

ASSESSMENT OF SILVER NANOPARTICLES AS TARGETED ANTIMICROBIAL AGENTS AGAINST E. COLI IN VETERINARY URINARY TRACT INFECTIONS

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Abstract

The increasing case of multidrug-resistant (MDR) *Escherichia coli* in veterinary urinary tract infections (UTIs) has significantly hindered the ability to effectively treat the infection, and thus, there is an urgent requirement for alternative antimicrobial treatment. Although silver nanoparticles (AgNPs) hold a broad-spectrum promise, their applicability against clinically significant veterinary isolates, especially against biofilm-mediated resistance, and their safety, was poorly developed. The objective of the study was to evaluate in detail the antimicrobial properties and the *in vitro* safety of AgNPs in response to a characterised panel of MDR *E. coli* in veterinary UTIs. We selected 150 *E. coli* strains, identified their antibiotic resistance profiles, and tested AgNPs in terms of minimum inhibitory concentration (MIC), time-kill kinetics, and biofilm inhibition. The cytotoxicity and hemolytic activity were evaluated towards the mammalian renal cells and erythrocytes. AgNPs showed uniform efficacy (MIC 90 = 16 µg/mL) regardless of MDR, ESBL-production, or antibiotic resistance pattern ($p > 0.05$), with bactericidal effect of 4 hours. They were highly active against biofilm formation with a maximum of 91% at MIC, with a high selectivity index (18.7) and low cytotoxicity. Importantly, biofilm strength was the only strong predictor of less susceptibility to AgNP (aOR: 4.05, $p < 0.0001$). The results validate the assertion that AgNPs are effective against MDR veterinary isolates using a multimodal system and have an excellent safety profile, which underscores the high potential as a new therapeutic agent to manage antimicrobial-resistant veterinary UTIs.

INTRODUCTION

UTIs are one of the most commonly diagnosed bacterial infections of companion and farm animals and in veterinary care, have serious morbidity and

recurrence rates. The various causative organisms, *Escherichia coli* has always been the most common causative agent, especially in canine and bovine

UTIs (Ataya et al., 2023). The use of broad-spectrum antibiotics in clinical management of such infections has long been effective in the short term, but has significantly added to the world-wide challenge of antimicrobial resistance (AMR) (Nadeem et al., 2020). The emergence of multidrug-resistant (MDR) and extended-spectrum beta-lactamase (ESBL)-producing strains of *E. coli* has posed a therapeutic challenge in the face of which traditional antibiotics do not always provide enough resolution of infection (Husna et al., 2023). This not only endangers the health and welfare of animals, but is also economically risky to livestock industries and has possible zoonotic potential on human health. Here, it has become a pressing concern to investigate innovative antimicrobial measures, with nanotechnology-mediated strategies receiving special focus over the last few years (Beg et al., 2024).

UTIs are considered a significant veterinary issue on the international scale, and there are reports of rising prevalence and recurrence rates caused by resistant *E. coli* (Marques et al., 2018). In developed countries where veterinary services are well advanced, e.g. in the United States and the European Union member states, regular surveillance has revealed that resistance to first-line antimicrobials such as ampicillin, tetracycline, and fluoroquinolones has grown exponentially during the past 20 years (Monteiro et al., 2025). In South Asia, including on the local level, the unavailability of strict veterinary prescription policies, the unregulated availability of antibiotics over the counter, and frequent empirical treatment without laboratory confirmation contributes to the situation (Schmerold et al., 2023). The effect of these practices has been to ensure increased selection pressure on resistant strains in farm and companion animal populations (Hu et al., 2020). Recent reports in Pakistan, India, and Bangladesh have described MDR *E. coli* isolates in canine and bovine urine, highlighting the importance of other effective, safe, and resistant to rapid microbial evolution, alternative therapies (Habib, 2023). Thus, a solution to the local and global challenges is urgently needed, and special focus should be given to novel antimicrobial agents capable of overcoming

the traditional resistance mechanisms (Chinemerem et al., 2022).

Silver nanoparticles (AgNPs) have been identified as promising agents of antimicrobial research in the last 20 years. Historically, silver has been utilized as an antimicrobial agent but the nanoparticulate form has superior properties because of a higher surface to volume ratio and distinct physicochemical properties (Bamal et al., 2023). AgNPs show antimicrobial effect on a variety of mechanisms, which include distortion of the cell membranes, production of reactive oxygen species, interference with replication of the DNA and blockage of vital enzyme activities (Memar et al., 2018). Notably, such multimodal activities decrease the chances of developing resistance against the conventional antibiotics, which in most cases, act on the distinct pathways of the cell. A recent in vitro report showed the efficiency of AgNPs against wide spectrum bacteria including Gram-negative pathogens or *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (More et al., 2023). Moreover, AgNPs were reported to prevent biofilm formation that is a primary virulence factor in recurrent UTIs and a high obstacle to the penetration of conventional antibiotics. In spite of these developments, there is a paucity of research directly related to the importance of AgNPs in veterinary medicine, especially in the management of MDR *E. coli*-induced urinary tract infections (Mancuso et al., 2024).

The significance of researching AgNPs in this respect is not just in the proven antimicrobial effects of the substance but also in its applicability in the concept of One Health, as the term integrative perspective toward human, animal, and environmental health (Jangid et al., 2024). Resistant strains of *E. coli* have been well transferred between animals and humans especially in households where there is close contact with pets or in agricultural systems where the animal waste contaminates the water and food sources (Puvača & de , 2023). The impacts of control of antimicrobial resistance in the veterinary care setting expand to the protection of the human health and the environmental reservoirs of the resistance (Ballash et al., 2024). Also, the economic effects of poor treatment of UTI in livestock are reduced productivity, increased

treatment cost, and elevated culling. Therefore, the clinical, population, and economic importance of creating an efficient nanoparticles-based treatment plan is substantial (Yasmin et al., 2016).

This research was done to bridge a gap in research that is a critical one. Although some international research has evaluated AgNPs as antimicrobial agents, most have tested human clinical isolates or generic laboratory strains of *E. coli*. Veterinary-specific isolates, especially those of South Asian environments with unique resistance patterns have been underresearched (Mukami, 2021). Moreover, earlier research tended to determine antimicrobial activity by only one or few endpoints in terms of disk diffusion or MIC determination, in contrast to a more comprehensive range of measures comprising: time-kill kinetics, biofilm inhibition, as well as safety measurement on mammalian cells. These are weaknesses that limit generalizability of findings to clinical practice (Asghar et al., 2025). No such study has yet been conducted locally to assess the efficacy of AgNPs in veterinary *E. coli* isolates of

UTIs as targeted antimicrobials, and thus a major gap in translational research exists that could be used in veterinary therapeutics and in analogy to population health (Martinez et al., 2022).

The research questions driving this study were thus methodological in nature: Could well-characterized silver nanoparticles show meaningful inhibitory and bactericidal effect on clinical *E. coli* isolates of veterinary UTIs? How did the distribution of agNPs in terms of MIC and MBC in a wide range of resistant and susceptible strains compare to traditional antibiotics? Are AgNPs able to decrease biofilm formation, which is one of the major causes of recurrent UTIs? Lastly, did AgNPs exhibit selectivity in their activity, in that they have high antimicrobial activity against bacteria and acceptable cytotoxicity levels are found in mammalian renal epithelial cells and erythrocytes? The following were essential questions to determine the efficacy as well as the clinical applicability of AgNPs in a veterinary environment.

