Study of the antimicrobial effect of the silver nanoparticles against biofilm producing *Staphylococcus aureus* strains

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Abstract: *Staphylococcus aureus* is a leading cause of nosocomial infections and the etiologic agent of a wide range of diseases associated with significant morbidity and mortality. Some of the diseases mediated by this species include endocarditis, osteomyelitis, toxic shock syndrome, food poisoning, and skin infections. Biofilms are well organized and complex aggregate of microorganisms, surrounded by a protective matrix of exo-polysaccharides and can adhere to each other on various surfaces. Because of increase the drug resistance and the side effect of over use of antibiotic, it is necessary to find a suitable agent to reduce the growth of microorganism. Biofilm are known as a significant problem because biofilm formation protects pathogenic bacteria against antibiotics and is one of the main causes of development of chronic infections. Thus biofilm is the main cause of drug resistance in microbial strains. These strains cause pathogenicity to a maximum extent and thus cause resistance against the conventional antibiotics/drugs. Silver is known as an antimicrobial agent and is utilized in several antimicrobials and medications. Staphylococcus aureus strains were isolated and screened for biofilm production. In the present investigation, the nano-particles were prepared of Ag^{+}. The biosynthesized silver nanoparticles (AgNPs) were characterized by UV–Vis spectroscopy and Transmission electron microscopy (TEM). UV–Vis spectra of silver nano-particles showed absorption spectra at 450 nm corresponding to the surface plasmon resonance of silver nanoparticles. The size and morphology of the fused nanoparticles were determined by TEM, which shows the formation of spherical nanoparticles in the size range of 5–20 nm. The antibacterial activity of biosynthesized AgNPs, were evaluated by measuring the diameter of zone of inhibition against pathogenic microbial strains and drug resistant *Staphylococcus aureus*. It were also observed that, these nanoparticles were having prominent antimicrobial activity against biofilm forming *Staphylococcus aureus*.

Key words: Nanoparticles, antimicrobial activity, silver, drug resistant, biofilm forming *S. aureus*

I. INTRODUCTION

Staphylococcus aureus is now seen as an important opportunistic pathogen. This bacterium has become the leading cause of infections related to indwelling medical devices such as vascular catheters, prosthetic joints and artificial heart valves, mainly due to its capacity to form biofilms on such materials thus causing persistent or recurrent infections. Infections of medical implants material are associated with considerable morbidity and costs. These infections are very difficult to eradicate since bacteria in biofilms can be up to 1,000-fold more resistant to antibiotic treatment than the same organism growing planktonically [1]. Another problem is the ability of bacteria to acquire resistance to antibiotics therapy. This arises from the frequent use of antibiotics and mainly those of broad-spectrum. Only a few antibiotics are relatively active against *S. aureus* biofilms, and rifampicin, a transcription inhibitor, is among the most effective molecules for treating biofilm-related infections.

A biofilm is a structured community of bacterial cells that is enclosed in a self-produced polymeric matrix and adheres to an inert or living surface. Biofilm formation on implant surfaces increases the resistance to antimicrobial agents and leads to the therapeutic failure of conventional antimicrobial agents. Vancomycin diffuses slowly into the inner layers of bacterial biofilms; the gradual exposure of the bacterial cells to low concentrations facilitates the development of vancomycin resistance. It should be noted that even among the more susceptible isolates, subpopulations develop resistance and/or intermediate susceptibility to vancomycin after exposure [2]. Bacterial biofilms can be defined as an assemblage of microorganisms. Biofilm formation represents the normal lifestyle of bacteria in the environment, and that all microbes can make biofilms. Bacterial biofilm formation is a two step process- 1. Attachment of bacteria to substrate surface; 2. Formation of multiple layers of biofilm due to cell-cell adhesion [3]. The molecular mechanisms underlying the bacterial biofilm formation is complex and variations exist among different strains. Bacterial teichoic acids play important role in initial step of biofilm formation on medical devices like- Artificial surfaces. Nanoscience is the study of atoms, molecules and objects whose size is on the nanometers scale (1-100nm). Nanotechnology is science, engineering & technology

