EJONS International Journal on Mathematic, Engineering and Natural Sciences ISSN 2602 - 4136

Article Arrival Date	Article Type	Article Published Date
25.06.2023	Research Article	20.09.2023

EFFECT OF ZINC OXIDE NANOPARTICLES ON THE GENE EXPRESSION OF THE BIOFILM FORMATION GENES IN PSEUDOMONAS AERUGINOSA ISOLATED FROM CLINICAL SAMPLES

ÇİNKO OKSİT NANOPARÇACIKLARININ KLİNİK ÖRNEKLERDEN İZOLE EDİLEN PSEUDOMONAS AERUGINOSA'DAKI BİYOFİLM OLUŞUM GENLERİNİN GEN EKSPRESYONU ÜZERİNE ETKİSİ

Ali Sultan MAALA, Lütfi TUTAR, Kais Kassim GHAIMA

Department, Faculty, University, City, Country

Department of Internal Medical Sciences, Faculty of Medicine, Gazi University, Ankara, Turkey

First Author: https://orcid.org/0009-0008-4434-5314

ABSTRACT

Background and aim: The use of antibiotics to treat Pseudomonas aeruginosa infections is limited by the bacteria's innate antimicrobial resistance. As a result, research has focused on developing advanced antibacterial agents to eradicate resistant bacterial strains. It has been discovered that various nanoparticles can be used as antibacterial agents. This study evaluated the role of zinc oxide nanoparticles (ZnO NPs) as an anti-biofilm agent.

Methods: We used six potent biofilm producer isolates of P. aeruginosa, which have two biofilm genes, to perform a quantitative PCR reaction experiment. These isolates were chosen because they have different sub-MIC values for ZnO-np. A quantitative RT-PCR assay was used to analyze the mRNA expression of biofilm genes in treated and untreated samples of bacterial growth with ZnO-np by using the concentration below the MIC dose for each sample.

Results: The results revealed a significant down-regulation in biofilm genes in the presence of ZnO-np. Correlation analysis was used to analyze the results, which included all the tested isolates before and after treatment with ZnO-np. This analysis showed a significant positive correlation between gene expression of lasI, and rhlI genes and biofilm formation. These findings suggest that ZnO NPs could potentially treat P. aeruginosa infections. However, further research is needed to confirm these findings and to determine the optimal dose and concentration of ZnO NPs for treating P. aeruginosa infections.

Keywords: biofilm formation, *Pseudomonas aeruginosa*, Zinc oxide nanoparticles.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that targets individuals with conditions such as cancer, AIDS, cystic fibrosis, and burns. Its multidrug resistance has evolved through a process called Quorum sensing, a cell-to-cell communication system responsible for activating various virulence genes, including those related to pyocyanin, proteases, toxins, and biofilm formation (1).

To combat the virulence of P. aeruginosa, researchers are exploring compounds that can disrupt the Quorum sensing process and inhibit the expression of virulence genes associated with proteases, toxins, siderophores, swarming, and biofilm formation (7).

In the realm of medical science, nanotechnology is gaining recognition for its potential applications in infection treatment. Among the various nanomaterials, ZnO nanoparticles (NPs) stand out for their remarkable antimicrobial properties due to their small size and high surface-to-volume ratio, which facilitates enhanced interaction with bacteria (2). These unique characteristics make ZnO NPs promising candidates as disinfectants and antimicrobial agents, especially in tackling nosocomial infections.

The severity of the P. aeruginosa problem in local hospitals in Iraq has prompted the current study's focus. The objective is to investigate the impact of ZnO nanoparticles on the gene expression of biofilm-related genes, which play a crucial role in the virulence of Pseudomonas aeruginosa isolates. The hope is that by exploring alternative antimicrobial materials, these bacteria can be effectively eradicated.

