

Carbapenem-resistant *Citrobacter* spp. isolated in Spain from 2013 to 2015 produced a variety of carbapenemases including VIM-1, OXA-48, KPC-2, NDM-1 and VIM-2

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Received 26 April 2017; returned 27 July 2017; revised 4 August 2017; accepted 4 August 2017

Objectives: There is little information about carbapenemase-producing (CP) *Citrobacter* spp. We studied the molecular epidemiology and microbiological features of CP *Citrobacter* spp. isolates collected in Spain (2013–15).

Methods: In total, 119 isolates suspected of being CP by the EUCAST screening cut-off values were analysed. Carbapenemases and ESBLs were characterized using PCR and sequencing. The genetic relationship among *Citrobacter freundii* isolates was studied by PFGE.

Results: Of the 119 isolates, 63 (52.9%) produced carbapenemases, of which 37 (58.7%) produced VIM-1, 20 (31.7%) produced OXA-48, 12 (19%) produced KPC-2, 2 (3.2%) produced NDM-1 and 1 (1.6%) produced VIM-2; 9 *C. freundii* isolates co-produced VIM-1 plus OXA-48. Fourteen isolates (22.2%) also carried ESBLs: 8 CTX-M-9 plus SHV-12, 2 CTX-M-9, 2 SHV-12 and 2 CTX-M-15. Fifty-seven isolates (90.5%) were *C. freundii*, 4 (6.3%) were *Citrobacter koseri*, 1 (1.6%) was *Citrobacter amalonaticus* and 1 (1.6%) was *Citrobacter braakii*. By EUCAST break-points, eight (12.7%) of the CP isolates were susceptible to the four carbapenems tested. In the 53 CP *C. freundii* analysed by PFGE, a total of 44 different band patterns were observed. Four PFGE clusters were identified: cluster 1 included eight isolates co-producing VIM-1 and OXA-48; *bla*_{VIM-1} was carried in a class 1 integron (*intI*-*bla*_{VIM-1}-*aacA4*-*dfrB1*-*aadA1*-*catB2*-*qacEA1*/*sul1*) and *bla*_{OXA-48} was carried in a Tn1999.2 transposon.

Conclusions: We observed the clonal and polyclonal spread of CP *Citrobacter* spp. across several Spanish geographical areas. Four species of *Citrobacter* spp. produced up to five carbapenemase types, including co-production of VIM-1 plus OXA-48. Some CP *Citrobacter* spp. isolates were susceptible to the four carbapenems tested, a finding with potential clinical implications.

Introduction

The unceasing increase in infections due to carbapenemase-producing (CP) Enterobacteriaceae (CPE) is one of the most worrying threats to the health services worldwide. Frequently, patients with CPE infections cannot be treated with effective antibiotics because of the dearth of alternative drugs.^{1,2} Thus far, the main clinical load of CPE has been due to the increasing incidence of nosocomial infections caused by *Klebsiella pneumoniae*, but other

CP species, such as *Escherichia coli*, *Enterobacter* spp. and *Serratia marcescens*, have also been detected in nosocomial settings.^{3,4}

Citrobacter spp., mainly *Citrobacter freundii*, have been recognized as opportunistic pathogens responsible for healthcare-associated infections such as urinary and respiratory tract infections. However, little information is available about CP *Citrobacter* spp.; very few studies have reported *C. freundii* producing VIM or KPC.^{4–8} Some Enterobacteriaceae species, such as *Citrobacter* spp.

or *Enterobacter* spp., often present decreased susceptibility to carbapenems, mainly ertapenem, due to the overproduction of chromosomal AmpC β -lactamase plus reduced outer membrane permeability,⁹ as a consequence, production of carbapenemases in these species may be underdiagnosed in clinical microbiology laboratories.

The aim of this study was to gain insight into the microbiological features and molecular epidemiology of CP *Citrobacter* spp., mainly *C. freundii*, submitted to the Spanish National Reference Centre of Microbiology from 2013 to 2015.

Materials and methods

This study was performed by the unrestricted and non-mandatory national Spanish Antibiotic Resistance Surveillance Programme operated by our official public health institute (Instituto de Salud Carlos III). When this non-mandatory programme was launched in 2009, all Spanish clinical microbiology laboratories and health-associated professionals were personally contacted and encouraged to submit their carbapenem-resistant Enterobacteriaceae to our antibiotic reference laboratory for molecular and epidemiological characterization. From January 2013 to December 2015, a total of 115 public hospitals (about 35% of the total Spanish public hospitals) participated in the programme. In this study we included all *Citrobacter* spp. isolates submitted to our reference laboratory from 2013 to 2015 suspected of being carbapenemase producers according to the EUCAST screening cut-off values for CPE.¹⁰ Only the first isolate per patient was analysed. Isolate identification was performed using MALDI-TOF MS (Bruker Daltonik GmbH, Leipzig, Germany).

