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Comparative infectivity, virulence and molecular epidemiology of MDR and XDR *Acinetobacter baumannii* isolates emerging from war-related injuries in Ukraine

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SUMMARY

Background: Multidrug resistant Acinetobacter baumannii remains a global concern with its ability to adhere, disperse and sustain in areas involving suboptimal infection control measures, particularly in war areas. In this study we aimed to evaluate the susceptibility profiles, molecular epidemiology and pathogenesis of A. baumannii isolates from conflict zones in Ukraine.
Methods: A. baumannii wound isolates (n=46) were tested to comparatively assess antimicrobial resistance, molecular epidemiology, pathogenesis and infectivity. Briefly, antimicrobial susceptibility testing, whole genome sequencing, biofilm and capsule formation, as well as serum survival and bacterial viability in G. mellonella and a mouse infection model were performed.
Results: Dominating sequence types were ST2, ST19, ST78 and ST400 while 65.21% of isolates were from international clones. All isolates were multidrug resistant (MDR), and 7 isolates were extensively drug resistant (XDR). ST19 isolates had higher encapsulation, lower serum survival and missing T6SS, omp33–36 and biofilm production associated genes. However, bacteria expressed higher virulence and lethality during early infection compared to later stages in vivo.
Conclusion: We report the first results characterizing the pathogenesis and infectivity of the emerging A. baumannii ST19. High MDR and XDR rates alongside clonally related isolates are concerning and highlight the importance of infection prevention and control measures in conflict zones.

Introduction

Acinetobacter baumannii is a global concern with its ability to adhere and persist on biotic and abiotic surfaces involving suboptimal infection control measures.[1,2] Especially, carbapenem-resistant *A. baumannii* remains as the top priority pathogen for the research and development of novel antimicrobials since 2017.[3] Emerging antimicrobial resistance and rapid dissemination of carbapenemase producing *A. baumannii* are narrowing down the available treatment options and making it unresponsive to novel antimicrobials.[4–6]

In addition to being a pathogen of concern due to its high morbidity, mortality and antimicrobial resistance rates, *A. baumannii* is a strictly aerobic, highly adhesive and capable of persisting under high temperatures and desiccation. These characteristics enable it to play a major role not only in nosocomial outbreaks but also in war zone outbreaks.[7] Alarmingly, it has been associated with hospital acquired infections, outbreaks and war-related injuries for decades.[8–11]

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Eventually, the alarming presence of excessively drug resistant and pandrug resistant gram-negative bacteria including *A. baumannii* infections in war victims in Ukraine was recently reported.[12–14]

It is being widely reported that international clones with well-characterized AMR genes dominate globally. However, novel lineages and sequence types of *A. baumannii* should also be studied further not only for AMR but for comparative pathogenesis and virulence.[4,15] Out of 9 international clones (ICs), IC2 is reported to be the most widespread clone in Africa, Asia, Europe and North America.[4] Although international clones of *A. baumannii* successfully segregate distinct allelic patterns, sequence types should still be considered when comparing resistance and virulence profiles. ST2 is currently the most concerning sequence type regarding carbapenemase carriage, virulence and persistence.[16,17] The most common STs that have been reported from Europe are ST2, ST1, ST15, ST79 and ST636 while ST2, ST1 and ST15 are especially reported to harbor numerous beta-lactamases that make *A. baumannii* non-susceptible to many classes of cephalosporins as well as carbapenems.[18,19] Carbapenem resistance in *A. baumannii* is reported to reach concerning rates worldwide and is mainly facilitated by the production of oxacillinases (OXA) and metallo-beta-lactamases.[17,19–21] One of the most common OXA groups can be listed as $bla_{\text{OXA-23}}$, $bla_{\text{OXA-66}}$, and $bla_{\text{OXA-72}}$.[19,22,23]

In terms of virulence, it is widely known that pathogenicity of *A. baumannii* is related to adherence, serum resistance, biofilm formation and capsule formation while infectivity of this pathogen mainly relies on outer membrane vesicles (OMVs) that play a role in initiation of TLR response, hence, mediating systematic inflammation.[24–27] Outer membrane vesicles are known to comprise high abundance of outer membrane proteins such as OmpA, CarO and Omp33–36 which not only enhances pathogenesis but also regulates cell integrity, efflux pumps and permeability of the cell hence plays a major role in survival of *A. baumannii*.[28] Alongside OMVs and OMPs, *A. baumannii* uses its secretion systems as interchangeable weapons to persist and kill even under oxidative stress.[28] Past studies have reported the importance of type I (T1SS), type II (T2SS), type IV (T4SS) and type VI (T6SS) secretion systems upon virulence of *A. baumannii*. Briefly, T1SS is known to transport biofilm like protein encoding genes (*bap*) and work in relation to T2SS which hydrolyzes long chain fatty acids to use as carbon source under lack of nutrients[29,30] while T6SS is known to boost bacterial competitiveness and mediate host killing while also enabling resistance to neutrophils.[28,31,32]

There are other studies evaluating *A. baumannii* based on its fitness cost upon emerging antimicrobial resistance rates alongside interchangeably used virulence genes and systems. Previous studies report that *A. baumannii* is losing its virulence score upon conferring antimicrobial resistance[33,34] while other studies have reported increased virulence alongside elevation in AMR rates despite clonality of isolates.[35] Hence, it is important to study *A. baumannii* isolates under the context of virulence, pathogenicity and antimicrobial resistance regardless of clonal relatedness due to strain specific behavioral pattern of this pathogen.

In this study we aimed to evaluate the molecular epidemiology, pathogenicity and infectivity of *A. baumannii* samples isolated from injured patients of conflict zones in Ukraine. In doing so, we performed *in silico, in vitro* and *in vivo* analyses to comparatively assess the sequence types and their variance in terms of genes, virulence and infectivity.

