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Bogdan, Maja; Drenjančević, Domagoj; Haršanji Drenjančević, Ivana; Bedenić, Branka; Zujic Atalić, Vlasta; Talapko, Jasminka; Vuković, Dubravka

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Antimicrobial Original Research Paper

In vitro effect of subminimal inhibitory concentrations of antibiotics on the biofilm formation ability of *Acinetobacter baumannii* clinical isolates

Maja Bogdan^{1,2‡} , Domagoj Drenjancevic^{2,3‡}, Ivana Harsanji Drenjancevic^{4,5}, Branka Bedenic^{6,7}, Vlasta Zujic Atalic^{1,2} , Jasminka Talapko², Dubravka Vukovic¹

¹Microbiology Service, Institute of Public Health Osijek-Baranja County, Osijek, Croatia, ²Faculty of Medicine, Department of Microbiology and Parasitology, University of Osijek, Osijek, Croatia, ³Department of Transfusion Medicine, Osijek University Hospital, Osijek, Croatia, ⁴Department of Anesthesiology, Reanimatology and Intensive Medicine, Osijek University Hospital, Osijek, Croatia, ⁵Faculty of Medicine, Department of Anesthesiology, Reanimatology and Intensive Medicine, University of Osijek, Osijek, Croatia, ⁶Department of Clinical and Molecular Microbiology, University Hospital Center Zagreb, Zagreb, Croatia, ⁷Department of Microbiology and Parasitology, School of Medicine, University of Zagreb, Zagreb, Croatia

The ability of *A. cinetobacter baumannii* strains to form biofilm is one of the most important virulence factor which enables bacterial survival in a harsh environment and decreases antibiotic concentration as well. Subminimal inhibitory concentrations (subMICs) of antibiotics may change bacterial ultrastructure or have an influence on some different molecular mechanisms resulting in morphological or physiological changes in bacteria itself. The aim of this study was to determine effects of 1/2, 1/4, 1/8 and 1/16 minimal inhibitory concentrations of imipenem, ampicillin-sulbactam, azithromycin, rifampicin and colistin on biofilm formation ability of 22 biofilm non-producing and 46 biofilm producing *A. baumannii* strains (30 weak producing strains and 16 moderate producing strains). Results of this study indicate that 1/2–1/16 MICs of imipenem, azithromycin, and rifampicin can reduce bacterial biofilm formation ability in moderate producing strains ($p < 0.05$), whereas 1/16 MIC of imipenem and 1/4–1/8 MICs of rifampicin reduce the biofilm formation in weak producing strains ($p < 0.05$). Statistically significant effect was detected among biofilm non-producing strains after their exposure to 1/16 MIC of azithromycin ($p = 0.039$). SubMICs of ampicillin-sulbactam and colistin did not have any significant effect on biofilm formation among tested *A. baumannii* strains.

Keywords: *Acinetobacter baumannii*, biofilm, subminimal inhibitory concentration, antibiotic, virulence factors

1. Introduction

Acinetobacter baumannii has nowadays emerged as an important opportunistic pathogen. It has become one of the leading causes of hospital-acquired infections primarily because of its remarkable ability to survive and spread in the hospital environment and to rapidly acquire resistance determinants agents.¹ These organisms have been implicated in diverse range of infections all over the world which create serious problem in intensive care units where numerous outbreaks associated with *A. baumannii* have been detected.² Once introduced in the hospital setting, *A. baumannii* easily spreads, persists in the environment and consequently

colonizes patients. It is usually associated with nosocomial infections, predominantly ventilator associated pneumonia, bloodstream infection or wound and urinary catheter infection in critically ill patients.¹ The precise mechanisms involved in the establishment and progression of *A. baumannii* infection are still unclear, because only few virulence factors have been identified so far.² Due to its amazing ability to persist on inanimate surfaces for a long period of time (encapsulated strains survive for more than four months on PVC, ceramics, rubber and steel), resist cleaning and disinfection as well as the extraordinary ability to develop resistance to even most potent antimicrobial compounds, it has become a very hard bug to control and eradicate.^{1,3} Adherence to host cells as an initial step during colonization and infection, followed by formation of microcolonies results in

Correspondence to: Domagoj Drenjancevic, Faculty of Medicine, Department of Microbiology and Parasitology, University of Osijek, Cara Hadrijana 10/E, HR-31000 Osijek, Croatia. Email: domagoj@mefos.hr
These two authors contributed equally to this work.

the development of highly structured microbial community, called the biofilm.⁴ In general, the ability of *A. baumannii* strains to adhere and form biofilm plays a pivotal role in the host–pathogen interactions and medical device-associated infections, involving a range of bacterial and environmental factors as well as multiple cell signals.⁵ The biofilm can be formed on both biotic and abiotic surfaces, solid–liquid and air–liquid surfaces, it resists desiccation and enables bacterial persistence in the hospital environment for a long period of time.⁶ Furthermore, its growth inside the biofilm confers bacterial resistance to the host mechanisms of clearance and antimicrobial therapy as well.⁷ Although, as previously described, antibiotics reduce bacterial biofilm formation, conversely, subminimal inhibitory concentrations (subMICs) of some antibiotics can even induce its formation and thus may play a significant role in colonization and progression to the development of acute or chronic infection.^{8–10}

Whereas information about the effect of subminimal inhibition concentrations of antibiotics on the biofilm formation ability of *A. baumannii* are scarce, this study is conducted with the goal of highlighting the effect of subMICs of imipenem, ampicillin-sulbactam, azithromycin, rifampicin and colistin on the biofilm formation of *A. baumannii* clinical isolates.

