Biologia Serbica, 2022, 44(2): 3-17

DOI: 10.5281/zenodo.7489087

Original paper

Acinetobacter calcoaceticus-A. baumannii complex: isolation, identification and characterisation of environmental and clinical strains

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Accepted: 27 December 2021 / Published online: 13 February 2022

Summary. Bacteria from *Acinetobacter calcoaceticus-A. baumannii* (Acb) complex are widespread, multi-drug resistant bacteria of great clinical importance. The aim of this study was to determine the most suitable sole carbon source for their isolation/cultivation from environmental samples, to facilitate easier isolation and to compare the properties of environmental strains and wound isolates, in order to reveal whether environmental isolates are a reservoir of infection. In addition to acetate, which is used in routine procedures, the most appropriate carbon sources for isolation, were alanine, L-histidine and L-tyrosine. Because none of the substrates enabled isolation from all samples, new carbon sources or their combinations should be further considered. In subculture, L-tyrosine, alanine, L-histidine and pyruvate were the most appropriate for bacterial growth. Environmental and clinical strains were identified using classical methods and multiplex PCR, and out of 31 strains two were not identified as *A. baumannii*, but as other members of the Acb complex (13 UT group). All strains possessed integron class 1, while all environmental isolates and one clinical strain possessed integron class 2. Integron class 2 should be further examined as a marker for environmental strains. The environmental strains were multi-drug resistant and there was no statistical difference in antibiotic resistance in comparison to clinical strains ($p = 0.36$). The detected similarity of RAPD-PCR fingerprints among *A. baumannii* environmental and wound isolates suggests their epidemiological relatedness, raising the urgency for finding adequate solutions for prevention of further resistance dissemination among strains of different origins.

Keywords: Acb complex, carbon sources, integrons, multiple-resistance, RAPD-PCR.

INTRODUCTION

Acinetobacter baumannii is a widespread, multi-drug resistant bacterium with specific genotypic and phenotypic (morphological, cultural and biochemical) features. Its taxonomic classification even at the genus level has been highly variable in the past. The genus *Acinetobacter* was previously classified into the Neisseriaceae family, containing one species, *Acinetobacter calcoaceticus* (Brenner et al. 2005). Later taxonomic studies have suggested that members of this genus should be included in the new family - Moraxellaceae, which includes the genus *Moraxella*, *Acinetobacter*, *Psychrobacter* and related bacteria, and represents a discrete phylometric branch in superfamily II Proteobacteria, based on studies of 16S rRNA and DNA-DNA hybridization (Bergogne-Berezin and Towner 1996). The current description of the genus and the name *Acinetobacter* (Greek, *akinetos* - nonmotile) was first used in 1954 to distinguish motile and non-motile microorganisms from the genus *Achromobacter* (Brisou and Prevot 1954; Peleg et al. 2008). This name was widely adopted in 1968, after publication of a report by Baumann and his associates, who isolated members of this genus from soil and water (Baumann et al. 1968; Peleg et al. 2008). Initially, the genus *Acinetobacter* included a heterogeneous group of non-motile, Gram negative, oxidase negative saprophytes, which could differ from other bacteria due to a lack of pigmentation. The bacteria that are now classified in the genus

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Acinetobacter have previously been classified under at least 15 different related names, of which the most famous are *Bacterium anitratum*, *Herellea vaginicola*, *Mima polymorpha*, *Achromobacter*, *Alcaligenes*, *Micrococcus calcoaceticus*, *Moraxella glucidolytica* and *Moraxella lwoffii* (Bergogne-Berezin and Towner 1996).

Today, this genus includes Gram negative coccobacili that are strictly aerobic, non-motile, catalase positive and oxidase negative. Most members of the genus grow on simple mineral media containing ammonia or nitrate salts as a source of nitrogen, and acetate, lactate or pyruvate as the only source of carbon and energy (Brenner et al. 2005). So far, at least 35 named and unnamed species have been identified within the genus *Acinetobacter*. Of the total number of species, 26 have defined names, while the other 9 are only temporary and they are designated as genomic species because they cannot be distinguished from other species based on their phenotypic properties (Brenner et al. 2005; Di Nocera et al. 2011). Namely, the term species in classical microbiology implies a group of strains showing a high degree of similarity in terms of phenotypic traits. Today, it is widely accepted that hybridization and sequencing of nucleic acids are the most rational methods for separating species and defining relationships between different organisms. On the basis of these criteria, within the genus *Acinetobacter*, 9 DNA-DNA homologous groups (genomic species) were determined. Genomic species 1 (*A. calcoaceticus*), genomic species 2 (*A. baumannii*), and unnamed genomic species 3 and 13TU are interconnected and comprise *A. calcocaeticus-A. baumannii* complex (Acb complex). The genomic species *Acinetobacter baumannii* was first identified by DNA-DNA hybridization performed in 1986 by Bouvet and Grimont.

Members of the genus *Acinetobacter* are ubiquitous organisms, and therefore, the species *A. baumannii* is also considered a ubiquitous, widespread microorganism. However, a study of the systematic examination of the natural habitats of certain species of this genus, including *A. baumanni*, has never been carried out (Baumann 1968; Fournier and Richet 2006; Peleg et al. 2008). *Acinetobacter baumannii* is present in the environment and is mostly found as a saprophyte in soil, water, food products, often as microbiota in humans (skin, respiratory and genital tract), and is often isolated from medical devices. Also, it is easily and most commonly isolated from blood, sputum, bronchial rinsings, manure, urine, and catheter drains (Espinal et al. 2012). It can also be found in raw, washed and frozen vegetables, in fresh, frozen and stored fish and meat products, as well as in milk and cheese (Brenner et al. 2005). In addition, this bacterium has the ability to survive up to several days on surfaces that are nutritionally poor, and is resistant to drying and disinfectants (Espinal et al. 2012). Generally, *A. baumannii* grows on mineral medium containing one source of carbon and energy, such as ethanol, acetate or lactate. Some organic compounds, such as amino-acids, can serve as a single source of carbon, energy and nitrogen. Several selective and differentiated media are currently in use for the isolation of this bacterium. However, it has remainded unknown which of them is the most suitable for the purposes of *A. baumannii* isolation from environmental samples.

The aim of the present study was to determine the most suitable media supplemented with 9 different substrates for the isolation/cultivation of bacteria of the *Acinetobacter calcoaceticus-A. baumannii* complex, with particular reference to *A. baumannii*, in order to facilitate easier isolation from the environment. The obtained strains were compared with clinical wound isolates, to reveal whether environmental isolates are a reservoir for wound infections. In this context, resistotypes, integron-1 and 2 class presence, and strain genetic relationships determined by RAPD-PCR were also examined.

MATERIAL AND METHODS

Isolation

Strains were isolated from the environment, more precisely from aquatic ecosystems. Samples were collected from 11 sampling sites in the Republic of Serbia (Table 1). Sampling was carried out in sterile containers, which were delivered to the laboratory and stored in a refrigerator at 4 °C until processing. Samples were analysed no later than 24 h from the moment of sampling.

Isolation of *Acinetobacter* spp. using the enrichment method has been described by Baumann (1968), and involves the use of Baumann enrichment medium, contain-

Table 1. Sampling sites for *Acinetobacter calcoaceticus-A. baumannii* complex strain isolation.

