In vitro efficacy of Rifampicin and nanoparticulate Rifampicin against *Mycobacterium tuberculosis.*

THESIS

Submitted

in partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY MICROBIOLOGY

BY

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Enrolment No : V/02/1409

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(INDIA)

2008

PGR - ANNEXURE - XIII

DECLARATION OF STUDENT

I hereby declare that the experimental Research work and interpretation of the thesis entitled **"*In vitro* efficacy of Rifampicin and nanoparticulate Rifampicin against *Mycobacterium tuberculosis*."** or part there of has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

Date : 25th August, 2008

Signature

Shivali

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Counter signed by

Dr. (Mrs) R. S. Gandge
Chairman, Advisory Committee, and
Assist. Professor of Microbiology,
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PGR - ANNEXURE - XIV

Declaration of Advisory Committee

Shri. **Gaikwad Shivali Pradeep** has satisfactorily prosecuted her course of research for a period of not less than one semester and that the thesis entitled, **"*In vitro* efficacy of Rifampicin and nanoparticulate Rifampicin against *Mycobacterium tuberculosis.*"** submitted by him is the result of research work is sufficient to warrant its presentation to the examination in the subject of **VETERINARY MICROBIOLOGY** for the award of **MASTER OF VETERINARY SCIENCE (M.V.Sc)** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part there of has not been previously submitted by him for a degree of any other University.

Place: Mumbai

Date : 25th August, 2008

Signature

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Advisor/Guide
Assistant Professor of Microbiology
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Advisory Committee

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4)	Dr. R. V. Gaikwad	Hospital superintendent, Bombay Vet. College	

PGR - ANNEXURE - XV

CERTIFICATE

This is to certify that the thesis entitled, **"*In vitro* efficacy of Rifampicin and nanoparticulate Rifampicin against *Mycobacterium tuberculosis.*"** submitted by Ms.**Gaikwad Shivali Pradeep** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **MASTER OF VETERINARY SCIENCE** in **VETERINARY MICROBIOLOGY** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

Name & Signature of
External Examiner

Signature with Seal
Head of the Department

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Dean/ Associate Dean

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Place : MUMBAI
Date 25th Aug. 2008

Shivali
(Ms. Shivali P. Gaikwad)

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LIST OF ABBREVATIONS

ATCC	American Type cell culture
CFU	Colony Forming Unit
DMSO	Dimethyl Sulphoxide
LJ Medium	Lowensten Jensen
MTT	3-[4,5-Dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide
MVM	Mean Viable Macrophage
Nano-Rif	Nanoparticulate Rifampicin
Rif	Rifampicin
RPMI	Roswell park memorial institute
SDS	Sodium Dodoecyl Sulphate
TB	Tuberculosis
UICT	Institute of chemical technology, university of Mumbai
UV	ultra violet
WHO	World health organization
Zn	Ziehl neelson

Introduction

1. INTRODUCTION

Tuberculosis (TB) is a major cause of worldwide morbidity and mortality (Raviglione *et al.*, 1995). It is estimated that over one-third of the World's human population harbours *Mycobacterium tuberculosis (Mtb)*, the bacterium that causes TB. The bacteria survive within macrophages and provide the organism with a resistant barrier to many common drugs (Katzung, 2001). The majority of TB cases (5–6 million) are observed in people of age group 15–49 years. India has almost 30% of the global burden of tuberculosis and one person dies of disease every minute. Currently, in India there are about 14 million suspected and about 3.5 million bacteriologically proven cases of pulmonary tuberculosis. Incidence of tuberculosis is about 1.5 per 1000 population (Kadri *et al.*, 2003). More recently, average prevalence of all forms of tuberculosis in India is estimated to be about 5.05 per 1000 (Chakraborty *et al.*, 2004). Likewise, bovine TB, caused by *Mycobacterium bovis*, is a significant veterinary disease that can spread to humans (Acha and Szyfres, 1987). In developing countries, animal TB is widely distributed, since control measures are either not applied or are applied sporadically. For instance, *M. bovis* infection is responsible for about 2% and 8% of new cases of human pulmonary and extra-pulmonary TB, respectively (Cosivi *et al.*, 1998).

Since the control measures for TB such as Bacillus Calmette-Guérin (BCG) vaccination and chemoprophylaxis appear to be unsatisfactory, treatment with anti-tubercular (anti-TB) drugs becomes the only option available. The goals of treatment are to ensure cure without relapse, to prevent death, to impede transmission, and to prevent the emergence of drug resistance. In the development of tuberculosis therapy, two points are important to consider. First, the metabolism of *M. tuberculosis* is slow, resulting in a generation time that is measured in hours . This means that drug regimens should ideally have a low level of toxicity for long-term administration, and if possible, should be bactericidal so that elimination of the organism is rapid and is not totally dependent on the immune system. Second, the tubercle bacillus is a facultative intracellular parasite ; therefore,

drugs should also be able to penetrate host cells. Thus, an ideal method for treating tuberculosis would be the one that not only is able to safely deliver drugs systemically for long term, but also would be able to target drugs to the intracellular environment in which the tubercle bacilli are found i.e., macrophages.

Anti tuberculosis drug like RIF (Rifampicin) is used in combination with PYZ (pyrazinamide) which has an important role in the sterilisation of lesions by eradicating organisms. These two drugs are crucial for successful 6-month treatment regimens. RIF kills low or non-replicating organisms and the high sterilising effect of PYZ serves to act on semidormant bacilli not affected by any other anti-TB agents in sites hostile to the penetration and action of the other drugs (Heifets *et al.*, 1999) . INH (isoniazide) and RIF, the two most potent anti-TB drugs, kill more than 99% of tubercular bacilli within 2 months of initiation of therapy (Iseman [illegible] 1993). Using these drugs in conjunction with each other reduces anti-TB therapy from 18 months to 6 months

Despite the availability of these highly effective treatments for TB, cure rates remain low, as commercial anti-TB formulations are inconvenient to administer and patients do not take the prescribed medications with sufficient regularity and duration to achieve a cure (Chen [illegible] 2000). Patients have to consume a large number of tablets (up to eight at one time), which is a common cause for non-compliance. It can be anticipated that non-optimal application of these short course regimens will result in the deterioration of their therapeutic potential, an escalation in the mortality rate and increased risk of developing acquired drug resistance (Kochi *et al.*,1993). Resistance of *M. tuberculosis* to anti-TB agents is a worldwide problem in both immunocompetent and HIV-infected populations (Edlin *et al.*, 1992).

Recent trends in controlled drug delivery have seen microencapsulation of pharmaceutical substances in biodegradable polymers as an emerging technology. Carrier or delivery systems such as liposomes and microspheres have been developed for the sustained delivery of anti-TB drugs and have demonstrated better chemotherapeutic efficacy when investigated in animal models, such as mice (Falk *et al.*,1997). Anti-TB drugs have been successfully entrapped and delivered in biodegradable polymers such as poly

(DL-lactide-co-glycolide) (PLG), which are biocompatible and release drug in a controlled manner at therapeutic levels (Kailasam *et al.*, 1994).

More recently, nanoparticle-based drug delivery systems have been shown to have considerable potential for treatment of tuberculosis (Svetlana *et al.*, 2005). The important technological advantages of nanoparticles used as drug carriers are their high stability, high carrier capacity, feasibility of incorporation of both hydrophilic and hydrophobic substances, and feasibility of variable routes of administration, including oral application and inhalation. Nanoparticles can also be designed to allow controlled (sustained) drug release from the matrix. These properties of nanoparticles enable improvement of drug bioavailability and reduction of the dosing frequency, and may resolve the problem of nonadherence to prescribed therapy, which is one of the major obstacles in the control of TB epidemics (Svetlana *et al.*, 2005).

In the case of pulmonary TB, delivering the drug directly to the site of infection through inhalation of an aerosolised delivery system has the inherent advantages of bypassing first-pass metabolism and maintaining local therapeutically effective concentrations with decreased systemic side effects (Zhou *et al.*, 2005). Liposomes coated with alveolar macrophage-specific ligands demonstrated preferential accumulation in alveolar macrophages, maintaining high concentrations of RIF in the lungs even after 24 hours (Vyas *et al.*, 2004).

A number of the aforementioned developments in drug delivery represent attractive options with significant merits and demerits. However, the need to develop an oral drug delivery system with improved patient acceptance is affirmed by the accelerated pace of oral drug delivery system development fostered by the need to deliver medications to patients more efficiently and with fewer side effects, especially in developing countries where controlled-delivery implants and injectables could be too expensive.

Taking into consideration the above points, for need of better treatment of TB the present research was planned to assess *in-vitro* intracellular delivery and efficacy of an improved antitubercular drug with following objectives.

Objectives:

I. To compare cytotoxic effect of plain Rifampicin and nanoparticulate Rifampicin on macrophage cell line.

II. To study internalization of nanoparticulate Rifampicin in human macrophage cell line.

III. To study efficacy of plain Rifampicin and nanoparticulate Rifampicin against *Mycobacterium tuberculosis.*

Review of Literature

2. REVIEW OF LITERATURE

2.1 Propagation and maintenance of human macrophage cell line

Jagannath *et al.* (1995) carried out studies on susceptibility methods using human monocyte-like cell line U937. The cells were maintained by *in vitro* passage in RPMI 1640 medium with 10% fetal bovine serum and addition of Gentamicin @ 50 µg /ml of growth medium.

Wright e*t al.* (1996) evaluated Mono Mac 6 (MM6) human monocytic cell line with the established J774 murine macrophage cell line to ascertain its effectiveness in determining the intracellular activities of antimycobacterial drugs. The MM6 cell line was maintained in RPMI 1640 containing 10% (vol/vol) fetal calf serum and other growth supplements.

Esther *et al.* (1998) studied the microsphere technology for targeted delivery of rifampin to *mycobacterium tuberculosis*-Infected Macrophages. They maintained MM6 cells in RPMI 1640 containing 10% (vol/vol) fetal calf serum and other growth supplements.

Serge *et al.* (1998) described a new compound, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid methyl ester (MX84), that was selectively activated in macrophages, leading to killing of only macrophage monocyte type cells *in-vitro*. They maintained RAW264.1 macrophage in RPMI 1640 medium with 10% fetal calf serum at a density of approximately 2 X 10^7 cells/ml.

Duman *et al.* (2003) studied the effects of rifampicin and fluoroquinolones on tubercle bacilli within human macrophages. The monocytic cells were propagated by washing and suspending to 1×10^7 cells/ml in RPMI-1640 (pH 7.2–7.3) culture medium containing serum. The cells were maintained throughout study using RPMI-1640 and serum.

Kalita *et al.* (2004) evaluated the combination of HNP-1, isoniazid, and rifampicin against *Mycobacterium tuberculosis* H37Rv *in vitro* on Murine

macrophage cell line J774.A1 obtained from National Centre for Cell Sciences (Pune). They maintained the cell line in RPMI 1640 medium supplemented with 5–10% fetal calf serum, 1 mg/mL Gentamicin and other growth supplements at 37^0C in 5% CO_2.

2. 2 Cytotoxic effect of nanoparticulate rifampicin and plain rifampicin on macrophage.

Mosmann (1983) developed a quantitative colorimetric assay using a tetrazolium salt MTT (3-[4,5- Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) for mammalian cell survival and proliferation. They reported that, MTT was cleaved by all living, metabolically active cells, but not by dead cells. They stated that the amount of formazan generated was directly proportional to the cell number over a wide range using a homogenous cell population.The signal generated was dependent on the degree of activation of the cells and the results could be read on a scanning spectrophotometer (ELISA reader). They reported that, the assay could be read a few minutes after the addition of acid-isopropanol, and the color was stable for a few hours at room temperature. They concluded that, the colorimetric MTT assay was used in wide range of applications for measuring survival/or proliferation of various cell lines.

Jagannath *et al.* (1995) evaluated poloxamer surfactant, CRL8131, for activity against *Mycobacterium tuberculosis.* CRL8131 was initially incubated at various doses with uninfected U937 cells. It showed cytotoxic effects of granularity, clumping, and loss of cell viability at doses of >25 µg/ml. No detectable sign of toxicity was observed at doses of below 10µg/ml of dose.

Esther *et al.* (1998) studied the microsphere technology for targeted delivery of Rifampin in *Mycobacterium tuberculosis*-infected macrophages. Release characteristics were examined *in-vitro and* also in two monocytic cell lines, the murine J774 and the human Mono Mac 6 cell lines. Cell viability was determined by means of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [thiazolyl blue]) cytotoxicity assay. Concentration of 0.25µg/ml of rifampin showed 91% cell viability, whereas 18ug/ml of microsphere showed 100% cell viability. They found that, delivery of rifampin

by means of microsphere formulations reduced the toxicity of rifampin for the human monocytic cell line MM6. Thus, they concluded that, not only can microsphere technology deliver rifampin more efficiently to host macrophages, it can deliver the drug at higher concentrations and with reduced toxicity.

Serge *et al.* (1998) described a new compound, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid methyl ester (MX84), that was selectively activated in macrophages, leading to killing of only macrophage monocyte type cells *in-vitro*. Either tRA or MX84 was applied at the desired concentrations (10211 to 1026 M) and following 6 days of incubation with the retinoids, MTT tests were performed for cell viability using a MTT kit. They found MX84 effectively killed macrophage/monocyte type cells.

Fischer *et al.* (2002) studied comparative *in-vitro* cytotoxicity of different water-soluble, cationic macromolecules which have been described as gene delivery systems. Cytotoxicity was performed in L929 mouse fibroblasts using the MTT assay. They allowed the following ranking of the polymers with regard to cytotoxicity results. Poly (ethylenimine) = poly (L - lysine) > poly (diallyl dimethyl ammonium chloride) > diethylaminoethyl dextran>poly (vinyl pyridinium bromide) >Starburst dendrimer>cationized albumin>native albumin. They concluded that, magnitude of the cytotoxic effects of all polymers were found to be time- and concentration dependent.

Takii *et al.* (2002) studied simple fibroblast-based assay for screening of new antimicrobial drugs against *Mycobacterium tuberculosis.* In their study, they proposed a simple and reproducible host-cell-based assay for the screening of antimycobacterial drugs that is suitable for drug discovery. The method evaluated both antimycobacterial activity of the drugs and their cytotoxicity to host cells. Human embryonic lung fibroblast cell line MRC-5. All of the detached cells were dead as determined by trypan blue dye exclusion. Results showed that, first-line antituberculosis drugs, such as isoniazide (INH-0.428 µg/ml), streptomycin (STR-1.816 µg/ml) and ethambutal (EMB-3.465 µg/ml), did not exhibit any cytotoxicity to host cells at the indicated concentrations.

Guggia *et al.* (2004) evaluated the toxicity profile of the resulting D-glucosamine-TBA (4-thiobutylamidine) conjugate, of chitosan-TBA conjugate *in-vitro*. Cytotoxity of 0.025, 0.25 and 0.5% solutions of the test compounds was evaluated on L-929 mouse fibroblast cells utilizing two different bioassays: the MTT assay (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide), which assesed the mitochondrial metabolic activity of the cells, and the BrdU-based enzyme-linked immunosorbent assay, which measures the incorporation in the DNA of 5-bromo-2'-deoxyuridine and consequently the cell proliferation. Data obtained by the MTT assay and the BrdU assay revealed a concentration dependent relative cytotoxicity for all tested compounds. They concluded that, TBA derivatives show a comparable toxicity profile to the corresponding unmodified compounds.

