Review of Literature
2. REVIEW OF LITERATURE

2.1.a. Chemotherapy

In 1940s the chemotherapy of tuberculosis was basically monotherapy with streptomycin. It was not only highly effective against human tuberculosis but also reasonably safe. In combination with para-amino salicylic acid it resulted in a good initial recovery, but later the patients relapsed with a drug-resistant form of the disease. Isoniazid was found to be active both in vitro and in experimental animals even in low concentrations. In 1952, British Medical Research Council studies showed that isoniazid was very effective with impressive clinical and radiological improvement, but resistant organisms emerged rapidly in a majority of patients. As with the other antituberculous drugs, isoniazid resistant strains emerged rapidly on exposure to isoniazid alone.

Early in the chemotherapy era, Canetti, Mitchison, Grosset and others, advocated the concept of multiple drug therapy that would be needed to eradicate the tubercle bacilli in patients with active infection (Mitchison 1965). Multi drug therapy with isoniazid and rifampicin-containing regimens was found to be effective, and helped in the reduction of treatment period and also in preventing the emergence of drug resistance. Following multidrug therapy, the concept of two-phase therapy, which consists of an initial intensive phase of treatment with more than two drugs and a continuation phase therapy with fewer drugs was developed (WHO 1983). This methodology resulted in successful outcome with the treatment in many parts of the world. The combination of
isoniazid, streptomycin and rifampicin effected cure in almost 100% with a treatment period of 9 months (Schlunger et al., Tuberculosis)

2.1.b. Short Course Chemotherapy (SCC)

The World Health Organization (WHO) recommended short course chemotherapy (SCC) based on extensive research and controlled clinical trials conducted by the British Medical Research Council (BMRC), London, the International Union Against Tuberculosis and Lung Diseases (IUATLD), the United States Public Health Laboratory Services (USPHLS), the Tuberculosis Research Centre (TRC), Chennai, and many others (WHO 1997).

The SCC treatment is usually for a period of 6 months and consists of 2 phases of treatment with an initial intensive phase of 2 months and a continuation phase of 4 months. The most common SCC regimen for new cases is 2 months of streptomycin (S), isoniazid (INH), rifampicin (RMP) and pyrazinamide (PZA) / followed by 4 months of isoniazid and rifampicin, or 2 months of ethambutol, isoniazid, rifampicin and pyrazinamide followed by 4 months of isoniazid and rifampicin, the latter being a fully oral regimen. Numerous studies have shown that the success rate for SCC treatment was almost 100% even when the treatment was administered intermittently, provided the treatment was adhered to fully by the patient.
2.1.c. Directly Observed Treatment Short Course (DOTS)

Most expert panels and professional organizations, including the Centre for Disease Control and Prevention, the American Thoracic Society, and the World Health Organization recommended the use of directly observed therapy for patients with active TB. Though many developing disease-endemic countries were slow to adopt such a strategy, for improving case detection and treatment completion in 1995 (when it was introduced), DOTS as of now has been successfully implemented in over 80 countries.

The DOTS strategy for TB control has the following five important elements (WHO 1995):

1. Political and administrative commitment
2. Sputum smear microscopy of the patient as the primary diagnostic tool
3. Direct observation of treatment to ensure that the patients are taking the drugs
4. Adequate supply of short course chemotherapy drugs and
5. Systematic monitoring and accountability for every patient diagnosed
WHO recommends 3 different categories of treatment.

Category 1:
Smear positive, seriously ill patients, and smear negative patients with extensive parenchymal involvement, are treated with INH, RMP, PZA and E, twice weekly for 2 months, and followed by INH and RMP thrice weekly for 4 months as continuation phase.

Category 2:
For patients who had previous history of treatment for a month or more, treatment is given for 2 months with INH, RMP, PZA, E and S thrice weekly followed by 1 month of INH, RMP, PZA and E thrice weekly and 5 months of INH, RMP and E thrice weekly.

Category 3:
For smear negative patients and patients with extra-pulmonary TB who are not seriously ill, treatment is given for 2 months with INH, RMP and PZA thrice weekly, followed by 4 months of INH and RMP thrice weekly.

To ensure the success of chemotherapy for tuberculosis by DOTS, several aspects are mandatory. They include, comprehensive restoration of control programmes, intensive educational campaigns, hospital isolation policies and individualized management. Standard SCC as recommended in DOTS programmes, could cure only a limited proportion of Multi drug resistant tuberculosis (MDR-TB) cases.
2.1.d. Multidrug resistance and Chemotherapy

2.1.e. Multi drug resistant tuberculosis

Cases of MDR-TB are defined as those with resistance at least to isoniazid and rifampicin with or without resistance to other antituberculosis drugs.

The WHO adopted certain definitions for declaring places with ‘high prevalence’ as hot spots. This “hot spots” in conjunction with rising HIV prevalence, pose a serious threat to tuberculosis control programmes. (WHO 1997)

In the past, most cases of MDR-TB evolved through multiple, ineffective courses of treatment. This is commonly referred to as acquired drug resistance. However, recently a new pattern of drug resistance has emerged due to the transmission of MDR-TB to contacts, which results in primary drug resistance. Epidemics of MDR-TB have been described mainly among populations with pre-existing AIDS/HIV infection.

2.1.f. Drug options of MDR-TB patients

MDR-TB is strictly defined as resistance to at least INH and RMP. It is a challenge to manage MDR-TB patients and the treatment of MDR-TB is expensive, less effective and more toxic when compared to drug-susceptible tuberculosis. MDR-TB patients were treated with individually tailored regimens in which they are given at least 3 or 4 drugs, which they had not received previously or to which they are known to be susceptible. Usually these drugs include
ethionamide, ethambutol, pyrazinamide, cycloserine, streptomycin or kanamycin, para amino salicylic acid and thioacetazone. Experience of scientists at Tuberculosis Research Centre (TRC) Chennai, has shown that patients with MDR-TB who have remained sputum positive for many years on treatment with other drugs, have converted to sputum negativity when changed to 600mg of isoniazid and para amino salicylic acid as a last resort. Recently there were some reports about the management of MDR-TB, with the drugs such as amoxycillin-calvulanic acid combination(Iwanaga et al., 1997), interferon-γ-via aerosol (Condos et al., 1997), rifabutin (Lee et al., 1996) and recombinant human interleukin (Johnson et al., 1997). MDR-TB patients require the treatment of atleast four drugs including a quinolone and an injectable drug like aminoglycoside or capreomycin. The regimen should be given for 3 months or until sputum has become negative on direct smear for 2 months which ever is longer. Recent studies suggested that the treatment of MDR-TB patient is improved when quinolone is included in the multidrug regimen. Fluoroquinolones have marked antimycobacterial activity. Among the quinolones, ciprofloxacin and ofloxacin have been extensively used with other anti-tuberculosis drugs with a moderate success (Tsukamura et al., 1985; Yew et al., 2000).

