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Antibacterial activity of silver nanoparticles against carbapenem-resistant *Acinetobacter baumannii* clinical isolates

Asad Ali Shah[1], Ijaz Ahmad[2], Muhammad Shafique[2]*, Abu Baker Siddique[2], Bilal Aslam[2], Muhammad Usman Qamar[2]

[1]Department of Bioinformatics and Biotechnology, Faculty of Life Sciences, Government College University Faisalabad, Pakistan
[2]Department of Microbiology, Faculty of Life Sciences, Government College University Faisalabad, Pakistan

Abstract: Carbapenem-resistant *Acinetobacter baumannii* (CRAB) produce resistance to various classes of antibiotics and left limited options for treatment. This study was designed to determine antibacterial activity of AgNPs against CRAB. Total 100 *A. baumannii* were collected from a tertiary care hospital, Lahore. Isolates were subcultured on blood and MacConkey agar. Preliminary identification was carried out by morphological and biochemical tests. Antibiogram was done by Kirby-Bauer disc diffusion method. Antibacterial activity of AgNPs was performed by agar well diffusion method, while minimum inhibitory concentration and minimum bactericidal concentration were determined by micro broth dilution assay. Of 100 *A. baumannii*, 24 were confirmed as carbapenem-resistant. These isolates were mainly recovered from tracheal secretion (8; 33%), CSF (5; 20.8%), and urine (4; 16.8%). Antibacterial activity of AgNPs revealed a maximum zone of inhibition, 22mm at 50mg/mL and 18mm at 40mg/mL by agar well diffusion method. MIC of AgNPs determined that 14 CRAB were inhibited at 12.5mg/mL and 7 at 25mg/mL. However, MBC revealed that 13 CRAB were killed at 25mg/mL and 7 at 50mg/mL. This study concluded that most of the CRAB were inhibited and killed at 12.5mg/mL and 25mg/mL, respectively. AgNPs can be used as an alternative therapeutic agent followed by their pharmacokinetics and pharmacognosy.

Keywords: Carbapenem-resistant, *A. baumannii*, MIC, MBC, silver nanoparticles

INTRODUCTION

The emergence of carbapenem-resistant *Acinetobacter baumannii* (CRAB) are one of the main concerns related to public health globally. Despite many advances in the present decade, there is still a failure in combating antimicrobial resistance (AMR) (Wareth *et al*., 2021). Recently, *A. baumannii* have appeared extensively in epidemic and endemic infections in hospitals settings. They are normally present on the mucous membranes and skin of humans and can cause opportunistic infections including septicemia, meningitis, upper respiratory tract infections, pneumonia and urinary tract infections (Ejaz *et al*., 2021). Biofilm formation is the main virulence feature of several *A. baumannii* isolates that also include the carbapenem-resistant strains (Khalil *et al*., 2021). *Acinetobacter* are resistant to several antibiotics (fluoroquinolones, cephalosporins, penicillins and aminoglycosides) by intrinsic and acquired pathways. Carbapenems have been successfully used in many centers to combat multidrug-resistant *Acinetobacter* infections, but CRAB have posed serious remedial issues (Khurshid *et al*., 2019). A recent study revealed that more than 60% of pan-drug-resistant and CRAB isolates cause hospital-acquired pneumonia in Asian countries (Park *et al*., 2021). In the United States of America (USA) and Europe, OXA-23, oxacillinase is accountable for carbapenem resistance. The absence of remedial agents with no patient safety and expected activity against *A. baumannii* becomes a challenge for the entire healthcare system in general and particularly for a country like Pakistan (Lin and Lan, 2014). In the review of this global issue, there is an urgent need to develop alternative therapeutic agents to cope with the problems which are associated with these drug-resistant pathogenic bacteria (Ahmad *et al*., 2021). Over the past few decades, nanotechnology has established an advanced solution to overcome the problem of antimicrobial resistance by developing nanoparticles (Qureshi *et al*., 2021). Inorganic metals and their Oxides occupied much interest because of their stable, safe, and non-toxic nature (Chavali and Nikolova, 2019). Silver nanoparticles have become much more attractive by their applications in biology, for example, biological labeling, nanomedicine, delivery of genes, delivery of the drug and biological sensing (Patra *et al*., 2018a). Due to their antimicrobial activity, they are viewed to be a practical solution to prevent diseases caused by drug-resistant bacteria.

