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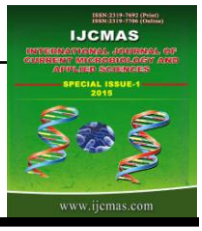


**Original Research Article**

# Antibacterial Activity of ZnO Nanoparticles against ESBL and Amp-C Producing Gram Negative Isolates from Superficial Wound Infections

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**A B S T R A C T**

Antibiotic resistance has become a serious problem that challenges the health of hospitalized patients. It is making a growing number of infections virtually untreatable. Therefore there is strong incentive to develop new bactericides. It is well known that inorganic nanomaterials are good antimicrobial agents. This study was done to look for antibacterial potentials of Zinc oxide nanoparticles to bacterial isolates of superficial skin infections. Total of 62 gram negative isolates from superficial wound infections were taken, which included 40 *E. coli*, 18 *P. aeruginosa* and 4 *Klebsiella spp*. Antibiotic susceptibility was done by Kirby Bauer disc diffusion method. MIC and MBC of ZnO nanoparticles against these isolates were calculated by agar dilution and broth dilution methods respectively and compared with that of standard strains. The bacterial growth curves were also plotted for 24 hours against these nanoparticles. Concentrations of ZnO nanoparticles used were 1000µg/ml, 2000µg/ml, 4000µg/ml, 8000µg/ml, 16000µg/ml and 32000µg/ml. Out of 62 isolates, 48 (77.7%) were multidrug resistant, 24 (38.7%) were AmpC producers while 26 (41.9%) were ESBL. The plotting of growth curves showed significant inhibition of growth at all concentrations. There was almost no rise in growth curve for 10hrs at concentrations of 1000µg/ml. MIC and MBC of standard strains were 1000µg/ml and 16000µg/ml respectively. Sixteen isolates showed MIC at 1000µg/ml while MBC at 4000µg/ml. Eighteen isolates showed MIC at 8000µg/ml while MBC at 16000µg/ml. ZnO nanoparticles can be used externally in the form of ointments, lotions and surface coating agents to prevent microorganisms from attaching, colonizing and spreading of bacterial infections.

**Keywords**

Nanotechnology, Antimicrobial resistance, ESBL, ZnO,

## Introduction

Antimicrobial drug resistance is a growing threat and a topic of intense research worldwide. Due to the extensive use of β-lactam antibiotics over the last several decades in clinical practice, various β-lactamases have emerged. The predominant mechanism for resistance to the β-lactam antibiotics in Gram-negative bacteria is the

production of β-lactamase. The production of extended-spectrum β-lactamases (ESBLs) is an important mechanism which is responsible for resistance to the third-generation cephalosporins (Bradford *et al.* 2001). New strategies are therefore needed to identify and develop the next generation of drugs or agents to control bacterial infections (Azam *et al.*, 2011).

During the last decade, development in the areas of nanotechnology has evolved to provide outstanding capabilities for understanding, fabricating, and manipulating structures at the atomic level. One of the most studied aspects of nanotechnology nowadays is their ability to offer the opportunity to fight microbial infections via the synthesis of nanoparticles (Luo *et al.*, 2007).

The considerable antimicrobial activities of metal oxide nanoparticles such as ZnO, TiO2, SiO2 and their selective toxicity to biological systems suggest their potential application as therapeutics, diagnostics, surgical devices and nanomedicine based antimicrobial agents (Sobha *et al.*, 2010; Reddy *et al.*, 2007; Laura *et al.*, 2006).

The antibacterial activity of ZnO has been studied largely with different pathogenic and non-pathogenic bacteria such as *Staphylococcus aureus and E. coli* (Brayner *et al.*, 2006; Jones *et al.*, 2008; Sawai, 2003). The previous comparative study on six metal oxide nanoparticles done by Jones *et al.* (2008) has shown that ZnO NPs significantly inhibit the growth of a wide range of pathogenic bacteria under normal visible lighting conditions.

The objective of the present study was to evaluate antibacterial activity of ZnO nanoparticles against ESBL and AmpC producing strains isolated from superficial skin lesions of patients attending a tertiary care hospital in J. N. Medical College, Aligarh Muslim University, Aligarh, India.

## Material and Methods

Total of 62 gram negative isolates from superficial skin infections were included in the study. Identification was done by using standard biochemical tests (Collee, 2006).

### Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed by Kirby Bauer disc diffusion method as per CLSI guidelines on Mueller Hinton agar (CLSI 2008). Antibiotics used were amikacin (30µg), gentamicin (10µg), levofloxacin (5µg), sparfloxacin (5µg), ceftriaxone (30µg), cefoperazone (75µg), cefoperazone-sulbactam (75µg, 1:1), cefixime (5µg) ceftriaxone-salbactam (30/15µg), piperacillin (100µg), piperacillin-tazobactum (100:10µg), cefotaxime (30µg) and tobramycin (10µg), cefoxitin (30µg), imipenem (10µg). All discs were obtained from HiMedia, India.

