Microb Health Dis 2022; 4: e703

BIOFILM ERADICATION METHODS AND EFFECT OF SILVER AND SELENIUM NANOPARTICLES ON HARMFUL BIOFILM FORMING *STAPHYLOCOCCUS AUREUS*

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Abstract – *Objectives:* *Staphylococcus aureus* (*S. aureus*) is a nosocomial bacterium that commonly causes several infectious diseases in humans and animals. *S. aureus* infectious diseases are difficult to treat due to their biofilm.

Material and Methods: In the present paper, we studied phenotypically biofilm eradication methods via three different methods, i.e., Congo red agar (CRA), tube method (TM), and tissue culture plate (TCP). The inhibition of Ag and Se nanoparticles was performed by well diffusion assay against clinically isolated biofilm-forming *S. aureus* strains.

Results: All *S. aureus* bacterial strains were found to be non-biofilm producers using the CRA method, whereas biofilm formation was performed by another method, namely TM. A total of 13.89% strains were shown strongly positive, while 55.56% isolates were unable to produce biofilm. On the other hand, with TCP method, 33.33% of strains were found to be high biofilm producer, while 44.44% were found non-biofilm producer.

Conclusions: The results of this study provided evidence of high inhibition growth of three biofilm-forming *S. aureus* (SA12, SA15, SA32) in the presence of silver nanoparticles (AgNPs) at 50 µl, whereas selenium nanoparticles (SeNPs) demonstrated low inhibitory effects (SA23).

Keywords: Biofilm forming, Nanoparticles, Selenium, Silver, *Staphylococcus aureus*, Well diffusion.

Abbreviations: *Staphylococcus aureus (S. aureus*), Congo red agar (CRA), Tube method (TM), Tissue culture plate (TCP), Nanoparticles (NPs), Silver (Ag), Selenium (Se), Biofilm forming (BF), Mannitol salt agar (MSA), Scanning Electron Microscopy (SEM), biofilm forming *S. aureus* (BF-SA), Zone of inhibition (ZOI).

INTRODUCTION

Clinical isolates of *Staphylococcus aureus* (SA) species have different capacities to form a biofilm. This may be due to the differences in the expression of biofilm-related genes, genetic makeup and physiological conditions[1]. SA biofilm type of development is closely regulated by complex genetic factors. However, in recent decades, some latest studies[1] have taken biofilm forming (BF) into account in terms of elucidating host immunity toward infection and may lead to the development of efficacious anti-biofilm SA therapies. A bacterial biofilm is a thin

but strong layer of mucilage that adheres to a solid surface and contains a population of bacteria and other microbes. Biofilms have different growth, gene expression, and protein synthesis phenotypes[2]. Biofilms can be as thin as a single cell layer or as thick as a large community wrapped in a viscous polymeric environment[3]. Silver nanoparticles are a non-toxic and safe antibacterial agent for the human body[1-4].

Applications of Ag and Se based NPs have been considered in the field of infectious disease therapy, such as biofilms on medical devices[2,5], and as an antibacterial in the previous several decades[2,6]. Haris and Khan[7] have discovered SeNPs-enhanced photodynamic therapy of toluidine blue O against BF *S. mutans* bacterial isolates. Haris and Ahmad[8] studied the impact of metal oxide (ZnO & TiO_2) NPs on beneficial soil microorganisms and their secondary metabolites. The antibacterial potential of NPs was determined by growth kinetics of *P. aeruginosa*, *P. fluorescens* and *B. amyloliquefaciens.* Biodegradable NPs can be used for delivering drugs as well as silencing multiple genes or gene activation in diabetic nephropathy Soni[9]. Chauhan et al[10] have examined the applications of nanotechnology in forensic inquiry and showed hidden shreds of evidence that can aid forensic scientists in reaching conclusions during investigations.

MATERIALS AND METHODS

Thirty-six coagulase-positive strains of SA strains were cultured on MSA at 37°C for 24 to 48 hrs. MSA is both a selective and differential medium used for the isolation of SA and was first identified by Bergey's Manual of Systemic Bacteriology[11].

Phenotypic analysis of biofilm formation of SA strains was proceeded by Congo red agar method by Freeman et al[12], Tube method by Christensen et al[13], and Tissue culture plate method by Gunti et al[14].