Tempone *et al.* (2004) verified the cytotoxicity of scavenger receptors (SR) ligands to peritoneal macrophages. Macrophages were seeded at 4 x 10^5/well (triplicate) in 96-well microplates and submitted to an incubation period of 48 h in the presence of cytochalasin B, chloroquine, dextran sulphate, LPS (5 µg/mL), polyinosinic acid and phosphatidylserine (150 nmol phospholipid/well) with macrophages for a period of 48 h to evaluate any cytotoxicity. Cytotoxicity was studied by using a MTT assay. They concluded that, the uptake of Sb-LP was reduced in infected macrophages, despite their effectiveness and targeting ability, suggesting a low metabolic rate in infected macrophages that could be overcome by the higher efficiency of the liposomal formulation.

Yoo and park (2004) studied folate-receptor-targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin–PEG–folate (doxorubicin–polyethylene glycol–folate) conjugate. MTT-based *in vitro* cytotoxicity assay was performed to compare anti-cancer effects of DOX nanoaggregates against KB and A549 cell lines. Various concentrations of DOX/FOL nanoaggregates, DOX nano-aggregates and free doxorubicin ranging from 1um, 10 µM,100µM was incubated for 48 hrs. At 10 µM concentration, cell viability for free DOX was 60 % whereas, for DOX nano-aggregates was 40 %. As the dose and incubation time increased viability was decreased. Free DOX showed less cytotoxicity against both cell lines

compared to DOX/FOL and DOX nano-aggregates, resulting from the reduced cellular uptake of DOX.

Lecaroz *et al.* (2006) studied the cytotoxicity of particles on THP-1(human myelomonocytic cells) monocytes by trypan Blue dye exclusion method and MTT assay. Viable cells were able to reduce MTT (3-[4,5- Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to coloured formazan serving as an indirect measurement of cell viability. PLGA micro- and nanoparticles containing gentamicin 1 mg/mL particle dispersion in RPMI complete medium were incubated with THP-1 for 24 h. Microspheres showed cell viabilities above 97%, and nanoparticles above 90%.

Loretz and schnurch (2006) carried out *in-vitro* evaluation of chitosan-EDTA conjugate polyplexes as a nanoparticulate gene delivery system. A lactate dehydrogenase (LDH) test was performed on Caco-2 cells with a cytotoxicity detection kit (LDH) . Cells were seeded at a density of 1×10^5 cells/mL in 12-well plates. Cytotoxicity on Caco-2 cells was investigated by testing membrane integrity via the release of intracellular LDH. LDH assay was done by incubating 260-µl nanoparticle suspension (10 µg pDNA) plus 740 µL MEM medium with Caco-2 cells for 2 and 4 hrs . The measured cytotoxicity for chitosan-EDTA particles was 0.5 %.

2.3 Internalization of nanoparticulate rifampicin

Haigler *et al.* (1978) studied binding and internalization of epidermal growth factor (EGF) in human carcinoma cells A-431. Cultures for fluorescence microscopy were prepared by plating the A-431 cells on glass cover slips at 6×10^4 cells per 35-mm dish. The binding of EGF to the surface of these cells was visualized by immunofluorescence using rabbit antibodies to EGF and rhodamine- labeled goat anti-rabbit antibodies. After binding of this derivative to cells, the cellular borders were prominently stained and the fluorescence on the remainder of the membrane was uniform. Upon warming of these cells to 37^0 for 10 min, the surface fluorescence diminished and

randomly distributed endocytotic vesicles appeared in the cytoplasm. After 20 min at 37^0 these fluorescent vesicles formed a perinuclear ring.

Steinberg *et al.* (1988) studied the intracellular transport of Lucifer Yellow in J774 macrophages and the nature of the cytoplasmic vacuoles into which this dye was sequestered. When the lysosomal system of J774 cells was prelabeled with a Texas red ovalbumin conjugate and Lucifer Yellow was then loaded into the cytoplasm of the cells by ATP-mediated permeabilization of the plasma membrane, the vacuoles that sequestered Lucifer Yellow after 30 min later were distinct from the Texas red-stained lysosomes. After an additional 30 min Lucifer Yellow and Texas red co-localized in the same membrane bound compartments, indicating that the Lucifer Yellow had been delivered to lysosomes. Cells viewed by fluorescence microscopy at this point demonstrated intense Texas red fluorescence at their plasma membrane. Control cells incubated with Texas red protein A alone did not fluoresce.

Arttamangkul *et al.* (2000) studied binding and internalization of fluorescent opioid peptide conjugates in living cells. Dermorphin (DERM), deltorphin (DELT), TIPP and endomorphin were conjugated to BODIPY TR iodoacetamide and Alexa Fluor 488 maleimide, two fluorescent dyes with distinct hydrophobic properties. They demonstrated that, these novel, fluorescent opioid peptide conjugates permit real-time visual tracking of receptor-ligand complexes, including their internalization and trafficking, in living cells.

Tempone *et al.* (2004) devised liposome-entrapped antimony with the negatively charged lipid phosphatidylserine—liposome-entrapped antimony (Sb-LP)—in order to improve their targeting to infected macrophages through the interaction with scavenger receptors (SRs). Uptake and internalization of [^{3}H] liposome-entrapped antimony (150 nmol phospholipid/well) by naive and *Leishmania*-infected macrophages was studied at 4℃ for 8 h. The uptake ratio expressed in percentage was determined by the intracellular radioactivity found in the control group (uninfected) measured in a liquid scintillation counter. A 45% decrease in the uptake of liposomes was observed compared with the control.

Yoo (2004) investigated selective cellular uptake of DOX/FOL nano-aggregates via folate-receptor-mediated endocytosis. KB cells and A549 cells were incubated with DOX/FOL nano-aggregates for 3 hrs in the presence or absence of folic acid in the culture medium. In order to visualize cellular uptake of doxorubicin nanoaggregates by cancer cells, the cells were examined by a confocal microscopy. Results showed that, the endocytic delivery of DOX within cells by using nanoparticulates maintains the intracellular DOX concentration to be high in the cytoplasm region. Cellular uptake extent of DOX/FOL nano-aggregates was significantly higher than those of free DOX and DOX nano-aggregates under the same condition.

Lecaroz *et al.* (2006) studied microspheres of 502H and 75:25H polymers and nanoparticles due to their lower cytotoxicity and higher drug loading compared with nanospheres of PLGA 502H. Microspheres showed cell viabilities above 97%, and nanoparticles above 90%. Microparticles of both polymers were successfully phagocytosed and no degradation was observed 24 h post-incubation.

Schrand *et al* (2008) examined the chemical and biological properties of Ag nanoparticles of similar sizes, but that differed primarily in their surface chemistry (hydrocarbon versus polysaccharide), in neuroblastoma cells for their potential use as biological labels. They observed strong optical labeling of the cells in a high illumination light microscopy system after 24 h of incubation. The internalization and localization of the Ag nanoparticles into intracellular vacuoles in thin cell sections with transmission electron microscopy were observed.

Gaikwad (2007) studied *in-vitro* effect of nanoparticulate drug delivery system with new anti tuberculosis drug. He studied the internalization of lipomer tagged with coumarine dye inside human macrophages. He observed that the internalization was appreciable within half an hour at 5 µg/ml.

2.4 Minimum inhibitory concentration (MIC) of nanoparticulate rifampicin and plain rifampicin:

Stottmeier *et al.* (1969) evaluated antimycobacterial activity of rifampin under *in-vitro* and simulated *in-vivo* conditions. Minimal inhibitory concentrations of rifampin for 62 strains of mycobacteria was determined in 7H-10 agar medium and Lowenstein-Jensen egg medium. The MIC of rifampin for *M. tuberculosis* was determined in both 7H-10 agar medium and Lowenstein- Jensen (LJ) egg medium. All strains were inhibited by 0.05 to 0.2 µg of rifampin per ml of 7H-10 medium or by 0.5 to 2.0 µg/ml of rifampin per ml of LJ medium, indicating a 90% inactivation of the drug in egg medium.

Heifets *et al.* (1986) determined the MICs of ethambutol for both *Mycobacterium avium* and *Mycobacterium tuberculosis* strains by using broth dilution (7H12 broth, radiometric method) and agar dilution (7H11 agar) methods. They found the MICs to be much lower in liquid than in solid medium. They proposed that the MICs, determined radiometrically in 7H12 broth, be considered as tentative criteria for susceptibility testing of *M. avium* isolates in future clinical trials. Ethambutol produced bactericidal effects against both *M. tuberculosis* and *M. avium*, and the MIC/MBC ratios were in the same range for both species when MICs and MBCs were tested in 7H12 broth by conventional sampling and plating.

Dhopale (1993) studied rapid susceptibility testing of *Mycobacterium tuberculosis* isolated from AIDS. They proposed to develop ATP assay technique to determine sensitivity of antibacterial compounds against Mycobacterium avium complex (MAC) and *M. tuberculosis*. Effect of antimycobacterial drugs was tested by using colony forming assay and ATP assay. Rifampicin at various concentrations of 0.005, 0.05, 0.5, 5, 10µg/ml were added to 7H9 broth and inoculated with *M. tuberculosis*. Aliquots were removed after 20 days for colony counts and ATP assays. They found 88 % reduction at concentration of 10 µg/ml.

Ohno *et al.* (1996) studied relationship between Rifampin (RFP) MICs for and *rpoB* Mutations of *Mycobacterium tuberculosis* strains isolated in Japan.

They analyzed the relationship between rifampin MICs and *rpoB* mutations of 40 clinical isolates of *Mycobacterium tuberculosis*. RFP susceptibility testing of all strains was performed by the broth microdilution method with Middlebrook 7H9 broth containing ADC supplement. Finally, 100 ml of the twofold concentrated bacterial suspensions containing $5X10^6$ CFU was inoculated into each well, with a final RFP concentration ranging from 0.063 to 512 mg/ml. The growth in RFP-free wells was observed after 7 or 10 days of incubation at 37^0C, and the MIC represented the lowest concentration of RFP that completely inhibited bacterial growth and showed a negative result in the nitrate reduction test. All 13 strains requiring MICs of RFP >64 mg/ml contained a point mutation in either codon 516, 526, or 531, while all 21 strains requiring MICs of RFP <1 mg/ml showed no alterations in these codons.

Heifets *et al.* (1999) developed rifapentine susceptibility tests for *Mycobacterium tuberculosis.* They used the agar proportion method and the radiometric BACTEC technique for testing the susceptibility of *Mycobacterium tuberculosis* to rifapentine. A critical concentration of 0.5 µg of rifapentine per ml was proposed for both methods The actual MICs of RPT (rifapentine) and RMP(rifampicin) for the resistant QC strain were 32.0 µg/ml, but in the reproducibility study they limited for practical purposes the range of concentrations to 8.0 µg/ml.

Sato and Tamioka (1999) studied antimicrobial activities of benzoxazinorifamycin (KRM-1648) and clarithromycin against *Mycobacterium avium–intracellulare* complex within murine peritoneal macrophages, human macrophage-like cells and human alveolar epithelial cells. MICs of test drugs were determined by either an agar dilution method using Middlebrook 7H11 medium or a broth dilution method using 7HSF medium (a broth medium with the same composition as 7H11 agar but without malachite green). After MIC determinations using 7HSF medium, MBCs were determined by inoculating 10 mL samples from wells in which test agents allowed no visible growth of the organisms, on to a 7H11 agar plate, followed by 14 day cultivation. MBCs were read as minimum concentrations of drugs causing 99.9% killing of the

inoculated organisms. The MIC for the parent *M. intracellulare*N-260 strain was 0.1 mg/L.

Palicova *et al.* (2000) studied susceptibility testing of *Mycobacterium tuberculosis* to anti-tuberculosis drugs such as rifampin (RMP) and isoniazid (INH). They found 28 strains susceptible (S), 10 strains resistant (R) and MIC of RIF for these strains was between 1-2 µg/ml.

Hwang *et al.* (2002) studied characterization of rifampicin-resistant *Mycobacterium tuberculosis* in Taiwan. To determine the MIC of rifampicin of each isolate, serial twofold dilutions of rifampicin were incorporated in 7H10 agar at concentrations that ranged from 0 to 256 µg ml^{-1}. Sets of quadrant Petri dishes (one quadrant in each plate contained drug-free medium) were inoculated with each isolate. Each plate was checked weekly and results were recorded after 3 and 4 weeks. The MIC was defined as the lowest concentration of drug that inhibited growth of the bacterial population by more than 99 %. The results revealed that, the mean MIC was 92.38 µg ml^{-1} for the 53 isolates with a mutation in the core region, whereas the mean MIC of the other 10 isolates without mutations was only 24.8 µg/ml^{-1}.

Duman *et al.* (2003) The effects of rifampicin and fluoroquinolones on tubercle bacilli within human macrophages. The objective of this study was to determine the activities of rifampicin, ciprofloxacin and sparfloxacin against *M. tuberculosis* H37Rv in human macrophages. The minimal inhibitory concentrations (MIC) were determined by agar macrodilution and broth microdilution methods and were 0.5, 1 and 0.125 mg/l, respectively. The minimal inhibitory concentrations (MIC) of rifampicin against *M. tuberculosis* H37Rv were 0.5 mg/l.

Coban *et al.* (2004) evaluated the broth microdilution method (BMM) for susceptibility testing of *Mycobacterium tuberculosis.* A total of 43 clinical isolates of *M. tuberculosis* and H37Rv as a control strain were studied. All isolates were tested by the proportion method and the BMM for isoniazid (INH), rifampicin (RIF), streptomycin (STR), and ethambutol (ETM). Antimycobacterial drugs were adjusted in the LJ medium to final concentrations of 0.2-1 µg/ml for isoniazid (INH), 1 µg/ml for rifampicin

(RIF).19 strains were resistant and 15 strains were succeptible to RIF at 1 µg/ml.

Deepa *et al.* (2005) studied detection and characterization of mutations in rifampicin resistant *Mycobacterium tuberculosis* clinical isolates. The drug susceptibility testing was carried out by the conventional absolute concentration method, which uses a standardized inoculum grown on drug free media and media containing graded concentrations of drug. Concentrations ranging from 4 ug/ml to 128ug/ml were tested. Of the 44 strains tested, 39 were sensitive to Rifampicin with a minimum inhibitory concentration of lesser than or equal to 5 µg/ml

Traore *et al.* (2007) developed low-cost rapid detection method of rifampicin resistant tuberculosis using bacteriophage. Minimal inhibitory concentrations (MIC) were determined by MTT assay. This assay is based on detection of bacterial growth by a redox dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) . A change in colour from yellow to violet indicated growth of bacteria and the MIC was interpreted as the lowest concentration that inhibited bacterial growth. The MIC of each isolate was found to be greater than 50 µg/ml compared to 2 µg/ml for the reference susceptible isolate (H37Rv) and all four isolates were judged to be truly resistant.

Raut *et al.* (2007) evaluated rapid MTT tube method for detection of drug susceptibility of *Mycobacterium tuberculosis* to rifampicin & isoniazid. Method utilizes the ability of viable mycobacterial cells to reduce MTT 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide. The method was standardized with known resistant and sensitive strains of *M. tuberculosis* and was then extended to 50 clinical isolates. Results were read visually and by colorimeter at 570nm. Results showed that, by proportion method, out of 50 strains tested, 38 were RIF resistant and 12 were RIF (1 µg/ml) sensitive.

