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Nanoparticles against resistant *Pseudomonas* spp.

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ABSTRACT

Pseudomonas spp. collected from areas where human regularly comes into contact with were tested for their susceptibility to antibiotics. Twenty-nine samples were collected and screened for *Pseudomonas* spp. Of the nine isolated strains *Pseudomonas* spp. six were resistant to antibiotics. A few were used for an antimicrobial study on the interaction with silver and zinc oxide nanoparticles individually and as a mixture. A mixture of silver and zinc oxide nanoparticles showed synergy against resistant *Pseudomonas* spp.

1. Introduction

It is believed that our body have more bacterial than human cells. Bacteria play a relevant role in our everyday life and have proven to be able to quickly adapt to constantly changing environment to ensure survival [1]. Since the discovery of the first antibiotics, bacteria have acquired resistance, limiting their effectiveness. Rise of antibiotic resistant strains are due to several factors including the misuse of antibiotics, and use in animal feed [2]. In this study, samples were collected from objects humans are regularly exposed to. This study focused on isolation of *Pseudomonas* spp. due to its prevalence in human diseases and its propensity to have resistance against common antibiotics [1]. After *Pseudomonas* spp. was isolated, they were tested against antibiotics that they typically would have susceptibility with. The resistant strains were identified and tested against silver (Ag) and zinc oxide (ZnO) nanoparticles (NPs) individually and as a mixture of both. Other studies have already shown antibiotic effectiveness of Ag NPs with concentrations as low as 0.156 parts per million (ppm, equivalent to μg/ml) against *Pseudomonas aeruginosa* [3]. ZnO NPs alone has shown a relatively weaker effect. In our previous study, we have shown the effectiveness of using a mixture of Ag and ZnO NPs as a bactericidal agent against *Mycobacterium tuberculosis* with low cytotoxicity in macrophage cells, Gram-positive bacteria, and Gram-negative bacteria [4–7]. In this research study, we tested individual silver and zinc oxide NPs and a mixture of both against antibiotic resistant *Pseudomonas* spp.

2. Results and discussion

Twenty-nine samples were collected and screened for *Pseudomonas* spp. Of the nine isolated strains *Pseudomonas* spp. six were resistant to antibiotics (Table 1). Of these six strains, two had mucoid colony morphology on an LB agar surface. We selected a few strains to test against Ag and ZnO NPs both as sole NPs and as a mixture. Mixed Ag and ZnO NPs showed synergy and had increased effectiveness over sole NPs against resistant *Pseudomonas* spp.

In this study, all the isolated *Pseudomonas* spp. strains were resistant to bacitracin, novobiocin and penicillin. Interestingly all of them had the maximum sensitivity to ciprofloxacin, gentamicin and imipenem. In other study, it was showed that the highest resistance to isolated *Pseudomonas* spp. was to ciprofloxacin and the lowest resistance to meropenem [8]. However, other studies have determined that the highest incidence of resistance was against carbapenems [9]. They also reported that *Pseudomonas* spp. is the second most frequently isolated pathogen in hospitals, especially in the intensive care units [9]. Table 1 lists the results of the antibiogram-antibiotic sensitivity tests with the strains used in the experiment. In this study, isolated strains 1, 2, 4 and 6 (Table 1) were resistant to kanamycin. It is noticeable that researchers have found that kanamycin is not only inactive in *Pseudomonas* spp. but also induces its biofilm formation [10].

Antimicrobial assay on antibiotic resistant *Pseudomonas* spp. using colloidal NPs showed ZnO NPs not only prevented all strains of *Pseudomonas* spp., but also killed them at the range of 15–30 ppm

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Table 1
Antibiogram-antibiotic sensitivity test on isolated antibiotic resistance *Pseudomonas* spp., using antibiotics from BD Biosciences. Inhibition zone is shown in millimeters. R = Resistant, S = Sensitive, I = Intermediate.

