Asian J Agric & Biol. 2024(4).
DOI: 10.35495/ajab.2024.096

AJAB

Original Article

Efficacy of silver oxide nanoparticles against multi-drug resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* in burn wound infections

Hira Ahsan[1,2], Maria Ayub[1], Mehraj Gul[3], Amber Qureshi[1], Hani Z. Asfour[4], Hafiz Muhammad Bilal[5], Muhammad Azeem[1], Ammara Wahid[1], Nadeem Ali[6], Rasheeha Naveed[7], Mudasar shabir[8], Nisreen Rajeh[9], Ayaz Mammadov[10], Abu Baker Siddique[1*]

[1]Institute of Microbiology, Government College University, Faisalabad, Pakistan
[2]Academy of Medical Sciences, College of Henan Medicine, Zhengzhou University, Zhengzhou, Henan, 450052, China
[3]Quality Control Laboratory, National Institute of Health, Islamabad, Pakistan
[4]Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah-21589, Saudi Arabia
[5]Department of Rehabilitation and Allied Health sciences, Riphah International University, Lahore Campus, Lahore, Pakistan
[6]Center of Excellence in Environmental Studies, King Abdulaziz University, Jeddah-21589, Saudi Arabia
[7]Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan
[8]Institute of Advanced Materials and Flexible Electronics (IAMFE), Nanjing University of Information Science and Technology, Nanjing, China
[9]Department of Clinical Anatomy, Faculty of Medicine, King Abdulaziz University, Jeddah-21589, Saudi Arabia
[10]Department of Life Sciences, Western Caspian University, Baku, Azerbaijan

Received:
May 24, 2024
Accepted:
September 22, 2024
Published Online:
October 13, 2024

**Corresponding author email:*
absiddique@gcuf.edu.pk

Abstract

Multi-drug resistant (MDR) bacterial infections rapidly increase morbidity, mortality, and treatment options. Therefore, the search for, development of, or discovery of antimicrobial drugs capable of combating MDR bacteria is urgently needed. The potential of nanotechnology to advance nanomedicine for human health is being studied. The purpose of the present research is to investigate the antimicrobial activity of silver oxide nanoparticles against carbapenem-resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*. For this purpose, a total of 240 pus and wound samples were collected from the burn patients and further processed for isolation and identification of *P. aeruginosa* and MRSA according to standard microbiological techniques. Using a species-specific primer for each bacterial strain, polymerase chain reaction was used for molecular detection. Antimicrobial susceptibility was performed according to the Kirby-Bauer Disk Diffusion method. Molecular detection of carbapenemase-producing *P. aeruginosa* was performed by PCR by using specific primers. The agar well diffusion assay was used to examine the antibacterial properties of silver oxide nanoparticles, and the broth dilution assay was used to estimate the minimum inhibitory concentration and bactericidal concentration respectively. Out of 240 samples, 42 (17%) were identified as *P. aeruginosa* and 32 were confirmed as *S. aureus* isolates. From positive isolates of *P. aeruginosa*, 25 (59%) were recorded MDR *P. aeruginosa* and from positive isolates of *S. aureus*, 18 (56.25%) were detected as MRSA. The highly resistant drug against *S. aureus* was Penicillin G (100%) followed by Gentamicin (84.37%) and Ciprofloxacin (81.25%). The highly resistant drug against *P. aeruginosa* was Meropenem (100%), Imipenem (100%) followed by piperacillin (71.42%), gentamicin (64.28%), and ciprofloxacin (64.28%). Out of 42 *P. aeruginosa* isolates, 8(19%) the prevalence of carbapenemase encoding was noted as *blaOXA* 3(37.5%), *blaNDM* 2(25%), *blaVIM* 1(12.5%) *blaKPC* 1(12.5%) and *blaIMP* 1(12.5%). Silver oxide nanoparticles were considered an effective antibacterial agent with 0.0065mg/mL-0.026mg/mL concentrations that highly inhibited the growth of MRSA and 0.39mg/mL-1.56mg/mL concentrations inhibited the growth of *P. aeruginosa*. The statistical analysis showed that the MIC and MBC for MDR *P. aeruginosa* were 0.96±0.43 µg/mL and 1.99±0.90 µg/mL, respectively, while for MRSA they were 0.01±0.008 µg/mL and 0.04±0.012 µg/mL, respectively. The MBC values were higher than MIC values for both pathogens. Silver oxide nanoparticles have such effective antibacterial properties that they can be used as an adequate source of antibacterial agents as alternatives to antibiotics.

Keywords: Silver oxide nanoparticles, Methicillin resistant, Gentamicin, Carbapenemase, Antimicrobial activity

How to cite this:
Ahsan H, Ayub M, Gul M, Qureshi A, Asfour HZ, Bilal HM, Azeem M, Wahid A, Ali N, Naveed R, Shabir M, Rajeh N, Mammadov A and Siddique AB. Efficacy of silver oxide nanoparticles against multi-drug resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* in burn wound infections. Asian J. Agric. Biol. 2024(4): 2024096. DOI: https://doi.org/10.35495/ajab.2024.096

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Introduction

Antimicrobial resistance (AMR) describes the ability that bacteria, fungi, viruses, and parasites have evolved to fight against and neutralize antimicrobial agents. The World Health Organization (WHO) (World Health Organization, 2017) states that the number and variety of resistant organisms have increased as a result of the extensive use and misuse of antibiotics. Recently, there has been an exponential rise in the number of microorganisms exhibiting multidrug resistance, which has been considered to be a serious public health concern (Roca et al., 2015). The need for novel antibacterial agents has become increasingly apparent as drug-resistant bacterial infections, particularly those caused by ESKAPE pathogens (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii, Enterobacter* spp*, and Enterococcus faecium.*), have become a major global health concern (Santajit and Indrawattana, 2016; Hassan et al., 2023).

As a last option, vancomycin is typically used to treat Gram-positive bacterial infections that are resistant to antibiotics. However, there is a challenge in clinical settings due to the development of vancomycin resistance in *Enterococcus* species, especially vancomycin-resistant *E. feacium* (VRE) (Mühlberg et al., 2020). Methicillin-resistant *S. aureus* is One of the most common Gram-positive, antibiotic-resistant pathogens identified in the last century, which is also the primary cause of surgical, orthopedic, and skin and soft tissue infections (Chen et al., 2020). Methicillin-resistant *S. aureus* isolates are those that are resistant to the β-Lactam drugs methicillin, cefoxitin, and cephalosporins; methicillin-susceptible *S. aureus* is defined as susceptible to these antibiotics (Naeem et al., 2021). Alternative antibiotics have become becoming increasingly common due to the growing prevalence of methicillin resistance in MRSA infections; however, this has restricted the number of antibiotics available and increased the level of multidrug resistance in MRSA (Shahid et al., 2023).

