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Research Article

EVALUATION OF ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND DETECTION OF VIRULENCE FACTORS - NUCLEASE, PHOSPHATASE, AND BIO FILM PRODUCTION

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ABSTRACT

Objective: To determine the virulence factors-biofilm, nuclease and phosphatase production in *Staphylococcus aureus* isolates. To determine the effect of silver nano particles and antibiotics on MRSA by MIC determination and kirby baeur method respectively and finally to compare antibacterial activity of silver nano particles and antibiotics.

Methods: In the present study, we explore the antibacterial activity of silver nanoparticles (Ag-NPs) dispersion (10 nm) against reference strain and clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). We evaluated the antibacterial activity of Ag-NPs against MRSA isolated from patients in Saveetha Medical College and Hospital, a tertiary care centre in Chennai, Tamil Nadu. The bactericidal activity of different concentrations of Ag-NPs (200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µg/ml) was tested by determining MIC using microbroth dilution and MBC by agar dilution methods.. In addition, the virulence factors phosphatase, nuclease, and biofilm production were tested.

Result: The values of minimal inhibitory concentration and minimal bactericidal concentration of Ag-NPs against all clinical isolates of MRSA and a single of *S. aureus* were found in the range of 12.5–50 µg/ml and 12.5–25 µg/ml, respectively, indicating very good bactericidal activity. Ag-NPs with the highest concentration showed almost no growth for up to 16 h representing a bactericidal effect at this concentration. Effect was proportional to dose since 50.0 µg/ml was the most effective dose since the bacterial population did not recover and 12.5 µg/ml was the least effective. All the MRSA isolates were positive for the virulence factors.

Conclusion: The study result suggests that Ag-NPs could be used as an effective alternative antibacterial agent.

Keywords: Nanoparticles, Silver nanoparticles, Methicillin-resistant *Staphylococcus aureus*, Biofilm, Nuclease, Phosphatase.

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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is *S. aureus* resistant to all beta-lactam antibiotics. Infections caused by *Staphylococcus aureus* have a poorer prognosis when the infecting strain is an MRSA [1]. Professor Patricia Jevons first observed *Staphylococcus aureus* that was resistant to Methicillin in Guildford, Surrey. Genetic changes in the MRSA have led to the evolution of "fitter" strains that combine antibiotic resistance with transmissibility and virulence [2]. MRSA is the leading cause of potentially life-threatening bloodstream infections, surgical site infections, and pneumonia.

In the human body, the main site of colonization of *S. aureus* is the anterior nares. Evasion of host's immune system is the primary pathogenic mechanism for its transformation from a commensal to a pathogen. The postulated sequence of events which leads to infection is initiated with carriage of the organism. It is disseminated through hand carriage to body sites where infection may occur in areas with breaks in dermal surfaces such as intravascular catheterization, operative incisions or minor abrasions in eczema, or shaving associated micro-trauma.

The main mechanism of methicillin resistance is mediated by PB2a, encoded by mecA gene. PBP 2a blocks binding of all beta-lactams but allows transpeptidation to proceed. MRSA contains a resistance island called SCCmec, where SCC stands for staphylococcal cassette chromosome and mec stand for the mecA gene. Although the origin of SCC mec remains unknown, the cassette could have originated from *Staphylococcus sciuri*, since a PBP was found in *S. sciuri* that showed 87.8% amino acid sequence identity with PB2a [3]. The first strains of MRSA were reported in the United Kingdom in 1961.The first important outbreak in the United States was in 1968. The second wave of MRSA emerged in the 1970s and 1980s in Australia, Irish Republic, and United States. However, reports of the hospital and inter-hospital spreads were increasing [4]. Since 1990s in many countries, the prevalence of MRSA has increased dramatically [5].

Epidemiologically MRSA has been classified as hospital-acquired (HA-MRSA) and community-acquired MRSA (CA-MRSA). HA-MRSA is an MRSA infection which occurs after 48 h of hospitalization. CA-MRSA is strains isolated in an outpatient setting, from patients within 48 h of admission with criteria which includes patients having no history of MRSA infection or colonization, no admission to a health-care facility in the previous year, no history of dialysis, surgery or permanent indwelling catheters or medical devices that pass through the skin.

