

Anti-bacterial Activity of Polyvinyl Pyrrolidone Capped Silver Nanoparticles on the Carbapenem Resistant Strain of *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii has been identified by Infectious Diseases Society of America as one of the six pathogens that cause majority of hospital infections. Carbapenems are the most effective β -lactam antibiotics that are routinely prescribed by clinicians to patients infected with *A. baumannii*. Emergence of resistance against carbapenem makes it difficult to treat *A. baumannii* hence it is a threat to public health. Therefore it is very important to find a suitable alternative to the carbapenem. In the present work we have tried to establish silver nanomaterial as an alternative to carbapenem resistant strain of *Acinetobacter baumannii*. Silver nanoparticles have been prepared without aggregation by a modified chemical reduction method; using ethylene glycol mono methyl ether as solvent and polyvinyl pyrrolidone (PVP) as capping agent. Transmission electron microscopic study revealed silver nanoparticles in spherical shape with narrow size distribution. The average size of silver nanoparticles has varied by changing the metal precursor to capping agent ratio, and corresponding characteristic size dependent plasmonic properties were observed using UV-Vis spectroscopy. It was found that novel silver nanoparticles having particle diameter <10 nm exhibited a tremendous potential antibacterial activity against the carbapenem resistant strain of *Acinetobacter baumannii*. Therefore, PVP-capped silver nanoparticle can be developed as an alternative to carbapenem, the most prescribed antibiotic for the *Acinetobacter baumannii* infections.

Keywords: PVP-capped silver nanoparticles; Carbapenem resistance; *Acinetobacter baumannii*; Silver nanoparticles; Carbapenem

Introduction

Acinetobacter baumannii causes pneumonia, urinary tract infections, respiratory infections and meningitis [1]. Due to its pathogenicity, it is grouped into ESKAPE pathogens, a group of pathogens causing hospital-acquired infections [1,2]. *A. baumannii* has emerged as a threat to soldiers, wounded during military operations in Iraq and Afghanistan [3] as well as isolated from natural resources [4]. It can grow on artificial surfaces and can utilize ethanol as a carbon source [5], resist desiccation, survive at diverse temperatures and pH [6]. Prevalence of *A. baumannii* in clinical setup increases gradually [7,8]. Commonly prescribed drug against *A. baumannii* are carbapenems which belongs to the β -lactam group of antibiotics [9]. *A. baumannii* has developed resistance against carbapenem using very diverse mechanisms [10-18] which is a significant health problem and responsible for high morbidity and mortality [19]. Mortality due to carbapenem-resistant *Acinetobacter baumannii* is about 52% as compared to 19% when infected with carbapenem sensitive variant [20]. Therefore, there is an urgent need to find the suitable alternative for carbapenem.

Silver is one of the most universal antimicrobial substances. Nano-engineering enables us to expand the surface area of silver particles markedly. Silver nanoparticles have shown antimicrobial activity against a wide array of microbes, probably due to their multiple mechanisms of antimicrobial action [21]. Contrary to bactericide effects of ionic silver, the antimicrobial activities of colloid silver particles are influenced by the morphology of the silver particles. Therefore, in developing synthesis methodologies, emphasis should be given to control the size, shape, size distribution and local distribution of silver nanoparticles. Silver nanoparticles have been produced using different methods: thermal decomposition [22,23], swift heavy ion irradiation [24], atom beam irradiation [25], electron beam physical vapour co-

deposition [26] and chemical reduction method [27]. In the present article we have employed the chemical method. Small nanoparticles with a large surface area to volume ratio provide a more efficient means for antibacterial activity even at very low concentrations [28].

