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# Whole Genome Sequencing Analysis of *Klebsiella pneumoniae* Isolates from Health Care–Associated Bacteremia of Urinary Origin in Spain: Findings from the Multicenter ITUBRAS-2 Cohort Study

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***Background.*** The objective of this study was to assess the microbiological and clinical features of *Klebsiella pneumoniae* health care–associated bacteremia of urinary origin (HCA-BUO) in Spain, with a focus on third-generation cephalosporin-(3GCR-Kp) and carbapenem-resistant *K pneumoniae* (CR-Kp) isolates.

***Methods.*** A total of 96 (21.4%, 96/449) *K pneumoniae* blood isolates were prospectively collected from patients with HCA-BUO (n = 443) from 12 tertiary care hospitals in Spain (2017–2019). Antimicrobial susceptibility was determined (standard broth microdilution), and extended-spectrum β-lactamase, AmpC, and carbapenemase production was screened. A subset of 55 *K pneumoniae* isolates was analyzed by whole genome sequencing (Illumina) to determine population structure, resistome, and virulome. Additionally, 13 of these isolates were subjected to long-read sequencing (Nanopore) for plasmid characterization. Patients' baseline and clinical characteristics were reviewed.

***Results.*** 3GCR-Kp prevalence was 43.8% (42/96), mostly associated with extended-spectrum β-lactamase production (34/96, 35.4%; mainly CTX-M-15, 32/34, 94.1%) and the dissemination of sequence type (ST)-307 (15/34, 44.1%) and other globally spread multidrug-resistant high-risk clones. CR-Kp prevalence was 9.4% (9/96); all isolates belonged to different STs and were mostly associated with carbapenemase production (6/9, 66.7%; mainly OXA-48–like, n = 3). Additionally, 3GCR-Kp and CR-Kp isolates showed higher content of other antibiotic resistance genes. Altogether, these episodes were associated with prior antibiotic use and receipt of inadequate empirical treatment.

***Conclusions.*** There is a high prevalence of 3GCR and *CR-Kp* causing HCA-BUO in Spain, mainly driven by the dissemination of ST307/CTX-M-15 and other globally spread multidrug-resistant high-risk clones, challenging the selection of empirical and targeted treatments for these infections.

**Keywords.** carbapenemase-producing Enterobacterales; extended-spectrum β-lactamases; health care–associated infections; *Klebsiella pneumoniae*; ST307 clone.

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*Klebsiella pneumoniae* is one of the main etiologic agents of urinary tract infections, which may often progress to invasive bacteremia. This situation is particularly relevant in health care–associated (HCA) infections among patients who are compromised and hospitalized and those with frequent hospital contacts or residence in long-term care facilities [1–3]. Unfortunately, proper management and treatment of these infections are being severely compromised by the escalating rates of multidrug resistance observed worldwide in this species. In fact, according to the SENTRY Antimicrobial Surveillance Program, a significant increase in third-generation cephalosporin-resistant (3GCR) and carbapenem-resistant (CR) *K pneumoniae* has been observed in the last decades [4]. For this reason, the World Health Organization has recently reaffirmed 3GCR and CR *K pneumoniae* as a critical priority for which new antimicrobial agents are urgently needed [5]. This increasing threat mostly results from the dissemination of transferable resistance determinants, such as those encoding for extended-spectrum β-lactamases (ESBLs) and carbapenemase enzymes, embedded into mobile genetic elements that are frequently associated with some successful lineages known as multidrug-resistant (MDR) high-risk clones (HiRCs). Examples of such clones well established in *K pneumoniae* include ST11, ST14, ST15, ST258 and ST512, but others, such as ST147 and ST307, are emerging globally [3, 6, 7].

To monitor the expansion of these successful MDR HiRCs and to understand the clinical characteristics and risk factors associated with these infections, it is important to conduct surveillance studies at regional, local, and international levels. Information provided from these studies is also useful for establishing guidelines for empirical antimicrobial therapy. In this regard, we used data from the ITUBRAS-2 Spanish multicenter study to describe the clinical and microbiological characteristics of HCA bacteremia of urinary origin (HCA-BUO). In this cohort study, we collected blood isolates from HCA-BUO episodes between 2017 and 2019 across 12 tertiary care hospitals in Spain. Overall, *Escherichia coli* was the most prevalent pathogen (49.4%), followed by *K pneumoniae* (21.4%). Data from *E coli* were previously presented [8]. Herein we focused on *K pneumoniae* isolates, and we used phenotypic, molecular, and whole genome sequencing (WGS) methods to assess their microbiological characteristics, molecular epidemiology, and population structure. We also characterized clinical features associated with 3GCR and CR *K pneumoniae* infections.

## METHODS

### Study Design and Bacterial Isolates

This study was part of the ITUBRAS-2 prospective cohort, which included HCA-BUO episodes from 12 Spanish hospitals (2017–2019) [9]. A total of 443 HCA-BUO episodes with 449 bacterial isolates were included and classified according to the origin of infection as hospital-acquired (HA) and community-onset HCA (CO-HCA) following criteria from Friedman et al (HA, n = 215; CO-HCA, n = 234) [10]. Blood isolates were collected and sent to the reference laboratory (Hospital Ramón y Cajal) for microbiological studies.

The study was approved by the Clinical Research Ethical Committee of Hospital del Mar (registration 2016/6957/I) and by the local ethics committee of each participating center.

### Bacterial Identification and Antimicrobial Susceptibility Testing

Bacterial identification was confirmed by MALDI-TOF MS (Bruker Daltonics GmbH). Antimicrobial susceptibility was performed by a standard broth microdilution method with 96-well plates (Thermo Fisher Scientific) and interpreted with EUCAST-2023 clinical breakpoints (https://www.eucast.org/clinical_breakpoints/). The antibiotics studied are listed in Table 1. Colistin resistance was confirmed by the UMIC colistin kit (Biocentric). Isolates were classified according to their resistance profile as MDR, extensively drug-resistant (XDR), and pandrug-resistant (PDR) based on the criteria of Magiorakos et al [11]. Additionally, the difficult-to-treat resistance phenotype was defined as nonsusceptibility to all β-lactams and fluoroquinolones [12].

