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Evaluation of antibacterial activity of silver nanoparticles against MSSA and MRSA on isolates from skin infections

Ansari MA[1], *Khan HM[1], Khan AA[2], Malik A[1], Sultan A[1], Shahid M[1], Shujatullah F[1], Azam A[3]
[1]Section of Antimicrobial Agents & Drug Resistance Research, Department of Microbiology, Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, Aligarh-202002, U.P., India.
[2]Department of Anatomy, Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, Aligarh-202002, U.P., India.
[3]Centre of Nanotechnology, King Abdul Aziz University, Jeddah, Saudi Arabia.

***Corresponding Author:** harismk2003@gmail.com

Abstract
In recent years, skin and soft-tissue infections (SSTIs), particularly due to multidrug-resistant pathogens are increasingly being encountered in clinical settings. Due to the development of antibiotic resistance and the outbreak of infectious diseases caused by resistant pathogenic bacteria, the pharmaceutical companies and the researchers are now searching for new unconventional antibacterial agents. Recently, in this field nanotechnology represents a modern and innovative approach to develop new formulations based on metallic nanoparticles with antimicrobial properties. The bacterial growth curve, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of silver nanoparticles (Ag-NPs) towards *Staphylococcus aureus* ATCC25923, methicillin-sensitive *S. aureus* (MSSA), and methicillin-resistant *S. aureus* (MRSA) were examined in this study. The experiment results showed that the lowest MIC and MBC of Ag-NPs to MRSA was 12.5 µg/ml and 25 µg/ml, respectively. The obtained results suggested that Ag-NPs exhibit excellent bacteriostatic and bactericidal effect towards all clinical isolates tested regardless of their drug-resistant mechanisms.

Keywords: SSTIs; Multidrug-resistant; MIC; MBC; Silver nanoparticles; MSSA; MRSA.

Introduction

In recent years, skin and soft-tissue infections (SSTIs), particularly due to multidrug-resistant pathogens, are increasingly being encountered in clinical settings. These infections are typically initiated by some breach in the epidermis, resulting in infections by the organisms normally colonizing the skin like *Staphylococcus aureus* (Lopez *et al*., 2003). Some of the most common infections caused by *S. aureus* involve the skin, including furuncles, cellulitis, impetigo, and postoperative wound infections of various sites. Some of the most serious infections produced by *S. aureus* are bacteremia, pneumonia, osteomyelitis, endocarditis (Lowy, 1998; Bhatia and Zahoor, 2007), empyema, scalded skin syndrome, toxic shock syndrome (Salyers and Whit, 2002) and abscesses of the muscle and various intra-abdominal organs (Conterno *et al*., 1998; Romero-Vivas *et al*., 1995).

Methicillin-resistant *S. aureus* (MRSA) were first isolated in 1961 (Jevons, 1961), 2 years after the introduction of the drug to combat penicillin resistant isolates. MRSA are inherently resistant to all β-lactam antibiotics, but some lineages (clones) have additionally evolved resistance to multiple antibiotic classes. Within the species, resistance to all known antibiotic classes has occurred due to mutation and horizontal gene transfer and this has led to anxiety regarding the future availability of effective chemotherapeutic options. The prevalence of MRSA increased dramatically in many countries during the 1990s. In the UK, MRSA bacteremia rose from <2% in 1990 to 43% in 2001 (Johnson *et al*., 2005) and this trend was mirrored in other countries including the USA (NNIS, 2002) and Japan (Izumida *et al*., 2007).

Due to the outbreak of infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance, the pharmaceutical companies and the researchers are now searching for new antibacterial agents. In the present scenario, nanoscale materials have emerged up as novel antimicrobial agents owing to their high surface area to volume ratio and the unique chemical and physical properties (Morones *et al*., 2005; Kim *et al*., 2007). 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).

Silver has been used for thousands of years as a precious metal by humans in

different applications as jewellery, tools, coins, photographic material or explosives. Hippocrates described the use of silver powder for its application in wound healing and in the treatment of ulcers (Klasen, 2000). In the 17th and 18th centuries, silver nitrate was used for ulcer treatment and its antimicrobial activity was established in the 19th century. Nevertheless, after the introduction of the antibiotics in 1940 the use of silver salts decreased. Subsequently, silver salts and silver compounds have been used in different biomedical fields, especially in burn treatment (Klasen, 2000).

The antimicrobial activity of silver nanoparticles (Ag-NPs) appears significantly high. Silver is more toxic element to microorganisms than many other metals in the following sequence: Ag > Hg > Cu > Cd > Cr > Pb > Co > Au > Zn > Fe > Mn > Mo > Sn (Zhao and Stevens, 1998). Ag-NPs exert more efficient than silver ions and other silver salts in mediating their antimicrobial activity (Lok *et al*., 2006; Rai *et al*., 2009). Silver exhibits low toxicity to mammalian cells (Zhao and Stevens, 1998). Silver has a lower propensity to induce microbial resistance than many other antimicrobial materials (Kim *et al*., 2007; Silver *et al*., 2006). 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). 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).