MATERIALS AND METHODS

Extraction of genomic DNA

A commercial purification method, the Genomic DNA Extraction Mini Kit (iNtron®, Korea), was used to extract DNA from *P. aeruginosa* bacteria. This kit was made to isolate DNA from both Gram-positive and Gram-negative bacteria. The dsDNA Quantitation was done by Qubit 4.0. The assay is accurate for initial sample concentrations of 10 pg/ to 100 ng/µL and is highly selective for double-stranded DNA (dsDNA) over RNA. The test is run at room temperature, and the signal is consistent for three hours. The assay tolerates common impurities, including salts, free nucleotides, solvents, detergents, or proteins.

Primers used in this study:

Table (1): Primers used in this study (PCR and gene expression).

Target gene		Nucleotide sequence (5'—3')	Size product (bp)	The reference
lasI	F	TCG ACG AGA TGG AAA TCG ATG	363	**Int *et al.* (2021)**
lasI	R	GCT CGA TGC CGA TCT TCA G		
rhlI	F	CGA ATT GCT CTC TGA ATC GCT	143	**Int *et al.* (2021)**
rhlI	R	GGC TCA TGG CGA TGT A		
***rsp*L**	F	GCAACTATCAACCAGCTGGTG	241	**Gong *et al.*, (2014)**
***rsp*L**	R	TCAGCACTACGCTGTGCTCTT		

RNA isolation by TRIzol

RNA Quantitation by Qubit 4.0

The assay is highly selective for RNA and accurate at concentrations ranging from 10 pg/µL to100 ng/µL. The assay is carried out at room temperature, and the signal remains stable for three hours. The assay tolerates common contaminants such as salts, free nucleotides, solvents, detergents, and proteins.

Quantitative Real-time PCR Assay (RT-qPCR) protocol

The primary step of our project was divided into two phases. The first phase involved the synthesis of cDNA from RNA, focusing on specific transcripts such as lasI, rhlI, and rspL, using proscript cDNA and targeted primers. The procedure consisted of the following steps:

Five microliters of total RNA extracted from each sample were added to separate PCR tubes.

1. A 10ul proscript reaction mix containing a buffer, dNTPs, and other essential ingredients was added to each sample.
2. Two microliters of MuLV Enzyme were then added to each reaction.
3. Two microliters of specific primers for each gene were added, and the final volume was adjusted to 20ul by adding 1ul.
4. The reaction mixture was incubated in a thermocycler at 42°C for 1 hour, followed by heating to 80°C to inactivate the enzyme.
5. In the second phase of the process, the quantification of the cDNA product was carried out using Qubit 4.0, which utilized relative quantitative PCR. For this step, samples of bacteria and controls were selected to run simultaneously. Each sample had three PCR tubes, one for each of the target genes (lasI, rhlI, and rspL), while rspL was considered the housekeeping gene in this study. Detection of the cDNA amounts was based on the fluorescence power of SyberGreen.

Table (2): Volumes and concentrations of the qPCR reaction mix

component	20ul reaction
Luna universal qPcr master mix	10ul
Forward primer (10um)	1ul
Reverse primer (10 um)	1ul
Template DNA	5ul
Nuclease-free water	3ul

Quickly spin for PCR tubes to remove the bubbles and collect the liquid (1 minute at 2000g, then the program for Real-Time PCR was set up with indicated thermocycling protocol as shown in table (3):

Table (3): RT-PCR Cycling Program

Cycle Step	Temperature	Time	Cycles No.
Initial Denaturation	95 °C	60 seconds	1
Denaturation	95 °C	15 seconds	40
Annealing	60 °C	30 seconds	
Extention	60 °C	30 seconds	
Melt Curve	60-95 °C	40 minutes	1

Calculations of Delta Ct (ΔΔCt) method

The simplest method is this one, which directly compares the Ct values of the target gene and the reference gene. The selection of a calibrator sample is necessary for relative quantification. Any sample you want to compare your unknown to can be used as the calibrator sample, including the untreated and time=0 samples. First, for each sample, the Ct between the target gene and the reference gene is computed (for the unknown samples and the calibrator sample).