Antibacterial activity of silver nanoparticles (Ag-NPs) in dispersed state as well as in embedded state has been reported for their antibacterial activity against different bacteria *i.e.* *Pseudomonas aeruginosa* (ATCC-17934), *Staphylococcus aureus* (ATCC-25923), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 29249) [29-33]. Silver and other nanoparticles are reported to interact with certain proteins and thereby showed its activity [34]. There are few reports which initiated the research of silver nanomaterial as an antimicrobial agent for *Acinetobacter baumannii* [28,35]. Niakan et al., identified that selected bacterial strains of *A. baumannii* are sensitive to silver (uncapped) nanomaterial [28]. Capped silver nanoparticles (like citrate-capped, SDS-capped) have also been synthesised against Gram negative bacteria [36-38]. Similarly, Huang et al., identified the antimicrobial activity of AgNPs against *A. baumannii* by synergistic combination of chitosan acetate with silver nanoparticle [35]. Antimicrobial activity of PVP capped Ag-NPs have been determined only for few bacteria like *Salmonella*, *S. aureus* and *E. coli* [37,38]. However, the antimicrobial

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activity of the polyvinyl pyrrolidone (PVP) capped Ag-NPs has not studied so far on *Acinetobacter baumannii*. PVP is a neutral organic stabilizer and is less sensitive to the surface charge screening processes by ionic strength as compared to citrate [37]. Retention of PVP coated AgNP is more than citrate coated AgNP because particle size of PVP coated nanoparticles is less sensitive to changes in pH and ionic strength [37,39,40]. Recent report showed that PVP capped Ag-NPs are more stable than other AgNPs like citrate AgNPs and SDS AgNPs [37,38] hence PVP capped AgNPs is less toxic for mammalian cells [41] therefore PVP capped AgNPs can be more favorable as an alternative drug to carbapenem as compared to other capped AgNPs. Similarly, interaction of AgNPs with serum proteins also affects its antimicrobial activity *in-vivo*. It is also reported that PVP-capped AgNPs showed better antimicrobial activity than other capped AgNPs *in-vivo* [42]. Therefore, it is important to study antimicrobial activity of PVP capped AgNPs on carbapenem resistant strain of *Acinetobacter baumannii*. The present result will help to demonstrate the antibacterial properties of the PVP capped AgNPs against carbapenem resistance strain of *Acinetobacter baumannii*.

Methods

Preparation of the PVP-capped silver nanoparticle (Ag-NPs)

For the size dependent properties of silver nanoparticles, colloidal suspension of silver nanoparticles were prepared using modified chemical decomposition method. In this method, we started with 0.1N AgNO₃ as metal precursor, polyvinyl pyrrolidone (PVP) as a capping agent (powder form with average molecular weight of 29000) and 97% ethylene glycol mono methyl ether (CH₃OCH₂CH₂OH) as a solvent (All chemicals procured from Sigma-Aldrich). Firstly, 5 ml of AgNO₃ was dissolved in 10 ml of ethylene glycol mono methyl ether and stirred for 2 h, kept in temperature range 40-50°C. After this step, appropriate amount of PVP was added in this solution to maintain different metal to capping agent ratio. Stirring was continued further for 30 min and a yellow solution of Ag colloids was obtained. In Table 1, the amount of precursor, solvent and capping agent that was taken to prepare silver nanoparticle and corresponding physical appearance of colloidal solutions has been presented.

Characterization of the PVP-capped silver nanoparticle

Silver nanoparticle was characterised by the transmission electron microscope [2] and UV-Vis spectroscopy. For TEM study, colloidal particles are first dispersed at carbon coated mica sheet, and then carbon coating is floated on Cu grid. Then, these grids were investigated using an electron microscope (FEI Tecnai 20) operated at 100 keV beam energy. For investigating the plasmonic properties of colloidal silver nanoparticles, double beam UV-Vis spectrophotometer (Hitachi U3300) was carried out in spectral range of 200 to 800 nm taking solvent as reference.

Sample's name	Amount of metal precursor (AgNO ₃)	Amount of Solvent (CH ₃ OCH ₂ CH ₂ OH)	Amount of capping agent (PVP)	Physical appearance (Color)	Size of Ag NPs
A0	5 ml (0.1 N)	10 ml (97%)	0.025 gm	Gray	-
A1	5 ml (0.1 N)	10 ml (97%)	0.05 gm	Dark Yellow	10 ± 2 nm
A2	5 ml (0.1 N)	10 ml (97%)	0.1 gm	Yellow	8 ± 2 nm
A3	5 ml (0.1 N)	10 ml (97%)	0.2 gm	Light	6 ± 2 nm

Table 1: Colloidal silver nanoparticle solution having different concentration of polyvinyl pyrrolidone.

