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Patterns of biofilm production and antiseptic resistance in multidrug-resistant *Acinetobacter baumannii* clinical isolates

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Abstract

Acinetobacter baumannii is a notorious pathogen known for its extensive drug resistance and ability to form biofilms, making infections difficult to treat and control. This study investigated antibiotic resistance profiles, biofilm production, and environmental adaptability of 32 *A. baumannii* clinical isolates. Predominantly hospital-derived, the isolates showed a high proportion of antimicrobial drug resistance, with 93.75% classified as extensively drug-resistant (XDR), and the rest as multidrug-resistant (MDR). Notably, isolates demonstrated high resistance to amikacin and meropenem (MIC_{50} >4096 μg/mL and 64 μg/mL, respectively). Biofilm production analysis revealed 13 strong producers, 14 moderate, 4 weak, and 1 non-producer. Strong and moderate biofilm producers exhibited higher antibiotic resistance on average. The most favorable conditions for biofilm formation proved to be in glucose-supplemented BHI and at room temperature. Six selected strong biofilm producers displayed significant variability in biofilm production across different media and temperatures. In antiseptic and topical antibiotic persistence tests, isolates showed varied survival and biofilm production, with some thriving and enhancing biofilm in saline and boric acid. The findings emphasize the adaptability and resilience of *A. baumannii* in clinical settings, highlighting the challenges in treating biofilm-associated infections.

Key words: *Acinetobacter baumannii*, biofilm production, antibiotic resistance, antiseptics, environmental adaptability

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Introduction

Acinetobacter baumannii is a notorious Gram-negative pathogen known for its significant resistance to antibiotics and its ability to cause severe hospital-acquired infections. Strains of *A. baumannii* are usually extensively drug-resistant (XDR) and present a major challenge in clinical settings due to limited therapeutic options for treating *A. baumannii* infections. According to the Global Antimicrobial Resistance Surveillance System (GLASS) results from 2018, over 55% of *A. baumannii* isolates worldwide are resistant to carbapenems, with the most critical resistance levels observed in the Balkan countries and Turkey, where XDR strains comprise over 90% of all isolates (1). The treatment of infections caused by these strains is often limited to last-resort antibiotics, such as colistin, which is often unfavorable due to multiple factors (2).

The ability of *A. baumannii* to persist in hospital environments and spread resistance is greatly facilitated by its capacity to produce large amounts of biofilm. Biofilms are interactive communities of microorganisms embedded in a self-produced extracellular polymeric substance (EPS) matrix, which includes exopolysaccharides, extracellular DNA (eDNA), and proteins with specific functions (3). *A. baumannii* is known for its prolific biofilm production on a wide range of hospital materials and surfaces, including glass, porcelain, stainless steel, rubber, and various plastics (4). These biofilms serve as protective niches that shield the bacteria from environmental stresses, including desiccation and disinfectants, thus promoting the survival and dissemination of *A. baumannii* in hospital settings. The hospital environment, where *A. baumannii* coexists with other multidrug-resistant pathogens and is continually exposed to a variety of extended-spectrum antibiotics, creates an ideal breeding ground for the global spread of resistant strains (5, 6). Further, biofilms contribute to the resistance and tolerance of embedded microbial cells to antimicrobial agents through several mechanisms. Resistance refers to biofilm-dependent acquired mechanisms that are vertically transmitted and persist even in the absence of the biofilm structure, while tolerance refers to insensitivity to bactericidal or inhibitory concentrations of antimicrobial agents that are lost when transitioning to planktonic form (7). One example of tolerance is the reduced diffusion of antimicrobial agents due to sorption and inactivation within the densely populated matrix, preventing effective concentrations from reaching the deeper layers of the biofilm (8). Additionally, slow-metabolizing or metabolically inactive persister cells within the biofilm are insensitive to high concentrations of bacteriostatic antibiotics and can repopulate the infection site after the eradication of all metabolically active biofilm cells (9).

Importantly, biofilm production facilitates the ability of *A. baumannii* to establish infections through medical devices such as endotracheal tubes and intravascular and urinary catheters, bridging epidermal and epithelial barriers and causing serious infections like pneumonia, bacteremia, and urinary tract infections (5, 6, 10). Also on living surfaces, such as during skin and soft tissue infections, *A. baumannii* exists within robust biofilms on wounds and occlusive dressings (11). *A. baumannii* infections, typically manifesting as pneumonia or bacteremia, pose significant clinical challenges,

particularly in patients on mechanical ventilation (ventilator-associated pneumonia, VAP) and those with indwelling central venous catheters (12, 13). The impact of VAP and bacteremia on mortality is difficult to quantify due to the affected population often comprising elderly patients and individuals with pre-existing comorbidities and poor prognoses (14).

The persistence of *A. baumannii* in hospital settings is further facilitated by the tolerance to disinfectants. Despite the extensive use of disinfectants for topical hygiene and decontamination of inanimate objects in hospitals, improper use, such as incorrect dilution and suboptimal application, can promote the spread of viable microorganisms and the selection of resistant strains (15-17). Additionally, microorganisms can possess or develop resistance to disinfectants, leading to survival in otherwise microbicidal concentrations (18, 19). Given the increased use of disinfectants across various settings, including healthcare, cosmetic industries, and households, often without clear indications and proper application, there is a pressing need to address this issue more seriously (19, 20).

Understanding the interplay between drug resistance, biofilm formation, antiseptic resistance, and the persistence of multidrug-resistant *A. baumannii* strains is crucial for devising effective infection control strategies and preserving the efficacy of antimicrobial agents. Therefore, this study aimed to investigate the biofilm production patterns and antiseptic resistance in diverse multiresistant *A. baumannii* clinical isolates. The study included an examination of the biofilm production of tested strains under different incubation conditions reflecting a wide range of nutrient compositions and temperatures, as well as biofilm production in response to antiseptic treatment.