Venugopal *et al.* (2007) determined minimum inhibitory concentration (MIC) of various anti-tuberculosis drugs for *Mycobacterium avium* complex (MAC) strains isolated from clinical samples by using both by agar dilution and MIC method. The stock suspensions of the drugs were prepared in

Middlebrook 7H9 medium. Two-fold serial dilutions of the drugs were prepared directly in the microtitre plates so as to get final concentration of Rifampicin(0.03-0.4 mg/ml). The plates were inoculated with 5mL of inoculum in each well except the blanks.MIC values were analysed, both visually and by enzyme-linked reader at 7^{th} and 14^{th} day. They found 14 strains succeptible to RFP (0.03-0.4 mg/ml). Results of the study confirm the suitability of the rapid broth micro dilution (MIC) method as a simple yet reliable method to assay for the drug susceptibility of nontuberculosis mycobacterium.

2. 4 Infection of Human macrophage cell line *Mycobacterium tuberculosis* culture

Alblas *et al.* (1979) studied origin and kinetics of pulmonary macrophages during an inflammatory reaction induced by intravenous administration of heat-killed bacillus calmette-guerin. The pulmonary macrophages were isolated from lung and cells that had phagocytosed BCG were examined by Ziehl-Neelsen (ZN) staining for acid-fast microorganisms.. They observed intracellular acid fast bacilli by Z-N staining.

Jagannath *et al.* (1995) studied poloxamer surfactant, CRL8131 activity against *Mycobacterium tuberculosis* with antibiotics in a macrophage cell line assay. For infection studies, 10^8 no. of U937 cells were mixed with a sonicated suspension of *M. tuberculosis* containing 10^6 CFU (in 0.1 ml) in 5 ml of assay medium. Phagocytosis was allowed to occur during gentle mixing at 37^0C, and cells were washed six times with assay medium. The cells were then diluted to 10^6 cells per ml and plated out at 1 ml per well of a 24-well Costar plate. They found that, infection on day 0 of about 25% of macrophages was observed with 10^3 CFU per 10^6 macrophages. At day 7, 50 to 70% of the cells became infected, yielding 10^5 to 10^6 CFU per 10^6 macrophages. Microscopic examination of acid-fast stained infected macrophages on day 0 and serially for 7 days did not show any extracellular bacilli.

Bange *et al.* (1996) showed that, a BCG leucine auxotroph with a transposon disruption of the *leuD* gene was unable to grow in mice. Bacteria

and THP-1 cells were washed separately in RPMI 1640 with 10% FCS. Then, 10^6 THP-1 cells were infected with a multiplicity of infection between 20 and 50 bacteria per cell and activated with 15 ng of phorbol 12-myristate 13-acetate (PMA). After adsorption for 6 h at 37^0C and 5% CO_2, non internalized bacteria were separated from macrophages by low-speed centrifugation at 500 X *g*. Cells were washed and vortexed vigorously four times with cold medium and resuspended in medium supplemented with 10% FCS and 15 ng of PMA. On glass coverslips pretreated with acetone and fitted to a 12-well plate cells were allowed to adhere overnight. At different time points, cells on the coverslips were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin and finally stained with auramine and rhodamine stains. The infection was evaluated by using fluorescence microscopy. Intracellular growth of the BCG leucine auxotroph (mc2798) was less as compared to the Intracellular growth of the complemented mutant [mc2798(*pleuCD*) in PMA-activated THP-1 macrophages. At 4 days, 2 Mean AFB(acid-fast bacilli) of the BCG leucine auxotroph (mc2798) and 7 Mean AFB of the complemented mutant [mc2798(*pleuCD)* in PMA-activated THP-1 macrophages was observed. The staining was used to evaluate the bacillary growth inside macrophages at different time intervals.

Khanna *et al.* (1996) infected alveolar macrophages (AMs) by nonopsonized *Mycobacterium bovis.* Alveolar macrophages were incubated overnight at 37° C, under 5% CO_2 to reach quiescence and *Mycobacterium bovis* was added at a bacillus-to macrophage ratio of 10:1. At 18 h after infection, cells were washed five times with warm HBSS supplemented with 0.02% Tween 80 and then fixed with 4% paraformaldehyde. The technique used for determining uptake of *Mycobacterium bovis* was auramine-rhodamine staining of the cell-associated bacteria, using an auramine rhodamine staining kit. Bacilli were counted using a fluorescence microscope, and results were expressed as the percentage of infected AMs by counting at least 300 cells per treatment group.

Mehta *et al.* (1996) compared *in-vitro* models for the study of *Mycobacterium tuberculosis* invasion and intracellular replication. They infected human alveolar pneumocyte epithelial cell line, a murine macrophage

cell line (J774), and fresh human peripheral blood-derived macrophages *M. tuberculosis*. Their data indicated that the initial level of association of *M. tuberculosis* with human alveolar pneumocyte cells (2%) was less than that observed with fresh human peripheral blood macrophages (9%) or J774 murine macrophages (13%) within 6 h of the addition of the bacteria. *M. tuberculosis* replicated in association with the pneumocyte cells by more than 55-fold by day 7 post infection. Whereas, total bacterial growth in the J774 cells and human macrophages was considerably less, with increase of only fourfold and threefold, respectively, over the same 7-day period.

Reddy *et al.* (1996) studied antituberculosis activities of clofazimine (CFM) and its new analogs B4154 and B4157. Adherent monolayer of Macrophages of density 10^6 cells per well were infected with bacterial suspension adjusted to no. 1 McFarland standard (about 10^7 CFU/ml), and incubated for 2 h at 37^0C in a CO_2 incubator.

Zhang *et al.* (1997) evaluated the intracellular growth of *M. tuberculosis* strains in macrophages under conditions similar to those encountered *in-vivo*, They infected human monocyte-derived macrophages with H37Ra, H37Rv, or one of four isolates from tuberculosis patients at a low bacillus-to-macrophage ratio i.e at 10^4 bacilli/well. Based on 50% bacterial viability and an estimated 4×10^5 cells/well, the estimated multiplicity of infection was one live *M. tuberculosis* organism per 80 cells. They found that, H37Rv and the patient isolates grew significantly faster than H37Ra, based on the numbers of CFU and acid-fast bacilli.

Birkness *et al.* (1999) studied an *in-vitro* tissue culture bilayer model to examine early events in *Mycobacterium tuberculosis* infection. Infected 5×10^6 PBMCs (peripheral blood mononuclear cells) with *Mycobacterium tuberculosis* and incubated at 37℃ in 5% CO_2 for 4, 24, or 48 h. The majority of bacteria were associated with the host cells and membrane after 48 h, ranging from 7×10^6 CFU for *M. bovis* BCG with PBMCs to 5×10^7 CFU for *M. tuberculosis* Erdman without PBMCs.

Esther *et al.* (1998) infected MM6 cells of density 8 $X10^5$ cells per ml, with mycobacteria so as to achieve a final ratio of 20 mycobacteria per macrophage, with a density of $4X10^5$ MM6 cells per 1.0 ml per well. After infection for 4 h, the infected MM6 cells were collected by centrifugation (200 X*g*) and washed twice with DPBS to remove unphagocytosed mycobacteria.

Fratazzi *et al.* (2000) studied the role of CD43 (leukosialin/sialophorin), the negatively charged sialoglycoprotein of leukocytes, in the binding of mycobacteria to host cells. In this study, monolayers of adherent macrophages were prepared by plating 2 x 10^6 murine spleen cells on 13-mm-diameter plastic coverslips; these were incubated with *M. avium*, harvested ,washed and fixed with 4% paraformaldehyde in PBS. The mycobacteria in the fixed monolayers were stained with the rhodamine-auramine TB Fluorescent stain kit. The stained cells were examined by phase–contrast and fluorescent microscopy. They found that, when incubated at a 20:1 ratio, fluorescent micrographs showed multiple mycobacteria associated with all or most of the CD43+/+ macrophages and negligible association of mycobacteria with CD43-/ macrophages.

Sharma *et al.* (2000) standardized the optimum ratio of macrophage: mycobacteria. Phagocytosis was performed by the radiometric method. Infection of macrophages with radiolabelled *M. tuberculosis* H37Rv suspended in plain RPMI-1640 medium at different ratios of macrophages : mycobacteria was carried out. Plates were kept at 37 ^{0}C in 5% CO_2 for 2 hours. It was observed that with an increase in the ratio of macrophages: mycobacteria from 1:1 to 1:100, there was an increase in the percentage of phagocytos is from 14.44$\pm$0.32% to 31.01$\pm$0.38% respectively. The optimum ratio was found to be 1:10 resulting in 23.68$\pm$0.11% phagocytosis. Acid fast staining revealed that 60-80% macrophages contained 2-3 bacilli per macrophage.

Takii *et al.* (2002) studied simple fibroblast-based assay for screening of new antimicrobial drugs against *Mycobacterium tuberculosis.* Antimycobacterial effect of INH on internalized bacilli was also observed at 24

h after infection These results showed that, phagocytosis occurred even at 48 h and there was almost no multiplication of bacilli at the indicated time.

Duman *et al.* (2003) observed effects of rifampicin and fluoroquinolones on tubercle bacilli within human macrophages. They infected monocytic cells of density 1×10^7 cells/ml with mycobacterial suspension diluted to a 0.5 Mac-Farland with Middlebrook 7H9 medium so as to obtain about 5×10^6 bacilli/ml After incubation for 40 min, the plates were washed twice to remove the extracellular bacteria.

Ghosh *et al.* (2004) infected macrophages with *M. tuberculosis and* pre-stained with DiLC18, a fluorescent dye which binds the bacterial cells irreversibly. Macrophages with or without fluorescent *M. tuberculosis* could be gated on a flow cytometer and expression of Mac-1 and ICAM-1 populations could be studied on both populations of cells. Results showed that, about 64% macrophages had taken up *M. tuberculosis* 8 h post infection. By 48 h more than 95% macrophages were positive for DilC18 stained *M. tuberculosis.*

Sander *et al.* (2004) infected mouse macrophage cell line with *M. tuberculosis.* Aggregates of bacteria were removed by low speed centrifugation (5 min at 20 *g*). An aliquot from the top representing a suspension of single bacteria was withdrawn and used to infect host cells at a multiplicity of infection (MOI) of one bacterium per macrophage. At 1 h after infection, the overlay medium was removed, and the numbers of intracellular bacteria were determined by plating after lysis of host cells. Growth of intracellular bacteria was followed for 7 days.

Mehta *et al.* (2006) examined attachment, internalization, and intracellular replication of *M. tuberculosis* bacilli in an immortalized human lung microvascular endothelial cell line (HULEC). By 6 h post-infection, 12% of infecting bacilli were associated with the HULEC monolayer cells. Using electron microscopy, large numbers of bacilli were visible in the vacuoles of HULEC cells after 48 h post-infection. These *in-vitro* findings support the hypothesis that lung endothelial cells have the potential to participate in *in-vivo* lung infections.

Torrado *et al.* (2007) infected The J774A.1 mouse macrophage cell line of density 2 x 10^5 cells/well with *M. tuberculosis* H37Rv and *M. bovis* with multiplicity of infection (MOI) of 1:1 and incubated at 37°C for 4 hrs. They found that, phagocytosis index of *M. tuberculosis* H37Rv and *M. bovis* BCG by macrophage murine cell line J774A.1 was found to be 30.3% ± 11.4% and 24.9% ± 5.7%, respectively.

2.6 Efficacy of nanoparticulate rifampicin and plain rifampicin

Jagannath *et al.* (1995) infected 10^8 U937 cells by mixing with a sonicated suspension of *M. tuberculosis* containing 10^6 CFU (in 0.1 ml). Addition of Rifampicin at concentration of 0.15 µg/ml, 0.3µg/ml, 0.6 µg/ml,1.25µg/ml 2.5 µg/ml and 5µg/ml in triplicate for each concentrarion was done and incubated at 37^0C in 5% CO_2. Aliquots of macrophages aspirated from wells on day 0 were used for determination of baseline CFU counts. Infected macrophages in drug-free wells of incubated plates served as controls for growth of *M. tuberculosis* over 7 days. On day 7, macrophages from individual wells were collected and pelleted. Lysates were prepared by the addition of 0.5 ml of sterile 0.25% sodium dodecyl sulfate Lysates were diluted in sterile saline,10-fold dilutions were plated on 7H11 agar, and CFU counts were determined after 4 to 6 weeks of incubation of the plates at 37^0C. They found a significant decrease in cfu i.e from 3 X 10^5 in control to 1.5 X 10^5(0.6 µg/ml of rifampicin) and at 1.2µg/ml, cfu was 10^5. There was total inhibition at concentrations of 2.5 µg/ml and 5ug/ml.

Mor and Esfandiari (1997) studied synergistic activities of clarithromycin (CLA) and pyrazinamide (PZA) against *Mycobacterium tuberculosis* in Human Macrophages. The macrophages were incubated with an *M. tuberculosis* suspension containing 2 X10^6 CFU/ml in RPMI-1640. The ratio of macrophages to bacteria was 1:10 and incubation was carried for 1 h. After incubation, the plates were washed three times to remove the extracellular bacilli. The infected macrophages were then incubated in RPMI-1640 supplemented with 1% human serum and various concentrations of the drugs i.e CLA (32.0µg/ml), PZA (64.0µg/ml), RMP (0.25µg/ml). Infected macrophage culture plates were sampled immediately after infection (time 0) and 4 and 8 days after infection to ascertain the numbers of viable bacilli. The

macrophages were lysed, and the numbers of CFU were determined from the lysate. The RMP at concentration 0.25 µg/ml caused total inhibition in growth of H37Rv.

Esther *et al.* (1998) infected MM6 cells of density 8 $X10^5$ cells per/ml with mycobacteria to achieve a final ratio of 20 mycobacteria per macrophage. After infection for 4 h, the infected MM6 cells were collected by centrifugation (200 *g*) and washed twice with DPBS to remove unphagocytosed mycobacteria. Following infections of monocytic cell lines with *M. tuberculosis* H37Rv, cells were treated with rifampin added either directly to culture medium or by means of drug-loaded microspheres. Infected cells were treated with rifampin concentrations equal to the MIC (i.e., 0.25 mg of rifampin/ml) and to 3X and 8X MICs (i.e., 0.75 and 2.0 mg of rifampin/ml, respectively). The total amounts of microspheres added in these experiments were 18, 54, and 143 mg/ml, for MIC, 3X MIC, and 8X MIC, respectively. Determination of CFU at zero hour was conducted by first lysing the monocytes with 0.25% SDS in DPBS and then plating serial dilutions onto 7H10 agar plates The plating procedure was repeated at 7 days, and numbers of CFU were enumerated after 10 to 14 days of incubation of cultures. There was decrease in CFU from 10^5 to total inhibition with 0.75 and 2.0 mg of rifampin/ml, and with the MM6 cell line, a significant reduction in numbers of CFU was observed for all concentrations of free rifampin as well as equivalent concentrations of rifampin-loaded microspheres MIC, 3X MIC, and 8X MIC, respectively.

