hospitals in Spain Reveals High In Vitro Susceptibility to Colistin and Meropenem

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The aim of this study was to determine the impact of carbapenemase-producing Enterobacteriaceae (CPE) in Spain in 2013 by describing the prevalence, dissemination, and geographic distribution of CPE clones, and their population structure and antibiotic susceptibility. From February 2013 to May 2013, 83 hospitals (about 40,000 hospital beds) prospectively collected nonduplicate Enterobacteriaceae using the screening cutoff recommended by EUCAST. Carbapenemase characterization was performed by phenotypic methods and confirmed by PCR and sequencing. Multilocus sequencing types (MLST) were determined for 0.04% cases of CPE, which were mostly VIM-1 and IMP-22 (4). Systems have been affected and the carbapenemase types that are resistant to almost all available antibiotics (1, 2), so there are few alternative treatment options. The most common carbapenemases are KPC (class A); VIM, IMP, and NDM (class B); and the OXA-48 types (class D). However, the extent to which health care systems have been affected and the carbapenemase types that are prevalent differ substantially from country to country (3).

A multicenter study performed in Spain in 2009 revealed 43 (0.04%) cases of CPE, which were mostly VIM-1 and IMP-22 (4). After that, we reported a rapid increase in the number of cases of CPE, mainly OXA-48-producing K. pneumoniae, in this country from 2010 to 2012 (5–7).

Because previous studies (5, 6) were based on voluntary reports without taking into account key important issues, in this paper, we present data on the impact of CPE as obtained from a prospective, multicenter, and population-based study. We show that carbapenemase production in this country is widely and irregularly distributed; however, the rates of susceptibility to meropenem and colistin were still high.

(Carbanapenem-producing Enterobacteriaceae (CPE), mainly Klebsiella pneumoniae, are an emerging threat to public and individual health worldwide. These microorganisms are often resistant to almost all available antibiotics (1, 2), so there are few alternative treatment options. The most common carbapenemases are KPC (class A); VIM, IMP, and NDM (class B); and the OXA-48 types (class D). However, the extent to which health care systems have been affected and the carbapenemase types that are predominant differ substantially from country to country (3).

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MATERIALS AND METHODS

Study design and bacterial isolates. A prospective multicenter study was designed to identify Enterobacteriaceae isolates with decreased susceptibility to carbapenems. The isolates were collected from clinical infections and carriers between February and May 2013. Eighty-three Spanish hospitals from 33 out of the 50 Spanish provinces participated in the study; these 33 provinces belonged to 15 of the 17 Spanish Autonomous Communities. Eight of the hospitals acted as coordinating centers. The estimated catchment population was approximately one-half of the Spanish population and consisted of approximately 21.7 million individuals and 40,100 hospital beds. The participating hospitals registered the total number of infections caused by Enterobacteriaceae during the study period so that the values for CPE prevalence could be estimated; the presence of infections was established according to previously defined criteria (9).

EUCAST screening cutoff values were used to identify CPE (10). The inclusion criteria were all Enterobacteriaceae isolates presenting either MICs of $>$0.125 mg/liter to meropenem and/or ertapenem and/or $>$1 mg/liter to imipenem, or disk inhibition zones obtained using the disk diffusion method of $<$25 mm to meropenem and/or ertapenem and/or $<$23 mm to imipenem. Only one isolate per patient and species was considered for further analysis. Isolates from the genera Proteus, Providencia, and Morganella that had reduced susceptibility to imipenem but were susceptible to ertapenem and meropenem were not included in the analysis; in addition, Enterobacter isolates that had reduced susceptibility to ertapenem but were susceptible to imipenem and meropenem were excluded.

Bacterial identification and drug susceptibility testing. The initial assays on the isolates were performed at each participating hospital using standard microbiological methods. Each hospital also submitted their isolates to one of the eight coordinating centers, where carbapenem production was confirmed using phenotypic and genotypic methods. Finally, all study isolates were submitted to the antibiotic laboratory of the Spanish National Centre of Microbiology, which acted as a central reference laboratory. All isolates meeting the phenotype inclusion criteria (10) were classified using the algorithm for phenotypic carbapenemase detection recommended by EUCAST (10). A modified Hodge test using a meropenem disk with cloxacillin (600 μg) was performed on all isolates. In addition, the degree of inhibition of carbapenemase activity was determined by comparing the inhibition zones obtained from meropenem disks with or without EDTA (10 μl of a 0.5 M solution), phenylboronic acid (400 μg), and cloxacillin (600 μg) in all isolates. The Carba NP method was used as a confirmatory test of carbapenemase activity when unclear phenotypic results or discrepancies between the phenotypic and genotypic results were observed (11).