The prevalence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections is high in Pakistan, especially in medical settings. A major concern is the prevalence of these bacteria in hospital-acquired infections, which is made severe by high rates of antibiotic resistance (AMR) (Bilal et al., 2021). Different studies reported that methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant (MDR) strains of *P. aeruginosa* are highly prevalent in different clinical settings across Pakistan. The study conducted by (Khan et al., 2019) in Pakistan indicated 8.1%. prevalence of MRSA Another study carried out by (Farooq et al., 2019) showed the prevalence of *P. aeruginosa* was 55% in tertiary care hospital in Pakistan. AMR prevalence in Pakistan is exacerbated by abuse of antibiotics, insufficient infection control procedures, and a lack of effective antimicrobial stewardship programs. These challenges make it more difficult to treat and control infections brought on by these pathogens, which raises morbidity, mortality, and healthcare expenses (Bilal et al., 2021).

Carbapenem has been considered the last line of defense against Gram-negative bacterial infections, such as bacteremia, pneumonia, and UTIs (Codjoe and Donkor, 2017). Owing to the extensive use of carbapenem, there are fewer treatment options available for infections caused by Gram-negative bacteria, including carbapenem-resistant *A. baumannii* (CRAB), carbapenem-resistant *P. aeruginosa* (CRPA), and carbapenem-resistant *K. pneumoniae* (CRKP). (Yang et al., 2021; Engeman et al., 2021). One of the main concerns for public health is the widespread dissemination of carbapenem-resistant *P. aeruginosa* (CRPA). worldwide (Botelho et al., 2019). Because CRPA is becoming more and more isolated, the World Health Organization declared in 2017 that it should be prioritized as a critical pathogen (World Health Organization, 2017). In *P. aeruginosa*, resistance to carbapenem is

primarily linked to the development of carbapenemase (*blaVIM, blaIMP, blaKPC, blaNDM, blaGES*, etc.), however, it is also linked to the loss or reduced production of the outer membrane barrier pore protein (OprD) or the overexpression of the efflux pump (MexAB-OprM) (Halat and Moubareck, 2022). As bacterial resistance is a major concern it's critical to develop an alternate antibacterial drug to prevent bacterial infection. Drug-resistant bacterial infections required the use of high dosages of antibiotics, which raises drug toxicity and increases hospital stays and mortality. Antibiotic-resistant bacterial infections cost the US economy and healthcare system $35 billion and $20 billion, respectively, each year (Luepke et al., 2017). Therefore, one of the key objectives for preventing bacterial infections is the efficient management or removal of drugs resistance. During the recent past, nanoparticles are frequently employed in biomedical research for disease diagnosis, photodynamic therapy, drug delivery, gene therapy and imaging (Ullah et al., 2023; Ali et al., 2024; Khan et al., 2024). Studies have indicated that nanomaterials are persistently used for drug delivery (Yu et al., 2023), feed additive for aquatic animals (Hussain et al., 2024), antimicrobials (Saif et al., 2023) and toxicological investigations (Khan et al., 2022; Khan et al., 2023; Ali et al., 2023). Nowadays, a lot of study is done on nanoscale science to develop antibacterial efficient nanoparticles (Moodley et al., 2018).

Nevertheless, several studies have indicated that AgNPs may be effective in treating infections brought on by MDR bacterial strains (Dong et al., 2017; Rasool et al., 2016; Basit et al., 2021). Silver nanoparticles (AgNPs) have garnered interest in recent years due to their significant significance in nanoscience and nanotechnology, particularly in the field of nanomedicine. The clinical properties of silver nanoparticles, including their antifungal (Hasan et al., 2020), antibacterial, and anticancer characteristics, are currently drawing attention in biomedical research (Huang et al., 2023). The broad-spectrum antibacterial characteristics and potency of silver nanoparticles (AgNPs) against multidrug-resistant (MDR) bacteria are what make them significant (Khatoon et al., 2017). Silver nanoparticles (AgNPs) have low toxicity profiles but strong antibacterial and antiseptic properties against both Gram-negative and Gram-positive bacteria. AgNPs exhibit antibacterial characteristics by going through the organism cell wall and detaching the respiratory chain through oxidative phosphorylation or by stimulating the destruction of proton-motive force over the cytoplasmic membrane (Boateng and Catanzano, 2020; Mwafy et al., 2023). AgNPs' complex mechanism of action, which includes physical interactions with bacterial cell membranes, the release of silver ions that attach to proteins, DNA, or other bacterial components, and the production of reactive oxygen species (ROS), inhibiting biofilm explains why Ag NPs have an antibacterial effect. Their significance for developing novel antibacterial agents is further highlighted by their low toxicity profile and prospective uses in a variety of applications (Hasan et al., 2022: Mansoor et al., 2023).

The synthesis of nanoparticles by several methodologies, including physical, chemical, and biological processes, has emerged as a top approach within their respective fields (Zulfiqar et al., 2019). However, chemical procedures produce hazardous byproducts that are absorbed at the surface and can limit their use in medical applications; physical methods, on the other hand, have low production rates and considerable energy consumption (Rajoka et al., 2020; Wu et al., 2021). Additionally, the practical application of physicochemically synthesized silver nanoparticles in antibacterial activity is typically limited by their intrinsic characteristics, such as low stability, which are typically gathered as a result of inter-particle interactions, which reduce surface area, reactivity, and interfacial free energy (Inbaraj et al., 2020). By using organisms like fungi, bacteria, and plants to produce nanoparticles, these techniques lessen the need for dangerous chemicals (AlRashdi et al., 2023). The green synthesis method supports environmental sustainability and safety by adhering to green chemistry principles and offering a biocompatible and affordable substitute. Thus, a green synthesis of Ag NPs that is both economically feasible and environmentally benign is becoming more and more important in science (Huang et al., 2023).

The research gap indicates that antibiotic resistance is a significant concern. This research was conducted to investigate the alternative therapies to overcoming bacterial resistance or investigate whether the silver nanoparticles are effective against multidrug resistance bacteria. The primary goal of this research was genotypic characterization of carbapenemase resistance genes in *P. aeruginosa* and Methicillin-resistant *S. aureus*, determine their antibiogram potential, and assess the antibacterial potential of

Silver-oxide nanoparticles against carbapenem resistant *Pseudomonas aeruginosa* and Methicillin resistant *Staphylococcus aureus* (MRSA).

Material and Methods

Ethical permission

Prior to conducting the research, the ethical review committee's approval was obtained from Government College University Faisalabad (GCUF/ERC/15/166). The patient was provided with written informed consent.

Sample collection, isolation, and identification

A total of 240 pus and wound samples of burn patients, 120 each from Allied Hospital, Faisalabad and Aziz Fatima Hospital, Faisalabad, were collected under aseptic conditions using sterile swabs (Shah et al., 2022) and subjected to bacterial isolation and identification. For the isolation of *Pseudomonas*, samples were swabbed on cetrimide agar (selective for *Pseudomonas aeruginosa* and contains cetrimide, which inhibits the growth of other bacteria) and for *S. aureus*, on Mannitol Salt agar (selective for *Staphylococcus aureus* and contains high salt concentration (7.5%), which inhibits the growth of most bacteria except for *Staphylococci)*. Identification of bacteria was done by Gram staining and biochemical testing (Catalase, Mannitol fermentation, and Coagulase for *S. aureus* and Catalase, Oxidase, and Citrate for *P. aeruginosa*) (Abdulhaq et al., 2020; Naeem et al., 2021).