Tomioka *et al.* (2002) studied that intramacrophage passage of *Mycobacterium tuberculosis* and *M. avium* complex alters the drug susceptibilities of the organisms. MM6 macrophages cultured and were infected with *M. tuberculosis* and incubated at 37℃ in a CO_2 incubator for 4 h. They incubated infected cells (4 $X10^4$) in the presence or absence of test drugs at the concentration of rifalazil (RLZ -0.05µg/ml) clarithromycin (CLR 2.3µg/ml; levofloxacin (LVX-2.0µg/ml) At intervals of 0, 5 and 7 days, the cells were lysed with 0.07% sodium dodecyl sulfate, and released organisms were collected and washed with distilled water by centrifugation (2,000 *g*, 30 min). The CFU of recovered organisms were counted on 7H11 agar plates. At

intervals, a CFU counting assay was done. Results showed that, RLZ and LVX exhibited CFU-reducing activity, and CLR caused growth inhibition of both types of *M.tuberculosis*.

Duman *et al.* (2003) studied the effects of rifampicin and fluoroquinolones on tubercle bacilli within human macrophages. They infected macrophages with *M. tuberculosis* H37Rv. After incubation for 40 min, the plates were washed twice to remove the extracellular bacteria. The infected macrophages were incubated in RPMI-1640 and 1.5% unheated autologous serum containing different antibiotic concentrations (Rifampicin 0.5 and 2.5 mg/l) and a control with no antibiotic. On days 4 and 7, macrophages on the culture plates were lysed using 0.25% sodium dodecyl sulphate prepared in PBS. The macrophage lysate was plated on the Middlebrook 7H10 agar containing petri dishes. These plates were incubated for 14 days at 37 ℃. The results were reported as mean colony forming units (CFU). Rifampicin was found to be effective against *M. tuberculosis* H37Rv at 0.5 and 2.5 mg/l concentrations which inhibited the growth of *M. tuberculosis* H37Rv.

Pandey *et al.* (2003) studied nanoparticle encapsulated antitubercular drugs as a potential oral drug delivery system against murine tuberculosis. They demonstrated that the nanoparticles provided sustained release of the antituberculosis drugs and considerably enhanced their efficacy after oral administration. Three frontline drugs, rifampin (RMP), isoniazid (INH), and pyrazinamide (PZA) were co-encapsulated in poly(lactide-co-glycolide) (PLG) nanoparticles. Following a single oral administration of this formulation to mice, the drugs could be detected in the circulation for four days (RMP) and nine days (INH and PZA); therapeutic concentrations in the tissues were maintained for 9-11 days. In contrast, free (unbound) drugs were cleared from the plasma within 12-24 h after administration. Treatment of *M. tuberculosis*-infected mice with the nanoparticle-bound drugs (five oral doses every 10^{th} day) resulted in complete bacterial clearance from the organs. Free drugs were able to produce bacterial clearance only after daily administration of 46 doses.

Skidan *et al.* (2003) studied enhanced activity of rifampicin loaded with polybutyl cyanoacrylate nanoparticles in relation to intracellularly localized

bacteria. *In-vitro* nanoparticle-loaded rifampicin was more active against *Staphylococcus aureus* and *Mycobacterium avium*, localized in isolated alveolar macrophages. Level of rifampicin in macrophages increased 2-3-fold after incubation with rifampicin loaded nanoparticles comparing to the free drug. Use of nanoparticles provided 2-fold increase in rifampicin efficacy, comparing with the free drug at treatment of staphylococcus sepsis in mice. Single administration of nanoparticulate rifampicin in the dose 25 mg/kg resulted in 80% survival of mice with salmonellosis, while 50 mg/kg of free rifampicin could provide only 10% survival.

Kalita *et al.* (2004) evaluated role of human neutrophil peptide–1 as a possible adjunct to antituberculosis chemotherapy. The effect of HNP-1, isoniazid, and rifampicin was evaluated against intracellular *M. tuberculosis* H37Rv by use of the murine macrophage cell line J774. Macrophage cells of density $2X10^6$ cells/mL/well was infected with *M. tuberculosis* H37Rv in the ratio of 1:10 and incubated for 2 h. The infected monolayer was washed 3 times with RPMI 1640 medium, to remove nonphagocytosed mycobacteria. The infected monolayers were then treated with HNP- 1 and anti-TB drugs at different concentrations (10–40 mg/mL HNP-1 and 5–25 mg/mL each anti-TB drug) for 3 days. At the end of the treatment period, infected monolayer cells were lysed with 500 uL of chilled 0.25% SDS. Lysates were approximately diluted and plated in solidified Youman's medium supplemented with 1% BSA. After incubation for 4–6 weeks, the number of colony forming units was counted in control and test plates, to determine the MICs of HNP-1, rifampicin, and isoniazid. Isoniazid, rifampicin, and HNP-1, when used individually, showed a significant reductions in colony-forming units, compared with control cells. A significant decrease in the viability of *M. tuberculosis* H37Rv (2.69 ± 0.08 log cfu) was observed in the presence of the combination of anti-TB drugs and HNP-1, compared with cells treated with anti-TB drugs only (isoniazid + rifampicin, 3.82 ± 0.05 log cfu), even at 1/4 MICs.

Sharma *et al.* (2004) studied lectin-functionalized poly (lactide-co-glycolide) nanoparticles as oral/aerosolized antitubercular drug carriers for treatment of tuberculosis. They studied the efficacy of PLG-based

formulations of antituberculosis drugs which was improved by covalent attachment of wheat germ agglutinin (WGA). Oral administration of WGA-coated PLG-NPs loaded with RIF, INH, and PZA in mice produced considerably extended serum half-life. Detectable RIF serum levels were observed for 6-7 days and INH and PZA for 13-14 days (versus 4-6 days and 8-9 days for non-modified nanoparticles). All three drugs were present in lungs, liver and spleen for 15 days. The lectin modified formulations produced bacterial clearance in these organs after three oral doses administered every 14 days (versus 45 daily doses of free drugs). They suggested the prolonged circulation of drugs encapsulated in WGA-grafted nanoparticles might be attributed to the fact that lectins enhance prolonged adhesion of the particles to the intestinal surface to allow an increase in the time interval available for absorption, and a localized increase in the concentration gradient between luminal and serosal sides of the membrane.

Vyas *et al.* (2004) designed liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. Their study was aimed at preparation, characterization, and performance evaluation of rifampicin-loaded aerosolized liposomes for their selective presentation to alveolar macrophages, that being the most dense site of tuberculosis infection. They observed that, percent viability of *Mycobacterium smegmatis* inside macrophages after administration of drug was in the range of 7-11% in the case of ligand-anchored liposomal aerosols, while it was recorded to be 45.7 and 31.6% in case of plain drug. Their results suggest that the ligand-anchored liposomal aerosols are not only effective in rapid attainment of high-drug concentration in lung with high population of alveolar macrophages but also maintain the same over prolonged period of time.

Wang *et al.* (2005) studied nanoparticulate formulation of rTS mimetic OHL improves drug stability and anticancer efficacy. They studied the *in-vitro* drug activity of 3-oxododecanoyl homoserine lactone (3-oxo-C12-HSL, or OHL) nanoparticles and a solution of OHL in DMSO, either as single agent or in combination with 5 fluorouracil, or paclitaxel, in several cancer cell lines (colon H630 and HT29, prostate PC3, and pancreatic MiaPaCa-2), using the MTT assay. Formulation of OHL in nanoparticles prolonged the half-life of OHL in cell culture medium from 1.9 hr to 3.5 hr. *In-vitro* drug activity assay

showed that formulation of OHL in nanoparticles enhanced its antitumor activity as single agent or in combination with 5-FU or paclitaxel.

Diane (2006) evaluated an intracellular pharmacokinetic in vitro infection model as a tool to assess tuberculosis therapy . *In-vitro* intracellular infection models have been used to evaluate drug therapy against *Mycobacterium tuberculosis*. This study demonstrated the intracellular and extracellular killing activity of antimycobacterial drugs in a pharmacokinetic intracellular in vitro model. The intracellular organism counts were reported as CFU/mL of cell suspension and the extracellular organism counts were reported as CFU/mL of fluid. Rifampicin was the most active drug both against extracellular and intracellular organisms and was mycobactericidal within 3 days and 5 days, respectively. Neither isoniazid nor levofloxacin achieved 99.9% killing activity against intracellular or extracellular organisms; however, both were trending towards mycobactericidal activity.

Zahoor *et al.* (2006) studied alginate nanoparticles as antituberculosis drug carriers. Alginate (a natural polymer) based nanoparticulate delivery system was developed for frontline ATDs (rifampicin, isoniazid, pyrazinamide and ethambutol). They observed that a single oral dose resulted in therapeutic drug concentrations in the plasma for 7-11 days and in the organs (lungs, liver and spleen) for 15 days. In comparison to free drugs (which were cleared from plasma/organs within 12-24 h), there was a significant enhancement in the relative bioavailability of encapsulated drugs. In TB-infected mice three oral doses of the formulation spaced 15 days apart resulted in complete bacterial clearance from the organs, compared to 45 conventional doses of orally administered free drugs.

Devalapally *et al.* (2007) evaluated paclitaxel and ceramide co-administration in biodegradable polymeric nanoparticulate delivery system to overcome drug resistance in ovarian cancer. The study was done to overcome drug resistance upon systemic administration of combination paclitaxel (PTX) and the apoptotic signaling molecule C_6-ceramide (CER) in biodegradable poly(ethylene oxide)-modified poly(epsilon-caprolactone (PEO-PCL) nanoparticles. Subcutaneous sensitive (wild-type) and multidrug resistant (*MDR-1* positive) SKOV-3 human ovarian adenocarcinoma

xenografts were established in female *Nu/Nu* mice. PTX and CER were administered intravenously either as a single agent or in combination in aqueous solution and in PEO-PCL nanoparticles to the tumor-bearing mice. There was significant tumor growth suppression in both wild-type SKOV-3 and multidrug resistant SKOV-3_{TR} models upon single dose co-administration of PTX (20 mg/kg) and CER (100 mg/kg) in nanoparticle formulations as compared to the individual agents and administration in aqueous solutions.

Jurriaan *et al.* (2007) studied targeted drug delivery to enhance efficacy and shorten treatment duration in disseminated *Mycobacterium avium* infection in mice. They studied the effect of the addition of targeted delivery of amikacin using stabilized liposomes to the infected tissues in the initial phase of treatment of *Mycobacterium avium* infection in mice. They found that, treatment with clarithromycin alone daily (6 days a week) slowly killed most of the mycobacteria in the lung, liver, spleen, inguinal and mesenterial lymph nodes. However, after 24 weeks of treatment, persistence of substantial numbers of mycobacteria in the infected organs was observed. The addition of ethambutol to the clarithromycin regimen did not significantly enhance the efficacy of treatment, neither did rifampicin as a third agent. In contrast, the addition of liposomal amikacin in the initial phase of therapy resulted in rapid and complete elimination of the mycobacteria in all infected organs within 12 weeks of treatment without relapse of infection. As a result, total treatment duration significantly reduced to 12 weeks.

Senthilkumar *et al.* (2008) investigated the ability of PEGylated poly(d,l-lactide-*co*-glycolide) nanoparticles (NPs) to deliver Docetaxel (DTX) (an anticancer agent) to solid tumors. The therapeutic efficacy and biocompatibility of NP formulations were evaluated in there study. For *in-vitro* cytotoxicity MTT assay was performed using MCF-7 and C26 cell lines. They found that, DTX-loaded PEGylated NPs increased the drug's biological half-life while providing substantial accumulation at the solid tumors. PEGylated NPs appear to be a promising alternate carrier for DTX having greater efficacy in inhibiting tumor.

Materials & Methods

3. MATERIAL AND METHODS

3.1 Material

3.1.1 Human macrophage cell line

Human macrophage cell line U-937 of histiocytic lymphoma origin was procured from National Centre for Cell Science (NCCS), University of Pune campus, Pune.

3.1.2 *Mycobacterium tuberculosis* culture

Lyophilized American Type Cell Culture (ATCC) of *Mycobacterium tuberculosis* H37Rv was procured from IVRI, Izatnagar.

3.1.3 Growth Medium for human macrophage cell line

Roswell Park Memorial Institute (RPMI 1640) medium with growth supplements was procured from Hi Media laboratories, Mumbai. Bovine fetal calf serum required to prepare the growth medium was purchased from Genetix, Mumbai.

3.1.4 Growth Medium for *Mycobacterium tuberculosis*

Middle brook 7H9 broth, Lowenstein Jensen (LJ) medium, Middle brook medium, Dorset egg medium, Acid egg medium required for study were purchased from Hi media laboratories, Mumbai.

3.1.5 Chemicals and reagents

Acid Fast staining kit and auramine staining kit were purchased from Hi media laboratories. Chemicals required for MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay were obtained from Sigma-Aldrich, USA. Sodium dodoecyl sulphate (SDS) and dimethyl sulfoxide were purchased from SRL.

3.1.6 Anti Tuberculosis drugs

Nanoparticulate rifampicin entrapped in polymer (Gentrez AN119) of size 400-450 nm and plain rifampicin required for the present study were supplied by Dept. of Pharmacy, Institute of Chemical Technology, University of Mumbai (UICT), Matunga, Mumbai.

3.1.7 Equipments and Laboratory wares

ELISA reader and CCD camera from BIORAD, Inverted microscope and Fluoroscent microscope from OLYMPUS were used in present study.

The tissue culture bottles, 24 and 96 well microtitre plates, micropipettes, filters of grade 0.22µm were purchased from Genetix and Millipore (I) ltd. Glass wares used were from Borosil.

3.2 Methods

All the procedures were carried out aseptically in Laminar flow of BSL-II grade. Proper protection and precautions were taken using masks of N-95 grade, gloves and goggles. Used material and cultures were disposed in either 5 % glutaraldehyde or 5 % formaline solution in biohazard disposal bags and autoclaved before discarding as biohazardous waste as per guidelines.

3.2.1 Propagation and maintenance of U937 human macrophage cell line

Propagation and maintenance of U-937 human macrophage cell line was carried as per the method of Wright *et al.* (1996). Human macrophage cell line U-937 of histiocytic lymphoma origin was maintained in RPMI 1640 medium with growth supplements, gentamicin @ 200 mg/l and 10 % bovine fetal calf serum by replacement of old medium every 2-3 days. Alternatively, cell culture was propagated by centrifugation and subsequent resuspension of cell pellet in growth medium maintaining cell density of 2×10^6 viable cells/ml. The growth medium was changed

every 2 to 3 days during maintenance of cell line. The standard growth conditions e.g. 37^0 C temperature and 5% carbon-dioxide atmosphere were maintained during incubation. Viability of cells was checked by trypan blue dye exclusion technique, carried out as per the method of Shivkumar (2003). Cells from human macrophage cell line U937 were taken in 100 µl quantity and mixed with 100 µl of working solution (0.16%) of Trypan Blue Dye. After 3 minutes of incubation at room temperature, the viable cells were counted by using haemocytometer.

$$\text{No. of viable cells} = N \times 10 \times 10^4$$

N = Average of total no. of viable cells counted in WBC chamber of haemocytometer.