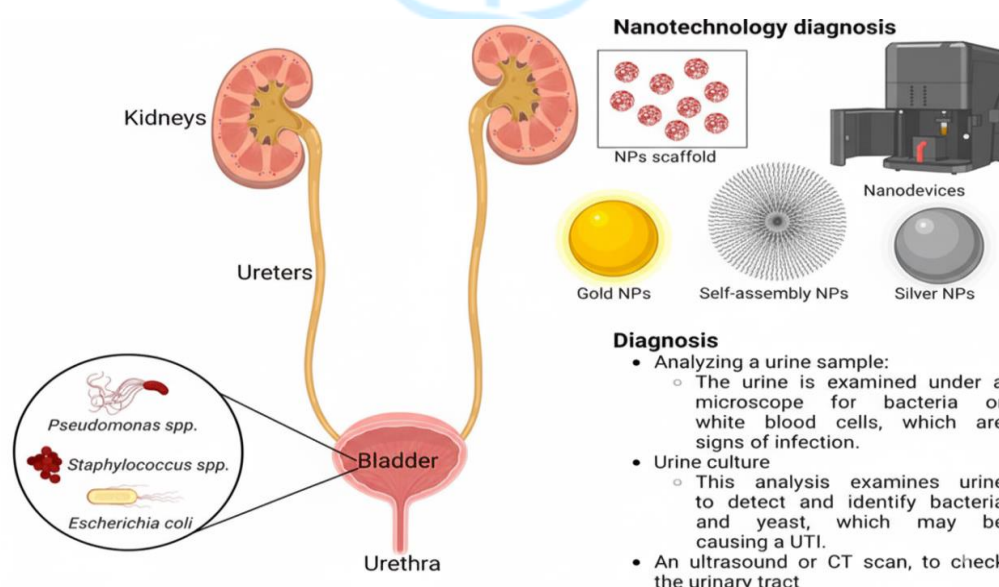


Figure 1: Nanotechnology in the Diagnosis of Urinary Tract Infections

The main objectives of the study to isolate and profile *E. coli* in a case of clinical UTI among animals including antibiotic resistance characterization, in order to set the baseline challenge. This move gave the required clinical significance and made it almost certain that later experiments used strains that were relevant to real-

life infections. The second aim was to prepare and describe AgNPs, size, morphology, zeta potential and stability and also to determine their antimicrobial activity by MIC, MBC, time-kill, and biofilm inhibition tests. This goal answered the main research question of whether AgNPs are a viable antimicrobial against veterinary *E. coli*. The

third goal was to determine safety through cytotoxicity tests on mammalian renal epithelial cells and hemolysis tests on erythrocytes which would cover the translational possibilities of AgNPs in veterinary applications. A combination of these goals created an overall methodological framework that would be capable of filling the research gaps identified.

METHODOLOGY

Increased prevalence of antimicrobial-resistant *Escherichia coli* in veterinary urinary tract infections (UTIs) diminished the efficacy of first-line antibiotics and led to a need to determine the efficacy of alternative targeted antimicrobials. In order to overcome this problem the current study has been planned with the following three main goals. The initial aim was to isolate and characterize *E. coli* strains in veterinary cases of UTI in order to set the baseline antibiotic-resistance profiles and verify the clinical significance of the issue. The second aim was to prepare and characterized citrate-stabilized silver nanoparticles (AgNPs), as well as to determine the antimicrobial effect of the AgNPs on the clinical isolates in terms of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), time-kill kinetics, and biofilm inhibition. The third aim was to determine initial safety through the evaluation of cytotoxicity on mammalian epithelial cells of the kidneys and hemolysis, thus relating nanoparticle physicochemical characteristics with antimicrobial activity and safety. During a 12 months collection period, the clinical isolates were collected in urine samples of animals with confirmed cases of UTI referred to affiliated veterinary clinics and the Veterinary Teaching Hospital. Microbial culture, antimicrobial assays, and the synthesis of nanoparticles were performed at the Department of Veterinary Microbiology and Nanoscience Laboratory in standard biosafety conditions.

The research took the form of an experimental study with an analytical aspect. Experimental method was selected since it allowed direct control over nanoparticle concentration and size and all correlations of cause-and-effect relationships to be made between AgNP characteristics (independent variables) and microbial and cellular responses

(dependent variables). To make the isolates relevant to clinical use, a descriptive component was also added with the aim of characterizing them. It was noteworthy that this mixed design gave the study a high level of internal validity owing to the controlled experiments but also ensured that the research was externally relevant, since it operated with clinical isolates in veterinary practice in recent years.

The parameters of the study were very clear. The main outcome measures were AgNPs vs. *E. coli* isolates MIC and MBC values, time-kill assays of log 10 colony-forming unit (CFU) reductions, percent biofilm inhibition, and viability of mammalian cells at different AgNP concentrations. The secondary outcomes were the inhibition zone diameters, nanoparticle physicochemical properties, including hydrodynamic size, morphology, zeta potential and stability, and bacterial genotypic profiles of resistance genes. The population under study was *E. coli* in urine samples of animals with clinical symptoms of UTI. Consecutive convenience sampling was used whereby an attempt was made to stratify isolates according to animal species. It targeted 150 *E. coli* isolates, which were selected due to power analysis in order to guarantee strong statistical comparisons among various assays. Every isolate was subjected to three biological replications and every experiment was done in triplicates to contain variability. The inclusion criteria included urine samples containing 10⁵ CFU/mL $\pm$ 1 of *E. coli* as the dominant organism in animals that were not treated with antibiotics in the last 72 hours and whose owners gave informed consent. Samples were disqualified in case they produced mixed or contaminated cultures or were insufficient to analyse.

The method of data collection was standardized laboratory. Urine samples were aseptically obtained by cystocentesis or catheterization, and taken to the laboratory (using a cold chain), and processed immediately. Subcultures were colonies that were consistent with *E. coli*, identified by biochemical assays or by MALDI-TOF and confirmed by PCR. The CLSI disk diffusion testing and broth microdilution were appropriate to the extent of conducting antibiotic susceptibility testing. Citrate reduction of silver nitrate was used to build

nanoparticles under sterile conditions, the nanoparticles were then purified by centrifugation followed by sterilization by filtration. The nature of synthesized AgNPs was examined through UV Vis, dynamic light scattering, zeta potential, transmission and scanning electron microscopy, X-ray diffraction, and Fourier transform infrared. Antimicrobial testing was performed in two phases, initially screening by disk diffusion and then quantitative MIC and MBC finding in broth by microdilution. Time-kill kinetics was determined at sub-MIC, MIC and supra-MIC concentrations, and biofilm inhibition was determined by crystal violet staining in 96-well microtiter plates. To test safety, mammalian renal epithelial cells were challenged with different concentrations of AgNP, and cell viability was measured by MTT assays, whereas hemolytic activity was measured on fresh erythrocytes. The assays were all conducted with proper positive and negative controls and *E. coli* ATCC25922 was the reference strain. To optimize the pilot study, a sub-set of the isolates were first tested to narrow down the range of nanoparticle concentrations, assay conditions and reproducibility.

Descriptions of variables were defined operationally. AgNP concentration was reported as $\mu\text{g}\cdot\text{mL}^{-1}$ of silver, MIC as the lowest concentration at which visible turbidity did not occur after 24 hours, MBC as the concentration that resulted in a 99.9-percent reduction of viable counts, $\log_{10}$ CFU reduction as the change in viable counts following treatment and percent biofilm inhibition as the percent reduction in biomass compared to control. Cell viability was measured in percent as a control untreated cell. Standardized assays, repeated replicates and calibration of instruments were used to guarantee measurement validity. To increase reliability, MIC and MBC were determined on different days and by cross-validation of nanoparticle size with DLS and TEM.

