UMSHA Press

AJCMI
Avicenna Journal of Clinical Microbiology and Infection

Avicenna J Clin Microbiol Infect, 2024; 11(3):107-112. doi:10.34172/ajcmi.3532

http://ajcmi.umsha.ac.ir

Original Article

The Application of *Spirulina platensis*-Based Green Synthesized Silver Nanoparticles Demonstrating Potent Anti-*Shigella flexneri* Effects

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Article history:
Received: March 22, 2024
Revised: May 7, 2024
Accepted: August 9, 2024
ePublished: September 30, 2024

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Abstract

Background: The emergence of antibiotic-resistant strains of *Shigella flexneri*, an important cause of Shigellosis, has led to extensive research to find alternative treatment approaches. Therefore, the current study investigated the antibacterial effects of the green synthesized silver nanoparticles (AgNPs) using *Spirulina platensis* on *S. flexneri* and the expression of pathogenic genes *ipaB*, *ipaD*, *ipaH*, and *qnrS*.

Methods: After synthesizing AgNPs using *S. platensis*, its antibacterial effects on *S. flexneri* were studied using the microdilution method with 96-well plates. In addition, to determine the minimum bactericidal concentration (MBC), 10 µL of the contents of the minimum inhibitory concentration (MIC) well and the like were swapped on the nutrient agar medium. After RNA extraction, complementary DNA (cDNA) synthesis, and primer design, the expression levels of *ipaB*, *ipaD*, *ipaH*, and *qnrS* genes were evaluated using the real-time polymerase chain reaction (PCR) technique. The data were analyzed by GraphPad Prism 8.

Results: The MIC of the green synthesized AgNPs was measured as 0.0625 µg/mL, and its MBC was 0.125 µg/mL. The results of RT-PCR analysis indicated a significant decrease in the expression levels of pathogenic genes *ipaB*, *ipaD*, *ipaH*, and *qnrS* in AgNP-treated *S. flexneri*.

Conclusion: The green synthesized AgNPs using *S. platensis* had strong antibacterial effects on *S. flexneri*, and the action mechanism was attributed to the downregulations of *ipaB*, *ipaD*, *ipaH*, and *qnrS* genes. *In vivo* and clinical studies are needed in this respect.

Keywords: Gene Expression, Nanoparticles, *Shigella*, Silver

Please cite this article as follows: Karami S, Heidary Z, Khaleghi S, Falsafi S,Rahimi MK, Hajrasouliha S. The application of *Spirulina platensis* based green synthesized silver nanoparticles demonstrated potent anti *Shigella flexneri* effects by specifically targeting pathogenic gene expressions. Avicenna J Clin Microbiol Infect. 2024; 11(3):107-112. doi:10.34172/ajcmi.3532

Introduction

Infectious diseases are among the most important and common diseases in the world, causing many problems to the health systems of most countries, especially developing countries (1). One of these kinds of diseases is shigellosis, which is caused by *Shigella* sp. bacteria, especially *Shigella flexneri* (2), and is an important cause of bacterial gastroenteritis and dysentery (3). About 12.5% of deaths caused by diarrheal diseases are due to *Shigella*, and its mortality rate is higher in children under 5 years of age (4). Clinical manifestations include diarrhea, dysentery, high fever, abdominal cramps, myalgia, and rectal tenesmus or spasm (5).

The ability of *S. flexneri* to penetrate epithelial cells is due to the presence of its large invasive plasmid, whose genes are responsible for the coding of invasive proteins (6), including *ipaA*, *ipaB*, *ipaC*, *ipaD*, and *ipaH* (7). After the bacteria contact the host cells, IpaB and IpaC inject inosines into the cytoplasm of the cell by creating a pore on the plasma membrane (8). IpaD provides bacteria with the ability to phagocytize (9), and IpaA causes the depolymerization of F-actin by binding to vinculin (10). On the other hand, ipaH protects *Shigella* from macrophages (11), which can move to the nucleus of the host cell and stimulate the secretion of *Shigella* proteins (12). One of the acquired genes involved in creating relative resistance to quinolones in *Shigella* is *qnrS*,

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and it protects bacterial DNA by inhibiting the binding of quinolones to DNA gyrase and topoisomerase 4 (13).

