



The alarming rate of carbapenem-resistant *Klebsiella pneumoniae* in Serbia: A deep dive into genomic diversity, resistome, hypervirulence, and expansion of high-risk clone ST147/*bla*_{NDM-1}

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ABSTRACT

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is an emerging global threat. This study aimed to determine the prevalence of CRKP, the genetic basis of antimicrobial resistance, including beta-lactamase production, efficacy of novel beta-lactam/beta-lactamase inhibitor (BLBLI) combinations, hypervirulence, and genetic diversity of circulating clones in a Serbian hospital setting. From 2022–2023, 2001 *K. pneumoniae* isolates were collected from 16 hospitals across Serbia. The prevalence of CRKP was 53.4% (N = 1069). Among these, 191 randomly selected CRKP isolates were subjected to expanded antimicrobial susceptibility testing, string test, and carbapenemase production, with 150 further randomly chosen for whole-genome sequencing. Resistance rates to ceftazidime-avibactam, imipenem-relebactam, and meropenem-vaborbactam were 50.8% (N = 97), 84.8% (N = 162), and 90.6% (N = 173), respectively. The majority of CRKP isolates (N = 146; 97.3%) harboured carbapenemase-encoding genes: *bla*_{NDM-1} (N = 65; 44.5%), *bla*_{OXA-48} (N = 64; 43.8%), and *bla*_{KPC-2} (N = 11; 7.5%). Additionally, six CRKP isolates co-harbored *bla*_{NDM-1} and *bla*_{OXA-48} (4.1%). This study revealed ten sequence types (STs) and six clonal complexes (CCs), with ST147/CC147/*bla*_{NDM-1} being the most prevalent (N = 44; 29.3%) followed by ST101/CC101/*bla*_{OXA-48} (N = 40; 26.7%). One CRKP isolate, ST101/*bla*_{NDM-1}/*bla*_{SHV-1}/*bla*_{CTX-M15} was resistant to cefiderocol. The predominant hypervirulence-associated genes were *ybt* (N = 138; 92%) and *iuc* (N = 80; 53.3%). According to the genotypic analysis, 75 out of 150 (50.0%) CRKP

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isolates had *iuc* and *rmpA2/rmpADC* genes, whereas 26 (13.6%) strains exhibited the hypermucoviscous phenotype.

The emergence of hypervirulent, *K. pneumoniae* clone ST147/*bla*_{NDM-1}, suggests a high potential for regional spread of a high-risk clone resistant to last-line antibiotics.

1. Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections have emerged in hospital settings worldwide, posing significant challenges due to limited treatment options and high mortality rates (Antimicrobial Resistance Collaborators, 2022; Wang et al., 2022). *K. pneumoniae* is a member of the "ESKAPEE" group of infamous bacterial pathogens known for their ability to escape the effects of antibiotics (i.e. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter species*, and *Escherichia coli*) (Rice, 2008). Consequently, the World Health Organisation has designated carbapenem-resistant *Enterobacterales* (CRE), including CRKP, as critical priority pathogens for the development of new treatment options (WHO, 2024).

Although carbapenems were previously the drug of choice for infections caused by *Enterobacterales*, their efficacy has diminished due to the widespread presence of carbapenemase-producing enterobacteria. In response to this urgent public health threat, various treatment strategies have been explored, including the reintroduction of older antibiotics and the development of novel beta-lactam/beta-lactamase inhibitor (BLBLI) combinations. Indeed, having been largely abandoned in the 1970s, polymyxins, including colistin, have reemerged as a "last-line" treatment option for various multidrug-resistant (MDR) gram-negative bacilli, despite associated nephrotoxicity and suboptimal outcomes (Qureshi et al., 2015). With the increased consumption of colistin, cases of colistin-resistant *K. pneumoniae* strains producing carbapenemases are being reported worldwide (van Duin and Doi, 2015). Besides polymyxins, commonly prescribed antibiotics, such as aminoglycosides, tigecycline, fosfomycin, and a combination of two carbapenems have also been proposed, although yielding varying results (Oliva et al., 2014). The other strategy to combat carbapenem resistance and to restore the effectiveness of beta-lactam antibiotics is to use beta-lactamase inhibitors. Thus, novel combinations of BLBLIs have resulted in many combinations approved for therapeutic use, such as ceftazidime-avibactam, ceftolozane-tazobactam, imipenem-relebactam, and meropenem-vaborbactam (Yahav et al., 2020). Currently, BLBLI combinations have been recommended as the first-choice treatment for class A-producing CRE (KPC), including CRKP (Paul et al., 2022; Tamma et al., 2024). Ceftazidime-avibactam uniquely demonstrates activity against *K. pneumoniae* strains producing the OXA-48 enzyme. Noteworthy, none of these BLBLI combinations are effective against class B beta-lactamases or metallo-beta-lactamases (MBLs) (Yahav et al., 2020). Nevertheless, the novel BLBLI combinations, aztreonam-avibactam and siderophore cephalosporin, cefiderocol, both had activity against CRE, including those producing MBLs. All aforementioned sensitivity profiles of CRKP may be compromised due to the functional loss of outer membrane porin and development of mutations in the genes encoding carbapenemases (Bovo et al., 2023). Of note, in 2022, several European countries demonstrated that NDM carbapenemase has emerged as the most common enzyme in CRKP isolates, with the dominant sequence types (STs) being ST147, ST307, and ST395 (Sandfort et al., 2022).

Hypervirulent *K. pneumoniae* (hvKP) is most commonly associated with community-acquired infections and specific clonal lineages, particularly ST23 (European Centre for Disease Prevention and Control, 2024). In contrast, hospital settings are predominantly affected by CRKP strains belonging to classical *K. pneumoniae* (cKP) lineages, which are typically multidrug resistant. In recent years, increasing attention has been focused on the emergence of hospital-acquired CRKP clones from cKP lineages that have acquired virulence plasmids characteristic of

hvKP, resulting in the evolution of hypervirulent carbapenem-resistant *K. pneumoniae* (hv-CRKP) (Tian et al., 2022). This evolving pathotype has raised clinical vigilance and has become one of the major public health concerns due to its ability to cause liver abscess, endophthalmitis, and meningitis, often resulting in infections at multiple body sites (Hallal Ferreira Raro et al., 2023; Wahl et al., 2024).

In Serbia, polymyxin-based therapies remain the first-line and often the only treatment for CRKP. Only exceptionally, ceftazidime-avibactam, imipenem-relebactam, and meropenem-vaborbactam are available for some patients with life-threatening infections. The only multicenter study to date on *K. pneumoniae* in Serbia focused on strains resistant to both carbapenems and colistin, identifying ST101/*bla*_{OXA-48} as the dominant genotype from 2013 to 2017 (Palmieri et al., 2020). With studies indicating a global increase in NDM metallo-beta-lactamases in *K. pneumoniae*, it is crucial to investigate the evolutionary changes in CRKP populations in Serbia and their implications on the currently recommended treatment options.

Therefore, the study aimed to: 1) determine the overall prevalence of infections caused by *K. pneumoniae* and prevalence of CRKP in Serbian hospitals; 2) evaluate CRKP resistance rates to last-line antibiotics, such as novel BLBLIs, colistin, and tigecycline; 3) investigate the genetic basis of antimicrobial resistance in CRKP and its correlation with resistance phenotypes; 4) assess the prevalence of hv-CRKP; 5) examine the genetic diversity of CRKP isolates; and 6) discern their relatedness to reported genomes in the NCBI database.

2. Methods

2.1. Study design, bacterial isolates, and data collection

In this multicenter, prospective observational study, a total of 11,768 non-redundant bacterial isolates were collected from five hospitals and clinical centers in the Vojvodina region, five hospitals in the Belgrade region, five hospitals and clinical centers in Central Serbia, and one clinical center from the Serbian enclave in Kosovo (Fig. 1), during the first week of each month between January 2022 and July 2023. These institutions are located across all five regions of the country, which have an estimated population of approximately 6.5 million. To ensure the sample's representativeness, isolates were obtained from hospitals and clinical centers, which collectively serve around 80% of Serbia's population (5.2 million) (Fig. 1). All 2001 *K. pneumoniae* isolated from all participant hospitals were included in the analysis. Out of them, 1069 non-duplicate clinical isolates were resistant to carbapenems. A total of 191 CRKP isolates were randomly selected for detailed analysis, taking into consideration the regional population distribution and the structure of the healthcare system, supporting the representativeness of the dataset and ensuring that it captures the geographic and epidemiological diversity of CRKP in Serbia. Specifically, 50 isolates were obtained from the Vojvodina region, 80 from Belgrade, and 55 from Central Serbia. Additionally, 5 isolates were collected from Serbian enclaves in Kosovo and Metohija region. Importantly, two major clinical centers located in Belgrade serve as national referral institutions and routinely admit patients from across the entire country. A flowchart showing inclusion and exclusion criteria for the selection of bacterial isolates is shown in Fig. 2.

