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Nanoparticles as Promising Anti-biofilm and Antibacterial agents of Multidrug-Resistant *Klebsiella pneumoniae*: Molecular Study and Future Prospects

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Abstract :

The rise of multidrug-resistant (MDR) *Klebsiella pneumoniae*, especially its capability to biofilm formation, poses a substantial problem in managing healthcare-associated illnesses. Biofilm production augments bacterial survival, provides protection against immunological responses, and complicates antibiotic treatment. This study sought to assess the efficacy of metal oxide nanoparticles—namely zirconium oxide (ZrO_2), zinc oxide (ZnO), and cerium oxide (CeO_2)—as innovative anti-biofilm and antibacterial agents against multidrug-resistant *K. pneumoniae*. Quorum-sensing mechanisms regulate the virulence characteristics and biofilm development of bacteria. Quorum-sensing systems often regulate antimicrobial production; hence, targeting these systems with metal-based or combination nanoparticles may serve as potent antibacterial therapies due to their distinct physicochemical and therapeutic attributes. The research examines the molecular processes by which these nanoparticles impede biofilm development, alter bacterial virulence factors, and improve antibiotic penetration into biofilms. The research seeks to evaluate the effectiveness of these nanoparticles against the *mrkA*, *mrkD*, and *luxS* genes associated with biofilm development. The study aims to assess the efficacy of these nanoparticles in diminishing bacterial proliferation and biofilm integrity, as well as in targeting virulence-associated genes, using biofilm tests and gene expression studies. This research seeks to advance alternative treatment options for addressing chronic *K. pneumoniae* infections, emphasizing optimizing nanoparticle-based medicines for clinical use.

Key words: *Klebsiella pneumoniae*, Nanoparticles, Antibiofilm, Real-Time PCR, Gene expression, Quorum sensing, *mrkA, mrkD, luxS*.

الجسيمات النانوية عوامل واعدة ضد الاغشية الحيوية لبكتريا الكليبسيلا الرئوية متعددة المقاومة للمضادات الحيوية: دراسة جزيئية وافاق مستقبلية

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مستخلص:

إن ارتفاع معدلات مقاومة بكتيريا **الكليبسيلا الرئوية** تجاه المضادات الحيوية، بالإضافة إلى قدرتها العالية على إنتاج الأغشية الحيوية، يمثل مشكلة صحية عالمية متزايدة. فبفضل قدرتها على تكوين الأغشية الحيوية، تتمكن هذه البكتيريا من تعزيز بقائها وحمايتها من الاستجابة المناعية للكائن الحي، مما يعقد علاجها باستخدام المضادات الحيوية. بناءً على ذلك، سعت هذه الدراسة إلى تقييم فعالية الجسيمات النانوية مثل **أوكسيد الزركونيوم**، **أوكسيد الزنك**، و**أوكسيد السيريوم** كعوامل مبتكرة لمكافحة بكتيريا **الكليبسيلا الرئوية** وأغشيتها الحيوية. يعد نظام **تحسس النصاب** من أبرز الأنظمة المسؤولة عن تطوير مقاومة وضراوة البكتيريا وتنظيم تكوين الأغشية الحيوية. لذلك، فإن استهداف هذه الأنظمة باستخدام الجسيمات النانوية، سواء بشكل فردي أو مشترك، يوفر طرقًا علاجية فعّالة نظرًا لخصائصها الفيزيائية والكيميائية المتميزة، حيث تساعد في إعاقة تطوير الأغشية الحيوية، تسهيل اختراق المضادات الحيوية، وتعديل عوامل الضراوة من خلال تأثيرها على الجينات المرتبطة بهذه العوامل. هدفت دراستنا إلى تقييم فعالية الجسيمات النانوية في تقليل تكاثر البكتيريا وتدمير الأغشية الحيوية، بالإضافة إلى استهداف الجينات المرتبطة ببعض عوامل الضراوة عبر دراسة التعبير الجيني. كما يسعى هذا البحث إلى تقديم خيارات علاجية بديلة لمعالجة العدوى ببكتيريا **الكليبسيلا الرئوية** وتحسين الأدوية المعتمدة على الجسيمات النانوية لاستخدامها في المجالات الطبية.

الكلمات المفتاحية: الكليبسيلا الرئوية، الجسيمات النانوية، مضادات الأغشية الحيوية، التعبير الجيني، تحسس النصاب .

