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

Jesús Oteo,[a] Adriana Ortega,[a] Rosa Bartolomé,[b] Germán Bou,[c] Carmen Conejo,[d] Marta Fernández-Martínez,[e] Juan José González-López,[b] Laura Martínez-García,[f] Luis Martínez-Martínez,[e,g] María Merino,[c] Elisenda Miró,[h] Marta Mora,[i] Ferran Navarro,[h] Antonio Oliver,[j] Álvaro Pascual,[d,k] Jesús Rodríguez-Baño,[k,l] Guillermo Ruiz-Carrascoso,[m] Patricia Ruiz-Garbajosa,[f] Laura Zamorano,[j] Verónica Bautista,[a] María Pérez-Vázquez,[a] José Campos,[a,n] on behalf of GEIH-GEMARA (SEIMC) and REIPI

Laboratorio de Antibióticos, Bacteriología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain[a]; Servei de Microbiologia, Hospital Vall d'Hebrón, Universitat Autònoma de Barcelona, Barcelona, Spain[b]; Servicio de Microbiología-INIBIC, Complejo Hospitalario Universitario A Coruña, A Coruña, Spain[c]; Departamento de Microbiología, Universidad de Sevilla, Seville, Spain[d]; Servicio de Microbiología, Hospital Universitario Marqués de Valdecilla-IDIVAL, Santander, Spain[e]; Servicio de Microbiología, Hospital Universitario Ramón y Cajal e Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain[f]; Departamento de Biología Molecular, Universidad de Cantabria, Santander, Spain[g]; Servei de Microbiología, Hospital de la Santa Creu i Sant Pau, Institut d'Investigació Biomèdica Sant Pau, Barcelona, Spain[h]; Unidad de Microbiología Clínica y Enfermedades Infecciosas, Hospital Universitario La Paz-IdiPAZ, Madrid, Spain[i]; Servicio de Microbiología, Hospital Son Espases, Instituto de Investigación Sanitaria de Palma (IdISPa), Palma de Mallorca, Spain[j]; Unidad Clínica de Enfermedades Infecciosas, Microbiología y Medicina Preventiva, Hospitales Universitarios Virgen Macarena y Virgen del Rocío, Seville, Spain[k]; Departamento de Medicina, Universidad de Sevilla, Seville, Spain[l]; Servicio de Microbiología, Hospital Universitario La Paz-IdiPAZ, Madrid, Spain[m]; Consejo Superior de Investigaciones Científicas, Madrid, Spain[n]

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 *Klebsiella pneumoniae* and *Escherichia coli*. A total of 702 *Enterobacteriaceae* isolates met the inclusion criteria; 379 (54%) were CPE. OXA-48 (71.5%) and VIM-1 (25.3%) were the most frequent carbapenemases, and *K. pneumoniae* (74.4%), *Enterobacter cloacae* (10.3%), and *E. coli* (8.4%) were the species most affected. Susceptibility to colistin, amikacin, and meropenem was 95.5%, 81.3%, and 74.7%, respectively. The most prevalent sequence types (STs) were ST11 and ST405 for *K. pneumoniae* and ST131 for *E. coli*. Forty-five (54.1%) of the hospitals had at least one CPE case. For *K. pneumoniae*, ST11/OXA-48, ST15/OXA-48, ST405/OXA-48, and ST11/VIM-1 were detected in two or more Spanish provinces. ST11 isolates carried four carbapenemases (VIM-1, OXA-48, KPC-2, and OXA-245), but ST405 isolates carried OXA-48 only. A wide interregional spread of CPE in Spain was observed, mainly due to a few successful clones of OXA-48-producing *K. pneumoniae* (e.g., ST11 and ST405). The dissemination of OXA-48-producing *E. coli* is a new finding of public health concern. According to the susceptibilities determined *in vitro*, most of the CPE (94.5%) had three or more options for antibiotic treatment.

Carbapenemase-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).

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.

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Address correspondence to Jesús Oteo, jesus.oteo@isciii.es.

