

The effect of Zinc Oxide Nanoparticles on Pseudomonas Aeruginosa Biofilm Formation and Virulence Genes Expression

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Abstract

Background: Due to increased resistance to antimicrobial agents, infectious disease remains a public health problem worldwide.

Aim: The current study was designed to examine the effect of ZnO nanoparticles (ZnO –np) against the biofilm formation ability of *P.aeruginosa* clinical isolates and to study its effect on the expression level of the genes involved in biofilm formation and virulence factors production.

Methodology: The MIC of ZnO –np against *P. aeruginosa* was determined by the broth microdilution method. The Effect of ZnO –np on the biofilm-forming isolates of *P. aeruginosa* was monitored by the microtiter plate method. *P.aeruginosa* isolates were tested for the expression of different biofilm and virulence genes using real-time rt-PCR.

Results: ZnO –np significantly downregulated the expression level of all biofilm and virulence genes of *P.aeruginosa* clinical isolates except the *toxA* gene.

Conclusions: This study demonstrates the promising use of ZnO –np as an anti-biofilm and anti-virulence compound.

1. Background

P.aeruginosa is an important cause of community and hospital-acquired infections, especially in immune-compromised patients. The formation of biofilm by *P.aeruginosa* is the key to its chronic colonization in human tissues. Due to the many clinical implications, biofilms formed by *P.aeruginosa* are the most frequently studied biofilm models [1].

Bacteria within biofilm shows marked resistance to antibiotics, reduction in growth rates and secretion of different surface molecules and virulence factors, which can enhance their pathogenicity by several hundred folds [2].

Increased resistance to antimicrobial agents is a major public health problem worldwide [3]. One of the most promising strategies for overcoming microbial resistance is the use of nanoparticles [4]. The exact mechanisms of action of nanoparticles are not yet known, it may be dependent on factors such as composition, surface changes, properties and concentration of nanoparticles [3].

One of the famous nanoparticles is ZnO –np which is one of metal oxide nanoparticles. Zinc oxide is a polar inorganic compound. It appears as a white powder, nearly insoluble in water with many applications, such as antimicrobial, wound healing, UV filtering properties, high catalytic and photochemical activity, due to its unique combination of interesting properties such as selective toxicity toward bacteria, with minimal effects on human and animal cells, stability in a hydrogen plasma atmosphere and low price [5].

ZnO disrupts membrane integrity via the production of reactive oxygen species that destroy bacteria [6]. In addition, the production of hydrogen peroxide and Zn^{2+} has shown a key role in the antibacterial activity of nanoparticles [7].

However pathogenic microorganisms are able to protect themselves against inhibitory compounds by the formation of biofilms [8]. Therefore, the current study was designed to examine the effect of ZnO -np against the biofilm formation ability of *P.aeruginosa* clinical isolates and to study its effect on the expression level of the genes involved in biofilm formation and virulence factors production of *P.aeruginosa* clinical isolates.

2. Methods

Written informed consents were obtained from all individuals. The study was approved by the Ethical Committee of Minia University, Faculty of Medicine (code number: 32 A).

2.1. Bacterial strains

The study was carried out on 100 biofilm-forming *P. aeruginosa* clinical isolates obtained from different clinical samples from different patients at Minia University Hospital. *P. aeruginosa* PAO1 as standard biofilm-producing strain and P1A isolate as non-biofilm producing strain were included as positive and negative controls respectively.

2.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

ZnO nanoparticles (20 ± 5 nm diameter) were purchased from Sigma–Aldrich. In order to examine the antibacterial activity of the ZnO -np, ZnO -np were suspended in sterile normal saline and constantly stirring until a uniform colloidal stock suspension was formed at a concentration of 1024 $\mu\text{g/ml}$. The stock suspensions were stored at 4 C. Before each experiment stock suspensions were stirred for a ~ 2 h. The MIC of ZnO -np against *P. aeruginosa* was determined by using the broth microdilution method in 96-well microplates according to CLSI guidelines. Serial dilutions were prepared in 10 wells with concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 $\mu\text{g/ml}$ and two wells were positive (including culture media and microbial suspension) and negative (including culture media) controls. Then, 10 microliters of bacterial suspension ($OD_{620} = 0.01$) were added to wells containing different concentrations of nanoparticles and the plates were then incubated overnight at 37 °C. MIC is the lowest concentration of the nanoparticles that inhibit visible bacterial growth. The concentration of ZnO -np that inhibited 50% and 90% of the isolates were measured as MIC50 and MIC90. The minimum bactericidal concentration was established by the lack of growth after re-inoculation from ZnO -np- treated media to agar medium without nanoparticles. All experiments were carried out three times [9].

