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Silver nanoparticles: A potential antibacterial and antibiofilm agent against biofilm forming multidrug resistant bacteria

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ABSTRACT

Background: Multi-drug resistant (MDR) bacteria are seriously endangering the antibiotics. Different alternative strategies are needed to reinforce antibiotics, of, these; nanostructured materials may play a fruitful role. This study aimed to investigate the antibacterial and antibiofilm activity of silver nanoparticles (AgNPs) against MDR bacteria. **Methods:** In a cross-sectional study, a total of 33 methicillin resistant *Staphylococcus aureus* (MRSA) and 52 MDR *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates were recovered from intensive care units' (ICUs) admitted patients over a period of 9 months, from December 2017 to August 2018. The antibacterial activity of AgNPs on the clinical isolates of MRSA and MDR *P. aeruginosa* was assessed by minimum inhibitory concentrations (MICs) using broth microdilution method. The minimum bactericidal concentrations (MBCs) were determined as the lowest concentrations required to kill 99.9% of the initial inoculum. Tissue culture plate method was used to evaluate the antibiofilm activity. **Results:** The MIC and MBC values ranged from 1 to 16 µg/ml and 2 to 64 µg/ml, respectively. Silver nanoparticles exerted a significant antibiofilm activity against MRSA and MDR *P.aeruginosa* at all tested concentrations, recording a maximum inhibition value of (82%) and (91%), respectively. **Conclusions:** AgNPs exhibited a considerable antibacterial and antibiofilm, effect; it could represent a promising weapon in the fight against biofilm forming MDR organisms.

Introduction

The ever-increasing number of MDR bacteria is seriously endangering the worth of antibiotics, effacing its golden era for combating infections. The extremely resistant MDR bacteria are currently threatening the efficiency of healthcare systems all over the world [1]. Methicillin resistant *S. aureus* and MDR *P. aeruginosa* are two of the most notorious bacteria with MDR activity [2].

Methicillin resistant *S. aureus* is responsible for a wide variety of human diseases. It is one of the notable contributors to morbidity and mortality among hospitalized patients all over the world [3]. Compared to other African countries and eastern Mediterranean countries, the highest rates of MRSA among *S. aureus* clinical isolates were recorded in Egypt [4,5].

Pseudomonas aeruginosa is one of the five most frequent causative agents responsible for urinary tract, bloodstream, soft tissue and surgical site infections, added to that, ventilator-associated pneumonia and wound infections in ICUs [6].

The ability of microorganisms to produce biofilms, can be considered as one of the major

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factors contributing to antibiotic resistance. Biofilm associated infections remain a major challenge to human health and represent one of the major threats of modern medicine, where current treatment regimens are not effective [7].

This complex situation of biofilm associated MDR infections has forced researchers and pharmaceutical companies to search for entirely novel antimicrobial strategies capable of controlling the current crisis [8].

Among nanoparticles, AgNPs have been widely used in a range of biomedical applications. Silver nanoparticles have gained the most attraction because of their distinctive chemical and physical properties, added to that, their effective antibacterial, anti-viral, antifungal and anti-inflammatory actions [9]. Furthermore, a notable efficacy of AgNPs against bacterial biofilms has been elucidated in a few studies [10]. Silver nanoparticles can be considered as a promising nominee for unconventional antimicrobial application. Besides their well-known ability to form biofilms [11], *S.aureus* and *P. aeruginosa* were the most prevalent isolated MDR as per our facility records. Thus, the aim of this study was to assess the antibacterial and antibiofilm effect of AgNPs against MRSA and MDR *P. aeruginosa* isolates from Zagazig University Hospital.

Methods

This cross-sectional study was carried out in Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University according to the international guidelines of Strengthening the Reporting for Observational Studies in Epidemiology (STROBE) check list and in accordance with the Declaration of Helsinki. The study was approved by the Institutional Reviewer Board (IRB), Faculty of Medicine, Zagazig University. Informed written consents were obtained from patients or their relatives.

From December 2017 to August 2018, the study included only *S. aureus* and *P. aeruginosa* isolates that met the Centres for Disease Control and Prevention (CDC) definitions of MRSA and MDR-*P.aeruginosa*, respectively.

Case definitions:

Methicillin resistant *S.aureus* is *S.aureus* that has tested Resistant (R) to at least 1 of the following: methicillin, oxacillin, or cefoxitin., MDR-*P.aeruginosa* is *P. aeruginosa* that has tested either Intermediate (I) or Resistant (R) to at least 1 drug in at least 3 of the following 5 categories: 1. Extended-spectrum cephalosporin (cefepime, ceftazidime) 2. Fluoroquinolones (ciprofloxacin, levofloxacin) 3. Aminoglycosides (amikacin, gentamicin, tobramycin) 4. Carbapenems (imipenem, meropenem, doripenem) 5. PIP/PIPTAZ (piperacillin, piperacillin/tazobactam) [12].