Sample designation	Origin of the sample	Locality of the sample
S	surface water	Srnjanska river, Kruševac
M		West Morava, Kruševac
DN		Danube – before the sewage inflow, Novi Sad
DZ		Danube – after the sewage inflow, Novi Sad
DP		Artificial lake of the Danube Park, Novi Sad
DTD		Danube-Tisa-Danube Canal, Novi Sad
B		Begeč pit, Begeč
DB		Danube, Begeč
P1	groundwater	Novi Sad
P2		Novi Sad
P3		Novi Sad

ing the components dissolved in 0.04 M KH_2PO_4/Na_2HPO_4 buffer (pH 6.0), in a volume of 1 litre. For cultivation of bacteria of the *Acinetobacter calcoaceticus-A. baumannii* complex (Acb complex), 8 different carbon sources were used by replacing the sodium acetate, characteristic of the original Baumann medium, with an appropriate alternative substrate: β-alanine, ethanol, L-phenylalanine, L-histidine, sodium-L-lactate, L-leucine, sodium pyruvate or L-tyrosine.

The enrichment medium was prepared as double-concentrated, and a sample (1:1, v/v) was added to each of the substrates to a final volume of 10 mL. The substrates were incubated for 24 hours and 48 hours at 37 °C with agitation at 200 rpm on an orbital shaker (Heidolph UNIMAX 2010, Germany). After the end of the incubation period, the appearance of turbidity was determined visually and by quantification of the turbidity, expressed as medium light transmission value. As light transmission values became smaller, bacterial growth was higher and *vice versa*, with a 100% transmission indicating a lack of growth.

Herella agar was used for the isolation of *Acinetobacter baumannii* species from enriched samples. After 24 hours incubation, 10 μL of enrichment medium was plated on Herella agar using a streak-plating method. Plates were incubated for 24 hours at a temperature of 37 °C. The procedure was repeated after 48 hours of enrichment. Pale-purple colonies grown on the Herella agar, corresponding to colonies of Acb complex, were transferred to Nutrient agar (Torlak, Serbia) and incubated for 24 hours at a temperature of 42 °C. After the end of the incubation period, the presence of growth was determined.

Identification

Strains were identified according to Gram reaction, morphological, cultural, and physiological properties, and/or using the VITEK2 system (Biomerieux, France).

Physiological traits of the isolated environmental strains were determined using preliminary (catalase test, oxidase test, oxidative-fermentative test, and a test to prove denitrification) and confirmatory biochemical tests (indol test, gelatin liquefaction test, test for citrate uptake, growth on Endo and MacConkey agar). In the case where confirmatory tests yielded positive results, the isolate was identified as member of the Acb complex. Resistance of isolates to penicillin and cefaclor was examined according to CLSI (2015), as an additional trait of the bacteria from the Acb complex. When results were ambiguous, confirmatory identification of the Acb complex isolates was carried out using the VITEK2 system.

More precise identification of the isolated strains from Acb complex was performed by molecular-genetic methods, enabling identification of isolates to the genomic species level. Identification Acb complex genomic species 2, i.e. *Acinetobacter baumannii* was conducted using a multiplex PCR technique, with reference strains used as a positive control. Bacterial DNA was extracted using a Gene JET Genomic DNA Purification Kit (Thermo Scientific, Pittsburgh, US) following the protocol recommended by the manufacturer for Gram negative bacteria. The isolates were identified to the genomic species level by ITS sequence analysis, based on the method of Chen et al. (2007). Briefly, multiple PCR was carried out using primers P-rA1 and P-rA2, which target a highly conserved 425-bp region of the *recA* gene of the genus *Acinetobacter*, and a second pair of primers P-Ab-ITSF and P-Ab-ITSB, used to amplify an internal 208-bp fragment from the ITS region of *A. baumannii* genomic species (Table 2). Multiplex PCR was performed as described previously and amplicons were separated by electrophoresis on agarose gels (1.5%, w/v) amended with ethidium bromide. The gels were visualized using a gel documentation system and analysed by the corresponding software (BioDocAnalyse, Biometra GmbH, Goettingen, Germany). Identified *A. baumannii* environmental isolates were stored in Luria Bertani broth (LB) containing glycerol (v/v 10%) at -70 °C. For experimental purposes, strains were inoculated into LB and grown overnight at 37 °C.

Growth using different carbon sources

The growth kinetics of *A. baumannii* strains using nine different carbon sources were examined using Baumann medium as a base. As carbon sources, Na-acetate, β-alanine, ethanol, L-phenylalanine, L-histidine, Na-L-lactate, L-leucine, Na-pyruvate and L-tyrosine were used to determine the most optimal carbon source for growth of this bacterial species. The growth of three strains was examined, two of which were reference strains (ATCC 19606 and ATCC BAA747) and one originating from the environment (Aba-DN-Ace). The bacterial suspension was added to double-concentrated enrichment media (1:1, v/v) so that the final suspension volume was 10 mL and the final bacterial count was approx. 1×10^3 CFU mL^{-1}. Inoculated substrates were incubated at 37 °C and growth kinetics were monitored by measurement of optical density at 620 nm (OD_{620}) after 3, 6, 12, 24, 36 and 48 hours of incubation. The experiment was conducted in triplicate and in three independent repetitions. The obtained absorbance values were expressed as a mean value with standard deviation and presented graphically using Origin 6.0 software (Microcal Software, USA).

Sensitivity to conventional antimicrobial agents

To determine the antibiotic sensitivity of Acb complex strains, commercially available antimicrobials relevant to *A. baumannii* were used: ceftriaxone, imipenem, netilmicin, to-

Table 2. Oligonucleotide sequence of primers for PCR analysis of *Acinetobacter baumannii*.

Primer purpose	Primer	Nucleotide sequence	Product size (bp)	Ref.
Genus *Acinetobacter*	P-rA1	5' - CCTGAATCTTCTGGTAAAAC - 3'	425	Chen et al. 2007
	P-rA2	5' - GTTTCTGGGCTGCCAAACATTAC		
Species *A. baumannii*	P-Ab-ITSF	5' - CATTATCACGGTAATTAGTG - 3'	208	
	P-Ab-ITSR	5' - AGAGCACTGTGCACTTAAG - 3'		
Integron class 1	Aba_Int1-F	5' - CAGTGGACATAAGCCTGTTC - 3'	160	Koeleman et al. 2001
	Aba_Int1-R	5' - CCCGAGGCATAGACTGTA - 3'		
Integron class 2	Aba_Int2-F	5' - TTGCGAGTATCCATAACCTG - 3'	228	
	Aba_Int2-R	5' - TTACCTGCACTGGATTAAGC - 3'		
RAPD-PCR	OPA-08	5' - GTGACGTAGG - 3'	-	-
	OPB-06	5' - TGCTCTGCCC - 3'		
	OPB-11	5' - GTAGACCCGT - 3'		
	OPN-02	5' - ACCAGGGGCA - 3'		
	K15	5' - CTCCTGCCAA - 3'		

bramycin, gentamicin, kanamycin, amikacin, trimethoprim/sulfamethoxazole, ciprofloxacin, polymyxin B, tetracycline and chloramphenicol.

