

# Correlation Between Biofilm-Formation and the Antibiotic Resistant Phenotype in *Staphylococcus aureus* Isolates: A Laboratory-Based Study in Hungary and a Review of the Literature

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**Introduction:** *Staphylococcus aureus* (*S. aureus*) is an important causative pathogen in human infections. The production of biofilms by bacteria is an important factor, leading to treatment failures. There has been significant interest in assessing the possible relationship between the multidrug-resistant (MDR) status and the biofilm-producer phenotype in bacteria. The aim of our present study was to assess the biofilm-production rates in clinical methicillin-susceptible *S. aureus* [MSSA] and methicillin-resistant *S. aureus* [MRSA] isolates from Hungarian hospitals and the correlation between resistance characteristics and their biofilm-forming capacity.

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**Methods:** A total of three hundred (n=300) *S. aureus* isolates (corresponding to MSSA and MRSA isolates in equal measure) were included in this study. Identification of the isolates was carried out using the VITEK 2 ID/AST system and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method and E-tests, confirmation of MRSA status was carried out using PBP2a agglutination assay. Biofilm-production was assessed using the crystal violet (CV) tube-adherence method and the Congo red agar (CRA) plate method.

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**Results:** There were significant differences among MSSA and MRSA isolates regarding susceptibility-levels to commonly used antibiotics (in case of erythromycin, clindamycin and ciprofloxacin: p<0.001, gentamicin: p=0.023, sulfamethoxazole/trimethoprim: p=0.027, rifampin: p=0.037). In the CV tube adherence-assay, 37% (n=56) of MSSA and 39% (n=58) of MRSA isolates were positive for biofilm-production, while during the use of CRA plates, 41% (n=61) of MSSA and 44% (n=66) of MRSA were positive; no associations were found between methicillin-resistance and biofilm-production. On the other hand, erythromycin, clindamycin and rifampin resistance was associated with biofilm-positivity (p=0.004, p<0.001 and p<0.001, respectively). Biofilm-positive isolates were most common from catheter-associated infections.

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**Discussion:** Our study emphasizes the need for additional experiments to assess the role biofilms have in the pathogenesis of implant-associated and chronic *S. aureus* infections.

**Keywords:** *Staphylococcus aureus*, MSSA, MRSA, biofilm, antibiotic resistance, crystal violet, Congo red agar, phenotypic assay

## Introduction

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive, catalase-positive and bacitracin-resistant coccus, which is a common colonizer of the human body. These bacteria are frequently found on mucosal surfaces (eg, the nares, the throat and the rectum)

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and moist regions of the skin (eg, axilla, groin and perineum).<sup>1,2</sup> According to recent data, 60% of the population is transiently colonized, while in 30%, this colonization is persistent.<sup>3</sup> *S. aureus* (both MSSA [methicillin-sensitive *S. aureus*] and MRSA [methicillin-resistant *S. aureus*]) is an exceptionally successful and adaptable pathogen, relevant in both community-associated and nosocomial infections.<sup>4</sup> They are an important cause of skin and soft tissue infections (SSTIs), osteoarticular infections, medical device-related infections, pneumonia, infective endocarditis and bacteremia (in addition, through hematogenous spread, this microorganism may cause a wide range of secondary pathologies).<sup>5,6</sup> MRSA was first identified in 1961 and has emerged as the first multidrug-resistant (MDR) bacterium in human medicine.<sup>7</sup> MRSA is resistant to all  $\beta$ -lactam antibiotics (with the exception of **fifth-generation** cephalosporins), severely narrowing safe and effective treatment options; additionally, these strains often possess a battery of resistance-determinants against other antibiotic groups (eg, fluoroquinolones, macrolides, tetracyclines, aminoglycosides), thus earning the name “superbug” for the first time.<sup>8</sup> Methicillin-resistance is mediated by modifications in penicillin-binding proteins (namely PBP2a/2c/2'), owing to the presence of *mecA* or *mecC* genes. Initially, MRSA was mainly associated with nosocomial infections (hospital-associated MRSA; HA-MRSA), however, some 20–30 years after its initial description, community-associated MRSA (CA-MRSA) infections have also emerged.<sup>4,9</sup> Since the 2000s, extensive research regarding livestock-associated MRSA (LA-MRSA) has been published, both due to its impact in veterinary medicine (for animal husbandry and for companion animals) and due to the possible relevance of animals as vectors for MRSA transmission.<sup>10</sup> The prevalence of MRSA infections shows large geographical differences: it is around 1–10% in Northern Europe, 15–30% in the United States, 40–50% in Southern and Eastern Europe, while it may exceed 80% in some parts of Asia.<sup>11</sup> Risk factors associated with acquiring an MRSA infection **include** advanced age ( $\geq 60$  years), prolonged hospital stay, prior antimicrobial treatment and the use of nasogastric tubes or endovascular catheters.<sup>12</sup> MRSA infections are associated with decreased quality of life (QoL), excess mortality and substantial economic costs, compared to MSSA infections.<sup>4,12</sup>

