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Short Communication

Phenotypic and molecular characterizations of carbapenem-resistant *Acinetobacter baumannii* isolates collected within the EURECA study

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ABSTRACT

Multi-drug-resistant *Acinetobacter baumannii* isolates are key pathogens that contribute to the global burden of antimicrobial resistance. This study aimed to investigate the phenotypic and molecular characteristics of carbapenem-resistant *A. baumannii* (CRAB) isolates from the EURECA clinical trial. In total, 228 CRAB clinical strains were recovered from 29 sites in 10 European countries participating in the EURECA study between May 2016 and November 2018. All strains were reconfirmed centrally for identification and antimicrobial susceptibility testing, and were then subjected to DNA isolation and whole-genome sequencing (WGS), with analysis performed using BacPipe v.1.2.6. K and O typing was performed using KAPTIVE. Overall, 226 (99.1%) strains were confirmed as CRAB isolates. The minimum inhibitory concentration (MIC_{90}) results of imipenem and meropenem were >16 mg/L. WGS showed that the isolates mainly harboured bla_{OXA-23} (n=153, 67.7%) or bla_{OXA-72} (n=70, 30.1%). Four bla_{OXA-72} isolates from Serbia co-harboured bla_{NDM-1}. An IS*5* transposase family element, IS*Aba31*, was found upstream of the bla_{OXA-72} gene harboured on a small (~10-kb) pSE41030-EUR plasmid. The majority of isolates (n=178, 79.1%) belonged to international clone II. Strains belonging to the same sequence type but isolated in different countries or within the same country could be delineated in different clusters by core-genome multilocus sequence typing (MLST). Whole-genome/core-genome MLST showed high diversity among the isolates, and the most common sequence type was ST2 (n=153, 67.7%). The EURECA *A. baumannii* strain collection represents a unique, diverse repository of carbapenem-resistant isolates that adds to the existing knowledge of *A. baumannii* epidemiology and resistance genes harboured by these strains.

1. Introduction

Pan-drug-resistant Gram-negative bacteria have become a major public health concern [1], and are one of the leading causes of increased mortality in patients with infections caused by multi-drug-resistant (MDR) pathogens [2]. Among these, *Acinetobacter baumannii* is a common opportunistic pathogen, and is responsible for a wide array of healthcare-associated infections which often take place in an epidemic context. *A. baumannii* isolates are frequently MDR or extensively drug-resistant (XDR), mainly due to their intrinsic resistance to most antimicrobial agents and their ability to easily accumulate multiple mechanisms of resistance towards various antibacterials [3]. As such, this pathogen represents a serious challenge in terms of treatment of infections, and often has very few or no targeted therapeutic options available.

Carbapenem resistance in *A. baumannii* is predominantly linked to the production of carbapenemases, and rarely to the loss or modification of outer membrane proteins and/or the overexpression of active efflux pump systems [4]. In addition to the naturally occurring class D β-lactamases, several acquired carbapenem-hydrolysing class D β-lactamases (CHDLs) have been identified in

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A. baumannii, among which OXA-23-like enzymes are the most common. Other commonly found CHDLs include OXA-24-like and OXA-58-like enzymes. Most acquired CHDLs display weak carbapenemase activity, but the presence of specific insertion sequences upstream of the CHDL gene leads to their overexpression conferring carbapenem resistance. Class B carbapenemases have also been reported in *A. baumannii* isolates [5,6], but these occur at much lower frequency than CHDLs. Class A serine β-lactamases with carbapenemase activity have been reported occasionally. Multiple reports have highlighted higher resistance percentages of carbapenem-resistant *A. baumannii* from southern and eastern Europe than northern Europe. Of note, very high carbapenem resistance rates have been reported in the Balkan countries [6–10].

A. baumannii exhibits genetically highly diverse clones that present a limitation for typing methods such as multi-locus sequence typing (MLST). Due to this low-level conservation at genetic level, the seven housekeeping-gene-based typing schemes have low discriminatory power. Thus, epidemiological markers are essential to determine and differentiate any MDR clones. Also, capsular polysaccharide and outer core (OC) lipo-oligosaccharide compositions and sugar arrangements are a very important barrier to the determination of virulence, antibiotic resistance and serotypes [11]. They can be one of the potential targets for vaccine development, and OC and K loci can be used as epidemiological typing markers.

