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Bacteria and Bacterial Diseases

Pandrug-resistant *Klebsiella pneumoniae* isolated from Ukrainian war victims are hypervirulent

Oskar Ljungquist [a,1], Michal Magda [b,1], Christian G. Giske [c,d], Chaitanya Tellapragada [d], Oleksandr Nazarchuk [e,f], Dmytro Dmytriiev [e,g], Oskar Thofte [h], Valdemar Öhnström [h], Erika Matuschek [i], Anna M. Blom [b], Kristian Riesbeck [h,j,*,2]

[a] *Division of Infection Medicine, Department of Clinical Sciences, Faculty of Medicine, Lund University, Lund, Sweden*
[b] *Protein Chemistry, Department of Translational Medicine, Faculty of Medicine, Lund University, Malmö, Sweden*
[c] *Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden*
[d] *Division of Clinical Microbiology, Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden*
[e] *National Pirogov Memorial Medical University, Vinnytsia, Ukraine*
[f] *Intensive Care Unit, Clinical Center for Thermal Injury and Plastic Surgery, MNPE Vinnytsia Regional Clinical Hospital Vinnytsia Regional Council, Vinnytsia, Ukraine*
[g] *Bogomolets National Medical University, Kyiv, Ukraine*
[h] *Clinical Microbiology, Department of Translational Medicine, Faculty of Medicine, Lund University, Malmö, Sweden*
[i] *EUCAST Development Laboratory, Växjö, Sweden*
[j] *Clinical Microbiology, Laboratory Medicine Skåne, Lund, Sweden*

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SUMMARY

Objectives: Carbapenem- and colistin-resistant *Klebsiella pneumoniae* were isolated from war victims treated in hospitals in Ukraine. The question was whether these pandrug-resistant *K. pneumoniae* are pathogenic and capable of causing disease in a broader context.
Methods: *Klebsiella pneumoniae* clinical isolates (n = 37) were tested for antibiotic resistance and subjected to whole-genome sequencing (WGS). In addition, their pathogenicity was tested by serum resistance and two separate animal models.
Results: Isolates belonging to the sequence types (ST) 23, 147, 307, 395, and 512 were found to harbor resistance genes against carbapenems and cephalosporins. Nine isolates carried point mutations in *pmrB* and *phoP* genes associated with colistin resistance. All bacteria were equipped with multiple virulence genes, and the colistin-resistant isolates each carried 10 different genes. Colistin-resistant *K. pneumoniae* were more serum-resistant, more virulent against *G. mellonella* larvae, and displayed an increased survival in mice compared to colistin-susceptible bacteria. The *iucA*, *peg-344*, *rmpA*, *rmpC*, and *rmpD* genes were associated with increased virulence in animals.
Conclusions: Pandrug-resistant *K. pneumoniae* in Ukraine are hypervirulent and retain their pathogenicity, highlighting the need to prevent disseminated spread.

Introduction

Klebsiella pneumoniae is one of the leading bacterial causes of mortality globally, responsible for 20% of all deaths attributable to antimicrobial resistance (AMR).[1] These multidrug-resistant organisms (MDRO) are often carbapenemase-producing, resulting in extensive drug resistance (XDR) with few treatment options. The dissemination is dominated by nosocomial spread by a few clonal lineages.[2] To reduce such spread, infection prevention and control measures, including rapid identification of XDR strains and isolation of colonized patients, have been implemented throughout healthcare systems in many countries.[3] Despite these strategies, healthcare-associated outbreaks of highly resistant clones regularly occur, disseminating between hospitalized patients.[4] Antimicrobial susceptibility testing and sequencing should be done on all strains

* Corresponding author at: Clinical Microbiology, Department of Translational Medicine, Lund University, Wallenberg laboratory, Inga Marie Nilssons gata 53, Malmö SE-214 28, Sweden.
E-mail address: kristian.riesbeck@med.lu.se (K. Riesbeck).
[1] These two authors contributed equally.
[2] https://orcid.org/0000-0001-6274-6965

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suspected or confirmed as carbapenemase-producing to monitor outbreaks and prevent further dissemination.

A major question is whether XDR bacterial species lose their virulence, and hence the ability to cause disease and spread in the community. Antimicrobial-resistant *K. pneumoniae*, carrying carbapenemase (KPC), are frequently less virulent compared to susceptible non KPC-producing strains.[5] This aligns with the theory of the decreased pathogenicity of acquiring antimicrobial resistance. Carrying plasmids with AMR genes may impair bacterial virulence and reduce essential cellular functions.[6] For instance, introducing colistin resistance in *K. pneumoniae*, using the plasmid-borne mobile colistin resistance gene (*mcr-1*) reduces the biological virulence and growth of *K. pneumoniae*.[7] However, certain clonal lineages of multidrug-resistant *Escherichia coli* and *K. pneumoniae* may be able to retain their virulence while also maintaining the dissemination of plasmids.[8] Virulence traits of *K. pneumoniae* include overproduction of capsular polysaccharides and hypermucoviscosity, and genes expressing these phenotypes are carried on the chromosome or on plasmids.[9] Sequence type (ST) 23 is, for example, associated with hypermucoviscosity.[10]

We recently reported the presence of highly XDR gram-negative bacterial infections in war victims in Ukraine, of which several strains of *K. pneumoniae* were pandrug-resistant (PDR), i.e., resistant to all antimicrobials tested.[11] Since our initial report of emerging XDR and PDR Enterobacterales from war-torn Ukraine, there have been multiple accounts of the secondary spread of carbapenemase-producing gram-negative bacilli to countries caring for wounded victims of the war.[11–14] These studies all support the presence of a significant issue involving the spread of carbapenemases, particularly those belonging to the NDM- and OXA-48-groups, within the Ukrainian healthcare system. These isolates cause severe, difficult-to-treat nosocomial infections within a resource-scarce healthcare system under immense pressure.[15] The aim of this study was to investigate these XDR and PDR *K. pneumoniae* regarding genes encoding for antimicrobial resistance and virulence factors as well as pathogenicity.

