# Targeting the MDR P.aeruginosa Biofilm Formation with Drug-Nanoparticle Conjugates;An Insight into Variation at Gene Level

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## BACKGROUND

*Pseudomonas aeruginosa* has been ranked the number one priority pathogen in the final 2017 report by the World Health Organization (WHO) and has been grouped into ESKAPE members due to its particular multi-drug resistant characteristics that possess a serious threat to public health (Yang *et al*. 2018). In Pakistan, Multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) has emerged as the most important pathogen due to its antimicrobial resistance and virulence (Farooq *et al*. 2019). Studies suggested the most important virulence factor of *P. aeruginosa* is the biofilm formation on biotic and abiotic surfaces that plays a key role in causing infections and antibiotic resistance such as surgical sites, medical implants, lungs, urine catheters and causes ventilator-associated pneumonia, cystic fibrosis, skin, and soft tissue infections, surgical infections, urinary tract infections and burn infections (Syed *et al*. 2020). The most important system that helps this organism to produce biofilm is the Efflux Pump System i.e. RND efflux pump system (Resistance-Nodulation and Division superfamily) that helps the bacteria to resist antimicrobial drugs by using efflux pump genes i.e. *MexAB-OprM*. The ineffectiveness of conventional antibiotics against multi-drug-resistant bacteria has led to the emergence of nanomedicines (nanoparticles), which is a growing approach in the field of antimicrobials to put an end to resistant microbes (Lee *et al.* 2019). Specifically, Silver Nanoparticles (AgNPs) have received promising importance for their bactericidal activity against broad-spectrum bacterial infections as can prevent DNA formation, penetrate the cell membrane, and inhibit biofilm formation in *P. aeruginosa* (Danial *et al*. 2020). Additionally, AgNPs have been reported to be biocompatible drug delivery nanocarriers with less toxicity to mammalian cells. Therefore, in this study, ciprofloxacin in combination with AgNPs will be used to inhibit *P. aeruginosa* biofilm-producing genes (Habash *et al*. 2017).

## OBJECTIVE

The aim of the study was to evaluate the anti-bacterial and anti-biofilm activities of silver nanoparticles in combination with ciprofloxacin against multidrug-resistant *Pseudomonas aeruginosa*. Genomic analysis identified the effect of these combinations upon genes that are involved in the formation of biofilm in *P. aeruginosa.* Hence, the outcomes of this study will be helpful to eliminate the risk of MDR pathogens and to observe the effect of these combinations in bringing the alteration at the gene level.

## METHODOLOGY

For this purpose, 120 clinical isolates of *P.aeruginosa* collected from various hospitals and diagnostic labs of Karachi from pus, urine, wound, tracheal aspirates, blood, and other body fluids samples, and identified by using standard microbiological techniques including morphological and biochemical characterization. Kirby-Bauer method was used for resistance profiling (CLSI 2020 guidelines) (Rasool *et.al*, 2019). To perform PCR, DNA was first extracted and purified following kit method (Thermo Scientific GeneJET Genomic DNA Purification Kit) and confirmed by PCR method to detect 16S RNA gene and carbapenem-resistant *blaVIM* gene using 1kb ladder and results were confirmed by 1.5% gel electrophoresis (Karunasagar, *et.al*. 2018). Congo red agar method was performed to confirm the biofilm formation (Hassan, *et.al.* 2011). AgNPs were

synthesized by chemical reduction method using 1.0mM silver nitrate and 2.0mM sodium borohydride and characterized by UV-visible spectrophotometry, FTIR, Scanning electron microscopy, and EDS (Solomon *et.al*. 2007). Synergistic effects of AgNPs with ciprofloxacin were analyzed by preparing drug-nanoconjugates with different concentrations of 1µM, 2µM, 3µM, 4µM, and 5µM by using the well-diffusion method and Calgary method (Mohanta *et.al*, 2020). RNA extraction was performed from samples of AgNPs and AgNPs+ ciprofloxacin treated 24-hour established biofilms using Purelink RNA Mini Kit by Invitrogen. High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (applied bio-system, life technologies), was used to create single-stranded cDNA from total RNA. rtPCR was carried out on *P.aeruginosa* to confirm the inhibition of *MexAB-OprM* gene expression (Arabestani, *et.al.* 2015, Singh, et.al. 2019).