The minimal inhibitory concentration (MIC) of conventional antibiotics was determined using a modified microdilution method (CLSI 2015). The final concentration of each antibiotic in the microtiter plates ranged from 0.125 to 512 mg L^{-1}, except for polymyxin B, the concentration of which ranged from 0.031 to 32 mg L^{-1}. The number of bacteria in each well of the microtiter plate was approx. 1×10^6 CFU mL^{-1}. The microtiter plates were incubated for 18 hours at 37 °C, after which 10 µL of a 1% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) was added to each well of the plate. The TTC was reduced by dehydrogenases present in viable bacterial cells to red colored formazan. Microtiter plates were additionally incubated for 2 hours at 37 °C, and the MIC for each antibiotic was read. The lowest antibiotic concentration required to prevent the formation of red colored formazan was considered to be the MIC. The experiment was conducted in at least three independent repetitions. The obtained results are presented as values of the geometrical mean.

For minimal bactericidal concentration (MBC), 10 µL from wells without obvious bacterial growth were subcultured on nutrient agar plates to determine if inhibition was reversible or permanent. MBC was determined as the highest dilution (lowest concentration) at which >99.9% reduction of initial CFU was obtained. Each experiment was performed in triplicate and in three independent experiments, with *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 used as internal quality control strains for antibiotic susceptibility tests. The results are represented as geometrical means of replications.

In addition, the resistance of environmental isolates to imipenem (10 µg), netilmicin (30 µg), tobramycin (10 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), ofloxacin (10 µg) and piperacillin (100 µg) (Bioanalyse Ltd., Turkey) was determined by the standard disc-diffusion method (CLSI 2015), because these antimicrobial agents were only available in the form of antibiogram discs.

The strains were characterized as sensitive, intermediate sensitive or resistant to tested conventional antibiotics according to recommended criteria (CLSI 2015).

The comparison of the environmental strains resistance levels with 20 previously characterized multi-drug resistant (MDR) *A. baumannii* wound isolates (Aleksic et al. 2014) was also done using the Wilcoxon test in the program Statistica 10 (StatSoft Inc. 2011). The determined differences were considered to be statistically significant when $p \leq 0.05$.

Integron class 1 and 2 detection

Multiplex PCR amplification of type 1 and 2 integrase genes was carried out using genomic DNA as a template and included initial denaturation at 94 °C for 5 minutes, followed by 35 reaction cycles (30 s of denaturation at 94 °C, 30 s of primers annealing at 55 °C and 30 s of extension at 72 °C) and a final extension of 7 minutes at 72 °C (Koeleman et al. 2001). The size of the products obtained for the *intI1* gene was 160 bp, and for the *intI2* gene was 288 bp. Products were visualized as described above.

RAPD-PCR analysis

For the analysis, the genomic DNAs of *A. baumannii* environmental strains, twenty previously characterized MDR *A. baumannii* outpatient and clinical wound isolates (Aleksic et al. 2014), as well as three reference *Acinetobacter baumannii* strains (ATCC19606, ATCC BAA747 and NCTC13423) were used to determine phylogenetic relationships of the strains of different origin. PCR-RAPD strain characterization was carried out using five primers: OPN-02, OPB-11, K15, OPB-06 and OPA-08, as recommended by the manufacturer (Table 2). Amplification of PCR fragments was performed in 15 μL of the total volume of the reaction mixture, containing 100 ng of each primer, 0.1 U Taq polymerase and 1 μL of bacterial DNA. PCR amplification was carried out in 39 reaction cycles (2 minutes denaturation at 94 °C, 1-minute primer binding at 36 °C and 2 minutes extension at 72 °C) and final extension of 10 minutes at 72 °C. Amplified fragments were detected by 1 % (w/v) agarose gel electrophoresis in Tris/acetate/EDTA buffer. Visualization and documentation of agarose gels was carried out as described above. Results obtained by the RAPD-PCR method were used to create a cluster based on the similarity between DNA profile patterns for each pair of strains, using the Free Tree software (Pavliček et al. 1999) and Tree View (Roderic 2001).

RESULTS

Isolation of the Acb complex environmental strains was carried out from different types of samples, as well as by application of various media, which according to the obtained results proved to be suitable for the purpose of the conducted research, with some being more favourable than others.

Acb complex strain isolation

After incubation of 9 enrichment media inoculated with original samples, growth was detected visually and by turbidity measurements. The detected values for light transmission ranged from 54% to 99% (Table 3). A decrease in the

Table 3. Percentage of light transmission of the enrichment media containing various carbon sources after 24- and 48-hours incubation at a temperature of 37 °C; samples with successful isolation and positive preliminary tests are underlined, while samples with isolates with positive confirmatory tests are in bold.

Sample designation	Light transmission (%)																		
	24 h									48 h									
	Ac	Al	E	Ph	H	La	Le	P	T	Ac	Al	E	Ph	H	La	Le	P	T
M	**87** *	82	98 *	89 *	85	89 *	90 *	85	89 *	**86**	**78**	98 *	84	**82**	84	82	81	81
S	**87**	77	**89** *	**88** *	**72**	87	**86**	90 *	**87** *	85	62	72	74	62	85	85	84	**85**
B	88 *	84	87 *	90 *	85	90 *	90 *	**86**	86	88 *	81	86	89 *	84	89 *	88 *	84	85
DTD	88	84	87	86	85	86	87	85	**88**	76	66	82	81	82	83	85	81	**83**
DN	**83**	**81**	85	**81**	**67**	77	97 *	78	**77**	**82**	62	67	54	60	72	**86**	74	54
DZ	79	79	85	79	68	81	96 *	**75**	**81**	**80**	**64**	63	**55**	**65**	75	76	73	**55**
DP	83	83	**87**	**84**	86	86	86	80	86	85	85	85	**83**	82	85	85	80	81
DB	88 *	86	87 *	87 *	85	86	84	85	87 *	82	85	86	86	77	81	83	84	84
P1	88 *	89 *	89 *	90 *	89 *	89 *	88 *	99 *	92 *	88 *	89 *	89 *	89 *	82	84	87	85	82
P2	87 *	89 *	88 *	90 *	87 *	90 *	90 *	87 *	88 *	86	87	83	89 *	85	83	86	84	80
P3	89 *	89 *	88 *	89 *	89 *	88 *	89 *	88 *	88 *	88 *	87 *	87 *	88 *	86	88 *	89 *	86	85

* - visual absence of growth; Ac - Na-acetate, Al - β-alanine, E - ethanol, Ph - L-phenylalanine, H - L-histidine, La - Na-L-lactat, Le - L-leucine, P - Na-pyruvate, T - L-tyrosine.

intensity of light transmission was detected during prolonged incubation. After incubation for 24 h, the light transmission value ranged from 67% to 99%, and after 48 h of incubation from 54% to 89%. The substrate with the highest light transmission was ethanol (85-98% after 24 h, and 63-98% after 48 h), as well as leucine (84-97% after 24 h and 76-89% after 48 h). The lowest light transmission, indicating the highest growth intensity, was detected on medium with histidine as a substrate after 24 h (60-86%), and on medium with tryptophan (54-85%) after 48 h. Groundwater samples (P1, P2, and P3) had the highest light transmission (87-99% after 24 h, 80-89% after 48 h), while the DN and DZ samples had the lowest (67-97% after 24 h, 54-86% after 48 h).

Acb complex strain identification

Following isolation from enrichment media, the isolates obtained after 24 hours and 48 hours of incubation were subjected to identification tests. By enrichment of 11 samples from the natural environment on 9 media supplemented with different substrates, a total of 66 strains from eight different samples were isolated, while for the remaining three samples isolation was not successful. In Table 3 an overview of the enrichment media that proved to be successful for isolation is presented.