Materials and methods

Bacterial growth conditions

All clinical XDR (n = 28) and PDR (n = 9) *K. pneumoniae* isolates were selected from sentinel testing of war victims in Ukraine between February and September 2022.[11] Clinical samples were mainly obtained from wounded soldiers and civilians including children having infected burns and shrapnel wounds when treated at tertiary hospitals in Ukraine. The exact locations cannot be revealed due to the ongoing conflict. *Klebsiella pneumoniae* were grown on blood agar plates at 37 °C. Bacteria were incubated overnight and subcultured on fresh blood agar plates for an additional 3 h at 37 °C. *Klebsiella pneumoniae* was collected, washed with PBS, and OD_{600} was measured followed by dilution in PBS to the required optical density. For mice infection experiments, bacteria were incubated overnight and then diluted 1:100 into fresh Brain Heart Infusion (BHI) broth and cultured to an optical density (OD_{600}) of 0.3. Samples were frozen at −80 °C in 10% glycerol, and after subculturing an aliquot was counted prior to infection. *Klebsiella pneumoniae* subsp. *pneumoniae* strain NCTC 9633 (ATCC 13883) was used as a control in mouse experiments.

Determination of AMR in K. pneumoniae isolates

Antibiotic susceptibility testing was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines at the EUCAST reference laboratory in Växjö (Sweden). Disc diffusion testing was performed, and for isolates that were either meropenem-resistant, or susceptible with increased exposure, broth microdilution was performed according to the International Organization for Standardization method.[11]

XDR and PDR were defined according to previously used definitions.[16,17] Briefly, XDR was defined as non-susceptible to at least one in all but 2 fewer antimicrobial categories including antimicrobials against Enterobacterales, i.e., aminoglyocsides, ceftaroline (anti-MRSA cephalosporin), anti-pseudomonal penicillins + β-lactamase inhibitors, carbapenems, non-extended spectrum cephalosporins (1st and 2nd generation cephalosporins), extended-spectrum cephalosporins (3rd and 4th generation cephalosporins), cephamycins, fluoroquinolones, folate pathway inhibitors, glycylcyclines, monobactams, penicillins (ampicillin), penicillins + β-lactamase inhibitors, phenicols, and polymyxins. *Klebsiella pneumoniae* was considered AMR against fosfomycin according to current guidelines from EUCAST (13). In addition, PDR was defined as non-susceptibility to all agents in all antimicrobial categories as outlined above (12).

Whole-genome sequencing and bioinformatics

DNA was extracted from freshly subcultured colonies of the study isolates using the DNA Tissue Kit (Qiagen, Hilden, Germany) on the EZ1 automated extraction system (Qiagen). Quantification of the extracted DNA was performed using the Qubit 4.0 assay (Life Technologies, Carlsbad, CA). Library preparation using the Nextera XT kit (Illumina, San Diego, CA) and paired-end, short-read sequencing of the study isolates was performed on the NovaSeq 6000 system (Illumina) at Biomarker Technologies (BMK) (Munster, Germany). The previously proposed virulence score, ranging between 0–5, was used as a marker for bacterial virulence.[18] Illumina reads were filtered using Trimmomatic (v0.32) and assembled using SPAdes (v3.15.5). Prokka v1.14.6 was utilized for genome annotation, providing functional annotations for the assembled contigs. The assembled genomes were analyzed using Kleborate (https://github.com/klebgenomics/Kleborate) to deduce the multilocus sequence types (MLST), antimicrobial resistance genes, and virulence profiles. PointFinder (https://bio.tools/PointFinder) was used for mutational analysis associated with antimicrobial resistance. Finally, prediction of the protein coding-genes was performed using Prodigal (https://github.com/hyattpd/Prodigal) and analyzed against reference protein sequences of PmrB (NCBI accession no. YP_005225933.1), PhoP (Uniprot id: A0A1Y0PZG2_KLEPN), PhoQ (UniProt id: A0A0H3GLK4_KLEPH), and MgrB (UniProt id: A0A2W0U461_KLEPN).

The core genome MLST scheme for *K. pneumoniae* was downloaded from cgMLST.org. The allele calling was performed using chewBBACA (v3.3.3). The pair-wise distances between isolates were calculated using GitHub: tseemann/ cgmlst-dists cgmlst-dists, and the tree was created in R (v4.3.0) using the hclust library. Plotting and annotations of the tree were performed in iTOL using the Kleborate output and AMRFinder databases.

Available sequence data

All sequences have been submitted to the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov); SUB14387023 and BioProject ID: PRJNA1102281.

Serum resistance

Normal human serum (NHS) was isolated from the blood of 14 healthy donors, pooled, and stored at −80 °C. Written consent was obtained according to the recommendations of the local ethics committee in Lund (Sweden; 2017/582). The antimicrobial peptide nisin A was from Handary (0301; Brussels, Belgium). Recombinant *Ornithodoros moubata* complement inhibitor (OmCI) blocking cleavage of C5 was expressed as described.[19] SYTOX Green DNA dye was

