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Determination of the effect of glucose, sucrose and sodium chloride addition in different culture media on biofilm formation of methicillin resistant *Staphylococcus aureus*

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ABSTRACT

Aim: *Staphylococcus aureus* is the most clinically important bacterium among *Staphylococci*, colonizing 15-36% of the entire population. Biofilm formation is an important virulence factor of *S. aureus*. Treatment of biofilm-associated *S. aureus* infections is difficult. This study aimed to investigate the effects of glucose, sucrose, and sodium chloride (NaCl) addition to seven different media on biofilm formation capacity of methicillin resistant *S. aureus* (MRSA) strains.

Material and Method: Biochemical and molecular methods (spa, nuc, coa, and mecA PCR) were used to identify *S. aureus* strains. Cefoxitin resistance was determined by the agar disc diffusion method. Biofilm formation of the strains was investigated in 7 different media (Tryptone soya broth (TSB), TSB+1% sucrose, TSB+1% glucose, TSB+4% NaCl, Brain Heart Infusion broth (BHI), BHI+1% glucose, and BHI+4% NaCl) using the microplate test. The growth of strains in 7 different media was determined at 600 nm, and then 96-well microplates were stained with crystal violet and their biofilm formation abilities were determined by measuring absorbance values at 590 nm.

Results: In this study, 53 strains containing spa, nuc, coa, and mecA genes were identified as MRSA with resistance to cefoxitin. When biofilm formation was examined in seven different media using the microplate test, the biofilm formation ability of MRSA strains increased significantly with glucose and sucrose addition to TSB and BHI ($P<0.05$), while the addition of 4% NaCl decreased the biofilm formation ($P<0.05$). When the media were compared, it was determined that BHI increased bacterial growth and biofilm formation compared to TSB.

Conclusion: It was concluded that the biofilm formation abilities of MRSA strains increased especially in the presence of glucose. The results showed that biofilm production capacity is affected by environmental factors, especially nutrient content in used media.

Keywords: MRSA, biofilm, microplate test

INTRODUCTION

Staphylococcus aureus, one of the most clinically important species among *Staphylococci*, is a commensal bacterium that colonizes 15-36% of the entire population (1). *S. aureus* may cause severe diseases such as wound infections, toxic shock syndrome, scalded skin syndrome, skin abscesses, deep tissue abscesses, styes, impetigo, pneumonia, emesis, meningitis, pericarditis, endocarditis, sinusitis, cellulitis, osteomyelitis, diarrhea, urinary tract infections, bacteremia, and sepsis (2-4). The mortality rate is quite high in invasive *S. aureus* infections. On the other hand, due to the resistance of MRSA strains to other antibiotics, the treatment of infections caused by multidrug-resistant *S. aureus* strains may be difficult, and the length of hospital stay may be prolonged (5).

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Biofilm forming ability is an important virulence factor of *S. aureus*. Biofilm is characterized as a collection of surface-associated microbial cells (6-7). *S. aureus* is accepted as one of the most important causes of biofilm-related infections (8). *S. aureus* adheres to material surfaces by using its rolling motion and sticky extensions, thus it constantly attaches to the surfaces, causing micro aggregation to form, resulting in a biofilm layer (9). *S. aureus* strains from different sources have been shown to produce biofilms (10). When *S. aureus* biofilm-related infections occur, it is very difficult to treat and eradicate with antibiotics and other disinfectants (11). Studies have reported that MRSA strains also form strong biofilms (12-13).

Nutrient-rich Brain Heart Infusion broth (BHI) and Tryptone soya broth (TSB), which is less nutrient-rich compared to BHI, are frequently used in *in vitro* biofilm studies (14). Previous studies have shown that the choice of medium strongly affects the biofilm development of *S. aureus* (15,16). It is known that substances such as glucose, sucrose, and NaCl at different concentrations added to the nutrient media have a significant effect on the biofilm production capacity (17).

In this study, the bacterial stocks that have been previously isolated from blood culture samples were used as the materials. The effects of glucose, sucrose, and NaCl addition to TSB and BHI media on biofilm formation of identified MRSA strains were investigated quantitatively.