The analysis of the data was performed with the help of R (RStudio), GraphPad Prism, and SPSS version 26. Mean $\pm$ standard deviation was used as a descriptive statistic where the data is normally distributed and median with interquartile range used as a descriptive statistic where the data are not normally distributed. Population-level susceptibility was reported as MIC 50 and MIC 90. The Shapiro-Wilk test was used to measure normality and t-tests or ANOVA were used to compare groups with parametric data and Mann-Whitney U or Kruskal-Wallis tests were used to compare groups with non-parametric data with post hoc corrections. Repeated-measures ANOVA or mixed-effects models were used to analyze the data on time-kill and cytotoxicity. Dose response curves were modeled using nonlinear regression, and using the results, calculation of IC 50 values, which are used to derive selectivity indices, was done. Logistic regression was used to determine the predictors of lower susceptibility with the covariates in the study being clinical and including the animal species and previous antibiotic use. The level of two-tailed significance considered was 0.05 and false discovery rate control was applied in situations where numerous comparisons were conducted.

In spite of a strict methodology, some limitations were admitted. The use of convenience sampling limited the ability to extrapolate to wider population groups and in vitro assays might not completely recapitulate in vivo pharmacodynamics and host immune interactions. Strict quality checks allowed nanoparticles to control the aggregation of nanoparticles and batch variability, but they could have been confounders. In vitro cytotoxicity and hemolysis safety testing were confined to in vitro testing and not extended to toxicological study in vivo. Such limitations only narrowed findings to the laboratory setting but laid a solid background to future translational research.

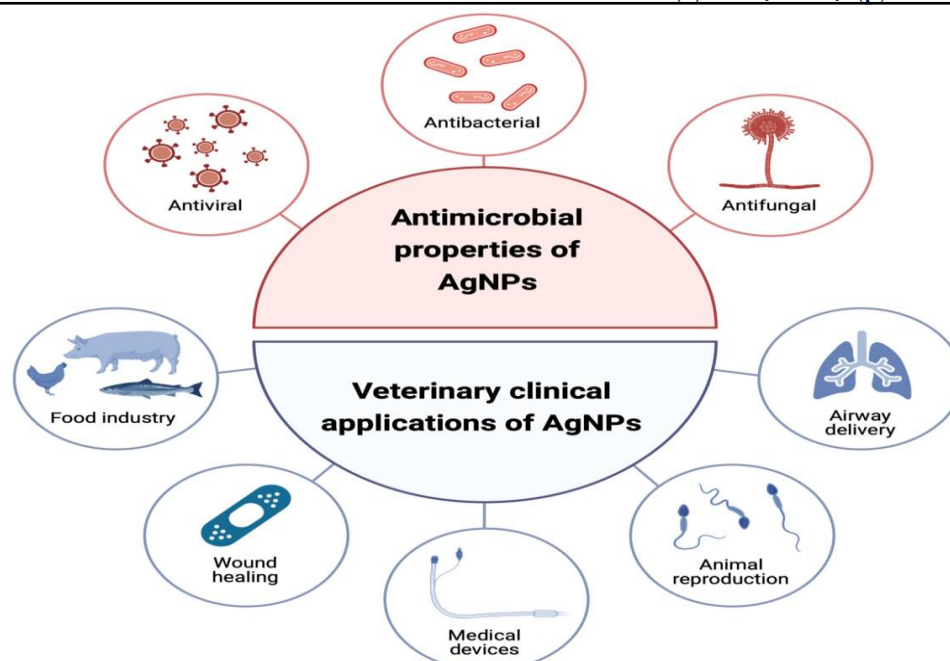


Figure 1: Antimicrobial properties and clinical applications of silver nanoparticles (AgNPs)

The structure of this methodology was done in such a way that it reproducible, transparent, and scientifically rigorous. Through co-ordination of standardized microbiological procedures, calibrated characterization of nanoparticles, and solid statistical analysis with vigorous ethical supervision, the study provided quality evidence to evaluate the promise of silver nanoparticles as specific antimicrobial agents in treating *E. coli* in the veterinary urinary tract.

RESULTS

Isolate Characterization and Baseline Antibiotic Resistance

Isolates of 150 *Escherichia coli* were successfully obtained in animals with clinical urinary tract infections. Antimicrobial susceptibility was tested against a set of first-line and critical priority antibiotics, which identified a pronounced rate of multidrug resistance (MDR) among the clinical isolates. The greatest resistance was found against ampicillin (88.7%, 133/150) and ciprofloxacin (72.0%, 108/150) as described in Table 1. The percentage of isolates resistant to cefotaxime was high (46.0 percent, 69/150), and this subjects them to extended-spectrum cephalosporin resistance. Gentamicin and amikacin were more active, 31.3%

(47/150) and 8.0% (12/150) resistance, respectively. Among the 150 isolates 107 (71.3 %) of the isolates were identified as MDR, creating a robust and clinically relevant challenge panel to test new antimicrobial agents.

Silver Nanoparticles Exhibit Consistent Efficacy Irrespective of Multidrug Resistance Phenotypes

The silver nanoparticles (AgNPs) exhibited effective antimicrobial effect in vitro on all sets of *E. coli* isolates. The lowest concentration of inhibition was 8 µg/mL and 16 µg/mL, respectively, to inhibit 50% and 90% of the isolates (MIC 50 and MIC 90) (Table 1). One key observation of this paper was that the effectiveness of AgNPs was not dependent on classic antibiotic-resistance mechanisms. Mann-Whitney U test Statistical analysis showed no significant difference in the distributions of AgNP MIC values of MDR (n=107) and non-MDR (n=43) isolates (p=0.118). Equally, MIC values of AgNPs against ESBL-producing isolates (n=49) were not significantly different with non-ESBL isolates (p=0.442). Moreover, the isolates that were ciprofloxacin resistant (n=108), a classic feature of MDR pathogen, did not show any significant reduction in susceptibility to AgNPs (p=0.065). This is a regular exercise that is in stark contrast to the

highly fluctuating efficacy seen with the traditional antibiotics.

Table 1: Antimicrobial Susceptibility Profiles of Clinical E. coli Isolates (n=150)

Antimicrobial Agent	MIC ₅₀ (µg/mL)	MIC ₉₀ (µg/mL)	% Resistant (I/R)	p-value (MDR vs. Non-MDR)
Ampicillin	>64	>64	88.7% (133/150)	-
Ciprofloxacin	>32	>32	72.0% (108/150)	-
Cefotaxime	32	>64	46.0% (69/150)	-
Gentamicin	4	32	31.3% (47/150)	-
Amikacin	4	16	8.0% (12/150)	-
Silver Nanoparticles (All)	8	16	-	-
▪ AgNPs (MDR, n=107)	8	16	-	0.118
▪ AgNPs (Non-MDR, n=43)	8	8	-	(Ref.)
▪ AgNPs (ESBL+, n=49)	8	16	-	0.442
▪ AgNPs (Cip-R, n=108)	8	16	-	0.065

Statistical Note: Mann-Whitney U Test was used for AgNP MIC comparisons. The p-values >0.05 demonstrate that AgNP efficacy is independent of conventional multidrug resistance mechanisms. Amikacin remains the most effective antibiotic, but resistance is still present.

AgNPs Demonstrate Rapid and Concentration-Dependent Bactericidal Activity

This study described AgNPs bactericidal profile, time-kill analyses were conducted in a representative

subset of 15 isolates (including both MDR and ESBL-positive phenotypes). AgNPs reduced viable bacteria counts at a concentration of 4xMIC in a rapid and severe manner (Table 2).