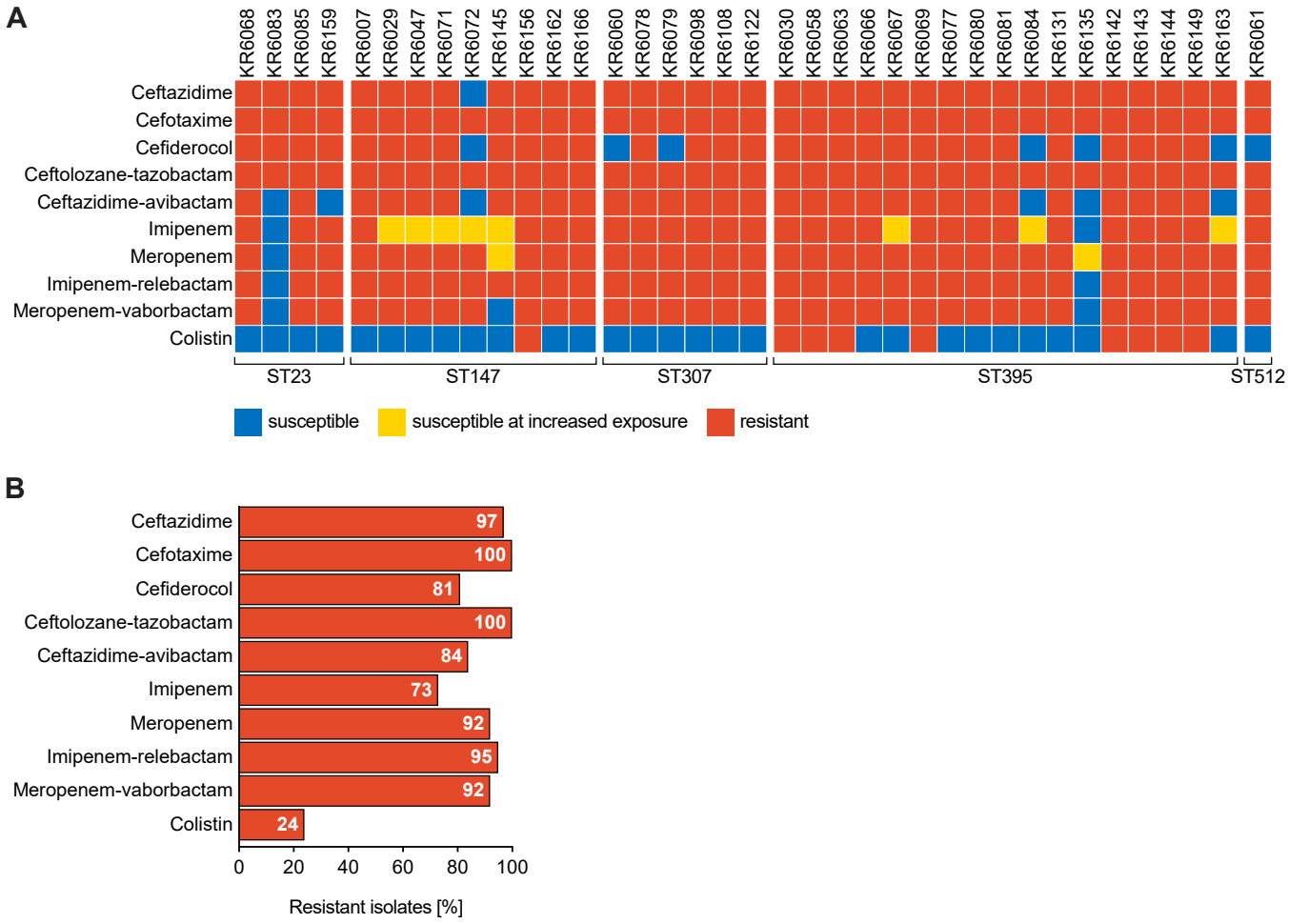

Fig. 1. *K. pneumoniae* from Ukraine are resistant to antibiotic. A) Heatmap of the antibiotic resistance in *K. pneumoniae* based on antibiograms. **B**) Percentage of *K. pneumoniae* isolates resistant to the tested antimicrobials.

obtained from Thermo Fisher Scientific (S7020; Waltham, MA). GVB^{+} $^{+}$ buffer used in experiments consisted of 5 mM Veronal buffer [pH 7.3], 140 mM NaCl, 0.1% gelatin, 1 mM $MgCl_2$, and 5 mM $CaCl_2$. To assess bacterial survival, bacteria were diluted in PBS to two concentrations [OD_{600} = 0.01 and 0.025 corresponding to 10^6 colony forming units (CFU) per mL and 2.5 × 10^6 CFU/mL for colony count and fluorescence measurement, respectively]. For colony count, bacteria were mixed with GVB^{++} buffer supplemented with 30% NHS or 30% NHS with 50 μg/mL OmCI (a negative control). After incubation at 37 °C for 1 h, bacteria were serially diluted and plated in triplicates onto blood agar. After overnight incubation, CFUs were counted and bacterial survival was calculated as a percentage relative to the control. For the fluorescence measurement GVB^{++} buffer with 30% NHS, 30% NHS with 10 μg/mL nisin A, or 30% NHS with 50 μg/mL OmCI (a negative control) was added to bacteria. All samples were supplemented with 2 μM SYTOX Green dye (a cell death indicator). Bacteria were incubated at 37 °C for 2 h, while the bactericidal activity of NHS was simultaneously monitored using the SYTOX Green signal (measured with BioTek Cytation5 at 504/523 nm excitation/emission).

Galleria mellonella infection model

Galleria mellonella larvae were purchased from Insekto Reptilfoder (Helsingborg, Sweden). Thirty isolates were collected from blood agar plates, and diluted in PBS to OD_{600} = 1 (ca. 10^8 CFU/mL) and OD_{600} = 0.1 (ca. 10^7 CFU/mL). The diluted bacteria were kept on ice until injection into *G. mellonella* larvae. Freshly delivered larvae were injected with 10 μl bacteria (10^6 or 10^5 CFU) or PBS into the last, left proleg using BD insulin syringes (Micro-Fine U-100 0.3 mL 30 G; Becton Dickinson, Franklin Lakes, NJ). Five larvae were transferred into the Petri dishes for each experimental condition (two concentrations of each isolate, PBS control, and uninfected larvae). Thereafter, larvae were incubated in Petri dishes at 37 °C for 5 days (120 h). The survival of the larvae was monitored every 12 h (at 8 am and 8 pm) while assessing the melanization and movement/reaction to the stimulus to determine the survival or death of the worms. The survival of the larvae was used as a proxy for bacterial virulence when comparing colistin- and carbapenem-resistant *K. pneumoniae* with colistin-susceptible and carbapenem-resistant *K. pneumoniae.*