Clinical strains of *Acinetobacter baumannii*

Clinical strains of *Acinetobacter baumannii* were taken from department of biochemistry, A.I.I.M.S., India. All clinical strains were isolated from sputum of patients admitted to Intensive Care Units (ICUs), A.I.I.M.S., India. All clinical strains were confirmed up to the species level (*baumannii*) by standard biochemical tests [8]. Four clinical strains (RS307, RS122, RS6694 and RS6053) of *Acinetobacter baumannii* are used in the present study.

Antimicrobial susceptibility testing and minimum Inhibitory concentration testing for carbapenem

Antimicrobial susceptibility test measures the ability of an antibiotic or other antimicrobial agent to inhibit bacterial growth *in-vitro*. Antimicrobial discs of the above drugs were prepared. After adjustment of an inoculum size, the inoculum was swabbed uniformly on Muller-Hinton Agar plate with the help of a sterile cotton swab where excess inoculum was removed after pressing the swab lightly on the inside wall of the test tube. After 15 min of swabbing, the antibiotic discs were carefully placed with the help of sterile forceps. After 5-10 min, the plates were incubated at 37°C. The inhibition zone against antibiotic discs was observed and interpreted as sensitive (S) or resistant (R) according to standards laid by CLSI. Minimal inhibitory concentration of clinical strains was determined for imipenem (a carbapenem) by agar dilution method under the CLSI guideline according to our previously described method [8].

Growth kinetics of resistance strain of the *Acinetobacter baumannii* under diverse condition of silver nanoparticle

Growth kinetics of different carbapenem resistant strains of *A. baumannii* have been determined by taking O.D. of different strain of *Acinetobacter* at 605 nm with the time using ELISA reader as well as UV-Vis spectroscopy. Growth curve has been prepared from the absorption data. The stock solution of silver nanoparticle with concentration of 33 mM was used and the final working concentration of silver nanoparticle was 165 µM. The experiments were repeated in the absence and presence of silver nanoparticle. The relative growth curve has been prepared for the comparison purpose.

Results and Discussion

To study the effects of silver nanomaterial on the carbapenem resistant strain of *Acinetobacter baumannii*. First step is the synthesis and characterisation of silver nanoparticle. This step is followed by antimicrobial testing of silver nanoparticle on the carbapenem resistant strain of *Acinetobacter baumannii*.

Characterization of polyvinyl pyrrolidone (PVP) capped nanoparticles

Transmission electron microscope is used to characterize silver nanoparticle. Figure 1 shows the representative micrograph of PVP capped Ag nanoparticles. From the TEM micrograph, it is evident that prepared nanoparticles were in spherical shape and uniformly distributed without any agglomeration. Their diameters were found in the range of 6-10 nm. For investigating the plasmonic properties of colloidal silver nanoparticles, UV-Vis spectroscopy was carried out in spectral range of 200 to 800 nm. The optical absorbance spectra of the colloidal silver nanoparticles are shown in Figure 2, where curves (a), (b), (c) and (d) are corresponding to the samples A0, A1, A2 and A3 prepared with capping amount PVP as 0.025 gm, 0.05 gm, 0.1 gm and 0.2 gm respectively. For all curves, two dominant peaks are observed;

