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ORIGINAL ARTICLE

Investigation the effects of silver nanoparticles and gold nanoparticles on expression of *bap* and *csu* genes in biofilm formation of *Acinetobacter baumannii*

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ABSTRACT

Background and Objectives: *Acinetobacter baumannii* is one of the main pathogens of the hospital and causes various infections. *csu A/BABCDE* involved in the initial surface attachment during biofilm formation and *bap* gene produces specific proteins at the cell surface that play a direct role in formation of biofilm and the infectivity of this bacterium. The aim of this study was to investigate the effect of silver nanoparticles and gold nanoparticles on the expression of *bap* and *csu* genes in the *Acinetobacter baumannii* biofilm formation.
Materials and Methods: The susceptibility test was performed to determine the MIC of silver nanoparticles, gold nanoparticles and gold- vancomycin nanoparticles performed by broth dilution method on *A. baumannii* strains. The ability of biofilms formation in strains treated by MIC of silver nanoparticles and gold- vancomycin nanoparticles were evaluated by microtiter plate method and *A. baumannii* ATCC19606 used as control. Expression of the *csu* and *bap* genes were determinded by measuring the cognate *mRNA* level by real-time PCR.
Results: In present study, gold nanoparticles could not prevent the growth and biofilm formation of *A. baumannii* strains. The MIC concentration of silver nanoparticles and vancomycin- gold nanoparticles were 6.25 µg/ml and 0.625 µg/ml respectively and MBC concenteration of nanoparticles for 70% of strain was 12.5 µg/ml and 1.25 µg/ml respectively. Real-time PCR and data analysis, determined that the expression of *bap, csuC* and *csuE* genes in *A. baumannii* strains treated with MIC concentration (6.25 µg/ml) of silver nanoparticles decreased compared to control groups. Also, the expression of *csuC* and *csuE* genes in strains treated with MIC concentration (0.625 µg/ml) of vancomycin -gold nanoparticles increased, however the expression of *bap* was decreased compared to the control groups.
Conclusion: Due to the inhibitory effect of silver nanoparticles and gold- vancomycin nanoparticles against *A. baumannii* biofilm formation and genes expression, they can probably be used for prevent of biofilm formation in medical instrument or can be use for treatment of infections with or without antibiotic.

Keywords: Gold nanoparticles; Silver nanoparticles; Gene expression; Biofilm formation; *Acinetobacter baumannii*

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INTRODUCTION

Acinetobacter baumannii is a Gram-negative, non-motile, strictly aerobic, non-fermentative, non-sporing, coccobacillus, and survive in a variety of environmental conditions. It causes various infections, including ventilator-associated pneumonia, urinary tract infections, skin and soft tissue infections or nosocomial meningitis (1). This organism is a public health risk recently rising to prominence due to the rapid increase in antibiotic resistance and infection rates (2, 3). Biofilm formation in *A. baumannii* is one of the most important problems associated to increased antimicrobial resistance (4).

Biofilms reduce the penetration of antimicrobial agents due to their special structure and the presence of extracellular polymeric materials. It is difficult to treat infections caused by biofilm-producing bacteria, and physician are facing many problems in this case (5). *A. baumannii* has the ability to form biofilms on a wide range of surfaces including abiotic surfaces, like stainless steel and polypropylene, as well as host epithelial cells (6). *Bap* (biofilm-associated protein) is required for the three-dimensional structure tower and water channel formation in biofilm (7). *Bap*, which is secreted via a type I secretion system, mediates *A. baumannii* biofilm formation and maturation (8). The majority of *A. baumannii* strains encode and produce a type I chaperone-usher pilus system designated csu pili (8). *csu* pili, which are regulated by the biofilm formation and cellular morphology two-component regulatory system (*BfmRS*) are not required for association with biotic surfaces, such as human epithelial cells, but are critical for biofilm formation and maintenance on abiotic surfaces, including polystyrene (9). Quorum sensing molecules have been linked with up-regulation in gene expression of the *BfmS* and *BfmR* system, which has been predicted to contribute to the enhanced biofilm-forming capacity of strains on abiotic surfaces (10). The possible role of *QS* signaling molecules to regulate pili formation and the ability of *A. baumannii* to form biofilms on abiotic surfaces investigated (10).