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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 carbapenemase 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 (bla_{OXA-48}, bla_{KPC}, bla_{VIM}, bla_{IMP}, and bla_{NDM}) (5) and ESBLs (bla_{TEM}, bla_{SHV}, and bla_{CTX-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/PF8/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) × 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 cloacae*, 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 the EUCAST criteria, were detected (42.2% and 35.2% were *Enterobacter* spp. and *K. pneumoniae*, respectively); of them, 340 (22.9%) isolates were carbapenemase 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.

TABLE 1 Distribution of carbapenemase-producing *Enterobacteriaceae*

Species	All isolates (no. [%])	No. (%) of isolates detected					
		CBP negative[a]	CBP positive	OXA-48 group	VIM group	KPC group	IMP group
K. pneumoniae	374 (53.3)	92 (28.5)	282 (74.4)	235 (86.7)	43 (44.8)	3 (37.5)	1(16.7)
E. cloacae	111 (15.8)	72 (22.3)	39 (10.3)	5 (1.8)	29 (30.2)	1 (12.5)	4 (66.6)
E. coli	97 (13.8)	65 (20.1)	32 (8.4)	25 (9.2)	7 (7.3)	0	0
K. oxytoca	16 (2.3)	5 (1.5)	11 (2.9)	1 (0.4)	10 (10.4)[b]	2 (25)[b]	0
C. freundii	14 (2)	7 (2.2)	7 (1.8)	2 (0.7)	3 (3.1)	2 (25)	0
S. marcescens	13 (1.8)	9 (2.8)	4 (1.1)	3 (1.1)	1 (1)	0	0
E. aerogenes	34 (4.8)	32 (9.9)	2 (0.5)	0	2 (2.1)	0	0
M. morganii	21 (3)	20 (6.2)	1 (0.3)	0	1 (1)	0	0
Enterobacter spp.	5 (0.7)	4 (1.2)	1 (0.3)	0	0	0	1 (16.7)
Other	17 (2.4)	17 (5.3)	0	0	0	0	0
Total	702 (100)	323 (100)	379 (100)	271 (100)	96 (100)	8 (100)	6 (100)

[a] CBP, carbapenemase.
[b] Two isolates produced both KPC and VIM carbapenemases.

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 (22). Compared with OXA-48 and VIM, KPC was identified very infrequently in this study. However, KPC-producing *Enterobacteriaceae* have already caused sporadic hospital outbreaks in Spain (3, 23). KPC enzymes are endemic in other European countries, such as Greece and Italy (1, 3, 24), and they produce nosocomial 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 ESBLs (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%]) isolates ($P < 0.0001$). 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).

TABLE 2 Comparison of OXA-48-group- and VIM-group-producing isolates

Variable	No. (%) of isolates producing:		Odds ratio	95% CI[a]	*P* value
	OXA-48 group	VIM group			
Age >65 yr	187 (69)	32 (47.4)	2.47	1.54–3.97	0.0002
K. pneumoniae	235 (86.7)	43 (44.8)	8.05	4.72–13.72	<0.0001
ST11	79 (33.6)	4 (9.3)	4.94	1.70–14.31	<0.0001
ST405	73 (31.1)	0	39.35	3.39– 648.40	<0.0001
ST15	22 (9.4)	15 (34.9)	0.19	0.089–0.41	<0.0001
ST326	22 (9.4)	1 (2.3)	4.34	0.57–33.08	0.22
E. coli	25 (9.2)	7 (7.2)	1.31	0.55–3.13	0.67
E. cloacae	5 (1.8)	29 (29.9)	0.044	0.016–0.12	<0.0001
K. oxytoca	1 (0.4)	10 (10.3)	0.032	0.004–0.25	<0.0001

[a] CI, confidence interval.