K. pneumoniae isolation and initial identification were performed in regional microbiological laboratories using conventional techniques, such as biochemical identification (API20, bioMérieux, France), Chromogenic agar (CPSE, bioMérieux, France), VITEK®2 system

(bioMérieux, France) or Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Bruker co., USA). Further characterization was done in the coordinating laboratory of the Institute of Microbiology and Immunology at the Faculty of Medicine, University of Belgrade. MALDI-TOF MS confirmation of the identification and antimicrobial susceptibility testing were performed for all isolates initially identified as *K. pneumoniae*. All isolates confirmed to be *K. pneumoniae* were stored at -80°C in skim milk broth (Merck, Germany) until further analysis. A total of 191 randomly selected CRKP isolates underwent extended antimicrobial susceptibility testing, including assessment of the last-line antibiotics, detection of the hypermucoviscous CRKP strains, and identification of carbapenemase production. All patients were de-identified in regional clinical laboratories and re-coded before the bacterial strains were sent to the coordinating laboratory. Relevant demographic and epidemiological data were obtained from medical records. The study was approved by the Ethics Committee of the Medical Faculty, University of Belgrade (25/V-18).

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility to amoxicillin-clavulanic acid, ampicillin-sulbactam, piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, amikacin, gentamicin, ciprofloxacin, levofloxacin, fosfomycin, and trimethoprim-sulfamethoxazole was performed using disk diffusion method (BioRad, UK). Susceptibility to colistin was evaluated by broth microdilution method, while susceptibility to tigecycline was tested using a gradient test (Liofilchem, Italy). To confirm carbapenem resistance, the minimum inhibitory concentrations (MICs) for imipenem and meropenem were determined using the gradient strip test (Liofilchem, Italy). Antimicrobial susceptibility to novel BLBLI combinations (ceftazidime-avibactam, ceftolozane-tazobactam, imipenem-relebactam and meropenem-vaborbactam) was performed by gradient test (Liofilchem, Italy). Cefiderocol susceptibility was tested using broth microdilution (Liofilchem, Italy). *K. pneumoniae* ATCC 13883 strain was used as a reference strain. Antibiotic susceptibility testing results were interpreted using EUCAST 2023 guidelines ([The European Committee on, 2023](#)). Breakpoints for *E. coli* were used to interpret antibiotic

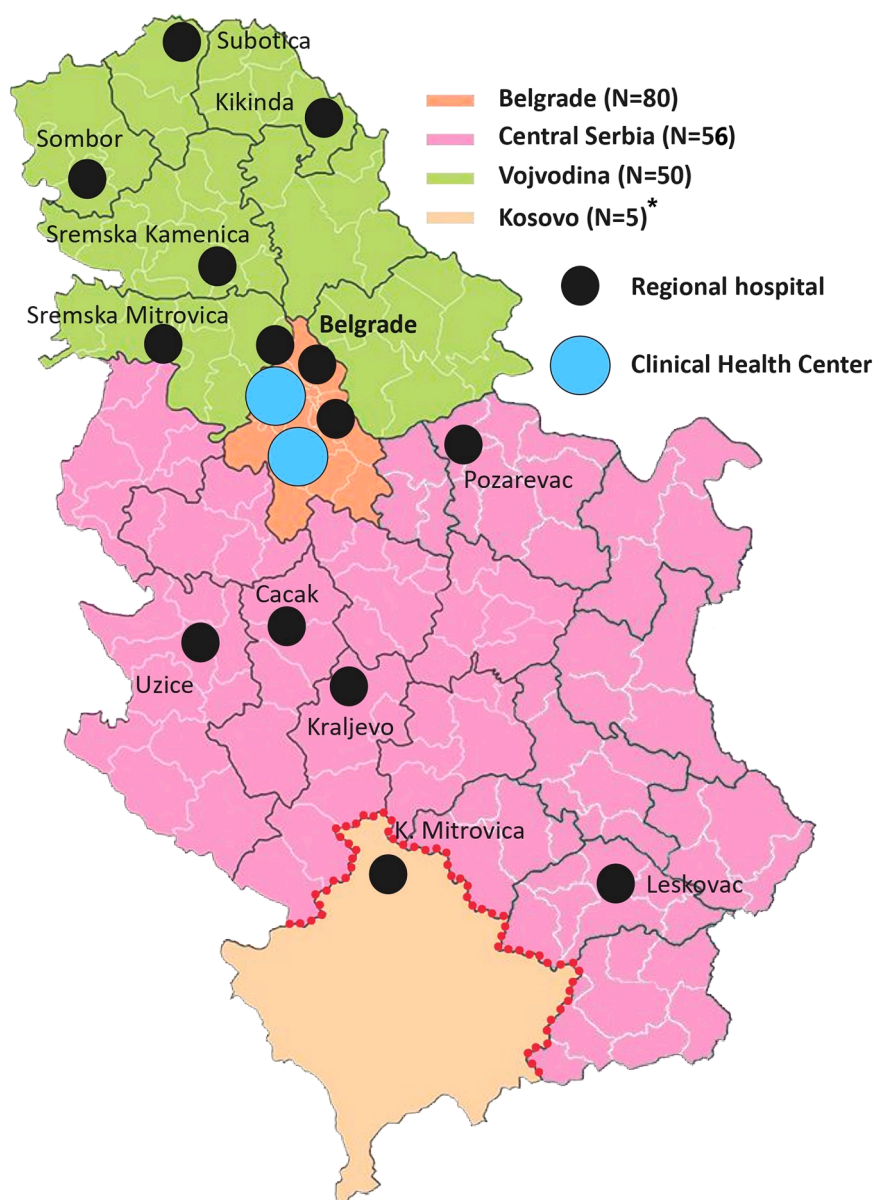


Fig. 1. Distribution of carbapenem-resistant *Klebsiella pneumoniae* isolates collected in Serbia, between January 2022 and July 2023 (N = 191). *Collected isolates from Kosovo and Metohija are from the Serbian enclaves. This designation is without prejudice to positions on status and is in line with UNSCR 1244 1999 and the ICJ Opinion on the Kosovo declaration of independence.

susceptibility testing for tigecycline and fosfomycin (Chirabundhu et al., 2024).

MDR, extensively drug-resistant (XDR), pandrug-resistant (PDR), and difficult-to-treat (DTR) *K. pneumoniae* were defined as follows: non-susceptibility to one or more agents in at least three antibiotic classes, non-susceptibility to at least one antibiotic in all but two or fewer antibiotic classes, resistance to all tested available antibiotics, and resistance to all classical β -lactams and fluoroquinolones, respectively (Magiorakos et al., 2012; Kadri et al., 2018; Karlowsky et al., 2021).

2.3. Identification of carbapenemase production

The KPC, NDM, VIM, IMP, and OXA-48-like carbapenemases were detected using immunochromatographic lateral flow assays (RESIST 5, CORIS BioConcept, Belgium) for 191 CRKP, according to the manufacturer's instruction.

2.4. Detection of the hypermucoviscosity phenotype

Hypermucoviscosity phenotype was evaluated for 191 CRKP using the string test, defined as positive if a viscous string longer than 5 mm formed when stretching an overnight colony from a blood agar plate (Li

