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Genome-wide analysis and longitudinal study of *Klebsiella pneumoniae* in Portugal: Tracing the evolution and spread of carbapenem resistance

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ABSTRACT

Background: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has high incidence in Portugal, causing severe and often fatal infections.
Objectives: Characterize the evolutionary history and epidemiology of CRKP in Portugal over a 40-year period.
Methods: WGS was performed using the Illumina platform. *In silico* multilocus sequence typing, surface antigen characterization, and resistance gene detection were subsequently carried out. Core and pan-genome analyses were conducted using Roary. Genomic clusters (GCs) were identified based on a 21-SNP threshold. To estimate the divergence times of the most prevalent sequence types (ST) in the dataset, Bayesian evolutionary analysis was performed using BEAST.

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Results: Nineteen GCs harboring carbapenemases were identified. The bla_{KPC-3} gene was the most prevalent carbapenemase, linked to strains circulating in both hospital and community settings, with dissemination patterns at regional, interregional, and international levels. ST15 was the most established sequence type in Portugal, with nine distinct GCs identified in both clinical and environmental samples. Towards the end of 2010s, ST147 and ST13 were responsible for significant outbreaks associated with bla_{KPC-3}.

Conclusions: This study underscores the value of genomic-based surveillance in understanding the evolution of high-risk clones coupled with the spread of AMR determinants. The data obtained highlights a shift in ST predominance across the country from an ST15-dominated period and strongly associated with ESBL dissemination, to the emergence of ST147 and ST13 CRKP clones, the latter associated with international transmission. This work further stresses the importance of cross-border surveillance efforts to monitor the emergence and dissemination of CRKP strains and inform risk assessment and prevention.

1. Introduction

Estimates suggest that by 2050, multidrug-resistant (MDR) infections could become the leading cause of death, resulting in 10 million fatalities annually if no decisive action is taken [1]. On May 2024, the World Health Organization (WHO) reinforced the carbapenem-resistant (CR) Gram-negative bacteria as the top priority in the global fight against MDR. This critical group includes *Acinetobacter baumannii*, and Enterobacterales species resistant to both carbapenems and third-generation cephalosporins (3GC), such as *Klebsiella* spp. and *Escherichia coli* [2]. On December 2017, Portugal's Infection and Antimicrobial Resistance Prevention and Control Program (PPCIRA) issued a national surveillance report identifying carbapenem-resistant *Klebsiella pneumoniae* (CRKP) as the most prevalent CR species in the country. Since 2010, the incidence of CRKP has quadrupled (from 1.6% to 6.4%), with a notable 73% surge between 2015 and 2016, despite a 23.3% reduction in carbapenem consumption during this period [3]. The upward trend continued into 2022, with Portugal recording the 7th highest increase in CRKP infections (from 1.6% to 11.6%) among the 29 European countries participating in the European Antimicrobial Resistance Surveillance Network (EARS-NET) [4]. Additionally, nearly half (47.6%) of *K. pneumoniae* strains exhibited resistance to 3GCs, with half of these strains also resistant to fluoroquinolones and aminoglycosides [4]. In addition to the chromosomally encoded class A β-lactamases from the bla_{SHV} family, *K. pneumoniae* is increasingly recognised as a major reservoir and hub for a variety of ESBLs from the bla_{SHV}, bla_{TEM}, and bla_{CTX-M} gene families, as well as carbapenemases such as bla_{KPC} and bla_{OXA} [5,6].

The clinical concern surrounding *K. pneumoniae* lies in its extensive diversity and rapid evolutionary dynamics, highlighting the critical role of genomics in understanding and combating this pathogen. Genome-wide studies have revealed that *K. pneumoniae* has a highly diverse population, with a genome size ranging from 5 to 6 Mbp, encoding over 5000 genes. Despite this diversity, the *K. pneumoniae* genome is well-structured, with approximately 1700 core genes providing a robust framework for investigating genetic variation [7]. Nevertheless, *K. pneumoniae* has a complex epidemiology demanding for high-resolution phylogenetic and population analyses to effectively manage its spread. This study aims to characterise the evolutionary history and dynamics of *K. pneumoniae* strains in Portugal over a 40-year period, with a particular focus on CRKP epidemiology.

2. Methods

2.1. Bacterial isolates

This study includes a convenience sample of 474 K. *pneumoniae* isolates from the Faculdade de Farmácia of Universidade de Lisboa's collection, obtained between 1980 and 2019 (Table S1), representative of historical healthcare-associated outbreaks and/or resistant isolates. The majority of the isolates (n = 425) are clinical samples obtained from 16 hospitals and laboratories located across northern (n = 43) and central (n = 382) regions of Portugal. The remaining isolates were sourced from two community settings: (I) wastewater treatment plants in the southern (WWTP1) and central (WWTP2) regions (n = 10), and (II) veterinary-associated samples (n = 39) from a previous study, which included 23 companion animals (dogs and cats) and 16 co-habiting humans [8].

2.2. Whole-genome sequencing

Genomic DNA was extracted from overnight growth at 37°C on Mueller-Hinton agar using the NZY Tissue gDNA Isolation kit (NZYTech, Portugal). WGS was performed by preparing indexed sequencing libraries using Covaris and the New England Biolabs (NEB) NEBNext Ultra DNA Library Prep Kit (NEB Inc., Massachusetts, USA E7370) following the manufacturer's recommended protocol. Sequencing was performed on an Illumina HiSeq 4 K high throughput platform (paired end 2 × 151 base pair reads) by The Applied Genomics Centre for Infectious Diseases at the London School of Hygiene and Tropical Medicine (genomics.lshtm.ac.uk; LSHTM).

2.3. Assembly and quality control of WGS data

The resulted reads were assembled by two strategies, *de novo* assembly and reference-based assembly using the *K. pneumoniae* NTUH-K2044 strain (GenBank Accession: NC_012731.1) as the reference genome. *De novo* assembly was carried out using the Unicycler pipeline (v0.4.4), and SPAdes (v.3.8) for selection optimization of the k-mer length [9]. Assembly completeness and contamination were evaluated by CheckM (v1.1.3), using 1 359 *Klebsiella* marker genes grouped in 348 sets [10]. The genomes were retained if completeness were ≥90% and contamination ≤5%. General assembly quality indexes were generated by QUAST (v5.0.2) [11]: N50, 101 150–740 541 bp; contig count, 31–218; largest contig, 1 594 573 bp; total length, 5 173 900 bp–5 929 545 bp.

