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ORIGINAL RESEARCH

Molecular Epidemiology and Genetic Characterization of Carbapenem-Resistant *Acinetobacter baumannii* Isolates from the ICU of a Tertiary Hospital in East China

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Purpose: To evaluate the clinical characteristics, antimicrobial resistance (AMR) phenotypes and genotypes, and homology features of carbapenem-resistant *Acinetobacter baumannii* (CRAB) in intensive care unit (ICU) and to provide basis for effectively prevention, control and treatment of nosocomial infections caused by CRAB.

Methods: A total of 39 CRAB strains isolated from hospitalized patients in the ICU and neurosurgical ICU (NICU) between 2020 and 2023 were subjected to antimicrobial susceptibility testing and whole-genome sequencing (WGS). Virulence factor genes (VFGs), antimicrobial resistance genes (ARGs), multilocus sequencing typing (MLST), complete genome multilocus sequencing typing (cgMLST), average nucleotide identity (ANI), and single nucleotide polymorphism (SNP) analyses were performed using WGS.

Results: All CRAB strains were 100% resistant to ciprofloxacin, ceftazidime, piperacillin/tazobactam, and ticarcillin/clavulanic acid. A total of 48 antimicrobial resistance genes (ARGs) were found in the 39 CRAB strains, including *bla*OXA-66, *bla*OXA-23, *bla*ADC-30, *bla*ADC-73, *gyrA, ant(3")-IIa, aph(3")-Ib, aph(6)-Id, tetB, tetR, sul1, sul2, LpxC and LpxA* which confered resistance to carbapenems, cephalosporins, fluoroquinolones, aminoglycosides, tetracycline and sulfonamides. There were 128 VFGs, including genes encoding the AdeFGH efflux pump, lipopolysaccharide (LpsBLC), outer membrane protein A (OmpA), penicillin-binding protein (PbpG), biofilm-associated proteins (bap, pgaBCD, CsuABCDE), type VI secretion system protein (Tss), quorum sensing protein (AbaI/AbaR). Six clonal lineages were identified by Oxford MLST method, whereas one sequence type (ST2) was identified using the Pasteur MLST method. ANI analysis, heat map of SNP analysis, and phylogenetic tree based on core SNP revealed six clusters, and the strain classification results were consistent with these different methods. Ten clonal lineages were identified by cgMLST.

Conclusion: The CRAB strains were ST2 clones accompanied by severe resistance to commonly used antibiotics and abundant ARGs and VFGs in genotype. Strict measures should be implemented to prevent and control transmissions and infections. CgMLST and SNPs analyses showed excellent discriminatory power in homology analysis.

Keywords: carbapenem-resistant *Acinetobacter baumannii*, drug-resistance genes, homology, multilocus sequencing typing

Introduction

Acinetobacter baumannii, a significant Gram-negative opportunistic bacterium, frequently leads to severe hospital-acquired infections, particularly in immunocompromised and elderly patients in ICU around the world.[1] This pathogen

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can cause a large number of infections, the most common of which are ventilator-associated pneumonia and bloodstream infections, with a mortality rate of up to 35%.[2,3] ICU infections caused by *A. baumannii* are associated with a 50% mortality rate.[4] In recent years, drug-resistant *A. baumannii* strains are increasing, including multiple drug-resistant (MDR, non-susceptibility to at least one agent in three or more antimicrobial categories), extensively drug-resistant (XDR, non-susceptibility to at least one agent in all but two or fewer antimicrobial categories) and pan-drug resistant (PDR, non-susceptibility to all agents in all antimicrobial categories) hospital isolates.[5–7] Increasing usage of β-Lactam antibiotics has led to the emergence of drug-resistant strains of *A. baumannii*. Carbapenem is used as a last-line antibiotic drug to treat infections caused by these MDR strains.[8] However, the development of carbapenem-resistant *A. baumannii* (CRAB) is limiting its use. CRAB has become a worldwide problem due to its resistance to commonly use antibiotics, in addition, pandrug-resistant *A. baumannii* emerged and posed the greatest challenge and required a comprehensive control program and treatment.[9] These isolates are difficult to treat with existing drugs, exacerbated the problem of nosocomial infection caused by the bacteria.[10]

CRAB strains have spread globally, and multiple drug-resistance characteristics and excessive virulence factors make it a serious threat to global public health.[11] CRAB was declared an urgent threat to public health by the US Centers for Disease Control and Prevention (CDC) in 2019 (https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf). Data from the China Antimicrobial Surveillance Network (CHINET) showed that from 2015 to 2021, the isolation rate of CRAB was relatively high, reaching 75.2% in tertiary hospitals, showing high resistance to commonly used clinical antibacterial drugs.[12]

The main mechanism of resistance to carbapenems in *A. baumannii* is the acquisition of carbapenem-hydrolyzing class D β-lactamases [oxacillinases (OXAs)], encoded by *bla*OXA-23, *bla*OXA−24, *bla*OXA-58, *bla*OXA-143 and *bla*OXA-235 variants.[13] In addition, ambler class A b-lactamases (*bla*CTX-M, *bla*TEM, *bla*KPC, *bla*SHV and *bla*GES-14) and metallo-ß-lactamases (*bla*VIM-like, *bla*IMP-like, *bla*NDM-1 and *bla*SIM-1) also lead to carbapenem resistance.[14] In addition to the complex mechanism of antimicrobial resistance, virulence factors of *A. baumannii* play important roles in invasiveness and pathogenicity. Even though virulence determinants of *A. baumannii* are not completely understood, the genes related to outer membrane proteins, biofilm formation, cell-associated and secretion systems, and micronutrient acquisition systems, as well as resistance to xeric stress, were regarded as important factors for successfully infecting its hosts.[15] The *A. baumannii* strains possesses a wide range of virulence factors with versatile abilities in recent study.[16] A better understanding of antimicrobial resistance mechanisms and virulence factors of CRAB isolates is essential for effective control and treatment.[17]

Recent studies based on whole-genome sequencing (WGS) have highlighted the diversity of CRAB strains across different geographical regions, revealing a complex interplay of genetic factors that contribute to their resistance profiles. A global study with CRAB strains isolated from 114 study centers in 47 countries across five continents revealed that global CRAB population currently comprised at least nine clonal lineages, isolates harboring *bla*OXA-23 and representing international clones 2 (IC2) are predominant strains in most parts of the world till date, but the distribution of carbapenem resistance determinants and ICs can vary widely among different geographical regions.[18] A prospective observational cohort study made by 46 hospitals in five global regions between 2017 and 2019 showed that although clonal group 2 (CG2) isolates remained predominant, non-CG2 strains were associated with higher mortality.[19] Two studies conducted in the United States illustrate the high prevalence of acquired *bla*OXA carbapenemase genes especially *bla*OXA-23 among CRAB isolates.[20,21] Research from Serbia identified multiple resistance genes among CRAB isolates, including *bla*OXA-72 and *bla*NDM-1, emphasizing the global spread of these resistance determinants.[22] Moreover, in the context of hospital outbreaks, the transmission dynamics of CRAB have been closely monitored in recent studies.[23–25] Overall, the molecular epidemiology of CRAB underscores the urgent need for continuous surveillance, molecular characterization, and the development of effective infection control strategies to combat this formidable pathogen. The ongoing research efforts are crucial for informing public health policies and improving clinical outcomes for affected patients.

The epidemiology of CRAB strains in East China is limited, particularly among ICU patients. Anqing First People's Hospital of Anhui Medical University was established in 1953. It is a large comprehensive teaching and tertiary hospital in the eastern region of China. The hospital currently has 2630 beds, nearly 2000 employees and 49 clinical medical technology departments. In this study, we conducted a four-year surveillance in the ICU to analyze the epidemiology and

antimicrobial resistance of CRAB strains. CgMLST and SNPs analyses were performed to determine the homology between CRAB strains isolated from different patients.

Materials and Methods

Bacterial Isolates

A total of 39 strains of carbapenem-resistant *Acinetobacter baumannii* (CRAB) bacteria clinically isolated from the ICU and neurosurgical intensive care unit (NICU) of Anqing First People's Hospital of Anhui Medical University, Anqing City, Anhui Province, China, from 2020 to 2023 were collected, and duplicate strains (if the same multidrug-resistant organism was detected multiple times in the same sample from a single patient, the isolate was considered only one strain)[26] were removed. Clinical information of the 39 CRAB isolates was obtained from Xinglin Real-Time Nosocomial Infection System, and the characteristics of age, sex, clinical specimen, primary ICU admission diagnosis, infection diagnosis, antibiotics used during ICU, invasive medical operation, infection type, and discharge method from the hospital were determined.

