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WGS characterization of MDR Enterobacterales with different ceftolozane/tazobactam susceptibility profiles during the SUPERIOR surveillance study in Spain

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Objectives: To analyse by WGS the ceftolozane/tazobactam (C/T) resistance mechanisms in *Escherichia coli* and *Klebsiella* spp. isolates recovered from complicated intra-abdominal and urinary tract infections in patients from Spanish ICUs (SUPERIOR surveillance study, 2016–17).

Methods: The clonal relatedness, the resistome and the virulome of 45 *E. coli* and 43 *Klebsiella* spp. isolates with different C/T susceptibility profiles were characterized.

Results: In *E. coli*, two (C/T susceptible) carbapenemase producers (VIM-2-CC23, OXA-48-ST38) were detected. The most relevant clone was ST131-B2-O25:H4-H30 (17/45), particularly the CTX-M-15-ST131-H30-Rx subline-age (15/17). ST131 strains were mainly C/T susceptible (15/17) and showed an extensive virulome. In non-ST131 strains (28/45), CTX-M enzymes [CTX-M-14 (8/24); CTX-M-15 (6/24); CTX-M-1 (3/24); CTX-M-32 (2/24)] were found in different clones. C/T resistance was detected in non-clonal *E. coli* isolates (13%, 6/45) with ESBL (4/6) and non-ESBL (2/6) genotypes. Among *Klebsiella* spp., *Klebsiella pneumoniae* (42/43) and *Klebsiella michiganensis* (1/43) species were identified; 42% (18/43) were carbapenemase producers and 58% showed a C/T resistance phenotype (25/43). OXA-48-ST11 (12/18), OXA-48-ST392 (2/18), OXA-48-ST15 (2/18), NDM-1-ST101 (1/18) and OXA-48+VIM-2-ST15 (1/18) isolates were found, all C/T resistant. Correlation between carbapenemase detection and resistance to C/T was demonstrated ($P < 0.001$). In non-carbapenemase-producing *K. pneumoniae* (25/43), C/T resistance (28%, 7/25) was detected in ESBL (3/7) and AmpC (2/7) producers. Overall, an extensive virulome was found and was correlated with carbapenemase carriage ($P < 0.001$) and C/T resistance ($P < 0.05$), particularly in OXA-48-ST11 strains ($P < 0.05$).

Conclusions: Prediction of antimicrobial susceptibility profiles using WGS is challenging. Carbapenemase-encoding genes are associated with C/T resistance in *K. pneumoniae*, but other resistance mechanisms might be additionally involved.

Introduction

The production of ESBLs and carbapenemases is the major mechanism of resistance to β-lactam antibiotics in Enterobacterales isolates. Since 2000, the global prevalence of ESBL enzymes, mostly CTX-M enzymes, has dramatically increased in both community and hospital settings.[1] Additionally, in the last two decades, the

worldwide dissemination of carbapenemase-producing Enterobacterales (CPE) has been recognized as one of the major challenges for public health due to their high mortality and morbidity and the elevated associated economic costs.[2] In Spain, CTX-M enzymes are considered endemic and are usually detected in nosocomial *Klebsiella pneumoniae* clones and community-acquired *Escherichia coli* non-clonal isolates.[3,4] Moreover, CPE prevalence is also increasing and is mostly related to OXA-48, VIM and KPC enzymes.[2] The dissemination of these carbapenemases is frequently associated with successful epidemic clones of *K. pneumoniae* and with healthcare exposure and prolonged hospital stays.[5,6]

The therapeutic options available to treat infections caused by MDR Enterobacterales isolates are limited and the development of novel antimicrobial agents is currently a priority. Ceftolozane/tazobactam is a cephalosporin/β-lactamase inhibitor combination useful in the treatment of complicated intra-abdominal infections (cIAIs) and complicated urinary tract infections (cUTIs) caused by MDR Gram-negative bacteria, with the exception of carbapenemase producers.[7] Several studies have demonstrated a higher *in vitro* activity of this novel combination against ESBL-producing Enterobacterales compared with currently available cephalosporins.[8,9] Nevertheless, low resistance rates to ceftolozane/tazobactam have been recently reported in ESBL-producing *K. pneumoniae* and *E. coli* isolates.[10–12]

In recent years, WGS has become a potent tool to characterize and understand the genetic diversity of bacterial populations. The use of genome-based technologies for the prediction of bacterial antibiotic resistance profiles in surveillance studies has also been proposed as a powerful alternative to antibiotic susceptibility testing.[13] However, discordances are still observed and the prediction of susceptibility or resistance to antimicrobial agents based only on the presence or absence of previously known genes is still under discussion.[14]

The aim of this work was to characterize, using WGS, the population structure, the resistome and the virulome of MDR *E. coli* and *Klebsiella* spp. clinical isolates recovered from patients admitted to eight Spanish ICUs between 2016 and 2017 as a part of the SUPERIOR surveillance study, focusing on the resistance genotype with respect to the ceftolozane/tazobactam antibiotic.

Methods

Study design and bacterial isolates

SUPERIOR is a prospective multicentre surveillance study designed to evaluate the *in vitro* activity of ceftolozane/tazobactam and comparator antimicrobials against Enterobacterales and *Pseudomonas aeruginosa* clinical isolates. A total of 400 Enterobacterales [52.3% *E. coli* (n = 209); 23.8% *Klebsiella* spp. (n = 95)] isolates were collected from cUTIs and cIAIs from patients (one isolate per patient) admitted to ICUs at eight Spanish hospitals between April 2016 and April 2017.[15] The participant hospitals were Hospital Universitario Ramón y Cajal (Madrid, centre A), Hospital General Universitario Gregorio Marañón (Madrid, centre B), Hospital Clínic i Provincial (Barcelona, centre C), Hospital Universitario Virgen Macarena (Sevilla, centre D), Hospital Universitario Marqués de Valdecilla (Santander, centre E), Complejo Hospitalario Universitario (A Coruña, centre F), Hospital General Universitario (Valencia, centre G) and Hospital Universitario Son Espases (Palma de Mallorca, centre H). Hospital Universitario Ramón y Cajal was the coordinator laboratory for the microbiological and molecular analysis. The study was approved by the ethics committee of Hospital Universitario Ramón y Cajal (Madrid, Spain) (Ref. 087–16) and the Spanish Medicines Agency (Ref. MSD-CEF-2016-01).