### Detection of β-Lactamase Enzymes

ESBL, AmpC, and carbapenemase production was screened by the double-disk synergy ESBL/AmpC confirm kit (Rosco Diagnostica) and the colorimetric Carba-NP test (bioMérieux), respectively [13, 14]. The presence of genes encoding ESBLs and carbapenemases was investigated by multiplex polymerase chain reaction and Sanger sequencing, as previously described [8].

### WGS and Bioinformatic Analysis

A subset of 55 *K pneumoniae* isolates was selected for genome characterization by short-read sequencing (2 × 250–base pair paired-end reads, Illumina NovaSeq 6000 platform; Oxford Genomics Centre). These isolates included all 3GCR isolates (2 plasmidic AmpC and 34 ESBL producers, including 3 ertapenem-resistant, non–carbapenemase-producing isolates), all carbapenemase producers (n = 7), and a control group randomly selected to represent different geographic areas (n = 12).

Sequencing processing was carried out as previously described [8]. Draft genomes were annotated by Prokka version 1.14.6 [15]. MASH version 2.3 and iTOL (https://itol.embl.de/) were used to generate and trace a similarity tree based on a neighbor-joining algorithm [16]. Additionally, the core genome was obtained from all sequenced *K pneumoniae* ST307 isolates and the ST307-KPN11 reference genome (GCA_002148835.1) with Snippy version 4.6.0. A maximum likelihood phylogenetic tree was reconstructed after the removal of recombined regions via IQ-TREE version 1.6.12 and visualized with iTOL.

**Table 1. Antimicrobial Susceptibility Among *Klebsiella pneumoniae* Complex Isolates.**

| | Minimum Inhibitory Concentration,[a] mg/L | | | EUCAST-2023, % | | |
|---|---|---|---|---|---|---|
| Organism/Antimicrobial Agent | MIC50 | MIC90 | Range | S | I | R |
| **Total *Klebsiella pneumoniae* complex (N = 96)** | | | | | | |
| Amoxicillin-clavulanate | >16 | >16 | <8 to >16 | 37.5 | … | 62.5 |
| Piperacillin-tazobactam | <8 | >32 | <8 to >32 | 52.0 | … | 48.0 |
| Ceftolozane/tazobactam | 0.5 | 2 | <0.12 to >64 | 90.6 | … | 9.4 |
| Ceftazidime | 1 | >32 | <0.12 to >32 | 55.2 | 3.1 | 41.7 |
| Cefotaxime | 0.5 | >32 | <0.25 to >32 | 56.3 | 1.0 | 42.7 |
| Cefepime | 0.5 | >32 | <0.12 to >32 | 56.3 | 3.1 | 40.6 |
| Aztreonam | <1 | >16 | <1 to >16 | 56.3 | 1.0 | 42.7 |
| Ertapenem | <0.06 | 0.5 | <0.06 to >4 | 90.6 | … | 9.4 |
| Imipenem | 0.5 | 2 | <0.25 to >16 | 92.7 | 4.2 | 3.1 |
| Meropenem | <0.12 | 0.5 | <0.12 to >16 | 94.8 | 2.1 | 3.1 |
| Trimethoprim/sulfamethoxazole | <1/19 | >4/76 | <1/19 to >4/76 | 54.2 | 0.0 | 45.8 |
| Ciprofloxacin | 0.5 | >2 | <0.06 to >2 | 47.9 | 3.1 | 49.0 |
| Gentamicin | <2 | >8 | <2 to >8 | 70.8 | … | 29.2 |
| Tobramycin | <2 | >8 | <2 to >8 | 59.4 | … | 40.6 |
| Amikacin | <8 | 16 | <8 to >32 | 85.4 | … | 14.6 |
| Colistin | <2 | <2 | <2 to >4 | 94.8 | … | 5.2 |
| **Non-ESBL/AmpC/carbapenemase (n = 53)** | | | | | | |
| Amoxicillin-clavulanate | <8 | >16 | <8 to >16 | 67.9 | … | 32.1 |
| Piperacillin-tazobactam | <8 | 32 | <8 to >32 | 79.3 | … | 20.7 |
| Ceftolozane-tazobactam | 0.5 | 1 | <0.12 to 2 | 100.0 | … | 0.0 |
| Ceftazidime | 0.5 | 1 | <0.12 to 2 | 98.1 | 1.9 | 0.0 |
| Cefotaxime | <0.25 | 0.5 | <0.25 to 1 | 100.0 | 0.0 | 0.0 |
| Cefepime | <0.12 | 0.5 | <0.12 to 2 | 98.1 | 1.9 | 0.0 |
| Aztreonam | <1 | <1 | <1 | 100.0 | 0.0 | 0.0 |
| Ertapenem | <0.06 | <0.06 | <0.06 to 0.25 | 100.0 | … | 0.0 |
| Imipenem | 0.5 | 1 | <0.25 to 2 | 100.0 | 0.0 | 0.0 |
| Meropenem | <0.12 | <0.12 | <0.12 to 0.25 | 100.0 | 0.0 | 0.0 |
| Trimethoprim/sulfamethoxazole | <1/19 | >4/76 | <1/19 to >4/76 | 84.9 | 0.0 | 15.1 |
| Ciprofloxacin | <0.06 | >2 | <0.06 to 2 | 84.9 | 1.9 | 13.2 |
| Gentamicin | <2 | <2 | <2 to >8 | 98.1 | … | 1.9 |
| Tobramycin | <2 | >8 | <2 to >8 | 98.1 | … | 1.9 |
| Amikacin | <8 | <8 | <8 | 100.0 | … | 0.0 |
| Colistin | <2 | <2 | <2 to >4 | 96.2 | … | 3.8 |
| **ESBL/AmpC (n = 36)** | | | | | | |
| Amoxicillin-clavulanate | >16 | >16 | 16 to >16 | 0.0 | … | 100.0 |
| Piperacillin-tazobactam | 32 | >32 | <8 to >32 | 19.4 | … | 80.6 |
| Ceftolozane-tazobactam | 0.5 | 2 | 0.25 to >64 | 91.7 | … | 8.3 |
| Ceftazidime | >32 | >32 | 2 to >32 | 5.6 | 0.0 | 94.4 |
| Cefotaxime | >32 | >32 | 32 to >32 | 0.0 | 0.0 | 100.0 |
| Cefepime | >32 | >32 | 0.25 to >32 | 2.8 | 2.8 | 94.4 |
| Aztreonam | >16 | >16 | 16 to >16 | 0.0 | 0.0 | 100.0 |
| Ertapenem | 0.12 | 0.5 | <0.06 to >4 | 91.7 | … | 8.3 |
| Imipenem | 0.5 | 2 | <0.25 to 4 | 97.2 | 2.8 | 0.0 |
| Meropenem | <0.12 | 0.5 | <0.12 to 0.5 | 100.0 | 0.0 | 0.0 |
| Trimethoprim/sulfamethoxazole | >4/76 | >4/76 | <1/19 to >4/76 | 11.1 | 0.0 | 88.9 |
| Ciprofloxacin | >2 | >2 | <0.06 to >2 | 2.8 | 5.6 | 91.7 |
| Gentamicin | >8 | >8 | <2 to >8 | 36.1 | … | 63.9 |
| Tobramycin | >8 | >8 | <2 to >8 | 8.3 | … | 91.7 |
| Amikacin | <8 | 16 | <8 to >32 | 66.7 | … | 33.3 |
| Colistin | <2 | <2 | <2 to >4 | 94.4 | … | 5.6 |
| **Carbapenemase (n = 7)** | …[b] | …[b] | | | | |
| Amoxicillin-clavulanate | | | >16 | 0.0 | … | 100.0 |
| Piperacillin-tazobactam | | | <8 to >32 | 14.3 | … | 85.7 |
| Ceftolozane-tazobactam | | | 0.5 to >64 | 14.3 | … | 85.7 |