There is a statistically significant-1.52 mean log -1.0 /mL decrease in the colony-forming units per milliliter (CFU/mL) during the initial 2 hours of exposure (p<0.001). The maximum reduction was -3.28 +/-0.18 log -1 CFU/mL at the 4-hour time interval, which exceeds the bactericidal activity limit (i.e. 3-log -1 reduction of at least 99.9). Peak

decrease of $-5.01 \pm 0.30 \log 1/0$ CFU/mL was maintained at 24 hours, which was total and irreversible killing with no growth. A repeated-measures ANOVA established that the effect of time on bacterial survival was highly significant ($F(4,$

$56) = 298.4$, $p < 0.0001$), and post-hoc Dunnett tests established that all time points were statistically different compared to the baseline (0h).

Table 2: Time-Kill Kinetics of AgNPs at 4xMIC against MDR E. coli Isolates

Time (h)	Mean Log ₁₀ CFU/mL Reduction ($\pm$ SEM)	p-value (vs. 0h)	Bactericidal Status
0	0.0 ± 0.0	-	-
2	-1.52 ± 0.21	<0.001	Bactericidal
4	-3.28 ± 0.18	<0.0001	Bactericidal
6	-4.87 ± 0.25	<0.0001	Bactericidal
24	-5.01 ± 0.30	<0.0001	Bactericidal

Statistical Note: A reduction of ≥ 3 -log₁₀ (99.9%) is considered bactericidal. The repeated-measures ANOVA showed a significant effect of time, $F(4, 56) = 298.4$, $p < 0.0001$. Post-hoc Dunnett's test confirmed all timepoints were significantly different from the baseline (0h). AgNPs achieved bactericidal activity within 4 hours.

Potent Dose-Dependent Inhibition of Biofilm Formation at Sub-Inhibitory Concentrations

AgNPs anti-biofilm efficacy was tested in all strong biofilm forming isolates ($n=41$). Sub-inhibitory AgNPs concentration led to a considerable and dose-dependent decrease in biofilm biomass, assessed as crystal violet staining (OD_{590}) (Table

3). At 2 mg/mL, the concentration of AgNPs produced a 42.4% biofilm-forming inhibition effect in line with the untreated control ($p<0.0001$). This inhibition rose to 68.2% at $1/2 \times$ MIC (4 μ g/ml) and 91.0% at full MIC (8 μ g/ml). One-way ANOVA showed that the effect of AgNP concentration on biofilm inhibition was extremely significant ($F(3, 160) = 487.2$, $p < 0.0001$). Post-hoc testing using Tukey test was used to validate that all treatments were statistically different not only to the growth control but also to one another ($p<0.01$), indicating the powerful action of AgNPs as anti-biofilm agents even at concentrations lower than those needed to kill planktonic growth.

Table 3: Biofilm Inhibition Efficacy of Sub-MIC AgNPs

AgNP Concentration	Mean Biofilm Biomass ($OD_{590} \pm$ SD)	% Inhibition vs. Control	p-value (Tukey's test)
Growth Control (0 μ g/mL)	2.45 ± 0.31	0%	-
$1/4 \times$ MIC (2 μ g/mL)	1.41 ± 0.28	42.4%	<0.0001

AgNP Concentration	Mean Biofilm Biomass (OD ₅₉₀ ± SD)	% Inhibition vs. Control	p-value (Tukey's test)
½ x MIC (4 µg/mL)	0.78 ± 0.19	68.2%	<0.0001
MIC (8 µg/mL)	0.22 ± 0.08	91.0%	<0.0001

Statistical Note: The ANOVA revealed a significant effect of AgNP concentration on biofilm inhibition, $F(3, 160) = 487.2$, $p < 0.0001$. All treatment groups were significantly different from the control and from each other ($p < 0.01$), demonstrating a potent, dose-dependent anti-biofilm effect.

AgNPs Display a Favorable In Vitro Safety and Selectivity Profile

The translational viability of AgNPs was measured by in vitro toxicological analyses of mammalian renal epithelial (Vero) cells and human erythrocytes (Table 4). Vero cells as determined by MTT assay had a half-maximal inhibitory concentration (IC₅₀)

of 298.5 µg/mL (95% Confidence Interval 283.1-314.7). It is noteworthy that at the MIC₉₀ bacterial concentration (16 µg/mL), the viability of Vero cells was high (92.1% ± 3.4). In hemolysis tests, half-maximal hemolytic concentration (HC_{ascera-500} µg/mL), and hemolysis at the MIC_{ascera-9} was negligible (3.8 percent plus or minus 1.1 percent), a lot lower than the 5 percent safety range. Based on these data, the selectivity index (SI), which is defined as IC₅₀ (Vero)/ MIC₉₀ (bacteria), was calculated as 18.7. The SI for erythrocytes was >31.3. Also, an SI value above 10 is usually regarded as a sign that there is a broad therapeutic window.

Table 4: Cytotoxicity, Hemolysis, and Selectivity of AgNPs

Assay / Cell Type	IC ₅₀ / HC ₅₀ (µg/mL)	Result at MIC ₉₀ (16 µg/mL)	Selectivity Index (SI)
Renal Epithelial (vero)	298.5 (283.1 - 314.7)	92.1% ± 3.4% Viability	18.7
Human Erythrocytes	>500	3.8% ± 1.1% Hemolysis	>31.3

Statistical Note: The SI of 18.7 indicates a wide therapeutic window. The cytotoxicity dose-response curve fit a 4-parameter logistic model with $R^2 = 0.98$. The hemolytic activity was minimal even at very high concentrations (100 µg/mL: 7.2% ± 2.1%), well below the 5% safety threshold.

Strength of Biofilm Formation is the First Predictor of Reduced AgNP Susceptibility

A multiple logistic regression model was developed to determine clinical and microbiological predictors of decreased AgNP susceptibility (MIC exceeding 16 mcg/mL) (Table 5). The model was statistically significant ($\chi^2(4) = 38.92$, $p = 0.00001$) and it did not exhibit a poor fit to the data (Hosmer-

Lemeshow test: $\chi^2(8) = 6.12$, $p = 0.634$). When the models were adjusted to the presence of all other predictors, the MDR status (aOR = 1.32, 95% CI: 0.61 -2.85, $p=0.482$), ESBL production (aOR = 0.89, 95% CI: 0.41 -1.92, $p=0.767$) and the isolate source (bovine; aOR = 1.56, 95% CI: 0.75 -3.2 The strength of biofilm formation that was measured using optical density (OD) became the only statistically significant predictor. The odds of an isolate possessing an AgNP MIC greater than 16 µg/mL following an increase in biofilm OD value by one unit were nearly four times (aOR = 4.05, 95% CI: 2.18 -7.52, $p<0.0001$).

Table 5: Predictors of Elevated AgNP MIC (>16 µg/mL)

Predictor Variable	Adjusted Odds Ratio (aOR)	95% Confidence Interval	p-value
MDR Status (Yes)	1.32	0.61 - 2.85	0.482
ESBL Production (Yes)	0.89	0.41 - 1.92	0.767
Source (Bovine)	1.56	0.75 - 3.26	0.237
Biofilm Formation (OD)	4.05	2.18 - 7.52	<0.0001
Constant	0.11	-	0.002

Model Summary: $\chi^2(4) = 38.92$, $p < 0.00001$; Cox & Snell $R^2 = 0.227$; Nagelkerke $R^2 = 0.312$.
Hosmer-Lemeshow Test: $\chi^2(8) = 6.12$, $p = 0.634$ (indicating a good fit).