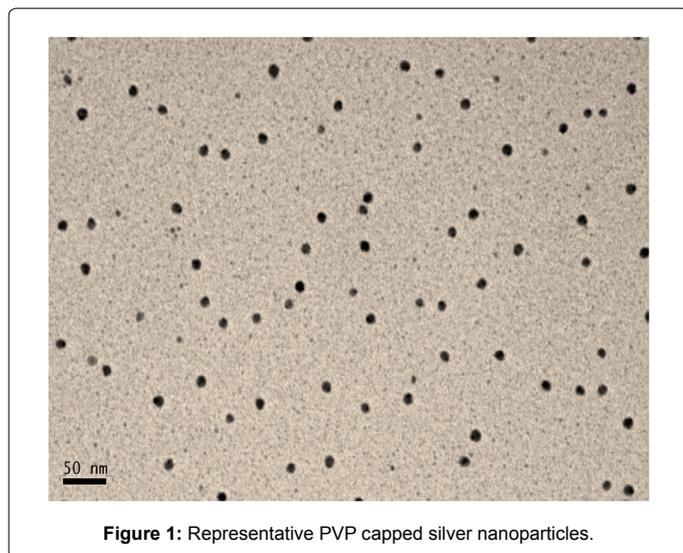


Figure 1: Representative PVP capped silver nanoparticles.

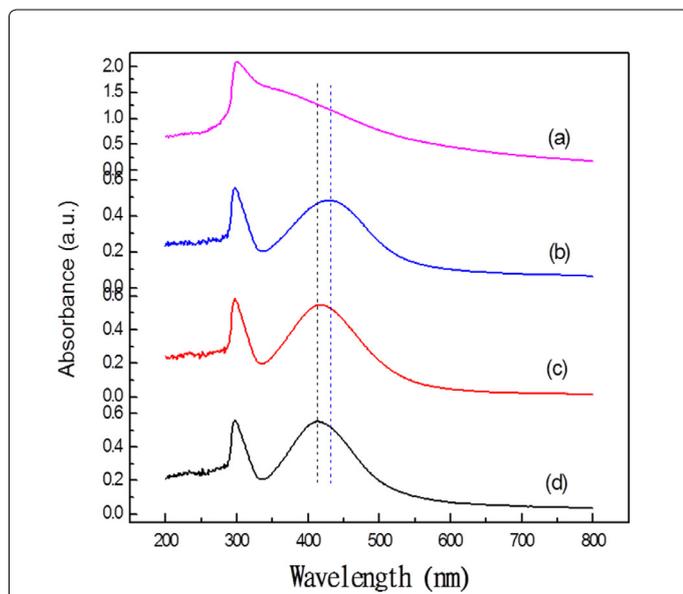


Figure 2: The optical absorbance spectra of the colloidal silver nanoparticles are shown, where curves (a), (b), (c) and (d) are corresponding to the samples A0, A1, A2 and A3 prepared with capping amount PVP as 0.025gm, 0.05 gm, 0.1 gm and 0.2 gm; respectively.

first peak centered at 296.65 nm and second peak centered in range from 412-431 nm. The first peak in spectrum is assigned to NO_3^- ions while second significant peaks centered in range from 412-431 nm provide clear evidence for the formation of silver nanoparticles. When silver nanoparticles are irradiated by visible radiation then surface plasmon resonance [43] occurs and provides absorbance peak in these wavelength regions. A decrease in full width at half maximum (FWHM) suggests an increase in silver nanoparticle core-diameter [44]. The broad plateau of A0 in wavelength region of 340-540 nm is an indication of agglomerated silver nanoparticles with wide size distribution, which suggests that amount of capping agent was too small to confine silver particle in monomodal nanoscale sizes. The characteristic peaks of silver nanoparticles in A1, A2 and A3 samples show narrow FWHM, which shows that average size distribution of silver nanoparticles is very small. This result is in agreement with the microscopic image shown in

