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Biofilm formation by *Staphylococcus aureus* on stainless steel surfaces and evaluation of its sensitivity to sanitizers

Mária Vargová[1], František Zigo[2], Jana Výrostková[3], Katarína Veszelits Laktičová[1], Šárka Bursová[4], Silvia Ondrašovičová[5]

[1]University of Veterinary Medicine and Pharmacy in Košice, Department of Public Veterinary Medicine and Animal Welfare, Košice, Slovakia
[2]University of Veterinary Medicine and Pharmacy in Košice, Department of Nutrition and Animal Husbandry, Košice, Slovakia
[3]University of Veterinary Medicine and Pharmacy in Košice, Department of Food Hygiene Technology and Safety, Košice, Slovakia
[4]University of Veterinary Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Animal Origin Food and Gastronomic Sciences, Czech Republic
[5]University of Veterinary Medicine and Pharmacy in Košice, Department of Biology and Physiology, Košice, Slovakia

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Abstract

This study aimed to assess the ability of adhesion, detachment, and biofilm formation of the reference strain *Staphylococcus aureus* CCM 4223 on a stainless steel surface, and the efficacy of three disinfectants: preparation A (based on peracetic acid, 20 mg/l), preparation B (newly developed disinfectant with a combination of two active ingredients, caprylic acid and hydrogen peroxide, 25 mg/l), and preparation C (based on sodium hypochlorite, 200 mg/l) in removing *S. aureus* cells adhered onto this surface. Cell detachment was around 5 log CFU/cm^2 (colony forming units) over the first 6 h and around 6 log CFU/cm^2 over 48 h characterizing a high persistence of cells on the tested surface. The number of cells (5–7 log CFU/cm^2) needed for biofilm formation was noted already after 6 h of incubation. The decrease in cell counts caused by preparation A was 1.12–2.07 log CFU/cm^2 ($P < 0.0001$), whereas for preparation C and preparation B, the decrease was 1.13–2.08 log CFU/cm^2 and 1.61–2.16 log CFU/cm^2, respectively ($P < 0.0001$). Preparation A was found to be the most effective (80.9%) in reducing the adhered cell count in the biofilm matrix. From these results, the assayed *S. aureus* strain revealed high capacities to adhere and form biofilms on stainless steel surfaces, and the cells in biofilm matrixes were resistant to total removal when exposed to the evaluated disinfectants.

Disinfectants, microorganism, ability of adhesion, reference strain

Microbial adhesion and biofilms are of great importance and occur on a high variety of contact surfaces which are good substrates for biofilm development. Bacteria may attach to the surface of the foreign material by surface charge attraction, hydrophilic or hydrophobic interactions, and by the specific attachment of fimbriae. Bacterial attachment is followed by growth, colonization, and maturation (Silverstein and Donatucci 2003). Multiplication of cells gives rise to colonies and biofilm is established when the cellular mass is thick enough to aggregate nutrients, residues, and other microorganisms (Zottola and Sasahara 1994). Biofilms are characterized by the environmental conditions and surfaces that favour their formation, the gene products that are required for their formation, the genes that are activated and required to maintain the biofilm, the architecture of the biofilm, and the types of extracellular products that are concentrated in the biofilm matrix (Beech et al 2006; Brady et al 2008; Bryers 2008; Pavithra and Doble 2008). They can be considered a deposit where microorganisms are highly adhered onto a surface by means of appendices of either protein or polysaccharide nature, referred to as glycocalyx.

Address for correspondence:
František Zigo
Department of Nutrition and Animal Husbandry
University of Veterinary Medicine and Pharmacy
Košice, Komenského 73, 041 81, Slovakia

Phone: +421 908 689 722
E-mail: frantisek.zigo@uvlf.sk
http://actavet.vfu.cz/

Such appendix protrudes externally either from the outer membrane of Gram-negative cells or from the peptidoglycan of Gram-negative ones (Criado et al 1994). There are as many different types of biofilms as there are bacteria; moreover, the same bacterium may make several different types of biofilms under different environmental conditions (Beech et al 2006; Brady et al 2008; Bryers 2008; Pavithra and Doble 2008).

