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Biofilm-forming ability and methicillin resistance among Staphylococci isolated from various surfaces in community settings

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Abstract

Biofilm-forming ability and sensitivity to antibiotic methicillin of staphylococci outside the healthcare facilities is not sufficiently explored. The aim of this study was to examine the biofilm-forming capacity of staphylococci on various metal, plastic and leather surfaces in our living and working environment and the association between their biofilm production and methicillin resistance. Detection of methicillin resistance in staphylococci isolates were examined by cefoxitin disc diffusion test. Production of bacterial biofilms was investigated by two phenotypic methods: crystal violet (CV) tube-adherence method and Congo red agar (CRA) plate method. A total of 88 staphylococci isolates, 47 (53.41%) of *S. aureus* and 41 (46.59 %) of *S. epidermidis* strains were recovered from 100 collected samples. Among them, 35 methicillin-resistant strains (20 MRSA and 15 MRSE) were detected. Applying the CV tube adherence method, 21 (39.62%) MRSA and MRSE isolates were positive for biofilm production, while using the CRA method 15 (44.12%) MRSA and MRSE strains were detected as biofilm producers. Statistical analysis did not find association between biofilm production ability and methicillin resistance in both applied methods for a significance level of 0.05. The study shows that different objects and surfaces contaminated with biofilm-forming staphylococci, including MRSA and MRSE, may serve as environmental source of infection for long periods of time.

Keywords: Methicillin-resistant, *Staphylococcus aureus*, *Staphyloccus epidermidis*, CV tube method, CRA method

1. Introduction

In natural environment bacteria usually live in biofilms and not as planctonic cells such as those commonly studied in the laboratory. Biofilm is an aggregate of microorganisms in which cells are stuck to each other and/or to a surface and produce matrix of extracellular polymeric substance (Verderosa et al., 2019). Bacteriaca form biofilms on living or non-living surfaces everywhere: in natural, industrial, community and healthcare setting. Biofilms may be composed of a single or mixed bacterial species. Within biofim, bacteria show remarkable organization and they communicate, coordinate and cooperate with each other. The matrix in which microbes in a biofilm are embedded protects them from UV exposure, metal toxicity, acid exposure, dehydration, salinity, phagocytosis and antimicrobial agents (Lebeaux et al., 2014). Biofilms have great significance for public health, because bacteria in biofilms are 10 to 1000 times more resistant to antibiotics compared to free living bacteria (Mah, 2012). Increasing evidence suggests that bacterial biofilms are major cause of persistent chronic infections like middle ear otitis (Akyıldız et al., 2013), periodontitis (Lasserre et al., 2018), rhinosinusitis, tonsillitis (Post et al., 2007), endocarditis (Lerche et al., 2021), urinary tract infections (Delcaru et al., 2016), colitis (von Rosenvinge et al., 2013), chronic wounds (Wu et al., 2019) and lung infections of patients with cystic fibrosis (Moreau-Marquis et al., 2008). In addition, many infections in patients with prosthetic and implantable medical devices are associated with biofilms (Khatoon et al., 2018). *Staphylococcus aureus* and *S. epidermidis* are recognized as the most frequent causes of biofilm-associated infections in healthcare settings (Otto, 2018). In clinical environment staphylococci biofilms were detected on various surfaces made of glass, stainless steel, polypropylene and polystyrene material (Lee et al., 2015).To our knowledge, the occurrence of staphyloccoci biofilms on various surfaces outside the health care facilities is not sufficiently explored. Therefore, the aim of this study was to examine the biofilm-forming capacity of staphylococci on different surfaces in our living and working environment and the association between their biofilm production and methicillin resistance.

2. Material and Methods

2.1. Bacterial Isolates

Environmental samples were taken from different places (offices, cafes, homes, gyms, pharmacyes, etc.) by swabbing each individual surface with a sterile polyester fiber-tipped applicator swab (Becton and Dickinson Company) moistened in 3 ml of 0.85% sterile saline solution. The area sampled varied

approximately 4 - 20 cm^2, depending on the object tested. A total of 100 samples were collected from various metal, plastic and leather surfaces (Table 1). After sampling, the swabs were immediately transported to the laboratory. All samples were plated within 3 hours after collection. The isolates were identified by standard microbiological techniques including Gram stain, cultural characteristics on blood agar, salted mannitol agar, catalase reaction, hemolysin and coagulase test, oxidase, nitrate reductase and urease test (Moraes et al. 2021). All cultures were maintained on TSA plates (Trypticase Soy Agar, Difco).

