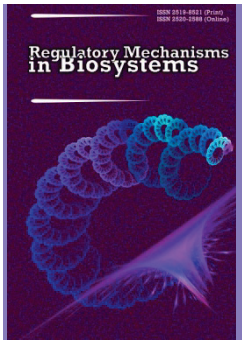

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Effect of nanoparticles on the expression of virulence and biofilm genes in *Klebsiella pneumoniae*

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Nanoparticles have received significant focus due to their extensive use in several industries and most notably as antimicrobial agents. The current work was dedicated to evaluating the role of different nanoparticles on the gene expression of defense mechanism genes in *Klebsiella pneumoniae*. Cultural characteristics and molecular identification identified 9/150 (6%) of the isolates from urine as *K. pneumoniae*. The 9 isolates were tested for their resistance to 12 antibiotics and the isolate, *K. pneumoniae* strain alamasfe, that showed the highest rate of resistance towards antibiotics, was submitted to NCBI under the accession number PQ126166. AgNPs, SiNPs, Clay NPs, and TiO_2NPs were tested for their effect on the growth of *K. pneumoniae*. The MIC of *K. pneumoniae* for AgNPs and SiNPs was 1000 µg/mL and for Clay NPs and TiO_2NPs it was 2000 µg/mL. *Klebsiella pneumoniae* strain alamasfe was exposed to sub MIC concentrations of AgNPs, Silicon NPs, Clay NPs, and TiO_2NPs to evaluate the effect of these nanoparticles on the expression of genes related to bacterial defense systems including ompC, ramA, soxS, luxS. Results showed that ramA was the most highly expressed gene among the genes studied in *K. pneumoniae* when exposed to AgNPs, Clay NPs, and TiO_2NPs. This gene was expressed 548.75 fold when exposed to TiO_2NPs followed by 319.57 fold when exposed to AgNPs, and 14.93 fold when exposed to clay NPs. The second most expressed gene tested was soxS. This gene was over expressed 76.11 fold when exposed to AgNPs and 131.6 fold when exposed to TiO_2NPs. Similar effects were noticed with the effect of nanoparticles on ompC but with lower fold changes. Down-regulation of ompC, ramA, soxS, and luxS genes was noticed in response to SiNPs which highlights the importance of SiNPs as effective antimicrobial agents that can impair bacterial defenses, resistance mechanisms, and communication pathways. The down-regulating of these critical genes may render bacteria more susceptible to environmental stresses and antimicrobial treatments, thereby reducing their pathogenicity and resistance.

Keywords: nanoparticles; gene expression; *Klebsiella pneumoniae*; MIC.

Introduction

Klebsiella pneumoniae, a Gram-negative bacterium with a protective capsule, is responsible for causing several diseases such as pneumonia, urinary tract infections, bacteremia, meningitis, and liver abscesses. Individuals that are at risk for obtaining *K. pneumoniae* infections include newborns, the elderly, and those with weakened immune systems. Nevertheless, the bacterium is also accountable for an increasing number of diseases that are contracted inside the population. *Klebsiella pneumoniae* is present in the environment, including soil and shallow waters, as well as on non-living surfaces such as medical instruments. *Klebsiella pneumoniae*, a bacterium that colonizes human mucosal surfaces, has significantly increased in prevalence over the past decade, leading to the need for a deeper understanding of its pathophysiology, as it has been found to invade other tissues from these sites (Fang et al., 2000; Guerra et al., 2022).

Biofilms are communities of bacteria embedded in an extracellular matrix, used by *K. pneumoniae* as a virulence factor. They provide increased resistance to external stresses and antimicrobial agents, allowing microbes to attach to both living and non-living surfaces and protect them from the effects of antimicrobial agents (Guerra et al., 2022). In addition to biofilm formation, *K. pneumoniae* has the ability to produce several other virulence factors, including the capsule, endotoxin, siderophore, and adhesins, which are essential for its disease-causing process (Riwu et al., 2022).