The emergence of MDR isolates in human infections considerably limits clinicians in administering adequate antimicrobial therapy.<sup>13,14</sup> A variety of resistance-determinants have been described in the literature (both

intrinsic resistance and genes acquired on mobile genetic elements), allowing bacteria to withstand otherwise lethal doses of antibiotics.<sup>15</sup> In addition to these resistance-determinants, the production of biofilms by bacteria is another important factor leading to treatment failures.<sup>16</sup> The first record on the existence of bacterial biofilms was published by Henrici (1933), while a recent publication by the National Institute of Health (NIH) suggested that in *in vivo* conditions, 60% of all infections are caused by bacteria embedded in biofilms.<sup>17,18</sup> Biofilms are aggregates of mono-species or multispecies bacterial communities, enveloped in a protective extracellular matrix.<sup>19,20</sup> This matrix is typically made up of secreted exopolysaccharides (EPS), environmental DNA (eDNA), proteins, surfactants, lipids and water.<sup>21</sup> Biofilms allow bacterial communities to attach to and persist on inanimate surfaces and inside the body. The initial step of biofilm-production is the attachment of bacteria to relevant surfaces (most commonly coarse or hydrophobic surfaces, such as catheters, implanted medical devices and other biomaterials), with the aid of EPS, surface proteins, fimbriae and pili.<sup>22</sup> After the development of the mature biofilm, bacteria residing inside this protective structure will be in different metabolic states: bacteria in the surface layer of the biofilm will be aerobic and **metabolically active**; while in the deeper layers, due to nutrient deficiency and lower oxygen concentrations, bacteria are fermentative and dormant.<sup>23</sup> In essence, biofilms provide double protection against antibiotics: as most antibiotics are only effective against actively-replicating (ie, planktonic) cells, the eradication of these persisters is an important challenge; additionally, the thick biofilm also **acts** as a pharmacokinetic barrier, limiting the diffusion of antimicrobials and other noxious agents in the vicinity of the pathogens.<sup>24,25</sup> This may result in minimal inhibitory concentrations (MICs) 10–10,000-times higher against bacteria embedded in biofilms.<sup>26</sup> Owing to this resistance against antibiotics and the protective effects of biofilms against harsh environmental stressors (eg, sheer forces, drying) and the immune system (eg, phagocytosis), it is unsurprising that biofilms are an important virulence factor in the development of skin and soft tissue infections, catheter- (intravascular or urinary) and medical device-associated infections, oral infections, dental caries and chronic infections.<sup>27–30</sup>

At present, the group of “ESKAPE” bacteria – which is a list **consisting of** MDR pathogens, including MRSA – is considered as the most concerning in respect to their resistance rates, clinical impact and mortality.<sup>31</sup> As most

of the ESKAPE-members are biofilm-producers, there has been significant interest in assessing the possible relationship between their MDR status and the biofilm-producer phenotype.<sup>32</sup> Although several studies have provided experimental data on the subject, corresponding to both MDR and wild-type Gram-negative (eg, *Escherichia coli*,<sup>33</sup> *Klebsiella* spp.,<sup>34</sup> *Pseudomonas aeruginosa*,<sup>35</sup> *Acinetobacter* spp.<sup>36</sup>) and Gram-positive bacteria (eg, MSSA/MRSA,<sup>37</sup> *Enterococcus* spp.<sup>38</sup>), the findings of these studies are often controversial. With this in mind, the aim of our present study was to assess the rates of biofilm-production in various clinical MSSA and MRSA isolates from Hungarian hospitals with phenotypic methods, and the potential correlation between the resistance characteristics and their biofilm-forming capacity.

## Materials and Methods

### Collection of Isolates

A total of three hundred (n=300) *S. aureus* isolates (corresponding to MSSA and MRSA isolates in equal measure; n=150 isolates, respectively) were included in this study, which were kindly provided by a tertiary-care teaching hospital and two smaller regional hospitals in Hungary. The study uses a cross-sectional study design; the microorganisms were isolated between 2019.01.01 and 2020.01.01., including n=100 isolates from catheter-associated infections (CAI-SA), skin and soft tissue infections (SSTI-SA) and urinary tract infections (UTI-SA). During our experiments, *S. aureus* ATCC 29213 (MSSA; positive for biofilm-production, *icaAB* gene negative), *S. aureus* ATCC 43300 (MRSA; positive for biofilm-production, *icaAB* gene positive), *S. aureus* ATCC 12600 (MSSA; non-biofilm producing, *icaAB* gene negative), *S. epidermidis* ATCC 35984 (positive for biofilm-production, *icaAB* gene positive) and *S. epidermidis* ATCC 12224 (non-biofilm producing, *icaAB* gene negative) were used as control strains, obtained from the American Type Culture Collection (ATCC; Manassas, VI, USA).<sup>39</sup> Stock cultures were stored at -80 °C in a cryopreservation medium (700 µL trypticase soy broth + 300 µL 50% glycerol).

### Bacterial Identification

Identification of *S. aureus* isolates was carried out using the VITEK 2 ID/AST automated system (bioMérieux, Marcy-l'Étoile, France) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics, Bremen,

Germany). During the MALDI-TOF assay, bacterial cells from fresh overnight cultures were transferred to a stainless-steel target. An on-target extraction was performed by adding 1 µL of 70% formic acid prior to the matrix. After drying at room temperature, the cells were covered with 1 µL matrix ( $\alpha$ -cyano-4-hydroxy cinnamic acid in 50% acetonitrile/2.5% trifluoro-acetic acid; Bruker Daltonics, Bremen, Germany). Mass spectrometry analyses were performed by the Microflex MALDI Biotyper (Bruker Daltonics, Bremen, Germany) in positive linear mode across the m/z range of 2 to 20 kDa; for each spectrum, 240 laser shots at 60 Hz in groups of 40 shots per sampling area were collected. The MALDI Biotyper RTC 3.1 software (Bruker Daltonics, Bremen, Germany) and the MALDI Biotyper Library 3.1 were used for spectrum analysis. As a result of the MALDI-TOF spectrum analysis, a log(score) value was provided, indicating the reliability of MALDI-TOF MS identification. The log(score) values were evaluated as follows: a log(score) <1.69 showed unreliable identification, 1.70–1.99 corresponded to probable genus-level identification, 2.00–2.29 corresponded to reliable genus-level identification, while a score  $\geq$ 2.30 corresponded to reliable species-level identification.<sup>40</sup> All isolates included in the study were re-identified as *S. aureus* before further experiments.

