An evaluation of bactericidal property of Zinc oxide Quantum Dots on a multiple-antibiotic and human serum resistant bacterium, *Klebsiella pneumoniae* MB45

6.1. Background

Klebsiella pneumoniae among all species of the genus *Klebsiella* derives special importance because of being pathogenic to human beings. Interestingly, they are omnipresent and have been isolated from diverse sources including animals, sewage, soils, vegetables, salt water, treated drinking water, sachet water and river water (Podschun *et al.*, 2001; Li *et al.*, 2004; Lal and Kaur, 2006; Banu and Menkuru, 2010; Xu *et al.*, 2010). The test strain MB45, described in this thesis, isolated from river water possessing oligotrophic characteristics, was resistant to ten different antibiotics. The strain MB45 was also found to be resistant to human serum. Like other *Klebsiella* strains, strain MB45 also contains polysaccharide capsule over cell which is the main determinant of pathogenicity in *Klebsiella*.

There are several reasons and mechanism (described elsewhere in the thesis) responsible for the spread of antibiotic resistance in the natural populations of bacteria. The fact remains that bacteria are becoming increasingly resistant to almost all known drugs/antibiotics invented or discovered. The prevalence of multiple antibiotic resistant bacteria among pathogens and by-standers (normally not pathogenic but becomes virulent under immunosuppressive conditions) poses a severe threat to public health worldwide. Hence, rise in incidence of antibiotic resistance among bacteria (pathogenic as well as environmental) have discouraged investment in R & D sector for searching new antibiotics. On the other hand, boom in infectious diseases caused by multiple-antibiotic resistant bacteria has created an unprecedented demand for novel antimicrobials and eradication strategies. This demand and challenge to innovate new therapies induced the researchers to look back to their civilization of the by-gone days when people used metals in the form of gold ashes for regaining health, silver for treating burns and chronic wounds or copper for making water potable (Moghimi, 2005). Gold is still used in the Indian Ayurvedas for rejuvenation and revitalization during old age under the name of Swarna Bhasma ("Swarna" meaning gold, "Bhasma" meaning ash) (Mahdihassan, 1985). Gold also has its long history of use in the western countries where it was used for revitalizing people suffering from nervous conditions (Higby, 1982). In recent years, various nanotized/nanosized (called as nanoparticles) metals like Ti, Mg, Ag, and Zn have been documented as important alternative candidate of antibiotics in controlling pathogenic bacteria (Joshi *et al.*, 2009). The potential application of nanoparticles as antimicrobials emanated from bacterial growth-inhibiting ability as well as their unique physiochemical properties. Recently, nanoparticles made out of such metallic compound are being characterised for novel physical and chemical properties; some of them are often shown to have biological effects like antimicrobials (Gajjar *et al.*, 1991). The antimicrobial activity of the nanoparticles is directly proportional to the surface area (size α 1/surface area) in contact with the microorganisms i.e. high surface to volume ratio means more interaction with the microbes (Rai and Bai, 2011) which micro- or macro-sized particles do not possess. Hence, researches on applications of nanoparticles as antimicrobials have gained momentum in the present century. The nanoparticles are broadly categorized in two groups (i) organic and (ii) inorganic nanoparticles. The latter one has received more attention due to their ability to withstand adverse processing conditions (Whitesides *et al.*, 2003). The nanosized oxide derivatives of metals are receiving increasing attention for a large variety of applications due to their superiority in terms of safety, durability and heat resistance in compare to conventional organic antibacterial agents. Oxides of titanium (TiO_2) and zinc (ZnO) nanoparticles (NPs) are included in toothpaste, beauty products, sunscreens, and textiles. Among the different oxide derivatives of nanosized metals, zinc oxide (ZnO) have been found as potent drug having antibacterial activity (Stoimenov, 2002; Hanley *et al.*, 2008; Nair *et al.*, 2008; Applerot *et al.*, 2009; Joshi *et al.*, 2009) not just because of their stability under harsh processing conditions, but also because they are generally considered as safe materials for human beings and animals (Stoimenov *et al.* 2002; Fu *et al.* 2005).