Introduction

Klebsiella pneumoniae is one of the opportunistic Gram-negative bacteria that cause a large portion of complicated UTIs and nosocomial infections around the world **(1)**. The complexity of treating bacterial infections leads to elevated morbidity and mortality **(2)**. The appearance of MDR strains producing extended-spectrum β-lactamases (ESBLs) and carbapenemases makes treatment difficult. Its virulence is polygenic and determined by a polysaccharide capsule, type 1 and type 3 fimbriae, siderophores, and a powerful biofilm formation, which apparently are important for fulminant persistence on urinary catheters and epithelial surfaces **(3)**. Biofilm development protects *K. pneumoniae* from the host environment (e.g., medical devices and host tissues). The EPS matrix shield is against the desiccation, host defenses, and antibiotic exposure, leading to chronic and recurrent infections **(4)**. This sanctuary makes it possible for the bacterial cells to withstand anti-microbial substances by diminishing the penetration. and metabolic change, and hence the cells became more tolerant than planktonic cells. Biofilms are strongly associated with *K. pneumoniae* virulence. They aid in adherence to the surfaces of the host, establishing infection and avoidance of the immune response of the host **(5)**. The biofilm mode of growth elicits expression of virulence factors, including capsular polysaccharides, fimbriae and siderophores, involved in pathogen adherence that is important for the establishment of infection and iron acquisition. Moreover, biofilm-formed infections can cause significant mortality and morbidity by being hard to be eradicated. Biofilm development on urinary catheters and endotracheal tubes, such as device-associated infections, increase the complexity of therapy **(6)**. Biofilm-related infections are typically not amenable to treatment and require lengthy courses of antibiotic therapy or explantation. Clarification of the process of biofilm formation by *K. pneumoniae* is important for the development of new therapeutic strategies. Current research focuses on: Anti-biofilm agents that inhibit initial adhesion or degrade the biofilm matrix, Quorum sensing inhibitors to prevent biofilm matu-

ration and, Innovative drug delivery systems to improve antibiotic penetration into biofilms. These strategies aim to overcome biofilm-associated resistance and reduce infection severity **(7)**. Nanoparticles, especially metal oxides such as CeO_2, ZnO, and ZrO_2, are promising alternatives due to their multifaceted bactericidal mechanisms, including ROS generation and biofilm inhibition **(8)**. This study aims to investigate the molecular mechanisms by which nanoparticles exert anti-biofilm and antibacterial effects against multi-drug-resistant *K. pneumoniae*. It seeks to evaluate the efficacy of selected metal oxide nanoparticles in disrupting biofilm formation, inhibiting key virulence factors, and overcoming antibiotic resistance. Furthermore, the study intends to explore the potential of these nanoparticles as innovative therapeutic agents, addressing challenges related to toxicity and targeted delivery, thereby advancing future clinical applications for combating persistent and resistant *K. pneumoniae* infections.

Materials and Methods

Study design and bacterial identification

The Research Ethics Committee (204-1223-2024) of the University of Anbar, College of Medicine/Microbiology department in Ramadi, Anbar governorate, Iraq, authorized this work. One hundred twenty-five samples of pathogenic bacteria isolates were collected from urine samples of patients admitted to Ramadi Teaching Hospital between 1st November 2024 and 15th January 2025. The inclusion criteria were patients of all age groups (over one month) from both genders who were not undergoing antibiotic therapy and had been medically diagnosed with a UTIs, then sent by physicians for urine culture analysis in the laboratory. Fifty isolates of *K. pneumoniae* were identified using routine bacteriological and biochemical testing, then verified by the Vitek 2 System.

Antimicrobial Susceptibility Testing

Fifteen antibiotic disks from HIMEDIA Limited, India, were utilized, comprising Nitrofurantoin, Gemifloxacin, Amikacin, Levofloxacin, Gatifloxacin, Cefazolin, Ceftriaxone, Trimethoprim, Ciprofloxacin, Gentamicin, Minocycline, Fosfomycin, Nalidixic acid, Meropenem, and Cefixime. Method in accordance with 35th Edition (CLSI),

2025 **(9)**. *K. pneumoniae* isolates were classified as Multi-Drug Resistant (MDR) if they exhibited resistance to at least one drug from three or more antibiotic classes **(10)**.

Experimental Design

Nanoparticles.

All nanoparticles (ZrO_2, ZnO, CeO_2) are procured from *Nanoshel* Company, USA, in powdered form (Purity: 99.9%, APS: 20 nm; Purity: 99.5%, APS: 10-30 nm; Purity: 99.99%, APS: 30 nm) correspondingly. Nanoparticles were validated using Scanning Electron Microscope (SEM) analysis, as seen in figure **(1)**.