Figure 1. The observed blue shift in this characteristic absorption peak from 431 nm to 412 nm is an indication of decrease in average particle sizes of silver nanoparticles, when we move from A1 to A2 and then further to A3. It also explains the PVP capping stabilizes the Ag-NPs [38]. The assignment of the absorption peaks are in agreement with other reported values in literature. Gasaymeh et al. reported NO_3^- peak at 256 nm [45]. Kumar et al. reported SPR peak of silver nanoparticle after adding NaBH_4 in aqueous solution at 389-402 nm corresponding to average nanoparticle size in 4 nm-10 nm and around 454 nm when Ag nanoparticles of average size 14 nm were embedded in ZrO_2 matrix [27,46]. Therefore the size of the PVP capped AgNPs is about 5 to 15 nm. The intermediate spectral location of the silver nanoparticles in present case is because of intermediate refractive index of solvent (1.43) in comparison to that of H_2O (1.33) and ZrO_2 (~2). The fine assignment of peaks can also be done using effective medium theories and considering the refractive indices of solvent as well as of capping layer along with the size dependent dielectric functions of silver. PVP capping on the surface of nanoparticles protects from changes in environmental conditions and prevents aggregation [38]. With these results, inference can be drawn that prepared silver nanoparticles, using PVP amount in range of 0.05 gm to 0.2 gm, were found spherical shaped, agglomeration-free and with narrow size distribution except by 0.025 gm. The prepared PVP capped silver particles A1, A2 & A3 have been used for the antimicrobial test.

Antimicrobial susceptibility test

This test was carried out in presence of various antibiotics namely amikacin, meropenem, imipenem, piperacillin, netilmycin, ciprofloxacin, aztreonam, ceftazidime, [piperacillin + tazobactam], [cefoperazone + sulbactam] and [ticarcillin + clavulanic acid], [amoxicillin + clavulanic acid]. Table 2 summarizes the result of the test for the selected four strains of *A. baumannii*. The data reveals that all the strains are resistant to most of the antibiotics that are usually used in clinical treatment. Only combination of β -lactams drugs with β -lactamase inhibitors have shown a slight potency in antimicrobial activity. All these *A. baumannii* strains also showed resistance against other groups like fluoroquinolones and aminoglycosides. The MIC of the strains for imipenem, a carbapenem has also been determined. Four resistant strains RS307, RS122, RS6053 and RS6694 have minimal inhibitor concentration of 64 $\mu\text{g/ml}$, 32 $\mu\text{g/ml}$, 8 $\mu\text{g/ml}$ and 8 $\mu\text{g/ml}$ respectively for carbapenem. Based on CLSI guidelines, RS307 is high

Strain no.	RS307	RS122	RS6053	RS6694
Amikacin	R	R	R	R
Meropenem	R	R	R	R
Imipenem	R	R	R	R
Piperacillin	R	R	R	R
Netilmycin	R	R	R	R
Ciprofloxacin	R	R	R	R
Aztreonam	R	R	R	R
Ceftazidime	R	R	R	R
Amoxicillin + Clavulanic acid	R	R	S	S
Pipra + Tazobactam	R	R	S	S
Cefope + Sulbacum	R	S	S	S
Ticarcillin + Calvulanic acid	R	R	S	S
MIC for Imipenem (Carbapenem)	64 $\mu\text{g/ml}$	32 $\mu\text{g/ml}$	8 $\mu\text{g/ml}$	8 $\mu\text{g/ml}$
Resistance level	High	Intermediate	Intermediate	Intermediate

Table 2: Antibiotic sensitivity test using various antibiotics for carbapenem resistant strains and MIC for the Imipenem.

resistance while RS122, RS6053 and RS6694 are intermediate resistance to carbapenem.

Differential growth kinetics of resistance strain of the *Acinetobacter baumannii* in the absence and presence of silver nanoparticle

After characterizations the nanomaterials (A1, A2 and A3) were further used to study the antimicrobial activity of the PVP capped silver nanoparticles on high resistant strains of *Acinetobacter baumannii* (RS307). The resistant strains of *Acinetobacter baumannii* was cultured at different silver nanoparticle prepared at different conditions and growth of the bacterium is monitored by ELISA reader. The result showed that there is a difference in the effect of different silver nanomaterial on the bacterial growth that means effectiveness of silver nanoparticle change with the change in the method of preparation. Figure 3 showed the effect of three types of silver nanomaterial (A1, A2, A3) on the growth of the carbapenem resistant strains of *Acinetobacter baumannii*. The experiment was run in triplicate and presented value in Figure 3 is the average of three. Although the A1 and A2 nanoparticles have similar inhibitory effect but A3 showed slightly higher antimicrobial activity. We found that among three nanomaterial's, A3 has more inhibitory effect therefore A3 nanomaterial has been selected for the further studies. PVP concentration was highest in A3 nanoparticle which also enlightens that the highly PVP-capped AgNPs have better reliability in terms of antimicrobial activity as compared to less PVP capped AgNPs.