Bacterial Identification of the Isolates

Clinical specimens from different patients were inoculated on sheep blood agar plates and incubated for 18–24 hours at 37°C. A pure culture was obtained from another blood agar plate by transferring the dominant strains from the first plate. Bacterial identification was performed using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and VITEK 2 Gram-Negative Identification Card (VITEK 2 GN Test Kit). All identified carbapenem-resistant *A. baumannii* isolates were stored at −80°C in a tube containing 20% glycerol.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing was performed according to the method recommended by the Clinical and Laboratory Standards Institute (CLSI), and the automated identification and susceptibility testing instrument VITEK2-Compact system (Bio-Merieux, France) and Antimicrobial susceptibility testing (AST) card (VITEK 2 AST-N335) were used for drug sensitivity interpretation, supplemented by KB method (Kirby-Bauer) according to CLSI guidelines (CLSI, 2020).[27] Standard strains were *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922. CRAB isolates were defined as *A. baumannii* strains which were multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR) as described previously.[6]

Bacterial DNA Extraction

All strains were obtained from a strain-storage refrigerator (−80°C, 20% glycerol). After balancing to room temperature, an appropriate amount of bacterial liquid was inoculated into blood culture dishes, and the plates were cultured in a CO_2 constant-temperature incubator for 18–24 hours. Before DNA isolation, the isolates were re-identified using MALDI-TOF. A single large colony was selected from a blood culture plate for DNA isolation. Genomic DNA of *A. baumannii* was extracted using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. The integrity and quality of genomic DNA were evaluated using 1% agarose gel electrophoresis. The yield and purity of the DNA were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA) and a TBS-380 fluorometer (Turner BioSystems Inc, Sunnyvale, CA). High-quality DNA (OD260/280=1.8–2.0, >1 μg) was used in subsequent experiments. *16S rRNA* and *16S-23S rRNA* genes were amplified and sequenced to confirm the identity of *A. baumannii*, as described previously.[28,29] Primers were listed in Table S1. Gel images and sequence confirmation information were shown in Figures S1 and S2.

Whole-Genome Sequencing (WGS), Draft Genome Assembly and Annotation

All isolates were sequenced by Shanghai Winnerbio Technology Co., Ltd. (Shanghai, China) on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA). At least 1μg of genomic DNA was prepared for each *A. baumannii* strain during the library construction. High-quality DNA(OD260/280=1.8–2.0, >1 μg) samples were sheared into 400–500 bp

DNA fragments using a Covaris M220 Focused Acoustic Shearer according to the manufacturer's protocol. Libraries for Illumina sequencing were created using fragmented DNA, which was subsequently utilized for paired-end sequencing with 2 × 150 bp reads using an Illumina NovaSeq 6000 instrument. Raw sequencing reads were evaluated using the FastQC software.[30] Except where otherwise, all software tools were run with default parameters. The data were trimmed by Trimmomatic v0.39 (LEADING:3; TRAILING:3; MINLEN:36) and assembled by SOAPdenovo v3.12.0.[31] All the sequences were submitted to the NCBI database.

The predicted gene sequences were translated and searched against the following database for annotation: National Center for Biotechnology Information (NCBI) non-redundant (NR) database, the UniProt/Swiss-Prot database, the protein families (Pfam), the Clusters of Orthologous Group (COG) database, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Additional annotation was carried out using Pathogen Host Interactions (PHI), Virulence Factors of Pathogenic Bacteria (VFDB), and the Antibiotic Resistance Genes Database (ARDB).

Multilocus Sequence Typing and Core Genome Multilocus Sequence Typing

Conventional MLSTs were performed with the MLST database for *A. baumannii* (https://pubmlst.org/organisms/acinetobacter-baumannii). Sequence type (ST) codes were generated by combining the *gltA, gyrB, gdhB, recA, cpn60, gpi*, and *rpoD* alleles detected under the Oxford scheme[32] and *cpn60, fusA, gltA, pyrG, recA, rplB*, and *rpoB* alleles detected under the Pasteur scheme.[33] Minimum spanning trees were drawn based on Pasteur typing results using Phyloviz software.

Core genome (cg) MLST classification was performed on 39 strains based on the cgMLST v1 classification scheme in the PubMLST database. The minimum spanning trees were marked by the sample ID, sample type, sample source, and separation time, respectively.

Whole Genome Average Nucleotide Identity Analysis

OrthANI v0.93.1 was used to calculate the Average Nucleotide Identity (ANI) values between the whole-genome sequences of the two samples, and R v4.0.2 was used to draw the ANI clustering heat map. The clustering method was "average".

Whole Genome Single Nucleotide Polymorphism Cluster Analysis and Core SNP Analysis

The genome sequences of all samples were mapped to the reference genome (the reference genome used in this project was the standard strain ATCC19606 (GCF_019331655) as it is a common reference strain). Phylogenetic trees based on SNPs were built using kSNP v3 (kmer = 19) with maximum likelihood algorithm, and the SNP tree was annotated using iTOL v6.9.1.

Statistical and Data Projection Analysis

Clinical data were obtained from Xinglin Real-Time Nosocomial Infection System (Xinglin Tec Inc., Hangzhou, China) and analyzed using SPSS software Version 26.0 (IBM SPSS Inc., Chicago, USA). The figures of AST and AMR profiles were generated by Excel (Microsoft Inc, Redmond., USA). The minimum spanning trees (MSTs), heat map ANI and SNP and phylogenetic tree were generated as described above.

Results

General Information of Isolates and Patients

The present study spanned from May 2020 to July 2023. A total of 39 CRAB strains, isolated from different sites, were collected from 38 patients. Detailed clinical characteristics of the 38 patients are summarized in Table 1. Most of the clinical specimens were obtained from the respiratory system (69.2%, 27/39). The most common diagnosis when these patients were admitted to the ICU was pneumonia. Nearly all patients used ventilators (94.9%, 37/39) and urinary catheters (94.9%, 37/39) during their ICU stay. Lower respiratory tract infections and ventilator-associated pneumonia (VAP) accounted for 51.3% (20/39) and 17.9% (7/39) of infection diagnoses, respectively. The detection rates of bloodstream, thoracic cavity, and intra-

Table 1 Clinical Characteristics of the Patients with CRAB Sequenced in This Study

Characteristics	
Age (years)	66.2 205.3
Gender, male	23 (59.0)
Clinical specimens	
Respiratory	27(69.2)
Wound secretion	3(7.7)
Ascitic fluid	3(7.7)
Hydrothorax	2(5.1)
Blood	2(5.1)
Catheter	1(2.6)
Midstream urine	1(2.6)
Primary ICU admission diagnosis	
Pneumonia	12(30.8)
Cerebral trauma and cerebral hemorrhage	11(28.2)
Hypertension	10(25.6)
Diabetes mellitus	6(15.4)
Chronic obstructive pulmonary disease	5(12.8)
Surgery procedure	19(48.7)
Infection diagnosis	
Lower respiratory tract infection	20(51.3)
VAP	7(17.9)
BSI	3 (7.7)
Thoracic cavity infection	3 (7.7)
Intra-abdominal infection	3 (7.7)
SSI	2 (5.1)
CAUTI	1 (2.6)
Antibiotics used during ICU	
Carbapenem	31(79.5)
Cefoperazone-sulbactam	29(74.4)
Piperacillin–tazobactam	16(41.0)
Glycopeptides	17(43.6)
Fluoroquinolones	15(38.5)
Oxazolidinones:Linezolid	15(38.5)
Tetracyclines	13(33.3)

(*Continued*)

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Table 1 (Continued).

Characteristics	
Cephamycins: cefoxitin	12(30.8)
Aminoglycoside	12(30.8)
Cephalosporin	6(15.4)
Colistin	4(10.3)
Antifungal	15(38.5)
Invasive medical operation	
Ventilator	37(94.9)
Urinary catheter	37(94.9)
Nasogastric tube	37(94.9)
Trachea cannula	27(69.2)
Central venous catheter	24(61.5)
Puncture drainage	3 (7.7)
Hemodialysis	1 (2.6)
Surgery procedure	19(48.7)
Infection type	
Hospital-acquired infection	26(66.7)
Community-acquired infection	7(17.9)
Colonization	6(15.4)
Discharge method	
Normal	25(64.1)
Transfer to another hospital	11(28.2)
Died	3 (7.7)

Notes: All the data are presented as the number, with the percentage in parenthesis, except for the ages, which are presented as the mean and SD.
Abbreviations: VAP, Ventilator associated pneumonia; BSI, Bloodstream infection; CAUTI, Catheter associated urinary tract infection.

abdominal infections were 7.7%(3/39). As the most common infection type, hospital-acquired infection was observed in 66.7% (26/39) of patients. Various antimicrobial drugs have been used to treat infections. Among them, carbapenem (imipenem and meropenem) (79.5%, 31/39) and cefoperazone-sulbactam (74.4%, 29/39) were the two predominant antibiotics. Glycopeptides, fluoroquinolones, and oxazolidinones accounted for 43.6%(17/39), 38.5%(15/39), and 38.5%(15/39), respectively.