2.2. Methicillin susceptibility testing

For detection of methicillin resistance, all staphylococci isolates were tested by cefoxitin disc diffusion test recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2022a) using a 30 µg cefoxitin discs. Suspension of the isolate was adjusted to a 0.5 Mc Farland turbidity standard and inoculated on MHA (Mueller Hinton Agar plate, Difco). After incubation at 35 ± 1°C for 18 h, inhibition zone diameters were measured. Experiment was done in triplicate. According to EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2022b) recommendations, *S. aureus* and *S. epidermidis* isolates were considered resistant if inhibition zone diameter were < 22 mm and < 27 mm, respectively.

2.3. Biofilm detection

Production of bacterial biofilms was investigated by two phenotypic methods: crystal violet (CV) tube-adherence method (Dumaru et al., 2019) and Congo red agar (CRA) plate method (Melo et al., 2013).

2.3.1. CV tube adherence method

Polystyrene test tube containing 10 ml TSB (Tryptic Soy Broth, Sigma-Aldrich) with 1% glucose was inoculated with loopful of staphylococci from overnight culture plate and incubated for 24 hours at 37°C. After incubation period, the tube was decanted, washed with PBS buffer (pH 7.3) and dried. Interior of the tube was stained with 0.1% Crystal violet (Sigma-Aldrich). Excess stain was removed and tube was washed with distilled water, dried in inverted position and observed for biofilm formation. Adherent film visible on the inner surface of the test tube was taken as evidence of biofilm formation. Experiments were performed in triplicate.

2.3.2. CRA plate method

CRA medium was prepared with 37 g/l brain heart infusion broth, 50 g/l sucrose, 10 g/l agar and 0.8 g/l congo red. Congo red stain was prepared separately from the other medium constituents as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes and then added in previously sterilized agar cooled to 55°C. Inoculated CRA plates were incubated aerobically for 24 to 48 hours at 37°C. Results were interpreted according to colony phenotypes. Positive result for biofilm production was indicated by black colonies with dry crystalline consistency, while non-slime and weak producing strains developed red and pink colonies. Experiments were repeated three times. All plates were examined independently by two different observers.

2.4. Statistical analysis

Descriptive statistical analysis and Student t-test (p value<0.05) were conducted using Microsoft Excel 2013 (Microsoft Corp., Redmond, WA, USA).

3. Results

In this study, a total of 100 swab specimens were taken from various environmental surfaces: 30 from metal, 46 from plastic and 24 from leather surfaces. A summary of the objects tested and the number of samples with detectable staphylococci are presented in Table 1.

Table 1. Tested surfaces and isolated staphylococci

Surface	Tested objects	Swabs	*S. epidermidis*		*S. aureus*	
		N	N	%	N	%
Metal	ATMS, buttons in the elevator, bench in the park, doors in the car, wedding rings, entrance doors, door handles	30	9	30.00	11	36.37
Plastic	laptops, keyboards, desks, office tables intercoms, cellular phones, credit cards, benchboards, cofee makers, combs, dor knobs, entrance doors, servery counters, toilet seats, remote controls, fitness devices, button lights, air conditioners	46	22	47.82	19	41.30
Leather/ skin	wallets, slippers, purses, furniture, handrails, fitness devices, mats and human palms	24	16	66.67	11	45.93
	Total	100	47		41	

Among 88 staphylococci isolates, 47 (53.41%) of *S. aureus* and 41 (46.59 %) of *S. epidermidis* strains were recovered from collected swabs. Considering the number of analyzed samples from different surfaces, the most percentage of staphylococci was isolated from leather/skin surfaces, then the plastic one, while the least staphylococci was isolated from metal surfaces (Table 1.).

The cefoxitin disc diffusion test detected 35 methicillin resistant strains from all tested staphylococcal isolates (Figure 1.).

