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# *In vitro* study on the efficacy of zinc oxide and titanium dioxide nanoparticles against metallo beta-lactamase and biofilm producing *Pseudomonas aeruginosa*

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**ABSTRACT**

*Pseudomonas aeruginosa* contributes to a multitude of infections exhibiting intrinsic resistance to numerous antibiotics. Metallo beta-lactamase and biofilm production is the most worrisome resistant mechanisms observed in *P. aeruginosa*. Emergence of antimicrobial resistance by pathogenic bacteria is a major health problem in recent years. Zinc oxide and titanium dioxide nanoparticles comprises of well-known inhibitory and bactericidal effects. The present study is designed to determine the efficacy of zinc oxide and titanium dioxide nanoparticles against metallo beta-lactamase and biofilm producing *P. aeruginosa*. A total of 51 non-repetitive *P. aeruginosa* isolates were obtained from Bioline laboratory, Coimbatore. Biofilm and metallo beta-lactamase production was tested by combined disc test and tissue culture plate method. Commercially available zinc and titanium dioxide nanoparticles were obtained and tested against two metallo beta-lactamase and biofilm producing isolates. Both the nanoparticles showed appreciable activity at all tested concentrations. Thus, it is concluded that ZnO and $TiO_2$ nanoparticles may serve as a promising antibacterial agents in coming years.

## INTRODUCTION

*Pseudomonas aeruginosa* exhibits intrinsic resistance to several antimicrobial agents (Kiska and Gilligan, 1999) and the rapid increase in drug resistance among the clinical isolates of this opportunistic human pathogen is of worldwide concern (Babay, 2005; Ling *et al.*, 2006; Gupta *et al.*, 2006; Patzer and Dzierzanowska, 2007; Walkty *et al.*, 2008). Metallo-β lactamases (MBLs) have emerged as one of the most worrisome resistance mechanisms owing to their capacity to hydrolyze with the exception of aztreonam, all β-lactams including carbapenems and also because their genes are carried on highly mobile elements, allowing easy dissemination (Walsh *et al.*, 2005). MBL producing *P. aeruginosa* isolates have been responsible for several nosocomial outbreaks in tertiary centers in different parts of the world (Cornaglia *et al.*, 2000; Tsakris *et al.*, 2000; Pournaras *et al.*, 2003; Crespo *et al.*, 2004) and they are responsible for serious infections, such as septicemia and pneumonia (Wang and Wood, 2011). Another important factor contributing to *P. aeruginosa* pathogenesis in clinical settings is the biofilm mode of growth involved in chronic as well as in acute infections (Schaber *et al.*, 2007). Biofilms, a surface-associated bacterial community, are complex and they are capable of growing in connection with different biological or inert surfaces (Johnjulio *et al.*, 2012). One of the most studied aspects of nanotechnology is its ability to offer the opportunity to fight microbial infections via the production of nanoparticles (Luo *et al.*, 2007) and the considerable antimicrobial activities of metal oxide nanoparticles such as ZnO, $TiO_2$, MgO, $SiO_2$ with their selective toxicity to biological systems suggests their potential applications as therapeutics, diagnostics, surgical devices and nanomedicine based antimicrobial agents (Sawai, 2003; Reddy *et al.*, 2007; Sobha *et al.*, 2010; Laura *et al.*, 2006).

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The advantages of using these metal oxides nanoparticles as antimicrobial agents are their greater effectiveness against resistant microbial pathogens with less toxic effect. ZnO nanoparticles are multifunctional inorganic nanoparticles that have many significant features such as chemical and physical stability, high catalysis activity and effective antibacterial activity (Kalyani *et al.,* 2006; Matei *et al.,* 2008).

On the other hand, commercially available $TiO_2$ nanoparticles serve as antimicrobial agents which are highly efficient in inhibiting the bacterial growth (Shah *et al.,* 2008). This potent oxidizing power of TiO2 NPs is normally seen in the case of bacteria and other organic substances (Cho *et al.,* 2005; Fujishima *et al.,* 2000; Shiraishi and Hirai, 2008). Thus, the small nanometer-scale of TiO2 particles imposes several effects that govern its antibacterial action. Therefore, the present study was designed to determine the efficiency of ZnO and TiO2 NPs against MBL and biofilm producing *P. aeruginosa*.

