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THE POTENTIAL OF DIFFERENT BIOCIDES IN ERADICATION OF MATURE *ACINETOBACTER BAUMANNII* BIOFILM

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Abstract: *Biofilm production is an important bacterial virulence factor, which contributes to bacterial resistance mechanisms to antibiotics and enables them to survive adverse conditions. Acinetobacter baumannii is an excellent biofilm producer and it represents a great challenge in the treatment of hospital infections, because it facilitates bacterial survival in the hospital environment. Aim of this study was to compare the efficacy of three different biocides in eradication of mature, performed A. baumannii biofilm. Biofilm production of 30 clinical isolates of A. baumannii was performed by method of Stepanovic et al. Strains were classified as biofilm-non producers or weak, moderate and strong biofilm producers according to OD of crystal violet dye, measured by spectrophotometer. Afterwards, mature biofilm was treated for 10 minutes with different comercially available biocides, such as 1% of sodium hypochlorite (bleach), 70% alcohol and ecological disinfectant "Frosch". Out of a total of 30 isolates tested, 5 isolates did not produce biofilm, 20 isolates were weak and strong producers (10 isolates in both groups), and 5 isolates were classified as moderate producers. There was a significant eradication of biofilm in tested isolates after the treatment of all three disinfectants. After treatment the majority of isolates became non- biofilm producers and weak biofilm producers ($p<0.001$, $p=0.01$, $p=0.04$). The best effect in biofilm removal was found with bleach (biofilm removed completely in all isolates), while alcohol and environmental-friendly disinfectant were equally effective (they removed biofilm in 46% and 53% of isolates, respectively). Based on the obtained results, we can recommend bleach as the best biocide for removing the mature biofilm. Also, it should be emphasized that it is very important to achieve a contact period of 10 minutes between the surface and the biocide.*

Key words: *biocides, biofilm, Acinetobacter baumannii*

Introduction

Acinetobacter baumannii is the member of ESKAPE group of pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) well known for their antimicrobial resistance. *A. baumannii* is one of the most common agents that cause nosocomial infections such as respiratory, urinary and wound infections and sepsis. *A. baumannii* accounts for more than 12% of hospital acquired bloodstream infections in intensive care units (ICUs), with broad regional variations: it is common in Southern Europe, the Middle East, Asia and South America, but

uncommon in Northern Europe and Australia [1]. In the hospital environment, a reported mortality rate of 26%, raised up to 40 to 50% in intensive care units [2]. So, it is very important to prevent the transmission of this bacterium in nosocomial facilities. One of the most important methods of prevention is the cleaning and disinfection of surfaces and medical equipment with biocides.

Biocides are chemical agents utilized in order to clean and disinfect surfaces and human skin or mucosa. Their practical application mostly determines their categorization within certain classes, such as antiseptics (skin, mucosa) and disinfectants (non-host surfaces). Biocides have many target sites within the bacterial cell (proteins, membranes, nucleic acid etc) and they cause overall damage to bacteria, especially in planktonic forms. Besides the planktonic, individual form, bacteria can survive inside the community called the biofilm. Biofilm is a collective way of bacterial existence, in which cells stick to each other and also to a substratum, and embedded within a slimy extracellular matrix that is composed of extracellular polysaccharides. This protective matrix layer does not exist in planktonic forms and is responsible for most of the mechanisms that bacteria avoid eradication from the infection site.

The aim of this study is to compare the efficacy of the biocides with different chemical compounds to biofilm produced by clinical isolates of *A.baumannii*.

Material and methods

Bacterial strains

In this study we used 30 *A. baumannii* isolates that are part of the collection of Faculty of Medicine University of Banja Luka, collected during period 2019- 2021. Bacterial isolates were identified according to routine bacteriological methods, including colony apperance, Gramm staining, biochemical and physiological test.

