Silver Nanoparticles as a Non Alcoholic Hospital Disinfectant to Combat Nosocomial Pathogens

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SILVER NANOPARTICLES AS A NON ALCOHOLIC HOSPITAL DISINFECTANT TO COMBAT NOSOCOMIAL PATHOGENS

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ABSTRACT

Common disinfectants used in hospital settings include alcoholic disinfectants which are required to be used in high concentrations, possess a strong odour and cause allergic reactions. Since the antibacterial activity of silver nanoparticles (AgNPs) is well established, this study explores AgNPs as a non- alcoholic disinfectant against five common nosocomial pathogens – *S. aureus, E. coli, A. baumanii, S. pneumoniae* and *K. pneumoniae*. The antibacterial and disinfectant activities of AgNPs were tested over a range of concentrations for a short duration of 60 seconds. Determination of Minimum Inhibitory Concentration (MIC) and kill kinetics suggest that AgNPs have strong antibacterial activity with MIC ranging from 3.4µg for *S. aureus* and 66.66 µg for *K. pneumoniae*. Disinfection testing revealed high log reduction in colony forming units (CFU) by Surface contact assay and Surface disinfection assay and a phenol coefficient of 3.3 for *S. aureus, E. coli, S. pneumoniae* and *K. pneumoniae* and 5.5 for *A. baumanii*, thus indicating effective disinfection by AgNPs within 60 seconds. Analysis of mechanism of action by Scanning Electron Microscopy (SEM) and Propidium iodide (PI) uptake of bacterial cells revealed distorted morphology of treated cells and an average PI uptake of 54.79% at 10µg in 60 seconds indicating a loss of membrane integrity. Toxicity testing by MTT assay and Ames test revealed no cytotoxicity or mutagenicity on treatment with 100 µg AgNPs for 60 seconds. Overall, AgNPs can be a prospective disinfectant due to its activity at low concentration and time of exposure provided its toxic effects are well established.

KEY WORDS: Silver nanoparticles, Nosocomial infections, Antimicrobial effects, Disinfectant, Propidium Iodide uptake, Toxicity.

1. INTRODUCTION

Metallic nanoparticles have always been a highly useful tool in the field of biomedical sciences and engineering. Presently, nanoparticles of iron oxide, silver, gold and zinc are most widely studied [1]. AgNPs are particles of silver with size between 1 and 100 nm that are currently being used in several technological applications and are gaining popularity as a form of counter measure against several illnesses that cannot yet be treated by conventional means. Green synthesized AgNPs have been reported to have activity on various multi drug resistant bacteria [2]. Efficacy of AgNPs against dental infections like apical periodontitis has also been reported[3] and their relative low cost of manufacturing is a huge advantage in production of consumer materials like soaps, cosmetics and textiles thus increasing their market value. [4]

It has been observed across Europe and North America that nosocomial infections occur in 5%-10% of all hospitalizations.[5] While the impact of the infections varies according to the type of infection, site of infection in the body, age, etc, they all result in prolongation of hospital stay by various degrees. According to Hospital Infection Society of India (HIS), a significant 10-30% of hospitalizations in the country result in nosocomial infections. A study from 7 Indian cities revealed that 4.4% of patients admitted acquired nosocomial infections with 9.06 infections per 1000 ICU days. [6]

Hand hygiene is the principal factor in the prevention of nosocomial infections. On the other hand, compliance with recommended hand hygiene programs by hospital staff is quite low.[7] In fact, it has been reported that poor hand hygiene is responsible for 40% of infections transmitted in hospitals.[8] At present, prevention of nosocomial infections is centred on rational use of antibiotics and sterilization and disinfection of the hospital environment.

However, it has been reported that methicillin resistant *S. aureus* and *Enterococcus* sp exhibit resistance to certain commonly used disinfectants.[9] This highlights the need for the use of appropriate antibiotics and broad spectrum disinfectants.

To be acceptable in the hospital environment, disinfectants must act independently of the number of bacteria and must also be easy to use, non-volatile, not harmful to staff, patients and equipment, free from unpleasant smells and should be effective within a relatively short time.[10] Concentration of the disinfectant used and duration of action appear to play a major role in its effectiveness.[11]

Silver based nanomaterials exhibit strong bactericidal effect on many species of bacteria while exhibiting low toxicity towards animal cells. AgNPs are fast acting [12] and effective at quite low concentrations.[13] These characteristics make silver nanoparticles (AgNPs) an attractive candidate as a prospective disinfectant that can be used to prevent nosocomial infections.

In this study, the antimicrobial activity of AgNPs has been analysed over short time periods of 30 seconds and 60 seconds and low concentrations against five common nosocomial pathogens – *S. aureus*, *E. coli*, *A. baumanii, S. pneumoniae* and *K. pneumoniae.* AgNPs were evaluated by common disinfectant tests used to determine its effectiveness as a disinfectant and the mechanism of action of AgNPs against bacterial cells has also been explored. MTT assay was performed to check the toxic effects of AgNPs against normal mouse fibroblast cells.

2. EXPERIMENTAL SECTION

2.1 Synthesis of AgNPs

AgNPs were synthesised by a method previously reported in literature. [14,15] Briefly, calculated amounts of silver nitrate, CTAB (Cetyl trimethyl ammonium bromide), and

sodium borohydrate were weighed and were dissolved in double distilled water under constant stirring on a magnetic stirrer. Solution of silver nitrate and CTAB were mixed well for few minutes. Then under vigorous stirring sodium borohydrate solution was added drop wise to the mixed solution of silver nitrate and CTAB. The solution changed from transparent to dark brown colloid, indicating the formation of silver nanoparticles. Figure 1 depicts the protocol for preparation of AgNPs and Figure 2 illustrates the scheme for AgNP formation. The samples were then centrifuged at 10000 rpm for three times to wash the unreacted silver nitrate, CTAB and sodium borohydrate from AgNPs. The centrifuged colloidal solution of AgNPs was subjected to UV Visible absorbance studies to observe the formation of silver nanoparticles, Zeta potential analysis to analyse the surface charge and stability of the formed nanoparticles and HRTEM (High Resolution Transmission Electron Microscopy) to study the size and shape of the nanoparticles.