Materials and methods

Bacterial isolates and growth conditions

All clinical isolates were collected from wound injuries of soldiers and civilians during their treatment at tertiary hospitals in Ukraine in 2022–2023.[13] In total, 46 *A. baumannii* isolates were collected and grown on blood agar plates at 37 °C. For experiments that require mid-log or logarithmic phase of bacteria, isolates were subcultured in LB (Becton Dickinson, NJ) for 3 to 8 h and measurements of absorbance at OD_{600} were done. *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853 and capsule mutant and wild type of *A. baumannii* were used as control in biofilm formation assay and capsule abundance assay respectively.[36,37]

Determination of A. baumannii *antimicrobial resistance*

Antibiotic susceptibility testing was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines at the EUCAST reference laboratory in Växjö (Sweden). Determination of MDR and XDR status of isolates was done using previously accepted guidelines.[38–40] The considered antimicrobial categories to define the susceptibility profile of *Acinetobacter* spp. were aminoglycosides (amikacin), antipseudomonal carbapenems (imipenem, meropenem), antipseudomonal fluoroquinolones (ciprofloxacin), antipseudomonal penicillins + β-lactamase inhibitors (piperacillin-tazobactam), extended spectrum cephalosporins (ceftazidime), folate pathway inhibitors (trimethoprim-sulphamethoxazole), penicillins + β-lactamase inhibitors (ampicillin-sulbactam), polymyxins (colistin) and tetracyclines (tigecycline).[38] Briefly, MDR was defined as being non-susceptible to minimum of 3 or more (≥3) antimicrobial categories while XDR was defined as being susceptible to only 2 or fewer (≤2) categories of antimicrobials overall.

Whole-genome sequencing and bioinformatics

DNA was extracted from bacterial colonies on the EZ1 automated system (Qiagen, Hilden, Germany) using the DNA Tissue kit (Qiagen), as per the manufacturer's instructions. DNA libraries from the DNA were constructed using the Nextera XT kit (Illumina, San Diego, CA) and subjected to short-read, paired end sequencing on NovaSeq 6000 (Illumina) at Biomarker Technologies (Munster, Germany). QC of the sequencing reads was assessed using FastQC and assembled *de novo* using SPAdes (v3.15.5). Quality of the assembled contigs was determined using the QUAST. The open reading frames (ORFs) predicted using Prodigal (v 2.6.3) were searched against the UniProt database for the proteins of interest using Diamond (v 0.6.3). Multilocus sequence typing of the study isolates was conducted using the Pasteur Scheme. Antibiotic resistance genes (ARGs) and virulence determinants were identified using ABRIcate and Virulence finder database (VFDG). Fifty-three different protein sequences of interest were obtained from the UniProt (https://www.uniprot.org) database to serve as reference proteins. For each reference protein, a separate BLAST protein database was created using the predicted protein sequences from the samples. BLASTp was then performed for each reference protein against its corresponding custom database to identify homologous sequences within the samples. The capsule (K) and lipopolysaccharide (OC) locus types were identified using the Kaptive database (v 2.0.4).

Available sequence data

All sequences have been submitted to the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov); SUB15232350 and BioProject ID: PRJNA1246223.

Biofilm formation assay

Biofilm formation assay was studied using a crystal violet staining assay as described with slight modifications.[34,41] Briefly, bacterial isolates were adjusted to 1.5×10^7 CFU units (0.5 McFarland

units) and added to sterile 96 well flat bottom microtiter plate to be cultured at 37 °C for 24 h. The cells were washed twice with distilled water and 200 µl of 0.1% crystal violet dye was used to stain the wells for 30 min and stain was washed using PBS. Eventually, washed wells were dried at 60 °C for 15 min and stained biomass samples were dissolved in 95% ethanol. The OD_{540} reading for each well was determined using TECAN Microplate reader (Männedorf, Switzerland). All experiments were performed in triplicates. The OD values were interpreted and categorized according to Stepanović et al.[42] into four categories. The cut off value (OD_c) was defined as three standard deviations (SD) above the mean OD of the negative control; $OD \leq OD_c$=no biofilm producer, $OD_c < OD \leq 2*OD_c$=weak biofilm producer, $2*OD_c < OD \leq 4*OD_c$=moderate biofilm producer, $4*OD_c < OD$=strong biofilm producer.

Capsule production assay

The capsule gradient assay was performed as described by Valcek et al.[36,43] Bacteria were cultured in LB medium overnight at 37 °C and bacterial count was adjusted to 3×10^8 CFU/mL per strain in PBS. One milliliter of the bacterial suspension was centrifuged for 2 min at 7,000 relative centrifugal force (rfc). The supernatant was removed, and pellet was resuspended in PBS. This was followed by mixing 875 µl of PBS-resuspended bacteria with 125 µl of Ludox LS colloidal silica (30% [wt/wt]suspension in H_2O) by Merck (Søborg, Denmark). The mixture was then centrifuged for 30 min at 12,000 rcf and immediately photographically recorded. The distance of the center of the band from the bottom of the Eppendorf tube was measured. The experiment was performed in triplicates and capsule mutant *A. baumannii* with its wildtype strain was used as internal control.[37] The cellular density was categorized into three groups of high, medium and low density. The height of capsule mutant *A. baumannii* was used as cut off value on the interpretation of capsulation levels. The increase in level of density suggests the decrease in the capsulation abundance of the isolates.

Serum resistance

Normal human serum (NHS) from the blood of 7 healthy donors was pooled and stored at −80 °C. Written consent was obtained according to the recommendations of the local ethics committee in Lund (Sweden; 2017/582). Gelatin Veronal buffer (GVB++) was used to dilute NHS (5 mM Veronal buffer [pH 7.3], 140 mM NaCl, 0.1% gelatin, 1 mM $MgCl_2$, and 5 mM $CaCl_2$). Recombinant *Ornithodoros moubata* complement inhibitor (OmCI) blocking cleavage of C5 was mixed with NHS and used as negative control. OmCI was expressed as described.[44] Bacteria were mixed with GVB++ buffer consisting of 30% or 30% NHS with 50 µg/mL OmCI (negative control) and incubated at 37 °C for 1 h. After incubation, bacteria were serially diluted and plated in triplicates onto blood agar for CFU counts. Bacterial survival was calculated as a percentage based on CFU counts obtained from the negative control.