According to our previous antibiotic susceptibility testing results, *E. coli* and *Klebsiella* spp. isolates were classified into ESBL (22% of *E. coli* and 23.1% of *Klebsiella* spp.) and carbapenemase (0.9% of *E. coli* and 20% of *Klebsiella* spp.) phenotypes.[15] Ceftolozane/tazobactam showed overall good activity against *E. coli* (95.2% susceptible by EUCAST) and moderate activity in the *Klebsiella* spp. group (66.3% susceptible by EUCAST). A subset of 89 Enterobacterales isolates (46 *E. coli* and 43 *Klebsiella* spp.) were selected for subsequent genome analysis based on the WGS approach. All *Klebsiella* spp. (25/95, 26.3%) and *E. coli* (7/209, 3.3%) isolates that displayed resistance to the ceftolozane/tazobactam antibiotic based on the EUCAST 2020 interpretative criteria (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf) were sequenced, including all those isolates with a susceptibility testing phenotype compatible with carbapenemase production [*Klebsiella* spp. (18/95, 18.9%), *E. coli* (1/209, 0.5%)]. Furthermore, a representative subset of isolates with ESBL [*Klebsiella* spp. (21/95, 22.1%), *E. coli* (45/209, 21.5%)] and non-ESBL phenotypes [*Klebsiella* spp. (4/95, 4.2%)], all of them with different susceptibility to ceftolozane/tazobactam (susceptible, standard dose; resistant), were also included in the analysis (Table 1).

WGS and sequence processing

The commercial Chemagic DNA Bacterial External Lysis Kit (PerkinElmer, USA) was used for the total genome DNA extraction. Short-read sequencing was performed by the Illumina Hiseq4000 or the Illumina NovaSeq 6000 platforms (OGC, Oxford, UK), with 2 × 150 pb paired-end reads. Quality control and filtering of sequences was carried out using FastQC v.0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Prinseq-lite-0.20.3 (http://prinseq.sourceforge.net/) tools, respectively. SPAdes v3.11.1 and QUAST v5.0.2 (http://quast.sourceforge.net/download.html) were used for the *de novo* assembling of short reads and the subsequent assembly evaluation, respectively.[16] Bacterial identification was confirmed by the Taxonomic Sequence Classification System Kraken v.1.0.[17] The draft genomes were annotated by Prokka v.1.13.3.[18] The MASH and iTOL applications (https://itol.embl.de/) were used to generate and trace a similarity tree based on a neighbour-joining algorithm.[19]

Molecular typing

In silico MLST assignment was performed using MLST v2.16.1 (https://github.com/tseemann/mlst) and clonal diversity was determined by calculating the dominant Simpson diversity index (SDI) [SDI = 0 (maximum

Table 1. Selection of Enterobacterales isolates for WGS during the SUPERIOR study

		Phenotype			
Microorganism	C/T susceptibility	ESBL	CP	non-ESBL non-CP	Total
E. coli	susceptible	39	0	–	39
	resistant	6	1[a]	–	7
	total	45	1	–	46
Klebsiella spp.	susceptible	16	0	2	18
	resistant	5	18	2	25
	total	21	18	4	43

C/T, ceftolozane/tazobactam; CP, carbapenemase.
[a]This isolate was contaminated during storage and was not finally included in the genome study.

clonality); SDI = 1 (maximum diversity)].[20] Phylogroups, serotypes and *fimH* alleles of *E. coli* isolates were determined using the ClermonTyping, SerotypeFinder and FimTyper (https://cge.cbs.dtu.dk/services/) tools, respectively.[21] In the *E. coli* ST131-H30 subclone, the C2-H30Rx subclade was identified based on the G723A point mutation in the *ybbW* gene (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Kleborate software v.0.4.0-beta was used for the inference of *K. pneumoniae* serotypes based on the *wzi* gene and the K (capsule) and O (LPS) antigens.[22,23]

Resistance and virulence gene characterization

Resistance mechanisms and virulence factors were detected using Abricate v0.8.11 and ARG-ANNOT and VFDB databases (threshold, 95% identity; 90% coverage). PointFinder software (https://cge.cbs.dtu.dk/services/) was used for the detection of chromosomal point mutations related to quinolone resistance in *E. coli* isolates. *K. pneumoniae* integrative conjugative elements (ICE*Kp*) and acquired factors, including siderophores (yersiniabactin, salmochelin and aerobactin), regulators of hypermucoidy (*rmpA*/*rmpA2* genes) and the genotoxin colibactin were also identified using Kleborate.

Statistical analysis

Associations between categorical variables were analysed by Fisher's exact test. The kappa index (κ) index was used to evaluate the concordance between genotypes based on WGS analysis and the *in vitro* antibiotic activity results ($\kappa < 0.40$, weak agreement; $\kappa = 0.41$–0.60, moderate; $\kappa = 0.61$–0.80, accurate; and $\kappa > 0.81$, very accurate). Statistical analysis was carried out using R software (RStudio Team 2016 version 1.0.44, RStudio, Boston, MA, USA). A P value <0.05 was considered statistically significant.

Accession numbers

All complete sequences were deposited at DDBJ/ENA/GenBank under the BioProject accession number PRJNA609897 (Table S1, available as Supplementary data at *JAC-AMR* Online).

Results

Genome characteristics and taxonomic classification

Genomic information about all isolates is shown in Table S2 (*E. coli*) and Table S3 (*Klebsiella* spp.). All *Escherichia* spp. isolates were classified as *E. coli* species. *K. pneumoniae* bacterial identification was confirmed in 97.7% of *Klebsiella* spp. isolates (42/43). One *Klebsiella michiganensis* strain was also detected (1/43).