The administration of water and electrolytes and prescription of antibiotics such as ampicillin, tetracycline, erythromycin, trimethoprim/sulfamethoxazole, and in severe cases, ciprofloxacin are among the treatment approaches (14). However, the emergence of antibiotic-resistant strains of *S. flexneri* has reduced the effectiveness of treatments (15,16). Therefore, there is a need for new approaches to the treatment of shigellosis.

Nanoparticles (NPs) have many applications in medicine due to their unique physicochemical and biological properties (17). The synthesis of NPs using chemical approaches is associated with side effects and environmental harms, which limit their application (18). To overcome these problems, NP green synthesis methods using plant extracts have been introduced, which are cost-effective and environmentally friendly (18). One of the widely used NPs is silver (Ag) NP, whose anticancer and antimicrobial properties have been investigated in many studies (19-21). AgNPs have antibacterial properties against gram-positive and gram-negative bacteria, and this compound has shown antimicrobial effects against antibiotic-resistant bacteria (22). For example, AgNPs have shown antibacterial effects on *Escherichia coli*, *Salmonella Typhimurium*, *Staphylococcus aureus*, and *Bacillus subtilis*, and it seems that the smaller size of this NP is associated with increased antimicrobial activity (23).

Therefore, the current research aimed to investigate the antibacterial effects of green synthesized AgNPs on *S. flexneri* and evaluate the pathogenic *ipaA*, *ipaD*, *ipaH*, and *qnrS* gene expressions.

Materials and Methods

Silver Nanoparticle Synthesis

Ethanol extract of *Spirulina* algae (Spirulife, Esfahan, Iran) was used for the synthesis of AgNPs. For this purpose, 20 g of dry *Spirulina* powder was dissolved in 200 mL of 96% ethanol and placed in a shaker at 140 rpm for 35 minutes. Then, the solution was filtered with Whatman paper, and the obtained extract was centrifuged at 13 000 rpm for 20 minutes. In addition, 340 mg of $AgNO_3$ (Merck, Germany) was mixed in 100 mL of distilled water and 100 mL of *Spirulina* extract and placed in a shaker for 24 hours. After observing the color change of the solution and ensuring the complete reduction of Ag ions to AgNPs, the sediment was washed three times using a centrifuge at 13 000 rpm for 20 minutes. Finally, the final sediment was collected after drying at 40 ˚C for 120 minutes.

Shigella flexneri Culture

Shigella flexneri (ATCC 12022) was obtained from the Microbiology Department of the Pasteur Institute of Iran and cultured in nutrient broth at 32 ˚C for 24 hours. Then, the bacteria were separated by centrifugation at 4000 rpm, and the McFarland method was used to determine the microbial population. The initial turbidity of the microbial suspension was determined using a 0.5 McFarland solution. The physiological serum was utilized to prepare a microbial population equal to 1.5×10^6 bacteria/mL.

Minimum Inhibitory and Bactericidal Concentrations

The microdilution method based on the CLSI 2017 standard was employed to measure the minimum inhibition concentration (MIC) of $AgNO_3$-NPs (19). Briefly, successive dilutions of $AgNO_3$-NPs in the concentration range of 0.063–32 mg/mL were poured into the wells of 96-well plates, and then 1 mL of the nutrient broth was added to it, along with 1 mL of microbial inoculum (1.5×10^6 bacteria). The plates were incubated for 24 hours at 37 ˚C. The well containing nutrient broth culture medium with bacteria and the well containing culture medium without bacteria were considered positive and negative controls, respectively.

To determine the minimum bactericidal concentration (MBC), 10 µL from the last well showing no bacterial growth was taken and cultured in the *Mueller-Hinton* agar medium. The plates were incubated for 24 hours at 37 ˚C.

Gene Expression

RNA was extracted using the RNX-PLUS method. Briefly, bacteria were trypsinized and separated by centrifuge 48 hours after treatment with AgNPs, and 500 µL of the RNX-PLUS solution was added to the samples. Then, 200 µL of chloroform was added and incubated at 4 ˚C for 5 minutes and centrifuged at 12 000 rpm for 15 minutes. Complementary DNA synthesis was performed using a kit (BioFact, South Korea) according to the manufacturer's instructions.