Bacterial biofilm is resistant to many antimicrobials. Although strict cleaning and disinfection procedures can generally assure suitable hygienic conditions by destroying planktonic cells and biofilms just starting to be formed, they may fall short for the elimination of biofilms that are already well developed. These settle on sites that are difficult to clean due to uneasy access or surface irregularities (Shi and Zhu 2009). The most important peroxygens are hydrogen peroxide (HP), peracetic acid (PAA) and ozone (O_3) (Block 2001; Weavers and Wickramanayake 2001).

The effect of HP is based on the production of free radicals, which affect the biofilm matrix (De Carvalho 2007; de Carvalho and de Fonseca 2007). Penna et al. (2001) reported that HP is a powerful non-toxic oxidizing agent. It is a mainstay in metal surface treatment, causing no damage in the disinfection of medical and dental devices in routine health care. It is also used for treating large volumes of wastewater, as well as in water and food disinfection applications. Also, Rideout et al. (2005) reported that H_2O_2 solution is safe when used and did not cause allergic reactions or bronchial asthma.

Peracetic acid is the most potent peroxygen (Popham et al. 1995). It is bactericidal, fungicidal and sporicidal but fungi such as *Aspergillus niger* are less susceptible to PAA than yeasts or non-sporulating bacteria (Block 2001). The major targets of its action is the oxidation of enzymes and protein thiol groups (Russel and Chopra 1996; Denyer and Stewart 1998). Multitargeted effects of peracetic acid suggest that similar mechanisms might be responsible for microbial inactivation.

Sodium hypochlorite disinfectant products irreversibly kill bacterial cells in biofilms by denaturing proteins in the biofilm matrix and inhibiting major enzymatic functions in bacterial cells. Although sodium hypochlorite disinfectants at concentrations as low as 0.0219% are effective against the formation of *S. aureus* biofilms (Barnes and Greive 2013), the use of sub-lethal concentrations of some sodium containing disinfectants could actually promote the formation of biofilms on environmental surfaces (Cincarova et al. 2016). In a study conducted by West et al. (2018), sodium hypochlorite products were more effective against both *S. aureus* and *Pseudomonas aeruginosa* planktonic cells compared to quaternary ammonium.

Caprylic acid (CAP) is a medium-chain fatty acid naturally present in human breast milk. It has been used intravenously in some total parenteral nutrition formulations (Wanten and Calder 2007). It is also readily absorbed following oral administration Haidukewych et al. 1982). Protonated CAP has been reported to have antimicrobial properties (Skrivanova and Marounek 2007; Yang et al. 2010).

A considerable number of spoilage and pathogenic microorganisms are able to participate at a lower or higher intensity in the adhesion process but also on biofilm formation. *Staphyloccocus aureus*, a leading cause of nosocomial infections worldwide, is the aetiologic agent of a wide range of diseases, from relatively benign skin infections to potentially fatal systemic disorders. Many of these diseases, including endocarditis, osteomyelitis, and foreign-body related infections, appear to be caused by biofilm-associated *S. aureus* (Mayberry-Carson et al. 1984; Gotz 2002; Donlan and Costerton 2002; Shirtliff et al. 2002).

The aim of this study was to verify the effectiveness of tested disinfectants, preparation A (active ingredient PAA), preparation B (active ingredients CAP and HP), and preparation C (active ingredient sodium hypochlorite) on the viability of selected bacterial biofilm.