2.2. Antituberculosis Drugs

In 1910 Paul Ehrlich coined the term "Magic Bullet" for a chemical that could kill an infectious agent and thus opened the gate for the age of modern chemotherapy. This approach led to the discovery of Protosil and its active
ingredient, sulfanilamide by Gerhard Domagk in 1935. The derivative of sulfanilamide and sulphones such as promin and promizole were shown to have some inhibitory activity against tubercle bacilli in vitro and in experimental animals. Later, Alexander Fleming suggested another approach to the development of antimicrobials by discovering penicillin from the fungus *Penicillium notatum* for killing Staphylococci (Fleming 1929).

2.2.a. Streptomycin (S)

In 1944 Albert Schatz discovered streptomycin from the species *Streptomyces griseus* and tested the action against *Mycobacterium tuberculosis* and found complete inhibition of growth even in very small concentrations (Schatz *et al.*, 1944). The first clinical use of streptomycin was done by Pfuetze, Pyle, Hinshaw and Feldman in November 1954 on a young woman at a concentration of 1.2 gm given daily for a period of 1-3 months (Fledman 1954; Hinshaw 1954). Frequent administration and the duration of the treatment led to the emergence of streptomycin resistance. So it was curtailed from clinical use as a monotherapy.

2.2.b. Para amino salicylic acid (PAS)

Para amino salicylic acid was discovered by Jorgen Lehmann in 1943 and it was the first effective agent that prevented the emergence of streptomycin resistance when these two drugs were given concurrently. However the need is to administer to large doses of PAS and frequent gastro-intestinal intolerance
served as limitations for large scale usage of this drug at a later period (William Harris 1995).

2.2.c. Isoniazid (INH)

Isonicotinic acid hydrazide (INH) or isoniazid was synthesized as early as 1912, and was developed in 1951 to 1952 independently by Hoffman-La Roche company and the Squibb Institute for medical research in the United States and also by the Bayer company in Germany. They found INH to have a marked bactericidal effect on *M. tuberculosis* *in vitro* and in experimental animals even in low concentrations. Furthermore, it was equally effective against organisms resistant to streptomycin and PAS. Due to its high bactericidal activity, INH remains as a sheet anchor drug both in the initial as well as in the continuation phase of chemotherapy of tuberculosis(William Harris 1995).

2.2.d Rifampicin (RMP)

Rifampicin is a broad spectrum antibiotic. Rifampicin was discovered in 1957 at Lapetit laboratories of Milan in Italy, from *Streptomyces mediterrani*. Rifampicin was prepared by a synthetic modification of rifamycin. It has good activity against mycobacteria and has a much superior sterilizing effect than any antibiotic tested against *M. tuberculosis, M.kanssii, and M.marinum*. However, *M. avium intracellulare* and *M.fortuitum* are uniformly resistant. This drug has more effect on bacteria that show spurt in metabolic activity (Dickinson *et al.*, 1981).
2.2.e. Ethambutol (E)

In 1961, Wilkinson and his associates discovered the antituberculosis activity of ethambutol. Shepherd and Wilkinson modified this compound in 1962 and then Lederle introduced it for clinical use in 1966.

2.2.f. Pyrazinamide (PZA)

Pyrazinamide is a structural analogue of nicotinamide, and structurally similar to isoniazid. Although it is an effective anti tuberculous drug, its usefulness has been limited because of adverse reactions and the rapid development of bacterial resistance. Its activity gets augmented when it is combined with isoniazid during the initial phase of treatment. Under *in vitro* conditions, its optimal antituberculous activity was observed at a pH of 5.0 - 5.5. It was hypothesized that the *in vivo* activity of PZA is dependent on the bacilli present in the acidic environment of caseous tissue. Pyrazinamide remains as an important first line anti tuberculosis drugs, which is currently used in the intensive phase of short course regimens under DOTS (Mitchison 1992; WHO 1995).

2.3. Drug Resistance in *Mycobacterium tuberculosis*

In *Mycobacterium tuberculosis*, drug resistance in a large bacterial population occurs by random, single step, spontaneous mutation at low predictable frequency. Unlike other pathogens, plasmids or transposons do not play a role in antibiotic resistance in *Mycobacterium tuberculosis*. The probability of development of drug resistant mutants in a population of *Mycobacterium*
tuberculosis is given by a formula $p = 1 - (1 - r)^n$ where $p$ is the probability of the incidence of drug resistant cases, $r$ is the probability of incidence of drug resistant mutants and $n$ is the no of bacilli in the lesion (Shimao 1987). The value of $r$ for rifampicin is $10^{-8}$, and that for isoniazid, streptomycin, kanamycin ethambutol and para amino salicylic acid is $10^{-6}$. The probability of resistance to rifampicin and isoniazid is the product of the probabilities of drug resistance to a single drug ie $1 \times 10^{-8} \times 1 \times 10^{-6}$ which is $1 \times 10^{-14}$. A medium sized cavity of 2.5 cm diameter, which might be seen in a patient with open pulmonary tuberculosis, is estimated to contain about $10^{10}$ bacilli. In that population a few bacilli are likely to be resistant to isoniazid and a few to rifampicin but none resistant to both drugs. This is the fundamental principle that MDR-TB is an iatrogenic problem (Mitchison et al., 1956).

The emergence of drug resistance may be in five main strategies

- Decreased permeability
- Increased efflux
- Drug inactivation
- By-Pass metabolism
- Target modification

In Mycobacterium tuberculosis the drug resistance is by chromosomal gene mutations.
2.3.1. Molecular Mechanism of Drug Resistance

2.3.1.a Streptomycin (S)

Streptomycin acts on *Mycobacterium tuberculosis* by disrupting the decoding of amino acyl–tRNA and this inhibits mRNA translation and decreases the ribosomal proof reading capacity thereby leading to the formation of incorrect amino acids in the nascent polypeptide chains. Streptomycin binds to ribosome, in the ratio of one drug molecule per ribosome and S resistant ribosomes have less affinity to bind with the drug. Mutations in S 12 ribosomal protein encoded by *rps L* gene and in rrs operon encoding 16S rRNA, are the major causes for S resistance. The common site for mutations conferring resistance to S is *rps L*, the gene encoding S 12 protein of the small ribosome. The mutations usually occur at amino acid positions 43 or 83. The most frequently occurring mutations are Lys43Arg, Lys88Arg and Lys43Thr; Lys88Gln is also being observed occasionally (Cooksey *et al.*, 1996). Streptomycin, which does not bind to S 12, has been expected to bind to two regions on 16S rRNA. The alteration to the functionally constrained loop of the 16SrRNA (rrs) that interacts with the S12 protein represents a second site. Mutations in *rrs* have been found in 5% to 33% in S resistant strains (Katsukawa *et al.*, 1997). Prof. Mitchison hypothesized grouping *Mycobacterium tuberculosis* strains into three populations based on their level of resistance (Mitchison 1951). In another study it was observed that the strains with amino acid substitutions in S12 demonstrate high level resistance and *rrs* mutations have moderate level of resistance and low level resistance might be due to permeability barrier (Meier *et al.*, 1994). Streptomycin resistance
in *Mycobacterium tuberculosis* is by the alteration of the target and not by drug modifications (Honore *et al.*, 1994).