Following application of preliminary and confirmatory standard biochemical tests, a total of 30 isolates originating from environmental samples that belong to the Acb complex were identified. In the case where confirmatory tests yielded the expected results, further identification of eight selected strains originating from different environmental samples was carried out.

The additional identification of selected strains was performed using VITEK2, which confirmed that the tested isolates belong to the Acb complex. The molecular-genetic methods enable identification of isolates to the level of the genomic species (Table 4). All of the isolates yielded both expected PCR products of the corresponding size, with the exception of Aba-B-Phe and Aba-DP-Phe (Fig. 1A). According to the results, these two strains are members of the Acb complex, but not the genomic species *A. baumannii*. According to growth temperature and PCR results, these two strains belong to 13 TU group. These isolates were used as members of the Acb complex originating from the environment in characterization tests, except in the RAPD-PCR analysis of *A. baumannii* isolates.

Acb complex strains growth using different carbon sources

Three tested *A. baumannii* strains (ATCC 19606, ATCC BAA747 and Aba-DN-Ace) have the ability to grow on media with different substrates as the sole carbon source (Fig. 2). Acetate has been shown to be the least suitable source of carbon for the growth of this bacterium, since the growth rate of *A. baumannii* on this substrate was low even after 48 hours of incubation. Similar results were obtained in the case of ethanol. After 24 hours, increasement factors for growth kinetics, measured as OD_{620}, were 1.2 for both substrates, and after 48 hours of incubation only ~1.4 and ~1.8 for acetate and ethanol, respectively. L-tyrosine proved to be the most suitable source for growth of all three strains, as it enabled a very high growth rate for *A. baumannii*. In the period from 0-24 h incubation, the growth rate increased by 1.8×, while after 48 hours of incubation it increased approximately 6.67× for all three strains. Alanine, pyruvate and L-histidine also proved to be very suitable sources of carbon for *A. baumannii* strains growth. After tyrosine, alanine caused the highest growth after 48 h (approximately 5.67× higher with respect to time zero), while pyruvate and L-histidine induced a similar growth intensity, with a total increase in growth of about ~5.0×. The growth intensity of *A. baumannii* on substrates L-phenylalanine, lactate and L-leucine could be characterized as moderate, since the multiplying factor in cell count after 24 hours of incubation was approximately 2.50, 2.8 and 2.9, respectively, and after 48 h was ~3.7 using the first two substrates, and ~3.9 on L-leucine.

Sensitivity of Acb complex strains to conventional antimicrobial agents

The sensitivity of *A. baumannii* environmental isolates

Table 4. Information about environmental isolates.

Strain designation	Locality	Enrichment media	Level of identification
Aba-S-Ace	Srnjanska river, Kruševac	Na-acetate	*A. baumannii*
Aba-S-Tyr	Srnjanska river, Kruševac	L-tyrosine	*A. baumannii*
Aba-DZ-Ace	Danube – after the sewage inflow, Novi Sad	Na-acetate	*A. baumannii*
Aba-DN-Ace	Danube – before the sewage inflow, Novi Sad	Na-acetate	*A. baumannii*
Aba-M-Ace	West Morava, Kruševac	Na-acetate	*A. baumannii*
Aba-DP-Phe	Artificial lake of the Danube, Park, Novi Sad	L-phenylalanine	Acb complex
Aba-DTD-Tyr	Danube-Tisa-Danube Canal, Novi Sad	L-tyrosine	*A. baumannii*
Aba-B-Phe	Begeč pit, Begeč	L-phenylalanine	Acb complex

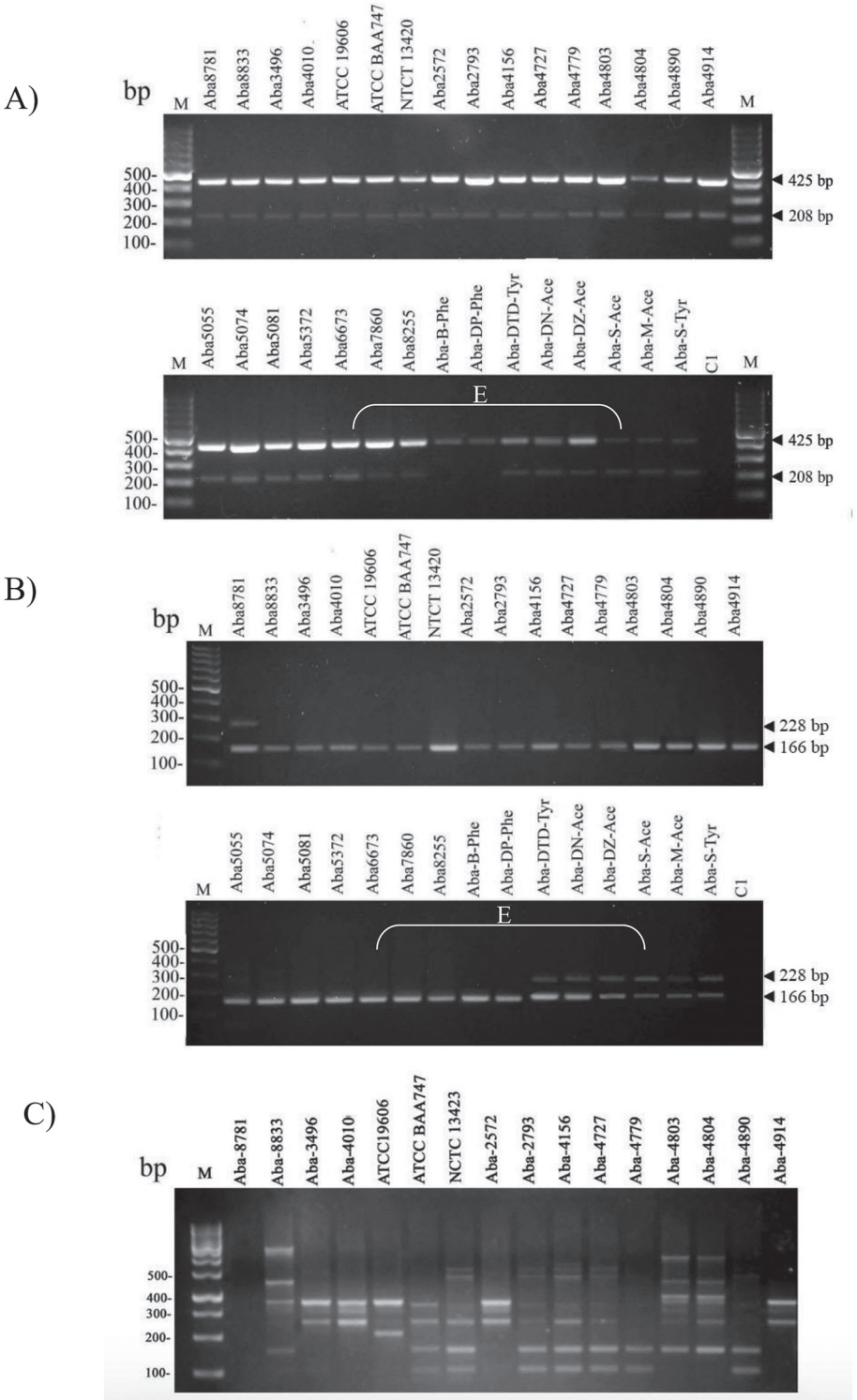

Fig. 1. PCR characterisation: (A) identification of genus and species; (B) IntI1 and 2 presence in strains; (C) example of RAPD-PCR profiles of individual *Acinetobacter baumannii* strains using primer OPN-02. E- environmental strains.

and two isolates of Acb complex to conventional antimicrobial agents is shown in Tables 5 and 6. The obtained MIC and MBC values confirm the fact that strains are highly resistant to many antibiotics. Namely, all of the tested strains were resistant to ceftriaxone, tetracycline and chloramphenicol, and high levels of resistance were also found for ciprofloxacin, gentamicin and kanamycin (63.6% strains), as well as for tobramycin and trimethoprim/sulfamethoxazole (54.5%). Among the examined conventional antibiotics, netilmicin, polymyxin B, and amikacin proved to be quite efficient, with 45% of the resistant strains. The most effective conventional agent was imipenem, to which 18.2% strains were resistant.