Isolation and identification of MRSA and MDR *P. aeruginosa*

Specimens were inoculated on mannitol salt agar, cetrimide agar and blood agar **(Oxoid, UK)** and incubated aerobically at 37°C for 24 hours [13]. Gram positive cocci were further identified for MRSA detection. Gram negative bacilli were further identified for MDR-*Pseudomonas* detection.

Detection of MRSA: strains of *S. aureus* were identified based on colony morphology, Gram's stain, and different biochemical tests [13]. Susceptibility of *S.aureus* isolates to cefoxitin (30 µg) was determined by modified Kirby-Bauer disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines [14].

Detection of MDR *Pseudomonas*: the isolates were presumptively identified by conventional tests, including colony morphology and pigment production, and positive oxidase test. API 20 NE was used for confirmed identification [15].

The antimicrobial susceptibility testing was performed by modified Kirby-Bauer disc diffusion technique using Mueller-Hinton agar (**Becton-Dickinson, Sparks, US)** following CLSI guidelines [14]. *Staphylococcus aureus* ATCC 43300 and *P. aeruginosa* ATCC 27853 strains were used as quality controls.

The antimicrobial efficacy of AgNPs

- **Minimal inhibitory concentration (MIC)**

The ready use AgNPs stock solutions (19±5 nm) (Nano-Tech, Egypt) were used. Minimum inhibitory concentrations of AgNPs were evaluated using the standard broth dilution method [16]. Briefly, serial two-fold dilutions of AgNPs in concentrations from 128 to 0.5 µg/ml were prepared. Initial bacterial inoculums of 1×108 CFU/ml was used. The time and temperature of incubation were 24 h at 37°C, respectively. Minimum inhibitory concentrations were evaluated by visual inspection of presence or absence of microbial growth and assayed using a microtiter plate reader by monitoring absorbance at 600 nm [17]. The MIC was determined as the lowest

concentration of AgNPs at which no visible growth was observed. This applies to the lowest concentration with an average optical density (OD) equal to or within three standard deviations (SDs) of the negative control well [18].

- **Minimal bactericidal concentration (MBC)**

After MIC determination, aliquots from wells that did not exhibit any visible growth were sub-cultured on Mueller Hinton agar plates and incubated for 24h at 37°C. The MBC is defined as the lowest concentration of AgNPs preventing the visible growth of bacteria on the agar plate [17]. Minimum inhibitory concentration and MBC tests were performed in triplicate.

Biofilm formation capacity

All MDR isolates were tested for biofilm formation potential by tissue culture plate assay [19]. Trypticase soy broth with 1% glucose were inoculated with the isolates and incubated for 18 h at 37 °C, then diluted (1 in 100) with fresh medium. Aliquots of 200µL of the diluted bacterial suspensions were added to each well of the 96-well flat bottom tissue culture plates **(Costar, USA)**. After 24 h of incubation at 37 °C, contents of the wells were gently removed, and the wells were rinsed three times with 200µL of phosphate buffered saline (pH 7.2) to remove the planktonic bacteria. Adherence of bacteria to culture plate was detected by 0.1% crystal violet solution, 200 µL/well, plates were incubated at room temperature for 10 min. Excess stain was then rinsed off by washing with deionized water (3 times with 200 µl/well), then plates were kept for drying 20 min. Solubilization of the crystal violet was done using 95% ethanol, 200µL/well. The absorbance at 600 nm was measured using a microplate reader. A well with 200µL sterile broth without isolates was considered as a negative control. *Staphylococcus aureus* ATCC 43300 and *P. aeruginosa* ATCC 15692 were used as positive controls. Experiments were performed in triplicate & the data were expressed as means ± SD.

- **Categorization of isolates based on biofilm-forming capacity**

The average OD to each isolate was calculated and compared with the control cut-off OD (OD_C); $OD_c = OD_{avg}$ of negative control + 3 SD_s of OD_s of negative control 19. Biofilm production among isolates was graded as follows: no production ($OD_{isolate} \leq ODC$), weak production ($ODC < OD_{isolate} \leq 2$ ODC), moderate ($2 ODC < OD_{isolate} \leq 4$ ODC), or strong ($4 ODC < OD_{isolate}$) [19].