## RESULTS

The morphological and biochemical identification examination confirmed the presence of *Pseudomonas aeruginosa*. Profiling antibiotic resistance revealed that, out of 120 isolates, 80 exhibited resistance to Ciprofloxacin, Carbapenem (Imipenem and Meropenem), Tobramycin, Trimethoprim/Sulfamethoxazole, Tazobactam, and Amikacin, while displaying susceptibility solely to Polymyxin B and Colistin B. PCR analysis confirmed the existence of the *blaVIM* gene in 14 samples, exhibiting a distinct band at 390bp against a 1KB DNA ladder, alongside the 16s RNA gene at 1500bp. The Congo red agar assay indicated biofilm formation in six *P. aeruginosa* strains. Conversely, the synthesis of silver nanoparticles (AgNPs) showed a yellow color and exhibited absorption spectra at 420nm wavelength in UV-vis spectrophotometry. Scanning electron microscopy revealed spherical nanoparticles ranging from 67 to 69nm in size, with Energy Dispersive X-ray Spectroscopy (EDS), confirming the presence of silver nitrate at 76% and sodium borohydride at 19% in the AgNPs solution. Fourier Transform Infrared (FTIR) spectra of AgNPs exhibited peaks at 3331.39cm-1 and 1634.32cm-1, indicating the interaction of OH groups with AgNPs. UV-vis spectrophotometry further validated the formation of AgNP-ciprofloxacin conjugates within the 1µM to 5µM range, evidenced by a broad absorption spectrum at 420nm wavelength. Agar well diffusion assays demonstrated the susceptibility of P. aeruginosa to AgNPs alone and in combination with ciprofloxacin, as evidenced by the measured zone of inhibition while exhibiting resistance to ciprofloxacin alone. Furthermore, the inhibition of P. aeruginosa biofilm formation was notably pronounced with AgNPs+ciprofloxacin conjugates, reaching 95%, compared to ciprofloxacin alone 20% and AgNPs alone 65% (figure **1**).

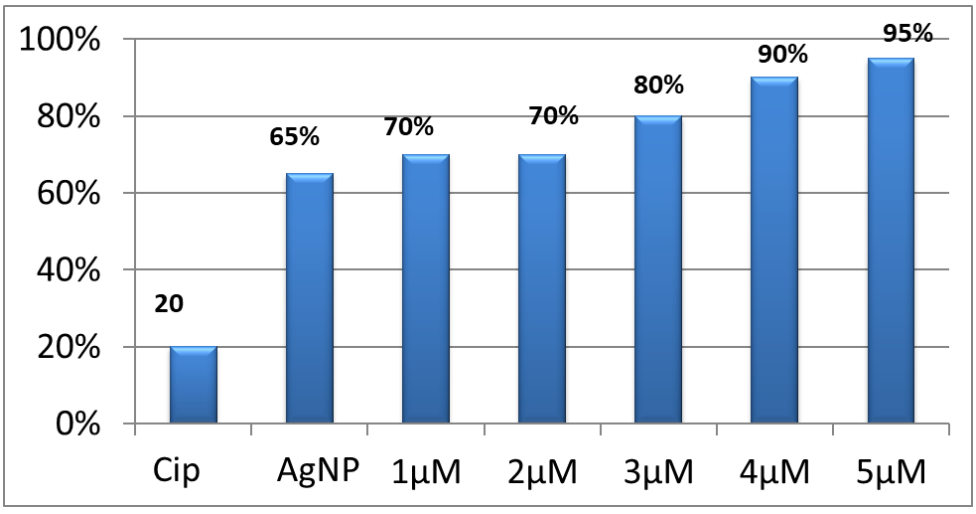


**Figure 1.** Biofilm inhibition of *P.aeruginosa* by Calgari method from AgNP alone, ciprofloxacin alone, and AgNPs+Cip conjugates ranging from 1µM to 5µM

