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Carbapenem-resistant *Acinetobacter baumannii*: Current status of the problem in four Bulgarian university hospitals (2014–2016)

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ABSTRACT

Objectives: A total of 226 carbapenem-resistant *Acinetobacter baumannii* (CRAB) isolates was collected during 2014–2016 from inpatients (age range 5–88 years) in four Bulgarian university hospitals (H1–H4) to assess their antimicrobial susceptibility and to explore carbapenem resistance mechanisms as well as the molecular epidemiology of the isolates.
Methods: Antimicrobial susceptibility testing, multiplex PCR, DNA sequencing and electrotransformation experiments were performed. Epidemiological typing by random amplification of polymorphic DNA (RAPD)–PCR was also performed.
Results: The resistance rates were as follows: imipenem, 90.7%; meropenem, 98.2%; doripenem, 100%; amikacin, 92.9%; gentamicin, 87.2%; tobramycin, 55.8%; levofloxacin, 98.2%; trimethoprim/sulfamethoxazole, 86.3%; tigecycline, 22.1%; colistin, 0%; and ampicillin/sulbactam, 41.6%. Intrinsic $bla_{\text{OXA-51-like}}$ genes were found in all of the isolates. The majority of the *A. baumannii* isolates harboured either $bla_{\text{OXA-23-like}}$ associated with the upstream-located IS*Aba1* (26.1%) or $bla_{\text{OXA-40/24-like}}$ (46.7%), 45 isolates (19.9%) harboured both genes, and 1 isolate harboured $bla_{\text{OXA-58-like}}$ surrounded by IS*Aba3C* upstream and IS*Aba3* downstream. The $bla_{\text{OXA-58}}$ gene was transferable by electroporation indicating its plasmid location. Epidemiological typing revealed the dissemination of nosocomial CRAB with high clonal relatedness (70% similarity threshold) belonging to six, four, three and two clusters in H1, H2, H3, and H4 hospitals, respectively.
Conclusions: The *A. baumannii* isolates studied were problematic nosocomial pathogens. Their multidrug resistance greatly limits therapeutic options. The persistence of endemic clones comprised of OXA carbapenemase-producing multidrug-resistant *A. baumannii* in the monitored hospitals over a period of ca. 3 years is of concern and requires continuous detailed investigations in the future.

1. Introduction

Acinetobacter baumannii is one of the most successful pathogens in the modern healthcare system, particularly in intensive care unit (ICU) settings where it causes a wide range of nosocomial infections and long-term outbreaks such as ventilator-associated pneumonia, bloodstream infection, urinary tract infection, wound infection and meningitis [1–3]. Over the last decades, infections caused by *A. baumannii* have also been reported outside of the ICU and have also affected patients with co-morbidities in the community [4].

A. baumannii infections are difficult to treat owing to innate and acquired resistance to multiple antimicrobial agents. Carbapenems, recognised as the therapy of choice, are no longer effective in some cases. Recent trends show that many infections are caused by carbapenem-resistant, multidrug-resistant or even extensively drug-resistant *A. baumannii* strains for which effective treatment options are severely limited [5]. Currently, the prevalence of carbapenem resistance has risen dramatically and is considered a global threat [6].

Carbapenem resistance in *A. baumannii* is most often mediated by carbapenem-hydrolysing class D β-lactamases (CHDLs), also called oxacillinase (OXA)-type carbapenemases, and less frequently

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by metallo-β-lactamases (MBLs) [7]. Until now, nine major subgroups of OXA carbapenemases have been identified based on amino acid homologies. Four subgroups, including the chromosomally-located intrinsic OXA-51-like and the acquired OXA-23-like, OXA-40/24-like and OXA-58-like, are prevalent in *A. baumannii* [8]. Outer membrane permeability defects are also implicated in carbapenem resistance [1]. In addition to its remarkable multidrug resistance, *A. baumannii* is characterised by a propensity for clonal spread. Eight international clones (IC-1 to −8) have been identified, from which IC-2 is the most widespread and is often associated with carbapenem resistance [9].

The purpose of this study was to explore the antimicrobial susceptibility and the main carbapenem resistance mechanisms among recent carbapenem-non-susceptible *A. baumannii* isolates collected from inpatients in four large hospitals in Bulgaria. The molecular epidemiology of these nosocomial infections was also evaluated.

2. Materials and methods

2.1. Bacterial isolates

A collection of 226 selected carbapenem-non-susceptible, multidrug-resistant *A. baumannii* (MDR-AB) isolates was studied. The isolates were collected during the period April 2014 to December 2016 from inpatients (89 females and 137 males; age range 5–88 years) of different types of ward in four multiprofile university hospitals (H1–H4) in Bulgaria, namely: Military Medical Academy, Sofia (800 beds) (H1); University Hospital 'St. Marina', Varna (1280 beds) (H2); University Hospital 'Alexandrovska', Sofia (880 beds) (H3); and University Hospital 'St. Ivan Rilski', Sofia (395 beds) (H4). The isolates were obtained from tracheobronchial aspirate or bronchoalveolar lavage ($n = 136$), surgical wounds or abscesses ($n = 48$), blood ($n = 23$), urine ($n = 12$), central venous catheters ($n = 4$) and cerebrospinal fluid ($n = 3$). The isolate distribution according to the receiving site was as follows: H1, 58 isolates; H3, 63 isolates; H2, 74 isolates; and H4, 31 isolates.