Antimicrobial activity of silver nanoparticle on different strain of *Acinetobacter baumannii* with diverse resistance level

The effects of highly PVP capped AgNPs (A3) on different resistant strains of *Acinetobacter baumannii* with different carbapenem resistance level have also been studied. Selected four carbapenem resistant strains RS307, RS122, RS6053 and RS6694 with the minimal inhibitor concentration of 64 µg/ml, 32 µg/ml, 8 µg/ml and 8 µg/ml respectively for carbapenem were used for the present study. The results of antimicrobial activity of A3 Ag-NPs on different strains of *Acinetobacter baumannii* are shown in Figures 4-6. The experiment is performed by taking absorbance at 605 nm using UV-Vis Spectroscopy. There are differences in the absorption maximum between different

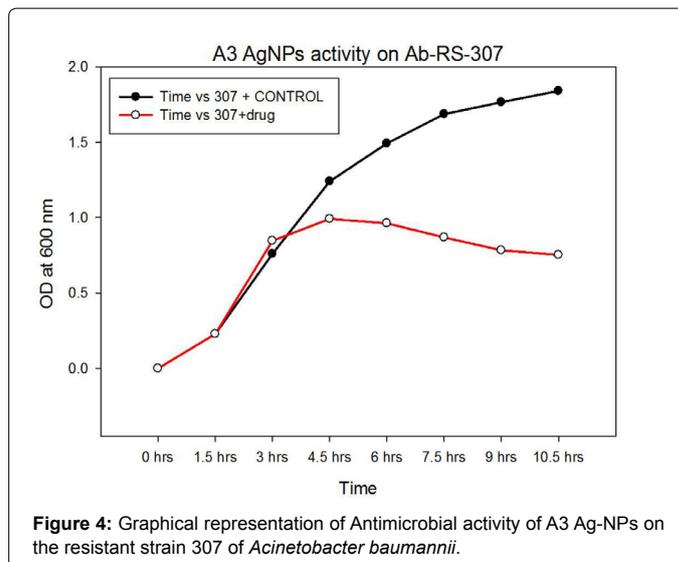


Figure 4: Graphical representation of Antimicrobial activity of A3 Ag-NPs on the resistant strain 307 of *Acinetobacter baumannii*.

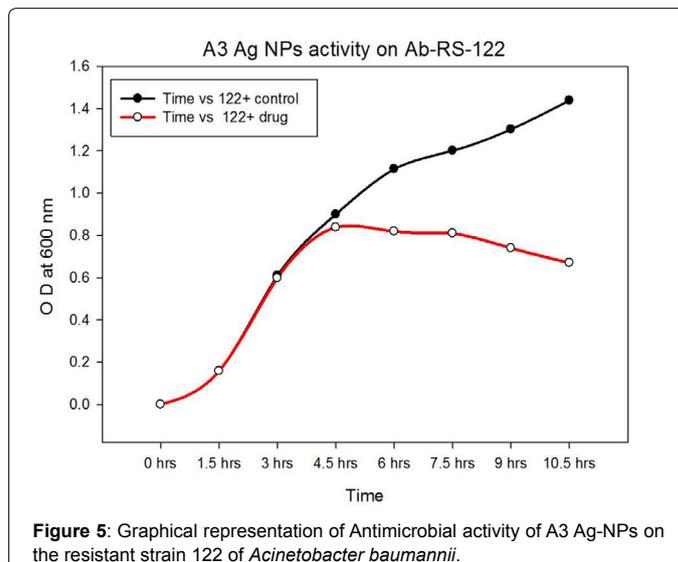


Figure 5: Graphical representation of Antimicrobial activity of A3 Ag-NPs on the resistant strain 122 of *Acinetobacter baumannii*.

