



The Effect of Silver Nanoparticles on Pyocyanin Production of *Pseudomonas aeruginosa* Isolated From Clinical Specimens

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Abstract

Background: *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen causing a wide range of human infections. The organism is resistant to a wide range of antibiotics. The purpose of this study was to investigate the effect of AgNPs on pyocyanin pigment production of *P. aeruginosa* bacteria isolated from clinical specimens.

Methods: In this study, 15 clinical isolates of *P. aeruginosa* were collected from different specimens of hospitalized patients. *P. aeruginosa* was detected by biochemical and molecular (detection of *pbo1* gene by colony PCR method) methods and the MIC and MBC of AgNPs were determined by agar dilution method. Inhibition of *P. aeruginosa* pyocyanin production at AgNPs concentrations of 0, 0.3, 0.5, 1 and 1.5 mg/ml of was studied with OD of 520 nm.

Results: The mean MIC and MBC of AgNPs were 1.229 and 1.687 mg/ml, respectively. Pyocyanin production was investigated for all isolates at different concentrations of nanoparticles, and their comparison showed that with increasing nanoparticle concentration, pyocyanin production significantly decreased ($p < 0.05$).

Conclusion: According to the results of this study, AgNPs had an inhibitory effect on *P. aeruginosa* and its pigment production and with increasing nanoparticles concentration, pigment production decreased; therefore, it seems that the nanoparticles can be used to treat and prevent diseases caused by *P. aeruginosa*.

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Introduction

One of the chief global problems is the infections caused by antibiotic-resistant bacteria, and controlling the spread of these infections, especially in health centers, has become a major challenge ¹. *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen causing a wide range of human infections. It is a common hospital-acquired pathogen and responsible for Urinary Tract Infections (UTIs), respiratory infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections, and a variety of systemic infections, particularly in patients with severe burns, bed ulcers, and in patients suffering from cancer or AIDS who are immunosuppressed ². The organism is resistant to a wide range of antibiotics ³; at present, the rate of nosocomial infections caused by antibiotic-resistant strains of *P. aeruginosa* shows a growing trend and is one of the leading causes of death in burn patients ⁴. In 1995, eighty-eight

thousand people died of nosocomial infections worldwide. Also, *P. aeruginosa* alone causes 10% of nosocomial infections. Accordingly, in recent years, the causes of nosocomial infections have been considered by many researchers ⁵. The ability of this bacterium to produce many virulence factors such as phenazines, alginate, proteases, phospholipase C, rhamnolipid, pili (for binding and colonization to host cells) and biofilm formation has made it one of the most important pathogens ⁶. Pigments produced by *P. aeruginosa* include pyocyanin (Blue), pyoverdine (Green), pyorubin (Red), and pyomelanin (Black) ⁷. Pyocyanin (N-methyl, L-hydroxyphenazine) is chemically in the group of phenazines. Phenazines are nitrogen-containing heterocyclic compounds produced by several bacterial species and have been studied for their role in pathogenicity ⁸. Pyocyanin is involved in biofilm formation as virulence factor; also, in eukaryotic cells as cellular signals, it

regulates gene expression that alters cellular responses. Concentrations of pyocyanin in the lungs of patients with chronic cystic fibrosis infection may impair epithelial cell function and reduce the immune response⁹. *P. aeruginosa* has two specific versions of the pyocyanin biosynthesis operon (pbo) called *phz A1 B1 C1 D1 E1 F1 G1 (phzI)* and *phz A2 B2 C2 D2 E2 F2 G2 (phzII)*. These two operons are 98.3% homologous at the DNA level but different in the promoter region. In addition, two other genes, called *phz M* and *phz S*, play a key role in the biosynthesis pathway of pyocyanin¹⁰. With the advent of antibiotic-resistant bacterial strains, researchers today are looking for a new method to treat and control diseases. The science of nanotechnology with a wide range of applications has been able to help researchers in biology and medicine. Silver nanoparticles (AgNPs) with significant antimicrobial effects are one of the most important products of nanotechnology^{11,12}. These particles by releasing sulfur-containing proteins on the surface of bacterial membranes change their morphology and by changing the bacterial respiratory chain, eventually lead to their death¹³. Since bacteria do not become resistant to nanoparticles, they affect a wide range of bacteria¹⁴. In previous research, the antimicrobial effects of the nanoparticles have been proven^{15,16}. But, research on the effects of nanoparticles on bacterial pigment production is scarce and so far few studies have been conducted on the effect of nanoparticles on the production of *P. aeruginosa*; moreover, since treatment of bacterial infections has inflicted a lot of cost and problems on the treatment department¹⁷ and given the special importance of pyocyanin in the pathogenesis of *P. aeruginosa*, the aim of this study was to investigate the antimicrobial effects of AgNPs and their effect on the production of pyocyanin pigment of *P. aeruginosa* isolated from clinical specimens (with *pboI* gene tracing). In this study, AgNPs synthesized by the US Research Nanomaterials, Inc. were used against the production of pyocyanin. Findings from the present study provide important insights into the potential of AgNPs as an effective new drug for controlling *P. aeruginosa*-related infections.

