RESEARCH ARTICLE

Study of Antimicrobial activity of Ag and Se Nanoparticles against Clinically Isolated Biofilm forming *Staphylococcus aureus*

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ABSTRACT

Introduction- Bacterial biofilm is a group of bacterial cells, covered by self-produced polymeric matrix film, which adheres very tightly to an inert or living surface constituting a protected mode of growth, allows survival in the unfavorable environment. Silver compounds have been used to treat burns, wounds, and infections. The effect of silver nanoparticles on the cell morphology of *Staphylococcus aureus* has been studied using SEM microscopy. Various salts of silver and their derivatives are used as antimicrobial agents against a wide range of pathogenic microbes. Selenium is an essential trace element. Selenium in the form of nanoparticles strongly inhibits the growth of *Staphylococcus aureus* on polycarbonate medical devices and no negative influence showed on osteoblastic cell growth.

Methodology- This study performed on thirty-six positive clinical samples of Urine, Blood and Pus. It was evaluated to phenotypic analysis test for biofilm forming *Staphylococcus aureus* strains by Congo red agar (CRA), Tube method (TM), and Tissue culture plate (TCP) method. Silver nanoparticles were synthesized by adopting chemical reduction, whereas Selenium nanoparticles (SeNPs) were synthesized by the reduction of sodium selenite by glutathione (reduced form). Antimicrobial activity was performed by well diffusion test of Ag and Se nanoparticles against biofilm forming *Staphylococcus aureus* strains.

Results- Distribution pattern showed the highest isolation rate from pus 30 (83.33%) followed by blood 4 (11.11%), and urine 2 (5.56%). Among the thirty-six positive samples screened for the present study, zero identified as biofilm-forming *S. aureus* and Intermediate, whereas all strains 36 (100%) showed as non biofilm-forming *S. aureus* by Congo red agar (CRA) method. Another method for Biofilm formation were performed by Tube method**,** 5 (13.89%) was strongly positive and 20 (55.56%) isolates unable to not shown any biofilm production, whereas via Tissue culture plate (TCP) method showed 12 (33.33%) high biofilm-producer while 16 (44.44%) were non biofilm producer. Ag-NPs showed morphology average size and shape with scanning electron microscopy (SEM) reveals spherical particles with the size of 80.32 nm whereas, Se-NPs showed the size of 74.29 nm with scanning electron microscopy.

Conclusion- For well diffusion inhibitory concentration test, 50 µl of the nanoparticles each (aqueous solution of silver and selenium) and 50 µl antibiotic (aqueous solution of amoxicillin as a positive control) were used. In this study amongst the two nanoparticles (Ag & Se) tested, silver nanoparticles were found to be the active inhibitory effects against biofilm forming *Staphylococcus aureus* strains (SA12, SA15, SA32), whereas selenium nanoparticles demonstrated the low inhibitory effects (SA23).

Key words- Microorganisms, Biofilm forming *Staphylococcus aureus,* SEM, Nanoparticles, Well diffusion test, Ag-NPs, Se-NPs

INTRODUCTION

Staphylococcus aureus is a gram positive cocci shape bacterium, is either commensal that colonizes healthy nasal mucosa (Williams, 1963) or pathogen of humans. The coagulase-positive species *Staphylococcus aureus* is well known as a human pathogen. Serious infections produced by *S. aureus*. Species of the *S. aureus* is identified on the basis of a variety of conventional physiological or biochemical tests. The key characters for *S. aureus* are colony pigment, heat-stable nuclease, clumping factor, free coagulase, and acid production from mannitol (Murray *et al*., 2003).

Clinical isolates of *Staphylococcus 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 (Verma and Maheshwari, 2017). *S. aureus* biofilm mode of growth is tightly regulated by complex genetic factors. Host immune responses against persistent biofilm infections are mainly ineffective and lead to chronic disease. However, last few decades studies have taken biofilm formation into the account in terms of elucidating host immunity toward infection and may lead to the development of efficacious anti-biofilm *S. aureus* therapies. A biofilm can be defined as a microbial derivative sessile community, typify by cells that are attached to a substratum, interface, are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with regard to growth, gene expression and protein production (Donlan and Costerton, 2002). Biofilm thickness can range from a single cell layer to a substantial community encased by a viscous polymeric milieu (Costerton et al., 1995).