MATERIALS AND METHODS

Bacterial strains

Clinical isolates of *A. baumannii* (n=100) were collected from the tertiary care hospital in Lahore from different clinical sources using aseptic techniques.

Bacterial strains identification

Isolates were reconfirmed using blood and MacConkey agar and the plates were incubated at 37^{o}C overnight.

**Corresponding author:* e-mail: drmshafique@gcuf.edu.pk

Bacterial isolates were identified by different biochemical tests such as triple sugar iron, indole, methyl red, citrate, and urease.

Antimicrobial susceptibility testing

Antimicrobial sensitivity testing was performed by the Kirby Bauer disc diffusion assay using the following Carbapenem antibiotic discs (imipenem 10µg, meropenem 10µg, and doripenem 10µg). The zones of inhibition were compared and interpreted according to the Clinical and Laboratory Standard Institute (CLSI) guidelines, 2020.

Silver nanoparticles

Silver nanoparticles were purchased from the US Research Nanomaterials, Inc, USA. They were black-colored fine powder with a spherical shape having a 99.99% purity level with 10.5g/cm^3 true density and 0.2wt% PVP as a stabilizing agent for low oxygen content and easy dispersion. They have a size of 20 nanometers (nm). They were characterized by using SEM and X-ray diffraction.

Antibacterial assay of Silver nanoparticles

An agar well diffusion assay was used to determine the antibacterial activity of silver nanoparticles (AgNPs) against CRAB (Qureshi *et al*., 2021). 5% dimethyl sulfoxide (DMSO) was the solvent used for the preparation of the AgNPs colloidal solution which was sonicated at 30°C for 2-3 hrs. DMSO was used as a negative control. The overnight culture of the bacterium was diluted in normal saline according to the 0.5 McFarland standard to achieve a concentration of 1.5×10^8 CFU/mL and then swabbing was performed onto the Muller Hinton agar plates. Wells of 6mm were shaped with the help of a cork borer on the swabbed agar plates. 100µL of AgNPs different concentrations i.e. (50, 40, 30, 20, and 10) mg/mL were poured into all the wells including DMSO (negative control) with the help of micropipette. The plates were incubated with the upright position at 37°C for 18-24 hrs.

Evaluation of Bacteriostatic Potential of Silver nanoparticles

The minimum inhibitory concentrations (MICs) of AgNPs were calculated against the said bacterium by the micro-broth dilution assay (Qureshi *et al*., 2021). Muller Hinton broth was used to carry out the two-fold serial dilutions of AgNPs (50mg/mL to 0.097mg/mL) in 96 well microtitration plates. Wells of microtitration plates having varying concentrations of AgNPs were inoculated with overnight bacterial culture suspensions that adjusted to 1.5×10^8 CFU/mL. The plates were covered to avoid contamination and incubated at 37°C for 18-24 hrs. After incubation, the plates were observed for color change from yellow to blue by adding a redox dye nitro blue tetrazolium chloride which indicates the viability of bacterial cells (blue color) and non-viability of bacterial cells (yellow color). The lowest concentration of AgNPs that failed to change the color of dye was considered as the MIC. All the experiments were performed in triplicate along with the placements of negative and positive controls.

Evaluation of Bactericidal Potential of Silver nanoparticles

Estimation of minimum bactericidal concentrations (MBCs) was performed by dispensing concentrations from the MIC and above wells onto the Muller Hinton agar plates. The plates were incubated at 37°C for 24 hrs. The concentration that did not show the growth (even a single colony) on the plate was considered as MBC. MIC and MBC values were shown as mg/mL (Qureshi *et al*., 2021).