The objective of present work was to evaluate the antibacterial activity of Ag-NPs against MRSA and MSSA strains recovered from patients with skin and soft-tissue infections. The antibacterial activity of the Ag-NPs was assessed by determining the minimal inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), and by measuring the dynamic growth curve of the bacteria.

Materials and Methods

Silver nanoparticles formulation

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 Milli Q water.

Bacterial strains, medium, and cultivation

The bacterial strains from the clinical specimens were isolated and characterized as MSSA and MRSA and were used as the test organisms to evaluate the antimicrobial effects of Ag-NPs. *S. aureus* ATCC25923 were used as reference strain. All the strains were cultured aerobically at 37^0C on Mueller-Hinton Agar (MHA) plates, Hi-Media (Mumbai, India). The present study was conducted in the Department of Microbiology, J. N. Medical College and Hospital, Aligarh Muslim University, Aligarh, India.

MIC and MBC determination

Minimal inhibitory concentration (MIC): Bacterial strains were grown overnight on MHA plates at 37^0C 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 200 to 1.5625 µg/ml, initial bacterial inoculums of 2×10^8 CFU/ml and the time and temperature of incubation being 24 h at 37^0C, respectively. The MIC is the lowest concentration of antimicrobial agents that completely visually inhibits 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, aliquots of 50 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 37^0C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills 100% of the initial bacterial population.

Bacterial testing of growth curve

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 until 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 25 µg/ml of Ag-NPs. All the flasks were put on rotatory shaker (150 rpm) and incubated at 37^0C. 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).

Statistical analysis

MIC and MBC tests were performed in triplicate, and the results were expressed as the mean ± the standard errors of the mean. Student's 't' test was used to compare these results. *P* values lower than 0.05 were considered significant.

Results

Bactericidal activity of Ag-NPs

The aim of present study was to evaluate the antibacterial effects of Ag-NPs against the MSSA and MRSA strains recovered from patients with skin and soft-tissue infections. The MIC and MBC values of Ag-NPs against MSSA and MRSA strains were observed very low (i.e. in the range of 12.5-100 µg/ml), indicating very well bacteriostatic (represented by the MIC) and bactericidal activity (represented by MBC) of the antibacterial agents (Table 1 & Fig. 1). In comparison with MSSA and MRSA, the MIC and MBC value of Ag-NPs for reference strain *S. aureus* ATCC25923 was found very low i.e. 12.5 µg/ml and 25 µg/ml, respectively; also indicating very good bacteriostatic and bactericidal activity of the antibacterial agents (Table 1).

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

MSSA Isolates (16)			MRSA Isolates (20)		
Number of isolates	**MIC (µg/ml)**	**MBC (µg/ml)**	**Number of isolates**	**MIC (µg/ml)**	**MBC (µg/ml)**
2	12.5	12.5	5	12.5	25
8	12.5	25	3	12.5	50
2	25	25	2	25	25
4	25	50	7	25	50
S. aureus ATCC25923	12.5	25	1	50	50
			2	50	100

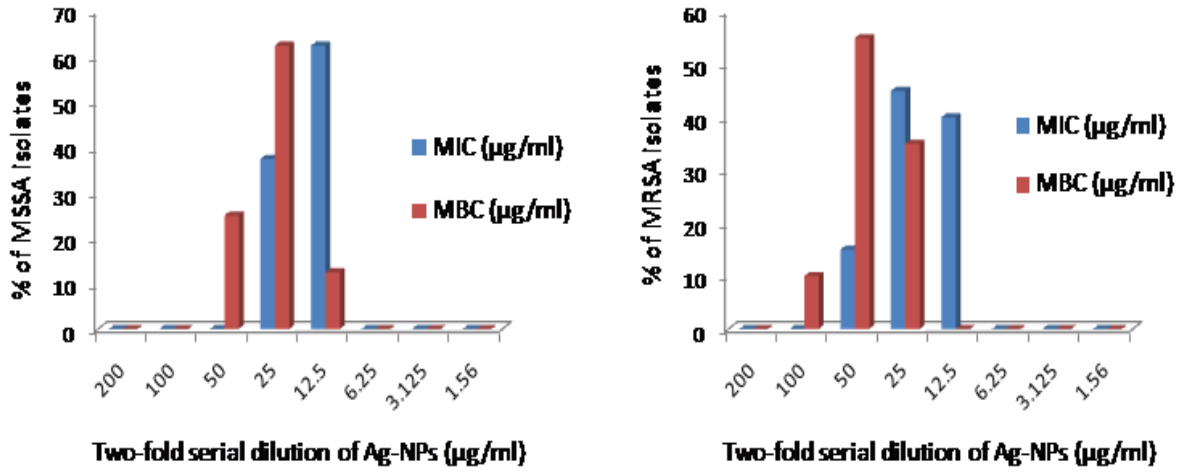

Figure 1. Clinical isolates of MSSA and MRSA showing MIC and MBC treated with serial two-fold dilution of Ag-NPs.