EFFICACY TESTING

2.2 Determination of minimum inhibitory concentration (MIC):

MIC of AgNPs against *S. aureus, E. coli, A. baumanii, S. pneumoniae* and *K. pneumoniae* was determined by preparing three-fold dilutions of AgNPs in a 96 well plate with sterile nutrient broth. 50µl of bacterial culture (10^6 cells/ml) was added to each well and incubated for 24 hours at 37°C. Four replicates were prepared for each bacterial species. The MIC was then determined by measuring bacterial growth at optical density ($OD_{600)}$ using a themoscan spectrophotometer.[16] All antimicrobial susceptibility tests were performed in accordance with the standards prescribed by Clinical and Laboratory Standards Institute- M7 A7.[17]

2.3 Time kill Assay:

The antibacterial efficacy of AgNPs was determined by time kill assay. 50µl of bacterial culture of *S. aureus, E. coli, A. baumanii, S. pneumoniae* and *K. pneumoniae,* were

treated with AgNPs of 1X MIC and 3X MIC for 60 seconds. At the end of the contact period, the cells suspensions were spread on sterile nutrient agar plates and incubated at 37°C for 24 hours. Three replicates were prepared for each concentration and antibacterial activity of AgNPs was estimated in terms of log reduction in colony forming units (CFUs) in comparison to untreated bacterial suspension.[18] Sterill® disinfectant solution (70% v/v of 2-propanol) was used as control agent.

MECHANISM OF ACTION

2.4 Cell morphology analysis using scanning electron microscope:

Morphology of bacterial cells treated with AgNPs was analysed by SEM. 10^6 cells of *A. baumanii* was added onto Whatmann No.1 filter paper and incubated at 37°C for 24 hours to allow biofilm formation. 11.11 µg of AgNPs (MIC of *A. baumanii*) was added onto the filter paper. After 60 seconds incubation, they were fixed by treatment with 2.5% gluteraldehyde for 20 minutes followed by an ethanol gradient.[19] The samples were coated with gold for 15 minutes. The changes in cell morphology were observed using a Zeiss Scanning Electron Microscope under a magnification of 34 KX.

2.5 Analysis of membrane integrity by propidium iodide (PI) uptake:

In order to analyse membrane damage caused by AgNP treatment, treated cells were incubated with the DNA-binding dye PI, which cannot pass through intact membranes. *S. aureus, E. coli, A. baumanii, S. pneumoniae* and *K. pneumoniae* cells were treated with AgNPs (2.5µg, 5µg, 7.5µg, 10µg, 50µg and 100µg) and 5µg of PI for 30 seconds and 60 seconds. Upon membrane damage, uptake of PI by the cells results in increased fluorescence intensity which was estimated by flow cytometry.[20] Four replicates of each concentration were prepared and Sterill® disinfectant solution (70% v/v - 2-propanol) was used as control agent.

DISINFECTION TESTS

QUALITATIVE TESTING OF DISINFECTANTS

2.6 In Use test

400µl of each bacterial culture of *S. aureus, E. coli, A. baumanii, S. pneumoniae* and *K. pneumoniae* were suspended in 500µl of sterile nutrient broth in sterile microfuge tubes. 100µl of AgNPs was added to each tube and mixed well. For positive control, 100µl of Sterill® disinfectant solution was used. From each tube, 20µl of suspension was placed at ten different spots on nutrient agar plates. Triplicates of each sample were prepared. The agar plates were incubated at 37°C for three days. At the end of incubation period, the spots were observed for bacterial growth. Compounds resulting in bacterial growth at less than five spots out of ten were considered to show acceptable disinfection. [21]

2.7 Kelsey Sykes test

200µl of AgNP suspension (500µg) was made up to 1ml with sterile water in a 15 ml tube. 10^6 cells of *S. aureus, E. coli, A. baumanii, S. pneumoniae* and *K. pneumoniae* were added to the AgNP suspensions and incubated at room temperature for eight minutes. At the end of the incubation period, 10 µl of the suspension was drawn and placed at five different spots on a sterile nutrient agar plate. This plate was labelled as 'Challenge 1'. This process was repeated two more times with a total of three challenges. The agar plates corresponding to each challenge were incubated at 37°C for 24 hours and observed for bacterial growth. The plates showing growth in less than three spots out of five were considered to pass the challenge. The compound that passes the three challenges is considered to pass the Kelsey – Sykes test.[21]

QUANTITATIVE TESTING OF DISINFECTANTS

2.8 Surface Contact Assay:

Bacterial cultures (10^6 cells) were treated with AgNPs (1X and 3X of MIC) for 60 seconds. The treated bacterial suspension was spread on sterile glass slides and allowed to

dry. 3ml of nutrient agar was aliquoted onto these slides and the slides were incubated at 37°C for 24 hours. Three replicates of each concentration were prepared and disinfectant efficacy of AgNPs was estimated by log reduction in CFUs of AgNP treated bacterial culture in comparison to untreated bacterial culture.[22] Sterill® disinfectant solution (70% v/v - 2-propanol) was used as control agent.

2.9 Surface Disinfection Test:

500µl of *S. aureus, A. baumanii, E. coli, K. pneumoniae* and S. *pneumoniae* cultures were aliquoted to into a sterile tube. 500µl of this bacterial suspension (10^6 cells/ml) was spread on a sterile ceramic tile and allowed to dry for 1 hour at room temperature. The tile was then swabbed with 1ml and 5ml of AgNPs (2.5mg/ml) and allowed a contact period of 10 min. The residual bacterial cells on the ceramic tile after AgNP treatment were harvested by swabbing the surface of tile and vortexing the swab in 10 ml of neutralizing broth. This suspension was spread on sterile nutrient agar and the disinfectant efficacy estimated by log reduction in CFUs.[23] Three replicates of each concentration were prepared and Sterill® disinfectant solution (70% v/v - 2-propanol) was used as control agent.

2.10 Determination of phenol coefficient (PC)

Dilutions in sterile nutrient broth were made from 5% (v/v) phenol and 1X MIC of AgNPs. 10µl of bacterial culture was added to each tube containing the final dilutions of AgNPs and phenol and allowed a contact time of 5 minutes and 10 minutes. At the end of the contact period, 10µl of the suspension was placed at five different spots on a sterile nutrient agar plate to check for the presence of bacterial growth. Five replicates of each dilution were prepared and these plates were incubated at 37°C for three days. The PC value was then determined by dividing the highest dilution of AgNPs that killed the test organism in 10 minutes, by the highest dilution of phenol for the same.[24]