A. baumannii ATCC 19606 and *Acinetobacter nosocomialis* (formerly *Acinetobacter* genomic species 13TU) ATCC 17903 were used as control strains for species identification and antimicrobial susceptibility testing.

2.2. Species identification of the isolates

Species identification was done using the BBL Crystal Enteric/Nonfermenter ID Kit (Becton Dickinson, Franklin Lakes, NJ) ($n = 94$ isolates), BD Phoenix™ automated system (Becton Dickinson) ($n = 74$ isolates) or VITEK®2 automated system (bioMérieux, Marcy-l'Étoile, France) ($n = 58$ isolates). Confirmation of species identity was performed using a PCR-based *gyrB* method as described previously [10].

2.3. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the investigated *A. baumannii* isolates was determined by the minimum inhibitory concentration (MIC) gradient test (MIC Test Strip; Liofilchem®, Roseto degli Abruzzi, Italy) or the broth microdilution method (Sensi-Test™ Colistin; Liofilchem) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [11] to the following antimicrobial agents: imipenem (IPM); meropenem (MEM); doripenem (DOR); amikacin (AMK); gentamicin (GEN); tobramycin (TOB); levofloxacin (LVX); trimethoprim/sulfamethoxazole (SXT); tigecycline (TGC); and colistin (COL). As previously described, the EUCAST MIC breakpoints listed for Enterobacteriaceae were applied to interpret TGC susceptibility [12]. In addition, susceptibility to ampicillin/sulbactam (SAM) was tested by the disk diffusion method and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [13].

2.4. Definition of multidrug-resistant A. baumannii (MDR-AB) and extensively drug-resistant A. baumannii (XDR-AB) isolates

According to previously described criteria [14], MDR-AB isolates were non-susceptible to at least one agent in three or more categories, whereas XDR-AB isolates were non-susceptible to at least one agent in all but two or fewer categories, including aminoglycosides (GEN, AMK, TOB, netilmicin), antipseudomonal carbapenems (IPM, MEM, DOR), antipseudomonal fluoroquinolones (ciprofloxacin, LVX), penicillins + β-lactamase inhibitors (SAM), folate pathway inhibitors (SXT), polymyxins (COL, polymyxin B) and tetracyclines.

Table 1
Oligonucleotides used as primers for PCR amplification and sequencing of carbapenem resistance genes in *Acinetobacter baumannii*.

Primer pair	Target	Sequence (5'→3')[a]	Product size (bp)	T_a (°C)	Reference
OXA23LF[b]	OXA-23-like CHDLs	GATCGGATTGGAGAACCAGA	501	58.3	[15]
OXA23LR[b]		ATTTCTGACCGCATTTCCAT			
OXA24LF[b]	OXA-40/24-like CHDLs	GGTTAGTTGGCCCCCTTAAA	246	58.3	[15]
OXA24LR[b]		AGTTGAGCGAAAAGGGGATT			
OXA51LF[b]	OXA-51-like CHDLs	TAATGCTTTGATCGGCCTTG	353	58.3	[15]
OXA51LR[b]		TGGATTGCACTTCATCTTGG			
OXA58LF[b]	OXA-58-like CHDLs	AAGTATTGGGGCTTGTGCTG	599	58.3	[15]
OXA58LR[b]		CCCCTCTGCGCTCTACATAC			
OXA143F	OXA-143 CHDL	TGGCACTTTCAGCAGTTCCT	146	60	[16]
OXA143R		TAATCTTGAGGGGGCCAACC			
OXA235F	OXA-235 CHDL	TTGTTGCCTTTACTTAGTTGC	768	56	[17]
OXA235R		CAAAATTTTAAGACGGATCG			
IMP-F[c]	IMP-type MBLs	GGAATAGAGTGGCTTAAYTCTC	232	58.5	[18]
IMP-R[c]		GGTTTAAYAAAACAACCACC			
VIM-F[c]	VIM-type MBLs	GATGGTGTTTGGTCGCATA	390	58.5	[18]
VIM-R[c]		CGAATGCGCAGCACCAG			
NDM-F[c]	NDM-type MBLs	GGTTTGGCGATCTGGTTTTC	621	58.5	[18]
NDM-R[c]		CGGAATGGCTCATCACGATC			

T_a, annealing temperature; F, forward primer; R, reverse primer; CHDL, carbapenem-hydrolysing class D β-lactamase; MBL, metallo-β-lactamase.
[a] Y = C or T.
[b] Primer pairs used in the multiplex PCR for CHDL-encoding genes.
[c] Primer pairs used in the multiplex PCR for MBL-encoding genes.