2. Materials and methods

2.1. Bacterial strains

Sixty-eight non-duplicate *A. baumannii* clinical strains isolated from various clinical sites were included in the testing. They were randomly selected from a collection of *A. baumannii* strains isolated during 2007–2012 from various clinical specimens obtained from patients hospitalized at different wards at Osijek University Hospital.

2.2. Biofilm formation

The ability of biofilm formation was determined in polypropylene flat bottom 96-microtiter well plate (Kartell, Italy) by modification of previously described method.^{11,12} Briefly, each well was filled with aliquots (50 μ L) of bacterial suspension adjusted to optical density of 0.5 McFarland standard and sterile Difco Luria-Bertani (LB) broth (Becton Dickinson, USA). The procedure was performed in triplicate. After overnight incubation at 35–37 °C, wells were twice washed with 300 μ L distilled water with drying in between by inversion. Staining of the washed wells was then performed for two minutes with 100 μ L 0.025% safranin water solution (Kemika, Croatia) as described previously.¹¹ Unbound dye excess was removed, again by washing twice with distilled water. After solubilization of bound dye with 200 μ L 96% ethanol, 125 μ L of suspension were transferred into wells of new microtiter plate and absorbance was measured in microtiter plate reader (Asys, Biochrom, UK) at 495 nm. The optical density (OD₄₉₅) of each well was measured. The mean OD₄₉₅ value for each isolate was calculated as well for negative control (uninoculated sterile LB broth)

which represent ODnc value. ODnc value is defined as sum of mean OD₄₉₅ and 3x standard deviation of negative control (ODnc = OD₄₉₅ negative controle + 3xSD).

The strains were then classified as: non producing (OD₄₉₅ \leq ODnc), weak (ODnc < OD₄₉₅ \leq 2xODnc), moderate (2xODnc < OD₄₉₅ \leq 4xODnc) and strong-biofilm-producing strains (OD₄₉₅ > 4xODnc).¹³ Biofilm producing strain *A. baumannii* ATCC 19,606 was used as a positive control.

2.3. Antimicrobial susceptibility testing

The determination of minimal inhibitory concentrations (MIC) of azithromycin (Pliva, Croatia), imipenem, ampicillin-sulbactam, rifampicin and colistin (Sigma, Biovit, Croatia) was performed by broth microdilution procedure in cation adjusted Mueller Hinton broth (Bio Rad, France) according to Clinical Laboratory and Standard Institute (CLSI) M7-A7 document.¹⁴ The stock solutions of antibiotics were prepared to the concentrations of 5120 mg/l and the starting concentration of 512 mg/l of each antibiotic for testing was obtained by diluting stock solutions in adequate diluents as described in CLSI document. *Pseudomonas aeruginosa* ATCC 27,853 served as quality control of MIC determination procedure. For imipenem and colistin, MIC values were interpreted according to EUCAST breakpoints while for ampicillin-sulbactam CLSI breakpoints were applied.

2.4. Exposure to subMICs of antibiotics

The effect of subMICs of the previously mentioned antibiotics were performed by mixing of aliquots (2 ml) of bacterial suspensions with concentration of antibiotic (1:1) at final bacterial concentration at $\sim 10^5$ cfu/ml within 1/2, 1/4, 1/8 i 1/16 of previously determined MIC of antibiotic. After 20 h of incubation at 37 °C the suspensions were pelleted for 10 minutes at 1500 g and the supernatant was decanted. The pellet was resuspended in 5 ml PBS after centrifugation, and the procedure was repeated twice. The obtained pellet of the bacterial cells was then subjected to the protocol of determining the biofilm forming ability according to the previously described procedure.

2.5. Pulsed-field gel electrophoresis

Clonal relatedness among examined bacterial strains was assessed by Pulsed-field gel electrophoresis (PFGE) as previously described using the CHEF-DRIII system (Bio-Rad Laboratories) with *Apa I* endonuclease. Gel images were analysed using GelCompare TM software (Applied Maths, Belgium) and based on the macro restriction profiles, grouped into clusters. Genotyping was performed at the Clinical Hospital Center Zagreb, Croatia.¹⁵

2.6. Statistical analysis

Statistical analysis was performed using χ^2 and Wilcoxon test where appropriate. Level of statistical significance was set at $p = 0.05$.

3. Results

3.1. Biofilm formation

According to the measured OD₄₉₅ for each strain and calculated OD_{nc} value (for each microtiter plate), they were divided into previously defined groups listed in Table 1. The mean OD_{nc} value ± standard deviation was 0.061 ± 0.008. There was no significant difference between the tested strains based upon the origin and the ability to form a biofilm ($X^2 = 11.596$, $df = 10$, $p = 0.313$). The OD₄₉₅ value for the *A. baumannii* ATCC 19,606 strain that served as a positive control within the procedure was 0.221 (±0.030) and the strain was categorized as the moderate producer.