Nanoparticles are biomaterials that have diameters ranging from 1 to 100 nm. Nanoparticles have received significant focus due to their extensive use in several industries such as agriculture, pharmaceuticals, consumer items, transportation, energy, cosmetics, and most notably as antimicrobial agents. Currently, they are considered to be feasible alternatives or additions to the antimicrobials that are already available. The behavior and function of these nanoparticles mostly rely on their chemical composition, shape, and size (Mba & Nweze, 2021). Silver, along with copper and zinc, have traditionally been utilized as potential antibacterial agents. The antibacterial efficacy of silver might vary based on its chemical composition. Metallic forms exhibit a continual release of a tiny quantity of ions, resulting in a slow-acting effect, whereas the ionic form demonstrates higher efficiency (Pareek et al., 2021). Zinc oxide nanoparticles have been shown to have anti-virulence effects on pathogenic bacteria through increasing the cellular hydrophilicity (Lee et al., 2014). In addition, inhibitory effects of quorum sensing, pyocyanin, and biofilm formation was noticed in *Pseudomonas aeruginosa* when exposed to zinc oxide nanoparticles suggesting that they possess a wide range of activity and could potentially serve as an alternative treatment for P. aeruginosa infections (García-Lara et al., 2015). Nano-titanium has been suggested as a substitute antibacterial agent for antibiotic-resistant microorganisms. Numerous studies have investigated the antibacterial properties of TiO_2 NPs and have shown that they possess a strong ability to eliminate bacteria, either on their own or when used in conjunction with antimicrobials (Ammendolia & De Berardis, 2022).

Many studies have focused on the effect of nanoparticles on the growth and biofilm formation of bacteria, however, very few studies have

focused on the effect of these nanoparticles on the expression of genes in *K. pneumoniae*. Therefore, this study was dedicated to measuring the effect of four different nanoparticles on the expression of biofilm and virulence related genes in *K. pneumoniae*.

Materials and methods

Sample collection and isolation of K. pneumoniae. Urine samples were collected from 150 patients suffering from UTI from different hospitals in Mosul city. Samples were streaked on MacConkey agar plates and incubated 37 °C for 24 hours. Mucoid colonies that fermented lactose were selected for further identification via 16S rRNA gene sequencing.

Molecular identification of K. pneumoniae. The genomic DNA of suspected *K. pneumoniae* isolates was obtained utilizing the genomic DNA isolation kit provided by Geneaid (Taiwan) and following the recommended steps by the manufacturing company. PCR was conducted in a 20 µL reaction volume using GoTaq G2 Green Master Mix supplied by Promega (USA). The whole area of the 16S rRNA gene was amplified using the universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1522R (5' AAGGAGGTGATCCARCCGCA 3'), as recommended by Ibrahim & Faisal (2024). The concentration of the primers was 1 µM, and the DNA template was added up to 100ng as illustrated in the guidelines provided by the manufacturer. The PCR protocol for amplifying the 16S rRNA gene was used as mentioned elsewhere (Khaleel et al., 2023). The PCR products that specifically target the 16S rRNA gene were purified and sent to the sequencing company Psomagene (USA) for the purpose of sequencing. The acquired sequences were analyzed for similarity with previously published genes in GenBank using the BLAST program at NCBI (Abdulrazzaq & Faisal, 2022).

Antibiotic susceptibility. Klebsiella isolates were tested for their resistance to 12 antibiotics including: ceftazidime (30 µg/mL), cefepime (30 g/mL), meropenem (10 µg/mL), piperacillin/tazobactam (100 µg/mL), cefotaxime (30 µg/mL), ciprofloxacin (10 µg/mL), gentamicin (20 µg/mL), ampicillin (50 µg/mL), chloramphenicol (25 µg/mL), amikacin (30 µg/mL), tetracycline (15 µg/mL), and doxycycline (30 µg/mL) using the disk diffusion method (Jorgensen & Turnidge, 2015).

Detection of MIC for Nanoparticles. Seven concentrations of nanoparticles were tested for their effect against *K. pneumoniae*. Double dilution concentrations were prepared including the concentrations 4000, 2000, 1000, 500, 250, 125, and 62.5 µg/mL. Dilutions were made in 2 mL of nutrient broth and 50 µL of the bacterial suspension at an optical density equivalent to the fifth tube of McFarland was added to each tube and incubated at 37 oC for 24 hours. The tube with the least concentration that prevented growth was marked as the minimum inhibitory concentration (Faisal & Younis, 2024).

Quantitative PCR experiment. Klebsiella pneumoniae isolate was cultivated in LB broth at 37 °C for 18–20 hours with shaking for 180 rpm. Subsequently, 1.5 mL of the culture was centrifuged at 4 °C for 10 minutes at 8000 rpm to collect the intact cells. The extraction of RNA was performed using the TransZol Up Plus RNA Kit from TransGen Biotech (China). Quantification of RNA concentrations and purities was performed using a NanoDrop spectrophotometer (BioDrop/UK). A value of approximately 2.0 was considered to indicate RNA of relatively high purity. The expression of ompC, ramA, saxS, and luxS was quantified by a two-step reverse transcriptase (RT)-PCR experimental procedure. Converting the RNA transcripts into complementary DNA (cDNA) was performed using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix/TransGen Biotech/China kit, which helped in purifying the samples from residual DNA contamination. Next, cDNAs were amplified using qPCR primers that specifically targeted the genes (Table 1). Analysis of gene expression was conducted using the Step One Plus real-time PCR instrument manufactured by Analytikjena (Germany). The 2−ΔΔCT equation was employed to determine the relative expression levels of genes (Faisal & Younis, 2024).