2.5. DNA isolation

Total DNA from all studied strains was isolated using an ISOLATE II Genomic DNA Kit (Bioline, London, UK) according to the manufacturer's instructions from 3 mL of overnight cultures inoculated with a single colony.

2.6. PCR amplification and sequencing of carbapenemase genes

PCR was performed to detect the presence of genes encoding different CHDLs (OXA enzymes) and MBLs. Oligonucleotides used as primers for PCR amplification and sequencing were synthesised by Alpha DNA (Canada) and are listed in Table 1 [15–18]. PCR mapping was then applied to seek the genetic elements [insertion sequence (IS) elements IS*Aba1*, IS*Aba2*, IS*Aba3* and IS*Aba3C*] associated with CHDL-encoding genes [19,20].

PCR was performed with 3 μL of template DNA, 0.1 μM of each primer, 0.2 mM deoxyribonucleoside triphosphates (dNTPs), 1 × reaction buffer, 2 mM $MgCl_2$ and 1.0 U of Prime *Taq* DNA Polymerase (GeNet Bio, Daejeon, South Korea) in a total volume of 25 μL. DNA was amplified in a Gene Pro Thermal Cycler (Bioer Technology, Hangzhou, China) using the following protocol: initial denaturation at 95 °C for 5 min; followed by 30 cycles of denaturation at 95 °C for 35–45 s, annealing at 56–60 °C from 45 s to 50 s and extension at 72 °C from 45 s to 60 s; and a single final extension at 72 °C for 7 min. PCR products were separated in 1.5% agarose gel for 50 min at 130 V, were stained with ethidium bromide (EtBr) (0.5 μg/mL) and detected by ultraviolet (UV) light (wavelength 312 nm). Amplified genes were identified on the basis of fragment size (Table 1).

Selected PCR products ($bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40/24-like}}$ and $bla_{\text{OXA-58-like}}$) were prepared for DNA sequencing by purification through an ISOLATE II PCR and Gel Kit (Bioline) and were sent to Macrogen Inc. (Seoul, South Korea). The sequences were subjected to BLAST analysis at the National Center for Biotechnology Information (NCBI) and were compared with the CHDL resistance gene sequences deposited at NCBI GenBank. MUSCLE [21] was applied for multiple alignment of nucleotide acid and amino acid sequences.

2.7. Plasmid transfer

To determine whether the acquired bla_{OXA} genes were plasmid-encoded, plasmid DNA was extracted using a PureLink™ HiPure Plasmid Filter Midiprep Kit (Thermo Fisher Scientific, Waltham, MA) and was transferred to the recipient isolate *A. baumannii* ATCC 19606 by electroporation [22]. Transformants were selected by plating on agar plates containing ticarcillin (100 μg/mL). The electrical pulse setting was 1.8 kV, 25 μF and 200 Ω using a Bio-Rad GenePulser Xcell™ system (Bio-Rad, Hercules, CA). In addition, plasmid DNA was used as a PCR template for the detection of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-40}}$ and $bla_{\text{OXA-58}}$ genes.

2.8. Random amplification of polymorphic DNA (RAPD)–PCR analysis

To ensure the maximum possible standardisation, all reaction mixtures were based on Ready-To-Go™ RAPD Analysis Beads (GE Healthcare, Chicago, IL). RAPD-PCR was carried out with primer M13 (5′-GAGGGTGGCGGTTCT-3′) using a previously published protocol [23]. The amplification products were compared by electrophoresis of 6 μL samples in 2% agarose gel stained with EtBr and photographed under UV light.

2.9. Unweighted pair-group method with arithmetic mean (UPGMA) analysis

Polymorphic RAPD patterns of the studied *A. baumannii* isolates were subjected to UPGMA analysis. The software GeneTools v.4.1 (Syngene, Cambridge, UK) was used for similarity matrices calculations and construction of UPGMA dendrograms using the 'profile' and 'band position' options. The dendrograms were based on the matrices of the similarity coefficients. Similarity of >70% was used as a threshold for clonal relatedness of the strains [23].

2.10. Statistical analysis

The rates of antimicrobial resistance of *A. baumannii* isolates from the different monitored hospitals were compared with each other as well as with recent results reported by other authors using Student's *t*-test. For simple comparison tests, a *P*-value of < 0.05 was considered statistically significant. To counteract the problem of multiple comparisons, when used, a Bonferroni correction with a critical value of $P = 0.00625$ was applied.

3. Results

3.1. Antimicrobial susceptibility of the investigated A. baumannii isolates

The results of antimicrobial susceptibility testing are presented in Table 2. The established rates of antimicrobial resistance, in increasing order, were: COL (0% resistance) < TGC (22.1%) < SAM

Table 2
Antimicrobial susceptibility of the multidrug-resistant *Acinetobacter baumannii* isolates ($n = 226$).