2.3. Effect of ZnO -np on biofilm formation:

The Effect of ZnO -np on the biofilm-forming isolates of *P. aeruginosa* was monitored by the microtiter plate method according to Samet *et al* instructions [10]. Briefly, 190 µl of bacterial suspension (OD₆₂₀ = 0.01) in Luria Bertani broth was inoculated in 96 microtiter plates. Sub-MIC concentrations of ZnO-np were added to each well excluding the positive and negative control wells. Plates were incubated at 37 °C for 24 hr. After incubation, the content of each well was gently removed. The wells were washed with phosphate-buffered saline solution to remove free-floating bacteria. Biofilms formed by bacteria were air and heat-fixed for one hour and stained with crystal violet (0.1%, w/v). Excess stain was rinsed off by washing with water and plates were kept for drying. Ethanol 95% was added to the wells and after 15 minutes the optical densities (OD) of stained adherent bacteria were determined with ELISA reader (model CS, Biotec) at 590 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiments were performed in triplicate, the data were then averaged.

2.4. Effect of ZnO -np on preformed biofilm

Individual wells of microplates were filled with 190 µl of bacterial suspension (OD₆₂₀ = 0.01). The MTPs were incubated for 24 h at 37°C. After incubation, 10 µl of ZnO -np dilutions were added to each well. The effect of ZnO nanoparticles on the preformed biofilms was tested after 2, 4 or 6 hours incubation at 37°C. The content of the microplates was gently removed at the end of the estimated time period and then examined as described above, OD of stained adherent bacteria in wells were read at 590 nm.

2.5. Effect of ZnO -np on relative genes expression:

P.aeruginosa isolates were tested for the expression of different biofilm and virulence genes using real-time reverse transcriptase-polymerase chain reaction (rt-PCR) according to the following steps.

2.5.1. RNA extraction:

Pure bacteria were inoculated in two tubes containing 2 ml LB broth. One of the tubes contained no nanoparticles and the other had ZnO -np. The ZnO -np concentration used herein was determined based on MIC and MBC results so that was less than MBC concentration (19). Tubes were incubated at 37 °C, shaking 200 rpm for 6 hours (19). Bacterial RNA was extracted by the Direct-zol RNA extraction kit (Zymo research CORP, Australian) according to the manufacturer's instructions. Absorbance was assessed by a spectrophotometer (Genova, USA), and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the extracted RNA. The result within the 1.8 to 2 range was considered as acceptable purity. The quality of the extracted RNA was evaluated via electrophoresis on 1.2% agarose gel at 100 V for 60 min.

2.5.2. Rt-PCR

According to manufacturer instructions quantitative real-time rt-PCR was done using one step Sybr green kits (SensiFAST SYBR Lo-ROX Kit, Meridian Life science, UK) in an ABI 7500 instrument (Applied Biosystems, USA). Real-time rt-PCR reaction was prepared with a final volume 20 µ (master mix: 10 µ, Forward primer: 0.8 µ, Reverse primer: 0.8 µ, Reverse transcriptase: 0.2 µ, RNase inhibitor, 0.4 µ, Water up

to 16 μ and Template: 4 μ). Different genes and primers were listed in Table 1. Four negative control samples contain deionized water instead of template, one for each gene were included in the same PCR run.

We analyzed PCR results with relative quantification to *pro C* (housekeeping gene) as a reference gene thus standards with known concentrations are not required. We calculated the fold changes of mRNA levels using the comparative cycle threshold ($\Delta\Delta C_t$). The fold change in gene expression was normalized to the reference gene (*pro C*) and relative to the control sample. Then the relative expression was confirmed by using free data analysis tools [11]. PCR products were analyzed by gel electrophoresis, to exclude any unspecific products are present.