Experimental part

Isolation and Identification of Clinical Strains

Clinical strains of *Acinetobacter* spp. were isolated from various healthcare facilities in Belgrade, including Clinical Hospital Center Zvezdara, Clinical Hospital Center "Dr. Dragiša Mišović", Beo-lab laboratories, Gynecology-Obstetrics Clinic of the Clinical Center of Serbia, and Emergency Department of the Clinical Center of Serbia, spanning from October 2017 to April 2018. The isolates originated from the wound (n=10), blood (n=7), aspiration catheter (n=6), bronchus aspirate (n=5), urine (n=1), sputum (n=1), tracheal swab (n=1), and tissue biopsy (n=1). Additionally, the standard *A. baumannii* strain ATCC 19606 (KWIK-STIK™, Microbiologics Inc., USA) served as a positive control in the experiments. Clinical isolates were initially identified as members of the *Acinetobacter calcoaceticus-baumannii* (Acb) complex using standard methods, including analysis of cultural characteristics, microscopic examination (Gram-staining), and biochemical analysis using the automated VITEK 2 system with GN ID cards (bioMérieux, France). Confirmation of species-level identification (*A. baumannii*) was conducted through two differential tests: growth at 44 °C and Fourier-transform infrared spectroscopy (FTIR) (21, 22). Comparison of FTIR spectra with the spectrum of the

standard *A. baumannii* strain ATCC 19606 confirmed the identity of 32 *A. baumannii* isolates, which were subsequently utilized in the experimental procedures.

Determination of Resistance Profiles

The resistance profiles of identified clinical isolates of *A. baumannii* were determined using a combined approach involving the VITEK 2 automated system (bioMérieux, France), the agar disk diffusion method, and the broth microdilution method. The VITEK2 system was applied by utilizing AST-GN76 card for G1108 strain and AST-N240 card for the remaining isolates. The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 11.0 (2021) (23). The agar disk diffusion method was used for imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg), and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 11.0 (2021) (23). The agar disk diffusion method was also done for piperacillin (100 µg), ampicillin/sulbactam (10/10 µg), piperacillin/tazobactam (100/10 µg), ceftazidime (30 µg), cefepime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), tetracycline (30 µg), and minocycline (30 µg), with interpretation according to the Clinical and Laboratory Standards Institute (CLSI) 2021 (24). The broth microdilution method was employed to determine the minimum inhibitory concentrations (MICs), MIC_{50} (MIC for 50% of clinical isolates), and MIC_{90} (MIC for 90% of clinical isolates) of key antibiotics, including amikacin (testing range: 2-4096 µg/mL), ciprofloxacin (testing range: 1-512 µg/mL), meropenem (testing range: 0.5-1024 µg/mL), and colistin (testing range: 0.125-4 µg/mL). These antibiotics were selected for the broth microdilution method due to their clinical relevance as commonly used or last-resort treatments for *A. baumannii* infections. Amikacin, ciprofloxacin, and meropenem were kindly provided by Galenika a.d. (Belgrade, Serbia), while colistin was purchased from Sigma-Aldrich (USA). The broth microdilution method (25) and agar disk diffusion method (26) were performed and interpreted (24) according to the CLSI guidelines. Incubations were performed for 24 h at 37 °C, using Müller-Hinton agar (MHA; HiMedia, India) and Müller-Hinton broth (MHB; HiMedia, India) as culture media. Detection of MICs was facilitated by the use of 2,3,5-Triphenyl-tetrazolium chloride (TTC) solution (Sigma-Aldrich, USA). Resistance profiles were determined based on standards established by Magiorakos et al. (27), with isolates classified as multidrug-resistant (MDR) or extensively drug-resistant (XDR), according to these guidelines.

Examination of Biofilm Production

Biofilm production in clinical isolates of *A. baumannii* was assessed using an *in vitro* static, colorimetric method as described by Stepanović et al. with some modifications (28). Bacterial colonies were suspended in saline to an inoculum equivalent to the 0.5 McFarland standard (1.5×10^8 CFU/mL). For each isolate, 20 µL of the bacterial suspension was added in triplicate to sterile, flat-bottomed 96-well polystyrene microtiter

plates containing 180 µL of tryptic soy broth (TSB; Torlak, Serbia) supplemented with 1% glucose (TSBG). After 24 hours of incubation at 37 °C, the plates were washed three times with 300 µL of phosphate-buffered saline (PBS) to remove planktonic cells, followed by fixation of biofilm cells in an incubator at 60 °C for 60 minutes. Biofilms were stained with 150 µL of 0.5% safranin, and after 15 minutes the stain was washed off with water and the plates were air-dried. The stain was extracted with 150 µL of 96% ethanol, and optical density (OD) was measured at 490 nm using an ELISA plate reader (EZ Read 400, Biochrom, USA). Biofilm production was classified as non-production (NP), weak production (WP), moderate production (MP), or strong production (SP) based on the OD values as described by Stepanović et al. (29). Additionally, correlations were estimated between the biofilm production and the source of isolation, as well as the resistance profile. Further biofilm production assays were conducted under different incubation conditions, including temperatures of 25 °C, 37 °C, and 45 °C and various media, such as MHB supplemented with 1% glucose (MHBG), Brain-Heart-Infusion (BHI) broth (HiMedia, India) supplemented with 1% glucose (BHIG), unsupplemented TSB, MHB, BHI broth, nutrient broth (NB; Torlak, Serbia), and peptone water (PW; Torlak, Serbia) (Table 1).

Table 1. Composition of nutrient media used for examination of biofilm production

Tabela 1. Sastav hranljivih medijuma primenjenih za ispitivanje produkcije biofilma

TSB[a]	BHI Broth	MHB	PW	NB
Tryptone, 17 g/L	Calf Brain Infusion, 12.5 g/L	Beef Infusion, 300 g/L	Peptone-4, 10 g/L	Peptone-1, 15 g/L
Soy Peptone, 3 g/L	Beef Heart Infusion, 5 g/L	Casein Hydrolysate, 17.5 g/L	Sodium Chloride, 5 g/L	Meat Extract, 3 g/L
Sodium Chloride, 5 g/L	Proteose Peptone, 10 g/L	Starch, 1.5 g/L	Lactose, 10 g/L	Sodium Chloride, 5 g/L
Dipotassium Phosphate, 2.5 g/L	Sodium Chloride, 5 g/L		Fuchsin S, 0.01 g/L	Dipotassium Phosphate, 0.3 g/L
Glucose, 2.5 g/L	Dipotassium Phosphate, 2.5 g/L Glucose, 2 g/L			

[a] TSB – Tryptic Soy Broth, BHI Broth – Brain-Heart-Infusion Broth, MHB – Müller-Hinton Broth, PW – Peptone Water, NB – Nutrient Broth

Investigation of Antiseptic Resistance Using Time-Kill Assay

The bactericidal effect of antiseptics and topical antibiotics on *A. baumannii* isolates from wound infections was evaluated using the time-kill assay according to the CLSI guidelines (30) with minor modifications. Bacterial colonies were suspended in saline to a density of approximately 1.5 x 10^8 CFU/mL. The prepared suspensions were diluted 1:100 (≈ 10^6 CFU/mL) in 5 mL of saline, 3% boric acid, 0.1% ethacridine lactate, 1% clindamycin, or MHB as a positive control. Samples were taken before incubation and after 2 and 24 hours of incubation at 37 °C. Serial dilutions (1:10^1 to 1:10^8) were performed, and 10 µL of each dilution was plated in triplicate on MHA and incubated at 37 °C for 18-24 hours. Colony counts were expressed as log CFU/mL. Survivors were isolated to evaluate the correlation between biofilm production levels (measured as described above) and persistence. Additionally, the ability of these isolates to grow and persist in saline was assessed.