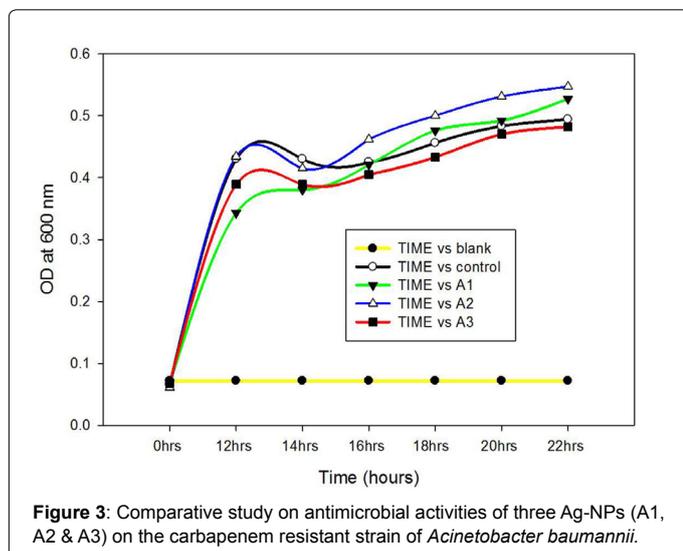


Figure 3: Comparative study on antimicrobial activities of three Ag-NPs (A1, A2 & A3) on the carbapenem resistant strain of *Acinetobacter baumannii*.

clinical strains (Figures 4-6) which showed the difference in growth kinetics of different carbapenem resistant strains of *A. baumannii*. Absorption maximum determined using ELISA (Figure 3) and UV-Vis Spectroscopy (Figure 4) are different but pattern of growth remains same. The PVP capped AgNPs (A3) have antibacterial activity on all the clinical strain of *A. baumannii* studied except the RS-6053. The antimicrobial activity of A3 silver nanoparticle on the RS-307 showed that it is inhibited by the A3 silver nanoparticle (Figure 4). It is very interesting because this strain was reported to have very high resistance to carbapenem [8,10,12]. Therefore PVP capped AgNPs can be used for the treatment of this type of high resistance strain. Similarly experiment with RS122 also showed the inhibitory effect of A3 Ag-NPs of this strain (Figure 5). Antimicrobial activity of A3 Ag-NPs was also studied for resistant strain 6694. The result showed that A3 Ag-NPs also have the antimicrobial activity on this strain (Figure 6). It is very interesting to note that resistant strain 6053 has very little inhibitory effect by the A3 Ag-NPs (Figure 7). It also enlighten that resistant strain 6053 might have naturally developed resistance against PVP capped AgNPs.

For selecting the PVP capped AgNPs as a drug of choice against

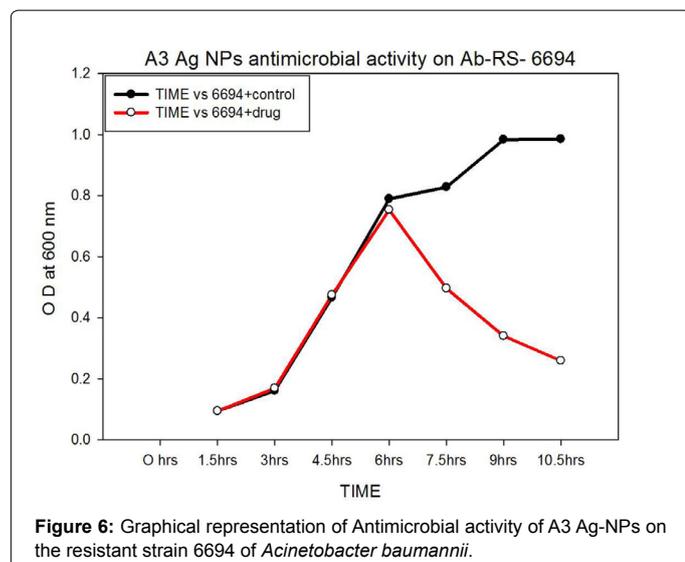


Figure 6: Graphical representation of Antimicrobial activity of A3 Ag-NPs on the resistant strain 6694 of *Acinetobacter baumannii*.