Galleria mellonella *infection model*

Galleria mellonella larvae were purchased from Imazo (Vara, Sweden). Bacterial count used for this experiment was 1×10^8 CFU/mL. Bacterial counts were adjusted in PBS and kept on ice until injection into *G. mellonella* larvae. Larvae were injected with 10 µl bacteria or PBS into the last, left proleg using BD insulin syringes (Micro-Fine U-100 0.3 mL 30 G; Becton Dickinson). Ten larvae were used per bacterial isolate. The infected larvae were incubated in Petri dishes at 37 °C for 5 days (120 h). The survival, movement, reaction to the stimulus and melanization of larvae were monitored every 12 h (at 9 am and 9 pm). The survival and melanization rates of larvae were used as a bacterial virulence scoring to comparatively assess different sequence types of *A. baumannii* strains.

Mouse acute pneumonia infection model

All mouse experiments were performed in compliance with Swedish animal protection laws and approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden (permit number: 5.8.18-19202/2023). Six to eight-week-old female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were maintained at the animal facility of Lund University in filter-top cages on standard laboratory chow and water *ad libitum* and daily light cycle of 12 h. This experiment was performed according to the previously published protocol[12] with slight modifications. Briefly, mice were anesthetized using inhalation of isoflurane (Forene; Abbott, Wiesbaden, Germany) and inoculated with 3×10^8 CFU *A. baumannii* in 50 µl PBS into the nares. Uninfected control mice received 50 µl of PBS only. Mice were examined for signs of distress twice daily. At 24, 48 and 72 h post-inoculation, animals were euthanized by CO_2 asphyxiation. For the bronchioalveolar lavage (BAL), animals were placed in a supine position and the trachea was incised. BALF was collected by two washes of 200 µl ice-cold PBS into the respiratory tract and kept on ice until plating. The lungs and nasopharynx were excised and mechanically homogenized in 200 µl ice-cold PBS and supplemented with ceramic beads using Minilys® Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Lung, nasopharynx homogenates and BAL fluid were plated on chocolate agar plates using appropriate dilutions with PBS and incubated overnight at 37 °C for CFU counts. All ST2 and ST19 isolates (n=18) were included in the experiment of 24 h of infection. Six mice were tested per isolate (n=108). Based on initial results, 2 strains causing lower-grade infection (ST2; KR6155 and ST19; KR6118) were picked for prolonged infection timepoints of 48 and 72 h. Three mice were used per isolate and per timepoint (48 and 72 h; n=12).

Statistics

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. A p-value of < 0.05 was considered statistically significant. Comparison of more than 2 groups was done using two-way ANOVA and Dunn–Bonferroni post-hoc tests. Statistical analyses were performed using GraphPad software 10 (Prism, La Jolla, CA).

Results

Wound isolates from war injuries are multidrug resistant and extensively drug resistant to antibiotics

Between 2022 and 2023, 46 *A. baumannii* samples isolated from wound injuries in Ukraine were collected. In total 15.2% (7/46) isolates were found to be XDR with susceptibility to only two classes of antimicrobials, polymyxin and tetracycline (Fig. 1A). Most the isolates belonged to ST2 (17.4%), ST19 (17.4%), ST78 (17.4%), ST400 (17.4%) while ST1077 and ST1 isolates were also identified with a lower ratio of 10.9% and 6.52%, respectively (Fig. 1A).

All isolates were resistant to amikacin, levofloxacin, ciprofloxacin, piperacillin-tazobactam and ceftazidime. Resistance to carbapenems was 71.7% and 84.8% for meropenem and imipenem, respectively (Fig. 1B). Susceptibility testing to cefiderocol revealed that 23.9% (11/46) of the isolates were resistant to cefiderocol. Eight out of 11 cefiderocol resistant isolates belonged to ST2 and 3 out of 11 isolates belonged to the ST19 cohort. Molecular resistome analysis revealed that the majority of the isolates were harboring beta-lactamases (Fig. 1C). Out of various beta lactamase genes,