### Antimicrobial Susceptibility Testing, Resistance Detection

Antimicrobial susceptibility testing (AST) was performed either using the Kirby–Bauer disk diffusion method or E-tests (Liofilchem, Teramo, Italy) on Mueller–Hinton agar (MHA) plates. During testing, the susceptibilities to erythromycin (ERY; 15 µg), clindamycin (CLI; 2 µg), ciprofloxacin (CIP; 5 µg), gentamicin (GEN; 10 µg), sulfamethoxazole/trimethoprim (SXT; 23.75/1.25 µg), vancomycin (VAN; E-test), tigecycline (TIG; 15 µg), linezolid (LZD; 10 µg), fusidic acid (FUS; 10 µg), quinupristin/dalfopristin (QDP; 15 µg), rifampicin (RIF; 5 µg) and ceftaroline (CFT; 5 µg) were determined. Interpretation of testing results and classification of isolates as MDR (being non-susceptible to at least one antimicrobial agent in three or more antimicrobial classes) was based on EUCAST standards and breakpoints v. 9.0. (<http://www.eucast.org>). VITEK 2 ID/AST (bioMérieux, Marcy-l'Étoile, France) was used for the verification of discrepant results. During data analysis, intermediate results were grouped with and reported as resistant. Inducible CLI

225 resistance was detected using the ERY-CLI D test; these strains were also reported as resistant.<sup>41</sup>

230 Methicillin-resistance was verified using mannitol salt agar (MSA) plates using cefoxitin (FOX) disks (zone diameters under 22 mm were considered positive for methicillin-resistance) and PBP2' Latex Agglutination Test (Thermo Fisher Scientific Hungary GmbH, Budapest, Hungary). A MRSA strain was automatically considered to be resistant to all  $\beta$ -lactam antibiotics other than CFT.<sup>41</sup> MSSA *S. aureus* ATCC 29213 and MRSA *S. aureus* ATCC 43300 were used as quality control strains.

### Detection of Biofilm-Production by the Tube-Adherence Method

240 Assessment of biofilm-formation was carried out in the tube-adherence method described previously.<sup>42</sup> In short, glass tubes containing 1 mL of sterile trypticase soy broth (bioMérieux, Marcy-l'Étoile, France) were inoculated with 1  $\mu$ L of the overnight culture of a respective bacterial strains. Respective tubes were then incubated statically for 24 h at 37 °C. Verification of planktonic growth was observed visually. After the incubation period, the supernatant was then discarded, the adhered cells were rinsed three times with phosphate buffer saline (PBS; Sigma-Aldrich; Budapest, Hungary) and the tubes were patted dry on a paper towel. The contents of the tubes were treated with a 1 mL solution of 0.1% crystal violet (CV; Sigma-Aldrich; Budapest, Hungary) to stain the adhered biomass; the tubes were incubated for 3 h at room temperature with the staining solution. The CV solution was then discarded and the tubes were again rinsed three times with PBS and the tubes were patted dry on a paper towel. Biofilm-formation was observed visually; in case of the appearance of visible biofilm lining at the bottom and on wall of the glass tubes, the strain was considered a biofilm-producer in this assay.<sup>33,42</sup> All experiments were performed in triplicate and were evaluated by two independent researchers.

### Detection of Biofilm-Production by the Congo Red Agar Method

265 Biofilm-formation of the isolates was also evaluated on Congo Red Agar (CRA) plates, based on the previously described protocol.<sup>43</sup> Briefly, CRA plates were prepared using trypticase soy agar supplemented with 5% sucrose and 40  $\mu$ g/mL Congo red dye (Sigma-Aldrich; Budapest,

270 Hungary). Congo red is a secondary diazo dye, which can be used as a pH indicator (with a detectable color change at pH 3.0–5.2). Strains were cultured on trypticase soy agar plates at 37 °C for 16–18 h; cells were resuspended in trypticase soy broth at a density of OD<sub>600</sub>=2; 10  $\mu$ l of the suspension was spotted on CRA plates. The inoculated CRA plates were incubated at 37 °C in aerobic conditions for 24 h, followed by incubation at room temperature before the reading of the plates for an additional 24 h. The isolates were assessed for their colony-morphologies: black colonies with a dry consistency and rough surface edges were considered as biofilm-producers in this assay, while red colonies with smooth, round and shiny surface were read as negative for biofilm-production.<sup>43</sup> All experiments were performed in triplicate and were evaluated by two independent researchers. 285

### Statistical Analysis

290 Descriptive statistical analysis (including means and percentages to characterize data) was performed using Microsoft Excel 2013 (Microsoft Corp.; Redmond, WA, USA). Additional statistical analyses were performed with IBM SPSS Statistics for Windows 24.0 (IBM Corp., Armonk, NY, USA), using the  $\chi^2$ -test.<sup>44</sup> p values  $\leq 0.05$  were considered statistically significant. Additionally, consistency-assessment was also performed between the results of the two different biofilm-production studies (CV assay vs CRA agar).<sup>30</sup> 295

### Ethical Considerations

300 Clinical, personal and epidemiological data pertaining to the affected patients were not collected or provided during the study, bacterial isolates were only identifiable based on their serial number; therefore, our present study was not subject to ethics review.

