American Journal of Infection Control ■■ (2017) ■■-■■

Contents lists available at ScienceDirect

American Journal of Infection Control

journal homepage: www.ajicjournal.org

Major Article

Sodium hypochlorite is more effective than 70% ethanol against biofilms of clinical isolates of *Staphylococcus aureus*

Shreshtha Tiwari MD [a], Shraddha Rajak MSc [a], Dehi Pada Mondal PhD, MSc [b], Debasis Biswas MD [a,*]

[a] *Department of Microbiology, AIIMS Bhopal, Madhya Pradesh, India*
[b] *CSIR-AMPRI, Bhopal 462026, Madhya Pradesh, India*

Key Words:
Scanning electron microscopy
Disinfectants

Introduction: Although disinfectants are used for eradication of bacteria from environmental surfaces, their antibiofilm efficacy is often not considered in determining the choice of disinfectant.
Aim: This study aimed to compare the effectiveness of 2 commonly used disinfectants, sodium hypochlorite and ethanol, against the planktonic and biofilm state of *Staphylococcus aureus* clinical isolates.
Materials and methods: Effect of 0.6% sodium hypochlorite and 70% ethanol was determined on the planktonic and biofilm states of 10 strong and weak biofilm formers through estimation of changes in colony forming unit counts and absorbance values. The morphologic changes were observed by scanning electron microscopy.
Results: Significant difference in the efficacy of sodium hypochlorite and ethanol was observed against the biofilm ($P = .004$) as well as planktonic ($P = .000$) states of *S aureus*. However, no significant difference was observed in their activity against strong and weak biofilm formers. On electron microscopy, sodium hypochlorite was found to induce significant formation of craters and irregular depressions on the surface of strong biofilm formers.
Conclusions: Sodium hypochlorite demonstrated superior efficacy in controlling both planktonic and biofilm states of growth in *S aureus*. Furthermore, the characteristic morphologic changes observed in strong biofilm formers hint at its biofilm-specific activity.

Staphylococcus aureus is an important nosocomial pathogen associated with a multitude of infections like chronic osteomyelitis, indwelling device-related infections, implant failures, catheter-associated bloodstream infections, and ventilator-associated pneumonia. These infections often follow a chronic indolent course owing to the characteristic ability of the bacteria to form biofilms and this leads to increased morbidity and mortality, prolonged hospital stay, higher treatment costs, risk of implant failure, and repeat surgery.[1-4] Hence, biofilm-forming ability constitutes a significant virulence factor for *S aureus* that protects the pathogen from host defense mechanisms and external antimicrobial agents. It has been estimated that 65% of infections in hospitals are associated with biofilm formation.[5,6]

In view of this, it becomes important to reduce the bacterial burden in biofilms to control cross- contamination of surfaces, equipment, and implanted medical devices. Strategies for removal of staphylococcal biofilms include treatment with enzymes that degrade polysaccharide intercellular adhesion like dispersin B, peptidoglycan-degrading enzyme lysostaphin, bactriocins, bacteriophages, antibiotics, and disinfectants.[7] Of these, vigorous cleaning accompanied by surface disinfection is the most commonly adopted strategy in all health care facilities. However, the success of this strategy is dependent on the choice of an appropriate disinfectant with proven anti-biofilm efficacy. In this regard, the present classification system of disinfectants; that is, Spaulding classification, is often adopted for selecting a suitable disinfectant. But this system fails to consider the activity of disinfectants against biofilms.[8] In addition, the various guidelines available for testing of disinfectants focus on planktonic cells that are far more susceptible than the more sturdy and adherent cells present in biofilms.[8,9]

Sodium hypochlorite and ethanol are intermediate-level disinfectants routinely used as surface disinfectants in hospitals and clinical laboratories. They are easily available, cheap, and include nonsporing vegetative bacteria like *S aureus* in their antimicrobial

* Address correspondence to Debasis Biswas, MD, Department of Microbiology, AIIMS Bhopal, Ground Floor, Saket Nagar, Bhopal 462020, Madhya Pradesh, India.
E-mail address: debasis.microbiology@aiimsbhopal.edu.in (D. Biswas).

0196-6553/
https://doi.org/10.1016/j.ajic.2017.12.015

spectrum. In view of the increasing medical relevance of biofilm-producing *S aureus* infections, we were interested in determining whether these 2 commonly used intermediate-level disinfectants were effective on the biofilm state of *S aureus* and also in characterizing the morphologic alterations caused by them on staphylococcal biofilms.

MATERIALS AND METHODS

Quantitative estimation of biofilm-forming ability

Eighty-nine isolates of *S aureus*, recovered from pus samples of patients with wound infections, were tested for biofilm formation by tissue culture plate method. Briefly, 50 μL of each isolate was inoculated in tryptic soy broth (TSB) to 0.5 McFarland opacity standard and was added to 150 μL TSB with 2% glucose taken in microtitre plates in triplicates and incubated at 37°C for 24 hours. Each well was washed with phosphate buffered saline (PBS) thrice and then stained with 3% crystal violet after fixation with methanol. The optical density of each well was observed by spectrophotometry (MultiscanFC; Thermo Scientific, Waltham, MA) at 492 nm wavelength, following solubilization with 33% glacial acetic acid. Uninoculated medium of same volume was used as control. As reported previously, the isolates were categorized as strong, moderate, weak, and nonbiofilm formers based on the following formula:[10,11] cut-off optical density value (OD_x) = mean of control + 3 $_C$ standard deviation.