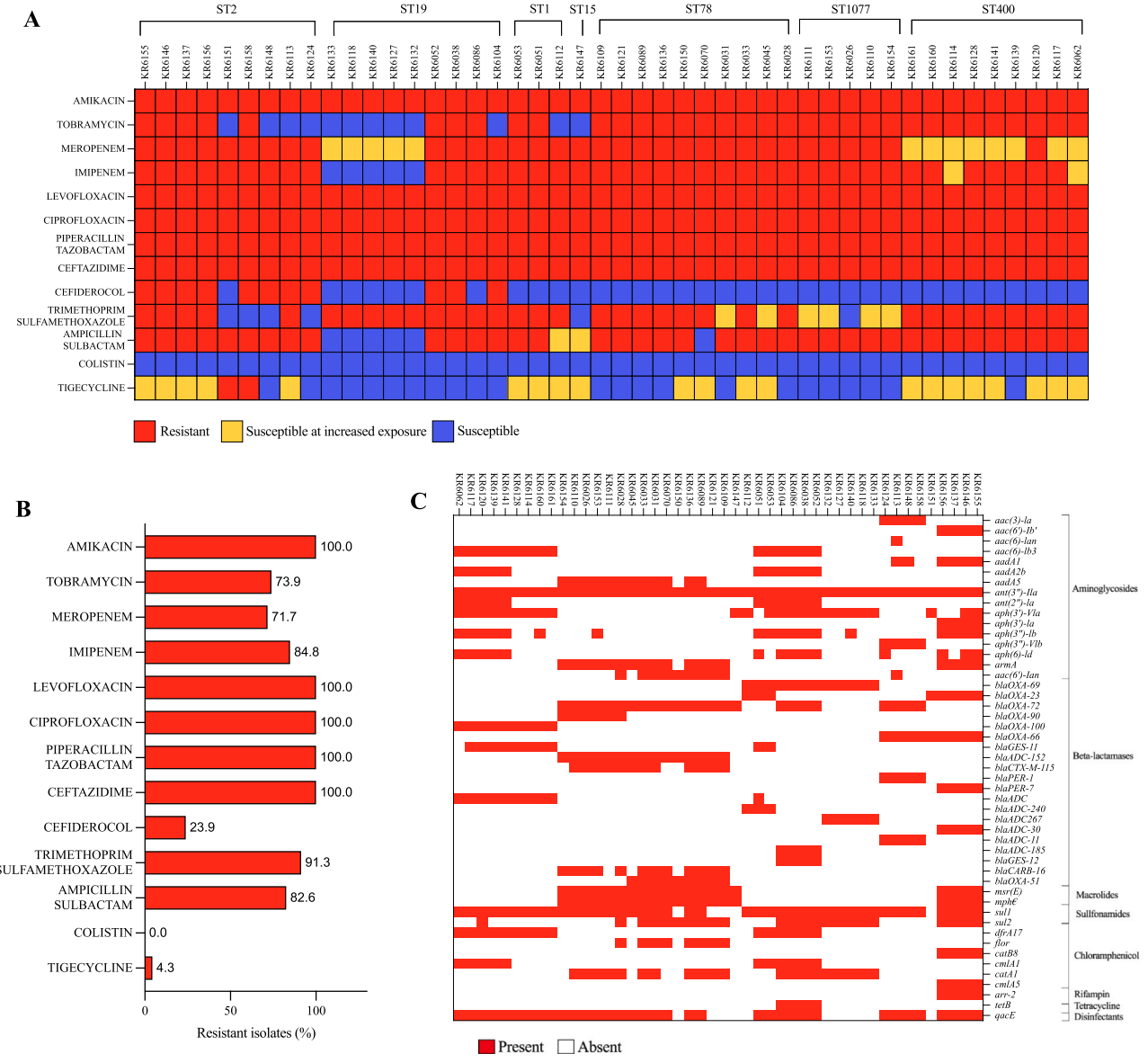

Fig. 1. Antimicrobial resistance and resistome analysis of *A. baumannii* isolated from wounded individuals in Ukraine. A) Susceptibility profile of *A. baumannii* isolates to 13 antibiotics from 8 different antimicrobial categories. B) Resistance rates of isolates (n=46) in percentage values to each antibiotic. C) Resistome evaluation of *A. baumannii* isolates listed based on clonal relatedness with depicting presence and absence of resistance genes for aminoglycosides, beta-lactamases, macrolides, sulfanomides, chloramphenicol, rifampin, tetracycline and disinfectants.

carbapenemases genes found were bla$_{OXA-69}$ (n=12), *bla*$_{OXA-23}$ (n=8), *bla*$_{OXA-72}$ (n=24), *bla*$_{OXA-90}$ (n=6), *bla*$_{OXA-100}$ (n=9), *bla*$_{OXA-66}$, (n=9) while the cephalosporinase genes found were *bla*$_{GES-11}$ (n=10), *bla*$_{GES-12}$ (n=4), *bla*$_{CTX-M-115}$ (n=12), *bla*$_{PER-1}$ (n=4), *bla*$_{PER-7}$ (n=4). Co-existence of *bla*$_{OXA-23}$ and bla$_{OXA-66}$ was observed on ST2 isolates while existence of bla$_{OXA-69}$ was specific to ST19 and ST1 isolates and was present in all samples of both STs (Fig. 1C).

Clonal relatedness, sequence types and virulome of wound isolates are of global concern

The ST1, ST19 (International Clone; IC1), ST2 (IC2), ST15 (IC4) and ST78 (IC6) isolates were observed to belong to international clones of concern. The two isolates that could not be identified in terms of sequence types were closely related to ST1 and ST78, respectively (Fig. 2). The K (capsule) locus and OC (lipopolysaccharide) locus analysis revealed that K locus variance is higher compared to OC locus. In total, 12 different K loci were identified for 46 isolates (Table 1). Eventually, identified OC locus for the sample cohort was mainly OCL1 while the OC locus for ST19 isolates and ST15 isolate were identified as OCL5 and OCL7, respectively. Virulome analysis revealed that Type 6 Secretion system (T6SS) encoding genes (*tssA, tssB, tssC, tssE, tssF, tssG, tssK, tssL, tssM*) were absent in ST19 isolates as well as biofilm formation encoding genes (*bap, blp1*), outer membrane protein encoding gene *omp33–36* and serum resistance associated gene *ata* (Fig. 2). Eventually, all isolates shared intact virulence genes