STATISTICAL ANALYSIS

The data were analyzed using SPSS version II. The average frequencies and standard deviations were calculated of all the data.

RESULTS

Bacterial confirmation

Of 100 clinical isolates, prominent sources included pus (n=19), tracheal secretion (n=19), wound swabs (n=16), sputum (n=8), blood (n=8) and urine (n=6). The frequency of male and female patient was 56% and 44%, respectively (Figure 1).

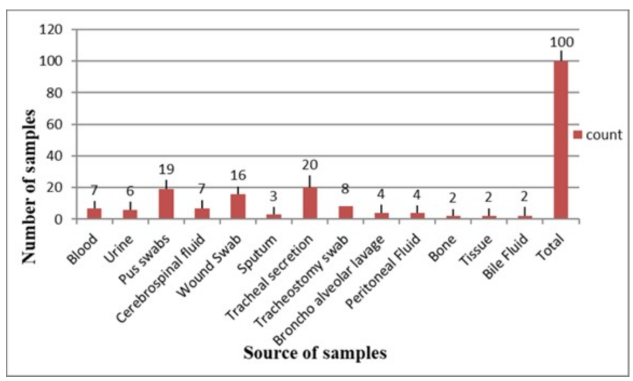

Fig. 1: Sources of samples from various sources

Antibacterial susceptibility

Of 100, 24 were resistant to carbapenem antibiotics such as imipenem, meropenem, and ertapenem.

Agar well diffusion method

The antibacterial activity of silver nanoparticles was investigated against *A. baumannii* using an agar well diffusion assay. The zone of inhibition against each concentration (i.e. 50, 40, 30, 20, 10 mg/mL) and DMSO as control, were measured. Table 1 shows that the maximum zone at a concentration of 50mg/mL was recorded as 22mm, at 40mg/mL was 18mm, at 30mg/mL was 15mm, at 20mg/mL was 13mm and at 10mg/mL was 12mm while DMSO (negative control) have no zone of inhibition. The mean and standard deviation of each concentration was calculated as 50mg/mL (18.04±1.756), 40mg/mL (15.25±1.452) 30mg/mL (13.17±1.007) 20mg/mL (11.63±0.924) and 10mg/mL (9.79±1.285) used in the agar well diffusion assay of silver nanoparticles against *A. baumannii*. Comparison of Imipenem and Meropenem drugs with silver nanoparticles was also carried out. It showed that the potent drugs had no killing effect on *A. baumannii* while Silver nanoparticles were found to be potentially active (fig. 2).

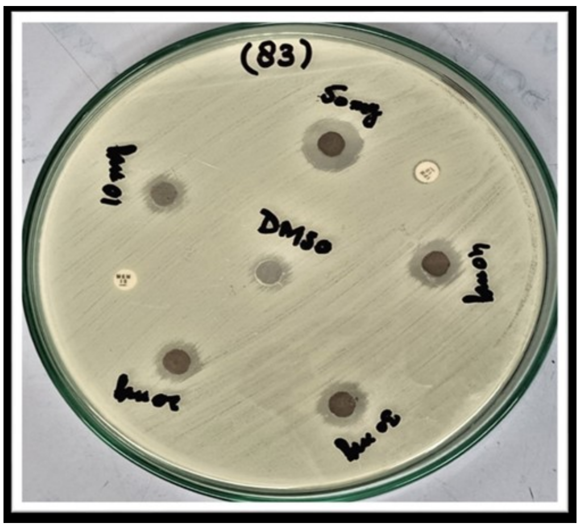

Fig. 2: Zone of inhibition (ZOI) of Silver nanoparticles against *A. baumannii* by agar-well diffusion assay