Effects of Ag-NPs on bacterial growth

The dynamics of bacterial growth curve was monitored in liquid LB broth. 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 different concentration 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. Nanoparticles with highest concentration showed almost no growth for upto 16 hrs representing a bactericidal effect at this concentration (Fig. 2a & 2b).

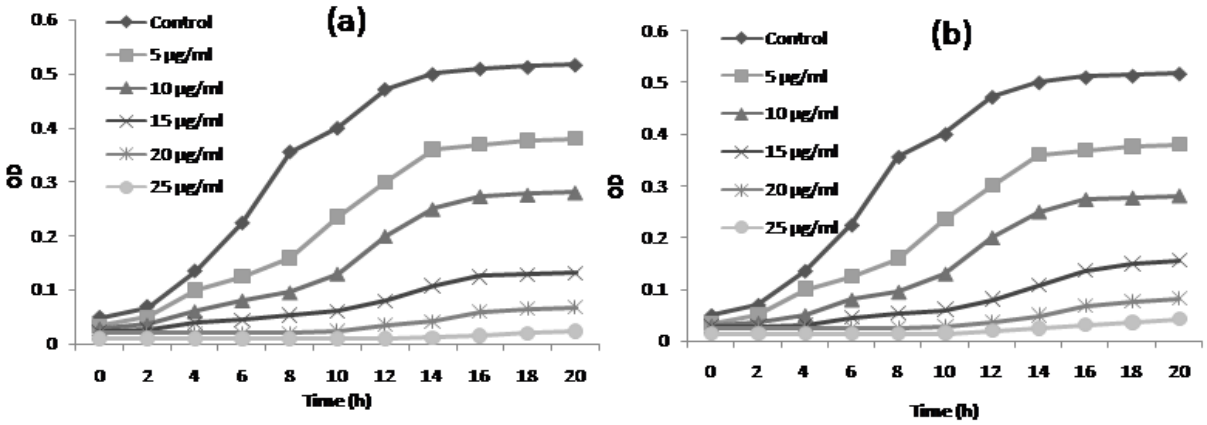

Figure 2. Dynamic growth curve of *S. aureus* ATCC 25923 (a) and MRSA (b) in the presence of different concentrations of Ag-NPs in liquid LB broth.

Discussion

Staphylococcus aureus accounts for 30-50% of skin and soft-tissue infections, followed by the *Enterobacteriaceae*, non-fermenters, *Streptococci* and anaerobes (Gales *et al*., 2000). Silver and silver-based compounds have been in use for centuries in the treatment of burns and chronic wounds (Castellano *et al*., 2007).

In our study, the values of MIC and MBC of Ag-NPS against all clinical isolates of MSSA, MRSA and single strain of *S. aureus* ATCC25923 were found in the range of 12.5-50 µg/ml and 12.5-100 µg/ml, respectively. Findings of Martinez *et al*. (2008) were similar to our findings. They reported that Ag-NPs were inhibitory at concentration of 16.67 µg/ml against *S. aureus* ATCC25923, but they used the Ag-NPs of size 29nm which was larger than the size used by us (5-10nm). Our results of antibacterial activity of Ag-NPs against *S. aureus* ATCC25923 are exactly in accordance with results shown by Fernandez *et al*. (2008). They showed MIC and MBC values of Ag-NPs of 12.5 µg/ml and 25 µg/ml, respectively for *S. aureus* ATCC25923, which is in agreement of our finding where the value of MIC and MBC of our Ag-NPs was same as shown by them. Our results showed better antibacterial activity as compared to earlier work of Ayala-Nunez *et al*. (2009). They have reported MIC and MBC values of Ag-NPs 1800 µg/ml and 2700 µg/ml, respectively.

The growth curve of standard strain of *S. aureus* ATCC25923 and MRSA were plotted in the presence of 0, 5, 10, 15, 20, and 25 µg/ml concentration of Ag-NPs. Figure 2 clearly indicates that as the concentration of Ag-NPs increases, reduction in bacterial growth was observed and this was even continued for 16 hrs. There was clear inhibitory action of Ag-NPs on *S. aureus* ATCC25923 and MRSA at all concentrations. Our results were better as compared to study done by Shrivastava *et al*. (2007) probably because the size of nanoparticles was smaller in our study. The finding of Li *et al*. (2010) showed a complete growth inhibition for *S. aureus* ATCC6538P at 20 µg/ml, while in case of our study no growth was observed upto 16 hrs at 25 µg/ml of Ag-NPs (Fig. 2). Thus, our result shows that there was very little difference between antibacterial activities of Ag-NPs against standard strain and methicillin-resistant strain, i.e. both were equally sensitive.

Conclusion

Finally, we conclude that nanobiotechnology is an important area of research that deserves all our attention owing to its potential application to fight against multidrug-resistant microbes. Therefore, further studies must be conducted to assess the genotoxic and cytotoxic effects in human cells and environmental microorganisms in order to evaluate the applications of Ag-NPs as a bactericidal agent.

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