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DETECTION OF CARBAPENEM-RESISTANCE AND BIOFILM FORMATION GENES, AND GENETIC RELATEDNESS OF *Acinetobacter baumannii* ISOLATES

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Acinetobacter baumannii is one of the most important nosocomial pathogen worldwide. This study aimed to investigate the virulence potential and genomic relatedness of *A. baumannii* strains isolated from patients hospitalized in the Military Medical Academy (MMA) by detecting OXA-type carbapenemases genes, biofilm-associated genes, and by RAPD analysis. PCR was used to detect the bla_{oxa} genes, IS*Aba-1* genetic element, and biofilm-associated genes. The genomic relatedness was determined by RAPD analysis using four different primers (AP2, DAF4. M13, and DECA). $bla_{oxa\text{-}51\text{-}like}$, $bla_{oxa\text{-}23\text{-}like}$, $bla_{oxa\text{-}24\text{-}like}$, and $bla_{oxa\text{-}58\text{-}like}$ were present in 100%, 34.0%, 62.4%, and 3.1% of isolates, respectively. All isolates had the IS*Aba1* sequence in their genome, in 35.1% of isolates it was associated with the $bla_{oxa\text{-}51\text{-}like}$, and in 97.0% with the $bla_{oxa\text{-}23\text{-}like}$ gene. Biofilm-associated genes *bap*, *ompA*, *epsA*, *csuA/BABCDE*, and *pgaABCD* were detected in 93.8%, 95.8%, 88.1%, 98.4%, and 98.9% isolates, respectively. RAPD analysis showed a high degree of genome similarity and clonal dispersion of the isolates. Detection of bla_{oxa} genes, especially biofilm-associated genes, in a high percentage of *A. baumannii* isolates indicated their great pathogenic potential. RAPD analysis revealed a high level of genomic similarity and clonal dispersion of the majority of isolates through MMA. Further, a continuous introduction of individual strains with different profiles contributes to the genetic diversity of *A. baumannii* isolates. These results can be useful for further management and tracking nosocomial outbreaks.

Key words: Acinetobacter baumannii; OXA-type carbapenemases; pandrug-resistant; biofilm-forming genes; RAPD

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INTRODUCTION

Acinetobacter spp. are Gram-negative, non-glucose fermenting, cocco-bacilli. The most important species, *Acinetobacter baumannii*, is primarily associated with hospital-acquired infections worldwide. This pathogen can cause different types of infection, especially in intensive-care units (ICU) and burn units that involve patients on mechanical ventilation. *A. baumannii* is frequently isolated from inanimate objects and the skin of healthcare personnel (ALMASAUDI, 2018).

Carbapenem-resistance in *A. baumannii* strains is a major health problem worldwide (HIGGINS *et al*., 2009). Production of carbapenemases, especially enzymes belonging to class D is the most common mechanism of resistance. Insertion sequences (IS), like IS*Aba1*, can enhance their resistance (NOWAK and PALUCHOWSKA, 2016).

A. baumannii produces different virulence factors and one of the most important is biofilm-formation. Biofilm plays a role in the interaction with the host and contributes to infections associated with medical devices. It protects bacteria from antibiotics, the immune system, and harsh environmental conditions (EZE *et al*., 2008).

Several methods have been developed for the genotyping of *A. baumannii* (PELEG *et al*., 2008). PCR-based methods, including Random Amplified Polymorphic DNA (RAPD), with different primers, are commonly used (RAVI *et al*., 2018).

In Military Medical Academy (MMA) the epidemiological surveillance of nosocomial infections caused by *Acinetobacter* spp. are conducted until 1999 (ŠULJAGĆ *et al*., 2011). In recent years, a higher frequency of *A. baumannii*, especially carbapenems-resistant strains, was observed. This study aimed to investigate the occurrence of bla_{oxa} genes, the presence of insertion sequence IS*Aba1*, and its association with the bla_{oxa} genes, to detect the genes associated with biofilm-formation, and to determine the genetic relatedness of *A. baumannii* strains isolated from patients hospitalized in the MMA.

MATERIALS AND METHODS

Bacterial strains

A. baumannii was isolated from various clinical specimens, originated from different wards in the Military Medical Academy in Belgrade, Serbia in a period between June 2017 and January 2019. All wards could be classified into four groups Critical Care Unit (CCU), Emergency Department (ED), Clinical Surgery Departments (CSD), and other Medical Care Departments (MCD). A total of 194 non-duplicated isolates were collected during routine clinical procedures. The bacteria are identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS system) (Vitek MS, bioMerieux, Marcy-l'Etoile France). Identification was confirmed by PCR detection of intrinsic $bla_{oxa\text{-}51\text{-}like}$ and *gltA* genes, as previously described (KOBS *et al*., 2016). Antimicrobial susceptibility testing was performed using the standard disk diffusion method and minimal inhibitory concentration (MIC) according to Clinical and Laboratory Standard Institute (CLSI, 2020) guidelines using ampicillin/sulbactam (SAM), piperacillin/tazobactam (TZP), cefepime (FEP), ceftazidime (CAZ), cefotaxime (CTX), ceftriaxone (CRO), doripenem (DOR), imipenem (IPM), meropenem (MEM), gentamicin (GEN), tobramycin (TOB), amikacin (AMK), netilmicin (NET),

tetracycline (TET), doxycycline (DOX), minocycline (MIN), ciprofloxacin (CIP), levofloxacin (LVX), trimethoprim/sulfamethoxazole (SXT), colistin (CS).

DNA extraction

DNA was extracted by the boiling method. A couple of colonies from the agar plate were transferred to Luria-Bertani Broth (LB) medium and incubated overnight. From overnight culture, 1500 µl of bacteria were centrifuged for 1 minute at 10,000 rpm, washed with saline solution, and centrifuged again. The pellet was resuspended in 300 µl of sterile, distilled water. Then, the pellet was boiled for 10 minutes and transferred at -20ºC for 10 minutes. Boiling and freezing were repeated one more time. Next, tubes were centrifuged for 2 minutes at 10,000 rpm and the supernatant containing the DNA was transferred to the new tube and kept at -20ºC until analysis.

