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ORIGINAL ARTICLE

Incidence of biofilm formation among MRSA and MSSA clinical isolates from hospitalized patients in Israel

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Abstract

Aim: To assess the biofilm-producing capacities of *Staphylococcus aureus* strains isolated from hospitalized patients in Israel.

Methods and Results: A total of 16 *S. aureus* (80 MRSA and 83 MSSA) from screening (nasal swab) and clinical samples (blood and wounds) were characterized. Biofilm-producing capacities were determined using two different biofilm detection assays: Congo Red agar (CRA) and microtiter plate (MtP). In addition, a real-time PCR analysis was performed to detect the presence of biofilm-associated genes (*ica*A and *ica*D) and *mec*A gene. The two assays showed similar biofilm production pattern (28.2% agreement). MRSA strains tended to be greater biofilm-producers than MSSA strains. The presence of *mec*A was associated with biofilm production ($p = 0.030$). Additionally, bacteria isolated from blood samples produced less biofilm compared to those from other sources. Finally, no association was found between *ica*A and *ica*D presence and biofilm production.

Conclusion: This study supports earlier assumptions that biofilm formation depends strongly on environmental conditions.

Significance and Impact of Study: This study significantly improved our knowledge on the biofilm production capacity of *S. aureus* strains in Israel. Moreover, it revealed an association between the *mec*A gene and biofilm production. Finally, this study underscores the importance of further research to evaluate risk factors for biofilm production.

KEYWORDS

biofilms, Congo red agar, *ica*A, *ica*D, microtiter plate, MRSA, *S. aureus*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a highly virulent pathogen that causes a wide variety of clinical manifestations. When *S. aureus* enters the bloodstream or internal tissues, it can cause serious infections (Taylor & Unakal, 2019), such as infective endocarditis, and skin, soft tissue, pleuropulmonary and device-related infections (Tong et al., 2015). It is currently considered the most common cause of infections among hospitalized patients (Archer, 1998), thereby imparting a significant burden on the healthcare system. Specifically, the emergence and spread of methicillin-resistant *S. aureus* (MRSA) strains in hospitals and subsequently in the community, have been associated with significant morbidity and mortality (Gnanamani et al., 2017).

One of *S. aureus*' virulence factors is its biofilm-forming capacity (Archer, 1998). Biofilm is a sessile microbial community embedded in a protective extracellular polymeric matrix, in which cells are attached to a surface or to other cells. This form of growth displays altered physiology, gene expression and protein production, which enables *S. aureus* bacteria to attach to medical implants and host tissues, and underlies its resistance to therapeutic treatment (Lister & Horswill, 2014).

In order to form biofilms, *S. aureus* cells create a polymer-based extracellular matrix composed of proteins, carbohydrates and extracellular DNA, which encases them within a sticky matrix and enables survival in hostile or extreme environments (Moormeier & Bayles, 2017). The biofilm formation decreases the bacterial susceptibility to antimicrobials and immune defences, thereby complicating their eradication. During infection, dispersal of cells from the biofilm can result in spread to secondary sites and worsening of the infection. This plays an important role in the persistence of chronic infections and leads to increased morbidity and mortality (Moormeier & Bayles, 2017). Therefore, early detection of *S. aureus* is an essential step toward prevention and management of infections (Jain & Agarwal, 2009; Melo et al., 2013).

Given the adverse impact of biofilm-mediated infections on patient health, and the lack of information regarding the biofilm-forming capacities of local *S. aureus* strains in clinical samples, this study aimed to determine the incidence of biofilm-production among *S. aureus* strains isolated from hospitalized patients in Israel. In addition, the work evaluated and compared two different biofilm detection assays. Finally, it examined the biofilm-forming capacities by source of collected strains (nasal, blood and wound), and by different antibiotic resistance groups, i.e., MSSA and MRSA.