Infections caused by multidrug-resistant (MDR) Gram-negative and Gram-positive bacteria such as *A. baumannii, P. aeruginosa, Enterobacter, Staphylococcus* spp., and *Enterococcus* spp. represent an increasing worldwide problem (11). The mortality of *A. baumannii* infections is due to the development of resistance against most of the antibiotics. Resistance against carbapenem (the most effective β-lactams antibiotic) used against *Acinetobacter*, is one of the major concerns (12). Nanoparticles are now considered a viable alternative to antibiotics and seem to have a high potential to solve the problem of the emergence of bacterial multidrug resistance (13). Nanotechnology is a multidisciplinary science focused on the wide-ranging properties of nanoparticles. Nanoparticles exhibit a broad range of physicochemical properties. Nanoparticles in the size range of 1-100 nm display unique and novel properties (14, 15). In particular, silver nanoparticles (AgNPs) have attracted much attention in the scientific field (16). Silver has always been used against various diseases; in the past it was used as an antiseptic and antimicrobial against Gram-positive and Gram-negative bacteria due to its low cytotoxicity (17, 18). Among the various types of metal nanoparticles produced, only a small number of them can be used for medical purposes. Because the nanoparticles produced must be compatible with the human body and have low toxicity. Accordingly, gold nanoparticles are considered as one of the most suitable options today. Gold has antibacterial, antifungal and antiviral properties (19).

Various methods have been proposed to control biofilm formation. Use of nanoparticles and metal cations, is one of the effective methods to control and eradicate the biofilm formation in bacteria. The aim of this study was to investigate the effect of silver nanoparticles and gold nanoparticles bonded to vancomycin on the biofilm formation of *A. baumannii* and the effect of these nanoparticles on the expression of genes effective in biofilm formation.

MATERIALS AND METHODS

Bacteria isolates. In this study, *Acinetobacter baumannii* ATCC19606 standard strain was ordered from Rhine Company. Twenty strains isolated from sputum, urine and blood of patients admitted to the ICU. The studied strain was cultured on Blood agar, MacConkey agar and differential tests such as TSI (Triple Suger Iron agar), SIM (Sulfide-Indol-Motility), MRVP (Methyl Red Voges Proskauer), Oxidase and Simons Citrate agar were performed to confirm the bacterium.

Biofilm formation in microtiterplate method. *Acinetobacter baumannii* ATCC19606 and clinical

isolates were cultured on TSB (Trypticase Soy Broth) medium with 2% glucose and incubated overnight. Suspensions of isolates were prepared in TSB medium supplemented with 2% glucose adjusted to 10^8 CFU/ml (McFarland standards turbidity) added to the 96 wells plate and 3 negative control wells (no bacterial growth and consequently no biofilm formation) and 3 positive control wells (bacterial growth and consequent biofilm formation) considered, then plate incubated at 37°C for 24 hours (7).

Biofilm staining. First, the culture medium was discarded from the wells and washed 1 to 2 times with PBS buffer. After washing, 95% methanol were added to the wells and incubated at room temperature. Then methanol discarded and the plate fixed to remove the alcohol. 1% Crystal violet was added to the wells and incubated at room temperature. Rinse with distilled water to remove excess color. Finally, 33% glacial acetic acid was added to each of the wells. After 20 minutes, the plates were read with an ELISA reader (Bio tech, USA) at a wavelength of 570 nm. Optical density cut-off (ODc) method was used to evaluate the results. In order to check, the calculation was performed according to the following formula (7):

ODc= Average OD of negative control wells + (3× Standard deviation of negative control wells).

After calculating the ODc or Cut F, the OD of the studied wells was classified according to Table 1.

Preparation of silver and gold nanoparticles. The nanoparticles used in this research were prepared based on water with a size of 10-20 nanometers of German Merck company.

Synthesis of gold nanoparticles bonded to vancomycin. One gram of vancomycin was mixed with 3 ml of chloromethane. Then 3 ml of Aminopropylsilane was added. After 10 hours, the dried sample was placed in haucl4 (German, Merck) in the presence of a 1% solution of gold nanoparticles (10 to 20 nm, German, Merck). Scanning electron microscopy (SEM) images of gold nanoparticles bonded to vancomycin were captured with a Hitachi SU-70 microscope.