In addition to antibiotic resistance, one of the virulence factors responsible for treatment failure and chronic and recurrent staphylococcal infections in burn patients is biofilm formation [6,7]. Since the ability of biofilm production by MRSA increases antibiotic resistance, hospital patients

infected with these strains are at serious risk for treatment failure [8]. Biofilm formation is considered to be a virulence factor because the microorganisms that establish in a burn wound biofilm fundamentally differ from suspended populations [9]. The signals that promote biofilm rapid formation are (i) presence of a suitable surface; (ii) increase of extracellular iron; and (iii) presence of indole, polyamines, calcium, and bile salts [10-12]. In the initial phase of biofilm formation, bacterial attacks proliferate, forming microcolonies and attracting surrounding cells. The mature biofilm is a real microbial community that exchanges and shares products in a dynamic manner [13]. The pathogenicity of biofilms can be summarized by the following properties: (i) Attachment to solid surfaces to high density; (ii) increased metabolic efficiency of the community; (iii) evasion of host-defenses; (iv) horizontal gene transfer; (v) antimicrobial resistance; and (vi) detachment of microbial aggregates able to colonize other sites. Biofilm acts as a barrier to antimicrobial agents and the host immune system that assists sustained bacterial colonization [14]. Biofilm formation is regulated by expression of polysaccharide intracellular adhesin (PIA) which mediates cell to cell adhesion and is the gene product of intracellular adhesion (ica) ADBC [15]. Various reports attest to the presence of ica ADBC gene in *S. aureus* and *Streptococcus epidermidis* isolated from infections associated with indwelling medical devices [16,17]. It was found that the ica locus is required for biofilm production [16]. Other studies have revealed that all strains bearing the icaA gene, a component of the ica locus, also bear icaD [17]. Thus, detection of one of these genes can be used to determine whether *S. aureus* is a potential biofilm producer. Production of ica ADBC-encoded, PIA/poly-N-acetylglucosamine, represents an important biofilm mechanism in staphylococci. Glucose-induced biofilm in MRSA was ica independent and apparently mediated by a protein adhesion (s) [18]. Experiments have been performed which shows that *S. aureus* has become resistant to methicillin as it is covered by biofilms [46].

A heat-stable nuclease or thermonuclease is produced by most strains of *S. aureus*. Phosphatase is probably produced by all staphylococci. A large percentage (95–100%) of the strains of *S. aureus*, *Staphylococcus hyicus*, *Staphylococcus Intermedius,* and *Staphylococcus schleiferi* produce detectable levels of thermonuclease [19]. The discovery of neutrophil extracellular traps (NETs) has opened a novel dimension in our understanding of how these specialized leukocytes kill pathogens. NETs consist of nuclear deoxyribonucleic acid (DNA) backbone associated with antimicrobial peptides, histones, and proteases that provide a matrix to entrap and kill various microbes. The study by Brenda *et al.* shows that *S. aureus* nuclease promotes resistance against NET-mediated antimicrobial activity of neutrophils and contributes to disease pathogenesis *in vivo* [20].

Phosphatase is probably produced by all Staphylococci. *S. aureus*, *S. epidermidis,* and *Staphylococcus xylosus* give positive results. The enzyme is repressed by inorganic phosphates in the medium in other species, so they give negative results by standard methods of testing [21].

In an era where antibiotics seem weakened and beginning to succumb to the battle against microbes, the field of nanobiotechnology holds a promising future. Nanotechnology offers opportunities to re-explore the biological properties of already known antimicrobial compounds by manipulating their size to alter their effect. Recent advances in the field of nanotechnology, particularly the ability to prepare highly ordered nanoparticulates of any size and shape, have led to the development of new biocidal agents [22]. In the study by Sunita and Palaniswamy showed The SN10 had shown the larger zone of inhibition as compared to SN5, from which it is concluded that smaller particle size gives better penetration and good antimicrobial activity [47].