conducted at the nanoscale, which is about 1-100nm. Nanoscience & nanotechnology are the study and application can be used in other science fields. Nanoparticle research is currently an area of intense scientific interest due to a wide variety of potential applications in biomedical, optical and electronic fields. Nanotechnology may penetrate to biofilms and reduce biofilm formation [4-6]. The dose dependent effect of silver nanoparticles (in the size range of 10-15 nm) on the Gram-negative and Gram positive microorganisms has been studied. Action of silver nanoparticles may be due to the interaction of silver ions with nucleic acids and impairment of DNA replication which results in loss of cell viability and eventually resulting in cell death of bacteria. Silver nanoparticles have been evaluated for their antimicrobial activities against a wide range of pathogenic organisms [7-10].

II. MATERIALS AND METHODS

Collection of samples

Samples viz. Urine, Blood (in sterile sample bottles), and Pus (in Stuart transport medium) were collected. Samples were collected from different hospitals of Delhi-NCR.

Processing of samples

Samples were processed for the isolation and identification of the organism preferably within 2 hours after the collection from the hospitals. Samples were stored at 4ºC till further processing.

Isolation of *Staphylococcus aureus*

S. aureus cultures were isolated and maintained in Mannitol salt agar (MSA) at 37°C. Mannitol salt agar (MSA) is both a selective and differential medium used in the isolation of *staphylococci* strains.

Identification and determination of cultural characteristics of *Staphylococcus aureus*

The isolates were identified on the basis of cultural, morphological, and biochemical characteristics as per Bergey's Manual of Systemic Bacteriology [11].

Morphological characteristics

A suspected colony were collected from the plate and smear preparation were made on clean glass slide and Gram staining were performed and observed under 100 X objective lens of microscope.

Biochemical characteristics

Various biochemical tests were performed for the identification of *Staphylococcus aureus.* Following biochemical tests were performed:

a) Urea hydrolysis test

Three test tubes were prepared with urea (20 g/L), agar (15 g/L), NaCl (5 g/L), KH_2PO_4 (2 g/L) and phenol red (0.012 g/L) in slant position and were divided into sample, urea control and blank group. Another one test tube were prepared with the same reagents but without urea as bacteria control group. Isolates were streaked on the slant in sample and bacteria tubes. All these four test tubes were kept in 37^0C for 20-22h. Reddish pink or red color of test tube was regarded as positive [12].

b) Starch hydrolysis test

The enzyme amylase was excreted out of the cells (an exoenzyme) into the surrounding media, catalyzing the breakdown of starch into sugars. At first, one starch agar plate were picked up and divided in half. Then the isolates were incubated on the one plate in either a straight line or a zig-zag at 37^0C overnight. After incubation and growth, the plates were flooded with iodine. The appearance of yellow or gold zone around the growth indicated positive result.

c) Galactose hydrolysis test

About 3.7 mg Eosin- ethylene- blue (EMB) media was suspended in sterile distilled water, sterilized in autoclaving at 15 lb (121^0C) pressure for 15 minutes. The autoclave mixture were cooled to (45-50) ^{0}C, poured into sterile petridishes and checked overnight at 37^0C. On the next day growing isolates in Mueller-Hinton broth (MHB) were streaked on overnight checked EMB plates and plates were kept at 37^0C overnight. The appearance of bacteria as green metallic sheen to brown colored colony were regarded as positive result.

d) Oxidase test

Filter paper was soaked with 2 drops of freshly prepared oxidase reagent. Colonies of the test organism were smeared on the filter paper using glass rod. Positive oxidases were indicted by the production of a deep purple/blue color within 10 seconds [13].

e) Catalase test

A drop of hydrogen peroxide was placed on a glass slide. With the use of a sterile wire loop, colonies of the test organism were emulsified in the hydrogen peroxide. Catalase positive reaction was seen by their immediate production of bubbles.