MATERIAL AND METHOD

Within the scope of the study, clinical bacteria (n:100) grown on blood agar medium from blood culture samples studied in the Clinical Microbiology Laboratory of Ege University Medical Faculty Hospital were used as the materials. The clinical bacterial stocks (n:100) containing 40% glycerol have been previously stored at -80°C. Ethics committee approval is not required as the study was conducted on the stock bacteria. Any clinical specimen were not used in this study. All procedures were performed in accordance with the ethical rules and principles of the Helsinki Declaration.

Identification of Bacteria

Bacterial stocks were cultivated on Mannitol Salt Agar (CM0085 Oxoid, UK) and grown bacteria were subcultured to obtain pure colonies in the same medium. Gram staining, microscopy, and catalase test using 3% (v/v) hydrogen peroxide were applied for the biochemical identification of the bacteria. Gram-positive, cocci-shaped, and catalase-positive bacteria, which were determined as Staphylococci, were included in the study. Then, Staphylococci were grown in TSB (CM0129, Oxoid), followed by DNA isolation using lysostaphin (L7386, Sigma, Germany) and proteinase K (P2308, Sigma) (18). Molecular identification of the isolates was performed by Polymerase Chain Reaction (PCR) using primers specific to the spa gene (19) encoding surface A protein, the nuc gene responsible for thermonuclease activity (20), the coa gene (21) responsible for coagulase activity, and mecA (22) genes responsible for methicillin resistance. The agar disc diffusion method was used to determine the methicillin resistance of the strains and the disc zone diameters of cefoxitin (30 µg) were measured and evaluated according to the CLSI 2020 standards (23). The reference strains *S. aureus* ATCC 25923 and MRSA ATCC 43300 were used as the positive control strains for antibiotic susceptibility and PCR experiments.

Biofilm Test

The microplate method was used to determine the ability of bacteria to form biofilms in different media (24). For this purpose, TSB, TSB containing 1% (w/v) sucrose, TSB containing 1% (w/v) glucose, TSB containing 4% (w/v) NaCl and BHI (CM1135, Oxoid), BHI containing 1% (w/v) glucose and BHI containing 4% (w/v) NaCl were used as test media. Bacterial strains were grown overnight on Tryptone Soya Agar (TSA, CM0131, Oxoid) at 37 °C and then their concentration was adjusted to McFarland0.5 using a densitometer (Den-1B, Biosan, Latvia) in 10 ml of 0.9% (w/v) NaCl. After that, 180 µl of each medium and 20 µl of bacterial suspension in McFarland 0.5 turbidity were inoculated in triplicate wells in a 96-well flat-bottom sterile microplate (3599 Corning Costar, USA). The microplates were incubated at 37 °C for 24 hours and then, bacterial growth was measured using a microplate reader (Epoch, BioTek, the USA) at 600 nm. After that, the microplates were emptied and washed 3 times with sterile 0.9% NaCl to remove bacteria that did not attach to the surface and did not form biofilm. The bacteria attached to the surface were fixed with 200 µl methanol for 15 minutes. The methanol in the microplates was removed by decanting. The microplates were then dried at 55 °C for 1 hour. The attached bacteria were stained with crystal violet (1159400 Merck, Germany) for 5 minutes to visualize biofilm formation, and excess dye was removed by washing the microplates with tap water. For quantification of the biofilm formation of bacteria on the surface, the microplates were dried at 55 °C for 1 h and 200 µl of 33% (v/v) acetic acid (Merck) was added to each well. The dye was completely dissolved in a microplate mixer (Vortex 4 digital, Ika, Germany) at 500 rpm for 5 minutes. The absorbance values of the microplates were measured at 590 nm using a microplate reader (Epoch, BioTek, USA). *S.*

epidermidis YT-169a strain, which produced biofilm in microplate tests, was used as a positive control strain (25).

Statistics

Mean, standard deviation, minimum and maximum values, frequency, and descriptive statistics were used for the data analysis. In addition, the Kruskal-Wallis test, which is a nonparametric test, was used to determine whether there was a statistically significant relationship between different media and glucose, sucrose and NaCl added to the media on the biofilm formation of isolated MRSA strains. Another nonparametric test, the Mann-Whitney test, was used to determine the significant relationship between different media and additives to these media. For all these tests, $p<0.05$ was considered statistically significant. These tests were performed in IBM SPSS statistical program version 22.0 for Windows (2020).