**Table 1. Continued**

| Organism/Antimicrobial Agent | Minimum Inhibitory Concentration,[a] mg/L | | | EUCAST-2023, % | | |
|---|---|---|---|---|---|---|
| | MIC50 | MIC90 | Range | S | I | R |
| Ceftazidime | | | 0.5 to >32 | 14.3 | 0.0 | 85.7 |
| Cefotaxime | | | 1 to >32 | 14.3 | 14.3 | 71.4 |
| Cefepime | | | 0.5 to >32 | 14.3 | 14.3 | 71.4 |
| Aztreonam | | | <1 to >16 | 14.3 | 14.3 | 71.4 |
| Ertapenem | | | 0.5 to >4 | 14.3 | … | 85.7 |
| Imipenem | | | <0.25 to >16 | 14.3 | 42.9 | 42.9 |
| Meropenem | | | <0.12 to >16 | 28.6 | 28.6 | 42.9 |
| Trimethoprim/sulfamethoxazole | | | <1/19 to >4/76 | 42.9 | 0.0 | 57.1 |
| Ciprofloxacin | | | 1 to >2 | 0.0 | 0.0 | 100.0 |
| Gentamicin | | | <2 to >8 | 42.9 | … | 57.1 |
| Tobramycin | | | <2 to >8 | 28.6 | … | 71.4 |
| Amikacin | | | <8 to >32 | 71.4 | … | 28.6 |
| Colistin | | | <2 to >4 | 85.7 | … | 14.3 |

Abbreviations: ESBL, extended-spectrum β-lactamase; I, susceptible–increase exposure; R, resistant; S, susceptible–standard dose.

[a]MIC50 and MIC90 refer to values that inhibit 50% and 90% of the growth of the tested bacterial isolates, respectively.

[b]Not applicable: number of isolates <10.

The genotyping tool Kleborate (version 2.3.2) was used for taxonomic identification and to determine in silico multilocus sequence typing (MLST), and detect truncations and point mutations in *OmpK35*/*OmpK36* porins [17]. Chromosomal point mutations in quinolone resistance–determining regions (QRDRs) were detected with PointFinder software [18]. Other resistance and virulence genes and plasmid replicons were detected using Abricate (version 1.0.1) via the ARG-ANNOT, ResFinder, vfdb, and PlasmidFinder databases, with a threshold of 80% coverage and >90% identity. *K pneumoniae* integrative conjugative elements (ICEKp), capsular polysaccharide K-loci, and acquired factors—including siderophores (yersiniabactin, salmochelin, and aerobactin), regulators of hypermucoid phenotype (*rmpADC* and *rmpA2*), and the genotoxin colibactin—were also identified with Kleborate.

Additionally, a subset of 13 ESBL/carbapenemase producers representing the most frequent HiRCs was randomly selected for long-read sequencing (MinION; Oxford Nanopore Technologies) for plasmid characterization. Libraries were prepared by the 1D Ligation Sequencing Kit (SQK-LSK109) in combination with the Native Barcoding Kit (EXP-NBD104; Oxford Nanopore Technologies) and were loaded onto an R9.4 flow cell (Oxford Nanopore Technologies). The run was performed on a MinION MK1b device. Collection of raw electronic signal data and live base calling was performed with MinKNOW 22.10.10 and the Guppy basecaller (Oxford Nanopore Technologies). Hybrid assemblies combining short- and long-read sequencies were obtained by Unicycler version 0.5.0 [19]. Plasmids were annotated and visualized through the Proksee server (https://proksee.ca/projects/new) [20]. A comparative map was drawn with Blast and EasyFig [21].