3.2.2 Propagation and purity testing of *Mycobacterium tuberculosis*

Lyophilized ATCC culture of *Mycobacterium tuberculosis* H37Rv, was rehydrated and inoculated on Lowenstein Jensen (LJ) medium, Acid egg medium, Dorset egg medium and Middle brook medium. Incubation of inoculated media was done at 37°C temperature in presence of 5% carbon-dioxide atmosphere for 4 weeks. The purity of propagated culture was checked by Ziehl Neelsen and fluorescent staining, performed as per the instructions given in Hi-Media manual.

3.2.3 Cytotoxicity assay of nanoparticulate rifampicin and rifampicin

3.2.3 a Trypan blue dye exclusion assay

The nanoparticulate rifampicin and plain rifampicin were screened at various dilutions for cytotoxic effect on U937 human macrophage cell line. Different dilutions of the nanoparticulate rifampicin ranging from 1 µg/ml to 200 µg/ml were prepared using RPMI 1640 in 96 well flat bottom tissue culture plate. Similar dilutions were prepared with plain rifampicin, initially dissolved in 100µl of methanol. Each dilutions were run in triplicates. Cell control with RPMI 1640 were also added in triplicates.

Human macrophage cell line U937 was added in 100 µl quantity with cell density of 2 x 10^6 cells/ml to each well and the plates were incubated upto 24 hrs. After the incubation period of 3, 6, 12, 18 and 24 hrs,100 µl of the cells from each well were mixed in 100 µl of working solution (0.16%) of Trypan Blue dye (Appendix II). Viable cells were counted in WBC chamber of haemocytometer and percent death was calculated as per the method of Sharma *et al.* (2000).

$$\text{Mean viable macrophage (MVM)} = N \times 1 \times 10^4$$

N = Average of total no. of viable cells counted in WBC chamber of haemocytometer.

$$\text{Percent Death} = \frac{\text{MVM in control} - \text{MVM in test}}{\text{MVM in control}} \times 100$$

3.2.3b MTT assay

MTT tetrazolium salt (3-[4,5-Dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide) assay was performed as per the method of Mosmann (1983) with some modifications, for testing cytotoxicity of nanoparticulate rifampicin and rifampicin

i. The nanoparticulate rifampicin and plain rifampicin were screened for cytotoxic effect on U937 human macrophage cell line. Different dilutions ranging from 1µg/ml to 200 µg/ml of the nanoparticulate rifampicin were prepared using RPMI 1640 in 100 µl quantity in 96 wells flat bottom tissue culture plate. Similar dilutions were also made with plain rifampicin, initially dissolved in 100µl of methanol. An of the dilutions was kept in triplicates. Cell control with RPMI 1640 was also kept in triplicates.

ii. Human macrophage cell culture U937 of 2 x 10^6 cells/ml cell density were added in 100 µl quantity to each dilutions of drug and in controls and the plates were incubated for 24 hrs at 37°C temperature in CO_2 incubator.

iii. At an interval of 3, 6 , 12 , 18 and 24 hrs, cells from triplicate wells were collected in microcentrifuge tubes and centrifuged at 1000 rpm for 5 min. Supernatant was removed and cells were resuspended in 300 µl of working MTT (Appendix III) and distributed in 100 µl quantity in triplicates in 96 well plate. Plates were wrapped with aluminium foil and incubated in CO_2 incubator at 37° C in presence of 5% carbon-dioxide atmosphere for 4 hrs.

iv. After 4 hrs, 100 µl of dimethyl sulfoxide was added to each well and the absorbance of each well was measured in an ELISA plate reader at a wavelength of 570 nm.

v. Percent viability was calculated as per following formula.

$$\text{Percent (\%) viability} = \frac{\text{Mean O.D of dilution}}{\text{Mean O.D of control}} \times 100$$

3.2.4 Internalization of nanoparticulate rifampicin

The internalization of nanoparticulate rifampicin tagged with coumarin dye was studied to explore its intracellular delivery inside U937 human macrophage cells. Various dilutions of the nanoparticulate rifampicin ranging from 0.1 µg/ml to 35 µg/ml were prepared in RPMI 1640. Human macrophage cells U937 were adjusted to the cell density of 2 x 10^6 viable cells. 100 µl of the cell suspension was mixed with 100 µl of each dilutions of the nanoparticulate rifampicin and incubated for 90 min. The internalization was studied under fluorescent microscope using ultra-violet light.

3.2.5 Minimal inhibibitory concentration (MIC)

Minimal inhibitory concentration of nanoparticulate rifampicin and plain rifampicin was determined by broth dilution as per the method of Venugopal *et al.* (2007) with some modifications. The details of the procedures used was as under.

Broth dilution method : the different dilutions of the nanoparticulate rifampicin and plain rifampicin, viz. 0.1, 0.2, 0.5, 1 and 5 µg/ml were prepared using 7H9 broth in 100 µl quantity in 96 wells flat bottom tissue culture plate in triplicates. The suspension of *Mycobacterium tuberculosis* culture was matched with the optical density of tube no. 1.0 of Mc Farland standard corresponding to 10^6 to 10^7 CFU/ml and 5 µl of this culture was added to each of the dilution of drug and incubated at standard physical conditions. Blank wells with only media and control wells containing organisms without drug were kept in triplicates. Readings were taken on 7^{th} and 14^{th} day of incubation using ELISA reader at a wavelength of 620 nm. The MICs were determined by using following formula.

$$\text{Percentage (\%) reduction of growth of } \textit{M.tuberculosis} = 100 - \left[(D-B) \times \frac{100}{(C-B)} \right]$$

B- Reading of wells containing media without organisms
D- Average reading of wells containing various drug dilutions with organisms
C- Average reading of control wells

3.2.6 Infection of U937 human macrophage cell line with *Mycobacterium tuberculosis*

Infection of U937 human macrophage cell line with *Mycobacterium tuberculosis* was carried out by following the procedure of Esther *et al.* (1998). The cell density of U937 human macrophage cell line was adjusted to 8 X 10^5 cells /ml. The Infection dose was determined in order to have a proportion of 20 *Mycobacterium tuberculosis* organism per macrophage cell. The desired density of culture was adjusted by matching the bacterial suspension with tube no. 1.0 of Macfarland standard and then cells were infected with above standard bacterial suspension and incubated for 4 hrs in incubator at 37^0 C in presence of 5% carbon-dioxide atmosphere. Internalization of *Mycobacterium tuberculosis* in U937 human macrophage cell line was observed for 4 hrs. Confirmation of internalization of organisms in macrophages was carried out by Ziehl-Neelsen and fluorescent staining. One side of a clean glass slide was thoroughly flamed and allowed to cool before making smear. The infected U937 human macrophage cell suspension in 10 µl quantity was uniformly spread on the glass slide and dried. The smears were fixed by using methanol and stained with Ziehl-Neelsen and Fluorescent staining as per instructions of Hi Media.

3.2.7 *In-vitro* efficacy of rifampicin and nanoparticulate rifampicin

In-vitro efficacy of rifampicin and nanoparticulate rifampicin against *Mycobacterium tuberculosis* were studied in infected macrophage cell line as per the method of Esther *et al.* (1998).

i. Infection of U937 human macrophage cell line adjusted to 8 x 10^5 cells /ml was carried out with ratio of 20 mycobacteria per cell.

ii. Infected cells were incubated for 4 hrs in a CO_2 incubator at 37^o C.

iii. After incubation of 4 hrs, cells were centrifuged and pellet was washed twice using RPMI media. The cell pellet was resuspended again in RPMI

maintainance media (without antibiotic) to adjust the density of 8 x 10^5 cells /ml.

iv. The infected cells of 8 x 10^5 cells /ml density were mixed with different dilutions i.e 0.1, 0.2, 0.5, 1, 5 µg/ml of the nanoparticulate rifampicin and plain rifampicin and incubated for 12 hrs at 37°C in presence of 5% carbon-dioxide atmosphere.

v. At an interval of 0, 3, 6 and 12 hrs, the cells were lysed using 0.06% of SDS (Appendix IV). After lysis, the lysates were washed once and bacteria were resuspended in PBS maintaining original quantity. The bacterial suspension from each dilution of drug was plated in 100 µl quantity on LJ medium plates (Appendix V) in triplicates and incubated at 37° C in presence of 5% carbon-dioxide atmosphere for 4 weeks.

vi. The plates were observed regularly for growth of *Mycobacterium tuberculosis*. Readings of control and test plates showing typical colonies of *Mycobacterium tuberculosis* were recorded and percent reduction in the growth was calculated.

Results and Discussion

4. RESULTS AND DISCUSSION

4.1 Propagation and maintenance of human macrophage cell line

Human macrophage cell line U937 procured from NCCS was propagated and maintained in RPMI 1640 growth medium containing growth supplements, 10% bovine fetal serum and gentamicin 200 mg/l. The morphology of normal cells was rounded, mononuclear with crenate borders when observed under inverted microscope (Plate 1).

The regular screening of cells for their viability and change in normal morphology was carried out by trypan blue dye exclusion technique. The dye exclusion test was used to determine the number of viable cells present in a cell suspension. By this method, 95 to 99 % viability of the cells could be detected. It was based on the principle that, live cells possess intact cell membranes which exclude trypan blue dye, whereas dead cells do not (Plate 2).

Human macrophage cell line could be maintained up to 15 passages with required viability of cells. After 15 passages, the cell death exceeded above 10 % and also morphological changes were seen in cells. Therefore, cell line for study was used within the limit of 15 passages.

The similar procedures for propagation and maintainance were used by Jagannath *et al.* (1995), Bange *et al.* (1996), Wright e*t al.* (1996), Esther *et al.* (1998), Serge *et al.* (1998), Duman *et al.* (2003) and Kalita *et al.* (2004). The cell lines which maintained proper viability percentage of cells were used for different studies by above authors.

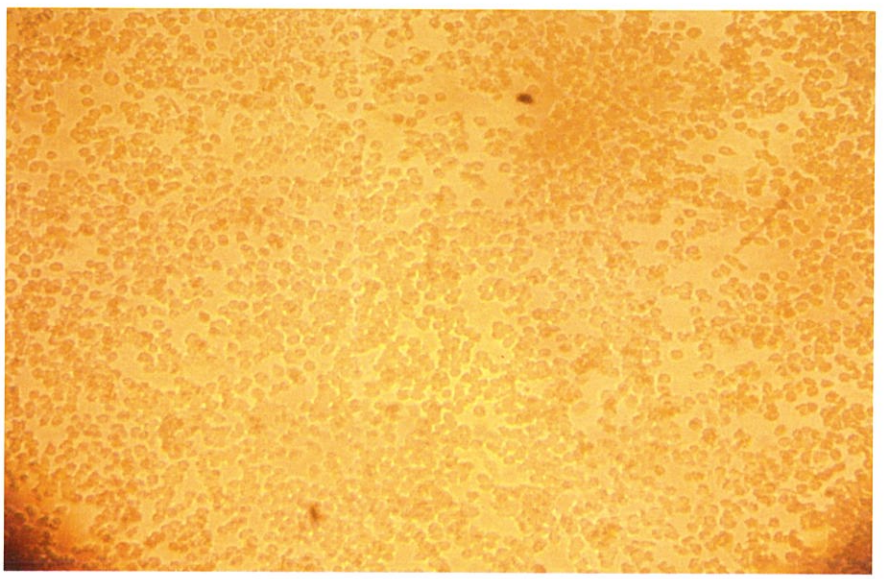

Plate 1. Human macrophage cell line U-937 maintained in RPMI growth medium with 10 % bovine fetal serum.

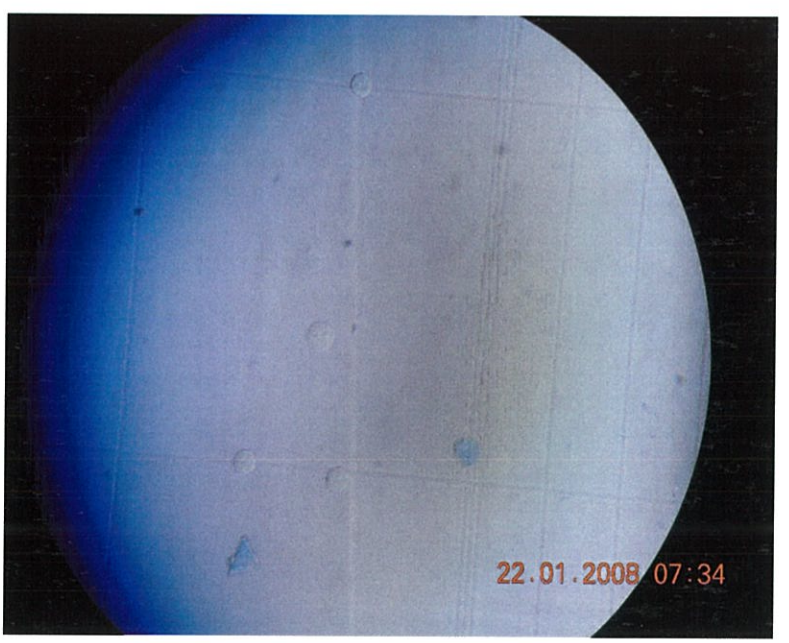

Plate 2. Trypan blue dye exclusion assay

4.2 Cytotoxicity assay

Nanoparticulate rifampicin and plain rifampicin were screened for cytotoxicity to observe adverse effect on macrophage cells if any, so as to find a safe dose range which would be non toxic to the cells.

Cytotoxiticity assay was carried out by trypan blue dye exclusion method and 3-[4,5-Dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide (MTT) assay (Plate 3 and 4). The result of cytotoxicity assay of nanoparticulate rifampicin and rifampicin are given in table 1.

The methods of trypan blue dye exclusion and MTT assay were similar to that used by Mosmann *et al.* (1983), Jagannath *et al.* (1995), Serge *et al.* (1998), Fischer *et al.* (2002) and Guggia *et al.* (2004) for studying cytotoxicity of different drugs or substances. They opined that, both the methods were suitable for testing the cytotoxicity of drugs.

Initially, nanoparticulate rifampicin and plain rifampicin were screened for cytotoxicity at broader range of concentrations i.e. from 1 to 200 µg/ml. For cytotoxicity tests different concentrations that were standardized i.e. 1, 5, 10, 15, 20, 25, 30 and 35 µg/ml for nanoparticulate rifampicin and plain rifampicin.

Lecaroz *et al.* (2006) studied cytotoxicity of nanoparticles by trypan blue dye exclusion and MTT assay. They observed similar values of the cytotoxicity determined by both the methods. In present study also, both the methods showed nearly similar results and were found reliable for cytotoxicity assay, although MTT was found to be slightly more sensitive.