Isolates were characterized as nonbiofilm formers if optical density (OD) ≤ OD_C. Weak biofilm formers had their OD in the range OD_C-2 OD_C, moderate biofilm formers had OD between 2 OD_C and 4 OD_C, and strong biofilm formers had OD > 4 OD_C.

Effect of disinfectants on biofilm produced by clinical isolates

Of these, 10 randomly selected strong and weak biofilm formers were chosen for further study. Each isolate was tested in triplicate in the presence of both the disinfectants, namely, 0.6% sodium hypochlorite and 70% ethanol. Control wells were similarly prepared in triplicate in the absence of the disinfectants. Briefly, after allowing biofilm to form in each well for 24 hours at 37°C, the planktonic cells were removed by washing each well thrice with PBS, and disinfectants were applied for 5 minutes at 16°C. After the contact period, each well was washed twice with PBS and the remaining biofilm was stained and quantitated, as described above. Percent reduction of the OD was calculated with reference to control by the formula: $(OD_C - OD_{Test} / OD_C) \times 100$.

Effect of disinfectants on the planktonic states of the clinical isolates

Effect of the disinfectants on the planktonic cells was also tested. For this 0.5 McFarland of the test isolate was treated with the disinfectants (1:10 dilution) for a period of 5 minutes at 16°C. The disinfectant was neutralized by addition of neutralization broth composed of 0.5% sodium thio-sulphate and 2% glycine in 1:10 dilution. Each tube was then subcultured on nutrient agar by using 10 μL broth in triplicates and colony count postexposure to disinfectant was determined. The $\log_{10}$ reduction in colony count following treatment with disinfectants was compared with control. Colony count in the control tube was ascertained by inoculating 10 μL10-fold serial dilution of the untreated tubes in nutrient agar.

Electron microscopy of the clinical isolates in the presence and absence of disinfectants

Sterile polystyrene coverslips were immersed in 20 mL selected *S aureus* strains inoculated in TSB medium with 2% glucose, which was adjusted to 0.5 McFarland opacity standard. The bacterial suspension was incubated at 37°C for 24 hours to allow biofilm to form on the surface. The coverslips were then removed and washed with sterile PBS (pH 7.4) to remove the nonadherent cells. To test for the action of the 2 disinfectants on the biofilm preparations, the coverslips were immersed in disinfectant solution for 5 minutes at 16°C. These were then washed twice with PBS in sterile petri dishes and air dried. All tests were performed in triplicate.

Each coverslip was immersed in fixing solution (2% glutaraldehyde) and kept for 2 hours at 4°C. After washing twice in PBS, each coverslip was dehydrated in a gradient series of ethanol solutions (35%, 50%, 70%, and 95% by volume, successively) for 10 minutes each and then in pure ethanol for 15 minutes and air dried. Completely air-dried coverslips were attached to metal holders with double-sided adhesive tape and finally coated in an evaporator with gold and palladium. The coverslips were then examined under a scanning electron microscope (Nova Nano SEM-430, D-9392, Lincoln, NE).

RESULTS

Eighty-nine clinical isolates recruited in the study were evaluated for their biofilm-forming ability and were categorized into 1 of 4 categories. These included strong biofilm producers (n = 33), moderate biofilm producers (n = 21), weak biofilm producers (n = 25), and nonbiofilm producers (n = 10). We selected a group of 10 strong and weak biofilm producers randomly from these 89 clinical isolates.

We compared the clinical details of patients yielding the respective groups of strong and weak biofilm producers (Table 1) and

Table 1
Demographic data and clinical details of the strong and weak biofilm producers

Parameter	Strong biofilm producers (n = 10)	Weak biofilm producers (n = 10)	*P* value
Age (mean ± standard deviation)	46 ± 22	43 ± 20	.8
Gender (male/female)	4/6	5/5	.7
Outpatient/inpatient	8/2	9/1	.5
MRSA/MSSA	8/2	8/2	1
Underlying clinical condition	Cellulitis (n = 2) Foot ulcer (n = 2) Osteomyelitis (n = 1) Folliculitis (n = 1) Lymph node discharge (n = 1) Arthritis (n = 1) Burns (n = 2)	Cellulitis (n = 2) Foot ulcer (n = 3) Osteomyelitis (n = 1) Folliculitis (n = 1) Lymph node discharge (n = 1) Arthritis (n = 1) Psoriasis (n = 1)	

MRSA, methicillin-resistant *Staphylococcus aureus; MSSA*, methicillin-sensitive *Staphylococcus aureus.*