Regarding mapping, raw sequencing reads were trimmed using Trimmomatic (v0.36). Subsequently, reads were mapped to the reference genome using the Burrows Wheeler Aligner tool (BWA-MEM algorithm) [12]. Following deduplication and local indel realignment by Picard Tools and GATK (v.3.6), variant calling was performed by both SAMtools/BCFtools and GATK (UnifiedGenotyper) software (mapping quality >23, depth of coverage >10) [13]. We retained only concordant variants between SAMTools/GATK, and an additional coverage validation step was performed where a missing call was assigned if the coverage depth

did not reach a minimum of 20 reads or none of the nucleotides reached 75% of the total coverage. SNP positions were removed if they: (i) showed an excess of 10% missing calls; (ii) were within 10 bp of other SNP positions; and (iii) yielded <49 bp unique k-mers.

2.4. Annotation and phylogenetic reconstruction

Two genome-wide phylogenetic trees were constructed using two distinct methodologies. First, all the 474 *de novo* assemblies were annotated using Prokka software (v1.14.6) [14]. The total core genome was assessed by Roary (v.3.13.0) using the GFF files generated by Prokka, with a 95% identity cut-off [15]. The core genome of STs comprising at least 12 isolates were also obtained. The number of conserved and total genes detected across the *K. pneumoniae* dataset, as well as for each of the STs with ≥12 isolates were plotted, and the images generated were modified in R (v4.2.2) using the ggplot2 package. A maximum likelihood phylogenetic tree was constructed from core genome SNP alignment, obtained by snp-sites (v.2.4.1) with 153 697 sites using the IQ-TREE (v2.1.3) and the statistical robustness of each clade was assessed by the approximate likelihood ratio test (aLRT) with 1,000 replicates [16]. The Interactive Tree of Life online tool was used to annotate and visualise the tree (iTOL, https://itol.embl.de/).

Additionally, the reference-based assembly approach produced an alignment of 272 899 sites across all 475 genomes (including reference genome) used to construct a second phylogenetic tree subsequently annotated with iTOL. The pairwise SNP distances based on the mapping alignment were calculated and genomic clusters (GC) were defined by using a 21 SNPs cut-off as established by the EuSCAPE Working Group and the ESGEM Study Group in the David et al. (2019) study [17], and contextualized within the relevant phylogenetic and epidemiological framework to ensure that clusters reflect true transmission dynamics. Population structure was carried out by Principal Component analysis (PCA) using the adegenet package in R.

2.5. Surface antigen biosynthesis, multilocus sequence type and resistome

Capsular locus (KL) and O-antigen locus (OL) types were inferred from the obtained assemblies with Kaptive as implemented in Kleborate [18]. To determine the sequence type (ST), genome assemblies were submitted to the institute Pasteur's website (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef) for *in silico* MLST. Genetic determinants of antimicrobial resistance were detected by the abritAMR pipeline that runs the AMRFinderPlus along with NCBI Bacterial Antimicrobial Resistance Reference Gene Database (Accession PRJNA313047) using 60% coverage and 90% identity thresholds [19]. The genomic context of the most common ESBL and carbapenemase genes were investigated using the contig-puller script (https://github.com/kwongj/contig-puller/) followed by a BLAST run. The detection of bla_{SHV} genes in the chromosome was inferred using Abricate (v0.8.13) by accessing the contig location of three core allelic profiles: MLST, rRNA and the strict cgMLST (downloaded from to the institute Pasteur's website), herein used as a proxy for chromosome contigs. bla_{SHV} genes mapping to these contigs were considered as chromosomally encoded.

2.6. Molecular clock dating

BEAST software (v2.6.7) was used to estimate the divergence years of the most abundant STs in this dataset (≥12 isolates) using isolates' year of isolation as tip dates [20]. Initial runs of 100 million iterations were carried out for the different molecular clock models and the Relaxed Clock Log Normal under a constant tree prior distribution, was the model to best fit our data. Using this molecular clock along with the HKY site substitution model, three independent runs for each ST were carried out by BEAST with 100 million (ST147 and ST231) or 200 million (ST13, ST14 and ST15) iterations and sampling at each 1000 iterations. Tracer was used to monitor the convergence of the independent runs, as well as the compliance of the minimum effective sample size threshold (ESS = 200), particularly for the likelihood statistics (tree and coalescent model). Due to lack of convergence of ST70, ST11, ST45, and ST348's independent runs, we were unable to proceed with BEAST estimations. The log and tree files produced for each ST were merged by LogCombiner. Final maximum clade credibility trees were produced from the combined trees using TreeAnnotator with median node heights, which were then annotated with GCs, carbapenemases and ESBL genes in iTOL.

3. Results and discussion

3.1. Surface antigen distribution and genetic diversity

A total of 79 distinct STs were identified across the 474 isolates, with the most prevalent being ST15 (n = 151; 31.9%), followed by ST147 (n = 34; 7.2%), ST13 (n = 27; 5.7%), and ST14 (n = 23; 4.9%). However, the majority of STs (n = 45, 57.0%) were represented by only a single isolate. Overall, a significant proportion of isolates (n = 187; 39.5%) belonged to STs previously associated with multidrug resistance (MDR), such as ST15 [21], and ST147 [22]. Among the 46 identified capsular K serotypes, the most prevalent were KL24 (n = 91; 19.2%), KL112 (n = 66; 13.9%), and KL64 (n = 34; 7.2%). In terms of O-antigens, 10 different OL types were identified, with the predominant ones being O1v1 (n = 204; 43.0%), O1v2 (n = 103; 21.7%), and O2v2 (n = 55; 11.6%) (Table S1, Fig. 1).