MATERIALS AND METHODS

Bacterial isolates

This study was performed at the clinical microbiology laboratory of the Baruch Padeh Poriya, Medical Center (PPMC), Israel. One-hundred and sixty-three *S. aureus* strains were collected for this study. Eighty strains (40 MSSA and 40 MRSA) were obtained from the *S. aureus* National Reference Center of the Israel Ministry of Health. The strains were originally isolated from blood and wounds samples of patients admitted to various medical institutes in Israel, between 2019 and 2020. In addition, 83 strains (40 MRSA and 43 MSSA) were isolated from screening samples of patients admitted to PPMC, between 2019 and 2020. Strains ATCC 35984 and ATCC 12228 were used as positive (biofilm-producing strain) and negative (non-biofilm-producing strain) control strains.

Detection of biofilm formation using the micro titre-plate (MtP) assay

The MtP assay was performed according to the protocol of Melo et al. (2013). *S. aureus* strains were cultured on blood agar (BD Diagnostics) and incubated for 24 h, at 37°C. Overnight specimens were diluted 1: 200 in trypticase soy broth (TSB; Sigma-Aldrich) containing 0.25% glucose; 200 µl were then seeded on sterile 96-well polystyrene tissue culture plates and incubated overnight at 37°C, for 18 h. Plates were then washed three times with phosphate buffered saline (pH 7.2), and dried for 1 h, at 60°C, before they were stained with 1% crystal violet (ELITechGroup Biomedical Systems,) and incubated for 1 min, at room temperature. After staining, the plate was washed three times with distilled water (DW) and left to dry at room temperature. Biofilm formation was measured at 490 nm ($O.D_{490}$) with a spectrophotometer ELISA reader (Thermo Fisher Scientific). TSB with no bacterium served as blanks, whereas ATCC 35984 served as a positive control and ATCC 12228 served as a negative control. Strains with an $O.D_{490}$ beneath 0.1 were classified as non-producers; strains with an $O.D_{490}$ above 0.1 were classified as weak biofilm producers, and strains with an $O.D_{490}$ above 1.0 were classified as strong biofilm producers (Figure 1). Each strain was tested for biofilm production in duplicates and the assay was repeated three times.

Detection of biofilm formation by CRA assay

Strains were cultured on CRA plates (50 $g\,L^{-1}$ sucrose, 37 $g\,L^{-1}$ brain heart infusion broth, 10 $g\,L^{-1}$ agar No.1, 8 $g\,L^{-1}$ Congo red indicator) and incubated for 24 h at 37°C, followed by an overnight incubation at room temperature. The isolates were characterized based on colony morphologies: rough black colonies were considered strong biofilm-producers, rough blackish red colonies were classified as moderate biofilm producers, pink colonies were considered weak producers and red smooth colonies were considered non-producers (Figure 1). Each strain was tested for biofilm production in duplicates.

Detection of biofilm-associated genes icaA and icaD by PCR

Molecular detection of *icaA/D* genes was performed using the real-time PCR (qPCR) technique. First, DNA was extracted from bacteria using the GenElute™ Bacterial Genomic kit (Sigma-Aldrich), according to the manufacturer's instructions. Briefly, bacteria were