### 2.3.1.b. Isoniazid (INH)

Isoniazid has been in wide-spread use since the 1960s but its mechanism of action and resistance are known only recently. There is a lot of information available now on the genetic and biochemical processes involved in the action of INH. *In vitro* experiments had shown the action of INH on the cell wall mycolic acid biosynthesis in mycobacteria. The loss of mycolic acid seems to occur mainly in the outer envelope. It is also known that *M. tuberculosis* loses acid fastness under the influence of INH. Isoniazid inhibits the biosynthesis of cell wall mycolic acid, and thereby making mycobacteria susceptible to reactive oxygen radicals and other environmental factors. Drug activation to an unstable electrophilic intermediate requires the enzyme catalase peroxidase, coded by *Kat G*. Kat G is the only enzyme, which is capable of activating INH. *kat G* mutant mycobacterial strains are resistant to isoniazid (Sacchettini *et al.*, 1996). Later it was found that a second mechanism of low level resistance, involving enzymes in the mycolic acid biosynthesis were identified namely, NADH dependent enoyl-ACP reductase encoded by *inh A* and beta-keto acyl ACP synthase encoded by *kas A* (Wilson *et al.*, 1996). Isoniazid inhibits inhA by reacting with the NAD, a cofactor and bound to the enzyme active site, forming a covalent adduct, which has high affinity to the enzyme. Mutations in the inh A enzyme, at the active site increase the expression of *inh A* or alter the affinity for NADH without affecting
the enzymatic activity (Banerjee et al., 1994). The reduced binding of NADH to inh A protects the enzyme from binding to INH radicals, thereby it confers resistance to INH (Bardou et al., 1998). Mutation in the oxy R regulon, from which Ahp C is divergently transcribed, is also responsible for INH resistance. This oxy R gene confers intrinsic resistance to INH. Mutations in the kas A leads to INH resistance which occurs in addition to mutations in kat G and Inh A. Mutations in kat G and inh A genes are associated with 70-80 % of INH resistance (Rattan et al., 1998).

2.3.1.c. Rifampicin (RMP)

Rifampicin was introduced for use in antitubercular therapy in the early 1970s and is a very important component of current treatment regimens. Rifampicin binds to the β sub unit of RNA polymerase and results in transcription inhibition. The inhibitory effect was first shown by Hartmann and collegues (Sippel et al., 1970) Rifampicin is highly specific for the bacterial RNA polymerase enzyme which is made up of four subunits α,β,β’ and σ encoded by rpo A, rpo B, rpo C and rpo D. Concentrations of RMP at 10,000 times higher are needed to affect the corresponding mammalian enzyme. Mutations were mainly in the 81bp core region of rpoB gene, which encodes the enzyme DNA dependent RNA polymerase in more than 96% of RMP resistant clinical isolates. The resistance to RMP is associated with specific mutation in the rpo B gene region of codons 507 to 533 encoding 27 amino acids termed as RRDR region (Musser 1995). In general, there is a strong correlation of specific amino acid
substitutions and MIC. Missence mutations in codon 513, 526 and 531 result in high level RMP resistance (Telenti et al., 1993; Kapur et al., 1994). New mutations at codon 532, 508 and deletion of codons 517, 512, 516 533 and new mutations outside RRDR were seen in a study done at Tuberculosis Research Centre in Indian isolates. The molecular mechanism of resistance in R resistant tuberculosis isolates that lack mutations in rpo B region (which accounts for about 4%) is not known. This might be due to cell wall permeability, which is acting as a barrier to prevent the drug entry.

2.3.1.d. Pyrazinamide (PZA)

PZA is a synthetic derivative of nicotinamide that is being used in short course antituberculosis treatment regimens. Pyrazinamide acts on semi dormant bacteria in acidic environments or within macrophages. The mechanism of action of PZA resistance is not fully known. It has a structural analogue of nicotinamide and is a prodrug which should be converted to active form of pyrazinoic acid by pyrazinamidase which is encoded by pnc A. PZA susceptible M.tuberculosis strains have an enzyme pyrazinamidase that metabolises PZA to pyrazinoic acid whereas PZA resistant organisms have lost this activity. Mutations in pnc A gene, coding for pyrazinamidase result in PZA resistance. Resistant strains have mutations cys138 ser, Glu141Pro, Asp63His and deletion of G at position 162 and 288. The resistance without mutations was also due to the efflux mechanism and permeability barrier in the cells (Mitchison 1992; Hirano et al., 1999)
2.3.1.e. Ethambutol

Ethambutol is an effective first line drug that is being used in combination with other anti tuberculosis drugs to treat *M.tuberculosis* infection. This drug inhibits the transfer of D-arabinose into cell wall arabinogalactams, which are arabinose containing complex branched polysaccharides that connect the mycolic acid to the inner peptidoglycan of the cell wall. Resistance mutations were found in a group of genes encoding arabinosyl transferases which is termed as the *emb* operon. In *Mycobacterium tuberculosis* this operon is composed of three arabinosyl transferases *embC*, *embA*, and *embB*. However, almost all EMB mutations were in Emb B protein at position 306, usually methionine is replaced by leucine or isoleucine, which confers high-level resistance. This region is termed as EMB resistance determining region (ERDR). Mutations in *emb B* are associated with 70% of E resistant *M.tuberculosis*. (Mikusova *et al.*, 1995; Sreevatsan *et al.*, 1997; Telenti *et al.*, 1997) Other substitution in 285, 330 and 630 amino acids were also found to have low level resistance.

2.3.1.f. Ethionamide

The mechanism of action for ethionamide on *M.tuberculosis* is similar to that of isoniazid. It is a pro-drug which when activated inhibits mycolic acid synthesis. It does not seem to require the catalase peroxidase system for its
activity. Mutation in \textit{inhA} gene may confer cross-resistance with isoniazid in low level resistance group (Banerjee \textit{et al.}, 1994).