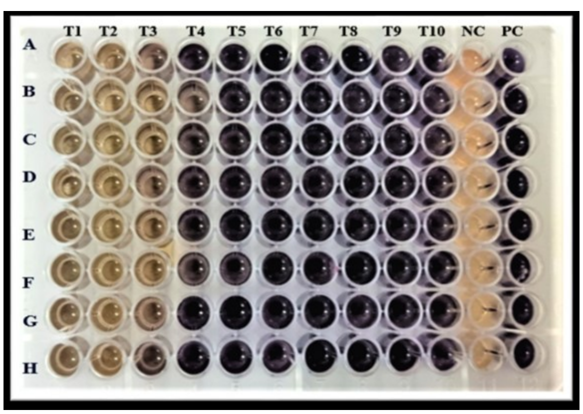

Fig. 3: MIC of Silver nanoparticles by broth micro-dilution against *A. baumannii* isolates using Nitro-blue Tetrazolium chloride

The minimum inhibitory concentration of AgNP

The MICs of metallic nanoparticles were determined by the Nitro-blue Tetrazolium chloride (NBT) dye. After adding the dye, a change in color from yellow to blue indicated the viability of bacterial cells and no change in color i.e. yellow showed that the cells were metabolically immobile (non-viable). About 3 isolates (12.5%) exhibited the MIC of 6.25mg/mL, 14 isolates (58.3%) showed the MIC of 12.5mg/mL and about 7 isolates (29.2%) showed the MIC of 25mg/mL (fig. 3 and table 2).

The minimum bactericidal concentration of AgNP

The minimum bactericidal concentration (MBC) of Silver nanoparticles was performed by the agar spread plate method. Fig. 3 shows different MBCs for different isolates (1:4 means 3rd dilution was 12.5mg/mL, 1:2 means 2nd dilution was 25mg/mL and 1:1 means 1st concentration was 50mg/mL). About 4 isolates (16.7%) exhibited the MBC of 12.5mg/mL, 13 isolates (54.2%) showed the MBC of 25mg/mL and about 7 isolates (29.2%) showed the MBC of 50mg/mL (Table 2).

DISCUSSION

Acinetobacter baumannii are nosocomial pathogens with elevated morbidity and mortality in clinical settings. The WHO has placed carbapenem-resistant *A. baumannii* on the top of priority organisms against which novel antibiotics are required. With the advent in recent years of antibiotic resistance, *A. baumannii* has become a significant health concern. It has emerged as a leading cause of nosocomial infections that cause a range of infections, especially in intensive care units (ICUs), including septicemia, urinary tract infections and, wound infections. Infections and diseases caused by CRAB are popular and have been recorded worldwide over the last 20 years (Qamar *et al*., 2020). *A. baumannii* isolates that are extensively drug-resistant and pan drug-resistant are commonly reported in medical settings (Ababneh *et al*., 2021). Due to rising resistance rates, carbapenems, the treatment of choice for treating *A. baumannii* infections, are becoming increasingly ineffective (Viehman *et al*., 2014). Resistance to the newer antibiotic tigecycline is also fast growing.

Colistin, a once-popular antibiotic, is now being used as a last resort. However, resistance to this medicine is increasing at an alarming rate around the world (Asif *et al*., 2018). Such dwindling medicinal options have prompted scientists to search for alternatives to antibiotics. These solutions are urgently needed now and will hopefully be available shortly (Qamar *et al*., 2019). To overcome these complications of carbapenem resistance and other antibiotics, researchers have focused on nanomaterials to solve this challenge for their use in the medical industry, for the last 30 years (Aslam *et al.,* 2018). In the present study, 24% of *A. baumannii* were resistant to carbapenem antibiotics which consider being the last report against MDR pathogens. Similar findings have been reported in different parts of the world. A review article has been published on the prevalence of CRAB that reflects the high spread of these pathogens (Poirel and Nordmann, 2006).