f) Nitrate Reduction Test

Nitrate reduction medium were inoculated with the organism and incubated aerobically at 37^0C for 24 to 48 hours. After incubation, α-napthylamine and sulphanilic acid were added. These two compounds react with nitrite and turn red in color. In few tubes nitrate were reduced to ammonia or nitrogen gas. To distinguish between these two reactions, zinc dust were added. Zinc reduces nitrate to nitrite. The tubes will turn red because α-napthylamine and sulphanilic acid are already present in the tube. The test organisms will reduce nitrate. Bright red color after the addition of α-napthylamine and sulphanilic acid and no color change upon the addition of zinc were recorded as positive nitrate reduction test.

g) Carbohydrate Fermentation

In this test, a test tube with an inverted Durham's tube containing a basal medium and the particular sugar (1% conc.) along with a suitable indicator (Bromocresol purple) was used. The medium were inoculated with the help of a loop containing the test organism and then incubated aerobically at 37^0C for 24 to 48 hours. Change in color of the medium from purple to yellow indicates acid production and the fermentation of the particular sugar. The presence of air-bubble in the Durham's tube indicates gas production. The organisms were tested for sucrose, maltose and mannitol fermentation.

h) Indole hydrolysis

In this test tryptone broth were used containing large amount of tryptophan. In presence of Tryptophanase enzyme tryptophan will hydrolyze into an indole and pyruvic acid. The isolated organisms were inoculated in tryptone broth. All the inoculated and un-inoculated (control) tubes were incubated at 37 ± 0.2^0C for 48 h. After incubation, 5 drops of Kovac's reagent were added, a red layer at the top of broth indicates positive test where as no change in color indicated as negative test.

i) Methyl red test

The Methyl Red test involves adding the pH indicator methyl red to MR-VP broth. If the organism uses the mixed acid fermentation pathway and produces stable acidic end products, the acids will overcome the buffers in the medium and produce an acidic environment in the medium. The isolated microorganisms were inoculated in Methyl Red-Voges Proskauer broth. All the inoculated and un-inoculated (control) tubes were incubated at 37 ± 0.2^0C for 48 h. After incubation, 5-6 drops of methyl red reagent were added. Red color of medium indicates positive test, while no color change in the medium indicated negative result.

j) Vogues- Proskauer test

This test was performed to determine the capability of microorganism to produce non-acidic end products such as ethanol and acetoin (acetyl methyl carbinol) from the organic acid. The isolated microorganisms were inoculated in Methyl Red-Voges Proskauer broth. All the inoculated and uninoculated (control) tubes were incubated at 37 ± 0.2^0C for 48 h. After incubation, 12 drops of freshly prepared VP-reagent I (naphthol solution), 2-3 drops of VP-reagent II (40% KOH) were added in all the inoculated and un-inoculated tubes. Development of crimson to pink (red) color indicated positive test where as no change in color indicated as negative test.

k) Motility test

This test was performed to check the motility of the bacterium. Tube containing motility agar was stab inoculated. Positive test is indicated by the growth around the stab line that has radiated outwards in all directions while no growth around the stab line indicates negative test.

Phenotypic analysis of slime production of *Staphylococcus aureus*

a) Tube method

S. aureus isolates were tested for biofilm production by a modification of the standard method of (Christensen *et al.*, 1982). Two milliliters of trypticase-soy broth (TSB) in 12 x 75 mm borosilicate test tubes were inoculated with a loopful of microorganisms from overnight culture plates and incubated for 48 hours at 37°C, after which the contents were decanted and washed with PBS (pH 7.3) and left to dry at room temperature. Afterward, the tubes were stained with 4% solution of crystal violet. Each tube were gently rotated to ensure uniform staining and then the contents were gently decanted. The tubes were placed upside down to drain and then observed for biofilm formation which was considered positive when a visible film lined the wall and bottom of the tubes. Ring formation at the liquid interface will not be regarded as indicative of biofilm formation [14].