Clinically isolated *Staphylococcus aureus* can express the *icaADBC*-encoded polysaccharide intercellular adhesin/poly-N-acetylglucosamine (PIA/ PNAG). The *icaADBC* dependent and independent pathways will be stimulated using different chemicals and level of biofilm formation as well as PIA/PNAG level will be assayed (Verma *et al*., 2013). In addition, proteomics and transcriptomics analysis will be performed to get insights in the interaction of various factors of the pathways involved in the biofilm formation in wild type as well as mutant strains (Verma *et al*., 2013). The biofilm formation in the MRSA is *ica* independent and involves a protein adhesin(s) regulated by *SarA* and *agr,* whereas *SarA*-regulated PIA/PNAG plays a more important role in MSSA biofilm development in *ica* dependent pathway. This further study will lead to the establishment of a comprehensive interactome of biofilm development (Verma *et al*., 2013).

In the human population, approximately 20-25% has become persistently colonized and 75-80% intermittently or never colonized (Kluytmans *et al.,* 1997; Dall'Antonia *et al*., 2005). Invading staphylococci are then either removed by the host innate immune response or attach to host extracellular matrix proteins and form a biofilm.

Nanoparticles have been widely used in various fields and the nanoparticles synthesized by the chemical processes are toxic in nature hence there is a growing need to develop environment friendly, cost effective and conveniently reproducible methods of nanoparticle synthesis. Furthermore, nanoparticles have increased surface area and therefore increasing the area of interaction with the pathogenic bacteria. They also are more likely to enter the bacterial surfaces than micron particles due to smaller size, exerting stronger effects on bacterial targets (Tran and Webstar, 2011).

The microbes have diversity in nature, which can be exploited usefully for the formation and harvesting of nanoparticles. These microorganisms when confronted with high concentrations of metal ions (like silver), they reduce them to their elemental state. The enzyme nitrate reductase reduces silver ion to the metallic silver. NADH dependant nitrate reductase enzyme is proven to be an important factor in the biosynthesis of metal nanoparticles, reducing silver metal ions to elemental silver nanoparticles. The possible mode of action can be the activity of nitrate reductase enzyme upon silver ion, when it is taken up by the cell and converting it to elemental silver (Kalimuthu *et al.,* 2008). Many biosynthesized metal nanoparticles like silver (Malarkodi *et al.,* 2013) are being used as antimicrobial compounds.

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 (Verma and Maheshwari, 2017).

In this context, selenium nanoparticles (SeNPs) possess antibacterial, antiviral and antioxidant properties, suggesting they could be suitable as therapeutic candidates to combat infectious diseases. In particular, nanostructured particles can be synthesized using bacterial and fungal cells as biological catalysts, providing a non-toxic and environmentally beneficial approach for the production of nanoparticles, including SeNPs (Xiangqian *et al*., 2011).

Selenium nanoparticles showed the highest bactericidal and antimicrobial properties. The antibacterial effects of silver and selenium nanoparticles were evaluated with respect to growth, biofilm formation of *Staphylococcus aureus* strains. 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 (Verma and Maheshwari, 2017).

METHODOLOGY

Collection of samples- Thirty-six positive strains of *S. aureus* isolated from clinical specimens (Urine, Blood, and Pus) at different hospitals of Dehradun, India and this study were also done in the department of Biotechnology, IFTM University, Moradabad, India.

Isolation and Identification of *Staphylococcus aureus*- *S. aureus* strains 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 37ºC for 24-48 hrs. Isolates bacteria were obtained from petri-plates were identified on the basis of cultural, morphological and biochemical characteristics as per as Bergey's Manual of Systemic Bacteriology (Holt *et al*., 1984).

Phenotypic Analysis of biofilm formation of *Staphylococcus aureus* strains

Congo Red Agar method- Freeman *et al.* 1989 had determined an alternative qualitative method of screening biofilm formation by *Staphylococcus* microbes, which need the use of a specially prepared CRA solid medium- Brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. 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 (non-biofilm) producers generally remained in pink, though occasional darkening at the centers of colonies was observed. A darkening of the black 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.* 1982. Ten 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 temperature. 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 a non-biofilm, moderate or high biofilm.

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 37^0C. 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 37^0C 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 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 (Christensen *et al.,* 1985).

Preparation and characterization of Silver and Selenium nanoparticles via SEM

Nanoparticles of the silver colloid were prepared by using chemical reduction method (Sileikaite *et al*., 2009) and Selenium nanoparticles (SeNPs) were synthesized (Tran and Webster, 2011) by the reduction of sodium selenite by glutathione (reduced form) and stabilized by bovine serum albumin (BSA). Nanoparticles (Ag and Se) were sterilized by ultra-violet light in laminar air flow. Scanning electron microscope (SEM) is a type of electron microscope that takes an image to the sample by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition, and other properties such as electrical conductivity. The sterilized Nanoparticles were carefully mounted on SEM stubs by using adhesive tape and uniformly coated with gold/palladium. The sample was placed in a sample chamber of SEM JEOL JSM-6490LV, Japan and scanning were performed under different magnifications ranging from 15,000x to 35,000x and voltage 20-30kV (Sileikaite *et al*., 2009; Razi *et al*., 2011). Scanning electron microscopy (SEM) reveals spherical with the size of particles were the range 80.32 nm for AgNPs whereas, rods shape with the size of particles were the range 74.29 nm for SeNPs.