Detection of genes associated with biofilm formation

The presence of *ompA* (ALI *et al*., 2017), *epsA* (TAYABALI *et al*., 2012), and *bap* (ALI *et al*., 2017) genes was detected by singleplex PCR as previously described. Genes grouped in chaperone-usher pili (*csuA/BABCDE*) and *pgaABCD* operons were detected by separate multiplex PCR as previously described (ALI *et al*., 2017).

Detection of OXA-type genes and ISAba1 element

All isolates were screened for the occurrence of four groups of bla_{oxa} genes, intrinsic $bla_{oxa\text{-}51\text{-}like}$ and acquired $bla_{oxa\text{-}23\text{-}like}$, $bla_{oxa\text{-}24\text{-}like}$, and $bla_{oxa\text{-}58\text{-}like}$ by multiplex PCR as previously described (KOBS *et al*., 2016). The screening for the IS*Aba1* element in the genome, and upstream of $bla_{oxa\text{-}51\text{-}like}$ and $bla_{oxa\text{-}23\text{-}like}$ genes was performed by singleplex PCR as previously described (KOBS *et al*., 2016).

PCR product detection and analysis

All PCR products were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator.

Epidemiology typing

RAPD analysis was performed with the primers AP2 (LI *et al*., 2016), DAF4 (GRUNDMANN *et al*., 1997), M13 (GRUNDMANN *et al*., 1997), and DECA (CARR *et al*., 2001). Results were analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide, under UV transilluminator. All primers used in the study are listed in Table 1.
Table 1.

Statistical analysis

Cluster analysis of genetic profiles and construction of dendrograms were performed by STATISTICA 10 software.

Table 1. Priers used in the study

Gene	Sequence	Size (bp)	Reference
gltA	F: 5'-AATTTACAGTGGCACATTAGGTCC-3′ R: 5'-GCAGAGATACCAGCAGAGATACACG-3'	722	(KOBS *et al*., 2016)
ompA	F: 5'-GTTAAAGGCGACGTAGACG-3' R: 5'-CCAGTGTTATCTGTGTGACC-3'	578	(ALI *et al*., 2017)
epsA	F: 5'-AGCAAGTGGTTATCCAATCG-3' R: 5'-ACCAGACTCACCCATTACAT-3'	451	(TAYABALI *et al*., 2012)
bap	F: 5'-TACTTCCAATCCAATGCTAGGGAGGGTACCAATGCAG-3' R: 5'-TTATCCACTTCCAATGATCAGCAACCAAACCGCTAC-3'	1500	(ALI *et al*., 2017)
csuA/B-csuA	F: 5'-ACCAGCACACTCGATCTG-3' R:5'-TTACTGGTCAGGTTGACG-3'	802	(ALI *et al*., 2017)
csuA-csuB	F: 5'-AATGCGGGTGAAATCGG-3' R: 5'-TGTAGGTGTTGTAGCAGG-3'	906	(ALI *et al*., 2017)
csuB-csuC	F: 5'-CTCATCTACAATCAGACG-3' R: 5'- -TATGCAGCAGATCCTCAG-3'	842	(ALI *et al*., 2017)
csuC-csuD	F: 5'-TTGAACCGCCTTGATAGG -3' R: 5'-GAGCAGTCATATCGTCTG-3'	1029	(ALI *et al*., 2017)
csuD-csuE	F: 5'-CGTAAAGCTACTCATGTC-3' R: 5'-AAGTGCCTGATGTTCTGG-3'	35	(ALI *et al*., 2017)
pgaA	F: 5'-ACCCGCATGATCAAAAGCTG-3' R: 5'-AGCATATGCAACCCGTACCA-3'	338	(ALI *et al*., 2017)
pgaB	F: 5'-AAATGTTGGGCTGTGCCTTG-3' R: 5'-GCCCGAAGCTTGCATTTCTC-3'	470	(ALI *et al*., 2017)
pgaC	F: 5'-GCTCAGGTCCGACAAACGAT-3' R: 5'-TTTTCTGCCAGGTGCACAAC-3'	158	(ALI *et al*., 2017)
pgaD	F: 5'-GGCTGCTTTTTCCACTCGTT-3' R: 5'-GCTGCGACGATCATCTCCAT-3'	197	(ALI *et al*., 2017)
$bla_{oxa\text{-}51\text{-}like}$	F: 5'-TAATGCTTTGATCGGCCTTG-3' R: 5'-TGGATTGCACTTCATCTTGG-3'	353	(KOBS *et al*., 2016)
$bla_{oxa\text{-}23\text{-}like}$	F: 5'-GATCGGATTGGAGAACCAGA-3' R: 5'-ATTTCTGACCGCATTTCCAT-3'	501	(KOBS *et al*., 2016)
$bla_{oxa\text{-}24\text{-}like}$	F: 5'-GGTTAGTTGGCCCCCTTAAA-3' R: 5'-AGTTGAGCGAAAAGGGGATT-3'	246	(KOBS *et al*., 2016)
$bla_{oxa\text{-}58\text{-}like}$	F: 5'-AAGTATTGGGGCTTGTGCTG-3' R: 5'-CCCCTCTGCGCTCTACATAC-3'	599	(KOBS *et al*., 2016)
ISAba1	F. 5'-CACGAATGCAGAAGTTG-3' R. 5'-CGACGAATACTATGACAC-3'	548	(KOBS *et al*., 2016)
ISAba1-$bla_{oxa\text{-}51\text{-}like}$	F: 5'-CAAGGCCGATCAAAGCATTA-3' R: 5'-GTGTCATAGTATTCGTCG-3'	359	(KOBS *et al*., 2016)
AP2	5'-GTTTCGCTCC-3'	Variable	(LI *et al*., 2016)
DAF4	5'-CGGCAGCGCC-3'	Variable	(GRUNDMANN *et al*., 1997)

M13	5'-GAGGGTGGCGGTTCT-3'	Variable	(GRUNDMANN *et al*., 1997)
DECA	5'-GCTTGTGAAC-3'	Variable	(CARR *et al*., 2001)

RESULTS

Bacteria identification

All 194 isolates were identified as *A.baumannii* by the MALDI TOF MS system and confirmed by PCR detection of intrinsic $bla_{oxa\text{-}51\text{-}like}$ and *gltA* genes.