Antibiotic susceptibility testing was performed using the disk diffusion and microdilution susceptibility methods, according to EUCAST guidelines (12, 13); in addition, susceptibility to ertapenem, imipenem, meropenem, and colistin was determined by a gradient test (bioMérieux, Marcy-l’Étoile, France).

Extended-spectrum β-lactamase (ESBL) production in OXA-48 and class B carbapenemase producers was suspected if activity of cefotaxime and aztreonam, respectively, was recovered in the presence of clavulanic acid. In the case of KPC producers, the molecular characterization of ESBL genes was carried out in all isolates.

Characterization of resistance mechanisms. The presence of genes encoding carbapenemases (blaOXA-48, blaKPC, blaNDM, blaVIM, and blaIMP) and ESBLs (blaTEM, blaSHV, and blaCTX-M) (14) was determined using PCR and DNA sequencing assays.

Molecular epidemiology. Multilocus sequence typing (MLST) was performed for all carbapenemase-producing K. pneumoniae isolates using the Institut Pasteur scheme (http://www.pasteur.fr/recherche/genopole/PFB/mlst/Kpneumoniae.html). Carbapenemase-producing Escherichia coli isolates were typed by MLST using the University of Warwick (Warwick Medical School, Coventry, United Kingdom) scheme (http://mlst.warwick.ac.uk/). The phylogenetic relationships among the different sequence types (STs) found in this study were established according to the eBURST program version 3 (http://eburst.mlst.net).

A simple diversity index (SDI) previously described by Gastmeier et al. (15) was applied to analyze the population diversity, calculated as (number of STs/total number of isolates) $\times$ 100.

We considered that two or more isolates of K. pneumoniae or E. coli (including clinical cases and carriers) were epidemiologically related if they belonged to the same species, had the same MLST, and produced the same carbapenemase type. For Enterobacter cloaceae, this epidemiological association was established when the genetic linkage was $>$85% using pulsed-field gel electrophoresis (PFGE) after total chromosomal DNA digestion with XbaI (14).

Statistical analysis. Differences in the prevalence values for resistance mechanisms between the different groups were assessed using Fisher’s exact test. Associations were determined by calculating odds ratios (ORs) with their 95% confidence intervals (CIs). The null hypothesis was rejected for $P$ values of $<$0.05. Statistical analysis was performed using the GraphPad Prism software, version 3.02 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

Bacterial isolates and carbapenemase types. A total of 702 Enterobacteriaceae isolates that were collected from an equal number of patients met the phenotypic criteria for inclusion, with K. pneumoniae (53.3%), E. cloacae (15.8%), and E. coli (13.8%) being the most common species (Table 1). Of these 702 isolates, 379 (54%) were confirmed to be CPE and were distributed as follows: 282 (74.4%) were K. pneumoniae, 39 (10.3%) were E. cloacae, 32 (8.4%) were E. coli, 11 (2.9%) were Klebsiella oxytoca, 7 (1.8%) were Citrobacter freundii, 4 (1.1%) were Serratia marcescens, 2 (0.5%) were Enterobacter aerogenes, 1 (0.3%) was Morganella morganii, and 1 (0.3%) was an Enterobacter species (Table 1). The percentages of CPE isolates significantly varied between species: 75.4% (282 of 374) of the K. pneumoniae, 35.1% (39 of 111) of the E. cloacae, and 33% (32 of 97) of the E. coli isolates were CPE isolates; the percentage obtained for K. pneumoniae was significantly higher than that for E. cloacae and E. coli ($P$ $<$ 0.0001). In a recent French study, 1485 Enterobacteriaceae isolates that were nonsusceptible to carbapenems, according to the EUCAST criteria, were detected (42.2% and 35.2% were Enterobacter spp. and K. pneumoniae, respectively); of them, 340 (22.9%) isolates were carbapenem producers (65.9% and 9.7% were K. pneumoniae and Enterobacter spp., respectively) (16).