### Detection of extended spectrum and AmpC beta lactamase

Screening of possible ESBL production was done by using ceftriaxone (30µg) and cefoperazone (75µg). Isolates showing zone diameter less than 25mm for ceftriaxone and less than 19mm for cefoperazone were subsequently confirmed by disc potentiation test using cefoperazone (CP) and cefoperazone-sulbactam (CPS) combination (Rizvi *et al.* 2009). Organism sensitive to cefoxitin and resistant to cefoperazone-salbactam and piperacillin-tazobactam combination was considered to be Amp C producers (CLSI, 2008).

**Evaluation of antibacterial activity of ZnO NPs**

**Assay for MIC and MBC determination of ZnO NPs**

***Minimal inhibitory concentration***

Bacterial strains (all clinical isolates) were grown overnight on MHA (HiMedia) plates at 35°C before being used. The antimicrobial activity of ZnO NPs was examined using the standard agar dilution method. Serial twofold dilutions of ZnO NPs in concentrations ranging from 16,000 to 125 µg/ml in Luria– Bertani (LB; HiMedia) agar were taken. Initial bacterial inocula of 2.5×105 CFU/ml were spot inoculated over the squares marked on agar plates, and incubated at 37°C. The minimal inhibitory concentration (MIC) was estimated visually on the surface of Luria–Bertani (LB; HiMedia) agar plates after 24 hrs of incubation.

The MIC is the lowest concentration of antimicrobial agents that completely visually inhibits the 99% growth of the microorganisms. Concentration of ZnO nanoparticles showing no growth at spot inoculation was considered as MIC of that particular isolate.

***Minimal bactericidal concentration***

Broth dilution method was used for estimation of MBC. Serial twofold dilutions of ZnO NPs in Luria-Bertani (LB; HiMedia) broth in concentrations ranging from 16,000 to 125 µg/ml were taken in test tubes and 50µl of bacterial inocula containing approx 2.5×105 CFU/ml were added to the tubes. Tubes were incubated for 24 h at 37°C.

After overnight incubation aliquots of 25 µl from all tubes of Luria-Bertani (LB; HiMedia) broth were plated on the surface of MHA plates not supplemented with ZnO NPs and were incubated for 24 h at 37°C. The minimal bactericidal concentration (MBC) endpoint is defined as the lowest concentration of antimicrobial agent that kills 100% of the initial bacterial population.

***Time-dependent growth inhibition assay***

Strains of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, ESBL and AmpC producing gram negative isolates were inoculated in 100 ml of Luria– Bertani (LB; HiMedia) culture medium. Growth was allowed until the optical density reached 0.1 at 600 nm (OD=0.1, which corresponds to 108 CFU/ml of the medium). Subsequently, 2×108 CFU/ml from above was added to 100 ml of liquid LB media supplemented with 0, 50, 200, 500 and 1,000 µg/ml of ZnO NPS. All the flasks were put on rotatory shaker (150 rpm) and incubated at 37°C.

Control broths were used without nanoparticles. Bacterial growth was determined by measuring the optical density after every 2 h (up to 16 h) at 600 nm using a spectrophotometer (VSP66, LOBA Life, India). Growth curves were plotted upto 24hrs.

## Result and Discussion

**Identification and Antibiotic susceptibility testing of bacterial isolates**

Of these 62 randomly selected gram negative isolates, 40 were *E. coli*, 18 were *P. aeruginosa* and 4 were *Klebsiella spp*. Among these 48 (77.7%) were multidrug resistant with 26 (41.9%) ESBL and 24 (38.7%) AmpC producing isolates. Sensitivity profile of these isolates is shown in table 1.

Infections caused by antibiotic resistant strains have become a global problem. The increasing prevalence of multi-drug resistance among Gram-negative bacilli such as extended spectrum beta-lactamase (ESBL) and AmpC production with few or no treatment options is a serious cause for concern.

As most of the skin infections are superficial or open, topical therapy can be effective and it has advantage of being given without the systemic side effects of the drug used. Topical therapy with nanoparticle solutions is an upcoming model in this context. ZnO powder has been used for a long time as an active ingredient for dermatological applications in creams, lotions and ointments on account of its antibacterial properties (Sawai, 2003). However, nanoparticles of ZnO are much more effective agents in controlling the growth of various microorganisms. We have taken mainly multi-drug resistant isolates so that we can determine the antibacterial activity of nanoparticles in condition where conventional chemotherapy is less effective or ineffective.