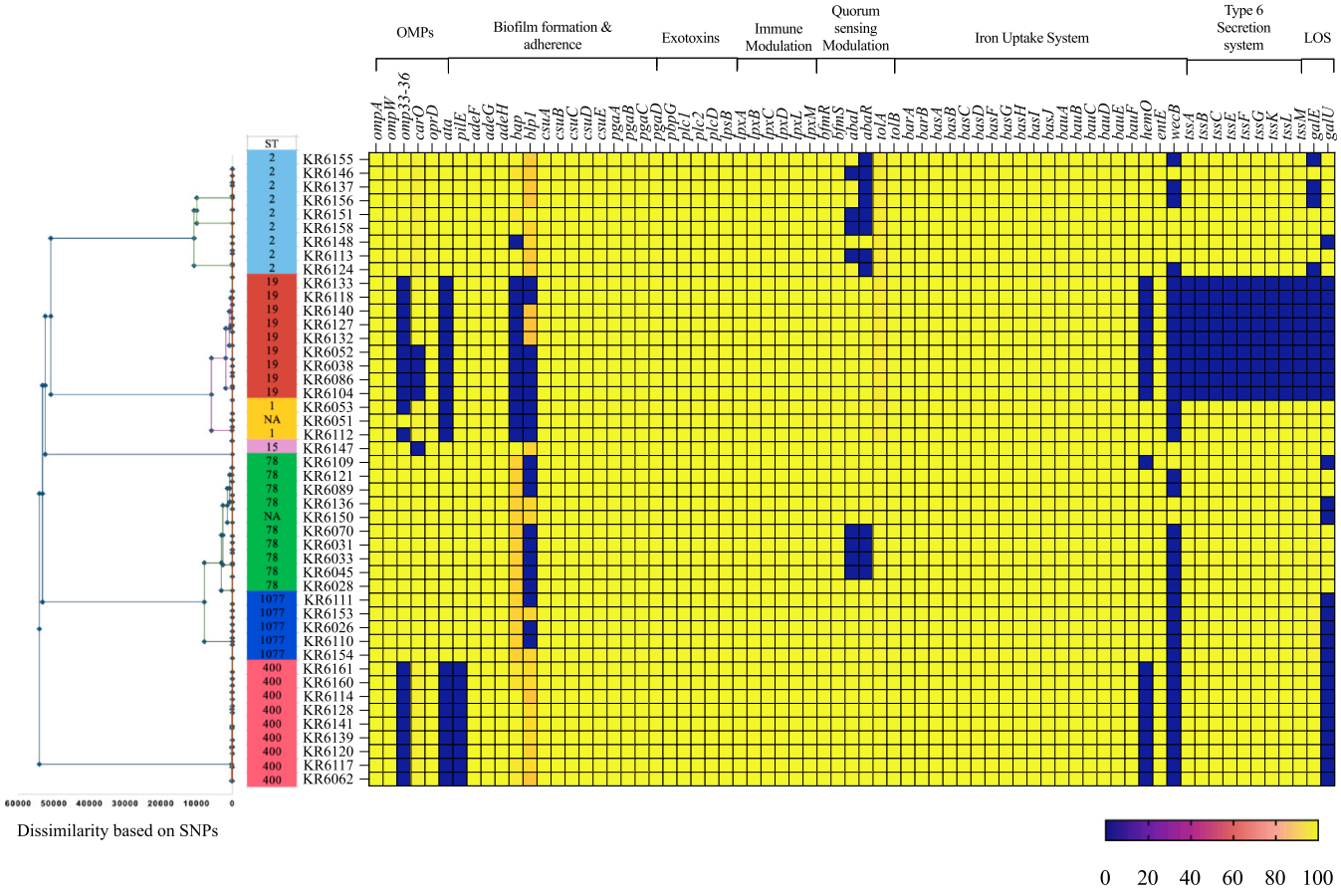

Fig. 2. Dendrogram and virulome analyses of *A. baumannii* isolates. Dendrogram of *A. baumannii* isolates constructed using a distance matrix based on SNP similarities. This is presented in parallel to the virulome profile, including genes encoding outer membrane proteins, biofilm formation and adherence factors, exotoxins and immune modulation, quorum sensing components, iron uptake systems and T6SS genes alongside genes involved in LOS production.

of *ompA, ompW, oprD, adeFGH* operon as well as *lpx* genes (*lpxA, lpxB, lpxD, lpxL*) that are mainly involved in immune response modulation. The complete set of virulence genes and their match score for the sample cohort is available in Supplementary File 1.

Sequence type 19 isolates are highly encapsulated and more susceptible to human serum

Serum bactericidal assay results revealed that ST19 and ST2 isolates are more susceptible to normal human serum *i.e.*, complement-mediated killing compared to the rest of the cohort (Fig. 3A). Although difference in susceptibility of the ST1 isolates (n=3) compared to the ST19 isolates (n=9) were not statistically significant, this may be based on sample size of ST1 cohort being low or molecular relatedness of ST1 to ST19 isolates (Fig. 3B). Additionally, ST19 isolates had a statistically significant capsule production compared to the rest of the cohort while the ability to produce biofilm was found to be unparalleled (Fig. 3C and D). The abundance of biofilm production was higher in ST2, ST400, ST1 and ST15 isolates compared to the ST19, ST1077 and ST78 isolates. Eventually, there was no correlation between capsule production and biofilm formation.

Sequence type 19 isolates are more virulent at the initial infection

A *G. mellonella* viability assay and a mouse infection model were performed to comparatively assess the lethality and infectivity of the *A. baumannii* wound isolates *in vivo*, respectively (Fig. 4). The *G. mellonella* viability assay showed that amongst 120 h of infection, ST400 isolates are the most virulent followed by ST2 isolates (Fig. 4A). On the other hand, ST19 isolates were found to have the highest virulence score at the first examination for the *G. mellonella* viability (12 h) while being the less virulent sequence type by the end of 120 h. This finding was further verified by the mouse infection model. Here, the CFU counts for ST19 isolates (n=9) were significantly higher in lung and nasopharynx homogenates as well as in bronchoalveolar fluid (BALF) compared to the ST2 isolates (n=9, $p < 0.0001$) (Fig. 4B). At 24 h post infection, only mice inoculated with ST19 isolates KR6127 (50% of mice), KR6086 (83%) and KR6140 (50%) showed signs of infection (piloerection, hunched posture and immobility) upon inspection. Additionally, the results from prolonged infection timepoints (48 and 72 h) that were performed with low-grade infection causing strains (ST2; KR6155 and ST19; KR6118) revealed that, ST19 isolates were cleared from the lungs and BALF followed by a drastic decrease in CFU counts for nasopharynx

Table 1
Identification of phenotypic susceptibility profile, sequence types, K locus and OC locus of wound isolates.