Antimicrobial agent	MIC (mg/L)			*n* (%) of isolates[a]		
	Range	MIC_{50}	MIC_{90}	S	I	R
Imipenem	8 to >32	>32	>32	0 (0)	21 (9.3)	205 (90.7)
Meropenem	4 to >32	>32	>32	0 (0)	4 (1.8)	222 (98.2)
Doripenem	>32	>32	>32	0 (0)	0 (0)	226 (100)
Amikacin	1.5 to >256	>256	>256	10 (4.4)	6 (2.7)	210 (92.9)
Gentamicin	0.75 to >256	96	>256	23 (10.2)	6 (2.7)	197 (87.2)
Tobramycin	0.38 to >256	128	>256	89 (39.4)	11 (4.9)	126 (55.8)
Levofloxacin	4 to >32	12	>32	1 (0.4)	3 (1.3)	222 (98.2)
SXT (1:19)	0.38 to >256	64	>256	31 (13.7)	0 (0)	195 (86.3)
Tigecycline[b]	0.125 to >256	1.5	16	94 (41.6)	82 (36.3)	50 (22.1)
Colistin	0.75–1	1	1	226 (100)	0 (0)	0 (0)
SAM (1:1)	N/A	N/A	N/A	86 (38.1)	46 (20.4)	94 (41.6)

MIC, minimum inhibitory concentration; $MIC_{50/90}$, MICs at which 50% and 90% of the isolates are inhibited, respectively; S, susceptible; I, intermediate; R, resistant; SXT, trimethoprim/sulfamethoxazole; SAM, ampicillin/sulbactam; N/A, not applicable.

[a] According to European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2016 criteria [11], except for ampicillin/sulbactam that was interpreted according to Clinical and Laboratory Standards Institute (CLSI) 2016 criteria [13].

[b] Interpreted according to the criteria for Enterobacteriaceae.

(41.6%) < TOB (55.8%) < SXT (86.3%). As shown, 90.7% of the isolates were resistant to the three antipseudomonal carbapenems. A total of 198 isolates (87.6%) were classified as MDR-AB and the remaining 28 isolates (12.4%) possessed an XDR resistotype with resistance to all antimicrobial agents tested except COL.

The nosocomial *A. baumannii* isolates from H2 in Varna showed significantly greater susceptibility ($P < 0.001$) to TOB (93.3% susceptible isolates) than those from the three hospitals (H1, H3 and H4) in Sofia (13.3–20% susceptible). Also, the isolates from H2 in Varna were significantly more susceptible to TGC than the isolates from H1 (60% vs. 20%; $P < 0.001$).

3.2. Molecular genetic investigation of carbapenem resistance mechanisms

The frequencies of distribution of the most prevalent CHDLs in the studied carbapenem-resistant *A. baumannii* (CRAB), as determined by a multiplex PCR, were as follows: OXA-51-like, 100%; OXA-40/24-like, 46.7%; OXA-23-like, 26.1%; combined OXA-40/24-like + OXA-23-like, 19.9%; and OXA-58-like, 0.4%.

All isolates (100%) harboured $bla_{\text{OXA-51-like}}$ genes. Of the 226 investigated CRAB isolates, 104 (46.0%) possessed $bla_{\text{OXA-23-like}}$ genes (26.1% alone and 19.9% together with $bla_{\text{OXA-40/24-like}}$) and 150 (66.4%) possessed $bla_{\text{OXA-40/24-like}}$ (46.7% alone and 19.9% together with $bla_{\text{OXA-23-like}}$). Only one isolate was identified as an OXA-58-producer. Structural genes for four of the major OXA-type carbapenemases (OTCs) were found, with the frequency of genes for acquired OTCs being 93% and those determining the production of intrinsic chromosomal enzymes (OXA-51-like), as expected, was absolute (100%). The studied isolates were negative for other CHDL genes and production of any MBLs, including the $bla_{\text{OXA-143}}$, $bla_{\text{OXA-235}}$, bla_{IMP}, bla_{VIM} and bla_{NDM} genes. A total of 199 CRAB isolates (88.1%) had IS*Aba1*. IS*Aba1* preceding $bla_{\text{OXA-51-like}}$ was determined to be the only enzymatic resistance mechanism to carbapenems in 16 isolates (7.1%).

Selected PCR products of the group genes for acquired OTCs [$bla_{\text{OXA-23-like}}$ ($n = 5$), $bla_{\text{OXA-40/24-like}}$ ($n = 5$) and $bla_{\text{OXA-58-like}}$ ($n = 1$)] were subjected to DNA sequencing to determine the exact variant of the enzyme as well as the location of the associated gene elements (IS), which play a direct role in gene expression. Sequencing established 100% homology with known $bla_{\text{OXA-23}}$ (GenBank accession no. **AJ132105AJ132105**), $bla_{\text{OXA-72}}$ (GenBank accession nos. **NG_049813NG_049813**, **KU870994**, **JX968505JX968505** and **HQ219688**) and $bla_{\text{OXA-58}}$ sequences (GenBank accession nos. **NG_049798NG_049798**, **KY202456** and **KF740448KF740448**). Insertion sequence IS*Aba1* was located upstream of the structural gene in all $bla_{\text{OXA-23}}$-positive isolates. The genetic environment of the $bla_{\text{OXA-58}}$ gene was defined as IS*Aba3*-like and IS*Aba3*, located upstream and downstream, respectively.