Preparation of silver nanoparticles

For the preparation of silver nanoparticles two stabilizing agents, sodium dodecyl sulphate (SDS) and sodium citrate were used. For the synthesis of silver nanoparticles, silver nitrate solution (from 1.0 mM to 6.0 mM) and 8% (w/w) Sodium Dodecyl Sulphate (SDS) were used as a metal salt precursor and a stabilizing agent, respectively. Hydrazine hydrate solution with a concentrate ranging from 2.0 mM to 12 mM and Sodium citrate (1. 0 mM to 2.0 mM) were used as reducing agents. Citrate of sodium was used as stabilizing agent at room temperature. The transparent colorless solution was converted to the characteristic pale yellow and pale red colour, when citrate of sodium was used as stabilizing agent. The occurrence of colour was indicated by the formation of silver nanoparticles. The silver nanoparticles were purified by centrifugation. To remove excess silver ions, the silver colloids were washed at least three times with deionized water under nitrogen stream. A dried powder of the nanosize silver was obtained by freeze-drying. To carry out all characterization methods and interaction of the silver nanoparticles with bacteria, the silver nanoparticles powder in the freeze-drying cuvette were resuspended in deionized water; the suspension were homogenized with a ultrasonic cleaning container [15].

Characterization of prepared nanoparticles via UV- absorption spectra and Transmission electron microscopy (TEM)

The formation of AgNPs and TR-AgNPs by the bioreduction of Ag+ to Ag0 was easily monitored using UV–Vis spectroscopy. The scanning was performed in the range of 200–700 nm. The morphology and size were determined by TEM.

Antimicrobial activity of silver nanoparticles against Biofilm forming *Staphylococcus aureus*

1. Agar well diffusion method

The agar well diffusion method was modified [16]. Nutrient agar medium (SCDM) is used for bacterial cultures. The culture medium is inoculated with the bacteria separately suspended in nutrient broth. A total of 8 mm diameter wells were punched into the agar and filled with silver nanoparticles. Standard antibiotic (Erythromycin 1 mg/ml) were simultaneously used as the positive control. The plates were incubated at 37^0C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. The procedure for assaying antibacterial activity was performed in triplicates to confirm the readings of diameter of zone of inhibition observed for each of the test organism.

2. Measurement of minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

Silver nanoparticles were added in LB medium, respectively. Each bacterium culture *Staphylococcus aureus* were controlled at 10^5-10^6 Cfu/mL and incubated at 37ºC. To establish the antimicrobial activity of nanoparticles on the bacterial growth, the minimum inhibitory concentration of nano particles for *Staphylococcus aureus* were determined by optical density of the bacterial culture solution containing different concentration of nano particles after 24h. All of the experiments (MIC) were triplicate, on three different days [17]. The minimum bactericidal concentration (MBC), i.e., the lowest concentration of nanoparticles that kills 99.9% of the bacteria were also determined from the batch culture studies. For growth inhibitory concentration (PMIC) the presence of viable microorganisms were tested and the lowest concentration causing bactericidal effect were reported as MBC as suggested [18]. To test for bactericidal effect, a loopful from each flask were inoculated on nutrient agar and incubated at 35ºC for 24 h. The nanoparticles concentration causing bactericidal effect was selected based on absence of colonies on the agar plate.

III. RESULTS AND DISCUSSION

In the present study, amongst 158 clinical samples collected, about 56 samples were found positive of *S. aureus*. The studies were performed to reveal the culture characteristics, gram staining and biochemical characteristics criteria. White colored colonies were observed on nutrient agar medium **(Figure 1a)** while yellow colored colonies were observed on mannitol salt agar medium **(Figure 1 b)**. Gram staining of the pure isolates showed cocci shape and was found to be gram positive **(Figure 1c)**. The study to reveal the bio-film producers from total positive 56 samples, showed the presence of 12 bio-film producers while rest 44 were found non-biofilm producing *S. aureus* strains. The results are shown in **Table 1** and **Figure 2**. The biochemical characteristics of the S. aureus strains