Molecular identification of isolates

All the confirmed isolates were subjected to boiling (Qurat-ul-Ain et al., 2021). The bacterial pellets were boiled for 15 minutes after being suspended in 200 µl of TE buffer (Tris-HCl [10 mM]: EDTA [1 mM]). The microfuge tubes were boiled, immediately followed by 15 minutes in an ice bath and 5 minutes at room temperature centrifuging at 14,000 rpm. The DNA-containing supernatant (100 µl) was transferred to a different clean tube and kept cold, at –20°C (Junior et al., 2016). Molecular detection of this heat inactivated biomass was performed by Polymerase Chain Reaction (PCR) by using specific primers against *oprl*, *oprL* for *Pseudomonas* and *nuc* and *mecA* genes for *Staphylococcus* (given in table 1)

Antibacterial susceptibility testing

All isolates were tested for antibiotic susceptibility using the Kirby-Bauer disc diffusion method. The antibiotics Amikacin (30µg), Tobramycin (10µg), Piperacillin (100µg) and Cefepime (30µg), Imipenem (10µg), Ceftazidime (30µg), Meropenem (10µg), Ciprofloxacin (5µg), and Gentamicin (10µg) were the drugs against which the susceptibility of *Pseudomonas aeruginosa* strains was assessed. For *S. aureus*, the antibiotics like Norfloxacin (10µg), Ciprofloxacin (5µg), Trimethoprim sulphamethoxazole (25µg), Clindamycin (2µg), Doxycycline (30µg), Penicillin G (1µg), Vancomycin (30µg), Levofloxacin (5µg), Gentamycin (10µg), Rifampin (5µg), Chloramphenicol (30µg), Erythromycin (15µg) and Tetracycline (10µg) were the drugs of choice. Results were interpreted as resistant, intermediate, and sensitive according to CLSI guidelines (Emamie et al., 2023).

Table-1. List of primers used in the study. All the sequences are listed 5' to 3'

Gene	Sequence	Primer	Product size	Reference
Nuc	TAC AGG TGA CTG CGG GCT TATC-3	Forward	484 bp	(Boukharouba et al., 2022)
	CTT ACC GGG CAA TAC ACT CACTA-3	Reverse		
mecA	AAA ATC GAT GGT AAA GGT TGG C	Forward	162 bp	(Shahid et al., 2023)
	ATC TGT ACT GGG TTA ATC	Reverse		
oprL	ATG GAA ATG CTG AAA TTC GGC-3	Forward	504 bp	(Algammal et al., 2023)
	CTT CTT CAG CTC GAC GCG ACG-3	Reverse		
Oprl	ATG AAC AAC GTT CTG AAA TTC TCT GCT	Forward	249 bp	(Ahmed et al., 2022)
	CTT GCG GCT GGC TTT TTC CAG	Reverse		
blaKPC	CGTCTAGTTCTGCTGTCTTG	Forward	798bp	(Shahcheraghi et al., 2017)
	CTTGTCATCCTTGTTAGGCG	Reverse		
blaVIM	GTT TGG TCG CAT ATC GCA AC	Forward	382	(De Sousa et al., 2021)
	AAT GCG CAG CAC CAG GAT AG	Reverse		
blaNDM	GGT TTG GCG ATC TGG TTT TC	Forward	561	(Mohan et al., 2015)
	CGG AAT GGC TCA TCA CGA TC	Reverse		
blaIMP	GAA GGA GTT TAT GTT CAT AC	Forward	432	(Al-Ouqaili, 2018)
	GTA CGT TTC AAG AGT GAT GC	Reverse		
blaOXA	GGT TAG TTG GCC CCC TTA AA	Forward	246	(Rouhi and Ramazanzadeh, 2018)
	AGT TGA GCG AAA AGG GGA TT	Reverse		

Molecular detection of carbapenem-resistant *Pseudomonas aeruginosa*

Isolates that have been confirmed as *P. aeruginosa* and show resistance to carbapenems were subjected to the detection of carbapenemase by PCR. First, denaturation for three minutes at 95°C, then thirty cycles of denaturation for thirty seconds at 95°C, and finally an annealing temperature of 65°C for *NDM*, 61°C for *KPC*, 60°C for *IMP*, 52°C for *VIM*, and 55°C for *OXA* for 45 sec, extension at 72°C for 45 sec, and final extension at 72°C for 5 min were the standard PCR conditions for the various genes (Sankar et al., 2022). The primer sequence of specific genes is mentioned in Table 1. The PCR amplicons undergo agarose gel electrophoresis in a subsequent step.

Preparation of sliver nanoparticles

Silver oxide nanoparticles solution was prepared by dissolving 50mg/mL of Silver nanoparticles in the DMSO in a container/tube. After that, the container/tube was placed in the sonicator to dissolve/dispersed the Silver nanoparticles completely (Shashiraj et al., 2023).

Antibacterial potential of AgO nanoparticles

Antibacterial assay

The confirmed isolates of *P. aeruginosa and S. aureus* were subjected to the antibacterial activity of AgNP by using the Agar well assay. The viability of the bacterial suspensions, containing 10^8 CFU/ml from 18 to 24 hours, was determined by measuring the OD_{600}, which ranged from 0.06-0.08. Using a swab, the bacterial lawn was prepared using sterilized MH agar plates. Six mm-diameter wells made by the sterile steel borer were filled uniformly (100µl) with AgNPs at three different concentrations (25 g/ml,, 20 g/ml, 15 g/ml, 10 g/ml, and 5 g/ml). The inhibitory zone diameter was measured after the plates were incubated for 24 hours at 37 °C. Negative control was provided by DMSO solution (Mishra and Padhy, 2018; Feroze et al., 2020).

Determination of Minimum Inhibitory Concentration

The agar well diffusion technique was used to measure the MIC of the AgNPs in accordance with the guidelines from a prior study (Patra and Baek, 2016). 50 mg/mL of AgNPs were added into the first well, and up to the tenth well (discard 100 from that tenth well), the AgNPs were serially diluted using a two-fold serial dilution procedure. Following this, the inoculum suspension (~1.5×10^8 CFU per mL) was pipetted into the 10th and 12th wells (keep in mind that the 11th well is for sterility control and the 12th well is for positive control). Whereas the 12th well has both media and bacterial suspension, the 11th well only contains media. Each of the micro-titration plate was set up similarly, and it was incubated at 37°C for 18 to 24 hours. Following the incubation period, 50µl of NBT dye (0.02% Methanol) was introduced into each well. An hour later, the change in color was monitored to determine whether any bacterial growth was present and the lowest dilution that inhibited the growth. The final well the least diluted was determined to be the MIC (Abootalebi et al., 2021).

Calculating Minimum Bactericidal Concentration

Minimum Bactericidal Concentration (MBC) is the lowest concentration of antimicrobial agent essential to kill a certain type of bacteria. MBC was performed by adding 100µL from the wells with no visible growth of bacteria and sub-culturing it onto plates of Muller Hinton agar. The plates were placed for 24 hour's incubation at 37°C. Muller Hinton agar plates with no visible growth will be considered MBC (Abootalebi et al., 2021).