Antibiotic resistance determinants

A diverse antibiotic resistance gene content was detected among the *E. coli* isolates (Figure S1). Despite the fact that susceptibility rates showed a phenotype non-compatible with carbapenemase production, carbapenemase-encoding genes were confirmed in two *E. coli* isolates from centres D (D36-bla_{VIM-1}) and E (E30-bla_{OXA-48}) (Figure 1). Both strains were also susceptible to ceftolozane/tazobactam (MIC = 0.5/4 mg/L). Concordance between the detection of carbapenemase genes using the WGS approach and resistance to ceftolozane/tazobactam could not be established ($P = 1$; OR = 0.0; 95% CI = 0.0–36.8). ESBL genes were identified in 91.1% (41/45) of the *E. coli* isolates (Figure 1). CTX-M (80%, 36/45) and SHV (13.3%, 6/45) groups were found and CTX-M-15 was the predominant enzyme (48.9%, 22/45). Ceftolozane/tazobactam resistance (MIC >2 mg/L) was observed in six *E. coli* strains (13.3%) from two centres (G and H) and related to ESBL production in four cases ($bla_{CTX-M-15}$, $bla_{CTX-M-1}$ and bla_{SHV-12}) and to an AmpC gene (bla_{CMY-17}) in another (Figure 1). Correlation between ceftolozane/tazobactam resistance and the carriage of ESBL-encoding genes was not observed ($P = 0.08$; OR = 0.18; 95% CI = 0.01–2.03). Additionally, three *E. coli* isolates (6.7%) showed colistin resistance (MIC = 4 to >4 mg/L), but only one strain (C63) carried the *mcr-1* gene (Figure 1). One of these isolates (H38) displayed non-susceptibility to ceftolozane/tazobactam (MIC = 32/4 mg/L), but resistance mechanisms against β-lactam antibiotics were not detected.

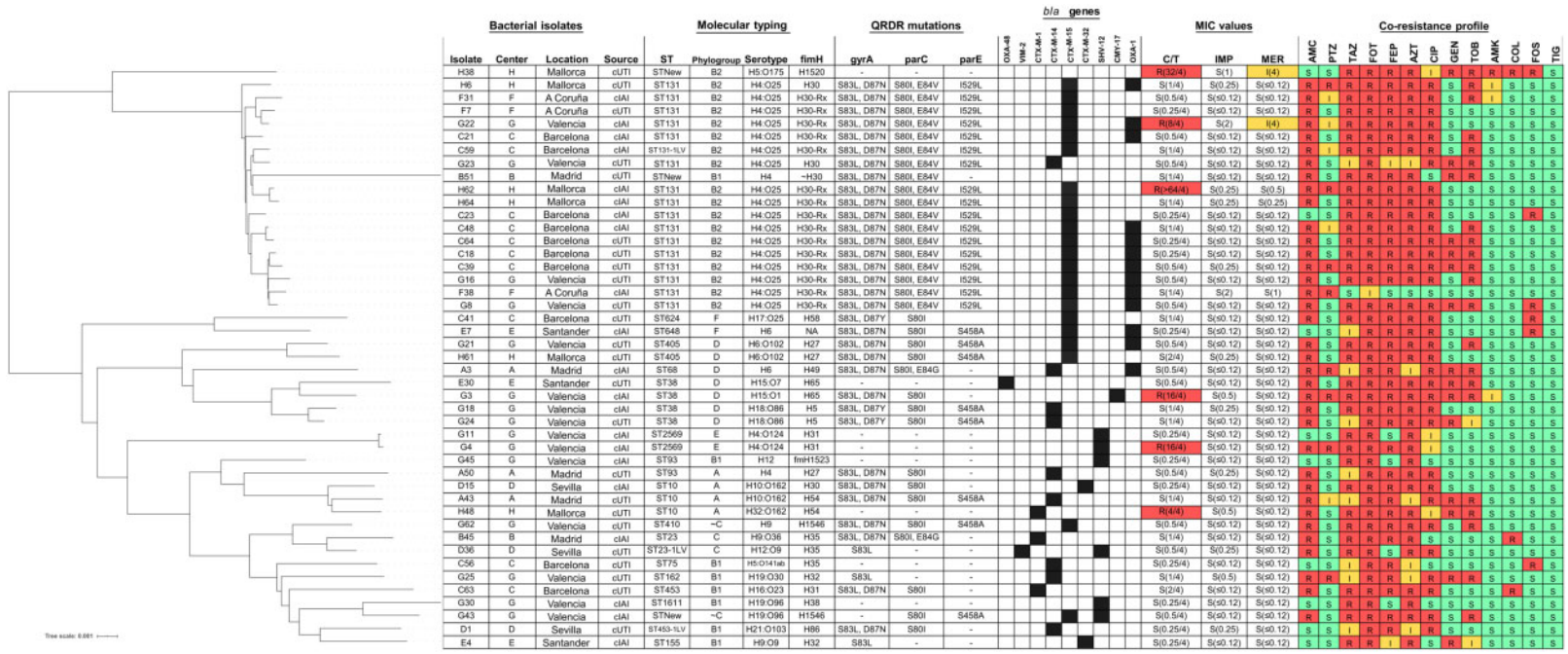
The resistome of all sequenced *Klebsiella* spp. strains is shown in Figure S2. Carbapenemase genes were identified in 41.9% (18/43) of the isolates; all of them belonged to the *K. pneumoniae* species. OXA-48 was the most frequent carbapenemase (88.9%, 16/18), but one NDM-1 producer (G19) and one OXA-48+VIM-2 co-producer (F65) were also identified. Carbapenemase-producing *K. pneumoniae* isolates were encountered in five hospitals (A, B, E, G and F). Interestingly, a weak agreement ($\kappa = 0.309$; $P = 0.0051$) was found between the detection of carbapenemase-encoding genes and the phenotypic resistance to carbapenems (27.8%, 5/18) (Figure 2). On the other hand, ceftolozane/tazobactam resistance was observed in 58.1% of *Klebsiella* spp. strains (25/43) (MIC range = 4/4 to >64 mg/L), including all carbapenemase-producing isolates (72%, 18/25) (MIC = 4/4 to >64 mg/L) (Figure 2). Concordance between the presence of carbapenemase genes and the lack of *in vitro* ceftolozane/tazobactam susceptibility was significant ($P < 0.001$; OR = 0.0; 95% CI = 0.0–0.13).