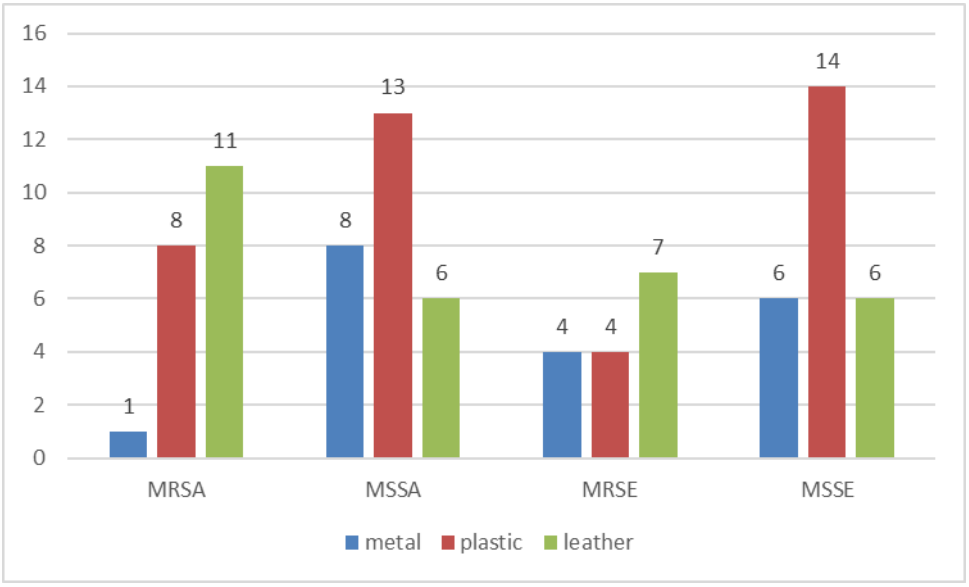

Figure 1. Numbers of methicillin-resistant and methicillin-sensitive isolates

Among 47 *S. aureus* isolates, 20 were methicillin-resistant *S. aureus* (MRSA) and 27 were methicillin-sensitive *S. aureus* (MRSA) strains. Fifteen out of 41 *S. epidermidis* isolates were methicillin-resistant *S. epidermidis* (MRSE) and 26 were methicillin- sensitive *S. epidermidis* (MSSE) strains. The most methicillin-resistant staphylococci were found on leather/skin surfaces, and the least MRSA and MRSE were isolated from metal surfaces (Figure 1.).

Biofilm production was evaluated for all 88 staphylococci isolates by the CV tube adherence and CRA plate methods (Figure 2.).

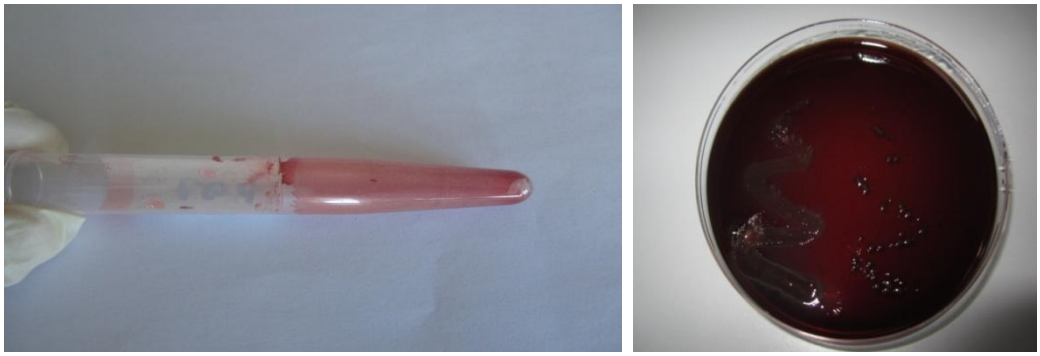

Figure 2. Positive results of CV tube adherence method (left) and CRA method (right)

Applying the CV tube adherence method was found that 53 (60.23%) of investigated staphylococcal strains were positive for biofilm production. Among them, 33 were *S. aureus* and 20 were *S. epidermidis* isolates. The CRA plate method detected a biofilm-forming ability in 34 (38.64%) bacterial isolates, of which 21 were *S. aureus* and 13 were *S. epidermidis* strains (Figure 3.).