Statistical analysis

The t-test analysis was used for mean comparison and Two-Way ANOVA was used to analyze the significance difference between MIC and MBC for silver nanoparticles efficiency against *P. aeruginosa* and MRSAA. statistically significant P-value of 0.05 was used.

Results

Isolation and molecular characterization of *P. aeruginosa* and *S. aureus*

Based on phenotypic, standard microbiological techniques including Gram staining and Biochemical tests (Catalase, Mannitol fermentation, and Coagulase for *S. aureus* and Catalase, Oxidase, and Citrate for *P. aeruginosa*) and molecular characteristics, out of 240 specimens, 42 (17%) isolates were confirmed *P. aeruginosa* and 32 (13%) isolates were confirmed *S. aureus.* Figure 1 represent the results. All 32 isolates of *S. aureus* were subjected to PCR. Of 32, 32(100%) were confirmed positive for *S. aureus* against the *nuc* gene. With the

use of particular primers and the outcomes of PCR amplification, *P. aeruginosa* was found to be present in all 42 morphologically verified isolates of the bacterium.

Antimicrobial susceptibilities of *P. aeruginosa* and *S. aureus* clinical isolates

The highest resistance of *P. aeruginosa* isolates was observed against Imipenem (100%), meropenem (100%), and Piperacillin (71.42%) followed by Ciprofloxacin and Gentamycin i.e., (64.28%) and Tobramycin i.e., (61.90%). Whereas, in the case of *S. aureus*, Penicillin G was 100% resistant, followed by Gentamycin (84.37%), Ciprofloxacin (81.25%), Tetracycline (78.12%), Levofloxacin (75%) and Doxycycline (71.87%). Out of 42 positive isolates of *P. aeruginosa*, 25 (59%) were recorded MDR as they showed resistance against various antibiotics, and out of 32 positive isolates of *S. aureus*, 18 (56.25%) were recorded MRSA as the amplification of *mecA* gene detected them. The prevalence of MDR *P. aeruginosa* and MRSA was found to be significantly higher in burn wound patients. Detailed susceptibility of isolates using different drugs is mentioned in Figure 2 and Figure 3.

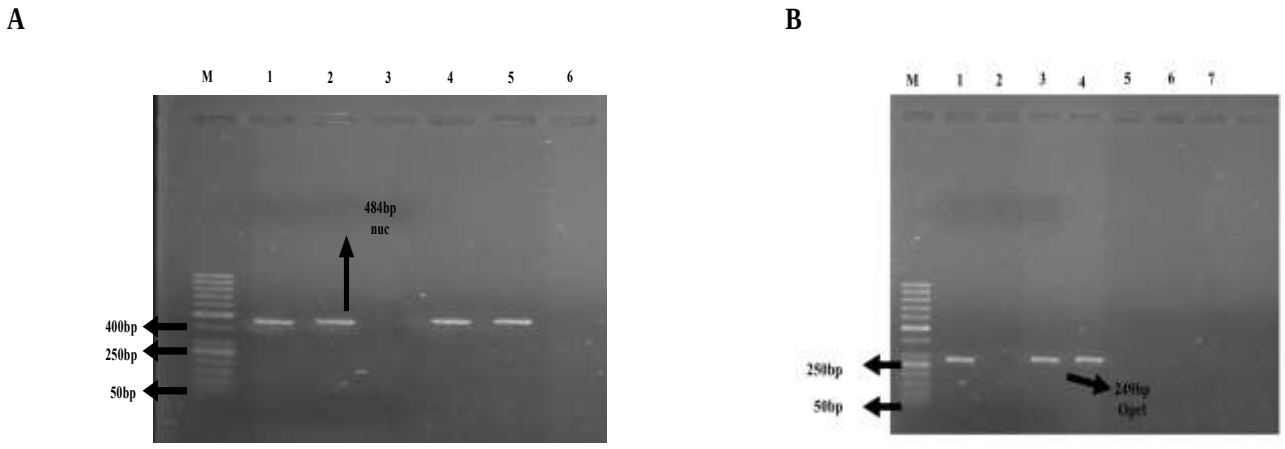

Figure-1. Molecular identification of *P. aeruginosa and S. aureus*

A) Gel electrophoresis analysis of *S. aureus*, ladder size: 50 bp, lanes 1, 2, 4, and 5 represent the positive sample of *nuc* (484bp). B) Gel electrophoresis analysis of *P. aeruginosa* Lanes 1, 3, and 4 correspond to the positive sample of *Oprl* (249 bp) with a ladder size of 50 bp.

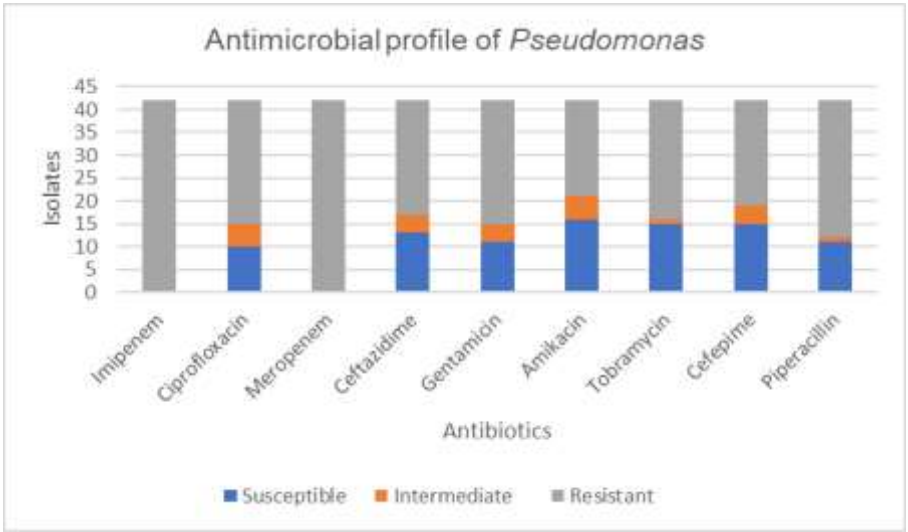

Figure-2. Antibiotic resistance pattern *P. aeruginosa*

This bar graph illustrates the antimicrobial profile of *P. aeruginosa* against different antibiotics. The x-axis showed the antibiotics tested while the y-axis showed the no of isolates. The bars are divided into 3 categories. Blue (the portion of isolates that are susceptible to antibiotics. Orange (the portion of isolates that show the intermediate susceptibility to antibiotics. Grey (the portion of isolates that are resistant to antibiotics).