## MATERIALS AND METHODS

### Bacterial Isolates

A total of 51 non-repetitive *P. aeruginosa* isolated from various clinical samples were obtained from Bioline Laboratory, Coimbatore was included in the study. The isolates were identified as *P. aeruginosa* by conventional methods (Collee *et al.,* 1996). The study was conducted at the Microbiology Division, Department of Biotechnology, Karunya University, Coimbatore.

### Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of all the *P. aeruginosa* isolates was performed according to the CLSI guidelines (CLSI, 2012). The following antibiotics were tested by the disk diffusion method, ampicillin (30 µg), cefoperazone (75 µg), ceftazidime (30 µg), cefuroxime (30 µg), ertapenem (10 µg), gentamicin (30 µg), imipenem (10 µg), lomefloxacin (10 µg), meropenem (10 µg), netilmicin (30 µg), perfloxacin (5 µg), piperacillin (10 µg) and tobramycin (25 µg) obtained from Hi-Media Laboratories, India.

### Screening for MBL production

### Imipenem (IMP)-EDTA Combined Disc test

The IMP-EDTA combined disk test was performed as previously described by Yong *et al.,* 2002. Test organisms were inoculated on to plates with Mueller Hinton agar as recommended by the CLSI, 2012. Two 10 µg imipenem disks (Hi Media, Mumbai) were placed on the plate, and appropriate amounts of 10 µL of EDTA solution were added to one of them to obtain the desired concentration (750 µg). The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 hours of incubation in air at 35° C. In the combined disc test, if the increase in inhibition zone with the Imipenem and EDTA disc was ≥ 7 mm than the Imipenem disc alone, it was considered as MBL positive.

### Detection of Biofilm production

Overnight growth of bacteria in Trypticase soya broth (TSB) with 1% glucose was diluted (1:100) and 200 µl portions were inoculated in 96 -well flat bottom polystyrene microtitre plates (Tarsons, India) (Christensen *et al.,* 1985). Incubation was carried out at 35º C for 24 hours. Cultures were then aspirated and the wells were washed 3 times with phosphate buffer saline (pH 7.2). The plates were then air dried overnight and stained with 0.1% crystal violet. The optical density of the wells was measured at 570 nm using micro ELISA auto reader. An optical density of < 0.120 for non biofilm producer, 0.120 – 0.240 for moderate biofilm producer and > 0.240 was chosen to distinguish strong biofilm producers. Sterile TSB was used as a negative control (Blank). To compensate for background absorbance, the OD reading value of blank was deducted from the test values. Intensity of biofilm was classified as described by Mathur *et al.,* 2006.

### ZnO and $TiO_2$ NPs

Commercially available ZnO and $TiO_2$ NPs were purchased from Sigma-Aldrich, St. Louis, MO. The reported "as manufactured" sizes were: ZnO NPs < 100nm and $TiO_2$ NPs < 50nm. The subsequent dilutions were made in autoclaved double distilled water

### Preparation of ZnO NPs and $TiO_2$ NPs Suspension

One hundred milligrams of ZnO and $TiO_2$ NPs was added to 10 ml of sterile MQ water separately and shaken vigorously. The suspending solution was treated by ultrasound (100w, 40 kHz) for 30 minutes, autoclaved at 121°C for 20 min and then cooled down to room temperature.

### Determination of antimicrobial activity by well-diffusion method

The ZnO and $TiO_2$ NPs were tested for antimicrobial activity by well-diffusion method. The pure cultures of *P. aeruginosa* were subcultured onto sterile Luria-Bertani broth and incubated at 37 °C for 16-18 hours. After 16-18 hours, the optical density of the culture was checked to be around 0.1 at 600 nm to obtain 1 x $10^8$ CFU/ml. Wells of 6-mm diameter were made on Mueller-Hinton agar plates using gel puncture. Each isolate was swabbed uniformly onto the individual plates using sterile cotton swabs. Using a micropipette, 100, 200 and 500 µg/ml of ZnO and $TiO_2$ nanoparticles solution was poured onto each of three wells on all plates. After incubation at 35 °C for 18 hours, the different levels of zone of inhibition were measured.