AgNPs was prepared by using the chemical reduction method (Sileikaite et al[15]) and SeNPs were made by reducing sodium selenite with glutathione (reduced form) and stabilizing it with bovine serum albumin[16].

A scanning electron microscope creates an image by scanning a focused electron beam across a surface. Both NPs scanning were performed under different magnifications ranging from 15,000x to 35,000x and voltage 20-30kV.

After the preparation and characterization of the Ag and SeNPs, their antimicrobial activity (well diffusion) was tested against the clinically isolated SA pathogens. The antibacterial activity of Ag and Se-NPs has been determined by the diameter of the inhibition zone formed around the well plates[16].

RESULTS

Among these SA isolates, three different phenotypic analyses (CRA, TM, and TCP) were used for the evaluation of biofilm formation.

Out of the 36 isolates of SA strains investigated for biofilm formation by the CRA method, no isolate produced a black colony with a dry crystalline consistency (i.e., strong), and black colonies without dry crystalline consistency indicated moderate biofilm producer. However, overall, 36 (100%) isolates produced pink colonies, which were taken as negative biofilm formation (Table I).

A total of 36 isolates were tested for biofilm formation by tube method, 5 (13.89%) were shown strongly positive, whereas 11 (30.56%) isolates produced moderate biofilm formation while 20 (55.56%) of them produced weak or non-biofilm formation (Table I).

A total of 36 isolates was tested for biofilm formation by TCP method, 12 (33.33%) strains were shown strongly positive, whereas 8 (22.22%) isolates produced moderate biofilm formation and 16 (44.44%) isolates produced weak or non-biofilm formation (Table I).

The shape and size of the Ag and Se-NPs were measured by Scanning Electron Microscopy (SEM). Particle morphology shape and size with SEM reveals spherical shape with the size of particle as 80.32 nm for AgNPs (Figure 1A) whereas, rods shape particles with the size of particle were 74.29 nm for SeNPs (Figure 1B).

TABLE 1. BIOFILM FORMATION ABILITY OF DIFFERENT SA STRAINS BY CRA, TM, TCP METHODS.

S. No	Biofilm forming methods	Biofilm production		
		H (%)	M (%)	L (%)
1	CRA	0 (0%)	0 (0%)	36 (100%)
2	TM	5 (13.89%)	11 (30.56%)	20 (55.56%)
3	TCP	12 (33.33%)	8 (22.22%)	16 (44.44%)

Antibacterial activity (by well diffusion assay) of these two Ag and Se-NPs varied among the SA species tested and showed an expected gradual result with the same NPs concentrations (50 µl). Out of the total 36 strains, only 9 strains of BF-SAs were shown to the zone of inhibition (ZOI) against Ag and Se-NPs, while the other remaining 27 strains were unable to show any ZOI against both NPs, so they were further studied with respect to the 9 strains of BF-SAs. According to the CLSI standard, an antibiotic (Amoxicillin), ZOI diameter (mm) resistant was seen at ≤19 mm, intermediate-not mentioned, and sensitive at ≥20 mm. Among 36 strains of SA, only 8 strains (SA12, SA15, SA20, SA22, SA23, SA24, SA26, and SA32) showed antimicrobial activity for AgNPs and only 8 strains (SA15, SA20, SA22, SA23, SA24, SA25, SA26, and SA32) for SeNPs against BF-SA strains.

DISCUSSION

Among these isolates, three different phenotypic analyses were used to the evaluation of biofilm formation in SA isolates. Overall, a total of 36 isolates of SA strains was investigated for biofilm formation by the CRA method. Some studies in literature[17] reported dissimilar results for the CRA method for strong and moderate biofilm formations in SA ranging from 1.32% to 56.5%. However, all isolates produced pink color colonies that were taken as a negative BF in SAs ranging from 30% to 94.74%. Therefore, ur study reported similarity with Croes et al[17].

Out of the 36 isolates tested for BF by tube method, 5 (13.89%) showed strongly positive. A total of 11 (30.56%) isolates produced moderate BF. However, a maximum number of isolates (20-55.56%) did not show any BF. Concerning the observations found in the present study, some studies in literature[16-18] reported similar TM result of stronger BF in SA ranging from 12.30%-19%, whereas non-biofilm formation by SA ranged from 51%-58.55%. This report showed similarity with our result in respect to TM.