Table 1. Classification of biofilm types based on plate microtiter method

OD observed	The power of biofilm formation
OD>4×ODc	Strong biofilm
2×ODc<OD≤4×ODc	Medium biofilm
ODc<OD≤2×ODc	Poor biofilm
OD≤ODc	Negative biofilm

Minimum inhibitory concentration test (MIC). The broth microdilution method, as described in the Clinical and Laboratory Standards Institute 2020 (CLSI 2020) was used for the antibacterial susceptibility test. First, 100 microliters of MHB medium were added to all wells. Then 100 microliters of nanoparticles were poured into the first row wells. Dilution was performed by serial dilution (silver nanoparticles 50-0.39 µg/ml, gold nanoparticles 150-0.146 µg/ml, vancomycin- gold nanoparticles 5-0.0048 µg/ml) method (20). Bacterial suspension with a concentration of 10^6 (1/100 turbidity of half McFarland) was added to all wells. For each plate, a positive control (culture medium and bacterial strain) and a negative control well (culture medium and nanoparticles) were used. The plates were incubated at 37°C for 24 hours. Finally, the growth rate of bacteria was assessed by turbidity of wells by Eliza reader (Bio Tek, USA) at wavelength of 620 nm.

Effect of MIC concentration of silver nanoparticles and vancomycin- gold nanoparticles on biofilm formation. Biofilm formation of strains were evauated by microtiterpate method. 100 µl of MIC concentration of silver nanoparticles were added into microtiter plates. Suspension of all isolates of *A. baumannii* were made in TSB medium supplemented with 2% glucose adjusted to 10^8 CFU/ml (McFarland standards turbidity) added to the wells. The plates were incubated at 37°C for 24 hours. All of these steps performed for vancomycin- gold nanoparticles. Finally, the plates were stained and biofilm formation was evaluated by Optical density cut-off.

RNA extraction. RNA extraction of *A. baumannii* was performed to evaluate the expression of *csu* and *bap* genes for control and treatment strains. RNA extraction was performed manually using trisol. Bacteria cultured in presence (treatment groups) and the absence (control) of nanoparticles were collected in a tube and centrifuged for 10 minutes at 12000 rpm at 4°C. The precipitate formed at the bottom of the vial and the culture medium in each vial were discarded. 200 µl of trisol solution was added to each vial and then vortex was applied for 15 minutes. Then 200 µl of chloroform solution was added and the vial was

shakes until its color changed to milky. It was then centrifuged at 4°C at 12000 rpm for 10 minutes. In this stage, 2 phases were formed. The supernatant, which contains RNA, is transferred to another vial. 500 µl of isopropanol solution was added to the vial and the samples were incubated at -20°C for 24 hours. The samples were centrifuged at 4°C at 12000 rpm for 15 minutes. The supernatant was discarded and 1 ml of 70% ethanol was added and centrifuged at 12000 rpm for 4 minutes at 4°C. The ethanol was then discarded and dried. 15 µl of DEPC was added then vortexed. Optical density of the RNA was measured by nanodrap (Thermo, USA) at the wavelength of 260/280 nm, which should be between 1.8 and 2.8. The product was stored at -70°C and used for *cDNA* synthesis (10).

Real-time PCR. In this study, the expression of *csuE*, *csuC* and *bap* genes in *A. baumannii* were determined by real-time reverse transcription chain reaction (RT-PCR). The 16S RNA gene was used as internal control gene. *cDNA* synthesis was used according to the instructions of the manufacturer kit (GeenALL, South Korea), equal concentrations of RNA (1 µg in 20 µL) were subjected to *cDNA* synthesis using random hexamer primers and reverse transcriptase enzyme. This product was stored at -70°C and used for real-time PCR. The primer sequences were obtained of published paper and their sequence examined with BLAST software in the NCBI (Table 2).

Real-Time PCR was carried out using the SYBR green master mix (Applied Biosystems, USA) and ABI thermocycler (Real time, ABI, USA). PCR conditions were as follows: 95°C for 15 min, 40 cycles of 95°C for 15-30s, 60°C for 60s. The final extension was done at 72°C for 1min (10).

