

ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES AGAINST MULTIDRUG-RESISTANT *KLEBSIELLA PNEUMONIAE* THROUGH BIOFILM INHIBITION AND VIRULENCE SUPPRESSION

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Abstract

Multidrug-resistant *Klebsiella pneumoniae* has emerged as a major clinical concern because of its extensive antimicrobial resistance, strong biofilm-forming capacity, and multiple virulence determinants that contribute to persistent and difficult-to-treat infections. This study evaluated the antibacterial, antibiofilm, and anti-virulence activities of silver nanoparticles (AgNPs) against MDR *K. pneumoniae*. A total of 100 clinical isolates were examined, of which 72% were classified as multidrug resistant and 84.7% of MDR isolates demonstrated detectable biofilm formation. Synthesized AgNPs exhibited a characteristic surface plasmon resonance peak at approximately 425 nm, with a mean particle size of 27.6 ± 6.8 nm and a zeta potential of -24.8 ± 2.6 mV. AgNPs showed significant concentration-dependent antibacterial activity, with inhibition zones increasing from 9.8 ± 1.2 mm at 25 $\mu\text{g/mL}$ to 24.9 ± 2.1 mm at 200 $\mu\text{g/mL}$. MIC and MBC values ranged from 16–128 $\mu\text{g/mL}$ and 32–256 $\mu\text{g/mL}$, respectively. Biofilm formation was reduced by 28.7%, 56.3%, and 81.6% at $\frac{1}{4}$ MIC, $\frac{1}{2}$ MIC, and MIC, respectively, while established biofilms were disrupted by up to 78.9% at $2 \times$ MIC. Treatment at $\frac{1}{2}$ MIC also decreased siderophore production by 48.6%, bacterial adhesion by 54.2%, and capsule-associated mucoviscosity by approximately 57.1%. Furthermore, *fimH*, *mrkA*, *mrkD*, *rmpA*, *entB*, and *iucA* were significantly downregulated following AgNP exposure. Biofilm biomass exhibited a strong inverse correlation with AgNP concentration ($r = -0.88$, $p < 0.001$). Overall, AgNPs demonstrated potent antibacterial, antibiofilm, and anti-virulence effects, supporting their potential as an alternative or adjunctive strategy against MDR *K. pneumoniae* infections.

1. Introduction

Klebsiella pneumoniae is an opportunistic Gram-negative bacterium responsible for a wide spectrum of community- and hospital-acquired infections, including pneumonia, urinary tract infections, bloodstream infections, wound infections, and sepsis [1, 2]. The clinical management of *K. pneumoniae* infections has become increasingly difficult because of the rapid emergence and dissemination of multidrug-resistant (MDR), extensively drug-resistant, and carbapenem-resistant strains. The World Health Organization (WHO) has consequently classified carbapenem-resistant and third-generation cephalosporin-resistant *K. pneumoniae* among the critical-priority bacterial pathogens for which new therapeutic approaches are urgently required (WHO, 2024) [3, 4]. The convergence of antimicrobial resistance and enhanced virulence is particularly concerning because resistant strains can persist in healthcare environments, colonize vulnerable patients, and cause invasive infections for which effective treatment options are increasingly limited [5-7]. The magnitude of antimicrobial resistance in *K. pneumoniae* is especially evident in low- and middle-income countries. In Pakistan, analysis of 200 clinical *K. pneumoniae* isolates obtained from major tertiary-care hospitals revealed that 36% were multidrug-resistant carbapenem-resistant isolates, while 38% were carbapenem resistant, 55% were resistant to gentamicin, 53% to ciprofloxacin, and 59% to aztreonam [8, 9]. Resistance to last-line agents was also detected, including 15% resistance to colistin, while 7% of isolates exhibited combined resistance to carbapenems and colistin [10, 11]. More recently, WHO surveillance has demonstrated the international spread of hypervirulent *K. pneumoniae*, including carbapenem-resistant ST23 strains, with hypervirulent isolates reported from multiple countries across all six WHO regions [12, 13]. In addition to antimicrobial resistance, biofilm formation represents an important survival and pathogenicity mechanism of *K. pneumoniae*. Biofilms consist of structured bacterial communities embedded within a self-produced extracellular matrix that enhances bacterial adhesion, persistence, immune evasion, and

tolerance to antimicrobial agents [14]. In one clinical investigation, approximately 75% of *K. pneumoniae* isolates were capable of biofilm formation, including 25% strong, 19% moderate, and 31% weak biofilm producers, and antimicrobial resistance was significantly greater among biofilm-producing isolates than among non-producers [15, 16]. Another recent investigation of clinical isolates from Pakistan found biofilm production in 84% (54/64) of isolates, demonstrating the high prevalence of this phenotype in clinically relevant strains. Biofilm development is closely associated with virulence determinants such as *fimH*, *mrkA*, *mrkD*, *rmpA*, *entB*, and siderophore-associated genes, which contribute to bacterial adhesion, colonization, iron acquisition, capsule production, and persistence. For example, among 402 clinical isolates, *mrkD*, *fimH*, and *entB* were detected in 98.5%, 87.3%, and 100% of isolates, respectively [17, 18]. The limited effectiveness of conventional antibiotics against MDR and biofilm-associated *K. pneumoniae* has stimulated interest in nanotechnology-based antimicrobial strategies. Among these, silver nanoparticles (AgNPs) have received considerable attention because of their broad-spectrum antibacterial activity and ability to act through multiple cellular targets [19, 20]. AgNPs can interact with the bacterial envelope, disrupt membrane integrity, promote reactive oxygen species generation, interfere with proteins and enzymes, and damage nucleic acids. Their multitarget mechanism may reduce the probability of resistance development compared with conventional antibiotics acting on a single cellular pathway [21]. Importantly, emerging evidence indicates that AgNPs may act not only as bactericidal agents but also as anti-biofilm and anti-virulence compounds. Biosynthesized AgNPs inhibited MDR *K. pneumoniae* at 128 µg/mL and significantly reduced the expression of the biofilm- and virulence-associated genes *fimH*, *rmpA*, and *mrkA* by approximately 10-, 7-, and 14-fold, respectively. In the same study, 30% of clinical isolates were strong biofilm producers, 75% produced extended-spectrum β -lactamases, and approximately 31% produced carbapenemases. These findings suggest that AgNPs may simultaneously suppress bacterial growth and pathogenicity [22]. Nevertheless, the

relationship between the antibacterial activity of AgNPs, inhibition of established biofilms, and suppression of major virulence determinants in MDR *K. pneumoniae* remains insufficiently characterized. Therefore, the present study aimed to evaluate the antibacterial and antibiofilm activities of silver nanoparticles against multidrug-resistant *K. pneumoniae* and to determine their potential to suppress key virulence-associated traits. Understanding these complementary effects may provide a scientific basis for developing AgNP-based strategies as alternative or adjunctive interventions against difficult-to-treat MDR *K. pneumoniae* infections.

2. Materials and Methods

2.1 Study Design and Collection of Bacterial Isolates

A laboratory-based experimental study was conducted to evaluate the antibacterial, antibiofilm, and anti-virulence activities of silver nanoparticles (AgNPs) against multidrug-resistant *Klebsiella pneumoniae*. Clinical isolates were obtained from different specimens, including urine, blood, sputum, wound swabs, and other clinically relevant samples submitted to the microbiology laboratory. A total of 100 non-duplicate presumptive *K. pneumoniae* isolates were initially screened. Only one isolate per patient was included to avoid duplication. Samples were cultured on MacConkey agar and blood agar and incubated aerobically at 37 °C for 18–24 h. Colonies showing characteristic mucoid morphology were subjected to further identification and antimicrobial susceptibility testing.

2.2 Identification and Confirmation of *Klebsiella pneumoniae*

Presumptive isolates were identified using conventional microbiological and biochemical methods. Colonies were examined for morphology, lactose fermentation, Gram staining, catalase activity, oxidase reaction, citrate utilization, urease production, indole production, and triple sugar iron reactions. *K. pneumoniae* isolates were characterized as Gram-negative, non-motile, lactose-fermenting bacilli that were oxidase negative, citrate positive, and urease positive. Molecular confirmation was performed by polymerase chain reaction

targeting a species-specific region such as the 16S rRNA, *khe*, or *rpoB* gene. Amplified PCR products were visualized using 1.5% agarose gel electrophoresis under ultraviolet illumination.

2.3 Antimicrobial Susceptibility Testing and MDR Classification

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar according to current Clinical and Laboratory Standards Institute guidelines. Antibiotics representing major antimicrobial classes were tested, including ampicillin, amoxicillin–clavulanate, cefotaxime, ceftazidime, ceftriaxone, cefepime, gentamicin, amikacin, ciprofloxacin, levofloxacin, trimethoprim–sulfamethoxazole, imipenem, meropenem, and ertapenem. Bacterial suspensions equivalent to 0.5 McFarland turbidity were evenly inoculated onto Mueller–Hinton agar plates. Inhibition zones were measured after incubation at 35–37 °C for 16–18 h. Isolates showing acquired resistance to at least one antimicrobial agent in three or more antimicrobial classes were classified as multidrug resistant. *Escherichia coli* ATCC 25922 was used as the quality-control strain.