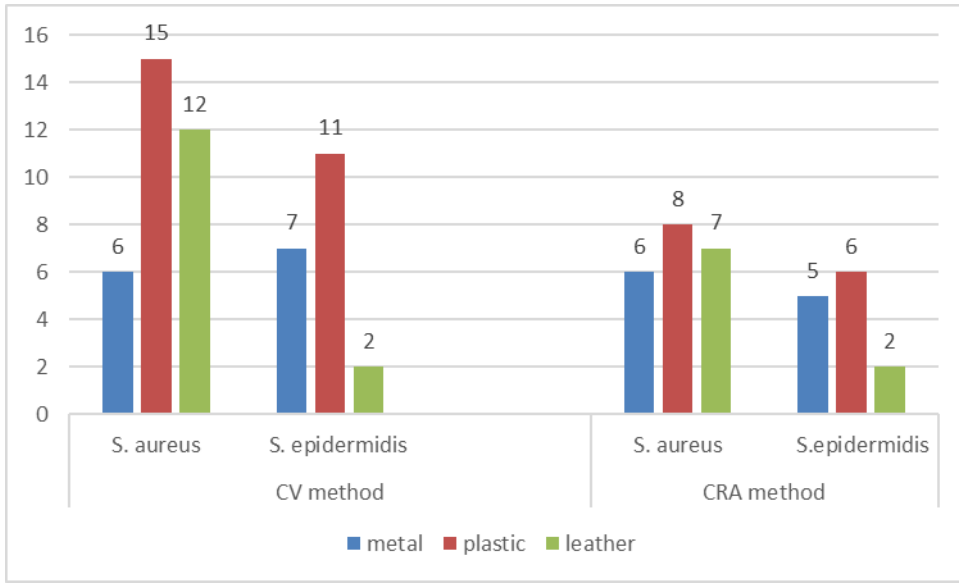

Figure 3. Number of biofilm-producing staphylococcal isolates from different surfaces

In the CV tube adherence method, 21 (39.62%) MRSA and MRSE isolates were positive for biofilm production. During the use of CRA plate method, 15 (44.12%) methicillin resistant strains of *S. aureus* and *S. epidermidis* were detected as biofilm producers (Figure 4.).

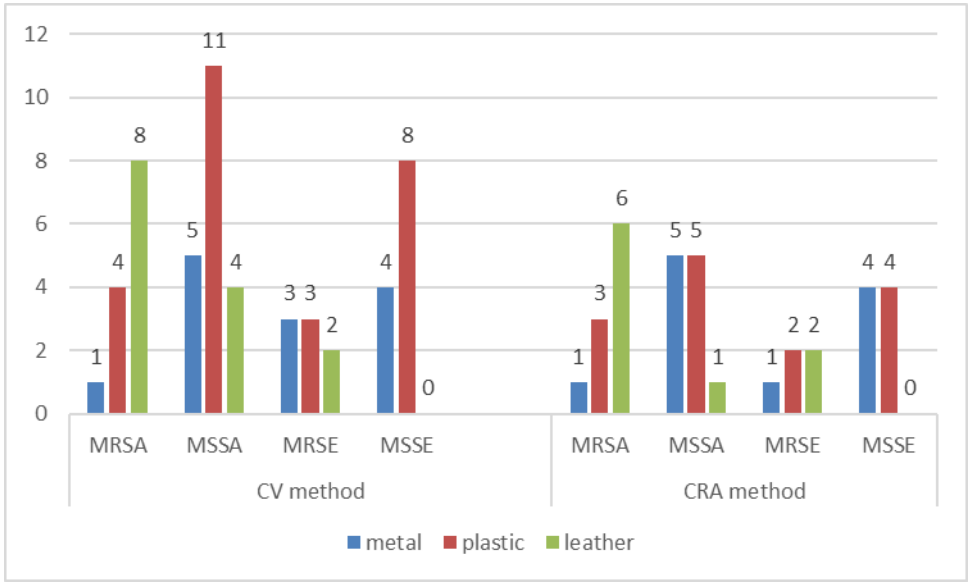

Figure 4. Methicillin resistant and methicillin sensitive biofilm-forming staphylococcal isolates

Based on statistical analysis, no association was found between biofilm production and methicillin resistance in both applied methods. For CV tube adherence method t values for *S. aureus* and *S. epidermidis* isolates were -0.78262 and -0.57143, respectively and for CRA method t values for *S. aureus* and *S. epidermidis* isolates were -0.16903 and -0.72761, respectively. In all cases results were not statistically significant at $p < 0.05$.