### Antibiofilm activity of ZnO and $TiO_2$ nanoparticles using Congo red agar (CRA) method

Four strong biofilm producing *P. aeruginosa* isolates were subjected to the antibiofilm study using ZnO and $TiO_2$ NPs. One hundred milligrams of ZnO NPs and $TiO_2$ NPs was added to 10 ml of sterile MQ water and shaken vigorously. Brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red were used for screening the formation of biofilm by *P.*

*aeruginosa*. Following autoclave, the concentrated solution of nanoparticles at different concentrations (100, 200 and 500 µg/ml) was added to the Congo Red Agar. Plates were streaked with the isolates and incubated aerobically for 24–48 h at 37°C. After incubation, absence of dry crystalline black colonies indicated no biofilm formation.

## RESULTS

### Antimicrobial Susceptibility Testing

The antibiogram pattern suggests that *P. aeruginosa* remained sensitive to a number of antibiotics. All the isolates subjected to the study exhibited different resistant pattern against 13 commonly used antibiotics (Table 1). The percentage of resistance to antibiotics such as ampicillin, cefuroxime, lomefloxacin, ceftazidime, cefoperazone, piperacillin, pefloxacin, netilmicin, tobramycin, gentamicin, meropenem imipenem and ertapenem were found to be in the order of 100 %, 100 %, 64.7 %, 60.7 %, 54.90 %, 52.94 %, 47.05 %, 43.3 %, 41.18 %, 37.26 %, 35.29 % 23.53 %, and 21.6 %. None of the *P. aeruginosa* isolates exhibited 100 % sensitivity to all the tested antibiotics.

**Table. 1:** Antimicrobial Susceptibility Pattern of *P. aeruginosa* (n=51).

| S.No. | Antibiotics | Resistant (%) | Intermediate (%) | Sensitive (%) |
|---|---|---|---|---|
| 1 | Ceftazidime (30µg) | 60.7 | 9.9 | 29.4 |
| 2 | Pefloxacin (5µg) | 47.1 | - | 52.95 |
| 3 | Netilmicin (30µg) | 37.3 | 3.9 | 58.8 |
| 4 | Gentamicin (30µg) | 37.3 | 2 | 60.7 |
| 5 | Cefoperazone (75µg) | 54.9 | 29.6 | 15.5 |
| 6 | Lomefloxacin (10µg) | 64.7 | 13.7 | 21.6 |
| 7 | Cefuroxime (30µg) | 100 | - | - |
| 8 | Piperacillin (100µg) | 52.9 | - | 47.1 |
| 9 | Imipenem (10µg) | 23.5 | - | 76.5 |
| 10 | Meropenem (10µg) | 35.3 | 5.8 | 58.9 |
| 11 | Ertapenem (10µg) | 21.6 | 13.7 | 64.7 |
| 12 | Tobramycin (10µg) | 41.2 | - | 58.8 |
| 13 | Ampicillin (25µg) | 100 | - | - |

### Multi Drug Resistant Pattern of *P. aeruginosa*

The multi- drug resistant pattern of the biofilm producing *P. aeruginosa* is shown in Table 2. In the present study, 47 isolates (92.15%) were found to be multi drug resistant and *P. aeruginosa* isolates at a range from 39.21 % to 92.15 % were found to be resistant to 3 to 7 antibiotics.

**Table. 2:** Multi Drug Resistant Pattern of *P. aeruginosa.*

| No. of drugs | No. of isolates (n=51) | Resistance (%) |
|---|---|---|
| ≥ 3 | 47 | 92.15 |
| ≥ 5 | 32 | 62.74 |
| ≥ 7 | 20 | 39.21 |
| ≥ 9 | 17 | 33.33 |
| ≥ 11 | 11 | 21.56 |
| ≥ 13 | 2 | 3.92 |

### Screening of metallo-β-lactamase producing *P. aeruginosa*

#### *Imipenem-EDTA Disk Potentiation Test*

In this study, 47.05% of *P. aeruginosa* isolates were found to be MBL producers. These isolates exhibited a ≥ 7mm zone enhancement for Imipenem and EDTA combined than the Imipenem disks alone. All the MBL producing isolates were multi-drug resistant, most of which showed resistance to more than ten antibiotics out of the thirteen antibiotics tested (Table 3).