ΔCt = Ct target – Ct reference gene

Then the difference between the ΔCt of the unknown and the ΔCt of the calibrator is calculated, giving the ΔΔCt value:

ΔΔCt = (Ct target – Ct reference) sample – (Ct target – Ct reference) calibrator

The normalized target amount in the sample is then equal to $\mathbf{2^{-\Delta\Delta Ct}}$, which can be used to compare expression levels in samples (3).

The samples were analyzed in triplicates and standardized against *16S rRNA* gene expression. The relative changes in mRNA expression levels were determined using the comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$) between the nanoparticles-exposed and non-exposed *P. aeruginosa.*

After adding the ZnO-np

Muller Hinton broth tubes were prepared with the appropriate peptide concentration and incubated at 37°C for 24 hours to monitor the bacterial growth in media. After growth, RNA extraction was done by the same steps by the SV Total RNA Isolation System kit. The same primers, RT master mix, and programs were used before adding the ZnO-np.

Results and discussion

Molecular detection of *rspL* gene and quorum sensing genes (*lasI and rhlI*)

The isolates of P. aeruginosa were the strong, moderate, and weak biofilm used in this study and detected by PCR technique using the **rspL gene** as the housekeeping gene of P. aeruginosa. All the tested P. aeruginosa clinical contained the **rspL gene** (241bp) (Figure 4-9).

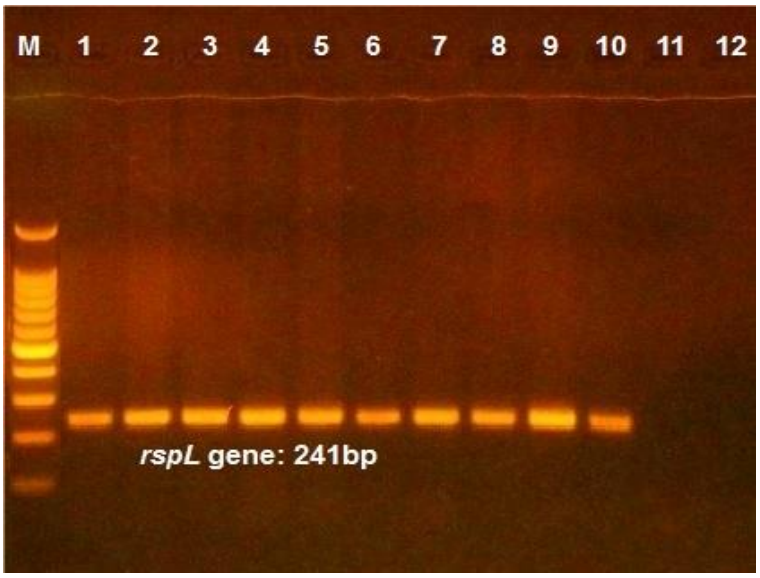

Figure (4-9). The amplification of the *rspL* gene of *Pseudomonas aeruginosa* samples was fractionated on 2% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1-10 resemble 241bp PCR products.

PCR technique detected two genes, lasI, and rhlI, that seem to be involved in the clinical quorum sensing of P. aeruginosa. The gel electrophoresis of amplified PCR product for lasI is shown in figure (4-10) and rhlI figure (4-11). The results showed that the lasI gene were found in 80% of isolates that produce strong biofilm, while the rhlI gene was found in all potent biofilm isolates.