3.3. Carbapenem-hydrolysing class D β-lactamase gene transferability

In the present study, $bla_{\text{OXA-58}}$ was transferred by a plasmid isolated from the IPM-resistant clinical isolate Aba24/2016 (IPM MIC >32 mg/L) into IPM-susceptible *A. baumannii* ATCC 19606 (IPM MIC = 0.38 mg/L) by electroporation, demonstrating its plasmid location. The IPM MIC for the transformant was 12 mg/L. A single plasmid band was visible following electrophoresis in 0.8% agarose gel stained with EtBr, and the plasmid DNA yielded a specific 599-bp PCR product, also suggesting the plasmid location of $bla_{\text{OXA-58}}$.

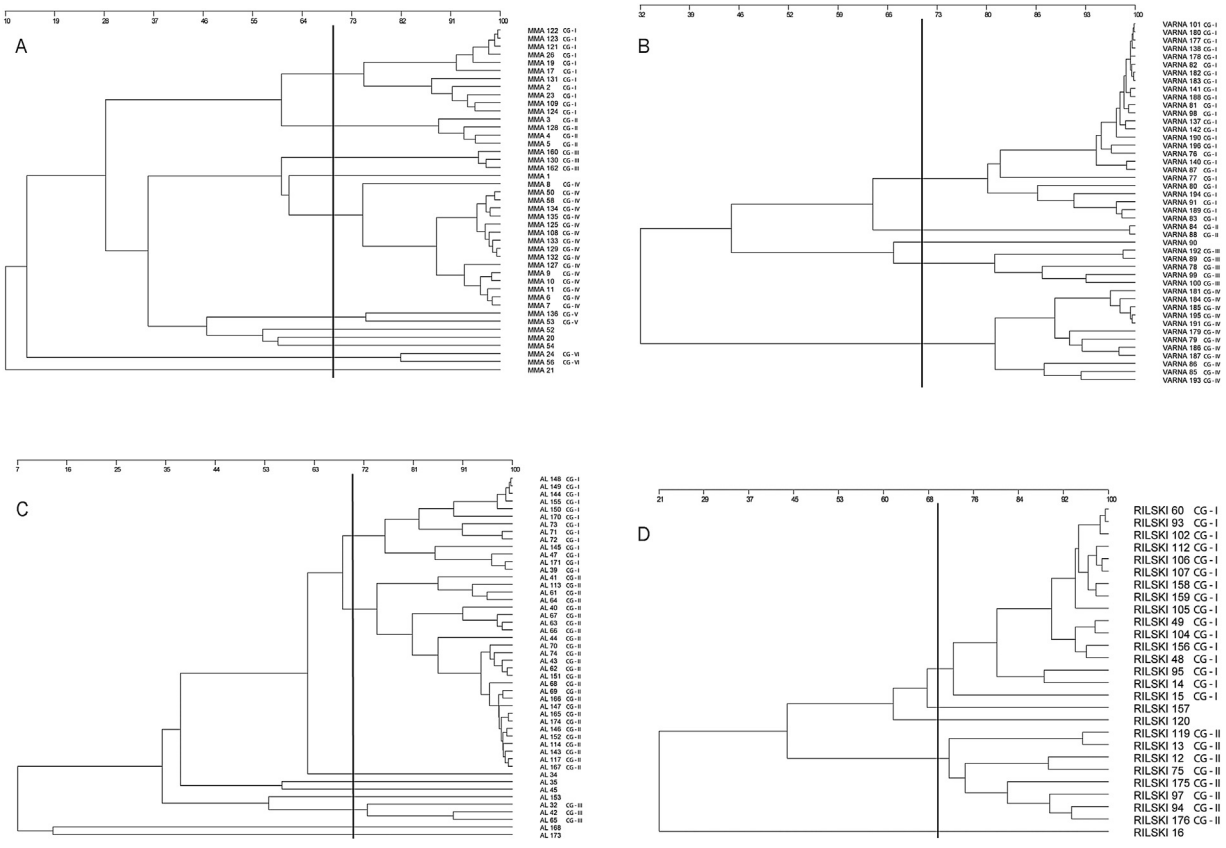

Fig. 1. Dendrograms illustrating the relationship between carbapenem-resistant *Acinetobacter baumannii* isolates in (A) Military Medical Academy (H1), (B) University Hospital 'St. Marina' (H2), (C) University Hospital 'Alexandrovska' (H3) and (D) University Hospital 'St. Ivan Rilski' (H4) based on analyses with the similarity coefficient (presented as percentage) of random amplification of polymorphic DNA (RAPD) profiles generated with the primer M13 [23]. CG, cluster group.

Electroporation experiments using plasmid extracts from bla_{OXA-23}- and bla_{OXA-72}-positive clinical isolates were unsuccessful in transferring carbapenem resistance to electrocompetent *A. baumannii* ATCC 19606. Bands were not visible following electrophoresis of plasmid DNA, and these plasmid preparations were PCR-negative for the respective genes, indicating the probable chromosomal location.