The pan-genome of this dataset consists of 25 428 genes, including 4061 which are very common (present in at least 95% of strains), 1771 shell genes (found in 15%–95% of strains), and 19 596 cloud genes (prevalence <15%). On average, each genome contains 5162 genes (ranging from 4797 to 5616), with 3593 genes constituting the core genome (shared by ≥99% of the strains). In larger STs (≥12 isolates), gene content was conserved, leading to a reduction of approximately 60% in pan-genome size (from 9920 genes in ST15 to 5659 genes in ST348). Meanwhile, the core genome expanded by around 1000 genes (from 3966 genes in ST15 to 4884 genes in ST348) (Table 1, Fig. S1).

The large accessory genome is partly attributed to the acquisition of antimicrobial resistance (AMR) determinants. Using AbritAMR, 107 acquired resistance genes and 13 chromosomal mutations were identified (n = 120), conferring resistance to 11 antibiotic classes. The most common were β-lactams (n = 50, 41.7%), followed by aminoglycosides (n = 19, 15.8%), fluoroquinolones (n = 13, 10.8%), trimethoprim (n = 11, 9.2%), chloramphenicol (n = 8, 6.7%), tetracyclines (n = 5, 4.2%), sulphonamides (n = 5, 4.2%), polymyxins (n = 4, 3.3%), macrolides (n = 4, 3.3%), rifampicin (n = 2, 1.7%), and streptomycin (n = 1, 0.8%). All isolates carried, at least fosfomycin resistance (*fosA*) and a multidug efflux pump (*oqxAB*). Chromosomal narrow-spectrum β-lactamases from the bla_{SHV} family were detected in 73.0% (n = 346) of the isolates, including bla_{SHV-28} (n = 135; 39.0%), bla_{SHV-11} (n = 89; 25.7%), and bla_{SHV-1} (n = 58; 16.8%), all previously reported [23]. Additionally, three bla_{SHV} alleles with extended-spectrum β-lactamase (ESBL) activity, $bla_{SHV-106}$ (n = 7), bla_{SHV-55} (n = 3), and bla_{SHV-2} (n = 1), were detected in the chromosome [23]. Overall, more than half of the isolates (n = 277; 58.4%) carried ESBL genes, with bla_{CTX-M} variants being the most prevalent (n = 235; 84.8%). Moreover, carbapenemase genes were found in a quarter of the isolates (n = 110; 23.2%), including bla_{KPC-3} (n = 100), $bla_{OXA-181}$ (n = 6),

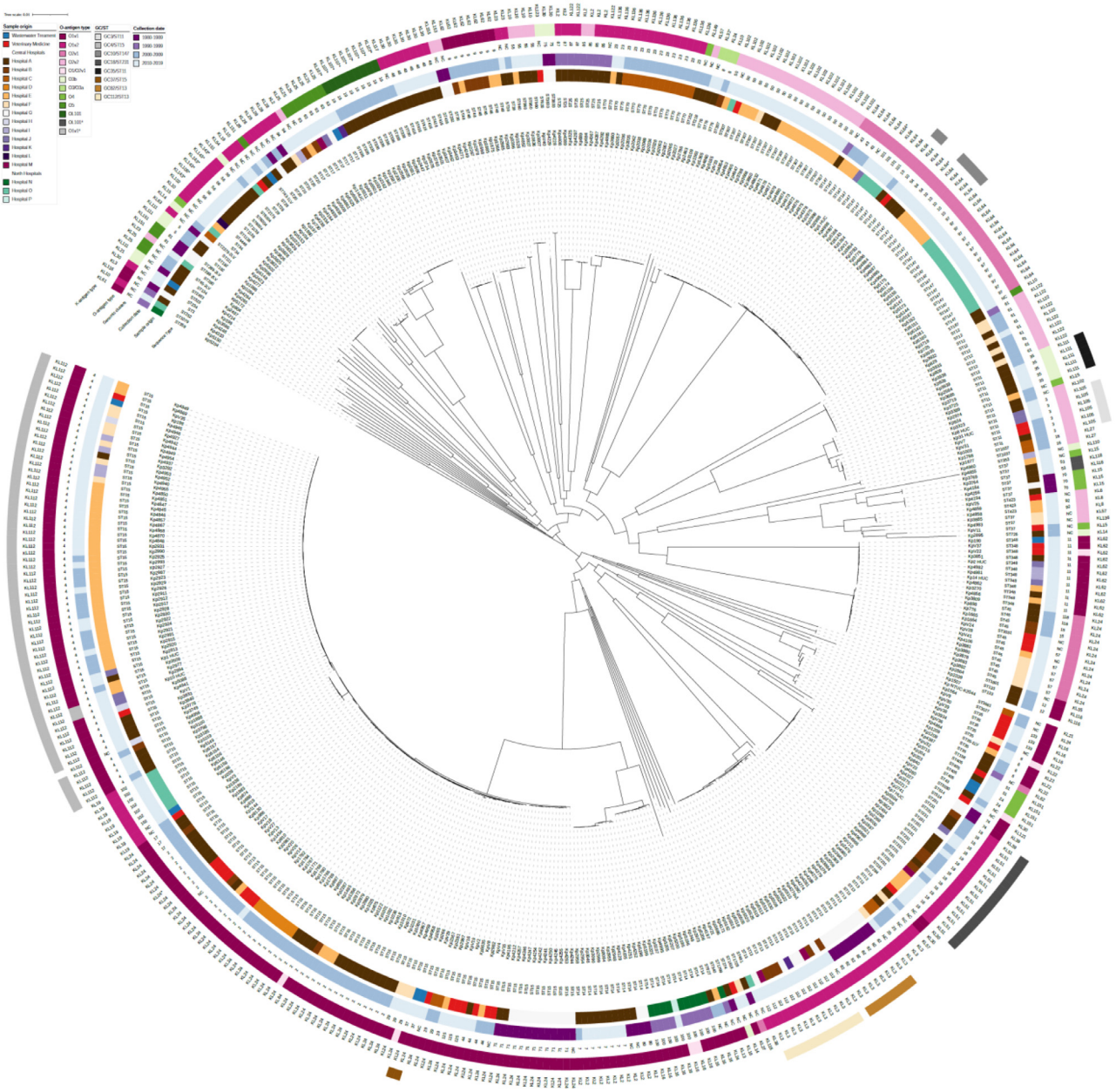

Figure 1. Genome-wide phylogenetic tree for the 474 *Klebsiella pneumoniae* isolates. The tree was annotated using the Interactive Tree of Life online tool (iTOL) with sequence type (ST), sample origin, collection date, genomic clusters (GC), KL and OL serotypes. The most relevant GCs are highlighted in the outer ring: GC3/ST11, GC4/ST15, GC10/ST147, GC18/ST231, GC35/ST11, GC37/ST15, GC82/ST13 and GC112/ST13.