## Results

### Antibiotic Susceptibility of MSSA and MRSA Isolates Included in the Study

305 Out of the tested *S. aureus* isolates, the following susceptibilities were detected overall: complete susceptibility (100%; n=300) was seen for VAN, CFT, QDP, FUS, LZD and TIG 100%; on the other hand, varying levels of resistance were observed for other antibiotics, such as 310 GEN 90% (n=271), SXT 89% (n=268), RIF 86% (n=257), ERY 54% (n=162), CIP 51% (n=153) and CLI 48% (n=144). The detailed susceptibilities for every group

of isolates (namely CAI-SA, SSTI-SA and UTI-SA) are presented in Table 1. There were significant differences among MSSA and MRSA isolates regarding susceptibility-levels to commonly used antibiotics (ERY:  $p < 0.001$ ,  $\chi^2 = 156.52$ , degrees of freedom [DOF]: 1; CLI:  $p < 0.001$ ,  $\chi^2 = 155.63$ , DOF: 1; CIP:  $p < 0.001$ ,  $\chi^2 = 192.03$ , DOF: 1; GEN:  $p < 0.023$ ,  $\chi^2 = 12.03$ , DOF: 1; SXT:  $p = 0.0027$ ,  $\chi^2 = 8.95$ , DOF: 1; RIF:  $p = 0.0037$ ,  $\chi^2 = 8.95$ , DOF: 1). Similarly, the subset of MRSA isolates could be classified as MDR more commonly (85% [n=128] vs 8% [n=12]), compared to isolates from the MSSA group.

### Association of MSSA/MRSA Status and Resistance to Other Antibiotics with Biofilm-Production

In the CV tube adherence-assay, 37% (n=56) of MSSA and 39% (n=58) of MRSA isolates were positive for biofilm-production, while during the use of CRA plates, 41% (n=61) of MSSA and 44% (n=66) of MRSA were positive; no associations were found between methicillin-resistance and biofilm-production ( $p > 0.05$  in both cases). The agreement between the results of the two phenotypic testing methods was 0.897 (89.7%) overall (91.8% in case of MSSA and 87.8% in case of MRSA isolates). Interestingly, biofilm-production was more commonly detected from both MSSA and MRSA CAI-SA isolates ( $p < 0.0001$  and  $p = 0.0018$  for MSSA,  $p = 0.02$  and  $p = 0.015$  for MRSA, respectively), compared to the isolates from other origins. The detailed distribution of biofilm-positive *S. aureus* among the isolates of different origin, the results of the statistical analyses and the agreements among the CV adherence assay and CRA plates are presented in Figure 1. Among the control strains, *S. aureus* ATCC 29213, *S. aureus* ATCC 43300, *S. epidermidis* ATCC 35984 were positive for biofilm-production in both phenotypic assays, while *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 12224 were both negative.

The relationship between biofilm-production and resistance to other antibiotics was also assessed; during these analyses, only the results from the CRA plates were considered. It was found that resistance to ERY ( $p = 0.004$ ,  $\chi^2 = 8.12$ , DOF: 1), CLI ( $p < 0.001$ ,  $\chi^2 = 44.57$ , DOF: 1) and RIF ( $p < 0.001$ ,  $\chi^2 = 96.95$ , DOF: 1) was associated with biofilm-positivity; in fact, 37 out of 43 (86%) of RIF-resistant isolates were biofilm-producers. On the other hand, this association was not shown for other antibiotics (ie, CIP, GEN, SXT;  $p > 0.05$ ).

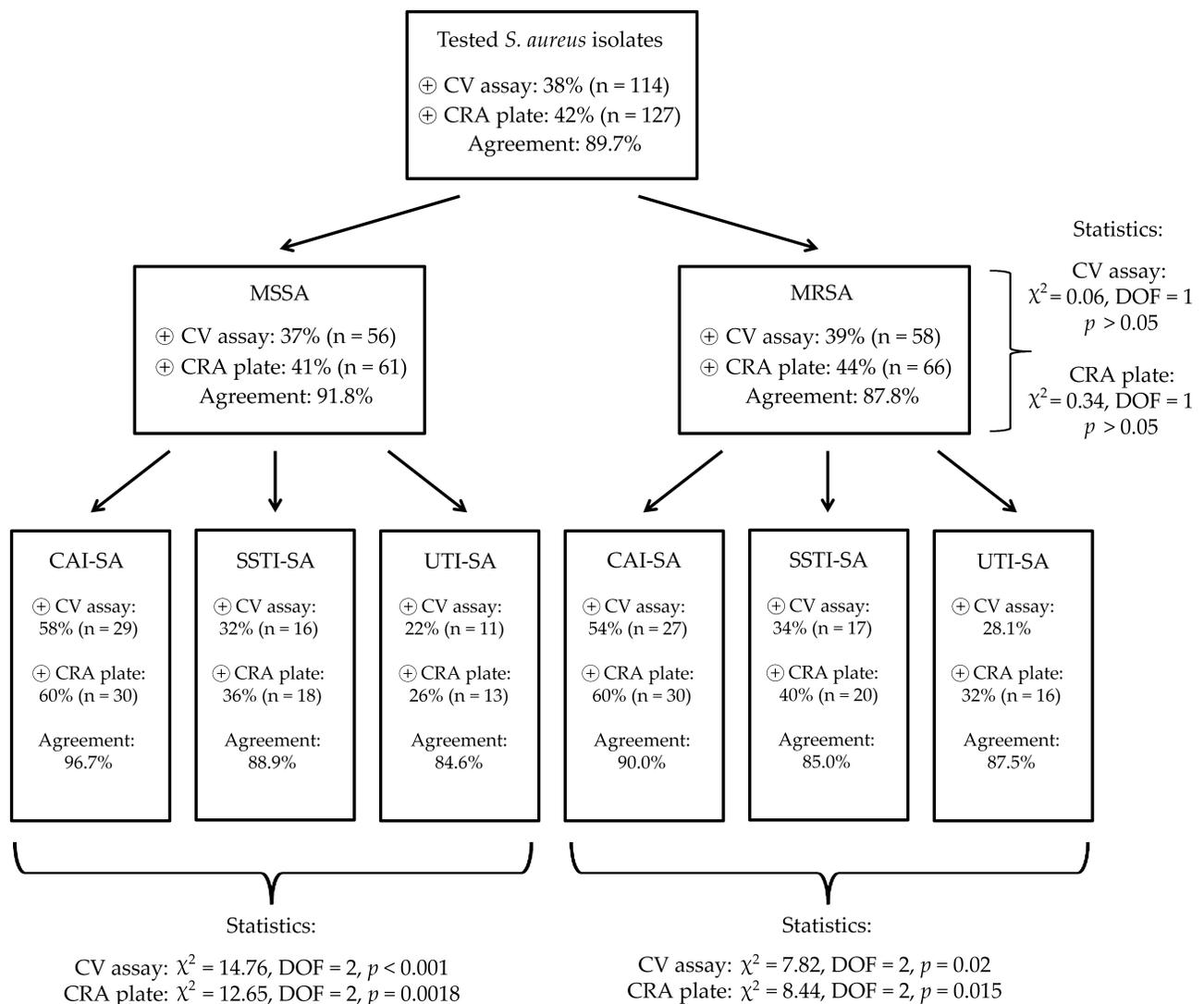
## Discussion and Review of the Literature