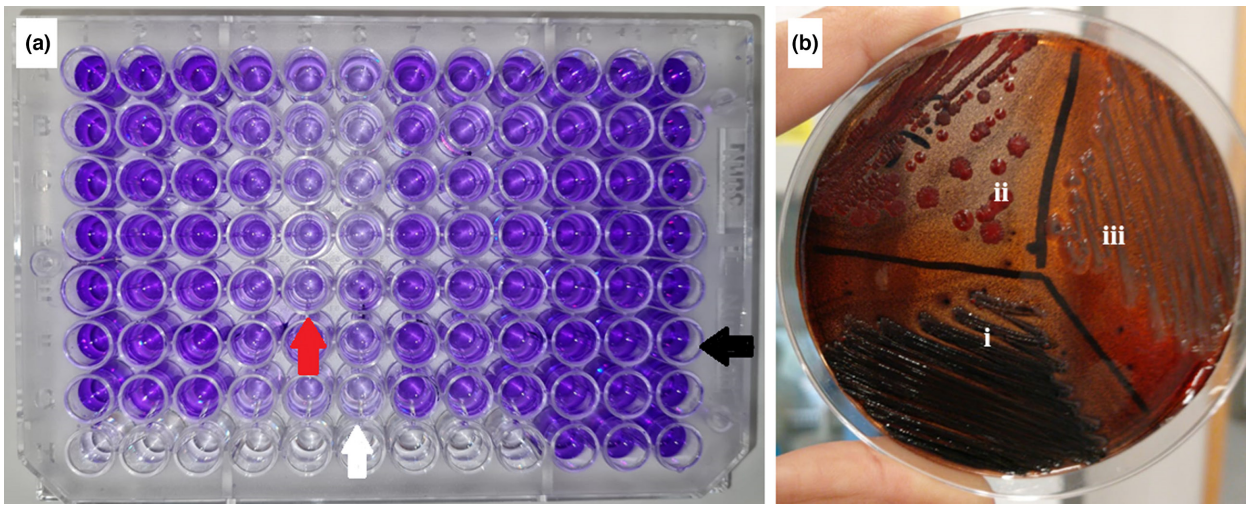

FIGURE 1 Screening of biofilm producing organisms by microtitre plate method (a), and by Congo red agar method (b). By the MtP method (a), strains were characterized for biofilm production according to colour absorbance intensity; strains with an $O.D_{490}$ beneath 0.1 were classified as non-producers (marked by a white arrow); isolates with an $O.D_{490}$ above 0.1 were classified as weak biofilm producers (marked by a red arrow), while strains with an absorbance above 1.0 were classified strong biofilm producers (marked by a black arrow). (b) Screening of biofilm by Congo red agar method, (i) rough black colonies were considered as strong biofilm-producers, (ii) rough blackish red colonies were classified as moderate biofilm producers, (iii) pink colonies were considered weak producers, and red smooth colonies were considered non-producers (not shown)

harvested from blood agar plates (BD Diagnostics) with a sterile loop. Cell lysis was performed by suspending the inoculating loop in a 2 ml Eppendorf tube containing 200 µl of lysostaphin (200 µg/ml) (Sigma-Aldrich), which was then incubated at 37°C for 30 min. Then, 20 µl of proteinase K (20 mg/ml) and 200 µl lysis solution were added, samples were vortexed and then incubated at 55°C, for 10 min. After addition of 200 µl ethanol (96%), samples were vortexed and transferred to a binding column and centrifuged (8000 x *g*, 1 min). The eluate was discarded and the column was transferred to a new collection tube. To separate the DNA from the other cell components, the column was washed with 500 µl Buffer Wash 1 and centrifuged (8000 x *g*, 1 min). Then, a second wash with 500 µl Buffer Wash 2 was performed in a fresh 2 ml collection tube, which was then centrifuged (12,000 x *g*, 3 min). Finally, the column was transferred to a fresh 2 ml Eppendorf tube and centrifuged (14,000 x *g*, 1 min). For DNA elution, the column was transferred to a fresh 1.5 ml Eppendorf tube, 100 µl elution buffer were added to the column, and tubes were incubated for 5 min at room temperature, and then centrifuged (12,000 x *g*, 1 min). DNA was stored at −80°C until further use.

To detect the presence of *ica*A/D genes, qPCR was performed using the primers previously used by Melo et al. (2013). The qPCR mix contained 10 µl SYBR green master mix (2×), 1 µl of each primer at a final concentration of 0.8 µM, 5 µl nuclease-free water and 4 µl DNA template (average of 100 ng/well). The qPCR reactions were performed using a real-time PCR system (CFX96 Real-Time system; BIO-RAD), under the following conditions: 94°C for 5 min, followed by 40 cycles of 92°C for 45 s, 49°C for 45 s, and 72°C for 1 min. Followed by a final elongation at 72°C, for 7 min.