Antibiotic susceptibility testing was performed by microdilution (MicroScan, Beckman Coulter, Inc.) and interpreted according to EUCAST breakpoints.^{10,11} According to EUCAST recommendations, inhibition of carbapenemase activity was determined by using EDTA, phenyl-boronic acid and cloxacillin. In addition, all the isolates were tested with both the Carba NP method¹² and the modified Hodge test¹³ with a meropenem disc containing 600 mg of cloxacillin.

The presence of genes encoding carbapenemases (*bla*_{OXA-48}, *bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{NDM}) and ESBLs (*bla*_{CTX-M}, *bla*_{SHV} and *bla*_{TEM}) was determined by PCR and DNA sequencing.^{14,15}

The genetic relationship among the CP *C. freundii* isolates was elucidated by PFGE after total chromosomal DNA digestion with XbaI.¹⁵

The genetic environment of *bla*_{VIM-1} was studied using PCR mapping and subsequent sequencing of the variable region of the class 1 integron; the genetic environment of the *bla*_{OXA-48} gene was determined by PCR as previously described.¹⁶

All the sequences obtained were compared with those available in GenBank (GenBank accession numbers KC354801.1, EF093146, FJ627181, GU086225.1 and FN396876).

Results and discussion

During the 3 year period of the study, 119 isolates of *Citrobacter* spp. suspected of being carbapenemase producers according to the EUCAST screening cut-off values for CPE¹⁰ were analysed. Sixty-three of them (52.9%) produced carbapenemases (1.5% of the 4129 CPE identified in the reference laboratory during the same period of time). An increase in the CP *Citrobacter* spp. isolates was observed: 1.3% in 2013 (10 out of 775), 1.7% in 2014 (25 out of 1431) and 1.5% in 2015 (28 out of 1923). The 63 CP *Citrobacter* spp. came from 27 Spanish hospitals located in 14 different provinces. Fifty-seven (90.5%) of them were *C. freundii*, four (6.3%) were *Citrobacter koseri*, one (1.6%) was *Citrobacter amalonaticus*

Table 1. Antibiotic susceptibility of 63 CP *Citrobacter* spp. isolates as determined by microdilution and according to EUCAST clinical breakpoints¹¹

Antibiotic	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)	MIC range (mg/L)	Susceptibility (%)
Colistin ^a	≤2	≤2	≤2 to ≤2	100
Fosfomycin	≤32	≤32	≤32–64	93.7
Tigecycline	≤1	≤1	≤1–2	92.1
Amikacin	≤8	>32	≤8 to >32	71.4
Imipenem	2	>8	≤1 to >8	58.7
Meropenem	2	>8	≤1 to >8	52.4
Gentamicin	8	>8	≤2 to >8	42.9
Doripenem	2	>4	≤1 to >4	39.7
Co-trimoxazole	>4	>4	≤2 to >4	33.3
Ciprofloxacin	>2	>2	≤0.5 to >2	24.4
Tobramycin	>8	>8	≤2 to >8	20.6
Ertapenem	>1	>1	≤0.5 to >1	14.3

^aThree isolates were resistant to colistin by a commercial microdilution method, but were susceptible by the in-house microdilution method recommended by EUCAST (http://www.eucast.org/ast_of_bacteria/warnings/).

and one (1.6%) was *Citrobacter braakii*. Thirty-two (50.8%) produced clinical infections as follows: 19 (59.4%) urinary tract infections, 8 (25%) wound infections, 1 (3.1%) bacteraemia and 4 (12.5%) other infections. The remaining 31 (49.2%) isolates were obtained from rectal samples.

Of the 63 CP *Citrobacter* spp., 37 (58.7%) produced VIM-1, 20 (31.7%) produced OXA-48, 12 (19%) produced KPC-2, 2 (3.2%) produced NDM-1 and 1 (1.6%) produced VIM-2; 9 *C. freundii* co-produced VIM-1 plus OXA-48. The modified Hodge test and the Carba NP test yielded positive results for all the 63 CP isolates.

