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ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES DISPERSION AGAINST MSSA AND MRSA ISOLATED FROM WOUNDS IN A TERTIARY CARE HOSPITAL OF NORTH INDIA

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ABSTRACT: *Staphylococcus aureus,* the main cause of nosocomial infection worldwide result in significant increases in mortality, morbidity, and cost related to prolong treatments. Silver compound has been in use since time immemorial for the treatment of burns, wounds and several other bacterial infections. In the present work, we explore the antibacterial activity of silver nanoparticles (Ag-NPs) dispersion (5-10 nm) against reference strain and clinical isolates of Methicillin-sensitive *S. aureus* (MSSA), and Methicillin-Resistant *S. aureus* (MRSA).The typical minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against standard reference strain as well as, MSSA and MRSA were observed in the range of 12-48 µg/ml and 12-96 µg/ml, respectively. The MBC/MIC ratios against all strains were found in the range of ≤1 to ≤4, which shows that Ag-NPs inhibit bacterial growth in a bactericidal rather than a bacteriostatic manner. Our finding suggests that Ag-NPs are effective broad-spectrum antibacterial agents regardless of their drug-resistance mechanisms.

Key words: MIC, MBC, Silver nanoparticles, MSSA, MRSA

INTRODUCTION

Staphylococcus aureus has been recognized as a major human pathogen ever since it was discovered by Sir Alexander Ogston in the 1880s as a major cause of wound suppuration (Archer, 1998). The introduction of large quantities of diverse antimicrobial agents into the human environment in the past century has presented a new set of challenges to pathogens such as *S. aureus*; which affected human beings and caused high mortality rates before the antibiotic era. Methicillin, originally called celbenine, is a semisynthetic derivative of penicillin, chemically modified to withstand the degradative action of penicillinase. The drug was introduced into therapy in Europe during 1959 -1960 and in the United States in 1961 (Oliveira *et al*. 2002; Rice, 2006). The first cases of MRSA were reported in the United Kingdom in 1961, followed soon by reports in other European countries, Japan, and Australia (Palavecino, 2004; Rice, 2006). Periodic outbreaks of MRSA were observed in various countries throughout the world in the 1970s, but it was not until the 1980s that MRSA became a significant problem in United States hospitals (Palavecino, 2004; Rice, 2006).

The re-emergence of infectious diseases and the continuous development of antibiotic resistance among a variety of diseases–causing bacteria pose a serious threat to public health worldwide (Desselberger, 2000). Among them MRSA is an important pathogen in the healthcare that has not been eliminated from the hospital nor community environment. In humans MRSA causes superficial lesions in skin and localized abscesses, septic arthritis, central nervous system infections, invasive endocarditis, osteomyelitis, septicemia and urinary tract infections (Velazquez-Meza, 2005).

During the last decade, developments in the area of nanotechnology have evolved outstanding capabilities for understanding, fabricating, and manipulating structures at the atomic level. Nanotechnology impacts a wide variety of disciplines, from materials science to engineering to biology, and has a wide variety of applications in every industry, from aerospace to medicine to agriculture. One of the most studied aspects of nanotechnology nowadays is their ability to offer the opportunity to fight microbial infections via synthesis of nanoparticles (Luo *et al.* 2007). The mechanism of prevention of bacterial growth by antibiotics is quite different from the mechanisms by which nanoparticles inhibit microbial growth. Therefore, nanoparticles have the potential to serve as an alternative to antibiotics and to control microbial infections such as those caused by MRSA (Luo *et al.* 2007).

Medicinal and preservative properties of silver have been known for over 2,000 years. The ancient Greek and Roman civilizations used silver vessels to keep water potable. Since the nineteenth century, silver-based compounds have been widely used in bactericidal applications, in burns and wounds therapy, etc. (Klasen, 2000). Over the last decades silver has been engineered into nanoparticles, structures from 1 to 100 nm in size. The antimicrobial activity of silver nanoparticles (Ag-NPs) appears significantly high than silver ions and other silver salts (Lok *et al*. 2006; Rai *et al*. 2009). The antimicrobial activity of Ag-NPs is comparable or better than the broad spectrum of most prominent antibiotics used worldwide (Roy *et al*. 2008). Silver has the highest bactericidal activity and biocompatibility amongst all the known antibacterial nanoparticles (Cubillo *et al*. 2006; Desai and Kowshik, 2009; Kloepfer *et al*. 2005; Yamamoto *et al*. 2004).