According to the results, it can be noticed that not all antibiotics had an effect on *A. baumannii* growth, so that a different level of strain resistance was detected. One isolate, Aba-DP-Phe, was resistant to all antibiotics, and Aba-DN-Ace isolate showed resistance to 11 antibiotics. Isolates from the same sample Aba-S-Ace and Aba-S-Tyr showed resistance to 10 and 9 antibiotics, respectively. A total of three isolates (Aba-DZ-Ace, Aba-M-Ace and Aba-B-Phe) were resistant to eight different antibiotics. Isolate Aba-DTD-Tyr was the most sensitive environmental isolate because it exhibited resistance to 6 out of 12 antibiotics tested. All the isolates demonstrated resistance to three or more conventional antibiotics, thus *Acinetobacter baumannii* strains and two strains belonging to Acb complex from the culture collection can be considered as MDR strains. A statistically significant difference in the degree of resistance of previously identified wound isolates (Aleksic et al. 2014) and environmental isolates was not found ($p = 0.36$).

Integron presence in environmental and clinical Acb complex strains

Genomic DNA analysis of *A. baumannii* isolates and two strains of Acb complex confirmed the presence of the *intI1* gene in all tested isolates, while the presence of the *intI2* gene was detected in 7 strains (24.1%). Interestingly, only one isolate was from human pathological material (Aba-8781), while six isolates originated from natural environments (Aba-DTD-Tyr, Aba-DN-Ace, Aba-DZAce, Aba-S-Ace, Aba-M-Ace, and Aba-S-Tyr) (Fig.1B).

Genetic variability of *A. baumannii* strains

The RAPD-PCR method was used to analyse the genetic variability, that is, the proximity or distance among 29 *A. baumannii* strains from the culture collection. The total number of detected products for all *A. baumannii* strains using five different primers was 485 gel bands. When primers K-15 and OPN-02 were used, the largest number of products was detected (total of 118 bands for all tested strains), while

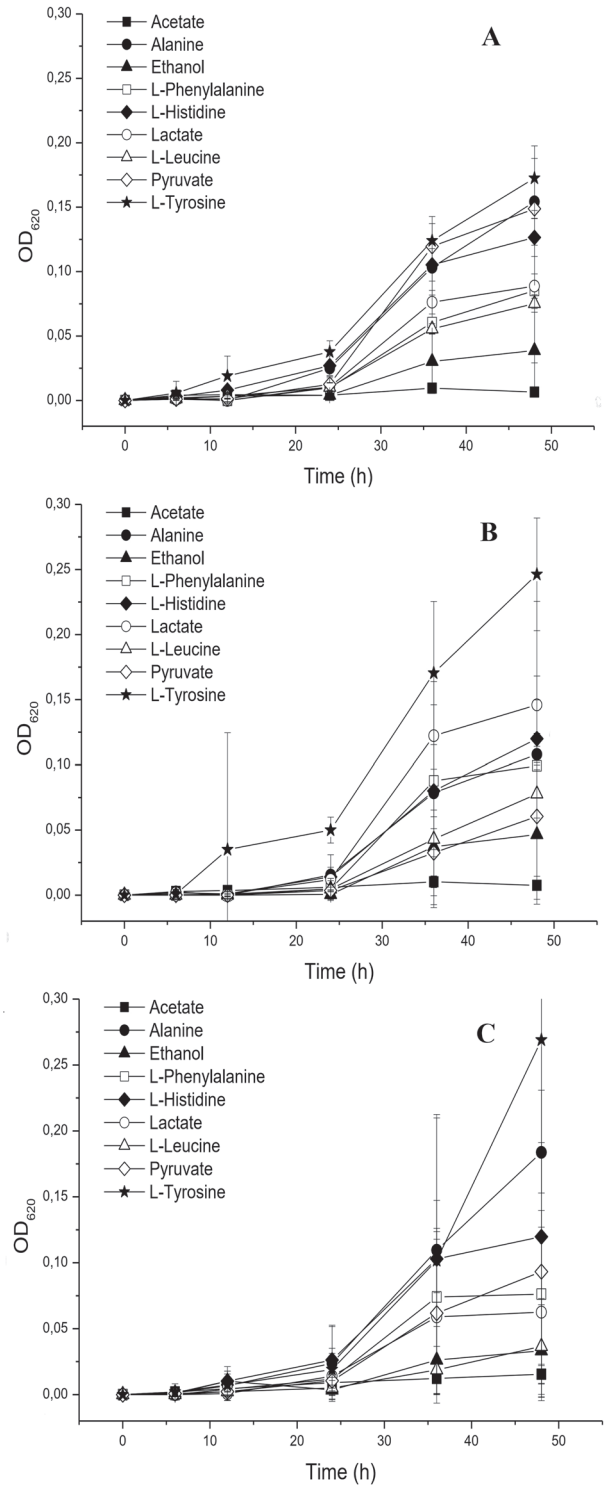

Fig. 2. Growth kinetics of *Acinetobacter baumannii* strains ATCC 19606 (A), ATCC BAA747 (B) i Aba-DN-Ace (C) on media with different substrates as sole carbon sources.

Table 5. MIC and MBC values of conventional antimicrobial agents against *Acinetobacter calcoaceticus-A. baumannii* complex strain.

A. baumannii strains		Conventional antimicrobial agents													
		Amikacin		Ceftriaxone		Ciprofloxacin		Chloramphenicol		Gentamicin		Kanamycin		Polymyxin B		Tetracycline	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Reference strains	ATTC19606	<0.25*	0.50	45.25	45.25	0.35*	0.35	64	128	16	16	4*	4	0.5*	0.5	2*	4
	ATCCBAA747	0.35*	0.35	45.25	90.51	0.35*	0.35	64	256	0.5*	0.5	2*	2	0.5*	0.5	5.66	11.31
	NCTC 13423	0.35*	0.35	22.63	45.25	0.35*	0.35	64	128	4*	16	2*	4	0.5*	0.5	2*	8
Environmental strains	Aba-S-Ace	16*	32	45.25	256	4	45.25	90.51	181.02	8	45.25	128	128	2	2	90.51	181.02
	Aba-S-Tyr	32	181.02	90.51	256	16	128	90.51	>256	4*	64	256	256	1.41*	2.83	>256	>256
	Aba-DZ-Ace	64	181.02	90.51	>256	11.32	128	90.51	>256	16	45.25	22.63	>256	1*	1	256	>256
	Aba-DN-Ace	>256	>256	>256	>256	64	>256	90.51	256	>256	>256	>256	>256	2	5.66	>256	>256
	Aba-M-Ace	64	>256	181.02	>256	22.63	256	90.51	181.02	64	45.25	256	>256	2.83	4	>256	>256
	Aba-DP-Phe	45.25	128	90.51	>256	5.66	128	90.51	>256	11.31	32	45.25	>256	2	4	181.02	>256
	Aba-DTD-Tyr	11.3*	256	16	256	5.66	128	90.51	>256	5.66	22.63	22.63	>256	1*	2.83	64	181.02
	Aba-B-Phe	11.3*	8	22.63	32	0.25*	5.66	90.51	181.02	0.5*	4	0.71*	4	2	2.83	90.51	>256
E. coli ATCC25922		2	-	0.25	-	<0.06	-	4	-	0.5	-	2	-	1	-	1	-
S. aureus ATCC 25923		1	-	<0.25	-	<0.06	-	4	-	0.25	-	2	-	0.5	-	1	-