The range of MIC values for imipenem was 0.125–128 mg/l (MIC₅₀ 1 mg/l; MIC₉₀ 32 mg/l), for ampicillin/sulbactam 1–32 mg/l (MIC₅₀ 8 mg/l; MIC₉₀ 16 mg/l) and for colistin 0.032–0.5 mg/l (MIC₅₀ 0.125 mg/l; MIC₉₀ 0.5 mg/l). The corresponding MIC range for azithromycin was 4–512 mg/l (MIC₅₀ 16 mg/l; MIC₉₀ 32 mg/l) and for rifampicin 0.25–8 mg/l (MIC₅₀ 16 mg/l and MIC₉₀ 32 mg/l). Two of the tested strains exhibited the MIC values for rifampicin >2560 mg/l and were not subjected to the testing. For imipenem, there was no statistically significant difference detected between the ability of forming biofilm (biofilm grade) and bacterial susceptibility ($X^2 = 4.156$, $df = 4$, $p = 0.385$). Conversely, the same difference was detected for ampicillin-sulbactam and the ability to form biofilm, with susceptible strains being more able to form any form of biofilm in comparison to resistant strains ($X^2 = 18.737$, $df = 4$, $p = 0.001$).

In Table 2 are summarized the mean OD₄₉₅ values for established groups after their exposure to the subMICs of tested antibiotics. Figure 1(a)–(e) present an effect of the subMICs of each antibiotic tested with corresponding control values (inoculated LB broth without antibiotic). As previously stated, the effect of antibiotics subMICs can be an induction or inhibition of biofilm production. SubMICs of imipenem, rifampicin and azithromycin showed overall

a statistically significant effect on the ability of biofilm formation. The reduction of biofilm was detected in the group of biofilm moderately producing (MP) strains for all tested subMIC concentration of imipenem, azithromycin and rifampicin. The same statistically significant effect was also detected in the weak producing group of strains (WP) at 1/16 MIC of imipenem and 1/4 and 1/8 MIC of rifampicin respectively. The statistically significant formation of biofilm was noted just for one group of non-producing strains (NP) at 1/16 MIC of azithromycin. The colistin and ampicillin/sulbactam did not exhibit any statistically significant effect among the tested group of *A. baumannii* strains at all.

3.2. Genotyping

According to PFGE the isolates belonged to four different clusters. First clusters comprised isolates 57, 56, 13, 12, 55, 53, 50, 28, 17, 54, 30, 31, 42, 35, 32 and 29. Five subclusters with highly related isolates were identified within the cluster. The second cluster was the largest and contained isolates 51, 49, 52, 47, 45, 48, 67, 64, 60, 26, 68, 5, 4, 33, 20, 19, 24, 23, 22, 25, 8, 6, 16, 15, 18, 14, 39, 58, 7, 34 and 44. Eight subclusters with pairs or triplets of highly related isolates were observed. Isolates 63, 62, 21, 59, 86, 11, 10, 9, 65, 37 and 61 were allocated to the third clone, whereas the fourth clone comprised only nine strains: 2, 38, 40, 3, 1, 36, 41, 46 and 43. Clonal relatedness of the tested *A. baumannii* strains is presented in Figure 2 and Table 3.

4. Discussion

The ability of *A. baumannii* to form a biofilm is multifactorial and diverse, dependent upon the surface which cells they interact with.¹⁶ Many of the molecular mechanisms by which these bacteria adhere to diverse, medically relevant surfaces and human host cells, remain obscure.¹⁶ Due to the presence of dormant cells, the environmental persistence of *A. baumannii* correlates with the ability of some clinical isolates to survive for a long time on abiotic surfaces under desiccated conditions.⁵ As already stated, it can confer the reason for successful colonization of patients followed by a variety of different clinical manifestations with implication on involvement of biofilm producing strains in chronic wound infections.^{1,17,18} In the literature, there are quite opposite observations regarding the correlation between different features of *A. baumannii* clinical isolates. Primary, it is noted that isolates from different clinical sites can exhibit different grade of biofilm forming ability. In this study, the distribution of biofilm producing and biofilm non-producing strains of *A. baumannii* among different clinical sites was not statistically significant. Half (8/15) of the tested *A. baumannii* isolates originated from blood stream infections (BSI) had the ability to form some level of biofilm, as well as both catheter-related isolates. That does not correlate with the results of Rodriguez-Bano et al. who detected the uniform

Table 1 The distribution of *A. baumannii* strains based upon the collection site and grade of biofilm forming ability

| Sample | Biofilm grade | | | Total | |
|------------------------|---------------|------|------|-------|-------|
| | NP | WP | MP | | |
| Blood | <i>N</i> | 7 | 5 | 3 | 15 |
| | % | 46.7 | 33.3 | 20.0 | 100.0 |
| Cerebrospinal fluid | <i>N</i> | 0 | 3 | 1 | 4 |
| | % | 0.0 | 75.0 | 25.0 | 100.0 |
| Respiratory tract | <i>N</i> | 6 | 9 | 5 | 20 |
| | % | 30.0 | 45.0 | 25.0 | 100.0 |
| Skin and soft tissue | <i>N</i> | 7 | 12 | 4 | 23 |
| | % | 30.4 | 52.2 | 17.4 | 100.0 |
| Urinary tract | <i>N</i> | 2 | 1 | 1 | 4 |
| | % | 50.0 | 25.0 | 25.0 | 100.0 |
| Intravascular catheter | <i>N</i> | 0 | 0 | 2 | 2 |
| | % | 0.0 | 0.0 | 100.0 | 100.0 |
| Total | <i>N</i> | 22 | 30 | 16 | 68 |
| | % | 32.4 | 44.1 | 23.5 | 100.0 |

Notes: NP – strain that does not produce biofilm, WP – weak producing strain, MP – moderate producing strain.