Mouse acute pneumonia infection model

Six to eight-week-old male C57BL/6 J mice (Jackson Laboratories, Bar Harbor, ME) were maintained in filter-top cages on standard laboratory chow and water ad libitum until use. Mice were anesthetized using inhalation of isoflurane (Forene; Abbott, Wiesbaden, Germany) and inoculated with 2.5 × 10^6 CFU *K. pneumoniae* in 50 μl PBS into the nares.[20] Uninfected control mice received 50 μl of PBS only, and in these mice no bacteria were isolated. At 48 h post-inoculation, lungs were excised and mechanically homogenized in 200 μl ice-cold PBS. The lung homogenate was diluted (1:10^2, 1:10^4, and 1:10^5) in PBS, plated on blood agar plates using glass beads, and incubated at 37 °C overnight. CFUs were counted using ProtoCOL 3^{HD} (SYNBIOSIS, VWR

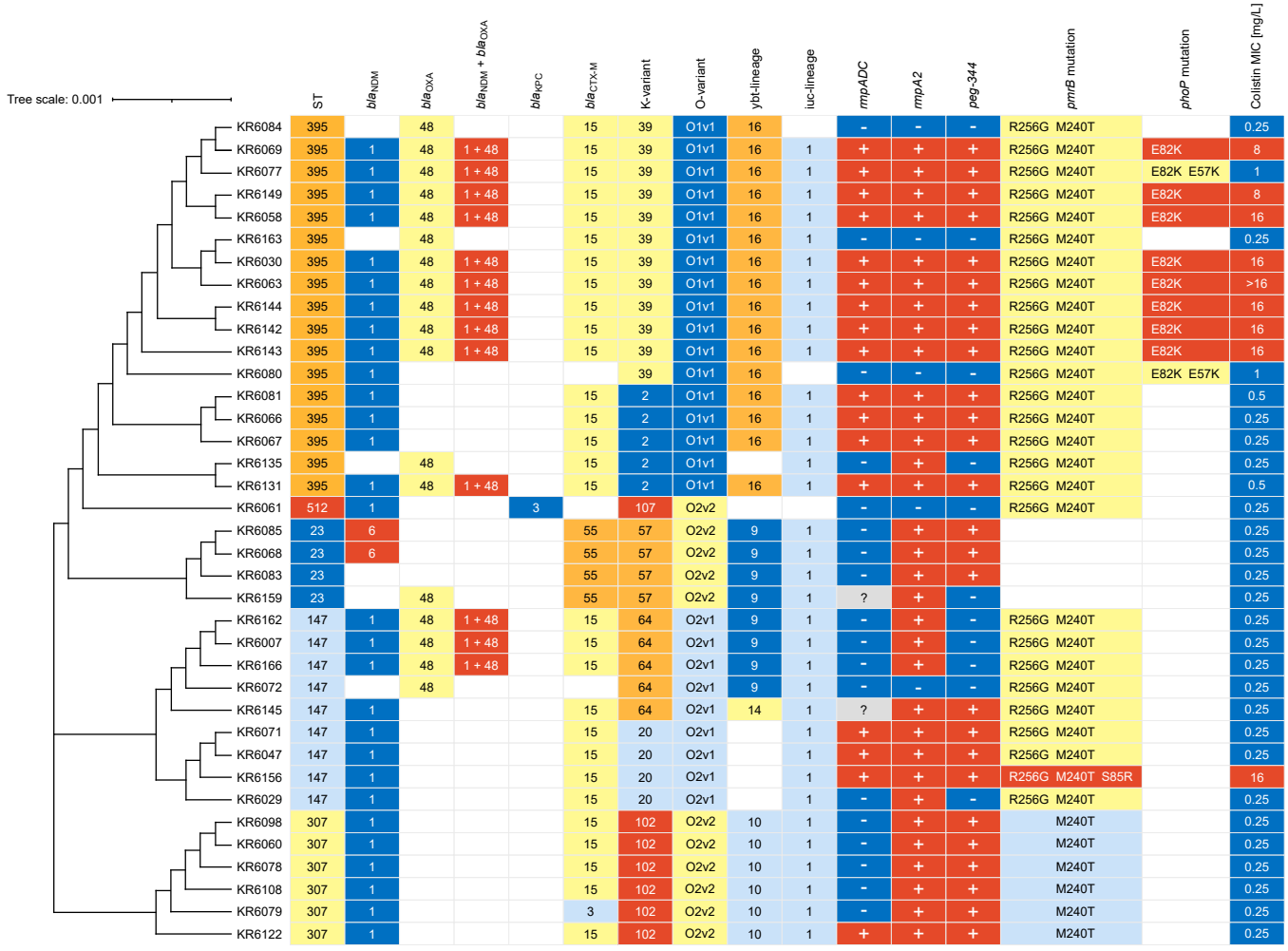

Fig. 2. *K. pneumoniae* carry many resistance and virulence genes. Bacterial genomes were sequenced and their relatedness was calculated using the cgMLST. Key genomic features of isolates were determined using the Kleborate-viz. MIC values for colistin are also shown in relation to point mutations in the *pmrB* and *phoP* genes.

International; Karlskoga, Sweden). All experiments were performed in compliance with animal protection laws and approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden (permit number: 5.8.18–19202/2023).

Statistics

One-way ANOVA test was used for comparisons of SYTOX Green signal between the two groups (colistin-susceptible vs. colistin-resistant), with Bonferroni post-test analysis. Two-way ANOVA test was used for comparisons of serum survival between the two groups with the Bonferroni post-test analysis. Kaplan-Meier curves were used to assess survival, and the log-rank test was used to determine statistical differences in survival. A Mann-Whitney test was used for the murine pneumonia model. The significance ($p = 0.005$) was calculated between colistin-susceptible and colistin-resistant isolates only. A p-value of < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad software 10 (Prism, La Jolla, CA).