Results

According to cultural characteristics and molecular identification only 9 isolates out of 150 isolates (6%) were identified as *K. pneumoniae*. The 9 isolates were tested for their resistance to 12 antibiotics and the isolate, *K. pneumoniae* strain ALAMASFE, that showed the highest rate of resistance towards antibiotics was submitted to NCBI under the accession number PQ126166 and used for further work.

AgNPs, Silicon NPs, Clay NPs, and TiO2NPs were tested for their effect on the growth of *K. pneumoniae*. The MIC of *K. pneumoniae* for AgNPs and Silicon NPs was 1000µg/mL and for Clay NPs and TiO_2NPs was 2000 µg/mL. The results indicated that ramA exhibited the highest level of expression among the genes examined in *K. pneumoniae* under exposure to AgNPs, Clay NPs, and TiO2NPs. Figures 1, 3, and 4 demonstrate that this gene exhibited a fold increase of 548.75 when exposed to TiO_2NPs, 319.57 when exposed to AgNPs, and 14.93 when exposed to clay NPs, in comparison to the housekeeping gene.

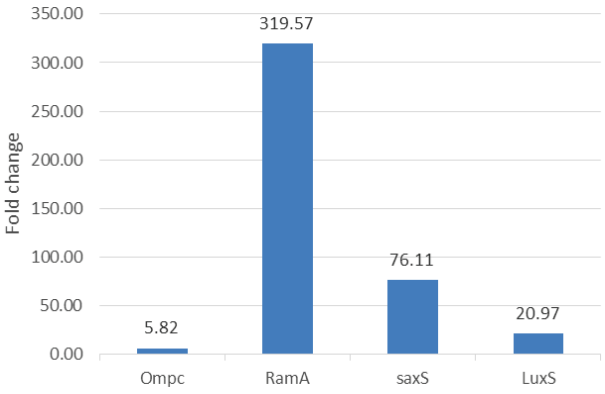

Fig. 1. Effect of Sub-MIC concentration of AgNPs on the expression of bacterial defense genes in *K. pneumoniae*: the gene 16S rRNA was used as the house-keeping gene and was set to 1

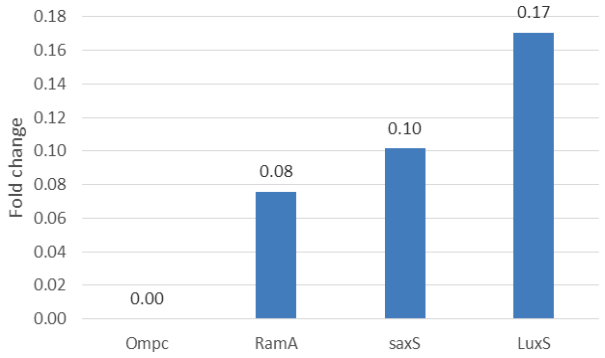

Fig. 2. Effect of Sub-MIC concentration of SiNPs on the expression of bacterial defense genes in *K. pneumoniae*: the gene 16S rRNA was used as the house-keeping gene and was set to 1

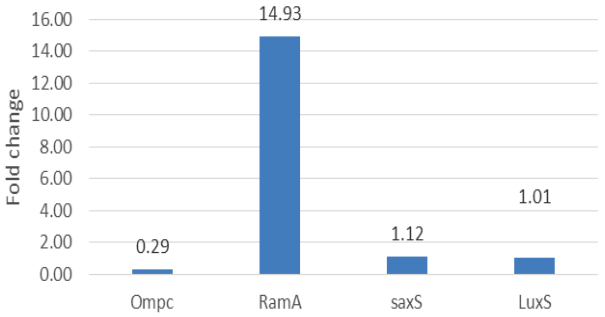

Fig. 3. Effect of Sub-MIC concentration of clayNPs on the expression of bacterial defense genes in *K. pneumoniae*: the gene 16S rRNA was used as the house-keeping gene and was set to 1

Our results also show that the second most expressed gene tested was soxS. This gene was over expressed 76.11 fold when exposed to AgNPs (Fig. 1) and 131.6 fold when exposed to TiO_2NPs (Fig. 4). Similar effects were noticed with the effect of nanoparticles on ompC but with lower fold

changes. SiNPs induced 5.82 fold with AgNPs and 5.28 fold in regards to TiO_2NPs as shown in Figure 1 and 4, respectively.