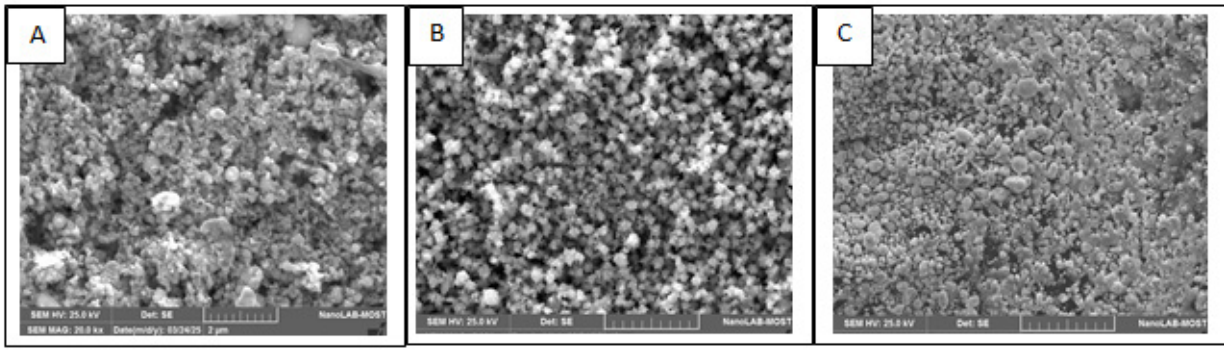

Figure (1): SEM images of the nanoparticles. **A- ZrNPs, B- ZnNPs, C- CeNPs**

Bacterial Culture and Treatment

Antimicrobial test and minimum inhibitory concentration (MIC) determination

At this step, 96-well plates were prepared with 100 µL serial dilutions (3.9, 7.8, 15.625, 31.25, 62.5, 125, 250, and 500 µg/mL) of ZrO2, ZnO, and CeO2. 100 µL of brain-heart infusion broth, with the same concentration of the examined compounds, was added to each well. Subsequently, utilizing the above estimations, compute the anticipated bacterial cell density (number of CFU of *K. pneumoniae*): A McFarland standard of 0.5, equivalent to 1.5×10^{8} cells/ml, was incubated at 37 C°. Subsequent to incubation, 30 µL of resazurin at a concentration of 0.15 mg/ml was introduced to each well. The wells were subsequently re-incubated for 24 hours prior to the reporting of the findings. Blank or negative controls consisted of the identical liquid medium containing the same quantity of the examined chemicals, devoid of microorganisms. Bacterial cultures that received no treatments acted as the positive con-

trol. A transition in hue from blue to pink indicated a reduction in resazurin and, consequently, bacterial growth. Each experiment was conducted thrice **(11)**. As previously described **(12)**, **(13)**, the microdilution checkerboard plates were prepared in triplicate with specific alterations to the *K. pneumoniae* strains, utilizing the interaction between the two treatments.

Biofilm Formation

The method employed to investigate biofilm formation by *K. pneumoniae* were as follows:

1. Inoculum Preparation: Overnight cultures of bacterial isolates were standardized to a 0.5 McFarland concentration in Tryptic Soy Broth (TSB) enriched with 1% glucose to enhance biofilm formation.

2. Microtiter Plate Assay: Inoculation entailed introducing 200 µL of bacterial suspension into each well of a 96-well microtiter plate. Biofilm development was encouraged by incubating the MTP at 37 C° for 24 hours. Following incubation, the wells were rinsed thrice with PBS to exclude non-adherent cells, and biofilms were fixed using 200 µL of 99% methanol.

3. Staining and Quantification: Crystal violet staining entailed applying 0.1% crystal violet to air-dried wells for 15 minutes, thereafter washing away the surplus pigment. The bound dye was dissolved in 33% acetic acid. Biofilm was assessed by measuring absorbance at 590 nm with a microplate reader. The biofilm strength was categorized based on optical density (OD) readings as follows: OD ≤ OD.c (OD.c of the negative control) = non-adherent; OD.c < OD ≤ (2 times OD.c) = weakly adherent; (2 times OD.c) < OD ≤ (4 times OD.c) = moderately adherent; and (4 times OD.c) < OD = strongly adherent **(14)**.

Resazurin's application in the microdilution checkerboard tests

In the microdilution checkerboard assays, resazurin was administered to each bacterial microtiter plate after a 24-hour incubation period. Color change was seen 4-hour later. This dye contact duration was selected based on the optimal results from the MIC tests. The formula utilized to calculate the fractional inhibitory concentration index (FICI) is as follows: FICI = (MICBcombi/MICBalone) + (MICAcombi/MICAalone). A FICI below 0.5

signifies a synergistic impact, a FICI between 0.5 and 4.0 denotes no interaction, and a FICI over 4.0 indicates antagonism **(15)**. Establish sub-inhibitory concentrations (sub-MIC) for each therapy to prevent bactericidal effects. The treatments were categorized into seven experimental groups as presented in **Table (1)**.

Table (1): *K. pneumoniae* treated with sub-MIC (1/2 MIC)

No.	Treatments
1	**Zr**NPs-treated
2	**Zn**NPs-treated
3	**Ce**NPs-treated
4	**Zr**NPs + **Zn**NPs
5	**Zr**NPs + **Ce**NPs
6	**Zn**NPs + **Ce**NPs
7	**Control** (untreated)

Antibiofilm evaluation (Microtiter plate assay)

Following the determination of the MIC, values of 1/2 MIC (sub-MIC) were employed to examine the antibiofilm efficacy of the metal nanoparticles. The antibiofilm activity of ZrO_2, ZnO, and CeO_2 Nanoparticles was assessed at sub-MIC concentrations alone and with combination. The test was conducted as previously outlined in the crystal violet assay. Wells containing NPs-free culture media served as the control **(16)**.

RNA Extraction & cDNA Synthesis

After 12 hours of treatment, collect bacterial cells, then isolate total RNA with TRIzol reagent. Measure RNA concentration and purity with a Thermo-Scientific Nano-Drop 8000 Microvolume UV-Vis Spectrophotometer. Reverse transcribe RNA into cDNA utilizing a simplified script, a single-step gDNA elimination process, and a cDNA synthesis super mix kit (Transgene-China).

Real-Time PCR

Design and validate primers for target genes **(*mrkA*, *mrkD*, *luxS*)** and ***16srRNA*** as a Reference gene table (2).

Conduct Real-Time PCR with SYBR Green and evaluate gene expression employing the Livak equation $2^{-\Delta\Delta Ct}$ **(17)**.

Statistical Analysis

Use SPSS statistical software. Perform one way ANOVA to compare gene expression levels between groups. Consider $p < 0.05$ as statistically significant.