Statistical analysis

All experiments were performed in triplicate and results are presented as mean values ± standard deviations (SDs). Statistical comparisons were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, with a significance threshold set at $P < 0.05$. All analyses were carried out using SPSS software, version 26.0 (IBM, USA)

Results

Clinical Isolate Sources and Antibiotic Resistance Profiles

Out of the 32 clinical isolates of *A. baumannii*, 30 were hospital isolates, and 2 were isolated in outpatient settings (Beo-lab laboratories). The majority of strains were isolated from biological samples of patients hospitalized at Clinical Hospital Center "Dr. Dragiša Mišović" (13 strains) and Clinical Hospital Center Zvezdara (11 strains). The highest number of strains was isolated from wounds (10 strains) and blood (7 strains) (Table 2).

A total of 30 isolates exhibited an XDR resistance profile, with only two isolates showing MDR profiles (Table 2). Among the MDR isolates, one was sensitive to aminoglycosides, and one to carbapenems. The following MIC_{50} and MIC_{90} values were obtained:

- Amikacin, MIC_{50} >4096 µg/mL, MIC_{90} >4096 µg/mL;
- Ciprofloxacin, MIC_{50} = 64 µg/mL, MIC_{90} = 128 µg/mL;
- Meropenem, MIC_{50} = 64 µg/mL, MIC_{90} = 192 µg/mL;
- Colistin, MIC_{50} = 0.5 µg/mL, MIC_{90} = 1 µg/mL.

Apart from colistin, to which all isolates were sensitive, some isolates also showed sensitivity to tobramycin, amikacin, ampicillin/sulbactam, and one isolate each to minocycline, gentamicin, piperacillin/tazobactam, meropenem, imipenem, and trimethoprim/sulfamethoxazole. It should be noted that not all isolates were tested for sensitivity to tobramycin, ampicillin/sulbactam, minocycline, and piperacillin/tazobactam.

Table 2. *A. baumannii* isolates sources, resistance profiles, and biofilm production types

Tabela 2. Poreklo, profili rezistencije i tipovi proizvodnje biofilma kod izolata *A. Baumannii*

Isolate	Sample	Resistance profile	Resistant[a]	Susceptible	Biofilm producer type[b]	Source[c]
Z6142	Bronchus aspirate	XDR	PEN^R, CEF^R, CB^R, TC^R, FQ^R	MIN^S, CST^S	MP	KBC Zvezdara
Z6228	Wound	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	TOB^S, CST^S	WP	KBC Zvezdara
Z6235	Wound	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	WP	KBC Zvezdara
Z6419	Bronchus aspirate	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Zvezdara
Z6420	Bronchus aspirate	XDR	CB^R, AG^R, FQ^R, SXT^R	SAM^S, CST^S	MP	KBC Zvezdara
Z6879	Bronchus aspirate	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Zvezdara
Z7085	Bronchus aspirate	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	MP	KBC Zvezdara
B101	Wound	XDR	PEN^R, CEF^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	Beo-lab
B102	Wound	XDR	CB^R, AG^R, FQ^R, SXT^R	TOB^S, CST^S	WP	Beo-lab
Z94	Wound	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Zvezdara
D345	Sputum	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	MP	KBC Dragiša Mišović
D371	Aspiration catheter	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Dragiša Mišović
G1108	Wound	MDR	PEN^R, CEF^R, AG^R, FQ^R, SXT^R	TZP^S, IPM^S, MEM^S, AMK^S, CST^S	MP	GAK-KCS
D423	Blood	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	MP	KBC Dragiša Mišović
D613	Aspiration catheter	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Dragiša Mišović
D710	Wound	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Dragiša Mišović
D755	Wound	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Dragiša Mišović

D809	Tracheal swab	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Dragiša Mišović
D812	Aspiration catheter	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Dragiša Mišović
D863	Tissue biopsy	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	MP	KBC Dragiša Mišović
D746	Aspiration catheter	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	MP	KBC Dragiša Mišović
D989	Aspiration catheter	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KBC Dragiša Mišović
D1107	Urine	XDR	PEN^R, CB^R, AG^R, FQ^R	SXT^S, CST^S	MP	KBC Dragiša Mišović
D1013	Aspiration catheter	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	MP	KBC Dragiša Mišović
Z838	Blood	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	WP	KBC Zvezdara
Z1213	Wound	MDR	CB^R, AG^R, FQ^R, SXT^R	SAM^S, AMK^S, CST^S	SP	KBC Zvezdara
Z881	Wound	XDR	PEN^R, CB^R, AG^R, FQ^R, SXT^R	CST^S	NP	KBC Zvezdara
K111	Blood	XDR	CB^R, AG^R, FQ^R, SXT^R	CST^S	MP	KCS-UC
K112	Blood	XDR	CB^R, AG^R, FQ^R, SXT^R	CST^S	SP	KCS-UC
K116	Blood	XDR	CBR, AGR, FQR, SXTR	CSTS	MP	KCS-UC
K118	Blood	XDR	CB^R, AG^R, FQ^R, SXT^R	CST^S	MP	KCS-UC
K119	Blood	XDR	CB^R, AG^R, FQ^R, SXT^R	CST^S	MP	KCS-UC

[a] R – resistant, S – susceptible, PEN – penicillins, CEF – cephalosporins, CB – carbapenems, AG – aminoglycosides, TC – tetracyclines, FQ – fluoroquinolones, SXT – trimethoprim/sulfamethoxazole, AMK – amikacin, MEM – meropenem, CST – colistin, MIN – minocycline, TOB – tobramycin, SAM – ampicillin/sulbactam, TZP – piperacillin/tazobactam, IPM – imipenem. Susceptibility to specific antibiotics was determined based on the results of the VITEK 2 system, agar disk diffusion method, and broth microdilution method. MIC breakpoints and inhibition zone diameters were interpreted according to the recommendations of EUCAST version 11.0 (2021) or CLSI, 2021 (M100).