The bactericidal effect of silver nanoparticles (Ag-NPs) is due to the following mechanism of actions. Ag-NPs attach to the surface of cell membrane, penetrate bacteria and release silver ions [23]. Binding to the bacterial membrane results in dissipation of the proton motive force [24].They target protein synthesis, nucleic acid synthesis and Gram-positive cell wall synthesis [25]. Ag-NPs inactivate DNA replicating ability [26]. They disrupt cell permeability and respiratory functions of the cell. Smaller Ag-NPs having a larger surface area available for interaction have a greater bactericidal effect [27]. The antibacterial activity was particle size dependent. Small particles exhibited higher antimicrobial activity than big particles [28]. This result can be due to high particle penetration when these particles have smaller sizes [29]. Electrostatic forces that develop when nanoparticles with a positive zeta potential encounter bacteria with a negative surface charge promote a closer attraction and interaction between the two entities and possibly the penetration in bacterial membranes. Indeed, the zeta potential along with the size of the nanoparticles is a fundamental parameter for controlling the antimicrobial activity, and more effective nanoparticles have a positive zeta potential and a reduced size. Ag-NPs can not only alter the expression in effect; nanoparticles have a higher antibacterial activity than the free ions of silver, whereby the antibacterial properties are attributed to both the physical properties of nanoparticles and the elution of silver ions to produce a strong antibacterial activity of broad spectrum [30]. Furthermore, the fact that bacterial resistance to elemental silver is extremely rare [31] emphasizes the presence of multiple bactericidal mechanisms that act in synergy. The Ag-NPs also damage membranes and induce the release of reactive oxygen species, forming free radicals with a powerful bactericidal action [32]. Moreover, Ag-NPs are also responsible for cytokine modulation in wound healing [33] and inhibition of the biofilm formation.

In theory, a bactericidal agent is preferred clinically because bacterial killing should produce a faster resolution of infection, improve clinical outcome and reduce likelihood of emergence of resistance and spread of infection. If pathogens are killed rather than inhibited, resistance mutations that might otherwise emerge as the result of antibiotic pressure are eliminated [34].One of the principal elements of bacterial infectivity is their rapid reproduction time. As shown by the time-kill assays, Ag-NPs were effective in inhibiting bacterial growth in a dose and time-dependent manner [25]. The highest sensitive antimicrobial activity was observed against MRSA followed by methicillin-resistant *S. epidermidis* and *Streptococcus pyogenes*; while with *Salmonella typhi* and *Klebsiella pneumoniae* moderate antimicrobial activity were observed [26]. Among all nanoparticles, Ag-NPs have proved to be the most effective antimicrobial agent against bacteria, viruses, and other eukaryotic microorganisms [35].

In view of this background, the present study has been carried out to find out the efficacy of silver nanoparticles against MRSA. This was done by comparing the minimal inhibitory concentration (MIC) of Ag-NPs and efficacy of antibiotics against MRSA.

MATERIALS AND METHODS

Before beginning the study, clearance from Institutional Ethics Committee was obtained. This prospective study of 6 months duration was conducted at Saveetha Medical College a tertiary care hospital in Chennai, Tamil Nadu, South India.

Sample size

Among isolates of *S. aureus* from exudates, blood, respiratory, and urine specimens, 30 MRSA were collected. The isolates were confirmed as *S. aureus* by the tube coagulase test, mannitol fermentation test, and urease test.

Confirmation of MRSA

Confirmation of MRSA strains was done using the cefoxitin disc diffusion. Kirby–Bauer method was used in accordance with the Clinical Laboratory Standards Institute (CLSI) using ATCC *S. aureus* 25923 as a control. The medium used was Cation-adjusted Mueller-Hinton Agar. Inoculum was direct colony suspension equivalent to 0.5 McFarland standards. The media were incubated at 37°C at ambient air for 16–18 h. The strength of the cefoxitin disc was 30 µg. The isolates were considered methicillin-resistant if zone of inhibition [43].