were determined. S. aureus isolates showed positive catalase, oxidase, nitrate, galactose and starch hydrolysis. The results are shown in **Table 2.** Further the phenotypic analysis of *S. aureus* was done for confirming positive and negative biofilm producers. The results showed, the high/potent; moderate and non biofilm producing strains as per the adhering capability of slimy biofilm layer within the test tubes when stained with 4% crystal violet dye. The results are shown in **Figure 3.** The silver nanoparticles were prepared as determined by the dark black colored solution from the colorless solution. The results are shown in **Figure 4.** The sizes of the nanoparticles were determined by SEM as 2µm. The results are shown in **Figure 5**. Antimicrobial susceptibility of the conventional antibiotics was determined against conventional antibiotics **(Figure 6** & **Table 3).** Antimicrobial activities of the silver nanoparticles were determined at different concentrations (5 and 10 ppm) against biofilm producing S. aureus strains. The results are shown in **Figure 7** and **Table 4**. The results of the present study are in correlation with the previous findings [19-23].

Further studies showed the antibacterial potential of silver nanoparticles against *V. cholerae* and *E. coli* [24]. Silver nanoparticles were investigated antimicrobial against *Pseudomonas aeruginosa* [25]. The shape of nanoparticles influences the aggression of antibacterial behavior [26].

IV. CONCLUSION

The proposed study attempts to describe the production of biofilm formation. Preparation of nanoparticles and its evaluation for antibacterial potential against biofilm producers and biofilm will leads to the stepping stone in area of research in Nano-biotechnology and medical Microbiology. This will lead to the establishment of possible therapeutic approaches to deal with resistant and sensitive strains of *Staphylococcus aureus*. The present study showed that the silver nanoparticles (AgNp) had prominent antibacterial activity against biofilm producing *S. aureus* strains in comparison to different antibiotics. The study thus illustrates the new prospects in area of nano-biotechnology through which different nano particles can be created and can be utilized in eradication of drug resistant and sensitive strains of *S. aureus*.

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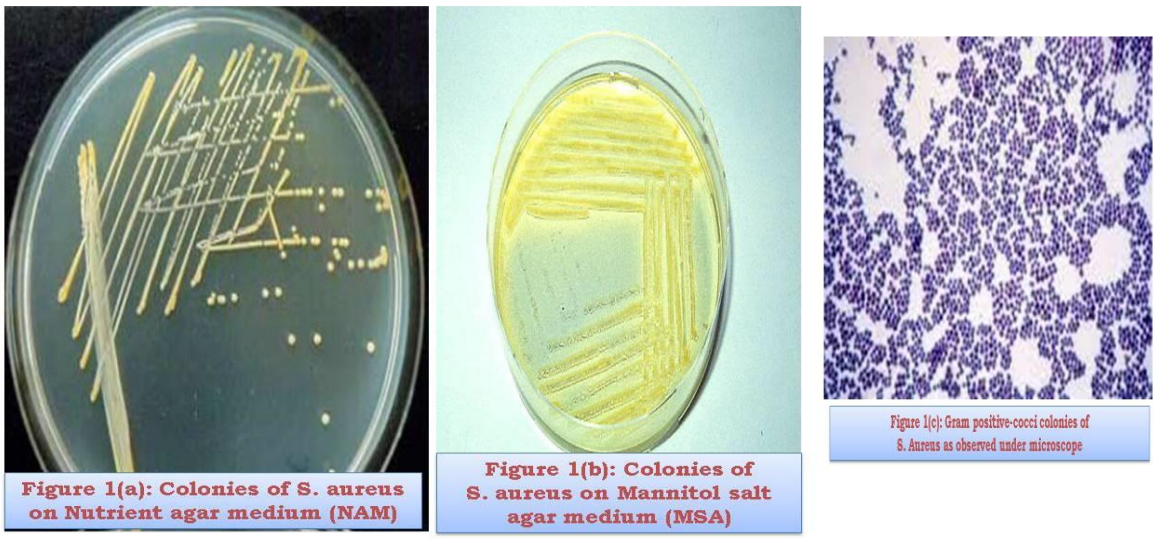