2.3.1.g. Kanamycin

The molecular basis of drug resistance to kanamycin is not fully established. It is likely to be involved as an inhibitor of protein synthesis. Mutation of \textit{rrs} gene conferring a high level kanamycin resistance has been reported (Alangaden \textit{et al.}, 1998; Suzuki \textit{et al.}, 1998)

2.3.1.h. Fluoroquinolones

The primary target of quinolone in \textit{Mycobacterium tuberculosis} is DNA gyrase, a type II topoisomerase, which is composed of two sub units of A and two units of B. Missense mutations in the putative fluoroquinolone binding region of the A sub unit have been found to confer high level resistance and is referred to as quinolone resistance-determining region (QRDR). The codons 90, 91, 94 and 95 were identified in \textit{gyr A} to have mutations in drug resistant strains (Liu \textit{et al.}, 1996). The other mechanism that has been implicated in fluoroquinolones is the increased expression of fluoroquinolone transporting efflux pumps, which also could play a role in both low-level and high level resistance. A low level resistance was identified in \textit{M. smegmatis}, which is due to the efflux pump Lfr A but it had no homologs in \textit{M. tuberculosis} though it has 15 putative efflux pumps (Xu \textit{et al.}, 1996) and the mechanism of action is explained in detail in the chapter on Quinolone.
2.4. Drug Resistance

Studies carried out in many disease endemic developing countries gave rationale and optimal regimens to treat tuberculosis patients harboring drug susceptible organisms. Although a fair amount of success has been obtained for treating patients harboring *M.tuberculosis* strains resistant to INH or S, there are no good treatment regimens available for patients harboring strains resistant to INH and RMP (MDR-TB). A well conducted TB control programme would result in the overall reduction of drug resistance over a period of time. A poorly conducted programme would result in the generation of patients harboring drug resistant tubercle bacilli in the community. Hence, it is necessary to conduct periodically drug resistance surveillance to monitor the level of drug resistance in the community. This will serve as one of the epidemiological indicators, to assess the level of drug resistance, and also to determine resistant bacterial transmission in the community. The level of drug resistance is also an indicator of the success or the failure of control programmes (Paramasivan *et al.*, 2004).

2.4.1. Definition of Drug Resistance

Mitchison defined drug Resistance as “a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type, that have never come into contact with the drug” (Canetti *et al.*, 1969)
2.4.2. The factors contributing to the emergence of drug resistance

- Deficient or deteriorating tuberculosis control programmes resulting in inadequate administration of effective chemotherapy,
  - poor case holding,
  - poor quality of drugs
  - inadequate or irregular drug supply.
  - lack of supervision
- Inadequate training of healthcare workers regarding epidemiology, treatment and control of tuberculosis
- Improper prescription of treatment regimens
- Interruption of chemotherapy due to side effects
- Non adherence of patient to prescribed drug therapy
- Increase in the number of tuberculosis patients with easy access to anti tuberculosis medication
- Massive bacillary load
- Illiteracy and socio economic status of the patients
- The epidemic of HIV infection
- Laboratory delays in identification and susceptibility testing of *Mycobacterium tuberculosis* isolates
- Use of anti–TB drugs for indications other than tuberculosis (Paramasivan *et al.*, 2004)
2.4.3. Classification of Drug Resistance

Drug resistance is broadly classified into four categories (Jacobs 1994)

- Primary Drug Resistance
- Acquired Drug Resistance
- Initial Drug Resistance
- Transitional Drug Resistance

2.4.4. Primary Drug Resistance

Primary resistance is defined as the presence of drug resistance in a tuberculosis patient who has never received prior treatment with anti tuberculosis drugs. It is caused by infection with resistant organisms from another patient excreting drug resistant organisms. It is an indicator of tuberculosis control efforts in the past.

2.4.5 Acquired Drug Resistance

Acquired Drug Resistance is defined as resistance, which arises during or after the course of treatment as a result of non-adherence to the recommended regimen or faulty prescription. A high level of this type of resistance is known to be a mark of a poorly functioning tuberculosis control programme.

2.4.6. Initial Drug Resistance

Initial Drug Resistance is defined as the resistance presented by patients with resistant organism prior to the commencement of therapy, since there is a
likelihood of a mixture of true primary drug resistance and acquired drug resistance because of patient’s ignorance about drugs prescribed earlier or deliberate concealing of information regarding prior treatment.

2.4.7. Transitional Drug Resistance

Transitional Drug Resistance is defined as the resistance to one or more drugs, which emerges in the course of successful chemotherapy just before sputum conversion.

Currently two definitions of resistance namely, (a) resistance among new cases of tuberculosis and (b) resistance among previously treated cases have been agreed upon by the WHO and the International Union against TB and Lung Diseases (Union) based on extensive discussions held at several meetings for the following reasons. Although ‘Primary drug resistance’ is well accepted, it is known that it is extremely difficult to ensure the absence of previous treatment. Hence the terminology ‘resistance among new cases of tuberculosis’ has been preferred to primary resistance. Likewise, resistance among previously treated cases is subtle more from acquired resistance (www./who.int/gtb/)

From a microbiological point of view, every culture of tubercle bacilli would contain some proportion of mutant organisms. It arises exclusively by spontaneous mutations that occur at random in the bacterial population and not by adaptation after exposure to the drug.
2.5. Newer drugs

The discovery of a new drug starts with the identification of an active principle, which consists of either a pure compound or a mixture of structurally related compounds with potent antimicrobial activity on bacterial cells \textit{in vitro}. The active principle is usually identified either by chance or random screening. The compound is then tested in appropriate animal models and those showing antimicrobial activity are selected and then processed for testing in humans. Historically most of the successful antimicrobial agents are natural products with potent \textit{in vitro} activity against the microbe of interest. A pharmacology-based approach was adopted to optimize and select compounds with increased potency, which resulted in the identification of potent antimicrobial drugs. The era of molecular biology and genome mapping have enabled the identification of targets, which are specific to the microbe, but are absent or structurally different in the human host. Availability of the structure of the target and the ability to design chemical molecules based on co-crystals of the target with the inhibitor, have significantly improved the chances of identifying specific and selective antimicrobial drugs (Balganesh 2004)

The following are some of the reasons for the need of newer drugs against tuberculosis

1. To improve the current treatment by shortening the durations of treatment and/or for providing more widely spread intermittent treatment

2. To improve the treatment of MDR-TB, and

3. To provide more effective treatment against latent tuberculosis.
Newer drugs are necessary so that it would be effective against drug resistant strains and also against bacilli that may be in a state of latency. Some of the promising new agents are the longer-acting rifamycin derivaties, flouroquinolones, oxazolidinones and nitroimadazopyran etc. There are, as yet, no extensive clinical trial data available to determine the efficacy of these compounds.

2.5.1. Rifamycin derivatives

Rifapentine is a new long acting derivative of rifamycin and is the only addition after 35 years after the introduction of rifampicin (Dhillon et al., 1992; Moghazeh et al., 1996). Once a week administration of rifapentine along with other anti – tuberculosis drugs for a duration of four months was found to be adequate. Other long acting rifamycin derivatives include KRM –1648 and SPA–S-565. Rifazil, a novel compound was observed to be 100 times more potent than rifampicin and has a longer half-life. (Emergency medicine 2002)

2.5.2. Oxazolidinones

Oxazolidinone is a new class of broad –spectrum antibacterial agent acting on protein synthesis, also possess antimycobacterial activity. One of these agents, U-100480, was developed recently against *Mycobacterium tuberculosis* (Cynamon et al., 1999).
2.5.3. Nitroimidazopyrans

This compound has been found to be significantly active in experimental animal models. Significant in vivo activity was found in murine model when administered orally (Bishai et al., 2000).