Microbes are unlikely to develop resistance against silver as they do against conventional and narrow-target antibiotics, because the metal attacks a broad range of targets in the organisms, which means that they need to develop a range of mutations simultaneously to protect themselves (Pal *et al*. 2007). As a result, Ag-NPs have been applied to a wide range of products, the most important current use is as antimicrobial agents to prevent infection, such as in burn and traumatic wound dressings, diabetic ulcers, coating of catheters, dental works, scaffold, and medical devices (Rai *et al*. 2009; Law *et al*. 2008; Silver *et al*. 2006; Kim *et al*. 2007; Thomas *et al*. 2007; Kim and Kim, 2006). Ag-NPs are also used in hygienic products including water purification systems, linings of washing machine, dishwashers, refrigerators, and toilet seats (Silver *et al*. 2006; Rai *et al*. 2009).

In the present work we investigated the antibacterial activity of Ag-NPs dispersion against clinical isolates of MSSA, MRSA stocks and reference strain of *S. aureus* ATCC25923. The antibacterial activity of the Ag-NPs was assessed by determining the minimal inhibitory concentration (MIC), the minimum bactericidal concentration and the MBC/MIC ratio; time-kill assay were also used. Furthermore, the bacterial-silver nanoparticles interaction was also analyzed by atomic force microscopy (AFM).

MATERIALS AND METHODS

Silver nanoparticles dispersion and bacterial strains

A stock solution of commercially available water soluble Ag-NPs (5-10 nm) were procured from Nanoparticle Biochem, Inc. (Columbia, USA). The subsequent dilutions were made in autoclaved double distilled water. The present study was conducted from December, 2009 to March., 2010, in the Department of Microbiology, J. N. Medical College and Hospital, Aligarh Muslim University, Aligarh, India. The bacterial strains from the clinical specimens (wounds) were isolated and characterized as MSSA and MRSA and were used as the test organisms to evaluate the antimicrobial effects of Ag-NPs. All the strains were cultured aerobically at 35 ^{0}C on Mueller-Hinton Agar (MHA) plates, Hi-Media (Mumbai, India).

Assay for MIC and MBC determination of Ag-NPs dispersion

Minimal inhibitory concentration (MIC): Bacterial strains were grown overnight on MHA plates at 35 ^{0}C before being used. The antimicrobial activity of Ag-NPs was examined using the standard broth dilution method (CLSI M07-A8).

The MIC was determined in Luria-Bertani (LB) broth Hi-Media (Mumbai, India) using serial two-fold dilutions of Ag-NPs in concentrations ranging from 192 to 1.5 µg/ml, initial bacterial inoculums of 2.5×10^5 CFU/ml and time and temperature of incubation being 24 h at 37 ^{0}C, respectively. The MIC is the lowest concentration of antimicrobial agents that completely visually inhibits the 99% growth of the microorganisms. The MIC measurement was done in triplicate to confirm the value of MIC for each tested bacteria.

Minimal bactericidal concentration (MBC): After MIC determination of the Ag-NPs tested, an aliquots of 25 µl from all tubes in which no visible bacterial growth was observed were seeded in MHA plates not supplemented with Ag-NPs and were incubated for 24 h at 37 ^{0}C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills 100% of the initial bacterial population.

Bacterial growth assays

To examine the bacterial growth curve in liquid broth, inoculations were given from fresh colonies on MHA plates into 100 ml of LB culture medium. Growth was allowed till the optical density reached 0.1 at 600 nm (OD of 0.1 corresponds to 10^8 CFU/ml of medium). Subsequently, 2×10^8 CFU/ml from above were added to 100 ml of liquid LB media supplemented with 5, 10, 15, 20 and 35 µg/ml of Ag-NPs. All the flasks were put on rotatory shaker (150 rpm) and incubated at 37 ^{0}C. Control broths were used without nanoparticles. The bacterial growth was determined by measuring optical density after every 2 hour (up to 20 h) at 600nm using spectrophotometer (VSP66, LOBA Life, India).