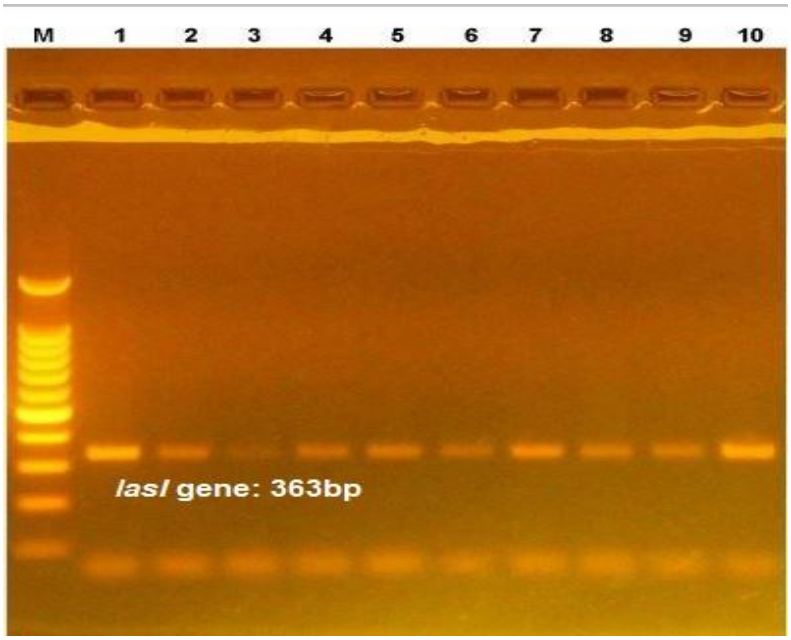

Figure (4-10): Gel electrophoresis amplification of *lasI gene* of *P. aeruginosa* samples were fractionated on 2% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1-32 resemble 363bp PCR products.

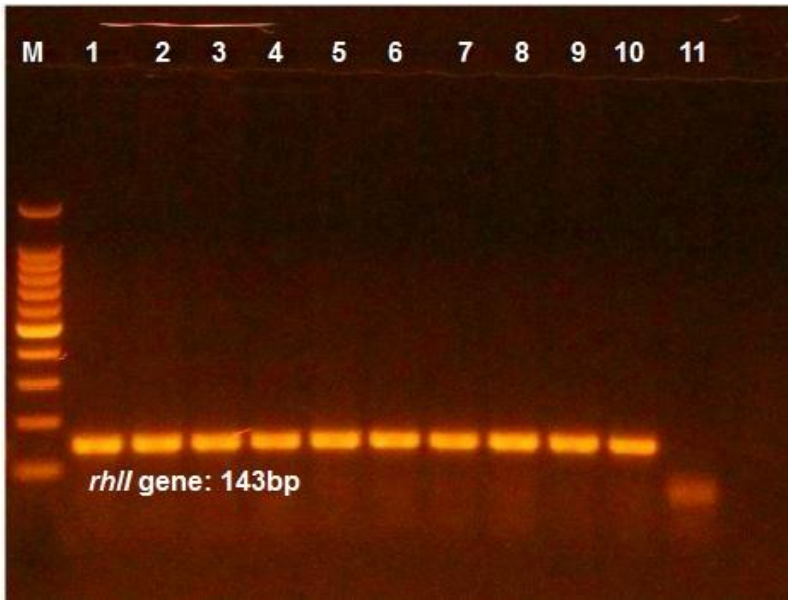

Figure (4-11): Gel electrophoresis amplification of *rhlI gene* of *P. aeruginosa* isolates were fractionated on 2% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1-32 resemble. 143bp PCR products.

Ten isolates from a total of 55 isolates were potent biofilm producers, and 8 contained both lasI genes (80%), while rhlI was found in all these isolates, as shown in Table (4-8).

Table (4-8): The existence of *lasI* and *rhlI* genes in potent biofilm isolates

Isolate code	*LasI*	*rhlI*
P.1	+	+
P.3	+	+
P.6	_	+

P.12	+	+
P.14	+	+
P.19	+	+
P.22	_	+
P.27	+	+
P.31	+	+
P.33	+	+

(P): *P. aeruginosa* isolate, (+): Present, (-): Absent

In a previous study conducted in Kastamonu, Turkey, researchers analyzed a total of 52 carbapenem-resistant Pseudomonas aeruginosa isolates. The study found that 51 isolates (98.1%) contained two Quorum Sensing (QS) system genes, with four isolates co-existing with both QS system genes. Additionally, 41 isolates (78.8%) were determined to have both lasI/R and rhlI/R genes, which are components of the QS systems.