Biofilm assay

Tissue culture plate method

This quantitative test described by Christensen *et al.* [36] is considered the gold-standard method for biofilm detection. 30 isolates from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37°C for 24 h. After 24 h incubation, the planktonic cells in MTPs were removed by rinsing the wells. The wells were rinsed twice with phosphate buffer saline P^H 7.2. The surface-adhered cells in the MTP wells were stained with 250 µl of 0.2% crystal violet solution (HiMedia, India). The solution was left in MTP wells for 10 min. Then, the excess crystal violet solution was removed. Hence, the crystal violet in stained cells was solubilized with 1 ml of 95% ethanol. The biofilm biomass was quantified by measuring the intensity of crystal violet solution. The intensity was measured at an optical density (OD) 650 nm using ultraviolet-visible spectrophotometer [37,44]. OD of stained adherent biofilm was obtained using micro ELISA auto-reader (model 680, Biorad) at wavelength 650 nm (Table 1).

Heat-stable nuclease (thermonuclease, DNase) test

Toluidine blue DNA agar is used. Colonies producing DNase hydrolyzes the DNA content of toluidine blue DNA agar medium located in their immediate vicinity. This test was carried out using commercially available DNase agar (Sisco). Spot inoculation of the organism was done on the DNase agar and incubated at 37°C for 18-24 hrs. After incubation, 1 N HCl was poured on the agar. Clearing around the bacterial growth was evaluated as positive [45].

Phosphatase test

Staphylococcal colonies are grown overnight at 37°C on phenolphthalein diphosphate agar. A few drops of ammonia solution are poured onto the lid and the plate is inverted. The colonies which turn bright pink within a few minutes are read as positive for phosphatase production.

Materials

Ag-NPs (product number from Sisco Research laboratories) of 10 nm particle size were used in this study. The stock concentration of 1 mg/ml solution was prepared freshly before using for MIC and MBC determination.

MIC determination

Initially, on MHA plates, bacterial strains were grown overnight at 35°C. Further, using the standard broth dilution method (CLSI M07-A8), antimicrobial activity of Ag-NPs was examined. Accordingly, by the microdilution method in Luria-Bertani (LB) broth (Hi-Media) using serial two-fold dilutions of Ag-NPs in concentrations ranging from 200 to 1.5625 µg/ml with bacterial inoculums of 2×108 CFU/ml. They were incubated at a temperature of 37°C for 24 h. MIC was determined.

Minimal bactericidal concentration (MBC)

Agar dilution method was used for estimation of MBC. Serial two-fold dilutions of Ag-NPs in saline were prepared in concentrations ranging from 200 to 1.625, which were taken in test tubes and mixed with the MHA agar medium, and poured onto plates. The 30 MRSA strains were streaked by single streak method and were incubated for 24 h at 37°C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills 100% of the initial bacterial population. After 24 h, the MBC was noted down for each of the strains.

Table 1: MIC and MBC values of MRSA

MRSA (30)			
Number of isolates	**MIC (µg/ml)**	**Number of isolates**	**MBC (µg/ml)**
16	12.5	17	12.5
14	25	13	25

MIC: Minimal inhibitory concentration, MBC: Minimal bactericidal concentration, MRSA: Methicillin-resistant *Staphylococcus aureus*

Antibiotic susceptibility testing

This was done by Kirby–Bauer method according to CLSI guidelines 2017. The antibiotics, which were tested, were Gentamicin 10 µg, Eythromycin15 µg, Clindamycin 2 µg, Ciprofloxacin 5 µg, and Linezolid 30 µg.

RESULTS AND DISCUSSION

Sample size

Out of 45 samples confirmed as *S. aureus* by tube coagulase test, mannitol fermentation and urease tests, 30 were determined as MRSA by the cefoxitin disc diffusion method. The strains were numbered from 1 to 30.

The 30 MRSA isolates were subjected to biofilm, nuclease, and phosphatase detection.