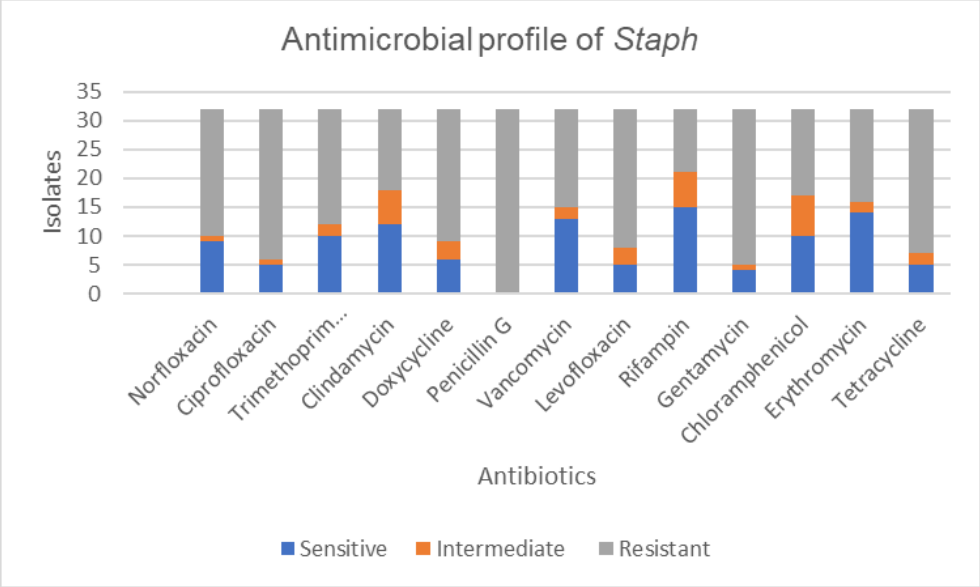

Figure-3. Antibiotic resistance pattern *S. aureus*

This bar graph illustrates the antimicrobial profile of *S. aureus* against different antibiotics. The x-axis showed the antibiotics tested while y-axis showed the no of isolates. The bars are divided into 3 categories. Blue (the portion of isolates that are susceptible to antibiotics. Orange (the portion of isolates that show the intermediate susceptibility to antibiotics. Grey (the portion of isolates that are resistant to antibiotics).

Genotypic characterization of Methicillin Resistant *S. aureus* and carbapenemase producing genes in *Pseudomonas aeruginosa*

All of the 42 isolates of *P. aeruginosa* were subjected to the molecular detection of carbapenemase. Out of 42, 8(19%) the prevalence of carbapenemase encoding was noted as blaOXA 3(37.5%), blaNDM 2(25%), blaVIM 1(12.5%) blaKPC 1(12.5%) and blaIMP 1(12.5%) as shown in figure 4. From 32 morphologically positive *S. aureus* isolates, 18 were confirmed as MRSA phenotypically as well as genotypically.

Antimicrobial potential of Silver nanoparticles against MRSA and *P. aeruginosa*

The antibacterial activity of silver oxide nanoparticles against *P. aeruginosa* and MRSA was assessed using the agar well diffusion method. Figure 5 illustrates the observation of clear zones following an overnight incubation period. Table 2 displays the maximum and minimum ZOI for each concentration. The static analysis is displayed in Figure 6.

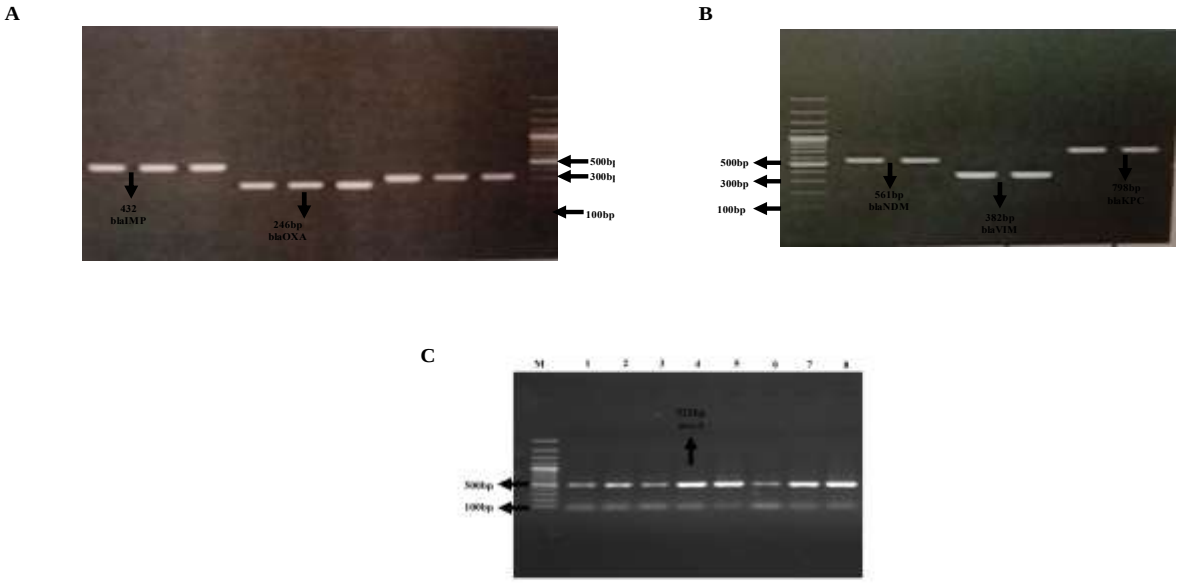

Figure-4. Genotypic identification of CRPA and MRSA.

A) Gel electrophoresis analysis of CRPA, ladder size: 100 bp, lanes 5 and 6 shows the positive sample of *blaOXA* (246bp). The *blaIMP*(432bp) positive samples are shown by lanes 7 through 9. B) Gel electrophoresis analysis of CRPA, ladder size: 100 bp, The *blaNDM* (561bp) positive sample is represented by lanes 1 and 2. The bla*VIM*(382bp) positive samples are shown by lanes 3 and 4. The *blaKPC* (798bp) positive samples are shown by lanes 5 and 6. C) MRSA gel electrophoresis analysis; lanes 1 through 8 correspond to the positive sample of *mecA* (533 bp); ladder size: 100 bp.

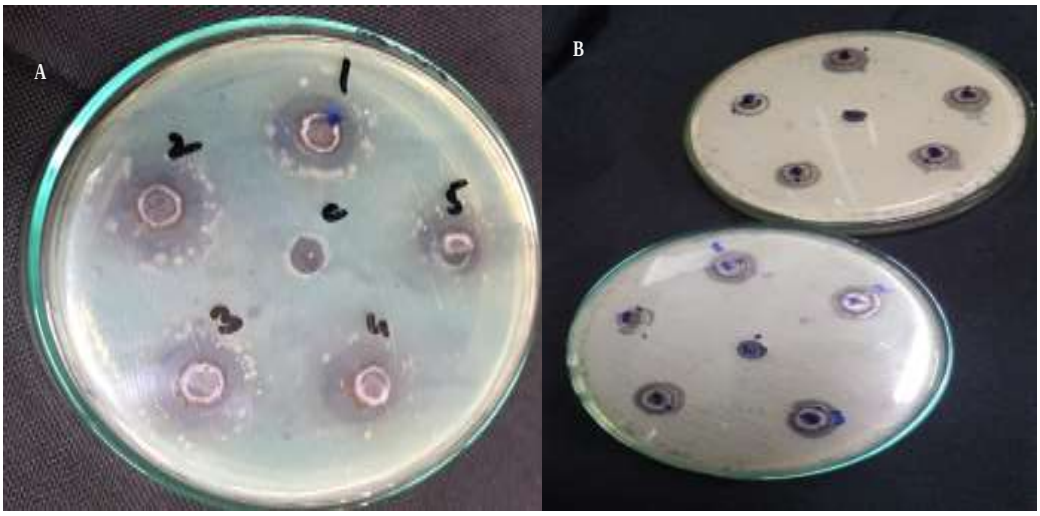

Figure- 5. Zone of inhibition (ZOI) of Silver oxide nanoparticles agar-well diffusion assay.