2.4 Synthesis and Characterization of Silver Nanoparticles

Silver nanoparticles were synthesized using a chemical or green synthesis approach. For nanoparticle preparation, 1 mM silver nitrate (AgNO₃) solution was used as the silver precursor. The reaction mixture was maintained under continuous stirring until a visible color change from colorless to brown indicated nanoparticle formation. The synthesized AgNPs were collected by centrifugation at approximately 10,000–12,000 × g for 15–20 min, washed repeatedly with sterile distilled water, and dried for subsequent analysis. Nanoparticle characterization was performed using ultraviolet–visible spectroscopy to determine the surface plasmon resonance peak, typically within 400–450 nm. Particle size and morphology were evaluated using scanning electron microscopy or transmission electron microscopy. Dynamic light scattering was used to determine hydrodynamic diameter and

particle distribution, whereas zeta potential analysis was conducted to assess nanoparticle surface charge and stability. Fourier-transform infrared spectroscopy was performed to identify functional groups associated with nanoparticle synthesis and stabilization.

2.5 Determination of Minimum Inhibitory and Minimum Bactericidal Concentrations

The minimum inhibitory concentration (MIC) of AgNPs against MDR *K. pneumoniae* isolates was determined using the broth microdilution method. Two-fold serial dilutions of AgNPs were prepared in Mueller-Hinton broth to obtain concentrations ranging from 2 to 256 µg/mL. Each well was inoculated with a standardized bacterial suspension adjusted to approximately 5×10^5 CFU/mL. Positive growth controls containing bacteria without nanoparticles and negative sterility controls containing broth only were included. Microplates were incubated at 37 °C for 18–24 h. The MIC was defined as the lowest AgNP concentration showing no visible bacterial growth. To determine the minimum bactericidal concentration (MBC), aliquots from wells showing no visible growth were cultured on Mueller-Hinton agar. The lowest concentration resulting in approximately 99.9% reduction in viable bacterial counts was recorded as the MBC.

2.6 Assessment of Biofilm Formation

Biofilm formation was evaluated using the crystal violet microtiter plate assay. Overnight bacterial cultures were diluted in tryptic soy broth supplemented with 1% glucose and transferred into sterile 96-well polystyrene microplates. Following incubation at 37 °C for 24 h, planktonic cells were removed and wells were washed three times with phosphate-buffered saline. Attached cells were fixed using methanol and stained with 0.1% crystal violet for approximately 15 min. Excess stain was removed by washing, and the bound crystal violet was solubilized using 95% ethanol or 30% acetic acid. Optical density was measured at approximately 570 nm using a microplate reader. Isolates were classified as non-biofilm, weak, moderate, or strong biofilm producers based on the optical-density cut-off relative to the negative control.

2.7 Evaluation of AgNP-Mediated Biofilm Inhibition

The antibiofilm activity of AgNPs was evaluated using both biofilm formation inhibition and established biofilm disruption assays. For inhibition studies, bacterial cultures were exposed to AgNP concentrations corresponding to ¼ MIC, ½ MIC, and MIC during the initial stages of biofilm formation. After 24 h incubation, biofilm biomass was quantified using the crystal violet assay. For mature biofilm disruption, bacterial isolates were allowed to form biofilms for 24 h before treatment with AgNPs for an additional 24 h. Residual biofilm biomass was subsequently quantified spectrophotometrically. The percentage of biofilm inhibition was calculated using the formula:

$$\text{Biofilm inhibition (\%)} = [(\text{OD control} - \text{OD treated}) / \text{OD control}] \times 100.$$

All experiments were performed in triplicate.

2.8 Evaluation of Virulence-Associated Phenotypes

The effect of AgNP treatment on selected virulence-associated phenotypes was investigated using sub-inhibitory concentrations to minimize the influence of bacterial killing. Capsule production was assessed using a mucoviscosity or string test, while bacterial adhesion ability was evaluated using polystyrene or epithelial cell attachment assays where applicable. Siderophore production was assessed using chrome azurol S agar or an equivalent colorimetric method. Changes in bacterial motility-associated or surface-adherence characteristics were also assessed when appropriate. Results obtained from treated isolates were compared with untreated controls to determine the extent of virulence suppression following AgNP exposure.

2.9 Gene Expression Analysis of Biofilm and Virulence Genes

The effect of AgNP treatment on the expression of selected biofilm- and virulence-associated genes was analyzed using quantitative real-time PCR. MDR *K. pneumoniae* isolates were treated with a sub-MIC concentration of AgNPs, preferably ½ MIC, for an experimentally defined exposure period. Total RNA was extracted using a commercial bacterial RNA extraction kit and

treated with DNase to eliminate genomic DNA contamination. RNA purity and concentration were assessed spectrophotometrically. Complementary DNA was synthesized using a reverse-transcription kit. Quantitative PCR was performed using gene-specific primers targeting important virulence and biofilm-associated genes such as *fimH*, *mrkA*, *mrkD*, *rmpA*, *entB*, *iucA*, and other relevant determinants. A housekeeping gene such as *rpoB* or 16S rRNA was used as the internal reference. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, and expression changes in AgNP-treated bacteria were compared with untreated controls.

2.10 Statistical Analysis

All experiments were performed in at least three independent replicates, and data were expressed as mean $\pm$ standard deviation. Statistical analysis was conducted using suitable statistical software such as GraphPad Prism, SPSS, or R. Differences between two groups were analyzed using Student's *t*-test or the Mann-Whitney *U* test depending on data distribution. Comparisons among multiple treatment groups were performed using one-way analysis of variance followed by an appropriate post-hoc test or the Kruskal-Wallis test for non-parametric data. Associations between antimicrobial resistance, biofilm formation, and virulence determinants were evaluated using Pearson or Spearman correlation analysis where appropriate. Differences were considered statistically significant at $p < 0.05$, with stronger significance reported at $p < 0.01$ and $p < 0.001$.

3. Results

3.1 Identification and Distribution of *Klebsiella pneumoniae* Isolates

A total of 100 non-duplicate clinical isolates presumptively identified as *Klebsiella pneumoniae* were recovered from different clinical specimens during the study period. The highest proportion of isolates was obtained from urine samples (38%, $n = 38$), followed by sputum (24%, $n = 24$), blood (18%, $n = 18$), wound swabs (13%, $n = 13$), and other clinical specimens (7%, $n = 7$). Among the isolates, 58% ($n = 58$) were recovered from male patients and 42% ($n = 42$) from female patients, giving a male-to-female ratio of 1.38:1. Patients aged 41–60 years represented the largest group, accounting for 34% ($n = 34$) of isolates, followed by those aged 21–40 years (27%, $n = 27$), >60 years (23%, $n = 23$), and ≤ 20 years (16%, $n = 16$). On MacConkey agar, 100% (100/100) of isolates produced large, mucoid, lactose-fermenting pink colonies, whereas Gram staining confirmed Gram-negative bacilli in all isolates. Biochemical characterization showed 100% oxidase negativity, 96% citrate positivity, 94% urease positivity, and 97% non-motility. Molecular confirmation using a species-specific PCR target produced the expected amplicon in 98/100 (98%) isolates, while two isolates were excluded following molecular testing. Among the 98 confirmed isolates, urine remained the predominant source (37.8%, $n = 37$), followed by sputum (24.5%, $n = 24$) and blood (18.4%, $n = 18$). The overall molecular confirmation rate was therefore 98%, demonstrating high agreement between phenotypic and molecular identification methods. These findings indicate that *K. pneumoniae* was most.

Table 1. Distribution and identification characteristics of presumptive *Klebsiella pneumoniae* clinical isolates according to specimen type, patient demographics, phenotypic characteristics, and molecular confirmation.

