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DISINFECTANTS ACTIVITY ON BIOFILM FORMATION AND AGAINST PLANKTONIC FORMS OF *ESCHERICHIA COLI* ISOLATED FROM DRINKING WATER

***Jelena Pantelić*[1], *Jovana Pantelić*[1], *Aleksandra Šmitran*[1], *Ljiljana Božić*[1], *Dragan Knežević*[2], *Dragan Kasagić*[2], *Bojan Golić*[2]**
[1]Faculty of Medicine, University of Banja Luka, Banja Luka, Bosnia and Herzegovina
[2]Public Institution Veterinary Institute of the Republic of Srpska "Dr. Vaso Butozan", Banja Luka, Bosnia and Herzegovina

Abstract. *Esherichia coli is one of the most important bacterium within the "One Health" concept due to its ability to colonise and infect various animal and plant hosts, as well as humans. Also, this bacteria can easily survive in environment, such as water. Biofilm formation allows E. coli to survive on nonliving and living subjects for prolonged period. Biofilm represents a highly organised bacterial sessile community embedded within a polysaccharide matrix, produced by bacteria only during biofilm formation. Biofilm is very resistant to antimicrobial drugs and can only be removed through physical means, such as thorough cleaning and treatment with high concentrations of chemical substances that may have a toxic effect. Our goal was to determine the disinfectants activity against planktonic E. coli and during biofilm formation. In this study were included 30 strains of E. coli, isolated in drinking water samples obtained from poultry farms. To examine the activity of disinfectants in preventing biofilm formation, we utilised doubly decreasing concentrations (7.5%-0.01% for H_2O_2, and 1%-0.001% for bleach) during overnight incubation in microtiter plates with the bacterial inoculum at 37°C. Disinfectants activity against planktonic forms was determined by broth microdilution method with the same concentration range of disinfectants used for biofilm prevention treatment. We have noticed statistically significant (Mann-Whitney U test) higher activity of H_2O_2 against both planktonic forms and in biofilm prevention. Average H_2O_2 MIC for planktonic forms was 0.022% and MICB (minimal inhibitory concentration for biofilm) was 0.021%. On the other hand, median bleach MIC for planktonic forms was 0.11% and MICB was 0.33%. H_2O_2 was statisticaly more effiecent both for inhibition of planktonic forms, as well as in preventing biofilm formation. Our results show that H_2O_2 was more effective both against planktonic E. coli forms and consenquently in bioflm formation than bleach*

Key words: *Escherichia coli, planktonic, biofilm, disinfectants*

Introduction

Escherichia coli is Gram negative bacterium which is one of the most common causes of human infections. Also, this bacterium can colonize and infect different animals, as well as plants and nonliving subjects. Due to its wide presence in our environment it is one of the most important bacteria within the "One Health" concept [1]. Additionally, this bacterium thrives in a variety of environments, including land and

water, particularly in fertilized soil. Besides its presence as individual bacteria in planktonic forms, *E. coli* can form sessile communities called biofilms. Biofilm represents a highly organised bacterial sessile community embedded within a polysaccharide matrix, produced by bacteria only during biofilm formation. Biofilm formation allows *E. coli* to survive on nonliving and living subjects for prolonged periods [2]. Bacteria embedded into biofilm matrix are protected against action of antibiotics and the immune system. Researchers are looking for active natural and chemical materials to eradicate mature, preformed biofilms as well as effective methods and materials to prevent the production of biofilms [3].

Disinfectants are often overlooked chemicals that can significantly reduce the production of biofilms. So our goal was to determine the disinfectants activity against planktonic *E. coli* and during biofilm formation.

Material and methods

Bacterial strains

In this study were included 30 strains of *E. coli*, isolated in drinking water samples obtained from poultry farms from 01.04.2024- 01.05.2025. Water samples was collected as a part of investigation in national project "Javnozdravstveni rizik učestalosti i antimikrobne rezistencije patogenih bakterija u vodi za piće porijeklom sa farmi živine" which is sponsored by Ministry of Scientific and Technological Development and Higher Education No. 19.032/961-101/23. Sampling and laboratory processing were performed according to guidelines: BAS EN ISO 6222:2003, BAS EN ISO 6887-1, BAS EN ISO 8199 and BAS EN ISI 7218 standards. Shortly, 1 mL of water sample and their appropriate dilutions were inoculated in nutrition agar and incubated at 36±2°C during 44 +/- 4 hours. Identification of E. coli was performed based to biochemical reactions (positive β-D- galactosidase and β-D- glucuronidase tests).

Antimicrobial activity of disinfectans against planktonic forms

Disinfectants activity against planktonic forms was determined by broth microdilution method in doubly decreased concentrations (7.5%-0.01% for H_2O_2, and 1%-0.001% for bleach) according to EUCAST recommendations [4]. Shortly, twofold serial dilutions of disinfectants were made using MH broth (Biorad, Watford, UK). Each well in the microtiter plates was filled with 100 μL of MH broth with disinfectants (final concentration ranging from 7.5%-0.01% for H_2O_2, and 1%-0.001% for bleach) and 5 μL of bacterial inoculum (concentration of 10^7 CFU/mL) to achieve a final concentration of 5×10^5 CFU/mL. The microtiter plates were incubated for 16–18 h at 37 °C. The MIC was defined as the lowest concentration which inhibited growth detected by the unaided eye.