Atomic Force Microscopy (AFM) analysis

A log phase culture of *S. aureus* ATCC25923 in LB broth was split into 1 ml aliquots. The cells were collected by centrifugation (10000 rpm, 5 min) and resuspended in Milli Q water. Two samples were prepared; one was untreated and another one was treated with Ag-NPs. The cells were collected by centrifugation (10000 rpm, 5 min) to remove the Milli Q water. The untreated and Ag-NPs treated bacterial cultures were washed and resuspended in Milli Q water. About 10 µl portion of the suspension was applied onto a glass microscope slide and allowed to dry at room temperature. For each sample duplicate slides were prepared. To image the samples Atomic Force Microscope (Veeco Innova) was used in tapping mode using commercial etched silicon tips as AFM probe with typical resonance frequency of ca. 300 Hz (RTESP, Veeco). The areas for imaging were chosen randomly from the total surface area.

Statistical analysis

MIC and MBC tests were performed in triplicate, and the results are expressed as the mean ± the standard errors of the mean. A student's test was used to compare these results. *P* values lower than 0.05 were considered significant.

RESULTS

Antibacterial activity of Ag-NPs dispersion

The aim of present study was to explore the antimicrobial activity of Ag-NPs dispersion against the MSSA and MRSA strains isolated from clinical specimens (wounds) in a tertiary care hospital. Standard serial two-fold broth dilution methods were applied to determine the MIC, MBC and MBC/MIC ratio of Ag-NPs dispersion against all the clinical isolates of MSSA, MRSA and reference strain. MIC is defined as the minimal concentration of antibacterial agents that inhibit the complete visible growth of the tested microorganisms after 24 h of incubation at 35 □C. The MBC is defined as the minimal concentration of antimicrobial agents that kills 100% of the initial bacterial inoculums after 24 h of incubation at 35 □C.

Both the MIC and MBC values of our Ag-NPs dispersion against MSSA strains were found very low (i.e., in the range of 12-48 µg/ml), indicating very good bacteriostatic (represented by the MIC) and bactericidal activity (represented by MBC) of the antibacterial agents (Table 1 & Fig. 1a). In case of clinical isolates of MRSA, the MIC and MBC values of Ag-NPs were observed in the range of 12-48 µg/ml and 24-96 µg/ml, respectively which also indicate good bacteriostatic and bactericidal activity of the antibacterial agents (Table 1 & Fig. 1b). In comparison to MSSA and MRSA, the MIC and MBC value of Ag-NPs for reference strain *S. aureus* ATCC25923 was also low i.e., 12 µg/ml and 24 µg/ml, respectively.

The bactericidal effects (MBC) of Ag-NPs against *S. aureus* ATCC25923, MSSA and MRSA were also assessed by a colony forming capacity assay. The value defined as MBC was the Ag-NPs concentration that completely inhibited visible colony growth in the MHA plates (Table 1 & Fig. 2).

Table 1. MIC and MBC of Ag-NPs tested against clinical isolates of MSSA, MRSA and reference strain *S. aureus* ATCC25923

MSSA Isolates (23)				**MRSA Isolates (32)**			
Number of isolates	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC ratio	Number of isolates	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC ratio
2	12	12	1	6	12	24	2
9	12	24	2	3	12	48	4
5	24	24	1	2	24	24	1
4	24	48	2	14	24	48	2
3	48	48	1	4	48	48	1
S. aureus ATCC25923	12	24	1	3	48	96	2

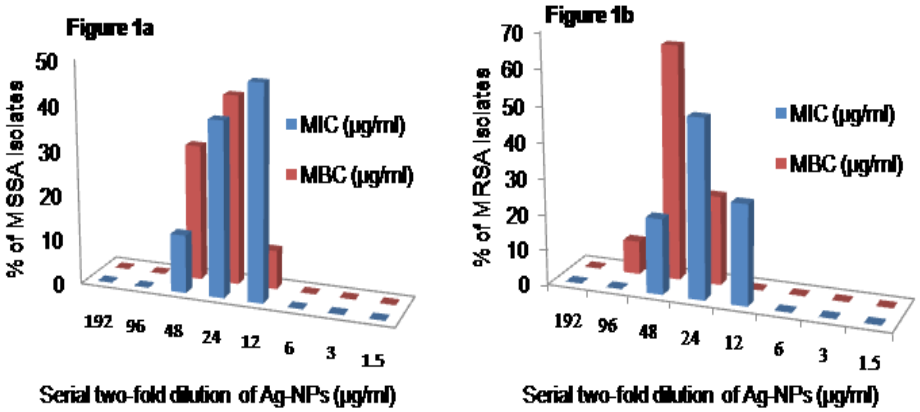

Fig 1. Clinical isolates of MSSA (a) and MRSA (b) showing MIC and MBC treated with serial two-fold dilution of Ag-NPs dispersion.