Gene expression analysis. Gene expression levels were determined by the ΔΔCt method using the REST software. For normalization of data 16S rRNA was used as housekeeping gene. Statistical analysis was performed SPSS ver.22.

RESULTS

Investigation of biofilm formation by plate microtiter method. *A. baumannii* ATCC19606 is a standard strains with strong biofilm. Biofilm formation of other isolates evaluated by microtiterplate were classified into four categories; strong, moderate, weak and negative (Fig. 1).

Determination MIC of gold and silver nanoparticles on *A. baumannii*. The growth inhibition of silver nanoparticles and gold nanoparticles was evaluated on *A. baumannii* ATCC19606 and isolated strains. The MIC concentration of silver nanoparticles was 6.25 µg/ml (Fig. 2) and MBC concentration for 70% of isolates was 12.5 µg/ml. Gold nanoparticles had no effect on the growth of *A. baumannii* strains as well as *A. baumannii* ATCC19606, so vancomycin- gold nanoparticles were synthesized (Fig. 4). The MIC concentration of vancomycin- gold nanoparticles was 0.625 µg/ml (Fig. 3) and MBC concentration for 75% of isolates was 1.25 µg/ml.

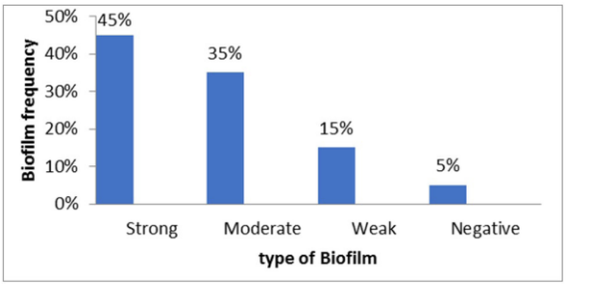

Fig. 1. Type of biofilms frequency in *A. baumannii* strains

Table 2. Primers used in Real-time PCR

Primer	Primer sequence (5'–3')	Length (bp)	Ref
Bap	F: TGCTGACAGTGACGTAGAACCACA	184	7
	R: TGCAACTAGTGGAATAGCAGCCCA		
csuE	F: TCAGACCGGAGAAAAACTTAACG	150	20
	R: GCCGGAAGCCGTAT GTAGAA		
csuC	F: AAAGCAGGCGAGAAGCATATG	100	20
	R: GGATCGGCAACTCATCTACAATC		
16S rRNA	F: TCGCTAGTAATCGCGGATCA	67	21
	R: GACGGGCGGTGTGTACAAG		