Figure 1 (a-c): Isolation of *Staphylococcus aureus* strains on Nutrient agar medium (NAM) and Mannitol salt agar medium (MSA)

Table 1: Number of biofilm and non biofilm S. aureus strains isolated

Total Positive Samples	Biofilm-forming *S. aureus*	Non-biofilm forming *S. aureus*
56	12	44

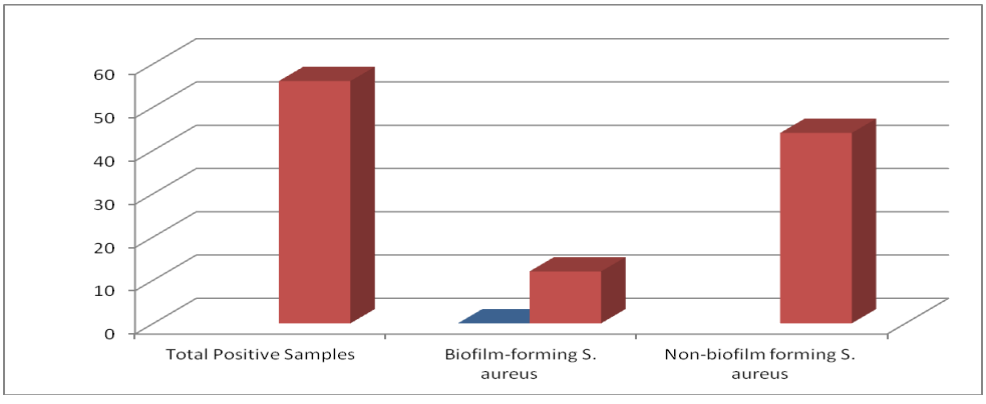

Figure 2: Prevalence of biofilm and non biofilm S. aureus strains isolated

Table 2: Biochemical tests of S. aureus positive colonies

Biochemical Tests	Result
Catalase	+
Oxidase	+
Nitrate	+
Galactose hydrolysis	+
Starch hydrolysis	+
Indole	-
M R	-
V P	-
Citrate	-
Motility	-
Urease	-

Figure 3: Screening of Biofilm and non film producing strains of *S. aureus*

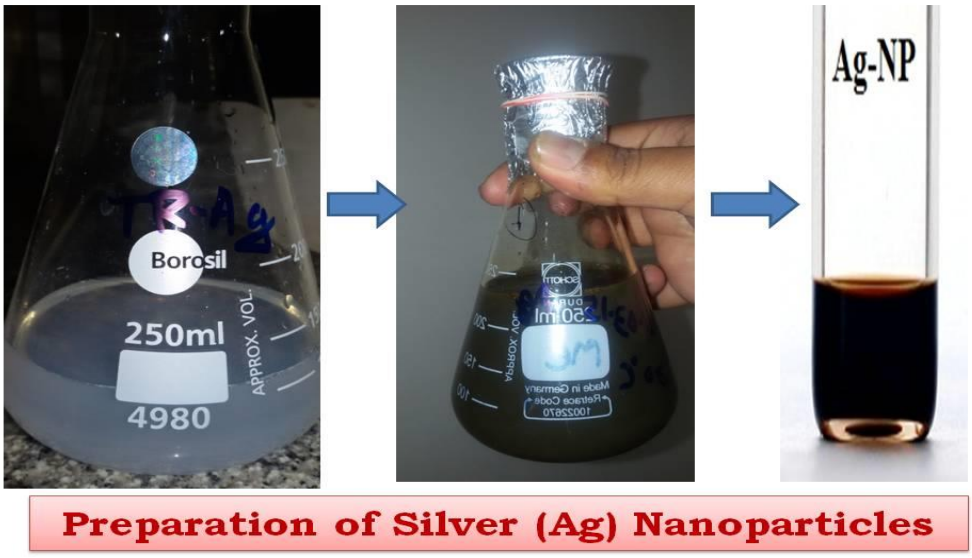

Figure 4: Preparation of silver nanoparticles (Ag-Np)