3.4. Epidemiological typing of carbapenem-resistant A. baumannii isolates in the monitored hospitals

Epidemiological typing of selected CRAB isolates (43, 45, 48 and 27 from hospitals H1, H2, H3 and H4, respectively) was performed by RAPD-PCR followed by UPGMA analysis. Selection criteria included different types of ward/clinic, different clinical specimens and different year of isolation. Dendrograms illustrating the relationship between the isolates in each hospital, based on analyses with the Dice similarity coefficient, are shown in Fig. 1.

Cluster analysis using the most widely applied criterion for clonal relatedness of the typified isolates (70% similarity threshold) [23] allocated the CRAB strains into six, four, three and two cluster groups (CGs) in H1, H2, H3 and H4, respectively (Table 3). The distribution of the isolates of each CG by year of isolation and by type of acquired CHDL produced is also shown in Table 3. In general, the majority of CRAB isolates were grouped into a small number of CGs, and only single isolates that generated unique RAPD profiles did not show cluster affiliations. Most CGs included CRAB isolated in different years (2014, 2015 and 2016) as well as producers of both main types of acquired CHDLs (OXA-23 and OXA-24).

Dissemination of clonally related strains was established in H1. The isolates formed six CGs as follows: CG-I was presented by 11 CRAB isolates (75% clonal relatedness); GC-II (4 isolates; 88% relatedness); GC-III (3 isolates; 95% relatedness); CG-IV (16 isolates; 75% relatedness); CG-V (2 isolates; 75% relatedness); and CG-VI (2 isolates; 80% relatedness). Five non-clustered isolates were also found. Four CGs and one non-clustered isolate were identified in H2 as follows: CG-1 (25 isolates; 80% relatedness); CG-II (2 isolates; 98% relatedness); CG-III (5 isolates; 81% relatedness); and CG-IV (12 isolates; 80% relatedness). Two major CGs (CG-I and CG-II), one minor CG (CG-III) and six non-clustered strains were found in H3. CG-I consisted of 13 CRAB isolates (76% relatedness), CG-II included 26 CRAB isolates (74% relatedness) and CG-III included 3 CRAB isolates (72% relatedness). Cluster analysis

Table 3
Grouping of selected *Acinetobacter baumannii* isolates from the four hospitals into cluster groups of clonally-related strains (70% similarity threshold)[a].

Cluster group (CG)	H1	H2	H3	H4
CG-I	Total number: 11 Year of isolation: 2014 (5), 2015 (1), 2016 (5) Acquired CHDLs: OXA-23 (6), OXA-24 (1), OXA–23 + OXA-24 (4) 75% relatedness	Total number: 25 Year of isolation: 2014 (4), 2015 (11), 2016 (10) Acquired CHDLs: OXA-23 (1), OXA-24 (19), OXA–23 + OXA-24 (5) 80% relatedness	Total number: 13 Year of isolation: 2014 (2), 2015 (3), 2016 (8) Acquired CHDLs: OXA-23 (2), OXA-24 (9), OXA–23 + OXA-24 (2) 76% relatedness	Total number: 16 Year of isolation: 2014 (3), 2015 (10), 2016 (3) Acquired CHDLs: OXA-23 (7), OXA-24 (1), OXA–23 + OXA-24 (3), none (5) 72% relatedness
CG-II	Total number: 4 Year of isolation: 2014 (3), 2016 (1) Acquired CHDLs: OXA-23 (3), OXA–23 + OXA-24 (1) 88% relatedness	Total number: 2 Year of isolation: 2015 (2) Acquired CHDLs: OXA-23 (2) 98% relatedness	Total number: 26 Year of isolation: 2014 (4), 2015 (13), 2016 (9) Acquired CHDLs: OXA-23 (12), OXA-24 (9), OXA–23 + OXA-24 (5) 74% relatedness	Total number: 8 Year of isolation: 2014 (2), 2015 (3), 2016 (3) Acquired CHDLs: OXA-23 (2), OXA-24 (2), none (4) 71.5% relatedness
CG-III	Total number: 3 Year of isolation: 2016 (3) Acquired CHDLs: OXA-23 (3) 95% relatedness	Total number: 5 Year of isolation: 2015 (4), 2016 (1) Acquired CHDLs: OXA-23 (1), OXA-24 (3), none (1) 81% relatedness	Total number: 3 Year of isolation: 2014 (2), 2015 (1) Acquired CHDLs: OXA-24 (1), OXA–23 + OXA-24 (2) 72% relatedness	
CG-IV	Total number: 16 Year of isolation: 2014 (4), 2015 (5), 2016 (7) Acquired CHDLs: OXA-23 (12), OXA-24 (2), OXA–23 + OXA-24 (2) 75% relatedness	Total number: 12 Year of isolation: 2014 (3), 2015 (1), 2016 (8) Acquired CHDLs: OXA-24 (7), OXA–23 + OXA-24 (4), none (1) 80% relatedness		
CG-V	Total number: 2 Year of isolation: 2015 (1), 2016 (1) Acquired CHDLs: OXA-23 (1), OXA–23 + OXA-24 (1) 75% relatedness			
CG-VI	Total number: 2 Year of isolation: 2015 (1), 2016 (1) Acquired CHDLs: OXA–23 + OXA-24 (1), OXA-58 (1) 80% relatedness			
No. of CGs	6	4	3	2
No. of non-clustered strains	5	1	6	3

H1, Military Medical Academy, Sofia; H2, University Hospital 'St Marina', Varna; H3, University Hospital 'Alexandrovska', Sofia; H4, University Hospital 'St Ivan Rilski', Sofia; CHDL, carbapenem-hydrolysing class D β-lactamase.