Fourteen (22.2%) CP *Citrobacter* spp. also produced ESBLs as follows: 8 (57.1%) produced CTX-M-9 plus SHV-12 (7 of them also producing VIM-1 plus OXA-48 and 1 producing VIM-1), 2 (14.3%) produced CTX-M-9 (1 of them also producing VIM-1 plus OXA-48 and 1 producing OXA-48), 2 (14.3%) produced SHV-12 (1 of them also producing OXA-48 and 1 producing VIM-1) and 2 (14.3%) produced CTX-M-15 (both also producing NDM-1). Interestingly, of the four CP *C. koseri*, three of them were OXA-48 producers without ESBL production and one co-produced NDM-1 and CTX-M-15. The remaining two non-*C. freundii* isolates (one *C. amalonaticus* and one *C. braakii*) produced VIM-1, but not ESBLs.

Antibiotic susceptibility data are displayed in Table 1. The OXA-48-producing isolates were more susceptible to ciprofloxacin (63.6%) and tobramycin (63.6%) than the isolates producing VIM-1 (32.1% and 0%, respectively) and KPC-2 (0% and 33%, respectively), although without statistical significance. Three isolates (4.8%) were resistant to colistin by the commercial microdilution method used, but susceptible by the in-house microdilution method recommended by EUCAST (http://www.eucast.org/ast_of_bacteria/warnings/, last accessed April 2017); by PCR, all three isolates were negative for *mcr-1*.

Of the 63 CP *Citrobacter* spp., 37 (58.7%) were susceptible to imipenem, 33 (52.4%) were susceptible to meropenem, 25 (39.7%)

were susceptible to doripenem and 9 (14.3%) were susceptible to ertapenem (Table 1). These data suggest that ertapenem has the highest sensitivity for the detection of CP *Citrobacter* spp. and imipenem the lowest, although specificity could not be determined. However, susceptibility to carbapenems varied in relation to the carbapenemase types as follows: ertapenem susceptibility was 0% in isolates producing OXA-48, 0% in OXA-48/VIM-1 producers, 16.7% in KPC-2 producers and 25% in VIM-1 producers, while imipenem susceptibility was 91%, 77.8%, 33.3% and 64.3%, respectively.

Interestingly, 8 (12.7%) of the 63 CP *Citrobacter* spp. isolates (6 of them producing VIM-1 and 2 producing KPC-2) were susceptible to the four carbapenems tested according to EUCAST clinical breakpoints,¹¹ but they were suspected of being CP by the screening cut-off values recommended by EUCAST.¹⁰ According to CLSI breakpoints,¹³ four of these eight isolates had an intermediate susceptibility to imipenem (MIC = 2 mg/L), but were susceptible to the other three carbapenems.

PFGE analysis revealed a high degree of genetic diversity because 44 different PFGE patterns were obtained from the 53 CP *C. freundii* analysed. However, four well-defined clusters were detected with a genetic linkage $\geq 84\%$ (Figure 1). Cluster 1 (C1) included nine VIM-1-producing *C. freundii* (eight of them also co-producing OXA-48) isolated from a single hospital (East Spanish area); all nine isolates also co-produced ESBLs (eight CTX-M-9 plus SHV-12 and one CTX-M-9). Cluster 2 contained five VIM-1-producing *C. freundii* isolated from three different hospitals located in three neighbouring provinces, two of them very close together. Cluster 3 consisted of five KPC-2-producing *C. freundii* isolated from two hospitals of the same province in the central Spanish area. Cluster 4 included four VIM-1-producing *C. freundii* isolated from the same hospital in the central Spanish area. These results point to a clonal dissemination of CP *C. freundii* within the same hospital or between different hospitals, but also to a polyclonal dissemination since 30 of the CP isolates had non-related PFGE patterns.

Interestingly, the nine C1 *C. freundii* isolates were collected from faecal carriers admitted to the onco-haematology unit of a university hospital. In the same hospital, during 2013–15, a total of 54 patients had rectal cultures positive for *C. freundii* co-producing VIM-1 plus OXA-48 although no clinical infections caused by this pathogen were observed; the 9 C1 isolates included in the study constituted a representative sample of the 54 mentioned isolates. The first identified carrier was a 60-year-old male diagnosed with AML transferred from another Spanish hospital in October 2013; this patient had a rectal culture positive for *C. freundii* producing VIM-1 plus OXA-48 in November 2013.