Antibiotic Family	Isolated antibiotic resistance *Pseudomonas* spp. strains						
	Antibiotic discs	1	2	3	4	5–Mucoid form A	6 –Mucoid form B
Beta-lactam	Ampicillin (AM10)	0 mm - R	0 mm - R	18 mm-S	0 mm - R	10 mm - R	17 mm - S
	Penicillin (P10)	0 mm - R	0 mm - R	0 mm-R	0 mm - R	0 mm - R	0 mm - R
	Cephalothin (CF30)	0 mm - R	0 mm - R	0 mm-R	0 mm - R	0 mm - R	19 mm - S
	Imipenem (IMP10)	20 mm - S	23 mm - S	24 mm-S	19 mm - S	24 mm - S	32 mm - S
Aminocoumarin	Novobiocin (NB5)	0 mm - R	0 mm - R	0 mm-R	0 mm - R	9 mm - R	0 mm - R
Aminoglycoside	Gentamicin (GM10)	18 mm - S	23 mm - S	18 mm-S	18 mm - S	24 mm - S	18 mm - S
	Kanamycin (K30)	0 mm - R	0 mm - R	20 mm-S	0 mm - R	19 mm - S	13 mm - R
Cyclic polypeptide	Bacitracin (B10)	0 mm - R	0 mm - R	0 mm-R	0 mm - R	10 mm - I	0 mm - R
Sulfonamide	Trimethoprim-sulphamethoxazole (SXT)	0 mm - R	0 mm - R	0 mm-R	0 mm - R	0 mm - R	32 mm - S
Fluoroquinolone	Nalidixic Acid (NA30)	0 mm - R	9 mm - R	0 mm-R	0 mm - R	19 mm - S	21 mm - S
	Ciprofloxacin (CIP5)	32 mm - S	26 mm - S	19 mm-I	18 mm - I	25 mm - S	27 mm - S
Chloramphenicol (C30)	0 mm - R	13 mm - I	8 mm-R	0 mm - R	25 mm - S	24 mm - S	

Table 2
MIC and MBC tests of Ag NPs, ZnO NPs and combine a mass ratio of 1_{Ag}:1_{ZnO} NPs against isolated strains of antibiotic resistance *Pseudomonas* spp. Concentration is measured in parts per million (ppm), equivalent to μg/ml.

Pseudomonas spp.	Serial micro-dilution		Ag (NPs)	ZnO (NPs)	$\mathbf{1_{Ag}:1_{ZnO}}$ **(NPs)**
Mucoid form A	MIC	Concentration (ppm)	0.095	15	0.312:0.187
		Mass Ratio	1:128	1:4	1:64
	MBC	Concentration (ppm)	0.390	15	3.125:7.5
		Mass Ratio	1:64	1:4	1:4
Mucoid form B	MIC	Concentration (ppm)	0.048	15	≤0.024:0.058
		Mass Ratio	1:512	1:4	≤1:512
	MBC	Concentration (ppm)	0.195	30	0.048:0.117
		Mass Ratio	1:128	1:2	1:256
Pseudomonas spp.	MIC	Concentration (ppm)	0.097	15	0.097:0.234
		Mass Ratio	1:256	1:4	1:128
	MBC	Concentration (ppm)	0.195	15	0.195:0.468
		Mass Ratio	1:128	1:4	1:64
Control (ATCC 15422)	MIC	Concentration (ppm)	0.048	15	0.048:0.117
		Mass Ratio	1:512	1:4	1:256
	MBC	Concentration (ppm)	0.097	15	0.097:0.234
		Mass Ratio	1:256	1:4	1:128

(Table 2). Other researchers, however, showed that ZnO NPs are able to inhibit antibiotic resistant *Pseudomonas* spp. at 512 ppm and kill it at ≥8192 ppm [6]. Per the results obtained in testing NPs, samples 2 and 3 showed the most sensitivity to Ag NPs. They were killed at 0.195 ppm (MBC ≈ 0.195 ppm) at the mass ratio of 1:128 by Ag NPs, while this colloidal NPs could eliminate the control laboratory strain (Sample 4) at 0.097 ppm at the mass ratio of 1:256 (MBC ≈ 0.097 ppm) (Table 2). By mixing the Ag and ZnO NPs with the mass ratio of 1_{Ag}:1_{ZnO}, a noticeable reduction in the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against antibiotic resistant *Pseudomonas* spp. (sample 2) was observed. This sample was shown to be resistant to penicillin, bacitracin, novobiocin and kanamycin. The MIC and MBC was observed ≤0.024 Ag: 0.058 ZnO ppm (ratio of ≤1:512) and 0.048 Ag: 0.117 ZnO ppm (mass ratio of 1:256), respectively. (Table 2). The results showed the synergetic antimicrobial effect of mixing the colloidal Ag NPs and ZnO NPs on kanamycin resistance *Pseudomonas* spp. found in this study.

