Egyptian Journal of Medical Microbiology Volume 26 / No.4 / October 2017 49-54

ORIGINAL ARTICLE

Inhibitory Effect of Silver Nanoparticles on Biofilm Production by Methicillin Resistant Staphylococci

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Key words:
Biofilm, Staphylococcus aureus, Staphylococcus epidermidis, MRSA, MRSE, Nanotechnology, silver nanoparticles

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ABSTRACT

Background: *The ability of bacteria to colonize surfaces and form biofilms is a major cause of antibiotic resistant infections. Biofilm formation is characteristic for Staphylococcus aureus and Staphylococcus epidermidis infections. Biofilm consists of several layers of bacteria encased within an exopolysachharide glycocalyx. Nanotechnology may help to penetrate such biofilms and reduce biofilm forming ability of the bacteria.* ***Objectives:*** *This study aimed to evaluate the anti-biofilm efficacy of silver nanoparticles against biofilm producing strains of methicillin resistant Staphylococcu aureus (MRSA) and methicillin resistant Staphylococcus epidermedis (MRSE).* ***Methodology****: biofilm formation by MRSA and MRSE strains was detected twice, before and after addition of Silver nanoparticles (AgNPs) using Congo Red Agar and tissue culture plate method to determine the anti-biofilm activity of AgNPs.* ***Results****: Addition of AgNPs by different concentrations reduced biofilm formation. For example, addition of 50µg ml of AgNPs, reduced biofilm formation. Percent of inhibition were 96.6 ± 1.85 for MRSA and 95.75 ± 4.18 for MRSE.* ***Conclusion****: AgNPs play a major role in the inhibition of biofilm formation by MRSA and MRSE.*

INTRODUCTION

Staphylococcus aureus (*S.aureus*) and *Staphylococcus epidermidis* (S.epider-midis) are members of the genus Staphylococcus that also includes a group of commensals that colonize on the skin or mucous membranes of humans. *S.aureus* causes superficial skin to deep seated infections including both hospital and community-acquired infections [1]. *S.epidermidis* is considered as a potential cause of infections due to its antimicrobial resistance[2]. Therefore, *S.aureus* and S. epidermidis are responsible for an overwhelming burden on the health care system 3.

Methicillin resistant *S.aureus* (MRSA) and Methillin resistant S.epidermedis (MRSE) have emerged as a significant threat in both the hospital and community acquired infections [4]. Transmission occurs mostly through direct contact with wounds, respiratory and feeding tubes, urinary catheters, or indwelling devices [5].

A biofilm can be defined as a microbial community where the cells are attached to an interface, embedded in an exopolysaccharides matrix [6]. Biofilms have been considered a problem in the medical field as they could delay wound healing. Biofilm forming bacteria can also cause chronic infections with persistent inflammation and tissue damage despite antibiotic therapy [7].

Biofilms formed by *Staphylococci* are of the most common etiologic agents of device related infections[8]. The ability of *S.aureus* and *S.epidermidis* to form biofilms on implanted medical devices or damaged host tissue is a key virulence factor for this pathogen especially in hospitals where antibiotic use is high. Subsequently, biofilm formation represents a survival mechanism for the bacteria [9].

Nanotechnology has been recently investigated to treat infections caused by resistant bacteria. Microbial cells are unlikely to develop resistance to nanoparticles (NPs), because they act by different mechanisms than that of conventional antibiotics [10]. Due to their extremely small size, NPs possess special characteristics. Their small size provides them enormous surface area, high reactivity and easy penetrability into the biofilm matrix and cell membranes[11].

Silver nanoparticles (AgNPs) are emerging as one of the fastest growing nanotechnology-based product categories[12], they have been known to exert inhibitory and bactericidal effects and to have a broad spectrum of antimicrobial activities against many Gram-positive, Gram-negative, and fungal pathogens [13], also they have a potential use to treat multi-drug resistant bacteria such as MRSA and MRSE as they act synergistically on distinct targets so it is expected that there will be no interference with antimicrobial resistance mechanisms[14].