Antimicrobial Susceptibility of *A. Baumannii* Strains

The antimicrobial resistance rates of the 39 clinical CRAB strains to 14 antibiotics are shown in Figure 1 and Table S2. Results revealed that CRAB were 100% resistant to carbapenems (imipenem and meropenem), piperacillin-tazobactam, ticarcillin/clavulanic acid, ceftazidime, and ciprofloxacin. The rates of antimicrobial resistance to other antibiotics were as follows: levofloxacin, 84.6%(33/39); cefepime, 82.1%(32/39); tobramycin, 76.9% (30/39); trimethoprim/

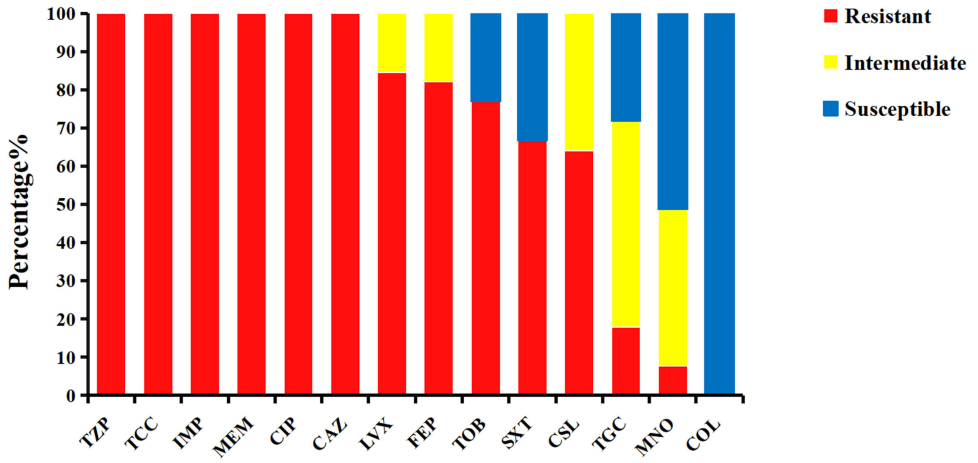

Figure 1 The antimicrobial susceptibility of 39 CRAB isolates from intensive care units.
Abbreviations: TZP, piperacillin/tazobactam; TCC, ticarcillin/clavulanic acid; IMP, imipenem MEM, meropenem; CIP, ciprofloxacin; CAZ, ceftazidime; LVX, levofloxacin; FEP, cefepime; TOB, tobramycin; SXT, trimethoprim/sulfamethoxazole; CSL, cefoperazone-sulbactam sodium;TGC, tigecycline; MNO, minocycline; COL,colistin.

sulfamethoxazole, 66.7% (26/39); cefoperazone-sulbactam sodium, 64.1% (25/39); tigecycline,17.9%(7/39); and minocycline, 7.7%(3/39). Colistins demonstrated the lowest resistance rate (0%, 0/39).

Characterization of Antimicrobial Resistance Genes (ARGs) in CRAB Strains

A total of 48 antimicrobial resistance genes (ARGs) were found in the 39 CRAB strains that responded to ten categories of antimicrobial drugs (Figure 2 and Table S3). A total of 58.3%(28/48) of the ARGs were detected in all 39 CRAB strains, and 28 ARGs shared by all strains encoded several types of antibiotics. *LpsB, LpxC*, and *LpxA* encoded resistance to peptide. *Bla*OXA-66 and *bla*OXA-23 conferred resistance to carbapenems. G*yrA* harbored resistance to fluoroquinolones. *Ant(3")-IIa, aph(3")-Ib*, and *aph(6)-Id* encoded resistance to aminoglycosides. *Ecol_EFTu_KIR* confers resistance to elfamycin. Many Efflux pump genes were 100% detected in all the strains, including *adeA, adeB, adeC, adeF, adeG, adeH, adeI, adeJ, adeK, adeL, adeN, adeR*, and *adeS. AbeM* and *abeS* were 100% contained by the strains. The detection rate of *blaADC*, which encodes resistance to cephalosporins, was 100%. Two types of *ADC* genes were identified: *bla*ADC-30 (30.8%,12/39) and *bla*ADC-73 (69.2%,27/39). The detection rates of *tetB* and *tetR*, which encode tetracycline resistance, were 97.4%(38/39) and 97.4%(38/39), respectively. Additionally, *sul1*(25.6%, 10/39) and *sul2*(38.5%, 15/39) harbored resistance to sulfonamides.

Virulence Factor Genes (VFGs) of CRAB Genomes

Table S4 presents the detailed VFG prediction information based on the virulence factor database (VFDB). The 39 CRAB strains contained 8 classes of VFGs. The VFGs identified in this study included regulation, stress survival, adherence, biofilm formation, immune modulation, nutritional/metabolic factors, exotoxins, and effector delivery systems. There were 128 VFGs among the 39 isolates, 69.5% (89/128) of them were found in all the 39 strains. All CRAB isolates contained genes encoding the AdeFGH efflux pump, lipopolysaccharide (LpsBLC), outer membrane protein A (OmpA), penicillin-binding protein (PbpG), biofilm-associated proteins (bap), and biofilm-associated proteins (pgaBCD). The detection rates of *CsuABCDE* (biofilm-associated proteins) genes, *tss* (type VI secretion system,T6SS) genes, *AbaI*/*AbaR* (quorum sensing) genes and *hemO* (heme oxygenase) gene and were 97.4%(38/39), 97.4%(38/39), 84.6%(33/39) and 71.8%(28/39), respectively.

Multilocus Sequence Typing and cgMLST Analysis

We used the MLST database (https://pubmlst.org/organisms/acinetobacter-baumannii) Pasteur and Oxford in the classification scheme to predict MLST information for the 39 samples in this study.

In the prediction results based on the Oxford typing scheme, because of the multiple copies of the gdhB gene (different copies had different allele numbers), most samples had two ST types. Six different ST types were identified

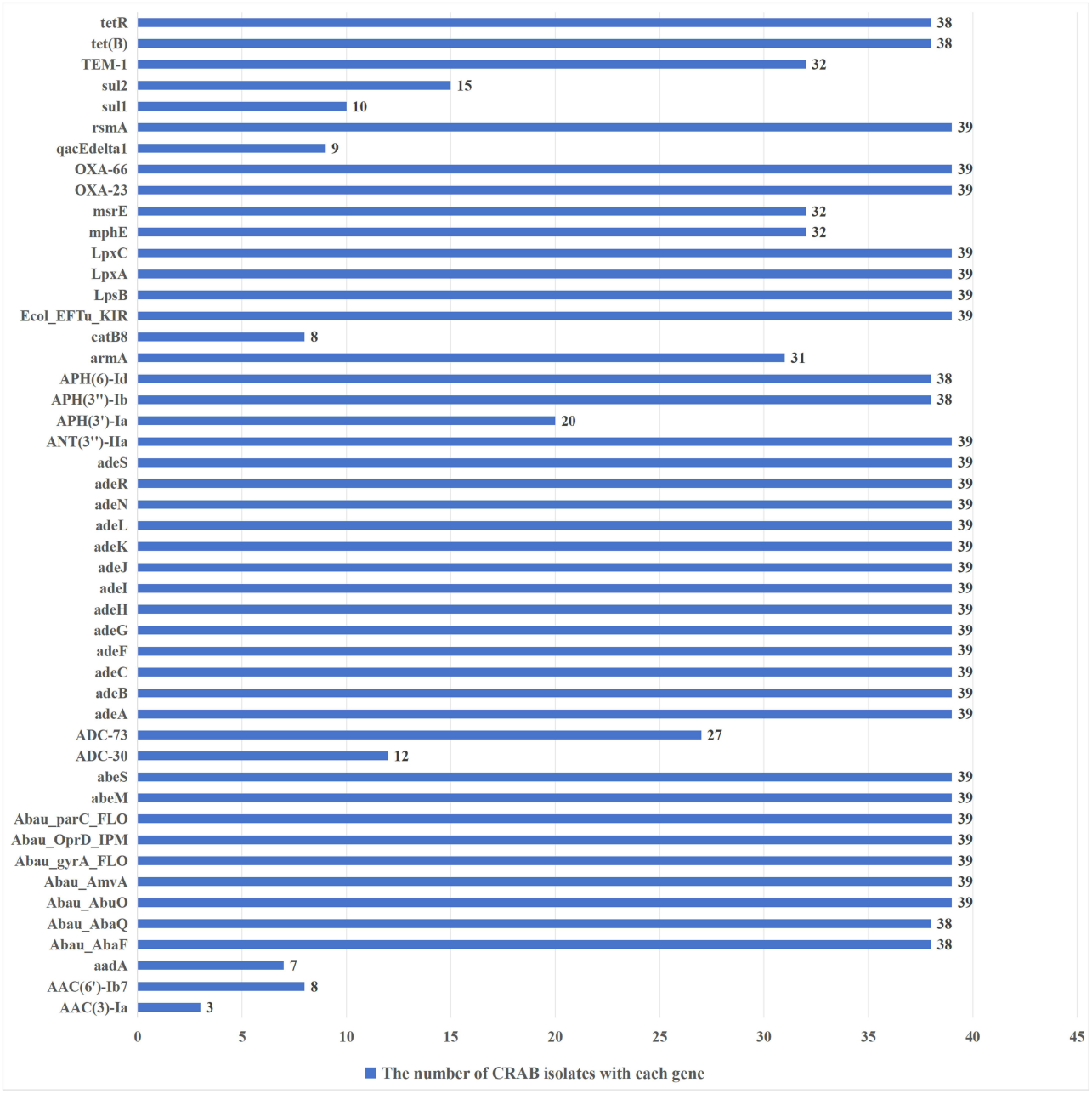

Figure 2 The number of CRAB isolates with each drug resistance gene.

based on the Oxford typing method (Table 2). The six ST types were ST2149 or ST938, ST191,ST136 or ST1851, ST1816 or ST195, ST540, ST1791 or ST2158.