**Bactericidal activity of ZnO NPs against ESBL and AmpC producing isolates**

Broth dilution and agar dilution methods were used to determine the MIC and MBC of ZnO NPs against ESBL and AmpC producing *E. coli* (n=40), *K. pneumoniae* (n=4), *P. aeruginosa* (n=18) and standard strains. The minimum and maximum MIC values recorded were 1000 and 8,000 µg/ml, respectively, whereas the minimum and maximum MBC values were found to be 2,000 and 16,000 µg/ml, respectively (Table 2 and Fig. 1). Other studies have also documented antimicrobial activities of ZnO nanoparticles against a number of bacterial strains: *E. coli* (Zhang *et al.*, 2010), *K. pneumoniae* (Lee, 2009), Staphylococcus spp. (Huang *et al.* 2008), Pseudomonas spp. (Jiang *et al.* 2009), etc.

Table 2 clearly shows that (4/24)20% AmpC and (6/26)23% ESBL producing isolates have MIC of 1,000 µg/ml whilst (7/24)29.1% AmpC and (7/26)26.9% ESBL producing isolates have MBC of 4,000 µg/ml. MBC at 8000 µg/ml was observed in (11/26)42.3% of ESBL and (8/24)33.3% of AmpC producing isolates. Recently, Zhang *et al.* (2010) reported a study on the inhibitory effect of ZnO nanoparticles against *E. coli*, but there is no report depicting the calculation of MIC and MBC values. In our study, the minimum MIC and MBC values for standard strain of *E. coli* were 1000µg/ml and 2,000µg/ml and that of *P. aeruginosa* were 8000µg/ml and 16000µg/ml, respectively. Almost similar results MIC and MBC values were obtained in the case of ESBL and AmpC producing isolates.

**Effects of ZnO NPs on bacterial growth**

Time-dependent growth inhibitory curves of ZnO NPs tested against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and ESBL and AmpC producing gram negative isolates. Optical densities were measured at different concentrations of ZnO NPs (0, 50, 200, 500 and 1,000 µg/ml) after every 2 h (up to 16 h) at 600 nm, shown in Figures 2, 3 and 4. Single ESBL and AmpC producing strain of *E. coli* and *P. aeruginosa*, randomly selected on the basis of having an MBC of 2,000 µg/ml, were used to determine the effects of ZnO NPs on bacterial growth. One isolate of *K. pneumoniae* was also selected for plotting growth curve against ZnO NPs. When cells was exposed to different concentrations of ZnO NPs, ZnO NP solutions of 50 and 200 µg/ml were enough to inhibit the growth of all tested strains within 2 h.

**Table.1** Antimicrobial sensitivity pattern of gram negative isolates (n=62)

| Antibiotics used for sensitivity testing. | No. of strains of *E. coli*(n=40) | | | No. of strains of *P. aeruginosa* (n=18) | | | No. of strains of *Klebsiella spp.* (n=4) |
|---|---|---|---|---|---|---|---|
| | ESBL (n=18) | Non-ESBL (n=8) | AmpC (n=14) | ESBL (n=8) | Non-ESBL (n=4) | AmpC (n=6) | AmpC (n=4) |
| Gentamycin | 9(50%) | 8(100%) | 0(0%) | 3(37.5%) | 4(100%) | 0(0%) | 2(50%) |
| Cefotaxime | 3(16.6%) | 6(75%) | 0(0%) | - | - | - | 0(0%) |
| Ceftriaxone | 3(16.6%) | 8(100%) | 0(0%) | 0(0%) | 4(100%) | 0(0%) | 0(0%) |
| Cefoperazone | 0(0%) | 8(100%) | 0(0%) | - | - | - | 0(0%) |
| Cefoperazone + clavulanate | 18(100%) | 8(100%) | 0(0%) | - | - | - | 0(0%) |
| Ceftazidime | 0(0%) | 8(100%) | 0(0%) | 0(0%) | 4(100%) | 0(0%) | 0(0%) |
| Ceftazidime + clavulanate | 18(100%) | 8(100%) | 0(0%) | 8(100%) | 4(100%) | 0(0%) | 0(0%) |
| Amikacin | 10(58.8%) | 8(100%) | 8(57%) | 3(37.5%) | 4(100%) | 3(50%) | 2(50%) |
| Tobramycin | - | - | - | 6(75%) | 4(100%) | 4(66.6%) | - |
| Piperacillin | - | - | - | 0(0%) | 4(100%) | 0(0%) | - |
| Piperacillin + tazobactum | - | - | - | 8(100%) | 4(100%) | 0(0%) | - |
| Imipenem | 18(100%) | 8(100%) | 14(100%) | 8(100%) | 4(100%) | 6(100%) | 4(100%) |