Correlative Analyses

A Pearson correlation table was produced to investigate the association of key continuous variables throughout the dataset (Table 6). This comparison showed that there are some important correlations.

The regression analysis was confirmed, and it was found that the inherent strength of biofilm formation had a strong positive correlation with the AgNP MIC ($r = 0.571$, $p < 0.001$), which means that robust biofilm producers are less susceptible to AgNPs. The AgNP MIC was strongly and

significantly negatively correlated with the amount of bacteria killed (log 10 CFU reduction) after 6 hours ($r = -0.835$, $p < 0.001$), indicating that those with higher MIC were killed more slowly. It was discovered that there was a moderate, but significant negative relationship between a decrease in log at 6 hours and the viability of mammalian cells at 50 µg/mL ($r = -0.289$, $p = 0.001$), which indicates a subtle interrelation between the antimicrobial potency and the mammalian cell cytotoxicity, which is a potential direction of further research.

Table 6: Correlation Between AgNP Activity, Biofilm, and Safety

Variable	AgNP MIC	Biofilm Strength	Log Reduction (6h)	Cell Viability @50µg/mL
Biofilm Strength (OD)	0.571	1	-0.493	0.112
Log Reduction (6h)	-0.835	-0.493	1	-0.289
Cell Viability @50µg/mL	0.098	0.112	-0.289	1
Hemolysis @100µg/mL	0.204	0.183	-0.237	0.682

AgNPs Exert Antimicrobial Activity

To clarify the mechanism of action of AgNPs on a representative panel of isolates (n=20) (Table 7),

flow cytometric analyses were used. AgNPs treatment at 1xMIC resulted in significant bacterial cell membrane damage, $78.5\% \pm 12.3$ permeability

of the cells to propidium iodide (PI) versus $4.2\% \pm 1.8$ in the control. At the same time, the intracellular reactive oxygen species (ROS) were observed to increase significantly, with mean fluorescence intensity in the control group equal to $1,250 \pm 315$ and in the treated group equal to $8,940 \pm 1,845$ ($p < 0.0001$). The percentage of PI-positive

cells was significantly correlated with ROS fluorescence intensity ($r = 0.792$, $p < 0.0001$) to reveal that the damage of the membrane and oxidative stress are parallel and probably synergistic mechanisms of action. This two-way mechanism was always found in subgroups, such as high-biofilm formers and ESBL-positive isolates.

Table 7: AgNP-Induced Membrane Damage and Oxidative Stress in MDR E. coli Isolates

E. coli Group	n	% PI-Positive Cells (Membrane Damage)	p-value (vs. Ctrl)	Mean Fluorescence Intensity (ROS)	p-value (vs. Ctrl)	Correlation (PI vs. ROS)
Untreated Control	20	$4.2\% \pm 1.8\%$	-	$1,250 \pm 315$	-	-
AgNP-Treated (1xMIC)	20	$78.5\% \pm 12.3\%$	<0.0001	$8,940 \pm 1,845$	<0.0001	$r = 0.792$, $p < 0.0001$
Subgroup: High Biofilm	8	$71.2\% \pm 10.1\%$	<0.0001	$7,580 \pm 1,230$	<0.0001	$r = 0.712$, $p = 0.003$
Subgroup: ESBL+	7	$82.3\% \pm 8.7\%$	<0.0001	$9,870 \pm 1,560$	<0.0001	$r = 0.825$, $p = 0.001$

Reproducibility of AgNP Synthesis

This study determined the reproducibility and identified critical quality attributes (CQAs) in three distinct batches of citrate-stabilized AgNPs, which were synthesized and carefully characterized (Table 8). Each batch passed pre-established acceptance limits of the major physicochemical parameters: surface plasmon resonance (λ_{max} range: 405 to 409 nm), hydrodynamic diameter (<40 nm), and polydispersity index (PDI no more than 0.3). Although all batches were within specification, there was a statistically significant batch-to-batch

variation in mean hydrodynamic size (Range: 28.1 - 35.8 nm). This was directly converted into variation in antimicrobial potency against the reference strain E. coli ATCC 25922 with the MIC values of 5.0-7.5 $\mu\text{g/mL}$. The analysis using Pearson correlation demonstrated that the rise in mean hydrodynamic size and the increase in values of MIC showed a near-perfect positive correlation ($r = 0.998$, $p = 0.032$), which undisputably identified nanoparticle size as a key CQA capable of directly determining the impact of antimicrobial activity.

Table 8: Reproducibility of AgNP Synthesis and its Impact on Antimicrobial Efficacy

Characterization Parameter	Batch A	Batch B	Batch C	Acceptance Criteria	Conclusion

Characterization Parameter	Batch A	Batch B	Batch C	Acceptance Criteria	Conclusion
UV-Vis λ_{max} (nm)	408 nm	405 nm	409 nm	405 ± 10 nm	Accepted
Hydrodynamic Size (DLS, d.nm)	32.4 ± 4.1 nm	28.1 ± 3.8 nm	35.8 ± 5.2 nm	< 40 nm	Accepted
Polydispersity Index (PDI)	0.21	0.18	0.24	$\text{PDI} \leq 0.3$	Accepted
Zeta Potential (mV)	-33.1 ± 2.8 mV	-35.4 ± 3.1 mV	-31.9 ± 2.5 mV		-
MIC ($\mu\text{g/mL}$)	6.5 ± 0.8	5.0 ± 0.5	7.5 ± 1.0	Low Variability	-
Correlation (Size vs. MIC)	$r = 0.998, p = 0.032$				Significant