Gene expression of the biofilm-associated genes icaA and icaD by PCR

RNA extraction

In order to detect gene expression, RNA was extracted using the using the RNAeasy Protect Mini kit (Qiagen), according to the manufacturer's instructions. Briefly, bacteria were harvested from blood agar plates (BD Diagnostics) with a sterile loop and collected into PBS. RNAprotect Bacteria Reagent (200 µl) was added to a new tube, into which 100 µl of the bacterial inoculum containing approximately 2×10^8 CFU was transferred. The tube was vortexed and incubated for 5 mins at room temperature and then centrifuged for 10 min at 5000×*g*. The supernatant was discarded and the pellet was suspended in 200 µl Tris-EDTA buffer, which contained 20 mg/ml Proteinase K and 200 µg/ml lysostaphin (Sigma-Aldrich). After vortexing for 10 s and incubated in a shaker for 10 min at room temperature, 700 µl Buffer RLT was added. Following vigorous vortex, 500 µl ethanol (96%–100%) was added and the tube was shaken. The lysate was transferred into an RNeasy Mini spin column placed in a 2 ml collection tube.

Following centrifugation for 15 secs at ≥8000× g, the flow-through was discarded and three wash steps were performed. Then, the RNeasy Mini spin column was placed in a new 1.5 ml collection tube and 50 µl of RNase-free water directly were added. The tube was centrifuged for 1 min at ≥8000× g to elute the RNA. Then, cDNA was synthesized from the purified RNA using the SuperScript™ III First-Strand Synthesis Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

Gene expression detection

The expression of *ica*A/D genes was performed with the same primers and reaction conditions as mentioned in the former section.

Statistical analysis

Univariate analyses were conducted to identify differences in biofilm-producing capacities of MSSA versus MRSA isolates, and of isolates from different sources. Comparisons of categorical and continuous variables between groups were made using the Chi-squared or non-parametric Wilcoxon-Mann–Whitney Rank Sum test for independent samples, respectively. Data were analysed using SPSS version 25. Statistical significance was defined as $p < 0.05$.

RESULTS

This study included 163 *S. aureus* clinical isolates, of which 83 were MSSA and 80 were MRSA. The isolates originated from various clinical sources, which included 40 blood culture isolates (20 MRSA, 20 MSSA), 40 wound isolates (20 MRSA, 20 MSSA), and 83 nasal isolates, for example, screening isolates (40 MRSA, 43 MSSA). According to the CRA method, 97 (59.5%) isolates were non-biofilm-producers, 6 (3.7%) isolates were strong biofilm-producers, and the remaining 60 isolates (36.8%) showed various degrees of biofilm production (Table 1). Using the MtP method, 66 (40.5%) isolates were non-producers and the remaining 97 (59.5%) were either strong or weak producers.

Seven (4.3%) isolates carried and expressed one or more of the *ica*A/D genes; most of these isolates were MSSA (5/7, 71.4%). No correlation was found between the existence of *iac*A/D genes and biofilm production. Five (71.4%) of these isolates were CRA-classified as non-producers, and five of the isolates were classified by the MtP method as weak biofilm producers (Figure 2).

Regarding methicillin resistance, a positive association was found between presence of the *mec*A gene, which is related to methicillin resistance, and biofilm production. MRSA strains showed higher biofilm-producing capacities when compared to MSSA strains (Table 2; $p < 0.001$). In the MtP assay, higher number of MRSA isolates were strong biofilm producers as compared to MSSA strains (8 vs. 2); however, this difference was not statistically significant ($p = 0.511$).

One of the study's aim was to compare the two methods for assessing biofilm formation. An agreement measures test was used to compare biofilm-producing capacity between the two qualitative assays (Table 3). Overall agreement (28.2%) and Krippendorff's alpha reliability (−0.02) were very low between the methods of detection, indicating no significant differences between them.