Isolate Code	XDR/MDR Status	Sequence Type	K locus	OC locus
KR6155	XDR	2	KL235	OCL1
KR6146	XDR	2	KL235	OCL1
KR6137	XDR	2	KL235	OCL1
KR6156	XDR	2	KL235	OCL1
KR6151	MDR	2	KL125	OCL1
KR6158	XDR	2	KL9	OCL1
KR6148	MDR	2	KL9	OCL1
KR6113	MDR	2	KL9	OCL1
KR6124	MDR	2	KL9	OCL1
KR6133	MDR	19	KL91	OCL5
KR6118	MDR	19	KL91	OCL5
KR6140	MDR	19	KL91	OCL5
KR6127	MDR	19	KL91	OCL5
KR6132	MDR	19	KL91	OCL5
KR6052	XDR	19	KL91	OCL5
KR6038	XDR	19	KL91	OCL5
KR6086	MDR	19	KL91	OCL5
KR6104	MDR	19	KL91	OCL5
KR6053	MDR	1	KL4	OCL1
KR6051	MDR	NA	KL4	OCL1
KR6112	MDR	1	KL4	OCL1
KR6147	MDR	15	KL9	OCL7
KR6109	MDR	78	KL125	OCL1
KR6121	MDR	78	KL125	OCL1
KR6089	MDR	78	KL3	OCL1
KR6136	MDR	78	KL3	OCL1
KR6150	MDR	NA	KL125	OCL1
KR6070	MDR	78	KL77	OCL1
KR6031	MDR	78	KL77	OCL1
KR6033	MDR	78	KL77	OCL1
KR6045	MDR	78	KL77	OCL1
KR6028	MDR	78	KL4	OCL1
KR6111	MDR	1077	KL40	OCL1
KR6153	MDR	1077	KL40	OCL1
KR6026	MDR	1077	KL40	OCL1
KR6110	MDR	1077	KL40	OCL1
KR6154	MDR	1077	KL40	OCL1
KR6161	MDR	400	KL15	OCL1
KR6160	MDR	400	KL15	OCL1
KR6114	MDR	400	KL15	OCL1
KR6128	MDR	400	KL15	OCL1
KR6141	MDR	400	KL15	OCL1
KR6139	MDR	400	KL15	OCL1
KR6120	MDR	400	KL15	OCL1
KR6117	MDR	400	KL15	OCL1
KR6062	MDR	400	KL15	OCL1

homogenates (Fig. 4C). In conclusion, ST19 isolates were found to be more virulent and caused a more efficient colonization at 12 and 24 h in *G. mellonella* and mouse infection model, respectively.

Discussion

In this study, we aimed to investigate the molecular epidemiology, comparative virulence traits, pathogenicity and infectivity of MDR and XDR *A. baumannii* wound isolates from war victims in Ukraine by using both *in vitro* and *in vivo* experimental set ups. It was observed that ST2, ST19, ST78 and ST400 were the dominating sequence types amongst wound isolates. Although ST2, ST78, ST400, ST1 and ST15 were widely reported previously not only from Europe but worldwide, ST19 has only been reported by a limited number of studies from Russia, Georgia, Moldova and Ukraine.[45–47] Dispersion of ST19 isolates in Eastern Europe is concerning. Additionally, the clonal relatedness of ST19 to ST2 isolates hints the fact that ST19 is the next candidate to be a sequence type of concern in terms of antimicrobial resistance and virulence in the context of *A. baumannii* infections (Fig. 2). In this study, we reported the carbapenem resistance rates of the isolates were as drastically high as 84.8%. This elevates the importance of studying carbapenem-resistant *A. baumannii* (CRAB) isolates based on the latest report of World Health Organization (WHO) pointing out CRAB as the first pathogen in line for emergency research and development of new antimicrobials.[3] This is one of the few reports to remark over 80% of resistance rate for carbapenem from Eastern Europe region. Although varying carbapenemase and cephalosporinase carriage was an expected outcome based on the antimicrobial susceptibility profiles of the isolates; coexistence of beta-lactamases is an alarming problem as it results in an elevation of breakpoint values which further limits combination therapies.[48] Additionally, although none of the isolates qualified as pandrug resistant (PDR) based on their susceptibility to colistin, it is known and reported that colistin is a highly nephrotoxic antimicrobial that is not favorably used and colistin resistance comes with its huge fitness cost on virulence of *A. baumannii*.[49,50] As an additional comment to the susceptibility profiles of the cohort, it was observed that cefiderocol resistance is emerging. The overall resistance rate of this study cohort to cefiderocol was 23.9%. As a siderophore based antimicrobial which has been in use only since 2020 in Europe, emerging resistance rates to cefiderocol are alarming.[51] It is known that molecular and proteomic mechanisms involved in resistance to cefiderocol consist of molecular alterations on iron binding sites, iron transporter proteins, penicillin binding proteins and coexistence of beta-lactamases.[52–55] In this study, cefiderocol resistance can be attributed to coexistence of $bla_{\text{OXA-23}}$ with $bla_{\text{PER-1}}$ and $bla_{\text{PER-7}}$ in ST2 isolates (Fig. 1A and C).

Regarding the conservation of virulence and pathogenicity of the isolates, numerous experimental set ups were performed to comparatively assess the wound isolates. Despite no positive correlation was observed between biofilm production and capsule production ability of the isolates, ST19 isolates were observed to produce low biofilm mass while being able to result in high capsule abundance (Fig. 3C and D). On the other hand, while the whole cohort tested highly serum resistant, ST19 and ST2 isolates were significantly less resistant to human serum compared to the remaining part of the cohort ($p < 0.001$) (Fig. 3B). Although clonal relatedness of our cohort may hint a parallel behavior of the isolates regarding serum resistance, it was previously reported that *A. baumannii* isolates from different regions tested highly serum resistant.[26]

Regarding *in vivo* studies, *G. mellonella* and mouse pneumoniae models have been widely used as infection models to assess the pathogenicity and infectivity of *A. baumannii* in previous studies.[56–60] In this study, we performed both models to assess the wound isolates on different infection models. Importantly, ST19 isolates were observed to be more lethal on *G. mellonella* and higher CFU counts were observed in the mouse infection model at 12 and 24 h of post-inoculation, respectively. However, a drastic decrease in the virulence and infectivity of ST19 isolates was observed both in *G. mellonella* and mice infection models upon prolonged infection timepoints (Fig. 4). This may further be explained by the absence of the complete T6SS operon and *omp33–36* in ST19 isolates. Previous studies have reported the failure of isolates on bacterial