RESULTS

Within the scope of the study, a total of 53/100 strains were identified as Staphylococci by biochemical tests, including spa, nuc and coa genes by molecular identification. The strains (n:53) were mecA gene positive and resistant to cefoxitin by agar disc diffusion method. When the growth of 53 MRSA strains in seven different media was examined with measurements performed at 600 nm. It was determined that MRSA strains developed better in BHI medium and BHI medium to which NaCl was added, compared to only TSB and TSB medium containing sucrose or glucose, and their OD 600 nm values were higher (**Figure 1**).

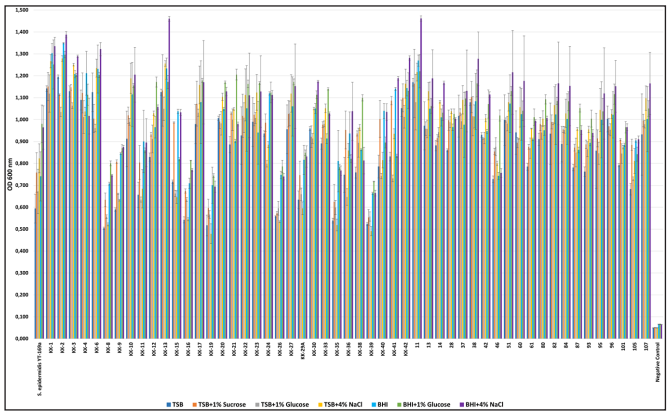

Figure 1. Growth of MRSA strains after incubation at 37 °C for 24 hours in seven different media. *S. epidermidis* YT-169a is indicated as the biofilm positive control strain and the bacteria-free medium as the negative control.

The biofilm formation abilities of the bacteria were determined using the microplate test. In general, higher adhesion and biofilm formation were detected in BHI medium containing 1% glucose (**Figure 2**). In the evaluation made based on the strains, it was determined that MRSA 38 strain showed the highest biofilm formation in BHI containing 4% NaCl.

As a result of the nonparametric Kruskal-Wallis and Mann-Whitney statistical tests based on the microplate test results at 590 nm, no significant relationship was found between TSB and BHI, in terms of biofilm formation ($P>0.05$). Only 1% sucrose and 1% glucose supplementation to TSB medium were found to be significantly associated with the biofilm formation ($P<0.05$), and 1% sucrose and 1% glucose added to TSB medium were found to increase the biofilm formation, compared to TSB alone (**Figure 2**). Compared to TSB, 4% NaCl added to TSB medium was also found to be significantly associated with the biofilm formation ($P<0.05$), and 4% NaCl added to TSB medium was found to reduce the biofilm formation. While there was no significant relationship between 1% sucrose and 1% glucose added to TSB medium on the biofilm formation ($P>0.05$), they were found to be significantly different with 4% NaCl ($P<0.05$) (**Figure 3**).

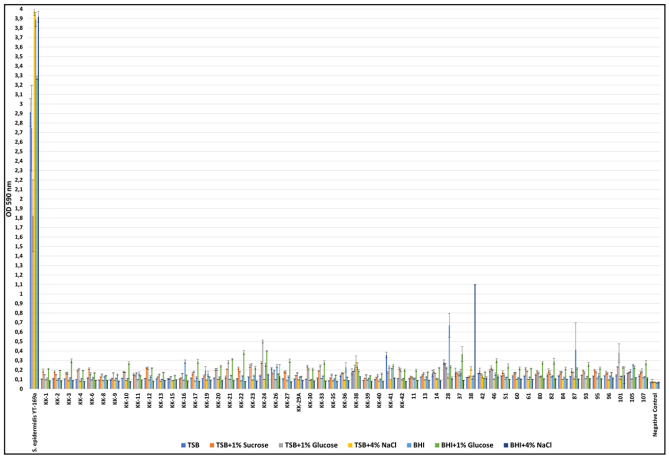

Figure 2. Determination of biofilm formation abilities of MRSA strains in seven different media by the microplate test. *S. epidermidis* YT-169a is indicated as a biofilm positive control strain and a bacteria-free medium as a negative control.