**Table.2** MIC and MBC of ZnO nanoparticles against ESBL and AmpC producing strains of gram negative isolates

| Organism | No. of strains | | | MIC (µg/ml) | MBC (µg/ml) |
|---|---|---|---|---|---|
| | Non-ESBL producing strains (n=12) | ESBL producing strains (n=26) | AmpC producing (n=24) | | |
| E.coli | 2 | 2 | 1 | 1000 | 2000 |
| | - | 2 | 2 | 1000 | 4000 |
| | - | 2 | 1 | 1000 | 8000 |
| | 2 | 3 | 3 | 2000 | 4000 |
| | - | 2 | 1 | 2000 | 8000 |
| | 2 | 4 | 4 | 4000 | 8000 |
| | 2 | 3 | 2 | 8000 | 16000 |
| *P. aeruginosa* | 2 | - | - | 1000 | 2000 |
| | 2 | 2 | 1 | 2000 | 4000 |
| | - | 2 | 1 | 2000 | 8000 |
| | - | 2 | 1 | 4000 | 8000 |
| | 2 | 2 | 3 | 8000 | 16000 |
| *Klebsiella spp* | - | - | 1 | 1000 | 4000 |
| | - | - | 3 | 8000 | 16000 |

**Fig.1** MBC of ZnO nanoparticles against bacterial isolates

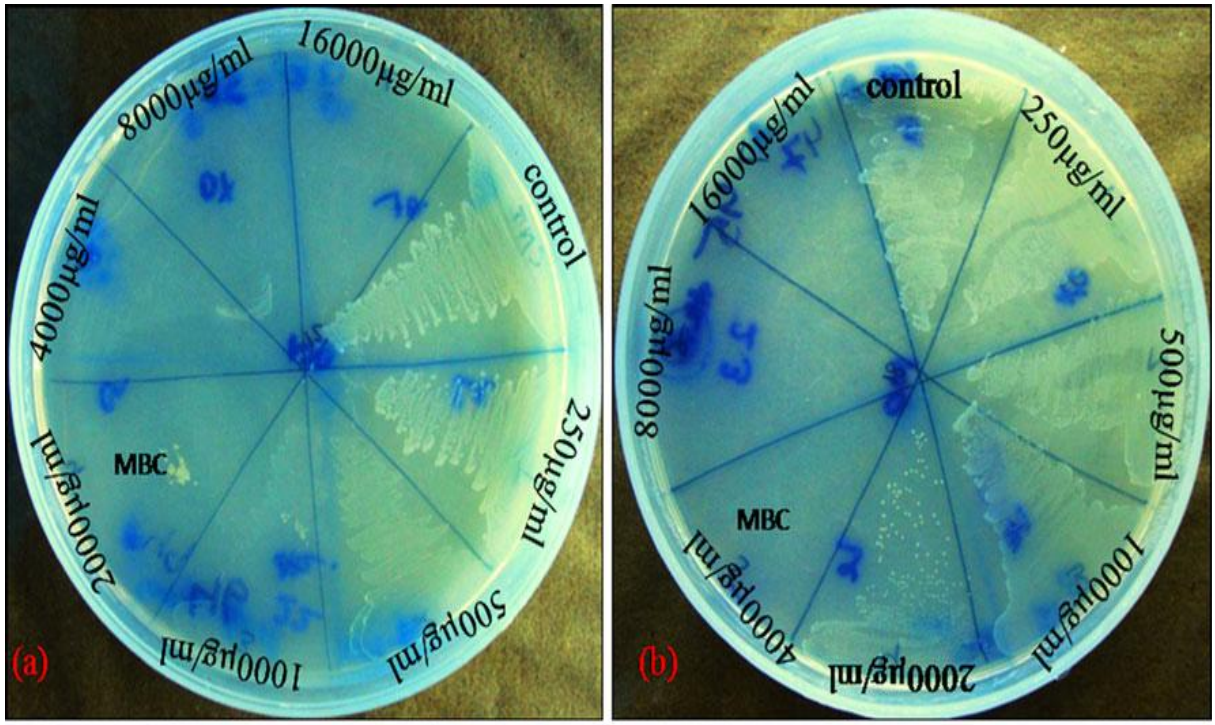
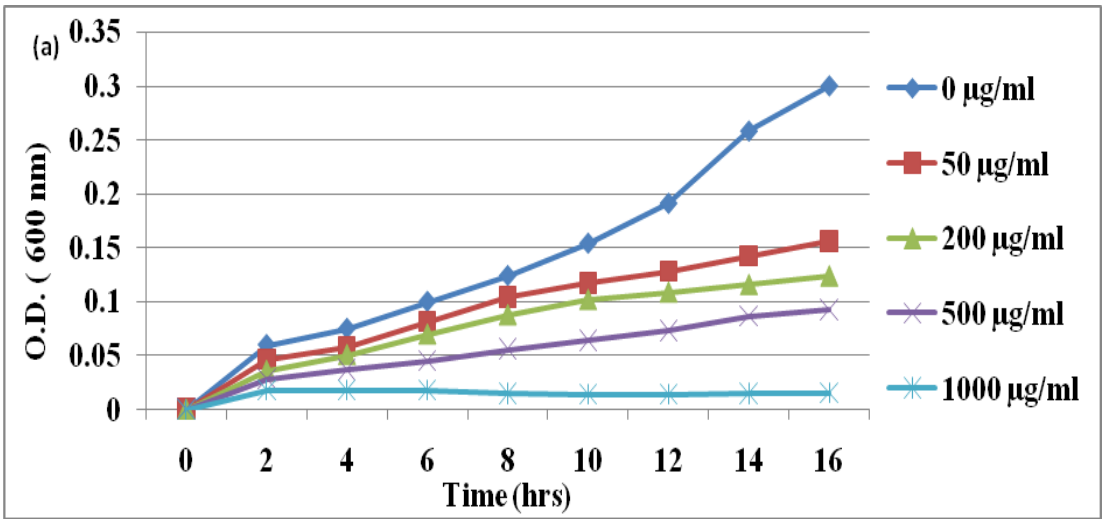