Biofilm assay

Tissue culture plate method

OD of stained adherent biofilm was obtained using micro ELISA auto-reader (model 680, Biorad) at wavelength 650 nm. The classification of bacterial adherence was done by TCP method [38].

Mean OD values	Adherence	Biofilm formation
< 0.12	Non	Non/weak
0.120–0.240	Moderately	Moderate
>0.240	Strong	High

OD: Optical density

The results of biofilm TCP method are tabulated in Table 2 and Fig. 1.

Nearly 50% of the MRSA isolates are strong producers of biofilm, 33.3% are moderate producers, and 16.7% are weakest producers. Our study results differ from that of Mathur *et al.,* where they have used 152 *S. aureus* isolates. They have compared different media such as TSB with 1% glucose and BHI. They have obtained the best results with TSB with 1% glucose. Out of the 152 isolates, 22 (14.5%) were strong producers of biofilm, 52 (34.2%) were moderate producers, and 70 (46%) were weak producers. The study of Grinholc *et al.* showed that 45% of MRSA were producers of biofilm which is comparable to our study [39].

Nuclease test

This was done on Toluidine blue DNA agar. Out of 30 MRSA isolates, 28 were positive for thermonuclease production. The strains number 13 and 20 did not produce thermonuclease. A positive zone of nuclease production was figured out in Fig. 2.

Test for phosphatase production

This test was done on phenolphthalein diphosphate agar. Out of the 30 MRSA isolates, 29 of them produced phosphatase (Fig. 3). Isolate number 5 did not produce phosphatase.

RESULTS AND DISCUSSION FOR MIC AND MBC

The bactericidal activity of Ag-NPs of concentrations (200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µg/ml) were tested and are shown in Fig. 4. A very low (i.e., in the range of 12.5–50 µg/ml) MIC and MBC values of Ag-NPs against MRSA strains recovered from patients from our tertiary care hospital were observed, indicating very good bacteriostatic and bactericidal activity of Ag-NPs (Table 1). Ag-NPs MIC and MBC value of reference strain *S. areas* ATCC 25923 was found to be very low, i.e., 12.5 µg/ml and 25 µg/ml, respectively, study, MIC and MBC of Ag-NPs against MRSA were estimated to 50 µL. The values of

Table 2: Biofilm assay

Number of MRSA	Strong (>0.240±0.022)	Moderate (0.120–0.240±0.020)	Weak (0.120±0.012)
30	15	10	5

MRSA: Methicillin-resistant *Staphylococcus aureus*

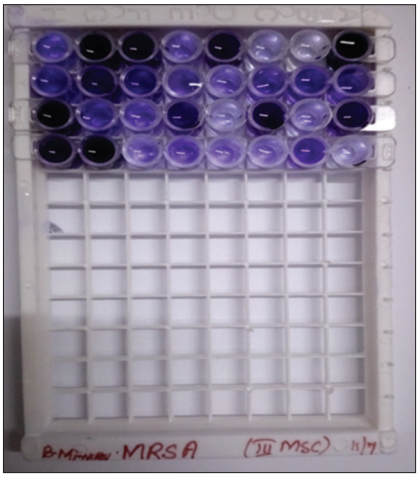

Fig. 1: Screening of biofilm producers by TCP method: High, moderate, and non slime producers differentiated with crystal violet staining in 96 well tissue culture plate