The presence of ESBL-encoding genes was confirmed in 74.4% (32/43) of the *Klebsiella* spp. isolates and 56.2% of them also carried a carbapenemase gene (18/32). CTX-M-15 (72.1%, 31/43) was predominant and only one CTX-M-1-producing isolate was identified. Among the non-carbapenemase producers with a ceftolozane/tazobactam-resistant phenotype (28%, 7/25), CTX-M-15 was detected in three cases (MIC = 4/4 to >16/4 mg/L), but a correlation was not established ($P = 0.65$; OR = 0.49; 95% CI = 0.054–3.91). Additionally, two *K. pneumoniae* isolates carrying the plasmid-mediated and inducible AmpC β-lactamase DHA-1 were detected among the ceftolozane/tazobactam resistant isolates (MIC = 4/4 and 32/4 mg/L) (Figure 2). On the other hand, nine *K. pneumoniae* isolates (20.9%) displayed colistin resistance (MIC = 4 to >4 mg/L); five of them also showed non-susceptibility to ceftolozane/tazobactam (MIC = 4/4 to >64/4 mg/L) and four isolates were OXA-48+CTX-M-15 co-producers (Figure 2). Furthermore, plasmid-mediated genes encoding colistin resistance were not found.

The distribution of all *E. coli* and *Klebsiella* spp. isolates according to the ceftolozane/tazobactam MIC values, the β-lactamase gene detected and the infection source is shown in Figure 3.

Genetic diversity

E. coli isolates showed a high clonal diversity ($SDI_{Ec} = 0.84$). The clonal complex CC131 was identified in 37.8% (17/45) of the isolates (16 ST131 and 1 single-locus variant of ST131) and was distributed in five different hospitals (B, C, F, G and H) in both cUTI (8/17) and cIAI (9/17) samples. All of them belonged to the serotype O25:H4 and were assigned to phylogroup B2. Moreover, all CC131 strains carried the *fimH30* allele and 15 out of 17 were identified as the sublineage C2/*H30-Rx* (88.2%). All CC131 isolates



Isolate	Center	Location	Source	ST	Phylogroup	Serotype	fimH	gyrA	parC	parE	MIC C/T	MIC IMP	MIC MER	AMC	PTZ	TAZ	FOT	FEP	AZT	CIP	GEN	TOB	AMK	COL	FOS	TIG
H38	H	Mallorca	cUTI	STNew	B2	H5:O175	H1520	-	-	-	R(32/4)	S(1)	I(4)	S	S	R	R	R	R	I	R	R	R	R	S
H6	H	Mallorca	cUTI	ST131	B2	H4:O25	H30	S83L, D87N	S80I, E84V	I529L	S(1/4)	S(0.25)	S(≤0.12)	R	R	R	R	R	R	R	S	R	I	S	S	S
F31	F	A Coruña	cIAI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	I	R	R	R	R	R	S	R	I	S	S	S
F7	F	A Coruña	cUTI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(0.25/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	S	S	S	S	S	S
G22	G	Valencia	cIAI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	R(8/4)	S(2)	I(4)	R	I	R	R	R	R	R	S	S	S	S	S	S
C21	C	Barcelona	cIAI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	S	R	S	S	S	S
C59	C	Barcelona	cIAI	ST131-1LV	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(1/4)	S(≤0.12)	S(≤0.12)	R	I	R	R	R	R	R	S	R	S	S	S	S
G23	G	Valencia	cUTI	ST131	B2	H4:O25	H30	S83L, D87N	S80I, E84V	I529L	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	S	I	R	I	I	R	R	R	S	S	S	S
B51	B	Madrid	cUTI	STNew	B1	H4	−H30	S83L, D87N	S80I, E84V	-	S(1/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	S	R	R	S	S	S	S
H62	H	Mallorca	cIAI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	R(>64/4)	S(0.25)	S(0.5)	R	R	R	R	R	R	R	S	S	S	S	S	S
H64	H	Mallorca	cIAI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(1/4)	S(0.25)	S(0.25)	R	S	R	R	R	R	R	S	S	S	S	S	S
C23	C	Barcelona	cIAI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(0.25/4)	S(≤0.12)	S(≤0.12)	S	S	R	R	R	R	R	S	S	S	S	R	S
C48	C	Barcelona	cIAI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(1/4)	S(≤0.12)	S(≤0.12)	R	I	R	R	R	R	R	S	R	S	S	S	S