**Table. 3:** Antibiogram-resistogram pattern of MBL and Non-MBL producing *P. aeruginosa*.

| S. No. | Antibiotics | MBL Producers | | Non MBL Producers | |
|---|---|---|---|---|---|
| | | Resistant (%) | Sensitive (%) | Resistant (%) | Sensitive (%) |
| 1 | Ceftazidime (30µg) | 95.8 | 4.2 | 48.2 | 51.8 |
| 2 | Pefloxacin (5µg) | 70.8 | 29.2 | 25 | 75 |
| 3 | Netilmicin (30µg) | 70.8 | 29.2 | 11.1 | 88.9 |
| 4 | Gentamicin (30µg) | 75 | 25 | 11.1 | 88.9 |
| 5 | Cefoperazone (75µg) | 100 | - | 63 | 37 |
| 6 | Lomefloxacin (10µg) | 83.4 | 16.6 | 77.8 | 22.2 |
| 7 | Cefuroxime (30µg) | 100 | - | 100 | - |
| 8 | Piperacillin (100µg) | 75 | 25 | 33.4 | 66.6 |
| 9 | Imipenem (10µg) | 37.5 | 62.5 | - | 100 |
| 10 | Meropenem (10µg) | 75 | 25 | 11.2 | 88.9 |
| 11 | Ertapenem (10µg) | 58.3 | 41.7 | 14.8 | 85.2 |
| 12 | Tobramycin (10µg) | 70.8 | 29.2 | 14.8 | 85.2 |
| 13 | Ampicillin (25µg) | 100 | - | 100 | - |

### Detection of Biofilm production

All the 51 *P. aeruginosa* isolates were subjected to Tissue Culture Plate Method, out of which 11.76% were strong, 41.19% were moderate and 47.05% were weak biofilm producers. The OD values are tabulated and are depicted in Table 4.

**Table. 4:** Biofilm producing *P. aeruginosa* using Tissue Culture Plate Method.

| Microorganism | OD values | | |
|---|---|---|---|
| | (>0.240) ++ (%) | (0.120-0.240)+ (%) | (<0.120) - (%) |
| *P. aeruginosa* (n= 51) | 6 (11.76) | 21(41.19) | 24(47.05) |

++→ Very Strong; + → Moderate; - → Negative

### Antimicrobial activity of ZnO and $TiO_2$ NPs by well-diffusion method

Two isolates (strong biofilm and MBL producer and weak biofilm and non MBL producer) were selected for the study. The antibacterial activity of ZnO and $TiO_2$ NPs were tested using well diffusion method for determining the zone of inhibition at 100, 200 and 500 µg/ml respectively. It was found that at all concentrations, ZnO and $TiO_2$ NPs was able to inhibit the bacterial growth (Table 5).

**Table. 5:** Zone of inhibition of ZnO and $TiO_2$ Nanoparticles against *P. aeruginosa* (n=2) at different concentrations.

| *P.aeruginosa* isolates (n=2) | Concentration (µg/ml)/Zone of inhibition (mm) | | | | | |
|---|---|---|---|---|---|---|
| | ZnO | | | $TiO_2$ | | |
| | 100 | 200 | 500 | 100 | 200 | 500 |
| PA1 | **11** | 12 | 17 | **10** | 15 | 16 |
| PA2 | **9** | 11 | 16 | **9** | 11 | 12 |

PA1 – Weak Biofilm producer, non MBL producer
PA2 – Strong Biofilm producer, MBL producer

### Antibiofilm activity of nanoparticles against biofilm producing *P. aeruginosa*

The antibiofilm activities of nanoparticles (ZnO and $TiO_2$ NPs) were tested against 4 isolates on the Congo Red Agar medium. When the colonies were grown on the CRA with ZnO and $TiO_2$ NPs of different concentrations such as 100, 200 and 500 µg/ml, the organisms did not survive. The ZnO and $TiO_2$ NPs inhibited the biofilm production in three isolates but, failed to inhibit the growth in the fourth isolate.

## DISCUSSION

*P. aeruginosa* emerged as an important pathogen and responsible for the nosocomial infections that is one of the important causes of morbidity and mortality among hospital patients (Khan *et al.,* 2008). In the present study, the carbapenems were the least active agents evaluated with only 76.5% and 58.9% of isolates being susceptible to imipenem and meropenem, respectively. Imipenem has been reported to be very active against *P. aeruginosa* in a number of earlier studies (Shenoy *et al.,* 2002; Rodriguez-Morales *et al.,* 2005; Ling *et al.,* 2006; Raja and Singh, 2007) while other researchers (Patzer and Dzierzanowska, 2007; Yetkin *et al*., 2006) have reported otherwise. The rate of susceptibility to ceftazidime was 47.6 % which was relatively low when compared to the previous studies (Shenoy *et al.,* 2002; Anupurba *et al.,* 2006; Farida and Asif, 2010; Nwankwo and Shuaibu, 2010). This may be related to its frequent use in hospitalized patients where the possibility of emergence of resistance is high. However, the rate was better than that reported from Salem, India (Mohanasoundaram, 2011).