4. Discussion

Microorganisms constantly find ways to protect themselves from antibiotics, disinfectants and the host immune system. Certainly, the most important forms of bacterial protection are the ability to form biofilm and multiple resistance to antibiotics. Bacterial biofilms pose a great threat to the public health and food industry sector (Rather at al., 2021). The biofilm lifestyle confers many advantages on microbial populations, and once formed, biofilms are very difficult to control. Staphylococci are common colonizer of human skin and mucous surfaces. The ability of biofilm formation seems to play an important role in the virulence of staphylococci (Silva et al., 2022). *Staphylococcus aureus* and *S. epidermidis* are opportunistic pathogens and one of the major causes of hospital-acquired infections (Kranjec at al., 2021) and infections on indwelling medical devices, which characteristically involve biofilms (Otto, 2008). The

resistance of *Staphylococcus* bacteria to all beta-lactam antibiotics is designated as methicillin resistance. The acronym MRSA stands for methicillin-resistant *Staphylococcus aureus*, although methicillin is no longer used to identify this type of resistance. In this study, we investigated the biofilm-forming ability and its association with methicillin resistance in *S. aureus* and *S. epidermidis* isolates from various commonly used metal, plastic and leather objects in community settings. In relation to the number of swabs collected from different surfaces, the most bacteria were found on leather/skin surfaces and the least on metal surfaces. The results obtained are expected, because the skin is the natural habitat of staphylococci. For the detection of biofilm-forming staphylococci two phenotypic methods were used: CV tube adherence method and CRA method. Comparing the results of these two methods, it was found that a greater number of staphylococcal biofilms were observed by the CV tube adherence method than by the CRA method. The agreement between the results of the testing methods was 64.15%. We believe that unlike the CV tube adherence test, the CRA test is a much more subjective method. Among the most significant factors that influence the staphylococci ability to form biofilm are the type of surface and its characteristics, the site of development, microenvironmental conditions (humidity, temperature, ionic strength and pH), nutrient type and concentration, network design and composition, strain type and heterogeneity (Kramer et al., 2014; Olar et al. 2022).This research has shown that the biofilm-forming ability was not connected with methicillin resistance in staphylococci and revealed a low percentage of staphylococcal strains (39.62% in CV tube adherence method and 44.12% in CRA method) that use both ways of protection against unfavorable external factors. These results are consistent with other studies that did not show association between antimicrobial resistance and the biofilm-forming capacity of staphylococci (Singhai et al., 2012, Cabrera-Contreras et al., 2013; Chon et al., 2020; Tahaei et al., 2021). Contrary to these researches, there are other studies that find the association between antibiotic resistance and the biofilm-forming ability of staphylococci (Jimi et al., 2017; Sun et al., 2018; Shah et al., 2019; Silva et al., 2022). It is possible that the species of staphylococci and antibiotics included in the tests may affect the results of the research. So, further experimental research is needed in order to clarify the connection between antibiotic resistance and biofilm production in staphylococci. According to literature data (Lei et al. 2017; O'Hara et al., 2019; Jabłońska-Trypuć et al., 2022), MRSA can survive on dry surfaces for up to several months. Therefore, commonly handled objects and environmental surfaces that are contaminated with biofilm-forming

staphylococci, including MRSA and MRSE, could act as reservoirs of bacteria for long periods of time and transfer potential infections to persons who come in contact with them. According to the estimates of Reynolds et al. (2005), about 80% of human infections are transmitted by hands, either through contact with contaminated hands or through contaminated objects. Dirty hands contaminated with bacteria play a key role in the transmission of infectious disease agents. For that reason, regular hand hygiene and appropriate treatment of all cuts and wounds on the skin are the best ways to prevent the spread of MRSA and MRSE infection, both in the hospital and in the community settings.

5. Conclusions

Out of a total of 100 swabs collected from different objects and surfaces in commmunity settings, 88 staphylococci isolates, 47 (53.41%) of *S. aureus* and 41 (46.59 %) of *S. epidermidis* strains were recovered. Of all tested isolates, 35 methicillin resistant strains (20 MRSA and 15 MRSE) were detected. Out of 88 staphylococci isolates, 60.23% and 38.64% of isolates were biofilm-producers based on the CV tube adherence and the CRA plate methods, respectively. Since only 39.62% in CV tube adherence method and 44.12% in CRA plate method methicillin- resistant strains were shown biofilm-forming ability, no association was found between methicillin resistance and biofilm production in our experiment. Staphyloccocal biofilms on commonly handled objects and surfaces in our environment certainly pose a risk for acquisition of infection.

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