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Antibacterial Effect of Silver Nanoparticles on Multi Drug Resistant Pseudomonas Aeruginosa

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Abstract**—**Multidrug resistant organisms have been taunting the medical world for the last few decades. Even with new antibiotics developed, resistant strains have emerged soon after. With the advancement of nanotechnology, we investigated colloidal silver nanoparticles for its antimicrobial activity against *Pseudomonas aeruginosa.* This organism is a multidrug resistant which contributes to the high morbidity and mortality in immunocompromised patients. Five multidrug resistant strains were used in this study. The antimicrobial effect was studied using the disc diffusion and broth dilution techniques. An inhibition zone of 11 mm was observed with 10 µg dose of the nanoparticles. The nanoparticles exhibited MIC of 50 µg/ml when added at the lag phase and the subinhibitory concentration was measured as 100 µg/ml. The MIC_{50} value showed to be 15 µg/ml. This study suggests that silver nanoparticles can be further developed as an antimicrobial agent, hence decreasing the burden of the multidrug resistance phenomena.

Keywords**—**Antimirobial activity, Multidrug resistance, *Pseudomonas aeruginosa*, Silver nanoparticles

I. Introduction

P SEUDOMONAS AERUGINOSA, A gram negative bacterium, is one of the bacteria found to be resistant to multiple antibiotics. It rarely causes problems to healthy individuals. However, in immunocompromised patients, it is one of the most important opportunistic pathogen that contributes to the high rate of morbidity and mortality [1]. The spectrum of diseases caused by this bacterium continues to expand from urinary tract infection to septicaemia, osteomyelitis and endocarditis, posing new challenges as resistance to current empirical therapy limits the available treatment options [2].

Silver is currently regaining interest for its renowned antibacterial properties. It has been used since ancient times for treating a wide range of illnesses from burn wounds, typhoid, anthrax to bacterial conjunctivitis in newborns [3]. Numerous studies have been conducted to uncover the antimicrobial properties of silver in its various forms. Currently, nanotechnology is gaining interest for its potential application in many fields including nanoantibiotics [4].

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This technology as its name implies, mainly enhances the potential of the material by manipulating the size to be in nanoscale. The smaller the particle, the greater the surface area for better interaction and contact with target sample.

Even though there have been numerous literatures reporting various studies evaluating the antimicrobial effect of silver, there has been minimal reports on its activity against the multidrug resistant *Pseudomonas aeruginosa*. Kim Kuk et al (2007) demonstrated antibacterial properties against yeast, *Escherichia coli* and S*taphylococcus aureus* [5]. Other studies have worked with *Bacillus .megaterium*, *Staphylococcus aureus, Escherichia coli, Proteus vulgaris* and *Shigella sonnei* [6]-[7]. In this study, we evaluated the potential of silver nanoparticles as an antimicrobial agent against multidrug resistant *P. aeruginosa*.

II. Materials And Methods

A. Characterisation of silver (Ag) nanoparticles

Silver (Ag) nanoparticles were purchased from Nano Silver Manufacturing Sdn Bnd, Malaysia. The nanoparticles used are of sizes that ranged from 20–30 nm. Briefly, colloidal silver were placed on copper grid and analysed by using Scanning electron Microscope (SEM).

B. Bacterial Strains and Culture Media

Ten clinical isolates of *P. aeruginosa* strains comprising of five multidrug resistant (MDR) strains were used in the study. Four strains that were susceptible to common empirical antibiotics (imipenem (IPM), ceftazidime (CAZ), cefoperazone (CFP), gentamicin (GM) and ciprofloxacin (CIP)) were used for comparison. *P. aeruginosa* (ATCC 27853) as a control. Brain heart infusion (BHI) and Muller Hinton (MH) agar were used as culture media.

C. Antimicrobial susceptibility test

This method was a modification of the agar disk diffusion method as described in Clinical and Laboratory Institute [8]. Approximately 10^8 CFU/ml of bacterial colonies were lawned onto MH agar plates. Sterile discs containing 10 µg Ag nanoparticles were placed onto the lawned agar plates. Disc containing 10 µg gentamicin and water was used as a positive and negative controls, respectively. The plates were incubated overnight (18-24 hr) at 37^{o}C. The diameter of zone of inhibition around the disc was observed and recorded. Experiments were conducted in triplicates and average inhibitory zone diameter with its standard deviation was determined.

D. Bacterial growth curve and inhibition assay

Bacterial suspension equivalent to 0.5 McFarland's Standard was pipetted into a 96 well-plate and kinetic optical density for 24 hours at 37^0C was recorded using Spectra-Max190 at wavelength 600 nm. A growth curve of the organism was plotted and the time taken for the bacteria to reach mid–log phase and the time taken to reach stationary phase was recorded.