C64	C	Barcelona	cUTI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(0.25/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	R	R	S	S	S	S
C18	C	Barcelona	cUTI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(0.25/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	R	R	S	S	S	S
C39	C	Barcelona	cUTI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(0.5/4)	S(0.25)	S(≤0.12)	R	R	R	R	R	R	R	R	R	S	S	S	S
G16	G	Valencia	cUTI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	S	R	S	S	S	S
F38	F	A Coruña	cIAI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(1/4)	S(2)	S(1)	R	R	S	I	S	S	S	S	S	S	S	S	S
G8	G	Valencia	cUTI	ST131	B2	H4:O25	H30-Rx	S83L, D87N	S80I, E84V	I529L	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	R	R	S	S	R	S
C41	C	Barcelona	cUTI	ST624	F	H17:O25	H58	S83L, D87Y	S80I		S(1/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	S	S	S	S	R	S
E7	E	Santander	cIAI	ST648	F	H6	NA	S83L, D87N	S80I	S458A	S(0.25/4)	S(≤0.12)	S(≤0.12)	S	S	I	R	R	R	R	S	S	S	S	R	S
G21	G	Valencia	cUTI	ST405	D	H6:O102	H27	S83L, D87N	S80I	S458A	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	S	R	S	S	S	S
H61	H	Mallorca	cUTI	ST405	D	H6:O102	H27	S83L, D87N	S80I	S458A	S(2/4)	S(0.25)	S(≤0.12)	R	S	R	R	R	R	R	S	S	S	S	S	S
A3	A	Madrid	cIAI	ST68	D	H6	H49	S83L, D87N	S80I, E84G	-	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	R	I	R	R	I	R	R	R	S	S	S	S
E30	E	Santander	cUTI	ST38	D	H15:O7	H65	-	-	-	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	R	R	S	S	S	S
G3	G	Valencia	cIAI	ST38	D	H15:O1	H65	S83L, D87N	S80I	-	R(16/4)	S(0.5)	S(≤0.12)	R	R	R	R	R	R	R	R	R	I	S	S	S
G18	G	Valencia	cIAI	ST38	D	H18:O86	H5	S83L, D87Y	S80I	S458A	S(1/4)	S(0.25)	S(≤0.12)	R	S	R	R	R	R	R	S	S	S	S	S	S
G24	G	Valencia	cUTI	ST38	D	H18:O86	H5	S83L, D87Y	S80I	S458A	S(1/4)	S(≤0.12)	S(≤0.12)	R	S	I	R	R	R	R	R	I	S	S	S	S
G11	G	Valencia	cIAI	ST2569	E	H4:O124	H31	-	-	-	S(0.25/4)	S(≤0.12)	S(≤0.12)	S	S	R	R	S	R	I	S	S	S	S	S	S
G4	G	Valencia	cIAI	ST2569	E	H4:O124	H31	-	-	-	R(16/4)	S(≤0.12)	S(≤0.12)	R	R	R	R	R	R	I	S	S	S	S	S	S
G45	G	Valencia	cIAI	ST93	B1	H12	fimH1523	-	-	-	S(0.25/4)	S(≤0.12)	S(≤0.12)	S	S	R	R	S	R	S	S	S	S	S	S	S
A50	A	Madrid	cUTI	ST93	A	H4	H27	S83L, D87N	S80I	-	S(0.5/4)	S(0.25)	S(≤0.12)	R	S	I	R	R	R	R	S	S	S	S	S	S
D15	D	Sevilla	cIAI	ST10	A	H10:O162	H30	S83L, D87N	S80I	-	S(0.25/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	S	S	S	S	S	S
A43	A	Madrid	cUTI	ST10	A	H10:O162	H54	S83L, D87N	S80I	S458A	S(1/4)	S(≤0.12)	S(≤0.12)	R	I	I	R	R	I	R	R	R	S	S	S	S
H48	H	Mallorca	cUTI	ST10	A	H32:O162	H54	-	-	-	R(4/4)	S(0.5)	S(≤0.12)	R	S	R	R	R	R	I	R	R	S	S	S	S
G62	G	Valencia	cUTI	ST410	−C	H9	H1546	S83L, D87N	S80I	S458A	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	S	R	S	S	S	S	S
B45	B	Madrid	cIAI	ST23	C	H9:O36	H35	S83L, D87N	S80I, E84G	-	S(1/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	S	S	S	S	S	R	S
D36	D	Sevilla	cUTI	ST23-1LV	C	H12:O9	H35	S83L	-	-	S(0.5/4)	S(0.25)	S(≤0.12)	R	S	R	R	S	R	R	S	S	S	S	S	S
C56	C	Barcelona	cUTI	ST75	B1	H5:O141ab	H35	-	-	-	S(0.25/4)	S(≤0.12)	S(≤0.12)	S	S	I	R	R	I	S	S	S	S	S	R	S
G25	G	Valencia	cUTI	ST162	B1	H19:O30	H32	S83L	-	-	S(1/4)	S(0.5)	S(≤0.12)	R	R	I	R	R	I	R	R	R	S	S	S	S
C63	C	Barcelona	cUTI	ST453	B1	H16:O23	H31	S83L, D87N	S80I	-	S(2/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	R	S	S	S	S	R	S
G30	G	Valencia	cIAI	ST1611	B1	H19:O96	H38	-	-	-	S(0.25/4)	S(≤0.12)	S(≤0.12)	S	S	R	R	S	R	S	S	S	S	S	S	S
G43	G	Valencia	cIAI	STNew	−C	H19:O96	H1546	-	S80I	S458A	S(0.5/4)	S(≤0.12)	S(≤0.12)	R	S	R	R	R	R	S	S	S	S	S	S	S
D1	D	Sevilla	cUTI	ST453-1LV	B1	H21:O103	H86	S83L, D87N	S80I	-	S(0.25/4)	S(0.25)	S(≤0.12)	S	S	I	R	R	I	R	S	S	S	S	S	S
E4	E	Santander	cIAI	ST155	B1	H9:O9	H32	S83L	-	-	S(0.25/4)	S(≤0.12)	S(≤0.12)	S	S	R	R	I	R	S	R	I	S	S	S	S