In contrast, real-time polymerase chain reaction (rtPCR) confirmed the inhibition of *MexAB-OprM* efflux pump genes in samples treated with AgNPs alone and AgNPs+ciprofloxacin conjugates, as evidenced by the absence of bands during gel electrophoresis. Conversely, samples treated with ciprofloxacin alone and control gene samples displayed bands for *MexA* at 203bp, *MexB* at 244bp, and *OprM* at 205bp against a 100bp DNA ladder, visualized using a UV-transilluminator. The last well, containing the housekeeping gene *rPsL*, displayed a band near 203bp (figure **2**).

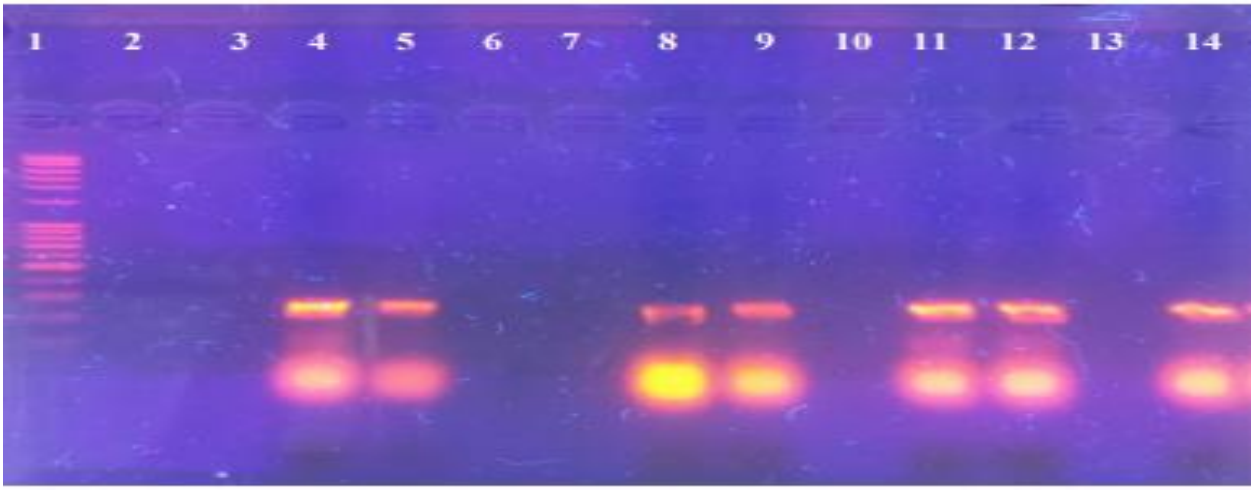


**Figure 2.** Well 1 contains 100bp DNA ladder, well 2 and 3 contain *MexA* gene treated with AgNP and AgNP+ CIP showed no bands, well 4 contains CIP alone treated *MexA* gene and well 5 contains control of *MexA* gene shows clear bands, well 6 and 7 contain *MexB* gene treated with AgNPs alone and AgNPs+ CIP conjugate showed no bands, well 8 contain CIP alone treated *MexB* gene and well 9 contain control of *MexB* gene shows clear bands, well 10 and 13 contain *OprM* gene treated with AgNPs and AgNPs+ CIP conjugate while well 11 contain CIP alone treated *OprM* gene and well 12 contain the control of *OprM* gene while well 14 contain *rPsL* gene as the housekeeping gene

## CONCLUSION

The findings demonstrated that by suppressing the efflux pump genes that give the bacterium antibiotic resistance, Drug-conjugated AgNPs can prevent MDR *P. aeruginosa* from generating biofilms when coupled with ciprofloxacin. Combining AgNPs with ciprofloxacin enhances antimicrobial activity, suggesting AgNPs as a potential alternative for treating bacterial infections. This approach holds promise for eradicating multi-drug resistant (MDR) pathogens, as indicated in the literature. Further research is required to explore the impact of nanoparticles on complex bacterial communities present in the environment.

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