The aim of this study was to investigate the phenotypic and molecular characteristics of carbapenem-resistant *A. baumannii* (CRAB) clinical isolates collected from 10 European countries within the EUropean prospective cohort study on Enterobacteriaceae showing REsistance to CArbapenems (EURECA). The EURECA study (NCT02709408) is a prospective, multi-centre, analytical observational study; it is part of the COMBACTE-CARE project and the New Drugs for Bad Bugs programme funded by the Innovative Medicines Initiative [12].

2. Materials and methods

2.1. Study design and patients

The EURECA study was designed to include three substudies focusing on infections caused by carbapenem-resistant Enterobacterales (CRE) [13], as well as a substudy on bacteraemia caused by CRAB to identify risk factors and assess mortality in patients with such infections. The study recruited patients with CRE (pneumonia, intra-abdominal, complicated urinary tract and bloodstream infections) and CRAB (bloodstream infections alone) from 50 sites in 10 European countries (Albania, Croatia, Greece, Italy, Kosovo, Montenegro, Romania, Serbia, Spain and Turkey) between May 2016 and November 2018.

2.2. Clinical samples and collection of bacterial isolates

Local laboratories in all 50 participating sites were instructed to process blood cultures from EURECA-enrolled patients according to their local standard procedures. Study-specific training was provided to all sites. All *A. baumannii* isolates (or *Acinetobacter* spp. if identification was not done to species level) from these cultures that were resistant to carbapenems according to the EUCAST clinical breakpoints (Version 6.0, 2016) [minimum inhibitory concentration (MIC) >8 mg/L for imipenem or meropenem and/or zone of inhibition <17 mm for imipenem and/or <15 mm for meropenem] were stored in microbanks frozen at -20°C or below until the end of the recruitment period, and shipped to the central laboratory at the University of Antwerp for confirmation and further testing.

2.3. (Re-)identification and further confirmation of bacterial isolates

All *A. baumannii* isolates transferred to the central laboratory were (re-)identified by matrix-assisted laser desorption/ionization-time of flight (Bruker Daltonics, Bremen, Germany) (library updated to v.9.0 which contains a total of 8468 reference spectra), and checked for carbapenem susceptibility by the disc diffusion method using imipenem 10-µg (Becton Dickinson, Franklin Lakes, NJ, USA) and meropenem 10-µg (Rosco Diagnostica, Taastrup, Denmark) discs. Antimicrobial susceptibility testing results were interpreted according to the EUCAST guidelines (v.9.0, 2019) (imipenem susceptible ≥24 and resistant <21 mm; meropenem susceptible ≥21 and resistant <15 mm). A check for consistency with data reported above for screening was performed.

2.4. Determination of minimum inhibitory concentrations

MIC testing was performed using the broth microdilution method at a EURECA-partner laboratory (Ramón y Cajal University Hospital-SERMAS, Madrid, Spain). A 96-well plate (Thermofisher Scientific, Waltham, MA, USA) with various dilutions of 16 different antibiotics was designed (Fig. S1, see online supplementary material). The panel was used to test all isolates from the EURECA study (Enterobacterales and *A. baumannii*). Results were interpreted using the EUCAST guidelines (v.9.0, 2019).

2.5. Genomic DNA isolation and whole-genome sequencing

Genomic DNA was extracted using the MasterPure DNA Purification Kit (Epicentre Technologies Inc., Madison, Wisconsin, USA.), and library prepared using Nextera XT, sequenced via 2 × 250 bp V2 500 cycle (Miseq, Illumina Inc., San Diego, CA, USA). Secondary data analysis, such as information on carbapenemase genes, was extracted from whole-genome sequencing (WGS) analysis using in-house-developed pipeline BacPipe v.1.2.6 [14]. In a first step, a plasmid finder was used to identify the plasmid replicon type. Specific contigs were selected using a BLAST search against the National Center for Biotechnology Information (NCBI) databases. Identifying the highest similar and query covered plasmid were selected for comparative analysis. Insertion sequence (IS) type and family were defined using IS finder (https://isfinder.biotoul.fr/).