Antimicrobial susceptibility testing

Definitions for multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) bacterial isolates are according to MAGIORAKOS *et al*. (2012). The majority of the isolates, 149/194 (76.8%), showed XDR phenotype, PDR phenotype showed 38/194 (19.6%), and, MDR phenotype showed 7/194 (3.6%) isolates.

Detection of OXA-type genes and ISAba1 element

All isolates harbored the $bla_{oxa\text{-}51\text{-}like}$ gene. In 6/194 (3.1%) isolates this was the only bla_{oxa} gene. The $bla_{oxa\text{-}23\text{-}like}$ gene was present in 66/194 (34.0%), the $bla_{oxa\text{-}24\text{-}like}$ gene in 121/194 (62.4%), and the $bla_{oxa\text{-}58\text{-}like}$ gene in 6/194 (3.1%) isolates. The co-existence of $bla_{oxa\text{-}23\text{-}like}$ and $bla_{oxa\text{-}24\text{-}like}$ was detected in only four 4/194 (2.1%) isolates. One (0.5%) isolate harbored both $bla_{oxa\text{-}24\text{-}like}$ and $bla_{oxa\text{-}58\text{-}like}$.

All isolates were positive for the IS*Aba1* sequence in their genome. Upstream of the $bla_{oxa\text{-}51\text{-}like}$ gene IS*Aba1* element was detected in 68/194 (35.1%) isolates. Upstream of the $bla_{oxa\text{-}23\text{-}like}$ gene IS*Aba1* element was detected in 64 (97.0%) of 66 $bla_{oxa\text{-}23\text{-}like}$ positive isolates.

Detection of genes involved in biofilm formation

Bap gene was detected in 182/194 (93.8%), *ompA* gene in 186/194 (95.8%), and *epsA* gene in 171/194 (88.1%) isolates. Genes belonging to the *csu* operon were detected in 191/194 (98.4%) isolates. One isolate didn't show the *csuA* band, and in three isolates none of these genes were detected. Genes belonging to the *pgaABCD* locus were detected in 192/194 (98.9%) isolates. One of these isolates didn't show a *pgaB* band.

RAPD

All *A. baumannii* isolates were tested by the RAPD method using four different primers (AP2, DAF4, M13, and DECA). The bands obtained after analysis were scored based on their presence (+) or absence (-). AP2, DAF4, and M13 primers generated satisfactory results, while DECA primer generated the simplest profiles. Cluster analysis was performed using Ward's method and distance was calculated using squared Euclidean distances. Based on the RAPD data, a dendrogram was constructed separately for each primer. All isolates that were clustered at a similarity level $\geq$ 80 % were considered the same strain. RAPD profiles and dendrograms obtained with each primer are represented in Figure 1.

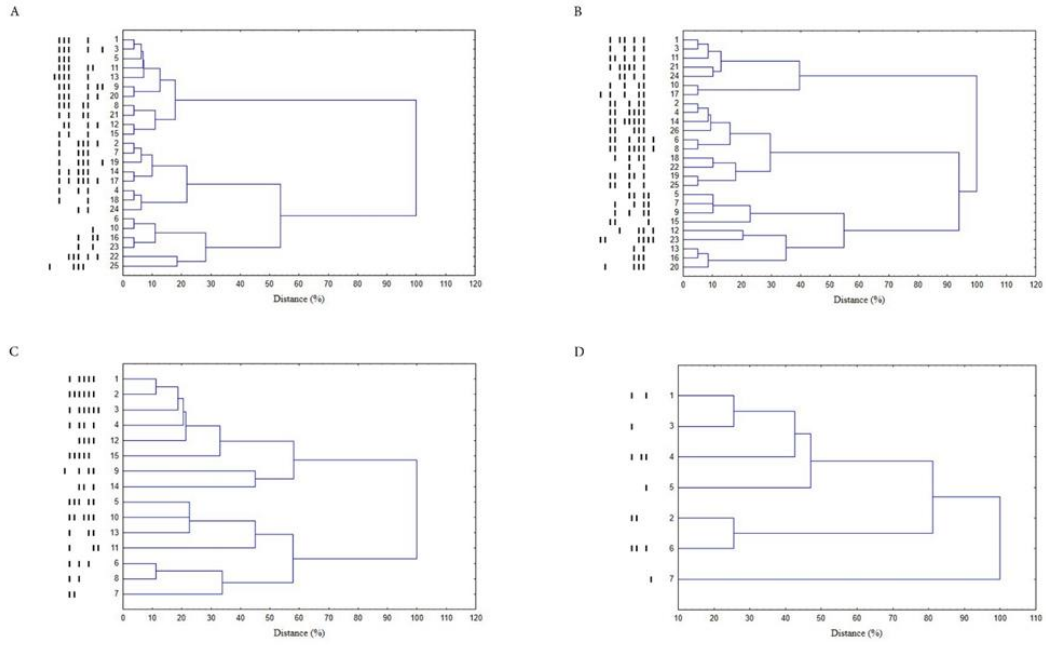

Figure1. Profiles and dendrograms derived from RAPD analysis of *Acinetobacter baumannii* isolates. A, AP2 primer; B, DAF4 primer; C, M13 primer; D, DECA primer.