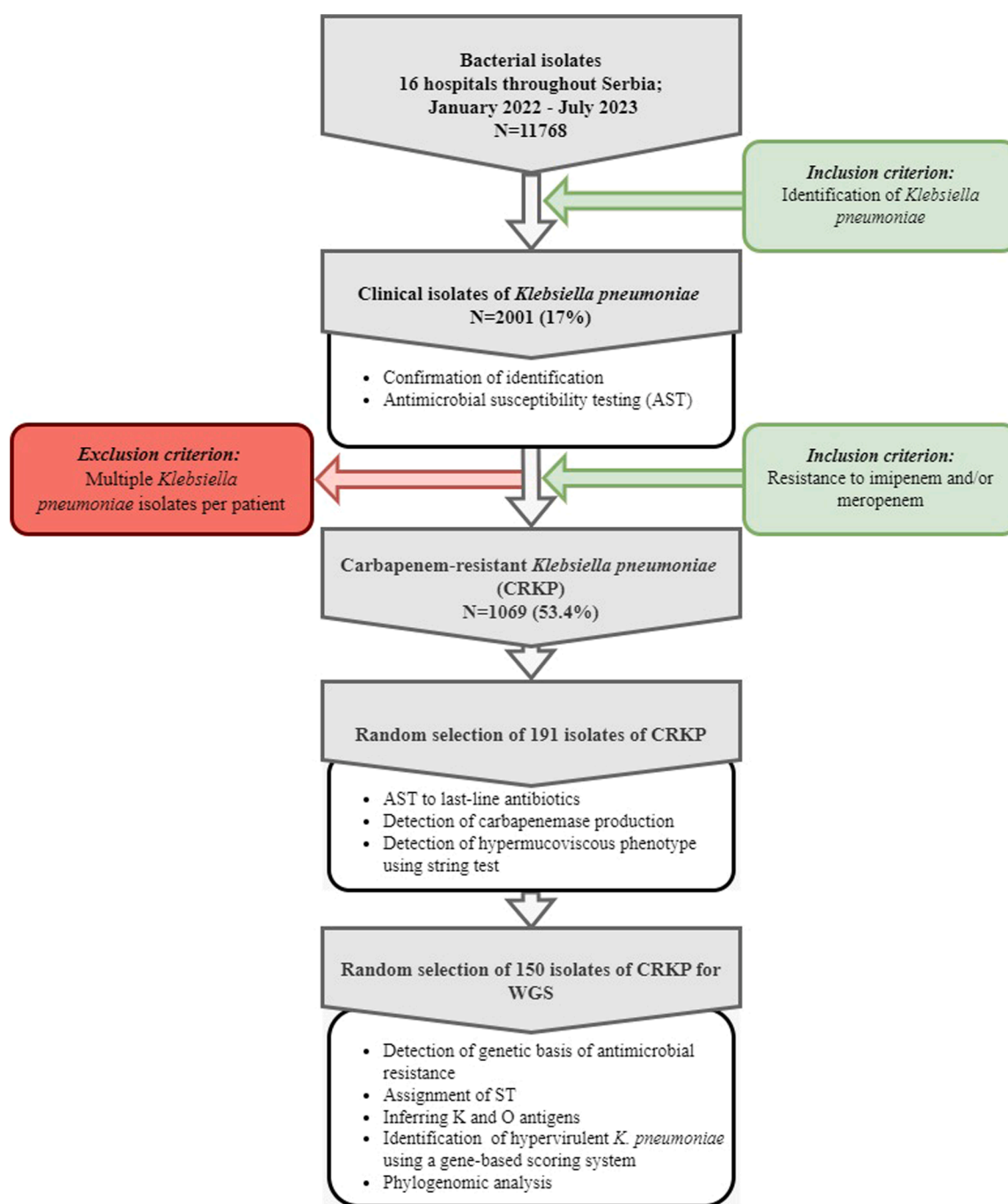


Fig. 2. Flowchart of the process of bacterial isolates selection for phenotypic and genotypic characterization; inclusion criteria were the isolation of *Klebsiella pneumoniae* obtained during the routine laboratory work and detection of carbapenem resistance. The exclusion criterion was multiple *K. pneumoniae* isolates per one patient. CRKP – carbapenem-resistant *Klebsiella pneumoniae*; ST– sequence type; WGS – whole genome sequencing, AST – antimicrobial susceptibility testing.

et al., 2014).

2.5. DNA extraction, whole-genome sequencing (WGS) and bioinformatic analysis

Of the 191 CRKP isolates, 150 were subjected to WGS using the Illumina NovaSeq 6000 PE150 platform. The isolates were randomly selected to reflect regional distribution across the country and to preserve the proportions of phenotypically detected carbapenemases. This strategy intended to minimize regional or genetic bias and to offer a more accurate and comprehensive view of the national epidemiological landscape.

DNA extraction was performed using GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) and quantification of genomic DNA was performed using BioSpec-nano (Shimadzu Corporation, Japan). The 150 *K. pneumoniae* genomes were assembled using the SPAdes Assembler algorithm and Reference-guided genome assembly using Geneious Prime 2024.0 software (<https://www.geneious.com>).

The quality of assembled genomes was assessed using QUAST software (Quality Assessment Tool). Genome annotation was performed using RAST software (Rapid Annotation using Subsystem Technology, available at: <https://rast.nmpdr.org/rast.cgi>). MLST analysis was performed using *K. pneumoniae* MLST database maintained by Pasteur Institute (Available at: <http://bigsdbs.pasteur.fr/klebsiella/klebsiella.html>). ResFinder v.4.1 (Available at: <https://cge.food.dtu.dk/services/ResFinder/>) was used to identify genetic determinants of resistance. In the cefiderocol-resistant CRKP isolate, known genomic determinants relevant to cefiderocol susceptibility were investigated, including carbapenemase genes, mutations in porin-encoding genes, and selected regulatory genes (*cirA*, *fiu*, *baeS*, *envZ*, and *mrcB*) previously reported to be associated with reduced susceptibility (Kriz et al., 2024; McElheny et al., 2021). The aforementioned genes, through alterations in siderophore receptors, multidrug efflux regulators, mutations in iron transport-associated loci, and modifications of penicillin-binding protein 1B, may contribute to decreased susceptibility to cefiderocol in *K. pneumoniae* (Kriz et al., 2024; McElheny et al., 2021). Analyses were performed by comparison with reference sequences using Geneious Prime 2024.0 software.

Hypervirulent *K. pneumoniae* was defined by the presence of virulence plasmid-associated loci, including the aerobactin gene cluster (*iuc*) and regulators of the mucoid phenotype (*rmpA2/rmpADC*) (Sun et al., 2025; Russo et al., 2024). Virulence genes frequently associated with hypervirulence *ybt* (yersiniabactin), *iucA* (aerobactin), *iroB* (salmonchelin), *rmpA* (regulator of mucoid phenotype A gene), and *clb* (colibactin), capsular (K antigen), and lipopolysaccharide (O antigen) types were inferred using Kleborate available at Pathogenwatch (Lam et al., 2021). Based on the presence of the *iuc*, *ybt*, and *clb* genes, *K. pneumoniae* isolates were assigned a virulence score (Lam et al., 2021). Strains harboring only the *ybt* gene were given a score of 1. Those carrying only the *clb* genes scored 2, while strains possessing only the *iuc* genes scored 3. A score of 4 was assigned to strains containing both *ybt* and *iuc* genes but lacking *clb*. The presence of all three genes resulted in a score of 5. Strains lacking all the mentioned genes received a score of 0 (Lam et al., 2021).

The phylogenomic analysis was performed using Roary v3.13.0 (Page et al., 2015), with a 99% core definition threshold and a minimum 95% blastp identity. Afterward, SNP-sites v2.4.1 (Page et al., 2016) was used to identify core genome single-nucleotide polymorphisms (SNP). A core-SNP-based phylogenetic tree was constructed using raxmlHPC-PTHREADS v8.2.12 with the neighbor-joining method using 1000 bootstraps (Stamatakis, 2014) and was visualized using iTOL software (Letunic and Bork, 2021).

To infer the epidemiological link of *bla*_{NDM-1}-positive isolates from Serbia to globally circulating *bla*_{NDM-1}-positive *K. pneumoniae* isolates, the cgMLST analysis of all *bla*_{NDM-1}-positive genomes from this study and all available *bla*_{NDM-1}-positive genomes belonging to the same STs in the

NCBI Genome database (Supplementary Table 1) was performed. The minimum spanning trees were constructed based on the cgMLST profiles using the RidomSeqSphere+ program. A cluster distance threshold of 15 cgMLST allele differences and a cluster quality threshold of at least 95% good cgMLST targets were used to detect closely related isolates (Miro et al., 2020).

2.6. Statistical analysis

Statistical analysis was performed using SPSS version 24.0 (SPSS Inc., Chicago, IL, United States). Pearson's chi-squared test or Fisher exact test was used to compare categorical variables. *p*-values of < 0.05 were statistically significant. Cohen's kappa coefficient was used to determine the agreement between the phenotype- and WGS-based hypervirulence results.

3. Results

3.1. Bacterial isolates and patients' characteristics

During the study period, out of 11,768 clinical isolates, *K. pneumoniae* accounted for 2001 isolates (17%). Among them, 1069 isolates (53.4%) were CRKP. A total of 191 CRKP strains were randomly selected for further analysis. They were isolated from 77 females (39.7%) with a median age of 69 years (95% CI: 67–72 years; range: 33–90 years) and 114 males (60.3%) with a median age of 71 years (95% CI: 68–73 years; range: 21–90 years). CRKP strains were isolated from urine (N = 88; 46.1%), blood culture (N = 36; 18.8%), wound specimen (N = 32; 16.7%), lower respiratory tract (N = 29; 15.2%), central venous catheter (N = 4; 2.1%), and peritoneal fluid (N = 2; 1%).

3.2. Antimicrobial susceptibility testing (AST)

All 191 CRKP isolates were resistant to amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftazidime, and cefepime (Fig. 3). One hundred and three (53.9%), 7 (3.7%), and 186 (97.4%) CRKP isolates were XDR, PDR, and DTR, respectively. MICs of novel BLBLI combinations, colistin, and tigecycline of CRKP isolates and their resistance rates are shown in Table 1. High resistance rates were observed for selected antimicrobial agents, including colistin (N = 100; 52.4%), tigecycline (N = 74; 38.7%), and ceftazidime-avibactam (N = 97; 50.8%). Among colistin-resistant CRKP isolates, 34 (34%) were resistant to ceftazidime-avibactam, 78 (78%) to imipenem-relebactam, and 89 (89%) to meropenem-vaborbactam.

3.3. Detection of hypermucoviscous isolates of carbapenem-resistant *K. pneumoniae* and production of carbapenemases

The string test was used to phenotypically screen for hypermucoviscous strains, a feature often associated with hv-CRKP. Of the 191 CRKP isolates tested, 26 (13.6%) yielded a positive result.