A) against *P. aeruginosa* agar-well diffusion assay. B) against MRSA

Table-2. Maximum and minimum zone measurement at different concentrations against *P. aeruginosa* and MRSA

AgO nanoparticle concentrations (mg/ mL)	*P. aeruginosa*			MRSA		
	No. of Isolates	Maximum zone (mm)	Minimum zone (mm)	No. of Isolates	Maximum zone (mm)	Minimum zone (mm)
25	25	18	15	18	19	15
20	25	17	12	18	18	13
15	25	15	11	18	16	11
10	25	14	10	18	15	11
5	25	12	08	18	14	09
DMSO	25	0	0	18	0	0

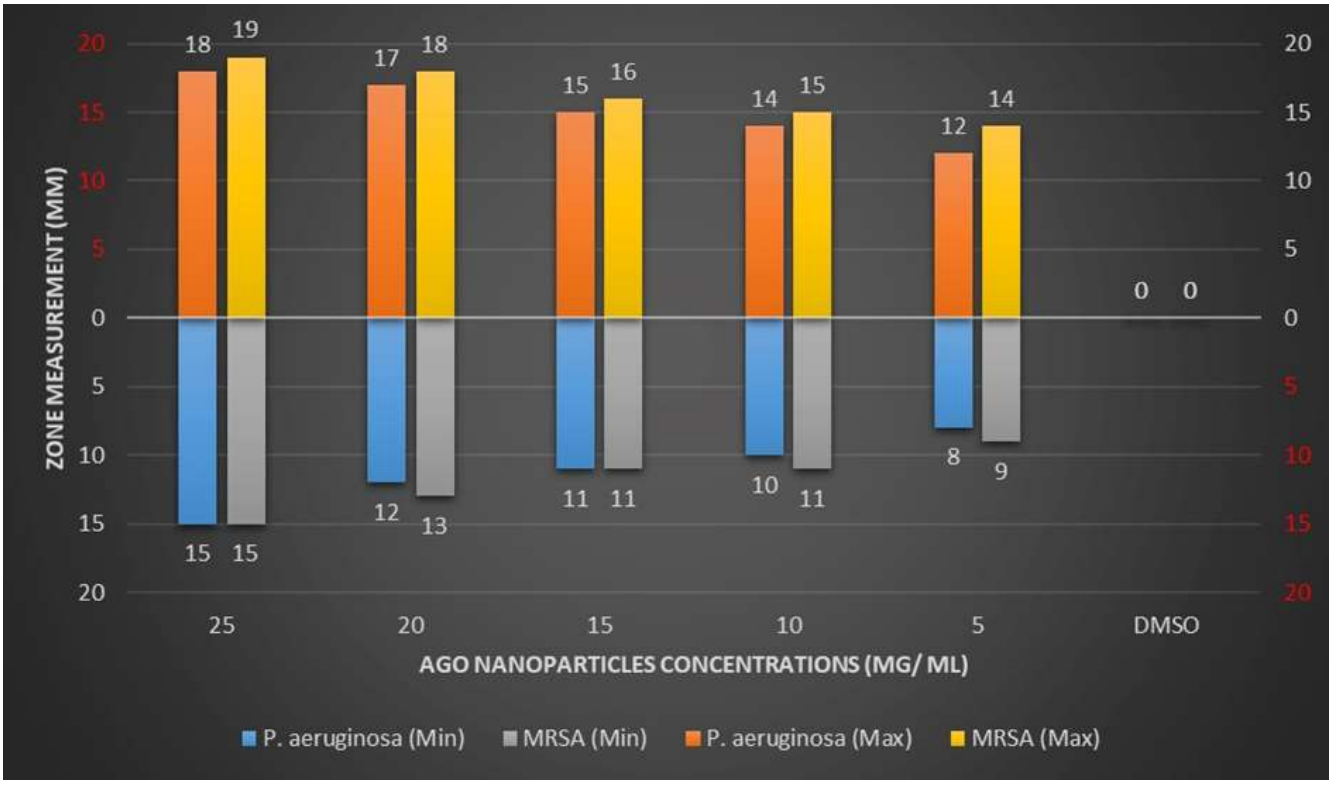

Figure-6. Zone measurements for *P. aeruginosa* and MRSA isolates at different AgO nanoparticle concentrations

The chart shows the measurement of zones for *P. aeruginosa* and MRSA isolates at different AgO nanoparticle concentrations. The MRSA showed the higher maximum zone measurement at 5mg/mL, 10mg/mL, 15mg/mL, 20mg/mL, and 25mg/mL concentration and had slightly significant differences between them. While at concentrations 5mg/mL, 10mg/mL, and 20mg/mL the MRS minimum showed a slightly higher difference than *P. aeruginosa* (min) and non-significant behavior shown by *P. aeruginosa* (min) and MRSA (min) at 15mg/mL and 25mg/mL concentration of AgO nanoparticle.

Estimation of Minimum Inhibitory Concentration (MIC) for *P. aeruginosa* and MRSA

MICs of metallic nanoparticles were ascertained using the Nitro-blue tetrazolium (NBT) dye. Following the addition of dye, bacterial cells are shown to be viable if their color changes from yellow to blue; if their color remains unchanged, it shows that the cells are not visible due to metabolism. The MIC of *P. aeruginosa* was found to be 0.39 mg/mL in about 4 isolates (16%), 0.78 mg/mL in 13 isolates (52%) and 1.56 mg/mL in approximately 8 isolates (32%). For MRSA five isolates (28%) demonstrated

a MIC of 0.0065mg/mL, four isolates (22.2%) demonstrated 0.013mg/mL MIC and nine isolates (50%) demonstrated 0.026mg/mL MIC. All the data have been mentioned in Table 3.