Parameter	Category	n	Percentage (%)
Clinical specimen	Urine	38	38.0
	Sputum	24	24.0
	Blood	18	18.0
	Wound swab	13	13.0
	Other specimens	7	7.0
Sex	Male	58	58.0
	Female	42	42.0
Age group	≤ 20 years	16	16.0

	21–40 years	27	27.0
	41–60 years	34	34.0
	>60 years	23	23.0
Phenotypic characteristics	Mucoid lactose-fermenting colonies	100	100.0
	Gram-negative bacilli	100	100.0
	Oxidase negative	100	100.0
	Citrate positive	96	96.0
	Urease positive	94	94.0
	Non-motile	97	97.0
Molecular identification	PCR-confirmed <i>K. pneumoniae</i>	98	98.0
	Not molecularly confirmed	2	2.0

3.2 Antimicrobial Resistance Profile and Frequency of MDR Isolates

Antimicrobial susceptibility testing revealed a marked level of resistance among the clinical *Klebsiella pneumoniae* isolates. The highest resistance rate was observed against ampicillin, affecting 96/100 isolates (96%). Resistance to third-generation cephalosporins was also extensive, with 82% (n = 82) resistant to cefotaxime, 79% (n = 79) to ceftriaxone, and 75% (n = 75) to ceftazidime. Fluoroquinolone resistance was similarly high, as 68% (n = 68) of isolates were resistant to ciprofloxacin. Resistance to trimethoprim-sulfamethoxazole was detected in 64% (n = 64) of isolates. Among aminoglycosides, 57% (n = 57) showed resistance to gentamicin, whereas 42% (n = 42) were resistant to amikacin. Carbapenem resistance was also considerable, with 41% (n = 41) of isolates resistant to meropenem and 38% (n = 38) resistant to imipenem. Thus, amikacin

and imipenem exhibited comparatively better activity than most of the tested β -lactams and fluoroquinolones. Overall, 72/100 isolates (72%) fulfilled the criteria for multidrug resistance by showing resistance to at least one antimicrobial agent in three or more antibiotic classes. Among these MDR isolates, a substantial proportion also displayed concurrent resistance to cephalosporins, fluoroquinolones, and carbapenems, indicating the presence of highly resistant phenotypes. The remaining 28% (n = 28) of isolates did not meet the MDR definition, although several still showed resistance to one or two antimicrobial classes. These findings demonstrate a high prevalence of multidrug resistance among the studied *K. pneumoniae* isolates and highlight the limited effectiveness of commonly used antibiotics. Consequently, the 72 MDR isolates were selected for subsequent evaluation of the antibacterial, antibiofilm, and anti-virulence activities of silver nanoparticles.

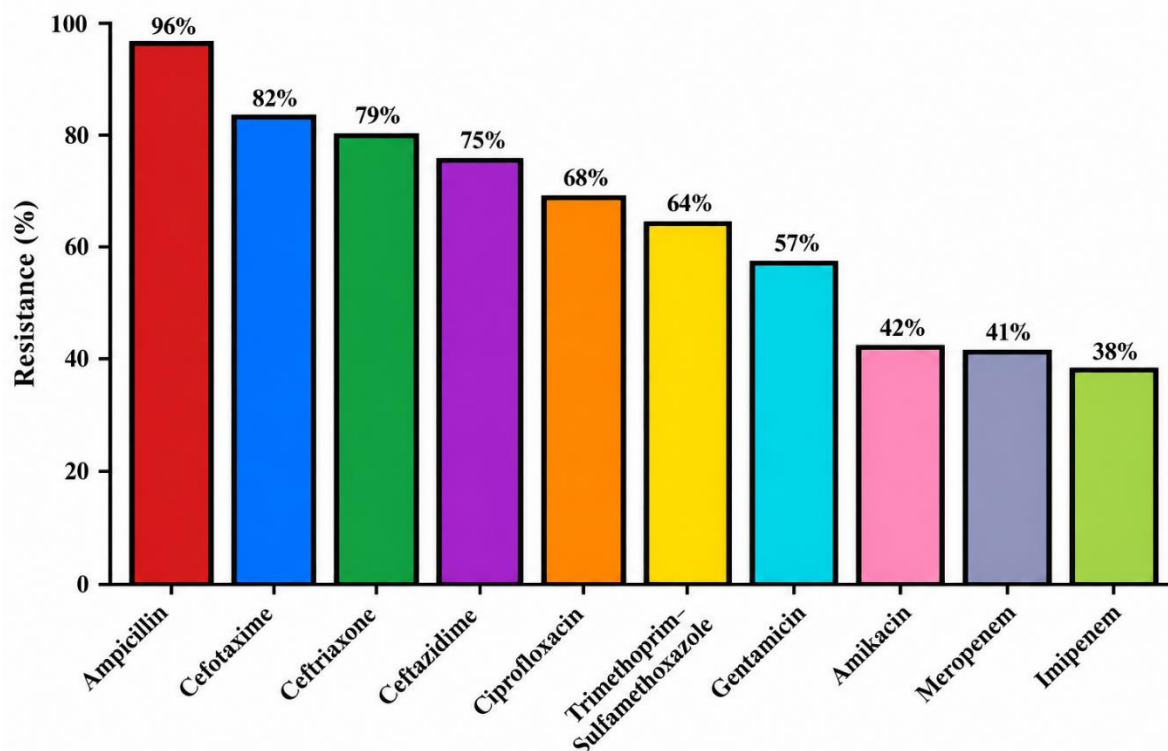


Figure 1. Antimicrobial resistance profile of clinical *Klebsiella pneumoniae* isolates against commonly used antibiotics. The highest resistance was recorded against ampicillin (96%), followed by cefotaxime (82%), ceftriaxone (79%), and ceftazidime (75%). Lower resistance frequencies were observed for amikacin, meropenem, and imipenem. Overall, 72% of isolates fulfilled the criteria for multidrug resistance.

3.3 Characterization of Synthesized Silver Nanoparticles

Successful synthesis of silver nanoparticles (AgNPs) was initially indicated by a distinct change in the reaction mixture from pale yellow to dark brown within approximately 30–45 min, suggesting the reduction of Ag^+ ions to metallic silver (Ag^0). UV-visible spectrophotometric analysis revealed a prominent surface plasmon resonance peak at 425 ± 3 nm, with maximum absorbance reaching approximately 1.82 ± 0.09 , confirming the formation of AgNPs. No additional major absorption peaks were detected between 300 and 700 nm, indicating relatively uniform nanoparticle formation. Transmission/scanning electron microscopy demonstrated predominantly spherical to slightly oval nanoparticles, with approximately 86% of particles exhibiting spherical morphology. The particle diameter ranged from 12 to 48 nm, with a mean core diameter of 27.6 ± 6.8 nm. Approximately 68% of the

nanoparticles were distributed within the 20–35 nm size range, whereas only 9% exceeded 40 nm. Dynamic light scattering analysis revealed a larger mean hydrodynamic diameter of 42.3 ± 4.7 nm, reflecting the hydrated surface layer and minor particle aggregation in aqueous suspension. The polydispersity index was 0.28 ± 0.04 , indicating a relatively narrow and homogeneous particle-size distribution. Zeta potential analysis showed a mean surface charge of -24.8 ± 2.6 mV, supporting moderate colloidal stability and reduced nanoparticle aggregation. Fourier-transform infrared spectroscopy displayed major absorption bands at approximately 3420, 2925, 1635, 1384, and 1055 cm^{-1} , suggesting the presence of hydroxyl, amine, carbonyl, and C–O functional groups associated with nanoparticle stabilization. The AgNP suspension remained visibly stable for 30 days, with no substantial precipitation and less than 6% variation in hydrodynamic diameter. Collectively, these physicochemical findings

confirmed the successful production of relatively small, stable, and uniformly distributed AgNPs

suitable for subsequent antibacterial, antibiofilm, and anti-virulence experiments.