Of the 379 CPE isolates detected, 300 (79.2%) were isolated from clinical samples and were mostly from urine (158 [52.7%]), wound (59 [19.7%]), respiratory (37 [12.3%]), and blood (28 [9.3%]) samples. The remaining 79 CPE (20.2%) isolates were from screening samples (81% from rectal or perianal samples). In total, 193 CPE cases (50.9%) were isolated from males, and 239 (63.1%) were from patients $>$65 years of age.

The carbapenemase types identified were 271 (71.5%) OXA-48 group (258 OXA-48 and 13 OXA-245), 96 (25.3%) VIM (95 VIM-1 and one VIM-2), 8 (2.1%) KPC-2, and 6 (1.6%) IMP (4 IMP-13 and two IMP-22). Each of the two K. oxytoca isolates produced both KPC-2 and VIM-1; these two isolates came from two different hospitals from the Madrid area and were isolated in March 2013 and May 2013. The isolates from carriers had the following carbapenemase types: 69.6% OXA-48 group, 30.3% VIM group, and 1.3% KPC. K. pneumoniae was more prevalent among the OXA-48 isolates (86.7%) than among the VIM isolates (44.8%) ($P$ $<$ 0.0001). A comparison of OXA-48-group- and VIM-group-producing isolates is shown in Table 2.
OXA-48 prevalence is also increasing in other European countries, such as France, Germany, Belgium, and the United Kingdom, where increasing numbers of outbreaks have been described (1, 17–21). However, OXA-48 is rarely identified in North America, where increasing numbers of outbreaks have been described, such as France, Germany, Belgium, and the United Kingdom. Enterobacteriaceae have already caused sporadic hospital outbreaks in North America (1). The number of carbapenemase-producing E. coli isolates identified in this study was much higher than that in previous studies (4, 5). This remarkable finding is of serious concern, because E. coli may facilitate the community spread of carbapenemases.

ESBL production was detected in 267 of the 379 CPE (70.4%) isolates; 227 (85%) produced CTX-M-15, 16 (6%) produced SHV-12, 15 (5.6%) produced CTX-M-9, 9 (3.4%) produced SHV-134, 1 (0.4%) produced CTX-M-14, and 1 (0.4%) produced CTX-M-1. Two CPE isolates had two different types of ESBLs. Carbapenemase-producing K. pneumoniae isolates more frequently coproduced KPC (235 of 282 isolates [83.3%]) than did E. cloacae (18 of 38 isolates [47.4%]) or E. coli (11 of 32 isolates [34.4%]) (P < 0.0001). The coproduction of ESBLs occurred in 90.6% and 28% of the OXA-48-producing K. pneumoniae and E. coli isolates, respectively (P < 0.0001), mostly of the CTX-M-15 type. Potron et al. (25) found that the coproduction of OXA-48 and CTX-M-15 occurred in 41.7% of OXA-48-producing E. coli isolates from 10 different European and African countries. Five OXA-48- and SHV-12-producing K. pneumoniae isolates belonging to ST147 were isolated in Asturias (northern Spain). Associations between OXA-48 and ESBLs of the SHV type have infrequently been reported (25).

**Antibiotic susceptibility testing.** Overall, the antibiotics showing the highest percentages of susceptibility were colistin (95.5%), amikacin (81.3%), meropenem (74.7%), ticaglycine (71%), and imipenem (67.6%) (Table 3). However, antibiotic susceptibility significantly varied between the OXA-48-producing and VIM-producing isolates (Table 3), with the VIM-producing isolates usually being more resistant.

A total of 182 CPE (48%) isolates were susceptible to colistin, of which 92 (50%) were susceptible to meropenem, 85 (47%) to ticaglycine, 78 (43%) to imipenem, 76 (42%) to gentamicin, 54 (30%) to ciprofloxacin, 45 (25%) to tobramycin, 41 (23%) to aztreonam, 38 (21%) to fosfomycin, 33 (18%) to trimethoprim-sulfamethoxazole, 18 (10%) to ceftazidime, 10 (6%) to cefotaxime, and 6 (4%) to ertapenem. The susceptibility of OXA-48-producing K. pneumoniae and E. coli isolates, respectively (P < 0.0001), mostly of the CTX-M-15 type. Potron et al. (25) found that the coproduction of OXA-48 and CTX-M-15 occurred in 41.7% of OXA-48-producing E. coli isolates from 10 different European and African countries. Five OXA-48- and SHV-12-producing K. pneumoniae isolates belonging to ST147 were isolated in Asturias (northern Spain). Associations between OXA-48 and ESBLs of the SHV type have infrequently been reported (25).