Effects of Ag-NPs dispersion on bacterial growth

The dynamics of bacterial growth was monitored in liquid LB medium; Ag-NPs were added in the medium at the beginning of bacterial cell growth. Time-dependent changes in the bacterial growth were monitored at a regular interval of 2 h (upto 20 h) by measuring the OD (at 600 nm) of the control and bacterial solutions supplemented with 5, 10, 15, 20 and 35 µg/ml of Ag-NPs are shown in Figure 2. Bacterial cell growth enhances the turbidity of the liquid medium and as a result the absorption increases. It is clear that at all these concentrations, the nanoparticles caused a growth delay of the bacterial cells; slope of the bacterial growth curve continuously decreased with increasing nanoparticles concentration and at concentration of 20 µg/ml and 35µg/ml; Ag-NPs were found as an effective bactericidal agent (Fig. 3a & 3b).

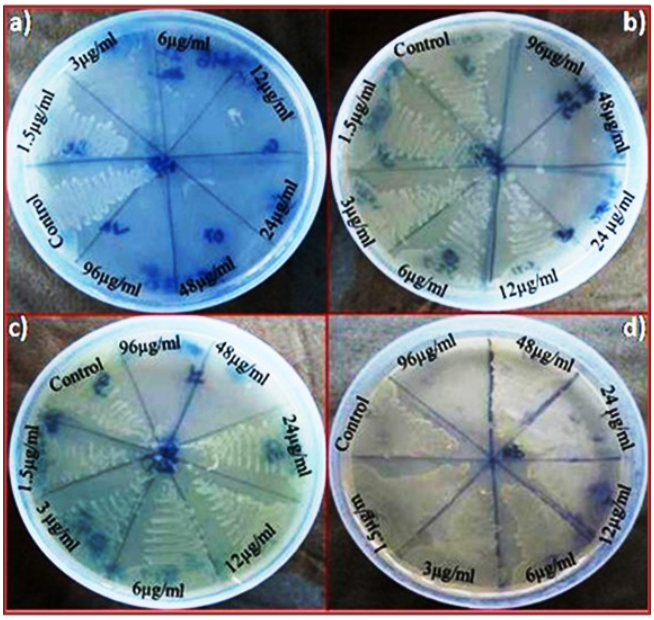

Fig 2. Bactericidal effect (MBC) of Ag-NPs dispersion against *S. aureus* ATCC25923, MSSA and MRSA. A colony-forming assay was performed to define the MBC of Ag-NPs against MSSA (**a, c**), *S. aureus* ATCC25923 (**b**) and MRSA (**d**), after 24-h treated with serial two-fold dilutions of Ag-NPs (5-10 nm), bacteria were grown in MHA plates, and colony growth was observed after 24 h of incubation at 37□C.

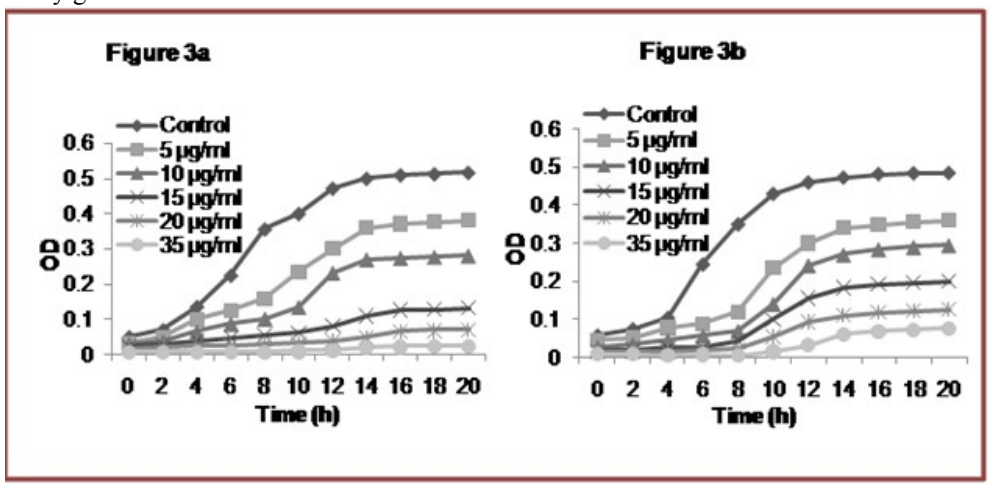

Fig 3. Dynamic growth curve of *S. aureus* ATCC 25923 (a) and MRSA (b) in the presence of different concentrations of Ag-NPs dispersion.