Another study's aim was to evaluate associations between sample source and biofilm production. Significant differences in biofilm-producing capacities were found between the isolates based on the sample clinical sources (blood, wound and nasal) (Table 4). Biofilm production, as measured using both methods, tended to be lower in isolates from blood as compared to other sources (nasal swab and wound).

Lastly, in efforts to assess a possible association between biofilm production and different factors which enable *S. aureus* antibiotic resistance, presence of the following factors was evaluated: Panton–Valentine leucocidin (*pvl*) gene, *mec*A gene, and mupirocin or chlorhexidine resistance. These tests were conducted only on 79 isolates obtained from the National Reference Center. The presence of *mec*A was the only parameter found to correlate with biofilm production; its presence was associated with increased biofilm production (Table 5).

TABLE 1 Distribution of biofilm-producing capacities of the study's isolates, using the CRA and MtP assays

Biofilm production/method	Non-producers*n* (%)	Weak producers*n* (%)	Moderate producers n (%)	Strong producers n (%)
CRA	97 (59.5)	14 (8.6)	46 (28.2)	6 (3.7)
Mtp	66 (40.5)	87 (53.4)		10 (6.1)

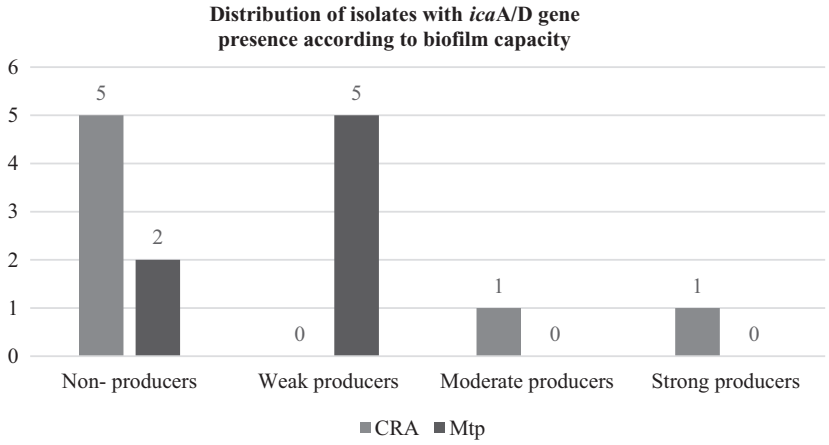

FIGURE 2 Distribution of isolates harbouring the *ica* genes according to biofilm-producing capacities

TABLE 2 Comparison of the biofilm-producing capacities of MRSA vs. MSSA strains, as measured using the CRA and MtP assays

	MRSA ($N = 80$)	MSSA ($N = 83$)	Total ($N = 163$)	*p* value
CRA				<0.001
No	41 (51.2%)	56 (67.5%)	97 (59.5%)	
Weak	1 (1.2%)	13 (15.7%)	14 (8.6%)	
Moderate	34 (42.5%)	12 (14.5%)	46 (28.2%)	
Strong	4 (5.0%)	2 (2.4%)	6 (3.7%)	
MtP				0.511
No	33 (41.2%)	33 (39.8%)	66 (40.5%)	
Weak	39 (48.8%)	48 (57.8%)	87 (53.4%)	
Strong	8 (10.0%)	2 (2.4%)	10 (6.1%)	

TABLE 3 Agreement measures and paired *t*-test results

	Agreement measures	
CRA vs. MtP	Simple agreement (%)	Krippendorff's alpha (ordinal)
Overall	28.2%	−0.02

DISCUSSION

Biofilm production is considered to be an important factor in *S. aureus* pathogenesis, antibiotics resistance, and immune evasion (Khasawneh et al., 2020). Biofilm-forming *S. aureus* causes many diseases including endocarditis, septicemia, osteomyelitis, pneumonia, pleuropulmonary and device-related infections (Lister & Horswill, 2014; Shivaee et al., 2019; Tong et al., 2015). Therefore, studying the biofilm-forming capacity of *S. aureus* is an important step toward understanding its severity and virulence.

