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The effect of Silver nanoparticles on biofilm production of vancomycin resistant *Staphylococcus aureus*

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Abstract

***Background & Aims*:** The increasing rate of vancomycin resistant *Staphylococcus aureus* (VRSA) with biofilm formation may become a new threat to humans. In such cases, finding an effective treatment strategy such as using Nanotechnology (Nano- drugs) to deal with these types of infections may be promising. This study aimed to investigate the inhibitory effects of silver nanoparticles (SNPs) on biofilm formation of VRSAs.

***Materials and Methods*:** Clinical *S. aureus* isolates were identified to the species level by conventional methods, and their identities were later confirmed by PCR. Following the determination of susceptibility patterns of the isolates; all the screened *S.aureus* isolates have been assessed regarding their susceptibility to vancomycin. Detection of *van*A gene and determination of minimum inhibitory concentrations (MICs) of VRSAs were carried out using PCR and Etest methods, respectively. The biofilm production was assessed on all VRSA isolates in the presence/absence of SNPs using micro-titer plate method.

***Results*:** In total, 11 (6.21%) VRSAs were identified among 177 *S. aureus* clinical isolates. These isolates were included in the biofilm production assay. All of the VRSAs were multidrug resistance and biofilm producers. The inhibitory effect of SNPs in concentration of 250 µg/ml on biofilm formation of VRSA isolates was significant ($Pv = 0.01$).

***Conclusion*:** Based on our findings, SNPs can prevent biofilm formation of VRSAs and applying of these nanoparticles may prohibit from the persistence and colonization of such resistant isolates.

***Key words*:** Vancomycin Resistant, *Staphylococcus aureus,* Biofilm, Inhibitory Effects

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Introduction

The increasing incidence of hospital-acquired infections caused by methicillin- resistant *Staphylococcus aureus* (MRSA) and recently vancomycin-resistant *S. aureus* (VRSA) correlate with excessive antibiotic therapy, the increasing use of medical apparatus such as catheters or endotracheal tubes and as well as cumulative underlying disease (1). Colonization and the formation of biofilm on the surface of the external devices is the most important phase in the initiation of staphylococcal infections. The biofilm consist of a group of microorganisms surrounded by a matrix, including polysaccharide, protein, and DNA. The biofilm characteristics like increased disinfectant

and antibiotic resistance along with anti-phagocytic properties provide context for chronic infections (2). In such cases, finding an effective treatment strategy to deal with these types of infections could be remedial.

Advances in nanotechnology have provided a ground for using metal- nanoparticles in various fields of medicine, for instance, in the diagnosis and treatment of different diseases (3). In the meantime, it seems that the researchers are shifting towards nanoparticles in general and the silver nanoparticles in particular to solve the problem of the emergence of MDR bacteria (4) such as MRSA and VRSA. Both the tube and the Microtiter-plate tests are available methods for measuring biofilm production, which is the main indicator for the pathogenicity of staphylococci (5). The present study aimed to evaluate the anti-biofilm ability of Silver nanoparticles (SNPs) among VRSAs, isolated from clinical specimens of hospitalized patients.

Materials and Methods

***Bacterial isolates and antibiotic susceptibility testing**:*

Bacterial isolates were obtained from different clinical specimens submitted to diagnostic laboratories of three university teaching hospitals of Urmia- Iran (2012- 2015). Initially, isolates were identified to the species level by conventional methods, and their identities were later confirmed by PCR. Isolates other than *S. aureus* were excluded from this study.

The susceptibility pattern of isolates was determined using the disk diffusion test (DDT) as described by the Clinical and Laboratory Standards Institute's (CLSI, 2014)(6) for 11 commonly prescribed antibiotics (MAST Co., England), namely; penicillin, gentamicin, erythromycin, rifampin, teicoplanin, ampicillin, mupirocin, ciprofloxacin, tetracycline, clindamycin and trimethoprim-sulfamethoxazole. *S. aureus* ATCC 35923 has been used as a quality control strain for susceptibility testing.