Materials and Methods

Stainless steel surfaces and biofilm preparation

The reference strain *S. aureus* CCM 4223 (Czech Collection of Microorganisms, Brno, Czech Republic) was used for biofilm preparation and the testing of its sensitivity to evaluated disinfectants. The reference strain was stored in Petri dishes on a medium Baird-Parker Agar (BPA; Sigma-Aldrich, St. Louis, MO, USA) at the temperature below 4 °C. The prepared culture was cultivated for 24 h at room temperature from 22 to 25 °C. The prepared bacterial suspension was diluted with sterile saline (0.85 g/100 ml) to have a final concentration of approximately 8 log of colony forming units per ml (CFU/ml) adjusted according to the turbidity of 0.5 McFarland standard tube (Oliveira et al. 2010). For the experiments, the following materials were selected: coupons made of stainless steel (STN 17 240, 17 241 W Nr. 1.4301 AISI 304) in the form of a plate with the dimensions of 20 × 20 × 10 mm). Stainless steel coupons were immersed in Petri dishes into a solution of 60 ml of Brain Heart Infusion (BHI) (HiMedia) (Thane, Maharashtra, India) and 10 ml of the above referred inoculum. The coupons were incubated in Petri dishes at room temperature for 3, 6, 9, 12, 24, 48 and 72 h. Subsequently, after each exposure time, the coupons (four for each treatment) were removed from the solution, and one plate was washed with sterile peptone water (SPW; 0.1 g/100 ml) for 15 s for releasing the non-adherent cells. The cells adhered to the coupons were collected by thoroughly rubbing their surfaces with moistened swabs, which were resuspended in SPW by vigorously vortexing (Benchmixer V2 Vortex Mixer, Benchmark Scientific, Sayreville NJ, USA) for 30 s. The mixture was serially diluted (10^{-1} to 10^{-5}) in SPW and aliquots of 100 µl were spread plated onto sterile BPA plates. The plates were incubated for 24 h at 37 °C (Rode et al. 2007; Herrera et al. 2007). After the incubation period, the number of viable cells was counted and the results were expressed in log CFU/cm^2.

Disinfectants

Three disinfectants with different active ingredients were evaluated: preparation A - 0.2% Persteril (OQEMA, Sokolov, Czech Republic) with the active ingredient PAA; preparation B - 0.25% two-component preparation belonging to the group of broad-spectrum disinfectants with bactericidal and fungicidal activity, with two active ingredients of CAP (Sigma-Aldrich, Oakville, Canada) and HP (Vulm, Bratislava, Slovakia); and preparation C - 2% Savo Original (Unilever, Bratislava, Slovakia) with the active ingredient sodium hypochlorite. For this, six stainless steel coupons were allowed to develop biofilm according to the above mentioned experimental conditions. After 12 days of incubation, the coupons were washed in SPW, immersed for 1 and 5 min in sterile Petri dishes containing the sanitizer solutions. Afterwards, the coupons were drawn of the sanitizer solution and immersed for 3 s in a neutralizing solution (0.1 M sodium thiosulphate, $Na_2S_2O_3$). The remaining cells were counted after scraping by using sterile moistened swabs, which were resuspended by vigorously vortexing in 9 ml of SPW. Serial dilutions were prepared in SPW and aliquots of 100 µl were spread plated onto BPA plates and incubated at 37 °C for 24 h (Ammor et al. 2004). After the incubation period, the number of viable cells was counted and the results expressed in log CFU/cm^2. The effectiveness of each disinfectant was calculated based on the difference between the counts obtained before and after using the disinfectant.

Data analysis

Counts were converted to decimal logarithmic values (log CFU/cm^2) to nearly match the assumption of normal distribution. Counts obtained for adhesion, detachment and biofilm formation and counts obtained for the effect of the sanitizers (before and after the application) on the biofilm matrix were submitted to Analysis of Variance (ANOVA) followed by *post hoc* Duncan test. Data were analyzed using the software Graph Pad Prism; probability value $P < 0.05$ was accepted as indicating significant difference.

Results

The number of *S. aureus* cells adhered on stainless steel surfaces after 3, 6, 9, 12, 24, 48 and 72 h of incubation is shown in Fig. 1. The number of adhered cells revealed by the evaluated isolate was between 4.26 and 6.59 log CFU/cm^2. The highest number of adhered cells (6.59 log CFU/cm^2) on stainless steel surfaces was found at 48 h. The incubation time and count of adhered cells were found to be in significant correlation ($r = 0.9183$, $P < 0.05$) (Fig. 1).