Results

Pandrug-resistant Klebsiella pneumoniae are isolated from wounded individuals in Ukraine

Between February and September 2022, 37 clinical isolates of *K. pneumoniae* were collected from patients with hospital-associated infections as described.[11] The isolates were tested for susceptibility to several antibiotics and their minimum inhibitory concentrations (MICs) were determined (Fig. 1A and Supplementary Table S1). Bacteria had alarming levels of AMR, ranging from 73% (n = 27) to imipenem and 100% (n = 37) to cefotaxime and ceftolozone-tazobactam (Fig. 1B and Supplementary Table S1). A fraction of *K. pneumoniae* isolates (n = 9; 24%) were resistant to colistin, classifying them as PDR. Whole genome sequencing (WGS) revealed that the bacteria belonged to the following STs: 23 (n = 4), 147 (n = 9), 307 (n = 6), 395 (n = 17), and 512 (n = 1) (Fig. 1A, Fig. 2, Supplementary Table S1). One colistin-resistant isolate was identified as belonging to ST147, while eight were categorized under ST395.

Klebsiella pneumoniae harbor numerous virulence factor genes

The WGS revealed the presence of multiple resistance and virulence genes (Table 1, Fig. 2, Supplementary Table S2-S5, and S7), as well as a set of plasmids carried by bacteria (Supplementary Table S6). The carbapenemase genes $bla_{\text{NDM-1}}$ (n = 15), $bla_{\text{NDM-1}}$ + $bla_{\text{OXA-48}}$ (n = 13), $bla_{\text{OXA-48}}$ (n = 5), $bla_{\text{NDM-6}}$ (n = 2) and, finally, $bla_{\text{NDM-1}}$ + $bla_{\text{KPC-3}}$ (n = 1) were found. The bla_{NDM} and $bla_{\text{OXA-48}}$ genes were predominantly associated with ST395 and ST147 (Table 1, Fig. 2). The isolates also carried cephalosporin resistance genes $bla_{\text{CTX-M-15}}$ (n = 29), $bla_{\text{CTX-M-55}}$ (n = 4), $bla_{\text{CTX-M-3}}$ (n = 1) and bla_{DHA} (n = 1). Additionally, AMR-associated resistance genes to aminoglycosides,

Table 1
Sequence types and AMR determinants in *K. pneumoniae* ($n = 37$).

ST	Isolate	Cephalosporinase genes		Carbapenemase genes	
23	KR6068	bla_{OXA-1}	$bla_{CTX-M-55}$	bla_{NDM-6}	-
	KR6083	bla_{OXA-1}	$bla_{CTX-M-55}$	-	-
	KR6085	bla_{OXA-1}	$bla_{CTX-M-55}$	bla_{NDM-6}	-
	KR6159	bla_{OXA-1}	$bla_{CTX-M-55}$	-	bla_{OXA-48}
147	KR6007	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6029	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6047	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6071	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6072	-	-	-	bla_{OXA-48}
	KR6145	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6156	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6162	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6166	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
307	KR6060	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6078	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6079	-	$bla_{CTX-M-3}$	bla_{NDM-1}	-
	KR6098	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6108	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6122	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	-
395	KR6030	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6058	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6063	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6066	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6067	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6069	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6077	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6080	-	-	bla_{NDM-1}	-
	KR6081	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	-
	KR6084	bla_{OXA-1}	$bla_{CTX-M-15}$	-	bla_{OXA-48}
	KR6131	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6135	bla_{OXA-1}	$bla_{CTX-M-15}$	-	bla_{OXA-48}
	KR6142	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6143	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6144	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6149	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}
	KR6163	-	$bla_{CTX-M-15}$	-	bla_{OXA-48}
512	KR6061	bla_{DHA}	-	bla_{NDM-1}	bla_{KPC-3}

fluoroquinolones, sulfonamide, trimethoprim were found in most isolates (Supplementary Table S3 and S4).

Interestingly, many isolates carried R256G and M240T mutations in the *pmrB* gene, but most of them did not show colistin resistance. Only one resistant ST147 isolate carried another point mutation (S85R). Most isolates ($n = 8$) carrying the E82K mutation in the *phoP* gene displayed colistin resistance (Fig. 2, Supplementary Table S5). Notably, isolates ($n = 2$) with a double mutation (E82K and E57K) in the *phoP* gene were still colistin susceptible. The colistin-resistant PDR *K. pneumoniae* all carried virulence genes *rmpA, rmpC*, *rmpD*, *iucA*, and *peg-344* (Fig. 2, Supplementary Table S7). In contrast, only 36% of colistin-susceptible isolates carried *rmpA* and *rmpC*, 21% had *rmpD*, 68% carried *iucA*, and, finally, 61% were equipped with *peg-344*. Virulence genes *ybt* and *iuc* encoding yersiniabactin and aerobactin, respectively, were prevalent in most isolates (Fig. 2, Supplementary Table S7).

Colistin-resistant Klebsiella pneumoniae are resistant to human serum

Another important trait for bacterial virulence is resistance to serum killing by the complement system. Complement activation results in the formation of the membrane attack complex (MAC), which can directly lyse gram-negative bacteria like *K. pneumoniae.* Since bacterial pathogenicity may correlate with serum resistance, we incubated *K. pneumoniae* for 2 h with 30% NHS and the cell death indicator, SYTOX Green dye. The cell membranes were completely intact in colistin-resistant bacteria exposed to NHS, whereas the colistin-susceptible bacteria showed increased staining with SYTOX Green (Fig. 3A, Supplementary Fig. S1A-B). Due to the MAC formation, the antibacterial peptide nisin A caused significantly more damage to the colistin-susceptible bacteria by passing through the MAC-mediated pores in the outer membrane. The survival of *K. pneumoniae* isolates was restored when the C5 inhibitor (OmCl) was added to NHS, indicating that the damage was mediated by MAC. After 1 h of incubation in 30% NHS, we also estimated the surviving bacteria by counting CFUs (Fig. 3B-C). In this experimental setup, all colistin-resistant isolates survived in NHS (Fig. 3B). In contrast, five colistin-susceptible isolates (18%) had statistically significantly reduced survival upon serum challenge (Fig. 3C).