With the AP2 primer, three major clusters were generated. In the first cluster, the most isolates were in profile 1 (133/194 or 68.5%), which differed from profile 3 (12/194 or 6.2%) in one band. Next to them was profile 5 (3/194 or 1.6%), 11 (1/194 or 0.6%) and 13 (1/194 or 0.6%). Also, profiles 9 (1/194 or 0.6%) and 20 (1/194 or 0.6%), 8 (2/194 or 1.0%) and 21 (1/194 or 0.6%), 12 (2/194 or 1.0%) and 15 (1/194 or 0.6%) showed the same similarity level. In the second cluster, the most similar profiles were 2 (4/194 or 2.1%) and 7 (13/194 or 6.7%). Next to them was profile 19 with one isolate. Profiles 14 (1/194 or 0.6%) and 17 (2/194 or 1.0%) differed in one band. Profiles 4 (5/194 or 2.5%) and 18 (2/194 or 1.0%) showed the same similarity level, Next to them was profile 24 (1/194 or 0.6%). In third cluster, profiles 6 (2/194 or 1.0%) and 10 (1/194 or 0.6%), 16 (1/194 or 0.6%) and 23 (1/194 or 0.6%) differed in one band. In this cluster, profiles 23 and 25, with one isolate each, showed a lower similarity level.

With the DAF4, primer, three major clusters were generated. In the first cluster, the most isolates were in profiles 1(14/194 or 7.3%) and 3 (25/194 or 12.9%). Next to them was profile 11 (1/194 or 0.6%). The same difference was between profiles 10 and 17 with one isolate each. From the second cluster, the most similar were profiles 2 (66/194 or 34.0%) and 4 (56/194 or 28.9%) that differed in one band. Next to them were profiles 14 and 26 with one isolate each. Profiles 6 and 8 also differed in one band, as profiles 19 and 25. Profiles 18 and 22 showed a lower similarity level. All these profiles had one isolate each. In the third cluster, the most

similar were profiles 13 and 16, and next to them was profile 20, with one isolate each. Profiles 5 (2/194 or 1.0%), 7 (5/194 or 2.5%), 9 (1/194 or 0.6%), 12 (1/194 or 0.6%) and 23 (1/194 or 0.6%) showed a lower similarity lever.

With the M13 primer, two major clusters were generated. Most isolates were in profile 1 (86/194 or 44.4%), which differed from profile 2 (77/194 or 39.6%) in one band. Next to them were profiles 3 (12/194 or 6.2%), 4 (7/194 or 3.6%), 12 (1/194 or 0.6%), and 15 (1/194 or 0.6%). Profiles 9 (2/194 or 1.0) and 14 (1/194 or 0.6%) showed a lower similarity level. In the second cluster profiles 6 and 8 differed in one band, and next to them was profile 7, with one isolate each. Profiles 5, 10, and 13 with one isolate each, had a lower similarity level, and next to them was profile 11 also with one isolate.

With the DECA primer, three major clusters were generated. Most isolates were in profile 1 (153/194 or 78.9%), which differed from profile 3 (15/194 or 7.8%) in one band. Next to them were profiles 4 (5/194 or 2.5%), and 5 (6/194 or 3.1%). In the second cluster, there were only two profiles 2 (13/194 or 6.7%) and 6 (1/194 or 0.6%) which differed in one band. The third cluster had only one profile with one isolate.

DISCUSSION

A. baumannii has become one of the major pathogens involved in hospital-acquired infections worldwide. It can quickly develop a resistant phenotype as a result of adaptability to environmental changes, and the ability to acquire foreign resistance determinants (ALMASAUDI, 2018).

We analyzed 194 *A. baumannii* isolates from clinical samples of patients treated in MMA, Belgrade in the period from June 2017 to January 2019. The majority of the isolates (149 or 76.8%) had XDR phenotype and were resistant to carbapenems. Seven isolates (3.6%) were MDR and only two of these were susceptible to carbapenems. We also detected 38 (19.6%) isolates resistant to all antibiotics, including colistin. With the emergence of carbapenem resistance in *A. baumannii*, colistin was considered to be the drug of choice (NOWAK *et al*., 2016). However, increasing resistance to colistin has been soon observed in many countries (QURESHI *et al*., 2015; OIKONOMOU *et al*., 2015). At the beginning of our study, from June 2017 to March 2018, the frequency of PDR strains was 8.8% and these were detected sporadically from the CCU and CSD. During the investigation period the frequency of these isolates raised, and from April 2018 to January 2019 the percentage of PDR raised to 38.3%. As to our knowledge, this is the first time that PDR *A. baumannii* strains were isolated in our country. In previous studies from Serbia, *Acinetobacter spp.* isolates were all colistin susceptible and came from patients with ventilator-associated pneumonia, from surgical wards, and the Clinic of Infectious Diseases from different parts of the country (INJAC *et al*., 2017; PANTOVIĆ *et al*., 2016; STEFAN-MIKIC, 2017).

The most prevalent mechanism of carbapenem resistance in *A. baumannii* is enzymatic degradation by carbapenemases, namely OXA type and metallo-β-lactamases (MBL) (NOWAK and PALUCHOWSKA, 2016). We tested *A. baumannii* isolates for the presence of the most common bla_{oxa} genes: intrinsic $bla_{oxa\text{-}51\text{-}like}$ and acquired $bla_{oxa\text{-}23\text{-}like}$, $bla_{oxa\text{-}24\text{-}like}$, and $bla_{oxa\text{-}58\text{-}like}$. The $bla_{oxa\text{-}51\text{-}like}$ gene was present in all isolates. In six isolates it was the only bla_{oxa} gene, yet these were carbapenems-resistant. Similar results were obtained in other studies, indicating the

existence of other mechanisms of carbapenem-resistance (KARMOSTAJI *et al*., 2013; SHOJA *et al*., 2016).

