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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Prospective multicenter study of carbapenemase producing Enterobacteriaceae from 83 hospitals in Spain: High in vitro susceptibility to colistin and meropenem

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Abbreviations: GEIH-GEMARA, Grupo de Estudio de Infección Hospitalaria-Grupo de Estudio de Mecanismos de Acción y Resistencia a Antimicrobianos; REIPI, Red Española de Investigación en Patología Infecciosa; SEIMC, Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica.

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Abstract

The aim of this study was to determine the impact of the carbapenemase-producing Enterobacteriaceae (CPE) in Spain in 2013 by describing their prevalence, dissemination and geographic distribution of CPE clones, their population structure and antibiotic susceptibility.

From February 2013 to May 2013, 83 hospitals (about 40,000 hospital beds) prospectively collected non-duplicate Enterobacteriaceae using the screening cut-off recommended by EUCAST. Carbapenemase characterisation was performed by phenotypic methods and confirmed by PCR and sequencing. MLST types 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 STs were ST11 and ST405 in *K. pneumoniae*, and ST131 in *E. coli*. Forty-five (54.1%) of the hospitals had at least one CPE case. In *K. pneumoniae*, ST11/OXA-48, ST15/OXA-48, ST405/OXA-48, and ST11/VIM-1 were detected in two or more Spanish provinces. ST11 carried four carbapenemases (VIM-1, OXA-48, KPC-2, and OXA-245), but ST405 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). Dissemination of OXA-48-producing *E. coli* is a new finding of public health concern. According to *in vitro* susceptibilities, most of the CPE (94.5%) had three or more options of antibiotic treatment.

Introduction

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 healthcare systems have been affected, and the predominant carbapenemase types, 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 2010–2012 (5-7).

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

Material and methods

Study design and bacterial isolates

A prospective multicentre study was designed to identify Enterobacteriaceae isolates with decreased susceptibility to carbapenems. 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; presence of infections was established according to previously defined criteria (8).

EUCAST screening cut-off values were used to identify CPE (9). The inclusion criteria were all Enterobacteriaceae isolates presenting either MICs >0.125 mg/L to meropenem and/or ertapenem and/or >1 mg/L to imipenem, or disk inhibition zones obtained using the disk diffusion method <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 also 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 centres, 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 (9) were classified using the algorithm for phenotypic carbapenemase detection recommended by EUCAST (9). A modified Hodge test using a meropenem disk with

cloxacillin (600 µg) was performed on all isolates. In addition, inhibition of carbapenemase activity was carried out by comparing the inhibition zones obtained from meropenem disks, with or without EDTA (10 µL 0.5 M solution), phenyl-boronic acid (400 µg), and cloxacillin (600 µg) in all isolates. Carba NP method was used as confirmatory test of carbapenemase activity when unclear phenotypic results or discrepancies between phenotypic and genotypic results, were observed (10).

Antibiotic susceptibility testing was performed by disk-difussion and microdilution susceptibility methods according to EUCAST guidelines (11,12) in addition susceptibility to ertapenem, imipenem, meropenem and colistin were carried out by 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 or aztreonam, respectively, was recovered in presence of clavulanic acid. In the case of KPC-producers molecular characterization of ESBL genes were carried out in all isolates.

Characterisation of resistance mechanisms

The presence of genes encoding carbapenemases (*bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{IMP,}}$ and *bla*$_{\text{NDM}}$) (5) and ESBLs (*bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{CTX-M}}$) (13) was determined using PCR and DNA sequencing assays.

Molecular epidemiology

MLST was performed for all carbapenemase-producing *K. pneumoniae* using the Institut Pasteur scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html; accessed January 2015). Carbapenemase-producing *E. coli* were typed by MLST using the

University of Warwick (Warwick Medical School, Coventry, UK) scheme (http://mlst.warwick.ac.uk; accessed January 2015). 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 (SD) previously described by Grundmann et al. (14) was applied to analyze the population diversity as follows: number of STs/total number of isolates x 100.

We considered that two or more isolates of *K. pneumoniae* or *Escherichia coli* (including clinical cases and carriers) were epidemiologically related if they belonged to the same species, had the same MLST type, and produced the same carbapenemase type. For *E. cloacae*, this epidemiological association was established when the genetic linkage was >85% using PFGE after total chromosomal DNA digestion with *Xba*I (13).