METHODOLGY

Patients and data collection

This study was conducted in Medical Microbiology and immunology Department, Faculty of medicine, Tanta University. The study included 122 different specimens, 108 were patient samples and 14 specimens were from medical devices.

Isolation and identification of Staphylococci

Endotracheal aspirates, Blood, Urine, samples from wounds, indwelling devices were inoculated followed by identification of the arising colonies according to standard microbiological methods. Cultures were maintained on tryticase soy broth containing 20 % glycerol at –80°C [15].

Antimicrobial susceptibility testing:

The disk diffusion method was carried out according to the Clinical and laboratory Standards Institute guidelines [16]. To determine the minimum inhibitory concentration (MIC) for oxacillin, E-test strips (LIOFILCHEM® - ITALY) were used. MIC of ≥4µg/mL and ≥0.5µg/mL was considered as resistant and MIC of ≤ 2µg/mL and ≤ 0.25µg/mL was reported as susceptible for *S.aureus* and *Staph.epidermides* respectively [17, 18].

Biofilm detection:

Congo red agar (CRA) method [19]:

Positive result was indicated by black colonies with a dry crystalline consistency. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result, non-biofilm producers usually remained pink [20].

Tube method [21]*:*

A total of 10 ml trypticase soya broth (TSB) with 1% glucose was inoculated with a loopful of microorganism from overnight culture plates and incubated for 24 h at 37°C. The tubes were washed with phosphate buffered saline (PBS) 0.1% (pH 7.3), dried, and stained with crystal violet (0.1%). Biofilm formation was considered as positive, when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation.

Tissue culture plate (TCP) method [21]:

A total of 10 ml of Trypticase soy broth (TSB) with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar. The broth was incubated at 37°C for 24 h then the culture was further diluted 1:100 with fresh medium. 96 wells flat bottom TCPs were filled with 0.2 ml of diluted cultures individually. Only sterile broth was served as blank (negative control). Reference strain of positive control *Staphylococcus epidermedis* ATCC 35983 was also diluted and incubated. After incubation, gentle tapping of the plates was done. The wells were washed with 0.2 ml of PBS (pH 7.2). Adherent biofilms were fixed with 2% sodium acetate and stained with 0.1% crystal violet. After drying the plates, optical densities (OD) of stained adherent biofilm were obtained with a micro ELISA autoreader at wave length 570 nm. Experiment was performed in triplicate. OD values greater than 0.240 were taken as Strong biofilm producer, OD values less than 0.120 as non-biofilm producer and those between 0.120 and 0.240 were taken as moderate biofilm producers [20].

Evaluation of antibiofilm effect of Silver nanoparticles:

A stock solution of water soluble silver nanoparticles (triangular) was purchased from Nano Tech, Egypt.

Using congo red agar [22]*:*

Silver nanoparticles at concentration of 20µg/ml was added with the congo red stain to other medium constituents then poured in plates.

Using tissue culture plates [23]:

Different concentrations of silver nanoparticles were prepared from stock 200 µg/ml, and then 0.1 ml of these dilutions was added to the wells after adding diluted cultures as follows: First column, silver nanoparticles were added with concentration 100 µg/ml to reach the desired concentration (50 µg/ml), Second, third and fourth columns, Ag NPs were added with concentration 80, 40, 20 µg/ml to reach (40, 20, 10 µg/ml) respectively, fifth column was served as negative control (untreated biofilm). The percentage inhibition of biofilm activity was calculated using the following equation [24]:

Biofilm inhibition (%) = 1- (absorbance of cells treated with AgNPs / absorbance of non-treated wells) × 100.

RESULTS

This study was conducted during the period from July 2016 to February 2017. It included 122 specimens, 108 were patient samples (60 males and 48 females), and their ages ranged from 5 years to 83 years and 14 specimens were from medical devices. Isolation and identification of organisms were done according to the standard microbiological methods.