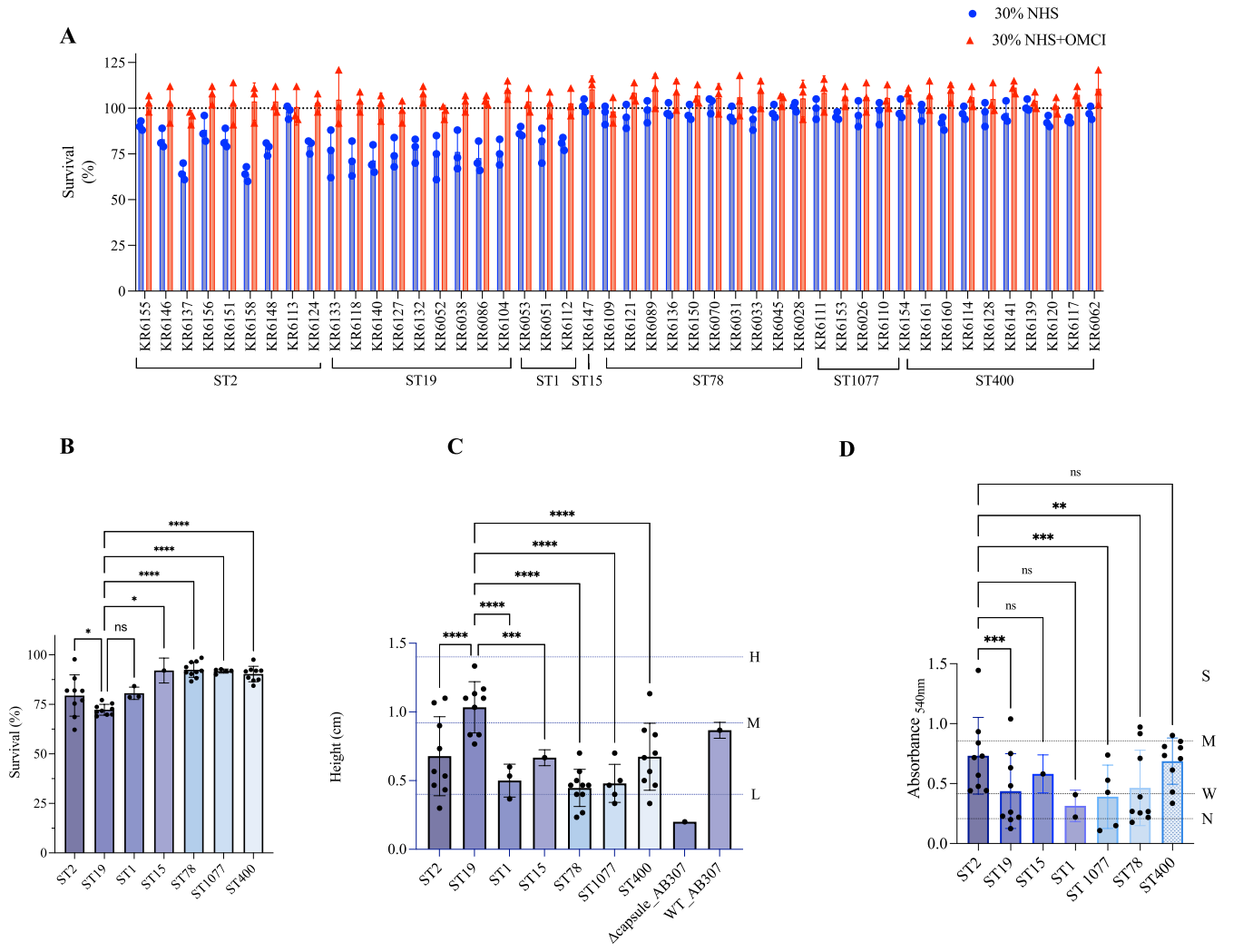

Fig. 3. Functional assays assessing serum survival, capsule abundance, and biofilm formation in *A. baumannii* isolates. A) Results on serum bactericidal assay results depicting percentage survival of *A. baumannii* isolates in the presence of 30% NHS and 30% NHS supplemented with the complement C5 inhibitor OmCI. B) Serum bactericidal assay graph with normalized serum survival values based on the survival rate of isolates upon incubation with 30% NHS+OmCI, segregated by sequence types. C) Comparative capsule abundance of different sequence types of *A. baumannii* isolates. A capsule mutant (AB307) was included as an internal control. D) Biofilm formation of different STs of *A. baumannii* isolates normalized based on the control ATCC strain.

competitiveness and persistence upon loss and alteration of T6SS sites.[32,61,62] The loss of the T6SS locus is attributed to fitness cost of *A. baumannii* which inactivates the T6SS in favor of antibiotic resistance that provides sufficient advantage of survival.[63–65] Additionally, it is known that Omp33–36 is involved in apoptosis and induces autophagy which differs functionally from OmpA that merely causes cytotoxicity by targeting host cell.[66] Hence, despite OmpA encoding genes remain intact in ST19 isolates, the absence of *omp33–36* may also be the possible cause of the decreased lethality score of ST19 on *G. mellonella* viability. Eventually, the survival of ST2 isolates on prolonged infection timepoints of 48 and 72 h in nasopharynx homogenates can be linked to the conservation of genes that are associated with adhesion and biofilm formation (*ata*, *bap* and *blp)* in all ST2 isolates.[67]

Our findings suggest that wounded and hospitalized war victims in Ukraine are infected by clonally related MDR and XDR *A. baumannii* that is alarming both in the context of high AMR rates and strong virulence profiles. Due to the ongoing war and shortage of hospital beds and facilities to cohort care of infected patients, there is a clear risk of healthcare associated outbreaks. Additionally, 65% (30/46) of isolates belonging to the international clones (IC1, IC2, IC4, IC6) approves that dispersal rate of carbapenemase carriage is at a highly detrimental rate.