bla_{GES-5} ($n = 6$), bla_{NDM-1} ($n = 2$), and bla_{OXA-48} ($n = 1$). Dissemination of bla_{KPC-3} was primarily driven by Tn4401*d* ($n = 93$; 97.9%), with Tn4401*b* ($n = 2$; 2.1%) contributing to a lesser extent. All $bla_{CTX-M-15}$ were linked to the insertion sequence IS*Ecp1*, $bla_{OXA-181}$ were associated with IS*3000*, and bla_{GES-5} were found in a class III integron (*intI*3) co-translocating bla_{BEL-1} (Table S1, Figure S2) [24]. Mutations in the *ompK35*/*ompK36* porins impair the activity of carbapenems by reducing their diffusion across the outer membrane. Partial or total deletion of *ompK35* (10 to 94%) and/or *ompK36* (21 to 100%) was observed in 19.0% of the isolates ($n = 90$). Among these, 31 isolates were carbapenemase producers, predominantly from ST147 ($n = 10$; 31.25%) (Table S1). A two-amino acid insertion in *ompK36* (Gly115–Asp116; OmpK36GD) was identified in an ST13 isolate (Kp5519) carrying bla_{KPC-3}, a combination previously associated with a 16-fold increase in meropenem minimum inhibitory concentration [25].

3.2. Population structure and phylogenetic reconstruction

Two genome-wide phylogenetic trees were constructed—one based on a core genome alignment (153 697 SNPs) (Figure S3) and the other using 272 899 genome-wide SNPs (Fig. 1). Both phylogenies exhibited similar topologies, with Sequence Types aligned with monophyletic branches, consistent with Bayesian population structure analysis (Fig. S4). A 21-SNP cut-off was used to define 53 genomic clusters (GCs), all but GC56 (ST6004 and ST1138) consisting of strains from the same ST. Carbapenemase genes were found in 19 GCs, while ESBL genes were detected in 37 GCs.

ST11 was distributed across two major clusters, GC3 and GC35. GC35/ST11 ($n = 5$, 2009–2011) isolates expressed the KL111 and O3b serotypes and carried bla_{KPC-3} and a 63% deletion of *ompK35*. Among these, two isolates from the same patient (Kp3719 and Kp3725) were collected 14 days apart (data not shown), indicating

Table 1
Number of total, core, soft core, shell and cloud genes across all 474 studied *K. pneumoniae* genomes and for the most predominant STs (≥12 isolates).

	Total (%)	ST15 (%)	ST147 (%)	ST13 (%)	ST14 (%)	ST307 (%)	ST231 (%)	ST70 (%)	ST11 (%)	ST45 (%)	ST348 (%)
No. of genomes	474 (100.0)	152 (32.1)	34 (7.2)	26 (5.5)	23 (4.9)	21 (4.4)	16 (3.4)	15 (3.2)	13 (2.7)	12 (2.5)	12 (2.5)
Core genes (strains ≥99%)	3593 (14.1)	3966 (40.0)	4598 (61.4)	4420 (60.7)	4587 (67.4)	4701 (75.6)	4790 (83.2)	4648 (82.0)	4693 (73.8)	4686 (73.7)	4884 (86.3)
Soft core genes (99% < strains ≥ 95%)	468 (1.8)	501 (5.1)	122 (1.6)	186 (2.6)	25 (0.4)	116 (1.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Shell genes (95% < strains ≥ 15%)	1771 (7.0)	1051 (10.6)	980 (13.1)	1051 (14.4)	1226 (18.0)	592 (9.5)	590 (10.3)	511 (9.0)	1168 (18.4)	1129 (17.8)	431 (7.6)
Cloud genes (15% < strains)	19 596 (77.1)	4402 (44.4)	1790 (23.9)	1628 (22.3)	966 (14.2)	807 (13.0)	371 (6.5)	509 (9.0)	500 (7.9)	539 (8.5)	344 (6.1)
Total genes	25428 (100.0)	9920 (100.0)	7490 (100.0)	7285 (100.0)	6804 (100.0)	6216 (100.0)	5751 (100.0)	5668 (100.0)	6361 (100.0)	6354 (100.0)	5659 (100.0)

a single infection episode that progressed to a bloodstream infection. The second cluster, GC3/ST11/KL105/O2v2 (n = 6), included four clinical isolates from hospitals A (n = 2, 2004–2011) and J (n = 2, 2016–2017), as well as two veterinary-associated samples (n = 2, 2007), one from a cat and the other from a co-habiting human. In fact, eleven genomic clusters encompass both hospital and community samples, GC4/ST15/KL112/O1v1 (n = 65) highlights dissemination across seven hospitals in Lisbon and Coimbra (2005-2017) and, further encompasses isolates from veterinary studies (n = 2, 2014–2015), and one from wastewater treatment plants (2017). Clinical isolates within this cluster frequently carried $bla_{\text{CTX-M-15}}$ (n = 64) and, in six cases, $bla_{\text{KPC-3}}$. In general, carbapenemase coding genes were present exclusively in hospital isolates. Of the 110 carbapenemase-producing *K. pneumoniae* strains analysed, only three recovered from wastewater samples, and none from animal-associated sources. All isolates from the GC63/ST17 (n = 6, 2017–2019) cluster, including the wastewater treatment sample (KpAFB), carried the $bla_{\text{OXA-181}}$ gene. Notably, GC37/ST15 include two isolates from wastewaters carrying both $bla_{\text{NDM-1}}$, a rare carbapenemase in Portugal, and $bla_{\text{CTX-M-15}}$ further highlighting the spread of highly resistant strains beyond clinical settings. The GC112/ST13/KL3/O1v1 (n = 12, 2018–2019) isolates, all harbouring $bla_{\text{KPC-3}}$, represent a concerning example of cross-border transmission (Fig. 1, Table S1). We recently linked these strains to patients from the Netherlands and France with prior hospitalizations in Portugal [26]. The analysis estimated the emergence of this clone around 2008 in Portugal, supporting the hypothesis of Portugal as the origin, underlining the critical need for strict infection control measures to prevent the dissemination of MDR strains between hospitals, communities, and countries.