Screening of VRSA among MRSA isolates and determination their minimum inhibitory concentration (MIC):

At first, methicillin resistance was evaluated by the cefoxitin (30 µg) (Mast Diagnostics, Merseyside-UK) disk diffusion method based on the CLSI (2014) recommendations (6) and correlated with the presence of the *mec*A gene using PCR. All the screened MRSA isolates have been assessed in respect of their susceptibility to vancomycin using BHI- vancomycin (6µg/ml) screening agar method (6). For all non-susceptible isolates, the minimum inhibitory concentration (MIC) of vancomycin was determined using the E-test method (Liofilchem, Italy) according to the manufacturer's instructions.

Biofilm formation assay:

The biofilm formation assay was performed on all VRSA isolates as described previously(7). Briefly, we adjusted the fresh growing bacteria (VRSAs) to obtain turbidity equivalent to 0.5 McFarland's standard (1.5×10^8 CFU/ml). Thereafter, the bacterial suspensions were diluted in 1:100 into fresh medium. A 100 µl of diluted suspensions were added to each well of 96- well (U shaped) Micro-titer plate. The medium without bacteria was considered as a negative control. *Burkholderia cepacia* (ATCC 25416) has been used as biofilm producer control strain. The plate was incubated for 24 h at 37°C. Then, the plate turns over and shakes out the liquid to remove unattached cells and medium components. Following that, the wells were washed three times with sterilized water to decrease of background staining. Then, 125 µL of a 0.1% solution of crystal violet in water was added to each well of the micro-titer plate. This plate was incubated at room temperature for 10-15 min. The extra stains were rinsed off by dipping in a tub of water, shake out and blot vigorously on a stack of paper towels. After drying of the plates, 125 µL of 30% acetic acid was added to the wells and incubated at room temperature for 10-15 min. Next, 125 µL of the solubilized crystal violet has been transferred to a new flat bottomed micro-titer dish and the optical densities (ODs) of stained adherent bacteria were determined with a micro plate reader at 550 nm. The 30% acetic acid in water was used as the blank. The obtained OD was measured as the formation of biofilm on the surface of the culture plate. In order to increase the accuracy and for assessable of tests, the experiment was performed in eight replicate for each isolate.

Interpretation of the results:

The biofilm production of each strain was calculated according to the criteria described by Stepanovic *et al*(8). Based on their recommendations; the biofilm producer strains are categorized in four groups; no biofilm producer (ODs ≤ ODc), weak biofilm producer (ODc< ODs ≤ 2 × ODc), moderate biofilm producer (ODc< ODs ≤4 × ODc) and strong biofilm producer (ODs > 4 × ODc). ODc and ODs stand for optical density of cut-off value and strains, respectively.

Biofilm inhibition assay:

To determine the anti-biofilm formation of the Silver nanoparticles (SNPs), the nanoparticles with 20 nm in size were diluted from a stock (US 1037; US Research Nanomaterials, Inc.) in four concentrations, including; 500, 250, 100 and 10µg/ml. The concentrations were chosen based on the inhibitory concentration values which only impeded the synthesis of the exopolysaccharides and following that biofilm formation but not to affect the viability of the organism. Different concentrations of SNPs were added to the 96-well micro-titer plate that each well contains 100 µl of overnight grown bacteria, which diluted in 1:100. Next steps were similar to the steps described in biofilm formation assay.

Hemolysis assay to evaluate the toxicity of SNPs:

In order to evaluate the toxicity of different concentrations of SNPs, hemolytic effects of SNPs in human erythrocytes have been assessed as mentioned by Jiang *et al* (9). 0.8 ml of distilled water and Phosphate-buffered saline (PBS) were added to 0.2 mL of diluted RBC suspensions as the positive and negative control, respectively.

