



Minimum Biofilm Eradication Concentration (MBEC) Assay of Silver and Selenium Nanoparticles against Biofilm forming *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is a most important bacterium that causes nosocomial infections and the etiologic agent of a wide range of diseases related with major mortality and morbidity. Total 36 positive clinical samples viz. urine, blood and pus collected from different patients were found to harbor *Staphylococcus aureus* with a maximum isolation from pus samples i.e. 30 (83.33%) and minimum isolation from urine samples i.e. 2 (5.56%). The degree of capacity to biofilm forming *Staphylococcus aureus* isolates to different phenotypic analysis of biofilm formation by Congo red agar (CRA) Nil, Tube method (TM) 5 (13.89%), and Tissue culture plate method (TCP) 12 (33.33%). The application of silver and Selenium nanoparticles as antimicrobials are gaining relevance in the medical field. Silver nanoparticles, due to their unique properties, use in day-by-day many applications in human life. The major uses of silver nanoparticles in the clinical and medical fields consist of investigative applications and curative applications. Selenium metal is an essential micronutrient for human beings and animals. Selenium nanoparticles showed the highest bactericidal and antimicrobial properties. Minimal biofilm eradication concentrations (MBEC) were determined by 96-well microtitre plate. The antibacterial effects of silver and selenium nanoparticles were evaluated with respect to growth, biofilm formation of *Staphylococcus aureus* strains. Among the three biofilm forming *Staphylococcus aureus* strains showed OD_{450} i.e. 0.019, 0.039, 0.075 value ≤ 0.080 for AgNPs whereas SeNPs couldn't showed any ≤ 0.080 value against biofilm forming *S. aureus* strains. In case of MBEC test, AgNPs showed more affective against biofilm forming *Staphylococcus aureus* strains compared of SeNPs. Finally we suggested that AgNPs showing best antimicrobial activity against SeNPs.

Keywords: Biofilm, Biofilm forming *Staphylococcus aureus*, Silver nanoparticles, Selenium nanoparticles.

INTRODUCTION

S. aureus is a most important cause of hospital acquired nosocomial infection of surgical wounds

and, with *S. epidermidis* causes infections associated with indwelling medical devices ^[1]. The treatment of bacterial infections becomes

extremely hard when bacteria develop in biofilm form for the reason that gene expression of bacteria in biofilm structure is altered, more than 20% of all bacterial genes are expressed another way leading a better protection against antibiotics compared to free living^[2-4].

Biofilm formation is a developmental process moving from attachment, to microcolony formation, and then to mature biofilm development under the control of specific biofilm genes^[5]. Attachment and colonization is the first step for *S. aureus* pathogenesis. Biofilm formation allows the bacteria to resist higher concentrations of antimicrobial agents, environmental conditions and the host immune responses^[6].

The production of a mucopolysaccharide on the surface, which further protects the biofilm, can often be seen with the naked eye^[7]. Microorganisms forming biofilms determined recalcitrance towards a wide range of antimicrobial treatments and have been reported to be 100 to 1000 un-susceptible than their planktonic counterparts^[8]. This resistivity is due to the occurrence of extracellular polysaccharide matrix, the physio-chemical heterogeneity developed within such acquiring of multi-antimicrobial resistance genes and the presence of cells of highly recalcitrant physiology (persisters)^[9].

Recently, silver nanoparticles exhibiting antimicrobial activity have been developed by^[10]. Antibacterial activity of the silver-containing materials can be used like: in medicine to reduce infections as well as to prevent bacteria colonization on prostheses^[11], catheters^[12-13], vascular grafts^[14], dental materials^[15], stainless steel materials^[16] and human skin^[17].

The use of silver nanoparticles as antibacterial agent is relatively new. Because of their high reactivity due to the bulky surface to volume ratio, nanoparticles play a crucial role in inhibiting bacterial develop in solid and aqueous media both. Silver containing equipment can be employed to eliminate microorganisms on textile fabrics^[18-19]. Contrary to bactericide effects of ionic silver, the

antimicrobial activity of colloid silver nano particles are influenced by the size of the particles the smaller the particles, the greater antimicrobial effect^[20]. Therefore, in developing routes of synthesis, an emphasis was made to control the size of silver nanoparticles.