Statistical analyses revealed significant positive correlations between the las and rhl QS systems, as well as strong and positive correlations between the rhl QS system and three virulence genes associated with slime production. Some other virulence genes also showed correlations with the QS systems (4). Another study, which included 24 P. aeruginosa isolates from Gazi University Faculty of Medicine, Turkey, identified QS genes (lasI, lasR, rhlI, and rhlR) using PCR amplification of chromosomal DNA. Nineteen isolates with moderate to strong biofilm formation were found to contain lasI, lasR, and rhlI genes, indicating their involvement in biofilm production (6).

Similarly, a study conducted in Iran with 140 clinical isolates of P. aeruginosa from Tehran and Ilam hospitals found that a majority of the isolates were capable of producing biofilms (87.15%), with a significant number forming strong biofilms (56.42%). PCR results showed that the frequency of lasI/R and rhlI/R genes was 93.57% and 83.57%, respectively. The presence of QS system genes was prevalent among these clinical isolates, and there was a significant correlation between biofilm formation and the presence of QS system genes (5). Overall, these studies highlight the importance of QS systems in P. aeruginosa virulence and demonstrate the significant association between biofilm formation and the presence of QS system genes in different P. aeruginosa isolates from various regions.

Effect of ZnO-np on gene expression of biofilm formation genes

In this step of the research, the main goal was to examine the gene expression of quorum sensing (QS) genes, particularly lasI and rhlI, in Pseudomonas aeruginosa isolates. The aim was to compare the gene expression levels when exposed to ZnO nanoparticles (ZnO-np) versus when not exposed, with a focus on how these nanoparticles might influence biofilm formation by affecting gene expression, especially in highly biofilm-forming strains. To achieve this, the researchers used a technique called reverse transcription-quantitative PCR (RT-qPCR), which is distinguished from other methods of gene expression analysis because it allows for relative quantification (8) . This means that the researchers were interested in comparing the expression levels of specific genes among different samples. During the PCR process, the amplification of genes was recorded as a cycle threshold (Ct) value. To ensure accurate comparisons, a housekeeping gene called RSPL was used as a reference gene, as its expression remains stable in the cells or tissues being investigated, even under different conditions. The study involved six potent biofilm-producing P. aeruginosa isolates, each containing two biofilm genes. These isolates were chosen with different sub-MIC

values of ZnO-np. The researchers then conducted quantitative RT-PCR to analyze the mRNA expression of biofilm and virulence genes in the presence and absence of ZnO-np, using concentrations below the minimum inhibitory concentration (MIC) for each sample. The gene expression fold change was calculated using relative quantification (RQ) based on the delta Ct value.

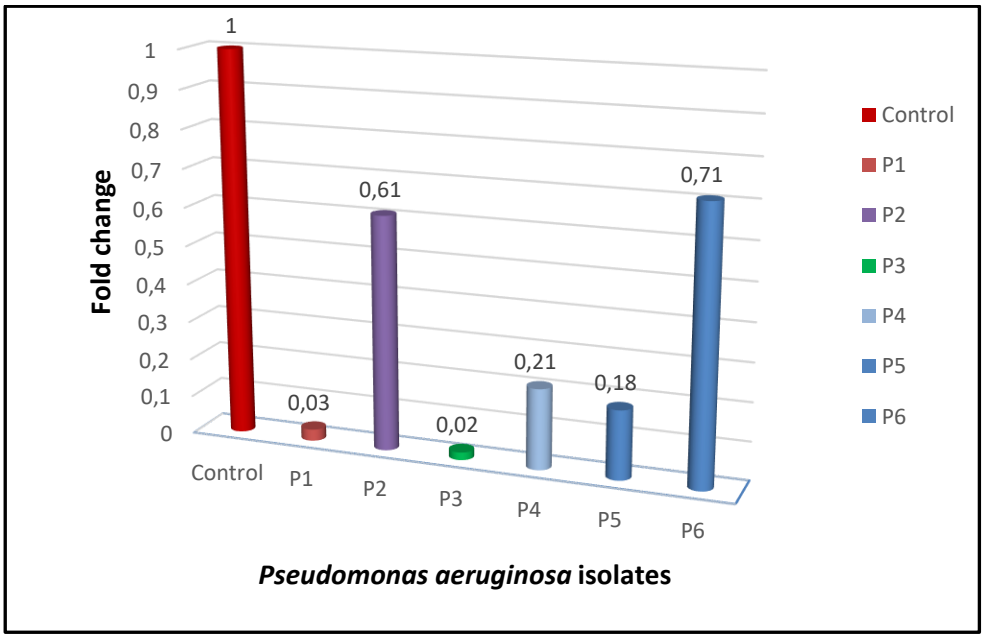

Figure (3): Gene expression (fold change) results for *lasI* before and after being treated with ZnO-np