Materials and Methods

Isolation and biochemical identification of *P. aeruginosa* from clinical specimens

In this study, different specimens of hospitalized patients who were suspected of *Pseudomonas* infection at Imam Reza and Ghaem Hospitals in Mashhad were transferred to the microbiology laboratory of Islamic Azad University of Mashhad under sterile conditions and next to an ice pack. To isolate *P. aeruginosa*, ceftrimide agar medium and biochemical tests including

Gram staining, oxidase, catalase, methyl-red, urea and culture in TSI agar and SIM were used¹⁸.

Molecular identification of *P. aeruginosa*

Colony Polymerase Chain Reaction (PCR) and *pboI* gene primers (Sinaclon, Iran) were used to confirm *P. aeruginosa* bacteria isolated from clinical specimens (Table 1). Exact sequences of *pboI* gene were extracted using bioinformatics tools and data from the NCBI database. To ensure the accuracy of the extracted sequences, their evaluation was performed by Blast toolkit at the NCBI database and primers specific for this gene were designed using oligo 7 software. The Blast toolkit was used to ensure that the primers were specific¹⁹.

The colony PCR reaction mixture (Sinaclon, Iran) was prepared with a final volume of 25 μ l comprising 3 μ l of 1 \times PCR buffer, 0.75 μ l of 0.2 mM MgCl₂, 0.5 μ l of dNTP Mix (0.5 mM each), 1 μ l of 2.5 pmol/ μ l of each primer, a template DNA, 0.25 μ l of Taq DNA polymerase (1.2 U/ μ l), and 17.5 μ l of distilled water. The amplification was carried out in a thermocycler (Kyratec, Korea) with the following cycling conditions: initial denaturation at 95°C for 5 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 5 min, and final extension at 72°C for 7 min. *P. aeruginosa* ATCC 1074 (Prepared by Iran's Scientific and Industrial Research Organization) was used as a positive control, and distilled water as a negative control. The colony PCR products were confirmed by electrophoresis on a 1.5% agarose gel containing 0.5 μ g/ml of ethidium bromide (CinnaGen, Iran) in TBE buffer and photographed with UV waves through gel documentation system (Kimiagene, Iran)²⁰.

Preparation of AgNPs

In this research, AgNPs powder with 99.99% purity, density of 210 nm and average size of 15 nm was obtained from the US Research Nanomaterials, Inc..

Determination of MIC and MBC of AgNPs for *P. aeruginosa* isolates by agar dilution method

To prepare the bacterial suspension, *P. aeruginosa* isolates were inoculated in Luria Bertani (LB) (Biomark, India) medium and incubated for 24 hr in the shaker incubator (37°C) (LaCopian, Korea). To prepare AgNPs suspension, 100 mg of the powder was mixed in 1000 μ g of sterile distilled water. Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of AgNPs were done on a 24-well polystyrene microplate. For this purpose, different concentrations of AgNPs (0 to 50 mg/ml) were prepared in the Mueller-Hinton Agar (MHA) (48°C) (Merck, Germany). After solidification of the culture medium, the bacterial suspension

Table 1. Characteristics of primers used to detect *pboI* gene in *P. aeruginosa* isolates

Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	PCR product length (bp)
CGCTCGGGATCGCTTCTG	GGACGCCTGACGCTGATC	400

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Table 2. Results of biochemical tests to detect *P. aeruginosa*

Test	Shape/Gram	Citratase	Urease	Methyl red	Grown in the cetrimide at 42°C	Move	Indole	Fermented sugars	Catalase	Oxidase
Result	Bacillus / -	+	-	-	+	+	-	-	+	+

(OD₆₂₀=0.01) with a volume of 10 μ l was inoculated on its surface. Finally, the microplate was incubated for 24 hr at 37°C. After 24 hr, the lowest concentration at which the bacterium did not grow was determined as the MIC. To determine the MBC, the surface of the wells in which the bacterium did not grow was inoculated into culture media without AgNPs and incubated at 37°C for 24 hr. After incubation, the lowest concentration at which the bacterium did not grow was identified as MBC²¹. This experiment was repeated 3 times for all isolates and standard strain (*P. aeruginosa* ATCC 1074) similarly.

Extraction of *P. aeruginosa* pyocyanin

First, 250 μ l of *P. aeruginosa* [Optical Density at 620 nm (OD₆₂₀)=0.01] suspension grown in LB medium was inoculated with 25 ml of Glycerol Alanine (GA) (Merck, Germany) medium and incubated for 24 hr in a shaker incubator at 200 rpm. Next, the samples were centrifuged (Sigma, USA) at 4°C and 10,000 rpm for 10 min. The supernatant was collected and passed through a filter with a pore size of 0.22 μ m. Next, 4.5 ml of chloroform was added to 7.5 μ l of the filtered supernatant and vortexed ten times, each time for 2 seconds. This step was repeated three times for each isolate.

Chloroform was placed at the bottom of the tube; because pyocyanin dissolves in chloroform, it changes to a greenish-blue color. After changing the color, the samples were centrifuged at 10,000 rpm for 10 min. Then, 3 ml of the blue layer collected at the bottom of the tube (Mixture of chloroform and pyocyanin) was transferred to a new tube and in order to acidify the mixture, 1.5 ml of 0.2 N hydrochloric acid was added to it and vortexed (changed blue to pink).

Then, the samples were centrifuged at 10000 rpm for 2 min and 1 ml of pink liquid was transferred to the cuvette and then OD was measured at 520 nm by a spectrophotometer. This experiment was repeated 3 times for all isolates and standard strain (*P. aeruginosa* ATCC 1074) similarly. The concentration of pyocyanin (μ g/ml) was calculated according to the following formula, (OD_{520 nm} × 17.072 [molar attenuation coefficient]) × 1.5 [volume of hydrochloric acid], to determine the dilution factor following transfer into the acidic phase^{22,23}.

Extraction of *P. aeruginosa* pyocyanin (Affected by AgNPs)

In the first step, 25 ml of GA medium with different concentrations of AgNPs (0 to 1.5 mg/ml) was prepared in erlenmeyer flask and inoculated with 250 μ l of *P. aeruginosa* suspension (OD₆₂₀=0.01). Erlenmeyer flasks were incubated at 200 rpm for 24 hr (37°C).

After the end of the incubation period, the steps of extracting the pyocyanin similar to the previous experiment were performed. This experiment was repeated 3 times for all isolates and standard strain (*P. aeruginosa* ATCC 1074) similarly.

Statistical analysis

Statistical analysis was performed using SPSS software version 21. Mean ± standard deviation was obtained by one-way ANOVA and p-values of less than 0.05 (p < 0.05) were considered statistically significant.

Results

Biochemical and molecular identification of *P. aeruginosa*

Based on the results of biochemical tests in table 2, 15 *P. aeruginosa* isolates were collected from clinical specimens. PCR results of *pbo1* gene confirmed all 15 *P. aeruginosa* isolates by biochemical tests (100% of clinical specimens). In figure 1, a gel electrophoresis PCR product of several isolates is shown next to the 100-bp marker.