[b] SP – strong producer, MP – medium producer, WP – weak producer, NP – non-producer

[c] KBC Zvezdara – Clinical Hospital Center Zvezdara, KBC Dragiša Mišović – Clinical Hospital Center "Dr. Dragiša Mišović", GAK-KCS – Gynecology-Obstetrics Clinic of the Clinical Center of Serbia, KCS-UC – Emergency Department of the Clinical Center of Serbia

Biofilm Production and Correlation with Source of Isolation and Antibiotic Resistance Profiles

Based on the results obtained from measuring ODs at 490 nm, following extraction with 96% ethanol of a 0.5% safranin-stained biofilm formation, isolates were classified into biofilm-producing types (Figure 1). A total of 13 isolates produced large amounts of biofilm and were classified as SP type. Fourteen isolates were classified as moderate producers (MP type), while four isolates produced small amounts of biofilm (WP type). Only one isolate did not produce biofilm and was classified as NP type. These results were obtained under control biofilm cultivation conditions, which included incubation in TSBG media at 37 °C.

The isolates from aspiration catheters produced the highest average biofilm quantities, with mean OD_{490} values of 0.473±0.214. Among samples from human tissues, isolates from wound swabs produced the most biofilm on average (mean OD_{490} = 0.383±0.116), followed by isolates from urine cultures (mean OD_{490} = 0.360±0.057) and lower respiratory tract samples (mean OD_{490} = 0.326±0.047). Isolates from blood cultures produced the least biofilm (mean OD_{490} = 0.290±0.036). A significant difference ($P < 0.05$) in biofilm production was observed between isolates from aspiration catheters and those from blood cultures.

SP and MP type isolates demonstrated higher resistance levels to amikacin and ciprofloxacin compared to WP and NP type isolates (Figure 2). The calculated MIC_{50} values for amikacin and ciprofloxacin for SP and MP isolates were >4096 and 64 μg/mL, respectively, while for WP and NP isolates these values were 256 and 32 μg/mL, respectively. Additionally, isolates that produced the highest amounts of biofilm (SP type) were generally the least sensitive to meropenem, with a MIC_{50} value of 128 μg/mL. However, no statistically significant differences were observed.

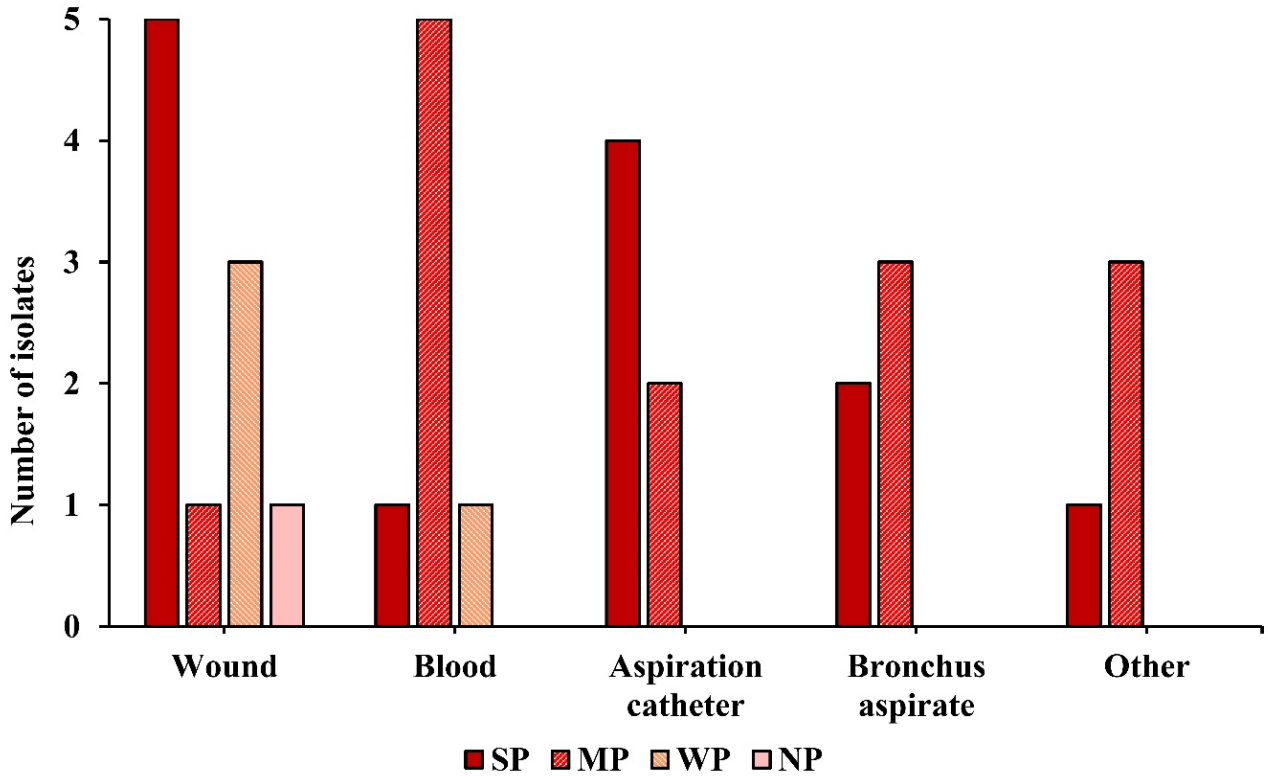

Figure 1. **Distribution of *A. baumannii* isolates by biofilm production categories across different sample types. Other sources of isolation include urine, sputum, tracheal swab, and tissue biopsy. SP – strong producer, MP – moderate producer, WP – weak producer, NP – non-producer**

Slika 1. **Distribucija *A. baumannii* izolata prema kategorijama produkcije biofilma kroz različite tipove uzoraka. U ostale izvore izolacije spadaju urin, sputum, bris traheje i biopsija tkiva. SP – izraziti proizvođač, MP – umeren proizvođač, WP – slab proizvođač, NP – ne proizvodi biofilm**