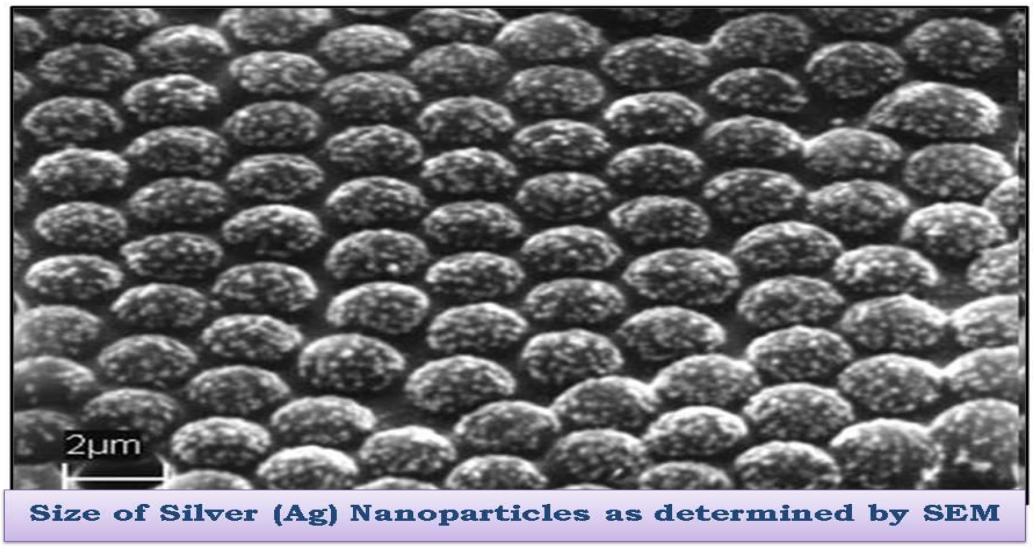

Figure 5: Size determination of silver nanoparticles (Ag Np) by SEM

Table 3: Antimicrobial susceptibility of conventional antibiotics against biofilm producing S. aureus

Biofilm forming S. aureus strains	Antibiotic	Conc. (µg)	Diameter of zone of inhibition (mm)
Strain 1	**Ofloxacin (Of)**	**30**	34
Strain 2	**Erythromycin (Er)**	**30**	36
Strain 3	**Amoxicillin (Am)**	**30**	25
Strain 4	**Ciprofloxacin (Cf)**	**30**	38

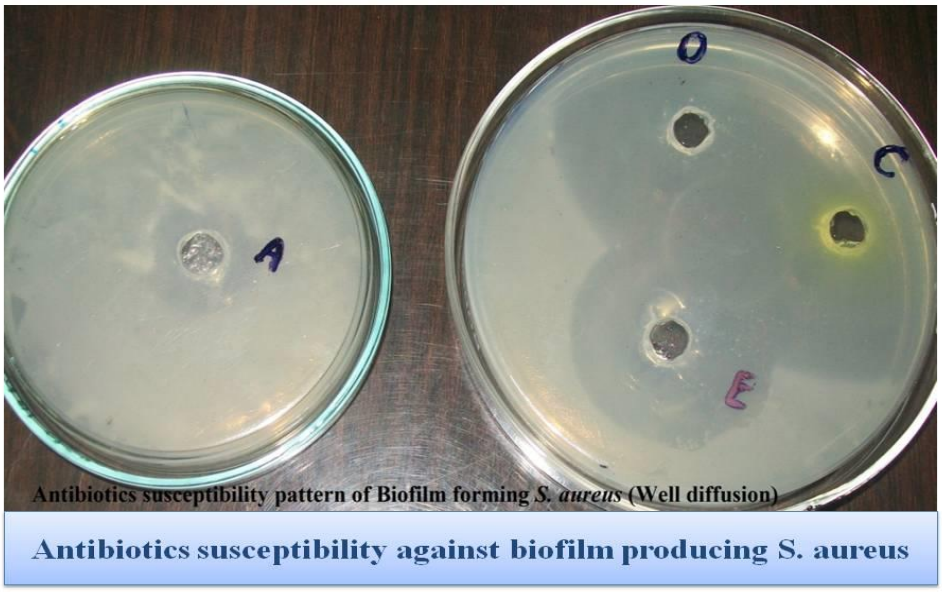

Figure 6: Antimicrobial susceptibility of conventional antibiotics against biofilm producing S. aureus