Information on WGS data is summarized in Supplementary Table 1. All complete sequences were deposited at the DDBJ/ENA/GenBank and Sequence Read Archive under BioProject PRJNA1070622.

### Clinical Features and Definitions

Patients' characteristics and baseline clinical variables were prospectively recorded, including comorbidity, antibiotic exposure in the previous 3 months, and clinical severity of the infection. Empiric therapy was defined as the antibiotic treatment administered before culture results were available, while targeted treatment referred to any antibiotic therapy administered after receiving definitive microbiological results. Antibiotic treatment was considered adequate when the blood culture isolate was susceptible to at least 1 of the antimicrobial agents prescribed and the dose was in accordance with the current medical standard (eg, EUCAST criteria).

### Statistical Analysis

Categorical variables were expressed as numbers of cases and percentages and were compared with the $\chi^2$ test or Fisher exact test. Continuous variables were expressed as median and IQR and compared by Student *t* test or Mann-Whitney *U* test. Statistical analysis was carried out in R version 4.1.2 (RStudio). A *P* value <.05 was considered statistically significant.

## RESULTS

### *K pneumoniae* Prevalence, Antimicrobial Susceptibility, and β-Lactamase Production

Overall, *K pneumoniae* accounted for 96 of 449 isolates (21.4%), all of which were obtained from unique patients. It was the second-most prevalent species in our cohort, following *E coli* (49.4%, 222/449), and was significantly associated with HA

episodes (HA, 55/215 [25.6%]; CO-HCA, 41/234 [17.5%]; $P = .037$).

Antimicrobial resistance rates among groups are shown in Table 1. High resistance rates to amoxicillin-clavulanate (62.5%), ciprofloxacin (49%), piperacillin-tazobactam (48%), and trimethoprim/sulfamethoxazole (45.8%) were observed. Resistance to at least one 3GC or carbapenem was detected in 43.8% (42/96) and 9.4% (9/96) of isolates, respectively. Among tested antibiotics, the most globally active were colistin, carbapenems, and ceftolozane/tazobactam (resistance <10%). In total, 52.1% of *K pneumoniae* isolates were categorized as MDR (50/96), 4.2% as XDR (4/96), and 1% (1/96) as PDR. Additionally, 3 isolates were classified as difficult-to-treat resistance (1 MDR, 1 XDR, and 1 PDR). Notably, the majority of isolates that were difficult-to-treat resistance (3/3), PDR (1/1), and XDR (4/5) were associated with HA episodes.

ESBL and AmpC producers collectively accounted for 37.5% (36/96) of isolates and showed higher resistance rates than nonproducers to tobramycin (91.7% vs 1.9%), ciprofloxacin (91.7% vs 13.2%), trimethoprim/sulfamethoxazole (88.9% vs 15.1%), piperacillin-tazobactam (80.6% vs 20.7%), gentamicin (63.9% vs 1.9%), and amikacin (33.3% vs 0%; $P < .01$ for all comparisons; Supplementary Table 2). Carbapenemase production was detected in 7.3% of isolates (7/96), 4 of them coproducing ESBL enzymes.

ESBL producers were equally distributed according to origin of infection (32.7% HA, 18/55; 39% CO-HCA, 16/41; $P = .4$). On the contrary, all AmpC and carbapenemase producers corresponded to HA episodes. Globally, resistance to ceftolozane/tazobactam (minimum inhibitory concentration >2 mg/L) was 9.4% (9/96), with all producing carbapenemase (n = 6), plasmid-encoded AmpC (n = 2), or ESBL (n = 1) enzymes.

#### WGS, Population Structure, and HiRCs

Supplementary Table 1 summarizes the WGS data of selected isolates (n = 55). Genotyping identified 3 species: *K pneumoniae sensu stricto* (n = 53, 96.4%), *K quasipneumoniae* subsp *quasipneumoniae* (n = 1, 1.8%), and *K variicola* subsp *variicola* (n = 1, 1.8%).

Seven-gene MLST grouped these isolates into 29 STs (Figure 1). In total, 56.4% (31/55) were assigned to internationally recognized MDR HiRCs: ST307 (n = 16), ST405 (n = 4), ST17 (n = 3), ST147 (n = 2), ST15 (n = 2), ST392 (n = 2), ST11 (n = 1), and ST101 (n = 1). The ST307 was predominant among ESBL producers (15/34, 44.1%). Other frequent STs in this group were ST405 (4/34, 11.8%), ST17 (2/34, 5.9%), and ST326 (2/34, 5.9%). In contrast, carbapenemase/AmpC producers and nonproducers belonged to a variety of STs, including 1 ST307. The predominant ST307 was found in 50% (6/12) of participant hospitals, with no differences in its prevalence between HA episodes (28.1%, 9/32) and CO-HCA episodes (30.4%, 7/23; $P = .85$). A phylogenetic analysis showed that these ST307 isolates clustered regardless of the geographic area and type of infection (HA or CO-HCA; Supplementary Figure 1).

#### Antibiotic Resistance Determinants

A schematic representation of resistance gene content detected among sequenced *K pneumoniae* complex isolates is shown in Figure 1 and Supplementary Figure 2.