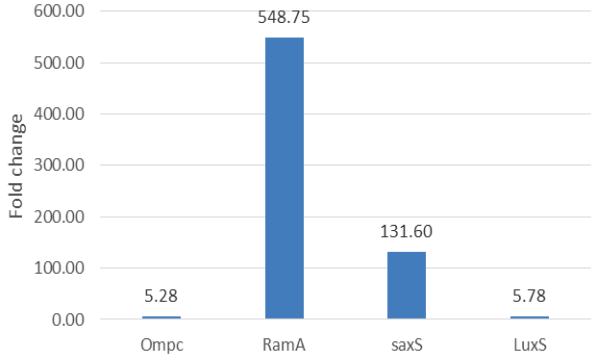

Fig. 4. Effect of Sub-MIC concentration of TiO_2NPs on the expression of bacterial defense genes in *K. pneumoniae*: the gene 16S rRNA was used as the house-keeping gene and was set to 1

Results obtained from the effect of SiNPs on the expression of the studied genes were different from results obtained from AgNPs, clay NPs, and TiO_2NPs. The genes tested were down-regulated when exposed to SiNPs (Fig. 2).

Discussion

Klebsiella pneumoniae strain alamasfe exposed to sub MIC concentrations of AgNPs, silicon NPs, Clay NPs, and TiO_2NPs to evaluate the effect of these nanoparticles on the expression of genes related to bacterial defense systems including an outer membrane porin protein ompC (Pareek et al., 2021), the intrinsic regulator ramA involved in antimicrobial challenge response (De Majumdar et al., 2015), the bacterial defense regulator soxS (Pareek et al., 2021), and the quorum sensing regulator for biofilm development luxS (Shafiei et al., 2021).

Pathogen strains of *K. pneumoniae* are of clinical significance and a common etiological agent of urinary tract infections acquired in hospitals and communities. The evolving resistance of this pathogen is resulting in restricted therapy alternatives (Caneiras et al., 2019). The incidence of *K. pneumoniae* in 6% of urine samples collected in this study is within the expected range observed worldwide. This urine pathogen is the second causative agent detected in UTI after *Escherichia coli* (Vachvanichsanong, et al., 2021). This opportunistic pathogen was found by Karampatakis et al. (2023) to be accountable for around 10% of bacterial infections acquired in healthcare settings.

Nanoparticles, particularly silver nanoparticles (AgNPs), have shown promising potential in antibacterial interventions, particularly against *Klebsiella* species. Factors such as size, shape, and coating determine the minimum inhibitory concentration (MIC) for AgNPs against different bacteria (Menichetti et al., 2023). Silver nanoparticles have demonstrated potent inhibition of over 650 types of harmful bacteria, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, dermatophytes, and other fungi. The prevailing belief is that silver nanoparticles liberate silver ions and stimulate the generation of reactive oxygen species (ROS). Further investigations have shown that silver nanoparticles possess inherent antibacterial properties and collaborate with the liberation of silver ions to exert their effects. The primary antibacterial mechanisms of silver nanoparticles, as now understood, mostly include modulating the bacterial habitat, disrupting cell membranes, and impeding DNA replication (Yin et al., 2020). The function of clay nanoparticles as an antibacterial is to adjust the pH and oxidation state of water to certain conditions that enhance the solubility of Fe^{2+} ions. The chemical analysis of *E. coli* cell death caused by aqueous leachates of an antibacterial clay reveals increased intracellular levels of iron (Fe) and phosphorus (P) compared to the control group. The cellular metabolism of phosphorus provides evidence for the regulatory function of polyphosphate or phospholipids in the control of Fe^{2+}. Studies have shown that extracellular activities do not result in cell death. Instead, Fe^{2+} molecules overpower outer membrane regulatory proteins and undergo oxidation upon entering the cell, resulting in the formation of Fe^{3+} ions and the generation of deadly hydroxyl radicals (Williams et al., 2011). On the other hand, titanium dioxide nanoparticles (TiO-NPs) are known for their photocatalytic properties and can be used for antibacterial applications. A study by Zhang et al. (2014) demonstrated that the MIC for TiO_2NPs against bacteria, including *Klebsiella* spp., typically falls between 500 and 1000 μg/mL, depending on the specific investigation and bacterial strain. This antibacterial effect is primarily due to the production of reactive oxygen species (ROS) when exposed to light, leading to cell damage and growth inhibition (Zhang et al., 2014).