Out of 191 CRKP isolates, 184 (96.3%) were found to produce carbapenemases. Among the isolates producing a single carbapenemase, NDM was the most common, detected in 82 out of 184 CRKP isolates (44.6%), followed by OXA-48 in 80 (43.5%) and KPC in 14 (7.6%) isolates. Additionally, eight CRKP isolates (4.3%) co-produced NDM and OXA-48. Notably, seven isolates (3.7%) tested negative for all evaluated carbapenemases. Neither VIM nor IMP production was observed in any of the tested CRKP isolates.

3.4. Whole-genome sequence-inferred genes encoding antimicrobial resistance to beta-lactams, sequence types, K and O genotypes

Out of 150 sequenced CRKP isolates, 146 (97.3%) were carbapenemase producers, as depicted in Fig. 4. The most frequently detected single carbapenemase genes were *bla*_{NDM-1} and *bla*_{OXA-48}, found in 65

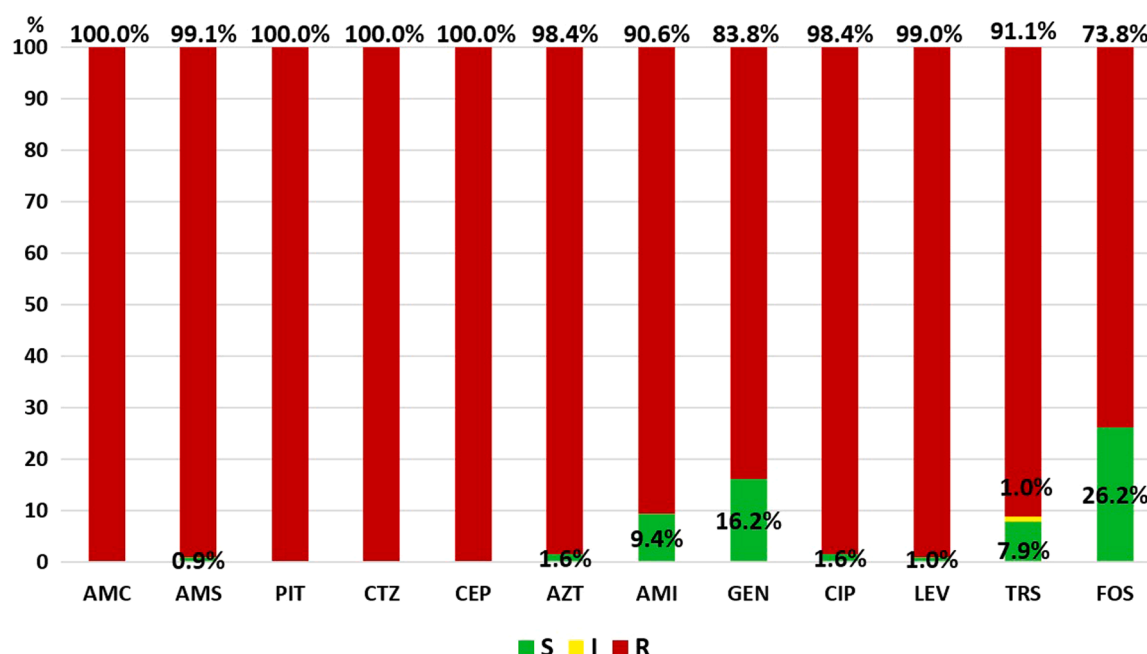


Fig. 3. Antimicrobial susceptibilities of 191 carbapenem-resistant *Klebsiella pneumoniae* isolated from hospitalized patients between January 2022 and July 2023. AMC – amoxicillin-clavulanic acid; AMS – ampicillin-sulbactam; PIT – piperacillin-tazobactam; CTZ – ceftazidime; CEP – cefepime; AZT – aztreonam; AMI – amikacin; GEN – gentamicin; CIP – ciprofloxacin; LEV – levofloxacin; TRS – trimethoprim-sulfamethoxazole; FOS – fosfomycin; S - Susceptible, standard dosing regimen; I - Susceptible, increased exposure; R - Resistant.

Table 1

Antimicrobial susceptibility categories and minimal inhibitory concentrations of 191 carbapenem-resistant *Klebsiella pneumoniae* isolates obtained from hospitalized patients between January 2022 and July 2023 to novel beta-lactam/beta-lactamase inhibitor combinations, cefiderocol, colistin, and tigecycline.

Antibiotic	MIC range (mg/l)	MIC ₅₀ (mg/l)	MIC ₉₀ (mg/l)	S N (%)	R N (%)
Ceftazidime-avibactam	0.38-> 256	12	> 256	94 (49.2%)	97 (50.8%)
Imipenem-relebactam	0.094-> 32	4	> 32	29 (15.2%)	162 (84.8%)
Meropenem-vaborbactam	0.047-> 256	16	64	18 (9.4%)	173 (90.6%)
Ceftolozane-tazobactam	4-> 256	> 256	> 256	0 (0%)	191 (100%)
Cefiderocol	0.25-4	0.5	2	190 (99.5%)	1 (0.5%)
Colistin	0.5-> 16	4	8	91 (47.6%)	100 (52.4%)
Tigecycline	0.094-64	0.75	2	117 (61.3%)	74 (38.7%)

MIC, Minimal inhibitory concentration; S, Susceptible; R, Resistant

(44.5%) and 64 (43.8%) of the 146 isolates, respectively, while 11 out of 146 isolates (7.5%) harbored *bla*_{KPC-2}. Additionally, six CRKP isolates (4.1%) co-harboured both *bla*_{NDM-1} and *bla*_{OXA-48}. None of the isolates harbored simultaneously genes encoding MBL and KPC. The most common extended spectrum beta-lactamase (ESBL) was *bla*_{CTX-M-15}, found in 141 out of 150 (94%) CRKP isolates (Supplementary Figure 1). In four CRKP isolates without genes encoding carbapenemases, several *bla* genes were identified, with the following genotypes: *bla*_{OXA-1}/*bla*_{CTX-M-15}/*bla*_{SHV-1}, *bla*_{TEM-1}/*bla*_{SHV-1}, *bla*_{OXA-1}/*bla*_{CTX-M-15}/*bla*_{SHV-11}, and *bla*_{CTX-M-15}/*bla*_{SHV-1} (Supplementary Figure 1). All four non-producer strains were susceptible to ceftazidime-avibactam, resistant to ceftolozane-tazobactam, while one was both resistant to meropenem-vaborbactam and imipenem-relebactam. As expected, all CRKP strains harboring only *bla*_{OXA-48} (N = 64) were resistant to meropenem-vaborbactam, while 12 strains (18.8%) were susceptible to imipenem-

relebactam. Of note, the rapid test for carbapenemase detection was consistent with genotypic data, except for two isolates that tested susceptible to meropenem phenotypically but were found to carry *bla*_{NDM-1} genes based on WGS data.

MLST analysis revealed ten STs belonging to six clonal complexes (CCs): ST147/CC147 (N = 44; 29.3%), ST101/CC101 (N = 40; 26.7%), ST11/CC11 (N = 22; 14.7%), ST437/CC11 (N = 22; 14.7%), ST258/CC11 (N = 6; 4%), ST307/CC307 (N = 6; 4%), ST39/CC39 (N = 5; 3.3%), ST16/CC16 (N = 2; 1.3%), ST395/CC395 (N = 2; 1.3%), and ST340/CC11 (N = 1; 0.7%), as shown in Table 2. Notably, ST11, ST258, ST340 and ST437 are all single locus variants within CC11. Tigecycline resistance was detected in all sequence types (STs) except ST16 and ST340, with the following distribution: ST147 (13 CRKP), ST11 (6), ST437 and ST101 (4 each), ST395 (2), and ST307, ST39, and ST258 (1 each). Colistin-resistant CRKP isolates belonged to these STs: ST101 (36 isolates), ST147 (16), ST437 (12), ST11 and ST258 (6 each), ST39 (4), ST307 (2), and ST395 (1) (Supplementary Figure 1). Genotype ST101 was significantly associated with CRKP strains isolated from respiratory tract samples (p = 0.01). Other STs were not linked to the source of the specimen.

The 65 *bla*_{NDM-1}-carrying isolates were assigned to five STs (Fig. 4). The presence of *bla*_{NDM-1} gene was significantly associated with ST147 (N = 43; 66.2%) and ST11 (N = 15; 23.1%) compared to ST307 (N = 5; 7.7%), ST101 and ST340 (N = 1; 1.5% each) (p < 0.05). The carriage of *bla*_{OXA-48} gene was observed in 64 CRKP genomes with five STs among which ST101 (N = 37, 57.8%) and ST437 (N = 21; 32.8%) were significantly more prevalent than types ST11, ST16, and ST395 (N = 2; 3.1% each) (p < 0.05). Eleven (7.2%) CRKP strains carried *bla*_{KPC-2} and were assigned to ST258 (N = 6; 3.9%) and ST39 (N = 5; 3.3%). Colistin resistance was significantly associated with ST101 CRKP strains carrying *bla*_{OXA-48} and ST258 carrying *bla*_{KPC-2} (p < 0.005). On the other hand, ST11 and ST147 CRKP strains exhibited higher susceptibility to colistin compared with other sequence types (p < 0.001).