Biofilm inhibition assay

Biofilm inhibition assay was carried out in 96 well plates by adopting a previously described method [20]. Briefly, AgNPs were diluted by serial two-fold dilutions from a stock concentration of 200 µg/ml with the lowest concentration used was 0.78 µg/ml. Diluted AgNPs were added to the wells after adding bacterial suspensions. Sterile broth was taken as a negative control. Bacterial suspensions without AgNPs were used as non-treated controls. Experiments were performed in triplicate.

The percentage of biofilm inhibition was calculated using the following equation [21]:

[1 − (A600 of cells treated with AgNPs/A600 of non-treated control cells)] × 100.

Statistical analysis

The data were statistically analysed using SPSS 24 (IBM; Armonk, New York, USA). Continuous data were presented as median (range). Categorical data were presented by the frequency and percentage. Mann-Whitney U test was used to determine if a difference exists between the medians of two independent groups. Wilcoxon signed-rank test was used to compare repeated measurements of the same sample. Simple linear regression was used to predict a continuous variable from another continuous variable. *P value <.05* was considered statistically significant.

Results

A total of 33 MRSA and 52 MDR *P.aeruginosa* isolates were investigated for the antibacterial and antibiofilm effect of silver nanoparticles.

Antibacterial activity of silver nanoparticles:

Minimum inhibitory concentration and MBC values of AgNPs against MRSA ranged from 4 to 64 µg/mL. Minimum inhibitory concentration and MBC values of AgNPs to MDR *P. aeruginosa* ranged from 1 to 64 µg/mL. Minimum inhibitory concentrations and MBCs for both bacteria are illustrated in **figures (1&2)**. A highly statistical significant difference in antibacterial effect was observed against Gram positive MRSA versus that of Gram negative MDR *P.aeruginosa* AgNPs *(P<.001).*

Minimum inhibitory concentrations were submitted for regression analysis with the related risk factors retrieved from patients' records. For MRSA, prolonged hospital stay and prolonged

medical device insertion, were significantly associated with high MIC's levels *(P<.05)*. None of the studied factors was significantly associated with the variations in the MICs of AgNPs against MDR *P. aeruginosa.*

Biofilm formation and inhibition

Biofilm formation was detected among all MRSA and 50/52 (96%) of MDR *P.aeruginosa* isolates. The majority of biofilm forming isolates were strong biofilm producers; 55% and 60% of MRSA and MDR *P.aeruginosa*, respectively. The highest activity of AgNPs was observed at concentration of 100 µg/ml, with an inhibition value around the 82% for MRSA biofilm. At 100 µg/ml, the inhibition value for biofilm forming MDR *P.aeruginosa* was around the 91 %. Variations in the biofilm formation profiles prior to and after treatment with AgNPs were furtherly evaluated. As illustrated in **tables (1&2)**, an obvious antibiofilm effect of AgNPs was observed as follows: at concentrations of 100, 50, 25 and 12.5 µg/ml of AgNPs, a median of strong biofilm production prior to treatment significantly shifted to no biofilm formation after treatment *(P<.01)*. Following exposure to 6.25 and 3.125 µg/ml of AgNPs, isolates shifted from strong to weak biofilm. At 1.56 µg/ml, the shift was found to be from a strong to weak among MRSA isolates and from strong to moderate among MDR *P.aeruginosa* . At 0.78 µg/ml, isolates with a strong production median changed to moderate following exposure to AgNPs.

Figure 1. AgNPs MIC values for both MRSA and MDR *P.aeruginosa* isolates.

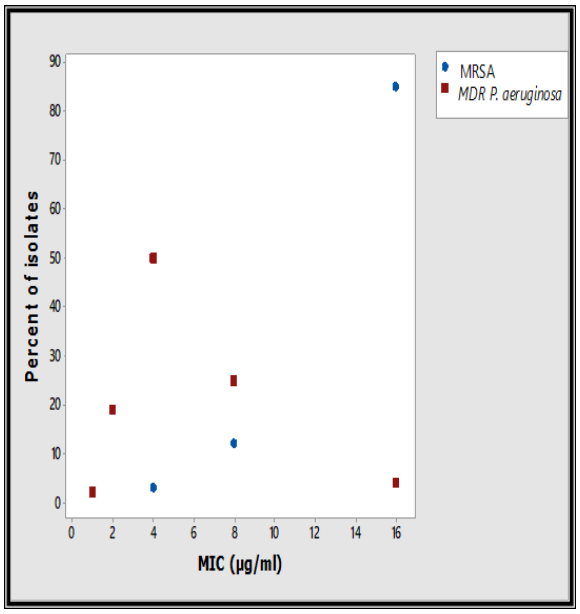

Figure 2. AgNPs MBC values for both MRSA and MDR *P.aeruginosa* isolates.