Figure 1. Similarity tree of *E. coli* isolates from the SUPERIOR study and molecular data obtained by WGS. Antimicrobial susceptibility results are also included.[15] Branch length is indicative of the MASH distance. C/T, ceftolozane/tazobactam; IMP, imipenem; MER, meropenem; AMC, amoxicillin/clavulanic acid; PTZ, piperacillin/tazobactam; TAZ, ceftazidime; FOT, cefotaxime; FEP, cefepime; AZT, aztreonam; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; FOS, fosfomycin; TIG, tigecycline; R, resistant; I, susceptible, increased exposure; S, susceptible, standard dose.

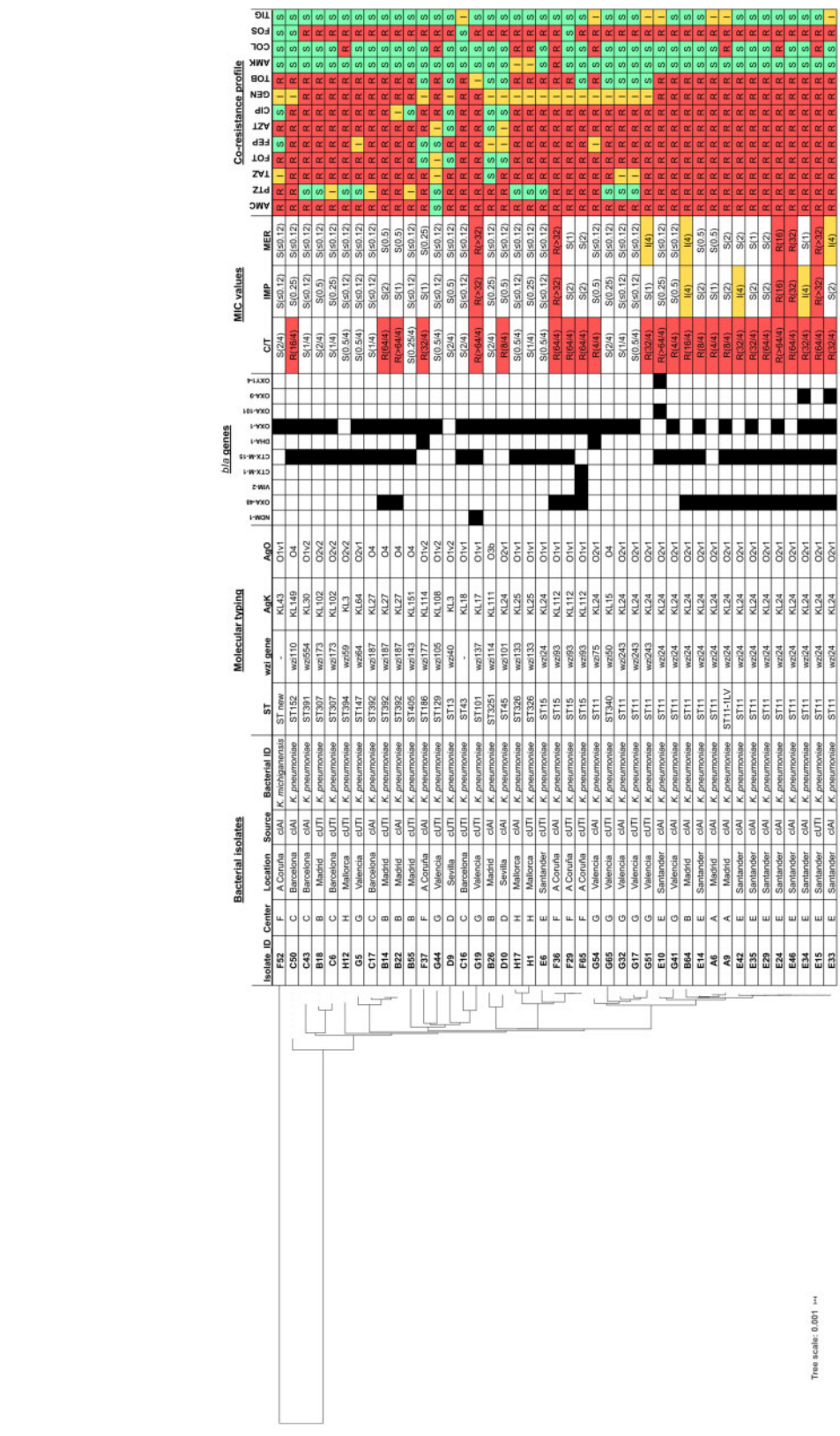


Figure 2. Similarity tree of *Klebsiella* spp. isolates from the SUPERIOR study and molecular data obtained by WGS. Antimicrobial susceptibility results are also included.[15] Branch length is indicative of the MASH distance. C/T, ceftolozane/tazobactam; IMP, imipenem; MER, meropenem; AMC, amoxicillin/clavulanic acid; PTZ, piperacillin/tazobactam; TAZ, ceftazidime; FOT, cefotaxime; FEP, cefepime; AZT, aztreonam; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; COL, colistin; FOS, fosfomycin; TIG, tigecycline; R, resistant; I, susceptible, increased exposure; S, susceptible, standard dose.

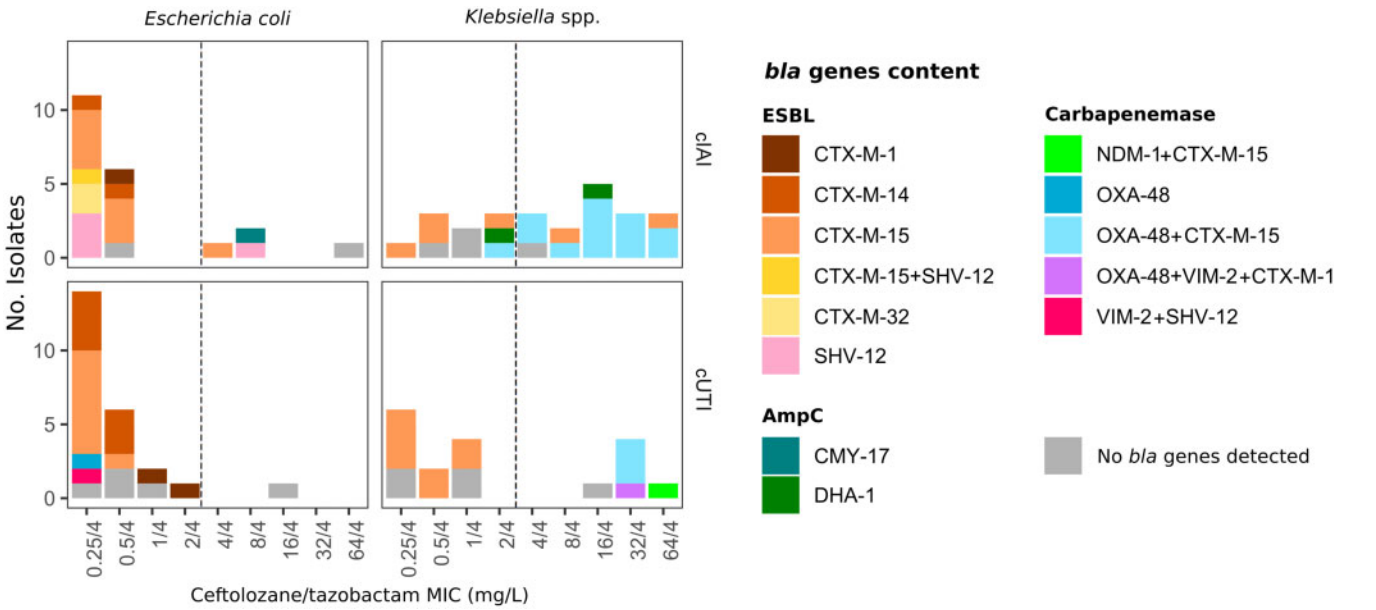


Figure 3. Distribution of *E. coli* and *Klebsiella* spp. isolates of the SUPERIOR study according to the different MIC values of ceftolozane/tazobactam[15], the β-lactamase genes detected and the infection source. Dotted lines represent the ceftolozane/tazobactam EUCAST 2020 breakpoint (susceptible ≤2 mg/L; resistant >2 mg/L).

harboured a $bla_{CTX\text{-}M}$ gene and the variant $bla_{CTX\text{-}M\text{-}15}$ was identified in 94.1% of cases (16/17). CTX-M-15 production was significantly related to the C2/*H30-Rx* subclade ($P < 0.001$; OR = 0.0; 95% CI = 0.0–0.12), which was only found in three hospitals (C, F and G). Additionally, all these ST131 *E. coli* strains carried identical fluoroquinolone resistance mutations in *gyrA* (S83L, D87N), *parC* (S80I, E84V) and *parE* (I529L) genes in the QRDR (Figure 1). Two major clusters of ST131 *E. coli* isolates were observed according to the resistome detected by WGS (Figure S1). Furthermore, for ceftolozane/tazobactam there was *in vitro* susceptibility (MIC = 0.25/4 to 1/4 mg/L) in all CC131 *E. coli* isolates except two [G22 (MIC = 8/4 mg/L) and H62 (MIC > 64/4 mg/L)] (Figure 1).