Results

Collection and identification

Among 125 patients, 67.2 % was female (N=84) while male was 32.8 % (N=41). There was a statistically significant difference ($p < 0.05$) favored female, the results showed 50 isolates were found to be *K. pneumoniae*. The microscopical features are Gram-negative, non-motile, straight, bacilli-shaped, non-spore-forming, cultural features large colonies, mucoid texture, lactose ferment on MacConkey agar that showed pink colonies, while on Eosin methylene blue (EMB) pale pink mucoid colonies and Hi-Chrome™ UTI Agar colonies appear Purple to blue color **figure (2)**. Biochemical characterization Catalase, Voges-Proskauer, citrate utilization lactose, and glucose

Table (2): Primer sequence for RT-PCR analysis

Virulence factor	Genes		Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Reference
Housekeeping	*16srRNA*	F	AGTCTGCAACTCGACTCCATG	122	Design in this study
		R	CTTTGCAACCCACTCCCATG		
Biofilm formation	*mrkA*	F	GATGACGTAAGCAAACTGGGC	175	Design in this study
		R	TGTCACCCGGGATGATTTTGT		
Biofilm formation	*mrkD*	F	GGCGCAACGGTAAATATCGTC	168	Design in this study
		R	CTCCCAGTCGTAGGAGGTGTA		
Quorum sensing (QS)	*luxS*	F	TACCGAACCAGGAAGTGATGC	160	Design in this study
		R	GGCGTACCAATCAGGCTCATA		

ferment are positive, but oxidase, and indole, methyl red, are negative. Triple sugar iron (TSI) Yellow/Yellow (A/A), Gas +ve, H_2S – ve **(18), (19)**.

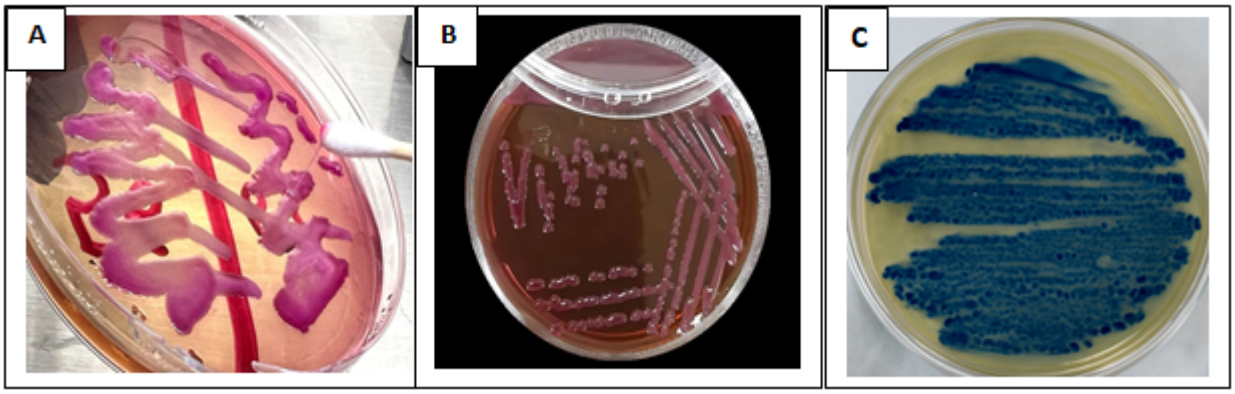

Figure (2): **A-** *K. pneumoniae* on MacConkey agar, **B-** *K. pneumoniae* on EMB, **C-** *K. pneumoniae* on UTI-Hi-Chrome agar.

The bacterial isolates diagnosis has been confirmed using the **VITEK2** system.

The Antibiotic Susceptibility Testing (AST)

In this study, antibiotics were used to establish a resistant pattern of *K. pneumoniae* to several broad-spectrum antibiotics associated with a high risk of infection. The results showed in figure **(3)** all isolate's resistance to Cefixime was around (100%), and approximately (98%), (98%), (94%) (and 90%) of them were highly resistant to Trimethoprim, Cefazoline, Ceftriaxone, and Nalidixic acid, respectively. Furthermore, around 86%, 84%, 82%, 76%, 74%, and 68 % of isolated *K. pneumoniae* were resistant to Gemifloxacin, Levofloxacin, Nitrofurantoin, Minocycline, Ciprofloxacin, and Fosfomycin, respectively. In addition, around 50% of this *K. pneumoniae* was resistant to Gatifloxacin and Gentamicin. In contrast, over 92% of *K. pneumoniae* were susceptible to Meropenem and (56%) to Amikacin.