Regarding acquired β-lactam resistance determinants, ESBL-encoding genes were detected in 34 isolates, 1 of them coexpressing 2 enzymes. CTX-M-15 was predominant (32/34, 94.1%), but other enzymes were also detected: CTX-M-9, CTX-M-14, and SHV-27 (n = 1 each). Additionally, plasmid-encoded AmpC β-lactamases were detected in 3 isolates (DHA-1, n = 2; CMY-2, n = 1), 1 of them coproducing CTX-M-15. Carbapenemase production was confirmed in 7 isolates (3 OXA-48–like, 1 OXA-244, 1 KPC-3, 1 KPC-33, and 1 VIM-1), 4 of them coproducing CTX-M-15. Remarkably, 1 of these isolates encoding $bla_{\text{KPC-33}}$ (isolate_0172/ST15) showed in vitro susceptibility to all carbapenems but resistance to 3GC and ceftolozane/tazobactam. $bla_{\text{OXA-1}}$ was detected in 52.7% of the isolates (29/55), all of them coproducing ESBL enzymes. Other broad- and narrow-spectrum acquired β-lactam resistance determinants were found: $bla_{\text{OXA-9}}$ (4/55), $bla_{\text{TEM-like}}$ (27/55, $bla_{\text{TEM-1-B}}$; 2/55, $bla_{\text{TEM-1A}}$), and $bla_{\text{LAP-2}}$ (2/55). Finally, 10 chromosomal-encoded SHV variants in *K pneumoniae sensu stricto* isolates were detected; $bla_{\text{SHV-28}}$ was the most frequent (35.8%, 19/53) and was associated with ST307 (84.2%, 16/19).

Aside from β-lactamases, a range of other acquired resistance genes (ARGs) conferring resistance to other antimicrobials was found, such as aminoglycosides, chloramphenicol, colistin, quinolones, sulfonamides, trimethoprim, and tetracyclines (Figure 1, Supplementary Figure 2). Altogether, ESBL, pAmpC, and carbapenemase producers presented significantly higher resistance gene content (mean, 14.3; median, 16; range, 5–19) than nonproducers (mean, 4.2; median, 4; range, 4–5; $P < .001$) and had a significantly higher frequency of some ARGs: *aac(3′)-IIa*, *aac(6′)-Ib-cr*, *aph(3′)-Ib*, *aph(6′)-Id*, *catB4*, *dfrA14*, *qnrB1*, *sul1*, *sul2*, *tet(A)*, and *tet(R)* ($P < .01$ for all comparisons).

Additionally, we identified at least 1 relevant mutation in QRDRs in 75% of fluoroquinolone-resistant (30/40) and 6.7% of fluoroquinolone-susceptible (1/15) isolates. Mutations in OmpK35 (n = 10) or OmpK36 (n = 3) porins were found in 20% (11/55) of sequenced isolates. These mutations were more frequent among ESBL, AmpC, and carbapenemase producers (8.3% [1/12] vs 69.8% [30/43], $P < .01$; 8.3% [1/12] vs 23.3% [10/43], $P = .4$; respectively). In vitro resistance to ertapenem among non–carbapenemase producers was observed in 3 ESBL/AmpC-producing isolates with the OmpK36 affected, either completely lacking this porin (n = 1) or presenting