TOXICITY TESTING

2.11 Cytotoxicity testing by MTT Assay:

L6 mouse fibroblast cells were seeded in a sterile culture flask with DMEM (Dulbecco's Modified Eagle's Medium) with 10% FBS until a confluent culture was obtained and the cells were harvested. 200µl of cell suspension carrying 20,000 cells was seeded into 96 well plates and incubated at 37°C with 5% CO_2. After 24 hours, the cells were treated with 10µg, 20µg, 40µg, 60µg and 100µg of AgNPs for 60 seconds. Five replicates of each concentration were prepared and 0.1% triton X-100 was used as control agent. At the end of the time period, 20µl of MTT dye (5mg/ml in PBS) was added and the plates were incubated at 37°C for 3 to 4 hours to allow the formazan production formation by MTT reduction. After 4 hours, the suspension in each plate was discarded and formazan crystals were dissolved by adding 200µl of DMSO. The OD was measured at 570nm and 695nm by a thermo scan spectrophotometer. The difference in OD between the two wavelengths was estimated and the cell viability was calculated from the following formula .[25]

$$\text{Cell viability (\%)} = \frac{\text{OD of treated cells}}{\text{OD of untreated cells}} \text{ X } 100$$

2.12 Testing for mutagenicity by Ames test

100µl of overnight cultures of *Salmonella typhimurium* – strains TA 98 and TA 100 (10^6 cells/ml) were added to 500µl of 0.1mM phosphate buffer (1.2mg sodium dihydro orthophosphate, 1.7 mg disodium hydrogen orthophosphate in 100 ml sterile water) and treated with 100 µg of AgNPs and incubated at 37°C for 60 seconds. The controls and AgNP-treated cells were mixed well with 2 ml of sterile soft agar (0.6% agar and 0.6% NaCl containing 0.5 mM histidine and 0.5 mM biotin) and evenly spread onto minimal glucose agar plates (1× Vogel-Bonner salts (0.2 g/L magnesium sulfate, 2 g/ L citric acid monohydrate, 10 g/L dipotassium hydrogen phosphate, and 3.5 g/L sodium ammonium phosphate, 2% glucose, and 1.5% agar).[26] 5µg of mitomycin C and 2µg of sodium azide

served as control agents for TA 98 and TA 100 respectively. Three replicates of each concentration were prepared and the plates were incubated at 37˚C for 48 hours after the numbers of revertant and surviving colonies were counted.

2.13 Data Analysis

Excel 2010 (Microsoft, Redmond, WA, USA) was used to analyze the data. The data from each assay were expressed as mean± standard deviations. Statistical analysis of the results obtained was performed by the Students t-test and *p* value was obtained using MedCalc®13 statistical software. Statistical significance was accepted at $p<0.05$.

3. RESULTS AND DISCUSSION

CHARACTERISATION OF THE AgNPs:

3.1 UV Vis absorbance studies:

UV Visible studies confirmed the formation of silver colloids. Due to surface plasmon resonance (SPR) colloidal silver nanoparticles show strong absorbance between 390 – 420 nm. The UV Vis spectra shown in Figure 3 clearly indicate the SPR peak around 400 nm, which is the SPR absorbance peak for silver nanoparticles. The sharp peak indicates the narrow size distribution of silver nanoparticles. Moreover the SPR peak around 400 nm denotes that the particles are nearly spherical in shape. The narrow size distribution and controlled shape of the nanoparticles was achieved by tuning the reducing agent (sodium borohydrate) and surfactant (CTAB).

3.2 Zeta potential analysis:

Zeta potential analysis was then performed to study the particle stability and surface charge. According to reports, the colloids possessing zeta potential values above ±30 mV are said to be good stable colloids. The zeta potential value for the prepared silver nanoparticles was found to be +54 mV as shown in Figure 4. The zeta potential value from the spectrum

states that the surface charge of the particles is positive and is highly stable. The stability and surface charge is attributed to the cationic surfactant (CTAB) used in the reaction.

3.3 HRTEM analysis:

HRTEM micrograph in Figure 5 shows that the AgNPs are in the size of 10 nm and is almost spherical. The SAED (Selected Area Diffraction) pattern indicates that the particles are single crystalline. The control in size and shape of the silver nanoparticles are due the cationic surfactant (CTAB) used in the reaction.

The yield of AgNPs obtained was 125mg.

EFFICACY TESTING

The antibacterial efficacy of AgNPs was analysed by determining the minimum inhibitory concentration of AgNPs against *S. aureus. E. coli, A. baumanii, S. pneumoniae* and *K. pneumoniae* and also by determining the killing kinetics of AgNPs against each of the strains.

3.4 Minimum Inhibitory Concentration

The minimum inhibitory concentration of AgNPs was estimated against *S. aureus, E. coli, A. baumanii, K. pneumoniae* and *S. pneumoniae.* The Spectrometric analysis of the treated samples revealed the MICs to be ranging from 3.7µg to 66.66µg with an average MIC of 19.93µg. Figure 6 displays the trend in OD values at different dilutions of AgNPs. The individual MICs against each organism were found to be 3.7 µg for *S. aureus,* 7.4µg for *S. pneumoniae,* 11.11µg for *E. coli,* 11.11µg for *A. baumanii,* and 66.66µg for *K. pneumoniae.* According to these MIC values, it could be inferred that the bacterial strains showed diverse sensitivity. *S. aureus* was found to have maximum sensitivity to AgNPs followed by *S. pneumoniae* while *K. pneumoniae* appeared to have the least sensitivity.

3.5 Time Kill Assay

The efficacy of AgNPs against each species was estimated by the time kill assay wherein the reduction in CFU was determined on AgNP treatment. As the study explores

AgNPs as a prospective disinfectant, the time of exposure of cells to AgNPs was restricted to 60 seconds. Here, the CFUs of the bacterial strains- *S. aureus, E. coli, A. baumanii, K. pneumoniae* and *S. pneumoniae* were rapidly reduced on treatment with AgNPs for 60 seconds. AgNPs were tested at 1X and 3X MIC for each bacterial strain. However, in case of *E. coli*, 20X of MIC was required to bring a significant log reduction. AgNPs had the best kill kinetics against *S. aureus* with > 6 log reduction in CFU at a concentration of 3X of MIC. *A. baumanii, S. pneumoniae* and *K. pneumoniae* showed similar kill kinetics with > 5 log reduction in CFU. Table 1 summarizes the log reduction in CFUs of each organism. $p<0.05$ was obtained in all strains tested. The efficacy testing of AgNPs revealed that antibacterial effects of AgNPs could be observed at concentrations as low as 3.4µg. Also, the kill kinetics resulted in high log reduction in CFU in case of all strains tested. From this, we could conclude that the AgNPs showed antibacterial efficacy at an exposure time of 60 seconds. Previous studies have also reported high log reduction in CFU on treatment of bacterial cells with AgNPs.[18] At 1X MIC, *E. coli* was found to have the least log reduction of 2.04 while *K. pneumoniae* was found to have the highest log reduction of 2.84. These findings indicate that even though the MIC value of *K. pneumoniae* was highest, it was more responsive to action of AgNPs. The log reduction values of *E. coli* and *A. baumanii* in time kill assay was found to vary significantly even though their MICs were found to be equal. To identify the cause of the high log reduction observed, the mechanism of action of AgNPs was explored.