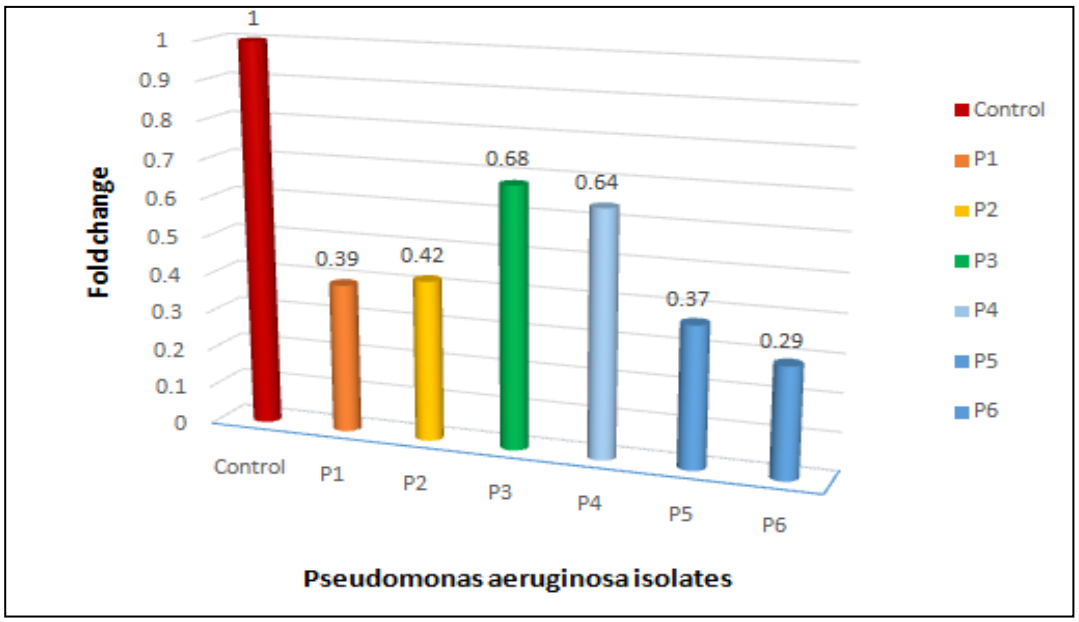

Figure (4): Gene expression (fold change) results for *rhlI* before and after being treated with ZnO-np.

The findings revealed that ZnO-np significantly down-regulated the expression of all biofilm and virulence genes in the P. aeruginosa clinical isolates. Targeting QS through the use of antimicrobial peptides, like ZnO-np, has emerged as a promising alternative in antimicrobial therapy, as it can control virulence factor formation and pathogenesis in P. aeruginosa. Unlike traditional antibiotics, anti-virulence agents like QS inhibitors do not impose stresses on bacterial growth, thus reducing the likelihood of microbial resistance. In the broader field of nanotechnology, researchers have been exploring the development of new nanoparticles targeting QS and virulence factors. In previous

studies, both ZnO nanoparticles and silver nanoparticles demonstrated the ability to reduce the expression of QS regulatory genes in P. aeruginosa isolates (9) .Furthermore, combining ZnO-np with meropenem showed synergistic antipseudomonal activity, and gold-nanoparticles and selenium-nanoparticles were found to inhibit QS-related virulence factors in P. aeruginosa. These nanoparticles achieve their antimicrobial effects by forming free radicals, causing oxidative stress, damaging DNA, and facilitating penetration through microbial cell walls. Overall, these research findings highlight the potential of ZnO-np and other nanoparticles as effective strategies for disrupting QS systems and controlling virulence traits in P. aeruginosa. This contributes to the development of alternative approaches to combat microbial diseases, and further studies are needed to fully understand the molecular mechanisms underlying the inhibition of QS by nanoparticles (10).