The *bla*$_{oxa\text{-}24\text{-}like}$ was the most common acquired *bla*$_{oxa}$ genes in our study, detected in 62.4% of the isolates. Two isolates harboring the *bla*$_{oxa\text{-}24\text{-}like}$ gene were the only carbapenem-sensitive isolates. Like in our study, *bla*$_{oxa\text{-}24\text{-}like}$ was the most prevalent oxacillinase gene in another research from our country (NOVOVIC *et al*., 2015). In a study from Taiwan, the authors pointed out the role of the *bla*$_{oxa\text{-}24\text{-}like}$ gene in high resistance to meropenem and imipenem (WANG *et al*., 2018). In other studies worldwide, the *bla*$_{oxa\text{-}23\text{-}like}$ was the most prevalent acquired oxacillinase gene (VIJAYAKUMAR *et al*., 2016; GOKMEN *et al*., 2016; PETROVA *et al*., 2017). It is demonstrated that the presence of this gene is enough to confer carbapenem-resistance (HANDAL *et al*., 2017). However, NIRWATI *et al*. (2018) showed that meropenem-sensitive isolates didn't have the *bla*$_{oxa\text{-}23\text{-}like}$ gene. Also, two isolates in our study, that were carbapenem-sensitive indeed were *bla*$_{oxa\text{-}23\text{-}like}$ negative. Both originated from CCD. In the high percentage, (96.9%), of isolates from our study, different combinations of the *bla*$_{oxa}$ genes existed in the same isolate. These, as well as a combination of several different mechanisms, could influence a level of carbapenem resistance.

The resistance to carbapenems could also be enhanced by the insertion sequences upstream of the *bla*$_{oxa}$ genes, namely *bla*$_{oxa\text{-}51\text{-}like}$ and *bla*$_{oxa\text{-}23\text{-}like}$ (VIJAYAKUMAR *et al* ., 2016). IS*Aba1* is the most common insertion sequence and it can contribute to resistance by providing additional promoters for these genes. It could be inserted into different locations in the genome influencing the expression of some other genes and contributing to the mobilization of the genes (RAIBLE *et al*., 2017). Investigation revealed that all isolates in our study possessed these sequences, but were not always associated with *bla*$_{oxa}$ genes. Upstream of *bla*$_{oxa\text{-}51\text{-}like}$ we detected it in 35.1% isolates, and upstream of *bla*$_{oxa\text{-}23\text{-}like}$ in 97.0% (64/66) of the isolates carrying this gene. The insertion of the IS*Aba1* sequence could explain carbapenem-resistance in these isolates. However, we detected carbapenem-resistant isolates that didn't have IS*Aba1* associated with *bla*$_{oxa\text{-}51\text{-}like}$ or *bla*$_{oxa\text{-}23\text{-}like}$ genes. Similar results were obtained in other studies (BIGLARI *et al*., 2015; KARUNASAGAR *et al*., 2011; POURABBAS *et al*., 2016). BIGLARI *et al*, (2015) detected IS*Aba1* upstream of the *bla*$_{oxa\text{-}51\text{-}like}$ gene in carbapenem-susceptible and non-susceptible isolates and concluded that IS*Aba1* did not influence the regulation of this gene. They showed that the presence of IS*Aba1* upstream of the *bla*$_{oxa\text{-}23\text{-}like}$ gene is sufficient to confer resistance. Similar results were observed in other studies (RAVI *et al*., 2018; GOKMEN *et al*., 2016). However, Pourabas et al (2016) showed no correlation between the presence of IS*Aba1* upstream oxacillinase gene and the level of carbapenem-resistant. The carbapenem resistance could be the result of other mechanisms (NOWAK and PALUCHOWSKA, 2016). To determine the exact resistance mechanisms further investigations are required.

The next aim of this study was to detect the genes associated with biofilm formation. We investigated the presence of *ompA*, *epsA*, *bap* genes, and genes belonging to the operons *csuA/BABCDE* and *pgaABCD.* We detected them at a high percentage in isolates from the different wards, samples, and rezistotypes. Three isolates didn't have *csu* genes. All these isolates came from different wards and showed XDR and PDR phenotypes. Also, one isolate didn't have any tested biofilm forming genes and had only *bla*$_{oxa\text{-}51\text{-}like}$ of all tested *bla*$_{oxa}$ genes, but showed PDR phenotype. In the studies of other authors, biofilm-forming genes have been

found in a higher percentage in clinical isolates compared to avirulent strains (ALI *et al*., 2017). Also, in another study, expression levels of these genes were higher in biofilm producers (AMIN *et al*., 2019). The higher prevalence of *A. baumannii* in hospital settings could be related to its biofilm-forming ability since it helps the bacteria to adhere to different surfaces including host cells (KARUNASAGAR *et al*., 2011).

Numerous studies attempt to determine the correlation between antibiotic susceptibility and the ability of *A. baumannii* to form a biofilm, and different results were obtained. (QI *et al*., 2016; THUMMEEPAK *et al*., 2016).

Many authors also attempt to establish any connection between different bla_{oxa} genes or carbapenem resistance and biofilm production and got different conclusions regarding the expression of different carbapenemase genes and their influence on biofilm formation in *A. baumannii* (AZIZI *et al*., 2015; WANG *et al*., 2018).

Considering all this, although the majority of the isolates in our study had biofilm-associated genes, further studies are necessary to investigate the biofilm forming capacity. Also, the analysis of transcription levels of these genes can provide useful information. It is also required to determine whether there is a correlation between the capacity to form biofilm and specific genotype, resistance pattern, wards, and sample type.

We performed a RAPD analysis to investigate the genetic similarity of *A. baumannii* isolates. Three of four used primers, AP2, DAF4, and M13, generated satisfactory results with a sufficient number of clear bands to distinguish different genotypes. DECA primer proved to be less discriminatory. Dendrograms generated after amplification with these three primers indicated a high level of similarity between certain RAPD profiles that differed from each other in only one or two bands. Also, the majority of the isolates were grouped in these clusters of highly similar profiles. These results suggest that the vast majority of isolates belong to the same lineage and have dispersed through MMA clonally. Small differences that exist between similar profiles could be the result of mutations accumulated during circulation in MMA.