As shown in Table 2, ST2149 or ST938 included ten strains: AB01-AB06, AB08-AB11. These strains were isolated from the ICU and NICU between 5–30-2020 and 8-5-2021. The ST-type AB07 strain isolated from patients in the old NICU was ST191. ST136 or ST1851 included five strains, AB12, AB13, AB15, AB17, and AB31, isolated from the ICU and NICU from 6–11-2021 to 5–23-2023. ST1816 or ST195 included six strains, AB14, AB16, AB18, and AB19, isolated from the ICU and old ICU from 11–27-2021 to 12–24-2021. ST540 included 11 strains: AB20-AB24, AB26, AB29, AB30 and AB32-AB34 isolated from the ICU and NICU from 10–10-2022 to 7–28-2023. ST1791 or ST2158 included eight strains from the NICU: AB25, AB27, AB28, and AB35-AB39, isolated from 10–25-2022 to 5–21-2023.

Table 2 Multilocus Sequence Typing Analysis Results Based on Oxford and Pasteur Classification Scheme

Sample ID	Isolation Date	Clinical Department	Clinical Specimen	ST type	
				Oxford	Pasteur
AB01	5-30-2020	ICU	Sputum	2149, 938	2
AB02	6-1-2020	ICU	Sputum	2149, 938	2
AB03	6-14-2020	ICU	Hydrothorax	2149, 938	2
AB04	7-17-2020	ICU	Sputum	2149, 938	2
AB05	7-20-2020	ICU	Wound secretion	2149, 938	2
AB06	6-14-2020	NICU	Sputum	2149, 938	2
AB07	7-9-2020	NICU(old)	Sputum	191	2
AB08	7-18-2020	NICU	Sputum	2149, 938	2
AB09	10-9-2020	NICU	Sputum	2149, 938	2
AB10	7-13-2021	ICU	Blood	2149, 938	2
AB11	8-5-2021	ICU	Wound secretion	2149, 938	2
AB12	12-8-2021	ICU	Sputum	136, 1851	2
AB13	12-22-2021	ICU	Wound secretion	136, 1851	2
AB14	12-24-2021	ICU	Hydrothorax	1816, 195	2
AB15	6-11-2021	NICU	Sputum	136, 1851	2
AB16	11-27-2021	ICU(old)	Sputum	1816, 195	2
AB17	12-6-2021	NICU	Sputum	136, 1851	2
AB18	12-6-2021	ICU(old)	Sputum	1816, 195	2
AB19	12-8-2021	ICU(old)	Sputum	1816, 195	2
AB20	10-10-2022	ICU	Ascitic fluid	540	2
AB21	10-26-2022	ICU	Sputum	540	2
AB22	11-20-2022	ICU	Ascitic fluid	540	2
AB23	2-23-2023	ICU	Sputum	540	2
AB24	2-27-2023	ICU	Sputum	540	2
AB25	10-25-2022	NICU	Sputum	1791, 2158	2
AB26	10-29-2022	NICU	Sputum	540	2
AB27	11-15-2022	NICU	Sputum	1791, 2158	2
AB28	11-18-2022	NICU	Midstream urine	1791, 2158	2
AB29	10-28-2022	NICU	Sputum	540	2
AB30	5-22-2023	ICU	Catheter	540	2
AB31	5-23-2023	ICU	Sputum	136, 1851	2
AB32	6-28-2023	ICU	Ascitic fluid	540	2

(*Continued*)

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Table 2 (Continued).

Sample ID	Isolation Date	Clinical Department	Clinical Specimen	ST type	
				Oxford	Pasteur
AB33	7-22-2023	ICU	Sputum	540	2
AB34	7-28-2023	ICU	Blood	540	2
AB35	4-16-2023	NICU	Sputum	1791, 2158	2
AB36	4-28-2023	NICU	Sputum	1791, 2158	2
AB37	5-12-2023	NICU	Sputum	1791, 2158	2
AB38	4-10-2023	NICU	Sputum	1791, 2158	2
AB39	5-21-2023	NICU	Sputum	1791, 2158	2

All 39 CRAB isolates were classified as ST2 based on the Pasteur typing formula. Minimum spanning trees were constructed based on the Pasteur typing results. In addition to the 39 samples in this study, reference strains from the Pasteur database were added for analysis, and the results were displayed in two parts. The first part included all samples from all over the world (Figure 3A and B), and the second part included all samples from China in the database (Figure 4A and B).

As shown in Figure 3A, North America had the largest sample size (5919), followed by Asia (3492). The largest clonal complex (CC) was CC1 (Figure 3B). ST2 was included in CC1 as the group founder. In CC1, 54.7% of the samples were from North America, 30.3% were from Asia, and the remaining 15% were from other regions. The main CC group of the samples from North America and Asia was CC1. It indicated that the main epidemic types in the two regions were similar. The ancestor ST2 may have been the most original source of other strains. Different ST strains were formed after different evolutionary processes. The diversity of the strains in Asia was higher than that in North America.

Regarding sample sources in China (Figure 4A and B), 699 (40.6%) samples were isolated from Hangzhou, Zhejiang Province, 453 (26.3%) samples had no clear source, 234 (13.6%) samples were isolated from Beijing, 71 (4.1%) samples were separated from Shanghai, and 66 (3.8%) samples were from Guangzhou. As shown in Figure 4A and B, ST2 was the main ST type, and isolates from Anqing City (39 CRAB strains in this study), Hangzhou City, Beijing City, Guangzhou City and so on clustered together and formed the largest circle in the figures.

According to the cgMLST classification (Table S5), 39 samples were divided into ten types, among which AB01, AB02, AB03, AB05, AB06, AB08, AB10, and AB11 belonged to the same cgMLST type. AB27 and AB28 had the same cgMLST. AB12, AB15, AB17, and AB31 belonged to the same cgMLST type. AB14, AB16, AB18, and AB19 belonged to the same type of cgMLST. AB20-AB24, AB26, AB29, AB30, and AB32-AB34 had the same cgMLST type. AB25, AB35 and AB36-AB39 belonged to the same cgMLST type. Four samples (AB04, AB07, AB09, and AB13) were classified into different types.

The minimum spanning tree was labeled with the sample ID (Figure 5A), sample separation department (Figure 5B), specimen type (Figure 5C), and isolation date (Figure 5D). The 20 isolates from the ICU included six STs, 15 isolates from the NICU contained six STs, three isolates from the old ICU had the same ST clone, and only one strain from the old NICU had adifferent ST clone compared with other isolates (Figure 5B). Nine strains isolated in 2020 and ten strains isolated in 2021 were assigned to 4 STs, 8 isolates from 2022, and 12 isolates from 2023 were assigned to 3 STs (Figure 5D).

Whole Genome Average Nucleotide Identity Analysis

As shown in Table S6 and Figure 6, there was high similarity among the 39 samples (ANI > 0.99). According to the level of ANI similarity, the 39 samples could be divided into six clusters, and the samples in each cluster were more similar.