Counts of *S. aureus* adhered to stainless surfaces after the application of the tested disinfectants, preparations A, B, and C, are shown in Table 1. When comparing the tested disinfectants, preparation A with PAA as the active ingredient (20 mg/l) was found to be the most effective (80.9%) in reducing the viable cell count in the biofilm after 5 min. The log reduction (fraction reduction) caused by preparation A ranged from 2.07 to 1.12 log CFU/cm^2; the log reduction caused by preparation C (based on sodium

Table 1. Effects of preparation A (peracetic acid, 20 mg/l), preparation B (caprylic acid + hydrogen peroxide, 25 mg/l) and preparation C (sodium hypochlorite, 200 mg/l) on the count (log CFU/cm^2) of *Staphyloccocus aureus* cells forming a 12-day biofilm on stainless steel surfaces.

Disinfectant	Initial count	Final count after 1 min	Final count after 5 min	Fraction reduction after 1 min (%)	Fraction reduction after 5 min (%)	*P*
A	5.89 ± 0.09^{AB}	3.82 ± 0.09^{AC}	4.77 ± 0.05^{BC}	2.07 (64.8)	1.12 (80.9)	< 0.0001
B	5.76 ± 0.18^{AB}	3.60 ± 0.08^{AC}	4.15 ± 0.06^{BC}	2.16 (62.5)	1.61 (72.1)	< 0.0001
C	5.85 ± 0.10^{AB}	3.77 ± 0.09^{AC}	4.72 ± 0.09^{BC}	2.08 (64.4)	1.13 (80.6)	< 0.0001

$^{A, B, C}$ - The same superscripts within rows mean significant differences of the achieved results at $P < 0.0001$

hypochlorite, 200 mg/l) was 2.08–1.13 log CFU/cm^2; whereas for preparation B (disinfectant with a combination of two active ingredients, CAP and HP, 25 mg/l), ranged from 2.16 to 1.61 log CFU/cm^2. However, each of the tested sanitizers largely decreased ($P < 0.0001$) the counts of cells adhered to the assayed surfaces.

Discussion

According to Marques et al. (2007), the number of adhered cells was 1.2 log CFU/cm^2 after 3 h of contact, and 4.27 log CFU/cm^2 after 6 h. The authors suggest that the count would probably increase, becoming a biofilm with prolonged contact time. This is supported also by our observation, as the incubation time and adhered cell count were in significant correlation ($r = 0.9183$, $P < 0.05$). In our study, the number of adhered cells was 4.26 log CFU/cm^2 after 3 h of contact, which is a similar to the number around 4.5 log CFU/cm^2 detected by Meira et al. (2012).

Souza and Siqueira-Junior (2014) studied biofilm formation and determined the highest number of adhered cells (5–6 log CFU/cm^2) on the stainless steel surfaces over 72 h, but accordind results of our study, the highest numbers of adhered cells (6.59 log CFU/cm^2) on stainless steel surfaces were already found at 48 h. The number of adhered cells decreased from 6.59 to 5.63 log CFU/cm^2 after 48 h of incubation. Meira et al. (2012) noted the same behavior in *S. aureus*, finding a decrease in the number of adhered cells after 48 h of incubation.

The result of our study is in agreement with some findings which suggested that under static conditions, adherent cells may be present in high numbers, but do not always increase over the incubation time. This behavior could be related to the cell division process and/or redistribution of adhered cells forming the biofilm (Stoodley et al. 2002; Kusumaningrum et al. 2003). Ronner and Wong (1993) and Wirtanen et al. (1995) state that a minimum of 5.0–6.0 log CFU/cm^2 is needed for the formation of biofilm, and lower counts could indicate only an adhesion process. The number of cells needed for biofilm formation was noted already after 6 h of incubation, however, Meira et al. (2012) state that three days of incubation are needed for biofilm formation. According to Heydorn et al. (2000) and Cheng et al. (2007), the maturation of a bacterial biofilm occurs between three to six days after the initial adhesion, and only after 10 days an increased population density with pronounced production and deposition of exopolysaccharide is reached as a mature biofilm. When assessing the biofilm formation by *S. aureus* on stainless steel and glass surfaces immersed in the Brain Heart Infusion broth, Marques et al. (2007) found bacterial counts of 7 and 8 log CFU/cm^2, respectively, after 15 days of cultivation.