* Strains sensitive to conventional antibiotics by MIC (μg mL^{-1}) values interpreted according to the standard (CLSI 2015). The results are presented as the geometric mean of three repetitions.

the lowest number was detected when the primer OPB-06 was applied (77 bands in total for all strains) (Suppl. 1). The greatest number of products were obtained using primer K-15 (1-10 bands per gel for each strain), slightly lower using OPN-02 (1-8 bands) (Fig. 1C), OPB-11 (1-7 bands) and OPB-06 (1-6 bands), while the OPA-08 primer gave the smallest number of RAPD-PCR products (0-6 bands per gel). When single *A. baumannii* strains were observed, the most products or gel bands were detected for the Aba-DTD-Tyr isolate from the natural environment (24 bands), but also for strains from wounds, Aba-4890 and Aba-8833 (23 bands), while the smallest number of products (only 9) were detected in the Aba-4914 strain (results are not shown).

A phylogram constructed using RAPD-PCR analysis of *A. baumannii* strains is shown in Fig. 3. The cophenetic correlation coefficient (r) of the shown phylogram is 0.87. The detected similarity between pairs of all individual strains, regardless of origin, ranges from 0% to 86.7%, where the highest detected similarity is grouped into two strains of Aba-2793 and Aba-4727. All the *A. baumannii* isolates are divided into two large clusters based on detected RAPD-PCR fingerprints, where the strength of their separation is very significant (bootstrap value is 100). The first cluster contains 8 *A. baumannii* strains (reference strain ATCC 19606, Aba-DZ-Ace strain from the environment and six wound isolates), and the other cluster contains remain *A. baumannii* strains (21 strains). The larger cluster is divided into two new clusters, in which a special branch identifies the species originating from the same environmental sample (Aba-S-Ace and Aba-S-Tyr), which according to the results presented on the phylogram are clonally different, and the established similarity is 27.6%.

DISCUSSION

The genomic species of the *Acinetobacter calcoaceticus-baumannii* complex (family Moraxellaceae) include species 1, 2, 3, and 13 TU. Although it is difficult to distinguish them phenotypically, only the representatives of *A. baumannii*, i.e. genomic species 2, grow at a temperature of 41 °C and 44 °C, with the exception of some representatives of 13 TU genomic species, which can also grow at these temperatures (Brenner et al. 2005).

One of the interesting features of the genomic species *A. baumannii* is the ability to grow on minimal substrates with different carbon sources, which were examined in the present study. Acetate as the sole source of carbon can be used by many bacteria, including bacteria from the Enterobacteriaceae family. This ingredient is routinely used in enrichment media intended for the isolation of bacteria of the Acb complex. Although enterobacteria can utilize acetate, after enrichment only bacteria from the Acb complex were

Table 6. *Acinetobacter calcoaceticus-A. baumannii* complex strain sensitivity determined by disc diffusion method.

A. baumannii strain	Imipenem		Netilmicin		Tobramycin		Trimethoprim/ Sulfamethoxasole	
	ZID (mm)	Int.*	ZID (mm)	Int.	ZID (mm)	Int.	ZID (mm)	Int.
ATTC19606	32	S	22	S	21	S	18	S
ATCCBAA747	34	S	23	S	19.5	S	17.5	S
NCTC 13423	28.5	S	19	S	18	S	0	R
Aba-S-Ace	19	S	0	R	0	R	0	R
Aba-S-Tyr	16	S	0	R	6	R	0	R
Aba-DZ-Ace	20	S	17	S	10	R	0	S
Aba-DN-Ace	24	S	0	R	6,7	R	16	R
Aba-M-Ace	22	S	17	S	15	S	13	I
Aba-DP-Phe	0	R	0	R	0	R	0	R
Aba-DTD-Tyr	22	S	16	S	15.5	S	11	I
Aba-B-Phe	0	R	0	R	0	R	0	R
E. coli ATCC25922	30	S	22	S	20	S	23	S
*S. aureus*ATCC 25923	32	S	23	S	22	S	25	S

* S-sensitive, I-intermediate, R-resistant, ZID- zone inhibition diameter, Int. – Interpretation.

isolated, indicating the high efficiency of this substrate in combination with Herella agar. However, use of medium with acetate as a carbon source was associated with very slow growth, even though acetate was proposed to be an appropriate substrate for isolation of the species *A. baumannii* by Baumann (1968). It is interesting to note that use of lactate in the enrichment medium did not result in isolation of any of the strains of this complex, although, like acetate, it is also recommended for routine use (Towner 2006). Successful isolation was obtained also with alanine, histidine and L-tyrosine, followed by phenylalanine, while use of ethanol and pyruvate for isolation was less efficient. Since no substrate enabled isolation from all of the samples, new carbon sources or their combinations should be further considered. This is probably a consequence of various other bacteria present in the samples that can utilise the substrates tested here, and thus compete with bacteria from the Acb complex.

Three *A. baumannii* strains (ATCC 19606, ATCC BAA747 and Aba-DN-Ace) were used to examine growth on media with different substrates as the sole carbon source (Fig. 2). According to the determined growth rate of *A. baimnannii*, the administered substrates can be divided into three groups: (1) substrates of high growth intensity, including L-tyrosine, alanine, L-histidine and pyruvate, which after 24 h resulted in an increase in absorbance >3.0 times and after 48 h an increase of >5.0 times; (2) substrates of moderate growth intensity, including L-leucine, L-phenylalanine and lactate, which resulted an increase in absorbance of 1.5-3.0 after 24 h and 2.0-5.0 times after 48 h, and (3) substrates of low growth intensity for *A. baumannii*, such as acetate and ethanol, which resulted in an increase in absorbance <1.5 after 24 h and <2.0 after 48 h. It is obvious that the success of isolation and growth in monoculture on the same substrate are not correlated to each other, indicating that optimal substrates for isolation and cultivation of bacteria from Acb complex are different.

Identification by molecular methods and the VITEK2 system confirmed the *A. baumannii* isolates previously identified by classical microbiological methods, with the exception of 2 strains from the environment. This means that the power of classical microbiological methods should not be disregarded or underestimated during the process of bacterial identification. In fact, it is not recommended to rely solely on one type of identification method, but instead to use a combination of standard microbiological, biochemical and molecular-genetic methods in order to identify strains as accurately as possible to the level of species; especially for identification to the level of genomic species, as is the case with *A. baumannii*. A collection of strains of *A. calcoaceticus-A. baumannii* complex at the Department of Microbiology, Faculty of Science, University of Novi Sad comprises a total of 31 strains, of which members of the genomic species *A. baumannii* are three reference strains, twenty clinical wound isolates and six environmental isolates, while two strains from the environment are identified as other members of the Acb complex, i.e. 13 UT group.