Table 1
List of primers used for amplification

Gene	Primer sequence	Reference
<i>Las R</i>	5'-AAGTGGAAAATTGGAGTGGAG-3' 5'-GTAGTTGCCGACGATGAAG-3'	[11]
<i>rhII</i>	5'-GTAGCGGGTTTGCGGATG-3' 5'-CGGCATCAGGTCTTCATCG-3'	[12]
<i>pqsR</i>	5'-CTGATCTGCCGTAATTGG-3' 5'-ATCGACGAGGAAGTGAAGA-3'	[12]
<i>Lec A</i>	5'-CACCATTGTGTTTCCTGGCGTTCA-3' 5'-AGAAGGCAACGTCGACTCGTTGAT-3'	[13]
<i>Pel A</i>	5'-AAGAACGGATGGCTGAAGG-3' 5'-TTCCTCACCTCGGTCTCG-3'	[14]
<i>toxA</i>	5'...GACAACGCCCTCAGCATCAACAGC 5'...CGCTGGCCCATTCGCTCCAGC	[14]
<i>exoS</i>	5'...AGGCATTGCCCATGACCTTG 5'...ATACTCTGCTGACCTCGCTC	[14]
<i>lasA</i>	TTCTGTGATCGATTCGGCTCGGTT ACCCGGGAAGACAACACTATCAGCTT	[13]
<i>Pro c</i>	5'CAGGCCGGGCAGTTGCTGTC-3' 5'-GGTCAGGCGCGAGGCTGTCT-3'	[15]

2.5.3. Statistical Analysis

All statistical analyses were performed using the SPSS program for Windows (version 20 statistical software; Texas instruments, IL, USA). A two-tailed p-value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. ZnO nanoparticles MIC and MBC:

The results of the broth microdilution method showed that MIC₅₀ and MIC₉₀ of ZnO -np that inhibits the growth of *P. aeruginosa* clinical isolates were 64 and 128 $\mu\text{g/ml}$, respectively. The range of MIC of ZnO nanoparticles for *P. aeruginosa* clinical isolates were 8-128 $\mu\text{g/ml}$. Anti-bacterial activity increased with the rising concentration of ZnO nanoparticles as shown in Fig. 1. The MBC of nanoparticles that kill 50 and 100% of the isolates were 128 and 256 $\mu\text{g/ml}$ respectively.

(Fig. 1: **MIC of ZnO -np**: X axis represent serial dilutions of ZnO -np that was studied in this study. Y axis represent the percent of the inhibited bacterial isolates at each concentration. The percent of the inhibited isoaltes increased ith rising ZnO -np concentration.)

3.2. Antibiofilm effect of ZnO nanoparticles

ZnO -np showed anti-biofilm activity on all tested isolates. The anti-biofilm activity increased with the rising concentration of nanoparticles as shown in Fig. 2. BIC₅₀ and BIC₉₀ (biofilm inhibitory concentration in 50 and 90% of the isolates respectively) were 16 and 32 $\mu\text{g/ml}$ respectively.

(Fig. 2: **BIC of ZnO -np**: X axis represent serial dilutions of ZnO -np that was studied in this study. Y axis represent the percent of the inhibited bacterial biofilms at each concentration. The percent of the inhibited bacterial biofilms increased ith rising ZnO -np concentration.)

3.3. Effect of ZnO -np on the preformed biofilm

In this study, *P. aeruginosa* was employed to evaluate the effect of ZnO -np on the removal of established biofilms. The OD at 590 nm shown in Fig. 3 corresponded to the amount of remaining attached biofilm biomass of *P. aeruginosa* isolates after 2, 4 and 6 h treatment with ZnO -np. Treating the preformed biofilm with ZnO -np resulted in significant OD reduction. The degree of reduction depends on the concentration of ZnO -np and the time of incubation between preformed biofilms and ZnO -np as shown in Fig. 3. Significant reduction on the OD value (mean \pm SD = 1 ± 0.03 ; P-value = 0.001) was reported at 64 $\mu\text{g/ml}$ concentration of ZnO -NP for 2 h incubation. The degree of reduction on the OD value was highly significant at 8, 16, 32 and 64 $\mu\text{g/ml}$ concentrations of ZnO -np incubated for 4 and 6 hours with the preformed biofilms (P-value ≤ 0.0001).