Selenium has been investigated for various medical applications such as anticancer applications. Selenium as a nutritional enhancement has been verified to reduce the risks of various types of cancers including prostate cancer^[21-22], lung cancer^[23] and esophageal and gastric- cardiac cancers^[24]. Selenium-enriched probiotics have been shown to strongly inhibit the growth of pathogenic *E. coli*, *in-vitro* and *in-vivo*^[25]. This study revealed a new type of antibacterial silver and selenium nanoparticles capable of decreasing Biofilm forming *S. aureus* growth.

METHODOLOGY

Collection of samples- A total of 36 positive clinical samples of Urine, Blood (sterile sample bottles), and Pus (using sterile swabs) were collected from different hospitals of Dehradun, India.

Isolation and Identification of *Staphylococcus aureus*- *S. aureus* cultures were cultured in Mannitol salt agar (MSA) at 37°C for 24 - 48 hrs. Mannitol salt agar (MSA) is both a selective and differential medium used for the isolation of *Staphylococcus aureus* (coagulase-positive Staphylococci) produced yellow colonies with yellow zones in the medium. Each sample was sub cultured and maintained into Nutrient agar medium they tend to be white, circular, entire, convex colonies on incubated aerobically at 37°C for 24 - 48 hrs. Isolates were obtained from petri-plates were identified on the basis of cultural, morphological and biochemical characteristics as per Bergey's Manual of Systemic Bacteriology^[26].

Analysis of biofilm formation of *Staphylococcus aureus* strains

Congo Red Agar method- Freeman *et al.*^[27] had determined an alternative qualitative method of screening biofilm formation by *Staphylococcus*

microbes, which need the use of a specially prepared solid medium- Brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red chemicals. This medium was composed of BHI (37 g/l), sucrose (50 g/l), agar (10 g/l) and congo red stain (0.8 g/l). Congo red was prepared by concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium components and then mixed when the agar had cooled to 55°C. Plates were inoculated and aerobically incubated for 24 - 48 hrs at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers generally remained in pink, though occasional darkening at the centers of colonies was observed. A darkening of the bacterial colonies with the lack of a dry crystalline colonial morphology indicated an intermediate result.

Tube method- A qualitative assessment of biofilm formation was determined as previously described by Christensen *et al.* [28]. 10 ml of Trypticase soy broth with 1% glucose was inoculated with loopful of microorganisms from overnight duration culture plates and incubated for 24 hrs at 37°C. The tubes were decanted and washed with PBS (pH 7.3) and dried test-tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Test- tubes were dried in invert position and determined for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm development. Test-tubes were observed and the amount of biofilm formation was scored as non biofilm, moderate or high biofilm (Fig 1).



Fig 1: Tube method by detection of Biofilm formation by *S. aureus*

Tissue culture plate method- 10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight cultured on nutrient agar media. The broth was incubated for 24 hours at 37°C. The culture was further diluted 1:100 with fresh medium. 96 wells flat bottom tissue culture plate was filled with 0.2 ml of diluted cultures individually. The plate was incubated at 37°C for 24 hrs. After incubation,

gentle tapping of the plate was done. The wells were washed with 0.2 ml of phosphate buffer saline solution (pH 7.2) four times to remove free floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was rinsed with deionizer water and plate was dried properly [29-30] (Fig 2).