Antibacterial assay of Nanoparticles (AgNPs & SeNPs) via Agar well diffusion against Biofilm forming *Staphylococcus aureus*-

After the preparation of silver and selenium nanoparticles were characterized, their antimicrobial activity was tested against the clinical pathogens *i.e. Staphylococcus aureus*. Petri plates containing 20ml Nutrient agar medium were seeded with 24hrs old culture of Biofilm forming *S. aureus* strains. Wells were cut and 50 µl of the nanoparticles each (aqueous solution of silver and selenium) and 50 µl antibiotic (aqueous solution of amoxicillin as a positive control) were added after that the plates were incubated at 37°C for 24 hrs. The nanoparticles (silver and selenium) activity was assayed by measuring the diameter of the inhibition zone formed around the well of Petri plates (NCCLS, 1993).

RESULTS AND DISCUSSION

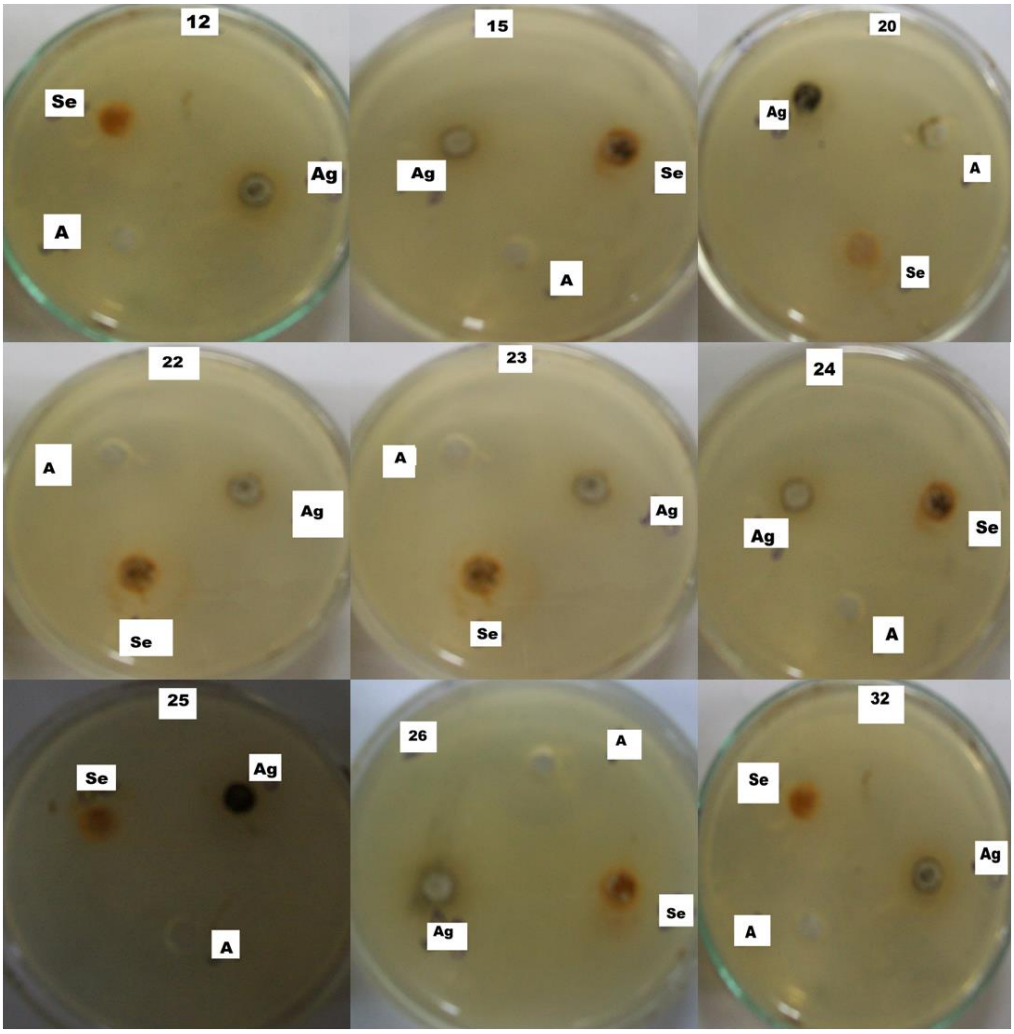
In the present study, the positive clinical samples were collected from different patients a high prevalence rate of bacterial 36 isolates was observed. *S. aureus* is the most frequent causes of nosocomial infection and infections on indwelling medical devices, which characteristically involved in Biofilms.