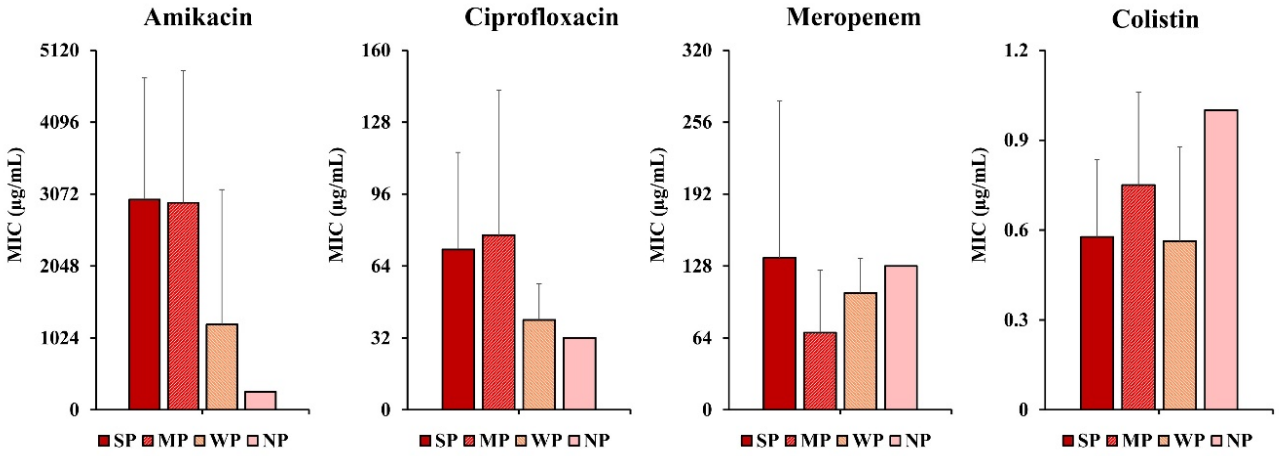

Figure 2. Minimum inhibitory concentrations (MICs) of amikacin, ciprofloxacin, meropenem, and colistin against isolates of SP, MP, WP, and NP types. Data are presented as mean values ± standard deviations (SDs). SP – strong producer, MP – moderate producer, WP – weak producer, NP – non-producer

Slika 2. Minimalne inhibitorne koncentracije (MIK) amikacina, ciprofloksacina, meropenema i kolistina u odnosu na izolate SP, MP, WP i NP tipova. Podaci su prikazani kao srednje vrednosti ± standardne devijacije (SD). SP – izraziti proizvođač, MP – umeren proizvođač, WP – slab proizvođač, NP – ne proizvodi biofilm

Influence of Culture Media and Temperature on Biofilm Production

A total of six clinical isolates of the SP type, exhibiting the highest levels of biofilm production (as tested using TSBG media at 37 °C), were selected to investigate biofilm production under different cultivation conditions to ensure the ability to observe significant variations in biofilm formation. The results showed that incubation in various nutrient media significantly affects the amount of biofilm produced (Figure 3). On average, *A. baumannii* strains produced 41.54% more biofilm after incubation in the presence of excess glucose. Incubation in BHIG resulted in the highest average biofilm production (11.34% more than in TSBG). Interestingly, for three strains, biofilm production was downregulated after incubation in this medium, leading to their reclassification as WP type. Among the unsupplemented media, the strongest biofilm production was recorded in TSB, with a slight difference compared to TSBG and 45.55% and 46.89% higher compared to unsupplemented BHI and MHB, respectively. The lowest biofilm production was observed in NB and PW, two media containing a high percentage of peptone without glucose. Regarding incubation at different temperatures, the strains produced the most biofilm at room temperature (9.81% more than at 37 °C), despite a slight decrease in production for two strains, which were reclassified as MP type. The lowest production was observed at 45 °C (23.35% less than at 37 °C).

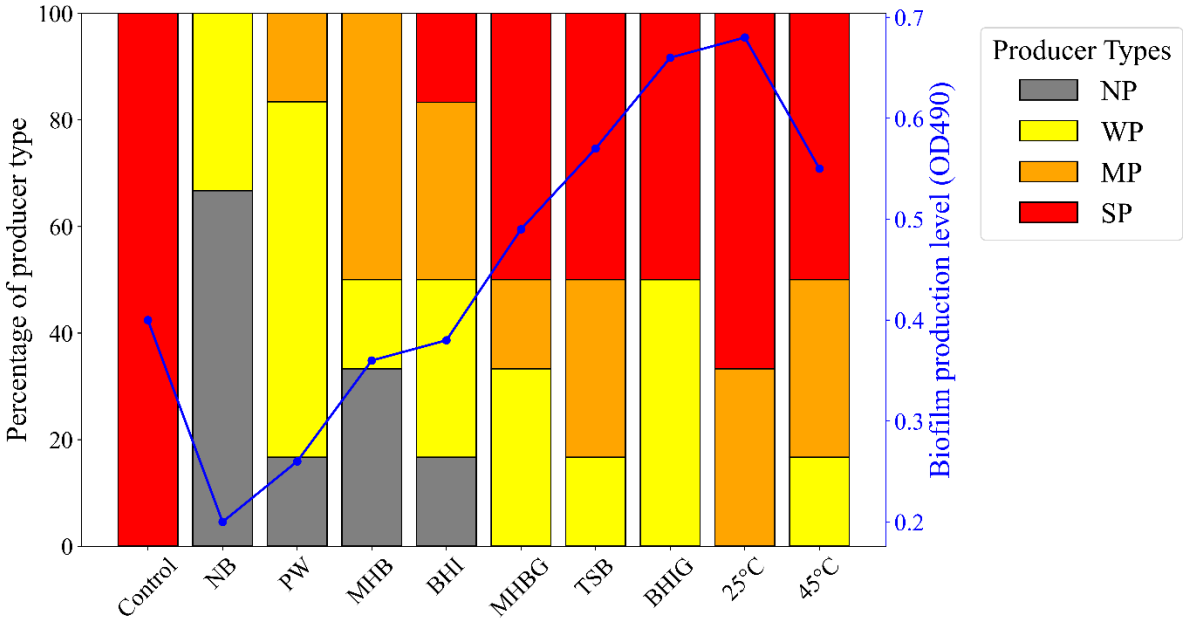

Figure 3. **Biofilm production types and levels of multiresistant *A. baumannii*, derived from six selected isolates, all strong biofilm producers, from different sources. The bar plots indicate the percentage distribution of NP, WP, MP, and SP, with gray, yellow, orange, and red colors, respectively. The blue line plot shows biofilm production levels (OD_{490} values) across various culture media and temperatures: Nutrient Broth (NB), Peptone Water (PW), Müller-Hinton Broth (MHB), Brain-Heart-Infusion (BHI), Müller-Hinton Broth with Glucose, 1% (MHBG), Tryptic Soy Broth (TSB), and Brain-Heart-Infusion with Glucose, 1% (BHIG). The 25 °C and 45 °C conditions represent the effect of temperature variation on biofilm formation. Control consisted of Tryptic Soy Broth with Glucose, 1% (TSBG) at 37 °C.**