Colistin-resistant Klebsiella pneumoniae are hypervirulent in Galleria mellonella and mouse infection models

Since colistin is one of the last remaining antimicrobial agents against *K. pneumoniae*, a set of colistin-resistant and -susceptible isolates were selected for further analysis in animal infection models. *Galleria mellonella* larvae were infected with 8 colistin-resistant and 22 colistin-susceptible isolates belonging to different STs and carrying different virulence genes (Supplementary Table S2). Larvae infected with colistin-susceptible *K. pneumoniae* survived significantly longer compared to larvae infected with colistin-resistant isolates, both at bacterial concentrations of 10^5 CFU and 10^6 CFU (both $p \leq 0.001$) (Fig. 4A-B).

The ultimate test for analyzing the bacterial pathogenicity of colistin-resistant *K. pneumoniae* involves infecting mice. We selected 8 resistant and 8 susceptible isolates each (Table 2). All of them belonged to ST395, except the colistin-susceptible isolate KR6029 which belonged to ST147. The colistin-resistant *K. pneumoniae* were armed with more virulence factors such as *iucA*, *rmpA*, *C*, and *D,* and *peg-344* than colistin-susceptible isolates. Mice were infected with *K. pneumoniae* (2.5×10^6 CFU) for 48 h. Importantly, colistin-resistant isolates were significantly more likely to infect the lungs of mice ($p = 0.005$) compared to colistin-susceptible bacteria (Fig. 4C). Taken together, colistin-resistant *K. pneumoniae* displayed an increased bacterial pathogenicity compared to colistin-susceptible isolates, as assessed by the killing of *G. mellonella*, and were also more prone to infect mice in the pneumonia model.

Discussion

In this study, we aimed to investigate the molecular characteristics and virulence traits of XDR and PDR *K. pneumoniae* isolated from war victims in Ukraine in 2022. We found that ST147 and ST395 were dominating among isolates, with most of the isolates carrying carbapenemases belonging to the NDM-1 and OXA-48 groups. These STs and associated carbapenemases have been reported in multiple previous reports on hospital-acquired *K. pneumoniae* originating from Ukraine.[21–24] However, we observed a variety of STs and a diversity of carbapenemases in our material, suggesting a high prevalence of carbapenem-resistance strains in Ukraine rather than a few transmission clusters.[24] Most of the isolates carried resistance genes that are associated with hypervirulence. The colistin-resistant *K. pneumoniae* isolates were highly serum resistant and more virulent in the *G. mellonella* infection model compared to colistin-susceptible isolates. A similar pattern was seen in mouse model suggesting that PDR *K. pneumoniae* have maintained their bacterial virulence. For meropenem-resistant *K. pneumoniae* or meropenem susceptibility in the I-group, our data indicate the presence of multiple lineages exhibiting diverse AMR profiles. However, clonal spread within hospitals cannot be excluded, due to the limited number of isolates included in this study. Two strains that exhibited susceptibility, increased exposure (I) towards meropenem, harbored carbapenemases. This also highlights the difficulties in identifying patients carrying carbapenemases, apart from the need for infection prevention and control measures in suspected cases. Alarmingly, most *K. pneumoniae* isolates show similarly vast characteristics of