Statistical analysis:

The data analysis carried out by using SPSS 16 software. Kruskal- Wallis test was used for detection of the effect of SNPs on biofilm production by VRSAs. In addition, the Mann- Whitney test was used for comparison of the corresponding between basic biofilm production by the bacteria and inhibitory effects of each concentration of SNPs.

Results

Bacterial isolates and antimicrobial susceptibility patterns of the VRSAs:

Of 177 isolated *S. aureus*, 95 (53.7%) were MRSAs. Among the MRSAs, 11 strains (11.57%) were VRSAs using both the phenotypic (E-test) and genotypic (PCR: the presence of *van*A gene) analyses. More information about the MICs and molecular characteristics of these isolates has been published earlier (10). The VRSA isolates included in this research to evaluated the effect of SNPs on biofilm formation. Antibiotic susceptibility patterns of the VRSA isolates have been shown in figure 1.

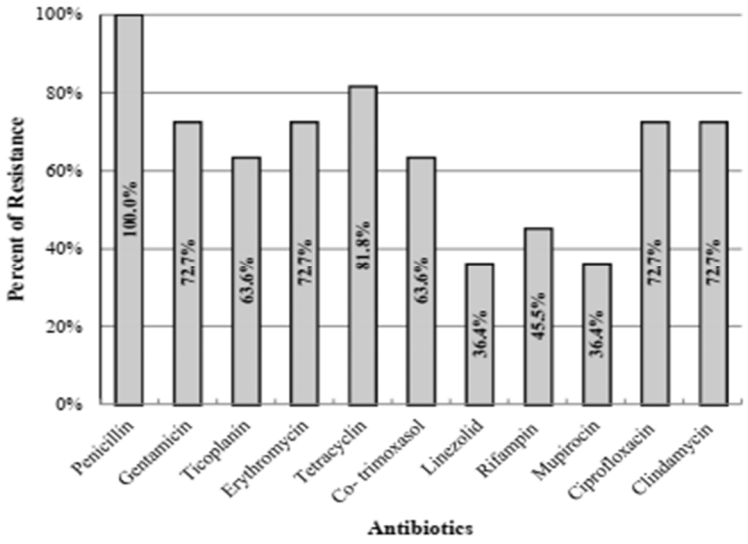

Figure 1. Antibiotic susceptibility patterns of vancomycin resistance S. aureus isolates

Biofilm formation:

Biofilm formation of VRSAs was investigated in vitro by monitoring the binding of 0.1% crystal violet to the bacterial cells adhered to a micro- titer plate by measuring optical density at 550 nm. Accordingly, six and five of the strains were strong and moderate biofilm producers (Figure 2).

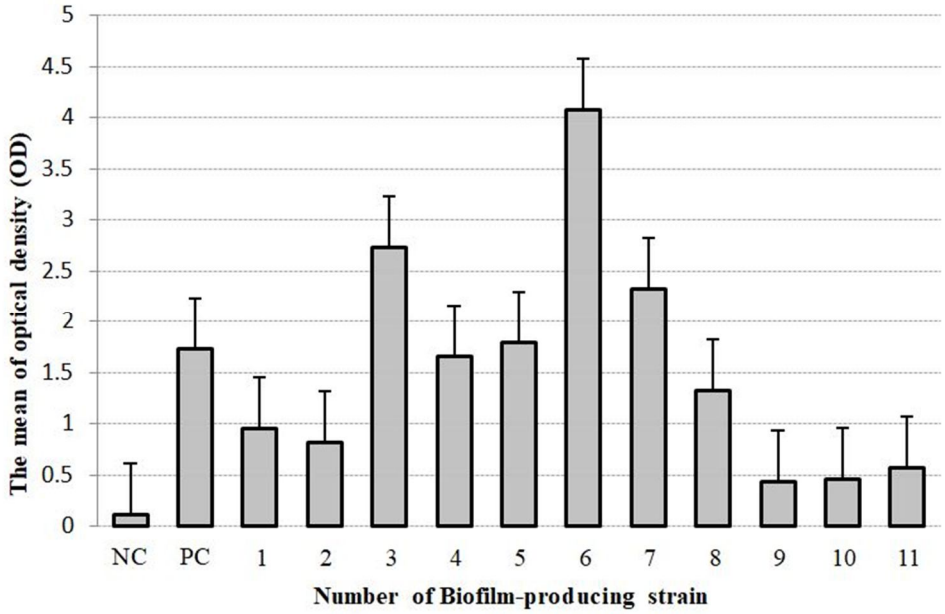

Figure 2. The results of biofilm production by vancomycin resistance isolates using micro- titer plate assay