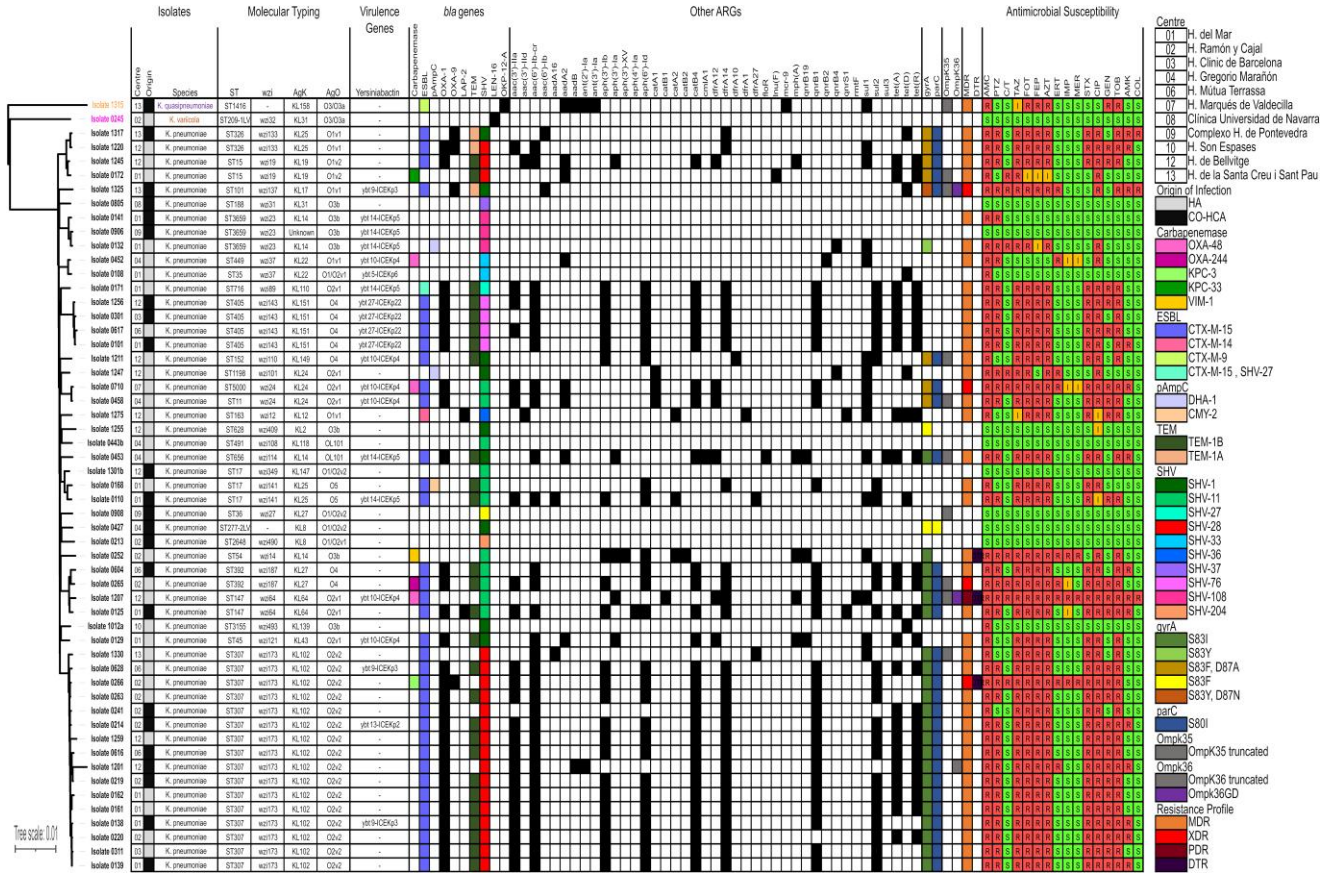


**Figure 1.** Similarity tree of sequenced *Klebsiella pneumoniae* complex isolates (n = 55) during the ITUBRAS-2 project and visualized with iTOL. Branch length is indicative of the MASH distance. Antimicrobial susceptibility results and molecular and epidemiologic data are also included. AMC, amoxicillin/clavulanic acid; AMK, amikacin; AZT, aztreonam; CIP, ciprofloxacin; COL, colistin; C/T, ceftolozane/tazobactam; ERT, ertapenem; FEP, cefepime; FOT, cefotaxime; GEN, gentamicin; IMP, imipenem; MER, meropenem; PTZ, piperacillin-tazobactam; STX, trimethoprim/sulfamethoxazole; TAZ, ceftazidime; TOB, tobramycin. I, susceptible–increase exposure; R, resistant; S, susceptible–standard dose.

the OmpK36GD insertion in conjunction with a truncated OmpK35 (n = 2).

Noteworthy, 1 ST147-PDR isolate harboring OXA-48–like + CTX-M-15 encoding genes (isolate_1207) carried the OmpK36GD mutation and the acquired 16S rRNA methyltransferase gene *rmtF1* (which confers high-level resistance to aminoglycosides) with other ARGs and QRDR mutations.

#### Characterization of Plasmids Carrying ESBLs and Carbapenemase Genes

Short- and long-read hybrid assemblies were obtained from 13 isolates for plasmid characterization (7 ST307, 3 ST405, 1 ST392, 1 ST147, and 1 ST716). Circular and linear representations of plasmids harboring ESBLs and carbapenemase genes are shown in Supplementary Figures 3 and 4. $bla_{\text{CTX-M-15}}$ was found in IncFIB plasmids carrying multiple ARGs in 11 of these isolates, while it was identified in the chromosome in 2 ST307 isolates (isolate_0241 and isolate_1201). $bla_{\text{KPC-3}}$ was found in 1 of these IncFIB plasmids alongside $bla_{\text{CTX-M-15}}$ and other ARGs, while $bla_{\text{OXA-244}}$ was identified separately in an IncL-OXA-48–like plasmid without any other ARG.

#### Virulence Gene Content

A schematic representation of virulence gene content detected among sequenced *K pneumoniae* isolates is shown in Supplementary Figure 5. Overall, 55 sequenced *K pneumoniae* complex isolates were classified into 22 capsular polysaccharide types. KL102 was the most prevalent K-locus and was associated with ST307 (16/16, 100%). Other frequent types were KL14, KL25, and KL151 (n = 4 each), as well as KL24 and KL27 (n = 3 each). Remarkably, capsule serotype K2 associated with enhanced pathogenicity was found in 1 HA fully antibiotic-susceptible isolate belonging to ST628 (isolate_1255).

Regarding acquired virulence factors, the siderophore yersiniabactin *ybt* loci were detected in 38.2% of sequenced isolates (21/55), with no significant differences between ESBL, AmpC, and carbapenemase producers and nonproducers. The most frequent *ybt* alleles were *ybt10*, harbored by the integrative and conjugative element ICEKp4, and *ybt14*-ICEKp5 (n = 6 each), followed by *ybt27*-ICEKp22 (n = 4) and *ybt9*-ICEKp3 (n = 3; Figure 1, Supplementary Figure 3). No isolates

**Table 2. Baseline and Clinical Characteristics of Patients With Health Care–Associated Bacteremia of Urinary Origin Caused by *Klebsiella pneumoniae* Complex.**

| | Total (N = 96) | Non-3GC/CR (n = 53) | 3GC/CR (n = 43) | *P* Value |
|---|---|---|---|---|
| Age y | 71.1 (64.5–80.0) | 71.8 (65.0–81.0) | 70.3 (64.0–77.0) | .333 |
| Sex: female | 29 (30.2) | 19 (35.8) | 10 (23.3) | .264 |
| Charlson index | 6.4 (5.0–8.0) | 6.5 (5.0–8.0) | 6.3 (5.0–8.0) | .877 |
| Any underlying disease | 85 (88.5) | 48 (98.6) | 37 (86.0) | .534 |
| McCabe: II or III | 33 (34.4) | 16 (30.2) | 17 (39.5) | .391 |
| Diabetes mellitus | 37 (38.5) | 17 (32.1) | 20 (46.5) | .206 |
| Chronic pulmonary disease | 10 (10.4) | 6 (11.3) | 4 (9.3) | >.99 |
| Chronic heart disease | 31 (32.3) | 19 (35.8) | 12 (27.9) | .511 |
| Chronic kidney disease | 34 (35.4) | 19 (35.8) | 15 (34.9) | >.99 |
| Chronic liver disease | 5 (5.2) | 2 (3.8) | 3 (7.0) | .654 |
| Vascular/degenerative brain disease | 17 (17.7) | 6 (11.3) | 11 (25.6) | .105 |
| Malignant disease | 41 (42.7) | 25 (47.2) | 16 (37.2) | .408 |
| Immunosuppressive therapy | 28 (29.2) | 16 (30.2) | 12 (27.9) | .826 |
| Recurrent UTI: >2 episodes/y | 22 (23.9) | 8 (15.7) | 14 (33.3) | .054 |
| Indwelling urinary devices[a] | 17 (17.7) | 12 (22.6) | 5 (11.6) | .188 |
| Community-onset HCA acquisition | 37 (38.5) | 23 (43.4) | 14 (32.6) | .300 |
| Prior antibiotic use: 90 d | 69 (71.9) | 32 (60.4) | 37 (86.0) | **.006** |
| Pitt score: >2 | 32 (33.3) | 18 (34.0) | 14 (32.6) | >.99 |
| Sepsis or shock | 14 (15.2) | 8 (15.7) | 6 (14.6) | >.99 |
| Inadequate empirical treatment | 12 (12.5) | 2 (3.8) | 10 (23.3) | **.005** |
| Antimicrobial therapy duration | 15.9 (12.0–17.0) | 15.9 (12.0–18.0) | 15.8 (12.0–17.0) | .593 |