Staphylococci were 44.3% from total specimens (21.3% were *S.aureus* and 23% were coagulase negative *staphylococci*). Identification of of MRSA and MRSE isolates was achieved by cefoxitin disc diffusion method and confirmed by oxacillin E test, For *S. aureus* isolates, E-test showed that 87 % were MRSA and 13% were MSSA while regarding *S.epidermedis* 95.2% were MRSE and only 4.8% were MSSE (table 1).

Detection of biofilm formation of clinical isolates of MRSA and MRSE was carried out by three methods: Congo red agar method (CRA), Tube method (TM) which is simple and fast and Tissue culture plate (TCP) method, which is the gold standard screening method. The percent of MRSA showing biofilm by CRA, TM and TCP methods were 90%, 85%, 75% respectively, however, regarding to MRSE, percent of biofilm

formation were 85% for both CRA and TM and 80% for TCP method (**table 2**).

The anti-biofilm effect of silver nanoparticles was observed by microtitre plate assay using different concentration, Silver nanoparticles with concentration of 50 µg/ml, recorded maximum antibiofilm effect (96.6 ± 1.85) followed by concentrations of 40,20, 10µg/ml which were able to eliminate the biofilm formation of MRSA on the plate surface by (89.11± 8.28), (78.35±16.57), (71.99±19.33) respectively. For MRSE isolates, the inhibition were (95.75 ± 4.18), (90.43 ± 6.08), (78.15 ± 11.14), (73.44± 14.04) respectively (**table 3**).

Table 1: Percentage of MRSA and MRSE by oxacillin E- test and cefoxitin disk

	Oxacillin E test	Cefoxitin disc						Chi-square	
		Resistant		Sensitive		Total			
		N	%	N	%	N	%	X^2	P-value
Staphylococcus aureus	Resistant	20	87	0	0.0	20	77	11.304	<0.001**
	Susceptible	3	13	3	100.0	6	23		
	Total	23	100.0	3	100.0	26	100.0		
Staphylococcus epidermedis	Resistant	20	95	0	0.0	20	74	13.238	<0.001**
	Susceptible	1	5	6	100.0	7	26		
	Total	21	100.0	6	100.0	27	100.0		

** Highly significant at p-value < 0.001.

Table 2: Comparison between three methods of biofilm detection (congo red agar, tube method and Microtitre plate method):

CRA	TM	MTP											
		MRSA						MRSE					
		Positive		Negative		Chi-square		Positive		Negative		Chi-square	
		N	%	N	%	X^2	P-value	N	%	N	%	X^2	P-value
Positive	Positive	15	75.0	2	10.0	10.294	<0.001**	16	80.0	1	5.0	11.922	<0.001**
	Negative	0	0.0	1	5.0			0	0.0	2	10.0		
Negative	Positive	0	0.0	0	0.0	0.567	0.837	0	0.0	0	0.0	0.687	0.924
	Negative	0	0.0	2	10.0			0	0.0	1	5.0		

** Highly significant at p-value < 0.001.

Table 3: Inhibition percentage of biofilm formation observed by different concentration of silver nanoparticles (10, 20, 40, 50 µg/ml)

AgNPs concentration	MRSA (15)			MRSE (16)		
	Mean	±	SD	Mean	±	SD
Nano 50 µg/ml	96.60	±	1.85	95.75	±	4.18
Nano 40 µg/ml	89.11	±	8.28	90.43	±	6.08
Nano 20 µg/ml	78.35	±	16.57	78.15	±	11.14
Nano 10 µg/ml	71.99	±	19.33	73.44	±	14.04
Paired t-test						
	T		P-value	t		P-value
50 - 40 µg/ml	4.057		<0.001*	7.470		<0.001*
40 - 20 µg/ml	5.064		<0.001*	7.822		<0.001*
20 - 10 µg/ml	5.129		<0.001*	6.570		<0.001*

DISCUSSION

Staphylococci infections are of particular concern due to their resistance to a wide range of antibiotics [25, 26]. Biofilm formation allows bacteria to persist and resist host defenses or antibiotics [20]. Infections associated with biofilm are difficult to treat as antimicrobials must penetrate the polysaccharide matrix to kill or remove biofilms. Nanotechnology may help to penetrate biofilms and reduce their formation [22].