This chapter briefly describes an attempt to evaluate zinc oxide nanoparticles (ZnO-Acetate quantum dots) for their bactericidal activities. The obvious choice of bacterium (on which the Nps to be tested) was the one which could resist several different antibiotics and human serum as well. Hence, the test strain was *Klebsiella pneumoniae* MB45.

6.2. Materials and methods

6.2.1. Isolation, selection, oligotrophic characteristics, and characterization of test strain MB45: described in chapter 5

6.2.2. Antibiotic susceptibility test and determination of resistance: described in chapter 5.

6.2.3. Serum bactericidal assay

Serum bactericidal assay was basically performed according to the methods described by Sharma *et al.* (1999). Normal human serum (NHS) obtained from healthy adult volunteers (having no record of antibiotic therapy for the last one year) was used for assay. Heat inactivated serum (56°C for 30 min) (HIS) was used as control. Bacterial culture for assay was prepared by transferring a loopful culture (24 h old grown on Luria agar plate) into 10 mL sterile Brain Heart infusion broth in 100 mL Erlenmeyer flask. The inoculated medium was incubated at 37°C for 4 h without agitation. The cells were harvested by centrifugation at 8000 rpm for 5 minutes at 4°C and re-suspended in phosphate buffer saline (PBS, pH 7.3). The cell suspension was diluted with PBS and optical density (at 600 nm) was adjusted to McFarland standard No. 0.5. Cell suspensions were further diluted with PBS to achieve a cell density of 1×10^{8} cells/mL. Aliquot of 0.1 mL of cell suspension (=1×10^{7} cells) was mixed with 0.2 mL of NHS and 0.1 mL of 10X peptone-glucose-bromothymol blue broth (composition: 10% peptone, 10% glucose and 0.075% of bromothymol blue). The volume was adjusted to 1 mL by adding 0.6 mL of sterile PBS. Control with HIS was also included. *Escherichia coli* K12 was also taken as control with NHS and HIS for testing serum susceptibility. Change in the color in NHS and HIS tubes at 5 h of incubation at 37°C indicated viability of cells as well as serum resistance while no change in color of NHS tube even at 8 h but change in HIS tube at 5 h was regarded as serum-sensitive.

6.2.4. Effect of ZnO QDs on viability and growth of MB45

The zinc oxide quantum dots (ZnO QDs) with surface adsorbed anionic species of acetate ions synthesized by wet chemical route were provided by Joshi *et al* (2009). Inoculum, for testing ZnO QDs efficacy, was prepared by the method as described in chapter 4. Aliquots of 10 μL of concentrated (4×10^{6} cells) cell suspension were added to 3.0 mL of Luria broth in 150×15mm (length × width) glass tubes amended with different concentrations of ZnO-Ac QDs. The tube containing all the ingredients except ZnO-Ac was taken as negative control. Luria broth containing ZnO-Ac but lacking cells were taken as positive control. The tubes were kept at 37°C with continuous agitation at 200 rpm throughout the period of investigation. Test was performed in triplicates and standard error was measured. The optical density was measured in spectrophotometer (Model-302, Electronic India) at 600 nm at different time intervals. Growth rate constant (μ) and mean generation time (g) of MB45 in absence and presence ZnO QDs were calculated.