MECHANISM OF ACTION

The mechanism of action of silver nanoparticles is a debatable topic. Most studies indicate loss of membrane integrity, formation of free radicals or release of silver ions form AgNPs.[27] In this study, mechanism of action was analysed by cell morphology analysis and Propidium Iodide uptake of AgNP treated cells.

3.6 Analysis of cell morphology under SEM

Morphology of *A.baumanii* cells treated with AgNPs for 60 seconds were observed under SEM and analysed for alteration in cell membranes. While untreated cells showed intact cell membranes and normal morphology, cells treated with AgNPs showed pits in the cell membrane associated with loss of membrane integrity as shown in Figure 7. Previous studies have also reported similar findings [28] indicating loss of membrane integrity.

3.7 PI uptake

Loss of membrane integrity was confirmed by estimation of PI uptake of AgNP treated cells. PI, a nucleic acid binding dye excluded by intact cells, is taken by cells with compromised cell membranes.[29] Bacterial cells treated with increasing concentrations of AgNPs were analysed for loss of membrane integrity by determination of PI uptake. Cells with compromised cell membranes exhibit permeability to PI and the PI uptake is determined by the resultant fluorescence intensity measured by flow cytometry. Flow cytometric analysis of the bacterial cells revealed an increase in fluorescence intensity with increasing concentrations of AgNPs in all organisms. Figures 8(a) -8(e) illustrate the increase in PI uptake by *S. aureus, E. coli, A. baumanii, K. pneumoniae* and *S. pneumoniae* with increasing AgNP concentrations. Also, PI uptake was found to vary between organisms with highest in *A. baumanii* at 98.5% and lowest in *E. coli* at 16.7% with an average of 54.79% on treatment with 10 µg AgNPs. Figure 6(f) shows the PI uptake of different organisms on treatment with 10µg of AgNPs. $p<0.01$ was observed in all strains tested. These results signify that the high log reduction in bacterial cell count that was observed can be attributed to cell membrane damage.

DISINFECTION TESTS

AgNPs were subjected to a further series of tests to determine their disinfectant efficacy. Basic qualitative tests – In Use dilution test (See Supplementary data Table 1 and Figure 1) and Kelsey Sykes test for disinfectants (See Supplementary data Table 2 and Figure

2) were performed to test for disinfectant activity of AgNPs by which it was observed that AgNPs showed high disinfectant activity. Quantitative estimation of disinfectant efficacy was performed by the Surface Contact assay, Surface disinfection test and determination of phenol coefficient.

3.8 Surface Contact Assay

Surface contact assay was performed to estimate the activity of AgNPs against bacterial cells present on a microscopic slide. Bacterial cells present on a sterile glass slide were treated with 1X and 3X MIC of AgNPs for 60 seconds and the log reductions in CFU were assessed. Significant log reduction was observed in case of *S. aureus, A. baumanii, K. pneumoniae* and *S. pneumoniae.* Table 2 summarizes the log reduction values of *S. aureus, A. baumanii, K. pneumoniae* and *S. pneumoniae* on treatment with 1X and 3X of AgNPs as well as the control agent. $p<0.01$ was observed in all strains tested. The log reductions as estimated by the surface contact assay were found to be lower than those estimated by the Time Kill assay. Maximum response was observed in case of *S. pneumoniae* which had a log reduction of 4.3. However, *E. coli* was excluded from this table as it was found to respond poorly to treatment with AgNPs with a mere 2.3 log reduction when treated with 20X MIC of AgNPs. This was consistent with the results from PI uptake and time kill assay wherein minimum response to AgNP treatment was observed in case of *E. coli*.

3.9 Surface Disinfection Test

In addition to the surface contact assay, surface disinfection test was performed wherein test surfaces harbouring bacterial suspension were swabbed with 1ml of AgNPs (2.5mg/ml) and 5ml of AgNPs (2.5mg/ml) and allowed a reaction time of 10 minutes. The surviving organisms were plated on nutrient agar and log reduction in the CFU was determined as summarized in Table 3. Maximum log reduction, close to 5 log reduction, was achieved on treatment of test surface with 5ml of AgNPs (2.5mg/ml). Comparable log

reduction value of 3.57 was obtained on treatment with 1ml of AgNPs (p<0.01). These results are in concordance with those obtained by the surface contact assay (4.91 log reduction for *K.pneumoniae* at 3X MIC).

3.10 Determination of phenol coefficient (PC)

To evaluate the disinfectant property of AgNPs, its Phenol Coefficient (PC) value was determined against each of the five bacterial species by the Rideal Walker method. 10^6 cells of each strain were treated to increasing dilutions of 1X MIC of AgNPs and 5% v/v Phenol for 5 min and 10 min. Table 4 indicates the dilutions at which bacterial growth was observed in case of *S. aureus* species. Figure 9 denotes the phenol coefficient values in case of each test strain. The phenol coefficient of AgNPs was found to have a constant value of 3.3 against the strains *S. aureus, E. coli, S. pneumoniae* and *K. pneumoniae*. *A. baumanii*, however, was observed to have an increased susceptibility and consequently, a higher PC value of 5.5 was obtained. From the results obtained, it could be inferred that AgNPs showed sufficient disinfectant activity against all bacterial strains tested. Since PC of AgNPs is largely unexplored, these findings were compared to the PC values of Polyhexamethylene guanidine hydrochloride (PHMGH), an antimicrobial biocide of the guanidine family against *S. aureus*. This study analysed the disinfectant activity of PHMGH over a time range of 0.5 minutes to 10 minutes. It was found that AgNPs have a lower PC value than PHMGH which had a PC value of 7.5.[24]

The surface contact assay, surface disinfection test and phenol coefficient tests were carried out as this study is focussed on AgNPs as a prospective hospital disinfectant. Surface disinfection tests have been used to assess hospital disinfectants as they reflect hospital conditions quite accurately [23] and the determination of phenol coefficient has also been carried out in studies testing disinfectants against nosocomial pathogens.[24] The favourable

results observed for AgNPs by these tests were also found to be comparable to those observed in previous literature and this can be said to confirm its efficacy in hospital settings.

TOXICITY TESTING.

While AgNPs have shown high bactericidal activity and disinfectant efficacy, toxicity of AgNPs on mammalian cell lines has also been reported.[26] Hence, cytotoxicity and mutagenicity of AgNPs were tested against mammalian cells by MTT assay and *Salmonella typhimurium* cells by Ames test respectively.