RAPD profiles with a lower level of similarity compared to the others were also observed. Probably, they represent strains recently imported in MMA. Another data that support this assumption is that majority of these profiles originated from the ED, and CSD the wards with the greatest number of newly received patients. One of the only two carbapenem-sensitive isolates in our study has a lower similarity level compared to others. This could be explained by recent importation in the hospital, and not acquired resistance genes, yet.

Analyzing RAPD profiles of PDR isolates we showed that the genetic similarity of *A. baumannii* isolates at the beginning of the study was probably less than in the total population of analyzed isolates. This indicated that PDR emerged from ancestors of different genetic backgrounds as a result of the selective pressure of antibiotics. On the other side, at least one strain that originated in ED expanded to CCU and other MCD in the period June to August 2018, contributing to the higher genetic similarity of PDR isolates.

CONCLUSIONS

In conclusion, the detection of PDR *A. baumannii* strains, especially increasing their frequency in MMA, is worrisome. The presence of particular bla_{oxa} genes, combinations of these genes, as well as the IS*Aba1* element upstream of these genes, were not connected to the

carbapenem resistance, indicating that other resistance mechanisms are also involved. Detection of genes responsible for biofilm formation in the majority of the isolates indicated the high pathogenic potential. RAPD analysis revealed a high degree of genome similarity in the majority of the isolates showing clonal dispersion of strains through all wards in MMA. Besides this, the existence of individual, genetically dissimilar strains, indicates a continuous introduction of *A. baumannii* strains in MMA contributing to their genetic diversity.

The results of our study could be useful for further understanding the *A. baumannii* epidemiology, sources of infection, control of cross-contamination, monitoring and prevention of outbreaks, elucidation of antibiotic resistance mechanisms, and more rational usage of antibiotics, and to prevent further dissemination of these resistance strains.

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REFERENCES

ALI, H.M., M.Z.M., SALEM, M.S., EL-SHIKH, A.A., MEGEED, Y.A., ALOGAIBI, I.A., TALEA (2017): Investigation of the virulence factors and molecular characterization of the clonal relations of multidrug-resistant Acinetobacter baumannii isolates. J AOAC Int., *100*:152–158.

ALMASAUDI, S.B. (2018): Acinetobacter spp. as nosocomial pathogens: Epidemiology and resistance features. Saudi J. Biol. Sci., *25*:586–596.

AMIN, M., T., NAVIDIFAR, F.S., SHOOSHTARI, M., RASHNO, M., SAVARI, F., JAHANGIRMEHR, M., ARSHADI (2019): Association between biofilm formation, structure, and the expression levels of genes related to biofilm formation and biofilm-specific resistance of Acinetobacter baumannii strains isolated from burn infection in Ahvaz, Iran. Infect. Drug Resist., *12*:3867–3881.

AZIZI, O., M.R., SHAKIBAIE, F., MODARRESI, F., SHAHCHERAGHI (2015): Molecular detection of class-D OXA carbapenemase genes in biofilm and non-biofilm forming clinical isolates of Acinetobacter baumannii. Jundishapur J. Microbiol., *8*:1–6.

BIGLARI, S., H., ALFIZAH, R., RAMLIZA, M.M., RAHMAN (2015): Molecular characterization of carbapenemase and cephalosporinase genes among clinical isolates of acinetobacter baumannii in a tertiary medical centre in Malaysia. J. Med. Microbiol., *64*:53–58.

CARR, E., H., EASON, S., FENG, A., HOOGENRAAD, R., CROOME, J., SODDEL, K., LINDREA, R., SEVIOUR (2001): RAPD-PCR typing of Acinetobacter isolates from activated sludge systems designed to remove phosphorus microbiologically. J. Appl. Microbiol., *90*:309–319.

CLSI (2020): *Performance Standards for Antimicrobial Susceptibility Testing.* 30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.

EZE, E.C., H.Y., M.E., EL ZOWALATY (2018): Acinetobacter baumannii biofilms: Effects of physicochemical factors, virulence, antibiotic resistance determinants, gene regulation, and future antimicrobial treatments. Infect. Drug Resist., *11*:2277–2299.

GOKMEN, T.G., B.,AKCIMEN, B., KAYAR, M., MARZI, F., KOKSAL (2016): The outbreak of Acinetobacter baumannii producing OXA-23 and OXA-51 type carbapenemases in a state hospital. J. Exp. Clin. Med., *33*:157–161.

GRUNDMANN, H.J., K.J., TOWNER, L., DIJKSHOORN, P., GERNER-SMIDT, M., MAHER, H., SEIFERT, M., VANEECHOUTTE (1997): Multicenter study using standardized protocols and reagents for evaluation of reproducibility of PCR-based fingerprinting of Acinetobacter spp. J. Clin. Microbiol., *35*:3071–3077.

HANDAL, R., L., QUNIBI, I., SAHOURI, M., JUHARI, R., DAWODI, H., MARZOUQA, M., HINDIYEH (2017): Characterization of Carbapenem-Resistant Acinetobacter baumannii Strains Isolated from Hospitalized Patients in Palestine. Int. J. Microbiol., *2017*:2017.

HIGGINS, P.G., C., DAMMHAYN, M., HACKEL, H., SEIFERT (2009): Global spread of carbapenem-resistant Acinetobacter baumannii. J. Antimicrob. Chemother., *65*:233–238.

INJAC, V., U., BATRANOVIĆ, J., MATIJAŠEVIĆ, M., VUKOJA, M., HADNADJEV, Z., BUKUMIRIĆ, G., TRAJKOVIĆ, S., JANKOVIĆ (2017): Etiology and resistance patterns of bacteria causing ventilator-associated pneumonia in a respiratory intensive care unit | Uzročnici pneumonije udružene sa ventilatornom potporom bolesnika i njihova rezistencija na antibiotike u pulmološkoj jedinici inte. Vojnosanit. Pregl., *74*:954–962.