Biofilm formation

The quantification of biofilm biomass was carried out following the protocol of Stepanovic et all [5]. Briefly, 100 μL of *E. coli* suspension in Trypticase soy broth (TS, Biorad, UK) with a final concentration of 10^6 CFU/mL was transferred to each well of a 96-well microtiter plate and incubated for 24 h at 37 °C. Biofilm growing in

the microtiter plate was dyed with 100 μL of 2% (w/v) crystal violet for 15 min. Glacial acetic acid at 33% (v/v) was used to dissolve the dye bound to the adhering cells inside the biofilm matrix. Using an automated microtiter plate reader, each well's optical density (OD) was determined at 570 nm (ICN Flow Titertek Multiskan Plus Reader, Meckenheim, Germany). TS broth was the only suspension in the negative control wells. Three standard deviations more than the mean OD of the negative control were designated as the cut-off OD (ODc).

The results were evaluated 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.

Antibiofilm activity of disinfectans against *E. coli* biofilm formation

The minimum inhibitory concentration for biofilm prevention (MICB) of disinfectants was determined following the protocol of Bagheri-Josheghani et al. [6], with a slight modification of the broth used in the experiment. Aliquots of 10 μL of *E. coli* isolates (concentration of 10^8 CFU/mL) were incubated overnight at 37 °C in microtiter plates containing 100 μL of doubled-strength MH broth and 100 μL of disinfectants (concentrations ranging from 7.5%-0.01% for H_2O_2, and 1%-0.001% for bleach). Afterwards, the microtiter plates were washed three times with PBS, dried at room temperature and dyed with 100 μL of 2% (w/v) crystal violet for 15 min. Biofilm production of treated isolates was measured according to the method of Stepanovic et al. [5].

Statistical analysis

PSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Categorical variables are presented as absolute frequencies and percentages (%). To compare variances without normal distribution between the two groups, the Mann–Whitney U test was used. Student t test was used for comparison of the frequency of occurrence of the analysed categorical variables. A p value less than 0.05 was considered to be significant.

Results

In this study we have detected 30 isolates during one year investigations from drinking water and water for other purposes in poultry farms in Republic of Srpska. Out of these 30 isolates, biofilm production was determined in 29 strains and classified as biofilm nonproducers (1), weak producers (8), moderate (14) and strong producers (7).

MIC values for planktonic forms of hydrogen peroxide ranged from 0.01 to 0.06%. For planktonic species, the average H_2O_2 MIC was 0.022%. MIC for biofilm prevention (minimal inhibitory concentration for biofilm- MICB) was ranged from 0.01 to 0.05% of H_2O_2, with median value of 0.021%. MIC and MICB for H_2O_2 was similar for both planktonic forms and bacteria embedded in biofilms (p=0.9).

On the other hand, median bleach MIC for planktonic forms was 0.11%, ranging from 0.03 to 0.25%. MICB for bleach ranged from 0.03% to 1%, with median value 0.33%. There was statistical significance between MIC and MICB values (p= 0.01)

H_2O_2 was statistically more efficient both for inhibition of planktonic forms, as well as in preventing biofilm formation ($p \leq 0.001$).

Disscusion

Bacterial resistance to antibiotics represents huge global problem in routine treatment of patients with infective diseases. According to some experts, the post-antibiotic age has come,and in the next two decades we will have the same mortality rate for cancer patients and for patients infected with multiresistant bacteria [7]. Preventive measures are also crucial, particularly in health facilities, the food industry and catering, where the conditions for human infection can be easily created through contact with hospital environment and contaminated food and drinks.

Biofilm production assurvival mode of life, enables bacteria to survive in hostile environments for prolonged periods. Biofilms are known as "double trouble" since bacteria inside of biofilms possess mechanisms of resistance originating both from planktonic forms and sessile community. Biofilms can be very easily removed by physical methods, such is cleaning or sterilization with wet and dry heat. Therefore, using specific chemicals for disinfection may be the best way to clean surfaces in medical institutions, the food sector, and catering. In this study we used bleach (as chlorine representatives) and hydrogen peroxide, which is an oxidizing substance. In our study, only one strain was not able to produce measurable biofilm biomass, and the majority of isolates were moderate producers. Eradication of biofilms formed in a moist environment is very problematic because of its resistance to antibacterial substances, in concentration safe for therapy of infected patients. Nearly all of our isolates are able to produce biofilm, which is extremely problematic. Such high percentage of biofilm productors was noticed by Baret et al [8].