Despite the advances in sanitation facilities in the hospitals and the introduction of a wide variety of antimicrobial agents with antipseudomonal activities still life threatening infections caused by *P. aeruginosa* is found to be increasing (Algun *et al*., 2004) and it is also notorious for biofilm formation (Sauer *et al.,* 2004) hence, it is used a model organism for biofilm studies (Caiazza *et al.,* 2007). In the present study, 11.76 % *P. aeruginosa* isolates produced biofilms by TCP method which was considered to be lower than 22.7 % as reported by Hassan *et al.,* 2011. Similarly, Mathur *et al.,* 2006 reported 53.9 % *P. aeruginosa* isolates to be biofilm producers.

MBLs have been identified from clinical isolates worldwide with increasing frequency over the past several years, and isolates that produce these enzymes have been responsible for prolonged nosocomial infections (Alaghehbandan *et al.,* 2001; Jiang *et al.,* 2006; Pagani *et al.,* 2004). Agarwal *et al.,* (2008) and Kalantari *et al.,* 2012 reported 8% and 12% of the *P. aeruginosa* isolates to be MBL producers and in this study, 47% were MBL producers. Moreover, 36% of MBL producers were resistant to imipenem which is contradictory to results obtained by Attal *et al.,* 2010 (11.4 %), Navneeth *et al.,* 2002 (12 %), Mendiratta *et al.,* 2005 (8.2 %), Hemalatha *et al.,* 2005 (14 %) and Agrawal *et al.,* 2008 (8.05 %) respectively from different parts of India.

Antimicrobial NPs offer many distinctive advantages in reducing acute toxicity, overcoming resistance and lowering cost, when compared to conventional antibiotics (Pal *et al.,* 2007; Weir *et al.,* 2008). Rizwan *et al.,* 2010 have stated that increasing the concentration of ZnO nanoparticles resulted in the increase of zone size. But in this study, there was no significant difference in the zone of inhibition at 100 and 200 µg/ml, but there was an increase in size at 500 µg/ml. Whereas, Ansari *et al.,* 2009 reported that the increased concentration of ZnO NPs did not show any steady increase in the zone size. Previous researchers have proposed that release of $H_2O_2$ might be one of the possible mechanisms for antibacterial activity of ZnO NPs. ZnO powder has been used for a long time as an active ingredient for dermatological applications. However, nanoparticles of ZnO are much more effective agents in controlling the growth of various microorganisms (Sawai, 2003). In this study, the ZnO NPs inhibited a maximum zone of 9mm and 16mm at 100 µg/ml and 500 µg/ml against MBL and strong biofilm producing *P. aeruginosa* isolates. Roy *et al.,* 2010 demonstrated that $TiO_2$ NPs failed to exhibit antibacterial activity but upon combination with antibiotics they were able to inhibit the growth of microorganism. But in this study, $TiO_2$ NPs without any kind of combination inhibited the microbial growth with a maximum zone of 12 mm and 16 mm at 500 µg/ml and a minimum zone of 9 mm at 100 µg/ml against strong biofilm producing *P. aeruginosa*.

Thus, the study indicates that, surveillance for the detection of MBL is necessary and may help in appropriate antimicrobial therapy and avoid the development and dissemination of these multidrug resistance isolates. The rapid dissemination of MBL producers is worrisome and necessitates the implementation of proper and judicious selection of antibiotics especially carbapenems. So, all isolates of *P. aeruginosa* resistant to imipenem should be screened for MBL production. The overall resistance demonstrated by clinical isolates of *P. aeruginosa* is high. This can be attributed to the inappropriate use of antibiotics. The present study may guide clinicians on the appropriate usage of antibiotics, furthermore regular monitoring of the use of antibiotics will help in preserving the effectiveness of antibiotics.

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