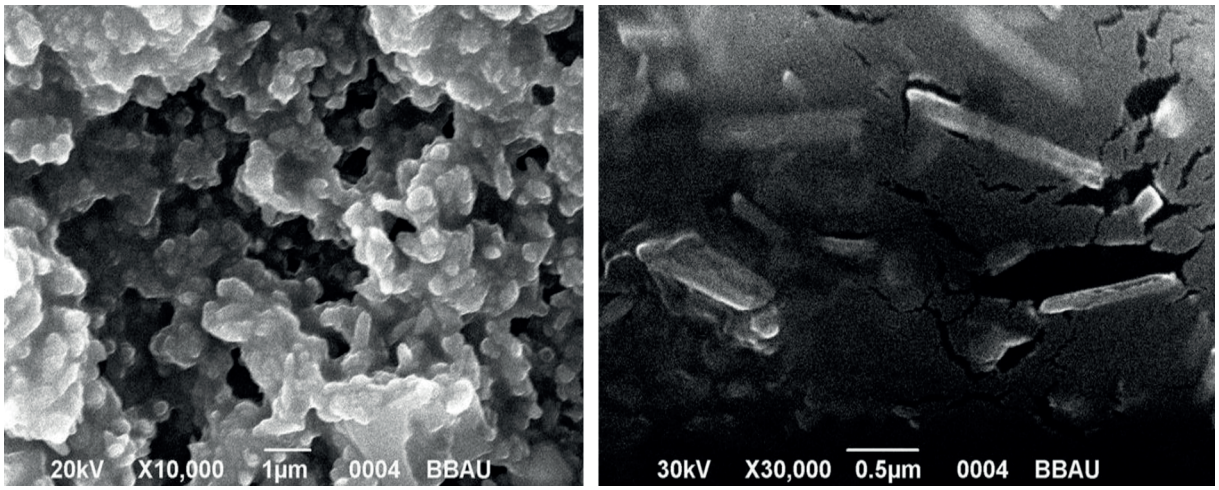

Figure 1. A, SEM image of Ag NPs16. **B,** SEM image of Se NPs16.

The tissue culture plate method showed 12 (33.33%) high biofilm-producer; 8 (22.22%) were moderate biofilm producers whereas non-biofilm producers were 16 (44.44%). Other studies[19] reported strong BF in SA ranging from 14.40% to 20.10% which is less than our percentage, whereas non-biofilm formation in SA ranging from 35.11% to 46%[19] reported similarity with our results.

SEM was used to determine the shape and size of the Ag and Se-NPs. The particle revealed spherical shape with AgNPs particle size in the range of 80.32 nm that also showed close similarity with our result ranging from 60-80 nm[20], whereas rods shape with SeNPs particle size in the range of 74.29 nm showed close similarity with our result ranging from 50-150 nm[2].

Amongst the 2 NPs tested, AgNPs were found to be the most active inhibitory effects against three biofilms forming SA strains (SA12, SA15 & SA32), whereas in the case of SeNPs, they demonstrated the most active strain, i.e., SA23 for BF-SA that showed inhibitory effects. Some scholars[21] have documented a similar pattern of sensitivity of AgNPs among SAs strains, so the antibacterial effect of the strains (SA12, SA15 & SA32) had a greater zone of inhibition than Amoxicillin for Ag NPs and SA23 strain showed greater ZOI than Amoxicillin for SeNPs[22].

CONCLUSIONS

AgNPs seem to be more promising and effective as an antibacterial agent against the clinically isolated BF-SA strains than SeNPs, which could be useful for the development of a new drug for antimicrobial, electronic and biomedical applications. AgNPs are indicated as a viable option for preventing BF-SA infections. NPs can be utilized as new agents in the fight against bacterial forming biofilms at the early stages of bacterial biofilm formation, such as reversible adhesion, biomaterial surface modification, or rupture of the mature biofilm matrix.

Conflict of Interest

The author declares no conflict of interest.

Authors' Contribution

All authors have equal contribution in this article.

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