*S. aureus* infections are associated with considerable morbidity, mortality and economic costs for the healthcare institutions worldwide.<sup>45</sup> Owing to the adaptability, the plethora of virulence factors and the increasing levels of antimicrobial resistance in *S. aureus*, treatment of these infections is a considerable challenge for clinicians.<sup>46</sup> Biofilm-formation has been classified as an important defense mechanism and pathogenic hallmark for both MSSA and MRSA isolates, both as a means to persist in the environment (eg, on a hospital ward) and in the host during infections.<sup>47</sup> It has been described that the staphylococcal colonization of the skin is dependent of the biofilm-formation of these bacteria; in addition, *S. aureus* strains adhere to damaged skin and mucosal surfaces more easily, leading to the development of SSTIs.<sup>48</sup> At the same time, all inserted and implanted medical devices (contact lenses, cardiac pacemakers, prosthetic valves, cerebrospinal fluid shunts, implanted catheters and synthetic joints) are at risk to be associated with *S. aureus* infections (eg, endocarditis, osteomyelitis, bacteremia).<sup>49</sup> In the present study, we have investigated n=150 MSSA and n=150 MRSA isolates – originating from clinical materials – regarding their antibiotic susceptibilities and their biofilm-forming capacities using two phenotypic tests, namely the CV tube-adherence assay and the plate-based CRA medium. There are a plethora of methods available for the characterization of the biofilm-forming capacity of bacteria – self-developed and chromogenic media (both in liquid and in plate form), staining methods, assessment via measurements with a spectrophotometer or electron microscopy and most recently, flow chamber systems – however, these are pronounced differences among these methods in the price, reproducibility, high-throughput nature and the in vivo adaptability of the results.<sup>50–52</sup> Our two utilized methods have been described for a number of years (the CV tube-based assay was described by Christensen et al in 1982,<sup>53</sup> while the CRA method was developed by Freeman et al in 1989);<sup>54</sup> although subsequent studies have demonstrated that these methods needed to be modified to improve accuracy and sensitivity, these methods are cheap, easy to perform, the criteria for their evaluation are straightforward and their results are comparable to other, more expensive assays.<sup>39</sup> In our study, no significant association was noted between MSSA/MRSA-status and biofilm-production in either

**Table 1** Antibiotic Susceptibility Rates Among *S. aureus* Isolates Included in This Study

	CAI-SA			SSTI-SA			UTI-SA			Overall	
	MSSA (n=50)	MRSA (n=50)	Overall (n=100)	MSSA (n=50)	MRSA (n=50)	Overall (n=100)	MSSA (n=50)	MRSA (n=50)	Overall (n=100)	MSSA (n=150)	MRSA (n=150)
<b>Erythromycin</b>	90% (n=45)	20% (n=10)	55% (n=55)	86% (n=43)	16% (n=8)	51% (n=51)	94% (n=47)	18% (n=9)	56% (n=56)	90% (n=135)	18% (n=27)
<b>Clindamycin</b>	84% (n=42)	14% (n=7)	49% (n=49)	80% (n=40)	10% (n=5)	45% (n=45)	86% (n=43)	14% (n=7)	50% (n=50)	83% (n=125)	13% (n=19)
<b>Ciprofloxacin</b>	92% (n=46)	14% (n=7)	48% (n=48)	90% (n=45)	10% (n=5)	50% (n=50)	90% (n=45)	8% (n=4)	49% (n=49)	91% (n=136)	11% (n=16)
<b>Gentamicin</b>	96% (n=48)	88% (n=44)	92% (n=92)	96% (n=48)	84% (n=42)	90% (n=90)	96% (n=48)	82% (n=41)	89% (n=89)	96% (n=144)	85% (n=127)
<b>Sulfamethoxazole/trimethoprim</b>	96% (n=48)	80% (n=40)	88% (n=88)	92% (n=46)	84% (n=42)	88% (n=88)	96% (n=48)	88% (n=44)	92% (n=92)	95% (n=142)	84% (n=126)
<b>Vancomycin</b>	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=150)	100% (n=150)
<b>Tigecycline</b>	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=150)	100% (n=150)
<b>Linezolid</b>	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=150)	100% (n=150)
<b>Fusidic acid</b>	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=150)	100% (n=150)
<b>Quinpristin/dalfopristin</b>	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=150)	100% (n=150)
<b>Rifampin</b>	90% (n=45)	80% (n=40)	85% (n=85)	88% (n=44)	80% (n=40)	84% (n=84)	92% (n=46)	84% (n=42)	86% (n=86)	90% (n=135)	81% (n=122)
<b>Ceftaroline</b>	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=50)	100% (n=50)	100% (n=100)	100% (n=150)	100% (n=150)

Abbreviations: MSSA, methicillin-resistant *S. aureus*; MRSA, methicillin-resistant *S. aureus*; CAI-SA, catheter-associated infections; SSTI-SA, skin and soft tissue infections; UTI-SA, urinary tract infections.