Investigation of morphological changes to bacteria induced by Ag-NPs using AFM

To further understand the interaction between the *S. aureus* ATCC25923 and Ag-NPs, AFM imaging experiments of the nanoparticles treated and untreated bacteria were carried out. Before imaging, they were dried at room temperature on glass slides. For each sample duplicate slides were made and numerous randomly chosen parts of each slide were imaged. The Figure 4a and 4b shows the morphology and bacteria-nanoparticles interaction in the absence (untreated) and presence (treated) of Ag-NPs.

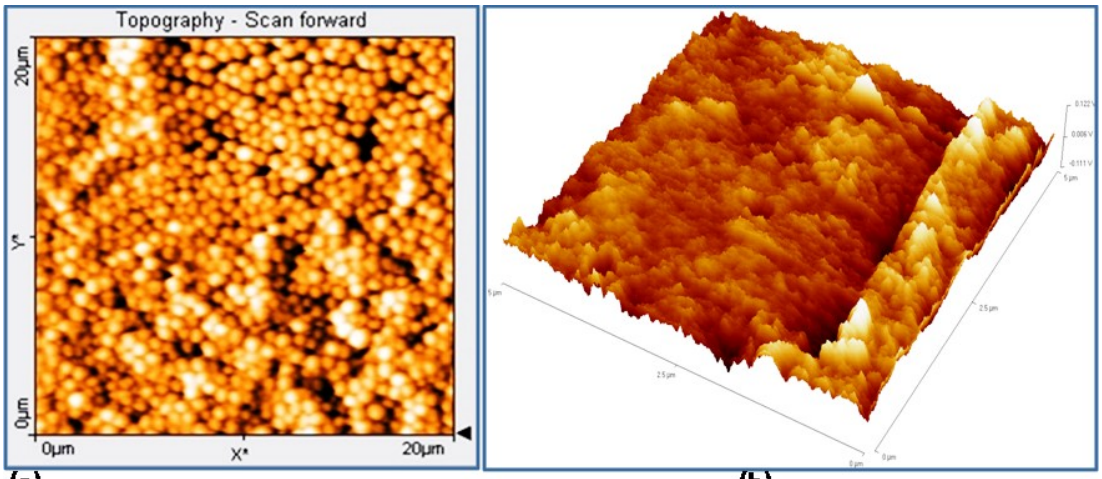

Fig 4. Three-dimensional topographic AFM image of *S. aureus* in the absence (a) of Ag-NPs and presence (b) of Ag-NPs dispersion.

DISCUSSION

A large number of synthetic compounds are known which exert antimicrobial activities and used as a bactericidal agents. Among these, silver compounds (salts and colloids) raise a potent antibacterial agent whose application is restricted to topical creams used to reduce the risk of wound infection and to treat infected wounds (Ayala-Nunez et al 2009).

Here in our study by utilizing various in-vitro susceptibility assays, we have evaluated the antibacterial activity of silver nanoparticles against clinical isolates of MSSA and MRSA; one of the most pathogenic bacteria that represent a constant threat in hospital and community environments. For reference strain *S. aureus* ATCC25923, the MIC value of our Ag-NPs observed was 12 µg/ml (Table1). This value is comparable with the earlier study carried out with Ag-NPs synthesized by different methods. They have reported a MIC value of 12.50 µg/ml (Fernandez et al 2008), 7.50 -33.67 µg/ml (Martinez-Castanon et al 2008) for *S. aureus* ATCC25923, and 12.50 µg/ml for *S. aureus* ATCC6538 (Jain et al 2009), whereas in other reports MIC was observed 80 µg/ml for *S. aureus* (Fayaz et al 2009) and 14.38 - 258.89 µg/ml for *S. aureus CCM* 3953 (Guzman et al 2009).