Table 2 The mean OD₄₉₅ values for *A. baumannii* biofilm grade groups after exposure to subMICs of antibiotics compared to control

| Anti-biotic concentration | Biofilm grade | N | Imipenem | | Ampicillin/Sulbactam | | Azithromycin | | Rifampicin | | Colistin | |
|---------------------------|---------------|----|------------------------|---------|------------------------|---------|------------------------|---------|------------------------|---------|------------------------|---------|
| | | | Mean OD ₄₉₅ | P value | Mean OD ₄₉₅ | P value | Mean OD ₄₉₅ | P value | Mean OD ₄₉₅ | P value | Mean OD ₄₉₅ | P value |
| 1/2 MIC | NP | 22 | 0.067 | 0.555 | 0.075 | 0.338 | 0.071 | 0.108 | 0.065 | 0.434 | 0.078 | 0.052 |
| | WP | 30 | 0.133 | 0.681 | 0.148 | 0.440 | 0.111 | 0.239 | 0.127 | 0.299 | 0.144 | 0.365 |
| | MP | 16 | 0.127 | 0.004* | 0.193 | 0.187 | 0.129 | 0.002* | 0.172 | 0.047* | 0.185 | 0.352 |
| 1/4 MIC | NP | 22 | 0.064 | 0.935 | 0.070 | 0.404 | 0.071 | 0.123 | 0.061 | 0.715 | 0.074 | 0.348 |
| | WP | 30 | 0.131 | 0.711 | 0.147 | 0.347 | 0.110 | 0.195 | 0.123 | 0.036* | 0.152 | 0.734 |
| | MP | 16 | 0.135 | 0.008* | 0.200 | 0.326 | 0.135 | 0.001* | 0.163 | 0.003* | 0.187 | 0.525 |
| 1/8 MIC | NP | 22 | 0.067 | 0.424 | 0.076 | 0.385 | 0.069 | 0.194 | 0.064 | 0.110 | 0.073 | 0.270 |
| | WP | 30 | 0.133 | 0.861 | 0.150 | 0.221 | 0.113 | 0.643 | 0.121 | 0.027* | 0.152 | 0.673 |
| | MP | 16 | 0.138 | 0.026* | 0.201 | 0.623 | 0.143 | 0.001* | 0.168 | 0.006* | 0.189 | 0.796 |
| 1/16 MIC | NP | 22 | 0.064 | 0.889 | 0.074 | 0.563 | 0.072 | 0.039* | 0.065 | 0.082 | 0.075 | 0.056 |
| | WP | 30 | 0.123 | 0.022* | 0.148 | 0.399 | 0.121 | 0.634 | 0.128 | 0.261 | 0.153 | 0.636 |
| | MP | 16 | 0.130 | 0.005* | 0.197 | 0.234 | 0.139 | 0.001* | 0.158 | 0.005* | 0.189 | 0.679 |
| Control (LB) | NP | 22 | 0.064 | | 0.071 | | 0.064 | | 0.062 | | 0.070 | |
| | WP | 30 | 0.135 | | 0.143 | | 0.120 | | 0.132 | | 0.147 | |
| | MP | 16 | 0.157 | | 0.205 | | 0.177 | | 0.188 | | 0.194 | |

Notes: NP – strain that does not produce biofilm, WP – weak producing strain, MP – moderate producing strain; Control (LB) – the same strain grown in sterile Luria Bertani (LB) broth and without an addition of any concentration of tested antibiotic.

*statistically significant, Wilcoxon test, $p < 0.05$.

ability of all tested BSI and catheter related *A. baumannii* strains to form the biofilm.¹⁹ Reviewing the strains related to respiratory tract infections (RTI), 14/20 had some form of ability to produce biofilm. The results of Kaliterna et al. support this feature with 28/61 isolates from endotracheal aspirates capable to form high level of biofilm.³ Comparing the clinical isolates from blood and sputum, Vijayakumar et al. found that most of the isolates were able to form varying degree of biofilm. They reported that blood isolates formed less robust biofilm in comparison to the sputum isolates which formed thicker biofilm. In that study, all of the 60 tested strains were MDR and resistant to meropenem.²⁰ Furthermore, we observed that almost one third of the tested biofilm producing strains (16/46) were associated with their isolation from some clinical manifestation of soft skin and tissue infection (SSTI), which is in concordance with the previously published results, which also recorded high percentage (44%) of biofilm producing *A. baumannii* strains among ones isolated from wounds.²¹