Overall, a single carbapenemase was detected per GC or isolate, although exceptions were noted in ST147, ST231, and ST15, where two carbapenemases were identified. The most recent isolates from GC10/ST147 (n = 5, 2016), linked to a polyclonal outbreak [24], both $bla_{\text{KPC-3}}$ and $bla_{\text{GES-5}}$, both class A carbapenemases. In line with findings by Perdigão et al. (2019) [24], four of these clones were associated with rectal colonisation rather than active infections. However, one of these patients subsequently developed a urinary tract infection with the same clone 17 days after the rectal swab. Similarly, $bla_{\text{KPC-3}}$ and $bla_{\text{GES-5}}$ were identified among GC18/ST231 isolates, some derived from rectal swabs. However, unlike GC10/ST147, these genes were not present together in the same strain. GC18/ST231 included multiple clones from the same patient (data not shown), with isolates such as Kp2454, Kp2705, and Kp2741 responsible for recurrent bloodstream infections over a nine-month period (31/12/2008; 20/07/2009; 04/09/2009). In 2018, carbapenemase genes from two distinct classes were found in GC102/ST15 isolates, specifically $bla_{\text{KPC-3}}$ (n = 5, class A) and $bla_{\text{OXA-48}}$ (n = 1, class D). These isolates were collected from patients at hospital O and were frequently linked to invasive infections, including hepatic abscesses and septicaemia (Fig. 1, Table S1).

The extensive diversity of antibiotic resistance genes identified in *K. pneumoniae* strains is highly concerning. Not only is this pathogen a frequent coloniser of human tracts capable of causing opportunistic and life-threatening infections, but these strains can also serve as reservoirs for the lateral transfer of AMR genes across different bacterial species, further exacerbating the public health threat.

3.3. Evolutionary dynamics and molecular dating of the ESBL and carbapenemase genomic clusters

The earliest ESBL genes identified in this dataset date back to the early 1990s. Specifically, in 1991, two isolates from northern (Kp5196; GC100/ST14) and central (Kp4287; GC85/ST25) regions