H_2O_2 was equally effective against planktonic forms and in biofilm prevention, with similar concentration of 0.02% or 200 ppm. Since bacteria require a "quorum," or a sufficient number of bacteria in the community, to make enough "quorum sensing" molecules—which serve as a trigger for bacterial association—this is a logical and expected outcome. Therefore, there will not be enough "quorum sensing" molecules to initiate the creation of biofilms when we stop bacterial growth and replication with disinfection. As a member of the reactive oxygen species family, H_2O_2breaks down the bacterial surface (cell wall and membrane) as well as any enzymes or genetic material in bacterial interior [9].

We used bleach or sodium hypochlorite in the second section of our experiment. Hypochlorous acid (HOCl), hypochlorite ions (OCl), and residual or free chlorine can all be produced when sodium hypochlorite breaks down in an aqueous environment during disinfection. All three compounds have a strong oxidizing effect, by firmly attaching and removing amino or thiol groups, as well as peptide bonds and double C=C bonds on various bacterial cell components (proteins, nucleic acids, and lipids). We needed a triple smaller concentration for planktonic isolates (0.1% or 1000 ppm)

than for bacterial community (0.3% or 3000 ppm), as we expected. Similar results are noticed by other authors [10, 11]. We can conclude that significantly higher concentration of bleach was needed for both inhibitory action against planktonic straind and in biofilm formation. Also, for eradication of mature biofilms, bleach concentration needed for eradication were much higher, up to 5000 ppm or 0.5% [12].

Conclusion

In conclusion, it is important to notice that *E. coli* strains isolated from drinking water samples are highly capable for biofilm formation. In prevention of biofilm formation hydrogen peroxide was significantly better than bleach, so we can reccomend this disinfectant for water treatment.

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EFIKASNOST DEZINFICIJENASA NA SPREČAVANJE FORMIRANJA BIOFILMA I NA PLANKTONSKE *ESHERICHIA COLI* FORME IZOLOVANE IZ VODE ZA PIĆE

***Jelena Pantelić*[1], Jovana Pantelić[1], Aleksandra Šmitran[1], Ljiljana Božić[1], Dragan Knežević[2], Dragan Kasagić[2], Bojan Golić[2]**

[1]Medicinski fakultet, Univerzitet u Banjoj Luci, Banja Luka, Bosna i Hercegovina
[2]JU Veterinsrski institut Republike Srpske "Dr. Vaso Butozan", Banja Luka, Bosna i Hercegovina

Sažetak. *Escherihia coli je jedna od najvažnijih bakterija u okviru koncepta „Jedno zdravlje" zbog svoje sposobnosti da kolonizuje i inficira različite životinjske i biljne domaćine, uključujići ljude. Takođe, ova bakterija može lako da preživi u spoljašnjoj sredini, npr. vodi. Formiranje biofilma omogućava bakteriji da preživi na neživim i živim objektima tokom dužeg perioda. Biofilm predstavlja visoko organizovanu bakterijsku sesilnu zajednicu ugrađenu u polisaharidni matriks, koju bakterije proizvode samo tokom formiranja biofilma. Biofilm je veoma otporan na antimikrobne lijekove i može se ukloniti samo fizičkim sredstvima, kao što su temeljno čišćenje ili tretman visokim koncentracijama hemijskih supstanci koje su često toksične. Naš cilj je bio da utvrdimo aktivnost dezinfekcionih sredstava na planktonske E. coli oblike, kao i njihovo preventivno desjtvo na formiranje biofilma. U ovu studiju je uključeno 30 sojeva E. coli, izolovanih iz uzoraka vode za piće sa živinarskih farmi. Da bismo ispitali aktivnost dezinfekcionih sredstava u sprečavanju stvaranja biofilma, koristili smo dvostruko opadajuće koncentracije (7,5%-0,01% za H_2O_2 i 1%-0,001% za varikinu) tokom prekonoćne inkubacije u mikrotitarskim pločama sa bakterijskim inokulumom na 37°C. Aktivnost dezinfekcionih sredstava na planktonske forme bakterija je određena bujon mikrodlucionom metodom sa istim opsegom koncentracija dezinfekcionih sredstava koji smo koristili za tretman prevencije biofilma. Uočena je statistički značajno veća aktivnost H_2O_2 i na planktonske forme bakterija i tokom formiranja biofilma, u odnosu na varikinu. Prosečan MIK za H_2O_2 za planktonske oblike je iznosio 0,022%, a MIKB (minimalna inhibitorna koncentracija za biofilm) bila je 0,021%. S druge strane, MIK varikine za planktonske oblike je bio 0,11%, a MIKB 0,33%. H_2O_2 je bio statistički značajno efikasniji kako za inhibiciju planktonskih oblika, tako i u sprečavanju formiranja biofilma. Naši rezultati pokazuju da je H_2O_2 bio efikasniji i protiv planktonskih oblika E. coli i posljedično bolje sprečavao formiranje biofilma nego varikina.*

Ključne riječi: *Escherichia coli, biofilm, planktonske forme, dezinficijensi*