(Fig. 3: **Anti-biofilm effect of ZnO -np**: The figure represents the degree of reduction of OD values after 2, 4, 6hs incubation of preformed bacterial biofilms with ZnO -np at 37°C.)

3.4. Effect of ZnO -np on relative genes expression:

The effect of ZnO nanoparticles on the expression of different genes responsible for biofilm and virulence factors production was studied by RT-PCR. ZnO nanoparticles significantly downregulated the expression level of all biofilm and virulence genes of *Paeruginosa* clinical isolates except the *tox A* gene which was up-regulated as shown in Fig. 4. The fold change decrease of the quorum sensing genes, *Las R*, *rhII* and *pqsR* after ZnO nanoparticles treatment were 10.4, 6.3 and 8.7 fold (P-value ≤ 0.0001) respectively. ZnO nanoparticles down-regulated other genes responsible for biofilm formation; *LecA* and *Pel A* genes by 4.7 and 5.6 fold (P-value ≤ 0.0004) respectively. ZnO nanoparticles also down-regulated *virulence genes*; *exoS* and *lasA* by 3.7 and 5.2 fold respectively (P-value ≤ 0.008). None statistically significant up-regulation of *tox A* gene after ZnO nanoparticles treatment by 1.9 fold was reported (P-value = 0.37).

(Fig. 4: **Effect of ZnO -np on relative genes expression:** The figure represents the fold change decrease (-) or increase (+) in the virulence genes expression of *P. aeruginosa* clinical isolates after ZnO -np treatment.)

4. Discussion

In our study, we have examined the antibacterial activity of ZnO -np (20 \pm 5 nm diameter) and its effect on the biofilm formation by *P. aeruginosa* isolated from hospitalized patients.

In the present study, ZnO -np were found to be effectively inhibiting the growth of *P. aeruginosa* and restrict the biofilm formation.

The antibacterial and anti-biofilm effect gradually increased with raising the concentration of ZnO -np. MIC50 and MIC90 of ZnO -np for the studied isolates were 64 and 128 $\mu\text{g/ml}$, respectively. BIC50 and BIC90 of ZnO -np for the studied isolates were 16 and 32 $\mu\text{g/ml}$ respectively. The MBC of nanoparticles was higher than the MIC indicates that ZnO -NPs can kill bacteria at higher concentrations. Also, treating the preformed biofilm with ZnO -np resulted in significant OD reduction.

ZnO -np treatment resulted in a significant reduction in the OD value of the preformed biofilms at a concentration of 64 $\mu\text{g/ml}$ for 2 h incubation. Also, a significant reduction was reported at lower concentrations for an extended time of incubation.

Overall, our results suggest that ZnO -np could inhibit the establishment and development of biofilm, also to remove pre-formed biofilm.

Some previous studies have shown the antibacterial activity of ZnO -np. Hosein Zadeh et al. have studied the antibacterial of ZnO -np with the average of 20 nm against some bacteria, the MIC for *P. aeruginosa* isolates was 156.25 $\mu\text{g/ml}$ [17].

Hassani et al. have studied the antibacterial and anti-biofilm effect of ZnO -np with the average of 20 nm against *P. aeruginosa* clinical isolates; they reported that MIC50 and MIC90 of their studied isolates were 150 $\mu\text{g/ml}$ and 175 $\mu\text{g/ml}$ [18].

Also, Hassani et al. reported that ZnO -np had an anti-biofilm effect at a concentration of 50 to 350 µg/ml. Also ZnO -np at a concentration of 100 to 350 µg/ml reduced pre-formed biofilm of *P. aeruginosa* [18].

Saadat et al. have studied the effect of ZnO -np with the size of 30–90 nm against *P. aeruginosa* and reported that the mean MIC of ZnO -np for the studied isolates was 300 µg/ml [19].

Pati et al showed that ZnO -np can disrupt bacterial cell membrane integrity, reduce cell surface hydrophobicity and down-regulate the transcription of oxidative stress-resistance genes in bacteria [20].

The toxicity of ZnO nanoparticles depends on concentration, bacterial species, and particle size [21].

We also assessed the relative expression of the genes regulating biofilm and other virulence factors production in ZnO-treated and untreated isolates using the $\Delta\Delta C_t$ method.