3.11 MTT Assay

Cytotoxicity testing of AgNPs was estimated by the MTT assay on L6 mouse fibroblast cell line at an AgNP concentration of 100µg – an amount considerably higher than the highest MIC obtained that is, 66.66µg for *K.pneumoniae.* Here, significant cell viability was observed when L6 cells were exposed to increasing concentrations of AgNPs for 60 seconds. 0.1% triton X-100 was used as positive control. No significant decrease in cell viability was observed with increase in concentration. Figure 10 shows the cell viability of L6 cells at concentrations 10 µg, 20µg, 40µg, 60µg and100µg on treatment period of 60 seconds. 80.7% cell viability was observed on treatment with the highest concentration of 100µg AgNPs for 60 seconds indicating no cytotoxicity. Morphology of L6 cells on AgNP treatment for 60 seconds is included in supplementary data (Figure 3).

3.12 Ames test

Bacterial mutagenicity of AgNPs was tested on *Salmonella typhimurium* strains TA 98 and TA 100. The bacterial cells were treated with 100µg of AgNPs for 60 seconds and the mutagenicity was estimated by the number of revertant colonies observed. In case of control agents- Mitomycin C and Sodium azide, the number of revertant colonies observed was very high which indicates that the test system responded appropriately. Relatively lesser number of colonies was observed in case of TA 98 and TA 100 cells treated with AgNPs indicating

no mutagenic tendency (p<0.0001). Table 5 shows the number of revertant colonies of TA 98 and TA 100 on treatment with 100µg and control agents. However, Ames test, being a preliminary screening test is insufficient to define mutagenic risk and hence, further study is required before the potential risk of AgNPs can be ascertained.

4. CONCLUSION

Hand hygiene and adequate disinfection in hospital settings are very important in preventing nosocomial infections. Furthermore, improvements in hand hygiene have shown decrease nosocomial infection rates.[30] AgNPs are prospective disinfectants as they exhibit broad spectrum antibacterial actions at low concentrations whilst being largely non-toxic to the human body.[31] AgNPs have shown to be effective against a number of drug-resistant bacteria [32], which opens doors for its use in therapeutic and pharmaceutical products and as a disinfectant.

While common disinfectants require a contact time of 60 seconds to 5 minutes [11], we could observe from this study that a high log reduction in bacterial count was obtained on a minimal contact time of 60 seconds.

Overall, it can be concluded that AgNPs, having high bactericidal and disinfectant efficacy can be a prospective disinfectant due to its activity at low concentrations in the range of 3.4µg to 66.6µg and low reaction time of 60 seconds. However, further preclinical studies are required to take AgNPs to the next level of testing.

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Figure 1: Flowchart for the preparation of AgNPs.