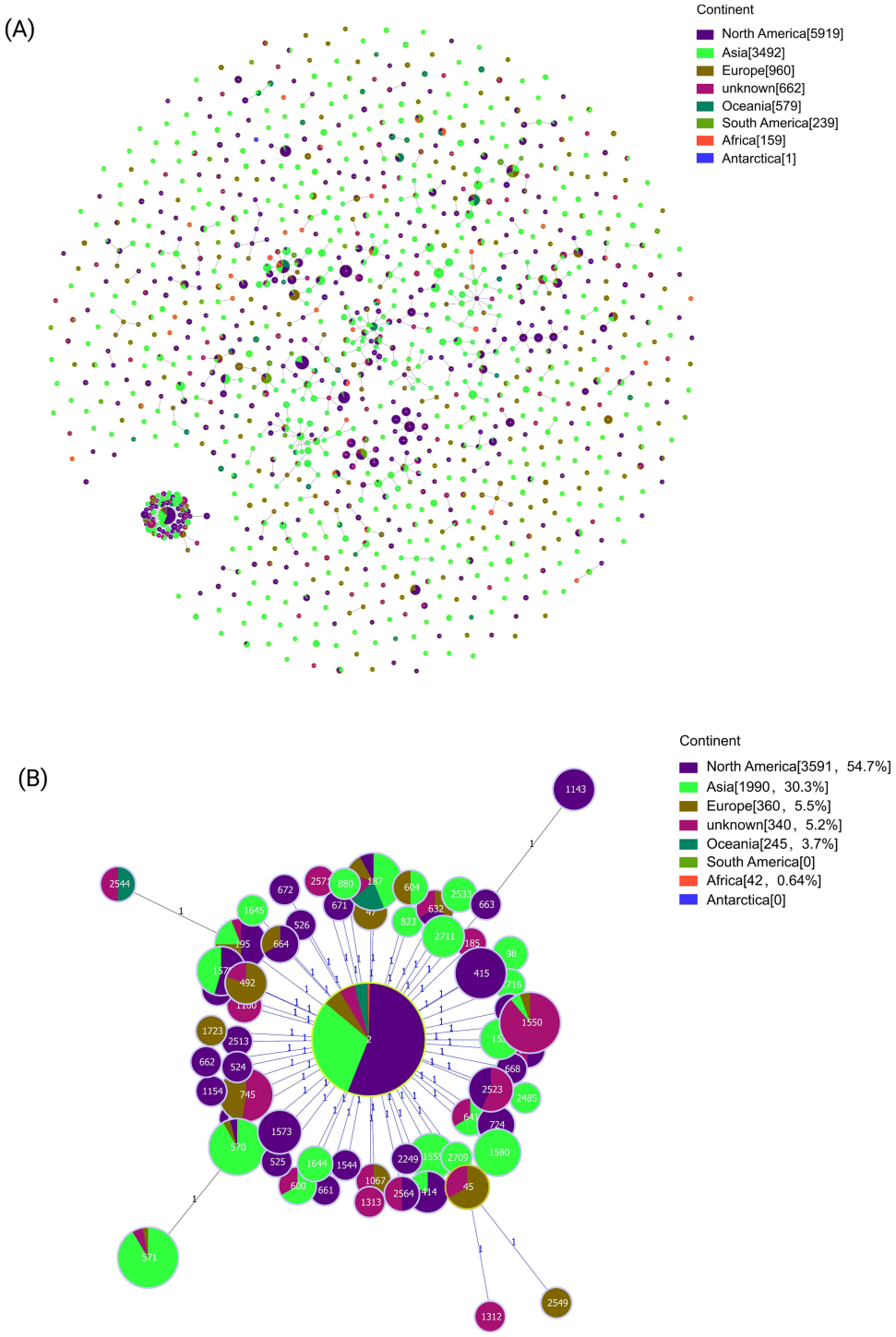

Figure 3 Minimum spanning tree using phyloviz software based on global data. (**A**) Minimum spanning tree using phyloviz software based on global data (Pasteur database). (**B**) Minimum spanning tree using phyloviz software based on global data (Pasteur database) of CC1.

Notes: (**A**) Each circle represents an ST type; the size of the circle is positively correlated with the number of samples, and the color of the circle indicates the origin of the sample. A single circle, which is not connected with other ST circles in a net-like structure, is a singleton, indicating that this ST is very different from other ST's and cannot be clustered into a cluster. A network structure formed by two or more circles is called CC, as shown in the figure above, with a total of 95 CC and 629 singletons. (**B**) Each circle represents an ST type; the size of the circle is positively correlated with the number of samples, and the color of the circle indicates the isolation information of the sample.

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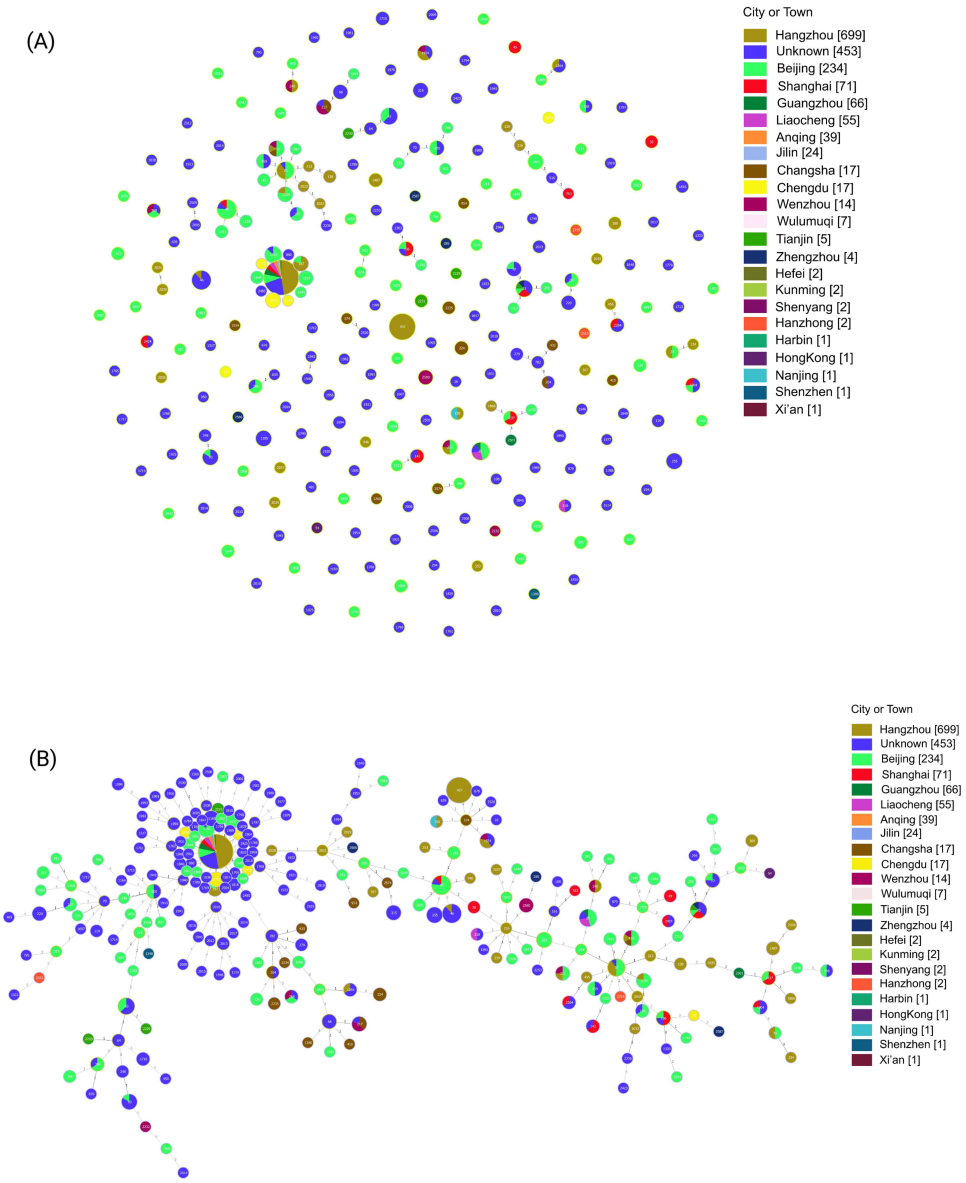

Figure 4 Minimum spanning tree using phyloviz software based on China data. (**A**) Minimum spanning tree of China based on Pasteur database using phyloviz. (**B**) Minimum spanning tree of China constructed at a high level based on Pasteur database using phyloviz.
Notes: (**A**) Each circle represents an ST type; the size of the circle is positively correlated with the number of samples, and the color of the circle indicates the clinical source of the sample, as detailed in the figure note. (**B**) Each circle represents an ST type; the size of the circle is positively correlated with the number of samples, and the color of the circle indicates the clinical source of the sample, as detailed in the figure note.

The results were basically consistent with the Oxford typing method (Table 2). AB07 showed a relatively large difference from the other samples and was a single group in the heat map.

Whole Genome Single Nucleotide Polymorphism Cluster Analysis and Core SNP Analysis

The similarities among the 39 samples were analyzed at the SNP level. As shown in Table S7 and Figure 7, the sample cluster obtained at the SNP level was consistent with the ANI results. The number of SNPs in the same cluster was less than 20. The SNP differences between AB07 and other samples were relatively large. This formed a single clade in the heat map of SNP.