Overall, 12 K types were identified in 150 sequenced CRKP isolates. The most frequent type was K64 found in 44 isolates (29.3%), while K17 was found in 33 (22%) isolates (Fig. 5). Overall, five O types were detected and the most common was O2a which was found in 67 isolates

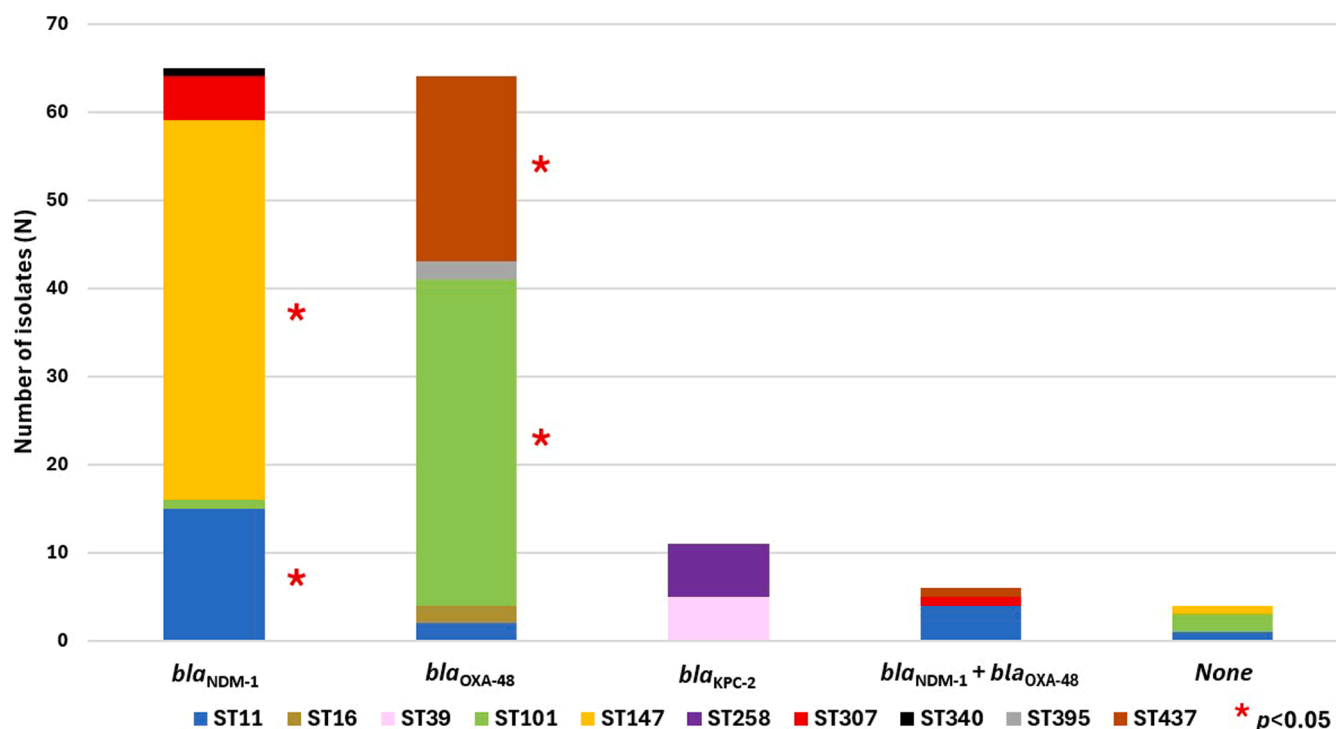


Fig. 4. Distribution of carbapenemase genes among 150 sequenced carbapenem-resistant *Klebsiella pneumoniae* isolated from hospitalized patients between January 2022 and July 2023. * $p < 0.05$.

Table 2

Antibiotic resistance of 150 carbapenem-resistant clinical isolates of *Klebsiella pneumoniae* belonging to ten sequence types.

ST	N (%)	Resistance to antibiotics N (%)									
		COL	CTV	IMI-REL	MER-VAB	TIG	AMI	GEN	CIP	TRS	FOS
ST11	22 (14.7)	6 (27.3)	19 (86.4)	21 (95.4)	21 (95.4)	7 (31.8)	21 (95.4)	21 (95.4)	22 (100)	21 (95.4)	15 (68.2)
ST16	2 (1.3)	0 (0.0)	0 (0.0)	2 (100)	2 (100)	0 (0.0)	1 (50.0)	0 (0.0)	2 (100)	2 (100)	1 (50.0)
ST39	5 (3.3)	4 (80.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (60.0)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
ST101	40 (26.7)	36 (87.8)	1 (2.4)	29 (72.5)	39 (97.5)	14 (35.0)	31 (77.5)	32 (80.0)	40 (100)	36 (90.0)	19 (47.5)
ST147	44 (29.3)	16 (36.4)	43 (97.7)	43 (97.7)	43 (97.7)	19 (43.2)	44 (100)	41 (93.2)	44 (100)	38 (86.4)	36 (81.8)
ST258	6 (4.0)	6 (100)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	5 (83.3)	0 (0.0)	6 (100)	3 (50.0)	5 (83.3)
ST307	6 (4.0)	2 (33.3)	6 (100)	6 (100)	6 (100)	2 (33.3)	5 (83.3)	4 (66.7)	6 (100)	6 (100)	4 (66.7)
ST340	1 (0.7)	0 (0.0)	1 (100)	1 (100)	1 (100)	0 (0.0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
ST395	2 (1.3)	1 (50.0)	0 (0.0)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
ST437	22 (14.7)	12 (54.5)	1 (4.5)	20 (90.9)	22 (100)	5 (22.7)	21 (95.4)	19 (86.4)	22 (100)	22 (100)	18 (81.8)

ST – sequence type; COL – colistin; CTV – ceftazidime-avibactam; IMI-REL – imipenem-relebactam, MER-VAB – meropenem-vaborbactam; TIG – tigecycline; AMI – amikacin; GEN – gentamicin; CIP – ciprofloxacin; TRS – trimethoprim-sulfamethoxazole; FOS – fosfomycin

Figure legends:

(44.7%). The most common clones detected in the tested CRKP isolates were ST147/*bla*_{NDM-1}/K64/O2a (N = 43; 28.7%), ST101/*bla*_{OXA-48}/K17/O1ab (N = 31; 20.7%), and ST437/*bla*_{OXA-48}/K36/O4 (N = 22; 14.7%), as depicted in [Supplementary Figure 1](#).

3.5. Non-beta-lactamase antimicrobial resistance determinants and their correlation with resistance phenotypes

The median number of resistance genes per isolate was 12 (range: 2–24), spanning a median of nine antibiotic classes (range: 3–12).

The mutations in Outer membrane proteins (Omps) were found in 116 (77.3%) CRKP isolates. The truncated *ompK35* gene was found in 92 (61.3%) isolates. On the other hand, in *ompK36* G133GGD insertion was observed in 75 (50%) isolates. All four CRKP isolates that lacked carbapenemase-encoding genes were also resistant to ceftolozane-tazobactam and displayed seven point mutations in the *ompK36* and *ompK37* genes. These included mutations associated with cephalosporin resistance (*ompK36*p.N49S, *ompK36*p.L59V, *ompK36*p.F207Y, and

*ompK36*p.E232R) and carbapenem resistance (*ompK36* p.A217S, *ompK37* p.I128M and *ompK37*p.I70M). Additional mutations linked to carbapenem resistance, *ompK36*p.N218H and *ompK37*p.N230G were found in two and one isolates, respectively. These non-carbapenemase-producing CRKP strains did not have point mutations known to confer resistance to BLBLIs. But each of them carried one of the following genes: *bla*_{SHV-1}, *bla*_{TEM-1}, and *bla*_{CTX-M-15}.

The prevalence of acquired genes mediating resistance to trimethoprim (*dfrA*) and sulfamethoxazole (*sul*) was high and was found in 122 (81.3%) and 129 (86%) CRKP isolates, respectively ([Supplementary Figure 1](#)). Of note, 4 and 6 CRKP isolates carrying *dfrA* and *sul* genes, respectively, remained susceptible to trimethoprim-sulfamethoxazole. Five *dfrA* genes were detected, with *dfrA5* being the most common (N = 72; 59%), followed by *dfrA14* (N = 65; 53.3%), *dfrA12* (N = 22; 18%), *dfrA30* (N = 4; 3.3%), and *dfrA1* (N = 2; 1.6%). Multiple *dfrA* genes were present in 41 (27.3%) CRKP isolates, most commonly *dfrA14* + *dfrA5* (N = 34; 27.9%). The predominant sulfamethoxazole resistance genotype was *sul1* + *sul2* (N = 80; 62%), with *sul1* alone in 32 (24.8%)