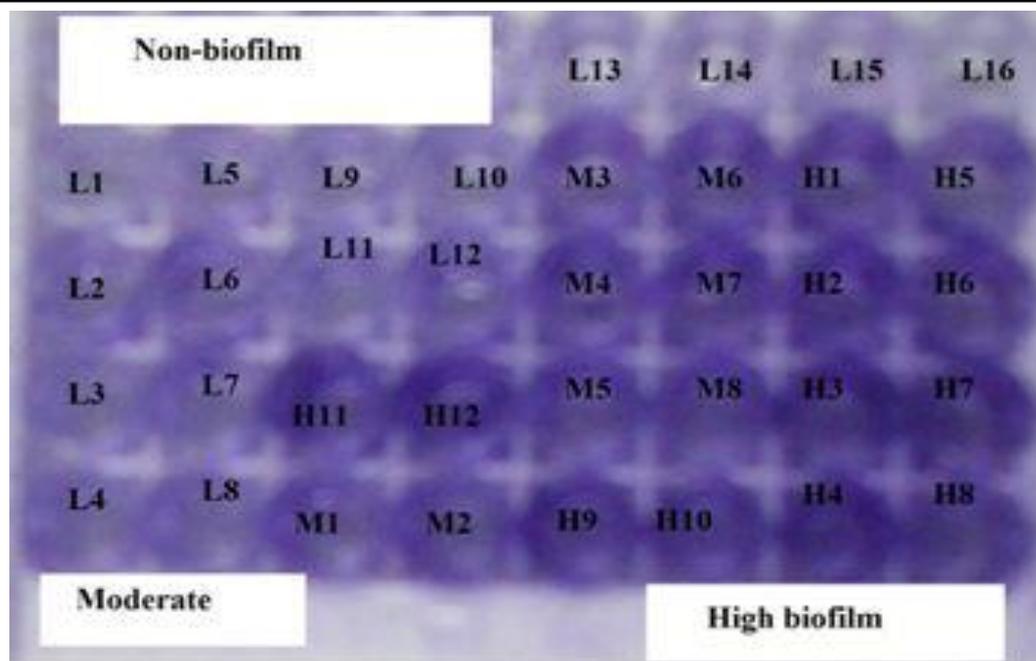


Fig 2: Tissue Culture plate method for detection of Biofilm formation by *S. aureus*

Preparation of Silver nanoparticles- The silver colloid was prepared by using chemical reduction method. All solutions of reacting materials were prepared in distilled water.

Solution 1: 0.0849 g of AgNO_3 was dissolved in 500 ml distilled water, & then the solution was heated to boiling.

Solution 2: Then 1 g of tri sodium citrate was dissolved in 100 ml distilled water.

Working Solution: 5 ml of tri sodium citrate were added to 500 ml of AgNO_3 after boiling (drop by drop). During the process, the solution was mixed vigorously. The solution was left on hot plate for 2 hrs at 90°C for heating (un-continuous manner), then it was cooled at room temperature, the color was reddish green^[31] appeared. Prepared AgNPs were collected in 1.5 ml Eppendorf tube and kept at room temperature for further experiment.

Preparation of Selenium nanoparticles- Selenium nanoparticles (SeNPs) were synthesized by the reduction of sodium selenite by glutathione (reduced form) and stabilized by bovine serum albumin in (BSA). Specifically, 3 mL of 25 mM Na_2SeO_3 , 3 mL of 100mM GSH, and 0.15 g BSA were added in 9 mL of double distilled water in a sterile cabinet. All solutions were made in a sterile

environment by using a sterile cabinet and double distilled water. After mixing the reactant solution, 1 M NaOH was added to bring the pH of the solution to the alkaline regimen. Selenium nanoparticles was formed immediately following the addition of NaOH as visualized by a color change of the reactant solution from clear white to clear red. Selenium nanoparticles were then collected by centrifuging at -4°C for 30 min at 13,000 rpm sterilized by ultra-violet light exposure before the use in bacteria experiments^[32].