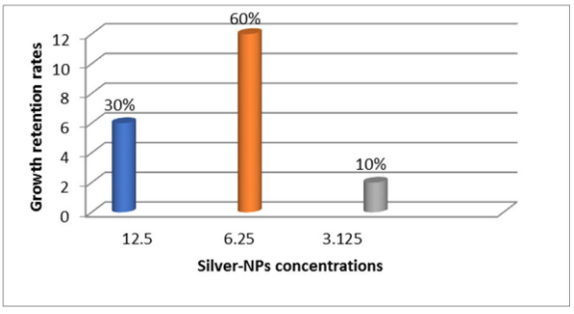

Fig. 2. Minimum inhibitory concentrations of silver nanoparticles

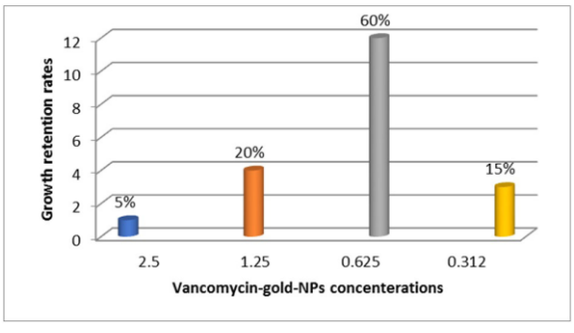

Fig. 3. Minimum inhibitory concentrations of vancomycin-gold nanoparticles

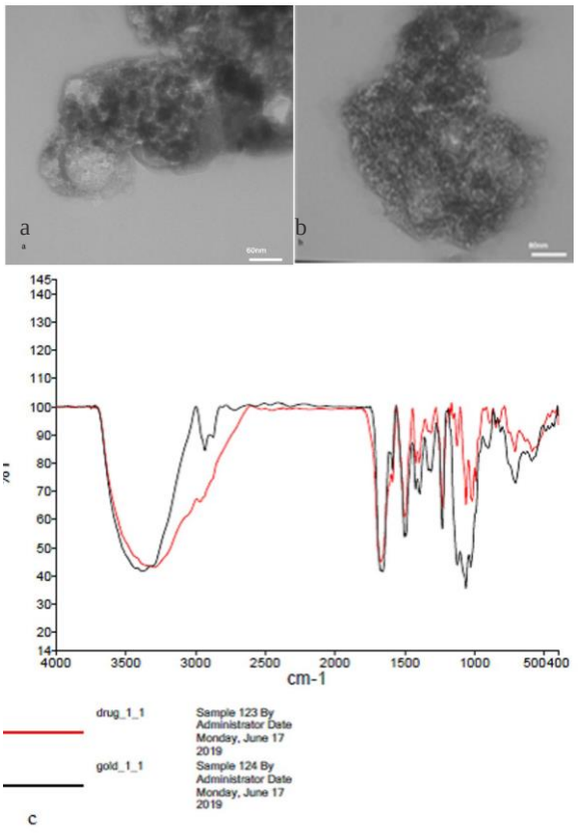

Fig. 4. Attachment of vancomycin to gold nanoparticles: a, b) Transmission electron microscopy images of vancomycin-AuNP c) Fourier transform infrared spectroscopy (FTIR) spectrum of vancomycin-AuNP

Results of the effect of nanoparticles on gene expression. Expression of *cusE*, *cusC* and *bap* genes in *A. baumannii* ATCC19606 and 12 biofilm producer isolates were evaluated by the effect of MIC concentration of silver nanoparticles (6.25 µg /ml) and MIC concentration of vancomycin-gold nanoparticles (0.625 µg/ml).

According to the results of real-time PCR, the expression of *cusE*, *cusC* and *bap* genes treated with MIC concentration of silver nanoparticles decreased compared to their control group. The amount of gene expression changes in strains have been calculated and displayed by Rest software (Fig. 5).

According to the results of real-time PCR, the expression of *bap* gene treated with vancomycin –gold nanoparticles MIC decreased and the expression of *cusC* and *cusE* genes increased compared to their control group. The amount of gene expression changes in ATCC19606 strain has been calculated and displayed by Rest software (Fig. 6).

DISCUSSION

In recent years, the extensive emergence of multi- and pan drug-resistant *A. baumannii* strains has revealed this organism's ability to quickly adapt to environmental changes (22). The biofilm formation is one of the hallmark characteristics of opportunistic pathogens. *A. baumannii* can produce a wide variety of virulence factors in the biofilm mode that contributes in the various steps of the attachment of biofilm cells to the biotic or abiotic surfaces (4).

For a long time, silver has been used as an antimicrobial agent for wound healing, both in its solid state and with salt solutions to clean wounds (23). Silver exhibits very interesting properties due to its chemical stability, good conductivity, catalytic, and antibacterial activity (24). Gold-NPs are colloidal or clustered particles composed of a gold core, an inert and biocompatible compound (25), the particle surface can bind thiols and amines, providing functional groups to the gold-NPs for labelling, targeting and conjugating pharmacologic molecules (26).