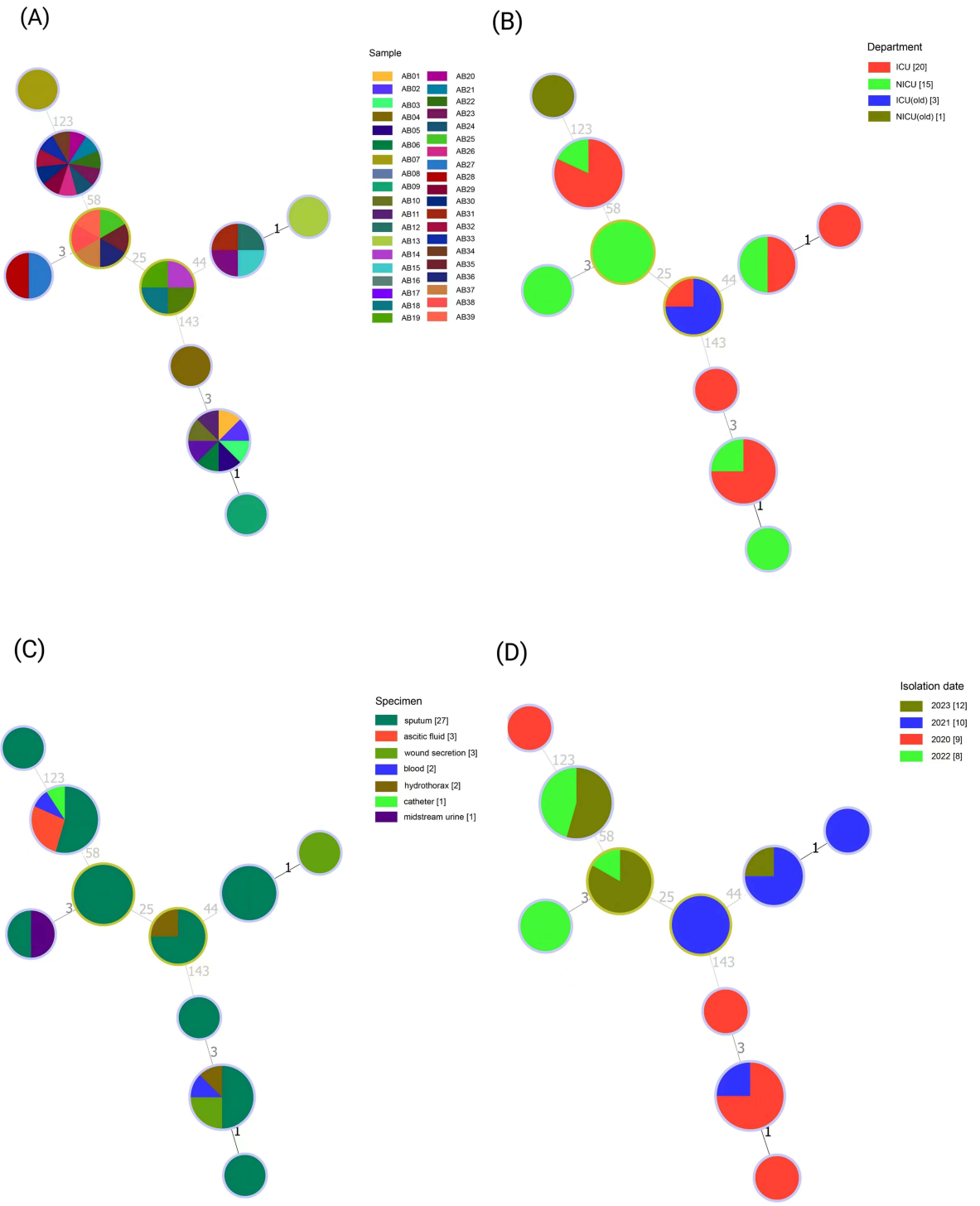

Figure 5 Minimum spanning tree constructed based on cgMLST allelic genes. (**A**) Minimum spanning tree constructed based on cgMLST allelic genes labeled with sample ID. (**B**) Minimum spanning tree constructed based on cgMLST allelic genes labeled with sample isolation department. (**C**) Minimum spanning tree constructed based on cgMLST allelic genes labeled with sample type. (**D**) Minimum spanning tree constructed based on cgMLST allelic genes labeled with sample isolation date.
Notes: The circles represent ST types, and the numbers on the lines represent allelic differences.

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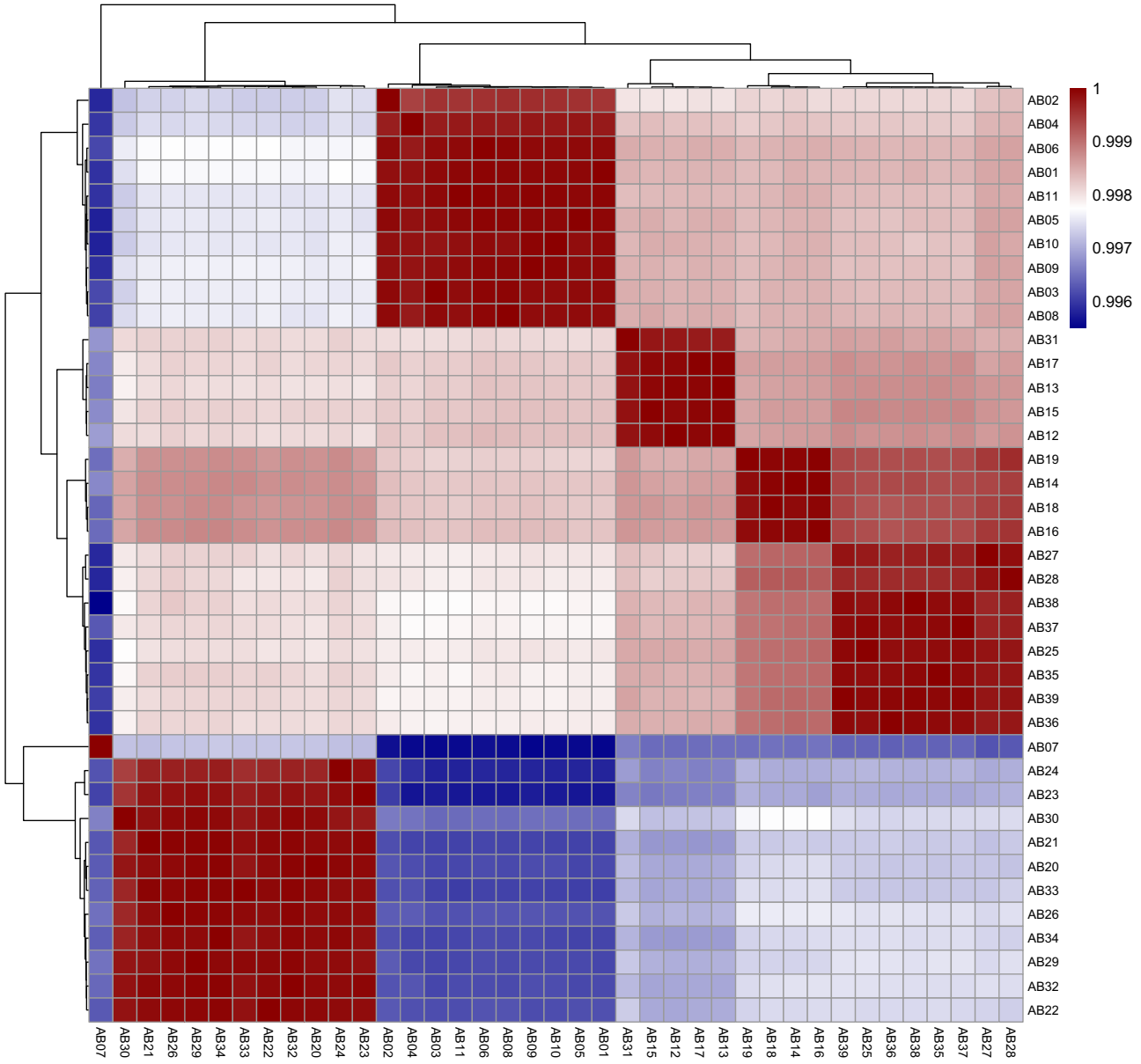

Figure 6 Heat map for 39 CRAB strains based on ANI among strains.

In Figure 8, the tree on the left was the phylogenetic tree of 39 CRAB strains. The distribution of drug-resistance genes and virulence genes of each sample were located in the middle and on the right of the figure, respectively. Consistent with previous results, the 39 samples were divided into six clusters. The evolutionary relationship between AB07 and the last cluster (from top to bottom in Figure 8) was relatively close. As can be seen from the figure, the distribution patterns of AMRs and VFGs were consistent with the results of whole-genome average nucleotide identity analysis and core-SNP evolutionary tree. We found that strains with similar whole-genome sequences had more similar distribution characteristics of resistance and virulence genes. AB20-AB24, AB26, AB29, AB32 and AB34 had more drug-resistance genes than other samples, while they had fewer virulence genes. Phylogenomic analysis revealed that the strains in the top five clusters did not contain seven resistance genes: *AAC(3)-Ia, blaADC-30, aadA, AAC(6')-Ib7, catB8, qacEdelta1*, and *sulI*. Phylogenetic analysis of the virulence genes showed that the sixth cluster (AB21, AB20, AB24, AB23, AB30, AB34, AB32, AB33, AB29, AB26 and AB22) did not detected with the *hemO, pseBCFGHI*, and *tviB* genes. The majority of the strains (AB20-AB24, AB26, AB29, AB32 and AB34) in this cluster were