It was also found that 1% glucose added to BHI medium was significantly associated with the biofilm formation compared to BHI alone ($P<0.05$), and 1% glucose added to BHI medium was found to increase the biofilm formation. According to BHI, 4% NaCl added to BHI medium was found to be significantly associated with the biofilm formation ($P<0.05$), and 4% NaCl added to BHI medium was found to reduce the biofilm formation. Significant differences were found on the biofilm formation between 1% glucose and 4% NaCl added to BHI medium ($P<0.05$) (**Figure 3**).

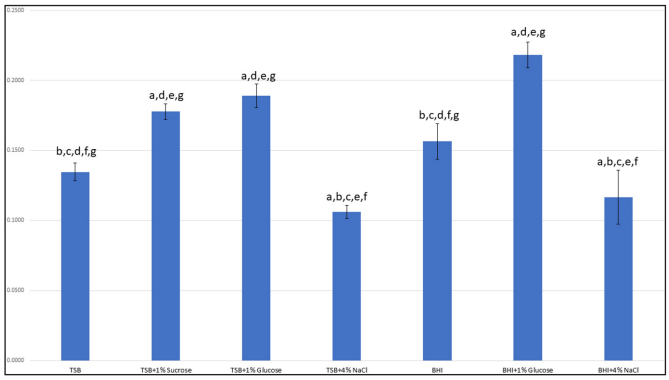

Figure 3. The relationships of glucose, sucrose and NaCl supplementation to TSB and BHI media with the biofilm formation of MRSA strains. (a) TSB, (b) TSB+1% Sucrose, (c) TSB+1% Glucose, (d) TSB+4% NaCl, (e) BHI, (f) BHI+1% Glucose, (g) BHI+4% NaCl. The results were recorded as mean values with±standard deviations of three independent measurements. Mean values with different letters show statistically significant difference ($P<0.05$).

DISCUSSION

The pathogenicity of *S. aureus*, which is the causative agent of various acute or chronic infections, depends on biofilm formation along with other virulence factors. The first stage of biofilm formation, which takes place in at least three stages, is the adhesion of microorganisms. The second stage is cell proliferation and exopolysaccharide synthesis on the surfaces, and the third stage is microaggregation and thick biofilm layer formation (26).

The choice of medium in which bacteria are cultivated and the use of glucose, sucrose, and NaCl at different concentrations influence the biofilm formation of *S. aureus* cells. The previous studies have reported that the increase in NaCl (27), sucrose (28), glucose (29), supplementation with combination glucose and NaCl (28-30) stimulate the biofilm formation capacity. It has also been reported that substances such as glucose and NaCl added to different media for MRSA strains, which are also known to form biofilms, significantly increase the biofilm production capacity of MRSA strains (31).