Because cluster C1 contained the highest number of CP isolates also producing two different types of carbapenemases, the genetic environments of *bla*_{VIM-1} and *bla*_{OXA-48} were studied in two representative C1 isolates. In the two isolates, VIM-1 was located in a class 1 integron containing five resistance genes (*intI*-*bla*_{VIM-1}-*aacA4*-*dfpB1*-*aadA1*-*catB2*-*qacEΔ1*/*sul1*). Also in the two isolates, upstream of *bla*_{OXA-48}, IS1999 was disrupted by the IS1R element, but downstream of *bla*_{OXA-48} this gene was flanked by IS1999 without the IS1R element, indicating the presence of a Tn1999.2.¹⁶

OXA-48 and VIM-1 are the most frequent carbapenemases detected in Spain and *K. pneumoniae*, *Enterobacter cloacae* and

E. coli are the most affected species.^{3,4,14} However, very little information is available about CP *C. freundii*.^{5,7,8} In addition, while some studies have described the co-production of two carbapenemases such as NDM-1 plus VIM-1 in *C. freundii*, *K. pneumoniae* and *E. cloacae*^{17,18} or KPC-2 plus VIM-2 in *K. pneumoniae*,¹⁹ very few studies have reported the co-production of VIM-1 plus OXA-48^{17,20} and none of them in *C. freundii*. We detected a moderate increase in CP *Citrobacter* spp. in Spain between 2013 and 2015; in addition, our data revealed several remarkable findings: (i) clonal and polyclonal spread of CP *C. freundii* across different geographical areas and hospitals; (ii) carriage of five different carbapenemase types by four species of *Citrobacter* spp., especially *C. freundii*; (iii) detection of *C. freundii* co-producing OXA-48 plus VIM-1 in carriers admitted to an onco-haematology hospital unit; and (iv) detection of carbapenemases in carbapenem-susceptible isolates by EUCAST clinical breakpoints, a finding that could have important clinical and epidemiological consequences. In our opinion, all these findings are of clinical, microbiological and epidemiological interest and deserve active surveillance.

Acknowledgements

We thank the Genomics Unit of the Centro Nacional de Microbiología for carrying out the DNA sequencing.

Other members of the Spanish Antibiotic Resistance Surveillance Programme Collaborating Group

Iris Gómez-Alfaro (Hospital Universitario i Politècnic La Fe, Valencia), José Esteban Aznar (Laboratorio BrSalud, San Sebastián de los Reyes, Madrid), Emilia Cercenado (Hospital General Universitario Gregorio Marañón, Madrid), Luis López-Urrutia (Hospital Universitario Río Hortega, Valladolid), Luisa García-Picazo (Hospital 'El Escorial', San Lorenzo del Escorial, Madrid), Ana Isabel López-Calleja (Hospital Universitario Miguel Servet, Zaragoza), Isabel Sánchez-Romero (Hospital Universitario Puerta de Hierro-Majadahonda, Majadahonda, Madrid), Pilar Zamarrón-Fuertes (Hospital Virgen de la Salud, Toledo), José Leiva (Clínica Universitaria de Navarra, Navarra), Juan Ignacio Alós (Hospital Universitario de Getafe, Getafe, Madrid), Sonia Solís (Hospital Universitario de Guadalajara, Guadalajara), M^a Dolores de Miguel (Hospital de Cabueñes, Gijón, Asturias), Belén Hernández (H. Infantil Universitario Niño Jesús, Madrid), Juan Romanyk (H. Universitario Príncipe de Asturias, Alcalá de Henares, Madrid), Alberto Delgado-Iribarren (Hospital Universitario Fundación de Alcorcón, Alcorcón, Madrid), Eva Ojeda Fernández (Complejo Asistencial Universitario de Burgos, Burgos), Gloria Trujillo (Hospital Sant Joan de Déu Fundació Althaia, Manresa, Barcelona), Luis Torroba (Complejo Hospitalario de Navarra, Navarra), José Luis Hernández Almaraz (Hospital de Cruces, Barakaldo, Vizcaya), M^a Antonia Remacha Esteras (Hospital de León, León), Santiago Salso (Hospital HM Montepíncipe, Boadilla del Monte, Madrid), Yolanda Gil (Hospital de Móstoles, Móstoles, Madrid), Irene Rodríguez-Conde (Hospital Povisa, Pontevedra), Teresa Alarcón (Hospital Universitario de La Princesa, Madrid),

Funding

This work was supported by Plan Nacional de I + D+i 2013–2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases