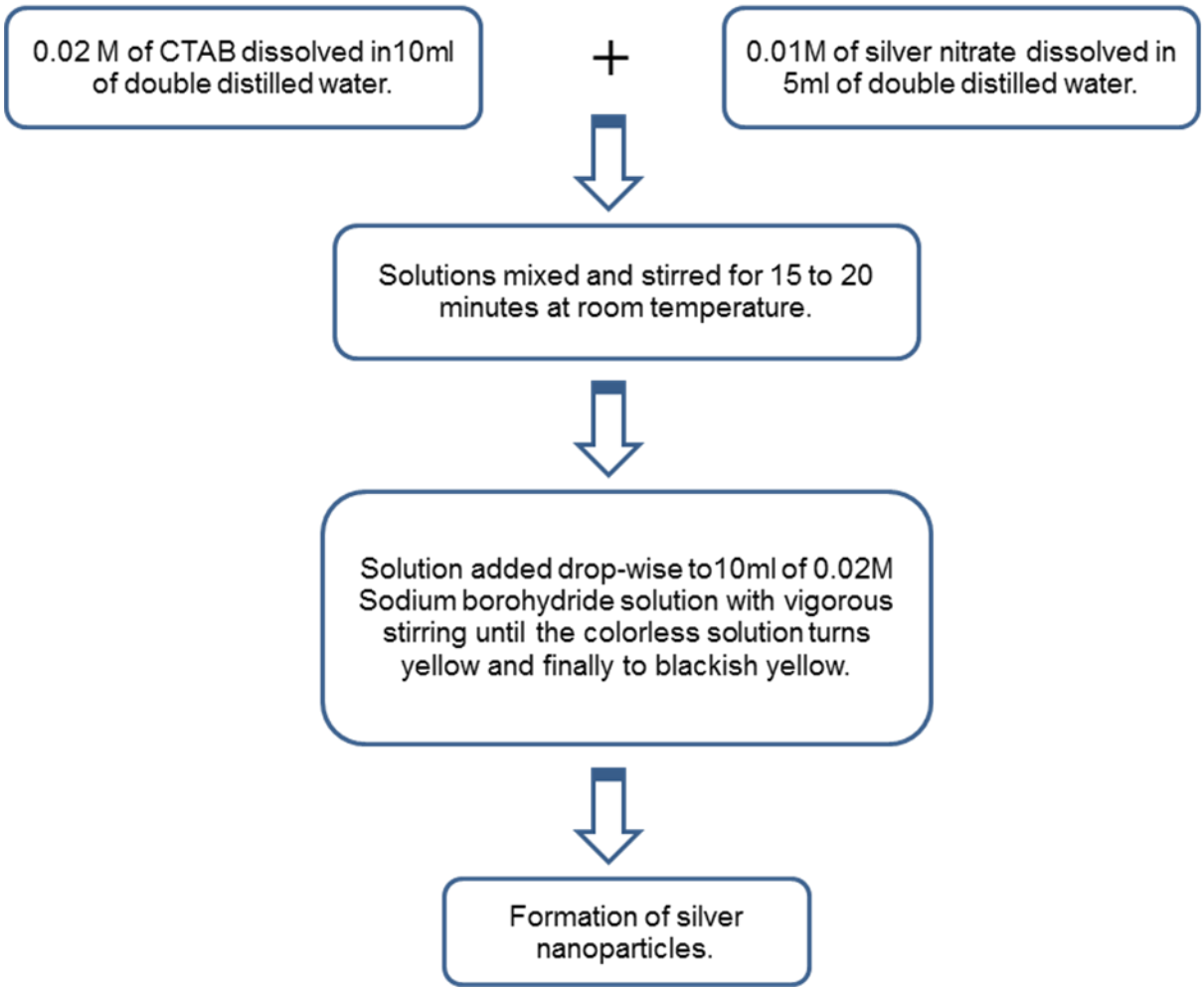

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Figure number: 1

Figure 2 : Schematic for formation of AgNPs

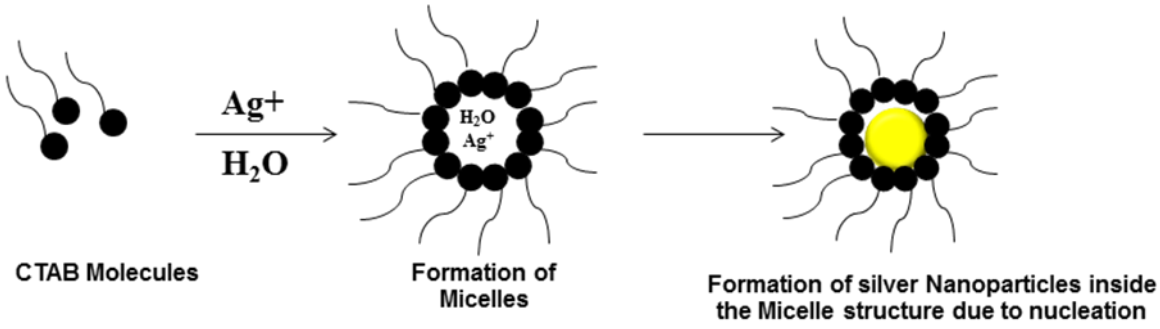

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Figure number: 2

Figure 3: UV-Vis Spectra for AgNPs.

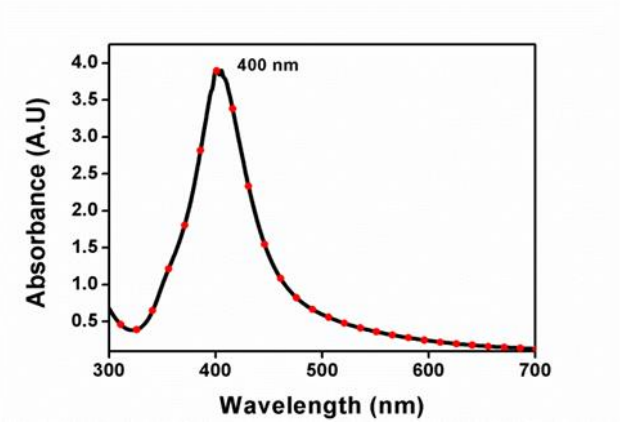

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Figure number: 3

Figure 4: Zeta potential spectra for AgNPs.

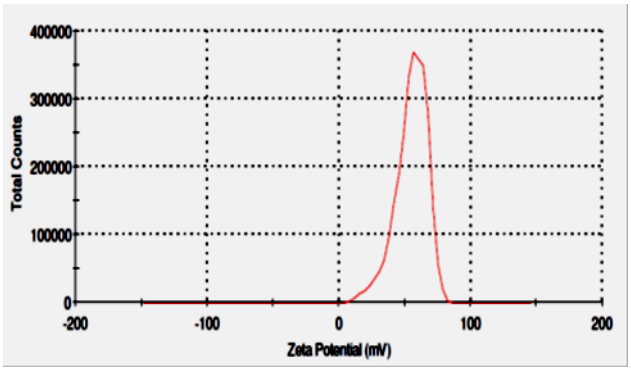

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Figure number: 4

Figure 5: HRTEM Micrograph of AgNPs.

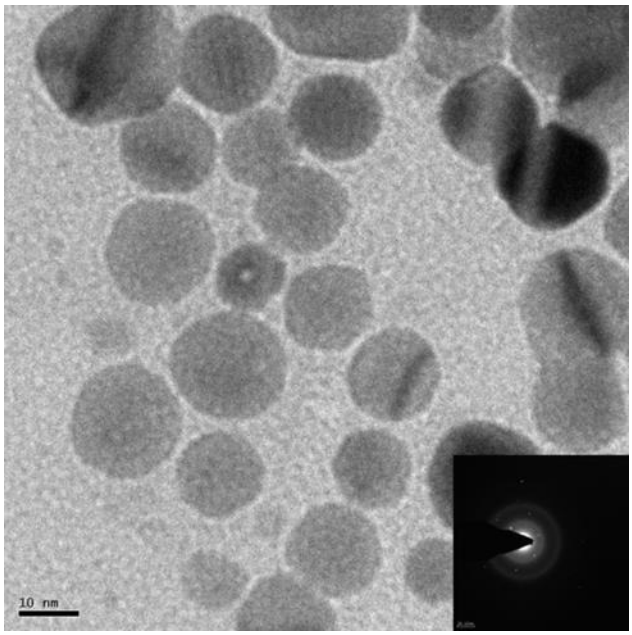

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Figure number: 5

Figure 6A: MIC of AgNPs against *S.aureus, E. coli* and *A. baumanii*.
Figure 6B: MIC of AgNPs against *S. pneumoniae* and *K. pneumoniae.*

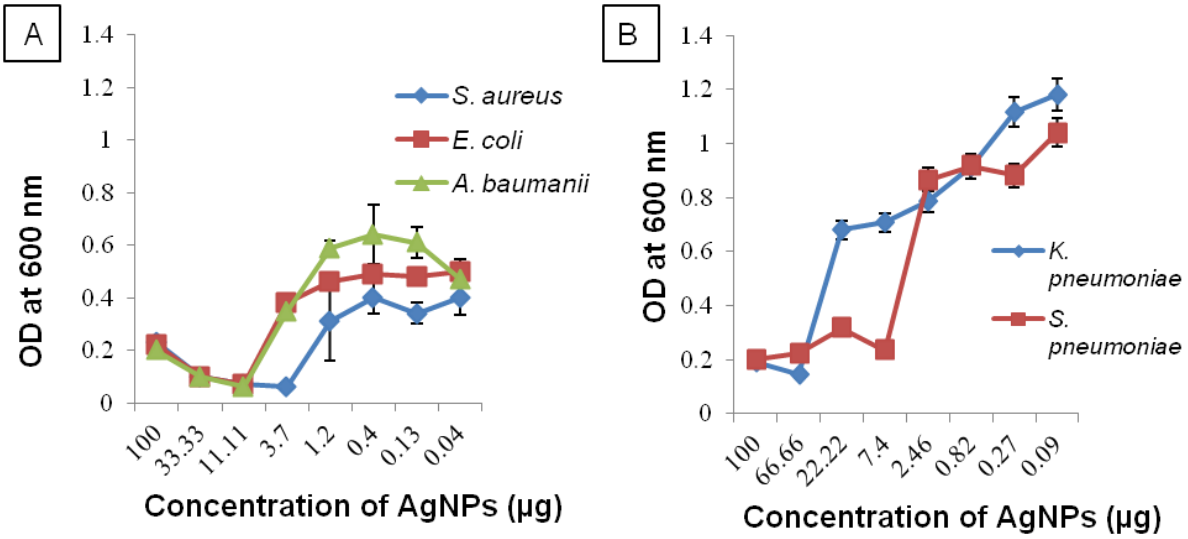

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Figure number: 6

Table 1: Efficacy testing of AgNPs by time kill assay with log reduction in CFUs after 60 seconds treatment with AgNPs. *In case of *E. coli*, the CFU was calculated for 20X instead of 3X due to poor response of the organism to AgNPs.