Data are presented as No. (%) or median (IQR). Statistically significant values are in bold.

Abbreviations: HCA, health care associated; non-3GC/CR, not third-generation cephalosporin or carbapenem resistant; UTI, urinary tract infection.

[a]Indwelling urinary device or an invasive urinary procedure within the last 30 days.

harboring aerobactin, colibactin, or salmochelin loci or the hypermucoid regulator locus *rmpADC* were found.

#### Clinical Variables Associated With *K pneumoniae* Episodes

Baseline and clinical characteristics of patients and main differences between 3GC/CR and nonresistant *K pneumoniae* episodes are shown in Table 2. Overall, 3GC/CR *K pneumoniae* infections were associated with prior antibiotic use (37/43 [86%] vs 32/53 [60.4%], $P = .006$) and receiving inadequate empirical treatment (10/43 [23.3%] vs 2/53 [3.8%], $P = .005$). Moreover, clinical history of recurrent urinary tract infection (>2 episodes/y) was more frequent among these episodes, although this difference did not reach statistical significance ($P = .054$).

Regarding antibiotic treatment, Supplementary Table 3 summarizes the empirical and targeted antibiotic treatments used to manage *K pneumoniae* complex BUO episodes during the ITUBRAS-2 study. Carbapenems were the most commonly used antibiotics for empirical treatment in our series: 41.5% of nonproducing episodes (22/53), 55.6% of ESBL/AmpC-producing episodes (20/36), and 42.9% of carbapenemase-producing episodes (3/7). Once antimicrobial susceptibility results were available, most of these nonproducing episodes were de-escalated to cephalosporins (9/20, 45%) or fluoroquinolones (6/20, 30%). In contrast, ESBL/AmpC-producing episodes were rarely de-escalated (1/20, 5%) or even had to be escalated to carbapenems in most cases receiving narrower-spectrum empirical treatments (15/16, 93.8%). In the end, 22.6% (12/53) of non-ESBL/AmpC, non–carbapenemase-producing episodes vs 94.4% (34/36) of ESBL/AmpC-producing episodes were ultimately treated with carbapenems ($P < .00001$). Target antibiotic regimens for carbapenem-producing episodes were as follows: aztreonam (n = 1; OXA-48–like), ertapenem (n = 1; KPC-33), meropenem in combination with gentamicin (n = 1; VIM-1 + SHV-12) or colistin (n = 1; OXA-48–like + CTX-M-15), and ceftazidime/avibactam (n = 3; CTX-M-15 + OXA-48–like, CTX-M-15 + OXA-244, and CTX-M-15 + KPC-3).

## DISCUSSION

In this study, we assessed the microbiological and clinical features of a collection of *K pneumoniae* isolates recovered from HCA-BUO episodes in a Spanish multicenter project (ITUBRAS-2). None of the antibiotics in the study showed full coverage of our isolates, with only colistin, carbapenems, and ceftolozane/tazobactam showing resistance rates <10%. Resistance to at least 1 commonly used 3GC or 1 carbapenem was detected in 43.8% and 9.4% of isolates, respectively. Our results show a remarkably higher prevalence to that recently reported from the European Antimicrobial Resistance Surveillance Network for our country. In that report, 3GCR among invasive *K pneumoniae* isolates in Spain was

consistently high at approximately 25% between 2018 and 2022, while CR *K pneumoniae* significantly increased from 3.8% in 2018 to 5.2% in 2022 [22]. These differences may be explained by the selection of isolates in our study, considering only 1 source of invasive infection (urinary) and HCA episodes. In this regard, previous studies conducted by our group showed that rates of CO-HCA-BUO were similar to those of HA infections in terms of patients' characteristics and microbiology and were even associated with worse clinical cure [8, 9]. In fact, here we did not find differences between these groups in the prevalence of MDR- or ESBL-producing *K pneumoniae*. Conversely, almost all difficult-to-treat resistance, XDR, PDR, AmpC, and carbapenemase producers were associated with HA episodes in our series, highlighting a preeminent role of this pathogen in HA infections.

Resistance to 3GC was mostly associated with ESBL production, mainly CTX-M-15. WGS analysis revealed that most of these isolates belonged to globally spread MDR HiRCs (76.5%), with ST307 being dominant (44.1%). This lineage likely emerged in the United States during the mid-1990s following the introduction of ciprofloxacin into clinical practice. Despite remaining largely unnoticed for almost 2 decades, this lineage has successfully disseminated worldwide in a relatively short time, being associated with characteristic substitutions in QRDRs (GyrA-S83I and ParC-S80I) as well as conserved IncF-type plasmids harboring the $bla_{\text{CTX-M-15}}$ gene [3, 6, 23, 24]. In recent years, this clone has been reported globally, causing nosocomial outbreaks associated with different carbapenemases (eg, OXA-48–like and KPC variants) and is considered to be replacing other MDR HiRCs in certain regions [7, 25–28]. Our study demonstrates that the ST307 clone with a high content of ARGs is disseminated in multiple Spanish geographic regions associated with HA and CO-HCA-BUO.