Table-3. Silver oxide nanoparticles' MICs and MBCs (mg/mL) against MRSA and multi drug resistant *P. aeruginosa*

Multi drug resistant *P. aeruginosa*			**MRSA**		
Isolates No.	**MIC (mg/mL)**	**MBC (mg/mL)**	**Isolates No.**	**MIC (mg/mL)**	**MBC (mg/mL)**
04	0.78	1.56	07	0.0065	0.031
07	0.39	0.78	13	0.013	0.026
08	0.78	1.56	21	0.013	0.026
09	1.56	3.12	39	0.026	0.0520
12	0.78	1.56	42	0.026	0.0520
15	0.78	1.56	44	0.0065	0.031
18	0.39	0.78	46	0.0065	0.031
23	0.78	1.56	51	0.026	0.0520
28	1.56	3.12	56	0.026	0.0520
35	0.78	1.56	58	0.026	0.0520
41	1.56	3.12	60	0.013	0.026
42	0.39	0.78	61	0.026	0.0520
49	0.78	1.56	63	0.026	0.0520
55	1.56	3.12	74	0.0065	0.031
63	0.78	1.56	77	0.026	0.0520
69	0.78	3.12	85	0.013	0.026
73	1.56	3.12	91	0.0065	0.031
78	0.78	1.56	96	0.026	0.0520
86	1.56	3.12	-	-	-
89	0.78	1.56	-	-	-
95	0.39	0.78	-	-	-
101	1.56	3.12	-	-	-
109	0.78	1.56	-	-	-
114	1.56	3.12	-	-	-
119	0.78	1.56	-	-	-

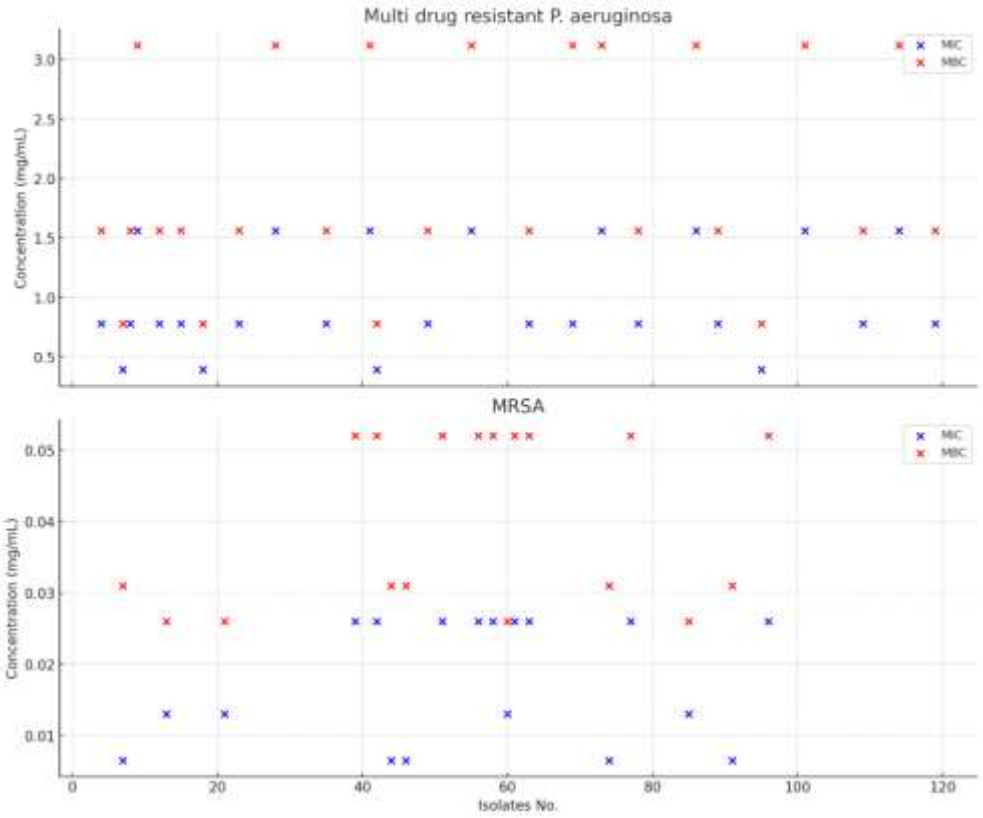

Figure 7. Silver oxide nanoparticles' MICs and MBCs (mg/mL) against MRSA and multi drug resistant *P. aeruginosa*.

Table-4. MICs and MBCs of AgNPs against *P. aeruginosa* and MRSA using the T-test and Analysis of Variance

	MDR *P. aeruginosa*		MRSA	
	MIC (mg/mL)	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**
Mean±SD	0.96±0.43	1.99±0.90	0.01±0.008	0.04±0.012
Minimum	0.39	0.78	0.0065	0.026
Maximum	1.56	3.12	0.026	0.052
Minimum Confidence	0.78	1.62	0.013	0.034
Maximum Confidence	1.14	2.36	0.022	0.046
Confidence Interval (95%)	0.18	0.37	0.0044	0.006

Table 4 shows the t-test and Two-way ANOVA for the MICs and MBCs of silver oxide nanoparticles against MDR *P. aeruginosa* and MRSA. The Mean±SD of MIC and MBC for MDR *P. aeruginosa* was 0.96±0.43 and 1.99±0.90 while 0.01±0.008 and 0.04±0.012 of MIC and MBC for MRSA respectively. The MBCs showed a greater index for MDR *P. aeruginosa* and MRSA rather than MICs. The same variation for minimum and maximum confidence values while the P-values for MRSA of MICs and MBCs were 0.004 and 0.006 which shows the highly significant difference between the nanoparticle efficacy against the MRSA P<0.05) while there was a non-significant difference shown against *P. aeruginosa* (P>0.05).

Discussion

Pseudomonas aeruginosa and *Staphylococcus aureus* are widespread, pervasive, and versatile bacteria that have caused mild and severe infections over the last few decades (Goldstein et al., 2018). Due to the

intricate nature of antibiotic resistance, heritable resistance to antibiotics usually arises as a result of evolutionary processes that normally take place during antibiotic therapy. Drug resistance may be propagated by horizontal gene transfer (HGT) via bacterial conjugation, transduction, transformation, or biofilm formation. The significant development of bacterial resistance to antimicrobials is becoming an important concern for all healthcare organizations to reduce the production of newly resistant isolates by constantly searching for alternatives to standard antibiotics (Mohanty et al., 2021). In the present study the prevalence of *P. aeruginosa* was observed as 17.5%. While the prevalence of *S. aureus* was noted as (13%). The results were compared with previously conducted studies. A study conducted by Ahmed et al. (2023) in Egypt explained the prevalence of *S. aureus* (62.5%) followed by *P. aeruginosa* (40%) which is greater than recent research. In another study the prevalence of *S. aureus* was (34%) and of *P. aeruginosa* (23%) in wound samples by Akgül and Bora, (2023). Geographical variations, patient demographics, sample collection procedures, healthcare environments, antibiotic use, infection control procedures, and detection techniques can all contribute to variations in bacterial prevalence across studies.