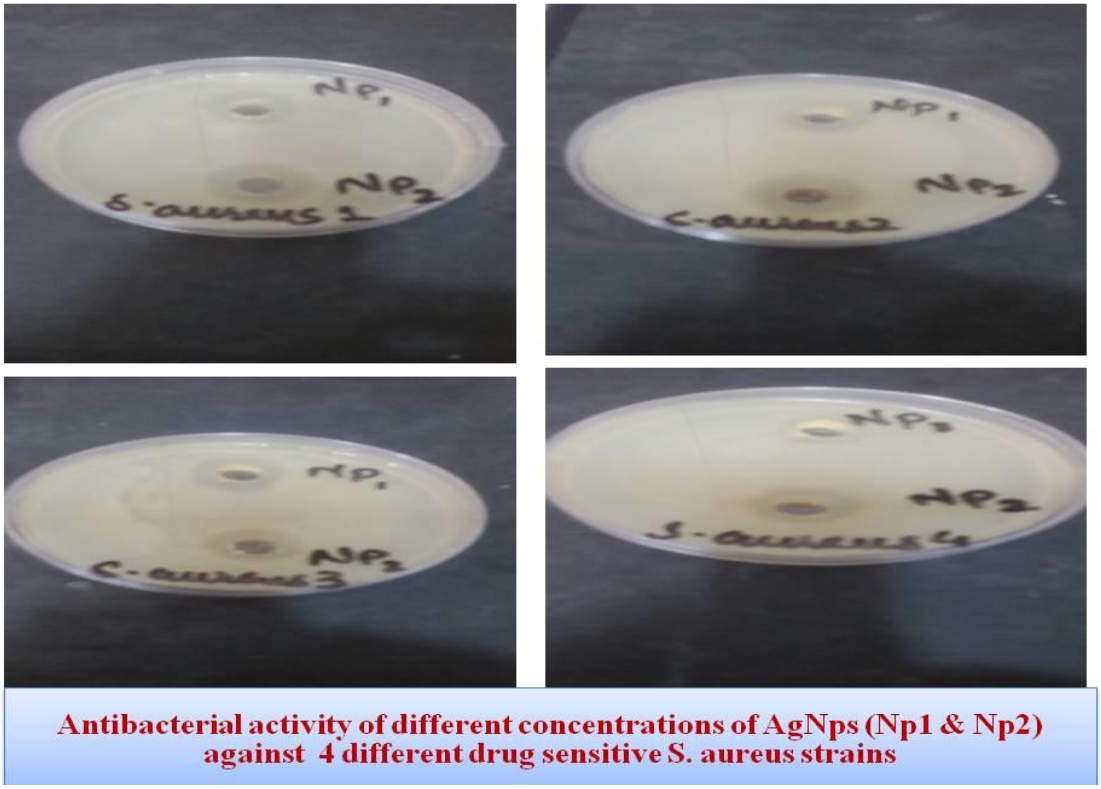

Figure 7: Antimicrobial activities of silver nanoparticles at 5 ppm (AgNp1) and 10 ppm (AgNp2) against biofilm producing S. aureus

Table 4: Antimicrobial activities of silver nanoparticles at 5 ppm (AgNp1) and 10 ppm (AgNp2) against biofilm producing S. aureus

Biofilm forming S. aureus strains	Diameter of zone of inhibition (mm) of AgNp-1 @ 5 ppm	Diameter of zone of inhibition (mm) of AgNp-2 @ 10 ppm
Strain 1	18	27
Strain 2	15	26
Strain 3	20	28
Strain 4	15	25