A gene-by-gene-approach-based allelic loci comparison was performed using chewBBACA [15] by generating a customized study-specific scheme along with two publicly available complete genome sequences (CP009257.1 and CP010779.1). Whole-genome/core-genome multi-locus sequence (cg/wgMLST) allelic loci distances were calculated and visualized using GrapeTree [16]. The tree can be further visualized in microreact. Relatedness criteria for wg/cgMLST clustering was considered at a threshold of eight or fewer allelic differences, as shown previously [17]. The capsular polysaccharides (K locus) and outer core biosynthesis (OC locus) were predicted using local KAPTIVE databases [11]. Comparing partitions analysis was performed using an online tool for Simpson's index diversity, adjusted Rand and adjusted Wallace. All sequences from this study have been deposited under BioProject ID PRJNA673068.

3. Results

3.1. Clinical isolates

In total, 228 CRAB strains from 29 laboratories (in 10 countries) were collected from blood cultures alone and transferred to the central laboratory. The majority of isolates originated from patients hospitalized in Balkan countries, predominantly Serbia

Table 1
Minimum inhibitory concentration values (MIC_{50} and MIC_{90}) of 226 *Acinetobacter baumannii* isolates from the EURECA study.

Antibiotic	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC ranges of tested isolates	%S	%R
Piperacillin-tazobactam	>64/4	>64/4	>64/4	No BP	No BP
Ceftazidime	>32	>32	16 –>32	No BP	No BP
Ceftazidime-avibactam	64/4	>64/4	2/4 –>64/4	No BP	No BP
Ceftolozane-tazobactam	32/4	>32/4	1/4 –>32/4	No BP	No BP
Aztreonam	>32	>32	≤0.5 –>32	No BP	No BP
Aztreonam-avibactam	>16/4	>16/4	0.5/4 –>16/4	No BP	No BP
Imipenem	>16	>16	4 –>16	0.4	99.6
Meropenem	>16	>16	8 –>16	0.4	99.6
Ciprofloxacin	>2	>2	>2	-	100
Gentamicin	>8	>8	≤2 –>8	7.1	92.9
Tobramycin	>8	>8	≤2 –>8	19.1	80.9
Amikacin	>32	>32	≤8 –>32	4.4	95.6
Colistin	≤1	2	≤1 –>4	90.7	9.3
Tigecycline	2	2	≤0.5 –>2	No BP	No BP

S, susceptible; R, resistant; BP, breakpoint.

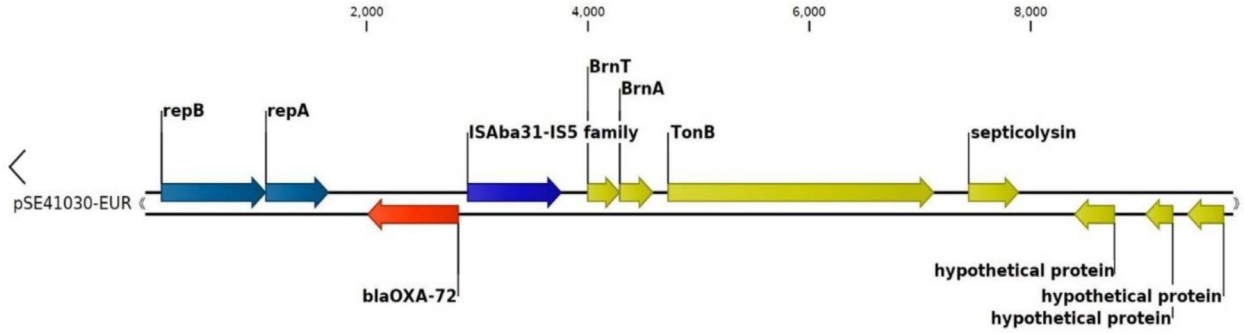

Fig. 1. Schematic diagram showing the genetic organization of plasmid pSE41030-EUR in ST492 *Acinetobacter baumanii* isolate (SE41030). The bla_{OXA-72} gene is flanked downstream by the IS*Aba31* element in the opposite direction, followed by the BrnT/BrnA type II toxin–antitoxin system, tonB-dependent outer membrane receptor (TonB), septicolysin (spI; pore-forming toxin involved in cytolytic activity) and three hypothetical proteins. Replication genes repB (primase) and repA (helicase) genes located upstream of bla_{OXA-72} code for proteins involved in the plasmid replication function.