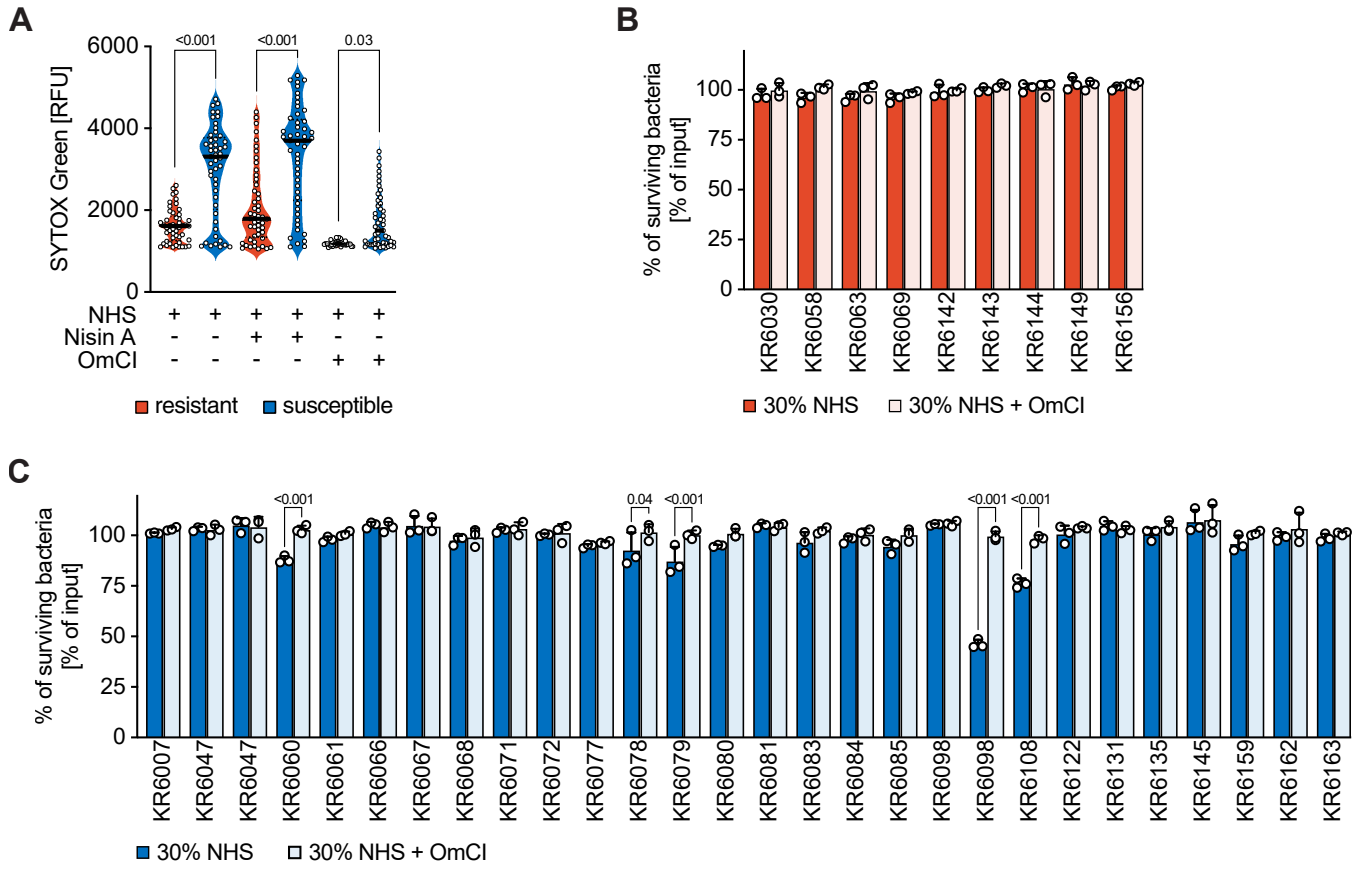

Fig. 3. Colistin-resistant *K. pneumoniae* are more serum and complement resistant. A) Serum susceptibility in the presence of NHS supplemented with the antimicrobial peptide nisin A or the complement inhibitor OmCI, using SYTOX Green as a cell death indicator. The graph represents the average of SYTOX Green [RFU] of colistin-resistant and colistin-susceptible isolates (median ± quartiles). Serum survival of **B**) colistin-resistant ($n = 9$) and **C**) colistin-susceptible isolates ($n = 28$). NHS with OmCI was used as negative control. Graphs represent % of surviving bacteria based on CFU. Data are shown from at least 3 independent experiments (mean ± SD); in **A**) one-way ANOVA + Bonferroni post-test, in **B-C**) two-way ANOVA + Bonferroni post-test.

hypervirulence. Hypervirulent *K. pneumoniae* can cause severe, life-threatening infections in healthy individuals and can be defined by the presence of the genes *rmpA*, *rmpA2*, *iucA*, *iroB*, and *peg-344*. These genes are typically acquired and mainly expressed via plasmids but could also be acquired or mutated chromosomally.[25,26] Whether the hypervirulent phenotype requires all or a combination of these genes remains to be defined. The pathogenicity of hypervirulent *K. pneumoniae* includes optimized capsular function, siderophore system, lipopolysaccharides (LPS), outer membrane proteins, and efflux pumps.[27] Previously, *rmpA* and *rmpA2* have been associated with hypermucoviscosity and pathogenicity.[28,29] Most of our isolates carried at least some of these genes on plasmids, as well as yersiniabactin and/or aerobactin. Yersiniabactin is a strong predictor of *K. pneumoniae* infection in contrast to colonization.[30] Fifteen out of 37

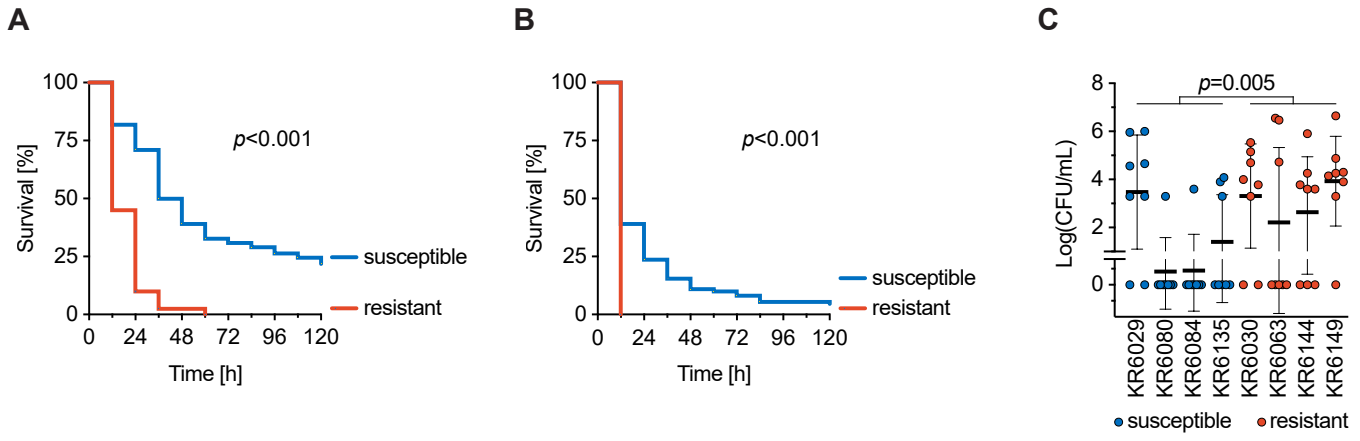

Fig. 4. Colistin-resistant *K. pneumoniae* are hypervirulent in animal infection models. Survival of *G. mellonella* larvae during the infection with **A**) 10^5 CFU and **B**) 10^6 CFU of *K. pneumoniae* (8 colistin-resistant and 22 colistin-susceptible isolates). All larvae survived injections with PBS, which was used as a negative control (not shown); Mantel-Cox test was used for statistical analyses. **C**) Survival of *K. pneumoniae* during lung infection in mice (4 colistin-resistant and 4 colistin-susceptible isolates). Dots represent mice for which lung bacterial load was analyzed (mean ± SD); Mann-Whitney test, *p*-value corresponds to the mean difference between colistin-susceptible and colistin-resistant isolates. Table 2 and S2 list the characteristics of each isolate used in the infection models.