KARMOSTAJI, A., S.N., PEERAYEH, A.H., SALMANIAN (2013): Distribution of OXA-type class D β-lactamase genes among nosocomial multi drug resistant Acinetobacter baumannii isolated in Tehran hospitals. Jundishapur J. Microbiol., *6*:1–5.

KARUNASAGAR, A., B., MAITI, M., SHEKAR, M.S., SHENOY, I., KARUNASAGAR (2011): Prevalence of OXA-type carbapenemase genes and genetic heterogeneity in clinical isolates of Acinetobacter spp. from Mangalore, India. Microbiol. Immunol., *55*:239–246.

KOBS, V.C., J.A., FERREIRA, T.A., BOBROWICZ, L.E., FERREIRA, R.C., DEGLMANN, G.A., WESTPHAL, P.A., CONDEIXA DE FRANÇA (2016): The role of the genetic elements blaoxa and ISAba1 in the Acinetobacter calcoaceticus-Acinetobacter baumannii complex in carbapenem resistance in the hospital setting. Rev. Soc. Bras. Med. Trop., *49*:433–440.

LI, X., Y., XIE, M., WANG, X., XIA, M., WANG, W., CAO, T., ZHANG, P., LI, M., YANG (2016): Phenotypic and genomic diversity in Acinetobacter baumannii stains random isolated from 2008 to 2012 in a teaching hospital in Hunan, China. Int. J. Clin. Exp. Pathol., *9*:7030–7039.

MAGIORAKOS, A.P., A., SRINIVASAN, R.B., CAREY, Y., CARMELI, M.E., FALAGAS, C.G., GISKE, S., HARBARTH, J.F., HINDLER, G., KAHLMETER, B., OLSSON-LILJEQUIST, D., PATERSON, L.B., RICE, J., STELLING, M.J., STRUELENS, A., VATOPOULOS, J.T., WEBER, D.L., MONNET (2012): Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect., *18*:268–281.

NIRWATI, H., M.S., HAKIM, S., DARMA, M., MUSTAFA, T., NURYASTUTI (2018): Detection of blaoxa genes and identification of biofilm-producing capacity of Acinetobacter baumannii in a tertiary teaching hospital, Klaten, Indonesia. Med. J. Malaysia, *73*:291–296.

NOVOVIC, K., S., MIHAJLOVIC, Z., VASILJEVIC, B., FILIPIC, J., BEGOVIC, B., JOVCIC (2015): Carbapenem-resistant Acinetobacter baumannii from Serbia: Revision of CarO classification. PLoS One, *10*:1–16.

NOWAK, J., E., ZANDER, D., STEFANIK, P.G., HIGGINS, I., ROCA, J., VILA, M.J., MCCONNELL, J.M., CISNEROS, H., SEIFERT ON BEHALF OF THE MAGICBULLET WORKING GROUP WP (2017): High incidence of pandrug-resistant Acinetobacter baumannii isolates collected from patients with ventilator-associated pneumonia in Greece, Italy and Spain as part of the MagicBullet clinical trial. J. Antimicrob. Chemother., *72*:3277–3282.

NOWAK, P., P., PALUCHOWSKA (2016): Acinetobacter baumannii: Biology and drug resistance — role of carbapenemases. Folia Histochem. Cytobiol., *54*:61–74.

OIKONOMOU, O., S., SARROU, C.C., PAPAGIANNITSIS, S., GEORGIADOU, K., MANTZARLIS, E., ZAKYNTHINOS, G.N., DALEKOS, E., PETINAK (2015): Rapid dissemination of colistin and carbapenem resistant Acinetobacter baumannii in Central Greece: Mechanisms of resistance, molecular identification and epidemiological data. BMC Infect. Dis., *15*:13–18.

PANTOVIĆ, V., M., DINIĆ, D., STANKOVIĆ ĐORĐEVIĆ, B., KOCIĆ, M., BOGDANOVIĆ (2016): Susceptibility Pattern of Carbapenem-Resistant Clinical Isolates of Acinetobacter Spp. Acta Medica. Median., *55*:86–91.

PELEG, A.Y., H., SEIFERT, D.L., PATERSON (2008): Acinetobacter baumannii: Emergence of a successful pathogen. Clin. Microbiol. Rev., *21*:538–582.

PETROVA, A.P., I.D., STANIMIROVA, I.N., IVANOV, M.M., PETROV, T.M., MITEVA-KATRANDZHIEVA, V.I., GRIVNEV, V.S., KARDJEVA, T.V., KANTARDZHIEV, M.A., MURDJEVA (2017): Carbapenemase Production of Clinical Isolates Acinetobacter baumannii and Pseudomonas aeruginosa from a Bulgarian University Hospital. Folia Med. (Plovdiv), *59*:413–422.

POURABBAS, B., R., FIROUZI, G., POULADFAR (2016): Characterization of carbapenem resistant acinetobacter calcoaceticus–baumannii complex isolates from nosocomial bloodstream infections in Southern Iran. J. Med. Microbiol., *65*:235–239.

QI, L., H., LI, C., ZHANG, B., LIANG, J., LI., L., WANG, X., DU, X., LIU, S., QIU, H., SONG (2016): Relationship between antibiotic resistance, biofilm formation, and biofilm-specific resistance in Acinetobacter baumannii. Front Microbiol., *7*:1–10.

QURESHI, Z.A., L.E., HITTLE, J.A., O'HARA, J.I., RIVERA, A., SYED, R.K., SHIELDS, A.W., PASCULLE, R.K., ERNST, Y., DOI (2015): Colistin-resistant acinetobacter baumannii: Beyond carbapenem resistance. Clin. Infect. Dis., *60*:1295–1303.