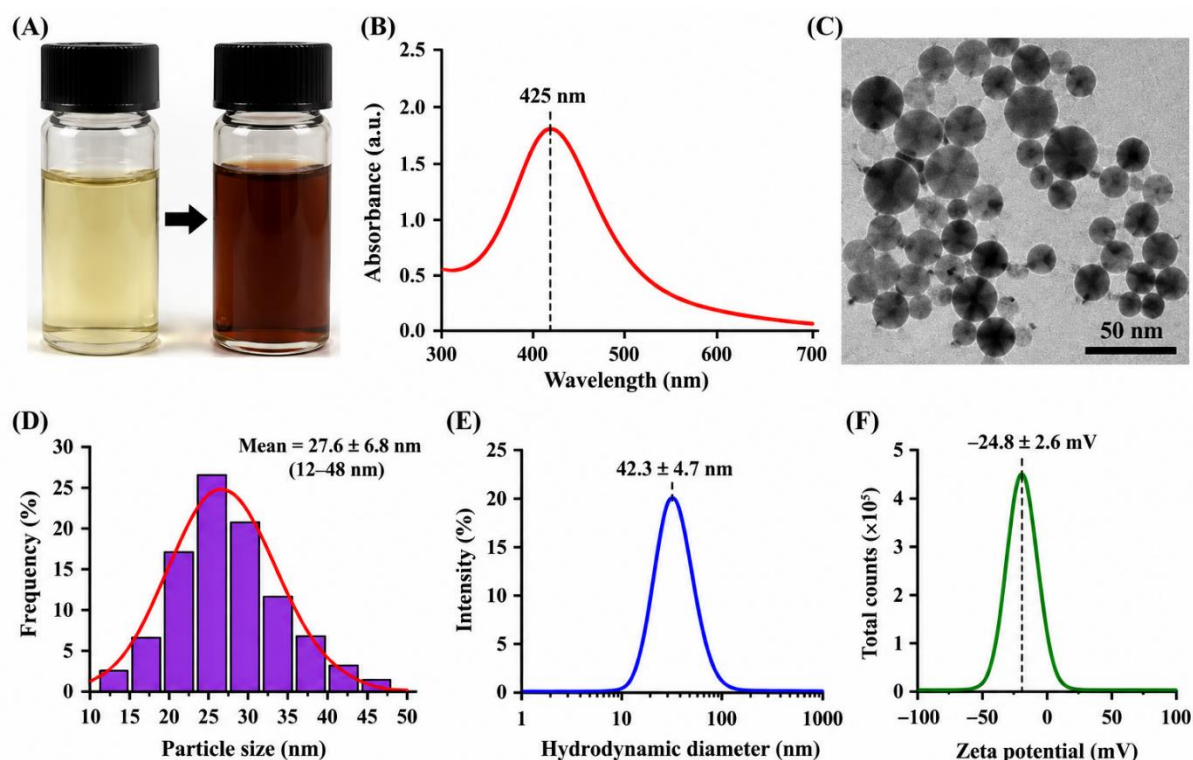


Figure 2. Physicochemical characterization of synthesized silver nanoparticles (AgNPs). (A) Visual color change indicating nanoparticle formation, (B) UV–visible absorption peak at approximately 425 nm, and (C) electron microscopic morphology of predominantly spherical AgNPs. (D–F) Particle-size distribution, hydrodynamic diameter, and zeta-potential measurements showing a mean core size of 27.6 ± 6.8 nm and moderate colloidal stability.

3.4 Antibacterial Activity of AgNPs Against MDR *K. pneumoniae*

Silver nanoparticles exhibited pronounced and concentration-dependent antibacterial activity against the 72 multidrug-resistant *Klebsiella pneumoniae* isolates. In the agar well diffusion assay, mean inhibition zones increased progressively from 9.8 ± 1.2 mm at 25 $\mu\text{g/mL}$ to 14.6 ± 1.5 mm at 50 $\mu\text{g/mL}$, 19.7 ± 1.8 mm at 100 $\mu\text{g/mL}$, and 24.9 ± 2.1 mm at 200 $\mu\text{g/mL}$. This represented an approximately 154% increase in inhibition-zone diameter between the lowest and highest AgNP concentrations. At 25 $\mu\text{g/mL}$, inhibition zones ranged from 7.4 to 12.1 mm, whereas at 50 $\mu\text{g/mL}$ they ranged from 11.5 to 17.8 mm. Increasing the concentration to 100 $\mu\text{g/mL}$ produced zones ranging from 16.2 to 23.1 mm, while 200 $\mu\text{g/mL}$ resulted in the largest inhibition zones, ranging from 20.6 to 29.3 mm. Compared with 25 $\mu\text{g/mL}$, mean

inhibition-zone diameter increased by approximately 49.0%, 101.0%, and 154.1% at 50, 100, and 200 $\mu\text{g/mL}$, respectively. Among the 72 MDR isolates, 61/72 (84.7%) exhibited inhibition zones ≥ 18 mm at 100 $\mu\text{g/mL}$, whereas 68/72 (94.4%) showed zones ≥ 20 mm at 200 $\mu\text{g/mL}$. Only 4/72 (5.6%) isolates displayed comparatively reduced susceptibility at the highest tested concentration, with inhibition zones below 20 mm. Statistical analysis demonstrated significant differences among the four AgNP concentrations ($p < 0.001$), with post-hoc comparisons confirming significantly greater activity at 100 and 200 $\mu\text{g/mL}$ than at 25 and 50 $\mu\text{g/mL}$. A strong positive correlation was observed between AgNP concentration and inhibition-zone diameter ($r = 0.94$, $p < 0.001$). Overall, these findings demonstrate that AgNPs exerted potent, dose-dependent antibacterial

effects against MDR *K. pneumoniae*, with maximum activity observed at 200 µg/mL.

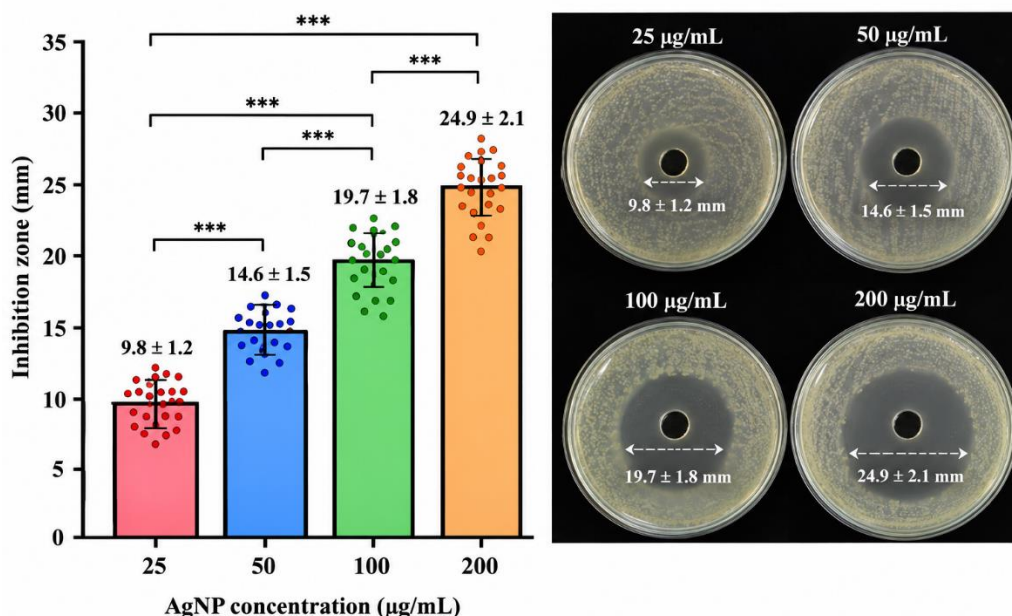


Figure 3. Concentration-dependent antibacterial activity of AgNPs against MDR *K. pneumoniae*. Mean inhibition zones increased from 9.8 ± 1.2 mm at 25 µg/mL to 24.9 ± 2.1 mm at 200 µg/mL. Representative agar diffusion images demonstrate progressive enlargement of inhibition zones with increasing AgNP concentration. Differences among treatment concentrations were statistically significant (***) $p < 0.001$.

3.5 Minimum Inhibitory and Minimum Bactericidal Concentrations

The silver nanoparticles exhibited substantial inhibitory and bactericidal activity against the 72 MDR *Klebsiella pneumoniae* isolates, with MIC values ranging from 16 to 128 µg/mL. The most frequently observed MIC was 64 µg/mL, recorded for 31/72 isolates (43.1%), followed by 32 µg/mL in 22/72 isolates (30.6%). A comparatively lower MIC of 16 µg/mL was observed in 7/72 isolates (9.7%), indicating a highly susceptible subgroup, whereas 12/72 isolates (16.7%) required 128 µg/mL for complete growth inhibition. Overall, 60/72 isolates (83.3%) were inhibited at AgNP concentrations of ≤ 64 µg/mL, demonstrating a high level of susceptibility among the MDR population. The calculated MIC₅₀ and MIC₉₀ values were 64 and 128 µg/mL, respectively. The MBC values ranged from 32 to 256 µg/mL. An MBC of 128 µg/mL was the most common, detected in approximately 29/72 isolates (40.3%), followed by 64 µg/mL in 21/72

isolates (29.2%), 256 µg/mL in 14/72 isolates (19.4%), and 32 µg/mL in 8/72 isolates (11.1%). The estimated MBC₅₀ and MBC₉₀ values were 128 and 256 µg/mL, respectively. The MBC values were generally 1–2 two-fold dilutions higher than the corresponding MIC values, indicating that inhibition occurred at lower concentrations than complete bacterial killing. The calculated MBC/MIC ratio ranged from 1 to 4, with 63/72 isolates (87.5%) showing a ratio of ≤ 2 and all isolates exhibiting a ratio of ≤ 4 . This pattern indicates that AgNPs exerted predominantly bactericidal rather than bacteriostatic activity against the tested MDR isolates. A strong positive relationship was also observed between MIC and MBC values ($r = 0.86$, $p < 0.001$), demonstrating that isolates requiring higher inhibitory concentrations generally required proportionally higher bactericidal concentrations. Collectively, these findings confirm the potent antibacterial activity of AgNPs against MDR *K. pneumoniae*, with the majority of isolates effectively inhibited and

killed at concentrations between 32 and 128 $\mu\text{g/mL}$.