Table 2
Sequence types, AMR, and virulence genes of *K. pneumoniae* ($n = 8$) used in the mouse pneumonia model.

ST	Isolate	Colistin[a]	Cephalosporinase genes		Carbapenemase genes		Virulence genes									
147	KR6029	S[b]	-	$bla_{CTX-M-15}$	bla_{NDM-1}	-	-	-	*iucA*	*iutA*	*iucB*	*iucC*	-	-	-	-
395	KR6080		-	-	bla_{NDM-1}	-	*ybtP*	*ybtQ*	-	-	-	-	-	-	-	-
395	KR6084		bla_{OXA-1}	$bla_{CTX-M-15}$	-	bla_{OXA-48}	*ybtP*	*ybtQ*	-	-	-	-	-	-	-	-
395	KR6135		bla_{OXA-1}	$bla_{CTX-M-15}$	-	bla_{OXA-48}	-	-	*iucA*	*iutA*	*iucB*	*iucC*	-	-	-	-
395	KR6030	R	bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}	*ybtP*	*ybtQ*	*iucA*	*iutA*	*iucB*	*iucC*	*peg-344*	*rmpA*	*rmpC*	*rmpD*
395	KR6063		bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}	*ybtP*	*ybtQ*	*iucA*	*iutA*	*iucB*	*iucC*	*peg-344*	*rmpA*	*rmpC*	*rmpD*
395	KR6144		bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}	*ybtP*	*ybtQ*	*iucA*	*iutA*	*iucB*	*iucC*	*peg-344*	*rmpA*	*rmpC*	*rmpD*
395	KR6149		bla_{OXA-1}	$bla_{CTX-M-15}$	bla_{NDM-1}	bla_{OXA-48}	*ybtP*	*ybtQ*	*iucA*	*iutA*	*iucB*	*iucC*	*peg-344*	*rmpA*	*rmpC*	*rmpD*

[a] S – susceptible, R – resistant.
[b] Susceptible to colistin when used in combination therapy.

(41%) isolates carried the *rmpD* gene, which is associated with hypermucoviscosity in *K. pneumoniae*.[9] While the siderophore virulence gene *iucA* was found in 34/37 (92%) of isolates, we did not find the siderophore virulence genes *entB* or *ybtS,* previously associated with the pathogenicity of carbapenem-resistant *K. pneumoniae*.[31,32] We also did not detect any colibactin genes, related to the hypermucoid phenotype.[18]

The *G. mellonella* infection model has previously been used as an infection model to investigate the virulence of *K. pneumoniae*.[33] Our results contradict a previous study in which hypervirulent XDR isolates exhibited low virulence; however, that study included only 12 strains in a murine model.[34] Another study failed to clearly differentiate between hypervirulent and classical *K. pneumoniae* strains in a *G. mellonella* infection model, whereas the distinction was accurately made in a murine model.[35] In our study, most isolates were hypervirulent strains, and we observed a statistically significant difference between the colistin resistance and the killing of larvae in the *G. mellonella* infection model. This is in accordance with a study from Egypt, where carbapenemase-producing *K. pneumoniae*, predominantly NDM-1 and OXA-48, produced biofilm with increased virulence.[36] We further proved the conserved bacterial virulence by demonstrating serum resistance in the presence of NHS in addition to an acute pneumonia model in mice. Finally, a previously published *G. mellonella* model, using carbapenem-resistant *K. pneumoniae* isolates from patients with ventilator-associated pneumonia (VAP), determined that their isolates were also hypervirulent, which was further confirmed with in vitro serum resistance tests.[37] Taken together, our data, supported by several lines of evidence, suggest that AMR *K. pneumoniae* most likely do not lose their capacity to cause disease.

Alarmingly, our findings show that war victims in Ukraine are affected by multidrug-resistant and hypervirulent *K. pneumoniae*. Moreover, the isolates exhibiting colistin resistance were also more virulent, which raises significant concerns, especially considering the added challenge of carbapenem resistance. None of the strains carried mobile colistin resistance genes; however, many isolates were observed to carry chromosomal point mutations in the *pmrB* gene resulting in R256G and M240T. Remarkably, one ST147 isolate carrying an additional mutation resulting in S85R presented colistin resistance. This point mutation was suggested in the literature to be correlated with the resistance to colistin.[38] Nonetheless, the *pmrB* mutation seems to be not strongly connected to the resistance as most of the bacterial carriers were susceptible to colistin (Fig. 2).[39] However, the *phoP* mutation resulting in E82K seems to be more related as all ST395 isolates with single E82K mutation were resistant.[40] Notably, two ST395 isolates with double E82K and E75K mutations within *phoP* were susceptible.

This study is limited by a small sample size, raising uncertainty about whether increased virulence truly indicates a hypervirulent phenotype of *K. pneumoniae* in a clinical context. Additional studies, ideally prospective, analyzing *K. pneumoniae* from sepsis and including patient outcomes, is crucial. Due to the ongoing conflict in Ukraine, clinical data on therapy and outcomes are not fully accessible. Investigating the link between colistin resistance and increased virulence could involve reversing key mutations potentially related to colistin resistance followed by evaluation of pathogenesis in animal models. This will be the impetus for future investigations.

In conclusion, carbapenem-resistant *K. pneumoniae* isolated from Ukrainian war victims were hypervirulent, and among them, the most multidrug-resistant were colistin-resistant isolates, which also demonstrated the highest bacterial virulence and pathogenicity.

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Author contributions

OL, CGG, AB and KR conceived the study. MM, OT and VÖ planned and conducted experiments. OL and MM performed statistical analyses and created images, and drafted the manuscript together with KR, which the other authors critically revised. CT performed bioinformatic analyses of sequencing data. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2024.106312.

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