**TABLE 3 Susceptibility to antibiotics in carbapenemase-producing Enterobacteriaceae isolates**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Total susceptibility (%), n = 379</th>
<th>Susceptibility (%), n = 270</th>
<th>OXA-48 group producing (n = 270)</th>
<th>VIM group producing (n = 97)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colistin</td>
<td>95.5</td>
<td>95.2</td>
<td>95.9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>81.3</td>
<td>84.8</td>
<td>73.2</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>74.7</td>
<td>80</td>
<td>63.9</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Ticaglycine</td>
<td>71</td>
<td>72.6</td>
<td>67</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>67.6</td>
<td>74.8</td>
<td>49.5</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>48</td>
<td>44.8</td>
<td>57.7</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>33.2</td>
<td>37.4</td>
<td>22.7</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Aztreonam</td>
<td>20.1</td>
<td>12.2</td>
<td>40.2</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>16.4</td>
<td>20.7</td>
<td>5.2</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>16.1</td>
<td>13.7</td>
<td>18.6</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>12.3</td>
<td>9.3</td>
<td>23.7</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>9.5</td>
<td>13.3</td>
<td>0</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>7.7</td>
<td>10.8</td>
<td>0</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Ertapenem</td>
<td>7.1</td>
<td>4.1</td>
<td>16.5</td>
<td>0.0002</td>
<td></td>
</tr>
</tbody>
</table>

"Table 3" is a table showing the susceptibility of various antibiotics to OXA-48 and VIM carbapenemase-producing Enterobacteriaceae isolates. The table includes columns for the antibiotic, total susceptibility, and susceptibility of OXA-48 and VIM group producing isolates, along with P values for the comparison between the two groups. The table also shows the percentage of isolates susceptible to each antibiotic, with notable differences in susceptibility between the two groups.
amikacin, tigecycline, and carbapenems (imipenem or meropenem); of them, 107 were also susceptible to fosfomycin. According to previous clinical experience (2), the use of a carbapenem (meropenem or imipenem) for the treatment of a CPE with an MIC of ≤8 mg/liter, in combination with another active agent, seems reasonable; according to these criteria, we identified 21 highly resistant CPE isolates (5.5% [12 OXA-48 isolates, 8 VIM-1 isolates, and 1 KPC-2 isolate]) presenting only one or two treatment options from which to choose for clinical purposes, mainly, colistin and carbapenems (∼5) or colistin and tigecycline (∼5).

Geographic distribution and prevalence of infections due to CPE in Spain. At least one case of CPE was identified at 45 (54.1%) of the 83 participating hospitals (Fig. 1). These hospitals were located in 18 of the 33 (54.5%) participating provinces. In 21 (46.7%) of the 45 hospitals with CPE isolates, potential outbreaks of epidemiologically related CPE isolates were detected, affecting 209 (55.1%) of the 379 CPE isolates included in this study.

Data about the total number of infections caused by Enterobacteriaceae during the study period were provided by 75 (90.4%) of the participating hospitals. A total of 120,808 single infections were identified by patient and species. The estimated overall prevalence of infection by carbapenemase-producing K. pneumoniae was 1.7% (range, 0 to 11.6%; 231/13,842), and 23 (30.7%) hospitals had a prevalence of >1%. For Enterobacter spp. and E. coli, these figures were 0.5% (range, 0 to 6.4%; 28/5,085) and 0.03% (range, 0 to 0.4%; 28/91,553), respectively. The prevalences of carbapenemase production in K. pneumoniae and E. coli in a multicenter study performed in Spain in 2009 were 0.2% and 0.001%, respectively (4).

Although the number of OXA-48-producing isolates was greater than the number of VIM-producing isolates (271 versus 96, Table 1), the VIM-producing isolates were more widely geographically distributed. VIM-producing isolates were detected in 16 (40.5%) provinces, and OXA-48-producing isolates were detected in 10 (30.3%) provinces. This finding might be related to the earlier detection of VIM in Spain that occurred in 2005 (26) compared with that of OXA-48 in 2009 (27).