6.3. Results and discussion

Phenotypic and 16S rRNA phylogeny revealed that the strain, MB45, belonged to the genus *Klebsiella*. The maximum homology (99%) of MB45 16S rRNA gene sequence was shared by the *Klebsiella pneumoniae* (detailed phylogenetic analysis described in chapter 5). *Klebsiellae* (especially *Klebsiella pneumoniae* among all) are opportunistic pathogens and can spread rapidly, which often leads to nosocomial outbreaks (Nordmann *et al.*, 2009). *K. pneumoniae* although being a respiratory pathogen, the other sites of infection reported include urinary tract, digestive tract, surgical wound sites etc. The invasiveness of infection takes place generally via contaminated respiratory support equipment and urinary catheters or via other surgical/non surgical equipments. Antibiotic-resistance genes are acquired by these bacteria through horizontal gene transfer and get selected in the environment where there is extensive use of broad-spectrum antibiotics. Massive use of drugs/ antibiotics resulted in development of multiple-antibiotic-resistant *Klebsiella* strains. *Klebsiella pneumoniae* strain MB45 is resistant to ten antibiotics (cotrimoxazole, ampicillin, gentamycin, netilmicin, tobramycin, chloramphenicol, cefotaxime, kanamycin and streptomycin) and could survive and multiply in nutrient poor medium (oligotrophic). An increase of 4.6 times of the initial cell number was noted in 2 days in nutrient poor medium which explains the oligotrophic nature of strain MB45. Since the existance of oligotrophic bacteria has already been reported previously from the clinical samples (Tada *et al.*, 1995), therefore, oligotrophs are of more interest in light of human health. Such bacteria normally do not appear on conventional rich laboratory media and thus their role if any in causing infections of hospital admitted patients generally goes undetected. Besides being resistant to ten antibiotics (as stated earlier), MB45 can remain viable in an environment without nutrient-luxury. Also it has been described in chapter 5 about its bearing of a novel dihydrofolate reductase gene. MB45 was capable to resist high concentration of trimethoprim (>1500 mg/L). Besides antibiotic resistance, serum resistance in clinical isolates of pathogenic bacteria is an additional threat. The present study showed that the test strain, MB45, was also resistant to human serum. Human serum functions as bactericidal agent in healthy persons and protect from invasion of pathogens. Pathogens which are successful in causing disease are generally equipped with the capability to resist normal human serum (NHS) resulting persistent infection. In an earlier study, conducted on more than hundred clinical isolates of *Klebsiella pneumoniae* for assessing serum bactericidal activity, it was shown that 50% of the strains were resistant to 20% normal human serum collected from different healthy humans (Sharma *et al.*, 1999). The strain MB45 cells could change the color in NHS and HIS tubes at 5 h and therefore inferred as serum resistant while control, *E. coli* K12 failed to change the color of NHS tube even at 8 h, but turned HIS tube yellow at 5 h, was regarded as serum-sensitive. The strong correlation between serum resistance and the ability of a variety of gram-negative bacteria to invade and survive in the human blood stream have been studied (Taylor, 1988).

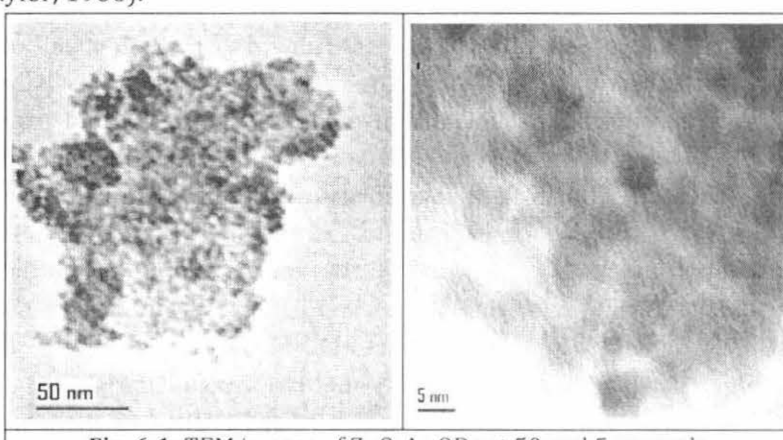

Fig. 6.1: TEM images of ZnO-Ac QDs at 50 and 5 nm scale.
(Fig. adapted from Joshi *et al.*, 2009)