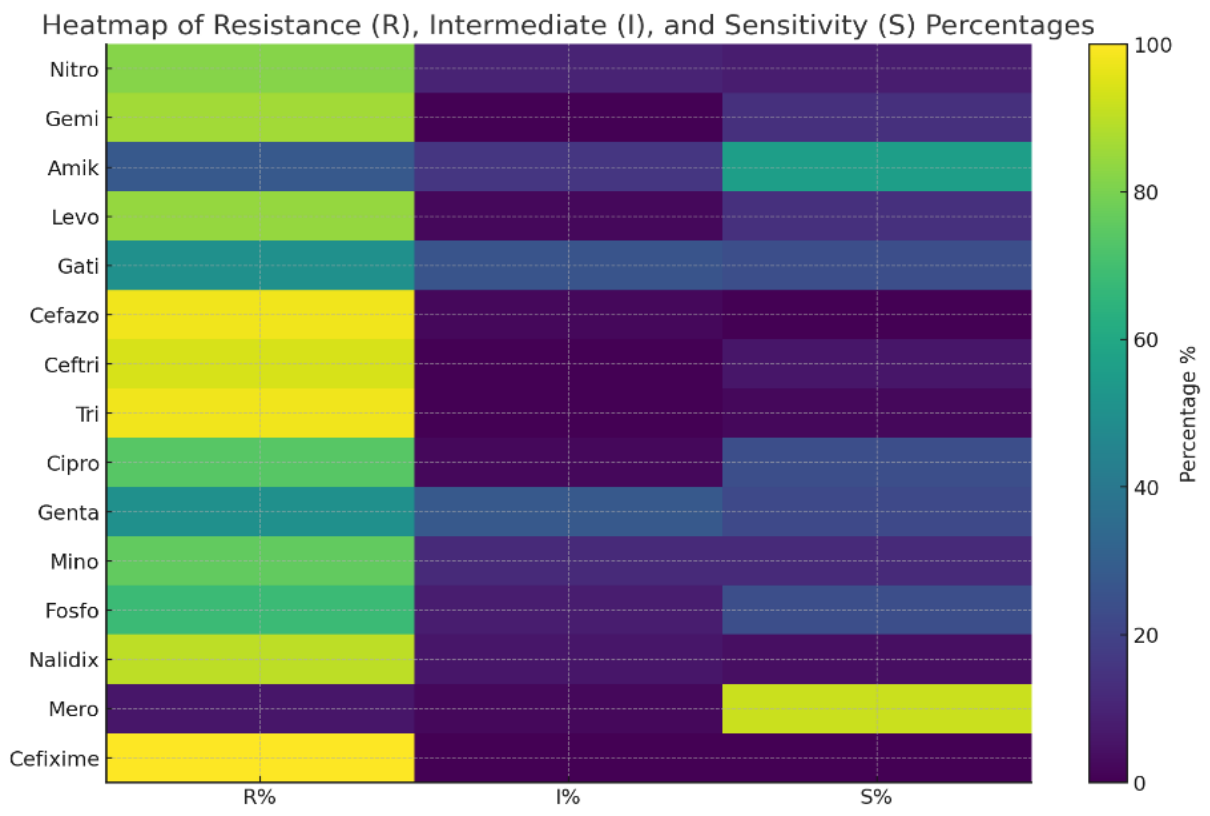

Figure (3). Percentage Distribution of Resistance (R), Intermediate (I), and Sensitivity (S) for Antibiotics against *Klebsiella pneumoniae* based on (CLSI) guidelines (2025).

Antimicrobial effect of Nanoparticles

The results showed minimum inhibitory concentration MICs of (ZrNPs, ZnNPs, and CeNPs,) at 125, 62.5, 125, µg/mL could inhibit the growth of the clinical isolates of *K. pneumoniae* respectively, whereases checkerboard results reveal in (table 3) the MIC of ZrNPs decreased to 15.625 µg/mL when mixed with ZnNPs or CeNPs. Both ZrNPs and ZnNPs are safe for biological use, but mixing them might create a better interface that lowers each nanoparticle's harmful effects while boosting their positive impacts in medical uses, like delivering drugs. Combined, these nanoparticles might interact in ways that enhance performance, such as improved antimicrobial activity, better material stability, or a more effective therapeutic effect. This study showed the best synergistic action between ZnNPs and ZrNPs by cal-

culating the value of Fractional inhibitory concentration FICI index equal to 0.25 Where it appeared that the value of FICI is less than 0.5 for *K. pneumoniae* and this indicates the synergistic action between these treatments, as shown in (table 3).

Table (3):
Combination between difference treatment against *K. pneumoniae*

	MIC alone (A)		MIC-Combination (A)		MIC alone (B)		MIC-Combination (B)		FIC (A)	FIC (B)	FICI	Conclusion
	ZrNPs	125	ZrNPs	15.625	ZnNPs	62.5	ZnNPs	7.812	0.125	0.125	**0.25**	Synergism
	ZrNPs	125	ZrNPs	15.625	CeNPs	125	CeNPs	62.5	0.125	0.5	**0.625**	Additive
	ZnNPs	62.5	ZnNPs	7.812	CeNPs	125	CeNPs	62.5	0.12499	0.5	**0.624992**	Additive

Biofilm formation of *K. pneumoniae* by microplate titer assay

The results of the microtiter experiment indicated that the isolates of *K. pneumoniae* demonstrated a significant potential for producing biofilm on plastic surfaces. The isolates were categorized into groups based on their biofilm formation capacities. Out of the 50 clinical bacterial isolates of *K. pneumoniae*, biofilm production was detected in 22 isolates (44%), indicating a high capacity to form biofilm on plastic surfaces (Strong). The remaining 28 isolates were classified as non-biofilm, weakly and, moderatly biofilm formation (4%), (22%), and (30%), respectively figure **(4)**.