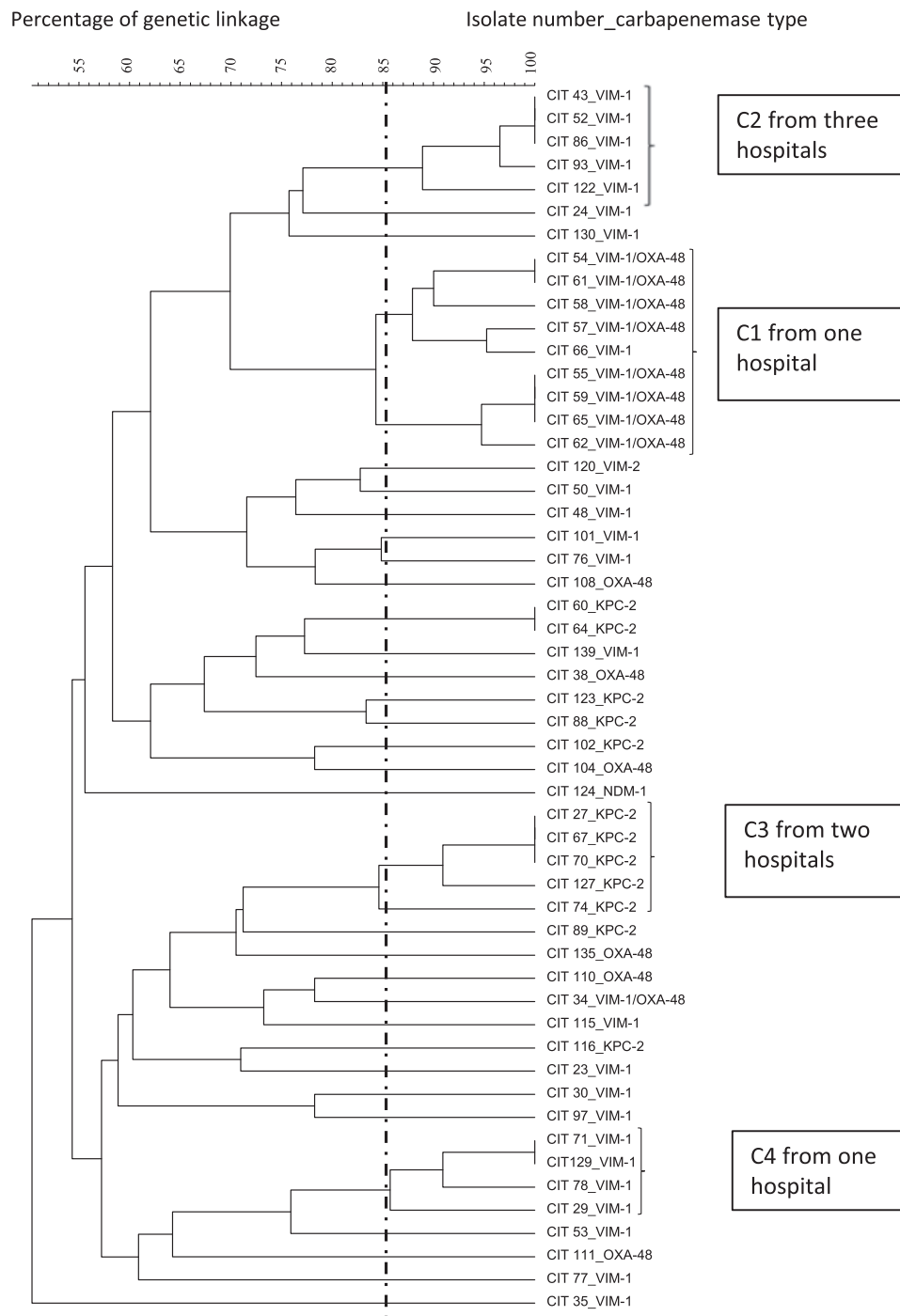


Figure 1. Dendrogram illustrating the PFGE profiles of 53 CP *C. freundii* isolates.

(REIPI RD16CIII/0004/0002) - co-financed by European Development Regional Fund (ERDF) 'A way to achieve Europe', Operative Program Intelligent Growth 2014–2020. This work was also supported by a grant from the Instituto de Salud Carlos III (grant number MPY 1135/16), by the Antibiotic Resistance Surveillance Program of the Centro Nacional de Microbiología (Instituto de Salud Carlos III, Ministerio de Economía y

Competitividad) of Spain and by a grant from the Agencia Española de Medicamentos y Productos Sanitarios (grant number MEG 1281/14).

Transparency declarations

None to declare.

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