The primer design was implemented using the NCBI (National Center for Biotechnology Information) database and Primer 3 software. The sequences of the primers of *ipaD*, *ipaB*, *ipaH*, and *qnrS* genes are presented in Table 1.

The expression levels of the studied genes were determined by the RT-PCR technique using the Cyber Green method (Q Rotor-Gene, Qiagen). The *16s rRNA* gene was used as the control. The reaction mixture included 7 µL of master mix, 0.5 µL of forward and reverse primers, 5 µL of deionized water, and 1 µL of complementary DNA.

The time-temperature schedule of the RT-PCR machine is provided in Table 2.

Statistical Analysis

The $2^{-\Delta\Delta Ct}$ method was utilized to analyze the expression levels of *ipaD*, *ipaB*, *ipaH*, and *qnrS* genes. Further, the gene expressions between the groups were analyzed by an unpaired Pearson *t*-test at probability levels of $P < 0.05$.

Results

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The microdilution method was employed to determine the AgNPs MIC against *S. flexneri*, and the results revealed that the growth of bacteria decreased with increasing the

Table 1. The Sequences of Primers Used for Measuring the Expression Levels of *ipaD*, *ipaB*, *ipaH*, and *qnrS* Genes by the RT-PCR Technique

Genes	Sequence [5'-3']	GC%	TM (°C)
ipaB	Forward: ACGACTGCTGCAACTAGGAC Reverse: GGAACAAGCCCTGAATCCGA	55	60
ipaD	Forward: ACGGAGTTTCCGTCGTTACC Reverse: GAAGCCGAGCTTGATGGAGA	55	60
ipaH	Forward: ACGACTGCTGCAACTAGGAC Reverse: TGAGATGCTGGAGAATGAGTACC	50	59.6
qnrS	Forward: TCACACATATCGGCACCACA Reverse: TCGCAAGTTGGCATTGTTGG	55	59.97

Note. RT-PCR: Real-time polymerase chain reaction; GC: Guanine-cytosine; TM: Melting temperature.

Table 2. The Time-Temperature Schedule of the RT-PCR

Steps	Temperature (°C)	Time
Denaturation and enzyme activation	95	10 minutes
Step 1: Denaturation	95	15 seconds
Step 2: Annealing	59	25 seconds
Step 3: Extension and *fluorescence* acquiring	72	30 seconds
Melting curve analysis	65-95	1°C each step

Note. RT-PCR: Real-time polymerase chain reaction.

concentration of AgNPs, and no bacterial growth was observed at the concentration of 0.0625 µg/mL. Therefore, this concentration was considered the MIC of AgNPs. Next, 10 µL of the wells containing 0.0312 µg/mL AgNPs and the like were removed and cultured in the nutrient agar medium, and after 48 hours, it was observed that the bacteria did not grow in the medium containing 0.125 µg/mL AgNPs and the like. Hence, the MBC of AgNPs against *S. flexneri* was considered at 0.125 µg/mL.

Gene Expression Analysis

ipaB and *ipaH*

Both *ipaB* ($P=0.006$) and *ipaH* ($P=0.004$) gene expressions in AgNP-treated *S. flexneri* were decreased significantly compared to the control (Figure 1). The expression level of *ipaH* in control was measured 1.18±0.3. However, in AgNP-treated *S. flexneri*, this expression level was measured 0.33±0.08, indicating the downregulation of *ipaH*. The same result was found for the *ipaB* gene, and the expression level was decreased ~3 times compared to untreated *S. flexneri* (control).

ipaD and *qnrS*

Significant differences in terms of *ipaD* ($P=0.005$) and *qnrS* ($P=0005$) gene expression were observed in *S. flexneri* treated with the MIC of AgNPs compared to untreated bacteria (Figure 2). The expressions of both genes decreased in AgNP-treated *S. flexneri*, highlighting the effect of AgNPs on reducing the expression of *S. flexneri* pathogenic genes.