Fig. 2: Clearing produced by nuclease production in positive strain

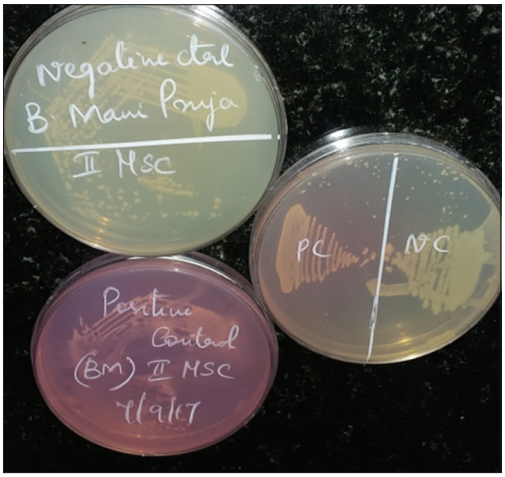

Fig. 3: Pink colour produced by phosphatase positive strains

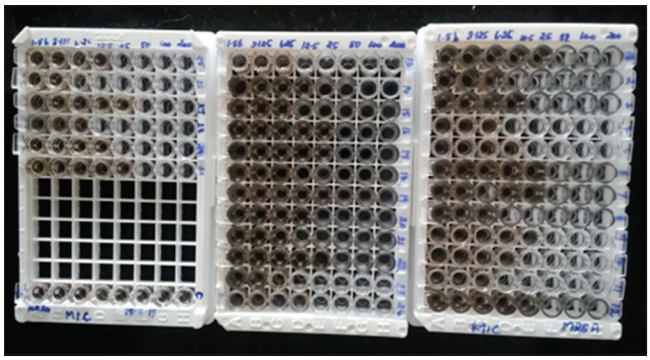

Fig. 4: Minimal inhibitory concentration determination

MIC and MBC of Ag-NPs against all clinical isolates of MRSA and a single strain of *S. aureus* ATCC25923 were found in the range of 12.5–50 µg/ml and 12.5–25 µg/ml, respectively. Figs. 4 and 5 show MIC and MBC results.

MIC and MBC of ATCC *S. aureus* 25923 were 12.5 and 25 (µg/ml).

Our study was comparable with that of Rangari *et al.*, they also had a similar MIC and MBC values for MRSA. In their study, Ansari *et al.* stated the values of MIC and MBC of Ag-NPs against all clinical isolates of MSSA, MRSA, and a single strain of *S. aureus* ATCC25923 were found in the range of 12.5–50 µg/ml and 12.5-100 µg/ml, respectively [40]. Their value of MBC is high compared to our study. The study by Li *et al.* concentrated on the antibacterial activity and mechanism of Ag-NPs (Ag-NPs) on *S. aureus* ATCC 6538P. The experiment results showed the MBC of Ag-NPs to *S. aureus* was 20 µg/ml. At a concentration of 50 µg/ml for 12 h, Ag-NPs reduce the enzymatic activity of respiratory chain dehydrogenase. Furthermore, the proteomic analysis showed that the expression abundance of some proteins was changed in the treated bacterial cell with Ag-NPs, formate acetyltransferase increased 5.3-fold in expression abundance, aerobic glycerol-3-phosphate dehydrogenase decreased 6.5-fold, ABC transporter ATP-binding protein decreased 6.2-fold, and recombinase A protein decreased 4.9-fold [41]. The study by Kim *et al.* revealed MIC of AgNPs against *S. aureus* was 100 µg/ml [42].

Comparison of antibacterial activity of Ag-NPs and antibiotics

The antibiotics tested were gentamicin 10 µg, erythromycin 15 µg, clindamycin 2 µg, ciprofloxacin 5 µg, and linezolid 30 µg.

Antibiotic susceptibility testing was done by Kirby–Bauer method according to CLSI guidelines 2017 for the 30 MRSA isolates.

All the isolates were susceptible to gentamicin. 70% of the isolates were susceptible to clindamycin. None of the isolates were susceptible to erythromycin, 90% of the isolates tested intermediate, and 10% of the isolates were resistant. 70% of the isolates were susceptible to ciprofloxacin. Moreover, all of the 30 isolates were susceptible to linezolid.