Moreover, one of the main known abilities of the biofilm matrix is to prevent antibiotics from reaching the cells, as well as the expression of individual genes that serve as a general stress response that enables cells inside the biofilm to respond to all kind of changes in the environment that they may encounter.²² In some extreme cases, concentrations of antimicrobials required to achieve bactericidal activity against adherent organisms can be three to four-fold higher than for those bacteria which do not produce biofilm, depending on the drug combination.^{17,23} Quite contradictory data on resistance of *A. baumannii* strains to antibiotics and their ability to create biofilms are available in the literature. It was published that *A. baumannii* strains capable of forming biofilms were often more resistant to aminoglycosides, carbapenems, tetracyclines and sulphonamides compared to those strains characterized

as weak biofilm producers.⁷ It was suggested that biofilm production may correlate with antibiotic resistance, so strains which are more susceptible to commonly prescribed antibiotic are better biofilm producers than those carrying drug resistance, as well.^{12,19,24} In addition, it was also published that isolates forming biofilm were less frequently resistant to imipenem and ciprofloxacin than the non-producing strains.¹⁹ Perez recently published the inverse relationship between meropenem resistance and biofilm production that supports this thesis. He found statistically significant difference ($p < 0.0001$) among meropenem resistant biofilm non-producing and meropenem susceptible biofilm producing isolates (73.7% vs. 90%, respectively).²⁵ That was also supported by the work of Qi et al. who found that non-MDR *A. baumannii* isolates tended to form stronger biofilms than MDR and XDR strains.²⁶ In this research, the proportion of strains with reduced susceptibility to imipenem among the groups of biofilm non-producing (NP) and biofilm-producing (WP and MP) strains were not statistically significant although we observed 30.4% (14/46) resistant biofilm producing *A. baumannii* strains and 63.6% (14/22) among ones resistant to imipenem. Apart of fact that all of the tested strains were susceptible to colistin and could not be compared between groups, we observed a correlation of ampicillin/sulbactam susceptible strains more capable to form a biofilm ($p = 0.001$). Complementary, a group of authors in Poland, did not find the correlation between the ability of biofilm formation and molecular type, carbapenem resistance or site of isolation of the clinical strains of *A. baumannii*.²⁷ Completely opposite results were recently published, regarding biofilm formation ability of 61 tracheal *A. baumannii* isolates, belonging to different EU clones.³ Strains resistant to carbapenems with lower MIC values expressed less ability to form a biofilm in comparison to the isolates

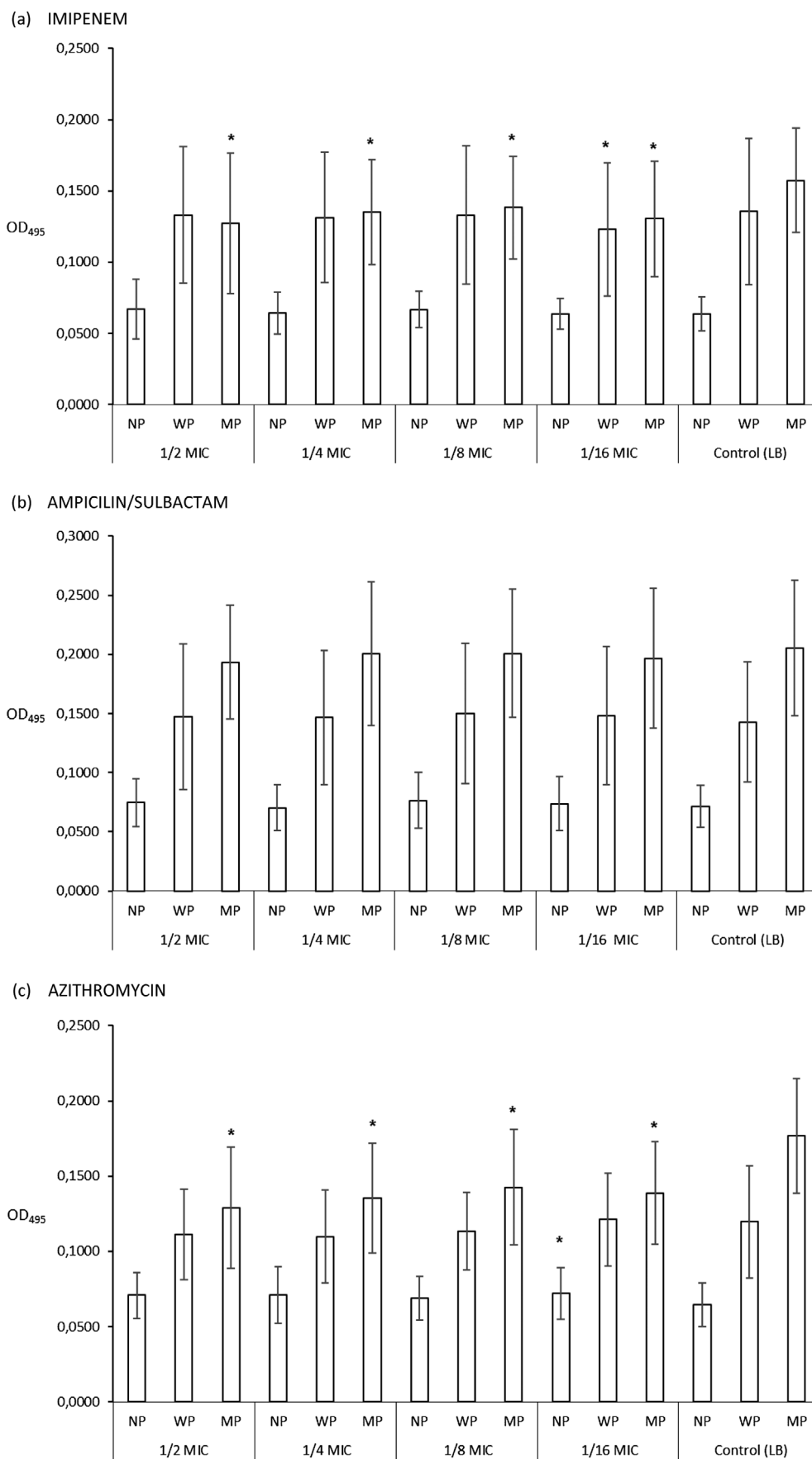


Figure 1 (a)–(e) The measured optical density (OD₄₉₅) values (mean ± standard deviation) for tested strains after their exposure to subMICs of tested antibiotic, stratified upon the grade of biofilm production.