We could detect biofilm formation in MRSA and MRSE isolates by three methods: Congo red agar (CRA) which is screening method, tube method (TM) which is simple and fast, and tissue culture plate (TCP) which is the gold standard method. The percent of MRSA showing biofilm by CRA was 90%. This finding

was matched with Moghadam et al.[27] who showed a rate of 85%. Contrarily, Namvar [28] reported a lower rate (65%). The percent of MRSE showing biofilm by CRA was 85%. Similarly, Saising et al. [29] found that 84.7% of *CoNS* isolated in their study were biofilm producing using CRA. However, Silva Filho et al.[30] found that, 35% of *S.epidermidis* were biofilm producers. Compared to our results, these lower rates may be due to geographical difference and still it is a screening method.

Our results for tissue culture plate method revealed that 75% of MRSA isolates exhibited biofilm formation while 25% could not produce biofilm. Nearly similar results found by Saising et al. [29]. Lower rates (57.1%) were obtained by Knobloch [31]**.**When testing MRSE, 80% were biofilm formers and only 20% were non-biofilm producers. In agreement with this, Salem-Bekhit [32] found that 76.9 % of *CoNS* in their study were biofilm producer and 23.1 were non biofilm producer. However, ***Sa***ising et al. [29] reported that 97.7% of their MRSE isolates were biofilm formers. Contrarily**,** Wojtyczka et al. [33] found that a lower rate (37.5%). The higher rates of our results in producing biofilms may be due to defect nursing care and empirical use of antibiotics.

Nanotechnology provides a useful approach in biofilm control. In our study, the antibiofilm efficacy of AgNPs was investigated by growing the organism on CRA with and without AgNPs. On the medium without AgNPs, the organisms appeared as dry crystalline black colonies, due to production of exopolysachharides. However, when the organisms were grown with AgNPs at concentration of (20µg/ml), the organisms continued to grow but with absence of dry crystalline black colonies because AgNPs inhibited the synthesis of exopolysachharide matrix. When the exopolysachharides synthesis is arrested, the organism cannot form biofilm. Similar results were suggested by Ansari and his colleagues [34] who revealed that the gum arabic coating of AgNPs can penetrate the biofilms.

We could also detect the inhibitory effect of silver nanoparticles on biofilm formation of MRSA and MRSE by microtitre plate assay using different concentrations; this clearly revealed that all the tested concentrations inhibited biofilm. Results were represented as inhibition percentage of biofilm development. The antibiofilm effect was observed as dose dependent manner. Silver nanoparticles with concentration of 50µg/ml, recorded maximum antibiofilm effect (96.6 %) followed by concentrations of 40, 20, and 10µg/ml which were able to eliminate the biofilm formation of MRSA on the plate surface by (89.11%), (78.35%), and (71.99%) respectively. Regarding MRSE, the inhibition was (95.75%), (90.43%), (78.15%), (73.44%) respectively. Similar results were also recovered by Kalishwaralal et al.[35] against *P.aeruginosa* and *S.epidermidis* biofilms who found that AgNPs resulted in a 95-98% reduction in biofilm. Ansari et al. [36] reported that 50 µg/ml of AgNPs resulted in about 95% reduction in biofilm formation in the clinical isolates of *E. coli* and *Klebsiella* spp. biofilms. Results of Martinez-Gutierrez et al. [37] showed that AgNPs were lethal to bacteria associated with a biofilm. To kill microbes within biofilms, high concentrations of AgNPs were needed as compared to those needed to kill planktonic forms. The previous work of Ashkarran et al. [38] also concluded that AgNPs should have high toxicity to bacteria and no/low toxicity to human cells. Also, Actis et al. [39] reported that all geometries of AgNP showed 0 % bacterial viability at the highest tested concentration, whereas lower concentrations could not reduce bacterial viability.

CONCLUSIONS

AgNPs can play a major role in the inhibition of biofilm formation by MRSA and MRSE and on turn makes its treatment by antibiotics much easier.

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