(*n*=105, 46%, four sites), Greece (*n*=41, 18%, five sites) and Kosovo (*n*=32, 14%, one site) (Fig. S2, see online supplementary material)

3.2. Identification and susceptibility testing

The identification and susceptibility testing results of almost all *A. baumannii* isolates (*n*=226, 99.1%) matched the baseline local laboratory data. The MIC results of imipenem and meropenem confirmed the initial results by disc diffusion method. The MIC_{90} values of both carbapenems were >16 mg/L (Table 1). The majority of isolates exhibited resistance to almost all tested agents where breakpoints exist, except for colistin (MIC_{90}=2 mg/L).

3.3. Genomic characteristics

WGS data analysis revealed that the majority of isolates harboured bla_{OXA-23} (*n*=153, 67.7%), while bla_{OXA-72} was the second most common CHDL coding gene (*n*=70, 30.1%) (Fig. 1). Four isolates from Serbia that had bla_{OXA-72} co-harboured bla_{NDM-1}. bla_{OXA-58} was present in six isolates (2.7%), always in conjunction with bla_{OXA-72}. The four isolates co-harbouring bla_{OXA-72} and bla_{NDM-1} belonged to ST492, a recently reported sequence type (ST) [18], and had the same genetic background. Also, detailed analysis of the bla_{OXA-72} harbouring contig suggested that bla_{OXA-72} was located on plasmid pSE41030-EUR, which showed the highest sequence similarity (99% identity) with plasmid pA105-2 (accession no. KR535993) recovered from an *A. baumannii* strain of ST636 from Sweden [19] and with pAB-MAL-1 from an *A. baumannii* strain of ST492 recovered from Serbia [8]. The bla_{OXA-72} gene is flanked by IS element IS*Aba31* (860 bp) which belongs to the IS*5* family. The analysis showed that the flanking regions immediately downstream of IS*Aba31* contain BrnA/BrnT toxin and antitoxin system and *tonB* gene receptors (Fig. 1). The flanking regions of *repB* were screened in the NCBI database and also showed high similarity to plasmid pAB0057 (CP001183, 8731 bp). However, in contrast to pSE41030-EUR, pSE41053, pSE41079, pSE41114 and pAB-MAL-1, pAB0057 plasmid does not harbour bla_{OXA-72} or the IS element IS*Aba31* region of 1767 bp (Fig. 1).

The Pasteur MLST scheme discriminated 12 different STs (Fig. 2); the most prevalent was ST2 (*n*=153, 67.7%), corresponding to international clone (IC) II. ST2 *A. baumannii* isolates predominantly carried bla_{OXA-23} (*n*=135, 60%), but also bla_{OXA-24} and bla_{OXA-72}. The bla_{OXA-72} gene was mainly present in ST1 (*n*=22, 9.7%) and ST492 (*n*=16, 7.1%). ST492, a single locus allele (*fusA*) mutant of ST2 belonging to clonal complex 2 (CC2), was only present in isolates from Serbia (*n*=13), Romania (*n*=2) and Montenegro (*n*=1). The bla_{OXA-72} gene was largely distributed in seven of the 12 ST lineages; the most common of these were ST1, ST492, ST2 and ST636.

The genetic relatedness of the study population based on cgMLST allelic loci distances showed that isolates were highly diverse and formed 11 distinct clusters despite belonging to the same ST (Fig. 2). In total, 4022 whole-genome allelic loci were identified, and among these, 2720 allelic loci were identified as core-genome loci among the study population. The maximum distance was 2575. cgMLST analysis revealed that isolates from the same ST were genetically diverse and formed different clusters. Highly labile genomes such as *A. baumannii* make it difficult to type the strain based on MLST alone as the core-genome content