Minimal Biofilm Eradication Concentration (MBEC) assay of Nanoparticles (Ag-NPs & Se-NPs) against Biofilm forming *Staphylococcus aureus*-

The MBEC assay was described by Moskowitz *et al.*^[33]. The isolates were cultured in Nutrient agar medium for 24 hrs at 35°C and then 20 μL of inocula of bacterial suspensions (10^8 cfu/ml) were prepared. These inocula were added to each well of a sterile 96-well microtitre plate that was then filled with 180 μL per well of TSB medium supplemented with 1% glucose for *S. aureus*. The plates were incubated for 18 hrs at 35°C and the non-adherent cells were removed by washing the wells with 200 μL of sterile saline. The remaining

attached bacteria was re-suspended in 100 μ L of cation adjusted nutrient broth and challenged with a solution of 100 μ L of Ag & Se Nanoparticles at different concentrations (12.5 μ g/ml, 25 μ g/ml, 50 μ g/ml, 100 μ g/ml, and 200 μ g/ml). The plates were incubated for 24 hrs at 35°C. The Nanoparticles (Ag & Se) were removed and the wells were rinsed three times with sterile saline. The consequent steps (i.e., fixation, staining and elution) were performed as in the biofilm formation assay. The MBEC was defined as the minimum concentration of nanoparticles required to eradicate the biofilm. Eradication of the biofilm given the OD₄₅₀ reading (UV-Visible Spectrophotometer) of approximately 0.080, which is similar to the readings for the negative control. All biofilm experiments were performed in triplicate for each isolate and repeated independently three times to minimize the variability in the OD measurements. Mean values and SDs were calculated.

RESULTS AND DISCUSSION

In the present study, the positive clinical samples were collected from different patients a high prevalence rate of bacterial 36 isolates was observed. *S. aureus* are the most frequent causes of nosocomial infection and infections on indwelling medical devices, which characteristically involved in Biofilms.

The different samples screened for Biofilm production was evaluated for a total of 36 *S. aureus* isolates included urine, blood, and pus showed highest isolation rate from Pus 30 (83.33%) followed by blood 4 (11.11%). The least number of isolates were obtained from urine samples 2 (5.56%) (Fig 3). Similar studies have been reported maximum isolation of *S. aureus* from pus varying the range of 61.70% - 89.28% [34-37]. Whereas, blood varying the range of 9% - 21% [38-40] and urine varying the range of 5.4% - 16.66% [34;36-37;41].

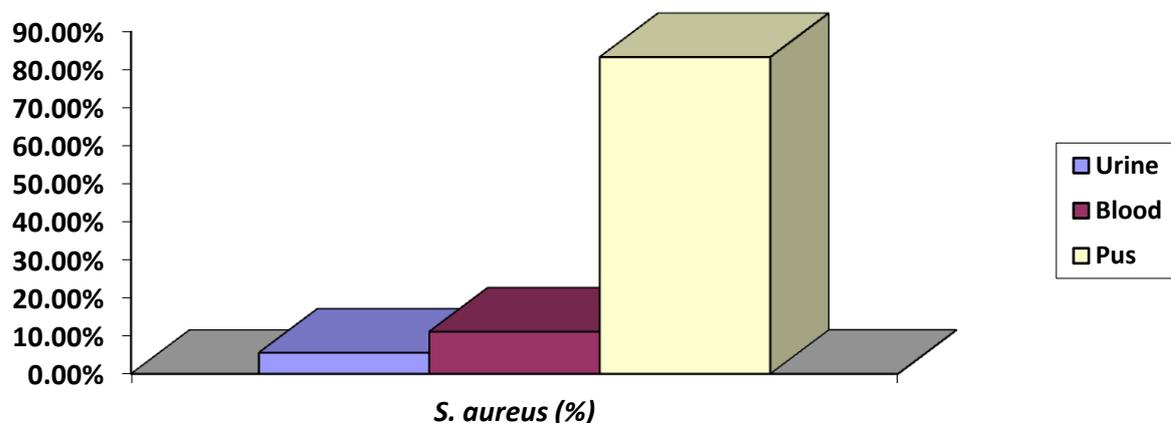


Fig 3: Distribution of *S. aureus* isolates in different sample sites

Among these isolates, 3 different phenotypic analyses used to evaluation of biofilm in *S. aureus* isolates. Out of the 36 isolates tested for biofilm formation by CRA method, none of these isolates produced black colonies. In contrast most literature showed a close similar report of CRA method of strong biofilm formation in *S. aureus* ranging from 1.32% - 56.5% [30;42-45]. However all strains 36 (100%) produced pink colonies, which were taken as negative for biofilm formation,