A number of nanosized metals itself or derivatives were found to be good antimicrobials. Among different oxides derivatives of metals (nano-particle), oxide derivative of zinc metal received more attention due to their long stability, effectiveness, and biocompatibility to humans and animals. The size of nanoparticles is the most important factor in its effectiveness. The mechanism of action of nanoparticle is still inconclusive. However, some authors proposed their views to explain the antibacterial activity of ZnO nanoparticles; for example, Yamamoto (2001) proposed generation of hydrogen peroxide from the surface of ZnO which causes inhibition of bacterial growth. Another possible mechanism was proposed by Brayner *et al.* (2006) where it was suggested that the released Zn^{2+} ions are responsible for damaging the cell membrane leading to further lethal interaction with other intracellular contents. It is known that the physical and chemical properties of the nanoparticles change with size (coming close to nanoscale). Typically the dimension of nanoparticles fell in the range of 0.2-100 nm. The average particle size of chemically synthesized (wet route) zinc oxide quantum dots with surface adsorbed anionic species of acetate ions (ZnO-Ac QDs) was estimated around 3–5 nm (Fig. 6.1) (Joshi *et al.*, 2009). The previous study on the ZnO-Ac QDs was conducted on *E. coli K12* bacterium by Joshi *et al.*, (2009) where they found that zinc nanoparticle with surface bound acetate ions (anionic species) had superior bactericidal activity than those described earlier (Hanley *et al.*, 2008). In this study, the antibacterial potency of ZnO-Ac QDs was tested against the multiple-antibiotic and serum resistant *Klebsiella pneumoniae* MB45. The bacterial growth rate was found to be inhibited with the increase in concentration of ZnO-Ac QDs under standard cultural conditions (Fig. 6.2). The growth rate constant (μ) and mean generation time (g) in Luria broth without ZnO QDs (μ =0.019 min^{-1}, g = 36.5 min) was significantly affected on addition of ZnO QDs (μ = 0.013 min^{-1}; g = 53.3 min; at 400 mg/L). Complete inhibition of growth of MB45 is observed at concentration of 500 mg/L ZnO-Ac QDs in the medium. Hence, in future ZnO-Ac QDs could be a good nanobiotic candidate for the control of multi-drug resistant pathogens as well as in disinfecting hospital environments, external wounds, medical and surgical devices; and also in suitable format may find application in drinking water treatment plants.

6.4. Conclusion

Apart from the general direction of the search elucidated in the preceding chapters, the final chapter leaves a ray of hope to curb the menace. Zn-based nanoparticles (quantum dots) may offer a possible solution to control human-serum-tolerant pathogens resistant to multiple antibiotics.

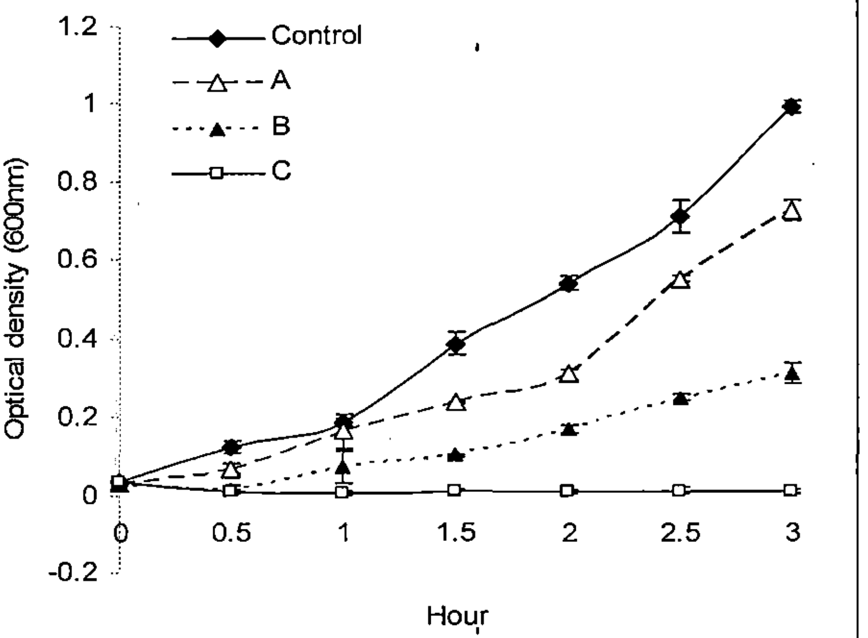

Fig. 6.2: Effect of ZnO nano-particles on the growth of MB45. Cultures were setup with an initial cell number 9×10⁶ cells in Luria broth amended with different concentration (♦, Control 0.0 mg/L; Δ, A 200mg/L; ▲, B 400mg/L; □, C 500mg/L) of colloidal solution of ZnO nano-particles. Cultures were incubated at 37 °C with constant shaking at 200 rpm under normal laboratory condition. Bars shows standard deviation.