**Fig.2** Growth curves of *Pseudomonas aeruginosa* ATCC 27853 (a), ESBL producing (b) and AmpC producing (c) strain of *P. aeruginosa* in LB medium in presence of different concentrations of ZnO nanoparticles



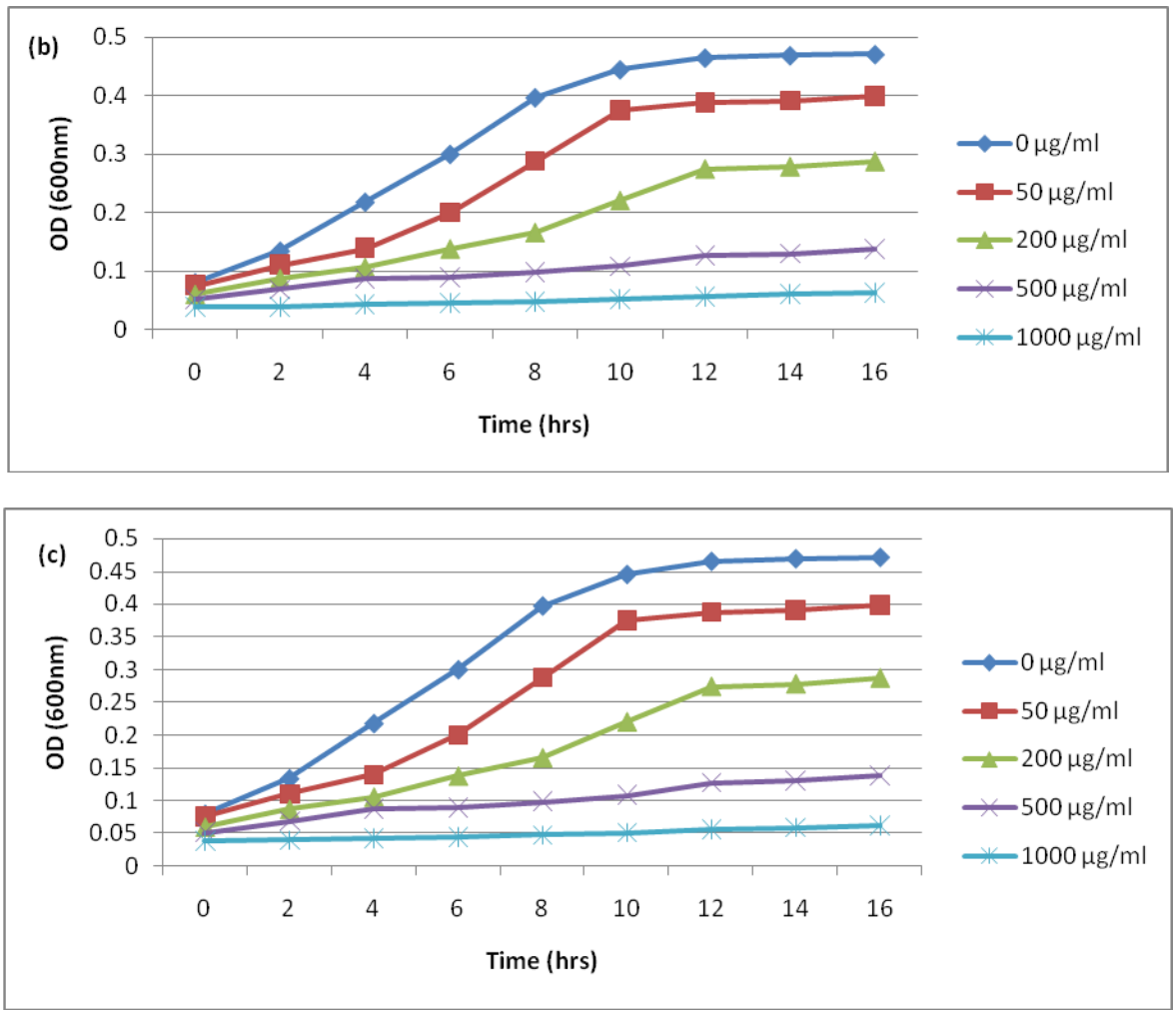
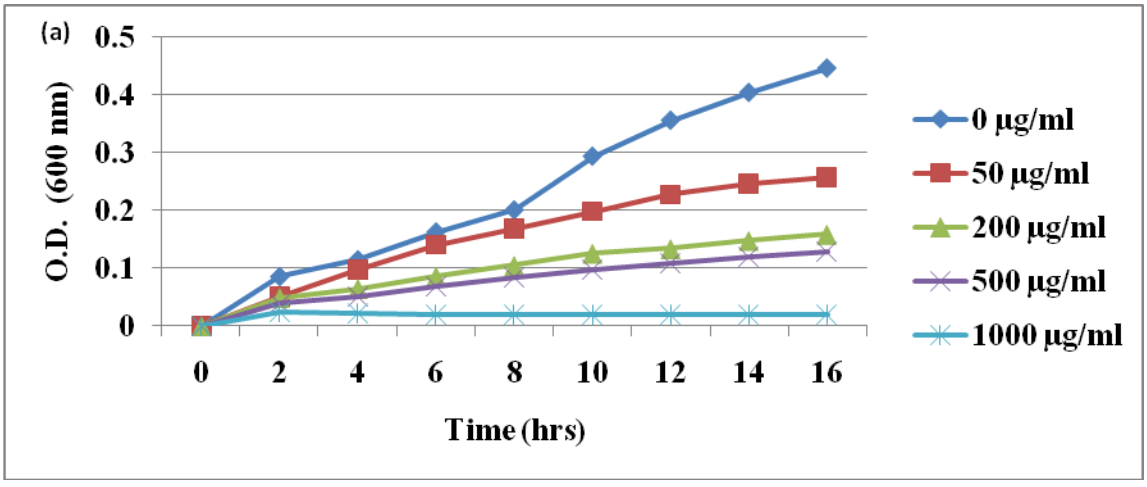