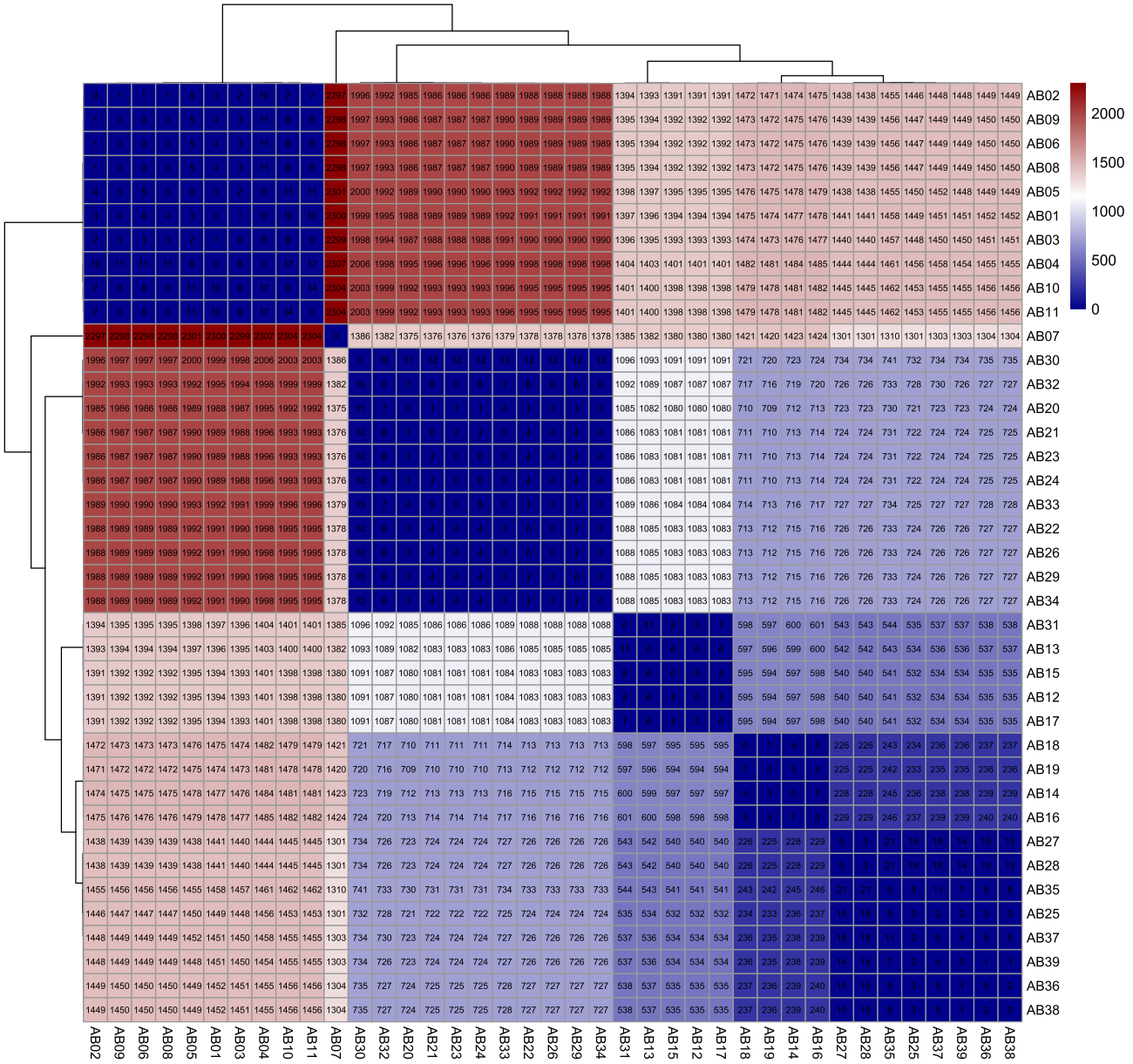

Figure 7 Heat map for 39 CRAB isolates based on SNP distance among strains.

mainly isolated from the ICU during 10–10-2022 and 7–28-2023, all of which were ST540 clones based on the Oxford MLST method (Table 2).

Discussion

It is becoming increasingly obvious that CRAB is a prominent cause of hospital-acquired infections among a variety of bacteria, especially in intensive care units (ICUs). Patients with critical illness are significantly hindered by its spread and multidrug resistance.[34] There have been many cases of outbreaks in hospital ICUs caused by CRAB strains, which pose a significant infection control challenge for healthcare facilities in recent years.[35,36] A report from US hospitals from 2014 to 2019 revealed that patients with CRAB infections have a significantly higher burden of illness and higher risk of mortality than those with carbapenem sensitive *A. baumannii* infections.[37] The resistance heatmap of China in the China Antimicrobial Surveillance Network (CHINET) shows that the detection rate of CRAB in Anhui Province and whole China

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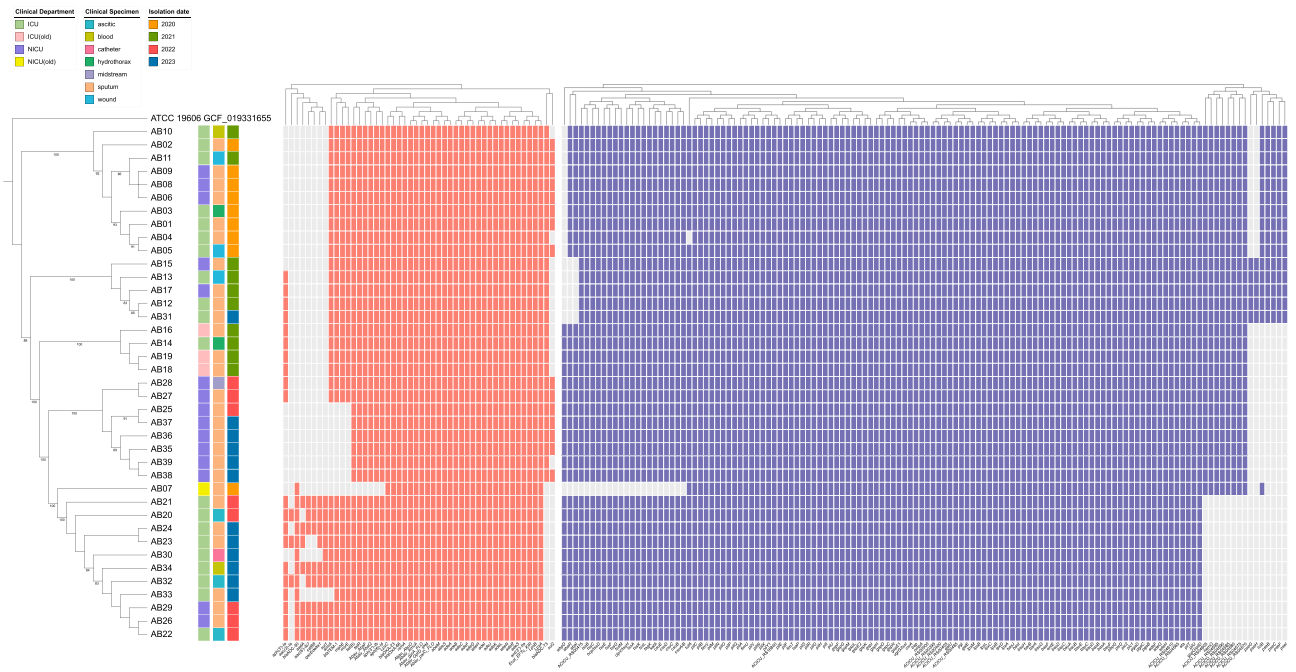

Figure 8 Phylogenetic tree based on core SNP and heat maps of the detection of ARGs and VFGs among the 39 CRAB isolates.

was 86.3% and 71.2%, respectively, in 2022 (https://www.chinets.com/Data/GermYear). Therefore, it is necessary to detect epidemiology of CRAB and implement effective measures to curb this trend. Based on the results of this study, CRAB resistance patterns and molecular phenotypes in this area were largely consistent over the four years.

The resistance rates of CRAB to several antibiotics in this study were extremely high, such as the resistance to carbapenems (imipenem and meropenem), β-lactam drugs (piperacillin-tazobactam, cefoperazone-sulbactam, ceftazidime), and quinolones (ciprofloxacin, levofloxacin). In recent years, carbapenem resistance has increased owing to its prior use in treating multidrug-resistant *A. baumannii* infections.[38] CRAB has emerged as an abuse of carbapenem, which poses a huge challenge to clinical treatment. Tigecycline, eravacycline, minocycline, cefiderocol, and polymyxins are currently available treatments for CRAB.[39–41] Pandrug-resistant *A. baumannii*, found worldwide, clearly illustrates the impact of emerging resistance to last-resort antimicrobials (for example, tigecycline and polymyxin).[42] Among which, tigecycline is considered a last-line antibiotic for the treatment of CRAB due to its strong bacteriostatic properties. Unfortunately, there has been an increase in worldwide reports of tigecycline-resistant *A. baumannii*.[43] The mechanisms underlying tigecycline resistance in *A. baumannii* are complex and multifactorial, involving various genetic mutations and the overexpression of efflux pumps, particularly the AdeABC system.[44,45] We found that nearly half of the CRAB strains showed intermediate to tigecycline and minocycline in this study. All isolates were susceptible to colistin. In recent years, colistin-resistant *A. baumannii* has emerged as a significant global health threat.[46] Infections caused by carbapenem-resistant *A. baumannii* (CRAB) are notoriously difficult to treat due to limited therapeutic options. Colistin, while often considered a last-resort antibiotic, is associated with nephrotoxicity, complicating treatment regimens.[47] Studies have shown that colistin resistance can develop rapidly, particularly in patients undergoing prolonged treatment, underscoring the need for effective antimicrobial stewardship.[48] The rise of CRAB, tigecycline-resistant *A. baumannii*, and colistin-resistant *A. baumannii* necessitates ongoing surveillance, research into resistance mechanisms, and the development of novel therapeutic strategies to combat these formidable pathogen.