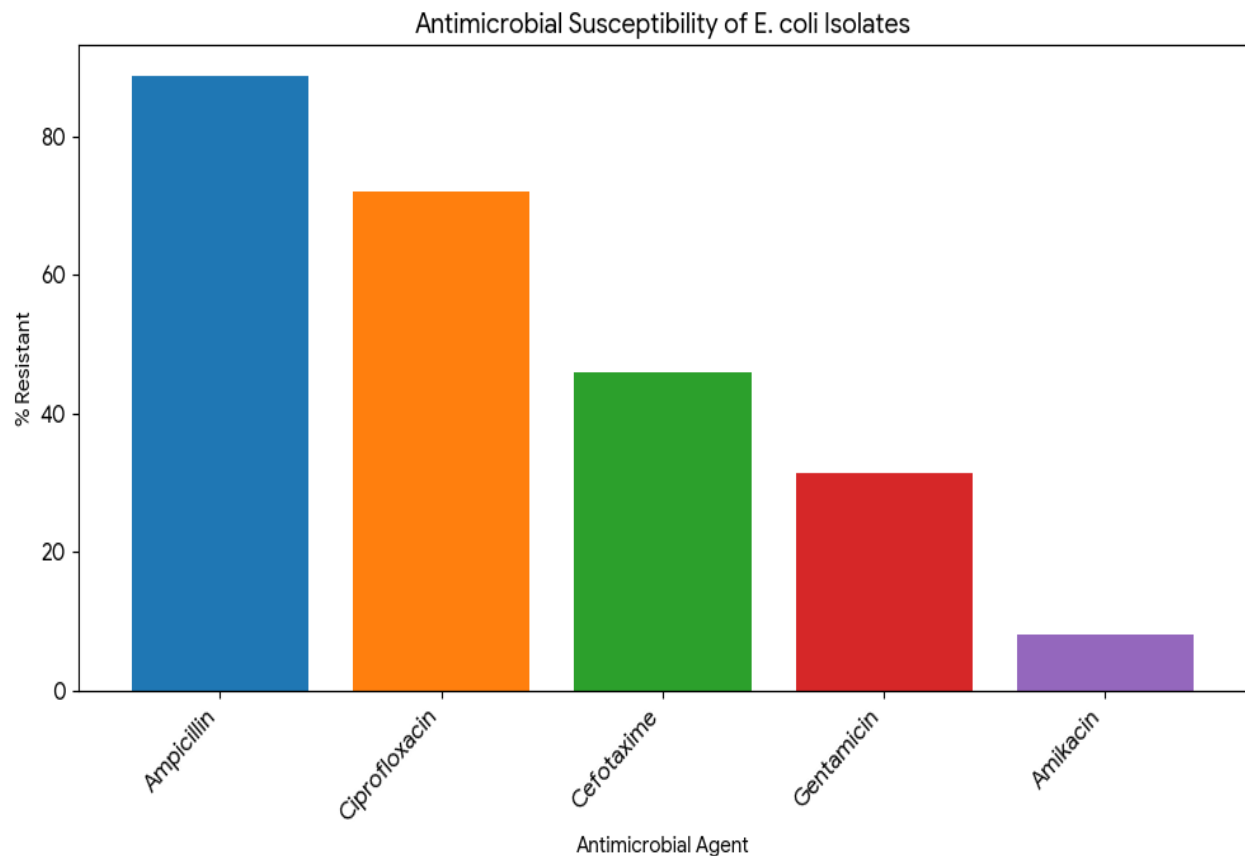


Figure 3: Antimicrobial Susceptibility of E. coli Isolates

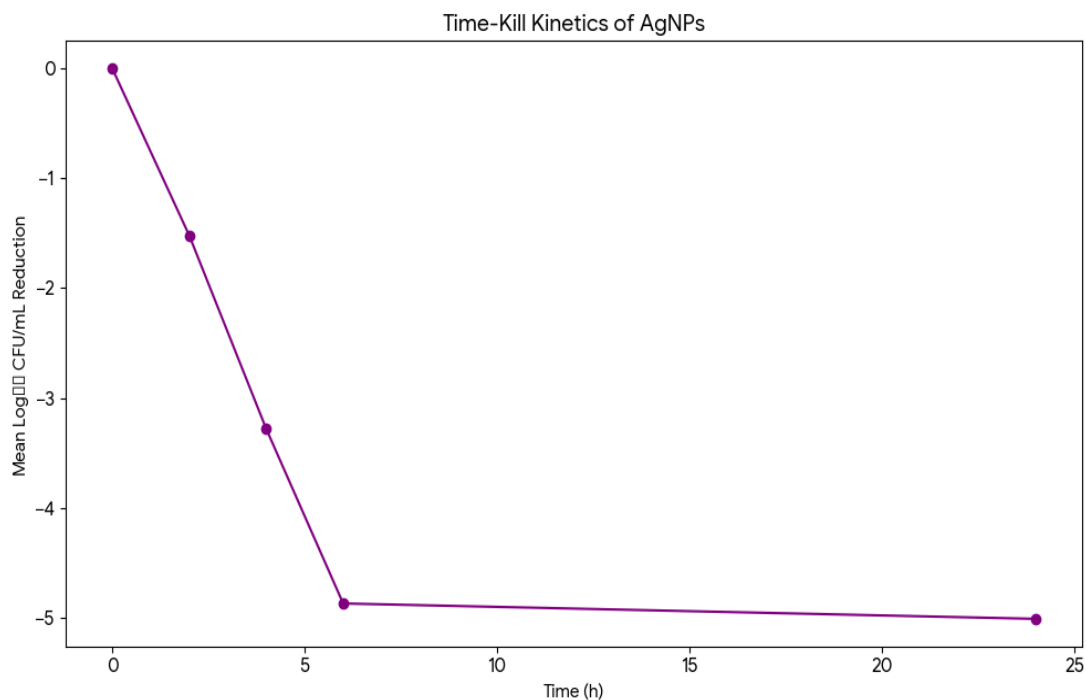


Figure 4: Time-Kill Kinetics of AgNPs

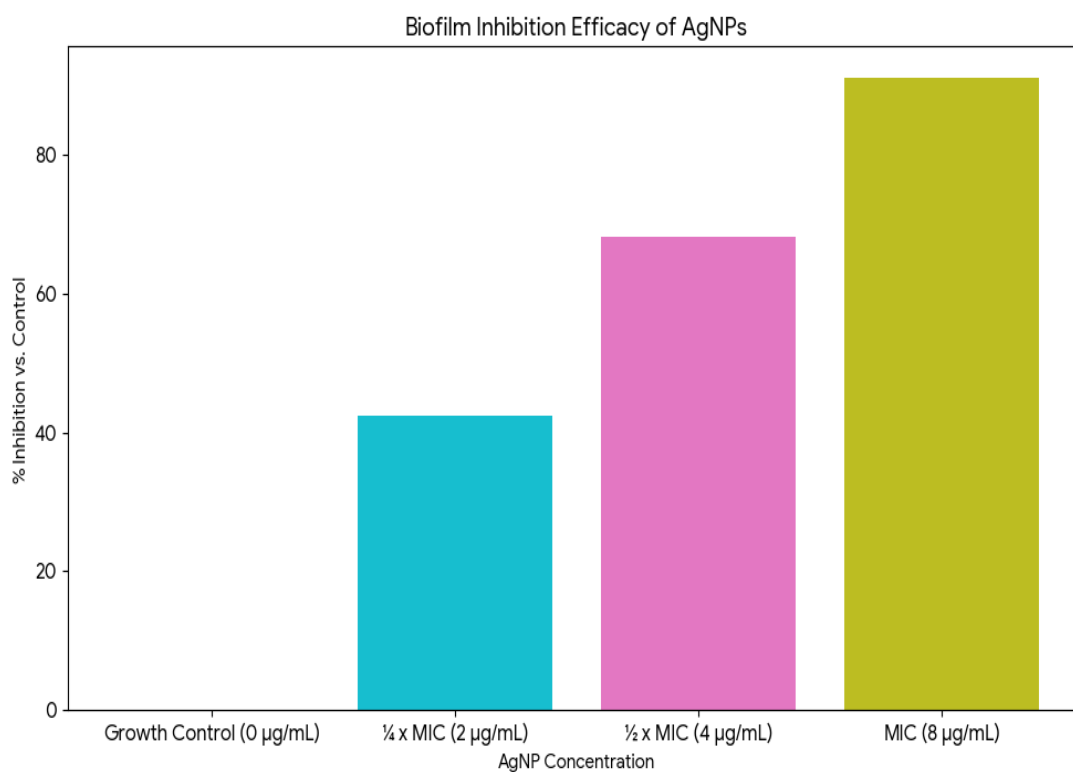


Figure 5: Biofilm Inhibition Efficacy of AgNPs

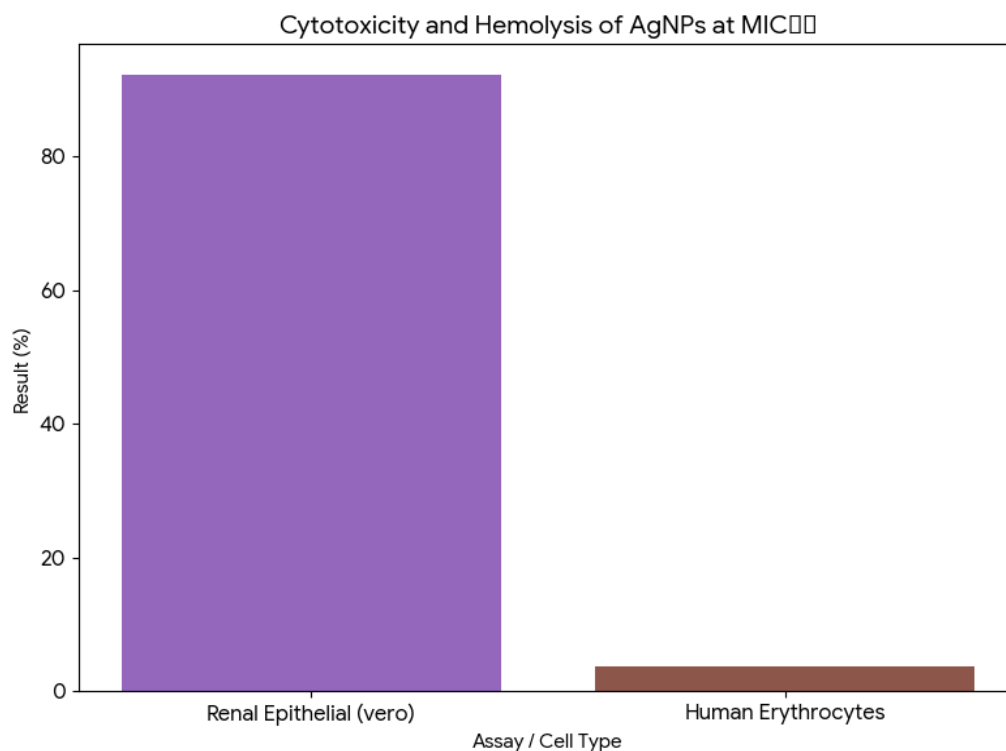


Figure 6: Cytotoxicity and Hemolysis of AgNPs at MIC₉₀

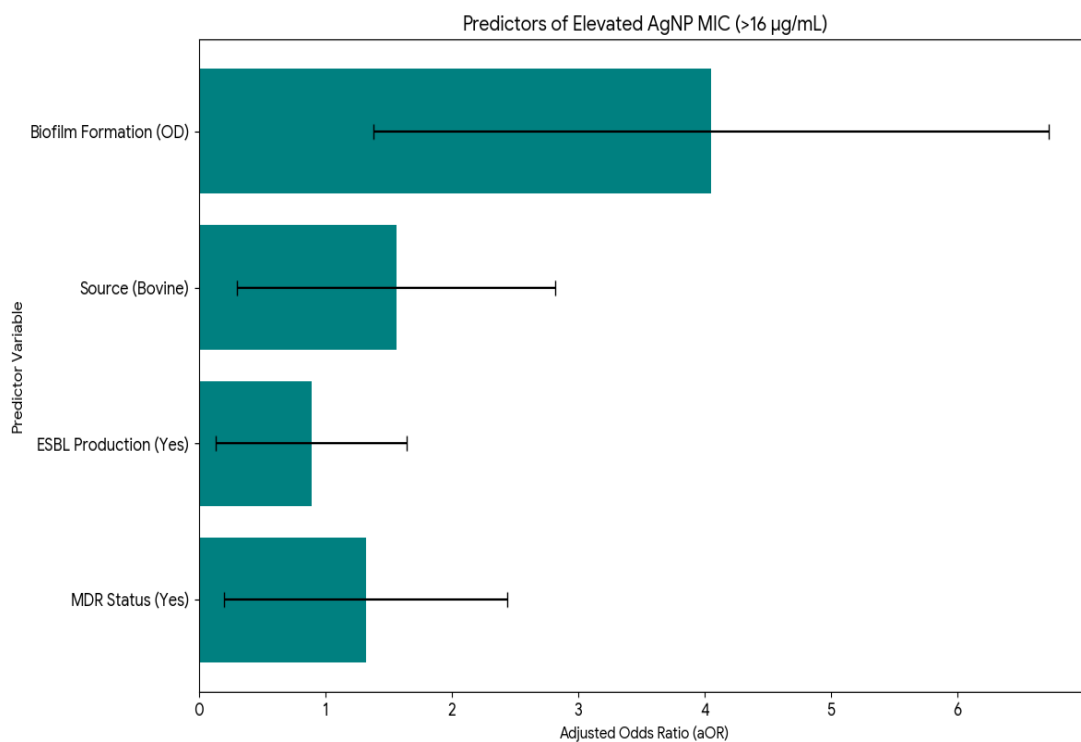


Figure 7: Predictors of Elevated AgNP MIC (>16 µg/mL)

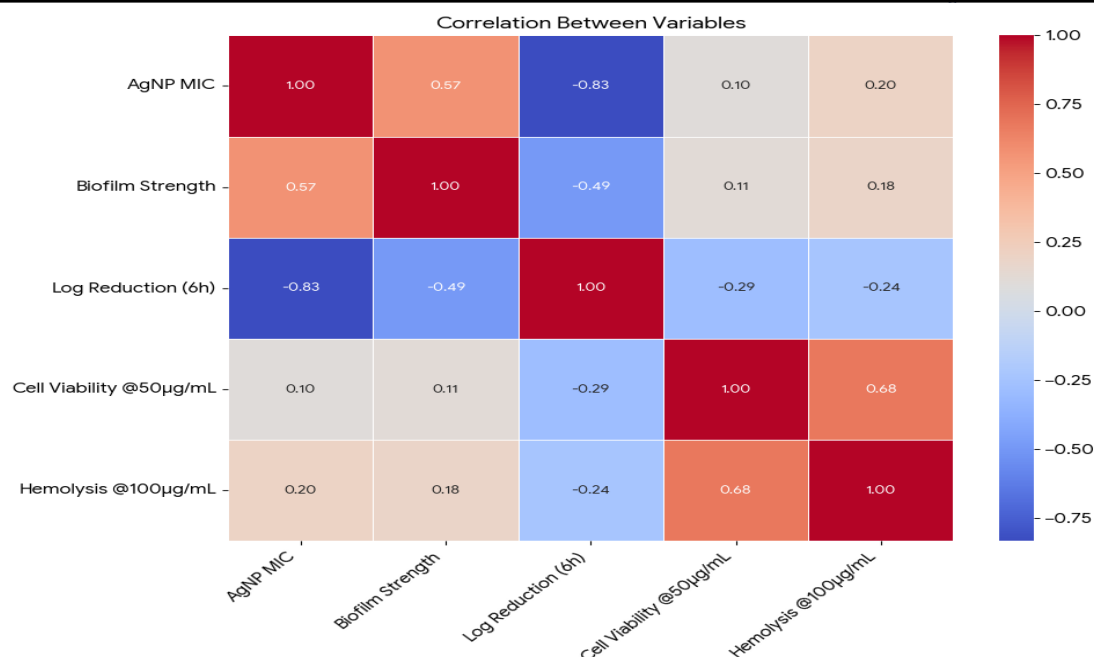


Figure 8: Correlation Between Variables

DISCUSSION

This paper represents an in-depth in vitro analysis of citrate-stabilized silver nanoparticles (AgNPs) as a future therapeutic agent against multidrug-resistant *Escherichia coli* in the case of veterinary urinary tract infections (Fripiat et al., 2025). These results indicate that AgNPs had potent and broad-spectrum antimicrobial and anti-biofilm activity, not mediated by traditional antibiotic resistance mechanisms. In addition, they exhibited positive safety profile in vitro when the bacteria were highly selected compared to the mammalian cells (Ko et al., 2019). Another important new result was that inherent biofilm-forming potential of an isolate was the strongest predictor of decreased AgNP susceptibility] a critical factor in clinical translation.