Slika 3. **Tipovi i nivoi produkcije biofilma kod multirezistentnih *A. baumannii*, izvedeni iz šest odabranih izolata, od kojih su svi bili jaki proizvođači biofilma, iz različitih izvora. Stubičasti grafikoni prikazuju procentualnu distribuciju NP, WP, MP и SP, sa sivom, žutom, narandžastom i crvenom bojom, respektivno. Plava linija grafikona pokazuje nivoe produkcije biofilma (OD_{490} vrednosti) kroz različite hranljive medijume i temperature: hranljivi bujon (NB), peptonska voda (PW), Miler-Hinton bujon (MHB), *Brain-Heart-Infusion* (BHI), Miler-Hinton bujon sa glukozom, 1% (MHBG), Tripton soja bujon (TSB), i *Brain-Heart-Infusion* sa glukozom, 1% (BHIG). Uslovi 25 °C и 45 °C predstavljaju efekat temperaturnih varijacija na formiranje biofilma. Tripton soja bujon sa glukozom, 1% (TSBG) na 37 °C je predstavljao kontrolu.**

Persistence and Biofilm Production of *A. baumannii* in Antiseptics and Topical Antibiotic Solutions

All tested isolates demonstrated survival in physiological saline after 24 hours of incubation, showing only a slight reduction in colony numbers (Table 3). For the standard strain *A. baumannii* ATCC 19606, the initial inoculum of approximately 10^6 CFU/mL decreased to 4.77×10^5 CFU/mL after 2 hours and 1.82×10^5 CFU/mL after 24 hours. Notably, one strain exhibited growth in physiological saline, reaching 4.79×10^6 CFU/mL after 24 hours.

Table 3. Growth and biofilm production of *A. baumannii* in various antiseptics or antibiotic solutions compared to the positive control (tryptic soy broth with glucose, 1%). The detection limit for viable bacteria was set at log CFU/mL <2.

Tabela 3. Rast i produkcija biofilma *A. baumannii* u različitim antisepticima i rastvorima antibiotika u poređenju sa pozitivnom kontrolom (tripton soja bujon sa 1% glukoze). Limit detekcije za vijabilne bakterije bio je log CFU/mL <2.

	Log CFU/mL	Biofilm (%)
Saline, 2 h	5.67 ± 0.34	98.14 ± 34.24
Saline, 24 h	5.26 ± 0.55	122.20 ± 50.35
3% boric acid, 2 h	5.11 ± 0.63	85.34 ± 47.16
3% boric acid, 24 h	< 4.34 ± 1.53	141.35 ± 92.36
0.1% ethacridine lactate, 2 h	< 2.34 ± 0.46	101.81 ± 56.75
0.1% ethacridine lactate, 24 h	< 2.02 ± 0.06	211.71 ± 0
1% clindamycin, 2 h	< 2.10 ± 0.17	143.01 ± 132.44
1% clindamycin, 24 h	< 2 ± 0	/[a]

[a] Due to the detection limit, no colony growth was observed, making it impossible to assess biofilm production.

In 3% boric acid, all isolates survived the 2-hour incubation period, but two of them (D710 and D755) did not survive the 24-hour incubation. For the surviving strains, colony counts slightly decreased to an average of $1.12-1.22\times10^5$ CFU/mL after 24 hours, suggesting limited bactericidal activity of boric acid over an extended period. Interestingly, three strains (B101, B102, and Z94) exhibited growth in 3% boric acid, with higher colony counts after 24 hours compared to 2 hours, further indicating their potential resistance to this antiseptic.

The bactericidal effect of 0.1% ethacridine lactate and 1% clindamycin was more pronounced. Six isolates (Z6228, B101, Z94, G1108, D710, and D755) survived 2 hours of incubation in 0.1% ethacridine lactate, but with a significantly reduced colony count, averaging 3.60×10^2 CFU/mL. Only one strain survived the 24-hour incubation. In 1% clindamycin, three strains survived the 2-hour incubation, with an average colony count of 1.78×10^2 CFU/mL, but no strains survived the 24-hour period. It is important to note that the detection limit was 10^2 CFU/mL, and strains may have persisted below this

detection threshold, suggesting a possible sub-detection level of persistence that could contribute to clinical challenges in treatment.

Biofilm production levels of colonies that survived incubation in physiological saline, antiseptics, or 1% clindamycin were further assessed and compared with positive control (biofilm production after incubation in TSBG) to evaluate the impact of biofilm on strain persistence. No consistent pattern of increased or decreased biofilm production was observed among the surviving colonies, indicating a varied response in biofilm synthesis that may not be solely related to antiseptic or antibiotic exposure.

Some colonies exhibited significantly stimulated biofilm production; for instance, one strain produced three times more biofilm after 24 hours in 3% boric acid, and another strain showed similar biofilm production levels after 2 hours in 1% clindamycin. Conversely, other colonies showed markedly inhibited biofilm production. One strain produced five times less biofilm after 2 hours in 1% clindamycin compared to the control, demonstrating a potential inhibitory effect of certain conditions on biofilm formation.

Discussion

Strains of *A. baumannii* used in this study were isolated between 2017 and 2018, primarily as pneumonia-causing pathogens from lower respiratory tract samples such as bronchial aspirates, tracheal swabs, or sputum, as well as from aspiration catheters. In addition, a significant number of strains were isolated from infected wounds and blood cultures. The sample origins are similar to the findings of Luković et al., and predominantly found in respiratory tract samples, wound exudates, and blood cultures, with rare cases isolated from urine cultures or from the tip of central venous catheters, and one strain from cerebrospinal fluid causing meningitis (31). The patients were usually older males, averaging 66 years, and frequently treated in intensive care units following invasive surgical procedures. Similarly, Gajić et al. studied 332 *A. baumannii* isolates from various locations in Serbia, Montenegro, and Bosnia and Herzegovina from 2016 to 2017 (32). Most of these isolates came from clinical samples of the lower respiratory tract, followed by surgical wound exudates and blood cultures. In both studies, the prevalence of carbapenem-resistant *A. baumannii* was over 92%, which aligns with our research findings. Interestingly, the MIC_{50} values for carbapenems in these studies were 8-16 μg/mL, significantly lower than the MIC_{50} values found in our study (64 μg/mL). However, it should be noted that a different testing method (Etest) was applied in other studies compared to broth dilution method in our study. Both studies showed that over 90% of strains were resistant to antibiotics from cephalosporin, aminoglycoside, and fluoroquinolone classes. Resistance rates were lower for ampicillin/sulbactam (<59%), tobramycin (<74%), tigecycline (<25%), and colistin. Interestingly, Gajić et al. found that nearly 40% of strains were susceptible to tetracycline (32).