The existence of numerous commercial food additives and antibiotics to control infections in humans often leads to an increase in bacterial antibiotic resistance. *Acinetobacter baumannii* is resistant to a large number of antimicrobial

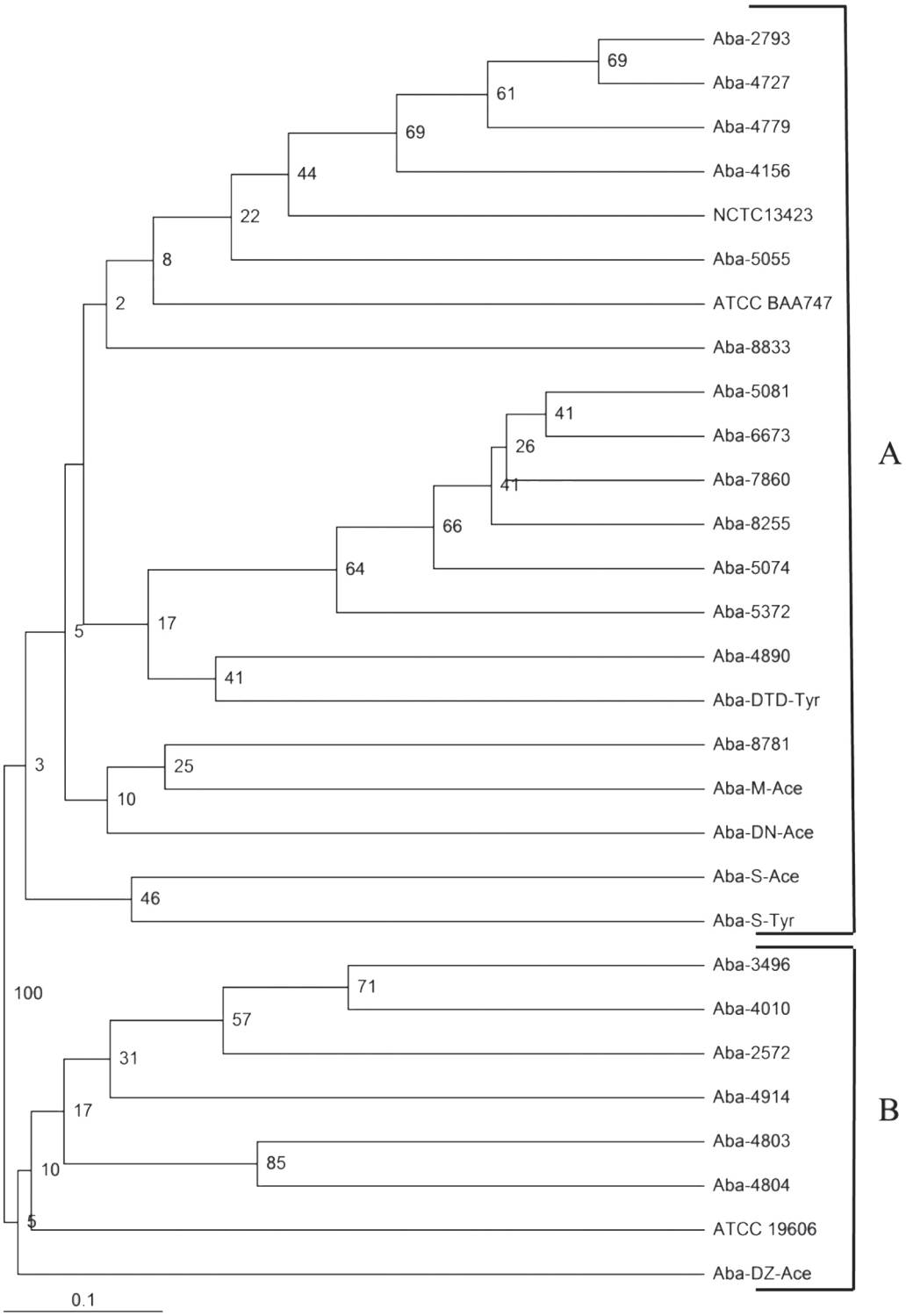

Fig. 3. Dendrogram constructed according to the RAPD-PCR analysis of *Acinetobacter baumannii* isolates.

drugs, which belong to different groups, while MDR isolates are spreading worldwide. The infections they cause represent a serious therapeutic problem (Peleg et al. 2008; Medić et al. 2011). In the present study, the detected resistance of *A. baumannii* isolates to conventional antimicrobial agents is very concerning, but is in accordance with the current situation in terms of the global expansion of multiple resistance in this genomic species.

Of all of the examined conventional antimicrobial agents, imipenem and polymyxin B displayed the most prominent antimicrobial activity, whereas all strains showed resistance to ceftriaxone, tetracycline and chloramphenicol. Considering the fact that the species *A. baumannii* is a common etiological agent of urinary infections, and fluoroquinolone antibiotics are the most common choice for the treatment of these infections, the high degree of resistance to ciprofloxacin that was detected in the present study is very worrying. A somewhat lower percentage of resistance to these antibiotics was detected by Karlowsy et al. (2003), while in the study of Medić et al. (2011), which also focused on isolates originating from Serbia (Vojvodina province), the disc-diffusion method revealed that this percentage is significantly higher: 100% for ciprofloxacin and cephalosporins of the third and fourth generations (ceftriaxone, ceftazidime and cefepime), 94.8% for amikacin, 88.9% for gentamicin and 87.4% for co-trimoxazole. Also, the resistance to the carbapenem imipenem was very high – 67.4% (Medić et al. 2011). Such results are mainly in agreement with the results obtained here for *A. baumannii* isolates, which certainly indicates an alarming situation in terms of the presence and spread of resistant strains of this species from the territory of Serbia.

Based on the criterion that the multiple resistance of *A. baumannii* implies resistance to three or more antibiotics from chemically different groups (Magiorakos et al. 2012) or antibiotics to which the bacteria develop various mechanisms of resistance, all *A. baumannii* isolates can be characterized as MDR. It is important to note that the class of phenicols, i.e. the antibiotic chloramphenicol, is included here, which according to Abbott et al. (2013) can also be used against *A. baumannii* isolates, and is not listed in the previous study of Magiorakos et al. (2012). Such results are in agreement with the results of Medić et al. (2011) which also analysed the strains of *Acinetobacter* spp. and characterized it as MDR, similarly to results from Italy (Bassetti et al. 2008), Slovakia (Babik et al. 2008), Greece (Giamarellou et al. 2008), America (Ray et al. 2010) and China (Jiang et al. 2008). Thus, the multiple resistant strains are detected not only in clinical strains, but also in environmental strains.