Prevention of biofilm formation by silver nanoparticles:

Inhibition of biofilm production in the presence of 500, 250, 100 and 10µg/ml of SNPs checked out and compared with the rate of biofilm production in the absence of nanoparticles in figure 3. The results revealed that the activity of SNPs is highest at the concentration of 250 µg/ml (Pv= 0.01).

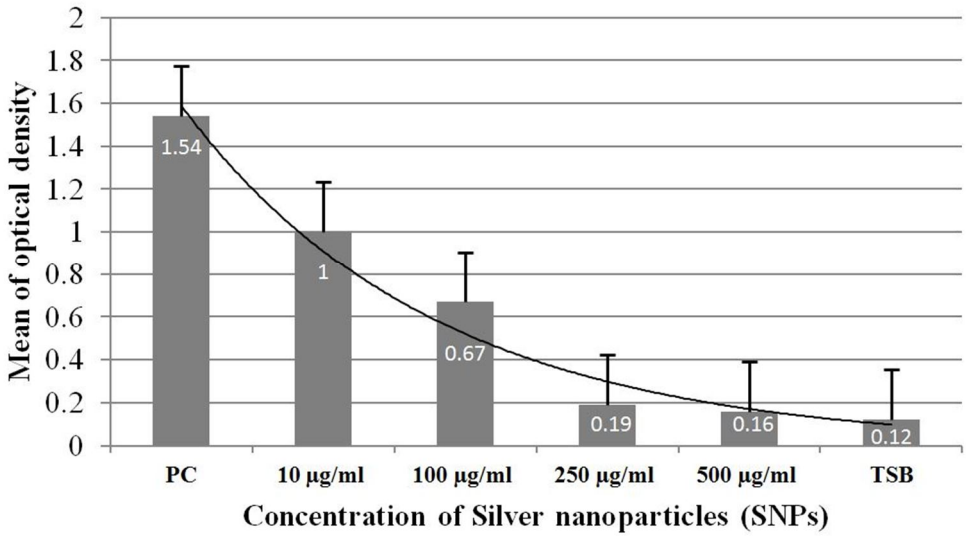

Figure 3. The results of inhibitory effect of different concentrations of Silver Nanoparticles on biofilm production

Hemolytic Effects of SNPs in human Erythrocytes:

As demonstrated in figure 4 and table 1, the percent of hemolysis has been augmented by the increased concentration of nanoparticles.

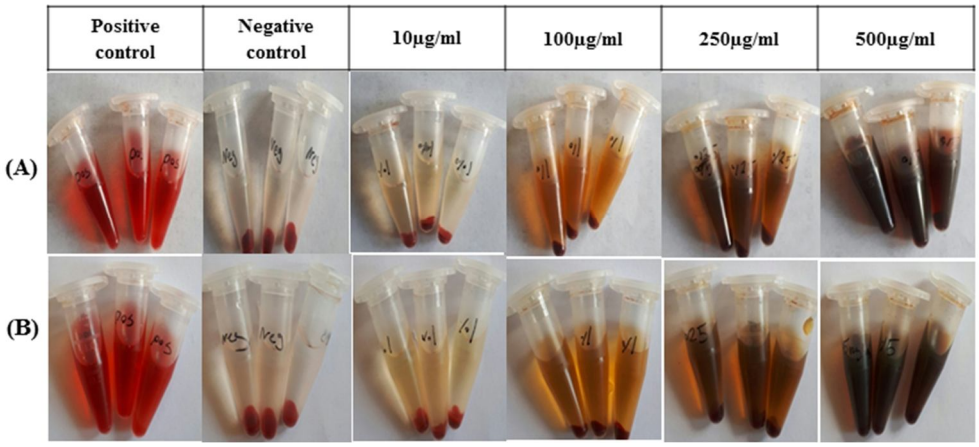

Figure 4. The results of hemolytic Effects of Silver Nanoparticles in Human Erythrocytes