In another study conducted on clinical strains of MRSA, MIC value of our Ag-NPs was observed in the range of 12 - 48 µg/ml (Table 1), whereas in the previous study the MIC values was found in the range of 1.8 to 10.79 mg/ml (Ayala-Nunez et al 2009) and 14.38-258.89 µg/ml, respectively (Guzman et al 2009) . Thus, the MIC value observed by us is much lower than the earlier reported values (Ayala-Nunez et al 2009). As a result, we could achieve an efficient growth inhibition of clinical strains of MRSA and MSSA at a very low and minimum concentration of Ag-NPs (Table 1). This large differences in MIC value might be because of the size and the methods by which Ag-NPs was synthesized and also genetic variation in the isolated strain.

The MBC/MIC ratio is a parameter that reflects the bactericidal capacity of a compound by relating both values. A ratio with a value superior to 1 (MBC>MIC) indicates that a great amount of compound is needed to reach the bactericidal effect and this compound could be considered as bacteriostatic agents (Ayala-Nunez et al 2009). For all the clinical isolates, the ratio of MBC/MIC was found in the range of ≤1 and ≤4, indicates that Ag-NPs have a bactericidal rather than a bacteriostatic effect on all the tested bacteria (Table 1).

Our results are in agreement with earlier reports where the ratio of MBC/MIC of Ag-NPs was ≤4 (Ayala-Nunez et al 2009). In theory, a bactericidal agent is preferred clinically because bacterial killing should produce a faster resolution of the infection, improve clinical outcome, and reduce the likelihood of the emergence of resistance and the spread of infection. If the pathogen are killed rather than inhibited, resistance mutations that might otherwise emerge as the result of antibiotic pressure are eliminated (French 2006).

No significant difference in bactericidal activity were found among the different clinical isolates of MSSA and MRSA strains (Table 1), demonstrating that activity of Ag-NPs was not affected by those resistant mechanisms that differentiate these strains and Ag-NPs are broad spectrum antibacterial agents. These results further agree with previous finding by other researchers, where it was proven that Ag-NPs exert the same effects on drug-resistant and drug- susceptible bacteria (Lara et al 2009).

The bacterial growth curve for *S. aureus* ATCC25923 and MRSA was monitored in LB broth supplemented with different concentrations of nanoparticles. Figure 3 clearly indicates that as the concentration of Ag-NPs increases, reduction in bacterial growth was observed and this was even continued for 14 h. A complete reduction in bacterial growth was observed at 20 µg/ml and no growth was observed at concentration of 35 µg/ml. Our results corroborates with the earlier finding where the Ag-NPs elicited only a partial growth inhibition of *S. aureus* at 100 µg/ml (Shrivastava *et al.* 2007), and complete growth inhibition was seen at 20 µg/ml (Li *et al*. 2010) for S. *aureus* ATCC6538P and *S. aureus* KCCM12256 at 40 µg/ml (Binupriya *et al.* 2010). Culture without nanoparticles (control) did not show any growth inhibition and reached stationary phase at the end of 16 h.

AFM is a suitable tool for investigating changes in cell membrane morphology and surface structure (Camesano *et al*. 2000; Mortensen *et al*. 2009; Suresh *et al*. 2010) and aid in elucidating early morphological changes induced by bacterial agents on bacterial cells (Suresh *et al*. 2010). Figure 4a (untreated) shows the three-dimensional representation of the topography of *S. aureus*. In this figure cocci form can be observed which is a typical characteristic of the bacteria. However, Figure 4b shows the three-dimensional representation of the topography of *S. aureus* treated with Ag-NPs, which clearly indicates that Ag-NPs were embedded on membrane of the bacteria. Though the exact mechanism of bacterial-nanoparticles interaction is still unclear, the nanoparticles do appear to cause structural changes to the cell surface that may eventually lead to cell death.

Finally, it can be concluded that our Ag-NPs dispersion (5-10 nm) shows excellent bacteriostatic and bactericidal activity against all the clinical isolates of MSSA and MRSA, regardless of their drug-resistance mechanisms. However, with the advent of Ag-NPs and its major use as an antibacterial agent, much experimental trials are needed to understand the toxicity and therefore, further studies must be conducted to examine the cytotoxicity of nanoparticles towards human cells before proposing their therapeutic use. Until our best knowledge this is the first reports that explore the antibacterial activity of silver nanoparticles dispersion on clinical stocks of MSSA and MRSA in a tertiary care hospital of North India.

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