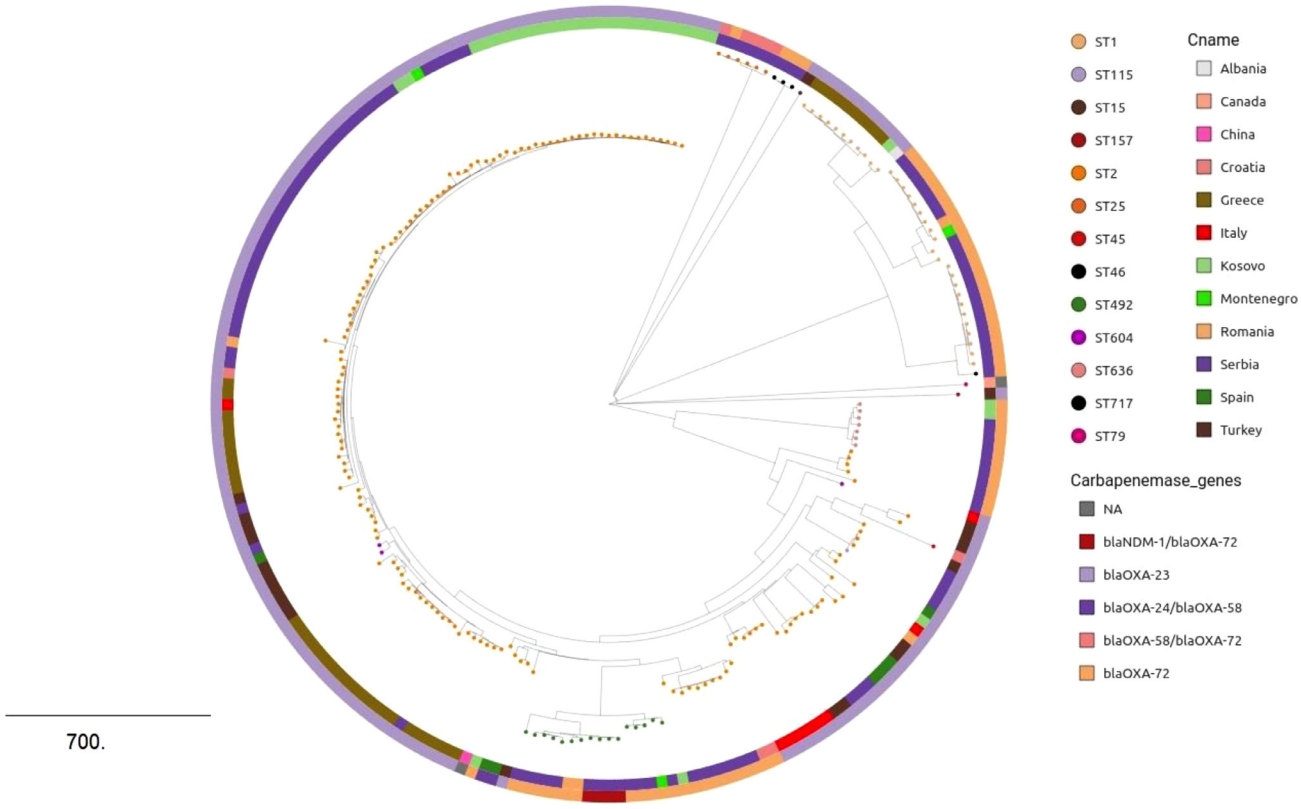

Fig. 2. Phylogenetic tree showing the clonal relatedness of *Acinetobacter baumannii* isolates within the EURECA study. Different colours represent either different sequence types (STs), countries or carbapenemase genes. The external ring represents carbapenemase genes while the inner ring pictures the country. The STs are indicated on the phylogenetic tree by the colour of each circle at the tip of the branch. Tree scale is 700. Cname, country name.

varies considerably. For instance, in strains from the same country belonging to ST2, the allelic loci distance could vary substantially (i.e. in Serbia, the maximum allelic loci distance was 247, compared with 54 in Greece, 13 in Kosovo and 80 in Turkey). In Serbia, six different core-genome clusters belonged to ST2 strains. ST492, a single allelic loci variant of ST2, showed a maximum allelic loci distance of 179. Similarly, ST1 isolates from Serbia showed a maximum allelic loci distance of 187 (Fig. 2).

3.4. *K and OC locus diversity in* A. baumannii

Among all 226 *A. baumannii* isolates, there were 22 different K locus variants. Among these, KL3 (n=63, 28%), KL9 (n=30, 13%), KL4 (n=25, 11%), KL40 (n=21, 9%) and KL1 (n=13, 6%) were the most prevalent K loci (Table S1, see online supplementary material). On the other hand, the OC locus was much more conserved among isolates, with only seven variants present. The majority of the isolates (n=193, 86%) belonged to OC1, followed by OC4 (n=12, 5%) and OC2 (n=11, 5%) (Table S1, see online supplementary material). Twenty-one of 22 different K loci identified in this study were found in isolates belonging to ST2 (CC2). The greatest diversity of the K locus was identified in CRAB strains from Serbia (n=15), Turkey (n=10) and Greece (n=6) (Table S1, see online supplementary material). Simpson's diversity index of KL locus [0.88, Jackknife pseudo-values confidence interval (CI) 0.85–0.90] and ST (0.51, Jackknife pseudo-values CI 0.44–0.59) were significantly higher than the diversity index of OC type or the country of origin of CRAB ($P \leq 0.05$). This suggests that the discriminatory capability of the K locus among the strains was significantly higher (Fig. S3, see online supplementary material). Similarly, the adjusted Wallace coefficient was also higher for the K locus (0.16) and ST (0.023).