Notes: NP – strain that does not produce biofilm, WP – weak producing strain, MP – moderate producing strain. *statistically significant, Wilcoxon test, $p < 0.05$.

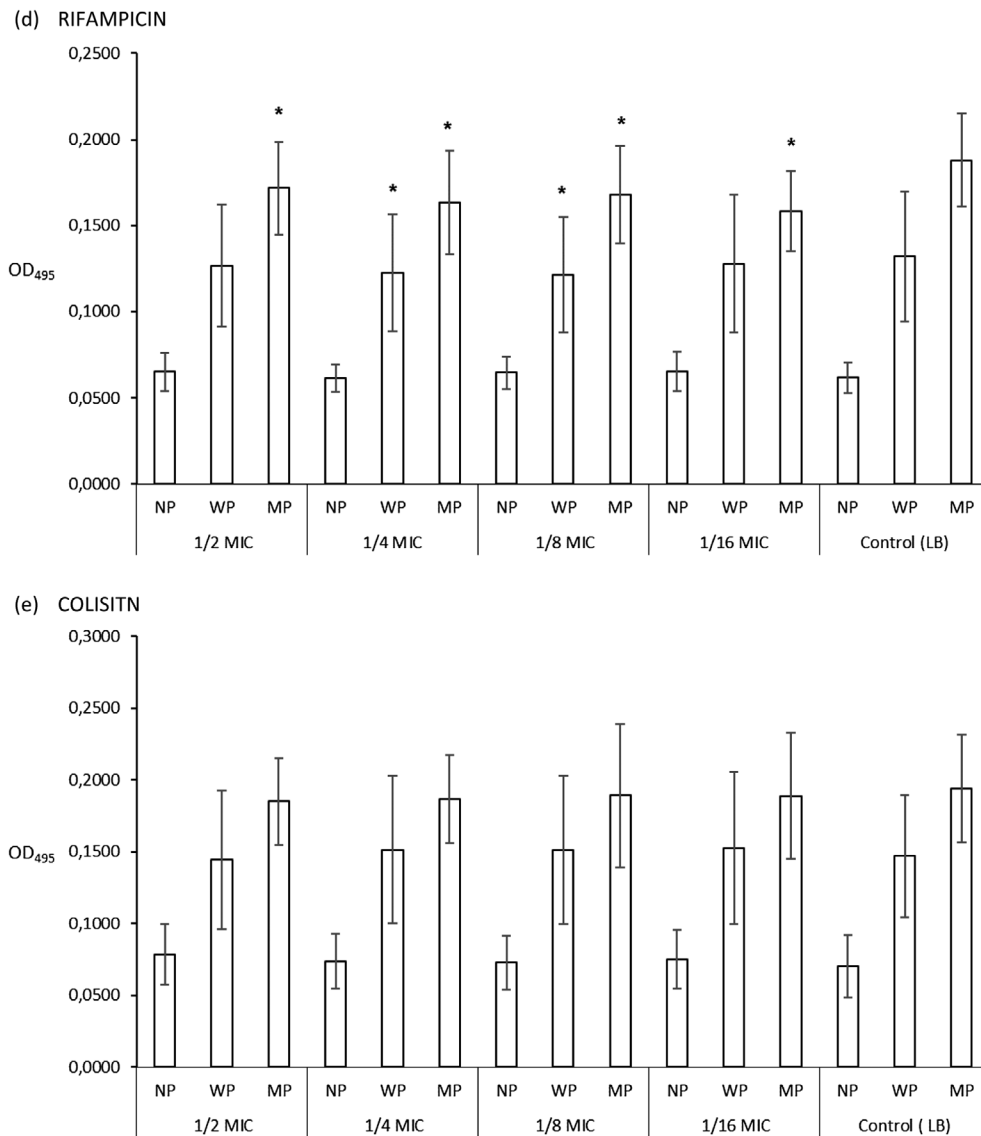


Figure 1 (Continued)

with higher MICs that exhibited greater ability to form a biofilm and were significantly more resistant to antibiotics with susceptibility only to sulbactam and colistin.³

Ability to form biofilm have been described as a unique feature of a single clon.^{3,28,29} As it is shown in Figure 2, in our research there were four different clusters of *A. baumannii*. Among this genetically diverse group of the tested isolates, there was no statistically significant difference due to the clinical site of isolation or biofilm production ability, before and after exposure to subMICs of all tested antibiotics (data not shown). Based on these different observations it is difficult to conclude, yet we can only speculate that the ability to form biofilms varies greatly, depending on the geographical distribution of the strains tested, and maybe, on the research methodology as well as heterogeneity of the methods used.^{3,10,27,30}