[a] The corresponding number of isolates, according to the year of isolation and the type of acquired CHDL, is given in parentheses.

detected three non-clustered strains and two CGs in H4, including CG-I (72% relatedness) and CG-II (71.5% relatedness) presented by 16 and 8 isolates, respectively.

4. Discussion

The investigated clinical *A. baumannii* isolates were problematic nosocomial pathogens. They were isolated from four hospitals where a high average CRAB rate was established for the study period (2014–2016), as follows: 92.6%, 70.6%, 89.7% and 98.1%, respectively at H1, H2, H3 and H4 (unpublished data).

Antimicrobial susceptibility testing showed the highest antimicrobial activity of COL (0% resistance), TGC (22.1%) and SAM (41.6% resistance). These antibiotics are involved in the recommended therapeutic regimens of severe infections caused by MDR-AB in the respective hospitals [24]. In line with world trends, combined antimicrobial therapy was increasingly being used, including the following schemes: COL + IPM or MEM, COL + TGC, COL + rifampicin, COL + SAM and IPM + SAM [1].

It is estimated that resistance to carbapenems alone is sufficient for *A. baumannii* to be considered a highly resistant and therapeutically problematic pathogen. In the current study, the prevalence of MDR-AB resistant to the three antipseudomonal carbapenems (90.7%) was higher than that observed among nosocomial MDR-AB isolates in the University Hospital 'St. Georgi' (Plovdiv) during the period 2010–2014 (84% resistant) and among older MDR-AB (2005–2012) isolated from patients in two hospitals in Varna (28% resistant; $P < 0.001$) [25,26]. Analysis of the antimicrobial susceptibility of carbapenem-non-susceptible MDR-AB isolated in H3 during the periods 2005–2011 and 2014–2016 showed a statistically significant chronological decrease ($P < 0.001$) in susceptibility for the newer isolates for several antibiotics: TCG (77.1% vs. 46.7% susceptible); SXT (64.3% vs. 26.7%); and LVX (17.1% vs. 0%) [27]. In H1, there were established similar resistance rates to IPM (93.3%), MEM (100%) and SXT (60%) for the current isolates compared with older isolates (2008) (83%, 85.8% and 53.1%, respectively), but a significantly higher resistance to aminoglycosides [86.7% vs. 2.85–25% (2008); $P < 0.001$] was found [28].

The European Antimicrobial Resistance Surveillance Network (EARS-Net) data for 2016, published by the European Center for Disease Prevention and Control (ECDC) [29], reported a very high prevalence of invasive carbapenem-resistant *Acinetobacter* spp. isolates in some Balkan and Mediterranean countries, e.g. Greece (95.4%), Croatia (94.5%), Romania (85%) and Italy (78.5%), which was directly correlated with the consumption of carbapenems in these countries. In Bulgaria, the frequency for 2016 was 74.8%, which was higher compared with the previous two years (73.8% in 2015 and 59.1% in 2014). Earlier retrospective cohort studies analysing the antimicrobial resistance of nosocomial *A. baumannii* from different parts of the world during 2006–2010 reported 63% resistance to carbapenems in Colombia [30], 55.8% in South Korea [31] and 54% in Turkey [32].

According to the EARS-Net annual report [29], the overall combined resistance to fluoroquinolones, aminoglycosides and carbapenems among invasive *Acinetobacter* spp. isolates in Europe for 2016 was 43.3%. The highest rate of MDR *Acinetobacter* spp. with cross-resistance to the three classes of antimicrobial agents was demonstrated in the Balkan countries, such as Greece (84% of resistant isolates, 2016), Romania (82.9%) and Croatia (81.1%) [29]. The prevalence of these isolates in Bulgaria was significantly higher than the average combined resistance for Europe (72.4% vs. 43.3%; $P < 0.001$) [29].

In the current study, the frequency of XDR-AB susceptible only to COL (12.4%) was lower ($P < 0.01$) than the recently established analogous antimicrobial resistance profile in another Balkan country, Bosnia and Herzegovina, where 23% of these isolates were found in 2011–2012 at the University Clinical Center in Sarajevo [33]. A longitudinal multicentre study in Taiwan reported a significant increase ($P < 0.001$; odds ratio = 1.970) in XDR-AB from 1.3% in 2002 to 41.0% in 2010 [34]. Typically, XDR-AB isolates retain their susceptibility to COL. In 2016, 4.0% of invasive *Acinetobacter* spp. isolates in Europe were resistant to COL, with the vast majority (70.7%) reported from Greece and Italy [29].