Determination of MIC and MBC of AgNPs

The mean (± standard deviation) MIC and MBC of AgNPs for *P. aeruginosa* isolated from clinical specimens were 1.229 ± 288 and 1.687 ± 288 mg/ml and for *P. aeruginosa* strain ATCC 1074 were 1.333 ± 288 and 1.333 ± 288 mg/ml, respectively (Table 3).

There was no significant difference in MIC of *P. aeruginosa* strains isolated from clinical specimens and standard strains (p < 0.05).

Extraction of *P. aeruginosa* pyocyanin

The average OD of *P. aeruginosa* pyocyanin after extraction without the influence of AgNPs and at different concentrations of AgNPs is shown in table 4.

All isolates could produce pyocyanin; mean OD of *P. aeruginosa* pyocyanin at 520 nm after extraction (without the effect of AgNPs) was 0.584 and the mean OD of *P. aeruginosa* pyocyanin at 520 nm after extraction with concentrations of 0 (Control), 0.3, 0.5, 1, and

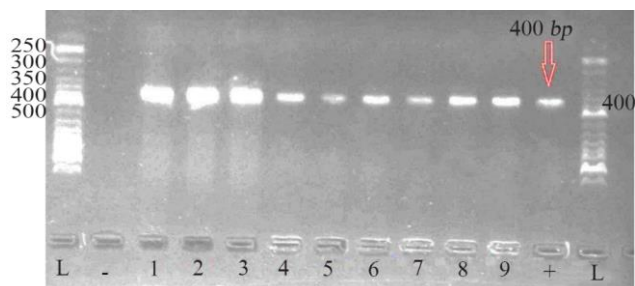


Figure 1. Gel electrophoresis of PCR product of *pbo1* gene from several *P. aeruginosa* isolates along with the standard 50-bp marker (1-9: Samples; L: Ladder; +: Positive control; -: Negative control).

Table 3. Mean MIC and MBC of AgNPs for 15 clinical isolates of *P. aeruginosa* and standard strains (data in mg/ml) (Repeated three times)

Bacteria	Mean (\pm SD) MIC	Mean (\pm SD) MBC
Standard	1.333 \pm 288	1.833 \pm 288
1	1.333 \pm 288	1.833 \pm 288
2	1.333 \pm 288	1.833 \pm 288
3	1.5 \pm 288	2.00 \pm 288
4	1.333 \pm 288	1.833 \pm 288
5	1.333 \pm 288	1.833 \pm 288
6	1.333 \pm 288	1.333 \pm 288
7	0.918 \pm 288	1.333 \pm 288
8	1.333 \pm 288	1.333 \pm 288
9	1.083 \pm 288	1.5 \pm 288
10	1.333 \pm 288	1.833 \pm 288
11	1.333 \pm 288	1.833 \pm 288
12	1.333 \pm 288	1.833 \pm 288
13	1.166 \pm 288	1.833 \pm 288
14	1.333 \pm 288	1.833 \pm 288
15	1.333 \pm 288	1.166 \pm 288
Total average	1.229 \pm 288	1.687 \pm 288

1.5 mg/ml was 0.556, 0.277, 0.109, 0.09 and 0, respectively. The highest OD (OD₅₂₀) at 0.576 nm was related to isolate 10 at a concentration of 0.3 mg/ml and all isolates at a concentration of 1.5 mg/ml had an OD equal or close to zero.

Discussion

The effect of AgNPs on pyocyanin production was investigated in 15 *P. aeruginosa* isolates of clinical specimens. Based on results of ANOVA, it was found that with increasing the concentration of AgNPs, the amount of OD of pyocyanin produced by *P. aeruginosa* and standard strains was significantly reduced compared to the control (Concentration 0) (p<0.05) which