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 ORs with 95% CIs. The null hypothesis was rejected for values of $P < 0.05$. Statistical analysis was performed using GraphPad Prism, version 3.02, software (GraphPad Software, Inc., San Diego, CA, USA).

Results and Discussion

Bacterial isolates and carbapenemases types

A total of 702 Enterobacteriaceae isolates that were collected from an equal number of patients met the phenotypic criteria for inclusion; being *K. pneumoniae* (53.3%), *E. cloacae* (15.8%), and *E. coli* (13.8%) the most common species (Table 1).

Of these 702 isolates, 379 (54%) were confirmed as CPE and were distributed as follows: 282 (74.4%) were *K. pneumoniae*, 39 (10.3%) *E. cloacae*, 32 (8.4%) *E. coli*, 11 (2.9%) *Klebsiella oxytoca*, 7 (1.8%) *Citrobacter freundii*, 4 (1.1%) *Serratia marcescens*, 2 (0.5%) *Enterobacter aerogenes*, 1 (0.3%) *Morganella morganii*, and 1 (0.3%) isolate *Enterobacter* sp. (Table 1). The percentages of CPE isolates significantly varied between species as follows: 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; the percentage obtained for *K. pneumoniae* was significantly higher than those for *E. cloacae* and *E. coli* ($P < 0.0001$). In a recent French study, 1485 *Enterobacteriaceae* isolates non susceptible to carbapenems according 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) (15).

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 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. 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%) ($P < 0.0001$). Comparison of OXA-48-group- and VIM-group-producing isolates is showed in Table 2.

OXA-48 prevalence is also increasing in other European countries as France, Germany, Belgium, and the United Kingdom, where increasing numbers of outbreaks have been described (1,16-20). However, OXA-48 is rarely identified in North America (21). 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 (3,22). KPC enzymes are endemic in other European countries, such as Greece and Italy (1,3,23), and it produces nosocomial outbreaks in North America (1). The number of carbapenemase-producing *E. coli* identified in this study was much higher than 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%); 227 (85%) produced CTX-M-15, 16 (6%) SHV-12, 15 (5.6%) CTX-M-9, 9 (3.4%) 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* more frequently co-produced ESBLs (235 of 282 isolates, 83.3%) than did *E. cloacae* (18 of 38 isolates, 47.4%) or *E. coli* (11 of 32, 34.4%) ($P < 0.0001$). Co-production of ESBLs occurred in the 90.6% and 28% of OXA-48-producing *K. pneumonia* and *E. coli*, respectively ($P < 0.0001$), mostly CTX-M-15. Potron *et al.* (24) found that co-production 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* belonged to ST147 were isolated in Asturias (northern

Spain). Associations between OXA-48 and ESBLs of the SHV-type have previously been infrequently reported (24).

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), the VIM-producing isolates usually being more resistant.

A total of 182 CPE (48%) were susceptible to colistin, amikacin, tigecycline, and carbapenems (imipenem or meropenem); of them, 107 were also susceptible to fosfomycin. According to previous clinical experience (2), use of a carbapenem (meropenem or imipenem) for the treatment of a CPE with a MIC of ≤8 mg/L, in combination with another active agent, seems reasonable; following this criteria, we identified 21 highly resistant CPE isolates (5.5%; 12 OXA-48 isolates, 8 VIM-1 and one KPC-2) presenting only one or two options to choose from 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 (Figure 1). These hospitals were located in 18 of the 33 (54.5%) participating provinces. In twenty one (46.7%) of the 45 hospitals with CPE isolates, potential outbreaks of epidemiological related CPE were detected affecting 209 (55.1%) of the 379 CPE isolates included in this study.

Data about the total numbers 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 by patient and specie were identified. The estimated overall prevalence of infection by carbapenemase-producing *K. pneumoniae* was 1.7% (231/13842; range: 0–11.6%), and 23 (30.7%) hospitals had a prevalence >1%. For *Enterobacter* spp. and *E. coli*, these figures were 0.5% (28/5085; range: 0–6.4%) and 0.03% (28/91553; range: 0-0.4%), respectively. The prevalence of carbapenemase-production in *K. pneumoniae* and *E. coli* in a multicenter study performed in Spain in 2009 was 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 *vs*. 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%). This finding could be related to the earlier detection of VIM in Spain that occurred in 2005 (25), compared with OXA-48 in 2009 (26).

Population structure of carbapenemase-producing K. pneumoniae and E. coli isolates causing clinical infections.