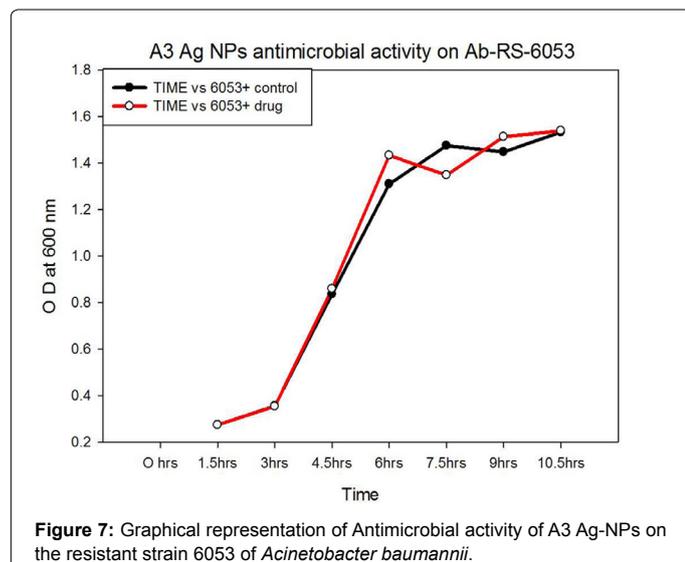


Figure 7: Graphical representation of Antimicrobial activity of A3 Ag-NPs on the resistant strain 6053 of *Acinetobacter baumannii*.

carbapenem resistance strain of *A. baumannii*, it is also important to know about the toxicity of PVP capped AgNPs on the mammalian cell i.e consumer of AgNPs. Toxicity of the AgNPs is brought via the modulation of reactive oxygen species in cells [41]. Increasing stability of AgNPs may lower toxicity in mammalian cells whilst not reducing their efficacy to fight bacteria which will result in a safer silver nanomaterial [41]. PVP capping on the surface of silver nanoparticles protects from changes in environmental conditions and prevents aggregation [38]. PVP capped Ag-NPs are more stable than citrate-capped AgNPs [37,38] and SDS-capped AgNPs [38] therefore PVP capped AgNPs is less toxic for mammalian cells [41]. Interaction of AgNPs with serum proteins also affects its antimicrobial activity *in-vivo*. It is also reported that PVP-capped AgNPs showed better antimicrobial activity than citrate capped AgNPs *in-vivo* [42]. Therefore, PVP-capped AgNPs are more favorable as an alternative drug to carbapenem. PVP-capped AgNPs bind and penetrate the cell wall of Gram-negative bacteria [47] and result into structural change in the cell membrane which could cause an increase in cell permeability, leading to an uncontrolled transport via cytoplasmic membrane which ultimately causes cell death. It is also been proposed that silver nanoparticles also produced free radical-

induced membrane damage. Silver ion also have greater tendency to strongly interact with thiol groups of vital enzymes and phosphorus-containing bases which further damage by interactions with DNA [47]. Therefore, the present study helps in the understanding of the bactericidal effect of PVP-capped AgNPs on carbapenem resistant strain of *Acinetobacter baumannii* as well as establishment of PVP-capped silver nanomaterial as a drug of choice for the carbapenem resistant strain of *Acinetobacter baumannii*.

Conclusions

Carbapenems are the most effective β -lactam antibiotics that are routinely prescribed by clinicians to patients infected with *A. baumannii* and other Gram negative bacteria. Emergence of resistant strains against carbapenem makes it difficult to treat *A. baumannii* and is therefore a threat to public health. Polyvinyl pyrrolidone (PVP) capped silver nanoparticles have been prepared which have antimicrobial property against carbapenem resistance strain of *Acinetobacter baumannii*. Therefore, PVP-capped AgNPs can be developed as an alternative choice to carbapenem, the most effective antibiotic against *Acinetobacter baumannii*.

Author Contribution

Conceived and designed the experiments: VT & MK. Performed the experiments: VT, MK, MKK & SB. Analyzed the data: VT & MK. Wrote the paper: MT, VT & MK.

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