Discussion

The results of the present study demonstrated that green synthesized AgNPs had antibacterial effects against *S. flexneri*, and the mechanism of antibacterial effects was attributed to the downregulation of pathogenic genes *ipaB*, *ipaD*, *ipaH*, and *qnrS*.

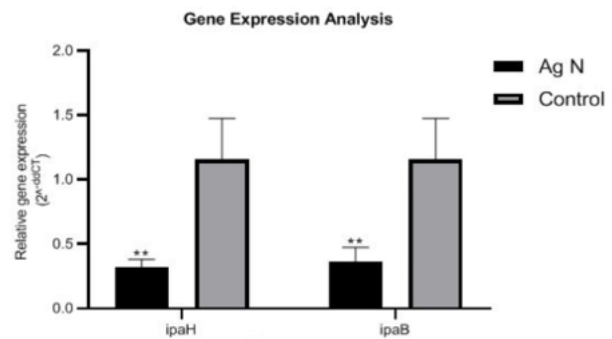

Figure 1. *ipaB* and *ipaH* Gene Expressions in *Shigella flexneri* Treated With or Without (Control) Silver Nanoparticles. The symbol ** displays significant differences at the probability level of $P<0.01$

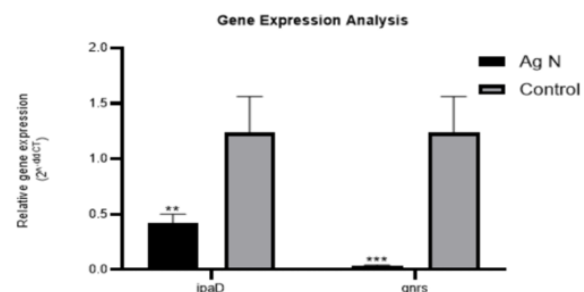

Figure 2. *ipaD* and *qnrS* Gene Expressions in *Shigella flexneri* Treated With or Without (Control) Silver Nanoparticles. The symbol ** shows significant differences at the probability level of $P<0.01$. Symbols ** and *** represent significant differences at the probability level of $P<0.01$ and $P<0.001$, respectively

Spirulina platensis was used to synthesize green AgNPs. The green synthesis of NPs can reduce its side effects on organisms and the environment (24). This algal shows good anti-inflammatory and antioxidant properties due to having various beneficial compounds such as vitamins and amino acids (25) and is widely utilized in the synthesis of most metal NPs (26,27). For example, Gunasundari et al employed ultrasonic-assisted *S. platensis* to synthesize metal NPs, including Zn, Fe, and Ag, and reported antimicrobial effects on Gram-positive and Gram-negative bacteria as well as *Aspergillus niger* (28). Likewise, Mahdieh et al used this alga for the synthesis of crystallized AgNPs (29). Therefore, *S. platensis* has great potential in the green synthesis of metal NPs due to its convenience to handle, low toxicity, and reduction of harmful effects on the environment (30), and the results of the present study confirm that this alga can be utilized in the green synthesis of AgNPs.

The synthesized green AgNPs revealed antibacterial effects on *S. flexneri*, which showed the potential of its application in the treatment of diseases caused by this pathogen. Its MIC was calculated as 0.0625 µg/mL, which is lower than the results of other studies investigating the anti-*Shigella* effects of AgNPs. This difference can be attributed to the NP synthesis method and bacterial species. For

example, Angamuthu et al estimated the MIC of *M. indica* AgNPs on the multi-drug-resistant strain *S. flexneri* to be 20 µg/mL (31), which is much higher than that of the present study. This difference can be related to different strains and the method for the synthesis of AgNPs. In the study of Bagherzade et al, the MIC of green AgNPs synthesized by the aqueous extract of the saffron plant on pathogenic bacteria was reported to be 250 µg/mL, representing a very high value (32). It seems that synthesis factors, bacterial strains, and toxicity criteria are important factors in this difference. In another study, Muthukrishnan et al reported the highest inhibitory concentration of pathogenic bacteria by AgNPs synthesized with *Ceropegia thwaitesii* at 100 µg/mL (33). They used the disk diffusion method to investigate the antimicrobial effects of AgNPs, while in the present study, the microdilution method was employed, which can explain the reason for this difference in the antibacterial concentration of this NP.