shows the inhibitory effect of AgNPs on the production of *P. aeruginosa* pyocyanin. The results of OD at 520 nm showed that the ability to produce pyocyanin in the clinical strains isolated in this study is higher than the standard strain. Finnan *et al*²⁴ isolated 12 *P. aeruginosa* bacteria from patients with cystic fibrosis and determined the frequency of *phzM*, *phzII*, *phzH* and *phzS* genes to be 91.6%, 100%, 100% and 8.3%, respectively; *phzII* and *phzH* genes like *pbo1* gene in the present study were detected in 100% of the clinical specimens. In another study by Dadmanesh *et al*²⁵ in 2013, the frequency of *phzI*, *phzII*, *phzS*, and *phzM* genes in 23 *P. aeruginosa* bacteria isolated from the urine of patients with urinary tract infections was 11.7, 28.4, 19.6, and 36.2 %, respectively. The frequency of all genes in their study was lower than the frequency of *pbo1* gene in our study. This difference may be due to research on a specific clinical specimen (Urine only). In 2016, Nasiri *et al*²⁶ reported 1 mg/ml MIC of AgNPs against *P. aeruginosa* isolated from urine cultures of hospitalized patients. Their MIC values were slightly lower than the ones in the present study. This difference in the two studies may be due to differences in the type of specimens tested. In a study, Khan *et al*²⁷ showed that the production of *P. aeruginosa* PAO1 KCTC 1637 pyocyanin at gold nanoparticles concentrations of 0.032, 0.128 and 0.256 mg/ml decreased 79.4, 81.9, and 87.7%, respectively compared to the control. The results of their study, similar to the results of the present study, proved the effects of nanoparticles on reducing the production of *P. aeruginosa* pyocyanin. *P. aeruginosa* virulence is a multifactorial process and has been attributed to cell associated factors or secreted virulence factors, pyocyanin and phenazine operons (*phzI* and *phzII*); the genes encode precursor proteins involved in the formation of three phenazine compounds (*phzH*, *phzM* and *phzS*)²⁵. Therefore, reduction

Table 4. OD of pyocyanin produced by clinical isolates of *P. aeruginosa* and the effect of different concentrations of AgNPs on the production of pyocyanin in these isolates based on OD at 520 nm

Bacteria	The concentration of AgNPs (mg/ml)					
	*	0 (Control)	0.3	0.5	1	1.5
Standard	0.503	0.503	0.258	0.12	0.051	0
Isolate 1	0.554	0.502	0.279	0.1	0.04	0.0003
Isolate 2	1.190	1.190	0.45	0.122	0.057	0.002
Isolate 3	0.495	0.554	0.300	0.112	0.054	0.001
Isolate 4	0.496	0.496	0.234	0.102	0.066	0.001
Isolate 5	0.678	0.680	0.303	0.102	0.04	0.0003
Isolate 6	0.287	0.281	0.141	0.09	0.001	0
Isolate 7	0.387	0.387	0.157	0.08	0.001	0.0003
Isolate 8	0.422	0.422	0.284	0.14	0.08	0.0003
Isolate 9	0.312	0.312	0.193	0.06	0.001	0.0003
Isolate 10	0.970	0.970	0.567	0.138	0.071	0
Isolate 11	0.264	0.264	0.174	0.08	0.001	0
Isolate 12	0.568	0.568	0.301	0.1	0.06	0
Isolate 13	0.279	0.302	0.133	0.086	0.0003	0
Isolate 14	0.553	0.553	0.281	0.095	0.044	0
Isolate 15	0.786	0.786	0.385	0.12	0.68	0
Average	0.584	0.585	0.277	0.109	0.083	0

*: OD of pyocyanin produced without the effect of AgNPs.

of pyocyanin can be considered to effectively attenuate the pathogenesis and colonization of *P. aeruginosa* without affecting bacterial growth or initiating resistance selection. Blue-green pigment pyocyanin essentially causes oxidative stress and cytotoxicity to the host tissues²⁷.

Conclusion

According to the results of this study, AgNPs had an inhibitory effect on *P. aeruginosa* and its pigment production and with increasing nanoparticle concentration, pigment production decreased. AgNPs may be a good alternative to antibiotics and chemical drugs and can be used to treat and prevent diseases caused by *P. aeruginosa*. Therefore, it is necessary to extend the scope of research to investigate the effects of nanoparticles on the production of *P. aeruginosa* pigments in the prevention and treatment of diseases caused by them and to prevent the emergence of antibiotic-resistant strains. Further studies should assess the combination of AgNPs and antibiotics against resistant strains for the development of new materials and substances for medical application.

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Conflict of Interest

There is no conflict of interest.

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