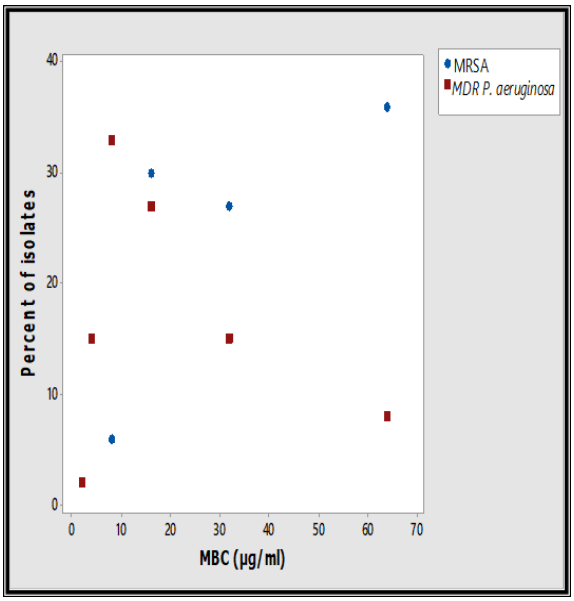

Table 1. Effect of AgNPs on MRSA biofilm formation profile

Before AgNPs (n=33) median(range)	**After AgNPs** (n=33) median(range)	**Wilcoxon signed rank test**	***P*-value**
At conc. 100 3(1-3)	0(0-3)	Z=-4.5	**<.001**
At conc. 50 3(1-3)	0(0-3)	Z=-4.2	**<.001**
At conc. 25 3(1-3)	0(0-3)	Z=-4.2	**<.001**
At conc. 12.5 3(1-3)	0(0-3)	Z=-4.1	**<.001**
At conc. 6.25 3(1-3)	1(0-3)	Z=-4.0	**<.001**
At conc. 3.125 3(1-3)	1(0-3)	Z=-3.8	**<.001**
At conc. 1.56 3(1-3)	1(0-3)	Z=-3.5	**<.001**
At conc. 0.78 3(1-3)	2(0-3)	Z=-2.9	**.004**

For easier interpretation: biofilm categories; ordinal categorical data (no biofilm, weak, moderate, and strong), were ranked on a numerical scale from 0→3, where 0= no biofilm formation, 1 = weak, 2= moderate and 3= strong. **P value of < 0.01 indicates highly significant results,

Table 2. Effect of AgNPs on MDR *P. aeruginosa* biofilm formation profile.

Before AgNPs	**After AgNPs**	**Wilcoxon signed rank test**	***P-value***
median(range)	median(range)		
At conc. 100		Z=-5.7	**<.001**
3(1-3)	0(0-3)		
At conc. 50		Z=-5.4	**<.001**
3(1-3)	0(0-3)		
At conc. 25		Z=-5.3	**<.001**
3(1-3)	0(0-3)		
At conc. 12.5		Z=-5.2	**<.001**
3(1-3)	0(0-3)		
At conc. 6.25		Z=-4.9	**<.001**
3(1-3)	1(0-3)		
At conc. 3.125		Z=-4.6	**<.001**
3(1-3)	1(0-3)		
At conc. 1.56		Z=-4.1	**<.001**
3(1-3)	2(0-3)		
At conc. 0.78		Z=-3.9	**<.001**
3(1-3)	2(0-3)		

For easier interpretation: biofilm categories; ordinal categorical data (no biofilm, weak, moderate, and strong), were ranked on a numerical scale from 0→3, where 0= no biofilm formation, 1 = weak, 2= moderate and 3= strong.

**P value of < 0.001 indicates highly significant results.

Discussion

As a global problem, the rising rate of biofilm forming MDR bacteria has gained the world's most attention lately [22]. Because of the complicated treatment regimens, novel alternative antimicrobial strategies became a must. In this area and owing to their unique properties, AgNPs have held great potentials in overcoming this growing problem [23].

Minimum inhibitory concentration values of AgNPs against MRSA ranged from 4 to 16 µg/mL. Within a similar range of 1.56-25 µg/ml, **Simon et al.** [24] reported the MIC values of AgNPs against MRSA. **Ibraheem et al.** [25] reported a higher MIC range of 20-160 µg/mL. On the other hand, **Holubnycha et al. [**26] reported AgNPs activity at much lower MIC range from 0.6-9.6 µg/mL.