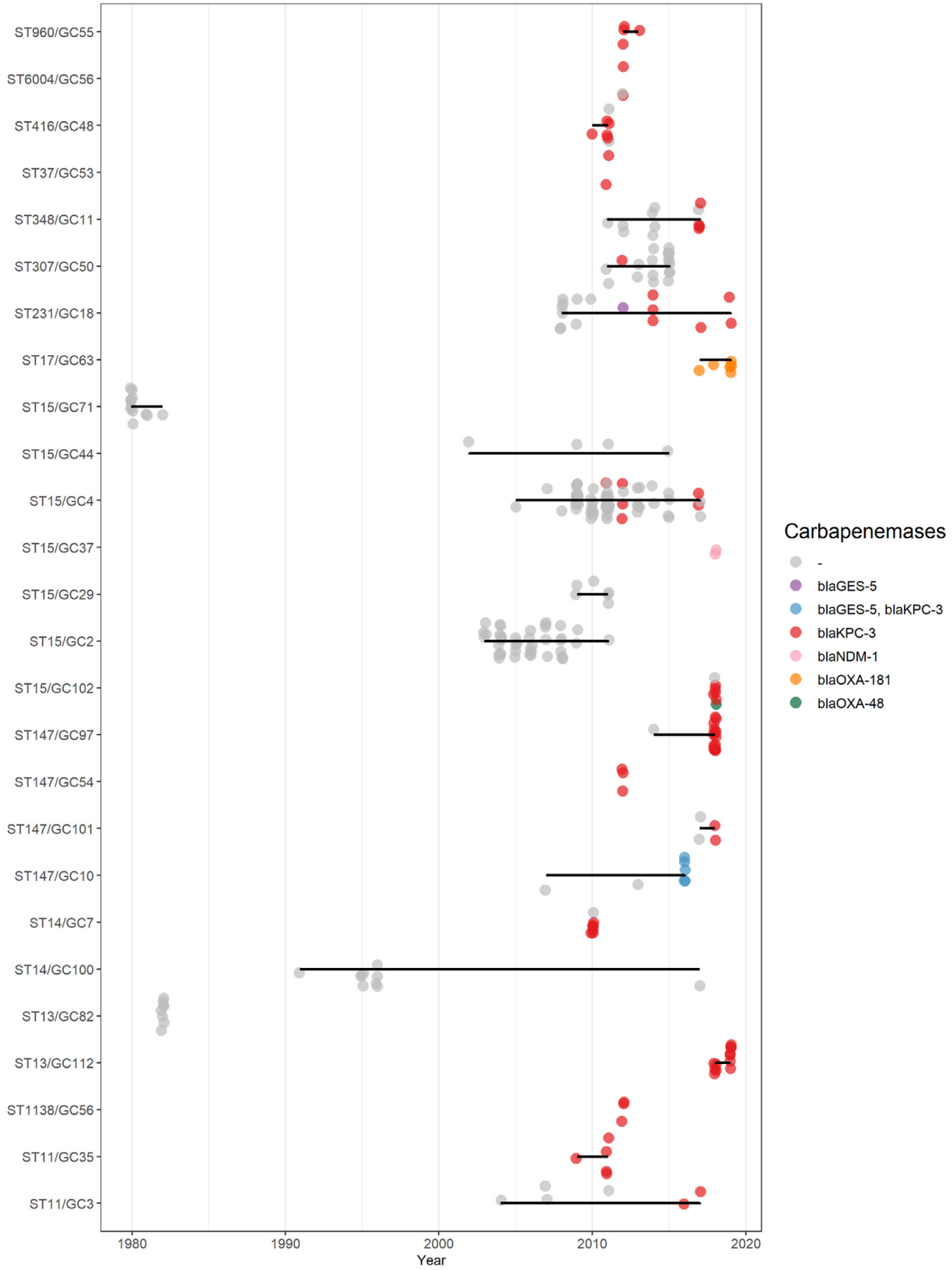

Figure 2. Distribution of carbapenemase producing genomic clusters and respective STs over time. Non-carbapenemase GCs with more than >3 isolates from the same STs were also represented. Each of the carbapenemase genes are coloured according to the colour palette on the right.

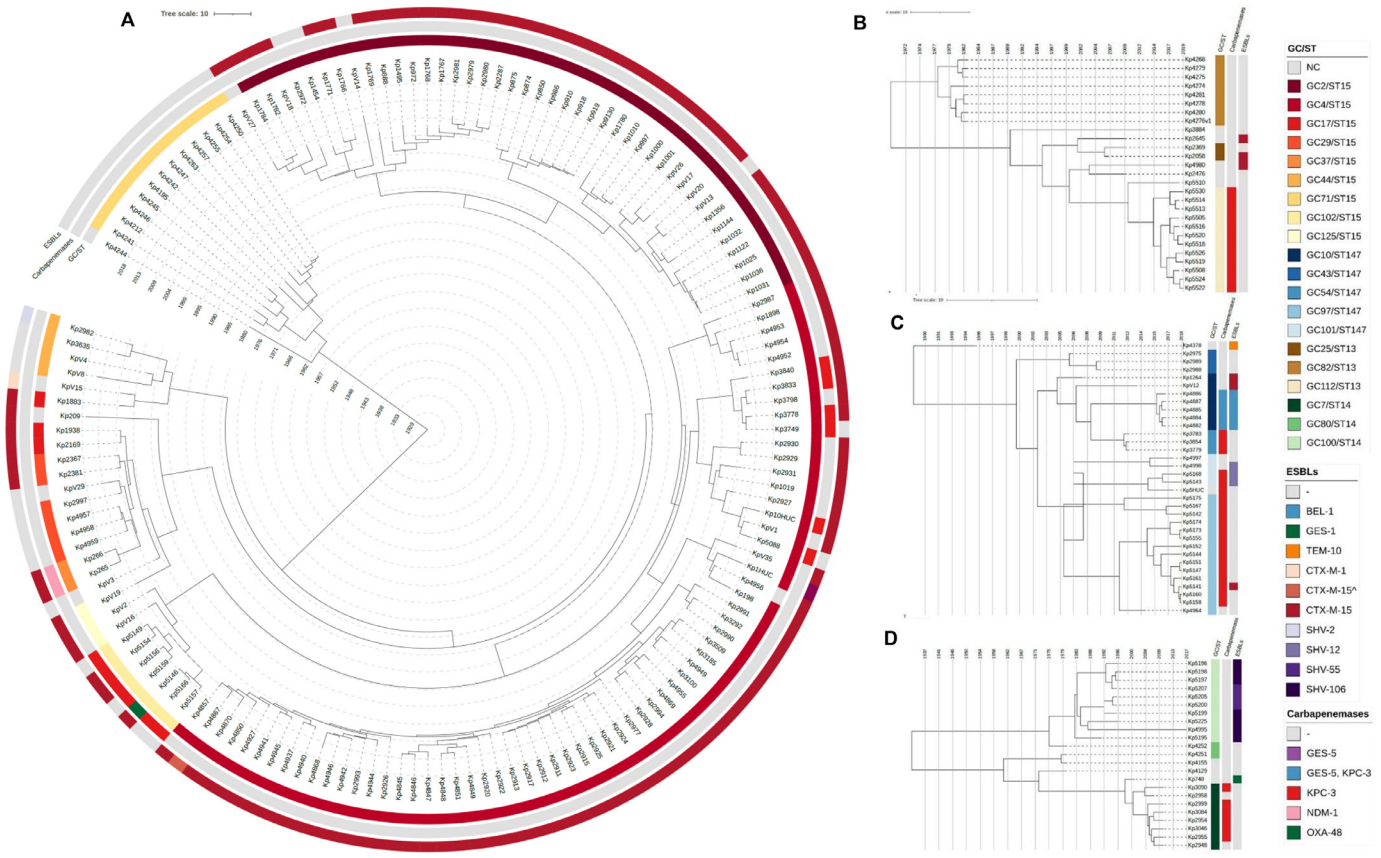

Figure 3. Time-scaled phylogenetic trees of ST15 (A), ST13 (B), ST147 (C) and ST14 (D). Trees were annotated with GCs, ESBLs and carbapenemase genes using iTOL.

of Portugal were identified carrying $bla_{SVH-106}$ and bla_{TEM-10}, respectively (Figure S5, Table S1). The first description of bla_{TEM-10} was made by Quinn et al. in 1989, in *K. pneumoniae* clinical isolates from Chicago, dating back to 1988 [27]. Interestingly, $bla_{SVH-106}$ was first reported in a Portuguese study in Northern Portugal in 2006, in three isolates, one of which also carried the bla_{TEM-1} gene, similar to Kp5196, despite a 15-year gap between the isolates (1991–2006) [28]. Notably, GC100/ST14 includes nine older isolates (1991–1996) bearing $bla_{SVH-106}$ or the closely related bla_{SHV-55} [23]. Phylogenetic analysis estimates that GC100 emerged in 1983 (1982.9; 95% HPD interval [1975.7–1990.2]), suggesting that $bla_{SHV-106}$ circulated in northern Portugal long before its first formal report in 2006 (Fig. 3D, Table S2). Since these early reports of ESBLs, the prevalence of these resistance genes in Portugal rose for two decades (n = 118; 73.8%) before declining in the 2010s (n = 136; 55.1%). This decline coincided with the rise of carbapenemase-producing isolates, which became more prevalent during the same period (n = 108; 43.7%). According to PPCIRA data from 2010 to 2020, the incidence of ESBLs in Portugal was 47.6%, comparable to our findings (55.1%). However, the incidence of CRKP strains was significantly lower (10.0%) compared to our estimates (43.7%), likely due to the broader geographic distribution and higher number of laboratories participating in the PPCIRA surveillance (n = 100) compared to our study (n = 16, primarily from central Portugal) [3,4].