The biofilm-associated protein (*Bap*) is one of the

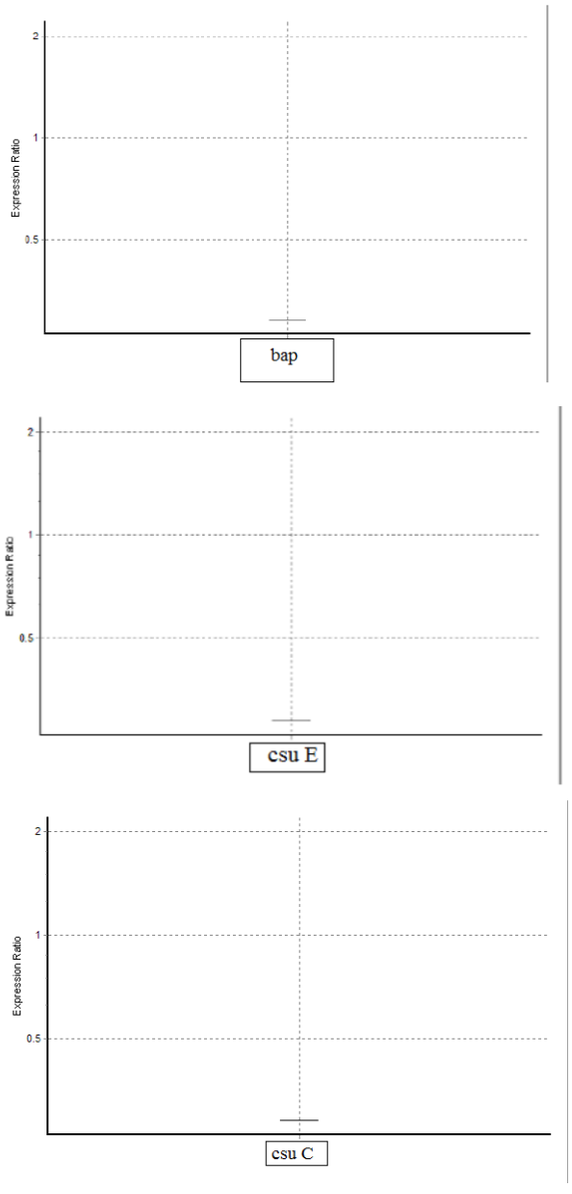

Fig. 5. Expression of a) *bap* b) *csuE* c) *csuC* genes treated with silver nanoparticles and control in *A. baumannii* strains.

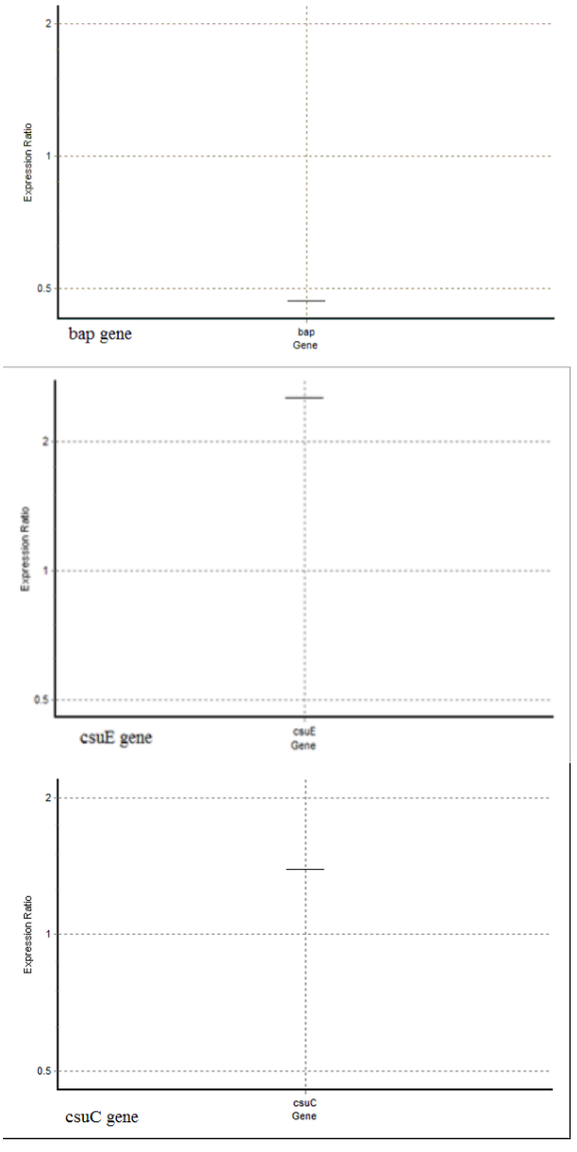

Fig. 6. Expression of a) *bap* b) *csuE* c) *csuC* genes treated with vancomycin- gold nanoparticles and control in *A. baumannii* isolates.