A. baumannii is known for its ability to produce significant amounts of biofilm, which enhances its persistence in the environment, tolerance to desiccation, and dissemination of epidemic multiresistant strains (33-35). The biofilm matrix protects *A. baumannii* cells from antimicrobial agents during infection and is considered a crucial

virulence factor (35). Chapartegui-Gonzalez et al. demonstrated that strains producing higher amounts of biofilm can survive in nutrient-limited conditions for up to 60 days, with increased biofilm production upon rehydration (36). Acb complex members typically produce three times more biofilm compared to other *Acinetobacter* species, which are weak pathogens and rarely found in hospital environments (37). Among the clinically significant *Acinetobacter* species, *A. baumannii* and *A. nosocomialis* produce the highest quantities of biofilm. Different studies have shown varying percentages of biofilm producers among *A. baumannii* hospital isolates, depending on geographic location and genetic lineage. For instance, in Hong Kong, only 60% of sporadic Sequence-Type (ST) strains produced biofilm (38). Eze and Zowalaty found that 32% of strains isolated from environmental samples in South African hospitals did not produce biofilm (39). In our study, out of 32 clinical isolates, only one did not produce biofilm. Among the biofilm producers, 44% were classified as strong producers (SP), another 44% as moderate producers (MP), and the remaining 12% as weak producers (WP). These results are consistent with those from a Taiwanese hospital where 45% were SP type, 32% MP type, and 16% WP type (40).

Biofilm formation has been linked to antibiotic resistance in several studies, though results are mixed. Some research indicates that strains producing more biofilm are more resistant to antibiotics (40, 41), while other studies report the opposite (35, 38, 42). Shenkutie et al. suggest that XDR and pandrug-resistant (PDR) strains produce less biofilm due to the reduced need for this protective mechanism (38). Their findings also show that biofilm-producing cells are significantly more resistant to imipenem, ciprofloxacin, and colistin compared to planktonic cells, likely due to increased β-lactamase production and efflux pump expression. Ciprofloxacin also showed a higher MBC for biofilm cells due to its ineffectiveness against dormant persister cells, while colistin showed the least change in MBC, potentially due to its increased activity in anaerobic conditions within the biofilm (38). Consistently, our study found that strains producing more biofilm were generally more resistant to amikacin and ciprofloxacin. Yang et al. reported similar findings, with biofilm production correlating with reduced sensitivity to aminoglycosides and some β-lactams (40). Although our study did not observe a linear increase in meropenem resistance with biofilm production, SP type strains were the least sensitive to this antibiotic. Vijayakumar et al. also reported similar results for clinical isolates from India (43). Inconsistent results regarding the relationship between biofilm production and antibiotic resistance may be due to methodological differences and the small proportion of low-resistance strains studied.

Biofilm production in external environments occurs under conditions different from those used in conventional biofilm testing methods. Specifically, *A. baumannii* forms biofilms at room temperature under nutritionally limited conditions. Our study aimed to investigate the effects of temperature and various nutrient media compositions on biofilm production in several clinical isolates of *A. baumannii*. Interestingly, we found that at room temperature, the tested strains collectively produced more biofilm than at human body temperature. This aligns with previous findings that *A. baumannii* biofilm

production is stimulated at lower temperatures (37, 44, 45). De Silva et al. demonstrated that thermoregulation plays a role in the increased biofilm production of strain *A. baumannii* ATCC 17978 at 28 °C, leading to differential expression of virulence factors associated with biofilm formation (45). At lower temperatures, hyper-expression of the *csu* operon, encoding pili involved in adhesion to abiotic surfaces, was observed (46). This enhanced adhesion contributes to increased biofilm production. Conversely, at 37 °C, strain ATCC 17978 produced less biofilm, with production regulated by increased expression of the *ompA* gene and *paa* operon, which significantly contribute to *A. baumannii* virulence in the human body (47). In our study, not all strains showed increased biofilm production at lower temperatures; two clinical isolates produced 52-57% less biofilm under these conditions. This could be due to the genomic variability of *A. baumannii*, as not all strains express Csu pili (48). Therefore, it is essential to include more *A. baumannii* strains in further investigations of the environmental temperature's impact on biofilm production. *A. baumannii* is among the few *Acinetobacter* species capable of persisting at temperatures of 44-45 °C (49), but the contribution of specific factors to this persistence has not been studied extensively. We examined biofilm production levels at 45 °C and found that, while biofilm production remained at a high level, it was reduced compared to the control.

Resistance to disinfectants and antiseptics is an increasing concern in the control of healthcare-associated infections, particularly with multiresistant organisms like *A. baumannii*. Disinfectants and antiseptics play a crucial role in infection control by reducing microbial load on surfaces and skin, respectively. However, *A. baumannii* has demonstrated remarkable resilience to these agents, partly due to its ability to form biofilms and intrinsic resistance mechanisms. Disinfectants such as quaternary ammonium compounds (QACs), chlorhexidine, and alcohols are commonly used in healthcare settings. However, *A. baumannii* has been shown to exhibit variable resistance to these agents. For instance, resistance to QACs and chlorhexidine can be attributed to the presence of efflux pumps and alterations in membrane permeability. The *qac* genes, which are associated with resistance to QACs, are frequently found in *A. baumannii* strains, contributing to their survival in environments with high disinfectant concentrations (50). The effectiveness of disinfectants is influenced by factors such as concentration, contact time, and environmental conditions. Suboptimal use, such as insufficient concentration or inadequate contact time, can lead to incomplete microbial eradication and foster the development of resistant strains. This issue is exacerbated by the persistence of *A. baumannii* in healthcare settings, where biofilms on surfaces can act as reservoirs of infection and contribute to the spread of resistant strains.