Using MLST, 24 different sequence-types (ST) were identified among the 221 carbapenemase-producing *K. pneumoniae* implicated in clinical infections (SD: 10.6; mean: 9.2 isolates per ST; range: 1–66). The most prevalent STs were ST11 (66 isolates, 29.9%), ST405 (65, 29.4%), ST15 (28, 12.7%) and ST326 (15, 6.8%). These four STs were found in 78.8% of the carbapenemase-producing *K. pneumoniae* isolates. ST405 and ST11 clinical isolates were isolated in 18 different each one. ST11 carried four different types of carbapenemases (VIM-1, OXA-48, KPC-2, and OXA-245), but

ST405 carried only OXA-48. Analysis with eBURST provided an overview of STs of the Spanish carbapenemase producing *K. pneumoniae* isolates from this study compared with all *K. pneumoniae* isolates from the MLST database. The major ST found are represented in Figure 2. Excluding the isolates from this study, ST11 was the second most frequent ST in the MLST database (103 of 2405, 4.28%) and ST405 was uncommon (4 of 2405, 0.17%). In this eBurst analysis, ST405 was shown as a singleton non-related with other classical STs (Figure 2). These results suggest that *K. pneumoniae* 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 ESBL- and carbapenemase production (27,28) , but ST405 has recently been associated with OXA-48 production in Spain, Belgium, and France (5,6,17,18).

Among the 27 carbapenemase-producing *E. coli* implicated in clinical infections, 16 different STs were identified (SD: 59.3; mean: 1.7 isolates per ST; range: 1–7). ST131 (seven isolates, 26%) and ST156 (three 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 previously demonstrated for other resistance mechanisms like ESBL (29). The acquisition of carbapenemases by the globally distributed *E. coli* ST131 that has been detected in this and other studies (30, 31) is a finding of serious concern. In a recent study of OXA-48-producing *K. pneumoniae* and *E. coli* in several European and north-African countries, the most common STs identified were ST101 and ST38, respectively (24).

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 be occurred; however, the possibility of OXA-48 encoding plasmids acquisition by two isolates of a same prevalent ST from different regions cannot be excluded. ST405/OXA-48 was detected in six different Spanish provinces, ST15/OXA-48 in four, and ST11/OXA-48 and ST11/VIM-1 in three each one. This interregional spread clearly indicates that further progress has occurred since the "independent hospitals outbreak" stage described by Canton et al. in 2010 (1).

In addition, these carbapenemase genes are plasmid encoded and therefore their spread is probably due to both the clonal dissemination of a few specific strains and the transmission of epidemic auto-transferred plasmids carrying them (1,6,22).

Conclusions

We found that there was a wide geographic distribution of CPE species, and a clear increasing trend in the number of infections caused to 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, 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 of antibiotic treatment.

The spread of CPE in Spain affected at least 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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Conflict of interest

L. M-M. was speaker for Merck, Pfizer, Janssen-Cilag, and Astra-Zeneca and received research support from Merck, Wyeth, and Janssen-Cilag and Astra-Zeneca.

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None of these poses a conflict of interest with this work.

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538 TABLE 1. Distribution of carbapenemase-producing Enterobacteriaceae.

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Species	**All isolates (%)**	**CBP[a] negative (%)**	**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)	2*(25)	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. morgannii	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)

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541 [a] CBP, carbapenemases

542 *Two isolates producing both KPC and VIM carbapenemases.

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

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Variable	OXA-48-group (%)	VIM-group (%)	Odds ratio	95% CI	P value
Age > 65 years	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

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551 TABLE 3. Susceptibility to antibiotics in carbapenemase-producing Enterobacteriaceae
552 isolates.
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Antibiotic	Susceptibility (%)			P value
	Total of isolates (n=379)	OXA-48-group-producing isolates (n=270)	VIM-group-producing isolates (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

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Figure legends

FIGURE 1. Geographic distribution of carbapenemase types detected during a prospective multicentre study in Spain (February–May 2013).

FIGURE 2. Population snapshot of sequence types (STs) of *Klebsiella pneumoniae* isolates from the multilocus sequence typing (MLST) database and STs carbapenemase-producing *K. pneumoniae* from this study considered altogether.

Footnote: The most important ST complexes found in this study (ST11, ST405, ST15, and ST 326) are emphasised. Some STs with single isolates from the MLST database have been excluded from the image for easy viewing.