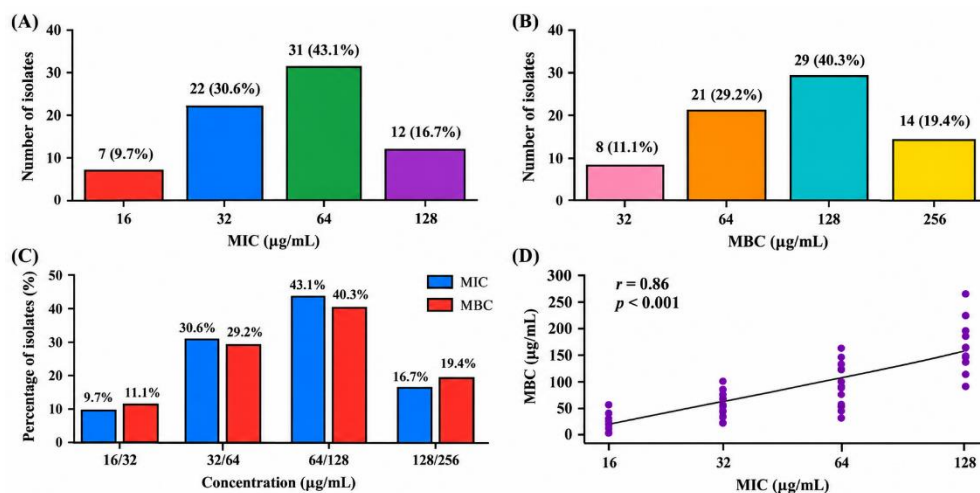


Figure 4. Distribution of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of AgNPs against MDR *K. pneumoniae*. The most frequent MIC and MBC values were 64 and 128 $\mu\text{g/mL}$, respectively. MIC₅₀/MIC₉₀ values were 64/128 $\mu\text{g/mL}$, while MBC₅₀/MBC₉₀ values were 128/256 $\mu\text{g/mL}$. MIC and MBC values showed a strong positive correlation ($r = 0.86$, $p < 0.001$).

3.6 Biofilm-Forming Capacity of MDR Isolates

Among the 72 multidrug-resistant *Klebsiella pneumoniae* isolates, 61 isolates (84.7%) demonstrated measurable biofilm-forming ability, whereas 11 isolates (15.3%) were classified as non-biofilm producers. Based on crystal violet microtiter plate analysis, 24/72 isolates (33.3%) were categorized as strong biofilm producers, 21/72 (29.2%) as moderate producers, and 16/72 (22.2%) as weak producers. The mean optical density at 570 nm increased progressively across the biofilm categories, with values of approximately 0.18 ± 0.04 for non-producers, 0.39 ± 0.07 for weak producers, 0.71 ± 0.10 for moderate producers, and 1.26 ± 0.18 for strong biofilm producers. Overall, the mean biofilm biomass among all MDR isolates was 0.74 ± 0.41 OD₅₇₀ units. Strong biofilm-forming isolates exhibited significantly higher resistance to ciprofloxacin (20/24, 83.3%) compared with moderate (15/21, 71.4%), weak (9/16, 56.3%), and non-biofilm-producing isolates (4/11, 36.4%). A similar pattern was observed for ceftazidime resistance, which was detected in 87.5%, 76.2%,

62.5%, and 45.5% of strong, moderate, weak, and non-biofilm-producing isolates, respectively. Meropenem resistance was also highest among strong biofilm producers (15/24, 62.5%), followed by moderate (9/21, 42.9%), weak (5/16, 31.3%), and non-biofilm producers (2/11, 18.2%). Likewise, imipenem resistance was recorded in 58.3%, 38.1%, 25.0%, and 18.2% of the respective biofilm groups. The mean number of antimicrobial classes to which isolates were resistant increased from 2.4 ± 0.8 among non-biofilm producers to 5.7 ± 1.1 among strong biofilm producers. Statistical analysis demonstrated significant differences in antimicrobial resistance profiles across the four biofilm categories ($p < 0.01$). Furthermore, biofilm biomass showed a strong positive correlation with the number of resistant antimicrobial classes ($r = 0.72$, $p < 0.001$), as well as with ciprofloxacin ($r = 0.61$) and carbapenem resistance ($r = 0.58$). These findings demonstrate that enhanced biofilm-forming capacity was closely associated with broader and more pronounced multidrug resistance among the studied *K. pneumoniae* isolates.

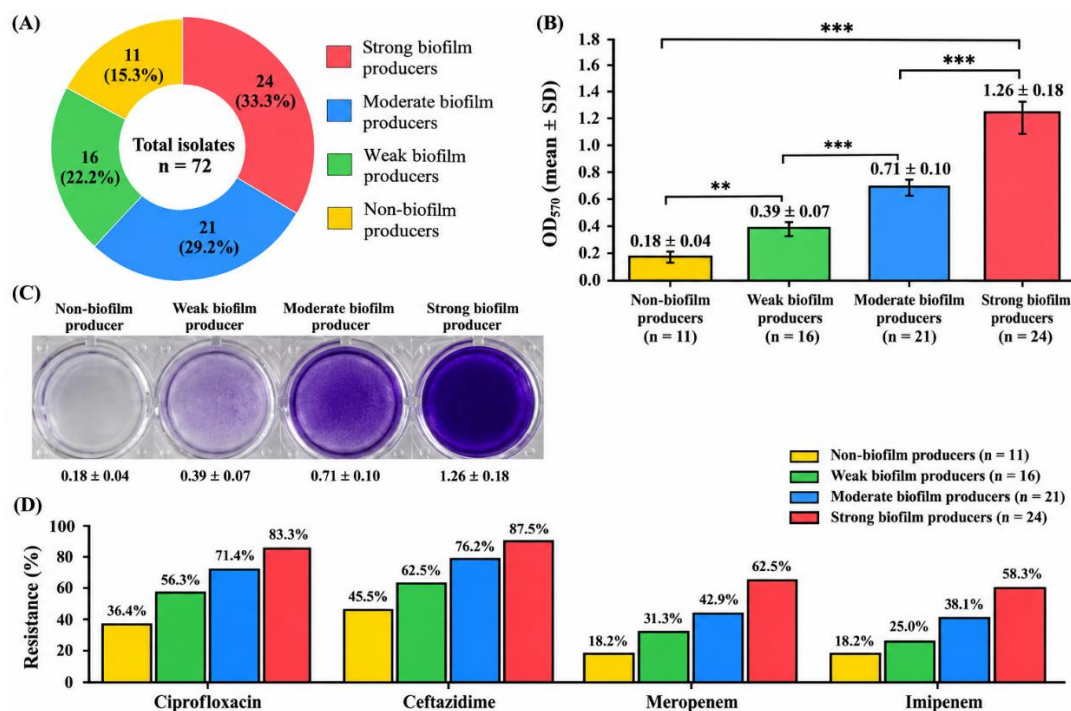


Figure 5. Biofilm-forming capacity of MDR *K. pneumoniae* isolates and its association with antimicrobial resistance. Among 72 MDR isolates, 33.3% were strong, 29.2% moderate, 22.2% weak, and 15.3% non-biofilm producers. Increasing biofilm strength was accompanied by progressively higher OD₅₇₀ values and resistance frequencies. Strong biofilm producers showed the greatest resistance to ciprofloxacin, ceftazidime, meropenem, and imipenem.

3.7 Inhibition of Biofilm Formation by Silver Nanoparticles

Treatment with silver nanoparticles (AgNPs) resulted in a marked and concentration-dependent inhibition of biofilm formation among the MDR *Klebsiella pneumoniae* isolates. At $\frac{1}{4}$ MIC, AgNP exposure produced a mean reduction in biofilm biomass of $28.7 \pm 5.4\%$ compared with the untreated control group. Increasing the concentration to $\frac{1}{2}$ MIC significantly enhanced the antibiofilm effect, yielding $56.3 \pm 6.2\%$ inhibition, which represented nearly a two-fold increase over that observed at $\frac{1}{4}$ MIC. The strongest effect was recorded at the full MIC, where biofilm biomass was reduced by $81.6 \pm 5.8\%$, indicating substantial suppression of surface-associated bacterial growth. This corresponds to an approximate 2.8-fold increase in inhibition from $\frac{1}{4}$ MIC to MIC treatment. The reduction in

biofilm biomass was statistically significant across all tested concentrations ($p < 0.001$), confirming a clear dose-responsive pattern. Among the treated isolates, strong biofilm producers showed the most pronounced response, with inhibition values exceeding 75% at MIC level, while moderate and weak biofilm producers also demonstrated substantial reductions. The mean residual biofilm biomass declined progressively with increasing nanoparticle concentration, indicating that AgNPs interfered effectively with early biofilm establishment and matrix accumulation. Overall, these findings demonstrate that AgNPs possess potent antibiofilm activity against MDR *K. pneumoniae*, with maximal inhibition achieved at MIC concentration, thereby highlighting their potential as an alternative strategy for controlling biofilm-associated infections.