Previous studies have shown that genes for naturally occurring OXA-51-like enzymes of *A. baumannii* are localised on the chromosome and their expression is regulated by IS*Aba1* [35]. Insertion of IS*Aba1* at the start of the promoter region, immediately upstream the structural gene, results in carbapenemase activity of the enzyme that primarily affects IPM [19]. This mechanism was involved in the carbapenem resistance in 7.1% of isolates in the current study where no genes for acquired OTCs were identified.

For the first time in Bulgaria, IS*Aba1* located upstream of the $bla_{\text{OXA-23}}$ gene was identified as a mechanism of acquired carbapenem resistance among nosocomial *A. baumannii* at the University Hospital in Pleven, isolated from 1999 to 2006 [36]. The authors reported a sustained hospital outbreak caused by OXA-23-producing CRAB belonging to two different clonal groups. Thereafter, widespread dissemination of clonally-related $bla_{\text{OXA-23}}$-positive *A. baumannii* was also found in other large hospitals in Bulgaria [26,27,37,38]. The prevalence of OXA-23 carbapenemase among *A. baumannii* isolates is global and covers countries of all continents, including France, Germany, Romania, Italy, Spain, the USA, Brazil, Australia, Taiwan, China, Korea, Singapore and others [1]. The $bla_{\text{OXA-23}}$ gene is either located on the chromosome or on plasmids and it is associated with different genetic structures [39].

$bla_{\text{OXA-40/24-like}}$ was the most prevalent gene for acquired OTCs in the present study (66.4%). Recently, $bla_{\text{OXA-24-like}}$ genes were detected in single nosocomial isolates of *A. baumannii* in several Bulgarian hospitals [40]. OXA-72 enzymes from the OXA-24-like group were found in 12.8% of CRAB isolated in 2013–2014 at H1 [38], and the incidence was significantly lower than the prevalence in the current study ($P < 0.001$). So far, except in Bulgaria, OXA-72 OTCs (chromosomally- or plasmid-encoded) have been reported in Taiwan, China, Brazil, Colombia, the USA, France, Poland, Italy, Lithuania, Sweden, Croatia and Serbia [1,41].

Plasmid-borne OXA-58 was detected only in one of the CRAB isolates in this study. We confirmed the typical genetic environment of $bla_{\text{OXA-58}}$ including IS elements [20]. Until now, single OXA-58-producing *A. baumannii* have been identified in two Bulgarian university hospitals in Sofia and Varna [26,37], which coincides with the low incidence of this carbapenemase found in the present study. Many OXA-58-producing *A. baumannii* isolates were reported worldwide, including isolates in Europe, Latin America, the USA, Asia and Australia. A number of outbreaks have also been reported in many countries, including Italy, Belgium, France, Turkey, Greece and the USA [1].

The rapid expansion of CRAB, MDR-AB and XDR-AB during the last decade is a worrying tendency in the monitored hospitals, especially in ICU wards, which underlines the necessity of elucidating the molecular epidemiology of circulating nosocomial isolates. To achieve this aim, RAPD-PCR was chosen owing to its undoubted advantages such as great flexibility, technical simplicity, cost effectiveness and rapid performance [23]. From the 1990s to the present, it has been successfully applied alone or in combination with other molecular techniques for epidemiological typing of nosocomial *A. baumannii* isolates [23,42]. RAPD-PCR is extremely useful in detecting outbreaks of healthcare-associated infections caused by MDR-AB. The present study illustrates the inter-ward dissemination of clonally-related CHDL-producing MDR-AB in the four Bulgarian hospitals. Demonstrating at least

two major cluster groups in each of the monitored hospitals (H1–H4) has given us reason to accept the persistence of more than one endemic clone in each of them for the period 2014–2016. These findings correlate with the data of apparent dissemination of a few successful MDR-AB clonal lineages worldwide [9].

5. Conclusion

The studied nosocomial CRAB isolates also exhibited cross-resistance to antimicrobial agents from other classes, which was the basis for categorising them as MDR-AB (87.6%) or XDR-AB (12.4%). Usually, the choices of antimicrobial treatment for MDR-AB and XDR-AB are severely limited. There are only a few effective options available, including COL, TGC and SAM.

Production of acquired CHDLs (OXA-40/24-like, OXA-23-like and OXA-58-like oxacillinases) was the main mechanism of carbapenem resistance in the investigated *A. baumannii* isolates (93%). The vast majority of them (88.1%) had IS*Aba1* upstream of the bla_{OXA} genes, indicating the prevalence of IS*Aba1* insertion. This study confirms the rare incidence of OXA-58-producing CRAB isolates in Bulgarian hospitals [25,36], but the plasmid gene location may enhance the spread of this carbapenem resistance marker in our country.

The persistence of endemic clones comprised of OXA carbapenemase-producing MDR-AB in the monitored hospitals over a period of ca. 3 years is of concern and requires continuous detailed investigations in the future.

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Competing interests

None declared.

Ethical approval

Not required.

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