The minimum inhibition concentration (MIC) of the Ag nanoparticles was determined by broth microdilution method. Bacterial suspension of 0.5 McFarland's Standard was pipetted into a 96 well-plate. The strains were incubated for 24 hr at 37^oC with a two-fold serial dilution of silver nanoparticles (Figure 1) added at 0 hr (beginning of lag phase) and after 4 hr (mid-log phase) of incubation. Following 24 hr incubation, the suspension was visualized for clarity.

E. Minimum bactericidal concentration (MBC)

Samples from wells used in broth microdilution assay that did not exhibit any visible growth after the 24 hr incubation was subcultured onto MH agar plates. The MBC was recorded as the lowest concentration of the Ag nanoparticles that did not allow a single colony to grow on the agar after further 24 hr incubation.

III. Results And Discussion

Organisms that are resistant to three or more classes of antibiotics are considered as multidrug resistant (MDR). Table I shows strains 19, 26, 38, 43a and 47 are MDR strains as they exhibit resistance against carbapenems, cephalosporin, aminoglycosides and fluoroquinolone.

TABLE I
Antibiotic Susceptibility of *P. Aeruginosa* Strains

	Antibiotic Class				
Strains	**Carb-apenem**	**Cephalo-sporin**		**Aminogly-coside**	**Fluoro-quinolone**
Antibiotics *	**IPM**	**CFP**	**CAZ**	**GM**	**CIP**
ATCC 27853	29	30	33	21	34
1	29	22	28	21	28
3	**12**	25	28	21	24
6	34	28	33	23	32
11	30	28	30	21	32
19	28	**0**	**0**	**0**	**0**
26	**0**	**10**	**0**	**9**	**0**
38	**0**	**9**	**0**	**8**	**0**
43a	**9**	**0**	**0**	16	**0**
47	21	**12**	**0**	**12**	**0**

***** Interpretation according to CLSI; R = resistant; I = intermediate; S = sensitive, IPM: R<14 mm, I:14-15 mm, S >16 mm; CFP: R<16, I:16-20, S>20; CAZ: R <15, I : 15-17, S > 17; GM: R <13, I : 13-14,S > 14; CIP: R < 16, I : 16-20, S > 20. Numerics in bold indicate resistant.

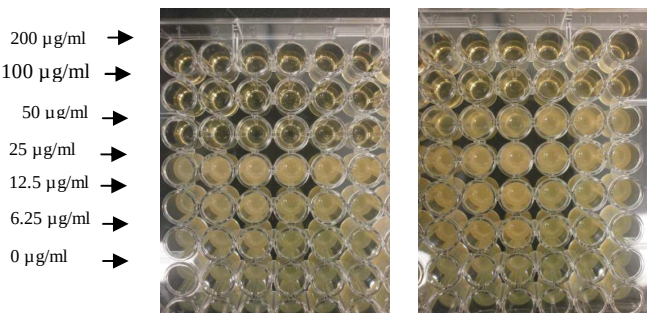
Table II shows the inhibition zone for all samples were of approximately 11.6 mm when the organism was tested against 10 µg of nanoparticles. The results were similar in MDR strains and the susceptible strains. Gentamicin showed a larger inhibition zone amongst susceptible strains but there was a slight reduction in the zone of inhibition among the MDR strains as compared to the Ag nanoparticles. This shows that the Ag nanoparticles have antimicrobial activity against the *P. aeruginosa* strains.

TABLE II
Antimicrobial Activity of Ag Nanoparticles in Comparison To Gentamicin

Strains	Antimicrobial activity		
	Ag NP (10 µg)	Gentamicin (10 µg)	Water
ATCC 27853	11.5 ± 0.6	16.7 ± 1.2	-
1	11.3 ± 1.0	15.3 ± 0.6	-
3	11.8 ± 0.5	14.3 ± 1.5	-
6	11.8 ± 0.5	19 ± 1.7	-
11	11.8 ± 0.5	16 ± 0	-
19	11.8 ± 1.0	10 ± 0	-
26	11.5 ± 0.6	10.3 ± 0.6	-
38	11.5 ± 0.6	10 ± 0	-
43a	11.5 ± 0.6	12.3 ± 1.2	-
47	11.8 ± 0.5	11 ± 0	-

AgNP: Silver Nanoparticles, Positive control: Gentamicin, Negative control: Water Inhibition zone (mm) Value = Mean ± SD

Using the broth microdilution method, the the MIC value was 50 µg/ml when the Ag nanoparticles were introduced at 0 hr, and the MIC was 100 µg/ml it was introduced after 4 hr of incubation. Minimum bactericidal concentration for the samples treated with the Ag nanoparticles at 0 hr and 4 hr are 100 µg/ml and 200 µg/ml, respectively. Fig 1(a) exhibit clarity at Ag nanoparticle concentration of 50 µg/ml while Fig 1(b) exhibit clarity at Ag NP concentration of 100 µg/ml. Experiments for all 10 samples were carried out in triplicates and results obtained were reproducible and consistent. The different inhibitory and bactericidal concentration between the four hours duration may be due to the bacterial load at the specific time point. At 0 hr, the colonies were at lag phase where the bacteria concentration is low and there is no significant multiplication of the bacteria, whereas after 4 hr of incubation, the bacterial growth has reached mid-log phase. At the mid-log phase there is exponential growth as the organisms are actively replicating. Thus, the concentration of silver nanoparticles needed to inhibit and kill the organisms is higher.