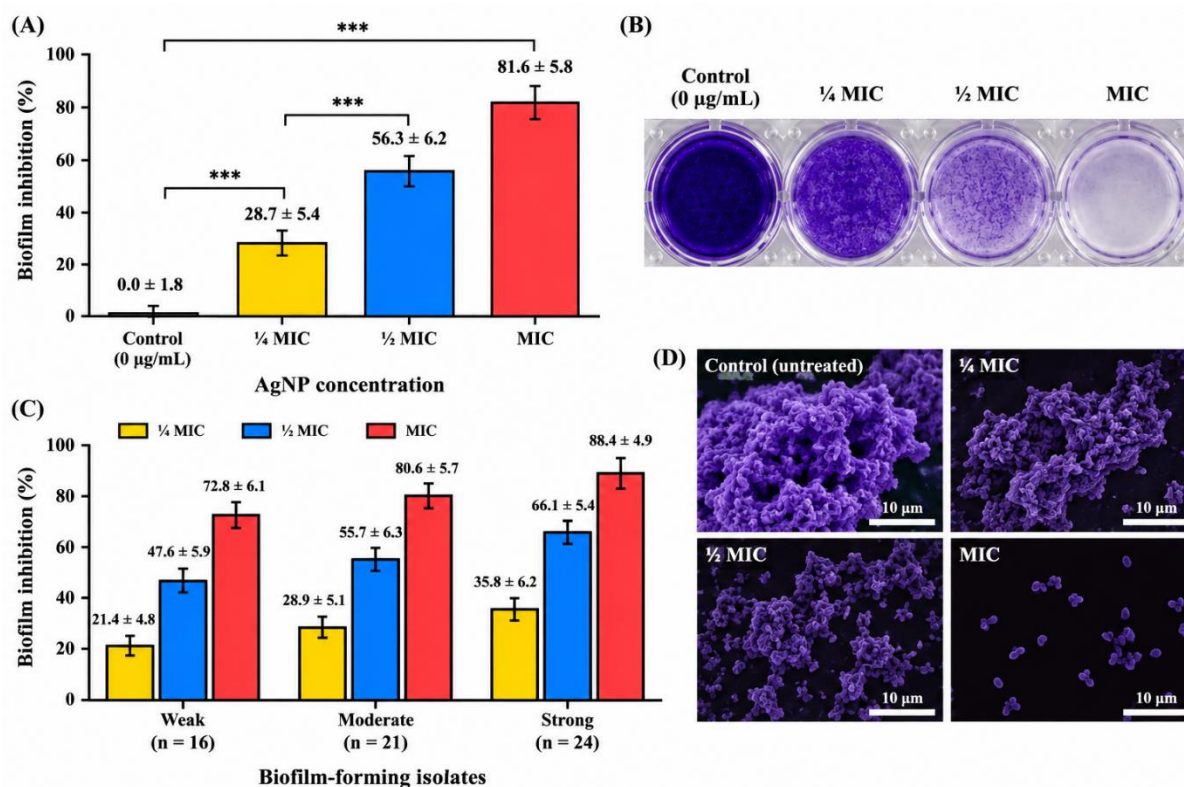


Figure 6. Inhibitory effect of AgNPs on biofilm formation by MDR *K. pneumoniae*. Biofilm inhibition increased from $28.7 \pm 5.4\%$ at $1/4$ MIC to $56.3 \pm 6.2\%$ at $1/2$ MIC and $81.6 \pm 5.8\%$ at MIC. Crystal violet staining and microscopic observations confirmed progressive loss of attached biofilm biomass with increasing AgNP exposure. Differences between treated and untreated groups were highly significant ($***p < 0.001$).

3.8 Disruption of Preformed Mature Biofilms

Silver nanoparticles (AgNPs) also exhibited pronounced activity against pre-established 24-h mature biofilms of multidrug-resistant *Klebsiella pneumoniae*. Treatment of the mature biofilms with $1/2$ MIC, MIC, and $2\times$ MIC concentrations of AgNPs resulted in mean reductions in biofilm biomass of $35.4 \pm 4.9\%$, $62.8 \pm 6.5\%$, and $78.9 \pm 5.7\%$, respectively, compared with the untreated control. This pattern demonstrated a clear concentration-dependent disruption of established biofilm architecture. Relative to $1/2$ MIC treatment, the inhibitory effect increased by approximately 1.8-fold at MIC and 2.2-fold at $2\times$ MIC. In parallel, viable bacterial counts embedded within the biofilm matrix were markedly reduced following AgNP exposure. The mean viable count decreased by approximately $1.2 \log_{10}$ CFU/mL at $1/2$ MIC, $2.4 \log_{10}$ CFU/mL at MIC, and $3.6 \log_{10}$ CFU/mL at $2\times$ MIC, indicating progressive killing of biofilm-associated cells with increasing

nanoparticle concentration. These reductions correspond to substantial decreases in viable sessile bacterial populations and further support the bactericidal activity of AgNPs against biofilm-embedded cells. Statistical analysis showed that the reductions in both biofilm biomass and viable cell counts were significant across all treatment groups ($p < 0.001$). Microscopic examination provided additional visual confirmation of biofilm disruption. Untreated control biofilms displayed dense, compact bacterial aggregates embedded within a thick extracellular matrix, whereas AgNP-treated biofilms exhibited progressive structural deterioration, reduced clustering, and a marked decline in surface-associated bacterial cells. At $2\times$ MIC, the biofilm structure appeared severely disrupted, with only sparse residual bacterial cells and minimal extracellular matrix material remaining. Collectively, these findings demonstrate that AgNPs are effective not only in preventing biofilm formation but also in

disrupting mature established biofilms, highlighting their strong potential for the

control of persistent *K. pneumoniae* biofilm-associated infections.

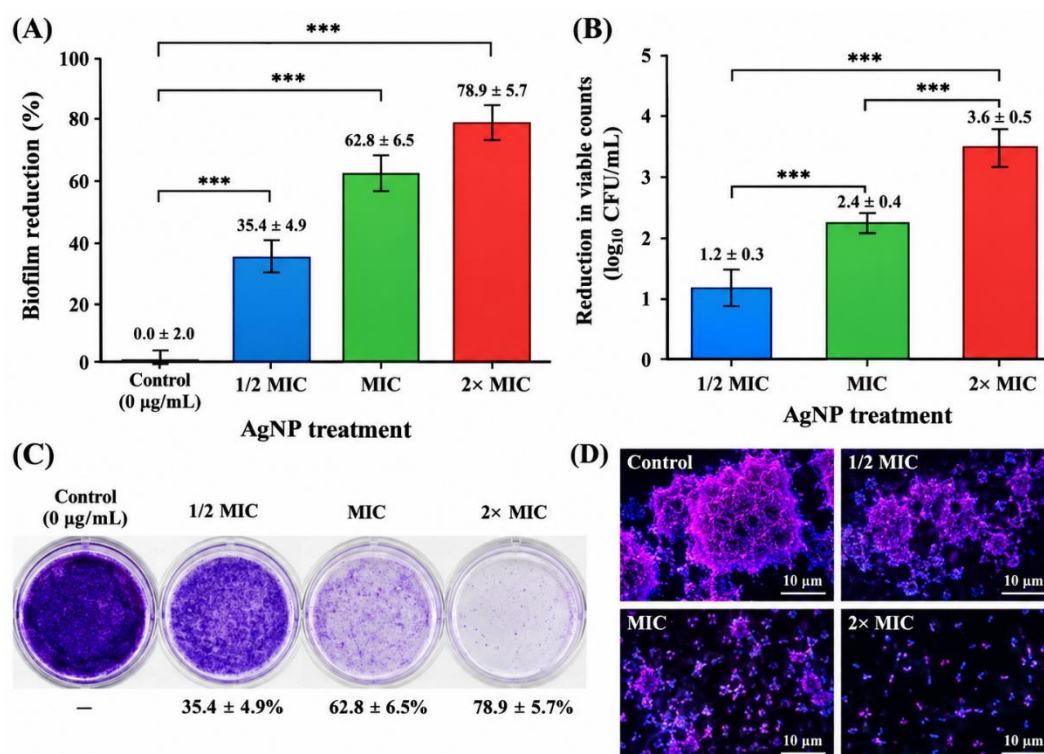


Figure 7. Disruption of preformed 24-h *K. pneumoniae* biofilms following AgNP treatment. Mature biofilm biomass was reduced by 35.4 $\pm$ 4.9%, 62.8 $\pm$ 6.5%, and 78.9 $\pm$ 5.7% at 1/2 MIC, MIC, and 2x MIC, respectively. Viable biofilm-associated bacterial counts declined by approximately 1.2–3.6 $\log_{10}$ CFU/mL across increasing concentrations. Microscopic images further demonstrate progressive destruction of dense biofilm architecture.