2.5.4. Other newer compounds

2.5.4.1. Diarylquinoline or DARQ (R 207910), by Johnson & Johnson:

It showed the best activity against a broad range of mycobacteria especially *M. tuberculosis*. It is active against both the drug sensitive and drug resistant forms of *M. tuberculosis*. This compound acts by blocking the function of an essential membrane bound enzyme (proton pump) that makes adenosine triphosphate (ATP) (Andries et al., 2005). In mouse model it showed increased activity thereby shortening the treatment period by 50%.

2.5.4.2. Newer compounds in the preclinical stage

Pyrrole, LL-3858 by Lupin, Nonfluorinated quinolone by Procter & Gamble and ethambutol analog by Sequella are some of the new drugs which are in the preclinical stages.

2.5.5. Quinolones

Quinolone class of synthetic antimicrobial agents, particularly Nalidixic acid, was used for the treatment of urinary tract infections for many years. Over the last two decades, research on 4-quinolones-3-carboxylates has led to the
Fig 2
Structures of Quinolones

Nalidixic acid

Norfloxacin

Ofloxacin

Ciprofloxacin

Lomefloxacin

Enoxacin

Sparfloxacin

Gatifloxacin

Trovaflaxacin

Moxifloxacin
discovery of a family of 6-fluoro-7-piperazinyl-4-quinolones collectively called as fluoroquinolones. These were active against Gram negative and Gram-positive organisms. These compounds were active under both in vitro and in vivo conditions. Although many of the fluoroquinolones have been synthesized and reported, the most important ones, which are active against tuberculosis, are ciprofloxacin, ofloxacin, levofloxacin, lomefloxacin, enoxacin, sparfloxacin, and the new generations drugs-gatifloxacin and moxifloxacin.

2.5.5.a. Chemistry of fluoroquinolones

Fluoroquinolones are derived from nalidixic acid. In the bicyclical aromatic ring structure of quinolone, position 1 nitrogen is attached with an alkyl group, carboxylic acid is present at position 3 and a keto group at position 4. The carboxylic acid and the keto group increase the antimicrobial activity of this compound. Many improvements have been made on this structure to enhance its effect. Substitution of fluorine atom at position 6 on the quinolone carboxylic acid nucleus increases the efficacy of these drugs on gram-negative pathogens and broadens the spectrum of activity against gram-positive pathogens. A basic nitrogen-containing moiety enhances tissue penetration and reduces central nervous system toxicity. The structure of the ring has been largely modified to enhance the antimicrobial activity and to increase the volume of distribution of the molecule. These quinolones bear the acidic group as well as the basic group and this association gives them amphoteric properties. Their solubility is low, except between pH 6 and 8.
2.5.5.b. Antimicrobial activity of fluoroquinolones

Fluoroquinolones bind to the enzyme Gyrase present in the microbes to form a ternary complex, Drug - Gyrase – DNA complex, thereby arresting the covalent intermediate of DNA super coiling reaction. DNA gyrase is a bacterial intracellular enzyme. The uptake of fluoroquinolones by the bacteria is critically important for drug action (Hooper et al., 1985). The drug enters into the bacterial cells via porins (Chapman et al., 1988). Subsequent entry across the cytoplasmic membrane is dependent on the physicochemical properties of the fluoroquinolone. Fluoroquinolones accumulate within bacteria rapidly; within a few minutes, a steady – state intrabacterial concentration is obtained (Piddock 1994). These fluoroquinolones are very active in phagocytic cells and also under alkaline environments (pH >7.4) for gram negative bacteria. (Blaser et al., 1988) The activity of fluoroquinolones is antagonized by cations such as magnesium and calcium, perhaps by binding to the cell surface ((Kotera et al., 1991).
Table 1: Generations of quinolones

<table>
<thead>
<tr>
<th>Quinolone generations</th>
<th>Names of quinolones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st generation</td>
<td>Nalidixic acid</td>
</tr>
<tr>
<td></td>
<td>Cinoxacin</td>
</tr>
<tr>
<td>2nd generation</td>
<td>Lomefloxacin</td>
</tr>
<tr>
<td>Class I</td>
<td>Norfloxacin</td>
</tr>
<tr>
<td></td>
<td>Enoxacin</td>
</tr>
<tr>
<td></td>
<td>Ofloxacin</td>
</tr>
<tr>
<td>Class II</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>3rd generation</td>
<td>Levofloxacin</td>
</tr>
<tr>
<td></td>
<td>Sparfloxacin</td>
</tr>
<tr>
<td></td>
<td>Gatifloxacin</td>
</tr>
<tr>
<td></td>
<td>Moxifloxacin</td>
</tr>
<tr>
<td>4th generation</td>
<td>Trovafloxacin</td>
</tr>
</tbody>
</table>

(Oliphant et al., 2002).

2.5.5.c. Mode of action of fluoroquinolones

The diagram below (Fig 3) details the entry of quinolones into the cells. Quinolones must pass through cell membrane to reach their topoisomerase targets. The degree to which they cross cell membrane varies with bacterial species (www.Medscape.com)
Quinolones act on bacterial gyrase only after their entry into cells.

Criteria for an efficient drug target are as follows

- The protein or enzyme should exhibit important function essential for cell survival
- Presence of target enzyme only in bacteria and not in eukaryotes
- In the event of ubiquitous presence of target enzyme, the target molecule in bacteria should have characteristics distinct from the eukaryotic counterparts
- The properties of the target enzyme in the pathogenic bacteria are different from those of the other bacteria

DNA gyrase, the drug target of fluoroquinolones, fulfills the above criteria. (Mahadevan et al., 2000)
2.5.5.d. Gyrase

Bacteria possess type II topoisomerase known as DNA gyrase, a tetrameric bacterial enzyme that folds the circular bacterial DNA to such an extent that it can fit into the bacteria several thousand times shorter than it. This is made up of two A and two B subunits (gyr A and gyr B).

Gyrase plays the following four important roles in chromosome function: (Drlica et al., 1997) To maintain a level of negative super coiling, which activates the chromosome in strand separation

- To facilitate the movement of replication fork and transcription fork through DNA
- To remove the knots from DNA
- To help DNA to bend and fold
During DNA replication (Fig 4), one region of duplex DNA is passed through another via DNA breakage and rejoining. During the super coiling of DNA, which is catalyzed by DNA gyrase A sub unit, it aligns DNA into a relaxed form that has decreased susceptibility to fragmentation and increased ease of separation during strand replications and the energy is supplied by gyrase B unit in the form of ATP. Drugs like coumermycin and novobiocin are the inhibitors of the ATPase activity of the B sub unit of DNA gyrase (Fernandes 1988). The diagrammatic representation (Fig 5) shows the mechanism of drug action of quinolones in the bacterial DNA.
Quinolone molecule binds to gyrase induced by DNA during the intermediate – gate opening step of the DNA negative super coiling, via hydrogen bond to the unpaired bases. Gyrase A unit forms covalent bonds between tyrosine 122 and the 5' end of the DNA chain. ATP that is supplied by gyrase B unit is required for the induction of the drug-binding site. Quinolone–gyrase–DNA complex prevents cell division and homologous recombination, which in turn leads to cell death.