Antibiotic susceptibility testing. Overall, the antibiotics showing the highest percentages of susceptibility were colistin (95.5%), amikacin (81.3%), meropenem (74.7%), tigecycline (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,

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

Antibiotic	Total susceptibility (%) ($n = 379$)	Susceptibility (%) of indicated isolate		*P* value
		OXA-48-group producing ($n = 270$)	VIM-group producing ($n = 97$)	
Colistin	95.5	95.2	95.9	1
Amikacin	81.3	84.8	73.2	0.014
Meropenem	74.7	80	63.9	0.002
Tigecycline	71	72.6	67	0.30
Imipenem	67.6	74.8	49.5	<0.0001
Fosfomycin	48	44.8	57.7	0.03
Chloramphenicol	39.6	46.7	23.7	<0.0001
Gentamicin	33.2	37.4	22.7	0.008
Aztreonam	20.1	12.2	40.2	0.0001
Tobramycin	16.4	20.7	5.2	0.0002
Trimethoprim-sulfamethoxazole	16.1	13.7	18.6	0.25
Ciprofloxacin	12.7	9.3	23.7	0.0007
Ceftazidime	9.5	13.3	0	<0.0001
Cefotaxime	7.7	10.8	0	0.0001
Ertapenem	7.1	4.1	16.5	0.0002