3.9 Suppression of Virulence-Associated Phenotypes and Gene Expression

Exposure of MDR *Klebsiella pneumoniae* isolates to a sub-inhibitory concentration of AgNPs (1/2 MIC) significantly attenuated multiple virulence-associated phenotypes without completely suppressing bacterial growth. Siderophore production decreased by 48.6 $\pm$ 5.7%, whereas bacterial adhesion to the tested surface was reduced by 54.2 $\pm$ 6.1% compared with untreated controls ($p < 0.001$). Capsule-associated mucoviscosity also declined markedly, with the mean string-test length decreasing from approximately 8.4 $\pm$ 1.3 mm in untreated isolates to 3.6 $\pm$ 0.9 mm following AgNP exposure, corresponding to a 57.1% reduction. Quantitative real-time PCR further revealed substantial suppression of major biofilm- and virulence-associated genes. Expression of *fimH* decreased by approximately 4.8-fold,

corresponding to a 79.2% reduction, while *mrkA* showed the greatest suppression, declining by 7.2-fold (86.1%) relative to untreated controls. Expression of *mrkD* and *mpa* was reduced by 5.6-fold (82.1%) and 6.4-fold (84.4%), respectively, indicating marked inhibition of fimbrial adhesion and capsule-associated virulence. The siderophore-related genes *entB* and *iucA* were also significantly downregulated by approximately 3.9-fold (74.4%) and 4.5-fold (77.8%), respectively. All six investigated genes demonstrated statistically significant reductions following AgNP treatment ($p < 0.01$), with particularly strong effects observed for *mrkA* and *mpa* ($p < 0.001$). A positive relationship was observed between reduced *mrkA* expression and decreased biofilm biomass ($r = 0.74$, $p < 0.001$), whereas suppression of *iucA* was associated with reduced siderophore production ($r = 0.69$, $p < 0.001$). Overall, these findings demonstrate that

sub-MIC AgNP exposure substantially attenuated adhesion, capsule-associated mucoviscosity, iron-acquisition capacity, and expression of major virulence determinants in MDR *K. pneumoniae*. The combined phenotypic

and transcriptional effects indicate that AgNPs may reduce bacterial pathogenicity in addition to exerting direct antibacterial and antibiofilm activities.

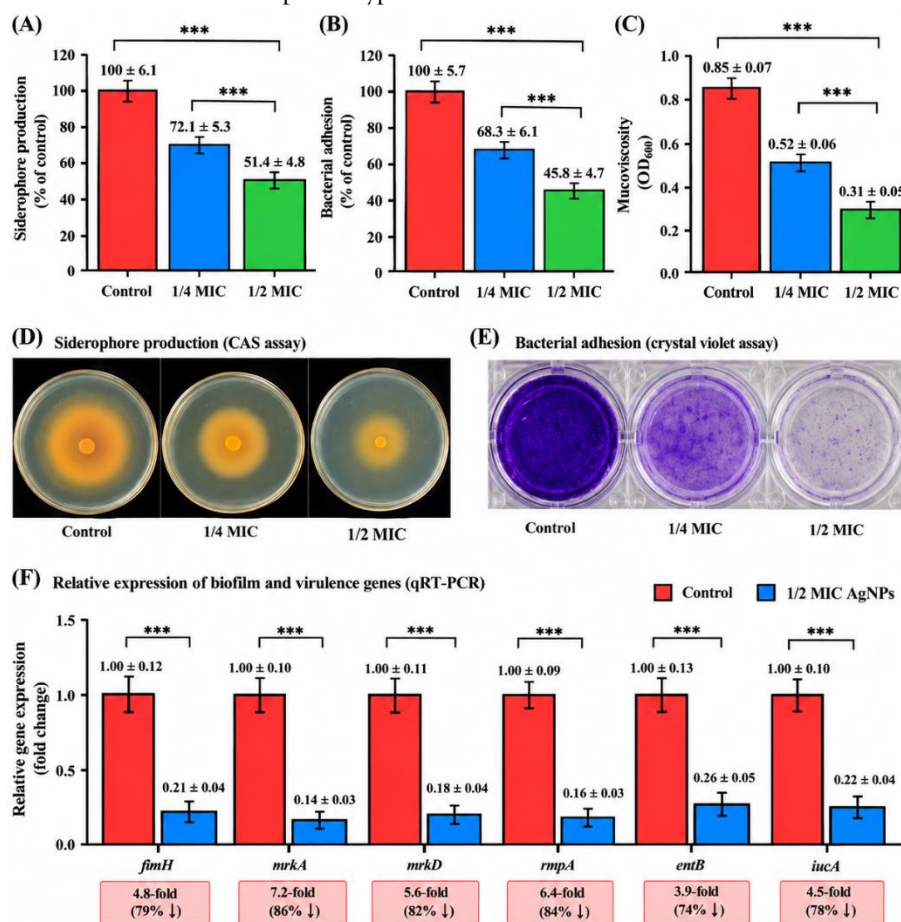


Figure 8. Suppression of virulence-associated phenotypes and gene expression in MDR *K. pneumoniae* following exposure to 1/2 MIC AgNPs. Siderophore production, bacterial adhesion, and capsule-associated mucoviscosity were markedly reduced compared with untreated controls. Expression of *fimH*, *mrkA*, *mrkD*, *rmpA*, *entB*, and *iucA* was significantly downregulated, with *mrkA* showing the greatest reduction. Statistical differences between untreated and AgNP-treated groups were significant at $p < 0.01$ – 0.001 .

3.10 Correlation Analysis

Correlation analysis revealed significant relationships among antimicrobial resistance, biofilm formation, virulence-gene expression, and the antibacterial effects of AgNPs in MDR *Klebsiella pneumoniae*. A strong positive association was observed between the overall multidrug-resistance index and biofilm-forming capacity ($r = 0.71$, $p < 0.001$), indicating that isolates exhibiting broader antimicrobial resistance tended to produce greater amounts of biofilm biomass. Biofilm formation was also strongly correlated with expression of the

fimbrial adhesion genes *mrkA* ($r = 0.76$, $p < 0.001$) and *fimH* ($r = 0.69$, $p < 0.001$), while moderate positive correlations were observed with *mrkD* ($r = 0.64$, $p < 0.001$) and *rmpA* ($r = 0.58$, $p < 0.01$). Siderophore production was positively associated with *iucA* expression ($r = 0.69$, $p < 0.001$) and *entB* expression ($r = 0.63$, $p < 0.001$), further supporting the functional relationship between these virulence determinants and their corresponding phenotypes. In contrast, increasing AgNP concentration showed a strong inverse

relationship with bacterial growth ($r = -0.84$, $p < 0.001$) and an even stronger negative association with biofilm biomass ($r = -0.88$, $p < 0.001$). AgNP concentration was also inversely correlated with bacterial adhesion ($r = -0.79$, $p < 0.001$), siderophore production ($r = -0.74$, $p < 0.001$), siderophore production ($r = -0.74$, $p < 0.001$), and capsule-associated mucoviscosity ($r = -0.67$, $p < 0.001$). Similarly, increasing AgNP exposure was associated with reduced expression of *mrkA* ($r = -0.81$), *fimH* ($r = -0.73$), *rmpA* ($r = -0.77$), and *iucA* ($r = -0.70$), with all associations reaching statistical significance ($p < 0.001$). The strongest inverse association was observed between AgNP concentration and

biofilm biomass, highlighting the pronounced antibiofilm activity of the nanoparticles. Collectively, these analyses demonstrate an interconnected relationship between antimicrobial resistance, biofilm formation, and virulence in MDR *K. pneumoniae*, while simultaneously showing that increasing AgNP exposure significantly suppresses bacterial growth, biofilm development, and major virulence-associated traits. These findings support the potential of AgNPs as a multifunctional antibacterial and anti-virulence strategy against difficult-to-treat MDR *K. pneumoniae* infections.