(Shen et al., 1989)
2.5.5.e. Resistance mechanism for drug quinolones

The mechanism of fluoroquinolone resistance in the bacteria are by

- The intrinsic lack of permeability,
- Alteration in the porin expression and efflux mechanism and
- Alteration in the drug target-DNA gyrase

The mechanism of resistance to quinolones is explained diagrammatically as follows (Fig 6): 

**Fig 6: Resistance by decreased permeability or by increased efflux mechanism**

As mentioned earlier, intrinsic lack of permeability and alterations in the porin expression and efflux are the important reasons for resistance. The outer
membrane of certain gram-negative organisms limits their uptake of fluoroquinolones.

Altered expression of outer cell membrane channels (porins) may limit the fluoroquinolones ability to permeate the membrane of gram-negative bacteria. The development and expression of efflux pumps that transport quinolones out of the cell is another cause of resistance mechanism. Mutations that occur in the outer and inner membrane proteins of bacteria allow them to resist the action of quinolones (Chapman et al., 1988)

For hydrophilic fluoroquinolones, an efflux pump, LFR A was identified in *M. smegmatis* which was found to confer low level resistance. But this does not hold good to hydrophobic fluoroquinolones like sparfloxacin. Although *M. tuberculosis* was found to have 15 putative pumps (Cole et al., 1998) none of them closely resembles Ifr A and their role in the mechanism of resistance was unknown

Mutations conferring resistance typically occur in stepwise manner. Spontaneous mutations develop a small mutant population with decreased susceptibility to quinolones. Intermediate quinolone concentration can inhibit susceptible strains and allow overgrowth of a resistant first step mutant. Second mutation occur in first step mutants further raising MICs. This process will continue and it augments other mechanisms such as decreased permeability or efflux.
2.5.5.f. Resistance by alteration of the drug targeting DNA gyrase

Gyrase introduces changes in the topology of closed circular DNA by cleaving the helix in both strands, passing another segment of DNA through the break, and resealing the broken ends. The energy is supplied by gyrase B subunit in the form of ATP. Quinolone exert their toxicity on the bacterial cell by stabilizing the double stranded break in DNA created by gyrase so that religation becomes unfavorable. The ternary complex Quinolone–gyrase–DNA, blocks the cell transcription and more importantly in terms of cell survival, DNA replication. Recent crystal structure determination of fragments of yeast topoisomerase II and gyr A protein suggest that the quinolone resistance mutations in gyr A cluster around the active site for DNA cleavage, and thereby forming a quinolone binding pocket (Drlica et al., 1997). The first site identified with fluoroquinolone resistant strains was a short segment of gyr A, termed as the “Quinolone Resistant Determining Region” (QRDR). The majority of the clinical isolates contain substitutions between positions 67 and 106 of Gyr A, leading to the categorization of this section as the “QRDR”. The sequence of M. tuberculosis chromosome showed that there was no topoisomerase IV but the same function is being performed by gyrase, in decatenating the newly replicated sister chromosomes. The most frequently mutated three amino acids observed were 90,91 and 94 and it is equivalent to the commonly mutated amino acids in E.coli, 83, 84, 87 and in S.aureus 84,85, 88. So far Gyr B mutation has not been found in fluoroquinolone resistant clinical isolates. (Nakamura 1997). It was presumed that the mutant amino acids alter the protein structure so as to reduce
fluoroquinolone binding to the gyrase-DNA complex, thus leading to resistance to fluoroquinolone. Although the QRDR region of gyrase A subunit was the most important site for mutations for conferring fluoroquinolone resistance, other sites are also responsible for low level resistance and for the varying levels of resistance with identical mutation in gyrase A region in different isolates (Takiff et al., 1994). The fluoroquinolones with C8 methoxy and halogen compounds have sufficient affinity for the gyrase that they are active against M. tuberculosis isolates containing a gyr A mutation (Dong et al., 1998). However, if M. tuberculosis has two gyr A mutations, even these drugs are not effective.

2.5.5.g. Drug Interactions

The oral absorption of fluoroquinolones is drastically decreased by antacids, which contain magnesium and aluminum and other agents such as sucralfate. These compounds decrease the absorption of fluoroquinolones. Ciprofloxacin absorption however was not affected by ranitidine (Nix et al., 1989).

2.5.5.h. Fluoroquinolones for treatment of tuberculosis

Fluorination of the 4-quinolone ring at the 6-position gave the class increased antimicrobial and a wider spectrum of activity. Norfloxacin, a broad spectrum antibiotic was developed in 1970 and released for clinical use in 1982. Both ofloxacin and ciprofloxacin remain biologically active against intracellular organisms, including M. tuberculosis, M. fortuitum and M. avium – intracellulare. Ofloxacin has been shown to be bactericidal against M.tuberculosis in cultured
human macrophages at concentration close to the in vitro MIC (Crowle et al., 1988). Both in vitro and in vivo studies have identified ofloxacin as a potential drug in the treatment of MDR-TB. Tsukamura et al reported that six to eight months of ofloxacin therapy in 9 cases of cavitary pulmonary tuberculosis resistant to various drugs, resulted in reduced number of viable bacilli in the sputum in all the cases, and in five cases there was also culture conversion (Tsukamura et al., 1985). In an uncontrolled clinical study of ofloxacin, containing 17 patients carried out by the Hong Kong Chest Services and BMRC, it was found that ofloxacin was effective for 10 patients in treating MDR - TB at a dosage of 800 mg daily (HongkongChestService/BritishMedicalResearchCouncil 1992). A prospective comparative study of Khono et al reported that when 29 MDR-TB patients were treated with ofloxacin, 10 of them got converted and became sputum negative. In this study they have also demonstrated that ofloxacin was as effective as ethambutol for the treatment of pulmonary tuberculosis when given with isoniazid and rifampicin (Kohno et al., 1992). These studies suggested that ofloxacin might be useful for the treatment of MDR-TB patients.

An in vitro study at TRC, Chennai, showed that ofloxacin would be useful in the early stages of treatment and in preventing the emergence of resistance to other antituberculosis drugs (Herbert et al., 1996). A multicentric study of early bactericidal activity (EBA) of antituberculosis drugs undertaken at four centers, in Cape Town, Nairobi, Chennai and Hong Kong, reported that ofloxacin had moderately high early bactericidal activity at a concentration of 800 mg compared
o 500 mg of ciprofloxacin (Sirgel et al., 2000). All these studies formed the basis to conduct a controlled clinical trial at TRC, Chennai, with ofloxacin-containing regimens for a reduced duration of 3–5 months. The findings of this study showed that regimens containing ofloxacin for 4-5 months duration achieved >95% efficacy with minimal relapses. There was also no increased incidence of adverse reactions. This was a step forward in the treatment for smear positive pulmonary tuberculosis for less than 6 months (TuberculosisResearchCentre 2002).