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**Figure 1** Antibiotic susceptibility rates among *S. aureus* isolates included in this study.

**Abbreviations:** MSSA, methicillin-resistant *S. aureus*; MRSA, methicillin-resistant *S. aureus*; CAI-SA, catheter-associated infections; SSTI-SA, skin and soft tissue infections; UTI-SA, urinary tract infections.

phenotypic tests; however, ERY, CLI and RIF resistance was more common in biofilm-producing *S. aureus* isolates. It is also interesting to note that 86% of isolates resistant to RIF were biofilm-producers, especially as RIF is considered an effective antimicrobial agent with good penetration into bacterial biofilms in vivo.<sup>55</sup> Although the exact reason behind this phenomenon is unknown, it has been described that the sub-inhibitory concentrations of several antibiotics may induce biofilm-formation in *S. aureus* isolates.<sup>56</sup> Interestingly, the interplay between sub-inhibitory doses of antibiotics has been most frequently published in relation to the MLKS (macrolide-lincosamide-ketolide-streptogramin); which are all protein synthesis inhibitors affecting the 50S ribosome) group of drugs and rifampin.<sup>57</sup>

For example, Lima-e-Silva et al reported that in sub-lethal doses (MIC/2 and MIC/4), rifampin strongly stimulated biofilm-formation (when measured by the CRA plate and CV microtiter plate assay), in contrast to minocycline, which did not have such inducing effects.<sup>58</sup> The effects of low antibiotic doses on the biofilm-forming capacity are thought to occur by differential expression of genes of interest due to the noxious agents.<sup>59</sup> The literature has shown that sub-MIC concentrations of tetracycline, quinupristin and dalfopristin were presented as strong inducers, while erythromycin was noted as a weak inducer of *ica*-gene expression. In contrast, gentamicin, chloramphenicol (which are also protein synthesis inhibitors), penicillin, oxacillin, ofloxacin and vancomycin did not

435 present with similar effects.<sup>60</sup> The clinical origin of the isolates also had an effect on their biofilm-producing capacity: they were the most prevalent in isolates from catheter-associated infections and the least common in strains isolated from the urinary tract. Finally, the agreement among the results of the two in vitro methods was 440 89.7%, which is a very good result, based on other studies from the literature.<sup>39</sup>

Many studies have aimed to assess the correlation of biofilm-production with antibiotic-resistance in *S. aureus* strains; nevertheless, the literature **has shown** conflicting data on the topic, thus, at present, we are unable to draw far-reaching conclusions. To make the interpretation of the currently available results even more troublesome, many different methodologies have been used to assess biofilm-formation, with or without the molecular characterization of the isolates. Similarly to our results, Arslan et al found no association between slime production and methicillin-resistance or resistance to other antibiotics in a sample of n=187 *S. aureus* isolates; in their report, CRA plates and the CV tube adherence assay **were** utilized, while molecular testing (for clonality or the presence of *agr* genes) was not performed.<sup>61</sup> Ghasemian et al assessed biofilm-production in n=209 *S. aureus* isolates, among which no relevant differences were shown for biofilm-positive and negative isolates, based on methicillin-resistance (36.1% and 28.9% for MRSA and MSSA, respectively). The extent of biofilm-production was assessed by a microtiter plate biofilm assay, and the genetic testing of the isolates was also performed: 58.3% and 22.0% of isolates belonged to the *agr* groups I and II, respectively, while 465 84.0% of MRSA isolates possessed the SCCmec III cassette.<sup>62</sup> Rodríguez-Lopez et al came to similar conclusions, when studying *S. aureus* isolates originating from animal and environmental samples at heavy swine farms in Italy. In this report, the same methodology was utilized 470 (plate-based quantitative measurements), and spa-typing was also performed: overall, isolates belonging to the ST398/t899 and ST398/t011 were the most common among biofilm-producers.<sup>63</sup> El-Nagdy et al detected biofilm-forming *S. aureus* (using the CRA plate method, complemented with scanning and transmission electron microscopy) from febrile neutropenic patients in Egypt; interestingly, they have found that 72.7% of isolates were biofilm-positive. Among the tested strains, 37.5% were positive for *icaA*, and 22.9% were positive for *icaD*; however, only 50% of biofilm-producers carried either 480 the *icaA* or *icaD* genes.<sup>64</sup> Similarly, no correlation

among methicillin-resistance and biofilm-production was seen in the reports of Knobloch et al (including n=128 *ica*-positive isolates, with utilizing CRA plates and the microtiter plate method)<sup>65</sup> and Mathur et al (where n=152 *Staphylococcus* spp. were tested using the microtiter plate method).<sup>66</sup>