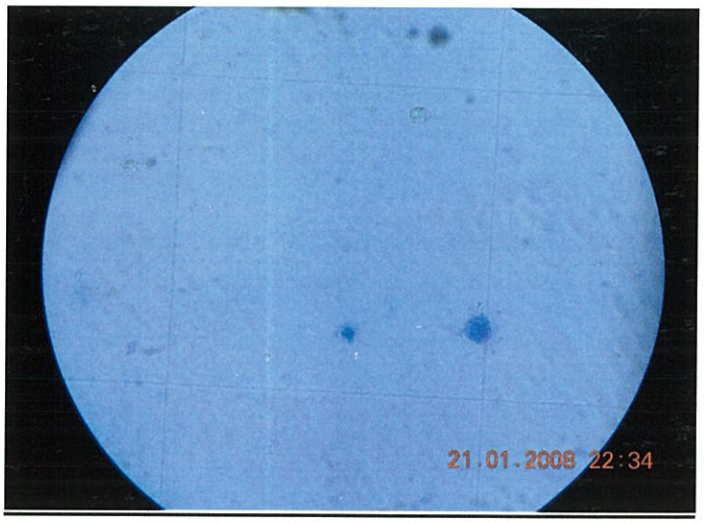

Plate 3. Trypan blue dye exclusion assay for cytotoxicity

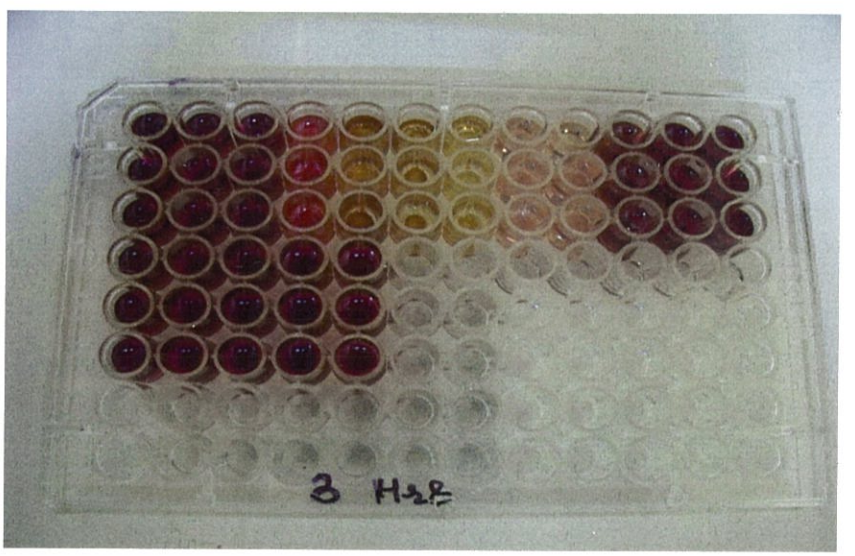

Plate 4. MTT Assay for cytotoxicity

A1- C1 : Cell control
A2 – C2 to A9 – C9 : Nanoparticulate rifampicin
A10 – C10 to A12 – C12 and D1-F1 to D5-F5 : Plain rifampicin

Table 1. Cytotoxicity of Nanoparticulate Rifampicin and Plain Rifampicin by MTT assay (% cell death)

Sr. no.	Contact time	Type of drug	Percent (%) death at concentration of									
			1 µg	5 µg	10 µg	15 µg	20 µg	25 µg	30 µg	35 µg	100 µg	200 µg
1.	3 hrs	Nano-Rif	1	4	31	32	46	54	63	67	100	100
		Rif	2	3	3	3	3	5	5	5	6	6
2.	6 hrs	Nano-Rif	3	3	37	48	59	64	64	68	100	100
		Rif	3	5	8	8	8	9	9	10	16	40
3.	12 hrs	Nano-Rif	6	33	40	51	73	92	93	94	100	100
		Rif	4	6	9	11	14	16	19	22	32	62
4.	18 hrs	Nano-Rif	8	39	50	77	86	94	95	100	100	100
		Rif	4	9	12	15	17	17	21	23	51	87
5.	24 hrs	Nano-Rif	21	62	79	91	93	100	100	100	100	100
		Rif	11	15	19	21	26	33	42	51	55	100

The cytotoxic effect of both the drugs were studied up to 24 hrs, since the viability of cells in control wells were maintained only up to 24 hrs. After 24 hrs, the cell death started even in control well without drug. The comparative cytotoxicity of nanoparticulate rifampicin and plain rifampicin are depicted in figures 1 to 4.

Cytotoxicity at 3 hrs and 6 hrs

There was 2-3% of cell death observed at lower concentrations up to 5 µg/ml and 67-68 % of cell death was seen at higher concentration i.e. 35 µg/ml of nanoparticulate rifampicin. However, plain rifampicin up to concentration of 35 µg/ml showed 6-7 % cell death. Cells in medium control showed 100% viability.

Cytotoxicity at 12 hrs

At concentration of 1 µg/ml, there was 6% of cell death observed and 33% of cell death was seen at 5 µg/ml concentration of nanoparticulate rifampicin. There was 94% of cell death seen at 35 µg/ml concentration of nanoparticulate rifampicin. Whereas, plain rifampicin up to 5 µg/ml showed 4-6% cell death and 22% of cell death was seen in concentration of 35 µg/ml. The viability of cells in medium control was 100%.

Cytotoxicity at 18 hrs

The cell death observed at 1 µg/ml was 8% whereas, 39% of cell death was found at 5µg/ml concentration of nanoparticulate rifampicin. There was 100% cell death found in 35 µg/ml concentration of nanoparticulate rifampicin. The plain rifampicin at concentration of 1 µg/ml caused 4% cell death and 8% cell death was seen at 5µg/ml. There was 23% of cell death seen in concentration of 35 µg/ml. Whereas, cells in medium control showed 100% viability.

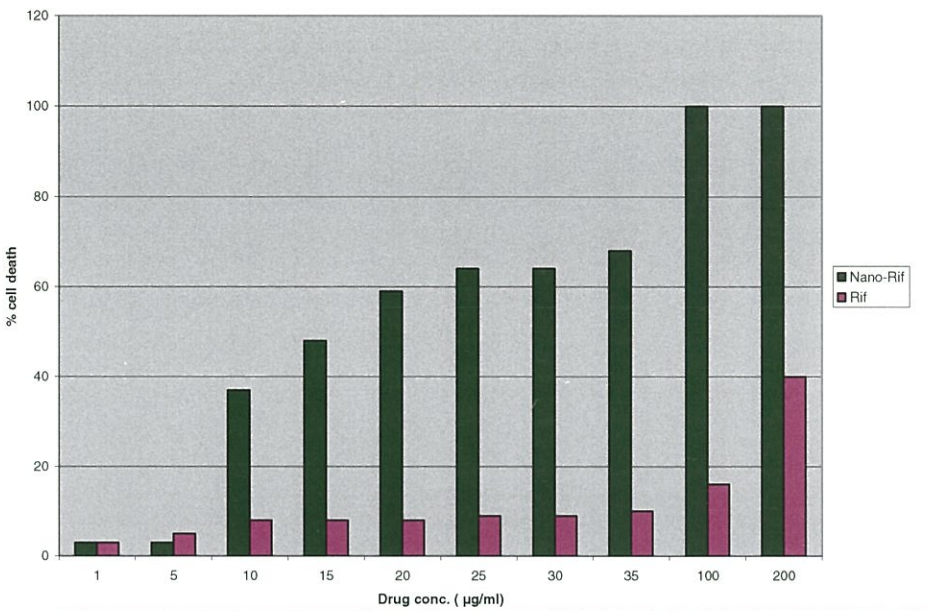

Figure 1. Comparative cytotoxicity of Nanoparticulat Rifampicin and Rifampicin at 6 hrs

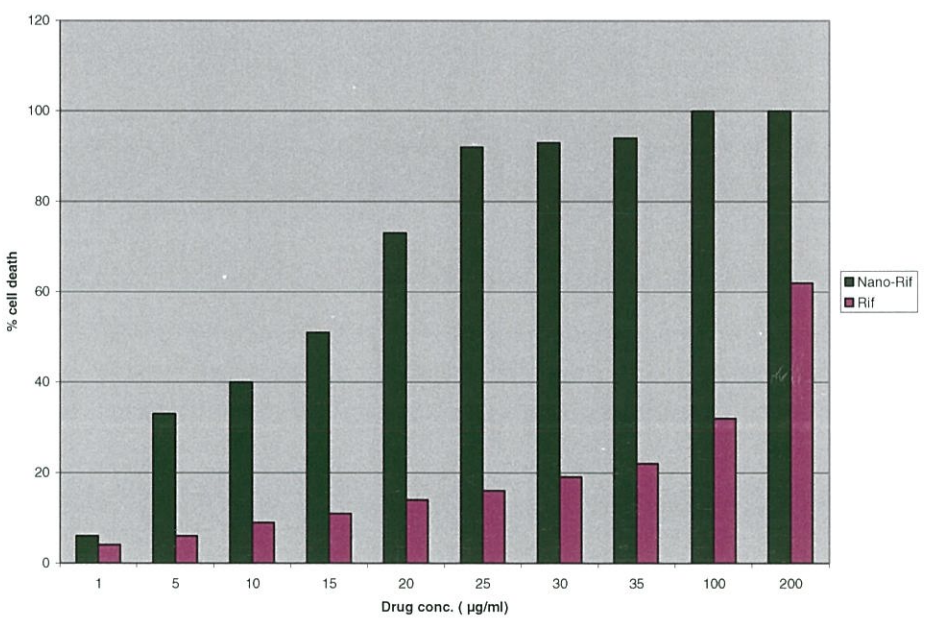

Figure 2. Comparative cytotoxicity of Nanoparticulat Rifampicin and Rifampicin at 12 hrs

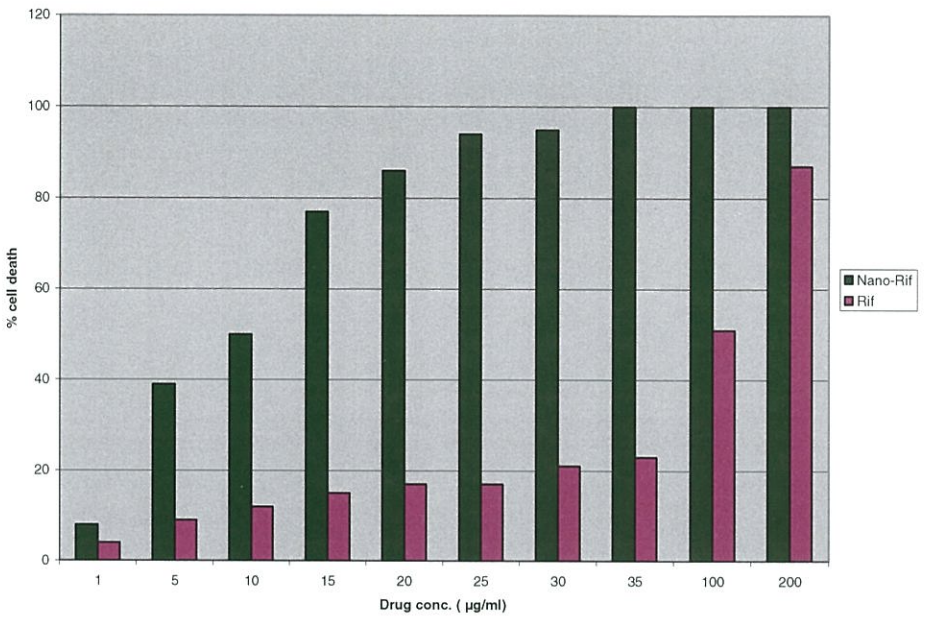

Figure 3. Comparative cytotoxicity of Nanoparticulat Rifampicin and Rifampicin at 18 hrs

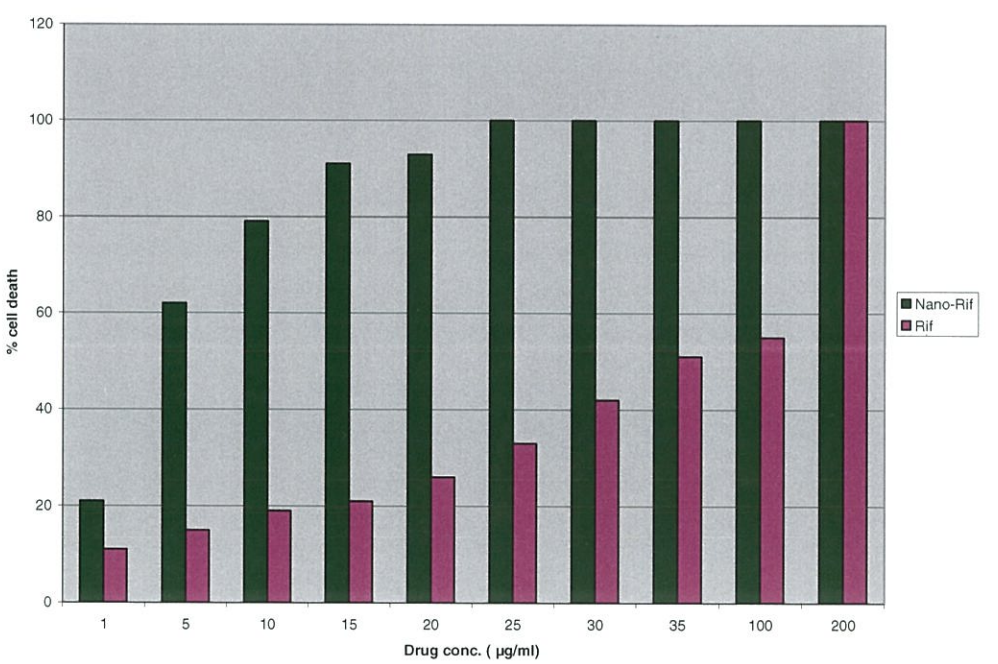

Figure 4. Comparative cytotoxicity of Nanoparticulate Rifampicin and Rifampicin at 24 hrs

Cytotoxicity at 24 hrs

There was 21% cell death observed at concentration of 1 µg/ml and 62% cell death at 5 µg/ml of nanoparticulate rifampicin. Whereas, plain rifampicin at 1µg/ml showed 11% cell death and 15% of cell death was seen at concentration of 5 µg/ml. There was 51% of cell death seen at 35 µg/ml of concentration. However, cells in medium control at this point also showed 6-7% death.

The nanoparticulate rifampicin by virtue of its property could internalize the macrophage quickly. The comparison of cytotoxicity of nanoparticulate rifampicin and plain rifampicin revealed that both formulations at the same concentrations reacted differently. The nanoparticulate preparation could produce the toxicity from within the cell.

In the present study, the cytotoxicity of nanoparticulate rifampicin was found higher as compared to plain rifampicin. The cytotoxicity increased proportionately with concentration and time. Cytotoxicity of nanoparticulate rifampicin was found in the range of 1 to 5 µg/ml. Therefore, the concentrations between 0.1 µg to 5 µg of both nanoparticulate rifampicin and rifampicin were selected for the further studies of MIC and efficacy against *Mycocobacterium tuberculosis*.

Similar finding of increased cytotoxicity with concentration and time was observed by Fischer *et al.* (2002) in the *in-vitro* cytotoxicity study of different polymers.

These findings of cytotoxicity of nanoparticles are corroborated with Yoo *et al.* (2004), where they reported, cell viability for free DOX was 60 % and for DOX nano-aggregates was 40 % i. e. nanoaggregates were more cytotoxic.

4.3 Internalization study of nanoparticulate rifampicin

The nanoparticulate rifampicin used in the present study was tagged with coumarin which had the fluorescence property under UV microscope (550 nm). The internalization of nanoparticulate rifampicin began within a minute and was appreciable in half an hour at 5µg/ml. This property was utilized to conjugate nanoparticulate with anti-tuberculosis drugs for targeted drug delivery. The results of internalization of nanoparticulate rifampicin are given in table 2.