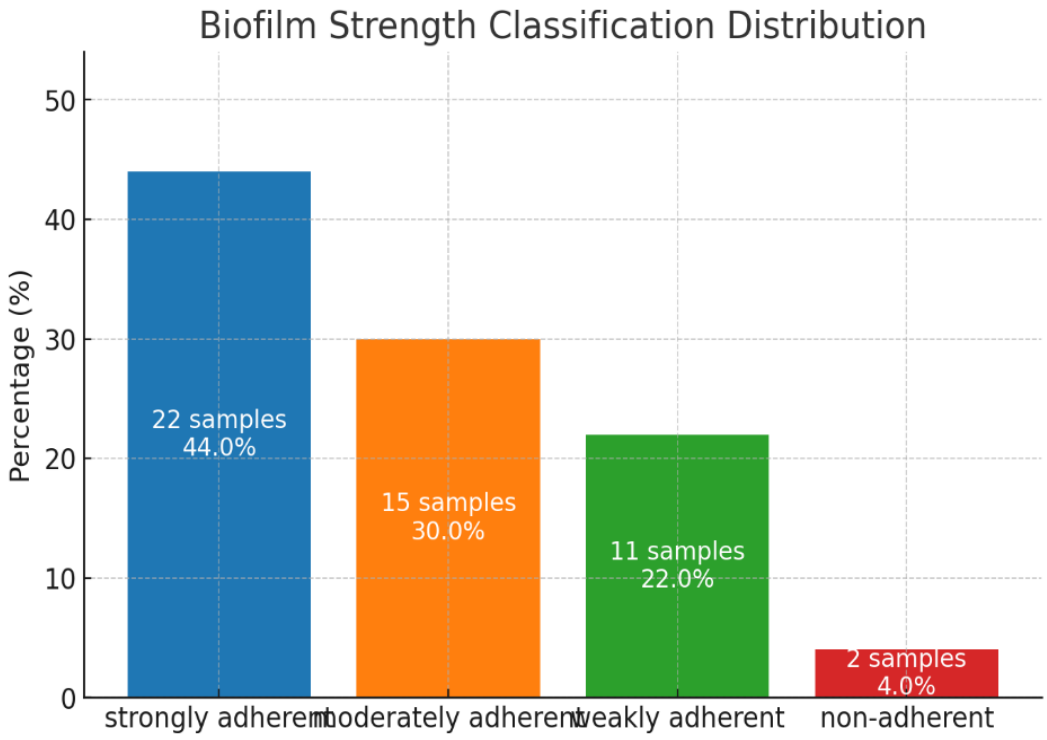

Figure (4):
Biofilm Production Strength across different groups.

Antibiofilm effect of Nanoparticles

Statistical analysis revealed significant differences at ($p < 0.05$) in biofilm development by *K. pneumoniae* after treatment with different nanoparticles figure **(5)**. The combined nanoparticle formulations, namely Zr+ZnNPs, Zr+CeNPs, and Zn+CeNPs, exhibited the most significant reductions in biofilm optical density (OD), with drops of 74%, 60%, and 56%, respectively, compared to the untreated control. Conversely, individual nanoparticle treatments had considerable inhibitory effects, with ZnNPs, CeNPs, and ZrNPs decreasing biofilm optical density by 45%, 27%, and 28%, respectively. The findings indicate that mixtures of nanoparticles demonstrate synergistic antibiofilm action, improving effectiveness beyond that of individual components. The findings highlight the potential efficacy of combination nanoparticle therapy in addressing *K. pneumoniae* biofilm-related resistance in clinical and pharmacological contexts.

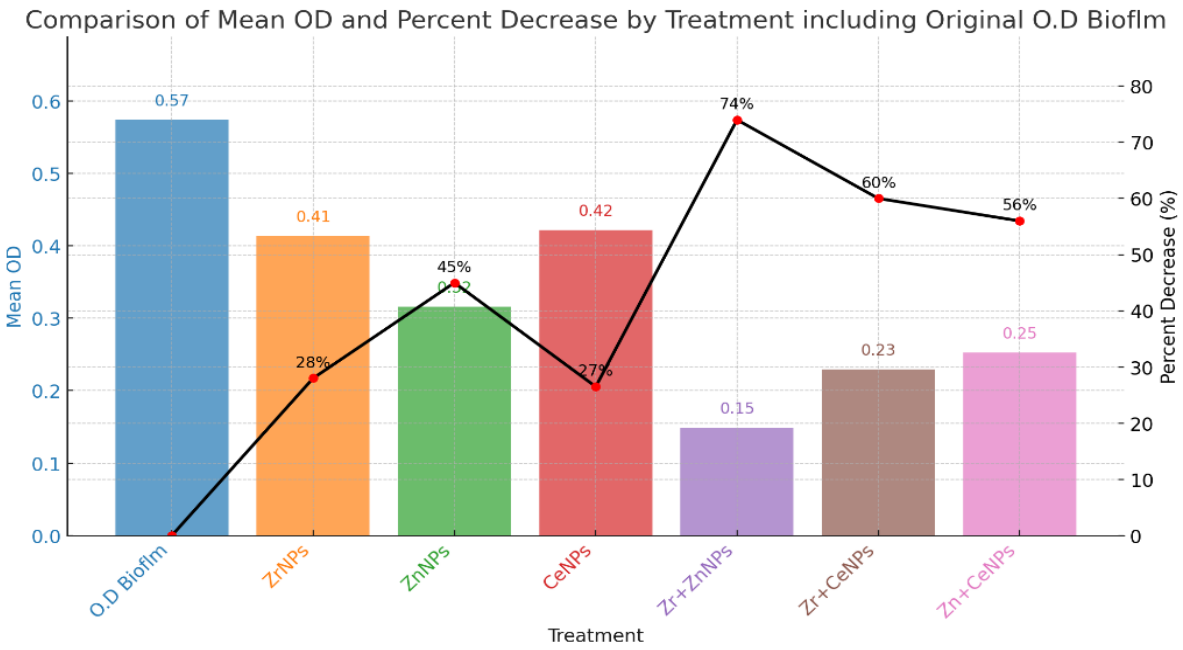

Figure (5).