485 However, there have been studies that identified differences in the rates of biofilm-production based on the phenotypic resistance of *S. aureus* isolates. In the study 490 by Bose et al (in which the authors have utilized similar methodologies to our study, in addition to the tissue culture plate method to quantify their results) found higher levels of antibiotic resistance in biofilm-producing *S. aureus* and *S. epidermidis* isolates.<sup>67</sup> Piechota et al 495 compared the biofilm-forming capacity of MSSA and MRSA isolates from Poland: in this report, MRSA isolates were stronger biofilm-producers overall in the microtiter plate-based assay (39.7% vs 36.8%) and the occurrence of *icaABCD* genes (51.5% overall) was also more common in 500 methicillin-resistant strains.<sup>68</sup> Cha et al characterized n=126 MRSA isolates from a Korean teaching hospital, during which they have found higher levels of MDR isolates (defined as MRSA+resistance to at least three non-β-lactam agents) among the biofilm-forming isolates. The 505 study group has used the microtiter plate assay to quantify biofilm-formation and has also performed molecular testing: they have shown that the majority of the isolates were ST5 (69.8%) and 64.0% of isolates are from the *agr* group II.<sup>69</sup> Souli et al **have** clearly demonstrated that strong 510 biofilm-producers (tested with the CV tube-based assay) among *S. epidermidis* isolates had also possessed higher levels of resistance in vitro.<sup>70</sup> Agarwal and Jain tested commensal, colonizing and invasive *S. aureus* isolates for their biofilm-forming capacity using the microtiter 515 plate assay; in their study, biofilm-producers were more frequently MDR in all groups, and 94.0% of biofilm-producers carried *ica*-genes.<sup>71</sup> De **Araujo** et al reported that methicillin-resistant and MDR *S. epidermidis* isolates were more frequent among biofilm-producers (tested by 520 the microtiter plate assay). They have found that 96.0% of biofilm-positive isolates carried *icaA* or *icaD* genes, while 86.0% were positive for *atlE* and *aap* genes.<sup>72</sup> In contrast, in a report concerning *S. aureus* isolates originating from pork, Zhang et al found 83.8% of the bacteria to be *agr*- 525 positive (*agr* I: 39.2%, *agr* IV: 32.3%) and noted the high prevalence of MDR isolates in moderate and weak biofilm-producers, when tested with the microplate method.<sup>73</sup> Our study demonstrated an association of CLI-resistance

530 and biofilm-production corresponding to our isolates, which has been identified in other reports as well, although these studies usually also showed a positive correlation with methicillin-resistance. In the study by Belbase et al, *S. aureus* isolates from pus/wound swab samples were assessed for the susceptibility and microtiter-based biofilm-production in a tertiary-care hospital in Nepal. Overall, methicillin-resistance and inducible CLI-resistance were more common in biofilm-producers.<sup>38</sup> In another study from Nepal, Manandhar et al also showed that the in vitro biofilm-production (assessed by CRA plates) of *S. aureus* isolates was associated with methicillin-resistance and inducible CLI-resistance.<sup>74</sup> Bhattacharya et al tested n=100 *S. aureus* isolates, including 47% MRSA isolates: biofilm-positivity was shown in 55.0% of isolates, with MRSA isolates in higher numbers among biofilm-producers; in addition, resistance-levels against CIP, RIF, ERY, CLI and SXT were also significantly higher in biofilm-positive isolates.<sup>75</sup> Finally, Neopane et al tested n=150 *S. aureus* isolates, originating from pus samples: in their study – although no clear association was seen with methicillin-resistance and biofilm-production, strong biofilm-production and the MDR status has shown good agreement.<sup>76</sup> In the latter three studies, only phenotypic characterization of resistance and biofilm-production was utilized. Overall, the literature findings suggest a possible relationship between the expression of antibiotic-resistance-determinants and biofilm-production, however, the clarification of nature of this association will require further studies.

560 Pulse-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) are not routinely performed in Hungary by diagnostic laboratories; thus, there is a scarcity of local data regarding the molecular epidemiology of *S. aureus* isolates, such data may be sourced from major national public health surveillance studies: the Hungarian clone (ST239-III, PFGE type E) – which was predominant before 2000 – was almost completely replaced by the Southern German clone (ST228-I, PFGE type B) and the New York/Japan epidemic clone (ST5-II, PFGE type A/C), and since 2006, the breakthrough of the EMRSA-15 (ST22-IV, PFGE type D) was described.<sup>77</sup> Based on the most recent data (2017–2018) available from the National Institute of Public Health in Hungary, ~45% of HA-MRSA isolates were ST22-IV clones, while ~24% of isolates were ST5-II; in case of CA-MRSA isolates, ST8-IV and ST80-IV clones were the most common, while among LA-MRSA, CC398 isolates were the most

580 prevalent.<sup>78,79</sup> Literature data also suggest that the clonal background of *S. aureus* isolates may play a major role in the biofilm-forming capacity, however (as demonstrated by the publications discussed in the previous section), the clonality of the isolates is seldom reported in these studies.<sup>80</sup> Croes et al reported that *S. aureus* isolates from MLST clonal complex CC8 were the most potent biofilm-producers – irrespective of the glucose-concentration (0–0.5%) in the media; at physiological glucose (0.1%) concentration, >60% of CC8 isolates were strong biofilm-producers, compared to 0–7% in other tested CCs.<sup>81</sup> These findings were also supported by the results of Luther et al: from n= 182 clinical MRSA strains, isolates belonging to the CC8 group and *spa* type t008 group were significantly more common among strong biofilm-producers (p=0.01), while *spa* type t895 and  $\beta$ -toxin-producing isolates showed a negative correlation with biofilm-production.<sup>82</sup> The study of Recker et al performed combined study, including laboratory assays and a data analysis regarding bacterial genotype and phenotype with available clinical metadata in a machine-learning framework, corresponding to n=300 *S. aureus*, including CC22 and CC30 isolates from bacteremia. Their results showed no relevant differences in biofilm-formation among MSSA and MRSA isolates or among CC22 and CC30 isolates. Although this report showed no association with biofilm-production and SCCmec-type, other studies highlighted that the SCCmec II element is associated with decreased capability to form biofilm.<sup>83</sup> On the other hand, Lim et al (who assessed n=465 clinical *S. aureus* isolates) and da Fonseca Batistao (during the study of fifteen isolates) both concluded that the presence of the SCCmec III cassette is a good predictor of strong biofilm-forming ability.<sup>84,85</sup> The importance of the SCCmec cassette and *mecA* genes in biofilm formation were highlighted by Pozzi et al, where  $\Delta$ SCCmec (deletion mutants) presented with decreased expression of virulence determinants, including biofilms; the authors of the study have concluded that these genes may have the potential to affect phenotypic characteristics mediated by other operons (ie, *agr* or *icaADBC*) to facilitate the adaptation of hospital-associated MRSA to the harsh environment in hospitals.<sup>86</sup> This may be the reason for the larger number of biofilm-producing isolates from catheter-associated (CAI-SA) infections.