The susceptibility testing demonstrated a high level of observable antimicrobial resistance, especially to different antibiotics; Imipenem (100%), meropenem (100%), and Piperacillin (71.42%) in *P. aeruginosa*. Whereas, in the case of *S. aureus*, Penicillin G was 100% resistant, followed by Gentamycin (84.37%), Ciprofloxacin (81.25%), Tetracycline (78.12%). Research conducted by Ali and Assafi (2024) in Iraq described that *P. aeruginosa* isolates showed high rates of resistance to aminoglycosides, including netilmicin (81.25%), tobramycin (81.25%), and gentamicin (84.37%). 70.83% and 91.67% of the *S. aureus* isolates, respectively, were resistant to azithromycin and penicillin G. More than 94% of *P. aeruginosa* isolates were resistant to ciprofloxacin, gentamicin, imipenem, and ticarcillin, according to another investigation done by (Shariati et al., 2019). Antibiotic misuse, bacterial mutation, the organism's genetic makeup, and the local environment all contribute to the rise in antimicrobial resistance Recently, there has been a rise in interest in nanotechnology and the medical applications of some metal nanoparticles, particularly in their usage as an antibiotic against MDR isolates (Sánchez-López et al., 2020). Nanomaterials have been suggested to have a promising role that allows them to interact with microbial membranes because of their distinctive physical and chemical functions, such as their large surface area to volume ratio. In recent years, the field of nanomedicine, industry, and academia have all become more and more interested in the use of nanomaterials in particular, AgNPs to supplement antibiotics. AgNPs are effective antibacterial agents against a variety of gram-negative and gram-positive bacteria (Yuan et al., 2017).

All 42 *P. aeruginosa* isolates were subjected to the molecular detection of carbapenemase-producing *P. aeruginosa*. on the other hand, *S. aureus* strains were subjected to *mac A* genes detection by polymerase chain reaction. The results showed that out of 42, 8(19%) the prevalence of carbapenemase encoding was observed as *blaOXA* 3(37.5%), *blaNDM* 2(25%), *blaVIM* 1(12.5%) *blaKPC* 1(12.5%) and *blaIMP* 1(12.5%). From 32 positive *S. aureus* isolates, 18 were confirmed as MRSA phenotypically and genotypically. The results were compared with previously conducted studies. The prevalence of carbapenemase encoding genes are as *blaIMP* (7.26%), *blaNDM-1* (32.96%), *blaOXA-48/blaVIM* (1.68%), *blaOXA48* (37.43%), *blaVIM* (5.03%), *blaNDM-1/blaOXA48* (13.97%), *blaKPC-2* (1.12%), and *blaVIM/blaIMP* (0.56%) by Gondal et al. (2024). Previous reports from Pakistan found that the CRPA contained the carbapenemases *blaNDM-1, blaIMP, blaVIM,* and *blaOXA-48* (Saleem and Bokhari, 2020). The findings indicate a potential hazard in treating clinical infections in both the human and veterinary sectors. Therefore, it is vital to eliminate the excessive and irrational use of antibiotics in both the human and animal sectors. The current national policies and standards could be the starting point for addressing this issue. Due to the diversity of bacterial species and the various genetic components involved, regular and comprehensive screening for the prevalence of carbapenem resistance using advanced molecular and culture-based approaches is necessary (Köck et al., 2018). The prevalence of MRSA was also explained previously by Tajik et al. (2019) in which (41.9%) of isolates were *mecA*-positive, which was considered MRSA. According to Parhizgari et al. (2016) molecular identification of the mec gene, 86.4% of MRSA strains have been identified.

The disk diffusion test was used to assess the in vitro antibacterial activity of Ag NPs. *Pseudomonas*

aeruginosa showed the largest observed inhibition zone of 18 mm, whereas methicillin-resistant *Staphylococcus aureus* (MRSA) showed the maximum ZOI 19 mm. The minimum ZOI 8 mm for *P. aeruginosa* and the lowest recorded inhibition zone was detected at 9 mm for MRSA. The activity likewise diminishes with concentration; for example, the zone size was only 8 mm at a dose of 5 mg/mL. the results were consistent with the previously conducted study by Huang et al. (2021)

MIC and MBC of Ag NPs against *P. aeruginosa* and *S. aureus* were assessed further at various concentrations. he majority of the *P. aeruginosa* cells have been killed at the concentration of 0.78 mg/mL. MBC of 0.78 mg/mL was found in approximately 5 isolates (20%), 1.56 mg/mL was found in 12 isolates (48%) and 3.12 mg/mL was found in approximately 8 isolates (32%). The results of *S. aureus* showed that 0.026mg/mL concentration mostly inhibited bacterial growth. Nine isolates (50%) demonstrated 0.026mg/mL MBC, six isolates (33.3%) demonstrated 0.013mg/mL MBC, and three isolates (16.6%) demonstrated 0.052mg/mL MBC. The results were compared with previously conducted study by (Huang et al., 2022) where the 99% Gram negative bacterial cells have been killed at the concentration of 0.4 mg/ml, MIC and MBC values Ag NPs against Gram negative bacterial pathogens are 0.04 mg/ml and 0.4 mg/ml, respectively. while the MIC value of Ag NPs against *S. aureus* bacterial is 0.04 mg/ml, and the MBC value decreases to 0.2 mg/ml. According to the results, Ag NPs exhibit remarkable antibacterial activity against clinically important Gram-positive and Gram-negative bacteria. According to the abovementioned data, Ag NPs had variable susceptibility to both Gram-negative and Gram-positive bacteria, demonstrating more potent antibacterial activity against *Pseudomonas aeruginosa* than *Staphylococcus aureus*. This could be explained by variations in the structure of the bacteria's cell walls, with *P. aeruginosa* having a thinner peptidoglycan layer that makes it more vulnerable to nanoparticle penetration, as well as the bacteria's unique susceptibility to reactive oxygen species and interference with the formation of biofilms (Rasool et al., 2016). Furthermore, the peptidoglycan layer of Gram-positive bacteria contains teichoic acids (Verlee et al., 2017), and the main cause of the antibacterial effects of silver particles is their strong reactivity with phosphorus and sulfur (Behravan et al., 2019).

Conclusion

This research concluded that the silver nanoparticles have been proved as a potential antibacterial agent and can be a choice of drug in therapeutic options. The effectiveness of silver oxide nanoparticles for use by humans in the future, however, requires more research. The results also show a concerning prevalence of carbapenemase and methicillin resistant isolates among *Pseudomonas* and *Staph aureus*, demanding immediate need for alternative treatments. In addition, next research should look at the interaction between antibiotics and silver oxide nanoparticles against hospital strains resistant to treatment to develop novel materials and compounds with potential medical uses.

Acknowledgment

This research work was funded by Institutional Fund Projects under grant no. (IFPIP: 2003-140-1443). Therefore, the authors gratefully acknowledge the technical and financial support from the Ministry of Education and King Abdulaziz University (KAU), DSR, Jeddah, Saudi Arabia.

Disclaimer: None.
Conflict of Interest: None.
Source of Funding: Ministry of Education and King Abdulaziz University (KAU), Jeddah, Saudi Arabia

Contribution of Authors

Ahsan H, Ayub M & Naveed R: Conducted the study and collected & analyzed data and manuscript write up
Gul M, Qureshi A, Asfour HZ, Bilal HM, Azeem M & Shabir M: Data analysis and interpretation
Wahid A: Conducted the study and collected data
Ali N: Data analysis and interpretation
Rajeh N, Mammadov A & Siddique AB: Conceptualization of study and supervision of research work
All authors read and approved the final manuscript

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