In this study, 83 MSSA and 80 MRSA samples isolated from different clinical sources were genetically and phenotypically characterized for biofilm formation. Biofilm production is usually genetically associated with the presence of elements of the *ica*ABCD operon (Cramton et al., 1999; Khasawneh et al., 2020; Knobloch et al., 2002; Rohde et al., 2001; Shivaee et al., 2019). While many studies have demonstrated *ica*ADBC frequencies of 77%–100%, most have not found a correlation between the presence of *ica* elements and biofilm formation phenotype (Cramton et al., 1999; Khasawneh et al., 2020; Knobloch et al., 2002; Rohde et al., 2001). In their characterization of 128 *S. aureus* isolates, Knobloch et al. (2002) found all to be *ica*-positive using PCR methods, while none displayed a biofilm-positive phenotype in the CRA assay, and only 4 (3.1%) were biofilm-positive in the tissue culture plate (TCP) assay. Similar results were observed by Cramton et al. (1999), who reported that 100% of the 25 tested isolates were *ica*-positive, but only a minority of isolates formed biofilms in a TCP assay. In contrast, McKenney et al. (1999) found 25% of their 207 tested isolates to be biofilm producers and only 8 (15.4%) to be *ica*-positive by PCR. In the current study, 40.5% of the isolates produced biofilm in the CRA method and 59.5% were found to be biofilm-positive using the MtP assay, whereas only 7 (4.3%) isolates were found to carry one or more of *ica*AD genes. This is compatible with the emerging evidence of the existence of *ica*-independent biofilm mechanisms in *S. aureus* isolates (Arciola et al., 2015; O'Gara, 2007; Shivaee et al., 2019). Another explanation for the varied incidence of *ica* in biofilm-forming isolates between the different studies may be due to the inconsistent choice of primers for PCR analysis. Khasawneh et al. (2020) and Arciola et al. (2015) characterized 100% and 78%, respectively, of their tested isolates as biofilm-forming using phenotypic assays, all of which were *ica*-positive. Overall, different phenotypic assays in conjunction with an inconsistent set of primers likely underlie the reported contradictory results relating to biofilm formation and its association with an *ica* genotype in *S. aureus* (Knobloch et al., 2002).

TABLE 4 Comparison of biofilm-producing capacities based on sample source

	Blood ($N = 40$)	Nasal swab ($N = 83$)	Wound ($N = 40$)	Total ($N = 163$)	*p* value
CRA					0.024
No	31 (77.5%)	44 (53.0%)	22 (55.0%)	97 (59.5%)	
Weak	3 (7.5%)	5 (6.0%)	6 (15.0%)	14 (8.6%)	
Moderate	5 (12.5%)	31 (37.3%)	10 (25.0%)	46 (28.2%)	
Strong	1 (2.5%)	3 (3.6%)	2 (5.0%)	6 (3.7%)	
MtP					0.014
No	9 (22.5%)	43 (51.8%)	14 (35.0%)	66 (40.5%)	
Weak	28 (70.0%)	36 (43.4%)	23 (57.5%)	87 (53.4%)	
Strong	3 (7.5%)	4 (4.8%)	3 (7.5%)	10 (6.1%)	

TABLE 5 Biofilm production according to presence of the *mec*A gene

	*mec*A-negative ($N = 36$)	*mec*A-positive ($N = 43$)	Total ($N = 79$)	*p* value
CRA				0.030
No	27 (75.0%)	25 (58.1%)	52 (65.8%)	
Weak	6 (16.7%)	3 (7.0%)	9 (11.4%)	
Moderate	2 (5.6%)	13 (30.2%)	15 (19.0%)	
Strong	1 (2.8%)	2 (4.7%)	3 (3.8%)	
MtP				0.372
No	11 (30.6%)	12 (27.9%)	23 (29.1%)	
Weak	24 (66.7%)	26 (60.5%)	50 (63.3%)	
Strong	1 (2.8%)	5 (11.6%)	6 (7.6%)	