LasI/R and *rhII/R* are two principle QS systems that regulate virulence genes production in *Ps. aeruginosa*. *LasI* and *rhII* synthases are responsible for the production of C12-AHL and C4-AHL autoinducers, respectively. At a threshold concentration of autoinducers, C12-AHL binds with *lasR* and induces the expression of genes that control the production of elastase and proteases and also activates the *rhII/R* system. In addition, C4-AHL binds with *rhIR* controlling the expression of genes encoding the production of elastase, and pyocyanin. If *lasI/R* and *rhII/R* are interrupted, virulence factors will be inhibited [22].

Our study reported that the relative expression levels of quorum sensing genes: *lasR*, *rhII*, and *pqsR* were significantly reduced under ZnO sub-MIC treatment.

The fold change decrease in the expression of *lasR*, *rhII*, and *pqsR* genes were 10.4, 6.3 and 8.7 fold (P-value ≤ 0.0001) respectively.

Adhesion factors are crucial for the attachment of bacterial cells to the surfaces. In *P. aeruginosa* biofilms, adhesion factors such as lectins (*lecA* and *lecB*) play an important role in adhesion and biofilm formation.

In this study, ZnO nanoparticles significantly down-regulated *LecA* gene expression (P-value ≤ 0.0004) in biofilm-forming *P. aeruginosa* clinical isolates by 4.7 fold change.

The presence of exopolysaccharides is an essential characteristic of the *P. aeruginosa* biofilm, which contributes to resistance and biofilm architecture. Pseudomonas biofilms are composed of at least three types of polysaccharides: Psl, Pel, and alginate. In the present study, the *pelA* gene was down-regulated in the presence of ZnO -np by 5.7 fold change (P-value ≤ 0.0004) in biofilm-forming *P. aeruginosa* clinical isolates. In accordance with our data, Saleh et al. reported that ZnO-np had a significant decrease in the relative expression of QS-genes *lasI*, *lasR*, *rhII*, *rhIR*, *pqsA* and *pqsR*. Additionally, ZnO significantly decreased the pathogenesis of *P.aeruginosa* in vivo [12].

Similarly, it was proved by Lee et al that ZnO -np (< 50 nm) inhibits *P. aeruginosa* biofilm formation and virulence factor production, they reported that ZnO -np at 1 mm inhibited biofilm formation by more than 95% on polystyrene surface. Also, Lee et al. showed that ZnO- np treatment resulted in significant regulation to most of the virulence genes of *P. aeruginosa* that were studied by microarray and qRT-PCR [23].

García-Lara et al. previously studied the effect of ZnO- np on the virulence factors production of clinical and environmental *P.aeruginosa* strains; they reported that ZnO- np were able to inhibit most virulence factors of the majority of the strains [24].

5. Conclusion

The results of our study showed that ZnO- np is highly effective against biofilm-forming *P. aeruginosa* isolates. In addition, this study also demonstrates the promising use of ZnO- np as an anti-biofilm QS inhibitor and anti-virulence compound. More studies are needed especially on animal models. Also, the possible harmful effects of ZnO- np should be investigated.

Declarations

Ethical Approval

Written informed consents were obtained from all individuals. The study was approved by the Ethical Committee of Minia University, Faculty of Medicine.

Consent to publish

The contents of this manuscript has not been published or submitted for publication elsewhere. Authors declare no conflicts of interest.

Declaration of interest: Authors declare no conflicts of interest.

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Author contributions:

Wedad conceived the study, perform the practical part, performed the data analysis, and drafted the manuscript. Ebtisam conceived the study purchased the required materials, assisted in performing the practical part and writing the manuscript.