Further one, subMICs of antibiotic can be often encountered *in vivo*. Numerous studies have shown that subMIC of some antibiotics although not able to kill bacteria, can inhibit the formation of biofilm.⁹ The results of our study

support this thesis as shown in Table 2 and Figure 1(a)–(e). Interestingly, the subMICs of antibiotics seem to deploy similar activity on biofilm forming ability which was low or modest. We observed statistically significant inhibition of biofilm formation for MP strains at all tested subMICs of imipenem, azithromycin and rifampicin. Additionally, for WP strains the same ability was also observed for imipenem at 1/16 MIC and rifampicin at concentrations of 1/4 and 1/8 MIC. The observed biofilm mean reduction rate among strains with statistical significance varies from 7 to 27%. For all tested concentrations of ampicillin/sulbactam and colistin, we did not observe any significant effect, although Pour et al. reported this effect when monitoring of subMIC effect on the bacterial adhesion to urinary catheter surfaces. In their study, they observed reduced adhesion at both 1/2 and 1/4 MICs of colistin, as well as reduction of biofilm formation.³⁰ Furthermore, as previously described, antibiotic subMICs can even induce biofilm formation.^{8,9} Such effect was also observed in this research. Biofilm formation was detected after the

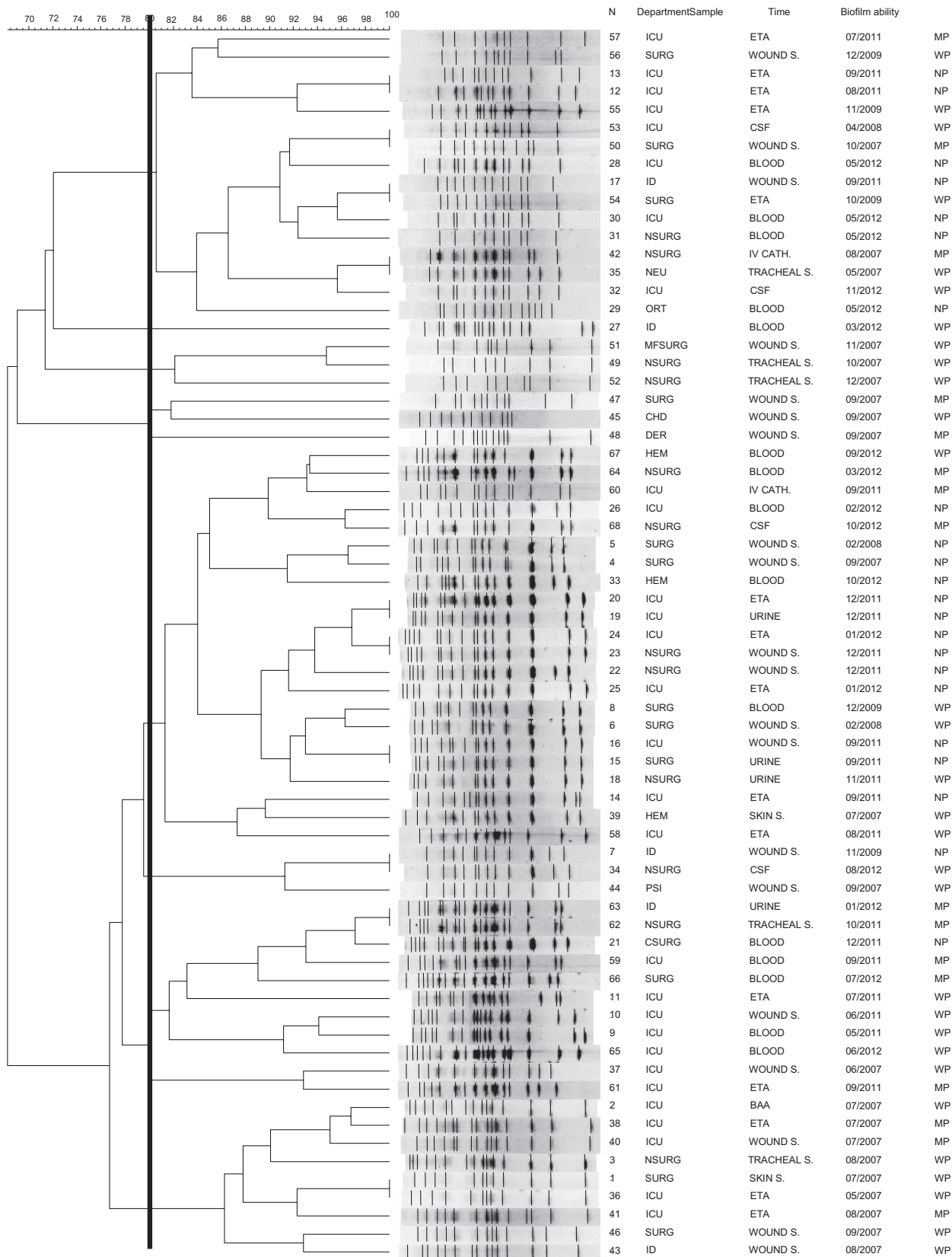


Figure 2 PFGE dendrogram of *A. baumannii* isolates. Cut-off value of 80% was applied to define a clone. The number of strain, department of isolation, specimen, date of isolation and biofilm formation ability are shown.