Biofilm production

Quantification of biofilm production to microtiter plates was based upon the protocol described by Stepanovic et al. [3]. The strains were incubated overnight in blood agar (Himedia, India) at 37°C, and then diluted in fresh Brain Heart Infusion (BHI) broth (Himedia, India) to achieve final concentration of 10^6 CFU/ml. Aliquots of bacterial suspension (100 μL) were transferred to each well of the 96-well microtiter plate and incubated for 24 hours at 37°C. The content of each well was then aspirated and wells were washed three times with sterile phosphate buffer solution (PBS). The plates were left overnight at room temperature for drying and air fixation. The plates were stained with 100 μL of 2% (w/v) crystal violet and, afterwards, the dye bound to the adherent cells was solubilized with 100 μL of 33% (v/v) glacial acetic acid. The negative control wells contained BHI broth only. *Staphylococcus epidermidis* ATCC 14990 was used as the positive control. The optical density (OD) of each well was measured at 570 nm using an automated microtiter plate reader. The cut-off optical density OD (ODc) was defined as three

standard deviations above the mean OD of the negative control. Strains with OD above ODc were considered adherent to microtiter plates. Strains were classified as follows:

OD ≤ ODc = non-biofilm producers, ODc < OD ≤ (2 × ODc) = weak biofilm producers, (2 × ODc) < OD ≤ (4 × ODc) = moderate biofilm producers and OD > (4 × ODc) = strong biofilm producers. All analyses were performed in triplicate and repeated at least two times.

Biocides treatment of mature *A. baumannii* biofilm

After biofilm production described above, we treated all isolates for 10 minutes with selected biocides:

1. 1% sodium/hipochlorite (bleach)
2. 70% alcohol
3. Comercial ecological disinfectant „Frosch“

After treatment, microtiter plates were aspirated and washed three times with sterile phosphate buffer solution (PBS). Following procedures and biofilm production clasiffication were described in the section above.

Statistical analysis

Data analyses were done with the SPSS version 20. McNemar test was used in order to compare the efficacy of different biocides on *A. baumannii* biofilm production. The differences were considered significant if $p < 0.05$, and highly significant if $p < 0.01$.

Results and discussion

Aim of this study was to determine potential of different biocides in eradication of the mature *A. baumannii* biofilm production. First, we needed to determine the biofilm production capacity of tested isolates. Out of a total 30 isolates, 5 isolates were not capable for biofilm production, while the majority of tested isolates (25/30) produced biofilm. Out of 25 biofilm producing isolates, 10 isolates were weak biofilm producers, 5 isolates were classified as moderate producers, and 10 isolates produced strong biofilm.

Biofilm production of *A. baumannii* is an important virulence factor that enables bacteria to evade immune defense as well as antibiotic and biocides treatment. *A. baumannii* is causing a wide range of infections mostly related to medical devices, eg, vascular catheters, cerebrospinal shunt, Foley catheters or artificial machines for ventilatory support, where this bacteria can form biofilm [4].

Within the biofilm, the bacteria strongly adhere to the abiotic plastic surface, produce a protective polysaccharide coat and thus gain a great advantage over antibiotic treatment or disinfection. Survival period for bacteria inside of the biofilm is much

longer than for planktonic forms, especially on the dry, abiotic surfaces, such as hospital equipment [5]. The only way to battle against this multiresistant biofilm-producing pathogen is to prevent its colonization and biofilm production. In healthcare facilities, the first line of prevention is cleaning and disinfection. Our study provides some preliminary results about the treatment of various biocides on the mature *A. baumannii* biofilm.

After biocides treatment, we have found that all three biocides were efficient in the mature biofilm eradication, as we shown in Table 1. There was a significant eradication of biofilm in tested isolates after the treatment of all three disinfectants. After treatment the majority of isolates became non- biofilm producers and weak biofilm producers ($p<0.001$, $p=0.01$, $p=0.04$). When comparing the efficacy of all three biocides, the best effect in the mature biofilm removal was found with bleach (biofilm removed completely in all isolates, $p<0.001$), while alcohol and environmental-friendly disinfectant were equally effective (they removed biofilm in 46% and 53% of isolates, respectively, $p=0.57$).