According to ARG analysis, CRAB isolates from the ICU and NICU contained a wide variety of resistance genes. All the CRAB isolates contained *bla*OXA-23, which encodes a class D carbapenemase gene in *A. baumannii*. There have been reports of *bla*OXA-23 in many countries worldwide, such as in China,[49] USA,[50] Turkiye,[51,52] Thailand[53] and Africa[54] that reported in recent five years. In addition, *bla*OXA-66 was detected accompanied with the *bla*OXA-23 gene in this study. *Bla*OXA-66 is a member of the *bla*OXA-51-like gene family, which is an intrinsic oxacillinase with low

carbapenemase activity, found on *A. baumannii's* chromosome. To date, the OXA-51 family is the most extensively mentioned group of OXA beta-lactamases and has numerous genes compared with other class D carbapenemase genes, such as OXA-23 family, OXA-24 family, OXA-58 family, OXA-143 family and OXA-134 family genes.[55] Extended-spectrum β-lactamase (ESBL) genes (*blaTEM-1*,32/39), other β-lactamase genes (*bla*ADC-30,12/39; *bla*ADC-73,27/39) were found in some CRAB isolates. 31/39 of the CRAB strains contained the *armA* gene, which was reported as giving high-level resistance to aminoglycosides, particularly amikacin, gentamicin, plazomicin, and tobramycin.[56] On the whole, these CRAB strains contained genes that encoded resistance to β-lactams, carbapenem, aminoglycoside, tetracycline, macrolide, fluoroquinolone, peptide, phenicol and sulfonamide. The tremendous resistance genes and severe phenotypic resistance rates detected in this study revealed that hospitals must take immediate action on the rational use of antimicrobial agents.

In the present study, we identified abundant virulence-factor-associated genes. VFGs include biofilm formation, adherence, stress survival, regulation, nutritional/metabolic factors, exotoxins, effector delivery systems, nutritional/metabolic factors and immune modulation. There may be a link between these virulence genes and greater pathogenicity and severity of infection among CRAB isolates.[57] The various VFGs found in this study were basically consistent with recent researches.[17,49,53,58] All the strains showed a lack of BfmRS which was known as an important factor in motility of *A. baumannii*.[59] Poly-β-(1, 6)-N-acetylglucosamine (PNAG) is encoded by a cluster of four genes (*pgaABCD*),[60] *pgaB, pgaC*, and *pgaD* were 100% found in all 39 CRAB isolates. It has been demonstrated in numerous studies that PNAG is crucial for maintaining the integrity of biofilms of *A. baumannii* in stressful and dynamic conditions.[61,62] OmpA (outer membrane protein A) is one of the most widely recognized and characterized virulence factors of *A. baumannii*. It is required for the development of robust biofilms on abiotic surfaces.[63] All the isolates in this study contained the *ompA* gene. A quorum sensing (QS) process allows bacteria to communicate with each other based on the density of the bacterial population. *AbaI*/*AbaR* regulates the QS system in *A. baumannii*. In *A. baumannii* ATCC 17978, a functional QS system is necessary for biofilm formation and surface-associated motility.[64] Except for AB07, the other 38 isolates possessed the type VI secretion system (T6SS), which is composed of 13 core *tss* genes and a variable number of tag genes (T6SS-associated genes) at least.[65] The T6SS system is a widely spread nanomachine used by bacteria to eliminate competition and give the bacteria growth advantages in settling natural habitats.[66,67] The multiple VFGs should be noticed in subsequent research.

During the four years of this study, the epidemiological characteristics of the CRAB isolates did not change significantly. All isolates harbored *blaOXA-23* and *blaOXA-66*, which mediate resistance to carbapenems. The epidemic sequence type of these isolates was ST2 based on the Pasteur MLST method. In addition to being the most prevalent clonal type reported worldwide and documented in various databases, ST2 is common in China.[68] In terms of resolution in distinguishing *A. baumannii*, the Oxford scheme showed a better ability than the Pasteur scheme. However, the copy of the Oxford scheme in this study makes us puzzle in the subsequent analyses. Based on the detection of seven housekeeping genes, MLST has been commonly used since 1998 for assessing clonal relationships among bacteria.[69] A comparative analysis of the two *A. baumannii* MLST schemes showed that they have complementary characteristics, each with their own advantages. The Pasteur method exhibits reduced discrimination but excels at identifying clonal lineages and generally performs better when comparing distant clones.[70] As ST2 is the most prevalent strain in China as shown in our research and related references, the Pasteur method might not be applicable for CRAB isolates.[49,71] Furthermore, the Oxford scheme better distinguishes between strains at short evolutionary distances and shows a higher degree of concordance with phylogenies. It should be noted, however, that an alternative gdhB locus appears in a majority (533/730) of the genomes of *A. baumannii* in the genomic era.[70] So in our research, we chose the Pasteur scheme for MLST classification of the CRAB strains for further minimum spanning tree construction based on databases of China and the World. However, a previous study showed that neither the MLST scheme for *A. baumannii* was able to recover the correct relationships among the isolates. As genome sequencing becomes increasingly affordable, it will serve as an ideal method for identifying bacterial species.[72] Molecular typing methods with higher discriminatory ability are crucial for elucidating the epidemiology of hospital outbreaks and pathogenic bacteria.

Whole-genome sequencing (WGS) has become a promising tool for isolate identification, molecular epidemiology, and nosocomial infection outbreak analysis in microbiological laboratories over the past decade.[73] The core genome

MLST (cgMLST) analysis based on genome sequencing results showed that all strains could be divided into ten groups. The six clusters obtained at the whole-genome SNP level were consistent with the ANI results, and the number of SNPs in each cluster was basically less than 20. In general, homologous and phylogenetic analyses based on the whole genome showed very strong similarity among the strains. The results imply several transmissions over four years. However, to date, among strains of *A. baumannii*, the transmission inference threshold for *A. baumannii* strains has not been established. Fourteen such strains collected from a single individual showed a range of 1–40 SNP differences between them which suggested that *A. baumannii* isolates have a higher threshold for inferring transmission.[74] In our study, SNPs distances of pair isolates less than 20 were regarded as closely related evolutionary isolates or genetically related clones. *A. baumannii* is capable of surviving in the environment for a long time and is capable of transmitting the infection.[75] Multi-drug resistant *A. baumannii* can be kept in contaminated environments and equipment.[76] In our research, isolates belonging to the same SNP cluster from ICU (such as AB12, AB13 and AB31) isolated from different patients were sustained for several years. The results suggested that some transmissions may have taken place indirectly via contaminated environments or healthcare personnel, which is consistent with several previous studies.[77,78] In addition, we observed transmission between the ICU and NICU, as some strains clustered together from the two departments. From the heat map of SNP, the phylogenetic tree and the separation time of strains, we can also speculate that several transmissions have occurred in the ICU and NICU, as some CRAB strains were detected in a short time with the same genotype.

To our knowledge, the advantage of this research is firstly reporting of epidemiology and genomic characterizations of CRAB isolated from ICU and NICU in East China. It reveals the huge carrying of ARGs and VFGs of CRAB. High-resolution cgMLST and SNP methods showed the transmissions of ST2 clone during the four years. Effective measures should be implemented for reducing the incidence of CRAB. In addition, we compare the two schemes of MLST method (Pasteur and Oxford) in distinguishing CRAB isolates. However, there are a few limitations to our research. First, the sample size of this study is limited. The CRAB strains were not fully collected and resuscitated in our hospital. Our next plan is to collect more CRAB strains in several hospitals in the area. Second, specimens from the environment of ICU were not collected at the same time when possible transmission occurred. Absent of environmental samples leaded the difficulty of explaining the pathway of dissemination of CRAB. Finally, the genetic environment of *bla*OXA23 gene was not described. The prediction of insertion sequence (IS) elements and the integrons were important to detailing the drug-resistance mechanism of CRAB.

Conclusion

In conclusion, this is the first report on the molecular epidemiology and genetic characterization of CRAB isolates from ICU and NICU in East China. All the CRAB strains showed high rates of antimicrobial resistance and possessed multiple antimicrobial genes and virulence factor genes. All CRAB were *bla*OXA-23-producing strains and ST2 clones characterized by MLST during four years. This study identified the emergence of several transmissions of *blaOXA-23* CRAB ST2 clones in hospital. All in all, our findings underscore the successful dissemination of ST2 clones in a tertiary hospital in East China. CRAB strains harboring the acquired *bla*OXA-23 β-lactamase gene in ICU and NICU deserve full attention in hospital as they exhibit strong phylogenetic homology. CgMLST and SNP analysis based on WGS showed excellent discrimination power in this transmission and homology research. Effective infection prevention and control measures need to be implemented in the ICUs. Further molecular epidemiology detection and containment activities in different areas are urgently needed.

Data Sharing Statement

The datasets generated in this research can be found in the NCBI Bioproject with the accession number PRJNA1111166.

Ethics Statement

This study was approved by the Medical Ethics Committee of Anqing First People's Hospital of Anhui Medical University (AQYY–YXLL–KJXM–41). This study was retrospective and associated with bacterial drug susceptibility and the genetic information of the specimens, our ethical petition for exemption from informed consent was waived. All

the patient's data in this study were collected anonymously and ensured the confidentiality of their information. This study was conducted in accordance with the principles of the Declaration of Helsinki.

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Disclosure

The authors declare that they have no conflicts of interest.

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