3. Conclusion

Pseudomonas spp. infections are a serious and important problem in hospitals. Patients who are critically ill can die from pneumonia caused by *Pseudomonas* spp. The elimination of *Pseudomonas* spp. in patients with infections is very difficult because of its resistance to a variety of antibiotics. *Pseudomonas* spp. currently shows resistance to the following antibiotics: penicillin G; aminopenicillin, including those combined with beta-lactamase inhibitors; first and second generation cephalosporins; piperacillin; piperacillin and tazobactam; cefepime; ceftazidime; aminoglycosides; the quinolones; and the carbapenems; as well as colistin and fosfomycin [11]. The increasing resistance of *Pseudomonas* spp. to numerous antibiotics, as a result of excessive antibiotic administration, is now leading to the accumulation of antibiotic resistance and cross-resistance between antibiotics and the appearance of multidrug-resistant (MDR) forms of *Pseudomonas* spp. [12,13]. These findings are relevant because antibiotic-resistance is a growing public health concern that will continue to be a problem until it is universally addressed. There is an urgent need to identify new antimicrobial agents against this pathogenic bacterium. We report that one of the new antimicrobial agent could be combination of 1_{Ag}:1_{ZnO} NPs. In our previous work, this same mass ratio of mixed metal NPs showed no toxic effects on human pulmonary cells [14]. Further studies into the antimicrobial activity of 1_{Ag}:1_{ZnO} NPs, their toxicity (if any), efficacy and delivery methods are warranted.

4. Experimental procedures

4.1. Sampling and isolating

A total of twenty-nine samples were collected from high traffic areas (such as coins, paper money, restroom sink handles, restroom towel dispenser handles, keys, cell phones, computer mice, gym equipment, trash can handle, desks, scrubs, hospital entrances, wheelchairs, elevator buttons, university cafeterias, and hospital lounges) and cultured on LB agar plates. *Pseudomonas* spp. was isolated through common biochemical tests [15]. Antibiotic resistance was then determined through the Kirby-Bauer disk diffusion method, in accordance with the standards set forth by the Clinical and Laboratory Standards Institute (CLSI) and the National Committee for Clinical Laboratory Standards. The control group was purchased from the American Type Culture Collection.

4.2. Synthesis of nanoparticles

The Ag NPs and ZnO NPs were synthesized via chemical reduction and deposition assay at the Institute for Tuberculosis Research,

University of Illinois at Chicago, College of Pharmacy. The characterization of NPs in aqueous solution was determined from a previous study [16]. The mixed NPs were prepared with a mass ratio of 1:1 (500 µl containing 12.5 ppm of Ag NPs plus 500 µl containing 30 ppm of ZnO NPs). The initial concentrations of Ag NPs and ZnO NPs had already been estimated by inductively couple plasma mass spectrometry at about 25 ppm and 60 ppm, respectively.

4.3. Antimicrobial assay using nanoparticles

The serial micro-dilution assay was performed to determine the MIC and MBC of NPs. The wells in row A, columns 1 to 6 and in row B, columns 1 to 5 of a 24-well microplate were filled with 1000 µl of Mueller Hinton Broth (MHB) (Merck, Germany). 1000 µl colloidal solutions containing solo and mixed NPs were added to wells A1 and B6, which had been already sonicated at room temperature at 28 kHz for 10 min. Consequently, a serial dilution was prepared (for each tested nanoparticle solution) in rows A in columns 1 to 6 and in rows B in columns 1 to 3. The range concentrations of the individual Ag and ZnO NPs were 12.5 ppm–0.048 ppm and 30 ppm–0.11 ppm, respectively. The range concentration of the Ag:ZnO mixture was from 12.5 ppm: 30 ppm–0.048 ppm: 0.11 ppm. 100 µl of the chosen strain of bacteria containing 1.5×10^8 CFU/ml was used to inoculate row A, columns 1 to 6 and row B, columns 1 to 4 and incubated at 37 °C for 18 h. The columns of 4, 5 and 6 in row B were defined as MHB plus bacteria, MHB and NPs controllers. The lowest concentration of NPs which visually inhibited the growth of isolated *Pseudomonas* spp. was considered to be the minimum inhibitory concentration (MIC).

To determine MBC, a loop-full of each well were inoculated on LB agar (Thermo-Fisher, U.S) and incubated at 35 °C for 24 h. The minimum bactericidal concentration (MBC), i.e., the lowest concentration of NPs that kills 99.9% of the bacteria. Each experiment had three trials [16].

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