A common form of infection caused by *A. baumannii*, after pneumonia and bacteremia, is deep wound infection, especially in cases of severe burns, surgical site infections, and diabetic wounds (13, 51). Antiseptics used for wound treatment differ in composition and are often applied at significantly lower concentrations compared to those used for skin decolonization. For example, chlorhexidine, due to its cytotoxicity, is used at a concentration of 0.05% (1:80 dilution of 4% chlorhexidine) (52), which often fails to

achieve microbicidal activity against *A. baumannii* (53-57). Other commonly used antiseptics for wound treatment include 3% boric acid and 0.1% ethacridine lactate solutions (52, 58-61). In this study, the microbicidal activity of these antiseptics against multiresistant *A. baumannii* clinical isolates was tested for the first time. Since the tested strains, including those from wounds and the ATCC 19606 strain, demonstrated persistence during short exposures to these antiseptics, further tests were performed to assess their survival over extended periods (2 and 24 hours). This is important, since a number of hospital outbreaks caused by contamination of disinfectants with multiresistant bacterial species (e.g. *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Serratia marcescens*, and *Stenotrophomonas maltophilia*) have been documented (16). In this regard, our study aimed to evaluate the potential for hospital outbreaks due to contamination of 3% boric acid or 0.1% ethacridine lactate with multiresistant *A. baumannii* strains. Results indicated that such potential exists, as 100% and 60% of the tested isolates survived incubation for 2 hours in 3% boric acid and 0.1% ethacridine lactate, respectively. Moreover, 90% of isolates survived in 3% boric acid for 24 hours, with some even multiplying in this disinfectant. Conversely, survival in 0.1% ethacridine lactate was much lower, with only one isolate surviving 24 hours and 92% showing a decrease in colony count below 3 log CFU/mL after 2 hours, which some authors interpreted as microbicidal effectiveness (53). Furthermore, we also tested *A. baumannii* survival in physiological saline, used in practice for wound irrigation, showing 100% survival after 24 hours. This aligns with previous findings (62). One strain even demonstrated growth, a phenomenon previously seen with other multi-resistant bacteria in distilled water or saline (62-64). Additionally, we tested 1% clindamycin, used topically for acne treatment, which exhibited the strongest bactericidal activity, with only 38% of strains surviving 2 hours (all below 3 log CFU/mL) and none surviving 24 hours.

Our study also explored the relationship between biofilm production and persistence in physiological saline or antiseptics. Although the literature suggests that biofilm production can increase resistance to disinfectants (chlorhexidine and QACs) and that some antiseptics can inhibit biofilm formation (octenidine dihydrochloride) (65, 66), no clear correlation was found in our analysis. The variability in biofilm production among strains may result from different gene expression levels, especially in the highly variable *A. baumannii* genome related to biofilm formation, indicating that strains may respond differently depending on the antiseptic tested (6). In summary, we observed that *A. baumannii* strains with high biofilm production on average exhibited increased resistance to commonly used disinfectants. This finding suggests that biofilm formation not only enhances bacterial persistence, but also may contribute to disinfectant resistance. The interaction between biofilm matrix components and disinfectants can reduce the efficacy of these agents, leading to the survival and propagation of resistant strains.

The current study revealed critical insights into the challenges posed by *A. baumannii* in healthcare settings. The high prevalence of carbapenem-resistant strains underscores the need for robust antimicrobial stewardship and infection control measures. Biofilm production by *A. baumannii* significantly contributes to its persistence and

resistance to both antibiotics and disinfectants. The high percentage of biofilm producers among resistant strains highlights the role of biofilms in enhancing bacterial survival and resistance mechanisms. Disinfectant resistance, particularly in strains with high biofilm production, presents a significant challenge in infection control. The presence of efflux pumps, *qac* genes, and biofilm matrix interactions contributes to the reduced effectiveness of disinfectants, potentially leading to persistent contamination and cross-resistance with antibiotics. Given these findings, it is crucial to implement stringent infection control measures, including effective disinfection protocols, regular monitoring of antimicrobial susceptibility, and active surveillance of patients in hospital settings. Active surveillance of patients includes taking samples in order to support early identification of resistant nosocomial pathogens. The development of new disinfectants and strategies to overcome biofilm-associated resistance could be pivotal in managing the spread of *A. baumannii* and other multidrug-resistant organisms. Continued research and surveillance are essential to address the evolving challenges posed by this resilient pathogen.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Dušan Ušjak: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Lidija Bošković: Investigation, Methodology, Resources, Writing – review & editing.

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Produkcija biofilma i rezistencija na antiseptike kod multirezistentnih kliničkih izolata *Acinetobacter baumannii*

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Kratak sadržaj

Acinetobacter baumannii je opasan patogen poznat po svojoj opsežnoj rezistenciji na lekove i sposobnosti da formira biofilmove, čineći infekcije teškim za lečenje i kontrolu. U ovoj studiji ispitani su profili rezistencije na antibiotike, produkcija biofilma i prilagodljivost različitim uticajima spoljašnjih faktora 32 klinička izolata *A. baumannii*. Izolati su poticali pretežno iz bolničkih sredina i pokazali su visoku rezistenciju na antimikrobne lekove, pri čemu je 93,75% klasifikovano kao ekstenzivno rezistentno (XDR), dok su ostali bili multi-rezistentni (MDR). Kao posebno značajna istakla se visoka rezistencija na amikacin i meropenem (MIC_{50} >4096 μg/mL i 64 μg/mL, respektivno). Analizom produkcije biofilma pokazano je da 13 testiranih izolata formira jak biofilm, 14 umereno formira biofilm, dok su 4 izolata slabi proizvođači biofilma, a samo 1 izolat nije proizvodio biofilm. Jaki i umereni proizvođači biofilma su u proseku ispoljili veću rezistenciju na antibiotike. Najpovoljniji uslovi za formiranje biofilma bili su BHI medijum sa dodatkom glukoze i sobna temperatura. Šest odabranih jakih proizvođača biofilma pokazalo je značajnu varijabilnost u produkciji biofilma u različitim medijumima i pri različitoj temperaturi kultivacije. U testovima perzistencije u rastvorima antiseptika i antibiotika za topikalnu primenu, izolati su pokazali različit nivo preživljavanja i produkcije biofilma, pri čemu se kod nekih broj kolonija i biofilm povećao, u fiziološkom rastvoru i u bornoj kiselini. Ovi rezultati ukazuju na izuzetnu prilagodljivost i otpornost *A. baumannii* u bolničkim uslovima, ističući izazove u lečenju infekcija povezanih sa biofilmom.

Ključne reči: *Acinetobacter baumannii*, produkcija biofilma, rezistencija na antibiotike, antiseptici, prilagodljivost na spoljašnje uslove