The older lineages circulating in Portugal, since the 1980s, have been ST15, ST14, and ST13. Previous studies on the global dynamics of ST15 and ST14 support this long-standing prevalence [29]. Our analysis estimates that the ancestral ST15 emerged in 1924 (1924.1; 95% HPD interval [1875.4–2004.7]), with the oldest genomic cluster, GC71/ST15, dating back to 1965 (1965.2; 95% HPD interval [1952.0–1975.6]; Table S2). Over time, eight additional GCs diverged, and ST15 strains predominantly acquired ESBLs (n = 120; 80.0%), particularly $bla_{CTX-M-15}$. A diverse set of carbapenemase coding genes, bla_{KPC-3} (n = 11), bla_{NDM-1} (n = 2), and bla_{OXA-48} (n = 1) were also identified but at a lesser extent (Fig. 2, Table S1). Among the 19 GCs identified with carbapenemases, bla_{KPC-3} was found in 17 of them. The first carbapenemase-producing isolate (Kp2564) in this study was detected in April 2009, carrying the bla_{KPC-3} gene and belonging to GC35/ST11, a single-locus allelic variant of ST258, which is widely recognized as a major driver in the dissemination of bla_{KPC} [30]. Notably, ST11 is the ancestral lineage of ST258, with the latter emerging through recombination of ST11 with ST442, involving approximately 1.1 Mbp of exchanged genetic material [30]. The first report of bla_{KPC-3} dates back to an outbreak at a New York Medical Centre (2000–2001), involving a KPC-3-producing *K. pneumoniae* strain [31]. During the early 2010s (2010–2012), GC35/ST11 (n = 5), GC7/ST14 (n = 8), and GC48/ST416 (n = 7) were the most prevalent carbapenemase-producing GCs, all of which exclusively harboured bla_{KPC-3}. GC7/ST14, the second major GC of ST14, is estimated to have emerged in 2001 (2000.7; 95% HPD interval [1995.8–2005.3]), 19 years after GC100/ST14/SHV-106 and without the $bla_{SHV-106}$ gene (Fig. 3D).

In the subsequent years, the predominant GCs shifted to GC4/ST15 (n = 65, 2005–2017) and GC18/ST231 (n = 16, 2008–2019), both of which spanned over a decade of collection, however, only 6 (GC4/ST15) and 7 (GC18/ST231) isolates bore carbapenemase genes. BEAST analysis suggests that GC18/ST231 and GC4/ST15 emerged in 2005 and between 1990 and 1995, respectively (Fig. 3A, Figure S6, Table S2). In 2016, GC10/ST147 (n = 5) caused an outbreak at hospital E [24], with all isolates carrying bla_{KPC-3}, bla_{GES-5}, and an ESBL gene (bla_{BEL-1}) (Fig. 2). BEAST analysis estimated that GC10 emerged between 2004 and 2005, while

the GC10/KPC-3/GES-5/BEL-1 clone diverged about a decade later (2015.2; 95% HPD interval [2014.1–2015.9]; Table S2), consistent with the timing of the outbreak [24].

Towards the end of the decade, five additional GCs emerged, introducing less common carbapenemases in Portugal (bla_{OXA-48}, $bla_{OXA-181}$, and bla_{NDM-1}): GC97/ST147, GC112/ST13, GC63/ST17, GC102/ST15, and GC37/ST15. Notably, the emergence of ST13 is particularly striking, as the temporal gap between GC112/ST13 (2018-2019) and the older GC82/ST13 (1980–1982) spans the entire collection period of this dataset. No carbapenemase or ESBL genes were detected in the older GC82/ST13, whereas all members of GC112/ST13 carried the bla_{KPC-3} gene. Dating analysis suggest that GC82/ST13 emerged in 1975 (1975.9; 95% HPD interval [1967.7-1981.2]), while GC112/ST13 emerged in 2013 (2013.4; 95% HPD interval [2009.0-2016.9]) (Figs. 2, and 3B, Table S2), which aligns with our previous study [26].

4. Conclusion

This genome-wide analysis has provided valuable insights into the dissemination of *K. pneumoniae* in Portugal, identifying both regional (e.g., GC3/ST11), interregional (e.g., GC4/ST15), and international (e.g., GC112/ST13) transmission events. These events involve isolates predominantly carrying the bla_{KPC-3} carbapenemase, which was the most prevalent resistance determinant in this dataset. In terms of population dynamics, ST15 stands out as the oldest and most established sequence type in Portugal, with nine distinct GCs detected over the entire study period. ST15 was identified in both clinical and environmental samples and was associated with a broader diversity of carbapenemases in this study, although the number of such isolates was limited (bla_{NDM-1} [$n = 2$] and bla_{OXA-48} [$n = 1$]). Towards the end of the 2010s, a marked decline in ST15 carbapenemase-producing GCs followed a concomitant rise in ST13 and ST147 strains, both sequence types causing distinct outbreaks. While ST15 was well established during the early introduction of carbapenem therapy in Portugal, our data suggest that ST13 and ST147 may have a selective advantage under current antimicrobial pressures, particularly in disseminating bla_{KPC-3}.

This study underscores the power of genomic surveillance in tracking the evolutionary dynamics of *K. pneumoniae* and the spread of AMR determinants. It also highlights the critical need for stringent infection control measures to prevent the transmission and emergence of high-risk clones across healthcare facilities, community settings, and even international borders.