620 It has been described that *S. aureus* strains usually form multilayered biofilms; this biofilm is useful in the

evasion of the non-specific and adaptive immune responses of the host, including decreased rate of opsonization, phagocytosis, killing by neutrophil granulocytes, and Toll-like receptor activation.<sup>87,88</sup> The latter is particularly relevant, because TLR-activation is important in facilitating a Th2-type immune response, which may act to prevent *S. aureus* biofilm-associated infections.<sup>89</sup> Biofilms are also protective against reactive oxygen species (ROS) in in vivo environments.<sup>90</sup> Biofilms also facilitate the metabolic transformation of *S. aureus* into the small colony variant (SCV; which may be seen as “dwarf colonies” on solid media) morphotype: in this sub-population, bacteria exist at a lower metabolic activity (leading to increased antimicrobial resistance), which also enables in vivo persistence and chronic infections.<sup>91</sup> In addition to metabolic switching, SCVs are also characterized by adaptation for intracellular survival in mammalian cells, where the pathogen acts similarly to other microorganisms with a strictly intracellular life cycle.<sup>92,93</sup> The relevance of eDNA in the stability and antimicrobial resistance of staphylococcal biofilms has been demonstrated, as DNase treatment (eg, in cystic fibrosis) clearly negatively impacts the biofilm structure.<sup>94</sup> Biofilm-formation in *S. aureus* is genetically mediated by the regulatory genetic locus staphylococcal accessory regulator (*sarA*); this controls two pathways – namely the intracellular adhesin (*ica*) operon and accessory gene regulator (*agr*) regulated pathways – both of which have been suggested as determinants of the extent of biofilm-formation in these bacteria.<sup>95,96</sup> The product of the genes of the *ica* operon (*icaADBC*) are the IcaA and IcaD transferase membrane proteins; these proteins have important roles in the biosynthesis of PIA (polysaccharide intercellular adhesion protein; or poly-β-1,6-N-acetylglucosamine [PNAG]), which is a major component of staphylococcal biofilms.<sup>97</sup> Although our study did not demonstrate pronounced differences among the biofilm-producing capabilities of the locally collected MSSA and MRSA isolates, there have been studies offering possible biological explanations to this phenomenon.<sup>38</sup> It has been suggested that biofilm-formation in MSSA is mediated by cell-cell adhesion via the production of PIA (encoded by *icaADBC*), while MRSA biofilm-production is PIA-independent, and rather, it is dependent on a protein adhesion, which is negatively regulated by the *agr* system.<sup>98,99</sup> O’Neill et al showed that media supplementation with NaCl results in the induction of biofilm-production in MSSA only, as this activated the expression of the *ica* operon.<sup>100</sup> In contrast,

Croes et al showed that the presence of excess glucose in the media represses the *agr* system (through the generation of low pH), which resulted in the induction of biofilm-production in MRSA only;<sup>81,101</sup> additionally, the deletion of the *agr* system also enhanced the biofilm-production of MRSA isolates, while it had no effect on its methicillin-susceptible counterparts.<sup>102</sup>

## Conclusions

The production of biofilm by pathogenic bacteria in vivo provides important protection from external forces and antimicrobials, in addition to facilitating chronicity. *S. aureus* is an exceptionally adaptable pathogen both in natural environment and in clinical situations. Biofilm-formation in both MSSA and MRSA isolates is an important step in the pathogenesis of implant-associated infections and leads to a synergistic interaction between “classical” resistance-determinants and the inability of antibiotics and immune cells to reach *S. aureus* isolates. The relationship between the MDR phenotype and biofilm-positivity has been studied for many relevant pathogens, however, the culmination of these results is inconclusive. In our study, one hundred and fifty MSSA and one hundred and fifty MRSA isolates (from a variety of clinical situations) were tested for their antibiotic-susceptibility and their biofilm-forming capacity to ascertain a possible relationship between the two. Among our tested isolates, we have found complete susceptibility to the last-resort agents, while there were significant differences in the resistance rates between MSSA and MRSA isolates regarding almost all other, commonly used agents. Thirty-eight percent and 42% of isolates were biofilm-producers based on the CV tube adherence assay and the CRA plate methods, respectively. Overall, no association was found between methicillin-resistance and biofilm-positivity in our settings; on the other hand, resistant isolates to erythromycin, clindamycin and rifampin were significantly more common among biofilm-producers.

Our study possesses some limitations: i) the cross-sectional nature of the study: although isolates were collected from different clinical specimen groups, they may not represent Hungarian *S. isolates*, their biofilm-forming capacity or susceptibility overall; ii) selection bias: isolates usually originated from tertiary-care centers, corresponding to patients with more severe conditions or underlying illnesses; iii) interpretation: both phenotypic methods were evaluated by organoleptic methods, therefore the reading of the results was

dependent on the expertise of the researchers; iv) lack of molecular methods: the molecular characterization of resistance determinants, clonal lineages (with PFGE or MLST) or genetic determinants of biofilm-production (eg, *agr*, *ica* genes) in the mentioned isolates was not performed. Nevertheless, our study provides additional data to the existing pool of literature on the association of drug resistance and biofilm-formation in *S. aureus*. Additional studies – with the inclusion of other isolates and utilization – are needed to provide clarity on this subject.

## Data Sharing Statement

All data generated during the study are presented in this paper.

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The authors declare no conflict of interest, monetary or otherwise. The authors alone are responsible for the content and writing of this article.

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