(a) :Ag NP added at 0 hr *1(b): Ag NP added after 4 hr*

Fig. 1 Broth microdilution assay on representative sample to determine minimum inhibitory concentration

The growth curves of the organism in the presence of Ag nanoparticles were plotted. In this study, the growth patterns of susceptible strains were similar to the MDR strains (Figs 2 and 3). Inhibition percentage is calculated when control sample (0 µg/ml) reaches optimal concentration (stationary phase) which is at 10.5 hr. 100% inhibition was observed at the concentration of ≥ 50 µg/ml. The presence of these particles at a concentration of 25 µg/ml inhibited bacterial growth by average of 84%. 50% growth inhibition (MIC_{50}) was found to be at concentration of 15 µg/ml. It was also observed that at the concentrations of 6.25, 12.5 and 25 µg/ml, the particles causes growth delay proportionate to the concentration of Ag nanoparticles (increased concentration, increased growth delay). Similar results have been reported on *E. coli* [9].

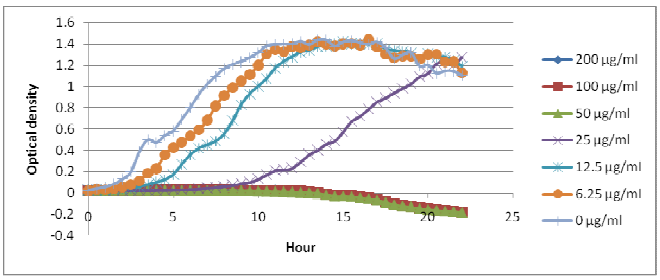


Fig. 2 The effect of Ag nanoparticles on the growth pattern of susceptible strain

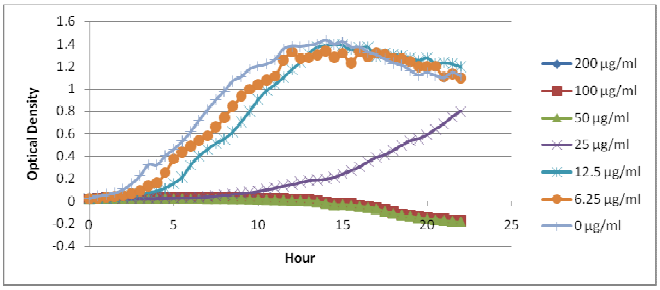


Fig. 3 The effect of Ag nanoparticles on the growth pattern of MDR Strain

The mechanism of drug resistance involving *P. aeruginosa* includes active efflux pumps, chromosomal and inducible beta-lactamases, plasmid-mediated ESBLs (Extended Spectrum Beta-Lactamases), and altered permeability. Loss of protein porin OprD results in resistance to carbapenems, specifically imipenem [10]. On the other hand, studies on the mechanism of action of Ag nanoparticles reported that silver ions penetrate bacterial cell, denature ribosomes and suppress the expression of enzymes and proteins essential for ATP production, thus leading to cell disruption [11]. Silver has also the ability to prevent DNA unwinding by binding to them, hence inhibiting the replication of bacteria [12]. Targeting the bacterial membrane also leads to dissipation of proton motive force [9]. The results obtained by our study have shown that both drug susceptible and resistant strains are affected in the same manner. This suggests that the mechanism of drug resistance developed by P. *aeruginosa* does not affect the action of Ag nanoparticles.

This study indicates the possibility of other pathways that may contribute to its effect. The preliminary data from this study could be used to carry out further studies on the mechanisms of action of Ag nanoparticles.

III. Conclusion

This study has shown that silver nanoparticles have significant antibacterial effect on *P. aeruginosa* regardless of its susceptibility to antibiotics. The data presented in this work are novel, thus could lead to new advances in development of antibacterial material that could overcome the multidrug resistance burden. However, further studies have to be conducted on the toxicity level and possible adverse effects that could arise in the use of Ag nanoparticles as an effective antibacterial agent in the health care system.

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Liew Jian Ping is a postgraduate student pursuing his Master's in the area of synthesis and rheological characterisation of silver nanoparticles based dense suspension.