Organism	**Agent**	**Average number of CFUs**	**Log Reduction in CFU**	***p* value**
S. aureus	Untreated	1712000	0	0.0236
	1X MIC AgNPs	3100	2.74	
	3X MIC AgNPs	0	6.53	
	Sterill®	0	6.53	
E. coli*	Untreated	926500	0	<0.0001
	1X MIC AgNPs	92667	2.04	
	20X MIC AgNPs	231	3.60	
	Sterill®	0	6.28	
A. Baumanii	Untreated	198137	0	0.0167
	1X MIC AgNPs	425	2.70	
	3X MIC AgNPs	0	5.61	
	Sterill®	0	5.61	

S. pneumoniae	Untreated	282334	0	<0.0001
	1X MIC AgNPs	817	2.52	
	3X MIC AgNPs	0	5.75	
	Sterill®	0	5.75	
K. pneumoniae	Untreated	381667	0	0.0032
	1X MIC AgNPs	560	2.84	
	3X MIC AgNPs	0	5.88	
	Sterill®	116	3.05	

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Table number: 1

Figure 7 : SEM Images showing morphology of cells treated with AgNPs. *A) A. baumanii* cells grown on Whatmann filter paper B) *A. baumanii* cells treated with AgNPs (2.5mg/ml) showing pits on the cell membrane.

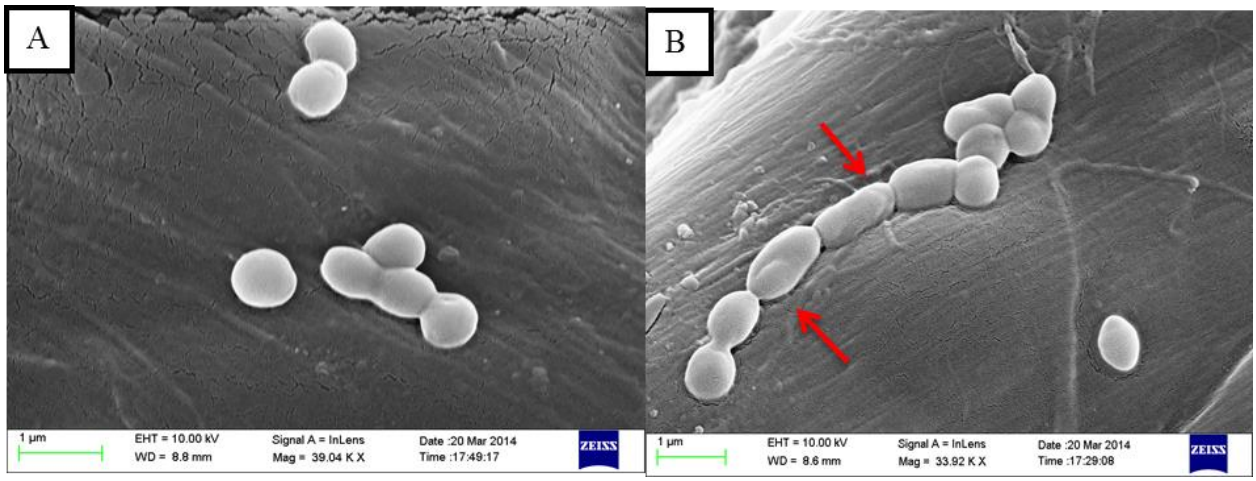

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Figure number: 7

Figure 8A -8E: Bacterial cells *of S. aureus, A. baumanii, E. coli, S. pneumoniae* and *K. pneumoniae* treated with AgNPs showed increased PI Uptake with increasing concentrations of AgNPs at 30 seconds and 60 seconds. Figure 6F compares the PI uptake of each bacterial species when treated with 10µg of AgNPs. UT= Untreated bacterial cells; UT+dye= Untreated bacterial cells+Propidium iodide; *S. a=S. aureus* ; *A. b=A. baumanii*; *E. c=E. coli; S. p=S. pneumoniae K. p= K. pneumoniae*