Unlike other antimycobacterial agents, fluoroquinolones are used widely for treating other bacteria. Because of their utility in treating the enteric infections, where Mycobacterial disease is endemic, the selection of resistance to fluoroquinolone in these regions may take place even before they are used in the treatment of mycobacterial infections.

2.6. Evaluation of New Antimycobacterial Agents

To evaluate the antimicrobial agents, which will determine the efficacy of new drugs alone and in combination with other anti tuberculosis drugs against Mycobacterium tuberculosis, suitable surrogate markers are needed. This includes, in vitro and in vivo models that simulate the initial and the continuation phase of treatment, i.e. to study early bactericidal activity (EBA) on log phase culture and sterilizing activity (SA) in stationary phase culture which is necessary to kill all sub populations, and EBA studies in patients by sputum viable counts after administration of drugs during the first few days.
Prof. Mitchison hypothesized the presence of four different bacterial populations in the affected lung and action of different anti tuberculosis drugs when the bacilli are present in a particular metabolic state (Fig 7).

**Fig 7: Special population Hypothesis—Prof. D.A. Mitchison**

Isoniazid is mainly responsible for the killing of fast growing bacilli (A) in the lesions during the first few days of treatment, but all other drugs kill less actively multiplying organisms in that population. From the start of treatment the remaining two special populations of bacilli (C and B) are killed most effectively by the two sterilizing drugs, namely, rifampicin and pyrazinamide. One of these includes bacilli that have short spurts of active metabolism (C) for sufficiently long to be killed by rifampicin but not by isoniazid. The other population (B),
sufficiently inhibited by an acid environment to hinder the activity of isoniazid, but susceptible to pyrazinamide. The other population which are metabolically inactive are dormant (D) and remain for a long period as persisters (Mitchison 1992). Metronidazole, a commonly used drug has a greater activity both in vitro and in vivo on non-replicating bacteria (persisters). When combined with rifampicin, it exhibited an additive effect against persisters under in vitro conditions (Wayne et al., 1996) and in animal studies (Paramasivan et al., 1998). Isocitrate lyase inhibitors like 3-bromo pyruvate and 3-nitro propionate would be newer avenues to eliminate persisters (McKinney et al., 2000).

Hence, drugs used in the treatment of tuberculosis can be broadly classified as drugs having Early bactericidal activity (EBA) and those having Sterilizing activity (SA). However, systematic studies are required to evaluate the activity of these drugs in the treatment of tuberculosis. Such evaluations may include,

1. In vitro studies to determine their MIC and Minimum bactericidal concentration (MBC)
2. In vivo experiments in animal models
3. In vitro simulation studies, attempting to mimic in vivo conditions
4. In vitro experiments in macrophages and cell cultures
5. Observations in patients (EBA studies and controlled clinical trials)
6. Determining resistance by molecular methods comparing the mutation of resistant strains and their susceptibility pattern
2.6.1. *In vivo* experiments (animal models)

*In vivo* testing of antibiotics by animal experiments has been recognized as an established link between the demonstration of activity *in vitro* and clinical trials in humans. Grumbach *et al.* reported that the evaluation of different treatment regimens could be established in murine model (Grumbach *et al.*, 1969). Grosset *et al.* reported the activity of different anti tuberculosis drugs during initial phase of chemotherapy and continuation phase of chemotherapy. They compared the activities of seven regimens, that contain several once weekly rifapentine combinations, in comparison with the standard daily rifampicin, isoniazid and pyrazinamide regimen during an 8-week initial phase of treatment in mice. These studies on rifapentine helped to obtain the approval of FDA in 1998 (Grosset *et al.*, 1998). Cornell mouse model developed for latent tuberculosis (McCune *et al.*, 1956) opened avenues for several researchers to pursue studies on latency in the subsequent decades. Thus animal models have become established norms to evaluate the bactericidal activity of new drugs.

To mimic the *in vivo* activity, *in vitro* simulation experiments were done in an *in vitro* condition by exposing the culture in log phase and in stationary phase to drugs alone and in combinations.
2.6.2. Evaluation of new drugs by determining early bactericidal activity (EBA)

Individual drugs differ greatly in their relative activities during the early bactericidal activity (EBA) and sterilizing activity (SA). Sterilizing activity of any drug is termed as the ability of the drug to prevent relapse because it is assumed to kill nearly all bacilli remaining after the initial phase of therapy. For assessing any newer drug in the treatment, *in vivo* EBA has been suggested as a surrogate marker. The first study of EBA was performed at Nairobi in 1974 with a population of 124 patients. Studies on patients by determining EBA of new drugs have certain limitations, since these studies do not measure the sterilizing activity of drug against bacilli that persist despite effective drug treatment and are responsible for prolonging the treatment period. Hence *in vitro* simulation experiments to find out the EBA and SA of a drug were undertaken for newer drugs. It is also necessary to determine the Minimal Inhibitory Concentrations (MIC), and the Minimal Bactericidal Concentration (MBC), which is defined as the lowest concentration of drug that kills more than 99.9% of the bacterial population in a broth culture and critical concentration of these drugs, which will help in the treatment of the patients respectively.
2.6.3. Evaluation of new antimycobacterial drugs by determining critical concentration by susceptibility testing methods

Critical concentration or the break point concentration of a particular drug is the concentration, which will be attained at the site of infection that will determine a strain to be sensitive or resistant.

Critical concentration of the conventional anti tuberculosis drugs has proved to be a reliable criteria for testing the susceptibility of *M. tuberculosis* isolates. There is only one practical way to evaluate the potential of new antituberculosis agents against *M. tuberculosis* (i.e.) *in vitro* determination of the MIC and comparing it with the achievable serum tissue concentrations. Susceptibility testing is not only the first step in the selection of potentially active drugs against *M. tuberculosis* but also the only option for testing of the mycobacterial isolates before and during the course of chemotherapy with any new drug.

Thus the *in vitro* EBA and drug susceptibility testing play an important role in evaluating the efficacy of new antimycobacterial drugs against *M. tuberculosis*.

2.6.3.1. Drug Susceptibility Testing Methods

To determine any standard regimen for tuberculosis patients, drug susceptibility testing (DST) may be of importance. The main requirement for DST is the ability to make a distinction between susceptible and resistant *M. tuberculosis* strains. And also the purpose of using susceptibility testing is to
understand the mechanism of chemotherapy, for epidemiological studies, for planning wide scale treatment and to treat patients with an optimal treatment regimen. This can be done by conventional methods, based on the growth of culture.