whereas non biofilm formation in *S. aureus* ranging from 30% - 94.74% reported similarity with our study. Out of the 36 isolates tested for biofilm formation by Tube method, 5 (13.89%) were showed strongly positive. The 11 (30.56%) isolates produced moderate biofilm formation. However, maximum number of isolates 20 (55.56%) did not show any biofilm production. By Tissue culture plate method (TCP), 12 (33.33%) were strongly positive for biofilm production, 8

(22.22%) were moderate biofilm producer, whereas 16 (44.44%) were negative (Fig 4). With respect to the observations made in the present study most literature report a similar result of Tube method of strong biofilm formation in *S.*

aureus ranging from 12.30% - 19% [30;42-44]. In respect of TCP, some other studies reported as a strong biofilm formation in *S. aureus* ranging from 14.40% - 20.10% *i.e.* less than our percentage *i.e.* 33.33% [30;42-45] (Fig 4).

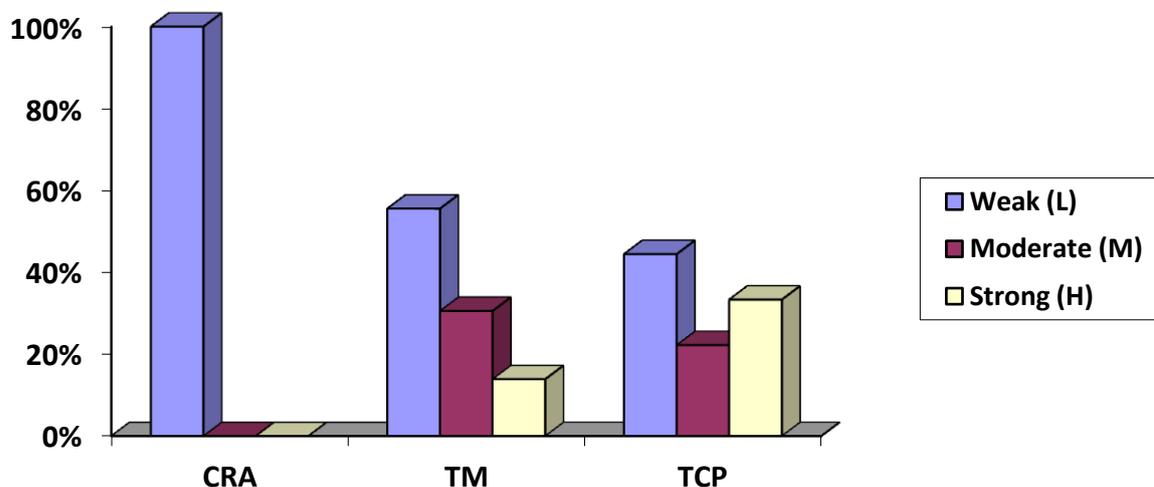


Fig 4: Biofilm ability of different *S. aureus* strains in CRA, TM, TCP methods

Antimicrobial activity of Minimal biofilm eradication concentration (MBEC) against biofilm forming *Staphylococcus aureus*

This study were evaluated the reliability of the *in vitro* biofilm model and the MBEC assay for antimicrobial susceptibility testing for bacterial biofilm in the anticipation that the MBEC would be more reliable for selection of clinically effective antimicrobials. MBEC values represent the lowest dilution at which bacteria fail to re-grow. The minimum biofilm eradication

concentrations (MBEC) of silver and selenium nanoparticles are summarized in Table 1. Result of the MBEC assay at 12.5µg/ml conc. of Ag nanoparticles showed less than 0.080 at OD₄₅₀ wavelength whereas 12.5µg/ml conc. of Se nanoparticles don't showed any less value than 0.080 against biofilm forming *S. aureus*. According our result for AgNPs, Mean±SD at OD₄₅₀ is 0.158±0.12, whereas Mean±SD of SeNPs at OD₄₅₀ is 0.266±0.08 *i.e.* not significant p≥0.47.