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Figures

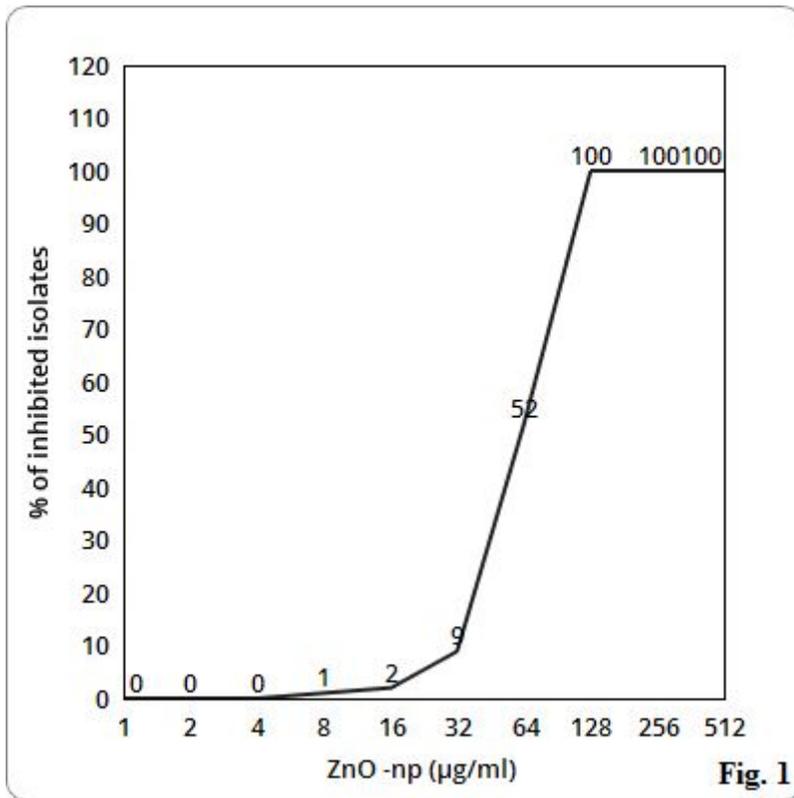


Fig. 1

Figure 1

MIC of ZnO -np: X axis represent serial dilutions of ZnO -np that was studied in this study. Y axis represent the percent of the inhibited bacterial isolates at each concentration. The percent of the inhibited isoaltes increased ith rising ZnO -np concentration.

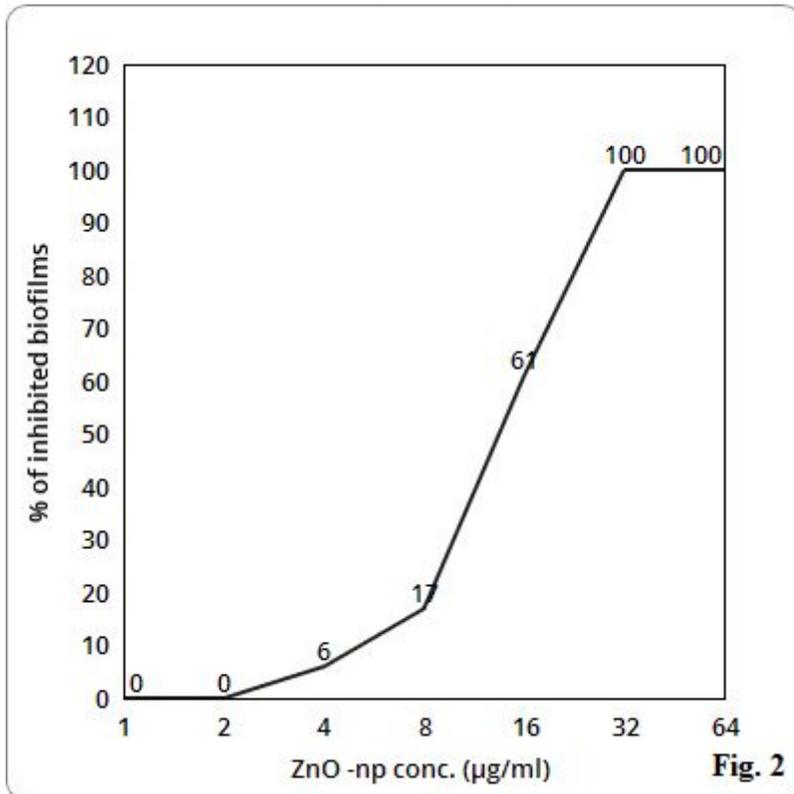


Fig. 2

Figure 2

BIC of ZnO -np: X axis represent serial dilutions of ZnO -np that was studied in this study. Y axis represent the percent of the inhibited bacterial biofilms at each concentration. The percent of the inhibited bacterial biofilms increased with rising ZnO -np concentration.