Among the non-CC131 *E. coli* isolates (62.2%, 28/45), a higher diversity of MLST clones ($SDI_{Ec\text{-}non\text{-}ST131} = 0.96$) related to different phylogroups, serotypes and *fimH* alleles was observed (Figure 1). The most frequent clones were ST38 ($n = 4$) and ST10 ($n = 3$), followed by other *E. coli* clones detected in different hospitals (CC23, ST405, ST93 and CC453). ESBL production was identified in 85.7% of non-ST131 isolates (24/28), distributed by all participant hospitals. $bla_{CTX\text{-}M}$ genes were also predominant (67.9%, 19/28), although a higher variety of enzymes was identified [CTX-M-14 ($n = 8$), CTX-M-15 ($n = 6$), CTX-M-1 ($n = 3$) and CTX-M-32 ($n = 2$)]. The $bla_{SHV\text{-}12}$ gene was also found in six isolates (21.4%), mainly from centre G (5/6). Furthermore, different mutations in QRDR genes were also identified in 71.4% (20/28) of non-CC131 isolates (Figure 1). Interestingly, *E. coli* isolates displaying colistin resistance (3/45) were typed as non-CC131.

Diversity of *K. pneumoniae* MLST clones ($SDI_{Kp} = 0.82$) was similar to that detected in the *E. coli* isolates. However, clonality was higher in the carbapenemase-producing *K. pneumoniae* isolates subset ($SDI_{Kp\text{-}CP} = 0.54$; $SDI_{Kp\text{-}non\text{-}CP} = 0.94$). Predominance of the ST11 *K. pneumoniae* high-risk clone was observed (41.9%, 18/43) and in 12 out of 18 cases (66.7%) the OXA-48-encoding gene was identified (Figure 2). ST11 was located in four hospitals (A, B, E and G) and mainly in cIAI samples (15/18). Moreover, OXA-48 production was only observed in ST11 isolates from three centres (A, B and E) and the presence of $bla_{CTX\text{-}M\text{-}15}$ was confirmed in all cases. The KL24-O2v1 serotype was inferred in all ST11 *K. pneumoniae* strains, although three different *wzi* genes were found (Figure 2). Moreover, ST11 *K. pneumoniae* strains were clustered according to the complete antibiotic resistance gene content (Figure S2). Among non-ST11 clones, the clonal diversity was higher ($SDI_{Kp\text{-}non\text{-}ST11} = 0.96$) and a wide variety of *wzi* genes, K and O antigens was observed (Figure 2). The most frequent clones were ST15 ($n = 4$), ST392 ($n = 3$), ST307 ($n = 2$) and ST326 ($n = 2$). Carbapenemase production was only confirmed in five isolates belonging to ST15 and ST392 clones [OXA-48-ST15 ($n = 2$), OXA-48+VIM-2-ST15 ($n = 1$) and OXA-48-ST392 ($n = 2$)] (Figure 2).

Virulence gene content

A high number of genes associated with the virulome was identified in the *E. coli* collection [14–110 virulence factors (VFs) belonging to 6–29 virulence loci (VL)], particularly in CC131 *E. coli* strains (53–88 VFs, 17–21 VL). Overall, *E. coli* strains were clustered by MLST clones according to the virulome detected (Figure S3). Furthermore, the *E. coli* isolates with a ceftolozane/tazobactam-resistant phenotype also presented a high virulence gene content (17–110 VFs, 10–29 VL), although similarities in the virulence profile were not found (Figure S3).

A high virulence gene content was also detected in all *K. pneumoniae* isolates [44–85 VFs, 17–35 VL], particularly in the ST11 clone (44–77 VFs, 25–35 VL). Virulence genes were not identified in the *K. michiganensis* strain (Figure S4). The siderophore yersiniabactin (*ybt*) was detected in 62.8% (27/43) of the

K. pneumoniae isolates and six distinct ICE*Kp* lineages were assigned: ICE*Kp2-ybt* 13 ($n = 3$), ICE*Kp3-ybt* 9 ($n = 1$), ICE*Kp4-ybt* 10 ($n = 17$), ICE*Kp10-ybt* 17 ($n = 1$), ICE*Kp12-ybt* 16 ($n = 3$) and ICE*Kp*-unknown ($n = 2$) (Figure S4). A positive association was established between the *ybt* virulence determinant and the presence of carbapenemase-encoding genes ($P = 0.0003$, OR = 23.6, 95% CI = 2.8–1127.2). Furthermore, correlation between the *ybt* and the OXA-48-ST11 high-risk clone ($P = 0.03$, OR = 5.2, 95% CI = 1.3–35.1), particularly with the ICE*Kp4-ybt* 10 lineage ($P = 0.013$; OR = 0.1; 95% CI = 0.01–0.76) and the ceftolozane/tazobactam-resistant phenotype, was also observed ($P = 0.01$, OR = 6.0, 95% CI = 1.3–31.0). The genotoxin colibactin *clb* 3 (2.3%, 1/43) and the siderophore aerobactin *iuc* 5 (4.6%, 2/43) were also found in non-carbapenemase producers with susceptible and resistant ceftolozane/tazobactam phenotypes, respectively (Figure S4).

Discussion

In this study we used genome-based technology to analyse the genetic diversity, the resistome and the virulome of a subset of *E. coli* and *Klebsiella* spp. isolates displaying different MDR profiles, including ceftolozane/tazobactam-resistant and -susceptible phenotypes, recovered from ICU patients admitted to eight hospitals distributed throughout Spain as a part of the SUPERIOR surveillance study.[15]

Among the sequenced *E. coli* isolates, the high-risk clone ST131 was the most prevalent. ST131 is the most frequent *E. coli* clone related to ESBL production and fluoroquinolone resistance and is considered worldwide as a major cause of urinary tract and bloodstream infections.[24] In Spain, the CTX-M-15-ST131-H30-Rx subclade has been reported as the main pathogen causing healthcare-associated ESBL-producing *E. coli* bacteraemia of urinary origin.[25] We found that the CTX-M-15-ST131-H30-Rx subclone is also predominant among the *E. coli* isolates causing cUTI and cIAI in patients admitted to Spanish ICUs. Moreover, we detected an extensive virulence gene content among all *E. coli* isolates, but particularly in the ST131 high-risk clone. The acquisition of virulence factors has been defined as a precursor in the evolution and global dissemination of this successful ST131 clone, conferring an increased capacity to cause human infections and to gain antibiotic resistance genes.[26] Note that the ST131 *E. coli* isolates detected in this collection came from five hospitals, although the hypervirulent H30-Rx subclade was only found in three centres, two of them located on the Mediterranean coast (Barcelona and Valencia).