In the present study, ramA was the most rapid overexpressed gene among them. ramA, a transcriptional activator in many members of the Enterobacteriaceae family, is an important regulator of bacterial responses to antimicrobial stress. While expression of ramA results in the increased production of efflux pumps, it may also regulate other MDR genes. Bacteria when exposed to nanoparticles (NPs) undergo severe stress and follow the adaptive process by over-expressing ramA gene. AgNPs, SiNPs, and TiO2NPs trigger up-regulation of ramA (Duval & Lister, 2013; Holden & Webber, 2020). Overexpression of ramA probably activates the expression of the pumps that eject harmful nanoparticles and their products generated from their exposure. Differential expression of ramA between nanoparticles may explain the difference in their toxic effect between bacteria (De Majumdar et al., 2015).

Overexpression of the soxS followed a pattern similar to ramA expression. soxRS in many microorganisms such as *K. pneumoniae* is activated in response to oxidative stress by the up(Outs) regulated soxS gene (a central regulator of the soxRS regulon). soxS is a XylS/AraC family member encoding a transcriptional activator. The soxR-dependent induction of soxS leads to expression of a number of genes whose products function in large part as antioxidants, detoxification enzymes, efflux pumps for redox-active substrates, membrane changes, and protection of DNA. This process preserves the bacteria from the oxidative stress mediated by overproduction of intracellular reactive oxygen species (ROS) in response to some nanoparticles (Anes et al., 2021).

The ompC gene codes for a porin protein, which regulates the permeability of the bacterial cell envelope and it forms a channel pathway through small molecules to pass as well as being able to serve as a pore for the entrance of drugs or antibiotics. Some bacteria express this protein to resist antimicrobials including antibiotics that would otherwise be lethal. Our results suggests that this porin may be involved in excluding both AgNPs and TiO_2NPs while not involved in the exclusion of clay NPs and SiNPs. The up-regulation of ompC with AgNPs and TiO_2NPs suggests that *K. pneumoniae* is using ompC to restore membrane function and maintain homeostasis. A study by Morones-Ramirez et al. (2013) showed that the expression of both soxS and ompC is increased in reaction to exposure to nanoparticles. When *E. coli* was exposed to silver nanoparticles (AgNPs), the expression of soxS gene was markedly increased, suggesting the activation of the oxidative stress response.

In contrast to this, we observed a distinct down- regulation of all investigated genes in response to SiNPs. SiNPs down-regulate ramA and soxS in bacteria which indicates that SiNPs reduce the expression of bacterial resistance mutators and make them more susceptible to antimicrobial agents. The possible mechanism for this suppression is through SiNPs interference in some signaling pathways or cellular structures which normally lead to ramA activation. Likewise, they also found down-regulation of soxS indicating that SiNPs might not cause similar degree of oxidative stress as other nanoparticles or may affect the pathway upstream which induce soxS for stress response (Varela et al. 2023).

Additionally, the down-regulation of luxS upon exposure to SiNPs indicates that these nanoparticles may interfere with quorum sensing systems, thereby compromising bacterial communication and coordination. These processes are critical for activities such as biofilm formation and the synthesis of virulence factors. Disruption of quorum sensing could weaken the bacteria's ability to form infections or resist antimicrobial treatments (Niazy, 2021; Faisal & Al-Shiti, 2023).

Conclusion

Nanoparticles are widely employed in numerous industries, particularly as antimicrobials, and have received attention. The current study loo-

ked at how different nanoparticles altered defense mechanism gene expression in *K. pneumoniae*. Sub-MIC concentrations of AgNPs, SiNPs, Clay NPs, and TiO_2NPs had varying effects on the expression of genes involved in bacterial defense systems, including ompC, ramA, soxS, and luxS. Overexpression of ramA in response to nanoparticle exposure demonstrates the complicated interaction between bacteria and antimicrobials. Antimicrobial nanoparticles may induce antibiotic resistance, thus they must be thoroughly tested. soxS defends against oxidative stress, while OmpC maintains membrane integrity, highlighting the dynamic nature of bacterial stress responses. SiNPs inhibited ompC, ramA, soxS, and luxS, proving their effectiveness as antimicrobials capable of disrupting bacterial defenses, resistance mechanisms, and communication routes. Down-regulating these genes may increase bacteria's sensitivity to environmental stresses and antimicrobials, reducing their pathogenicity and resistance.

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