Notes: **Departments:** ICU – intensive care unit, NSURG – neurosurgery, NEU – neurology, PSI-psychiatry, CHD – haemodialysis, HEM – haematology, ID – infectious diseases, SURG – surgery, CSURG – cardiothoracic surgery, ORT – orthopaedic surgery, MFSURG – maxillofacial surgery, DER – dermatology. **Specimens:** ETA – endotracheal aspirate, BAA – bronchoalveolar aspirate, TRACHEAL S. – tracheal swab, WOUND S. – wound swab, SKIN S. – skin swab, CSF – cerebrospinal fluid, IV CATH. – tip of intravascular catheter. **Biofilm formation:** NP – strain that does not produce biofilm, WP – weak producing strain, MP – moderate producing strain.

Table 3 PFGE clonal relatedness regarding to the biofilm production

| PFGE | NP | WP | MP | Total |
|-----------|-----------|-----------|-----------|-----------|
| | N (%) | N (%) | N (%) | N (%) |
| Cluster 1 | 7 (10.5) | 6 (8.9) | 3 (4.4) | 16 (23.8) |
| Cluster 2 | 14 (20.9) | 12 (17.9) | 5 (7.5) | 31 (46.3) |
| Cluster 3 | 1 (1.5) | 5 (7.5) | 5 (7.5) | 11 (16.5) |
| Cluster 4 | 0 (0) | 6 (8.9) | 3 (4.5) | 9 (13.4) |
| Total | 22 (32.9) | 29 (43.2) | 16 (23.9) | 67 (100) |

Notes: NP – strain that does not produce biofilm, WP – weak producing strain, MP – moderate producing strain. Biofilm strong producing strains were not detected. Strain 27 (WP) was out of clusters; (non-statistically significant, χ^2 , $p = 0.068$).

exposure to 1/16 MIC of azithromycin and was statistically significant as shown in Table 2. The same effect of imipenem subMICs to the biofilm-nonproducing *A. baumannii* strains were observed in the study of Nucleo et al.¹⁰ In this research the authors found that bacterial adhesion and consequently biofilm formation by *A. baumannii* SMAL clone was strongly affected by imipenem subMICs and both growth media and temperature, preferred at 30 °C in glucose based medium (LB1/4, and M9Glu), as well.¹⁰

In conclusion, the results of our study point towards the fact that imipenem, rifampicin and azithromycin subMICs expressed statistically significant effect against 68 strains of *A. baumannii* isolated from variety of clinical sites, as shown in Table 2. On the other hand ampicillin/sulbactam and colistin subMICs did not have any statistically significant effect at all. Although, there are no published extensive studies about the effect of subMICs of antibiotics on the virulence factors of *A. baumannii*, given the growing global problem of *A. baumannii* multiresistance to antimicrobial drugs, there are series of publications in the literature, on *in vitro* and *in vivo* effects of the different antibiotics combinations potentially useful in the treatment of the resistant Acinetobacter-related infections. There are contradictory reports on various possible combinations of imipenem, meropenem, colistin, doxycycline, rifampicin, azithromycin, vancomycin and other antibiotics used in the treatment of such infections with potentially synergistic effect. *In vitro*, this combinations can exhibit synergistic effect, but results of clinical studies are quite contradictory. In some of the studies authors did not report synergistic effect and in the other it was described. Similar results are reported for animal models as well, all in all suggesting the potential beneficial effect of subMICs, and possible better treatment outcomes.^{31–36}

According to the results of this study, subMIC concentrations of some antibiotics which are usually not recommended for infections associated with *A. baumannii* could be considered as an additional therapeutic option to decrease the virulence factor expression.

5. Conclusion

In conclusion, subMICs of antibiotics can reduce biofilm production ability among *A. baumannii* strains that exhibit this feature.

There are probably still some important virulence factors and mechanisms that serve as a conjunction between some steps in this process that remain obscure or unknown. This is the first study, to our knowledge, that was conducted to elucidate the effect of non-traditional antibiotics used in the treatment of the *A. baumannii* infections onto the biofilm formation ability.

Although we have come along the way towards looking to the insight of the *A. baumannii* virulence and pathogenicity, it is still much more in front of us to discover during evaluating the possible new targets and procedures in the path of controlling this worldwide spread pandrug resistant menace.

Conflict of interest

No potential conflict of interest was reported by the authors.

Notes on contributors

Maja Bogdan conceived and designed the study, obtained ethics approval, collected and analysed the data, wrote the article in whole, and revised the article.

Domagoj Drenjancevic conceived and designed the study, obtained funded and ethics approval, analysed the data, wrote the article in whole, and revised the article.

Ivana Harsanji Drenjancevic analysed the data, wrote the article in part, and revised the article.

Branka Bedenic collected and analysed the data, wrote the article in part, and revised the article.

Vlasta Zujic Atalic collected and analysed the data, wrote the article in part, and revised the article.

Jasminka Talapko collected and analysed the data, and revised the article.

Dubravka Vukovic obtained funded approval, collected and analysed the data, and revised the article.

ORCID

Maja Bogdan  <http://orcid.org/0000-0003-0692-5437>
Vlasta Zujic Atalic  <http://orcid.org/0000-0001-8033-4374>

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