The antibacterial activity of silver and Selenium nanoparticles were tested against the Biofilm forming *Staphylococcus aureus* strains using the well diffusion assay. The measured diameter of the zone of inhibition (mm) is directly proportional to the potency of inhibition. For the antibacterial activity of these two Ag and Se nanoparticles were varied among the *S. aureus* species tested and showed an expected gradual result with same NPs concentrations (50 µl).

Out of the total 36 strains, only 9 strains (SA12, SA15, SA20, SA22, SA23, SA24, SA25, SA26, and SA32) of *S. aureus* species were shown zone of inhibition against

Table 1: Antimicrobial activity of AgNPs and SeNPs against biofilm forming *Staphylococcus aureus* strains

S. No.	Diameter of zone of inhibition (mm)			
	Biofilm forming *S. aureus* isolates	**AgNPs**	**SeNPs**	**Amoxicillin (Positive control)**
1	SA1 - SA11 SA13 - SA14 SA16 - SA19 SA21 SA27 - SA31 SA33- SA36	NO	NO	NO
2	SA12	25	NO	14
3	SA15	**27**	19	18
4	SA20	13	22	31
5	SA22	26	28	39
6	SA23	14	28	23
7	SA24	15	16	14
8	SA25	NO	18	22
9	SA26	17	18	23
10	SA32	26	17	24



A: Amoxicillin, Ag: Silver nanoparticle, Se: Selenium nanoparticle

Fig 1: Antimicrobial activity of nanoparticles (Silver and Selenium) against biofilm forming *S. aureus*

Ag and Se nanoparticles (Fig 1) and other remains 27 strains (SA1, SA2, SA3, SA4, SA5, SA6, SA7, SA8, SA9, SA10, SA11, SA13, SA14, SA16, SA17, SA18, SA19, SA21, SA27, SA28, SA29, SA30, SA31, SA33, SA34, SA35, and SA36) unable to shown zone of inhibition or not observed zone against nanoparticles (Ag and Se) so that we were further studied with respect to the 9 strains (SA12, SA15, SA20, SA22, SA23, SA24, SA25, SA26, and SA32) of *S. aureus.*

CLSI Standard for an antibiotic (Amoxicillin, AMX), Diameter of Zone of inhibition (mm) for resistant *i.e.* ≤19 mm, intermediate *i.e.* Not mentioned, and sensitive *i.e.* ≥20 mm. Among the 36 strains of *S. aureus*, only 8 strains (SA12, SA15, SA20, SA22, SA23, SA24, SA26, and SA32) showed antimicrobial activity of AgNPs and only 8 strains (SA15, SA20, SA22, SA23, SA24, SA25, SA26, and SA32) of SeNPs against biofilm forming *Staphylococcus aureus* strains (Table 1).
Amongst the two nanoparticles (Ag and Se) tested, silver nanoparticles were found to be the most active inhibitory effects against three biofilm forming *Staphylococcus aureus* strains (SA12, SA15, SA32), whereas in case of selenium nanoparticles demonstrated the most active only for one biofilm forming *Staphylococcus aureus* strain (SA23) that showed inhibitory effects. Some studies have documented a similar pattern of sensitivity of AgNPs among *Staphylococcus aureus* strains (Salomoni *et al.,* 2015) so that the antibacterial effect of the strains (SA12, SA15, and SA32) greater zone inhibition than Amoxicillin antibiotic for silver nanoparticles and *Staphylococcus aureus* strain (SA23) showed the greater zone inhibition than Amoxicillin antibiotic for selenium nanoparticles (El-Kheshen and El-Rab, 2012).

CONCLUSION:

In coming years, increasing bacterial Multi Drug Resistance against many types of antibiotics. It is becoming very difficult to control infectious diseases and cure patients, resulting in serious morbidity and mortality. Nanoparticles are a viable alternative to antibiotics and appear to have more potential to solve the problem of the emergence of Multi Drug Resistant bacteria. Antibacterial activities of nanoparticles depend on two main factors: (i) Type of microbes (ii) Physicochemical properties of NPs. Smaller sized silver and Selenium nanoparticles have numerous positive attributes, eg: Antibacterial activity, which would make them suitable for many practical applications. Although there are excellent trends of correlation in a small number of aspects of the antibacterial activity of nanoparticles (e.g., for biofilms), individual studies are difficult to generalize. Silver nanoparticles are one of the more attractive nanomaterials in commercialization applications. They have been usually used for antimicrobial, electronic and biomedical products. We suggest in our results, an effective way to prevent biofilm forming *S. aureus* infections using silver nanoparticles.

Conflicts of interest: The authors stated that no conflicts of interest.

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