REFERENCES

Applerot G, Lipovsky A, Dror R *et al.* Enhanced antibacterial activity of nanocrystalline ZnO due to increased ROS-mediated cell injury. *Adv Funct Mater* 2009; 19: 842–52.

Banu N, Menakuru H. Enumeration of microbial contaminants in sachet water: a public health challenge. *Health* 2010; 2: 582-8.

Brayner R, Ferrari-Iliou R, Brivois N *et al.* Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. *Nano Lett.* 2006; 6: 866–70.

Fu G, Vary PS, Lin CT. Anatase TiO2 nanocomposites for antimicrobial coatings. *J Phys Chem* B 2005; 109: 8889–98.

Gajjar P, Pettee B, Britt DW *et al.* Antimicrobial activities of commercial nanoparticles against an environmental soil microbe, *Pseudomonas putida* KT2440. *J Biol Eng* 2009; 3: 9-22.

Hanley C, Layne J, Punnoose A *et al.* Preferential killing of cancer cells and activated human T cells using ZnO nanoparticles. *Nanotechnol* 2008; 19: 295103.

Higby GJ. Gold in medicine: a review of its use in the West before 1900. *Gold Bull* 1982; 15: 130–40.

Joshi P, Chakraborti S, Chakrabarti P *et al.* Role of Surface Adsorbed Anionic Species in Antibacterial Activity of ZnO Quantum Dots Against Escherichia coli. *J Nanosci Nanotechnol* 2009; 9: 6427-33.

Lal M, Kaur H. A microbiological study of bottled mineral water marketed in Ludhiana. *Indian J Public Health* 2006; 50: 31-2.

Li X, Zhang D, Chen F *et al.* *Klebsiella singaporensis* sp. nov., a novel isomaltulose-producing bacterium. *Int J Syst Evol Microbiol* 2004; 54: 2131-6.

Mahdihassan S. Cinnabar-gold as the best alchemical drug of longevity, called Makaradhwaja in India. *Am J Chin Med* 1985; 13: 93–108.

Moghimi SM. Nanomedicine: prospective diagnostic and therapeutic potential. *Asia Pacific Biotech News* 2005; 9:1072-7.

Nair S, Sasidharan A, Divya Rani VV *et al.* Role of size scale of ZnO nanoparticles and microparticles on toxicity toward bacteria and osteoblast cancer cells. *J Mater Sci Mater Med* 2008; 20: S235–41.

Podschun R, Pietsch S, Höller C *et al.* Incidence of *Klebsiella* Species in Surface Waters and Their Expression of Virulence Factors. *Appl Environ Microbiol* 2001; 67: 3325–7.

Rai RV, Bai JA. Nanoparticles and their potential application as antimicrobials. Science against microbial pathogens: communicating current research and technological advances. 2011.

Sharma SK, Fatma T, Thukral SS. A simple and rapid serum bactericidal assay and its evaluation in clinical isolates of *Klebsiella pneumoniae*. *J Microbiol Methods* 1999; 39: 45–8.

Stoimenov PK. Metal oxide nanoparticles as bactericidal agents. *Langmuir.* 2002; 18: 6679-86.

Tada Y, Ihmori M, Yamaguchi J. Oligotrophic Bacteria Isolated from Clinical Materials. *J Clin Microbiol* 1995; 33: 493-4.

Taylor PW. Bacterial resistance to complement. In Virulence Mechanisms of Bacterial Pathogenesis. Edited by: Roth JA. American Society of Microbiology, Washington, DC. 1988. p.107-20.

Whitesides GM. The 'right' size in Nanobiotechnology. *Nat Biotechnol* 2003; 21:1161-5.

Xu J, Li W, Chen X *et al. Klebsiella alba* sp. nov., a novel pesticidetolerant bacterium from a heavily polluted environment. *J Gen Appl Microbiol* 2010; 56:241-7.

Yamamoto O. Influence of particle size on the antibacterial activity of zinc oxide. *Int J Inorg Mater* 2001; 3: 643–6.