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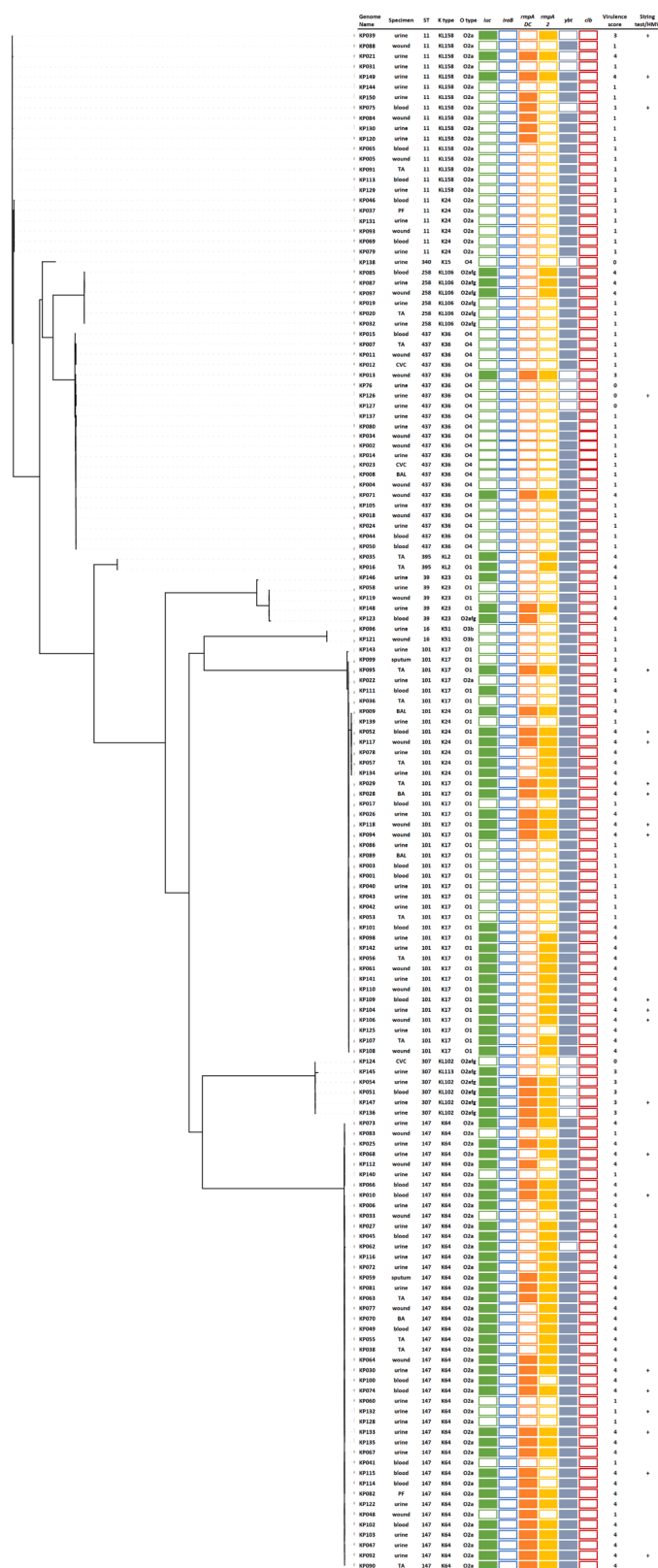


Fig. 5. The distribution of sequence types, K types, O types, virulence genes, and virulence scores among 150 sequenced carbapenem-resistant *Klebsiella pneumoniae* strains. BAL - bronchoalveolar lavage; *clb* - colibactin; CVC - central venous catheter; *iroB* - salmonelichine; *iuc* - aerobactin; HMV - hyper-mucoviscosity phenotype; PF - peritoneal fluid; *rmp* - regulator of mucoid phenotype gene; TA - tracheal aspirate; *ybt* - yersiniabactin.

isolates, *sul2* alone in 16 (12.4%), and one isolate (0.8%) harboring *sul1* and *sul3*.

All tested CRKP isolates had mutations in both *gyrA* and *parC* genes (Supplementary Figure 1). The most common mutation was *parC* S80I found in 148 (98.7%) sequenced strains. The remaining two strains (1.3%) carried a *parC* S84K mutation. Six *gyrA* mutations were identified, with *gyrA* S83I being the most frequent ($N = 86$; 57.3%). The most common fluoroquinolone resistance genotype was the *gyrA* S83I + *parC* S80I combination, present in 62 (41.3%) CRKP isolates. Plasmid-encoded *qnr* genes mediating fluoroquinolone resistance were found in 72 (48%) CRKP isolates, where *qnrS1* was the most frequent ($N = 51$; 34%).

The majority of sequenced CRKP isolates had aminoglycoside-resistance genes ($N = 145$; 96.7%), regardless of their susceptibility to aminoglycosides, as displayed in Supplementary Figure 1. AAC-encoding genes were found in 103 out of 145 CRKP isolates with aminoglycoside-resistance genes (71.0%). The *aac(6')-Ib-cr* was the most frequent, found in 63 (43.4%), followed by *aac(3)-IIa* detected in 37 (25.5%) CRKP isolates. 16S rRNA methyltransferase genes were detected in 61.4% of isolates (*armA*), with *rmtB* and *rmtC* less frequent (3.4% and 0.7%), while APH genes (*aph(3')-VI* 35.9%, *aph(3')-Ia* 29%), *strA* + *strB* (18.6%), and ANT genes (*aadA1* 29%) were also observed among CRKP isolates. The two most common aminoglycoside resistance genotypes among CRKP isolates were the isolated presence of *armA* and the co-presence of *armA* and *aac(3)-IIa*, each found in 12 isolates (8%).

The truncated *mgtB* gene was detected in 37 out of 150 CRKP isolates (24.6%). The plasmid-mediated *mcr* genes were not observed. Tetracycline resistance genes were found in 31 (20.7%) CRKP isolates, with *tet(D)* in 19 (61.3%) and *tet(A)* in 12 (38.7%); *tet(X)* was not detected. Chromosomal *fosA* gene was observed in all CRKP isolates, while plasmid-encoded *fos* genes were not detected.

One cefiderocol-resistant strain (MIC=4 mg/l) was assigned to ST101, carrying the *bla_{NDM-1}*, *bla_{SHV-1}*, *bla_{CTX-M15}*, and *bla_{OXA-1}*. Also, this strain had mutations in porin-encoding genes: *ompK36p.A217S*, *ompK36p.N218H*, *ompK37p.I70M*, and *ompK37p.I128M*. This strain carried a missense mutation p.D558N in the siderophore receptor *cirA*. Additionally, the *baeS* gene was truncated due to a premature stop codon at position 254. There were no mutations in *fiu*, *mrcB* and *envZ* genes.

3.6. Phylogenomic analysis

The phylogenomic analysis of CRKP isolates from Serbia revealed a complex population structure, with the presence of multiple high-risk STs, grouped into distinct phylogenetic clades, with ST11, ST101, ST147, and ST437 being the most prevalent (Fig. 6). Within these clades, isolates further clustered according to specific K and O antigen types. The distribution of carbapenemase genes among analysed genomes showed that *bla_{OXA-48}* appeared sporadically among multiple lineages, while *bla_{NDM-1}* was present in several STs, including ST147 and ST307, highlighting the polyclonal nature of *bla_{NDM-1}* dissemination. Meanwhile, *bla_{KPC-2}* was detected in all isolates belonging to ST39 and ST258.

Core-genome SNP analysis revealed that the similarity among the sequenced CRKP isolates ranged from 54.47% to 100% (Supplementary Figure 2). Eight distinct clonal groups circulated, with high intra-group similarity and inter-group diversity, as shown in the Supplementary Figure 2. Clusters of closely related isolates showed > 98.8% identity, with 0–641 core SNP differences. Nine CRKP isolates (6.0%) were grouped into 4 transmission lineages, characterized by fewer than 5 SNPs, indicating likely outbreak-related isolates.

The global cgMLST minimum spanning trees of the 71 *bla_{NDM-1}*-positive CRKP isolates from this study and 2118 *bla_{NDM-1}*-positive *K. pneumoniae* isolates from the NCBI database showed significant genetic diversity among strains worldwide (Supplementary Figures 3–7). Within the sequence types included in the global cgMLST analysis, the number of *bla_{NDM-1}*-positive genomes in the NCBI database was highest for ST147 ($N = 1148$), followed by ST11 ($N = 617$), ST307 ($N = 228$),