RAIBLE, K.M., B., SEN, N., LAW, T.E., BIAS, C.L., EMERY, G.D., EHRLICH, S.G., JOSHI (2017): Molecular characterization of β-lactamase genes in clinical isolates of carbapenem-resistant Acinetobacter baumannii. Ann. Clin. Microbiol. Antimicrob., *16*:1–10.

RAVI, N.S,. S., ANANDAN, S., VIJAYAKUMAR, R., GOPI, B.S., LOPES, B., VEERARAGHAVAN (2018): The potential of different molecular biology methods in tracking clones of acinetobacter baumannii in an ICU setting. J. Med. Microbiol., *67*:1340–1347.

SHOJA, S., M., MOOSAVIAN, S., ROSTAMI, F., ABBASI, M.A., TABATABAIEFAR, A., PEYMANI (2016): Characterization of oxacillinase and metallo-β-lactamas genes and molecular typing of clinical isolates of Acinetobacter baumannii in Ahvaz, South-West of Iran. Jundishapur J. Microbiol., 9(5) :e32388.

STEFAN-MIKIC, S. (2017): Antimicrobial susceptibility pattern of Acinetobacter spp in the period 2012-2015. Med. Pregl. Rev., *70*:99–106.

ŠULJAGIĆ, V., M., JEVTIĆ, B., DJORDJEVIĆ, P., ROMIĆ, R., ILIĆ, N., STANKOVIĆ, N., MILOVIĆ, M., NOVAKOVIĆ, J., KOZARSKI, Z., ROGANOVIĆ, Z., POPOVIĆ, A., JOVELIĆ (2011): Epidemiologija bolničkih infekcija/kolonizacija uzrokovanih Acinetobacter spp. u ratu i miru kod bolesnika iz šest hirurških klinika. Vojnosanit. Pregl., *68*:661–668.

TAYABALI, A.F., K.C., NGUYEN, P.S., SHWED, J., CROSTHWAIT, G., COLEMAN, V.L., SELIGY (2012): Comparison of the virulence potential of Acinetobacter strains from clinical and environmental sources. PLoS One, *7*(5): 37024.

THUMMEEPAK, R., P., KONGTHAI, U., LEUNGTONGKAM, S., SITTHISAK (2016): Distribution of virulence genes involved in biofilm formation in multi-drug resistant acinetobacter Baumannii clinical isolates. Int. Microbiol., *19*:121–129.

VIJAYAKUMAR, S., R., GOPI, P., GUNASEKARAN, M., BHARATHY, K., WALIA, S., ANANDAN, B., VEERARAGHAVAN (2016): Molecular Characterization of Invasive Carbapenem Resistant Acinetobacter baumannii from a Tertiary Care Hospital in South India. Infect. Dis. Ther., *5*:379–387.

WANG, T.H., YS., LEU, N.Y., WANG, C.P., LIU, T.R., YAN (2018): Prevalence of different carbapenemase genes among carbapenem-resistant Acinetobacter baumannii blood isolates in Taiwan. Antimicrob. Resist. Infect. Control, *7*:1–8.

WANG, Y.C., T.W., HUANG, Y.S., YANG, S.C., KUO, C.T., CHEN, C.P., LIU, Y.M., LIU, T.L., CHEN, F.Y., CHANG, S.H., WU, C.K., HOW, Y.T., LEE (2018): Biofilm formation is not associated with worse outcome in Acinetobacter baumannii bacteraemic pneumonia. Sci. Rep., *8*:1–10.

DETEKCIJA GENA ZA KARBAPENEMSKU REZISTENCIJU, GENA UKLJUČENIH U FORMIRANJE BIOFILMA I PROCENA GENETIČKE SRODNOSTI KLINIČKIH IZOLATA *Acinetobacter baumannii*

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Izvod

U studiji je analizirano 194 izolata poreklom od hospitalizovanih pacijenata na Vojnomedicinskoj akademiji u Beogradu. Cilj rada je bio da se utvrdi rezistencija na antibiotike, ispita prisustvo gena koji kodiraju oksacilinaze (*bla*$_{oxa\text{-}51\text{-}like}$, *bla*$_{oxa\text{-}23\text{-}like}$, *bla*$_{oxa\text{-}24\text{-}like}$, and *bla*$_{oxa\text{-}58\text{-}like}$), utvrdi prisustvo insercione sekvence IS*Aba1*, detektuju geni uključeni u formiranje biofilma kao i da se utvrdi genetička srodnost između izolata. Analiza rezistencije je urađena disk difuzionom metodom, detekcija gena PCR metodom a genotipizacija RAPD metodom primenom četiri različita prajmera (AP2, DAF4. M13 i DECA). Samo dva izolata su pokazala osetljivost na karbapeneme. Većina izolata je bila osetljiva samo na kolistin. Detektovano je 38 izolata koji su bili rezistentni na sve testirane antibiotike. Geni *bla*$_{oxa\text{-}51\text{-}like}$, *bla*$_{oxa\text{-}23\text{-}like}$, *bla*$_{oxa\text{-}24\text{-}like}$, and *bla*$_{oxa\text{-}58\text{-}like}$ su detektovani kod 100%, 34.0%, 62.4%, i 3.1% izolata, redom. Inserciona sekvenca IS*Aba1* je detektovana u genomu svih izolata, dok je kod 35.1% izolata bila asocirana sa *bla*$_{oxa\text{-}51\text{-}like}$, a kod 97.0% izolata sa *bla*$_{oxa\text{-}23\text{-}like}$ genom. Geni uključeni u formiranje biofilma *bap, ompA, epsA, csuA/BABCDE*, i *pgaABCD* su detektovani kod 93.8%, 95.8%, 88.1%, 98.4%, i 98.9% izolata, redom. RAPD metodom je utvrđen visok stepen srodnosti (> 90%) između dobijenih profila. Rezultati studije su pokazali veliki patogeni potencijal sojeva, kao i njihovo klonalno širenje kroz različite klinike. Po našem saznanju, po prvi put su u našoj zemlji detektovani panrezistentni sojevi *A. baumannii*.

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