Declaration of competing interest: None declared.

Availability of data and material:> Sequence data have been submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under study accession ERP125389.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2025.107583.

References

[1] Review on Antimicrobial Resistance. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. 2014.
[2] World Health Organization. WHO Bacterial Priority Pathogens List, 2024: bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. WHO: Geneva, Switzerland; 2024.
[3] Programa de Prevenção e Controlo de Infeções e de Resistência aos Antimicrobianos Programa de Prevenção e Controlo de Infeções e de Resistência aos Antimicrobianos. Lisboa, Portugal: PPCIRA; 2017.
[4] Programa de Prevenção e Controlo de Infeções e de Resistências aos Antimicrobianos. Infeções e resistências a antimicrobianosRelatório do Programa Prioritário PPCIRA. Lisboa, Portugal: PPCIRA; 2022.
[5] Haeggman S, Löfdahl S, Paauw A, Verhoef J, Brisse S. Diversity and evolution of the class a chromosomal beta-lactamase gene in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2004;48:2400–8.
[6] Navon-Venezia S, Kondratyeva K, Carattoli A. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. FEMS Microbiol Rev 2017;41:252–75.
[7] Wyres KL, Lam MMC, Holt KE. Population genomics of *Klebsiella pneumoniae*. Nat Rev Microbiol 2020;18:344–59.
[8] Marques C, Belas A, Aboim C, Cavaco-Silva P, Trigueiro G, Gama LT, et al. Evidence of sharing of *Klebsiella pneumoniae* strains between healthy companion animals and cohabiting humans. J Clin Microbiol 2019;57:e01537-18.
[9] Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLOS Computat Biol 2017;13:e1005595.
[10] Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res 2015;25:1043–55.
[11] Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies. Bioinformatics (Oxford, England) 2013;29:1072–5.
[12] Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics (Oxford, England) 2009;25:1754–60.
[13] Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. Bioinformatics (Oxford, England) 2009;25:2078–9.
[14] Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics (Oxford, England) 2014;30:2068–9.
[15] Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, et al. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics (Oxford, England) 2015;31:3691–3.
[16] Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, et al. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evolut 2020;37:1530–4.
[17] David S, Reuter S, Harris SR, Glasner C, Feltwell T, Argimon S, et al. Epidemic of carbapenem-resistant *Klebsiella pneumoniae* in Europe is driven by nosocomial spread. Nat Microbiol 2019;4:1919–29.
[18] Lam MMC, Wick RR, Watts SC, Cerdeira LT, Wyres KL, Holt KE. A genomic surveillance framework and genotyping tool for *Klebsiella pneumoniae* and its related species complex. Nat Commun 2021;12:4188.
[19] Sherry NL, Horan KA, Ballard SA, Gonçalves da Silva A, Gorrie CL, Schultz MB, et al. An ISO-certified genomics workflow for identification and surveillance of antimicrobial resistance. Nat Commun 2023;14:60.
[20] Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, et al. BEAST 2.5: an advanced software platform for bayesian evolutionary analysis. PLOS Computat Biol 2019;15:e1006650.
[21] Li R, Cheng J, Dong H, Li L, Liu W, Zhang C, et al. Emergence of a novel conjugative hybrid virulence multidrug-resistant plasmid in extensively drug-resistant *Klebsiella pneumoniae* ST15. Int J Antimicrob Agents 2020;55:105952.
[22] Peirano G, Chen L, Kreiswirth Barry N, Pitout Johann DD. Emerging antimicrobial-resistant high-risk *Klebsiella pneumoniae* clones ST307 and ST147. Antimicrob Agents Chemother 2020;64. doi:10.1128/aac.01148-20.
[23] Tsang K.K., Lam M.M.C., Wick R.R., Wyres K.L., Bachman M., Baker S., et al. Diversity, functional classification and genotyping of SHV β-lactamases in *Klebsiella pneumoniae*. bioRxiv. 2024:2024.04.05.587953.
[24] Perdigão J, Modesto A, Pereira AL, Neto O, Matos V, Godinho A, et al. Whole-genome sequencing resolves a polyclonal outbreak by extended-spec-

trum beta-lactam and carbapenem-resistant *Klebsiella pneumoniae* in a Portuguese tertiary-care hospital. Microb Genom 2021;7:000349.
[25] Wong JLC, Romano M, Kerry LE, Kwong H-S, Low W-W, Brett SJ, et al. OmpK36-mediated carbapenem resistance attenuates ST258 *Klebsiella pneumoniae in vivo*. Nat Commun 2019;10:3957.
[26] Elias R, Spadar A, Hendrickx APA, Bonnin RA, Dortet L, Pinto M, et al. Emergence of KPC-3- and OXA-181-producing ST13 and ST17 *Klebsiella pneumoniae* in Portugal: genomic insights on national and international dissemination. J Antimicrob Chemother 2023;78:1300–8.
[27] Quinn JP, Miyashiro D, Sahm D, Flamm R, Bush K. Novel plasmid-mediated beta-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*. Antimicrob Agents Chemother 1989;33:1451–6.
[28] Mendonça N, Ferreira E, Louro D, Caniça M. Molecular epidemiology and antimicrobial susceptibility of extended- and broad-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolated in Portugal. Int J Antimicrob Agents 2009;34:29–37.
[29] Rodrigues C, Lanza VF, Peixe L, Coque TM, Novais Â. Phylogenomics of globally spread clonal groups 14 and 15 of *Klebsiella pneumoniae*. Microbiol Spectrum 2023;11:e0339522.
[30] Chen L, Mathema B, Pitout JDD, DeLeo FR, Kreiswirth BN. Epidemic *Klebsiella pneumoniae* ST258 is a hybrid strain. mBio 2014;5. doi:10.1128/mbio.01355-14.
[31] Woodford N, Tierno PM, Young K, Tysall L, Palepou M-FI, Ward E, et al. Outbreak of *Klebsiella pneumoniae* producing a new carbapenem-hydrolyzing class A β-lactamase, KPC-3, in a New York Medical Center. Antimicrob Agents Chemother 2004;48:4793–9.