A high clonal diversity was found in non-ST131 *E. coli* isolates. An association with different $bla_{\text{CTX-M}}$ genes was observed and $bla_{\text{CTX-M-14}}$ was the most frequent ESBL, followed by $bla_{\text{CTX-M-15}}$. Both CTX-M-14 and CTX-M-15 have been reported as the most prevalent ESBL enzymes in our country in the last decade and have usually been related to community-acquired *E. coli* isolates.[1] In this work, the inter-hospital distribution of *E. coli* clones previously described in Spanish healthcare centres, such as ST10, ST23, ST38 or ST410, was also found to be related to CTX-M production.[27] Furthermore, carbapenemase-encoding genes were only detected in two *E. coli* isolates and both strains were typed as ST38 ($bla_{\text{OXA-48}}$) and CC23 ($bla_{\text{VIM-2}}$). The association of some of these clones with carbapenemase production, particularly OXA-48 type, is globally increasing and is currently a cause of great concern for public health.[28]

Among the *K. pneumoniae* isolates, carbapenemase production was frequently detected in ceftolozane/tazobactam-resistant isolates and $bla_{\text{OXA-48}}$ was predominant. Most OXA-48-producing isolates belonged to the ST11 high-risk clone and were also CTX-M-15 and OXA-1 (Figure 2) co-producers. CTX-M-15+OXA-48-ST11 is considered the most prevalent *K. pneumoniae* clone among both infected and colonized patients and has recently been involved in different healthcare-associated outbreaks in Spain.[5,6] In this work, the OXA-48-ST11 clone was found in three participating ICUs, two of them from the same geographical region (Madrid). Additionally, other *K. pneumoniae* epidemic clones previously reported in Spanish hospitals were identified among OXA-48 producers (ST15, ST392) and were also related to the production of ESBL enzymes (ST15, ST307, ST101, ST405).[5,6,29] Most of them were detected in at least two hospitals, showing the inter-hospital clonal dispersion among Spanish ICUs. The risk of endemicity of the OXA-48 enzyme is increasing and has been related to the successful dissemination and establishment of *K. pneumoniae* high-risk clones in the healthcare setting.[28] The role of certain *K. pneumoniae* clones such as ST11 in the spread of OXA-48 among different *K. pneumoniae* clones and also to other Enterobacterales species has been demonstrated recently.[30,31] In this study, we highlight that the OXA-48-ST11 *K. pneumoniae* clone is also predominant in ICU patients with cUTI and cIAI and that the risk of OXA-48 transmission to other Enterobacterales members may also be increasing in these units.

The key to controlling and avoiding the spread of resistance determinants such as carbapenemase-encoding genes among clinical isolates is their accurate and rapid identification and characterization. The contribution of WGS to the study of the emergence and evolution of resistant microbial populations has been clearly demonstrated.[32] Nevertheless, the surveillance of MDR microorganisms and the prediction of antimicrobial resistance profiles using DNA sequencing technology is still challenging for microbiology laboratories.[33,34] In this collection, we found discrepancies in both *E. coli* and *Klebsiella* spp. subsets between the genomic detection of carbapenemase-encoding genes and the antibiotic susceptibility testing results obtained for carbapenems in our previous work.[15] The detection of carbapenemases among the *E. coli* isolates was scarce and conclusive results could not be obtained. However, in the *Klebsiella* spp. collection, genotype/phenotype discrepancies could be explained by the predominance of the OXA-48 enzyme. OXA-48-type carbapenemases weakly hydrolyse carbapenems and their recognition and detection based on phenotypic profiles can be difficult.[35] In the same way, colistin resistance was identified in 7% and 21% of *E. coli* and *K. pneumoniae* isolates, respectively, although acquired resistance mechanisms such as the *mcr-1* gene were only detected in one *E. coli* isolate.

During the SUPERIOR study, good activity of ceftolozane/tazobactam was demonstrated in both *E. coli* (95%) and *Klebsiella* spp. (66%) populations. This novel combination is proposed as a therapeutic option for the treatment of infections caused by ESBL-producing Enterobacterales isolates in order to spare carbapenems.[7] In our collection, resistance to ceftolozane/tazobactam was detected among carbapenemase producers, but only in *K. pneumoniae* isolates, since both carbapenemase-producing *E. coli* isolates were susceptible to this antibiotic. On the other hand, in agreement with other studies, a low resistance rate to

ceftolozane/tazobactam was also detected among ESBL- and/or AmpC-producing *K. pneumoniae* and *E. coli* strains, although correlation with these resistance mechanisms could not be established.[10–12]

In conclusion, OXA-48+CTX-M-15-ST11-*K. pneumoniae* and CTX-M-15-*E. coli*-ST131-H30-Rx are the most frequent clones causing cUTI and cIAI in patients admitted to ICUs from Spanish hospitals. As expected, *in vitro* activity of ceftolozane/tazobactam was not demonstrated in carbapenemase-producing *K. pneumoniae* isolates, but this could be a therapeutic option to treat complicated infections caused by the hypervirulent ST131 *E. coli* high-risk clone. In addition, although further analysis should be performed, according to our results, the presence of carbapenemase genes in *E. coli* does not always correlate to ceftolozane/tazobactam resistance. This correlation was present in *K. pneumoniae*, but other resistance mechanisms might be involved when resistance to this β-lactam/β-lactamase inhibitor combination is present. The genome-based approach is a powerful tool to study the molecular epidemiology of circulating nosocomial pathogens, but the implementation of this technology to infer genotype/phenotype relationships is still a challenge for diagnostic laboratories.

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Transparency declarations

R.C., E.C. and G.B. have participated in educational programmes organized by MSD and Pfizer. J.D.-R. and D.L.-M. are employees of MSD Spain. All other authors: none to declare.

Supplementary data

Figures S1 to S4 and Tables S1 to S3 are available as Supplementary data at *JAC-AMR* Online.

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