The antimicrobial activity of the 10 nm Ag-NPs was evaluated using well diffusion method on Mueller-Hinton Agar (MHA). The inhibition zones were measured in mm ATCC *S. aureus* 25923 was used as a reference for antibacterial assay. Briefly, MHA agar plates were inoculated with

Table 3: Antibiotic susceptibility of MRSA

Antibiotic and nanoparticles	Strength	Zone size 15 to 19 mm (No. of isolates)	Zone size 20 to 24 mm (No. of isolates)	Zone size 25 to 30 mm (No. of isolates)
Gentamicin	10 µg	9	21	Nil
Clindamycin	2 µg	9	15	6
Erythromycin	15 µg	18	12	Nil
Ciprofloxacin	5 µg	9	15	6
Linezolid	30 µg	6	21	3

MM: Millimeter, MRSA: Methicillin-resistant *Staphylococcus aureus*

Table 4: Antibacterial activity of silver nanoparticles against MRSA

Ag-NPs	Strength	Zone size 25–30 mm (No. of isolates)
10 nm	12.5 µg	18
10 nm	25 µg	12

mm: Millimeter, MRSA: Methicillin-resistant *Staphylococcus aureus*

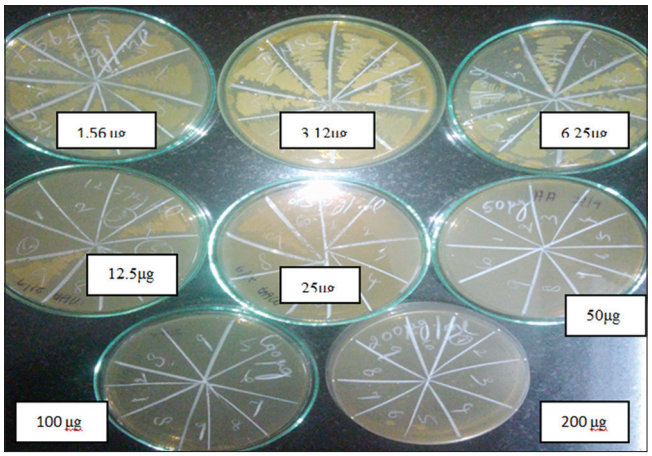

Fig. 5: Minimal bactericidal concentration determination

Fig. 6: Disc diffusion assay of antibiotics

bacterial strains under aseptic conditions, and 2 wells (8 mm) were filled with 12.5 µl and 25 µl of Ag-NPs and incubated at 37°C for 24 h. After the incubation period, the diameter of the growth inhibition zones was measured. 18–24 old single colonies on agar plates were used to prepare the bacterial suspension with the turbidity of 0.5 MacFarland (equal to $1.5*10^8$ CFU/ml).

The zone sizes of antibiotics for isolates are tabulated in Tables 3 and 4 and Fig. 6.

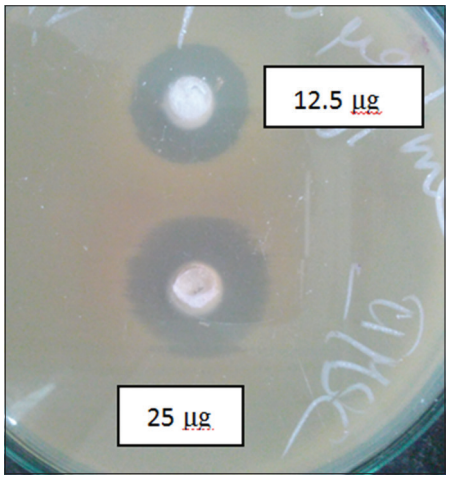

Fig. 7: Well diffusion assay of silver nanoparticles

By comparing the zone sizes of the Ag-NPs and the antibiotics it is evident that the zone size of the Ag-NPs is greater compared to that of the antibiotics tested which is shown in Fig. 7.

CONCLUSION

There has been an evolution of MRSA into filter strains that combine antibiotic resistance, transmissibility, and virulence. Biofilm formation results in chronic recurrent staphylococcal infections or treatment failure. Ag-NPs are effective bactericidal agents regardless of the drug-resistant mechanisms in MRSA. Therefore, Ag-NPs can be recommended as effective broad-spectrum bactericidal agents. Silver nanoparticles have been proven to inhibit biofilm formation in *S. aureus*. Hence, this multifaceted nanoweapon can be used not only for treatment but also for the prevention of drug-resistant microbes.

CONFLICT OF INTEREST

None.

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