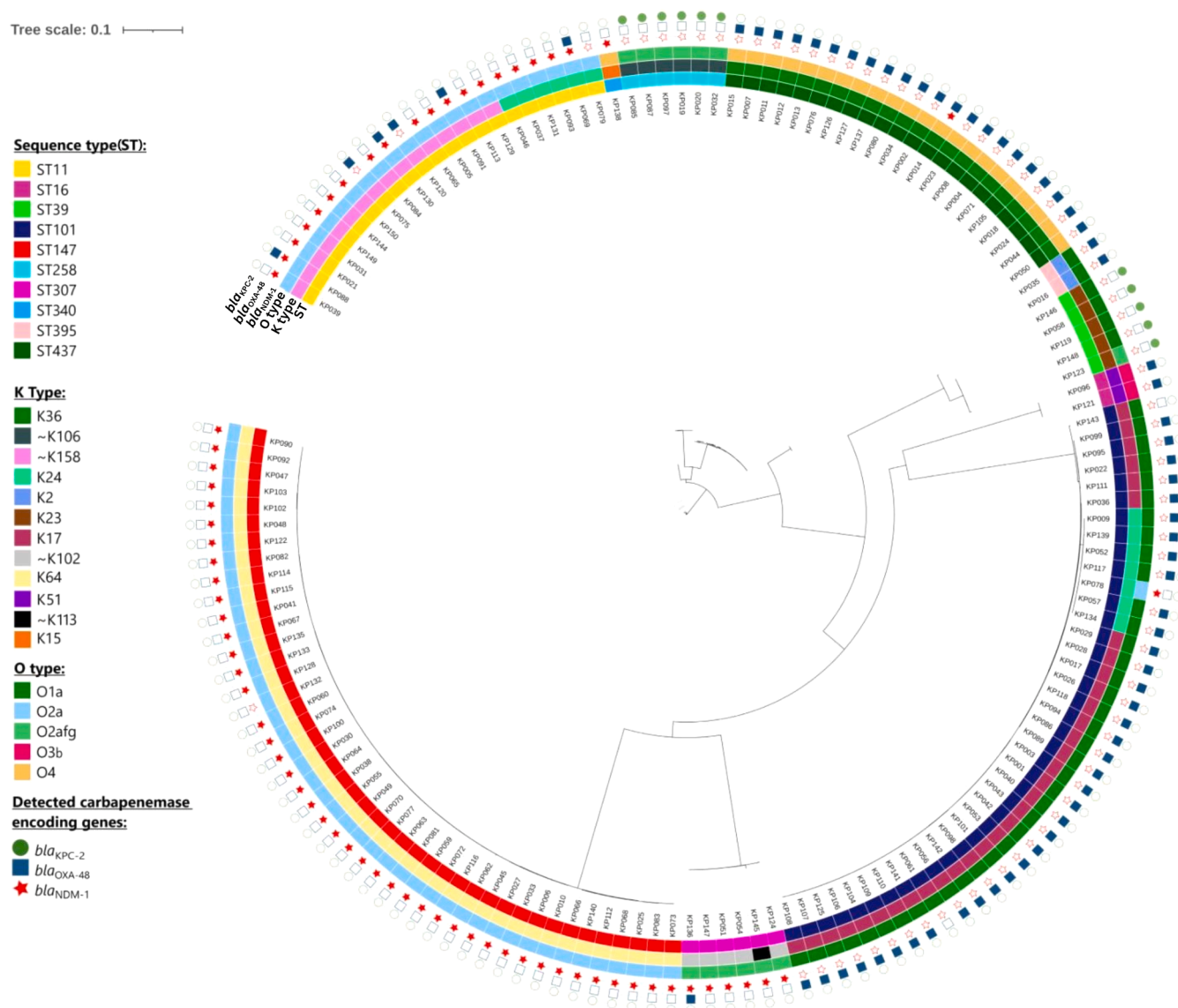


Fig. 6. SNP-based phylogenetic tree constructed on core gene alignment of the 150 *Klebsiella pneumoniae* clinical isolates from Serbia using the neighbor-joining method with 1000 bootstraps. The fragments with varying colors in the innermost ring represent corresponding sequence types (STs), the middle ring shows K types, and the outer ring represents the O types. The external-colored symbols corresponded to genes that encoded OXA-48 beta-lactamase, KPC, and NDM-1. Open symbols indicate the absence of genes.

ST101 (N = 102), ST340 (N = 62), and ST437 (N = 32).

Based on the cluster quality threshold of 15 cgMLST allele differences, the *bla*_{NDM-1}-harbouring CRKP ST147 isolates from this study were found to be genetically related to two distinct cluster types (CT), CT3125 and CT11308, neither of which were related to previously reported *K. pneumoniae* isolates from Serbia (Supplementary Figure 3). CT3125 emerged as the dominant cluster, encompassing isolates from all continents except South America, with a significant number originating from Italy. In contrast, CT11308 represented a smaller cluster, primarily comprising isolates from Serbia and Germany, with one also detected in the USA.

Similarly, *bla*_{NDM-1}-positive CRKP ST11 isolates were grouped into two CTs: the large cluster CT901, predominantly comprising isolates from Europe, and the smaller cluster CT8110, which included isolates from this study and one from Germany (Supplementary Figure 4). The *bla*_{NDM-1}-positive CRKP ST101 and ST437 isolates from this study clustered with other isolates previously reported in Serbia and neighboring countries, such as Slovenia and Turkey (Supplementary Figure 5). Notably, *bla*_{NDM-1}-positive CRKP ST307 isolates had not been previously

detected in Serbia. According to the cgMLST analysis, these newly identified isolates belong to clonal cluster CT606, which mainly includes isolates from the USA, United Kingdom, and Germany (Supplementary Figure 6). One ST340 isolate is grouped within a cluster CT1947 with seven already published *K. pneumoniae* genomes from Serbia (Supplementary Figure 7).

3.7. Genotypic detection of hypervirulent *Klebsiella pneumoniae* and its association with hypermucoviscosity

Based on a stringent genomic definition, 75 (50.0%) of the 150 sequenced CRKP isolates were classified as hv-CRKP due to the presence of *iuc* and *rmpA2/rmpADC*. All ST395 (N = 2; 100%) and 36 (81.8%) ST147 CRKP strains were classified as hypervirulent. In contrast, only two (9.1%) ST437 strains fulfilled the criteria for genotypic hypervirulence. Eighty CRKP isolates (53.3%) contained the gene responsible for producing siderophore aerobactin, while *rmpA2* was detected in 71 (47.3%) CRKP isolates (Fig. 5). On the other hand, the regulator of mucoid phenotype *rmpADC* was found in 51 (34%) CRKP isolates, while

salmonelichine and colibactin genes were absent.

Genotypic hypervirulence based on *iuc* and *rmpA2/rmpADC* was most frequently observed among non-invasive isolates from the respiratory tract (N = 15; 65.2%), specifically ST101, ST147, and ST395, with frequencies of 7 (46.7%), 6 (40%), and 2 (13.3%), respectively (Fig. 5). Virulence scores determined by Kleborate ranged from 0 to 4, with 80 isolates (53.3%) exhibiting scores of 3 or 4.

On the other hand, out of 150 sequenced CRKP isolates, 23 (15.3%) displayed a hypermucoviscous phenotype. These isolates were distributed across the following STs: ST101 (N = 10; 43.5%), ST147 (N = 9; 39.1%), ST11 (N = 2; 8.7%) ST307 (N = 1; 4.3%), and ST437 (N = 1; 4.3%). Furthermore, K17 and K64 were the most common K types among phenotypically hypermucoviscous isolates, detected among nine (39.1%) CRKP isolates each (Fig. 5). Both CRKP isolates with K2 were string negative. The hypermucoidy locus *rmpADC* was detected in 16 out of 26 (61.5%) hypermucoviscous CRKP isolates. The agreement between the positive string test and WGS-based hypervirulence (presence of *iuc* and *rmpA2/rmpADC*) was 61.3% (kappa 0.227).

4. Discussion

In this study, we thoroughly characterized antimicrobial resistance of CRKP to last-line antibiotics, including novel BLBLI combinations, as well as hypervirulence and genomic features of circulating isolates from healthcare facilities in Serbia, comparing them to globally disseminated CRKP strains.

The results obtained show that the CRKP prevalence (53.4%) exceeded the global estimate of 28.69% reported in a recent meta-analysis but was lower than the prevalence reported in Serbia during the COVID-19 pandemic (71.3%) (Lin et al., 2023; Gajic et al., 2023). Furthermore, this study found that the prevalence of PDR *K. pneumoniae* was 3.7% in 2023, lower than the 12.3% observed among hospitalized COVID-19 patients in Serbia in 2021/2022 (Gajic et al., 2023). Outbreaks of CRKP have been reported in many European regions, particularly in Southern European countries, like Greece, Spain, and Italy (European Centre for Disease Prevention and Control and World Health Organization, 2023). Additionally, the 2023 China Antimicrobial Surveillance Network (CHINET) reported an increase in meropenem resistance in *K. pneumoniae* from 2.9% in 2005–30.0% in 2023 (Qin et al., 2024).

The current study found high resistance rates in CRKP to commonly used antibiotics, including beta-lactams, fluoroquinolones, trimethoprim-sulfamethoxazole, and aminoglycosides. All CRKP isolates exhibited resistance to ceftolozane-tazobactam, underscoring the limited efficacy of this antibiotic combination against CRKP strains circulating in our healthcare facilities. As expected, it did not act against carbapenemase-producing organisms. However, two CRKP strains that did not produce carbapenemases but had resistance-associated point mutations in porins conferring resistance to carbapenems. These isolates were also resistant to ceftolozane-tazobactam. Overall, resistance to other BLBLI combinations, such as ceftazidime-avibactam, imipenem-relebactam, and meropenem-vaborbactam, varied, with ceftazidime-avibactam showing the highest efficacy against over half of the isolates. Indeed, the limited activity of imipenem-relebactam and meropenem-vaborbactam against OXA-48 producers has already been observed and is attributed to the poor inhibition of OXA-48-like enzymes by relebactam and vaborbactam (Bush and Bradford, 2019). Resistance rates to colistin and tigecycline exceeded 50% and 35%, respectively. Among colistin-resistant CRKP isolates, the resistance rate to ceftazidime-avibactam (34%) was lower than the resistance rates to imipenem-relebactam (78%) and meropenem-vaborbactam (89%).