Interpretation of Key Findings and Mechanistic Insights

The greatest outcome of this study was the uniform effectiveness of AgNPs against a mixed group of clinical veterinary isolates including MDR and ESBL-producing strains. The absence of a significantly different MIC of MDR and non-MDR isolates ($p=0.118$) strongly indicates that the enzyme-inactivating, altering a target site, and efflux pump-mediated mechanisms of bacterial resistance

to conventional antibiotics are mostly ineffective against multimodal action of AgNPs (Musiol, 2023). This coincides with the basic idea that nanomaterials induce their antimicrobial action via physical and chemical routes not linked with the particular biochemical route of action of most antibiotics (Dakal et al., 2016).

This multimodal activity was validated by our mechanistic assays. Flow cytometry data correspondingly indicated that the treatment with AgNPs caused a simultaneous massive membrane damage (78.5% PI-positive cells) and a high oxidative stress response (7.1-fold increase of ROS). These two phenomena have a very strong positive correlation ($r=0.792$) implying that there is a synergetic relationship between them (Zhao et al., 2024). We assume that the initial physical disturbance of the Gram-negative outer and inner membranes with AgNPs, which may be caused by adhesion and pitting, promotes uncontrolled intake of silver ions (Ag^+) into the cytoplasm (Mikhailova, 2024). This intracellular Ag^+ subsequently amplifies oxidative stress by preventing respiratory chain enzymes and Fenton-type reactions, causing the fatal build-up of ROS that causes the destruction of proteins, lipids, and DNA (Direksunthorn et al., 2025). This sequence of events underpins the rapid

and bactericidal outcome of the time-kill kinetics, in which over 99.9% viability reduction was attained after 4 hours.

Another essential result is the potent dose-dependent prevention of biofilm formation to sub-MIC levels. The main cause of recurring and chronic UTIs is biofilms that offer a protective shelter against antibiotics and host defense (Lila et al., 2023). We find that AgNPs are capable of penetrating the biofilm matrix to disrupt quorum sensing and adhesion pathways required to mature a biofilm. This could probably be attributed to the size and high surface reactivity of the nanoparticles and therefore they are able to penetrate the extracellular polymeric substance (EPS) and attack embedded bacterial cells (Tiwari et al., 2025). This anti-biofilm property contributes greatly to the treatment of chronic and device-related infections in which biofilms are common with AgNPs.

Comparison with Previous Studies

Our results regarding the wide-spectrum activity of AgNPs are corroborated with a mass of in vitro data. The effectiveness of AgNPs against a variety of pathogens (including *E. coli*) was demonstrated by earlier studies by Morones et al. (2005), who first attributed it to the high surface area and anchoring behavior of AgNPs, which damage the microbial membranes. We develop on this by specifically using clinically relevant and genotypically diverse veterinary isolates that have been under-explored population (Menichetti et al., 2023). This value of 16 µg/mL of MIC₉₀ is consistent with the range of 2-32 µg/mL of AgNPs against Gram-negative bacteria in other works (Haji et al., 2022), and confirms our synthesis and testing procedures.

The most conspicuous validation of our study is the nondependence of AgNP activity on antibiotic resistance markers. The same has been witnessed in other settings (Rasool et al., 2024), AgNPs were equally effective against both the methicillin-resistant and susceptible *Staphylococcus aureus* (MRSA/MSSA). Our data is a sound extension of this principle to MDR and ESBL-producing *E. coli* in the veterinary environment that supports AgNPs as an effective future-resistance-resistant antimicrobial approach (Laird et al., 2021).

Nevertheless, there is also one important nuance that our analysis brings. Although AgNPs circumvent conventional resistance, we discovered a new potential challenge, that of biofilm-associated tolerance (Almatroudi, 2025). The logistic regression analysis showed that the strength of biofilm formation was the sole significant predictor of an increased AgNP MIC (aOR=4.05). This is attributed to the presence of EPS matrix as diffusion barrier that has the strength to entrap AgNPs and Ag⁺ ions before they reach all the cells in the biofilm depths, a factor earlier on was proposed by Li et al. (2023). This result is vital because it alters the mechanism of genetic resistance to the effect of phenotypic tolerance as the response on AgNP efficacy.

Implications for Veterinary Medicine and One Health

The research has significant implications to the veterinary practice and to One Health program as a whole. The growing crisis of antimicrobial resistance in veterinary pathogens directly affects animal welfare and its zoonotic and food safety outcomes are severe. AgNPs hold a potential alternative or adjunct therapy, and especially in the treatment of UTIs induced by MDR *E. coli* where treatment options are extremely restricted (Qasim et al., 2024).

Translational potential is anchored on the high selectivity index (SI > 18). It shows that there is a broad therapeutic window in which antimicrobial activity may be attained at dosages that are grossly lower than those that cause significant toxicity to mammalian cells (Hardefeldt & Prescott, 2024). The low hemolytic activity (<4% at the MIC 90) also indicates that systemic toxicity or toxicity to the urinary tract epithelium is unlikely to occur at therapeutic dosages. This safety profile along with strong anti-biofilm activity makes AgNPs an appealing topical bladder instillation choice or a urinary catheter coating to prevent biofilm formation and CAUTIs (Catheter-Associated UTIs) (Fydrych et al., 2025).

In addition, the batch reproducibility experiment, although showing a weak positive size-potency relationship ($r=0.998$), showed that when the synthesis is controlled, it is possible to obtain

homogenous and high quality AgNPs. Critical Quality Attributes (CQAs), including a small hydrodynamic size distribution, is part of what is required to standardise nanotherapeutic use in the clinic (Dri et al., 2023).

Limitations and Future Research Directions

This research has strong in vitro evidence, a number of limitations have to be mentioned. To begin with, all the experiments were done in vitro that cannot completely replicate the complicated pharmacodynamics and immune interactions in a living animal. The urine components of the sample including urine proteins and salts may have a possible impact on AgNP stability, aggregation and bioavailability (Abbott et al., 2021).

Second, acute cytotoxicity and hemolysis were the only safety tests performed. An extensive toxicological data package, including in vivo experiments to determine renal clearance, systemic retention and possible long-term outcomes is an absolute requirement prior to clinical use. Finally, the correlation between biofilm strength and reduced susceptibility, while statistically significant, warrants deeper investigation. Future studies should aim to clarify the specific tolerance-inducing mechanisms of biofilm-attached AgNPs and to devise methods of overcoming it, e.g. combining AgNPs with biofilm-dispersing enzymes (e.g. DNase, dispersin B) or other nanoparticles (Rubio, 2025).

CONCLUSION

This paper has shown that silver nanoparticles (AgNPs) show strong and consistent bactericidal effects on clinical MDR E. coli isolates of veterinary UTIs. Most importantly, the AgNP efficacy was not dependent on the traditional antibiotic resistance phenotypes such as MDR and ESBL production. The nanoparticles demonstrated a high level of safety profile and were very selective with a high index of selectivity as they quickly killed the bacteria, prevented the formation of biofilms, which is a major virulence factor and inhibited the formation of biofilms. A synergistic action of membrane disruption and oxidative stress was the predominant mechanism of action. Inherent strength of biofilm formation was the only predictor of reduced AgNP susceptibility, rather than

resistance genes. The results validate that AgNPs is a promising substitute therapeutic agent, as it fulfills the main goal of treating resistant infections in animals. It is a critical basis of a translational research work that contributes to One Health approach against antimicrobial resistance. Future research should aim at standardization of AgNP synthesis to regulate key quality parameters such as size and development of in vivo models to confirm these efficacy and safety results.

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