Prolonged hospital stay and prolonged device insertion were the most important risk factors with high MICs of AgNPs against MRSA.

Regarding MDR *P.aeruginosa,* in this study , AgNPs' MIC values were observed to be in the range of 1-16 µg/mL, thus recording a better antibacterial activity as compared to the earlier work of, **Ali et al.** [27] and **Nasiri et al.** [28], who have reported AgNPs activity against MDR *P.aeruginosa* at higher concentration range of 20-40 µg/mL and 12.5-100 µg/mL, respectively. On the contrary, **Liao et al.** [29] reported AgNPs activities at much lower concentrations, in which the MIC range was found to be 1.406-5.624 µg/mL.

Among the suspected causes of the aforementioned variability is the difference in the size and shape of AgNPs used, the assessment methods, as well as, the tested bacterial strains.

Minimum inhibitory concentrations and MBCs of AgNPs against MDR *P.aeruginosa* were lower than against MRSA suggesting that the Gram-negative bacteria are more sensitive to AgNPs than the Gram-positive one. This was consistent with the findings of **Navarro-Gallón et al.** [30]. The major reason supporting this difference in the sensitivity to AgNPs, is the variation in cell wall composition between Gram-positive and Gram-negative bacteria.

All MRSA isolates were biofilm producers. **Abdel-Halim et al.** [31] also reported high percentage of biofilm production (86.5%) among MRSA isolates. The obtained result in this work was much higher than that reported by **Neopane et al.** [32], where biofilm formation was observed in (43.3%) of the isolates. This difference probably came from the diversity of isolates between different hospitals and different geographical locations as well as site variation of the clinical samples.

In the present study, out of 52 MDR *P. aeruginosa* strain, 50 (96%) were biofilm producers. This result nearly resembled that presented by **Elhabibi and Ramzy** [33], in which biofilm production was reported in (100%) of the isolates.

An evident high rate of biofilm production among MRSA and MDR *P. aeruginosa* could be noticed. For some bacteria, the expression of biofilm is mostly influenced by acquisition of resistance genes, this biofilm might have a role in the pathogenesis of infections caused by MDR [34]. Furthermore, antimicrobials used to treat biofilm-forming pathogens does not target the intractable biofilms; rather they target their planktonic counterparts, thus creating selective pressure on bacteria with subsequent increase in the resistant ones [35].

Being an important cause of drug resistance, infection control protocols should be revised and updated to reconsider biofilm prevention and control. Formulation of an effective antimicrobial policy for early treatment of biofilm associated infections could help. Furtherly, strict adherence to infection control bundles in healthcare settings would minimize device related biofilm infections.

The results of the present study revealed a highly significant antibiofilm effect of AgNPs against MRSA at all tested concentrations with the highest inhibition value around (82%) at 100 µg / mL. These results were better than **Zhang et al.** [36] who recorded maximum inhibition rate of (50.4%) at 500 µg/ml. However, **Abo-Shabha et al.** [37] recorded better results, in which, the highest antibiofilm effect (97%) was achieved at a concentration of 50 µg/ml.

In this study, a very highly significant antibiofilm effect of AgNPs against MDR *P. aeruginosa* was found at all tested concentrations with the highest inhibition value around (91%) at 100 µg / mL. Similar results were reported by **Singh et al.** [38], where 100 µg/mL resulted in (90%) reduction in biofilm. On the other hand, **Pompilio et al.** [39] recorded much better results, in which AgNPs with a concentration of 8.5µg/ml, reduced (98%) of the biofilm.

Biofilm inhibition at sub-MIC concentrations; 3.125, 1.56 and 0.78 µg/ml for MRSA and 0.78 µg/ml for MDR *P.aeruginosa*. might be due to nonlethal damage or due to inhibitory effect on expression of genes related to biofilm formation and Quorum sensing [40]. This could be supported by the significant antibiofilm effect on both organisms regardless of Gram positive and negative.

Given, the different methods used for AgNPs' synthesis and the lack of breakpoints guiding results interpretation, a major limitation would be anticipated in the current study. Yet, the reported data could set a base for further studies for much more exploration of the effect of AgNPs.

Conclusion

Silver nanoparticles exhibited a remarkable antibacterial activity against both MRSA and MDR *P. aeruginosa*, representing a possible alternative for antibiotics. Furthermore, AgNPs can also be a promising antibiofilm agent.

Recommendations

Further studies are needed to explore the molecular basis of AgNPs effect as well as the probable cytotoxicity of these materials. Development of guidelines for characterization of nanoparticles and unified standards for breakpoints have become indispensable both on the national and international levels.

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