Comparison of mean original OD biofilm and Percent decrease by treatment.

Effect of Treatments on Virulence Gene Expression

Extraction of RNA and cDNA synthesis

Ribosomal Nucleic Acid (RNA) was extracted from *K. pneumoniae* isolates before and after treatment with Sub-MIC (1/2 MIC) of different treatments (table 1), alone and in combination. All samples of RNA were extracted with a TRIzol™ Reagent purification kit. The concentration of RNA ranged between (142 – 157) ng/µl, and the purity fluctuated from 0.94 to 1.74.

Real-Time PCR (qPCR)

The highest biofilm-producing K. pneumoniae was selected in a two-step RT-PCR process was used to detect the effect of sub–MIC (1/2 MIC) of Metal Nanoparticles, alone and in combination on gene expression levels (fold changes) *of* ***mrkA***, ***mrkD***, **and** ***luxS*** compared to a reference gene *(16srRNA*), the amounts by which they influ-

ence gene expression were quantitated by SYBR green **figure (6)**. The results of this investigation showed down regulation of the gene expression levels when treated with all treatments alone or in all combinations as compared with untreated bacteria (control). The combination of Zr+ZnNPs and Zn+CeNPs simultaneously had the most effect on gene expression in all three target genes, with levels of expression between 0.12 and 0.24 compared to the control. This large downregulation shows that biofilm-related gene activity is strongly inhibited. Treatment with CeNPs alone produced a mild decrease in gene expression, with values between 0.25 and 0.29, indicating a less effective inhibitory effect. The Zr+CeNPs combination demonstrated a somewhat diminished impact, with expression levels remaining near control values, fluctuating between 0.47 and 0.85. The treatment with single nanoparticles of ZrNPs resulted in a moderate reduction in gene expression, with values ranging from 0.39 to 0.52. The ZnNPs treatment was ineffective in reducing gene expression and, for the *mrkA* gene, resulted in an enhanced expression level of 1.33, indicating a possible stimulatory impact rather than suppression.

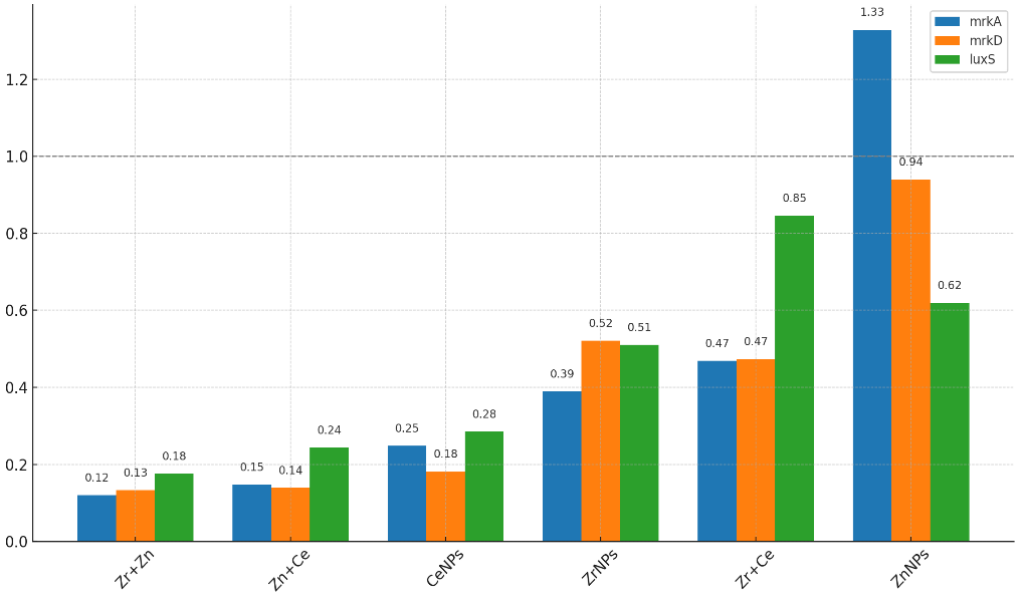

Figure (6): A gene expression (fold $2^{-\Delta\Delta CT}$) represented for *K. pneumoniae* by different treatments and for the expression levels of *mrkA*, *mrkD*, *and*, *luxS* genes. It is apparent from the chart that most treatments had down-regulation on the gene expression.