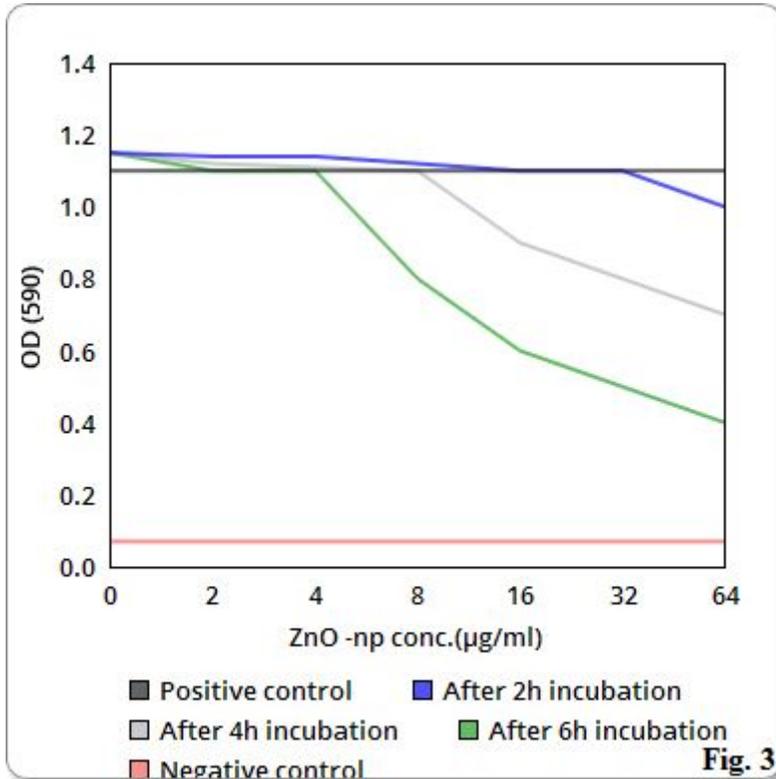


Figure 3

Anti-biofilm effect of ZnO -np: The figure represents the degree of reduction of OD values after 2, 4, 6hs incubation of preformed bacterial biofilms with ZnO -np at 37. Letter S represent significant reduction of OD values at certain concentration for certain time of incubation.

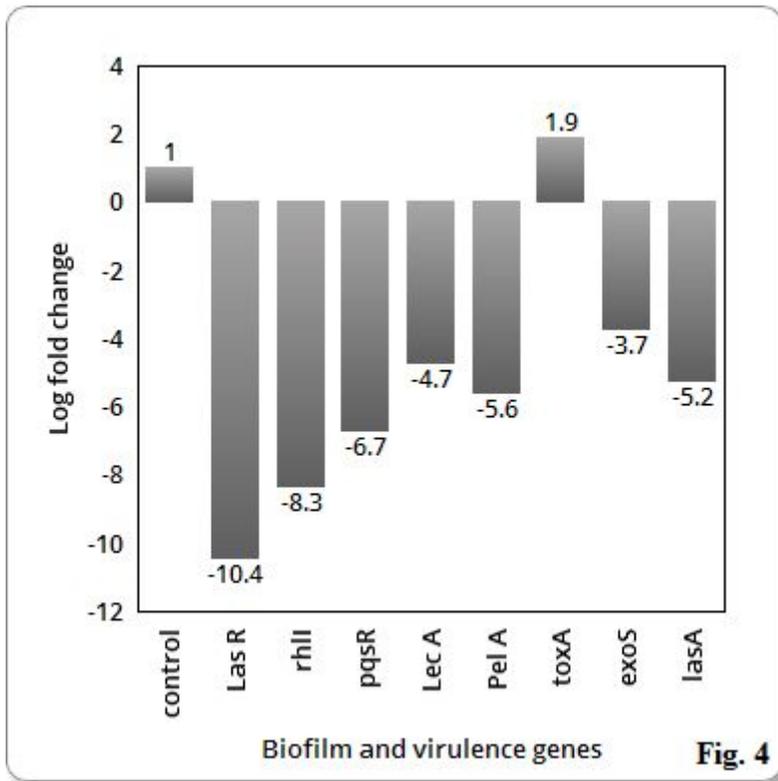


Figure 4

Effect of ZnO -np on relative genes expression: The figure represents the fold change decrease (-) or increase (+) in the virulence genes expression of *P. aeruginosa* clinical isolates after ZnO -np treatment.