**Fig.3** Growth curves of *Escherichia coli* ATCC 25922 (a), ESBL producing (b) and AmpC producing (c) strains of *E. coli* in LB medium in presence of different concentrations of ZnO nanoparticles



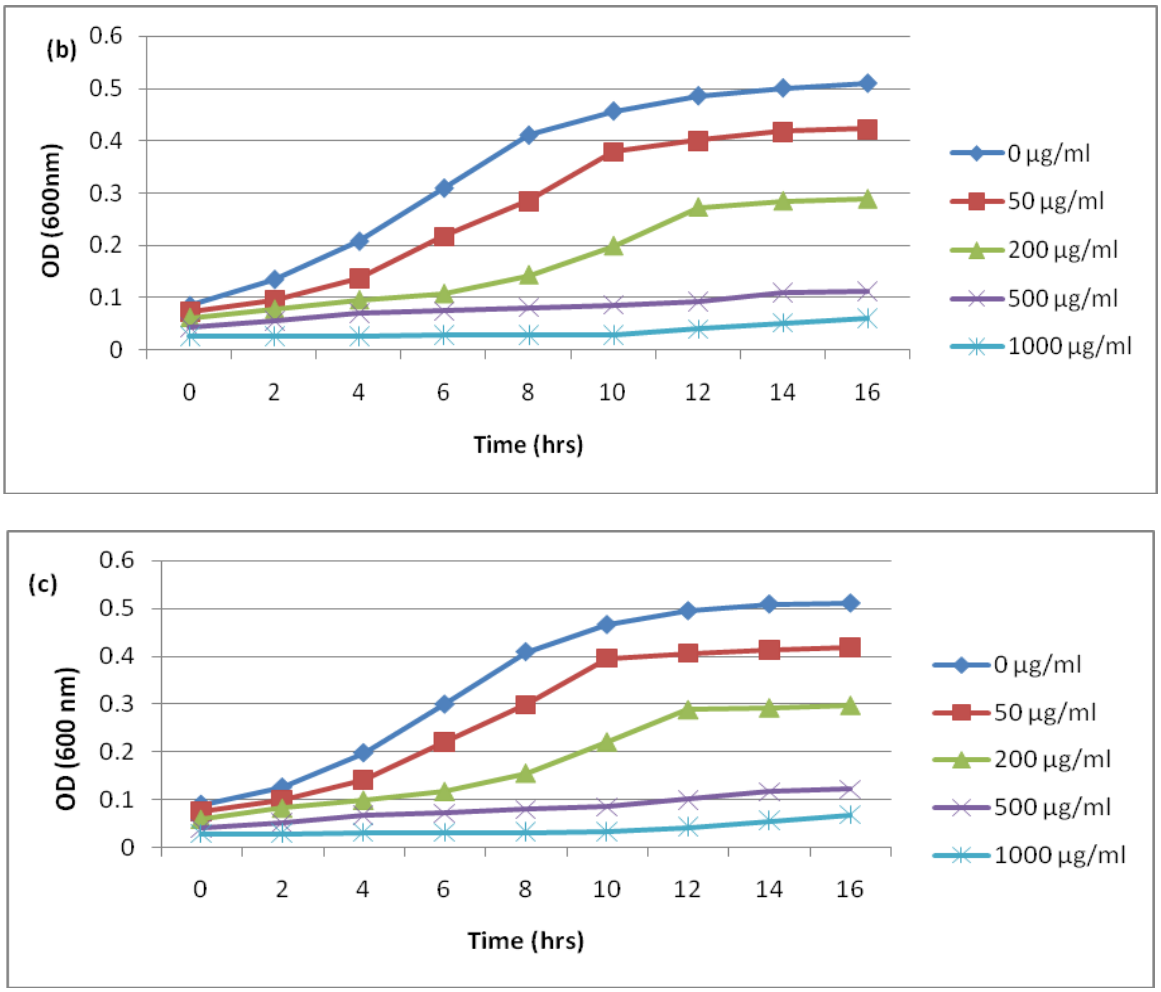
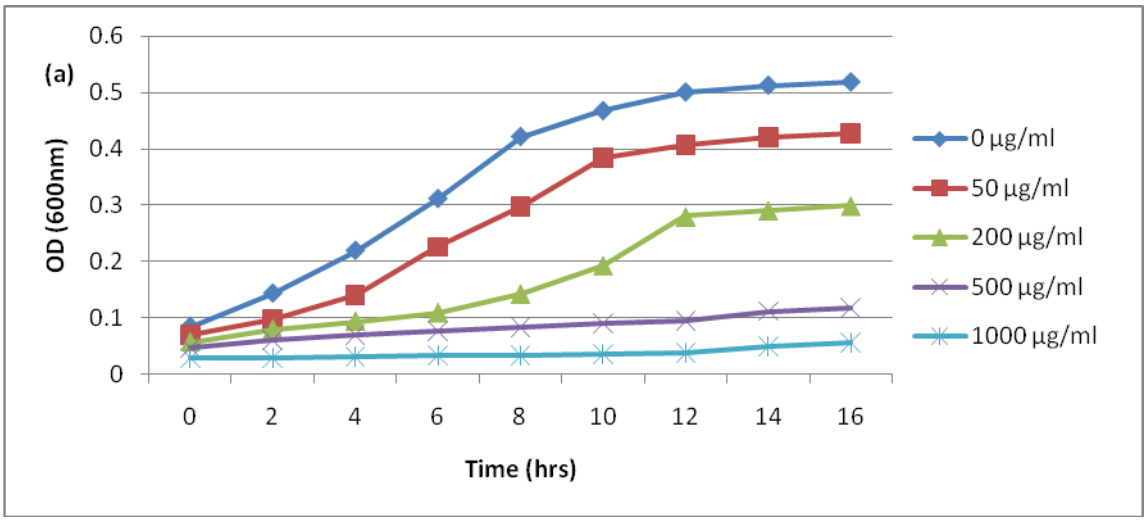



**Fig.4** Growth curves of AmpC producing strain of *Klebsiella sp*. in LB medium in presence of different concentrations of ZnO nanoparticles



However, ZnO NPs with the highest concentration (1,000 µg/ml) showed almost no growth for up to 8 h for *E. coli* ATCC 25922 and 16 h for ESBL and AmpC producing *E. coli* (Fig. 3).

In case of *P. aeruginosa* ATCC 27853 and ESBL & AmpC producing *P. aeruginosa* growth was inhibited for upto 16 hrs (Fig. 2), representing a bactericidal effect at concentration of 500µg/ml and 1000µg/ml.

It has been observed from the OD plot given in the figure that optical absorption in the growth medium decreased in comparison to the control with increasing concentrations of ZnO NPs. This has been attributed to the reduced growth of bacterial cells.

In our study, we achieved an antibacterial effect comparable to that of Zhang *et al.* (2007) at a slightly lower concentration of 100 µg/ml as compared to 250 µg/ml in their study.

Yamamoto (2001) and Sawai (2003), although a different method called conductance method was used by them. Though we have not used different sizes of ZnO NPs, in our study, better antibacterial activity was seen at a lower concentration, which may be because the size of ZnO NPs used in our study (19 nm) is smaller as compared with that of Zhang *et al.* (2007) (230 and 2417 nm).