In this study, we found that the addition of glucose and sucrose in TSB and BHI media increased the biofilm production capacity of MRSA strains, while the addition of NaCl to TSB and BHI media decreased the biofilm formation. In addition, no significant difference was found between the BHI medium and the TSB medium in terms of biofilm production. Singh et al. (32) investigated the effect of glucose, sucrose, and NaCl on the biofilm production capacity of *S. aureus* strains and they stated that these added substances did not have any effect on increasing biofilm production alone, but increased biofilm production when given together. Vázquez-Sánchez et al. (33) examined the biofilm formation of 26 *S. aureus* strains isolated from seafood in the presence of 5% glucose, 5% NaCl, their combination, and 0.1 mM and 1 mM $MgCl_2$. Similar to our glucose supplementation to test media, their results showed that the highest biofilm formation was obtained in 5% glucose supplementation of TSB alone. Meanwhile, glucose increased biofilm formation with increasing incubation temperature from 25°C to 37°C. NaCl had negative correlation with biofilm formation at 37°C. The combination of glucose with NaCl slightly increased the biofilm formation compared to the absence of supplements. Moreover, $MgCl_2$ did not affect significantly the biofilm formation of the strains concerning for to the absence of $MgCl_2$. Lade et al. (16) conducted a study with 40 strains including 21 MRSA and 19 methicillin-susceptible *S. aureus* (MSSA), selected from blood samples. Similar to our study, they stated that biofilm formations of both MRSA and MSSA strains increased in TSB + 0.5% glucose and TSB + 1% glucose media compared to TSB alone. Unlike us, they found that biofilm formations of both MRSA and MSSA strains increased at TSB + 1% NaCl and TSB + 2% NaCl concentrations. In addition, there was no significant difference between MRSA and MSSA at different glucose concentrations, while biofilm formation of MRSA strains was stronger than MSSA strains at TSB + 2% NaCl concentration. In another study, Sugimoto et al. (15) studied 47 clinical *S. aureus* strains (23 MRSA and 24 MSSA) and their results were demonstrated that glucose supplementation increased biofilm formation in most of the strains (42/47). Moreover, NaCl supplementation stimulated biofilm formation of mostly MSSA strains (15/24) than MRSA strains (1/23). The main difference in the results of Sugimoto et al. with our data was the stimulatory effect of NaCl on biofilm formation of MSSA strains rather than MRSA strains. Furthermore, Xu et al. (34) analyzed the effects of NaCl concentrations (0%, 2%, 4%, 6%, 8%, and 10%) on biofilm formation ability of *S. aureus* at 37 °C. Adherence of *S. aureus* cells to polystyrene microplate surface was starting to decrease after 4 days of incubation at 37 °C in all tested NaCl concentrations. Their results demonstrated that high concentrations of NaCl from 4% to 10% continuously increased adherence of bacteria. Ionic nature of NaCl could impact on biofilm formation of the strains. In this respect, surface charge of bacteria could be affected due to environmental Na and Cl ions. In our study, 4% NaCl tested and only one MRSA strain 38 was determined with inreased biofilm formation. Further studies are needed to elucidate the affect of NaCl on clinical MRSA strains with higher and lower concentrations in different growth media.

In the determination of biofilm formation abilities of bacteria, mostly microplate test is used. This test is easy, convenient, and gives quantitative results. Bacterial physicochemical properties especially surface charge, hydrophobicity, and auto-aggregation capacity play important roles in biofilm formation. When we compared our results with previously reported studies

the main difference is the bacterial surface characteristics. Adhesion capacity depends on each bacterial surface characteristic in both MRSA and MSSA strains. Another parameter is test conditions. Incubation time, temperature, test surface (polystyrene, polypropylene, glass, or metal) and surface properties (surface charge, hydrophobicity, and roughness), used media and their ingredients (supplementation with glucose, sucrose, and different type of salts) influence biofilm formation of bacteria (25, 35-36). These test conditions differ from lab to lab with the used test materials and chemicals. This is the main reason for discrepancies in the results.

CONCLUSION

As a result of our study, it was determined that 53 MRSA strains originating from blood culture had the potential to produce biofilms. While the biofilm feature of MRSA strains increases significantly with glucose and sucrose added to different media such as TSB and BHI, the addition of 4% NaCl decreases the biofilm feature. In only one MRSA strain, NaCl stimulated biofilm formation compared to other supplements. The main contribution of our results to the literature was to use different media (TSB and BHI) and supplements (glucose, sucrose and NaCl) together to test biofilm forming abilities of clinical MRSA strains isolated in Turkey. Biofilm formation was controlled both genetic and physicochemical properties of each bacteria. For this reason, each individual bacterium can behave differently to adhere material surfaces.

Contamination of polymeric catheter and percutaneous endoscopic gastrostomy equipment with Staphylococci, which can form a biofilm, poses a great risk of infection for patients. Our results also showed that biofilm production capacity was affected by environmental factors such as nutrient content and type. Using a large number of MRSA and MSSA strains, meanwhile analyzing their physicochemical properties, biofilm-forming ability of clinical strains should be elucidated. These results will be a guide in the development of anti-biofilm surfaces and materials.

ETHICAL DECLARATIONS

Ethics Committee Approval: Ethics committee approval is not required as the study was conducted on the stock bacteria. Any clinical specimen were not used in this study.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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