Our study is the first report on the virulence and pathogenicity of *Acinetobacter baumannii* ST19 isolates from Ukraine. Moreover, the ST19 isolates being highly virulent at the early infection is alarming. As all the isolates tested highly antimicrobial resistant and serum resistant, possible nosocomial spread of these isolates and the risk of bacteremia, ventilator associated pneumonia (VAP) and hospital associated pneumonia is concerning as the infection prevention and control measures remained suboptimal since the initiation of the war.[12,68] This study is limited by sample size and closely related

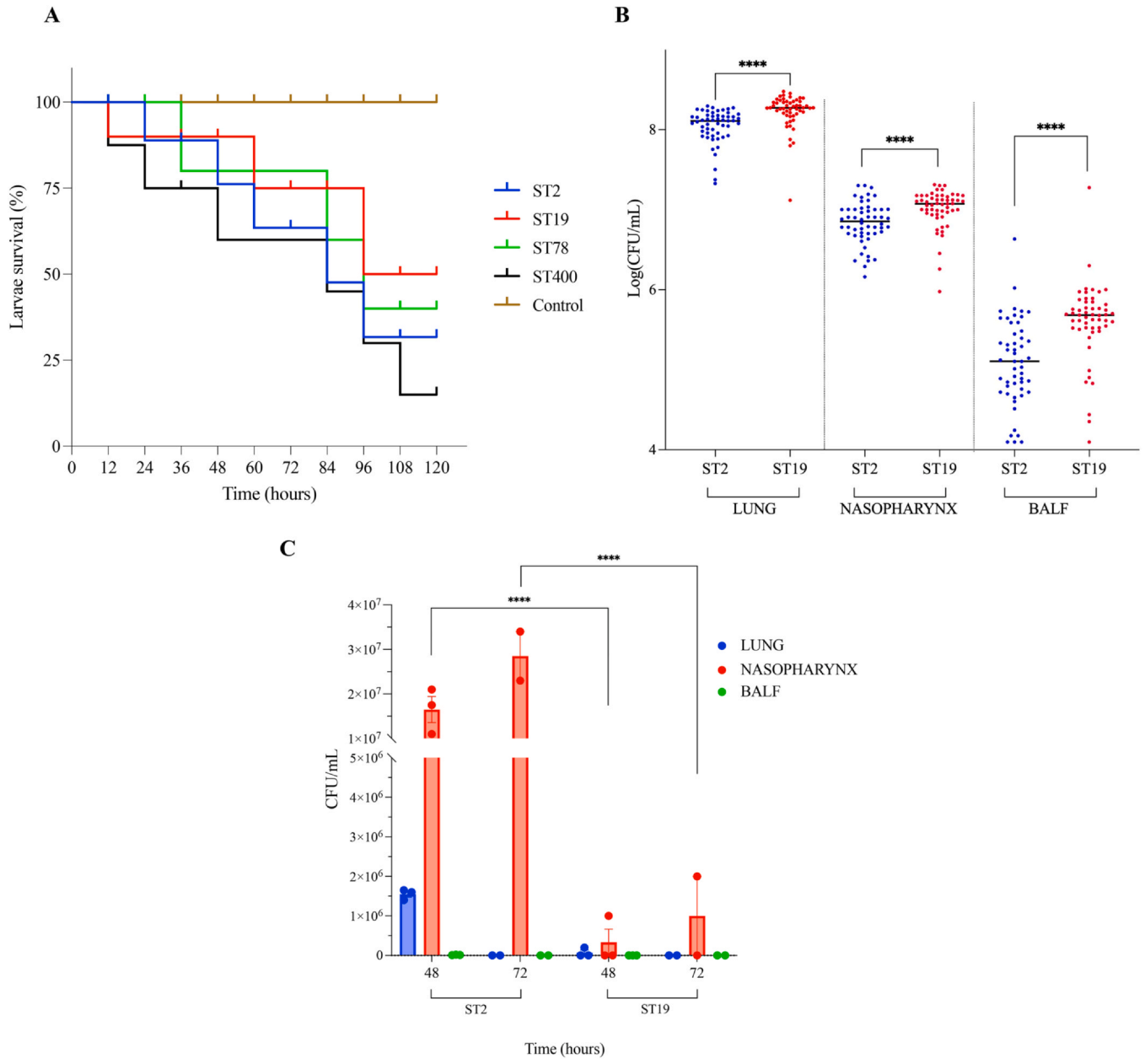

Fig. 4. *In vivo* infection models evaluating virulence and bacterial load of *A. baumannii* isolates across sequence types. A) Kaplan–Meier survival curve of *G. mellonella* infection model over 120 h. Each dataset consisted of 10 larvae per isolate and a total number of 46 isolates were tested. Median values of the grouped isolates based on sequence types (STs) are depicted. B) CFU counts of 24 h mouse infection model of lung and nasopharynx homogenates in addition to BALF with bacterial isolates grouped based on sequence types. Each dataset consisted of 6 mice per isolate ($n = 108$). C) CFU counts of lung, nasopharynx homogenates and BALF at 48 and 72 h of mice infection timepoints for lower-grade infection causing ST2 (KR6155) and ST19 (KR6118) isolates. Three mice were used per each infection timepoint of isolate ($n = 12$).

isolates in terms of clonality as well as isolates belonging to a single year (2022) and not to a broader spectrum of timespan. Additional studies that evaluate *A. baumannii* samples isolated from war injuries and hospitalized patients in Ukraine should be performed on a molecular and phenotypic basis to assess the clonality, susceptibility profiles and sequence types of isolates over a broader timespan to further comment on the divergent molecular epidemiology of *A. baumannii* in the conflict zone.

In conclusion, MDR and XDR *A. baumannii* wound isolates from Ukrainian war are closely related, have high AMR rates as well as concerning virulence and infectivity profiles. Instability and absence of T6SS, Omp33-36 and biofilm encoding genes and their effect on encapsulation in ST19 isolates should be studied further, as such traits may influence persistence and transmissibility, which in turn could inform the prioritization of infection control measures, including cohort care and isolation capacity.

Author contributions

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

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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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2025.106561.

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