CONCLUSION

The study's findings demonstrated a notable reduction in the expression of biofilm genes (lasI and rhlI) when Pseudomonas aeruginosa isolates were exposed to sub-minimum inhibitory concentration (sub-MIC) doses of ZnO nanoparticles (ZnO-np). This down-regulation indicated that ZnO-np had a suppressive effect on the genes responsible for biofilm formation in the bacteria.

Furthermore, the results showed a significant positive correlation between the gene expression of biofilm formation genes and the actual biofilm formation itself. This correlation implies that higher expression levels of the genes involved in biofilm formation were associated with increased biofilm formation by the P. aeruginosa isolates.

In summary, the study provided evidence that ZnO-np exerted an inhibitory effect on biofilm gene expression, which in turn correlated with reduced biofilm formation in Pseudomonas aeruginosa. These findings highlight the potential of ZnO-np as an anti-biofilm agent and suggest its role in regulating biofilm-related genes to control bacterial biofilm formation.

REFERENCES

1. **Wood SJ, Kuzel TM, Shafikhani SH**. Pseudomonas aeruginosa: Infections, Animal Modeling, and Therapeutics. Cells. 2023 Jan 3;12(1):199.
2. **Avershina E, Shapovalova V, Shipulin G. (2021).** Fighting Antibiotic Resistance in Hospital-Acquired Infections: Current State and Emerging Technologies in Disease Prevention, Diagnostics and Therapy. Front Microbiol. 21;12:707330.
3. **Schmittgen TD, Livak KJ.** Analyzing real-time PCR data by the comparative CT method. Nat Protoc. 2008;3(6):1101-8.
4. **Başkan C, Sırıken B, Tüfekci EF, Kılınç Ç, Ertürk Ö, Erol İ. (2022).** Presence of quorum sensing system, virulence genes, biofilm formation and relationship among them and class 1 integron in carbapenem-resistant clinical Pseudomonas aeruginosa isolates. Arch Microbiol. 8;204(8):464.
5. **Hemati S, Azizi-Jalilian F, Pakzad I, Taherikalani M, Maleki A, Karimi S, Monjezei A, Mahdavi Z, Fadavi MR, Sayehmiri K, Sadeghifard N.** (2014). The correlation between the presence of quorum sensing, toxin-antitoxin system genes, and MIC values with the ability of biofilm formation in clinical isolates of Pseudomonas aeruginosa. Iran J Microbiol. 6(3):133-9.
6. **Erdal B, Yalınay M, Elmas Ç, Yazıcı GN. (2020).** Piperasilin/Tazobaktam ve Siprofloksasin Sub-minimal İnhibitör Konsantrasyonlarında Pseudomonas aeruginosa Biyofilm Oluşumunun ve Quorum Sensing Genlerinin Araştırılması [Investigation of Pseudomonas aeruginosa Biofilm Formation and Quorum Sensing Genes in Piperacillin/Tazobactam and Ciprofloxacin Sub-minimal Inhibitory Concentrations]. Mikrobiyol Bul. 54(4):547-558.
7. **Thi MTT, Wibowo D, Rehm BH.** Pseudomonas aeruginosa biofilms. Int. J. Mol. Sci. 2020;21:8671.
8. **Derveaux S, Vandesompele J, Hellemans J. (2010).** How to do successful gene expression analysis using real-time PCR. Methods. 2010 Apr;50(4):227-30.

9. **El-Mowafy SA, Shaaban M, Abd El-Galil KH. (2014).** Sodium ascorbate as a quorum sensing inhibitor of Pseudomonas aeruginosa. Journal of Applied Microbiology. 117:1388–1399.
10. **Gellatly, S. L., & Hancock, R. E. (2013).** Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. Pathogens and disease, 67(3), 159-173.