Table 1. Biofilm production of tested *A. baumannii* isolates befor and after treatment

	Untreated	Bleach	70% alcohol	„Frosch“
Non-producers	5	30	16	14
Weak producers	10	0	18	8
Moderate producers	5	0	5	5
Strong producers	10	0	1	3

According to our experiment and other researchers' results, bleach or 1% sodium hypochlorite is the most potent biocides in the biofilm eradication [6]. Possible explanation is in their different mechanisms of action. The mechanism of action for ethanol is the degradation of cell membranes, resulting in cell lysis and further leading to the degradation of cellular proteins and enzymes [7]. On the other hand bleach is more efficient than alcohol as it is a potent oxidizer and disrupts cellular activities, such as protein synthesis [7]. Also, in the situations when bleach could not be used we can use 70% alcohol, which has the same eradication effect as environmental-friendly commercial cleaners. Also, in the future investigations we would like to analyze the effect of some others biocides that are could be used for disinfection of human tissues (povidone-jodid, boric acid) or abiotic surfaces (benzalkonium chloride, peracetic acid). It is noteworthy to mention, that the price of the biocide would be one of the elimination factors, especially in situations where a huge amount of biocide is needed for the disinfection of large surfaces, eg, in health-care facilities.

Conclusion

Based on the obtained results, we can recommend bleach as the best biocide for removing the mature biofilm. Also, it should be emphasized, that it is very

important to achieve a contact period of 10 minutes between the surface and the biocide.

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EFIKASNOST BIOCIDA U ERADIKACIJI *ACINETOBACTER BAUMANNII* BIOFILMA

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Sažetak: *Produkcija biofilma je veoma značajan faktor virulencije bakterija, koji dodatno doprinosi rezistenciji na antibiotike i omogućava im preživljavanje nepovoljnih uslova. Acinetobacter baumannii je odličan produktor biofilma, koji predstavlja veliki izazov u terapiji intrahospitalnih infekcija, jer mu omogućava preživljavanje u bolničkoj sredini. Cilj rada je bio da se uporedi dejstvo različitih biocida na uklanjanje zrelog, formiranog biofilma koji su produkovali A. baumannii klinički izolati. U ovoj studiji je ispitana sposobnost produkcije biofilma kod 30 A. baumannii kliničkih izolata, metodom po Stepanoviću i saradnicima u mikrotitarskoj ploči sa bojenjem formiranog biofilma pomoću biološke boje gencijana ljubičasta. Sojevi su bili klasifikovani kao neproduktori, slabi, umjereni i jaki produktori na*

osnovu adsorbance gencijana ljubičasta boje mjerene pomoću spektorfotometra. Nakon toga je isprodukovani biofilm bio tretiran 10 minuta sa komercijalno dostupnim biocidima različitog sastava (1% natrijum hipohlorit-varikina, 70% alkohol i ekološki dezinficijens marke "Frosch"). Od ukupno 30 testiranih izolata, 5 izolata nisu isprodukovali biofilm, po 10 izolata su bili slabi i jaki produktori, a 5 izolata su klasifikovani kao umjereni produktori. Nakon dejstva sva tri dezinficijensa došlo je do značajne eradikacije biofilma, jer nakon tretmana sa sva tri biocida najveći broj sojeva prešao u neproduktore i slabe produktore biofilma (p<0,001, p=0,01, p=0,04). Najveći stepen uklanjanja biofilma utvrđen kod varikine (biofilm uklonjen u potpunosti kod svih izolata), dok su alkohol i ekološki dezinficijens podjednako djelovali (uklonili su biofilm kod 46%, odnosno kod 53% izolata). Na osnovu dobijenih rezultata možemo preporučiti varikinu kao najbolje sredstvo za uklanjanje produkovanog biofilma, pri čemu je veoma važno da se ostvari dužina kontakta između površine i biocida od 10 minuta.

***Ključne riječi**: biocidi, biofilm, Acinetobacter baumannii*