*mec*A is part of the *Staphylococcus* cassette chromosome *mec* (SCC*mec*), which is usually found in MRSA strains, where it confers resistance to β-lactam antibiotics (Pozzi et al., 2012). Our CRA results showed higher prevalence of the *mec*A gene in biofilm-producing isolates (41.9%) as compared to non-biofilm forming isolates (25%). In parallel, the presence of *mec*A was associated with increased biofilm-producing strength, i.e., biofilm production was significantly more moderate (30.2%) and strong (4.7%) among *mec*A-positive isolates compared to *mec*A-negative isolates (5.6% and 2.8%, respectively). This finding is compatible with those reported by Pozzi et al. (2012), who highlighted the association between the presence of *mec*A and biofilm production in MRSA strains.

In addition, we used two methods to compare the biofilm-forming capacity of MSSA and MRSA isolates. The two methods showed higher and stronger biofilm-production in MRSA when compared to MSSA strains. While these findings are consistent with the results of Lee et al. (2016), Askhan et al. (2021) and Piechota et al. (2018), most previous studies found no significant differences regarding biofilm-forming capacity between *S. aureus* strains classified by antibiotic resistance phenotype (Arslan & Özkardeş, 2007; Askhan et al., 2021; Ghasemian et al., 2016).

The application of multiple and inconsistent methods for detecting biofilm formation leads to conflicting results between studies (Askhan et al., 2021; Knobloch et al., 2002). While we, as Khasawneh et al. (2020), found significant differences in biofilm formation between MSSA and MRSA strains using CRA method, the MtP method failed to detect any differences. On the other hand, Piechota et al. (2018) did identify significant differences between MSSA and MRSA using the MtP method. Given that biofilm formation has been shown to be affected by different ingredients, such as glucose and NaCl (Agarwal & Jain, 2013; Croes et al., 2009; O'Gara, 2007), the use of organic substrates may lead to different ingredient ratios across the reported studies, which may explain their discrepancies.

Furthermore, we have investigated the biofilm-forming capacities of isolated *S. aureus* strains originating from diverse clinical sources. In accordance with earlier studies (Agarwal & Jain, 2013; Piechota et al., 2018), we found significant differences between the different sample sources. These results support earlier assumptions that biofilm

formation depends strongly on environmental conditions (Askhan et al., 2021; Knobloch et al., 2002; Taj et al., 2012). In the current research, strains collected from nasal passages or wounds were stronger biofilm-producers than isolates collected from blood. These findings are in agreement with those reported by Piechota et al. (2018), but contrast those of Agarwal and Jain (2013), who showed higher biofilm production among blood isolates. On the other hand, Knobloch et al. (2002) found differences in biofilm-producing capacities between blood and nasal isolates that were attributed to the media type on which the isolates were grown, and argued that these contradictory results may indicate the involvement of additional regulatory mechanisms in biofilm activation and expression.

In conclusion, this was first characterization of the biofilm-producing capacity of *S. aureus* isolates collected in Israel from different clinical sources and with different antibiotics resistance profiles. Compared to MSSA, a higher percentage of MRSA strains had a biofilm-forming capacity, which was also stronger. The strains from blood cultures produced significantly less biofilm than strains from wounds and nasal swabs.

As the biofilm-forming capacity of *S. aureus* strains increases their ability to cope in difficult environmental conditions, as in hospitals, and decreases the effectiveness of antibiotic treatments, a further research should be performed to evaluate risk factors for biofilm development. Another important point is the resulting need for more consistent methods to characterize *S. aureus* biofilms to enable a better understanding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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