Antibiotic resistance in the Acb complex bacteria can be caused by the presence of integrons in bacteria. Integrons are transposable genetic elements that possess the ability to transition from plasmids to bacterial chromosomes and *vice versa*. Class 1 integrons consist of a 5′-conserved segment carrying a gene named *intI,* which encodes a site-specific integrase protein, and a variable region containing one or more antibiotic resistance genes, while the related class 2 integrons contain a mobile genetic element, *Tn7* transposon, and their structures resemble class 1 integrons (Cicek et al. 2013). Their insertion and excision are mediated by recombinase activity belonging to the integrase family that specifically recognize certain sites in the genome. In this way integrons serve as carriers of genes responsible for antibiotic resistance, and enable their transfer and insertion in the bacterial genome. *A. baumannii* genomic DNA showed the presence of integrase from both class 1 and class 2 integrons, and these genes are highly specific, in contrast to other genes within the integron gene cassettes, which are variable. According to the obtained results for multiple resistant *A. baumannii* isolates, all strains both from wounds and from the natural environment possessed genes for class 1 integron, while seven isolates, predominantly those from environmental origin, possessed the class 2 integron gene. This indicates that class 2 integron should be further examined as a marker for environmental strains. These results are in agreement with data available in the literature, because class 1 integrons were the most often detected integrons in most studies of *A. baumannii* isolates, while class 2 integrons were found in a smaller number of isolates of this species (Seward and Towner 1999; Ploy et al. 2000; Koeleman et al. 2001; Turton et al. 2005; Taherikalani et al. 2011; Koczura et al. 2014). The high prevalence of class 1 integrons in relation to class 2 integrons was detected in resistant hospital isolates originating from France, Greece and the Czech Republic (80% integron 1 positive strains) (Ploy et al. 2000), Iran (80%) (Taherikalani et al. 2011), Taiwan (71.4%) (Huang et al. 2008), and Poland (63.5%) (Koczura et al. 2014). In the United Kingdom, class 1 integrons were found in all epidemic isolates, but not in sporadic isolates, while the presence of class 2 integrons was not detected (Turton et al. 2005). In contrast to the results obtained in the present study for the collection of *A. baumannii* strains, as well as results reported in the aforementioned studies, in the study of Gonzalez et al. (1998) concerning *A. baumannii* strains originating from hospitals in Chile, only 5.1% of strains possessed the *intI1* gene, while the *intI2* gene was detected in 69.3% of strains of this species. Taken together, it can be assumed that the *intI1* gene is present in *A. baumannii* strains originating from Europe and Asia, while the *intI2* gene dominates among strains originating from South America. The results obtained in the present study for environmental isolates are very interesting, and indicate that these strains could be a reservoir of class 2 integrons, which

previously have not been frequently observed in Europe and should be further examined.

Comparing the similarity of RAPD-PCR profiles (fingerprints) of *A. baumannii* environmental and clinical isolates, it was found that all of the isolates were clonally different, even strains from the environment isolated from the same sample, by using different sources of carbon (e.g. Aba-S-Ace and Aba-S-Tyr). The lowest number of RAPD-PCR fingerprints was detected using OPB-06 primer, indicating the weaker discriminatory capacity of this primer. In addition, the obtained results indicate that, although the most discriminating primer for the analysis of *A. baumannii* strains is OPN-02, the application of a number of primers in RAPD-PCR analysis increases the discrimination of the method and allows for more precise construction of the phylogram. The obtained cophenetic correlation coefficient (0.87) for the constructed phylogram indicates that the similarity among *A. baumannii* strains is appropriate, i.e. realistically represents their relationships, as according to Rodrigues et al. (2002), values of the cophenetic correlation coefficient above 0.8 indicate a more realistic presentation of the similarities between the strains. Considering the dendrogram, two distinctive clades are present – clade A which is comprised of 21 strains, including reference strains BAA-747 and NCTC13423, and clade B that is comprised of 8 strains, including one environmental strain and ATCC19606. Some strains from the environmental and clinical samples had highly similar RAPD-PCR patterns. A similar phenomenon was previously detected by Wroblewska et al. (2004), who also tested *A. baumannii* strains from the environment and strains isolated from the cerebrospinal fluid of neurosurgical patients with meningitis, using two primers for RAPD-PCR analysis. The assumption of these authors was that environmental strains with the same profiles as human isolates are most likely epidemiologically related, in agreement with results obtained here in the present study for Aba-DTD-Tyr, Aba-M-Ace and Aba-DN-Ace strains having the same profiles as isolates from wounds, even though they were obtained with different primers. The high level of strain similarity is in accordance with the fact that *A. baumannii* is a genome species, with a high level of DNA similarity. The agreement between results obtained by genotypic and phenotypic strain characterization confirms the relevance and applicability of the RAPD-PCR method, since it provides an efficient and fast approach for determining the similarity of bacterial isolates, including genomic species such as *A. baumannii*.

ACKNOWLEDGEMENTS

The authors acknowledge financial support from the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 451-03-68/2020–14/200125).

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Suppl. 1. RAPD-PCR analysis data

	Detected products*						Detected fingerprints**				
Acinetobacter baumannii	K-15	OPB-06	OPB-11	OPN-02	OPA-08	Total	K-15	OPB-06	OPB-11	OPN-02	OPA-08
Aba-2572	1	3	3	3	3	13	b2	a1	b	b	a1
Aba-2793	5	1	1	5	2	14	b	a	c	c	b
Aba-4156	3	4	1	6	2	16	b1	a	c	c	b
Aba-4727	4	2	1	5	2	14	b1	a3	c	c	b
Aba-4779	5	1	1	2	2	11	b	a	c	d	b
Aba-4803	2	1	3	6	3	15	a1	b	d	e	a
Aba-4804	1	3	2	6	6	18	1	a1	d	e	c
Aba-4890	8	1	5	3	6	23	b1	a	c	c	f
Aba-4914	1	1	4	3	0	9	c	b	c1	b	g
Aba-5055	3	1	1	5	2	12	b	a	c	c	b
Aba-5074	7	2	2	6	2	19	b	a	c1	c2	b
Aba-5081	4	2	1	4	2	13	b1	a	c	d	b
Aba-5372	4	2	1	4	5	16	b1	a	c	c	f
Aba-6673	6	2	1	4	1	14	b	a	c	c	g
Aba-7860	6	4	2	4	2	18	b	a	c1	c	a2
Aba-8255	5	1	2	5	2	15	b	a	c1	c	b
Aba-8781	3	3	1	4	2	13	a	a2	a	b	b
Aba-8833	8	3	3	5	4	23	b	a2	a1	a	a
Aba-3496	1	2	6	2	4	15	c	a1	a3	b	c
Aba-4010	1	2	4	3	6	16	c	a1	a	b	e
ATCC 19606	3	3	3	2	2	13	f	a2	a2	b1	a1
ATCC BAA747	2	4	4	3	2	15	h	a1	a3	c1	b
NCTC13423	5	2	1	5	2	15	b1	a	c	c	b
Aba-B-Phe	1	2	6	2	5	16	d	c	e	b2	d
Aba-DP-Phe	4	2	7	3	1	17	d	c	e	b2	d1
Aba-DTD-Tyr	10	4	5	3	2	24	b	a2	c	f	c
Aba-DN-Ace	2	2	3	2	2	11	e	a1	c1	g	b
Aba-DZ-Ace	3	3	4	3	1	14	f	a2	a	b2	h
Aba-S-Ace	2	5	3	8	3	21	g	d	f	h	c
Aba-M-Ace	7	4	2	1	2	16	e	a2	c1	a	c1
Aba-S-Tyr	1	5	7	1	2	16	a	d	f	i	c1
Total	118	77	90	118	82	485	11	7	10	13	11

* Number of detected gel bends; ** Different RAPD-PCR fingerprints obtained using five primers; the detected profiles are marked in letters, where similar profiles (the difference is in a single gel band) are marked as a new profile in which the name of the add-on number.