Table 2. Internalization of Nanoparticulate Rifampicin in macrophage cells

Sr. no.	Conc. of drug (ug/ml)	Fluorescence at time interval of					
		1 min	5 min	10 min	30 min	60 min	90 min
1	0.1	+	+	+	+	+	++
2	1	+	+	+	++	++	+++
3	5	+	++	++	+++	+++	++++
4	10	++	++	+++	++++	++++	++++
5	25	++	+++	++++	++++	++++	++++

Poor Fluorescence (+)
Weak Fluorescence (++)
Good Fluorescence (+++)
Excellent Fluorescence (++++)

From the table it is clear that at 0.1 µg/ml, the cells fluoresced poorly and intensity of fluorescence was increased after longer incubation period. Internalization of 0.1 and 1 µg/ml concentration at 30 min is shown in Plate 5a and 5b. Although internalization of 5 µg/ml concentration at 30 min was good (Plate 5c), the best time-concentration combination was found at 5 µg/ml with 90 minutes of contact time. Higher concentration of 25 µg/ml showed excellent fluorescence with even lesser incubation periods (Plate 5d).

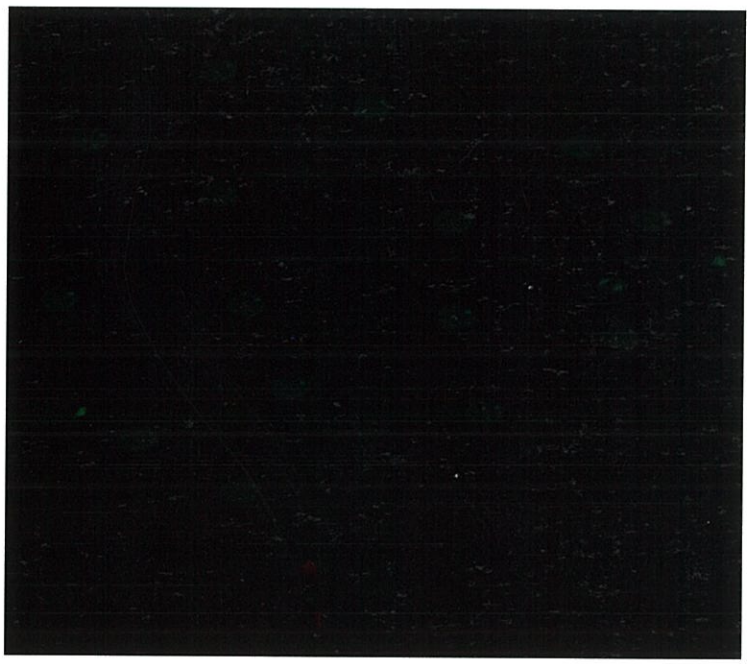

Plate 5a. Internalization of nanoparticulate rifampicin at 0.1 µg/ml in 30 min.

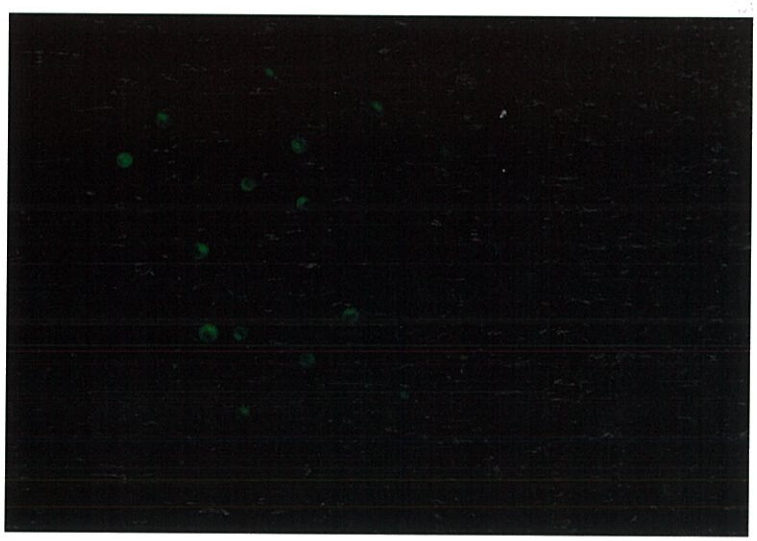

Plate 5b. Internalization of nanoparticulate rifampicin at 1 µg/ml in 30 min.

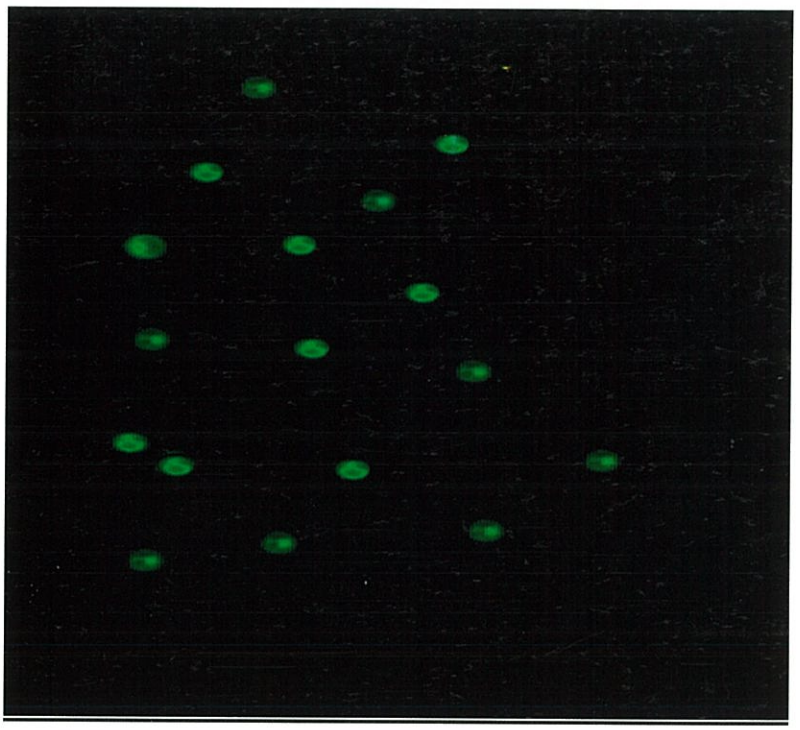

Plate 5c. Internalization of nanoparticulate rifampicin at 5 µg/ml in 30 min.

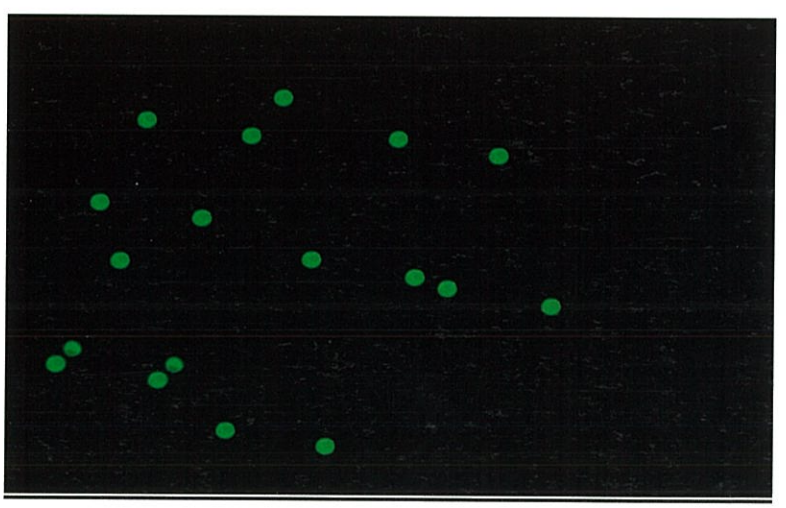

Plate 5d. Internalization of nanoparticulate rifampicin at 25 µg/ml in 30 min.

The similar method of fluorescent dyes for internalization studies of different drugs in cell lines was used by Gaikwad (2007). He also observed that internalization of lipomer occurred within minutes and was appreciable within half an hour at 5 µg/ml. Haigler *et al.* (1978) also observed fluorescence of internalized particle at 20 min.

4.4 Minimal Inhibitory Concentration

MIC of nanoparticulate rifampicin and plain rifampicin was estimated by broth dilution method to determine the therapeutic values of above drugs, since agar dilution method was found less sensitive and time consuming.

Since, the nanoparticulate rifampicin was found more cytotoxic above the of 5 µg/ml, a concentration range of 0.1 - 5 µg/ml was used in estimation of MIC. The different dilutions of both the drugs were prepared in 7H9 broth to obtain the concentration of 0.1, 0.2, 0.5, 1 and 5 µg/ml. The *Mycobacterium tuberculosis* suspension was added in 5 µl quantity to each dilution of both the drugs. The bacterial suspension and different dilutions of drugs were mixed properly and incubated for 2 weeks. The spectrophotometric / ELISA readings were taken on 7^{th} and 14^{th} day of incubation at 620 nm wavelength. (Table 3).

Similar method of broth dilution was used by Sato *et al.* (1999) Hwang *et al.* (2002), Duman *et al.* (2003) and Venugopal *et al.* (2007) for determining MICs of various drugs.

Table 3. MIC of Nanoparticulate Rifampicin and Plain Rifampicin

Sr. no.	Conc. of drug (µg/ml)	Percent (%) reduction in growth on 7th day		Percent (%) reduction in growth on 14th day	
		Nano-Rif	Rif	Nano-Rif	Rif
1.	0.1	59.55	64.40	66.62	68.64
2.	0.2	77.16	74.89	80.03	82.53
3.	0.5	82.28	85	83.04	86
4.	1	95.06	99.61	97	100
5.	5	100	100	100	100

Nanopaticulate rifampicin and plain rifampicin showed 100 % reduction in growth of *Mycobacterium tuberculosis* at concentration of 5 µg/ml. Thus, it was seen that, MIC values of nanopaticulate rifampicin and plain rifampicin were nearly same. These observations are in accordance with the findings of Deepa *et al.* (2005) suggested the MIC of rifampicin was 5µg/ml.

The MIC values recorded in present study for nanopaticulate rifampicin and plain rifampicin were similar in range

Whereas, lower values of MIC were observed by Palicova *et al.* (2000) i.e. 1-2 µg/ml of rifampicin. Whereas, the MIC of rifampicin was 2µg/ml for H37Rv strain was reported by Traore *et al.* 2007. Coban *et al* (2004) also obtained a similar MIC for rifampicin. Stottmeier *et al.* (1969), Ohno *et al.* (1996) and Duman *et al.* (2003) observed that the lower minimal inhibitory concentrations (MIC) of rifampicin was 0.5 µg/ml and Venugopal *et al* (2007) found MIC of rifampicin between 0.03 to 0.4 mg/ml.

On the other hand, higher MICs were reported by Dhopale *et al.* (1993) who found MIC of rifampicin at 10 µg/ml. Similarly, Heifets *et al.* (1999) and Hwang *et al.* (2002) also reported higher MIC values.

4.5 Infection of Human Macrophage cell line U 937 with *Mycobacterium tuberculosis*

Infection of human macrophage cell line was carried out with *Mycobacterium tuberculosis*. After 4 hrs of infection, the internalization was confirmed by Ziehl-Neelsen and fluroscent staining. In Ziehl-Neelsen stained smears, mycobacteria were observed pink within blue coloured macrophages (Plate 6). Acid-fast mycobacteria resisted decolourization by acid-alcohol after primary staining owing to the high lipid (mycolic acid) content in their cell walls. The staining of mycobacteria with auramine O was due to the affinity of the mycolic acid in the cell walls for the fluorochrome. The dye bound to the mycobacteria appeared as bright yellow, luminous rods against a dark background. The potassium permanganate helped to prevent non-specific fluorescence.

In the present study the infection of human macrophage cell line was carried out for 4 hrs and internalization could be observed within this time.

Similar range for infection of different cell lines by bacteria were used by Bange *et al.* (1996), Mehta *et al.*, (1996), Reddy *et al.* (1996) Esther *et al.*, (1998), Sharma *et al.* (2000), Ghosh *et al.* (2004), Sander *et al.* (2004) and Torrado *et al.* (2007).

After staining with Ziehl-Neelsen method, *Mycobacterium tuberculosis* present inside the macrophages were found to be more curved and longer and the clumps of bacilli were also observed in some of the macrophages. The clumping observed inside the macrophages might have been due to the cord factor.

Fluorescent staining with auramine was found to be specific for both *Mycobacterium tuberculosis* and macrophages. Mycobacterium fluoresced yellow while the macrophage fluoresced green after the staining. The organism appeared well internalized in macrophage cell after an incubation for 4 hours with the cell suspension (Plate 7).

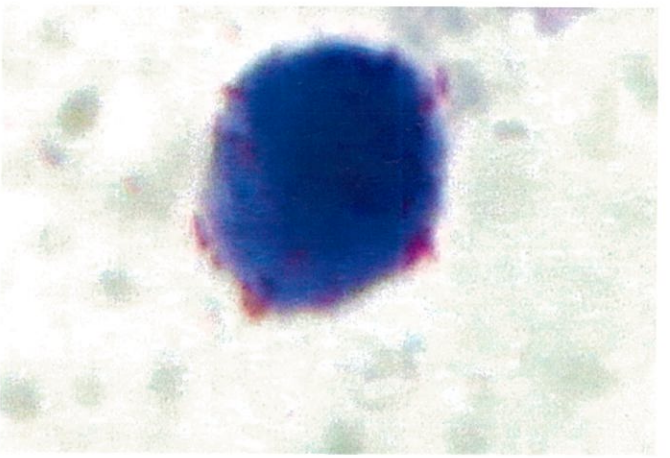

Plate 6. Internalization of *Mycobacterium tuberculosis* in macrophage by Zheil-Neelson staining.

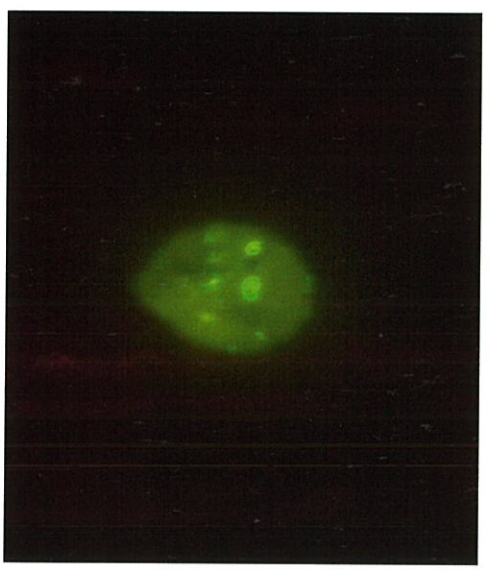

Plate 7. Internalization of *Mycobacteriumtuberculosis* in macrophage by Fluorescent staining.

Similar staining procedures were carried out by Albas *et al* (1979), Bange *et al.* (1996) and Khanna *et al.* (1996) for confirmation of internalization of bacteria in different cells after infection.

Youmans (1979) suggested that, the cord factor present in the mycobacterial cell wall was responsible for sticking of the cells together. The findings of present study were in agreement with Esther *et al.* (1998) who observed that, the bacilli were bright yellow and the macrophages were green when stained with auramine dye and observed under U.V microscope (550nm). Sharma *et al.* (2000) reported that, when observed by acid fast staining 60-80% macrophages contained 2-3 bacilli per macrophage. Similar studies of infection and internalization for Mycobacteria were carried out by Duman *et al.* (2003), Sander *et al.,* (2004), Mehta *et al.,* (2006) and Torrado *et al.* (2007).