key factors in the initial attachment and maturation of *A. baumannii* biofilm, so that can affect both the biofilm thickness and bio-volume (7). The majority of *A. baumannii* strains have a chaperon/usher pilus system, known as *csu* pili, regulated by the *BfmRS* two-component system, a network of molecules that influences gene expression and enables building a protective capsule in response to antibiotics. The system also mediates pili formation to facilitate cell attachment (6). The production of pili is essential for the initial steps of biofilm formation. The pili of *A. baumannii* are encoded by the *csuA/BABCDE* operon and the inactivation of the *csuE* gene resulted in the abolition of both pili production and biofilm formation (27). In this study we observed silver nanoparticle in 6.25 µg/ml decrease the expression of *csuC*, *csuE* and *bap* genes. In *A.baumannii* expression of *csu* genes cause to pili formation and create the biofilm (4), so decrease *csuE* and *csuC* genes expression is a reason of biofilm inhibition.

In a study conducted by Azizi, it was stated that biofilm formation and *bap* gene expression increased at 20 µM concentration of iron in *A. baumannii* (28).

Contrary to the results of the present study, the expression of bap gene and consequently the formation of biofilm with both of silver and gold nanoparticles bonded to vancomycin decreased in *A. baumannii* ATCC19606 and other tested isolates. Since *bap* is the most influential factor in the formation of *A. baumannii* biofilm, iron nanoparticles, unlike silver and vancomycin -gold nanoparticles, have an additive effect on the formation of *A. baumannii* biofilm.

Consistent with the results of the present study, the results of the Hetta study showed that treatment of *A. baumannii* strains with subMIC concentration of silver nanoparticles reduced *bap* gene expression (29).

In a study by Mahnaie, the results showed that treatment of clinical strains of *Pseudomonas aeruginosa* with silver nanoparticles (256 µg/ml) reduced *lasR* and *lasI* gene expression (30). The quorum sensing system in *P. aeruginosa* affects the overall development and formation of biofilms, which are controlled by the *lasR* and *lasI* genes. Since *lasR* and *lasI* genes are genes that affect the formation of *P. aeruginosa* biofilm by acting on the quorum sensing system, it can be concluded that they have a similar effect to the *csuC* and *csuE* genes in biofilm formation process in *A. baumannii*. According to the results of the present study, silver nanoparticles reduce the expression of these genes.

choi analysis showed that the combination of silver nanoparticles with silver nitrate has antibiophilic and antifungal effects against *Candida albicans* (31). Singh revealed that the MIC concentration of silver nanoparticles (6.25 µg/ml) had an antibiotic effect against *E. coli* (32). The results of the present study showed that silver nanoparticles at MIC concentration of 6.25 µg/ml have an inhibitory effect on biofilm and antibacterial effect on *A. baumannii*. Both *E. coli* and *A. baumannii* are Gram-negative and due to the lack of a thick peptidoglycan wall, more susceptible to antibacterial agents, which allows higher uptake of these agents (33).

Khan's showed that the MIC concentration of fucoidan-stabilized AuNPs (512 µg/ml) had antibacterial activity (sub-MIC) levels of F-AuNPs inhibited biofilm formation without affecting bacterial growth in *P. aeruginosa* (34). In this study vancomycin-AuNPs inhibited the growth and biofilm formation *A. baumannii* in MIC concentration (0.625 µg/ml), Vancomycin is a glycopeptide antibiotic that inhibits peptidoglycan synthesis, so at low concentrations cause destroying the wall and nanoparticles enter bacterial cells thus inhibit the growth and biofilm formation.

CONCLUSION

The results of the present study showed that silver nanoparticles and Vancomycin-gold nanoparticles significantly reduced *bap* gene expression. Silver nanoparticles also reduce the expression of *csuE* and *csuC* genes, while vancomycin- gold nanoparticles increase the expression of these two genes. Therefore, it seems that vancomycin- gold nanoparticles have an inhibitory effect on other genes in biofilm formation of *Acinetobacter baumannii*. We conclude that silver and Vancomycin-gold nanoparticles inhibit the growth, biofilm formation and biofilm-related genes, therefore can be use as antibiofilm drug in medical devices, food and environmental industries.

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