These findings underscore the importance of ongoing surveillance and judicious use of newer agents to mitigate further rise of resistance.

Genomic analysis of circulating CRKP in Serbia revealed insights into the genetic basis of antimicrobial resistance and virulence, allowing comparisons with globally disseminated clones. Two isolates exhibited

co-occurrence of carbapenemase genes, specifically *bla*_{OXA-48} and *bla*_{NDM-1}, highlighting the complexity of resistance mechanisms in CRKP. These findings align with reports from China, describing *K. pneumoniae* ST307, carrying both *bla*_{OXA-48} and *bla*_{NDM-1} (Xie et al., 2017). In contrast, other studies identified the international high-risk clone ST11 as the predominant NDM producer (Izdebski et al., 2022), which has spread in Asia and South America (Munoz-Price et al., 2013; Chen et al., 2014; Qi et al., 2011). Among less common genotypes, ST258 has been disseminated in North America, Latin America, and several countries in Europe (Munoz-Price et al., 2013; Chen et al., 2014; Deleo et al., 2014), while ST39, found in one CRKP in Serbia and harboring *bla*_{KPC-2}, is considered endemic in Greek hospitals, where it carries *bla*_{VIM-1} and *bla*_{KPC-2} (Chatzidimitriou et al., 2024). A high median number of resistance genes, identified as 12 and spanning a median of nine antibiotic classes, reflects a substantial multidrug resistance burden, emphasizing the importance of continuous molecular surveillance to uncover key molecular features essential for combating CRKP (Castro et al., 2024).

This study is the first to report the detection of ST147, ST11, ST39, and ST395 in Serbia, while ST101, ST437, ST258, ST16, ST340, and ST307 have previously been identified (Palmieri et al., 2020; Novović et al., 2017; Budia-Silva et al., 2024). The findings indicated that ST147/*bla*_{NDM-1} was the most common genotype, spreading both within and between hospitals across the country. This suggests a shift in the epidemiology of CRKP in the region, contrasting with previous research that identified ST101/*bla*_{OXA-48} as the dominant genotype among carbapenem- and colistin-resistant *K. pneumoniae* from 2013 to 2017 (Palmieri et al., 2020). Of note, the ST147 sublineage of *K. pneumoniae* is well known for its remarkable epidemic potential (Di Pilato et al., 2022) and associated resistance to β -lactams and fluoroquinolones (Peirano et al., 2020). The genotype ST147 with and without NDM was found in Spain and Italy (Martin et al., 2021). Moreover, in Thailand, ST147 carrying *bla*_{NDM} have been reported in several studies (Assawatheptawee et al., 2023; Kk et al., 2021). While not the predominant genotype, ST101/*bla*_{OXA-48} is still circulating in Serbia. In this study, one ST101 isolate carried *bla*_{NDM-1}, a MBL gene, which is present in only 5% of isolates of this genotype (Isler et al., 2023). Additionally, this isolate was resistant to cefiderocol, and the proposed mechanisms of resistance included the coexistence of the MBL and ESBL-encoding genes, *bla*_{SHV-28/106} and *bla*_{CTX-M15}, as previously reported (Kohira et al., 2020). Furthermore, mutations detected in the *opmK35* gene in this isolate may have additionally impaired outer membrane permeability, potentially contributing to reduced cefiderocol susceptibility (Moon and Huang, 2023). Although alterations in *envZ* and *mrcB* genes have previously been associated with cefiderocol resistance, no mutations in these genes were detected in the isolate from our study (Kriz et al., 2024). In contrast, this isolate harbored a truncated *baeS* gene, a multidrug efflux regulator. Although *baeS* mutations have not been directly linked to cefiderocol resistance, alterations in this system may impact cefiderocol susceptibility, especially when combined with other resistance mechanisms (Yang et al., 2024). While deletions of *cirA* have been associated with cefiderocol resistance in gram-negative bacteria, the functional impact of *cirA* D558N substitution remains uncertain (Ito et al., 2017). To the best of our knowledge, this is the first genetically characterized cefiderocol-resistant CRKP isolate identified in the Balkan region. We also observed a correlation between the O-locus, one of the major components of LPS, and MLST, revealing that ST147 and ST11 were related to the O2a locus, ST101 was related to O1, and ST437 was related to O4.

Of particular concern is the emergence of hv-CRKP, due to its combination of virulence and antimicrobial resistance. Although a standardized method for identifying hvKP does not exist, this study found a subset of CRKP isolates with increased virulence, characterized by hypervirulence-associated genes, predominantly ST147, which aligns with findings from Italy and Ghana (Falcone et al., 2022; Ofosu-Appiah et al., 2024). The agreement between the string test and WGS data in

detecting hypervirulent strains was low (61.3%), suggesting that phenotypic methods may underestimate hypervirulence. This finding reinforces the claim that hypermucoviscosity and hypervirulence, although often used interchangeably in medical literature, are distinct traits that may coexist within the same strain (Catalán-Nájera et al., 2017).

Notably, we did not observe the globally dominant hvKP ST23, already detected in Ireland, France, Latvia, and Lithuania (European Centre for Disease Prevention and Control, 2024). Importantly, the delineation between cKP and hvKP pathotypes is complicated by the inconsistent definitions of hypervirulence. Nevertheless, functional annotation identified a great abundance of genes associated with virulence, which may suggest enhanced fitness during infection progression, which might facilitate the spread of these clones. Thus, the study emphasizes the complexity and diversity of virulence factors in *K. pneumoniae*, revealing the importance of these genes in pathogenicity and the adaptability of these strains for establishing infections in hospital settings.

The phylogenetic analysis based on core gene alignment highlighted the genetic relatedness among the CRKP isolates, revealing clusters corresponding to different STs and resistance genes. The WGS, as a gold standard for typing, revealed a diverse array of STs and clonal groups, suggesting both clonal dissemination and horizontal transfer of resistance determinants among CRKP strains. Capsular or lipopolysaccharide typing is valuable as it offers a supplementary classification of *K. pneumoniae* beyond what is provided by ST sequence typing alone. Herein, we announced a strong association between *K. pneumoniae* ST147 and K64 and O2a types.

The main limitations of this study include: (1) Due to the limited number of sequenced isolates, the representativeness of CRKP genomes used for sequencing is affected; (2) The study design was based on genomic inference of antimicrobial resistance determinants, without functional validation of resistance mechanisms; (3) Due to limitations in the sequencing method used, we were unable to confirm the presence of all plasmids linked to antimicrobial resistance.

In conclusion, our study provides the first in-depth assessment of clinical isolates of CRKP, including susceptibility to last-line antibiotics, such as novel BLBLs and comprehensive insights into the genomic epidemiology of CRKP isolates, including their virulence, marking the first such analysis in Serbia. A substantial intraspecies diversity in the isolates was seen, as evidenced by MLST, capsular, and O typing. The emergence of ST147/*bla*_{NDM-1} CRKP with combined hypervirulence and resistance to last-line antibiotics is of major concern, as it can cause severe infections even in healthy individuals. Besides, a cefiderocol-resistant, MBL- and ESBL-positive CRKP isolate with genotype ST101/*bla*_{NDM-1}/*bla*_{SHV-1}/*bla*_{CTX-M15} was described, for the first time in Serbia, although this drug is not yet in use. Obtained results underscore the urgent need for a multifaceted strategy integrating antimicrobial stewardship, enhanced surveillance, stringent infection control measures to limit potential spread in the region, and innovative treatment options for these emerging pathogens.

CRedit authorship contribution statement

Lidija Boskovic: Resources, Investigation. **Dusan Kekic:** Methodology, Investigation. **Balint Kintses:** Writing – review & editing, Supervision, Investigation. **Milena Brankovic:** Resources, Investigation. **Tamas Stirling:** Writing – review & editing, Visualization, Methodology, Investigation. **Anita Sente Zigmanovic:** Resources, Investigation. **Marija Zdravkovic:** Writing – review & editing. **Snezana Delic:** Resources, Investigation. **Natasa Opavski:** Supervision, Resources. **Mirjana Hadnadjev:** Resources, Investigation. **Zoran Todorovic:** Supervision, Resources. **Anika Trudic:** Resources, Investigation. **Ljuban Blanus:** Resources, Investigation. **Ina Gajic:** Writing – review & editing, Supervision, Resources, Conceptualization. **Natasa Trifunovic:** Resources, Investigation. **Aleksandra Jencic:** Resources, Investigation.

Lazar Ranin: Supervision, Resources. **Milos Jovicevic:** Writing – original draft, Visualization, Methodology, Investigation. **Stanislava Senicic:** Resources, Investigation. **Jovana Kabic:** Visualization, Methodology, Investigation.

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Declaration of Competing Interest

The authors declare no competing interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2026.128543.

Data availability

Data will be made available on request.

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