Discussion

This study offers important information regarding the antibacterial resistance profile, biofilm forming capability, and the possible therapeutic use of metal nanoparticles for *K. pneumoniae* infections. These findings correspond to modern studies on antimicrobial resistance and new antimicrobial methods, stressing the importance of developing alternative treatment approaches due to increasing cases of multi-drug-resistant infections. The high resistance rates observed in *K. pneumoniae* isolates to antibiotics such as Cefixime (100%), Trimethoprim (98%), and Ceftriaxone (94%) underscore the growing challenge of MDR infections. These findings are consistent with global trends reported by **(20)**, who identified *K. pneumoniae* as a priority pathogen due to its resistance to third-generation cephalosporins and carbapenems. The susceptibility of isolates to Meropenem (92%) and Amikacin (56%) suggests that these antibiotics remain viable options, albeit with caution, as resistance to carbapenems is increasingly documented **(21)**. The resistance patterns observed in this study emphasize the necessity for continuous surveillance and adherence to CLSI guidelines to guide empirical therapy. The capability of *K. pneumoniae* to obtain and spread antibiotic resistance genes renders antibiotic resistance in this pathogen a significant issue. Consequently, extensively drug-resistant (XDR) and multidrug-resistant (MDR) bacteria have evolved. Extended-spectrum beta-lactamases (ESBLs) and carbapenemases, which inactivate many beta-lactam antibiotics, exemplify resistance mechanisms **(22)**, **(23)**, **(24)**. *K. pneumoniae* is a significant pathogen in healthcare environments, recognized for its acquisition of antimicrobial resistance (AMR) via mobile genetic elements (MGEs), including plasmids, transposons, and integrons **(25)**, **(26)**, **(27)**. The observed reduction in *K. pneumoniae* biofilm formation following nanoparticle treatments can be attributed to multiple mechanisms supported by recent scientific evidence. Nanoparticles, especially metal oxides like zirconium (Zr), zinc (Zn), and cerium (Ce) oxides, exhibit strong antimicrobial characteristics through the generation of reactive oxygen species (ROS), disruption of bacterial cell membranes, and interference with quo-

rum sensing—key processes in biofilm development **(28)**, **(29)**. In this study, the MIC values of ZrNPs, ZnNPs, and CeNPs against *K. pneumoniae* isolates were 125 µg/mL, 62.5 µg/mL, and 125 µg/mL, respectively, which indicates their ability to inhibit bacterial growth effectively. The combination of nanoparticles further enhanced their antimicrobial activity, as indicated by the synergistic effect observed between ZrNPs and ZnNPs. In this study, the combined nanoparticles (Zr+ZnNPs, Zr+CeNPs, and Zn+CeNPs) showed significantly enhanced antibiofilm activity compared to individual nanoparticles, with reductions in biofilm optical density of 74%, 60%, and 56%, respectively. These findings further indicate that co-combined nanoparticles can synergistically inhibit biofilm formation, which is vital to improve the efficiency of antimicrobial therapies. This combined effect is due to the ability of nanoparticles to target multiple bacterial targets and cells, enhance biofilm matrix collapse, and facilitate bacterial cell death. Our study findings are in accordance with those of **(30)**, which shows that there is a synergistic antibiofilm activity against *P. aeruginosa*, when combining zinc oxide (ZnO) and silver (Ag) nanoparticles. This synergy increases the formation of ROS that results in efficient oxidative stress and membrane destruction, thus impacting the biofilm's integrity and the viability of bacteria. The combined nanoparticles treatment showed stronger antibiofilm activity, presumably because of the synergy in imposing oxidative stress and membrane destruction that exceed the level induced when using a single nanoparticle type **(31)**. This synergy may also improve nanoparticle penetration into the biofilm matrix, overcoming protective barriers **(32)**. In addition, genes associated with adhesion and EPS production (as part of biofilm organization and maturation) can also be repressed **(33)**, **(34)**. The fact that OD of biofilm was remarkably decreased indicates the disturbance of these two mechanisms and impairment of biofilm formation and bacterial survival. This multi-functional antibiofilm activity makes these nanoparticle combinations attractive therapeutic/prophylactic candidates for chronic infections and industrial biofouling by *K. pneumoniae*.

Conclusion

Nanoparticles especially in consort have high potential for controlling MDR-*Klebsiella pneumoniae* with biofilm resistance. The observed synergistic tendencies, particularly with the ZrNPs-ZnNPs combination, emphasize the applicability of nanoparticle treatments as a new strategy for solving infections caused by *K. pneumoniae*. These results further validate the growing body of evidence that nanoparticles are effective in the targeting of biofilm formation, a fundamental component of chronic and persistent diseases, and in the inhibition of bacteria growth. Additional studies will be required to better determine how they can be utilized for in vivo applications and further investigate their *in vivo* safety and therapeutic efficacy.

Limitations of the Study

This study focused exclusively on one species of bacteria. This constraint limits the applicability of the findings to other species that may have varied reactions to metal nanoparticles. In addition, the study involved patients from specific healthcare facilities in Al-Ramadi City, Iraq, which might not represent a diverse patient population.

Future Directions

1. ***In Vivo* Validation:** the efficacy of combined nanoparticle treatments in murine urinary tract infection (UTI) models, specifically using C3H/HeN mice.
2. **Mechanistic Studies:** Use RNA-seq to identify global transcriptional changes.
3. **Formulation Optimization:** Develop nanoparticle-antibiotic conjugates for targeted delivery.

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