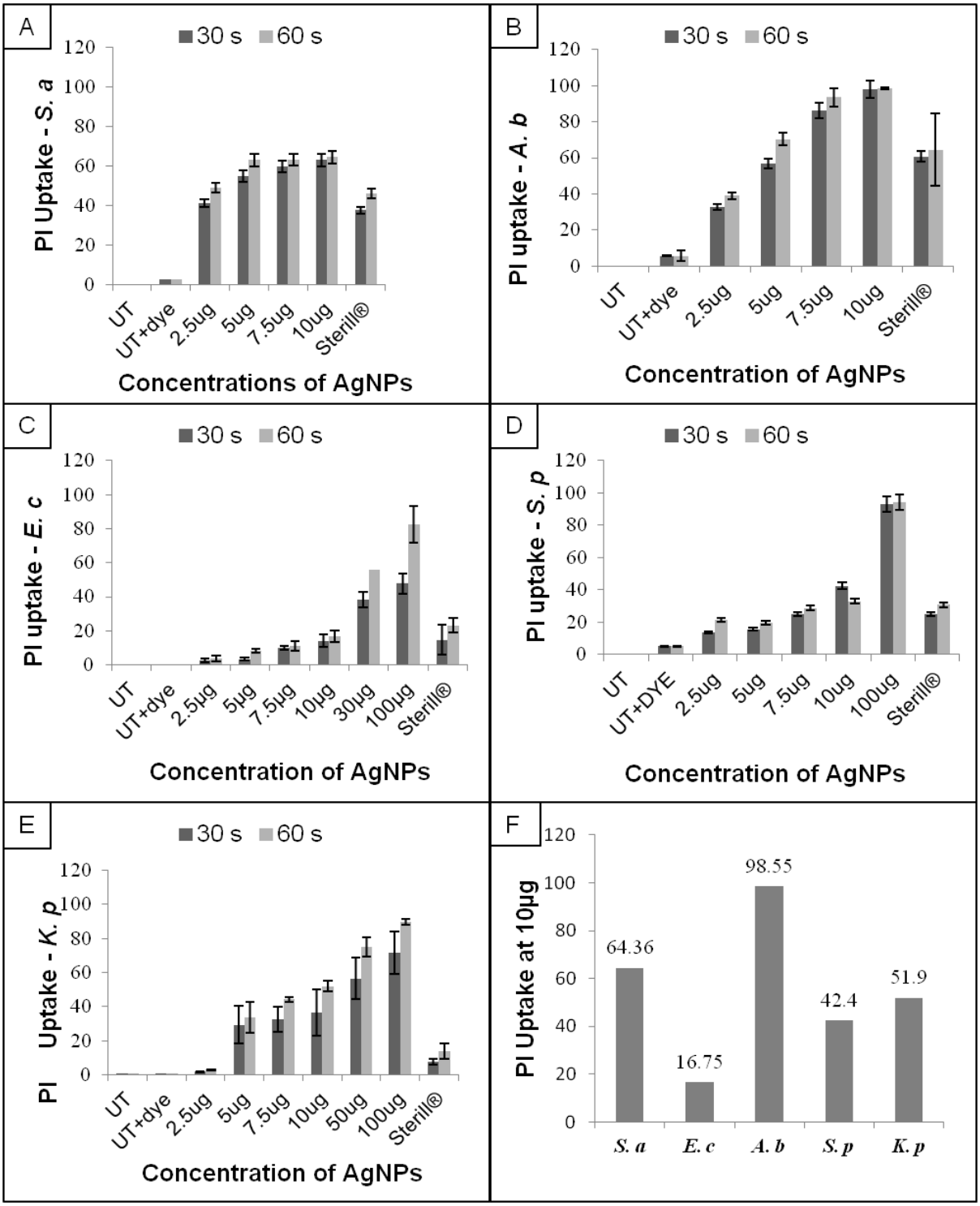

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Figure number: 8

Table 2 : Surface Contact Assay with log reduction in CFUs after 60 seconds treatment with AgNPs.

Organism	**Agent**	**Average number of CFUs**	**Log Reduction in CFU**	***p* value**
S. aureus	Untreated	25550	0	
	1X MIC AgNPs	80	2.54	0.0008
	3X MIC AgNPs	22	3.01	
	Sterill®	0	4.68	
A. Baumanii	Untreated	20880	0	
	1X MIC AgNPs	144	2.11	<0.0001
	3X MIC AgNPs	0	4.62	
	Sterill®	0	4.62	
S. pneumoniae	Untreated	8267	0	
	1X MIC AgNPs	0	4.23	0.0001
	3X MIC AgNPs	0	4.23	
	Sterill®	0	4.23	
K. pneumoniae	Untreated	44000	0	
	1X MIC AgNPs	116	2.54	0.0027
	3X MIC AgNPs	0	4.91	
	Sterill®	0	4.91	

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Table number: 2

Table 3: Log Reduction in CFUs of bacterial cells post treatment with AgNPs and control agents.

Conditions	**Average no. of CFUs**	**Log Reduction**	***p* value**
Untreated	2556	0	
1ml (2.5mg/ml) AgNPs	87	3.57	0.0049
5ml (2.5mg/ml) AgNPs	0	4.92	0.0043
Sterill®	8	4.66	

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Table number: 3

Table 4: Determination of phenol coefficient of AgNPs with *S. aureus*

Disinfectant	**Dilution**	**Exposure time**	
		5 min	**10 min**
AgNPs (1X MIC)	1:250	-	-
	1:300	+	-
	1:350	+	+
	1:400	+	+
	1:450	+	+
Phenol (5%v/v)	1:50	-	-
	1:60	-	-
	1:70	-	-
	1:80	-	-
	1:90	+	+

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Table number: 4

Figure 9: Comparison of Phenol Coefficient values for AgNPs on each bacterial strain

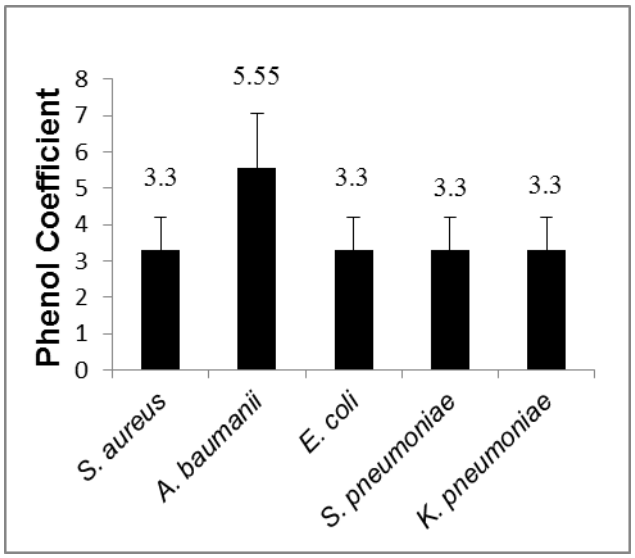

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Figure number: 9

Figure 10: Cytotoxicity of AgNPs by MTT assay - %viability of L6 cells treated with increasing concentrations of AgNPs for 60 seconds.

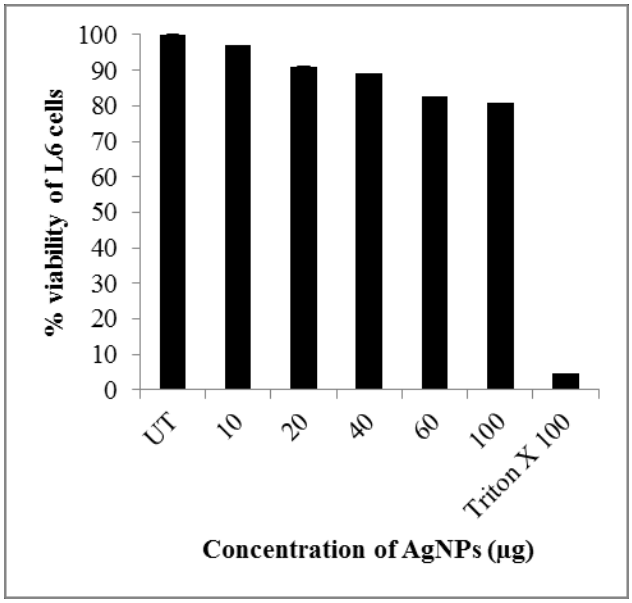

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Figure number: 10

Table 5: Number of revertant colonies observed on treatment with AgNPs for 60 seconds.

Agent	**Number Of Revertant Colonies**		***p* value**
	TA 98	**TA 100**	
PBS	255	253	
AgNPs (100µg)	291	305	<0.0001
Mitomycin C	1279	_	
Sodium Azide	_	1310	

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