Table 1: Status of AgNPs & SeNPs according to Minimal biofilm eradication concentration (MBEC) against biofilm forming *Staphylococcus aureus*-

S. No	Biofilm forming <i>S. aureus</i> strains	OD ₄₅₀ value	
		AgNPs	SeNPs
1	SA12	0.310	-
2	SA15	0.075	0.264
3	SA20	0.019	0.382
4	SA22	0.039	0.316
5	SA23	0.093	0.243
6	SA24	0.140	0.114
7	SA25	-	0.272
8	SA26	0.264	0.252
9	SA32	0.323	0.282
Mean±SD		0.158±0.12	0.266±0.08

Ag-NPs and Se-NPs observed with Scanning electron microscopy (SEM)-

Particle shape and size of silver nanoparticles measured by Scanning electron microscopy (SEM) reveals spherical particles with 80.32 nm size for AgNPs (Fig. 5) whereas, rod shape

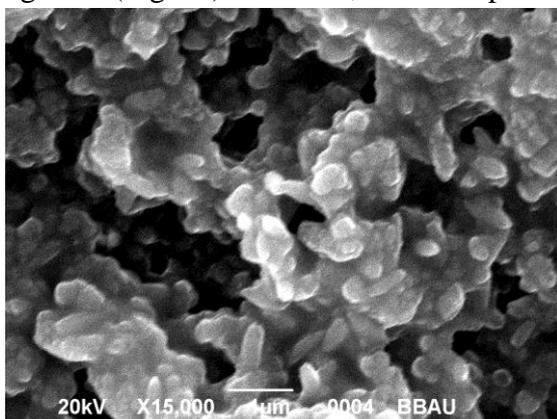


Fig 5: SEM image of synthesized Silver nanoparticles

particles with size 74.29 nm for SeNPs (Fig. 6). Smaller sized Ag and Se nanoparticles have many positive attributes, such as good conductivity, chemical stability, and catalytic and antibacterial activity, which would make them suitable for many practical applications.

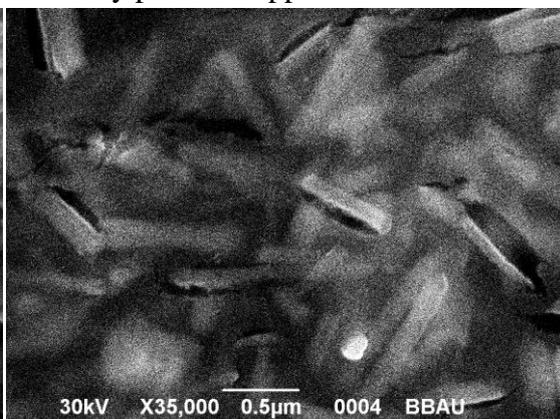


Fig 6: SEM image of synthesized Selenium nanoparticles

CONCLUSION

We concluded in our study that clinical isolates of *S. aureus* species have different capacity to formed biofilm. It might be caused by the differences in the expression of biofilm related genes, genetic make-up and physiological conditions. Three different biofilm forming *Staphylococcus aureus* strains for AgNPs having OD₄₅₀ values 0.019-0.075, *i.e.* ≤ 0.080 for Minimal biofilm eradication concentration (MBEC) assay whereas in case of SeNPs no any strain of *S. aureus* showed OD₄₅₀ values ≤ 0.080 . In this study Silver nanoparticles was more sensitive to selenium nanoparticles used for biofilm forming *S. aureus* by MBEC assay. Experiments for the Ag and Se nanoparticles surfaces effectiveness with the drugs are important, which will open new passages in medical biology. Also the quality of Ag and Se nanoparticles as a catalyst, and targeted drug-delivery vehicles is requisite.

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