The antibacterial activity of ZnO NPs increased with an increase in the concentration of nanoparticles. These findings suggest that ZnO NPs may potentially prove useful as nanomedicine-based antimicrobial agents at selective therapeutic dosing regimes.

In conclusion, the data presented here are novel in that they prove that ZnO NPs are effective bactericidal agents against ESBL and AmpC producing gram negative isolates regardless of the drug resistance mechanisms that confer the importance of these bacteria as an emergent pathogen. Overall, these findings suggest that ZnO nanoparticles can be used externally to control the spreading of bacterial infections. It would be interesting to determine if any derivatives of ZnO nanoparticles with various chemical groups or bio agents are more effective at eliminating various microorganisms. Therefore, in the future, ZnO nanoparticle containing formulations may be utilized for external uses as antibacterial agents in ointments, lotions, mouthwashes, and surface coatings on various substrates to prevent microorganisms from attaching, colonizing, spreading, and forming biofilms in indwelling medical devices.

## References

Bradford, P.A. 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.*, 14: 933–51

Brayner, R., Ferrari-lliou, R., Brivois, N., Djediat, S., Benedetti, M.F., Fievet, F. 2006. Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. *Nano Lett.*, 6: 866–870.

Clinical and Laboratory Standards Institute 2003. Performance standards for antimicrobial susceptibility testing: eighteenth informational supplement: Approved standards M100-S18. Clinical and Laboratory Standards Institute, Baltimore, USA. 2008.

Collee, J.G., Miles, R.S., Watt, B. 2006. Tests for the identification of bacteria.

In: Collee, J.G., Fraser, A.G., Marmion, B.P., Simmons, A., (Eds). Mackie and McCartney practical medical microbiology, 14th edn. Churchill Livingstone, New Delhi. Pp. 131–49.

Jiang, W., Hamid, M., Baoshan, X. 2009. Bacterial toxicity comparison between nano- and micro-scaled oxide particles. *Environ. Pollut.*, 157(5): 1619–1625.

Jones, N., Ray, B., Ranjit, K.T., Manna, A.C. 2008. Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. *FEMS Microbiol. Lett.*, 279: 71–76.

Laura, K.A., Delina, Y.L., Pedro, J.J.A. 2006. Comparative eco-toxicity of nanoscale TiO2, SiO2, and ZnO water suspensions. *J. Water Res.*, 40: 3527–3532.

Lee, S. 2009. Multifunctionality of layered fabric systems based on electrospun polyurethane/zinc oxide nanocomposite fibers. *J. Appl. Polymer Sci.*, 114(6): 3652–3658

Luo, P.G., Tzeng, T.R., Shah, R.R., Stutzenberger, F.J. 2007. Nanomaterials for antimicrobial applications and pathogen detection. *Curr. Trends Microbiol.*, 3: 111–128.

Mohammad Azam Ansari, Haris M. Khan, Aijaz A. Khan, Asfia Sultan, Ameer Azam. 2011. Synthesis and characterization of the antibacterial potential of ZnO nanoparticles against extended-spectrum β-lactamases-producing *Escherichia coli* and Klebsiella pneumoniae isolated from a tertiary care hospital of North India. *Appl. Microbiol. Cell Physiol.*, 94: 467–477.

Reddy, K.M., Kevin, F., Jason, B., Denise, G.W., Cory, H., Alex, P. 2007. Selective toxicity of zinc oxide nanoparticles to prokaryotic and eukaryotic systems. *J. Appl. Phys. Lett*., 90(21): 1–3.

Rizvi, M., Fatima, N., Rashid, M., Shukla, I., Malik, A., Usman, A., Siddiqui, S. 2009. Extended spectrum AmpC and metallo-beta-lactamases in Serratia and *Citrobacter* spp. in a disc approximation assay. *Infect. Dev. Countries*, 3(3): 177–186.

Sawai, J. 2003. Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay. *J. Microbiol. Methods*, 54:177–182.

Sobha, K., Surendranath, K., Meena, V., Jwala, K.T., Swetha, N., Latha, K.S.M. 2010. Emerging trends in nanobiotechnology. *J. Biotech. Mol. Bio Rev*., 5(1): 001–012.

Yamamoto, O. 2001. Influence of particle size on the antibacterial activity of zinc oxide. *Int. J. Inorg. Mater.*, 3: 643–646.

Zhang, L., Yunhong, J., Yulong, D., Nikolaos, D., Lars, J., Povey, M., O'Neill, A.J., York, D.W. 2010. Mechanistic investigation into antibacterial behavior of suspensions of ZnO nanoparticles against *Escherichia coli*. *J. Nanopart. Res.*, 12: 1625–1636.

Zhang, L., Yunhong, J., Yulong, D., Povey, M., York, D.W. 2007. Antibacterial behaviour of suspensions of ZnO nanoparticles (nanofluids). *J. Nanopart. Res.*, 9: 479–489.