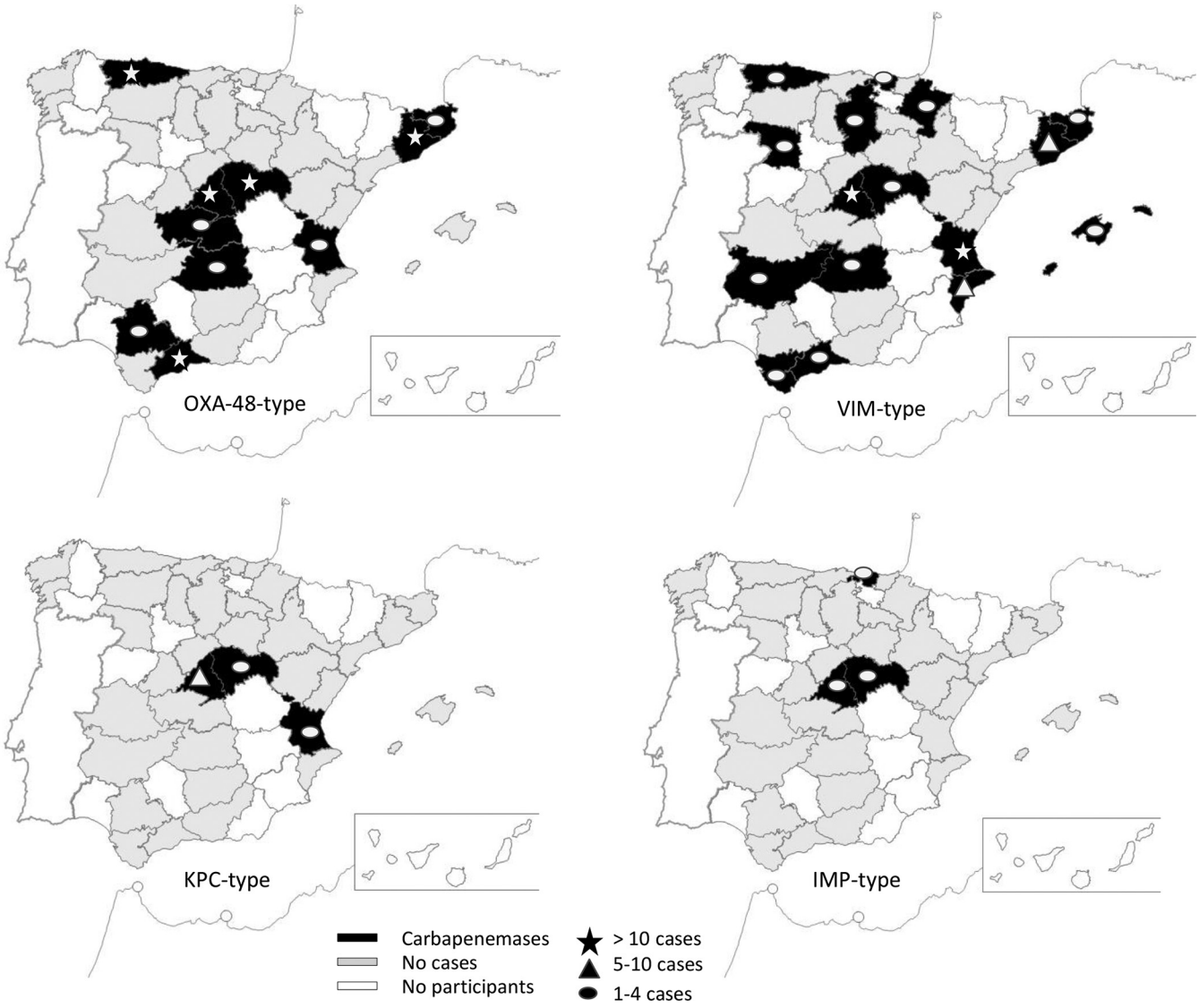

FIG 1 Geographic distribution of carbapenemase types detected during a prospective multicenter study in Spain (February to May 2013).

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 ($n = 5$) or colistin and tigecycline ($n = 5$).

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

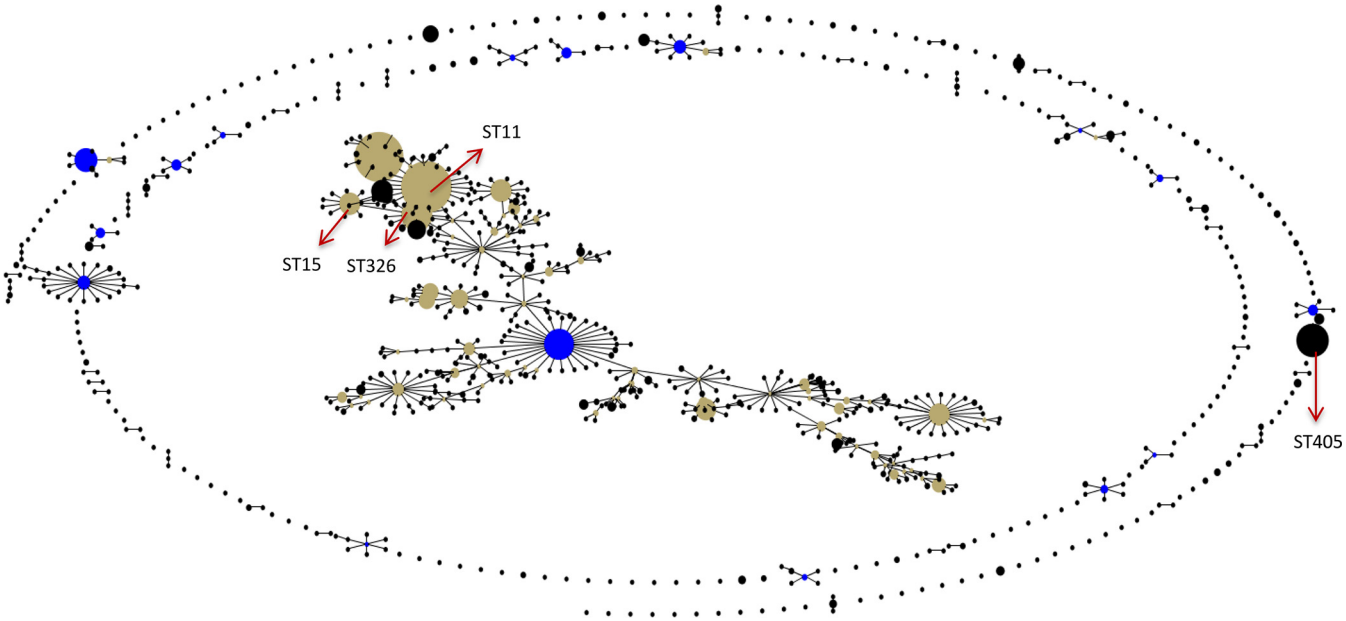

FIG 2 Population snapshot of sequence types (STs) of *K. pneumoniae* isolates from the multilocus sequence typing (MLST) database and STs of carbapenemase-producing *K. pneumoniae* isolates from this study considered together. The most important ST complexes found in this study (ST11, ST405, ST15, and ST 326) are emphasized. Some STs with single isolates from the MLST database have been excluded from the image for easy viewing. Each ST is represented by a dot, and lines connect single-locus variants. The size of the dots is related to the number of isolates. Blue dots represent the putative founders, and grey dots represent putative subgroup founders.

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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