Population structure of carbapenemase-producing K. pneumoniae and E. coli isolates causing clinical infections. Using
MLST, 24 different sequence types (STs) were identified among the 221 carbapenemase-producing *K. pneumoniae* isolates implicated in clinical infections (SDI, 10.6; mean, 9.2 isolates per ST; range, 1 to 66). The most prevalent STs were ST11 (66 isolates [29.9%]), ST405 (65 isolates [29.4%]), ST15 (28 isolates [12.7%]), and ST326 (15 isolates [6.8%]). These four STs were found in 78.8% of the carbapenemase-producing *K. pneumoniae* isolates. ST405 and ST11 clinical isolates were each isolated in 18 different isolates. ST11 carried four different types of carbapenemases (VIM-1, OXA-48, KPC-2, and OXA-245), but ST405 carried OXA-48 only. Analysis with eBURST provided an overview of the STs of the Spanish carbapenemase-producing *K. pneumoniae* isolates from this study compared with those of all *K. pneumoniae* isolates from the MLST database. The major STs found are represented in Fig. 2. Excluding the isolates from this study, ST11 was the second most frequent ST in the MLST database (103 of 2,405 [4.28%]), and ST405 was uncommon (4 of 2,405 [0.17%]). In this eBURST analysis, ST405 was shown as a singleton nonrelated to other classical STs (Fig. 2). These results suggest that *K. pneumoniae* isolates belonging to ST11 and ST405 strongly contribute to the dissemination of OXA-48-producing *Enterobacteriaceae* in Spain. ST11 has been identified as an international high-risk clone associated with ESBL and carbapenemase production (28, 29), but ST405 has recently been associated with OXA-48 production in Spain, Belgium, and France (5, 6, 18, 19).

Among the 27 carbapenemase-producing *E. coli* isolates implicated in clinical infections, 16 different STs were identified (SDI, 59.3; mean, 1.7 isolates per ST; range, 1 to 7). ST131 (7 isolates [26%]) and ST156 (3 isolates [11.1%]) were the most prevalent STs identified. These findings suggest that compared with *K. pneumoniae*, carbapenemase-producing *E. coli* isolates may have a more diverse and polyclonal population structure, as has been demonstrated for other resistance mechanisms, like ESBL (30). The acquisition of carbapenemases by the globally distributed *E. coli* ST131 detected in this and other studies (31, 32) is a finding of serious concern. In a recent study of OXA-48-producing *K. pneumoniae* and *E. coli* isolates in several European and North African countries, the most common STs identified were ST101 and ST38, respectively (25).

**Potential interregional spread of CPE strains.** One finding with epidemiological implications was that some of the more prevalent *K. pneumoniae* clones carrying carbapenemases were identified in more than one Spanish province, suggesting that potential interregional spread of these clones may have occurred; however, dissemination of OXA-48-encoding plasmids between isolates of the same prevalent ST cannot be excluded. ST405/OXA-48 was detected in six, ST15/OXA-48 in four, and ST11/OXA-48 and ST11/VIM-1 in three different Spanish provinces each. This interregional spread clearly indicates that further progress has occurred since the “independent hospitals outbreak” stage described by Cantón et al. (1).

In addition, these carbapenemase genes are carried by plasmids and, therefore, their spread is probably due to both the clonal dissemination of a few specific strains and the transmission of epidemic autotransferred plasmids carrying them (1, 6, 23).

**Conclusions.** We found that there was a wide geographic distribution of CPE species and a clear increasing trend in the number of infections caused by CPE in Spain. Two successful clones of *K. pneumoniae* (ST11 and ST405) carried mainly OXA-48, while ST15 more often carried VIM. Although still infrequent, the detection of a polyclonal dissemination of OXA-48-producing *E. coli* has serious implications for public health.

According to *in vitro* susceptibilities, most of the CPE (94.5%) had three or more options for antibiotic treatment.
The spread of CPE in Spain affected ≥68% of all provinces, with a potential interregional spread of CPE strains. This finding also suggests that the public health situation posed by CPE has worsened in the last few years in Spain.

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