Table 1: Zone of inhibition (ZOI) of AgNPs against *A. baumannii* by agar-well diffusion assay

Isolate No.	50mg/mL ZOI (mm)	40mg/mL ZOI (mm)	30mg/mL ZOI (mm)	20mg/mL ZOI (mm)	10mg/mL ZOI (mm)	DMSO ZOI (mm)
4	19	15	12	10	07	0
8	16	15	13	11	09	0
9	18	16	14	12	10	0
16	16	13	12	11	08	0
18	18	16	14	12	11	0
24	19	16	13	11	10	0
25	17	15	14	12	11	0
27	19	17	15	13	12	0
28	17	16	14	12	11	0
31	16	14	12	11	09	0
36	18	15	13	11	10	0
52	17	16	14	12	11	0
63	16	13	12	11	08	0
67	21	18	15	14	12	0
75	18	16	14	12	11	0
77	20	14	12	11	09	0
80	22	18	14	13	09	0
82	17	14	13	11	10	0
83	16	13	12	11	09	0
84	17	16	14	13	11	0
97	20	17	13	12	10	0
98	18	14	12	11	09	0
99	21	14	12	11	09	0
100	17	15	13	11	09	0

Table 2: MICs and MBCs (mg/mL) of Silver nanoparticles against carbapenem resistant *A. baumannii*

Isolate No.	MIC (mg/mL)	MBC (mg/mL)
4	12.5	12.5
8	12.5	25
9	12.5	25
16	6.25	12.5
18	25	25
24	12.5	12.5
25	12.5	25
27	12.5	25
28	12.5	25
31	12.5	50
36	12.5	25
52	12.5	12.5
63	12.5	25
67	25	50
75	25	25
77	6.25	25
80	25	25
82	25	50
83	25	25
84	25	50
97	6.25	25
98	12.5	50
99	12.5	50
100	12.5	50

Further, a study from Pakistan also reported a high 85% CRAB in clinical settings (Khalid *et al*., 2020). Similarly, another study from Pakistan also described 65% of CRAB were isolated from the clinical origin (Ejaz *et al*., 2021). The high spread of CRAB in our clinical settings is mainly linked with the fragile health system, inappropriate health facilities, lack of hygiene practices, sharing of beds, substandard practices, and lack of health care staff (Kim *et al*., 2021 and Qamar *et al*., 2021). Therapeutic and diagnostic procedures include the use of nanomedicines for a variety of diseases that invade multiple organs of the human body.

Nanocarriers have a significant effect on medicine development and infection control due to the secure distribution of drugs (Patra *et al*., 2018b). Nanoparticles have a significant role in different fields of biology and medicine. Nowadays, nanoparticles have been used for the treatment of various bacterial and fungal infections (Sharma *et al*., 2019). In this study, AgNPs revealed a maximum zone of inhibition, 22mm at 50mg/mL and 18mm at 40mg/mL by the agar well diffusion method. However, MIC of AgNPs determined that 14 CRAB were inhibited at 12.5mg/mL and MBC revealed that 13 CRAB were killed at 25mg/mL. Similarly, a study from Brazil reported 0.460 to 1.870 µg/ml against CRAB clinical isolates (Allend *et al*., 2021). Another study from Egypt documented 4 to 25 µg/ml against *A. baumannii* (Hetta *et al*., 2021). Another Saudi study determined the combinations of colistin and silver nanoparticles or imipenem and silver nanoparticles resulted in synergistic action that led to reduction of MICs against *A. baumannii* (Khaled *et al*., 2021). Therefore, it is the need of the hour to develop such alternative medicine especially nanoparticles. Recently, the Ministry of Health Pakistan develop the National Action Plan (NAP) on AMR, which emphasized the development of novel antibiotics and alternative medicines such as nano biotics, bacteriophages, medicinal plants extracts, and, bacteriocins (Saleem *et al*., 2021).

CONCLUSION

This study concluded a high prevalence of CRAB (24%) in clinical settings. The CRAB were inhibited and killed at 12.5mg/mL and 25mg/mL, respectively. AgNPs can be used as an alternative therapeutic agent followed by several clinical trials. Further, there is a need of the hour to implement the NAP on AMR in the countries.

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