In the present study, it was observed that green synthesized AgNPs using spirulina caused changes in the expression of pathogenic genes such as *ipaB*, *ipaD*, *ipaH*, and *qnrS* in *S. flexneri* bacteria and reduced their expressions. Therefore, in this research, it was found that the anti-*Shigella* mechanism of AgNPs is the effect on the expression of pathogenic genes. These genes play an important role in the penetration of bacteria into the epithelial cells and protect the DNA against destructive factors (12). Accordingly, the synthesized green AgNPs increase the sensitivity of *Shigella* flexneri to protective agents by reducing the expressions of *ipaB*, *ipaD*, *ipaH*, and *qnrS* genes, thus exerting anti-*Shigella* effects.

Conclusion

The green synthesized AgNPs using *S. platensis* had strong antibacterial effects on *S. flexneri*, and the action mechanism was attributed to the downregulations of *ipaB*, *ipaD*, *ipaH*, and *qnrS* genes. *In vivo* and clinical studies are necessary in this regard.

Antibacterial Mechanism of Nanosilver

AgNPs are recognized for their potent antibacterial properties, which are attributed to several interrelated mechanisms. Understanding these mechanisms is crucial for their application in medical and consumer products.

Interaction With Bacterial Cell Membranes

AgNPs primarily exert their antibacterial effects through direct interaction with bacterial cell membranes. The positively charged AgNPs adhere to the negatively charged components of the bacterial cell wall, facilitating their penetration into the cell. This interaction disrupts the integrity of the cell membrane, leading to increased permeability and eventual leakage of cellular contents, which can result in cell death (34, 35).

Electrostatic Attraction

The electrostatic interaction between AgNPs and the bacterial membrane enhances adhesion, promoting physical changes that compromise membrane integrity (35).

Size-Dependent Effects

Smaller NPs have a larger surface area relative to their volume, allowing for more effective contact with bacterial cells and facilitating deeper penetration into the cytoplasm (35).

Release of Silver Ions

Another significant mechanism is the release of Ag ions ($Ag+$), which can occur when AgNPs dissolve upon contact with bacteria. These ions are highly reactive and can interact with various cellular components:

Protein Interactions

$Ag+$ ions bind to thiol groups in proteins, disrupting their function by forming stable complexes that inhibit enzymatic activity (36, 37). This interaction can lead to oxidative damage and cellular dysfunction.

Reactive Oxygen Species Generation

The presence of AgNPs and $Ag+$ can induce oxidative stress within bacterial cells by promoting the formation of reactive oxygen species , damaging cellular components such as lipids, proteins, and DNA (34,37). This oxidative damage is a critical pathway leading to bacterial cell death.

Induction of Oxidative Stress

The generation of ROS is a key factor in the antibacterial action of AgNPs. Upon entering bacterial cells, AgNPs can catalyze reactions that produce free radicals:

Membrane Damage

ROS can oxidize fatty acids in the membrane, leading to increased permeability and structural failure (36,37).

DNA Damage

Inside the cell, ROS can interact with DNA, causing mutations and impairing replication processes (36).

Biofilm Disruption

AgNPs also play a role in preventing biofilm formation, which is a significant challenge in treating bacterial infections. By adhering to surfaces and disrupting the initial stages of biofilm development, AgNPs can reduce bacterial colonization on medical devices and tissues (35). This property is particularly beneficial in combating infections associated with implants and catheters.

The antibacterial mechanisms of silver nanoparticles (nanoAg) are multifaceted, involving direct membrane disruption, ion release, oxidative stress induction, and biofilm prevention. These properties make AgNPs a valuable tool in the fight against antibiotic-resistant bacteria and a promising candidate for various biomedical applications. Continued research into optimizing their

use while minimizing potential toxicity is essential for advancing their application in healthcare settings.

Authors' Contribution

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Supervision: Shadi Hajrasoulih.
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Competing Interests

The authors declare no competing interests.

Consent to Participate

Not applicable.

Consent to Publish

All authors agree to publish the article.

Data Availability Statement

Not applicable.

Ethical Approval

Not applicable.

Funding

This research was conducted with funding provided by the authors.

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