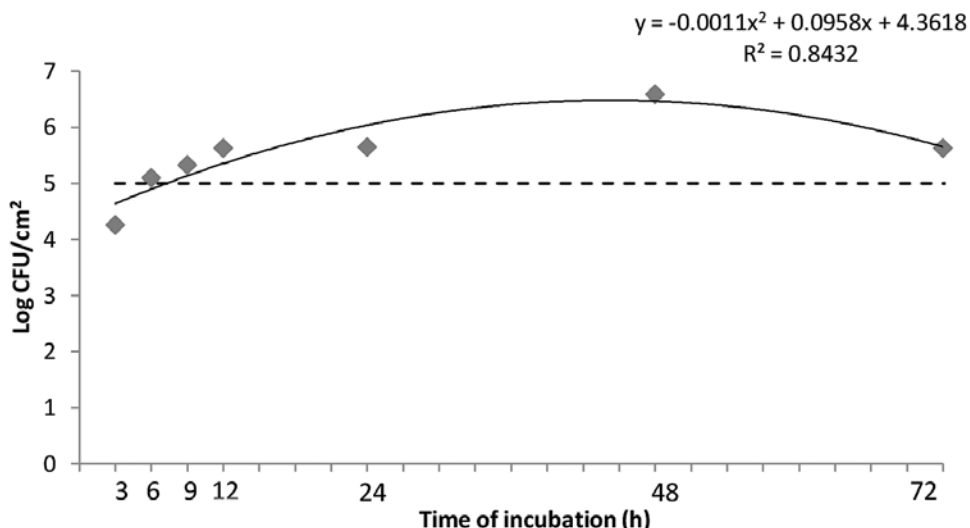

Fig. 1. Kinetics of adhesion of *S. aureus* to stainless steel surfaces after 3, 6, 9, 12, 24, 48 and 72 h. Dotted line means the lower bacterial count (log CFU/cm²) needed for biofilm formation according to Wirtanen et al. (1995).

CFU - colony forming units

Biofilm-associated bacteria show an innate resistance to antibiotics (Ceri et al. 1999), disinfectants (Oie et al. 1996), and clearance by host defenses (Shiau and Wu 1998; Donlan and Costerton 2002). Tested disinfectants with their active ingredients were not efficient in completely removing the cells of *S. aureus* adhered onto stainless steel surfaces. From these results, the assayed strain revealed high capacities to adhere and form biofilm on stainless steel surfaces and the cells in biofilm matrixes were resistant to total removal when exposed to the evaluated disinfectants. When studying biofilm formation on stainless steel surfaces, Meira et al. (2012) also found results similar to those of our study, determining that peracetic acid and sodium hypochlorite were not efficient in completely removing the cells of the *S. aureus* biofilm. Residual cells after the application of these sanitizers reinforce the biofilm as a likely source of cross-contamination.

In conclusion, our results demonstrate that the assayed reference strain *S. aureus* presented heightened capacity to adhere and form biofilm on stainless steel surfaces. These data regarding the detachment of *S. aureus* suggest a high-risk source for cross contamination and point to the ability of *S. aureus* to form biofilm which helps the bacteria to survive in hostile environments, which may be considered to be the reason for chronic or persistent infections. Moreover, the evaluated disinfectants during the exposure times of one and five minutes were not efficient in totally removing the adhered cells of *S. aureus* forming a mature biofilm. Our results encourage further research focusing on the capability of *S. aureus* to adhere and form biofilm on surfaces, and on the efficacy of disinfectants to reduce the cell count in the biofilm.

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