Yet, resistance to carbapenems in *K pneumoniae* is mainly associated with production of carbapenemase enzymes [22, 29]. Herein we observed a relatively high prevalence of polyclonally distributed carbapenemase producers (7.3%), mainly associated with $bla_{\text{OXA-48–like}}$, which is the most frequent carbapenemase enzyme found in clinical isolates from this species in Spain [30]. Remarkably, 1 of these isolates, belonging to HiRC-ST15 and producing KPC-33, showed in vitro susceptibility resembling an ESBL/AmpC phenotype, hence hampering its rapid laboratory detection [27]. The KPC-33 enzyme is a KPC-2 variant presenting an amino acid substitution (D179Y) that provokes a conformational change in the active site, which determines collateral susceptibility to carbapenems and resistance to ceftazidime-avibactam [31]. CR in *K pneumoniae* may also result from the production of ESBLs or cephalosporinases in combination with decreased membrane permeability due to loss or modification of porins [29, 32]. In fact, we detected 3 additional ertapenem-resistant, non–carbapenemase-producing isolates in our collection corresponding to 2 ESBL producers harboring the porin insertion OmpK36GD coupled with loss of OmpK35 and 1 AmpC producer.

These ESBL, AmpC, and carbapenemase enzymes found in *K pneumoniae* are associated with transferable resistance determinants embedded into mobile genetic elements, such as plasmids and transposons, often carrying multiple accessory ARGs [3]. Indeed, we observed a significantly higher prevalence of genes affecting other antimicrobial groups among ESBL-, AmpC-, and carbapenemase-producing isolates. Furthermore, mutations in QRDRs and loss/alterations in OmpK35/OmpK36 porins were significantly more frequent among these isolates. Altogether, this might explain their alarmingly higher resistance rates to other antibiotics, such as trimethoprim/sulfamethoxazole, ciprofloxacin, amoxicillin-clavulanate, piperacillin-tazobactam, and aminoglycosides, thus limiting the therapeutic options of these infections.

Nowadays and rightfully so, most studies are focused on CR infections. Nonetheless, within our cohort, the majority of MDR isolates were ESBL producers. According to current European Society of Clinical Microbiology and Infectious Diseases guidelines and Infectious Diseases Society of America guidance on the treatment of antimicrobial-resistant gram-negative infections, the use of carbapenems is recommended as a first-line treatment for ESBL-producing Enterobacterales bloodstream infections [33, 34]. However, to avoid selective pressure, both guidelines also encourage clinicians to minimize the use of such antibiotics once patients are stabilized, by using quinolones and cotrimoxazole as a step-down therapy based on the susceptibility pattern of the isolate. The use of piperacillin-tazobactam or amoxicillin-clavulanate for this purpose is recommended in the guidelines from the European Society of Clinical Microbiology and Infectious Diseases. Yet, almost all ESBL/AmpC-producing episodes in our cohort were treated with carbapenems, probably because de-escalation was not possible due to the high coresistance rates to these other antibiotic groups.

Furthermore, there is an increasing concern about the potential convergence of antimicrobial resistance and virulence in *Klebsiella pneumoniae*, which could significantly amplify the public health threat posed by this pathogen, as both traits are conferred by the presence of multiple resistance and virulence genes carried in MGEs that are mobile in the population [35]. In our study, we observed significant variability in capsular polysaccharide types, with only 1 isolate carrying the K2 serotype, which is frequently associated to hypervirulent clones. In contrast, KL102 was the predominant type, exclusively associated with ST307 clone, consistent with previous studies [3, 6]. The only acquired virulence factor consistently identified in our isolates was the siderophore yersiniabactin, in 38.2% of sequenced isolates, which was equally disseminated among ESBL-, AmpC-, and carbapenemase-producing and

nonproducing isolates and in a similar proportion in other studies considering invasive infections [36].

To the best of our knowledge, this work represents the largest study on the molecular epidemiology of *K pneumoniae* complex causing HCA-BUO in our country. The study's main strength is the inclusion of a large prospective cohort of HCA-BUO episodes from 12 tertiary care hospitals located in different geographic areas of Spain. However, several limitations should be considered, such as the fact that not all non–ESBL-, non–AmpC-, and non–carbapenemase-producing isolates were subjected to WGS. To minimize this problem, selection criteria were stablished, enabling comparative analysis between groups. Moreover, since our study was conducted between 2017 and 2019, it did not investigate other novel β-lactam/β-lactamase inhibitor combinations or cefiderocol. Yet, we believe that our results are relevant, as the most commonly used antibiotics in daily clinical practice were included; meanwhile, access to these "new antibiotics" is still not uniform across hospitals. Additionally, the majority of MDR isolates within our cohort were carbapenem-susceptible ESBL producers, and novel antibiotics are often reserved as last-line treatments for CR infections.

In summary, we describe a high prevalence of 3GCR and CR *K pneumoniae* causing HCA-BUO in Spain, mainly associated with the dissemination of the ST307/CTX-M-15 and other globally spread MDR HiRCs that are circulating in our country. These successful populations are often enriched in other ARGs conferring coresistance to other antibiotics, hence challenging the selection of empirical treatment for these infections and, in some cases, even the targeted approach. These results should be considered for antimicrobial stewardship programs to optimize the use of current antibiotics and minimize the emergence of resistance. Our findings also highlight the need for novel treatments and the usefulness of genomic tools for monitoring the expansion of MDR HiRCs and detecting novel antimicrobial resistance mechanisms.

## Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

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