4.6 Efficacy of nanoparticulate rifampicin and rifampicin

Efficacy of both the drugs was studied at concentrations of 0.1, 0.2, 0.5, 1 and 5 µg/ml. This range of concentration of drugs was selected since the nanoparticulate rifampicin was found cytotoxic above 5 µg/ml after 12 hrs.

Efficacy studies were carried out as per the method of Esther *et al* (1998). Infected cells were incubated with different concentrations of drugs up to 12 hrs. At an interval of 0, 3, 6 and 12 hrs, cells were lysed and plated on LJ medium. After incubation of 4 weeks, readings of control and test plates showing the typical colonies of *Mycobacterium tuberculosis* organisms were read (Plate 8a and 8b) and the percent reduction in the growth was calculated. (Table 4).

It was observed that, at 3 hrs of contact time, nanoparticulate rifampicin at concentration of 0.5 µg/ml and above inhibited the growth of *Mycobacterium tuberculosis* completely. Whereas, plain rifampicin inhibited the growth of *Mycobacterium tuberculosis* completely at concentration of 5 µg/ml.

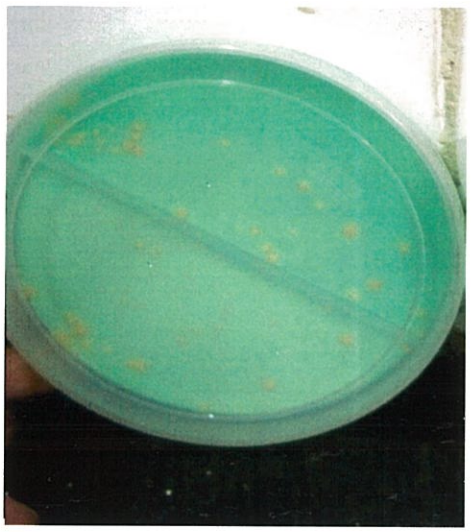

Plate 8a. Colonies of *Mycobacterium tuberculosis* on L. J. Medium

Plate 8b. Inhibition of *Mycobacterium tuberculosis* growth at 0.5 µg/ml and above concentration of nano-Rif.

At 6 hrs of contact time, 0.2µg/ml concentration of nanoparticulate rifampicin inhibited the growth of *Mycobacterium tuberculosis* completely. Whereas, rifampicin at concentration of 1 µg/ml and above inhibited the growth of *Mycobacterium tuberculosis* completely.

Table 4. Efficacy of Nanoparticulate Rifampicin and Plain Rifampicin

Sr. no.	Concentration of drug (µg/ml)	Type of Drug	Percent (%) inhibition of growth at contact time of		
			3 hrs	6 hrs	12 hrs
1	0.1	Nano-Rif	58	83	100
		Rif	33	40	75
2	0.2	Nano-Rif	86	100	100
		Rif	54	72	83
3	0.5	Nano-R	100	100	100
		Rif	72	93	100
4	1	Nano-R	100	100	100
		Rif	90	100	100
5	5	Nano-R	100	100	100
		Rif	100	100	100

At 12 hrs of contact time, 0.1 µg/ml and above concentration of nanoparticulate rifampicin inhibited the growth of *Mycobacterium tuberculosis* completely. However, the growth of *Mycobacterium tuberculosis* was completely inhibited at 0.5 µg/ml of rifampicin.

Similar values were obtained by Jagannath *et al.* (1995) who had observed total inhibition in growth at 2.5 and 5 µg/ml of rifampicin, Mor *et al.* (1997), found total inhibition in growth of H37Rv at 0.25 µg/ml concentration

of rifampicin. Also Duman *et al.* (1998) observed total inhibition at 0.5 and 2.5 µg/ml. Similar findings were reported by Kalita *et al.* (2004), Diane *et al.* (2006).

Thus, from these results it was observed that, nanoparticulate rifampicin was found to be 5-10 times more effective than plain rifampicin. This may be due to the property of nanoparticulate rifampicin to internalize the macrophage more efficiently and maintain an effective concentration for a long time.

Similar findings of greater efficacy of nanoparticulate drugs were observed by many authors. Pandey *et al.* (2003) reported better efficacy of nanoparticle encapsulated antitubercular drug in treatment of murine tuberculosis. Skidan *et al.* (2003) suggested that nanoparticles provided 2-fold increase in rifampicin efficacy. Zahoor *et al.* (2006) suggested that alginate (a natural polymer) based nanoparticulate delivery system resulted in complete bacterial clearance from the organs. Sharma *et al.* (2004) reported that nanoparticles loaded antitubercular drugs produces extended serum half life. Vyas *et al.* (2004) suggested that the ligand-anchored liposomal aerosols are effective in rapid attainment of high-drug concentration in lung alveolar macrophages. Wang *et al.* (2005) reported enhanced its antitumor activity of nanoparticles. Whereas, Jurriaan *et al.* (2007) suggested better in-vitro efficacy in treatment.

Devalapally *et al.* (2007) observed better efficacy of nanoparticle formulations as compared to the agents administered in aqueous solutions. Senthilkumar *et al.* (2008) also suggested DTX-loaded PEGylated nanoparticles increased the drug's biological half-life while providing substantial accumulation in the solid tumors.

Summary & Conclusions

5. SUMMARY AND CONCLUSIONS

It is estimated that over one-third of the world's human population harbours *Mycobacterium tuberculosis (Mtb)*, the bacterium that causes TB. Recent implementation of the World Health Organization's strategy Directly Observed Therapy DOT , (short-course) has been problematic, and TB remains a major burden in many developing countries. The resistance of *M. tuberculosis* to anti-TB agents is a worldwide problem in both immunocompetent and HIV-infected populations. Nanoparticle-based drug delivery systems have considerable potential for treatment of tuberculosis. Therefore an experimental study was conducted to determine the *in-vitro* efficacy of nanoparticulate rifampicin and plain rifampicin against *Mycobacterium tuberculosis.*

Human macrophage cell line U937 was maintained in the form of suspension culture in growth medium and the cell morphology was examined regularly to know the abnormalities and death. The morphology of normal cells was rounded, mononuclear with crenate border. The cell density was maintained at 2 x 10^6 cells per ml by counting cells in the haemocytometer. The viability of the cells were regularly assured by trypan blue dye exclusion technique. It showed nearly 95-98% viability of the cells during maintenance of cell line. The cell line could be maintained upto 15 passages without much morphological changes and cell death.

Lyophilized H37Rv *Mycobacterium tuberculosis* culture was rehydrated and inoculated in various commercial media like Lowensten Jensen medium, acid egg medium and 7H9 broth. The plates and broth were regularly examined for growth upto four weeks of incubation. The purity of the grown organisms was checked using Ziehl Neelsen and Fluorescent staining techniques. In Ziehl Neelsen stained smears, the organisms appeared short, straight or slightly curved rods, arranged singly or sometimes in strands. Fluorescent staining of smear showed *Mycobacterium tuberculosis* as yellowish green fluorescent rods when observed under UV microscope.

The cytotoxicity of nanoparticulate rifampicin and plain rifampicin on human macrophage cell line was studied using different concentrations. The

cytotoxic effect of both the drugs were determined by trypan blue dye exclusion assay and MTT assay. The cytotoxicity of nanoparticulate rifampicin and rifampicin was studied at an interval of 3, 6, 12, 18 and 24 hrs. Nanoparticulate rifampicin was found to be more cytotoxic than plain rifampicin. Nanoparticulate rifampicin was found non-cytotoxic at 1 µg/ml. However, plain rifampicin was found to be non- cytotoxic up to 5 µg/ml concentration. Cytotoxicity of both the drugs increased with increasing concentration and time.

Internalization of nanoparticulate rifampicin tagged with coumarine dye was studied using fluorescent microscope to know the intracellular delivery of drug inside macrophages. Internalization of nanoparticulate rifampicin was initially observed at 0.1 µg/ml in 1 min and optimized at 5 µg/ml at 90 min that indicated better intracellular delivery of drug in human macrophage cell line.

MIC values of both the drugs were determined using broth dilution method. There was 100 % reduction in growth of *Mycobacterium tuberculosis* at concentration of 5 µg/ml of nanopaticulate rifampicin and plain rifampicin Thus, it was seen that, MICs of both the drugs were found to be 5 µg/ml at 7 days.

Infection of human macrophage cell line with cell density 8 x 10^5 cells/ml was carried out with *Mycobacterium tuberculosis* culture and incubated for 4 hours. Internalization of microorganisms was observed by staining the infected cells with Ziehl Neelsen's and Fluorescent staining method. In Ziehl-Neelsen's stained smear, *Mycobacterium tuberculosis* were found to be more curved, longer and the clumps of bacilli were also observed in certain macrophages. Fluorescent staining revealed that, mycobacterium fluoresced bright yellow colour, while the macrophage fluoresced green after the staining. The organisms appeared well internalized inside the cells within 4 hours of incubation.

In-vitro efficacy of both the drugs was studied at concentrations of 0.1, 0.2, 0.5, 1, and 5 µg/ml. The infected human macrophage cell line was incubated with different concentrations of drugs upto 12 hrs. Effective concentrations of both the drugs were determined at an interval of 3, 6 and 12 hrs. It was found that, nanoparticulate rifampicin was more effective than rifampicin in killing intracellular mycobacteria. At contact time of 12 hrs, concentration of 0.1 µg/ml

of nanoparticulate rifampicin inhibited the growth of mycobacteria completely. Whereas, plain rifampicin inhibited growth of *Mycobacterim tuberculosis* completely at 0.5 µg/ml at same time interval.

The following conclusions could be drawn from the present study

1. Human Macrophage cell line U937 could be maintained in the RPMI medium with 10 % bovine fetal calf serum upto 15 passages without any abnormal changes.

2. Nanoparticulate rifampicin was found to be non cytotoxic up to 1 µg/ml. Whereas, plain rifampicin was non-cytotoxic at 5 µg/ml.

3. Although, nanoparticulate drug was internalized at 0.1 µg/ml, the concentration of 5 µg/ml with contact time of 90 minutes showed excellent internalization.

4. The MIC values of both the drugs were found to be 5 µg/ml.

5. The intracellular efficacy of nanoparticulate rifampicin was found better (5-10 times) than plain rifampicin.

To conclude, in the present study nanoparticulate rifampicin at 0.5-5 µg/ml was standardized as a dose that was non-cytototoxic, having in-vitro efficacy up to 3 hrs and was well internalized within human macrophages. Hence, rifampicin entrapped in polymer (gentrez AN119) could be exploited for clearance of intracellular *M. tuberculosis* from human macrophage. Further studies on efficacy of this preparation in animal model are obvious.

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Appendix

B. APPENDIX

Appendix - I

Reagents for propagation and maintenance of human macrophage cell line U937

RPMI 1640 medium

RPMI powder	: 10.3 g/lit (1 bottle)
HEPES buffer	: 25mM (6.51 g)
Gentamycin	:200 mg/l
Na bicarbonate	:2 g
Distilled water	:1 lit.
Fetal Bovine Serum	:5%
Sodium azide	:0.1% (sterilized by autoclaving and stored at 4 ^{0} C)

RPMI 1640 powder containing L-glutamine, HEPES buffer, antibiotics and sodium bicarbonate were added in sterile distilled water (autoclaved) and filtered through 0.22 µ filter and stored at 4 ^{0} C until its use. The pH was checked and adjusted to 7.4. The media was regularly checked for sterility throughout the experimentation period.

Appendix - II

Reagents and stains used for Trypan blue exclusion assay

A] Stock solutions

1) 4.5 % Normal saline solution:

Sodium chloride :4.5 gm

Distilled water :100ml

PH :7.0

2) 0.5 % Trypan blue dye:

Trypan blue dye :0.5 gm

Distilled water :100ml

Filtered through Whatman filter paper and stored at room temp.

B] Working solution of 0.16 % Trypan blue dye:

One part of 4.5 % NSS stock solution was added into three parts of 0.5% Trypan blue stock solution and used as a working concentration.

Appendix - III

Reagents for MTT assay

A) Stock solutions

1) Phosphate buffer saline solution:

Nacl	:8.0 gm
KCl	:0.2 gm
Na_2HPO_4	:1.13
KH_2PO_4	:0.2
Distilled water	:1000ml
pH	:7.0

2) MTT stock solution:

MTT reagent	:5 mg
PBS	:1ml

B) Working solution:

1 ml of stock solution of MTT was mixed with 9 ml of PBS to prepare working MTT solution for assay.

Appendix - IV

Reagents used for lysis of cells

1) Phosphate buffer saline solution:

Nacl	:8.0 gm
KCl	:0.2 gm
Na_2HPO_4	:1.13
KH_2PO_4	:0.2
Distilled water	:1000 ml
pH	:7.0

2) Sodium dodoecyl sulphate (0.06%) solution:

Sodium dodoecyl sulphate	:0.06 gm
PBS	:100ml

Appendix - V

Reagents for preparation of Lowenstein Jensen (LJ) medium plates

1) Media base:

LJ media base (from Hi media) :37.24 gm

Distilled water :600 ml

Autoclaved at 121^0 C and 15 lbs pressure for 15 min.

2) Whole egg emulsion:

Eggs were washed with detergent, swabbed with alcohol. Whole egg emulsion was prepared by mixing whole contents of eggs with help of glass beads and then filtered through single layer muslin cloth.

Whole egg emulsion in quantity of 1 lit. was mixed with 600 ml of media base after cooling. Media was dispensed in plates and sterilized in water bath at 85^0 C for 45 min for 3 consecutive days.

Vita

VITA

The author, Dr. Ms. Shivali Pradeep Gaikwad was born on 27th August, 1983 at Nasik. She was brilliant child from the beginning of her academic life. She completed his schooling from J. D. C. Bytco English medium High school, Nasik. Her primary and secondary school record were good and passed her SSC examination with distinction in year 1999. She was given an award of outstanding student of the year, as all round student. She completed her higher secondary education from R. Y. K. College, Nasik. She passed 12th std. with distinction. Her love for animal aspired her to join Bombay Veterinary College, Parel Mumbai -12. She passed her B.V.Sc & A.H. with first class distinction and was university topper in ladies and SC category. Her managerial qualities were highlighted throughout the academic years of her graduation. She was hostel representative of the ladies hostel in 2nd year B.V.Sc & A.H. and acted as ladies representative during Spandan 2005. She was the member of Women's Redressal Committee of college. Her enthusiasm and spirit during the N.S.S camp (Large animal vaccination camp) in Bhiwandi Taluka, Dist. Thane was commendable. She is registered with Maharashtra State Veterinary Council, Nagpur in the capacity of a Veterinary practitioner and worked as Veterinary Clinician at Goodman's Pet Clinic, Parel for 2 years.

After graduation, she sought admission for M.V.Sc. degree in Veterinary Microbiology, at Bombay Veterinary College, in 2006. Her zeal and desire to learn more encouraged her to actively involve in various departmental projects as well as Tissue Culture Training Programs and CVE trainings, organized by the College where she had also worked as demonstrator.

She has published as co-author technical abstract on "Effect of Deuterium oxide on human macrophage cell line" in International conference held at Chennai, in 2008.

Shivali

(Dr. Shivali P. Gaikwad)