4. Discussion

In line with prevalence data from the EURECA laboratories obtained during site selection [20], the majority (78.7%) of the *A. baumannii* strains were collected in Serbia, Kosovo and Greece. Overall, all participating countries in the site selection survey from the Balkan region (except Albania) had the highest rates of CRAB.

The majority of isolates in this study exhibited an XDR profile with almost no therapeutic alternatives other than colistin and tigecycline. Furthermore, some of the isolates (9%) were resistant to colistin.

$bla_{\text{OXA-23}}$ was the predominant carbapenemase-coding gene, being present in two-thirds of the isolates from sites from almost all countries. The $bla_{\text{OXA-23}}$ gene has been reported to be the most prevalent CHDL in CRAB in several studies in the Balkan region, including Croatia [21], Greece [6], Romania [10] and Turkey [22]. Overall, 30% of the CRAB isolates in the EURECA study, predominantly from Serbia, harboured $bla_{\text{OXA-72}}$, which is part of the $bla_{\text{OXA-40-like}}$ group and a single amino acid variant of OXA-24. The OXA-72 carbapenemase was reported in the Balkan region for the first time in Serbia [8] in 2016, followed by Bulgaria [23] in 2017. There have been numerous reports [24–26] on the emergence and spread of NDM-1-harbouring *A. baumannii* in many European countries and worldwide. The *bla*NDM-1 gene has been reported in association with *bla*OXA-23 [24], and also in conjunction with *bla*OXA-72 in a recent nationwide multi-centre study [18] from Serbia. The co-existence of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-72}}$ was found in four ST492 isolates from one Serbian hospital in the EURECA study. In

these strains, the bla_{OXA-72} gene was flanked upstream by IS*Aba31* (Fig. 1) and an IS element of 860 bp belonging to the IS*5* family. The IS*Aba31* element is relatively small, and a transposition event is highly likely as short inverted 5-bp repeat sequences flank the end of this element. The IS*5* family elements follow a 'copy–paste' mechanism and are highly mobile/transferrable.

Sequence analysis of the $bla_{OXA-51-like}$ genes undertaken in several studies [27,28] has been proposed as a useful typing method for *A. baumannii* isolates, given that close correlation has been established between particular $bla_{OXA-51-like}$ coded enzymes and particular epidemic lineages. The majority of the isolates in this study (79%) belonged to IC II because they encoded bla_{OXA-66}, while only 9% had bla_{OXA-69} which classifies them as IC I.

IC I–III correspond to CC1, CC2 and CC3, respectively. The most common ST in this study was ST2, a CRAB lineage that has been reported from various parts of the world [29,30]. Of note, ST2 has been described as endemic in certain Balkan countries [30,31], as well as the cause of multiple outbreaks in four Mediterranean countries (Greece, Italy, Lebanon and Turkey) [32].

Eight IC lineages of *A. baumannii* have been identified in previous studies [17], the most prevalent of which is IC II. This study showed that determination of the KL locus is very useful as a typing marker tool with very high discriminatory power, which allows *A. baumannii* to reveal diversity at genomic (and also at country) level within given lineages such as CC2 or CC1. For example, ST2 comprises 21 different KL loci, and ST1 comprises nine KL locus variants. However, some of the KL locus variants were common in both STs.

5. Conclusions

The *A. baumannii* strains from the EURECA study represent a unique and diverse collection of carbapenem-resistant isolates belonging to this species that add to existing knowledge on the epidemiology and resistance genes harboured by these strains. A high proportion of CRAB isolates was observed in several Balkan countries, particularly Serbia, which is likely to have resulted from the recent emergence and dissemination of several major epidemic lineages including ST2, ST1 and ST492. The presence of OXA-72 associated with a plasmid and transposable mobile genetic elements such as IS*Aba31* in these strains could explain the horizontal transfer of bla_{OXA-72} across different ICs and lineages. Further research is needed to unravel the main virulence factors and their mechanisms in these clinical isolates.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2021.106345.

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