Table 1. The percent of hemolysis by different concentrations of Silver Nanoparticles

Time		Concentrations of SNPs			
		10µg/ml	100µg/ml	250µg/ml	500µg/ml
Hemolysis (%)	30 min (FigA)	1.56	1.56	6.66	19.21
	2 h (FigB)	2.32	6.58	13.95	40.69

Discussion

In the past few years, a large number of clinical isolates of *Staphylococcus aureus* are methicillin resistant (resistant to all beta-lactams) followed by acquiring resistance to other classes of antibiotics (11). Subsequently, the growing rate of infections due to MRSAs has been a global concern. Despite the spending of more than fifty years from the introduction of vancomycin, this antibiotic has been used mainly for the treatment of invasive MRSA infections. However, the advent of VISA and following that VRSA isolates are limiting the use of the antibiotic to mentioned situations (10, 12). Acquisition of antibiotic resistant along with more virulence factor such as biofilm production can exacerbate disease caused by VRSAs; since biofilm helps the persistence of staphylococcal infections by protecting bacteria from host immune defenses, antibiotic therapies, and enhancing bacterial adherence (13). It is also thought that the transfer of *van*A gene from vancomycin resistant enterococci to *S. aureus* was completed within a polymicrobial biofilm (14). In this research, all studied VRSAs were multidrug resistant (resistant to three or more groups of antibiotics) and were biofilm producers. It seems that the biofilm production limits the penetration of antibiotics into the bacteria and following that increases the mortality rates by such bacteria. Among our cases, two cases (hospitalized in ICU) have been deceased. Consistent with our study, the desire to vancomycin resistance among MRSA strains has been reported from different countries (14, 15), that spreading of them may become a new threat to human being. In such circumstances, collaboration among the researchers by providing appropriate and applicable methods can solve this problem.

Recently, regarding to the use of nanotechnology, the studies on nanomaterial against the new multidrug-resistant bacteria and formed biofilms has been

increased. The effect of SNPs is highly influenced by the nanoparticles' size, shape and concentration (16). The experiments on *S.aureus* treated with SNPs revealed that damages on the cell wall and cell membrane, interfered with the normal metabolism of cells, following entrance into the cell can condense DNA to prevent DNA replicating (17). In the present study, we checked the different concentration of SNPs on biofilm producer VRSAs and found that more effective concentration is 250 µg/ml (*P*v= 0.01). In agreement with our findings, Kalishwaralal K. *et al.* (2010) revealed that SNPs almost completely inhibit the biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (18). Another study also revealed that SNPs as a medicament could potentially eliminate residual bacterial biofilms during root canal disinfection (19). Regarding to cytotoxicity effects of SNPs on human red blood cells, we found that SNPs with 10µg/ml is less cytotoxic than other concentrations tested in this study. Dose-dependent hemolytic effects also increased by the time of exposure (Figure 4).

Conclusion

Based on our finding, the VRSA isolates maintain the ability to produce biofilm, which is the severe threaten for the infected patients. Constant and precise efforts to the detection of such resistant strains via application of the proper infection control measures can prevent the spread of them in the hospital setting. Our finding indicated that SNPs can reduce the production of biofilm by VRSAs. It suggests that SNPs can be used in the form of antimicrobial agents as alternative treatments to conventional antimicrobial agents for control and prevention of spreading of *S.aureus.*

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Declaration of interest

The authors report no conflicts of interest.

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