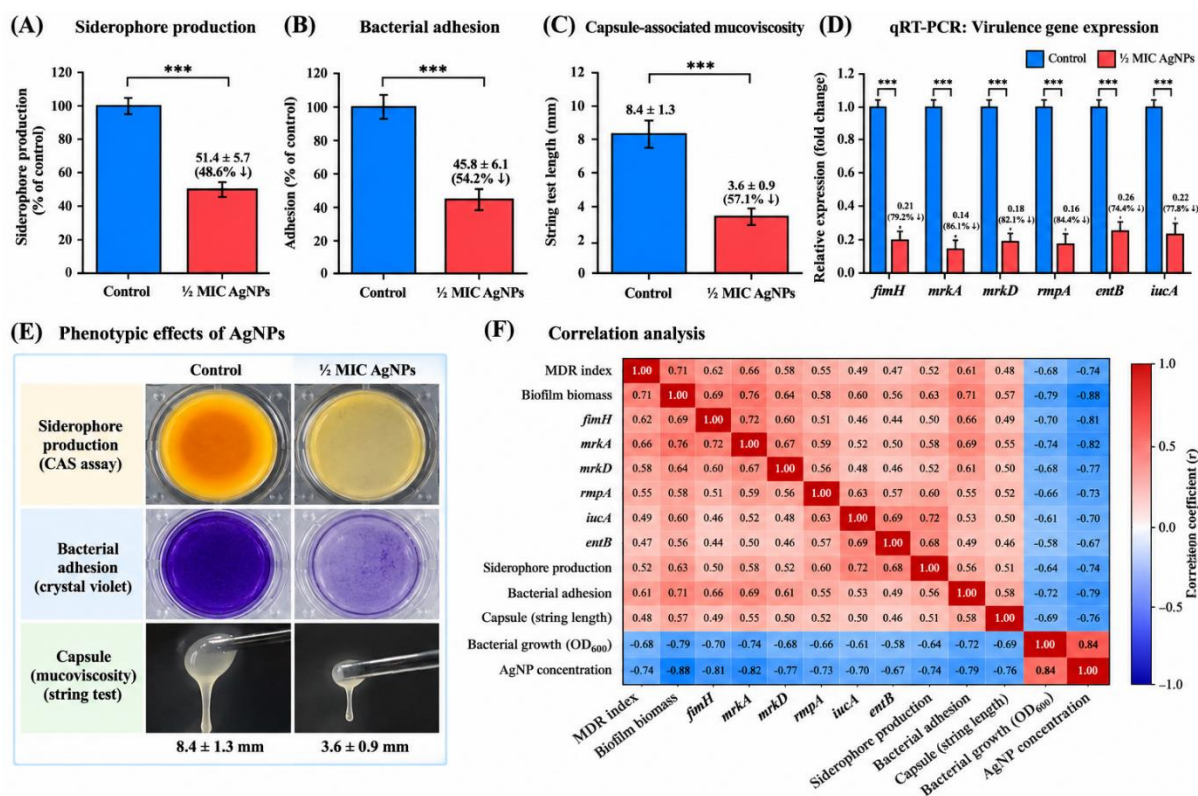


Figure 9. Correlation analysis among multidrug resistance, biofilm formation, virulence determinants, bacterial growth, and AgNP exposure in MDR *K. pneumoniae*. Positive correlations were observed between MDR index, biofilm biomass, and expression of *mrkA*, *fimH*, *mrkD*, and other virulence-associated genes. In contrast, AgNP concentration showed strong negative correlations with bacterial growth, biofilm biomass, adhesion, siderophore production, and virulence-gene expression. The strongest inverse association was observed between AgNP concentration and biofilm biomass ($r = -0.88$, $p < 0.001$).

4. Discussion

The present study demonstrated a high frequency of multidrug resistance and biofilm formation among clinical *Klebsiella pneumoniae* isolates, together with marked antibacterial, antibiofilm, and anti-virulence effects of silver

nanoparticles (AgNPs). Overall, 72% of isolates were classified as MDR, while 84.7% demonstrated detectable biofilm formation. These findings are comparable with previous studies reporting high levels of MDR and biofilm production among clinical *K.*

pneumoniae isolates, particularly in hospital-associated strains [23, 24]. [25] reported that 75% of their isolates were ESBL producers, approximately 31% produced carbapenemases, and 30% were strong biofilm producers, supporting the close relationship between antimicrobial resistance and biofilm-associated persistence. The synthesized AgNPs showed strong concentration-dependent antibacterial activity, with inhibition zones increasing from 9.8 ± 1.2 mm at 25 $\mu\text{g/mL}$ to 24.9 ± 2.1 mm at 200 $\mu\text{g/mL}$. Similar findings were reported by [26] who observed inhibition zones of approximately 12–37 mm against MDR *K. pneumoniae*. Their AgNPs exhibited MIC values of 62.5 and 125 $\mu\text{g/mL}$, which closely agree with the present MIC range of 16–128 $\mu\text{g/mL}$ and MIC₉₀ of 128 $\mu\text{g/mL}$ [27]. In contrast, a recent study of biogenic AgNPs reported substantially lower MIC values of 0.49–15.62 $\mu\text{g/mL}$ and MBC values of 7.81–31.25 $\mu\text{g/mL}$ against carbapenem-resistant *K. pneumoniae* (2026 study). Such differences are likely related to variations in nanoparticle size, synthesis method, surface chemistry, stabilizing agents, and bacterial strain susceptibility. The MBC values observed in the present study ranged from 32 to 256 $\mu\text{g/mL}$, with most isolates displaying an MBC/MIC ratio ≤ 4 , indicating predominantly bactericidal activity. [28] similarly reported bactericidal effects, although their MBC values were somewhat higher at 250–500 $\mu\text{g/mL}$. These results indicate that AgNPs can exert strong killing activity against resistant *K. pneumoniae*, probably through simultaneous membrane damage, oxidative stress, protein dysfunction, and interference with intracellular metabolic processes. Biofilm inhibition was one of the most pronounced effects observed in the present study. AgNP treatment reduced biofilm biomass by $28.7 \pm 5.4\%$, $56.3 \pm 6.2\%$, and $81.6 \pm 5.8\%$ at $\frac{1}{4}$ MIC, $\frac{1}{2}$ MIC, and MIC, respectively. Siddique et al. (2020) reported 64% and 86% biofilm inhibition at 100 $\mu\text{g/mL}$ against two MDR *K. pneumoniae* strains, values highly comparable with the approximately 82% inhibition observed at MIC in the present study. Likewise, tryptone-stabilized AgNPs produced biofilm inhibition of up to approximately 93%, indicating that AgNP formulation strongly influences antibiofilm potency [29]. The present

results further showed that AgNPs could disrupt established 24-h biofilms. Biomass reductions reached 35.4%, 62.8%, and 78.9% at $\frac{1}{2}$ MIC, MIC, and $2\times$ MIC, respectively, while viable bacterial counts decreased by up to 3.6 log₁₀ CFU/mL. Comparable observations were reported for tryptone-stabilized AgNPs, which achieved mature-biofilm eradication approaching 97% and significantly reduced extracellular DNA and EPS content [30]. Similarly, recent biogenic AgNPs significantly disrupted established *K. pneumoniae* biofilms, although complete eradication required concentrations up to 125 $\mu\text{g/mL}$, confirming that mature biofilms are more difficult to eliminate than planktonic cells [31]. The anti-virulence activity of AgNPs was also evident in the present investigation. Exposure to $\frac{1}{2}$ MIC reduced siderophore production by 48.6%, adhesion by 54.2%, and capsule-associated mucoviscosity by approximately 57%. These phenotypic changes were accompanied by significant downregulation of *fimH*, *mrkA*, *mrkD*, *rmpA*, *entB*, and *iucA*. [32] similarly demonstrated 10-, 7-, and 14-fold reductions in *fimH*, *rmpA*, and *mrkA*, respectively, following AgNP treatment. The present findings therefore support previous evidence that AgNPs suppress pathogenicity not only by inhibiting growth but also by directly interfering with adhesion, capsule formation, iron acquisition, and biofilm-associated gene expression. The strongest gene suppression in the present study was observed for *mrkA*, with an approximately 7.2-fold reduction, equivalent to an 86.1% decrease in relative expression. This is consistent with findings from AgNP-based chitosan–NaF nanomaterials, which significantly downregulated *mrkA*, *fimA*, *treC*, and *ecpA* while simultaneously disrupting *K. pneumoniae* biofilm architecture [33]. These observations emphasize the central importance of fimbrial genes in nanoparticle-mediated antibiofilm activity. Correlation analysis further demonstrated that biofilm biomass was positively associated with *mrkA* ($r = 0.76$) and *fimH* ($r = 0.69$), whereas AgNP concentration was negatively correlated with bacterial growth ($r = -0.84$) and biofilm biomass ($r = -0.88$). Similar biological relationships have been reported in studies

showing that increased expression of fimbrial and adhesion-associated genes promotes stronger biofilm development, whereas AgNP exposure reduces both biofilm biomass and expression of these determinants [34]. Collectively, the present findings are in good agreement with previous investigations demonstrating that AgNPs possess multiple complementary activities against MDR *K. pneumoniae*. However, the magnitude of antibacterial and antibiofilm activity varies considerably among studies, with reported MIC values ranging from below 1 µg/mL to approximately 128 µg/mL. Such variation probably reflects differences in nanoparticle composition, synthesis approach, particle size, stabilizing molecules, bacterial genotype, and experimental methodology. Overall, the strong antibacterial activity, substantial inhibition and disruption of biofilms, and suppression of major virulence determinants observed in this study support further evaluation of AgNPs as an alternative or adjunctive approach for controlling difficult-to-treat MDR *K. pneumoniae* infections.

5. Conclusion

The present study demonstrates that silver nanoparticles possess strong antibacterial, antibiofilm, and anti-virulence activity against multidrug-resistant *Klebsiella pneumoniae*. AgNP treatment significantly inhibited bacterial growth, reduced both initial and mature biofilm formation, and suppressed key virulence-related genes involved in adhesion, capsule formation, and siderophore production. The concentration-dependent activity of AgNPs, together with their bactericidal effect, highlights their potential as an alternative or adjunctive strategy against difficult-to-treat MDR infections. Further in vivo studies and toxicity assessments are required to confirm their safety and therapeutic applicability.

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