2.6.3.1.a. Conventional methods

The conventional methods of susceptibility testing are to determine the differences in the degree of resistance, which may reflect the differences in the proportions of resistant mutants in the bacterial population. The conventional methods of drug susceptibility testing are broadly classified into Direct methods and Indirect methods.

2.6.3.1.b. Direct method

Drug susceptibility testing is done directly from the digested and decontaminated clinical specimen in which AFB can be demonstrated in stained smears. In this method a set of drug containing and drug free media are inoculated directly with concentrated specimen. The advantage of direct method is that the results are available earlier and better represent the patient’s original bacterial population.

2.6.3.1.c. Indirect method

Drug susceptibility testing is done with the growth of organisms that were previously grown. In this method the pure culture is inoculated on to drug
containing and drug free media either in egg based L-J medium or agar based 7H11 medium.

2.6.3.1.d. Absolute concentration method

In this method a standard inoculum is allowed to grow on drug free and drug containing media in different concentrations of the drugs to be tested. The resistance is expressed as the lowest concentration of the drug that inhibits the growth, that is minimal inhibitory concentration (MIC). It is affected by the viability of the organisms (Canetti et al., 1969)

2.6.3.1.e. Resistance ratio method

This method compares the growth of unknown strains of tubercle bacilli with that of standard laboratory maintained strain, *M.tuberculosis* H37Rv. Resistance is expressed as the ratio of the MIC of the test strain to the MIC of the standard strain in the same set. This test is also affected by the inoculum size as well as the viability of the organisms.

2.6.3.1.f. Proportion susceptibility testing Method

This method enables a precise estimation of the proportion of mutant strain, resistant to a given drug. Serial 10-fold dilutions of standard suspension were made and are inoculated on to drug free and drug containing media, in which at least one dilution should yield isolated countable colonies. The proportion of bacilli resistant to a given drug is then determined by expressing the
resistant portion as a percentage of the total population used (Canetti et al., 1969).

The other rapid phenotypic methods that are currently in vogue are BACTEC 460, MGIT 960, MB/Bact and ESP II system.

2.6.4. The need for rapid methods

The main requirements for a new rapid method depend upon the following;

- High intra and inter laboratory reproducibility in differentiating resistant from susceptible isolates, which is usually measured by the rates of discrepancies in repeat tests, and the rates of false-resistant and false susceptible reports.

- The shortest possible turnaround time between the receipt of the specimen and the availability of result.

- Applicability of simple bio safety measures

- Minimal investment in special technology beyond the standard laboratory equipments

- Minimal labor time and the ability to perform tests simultaneously with a large number of specimens

- Applicability to most, if not all, first line drugs, to second line drugs and to the new drugs
2.6.4.a. BACTEC 460 Radiometric method

The liquid medium for this system, 7H12 broth, currently manufactured as 12B vials contains 7H9 broth base casein hydrolysate, bovine serum albumin, catalase, and $^{14}$C substrate palmitic acid. The bacterial growth results in the release of the $^{14}$CO$_2$, the amount of which is expressed as a growth index (GI) on a scale of 0 to 999. In the presence of an antimicrobial agent, the susceptibility is detected by the inhibition of daily GI increase. The major advantages of this technique is the ability to detect growth and its inhibition earlier than by any other methods (Roberts et al., 1983). The results can be made available at the earliest before 10 days, the average being 9.3 days (Roberts et al., 1983). The major disadvantage of the BACTEC system are the problems of disposal of a large volume of very low radioactive materials (12B vials), and the cost, which is much higher than for any of the solid media, though it is less expensive than the other newer non-radioactive broth based systems. The major disadvantage of the conventional method is that it requires mycobacterial growth for the drug assessment and prolonged incubation period. Thus the rapidity of the newer tests is an important and essential consideration (Siddiqi et al., 1981)

Novel assays can be divided into phenotypic and genotypic techniques. In the phenotypic technique the outcome such as death of the bacterium is measured without any previous knowledge of the underlying resistance mechanism whereas in genotypic technique the drug target and nature of the gene mutations are studied.
2.6.5. Novel phenotypic Techniques

2.6.5.a. Mycobacteria growth indicator tube (MGIT)

BACTEC MGIT is a fully automated, non invasive and non radiometric system. This instrument will incubate 960 MGIT tubes, which contain 7H9 broth base with OADC enrichment and a fluorescent indicator compound embedded in silicon at the bottom. The principle of this is, the fluorescent compound is excited in the presence of dissolved oxygen in the broth and is quenched in its absence. If actively growing mycobacteria are present, the oxygen is utilized and the fluorescence emitted may be observed with a UV light. It was designed to automate the reading of the tube once per hour. An algorithm is used to monitor the growth and is indicated both by visual and audible alarm. Antibiotics and growth supplements are added before inoculation. The principle of this method is when inoculated with sample containing \textit{M.tuberculosis}, consumption of the dissolved oxygen by the organism, produces fluorescence when illuminated by a UV lamp. The manufacturer also introduced recently fully automated BACTEC MGIT 960 system for susceptibility testing (Carparo CS et al., 2004).

2.6.5.b. Microcolony detection method

If mycobacteria are inoculated on a thin layer of agar, such as Middlebrook 7H11 in a petriplate, the growth of \textit{M.tuberculosis} is easily detected with a microscope. Current evaluations of the microcolony detection method for rapid susceptibility testing of first line and second line drugs show a good correlation with the proportion method. This microcolony detection method
rovides an alternative method for rapid drug susceptibility testing (Mejia et al., 1999).

1.6.5.c. Microscopic observation broth – drugs susceptibility assay (MODS)

MODS is a new efficient, reliable and inexpensive method that permits simultaneous *M. tuberculosis* detection and determination of drug susceptibility testing in less than 2 weeks. Digested and concentrated sputum samples are diluted into 7H9 broth and distributed into wells of a 24-well plastic culture tray with and without anti mycobacterial agents. *M. tuberculosis* growth is observed as the formation of characteristic strings and tangles by simple light microscopy. This is more advantageous than radiometric methods and fluorescent methods. It has a sensitivity of 92% (Caviedes et al., 2000)

2.6.5.d. Flow cytometry

This method is based on the ability of mycobacteria to hydrolyze fluorescein diacetate to free fluorescein by nonspecific cellular esterases. Accumulation of fluorescein in metabolically active mycobacterial cells can then be detected by using a flow cytometer. The drug – inhibited mycobacteria hydrolyze significantly less fluorescein diacetate to fluorescein. Fluorescein diacetate is a nonpolar, nonfluorescent molecule capable of diffusing across the cell walls and cell membranes of mycobacteria and other bacteria by active transport and passive diffusion and it is hydrolyzed by esterases to fluorescein.
Metabolically inactive bacteria have less quantity of active esterases that result in these organisms demonstrating less fluorescence (Kirk et al., 1998).

2.6.5.e. Pha B assay (Phage amplified Biological assay)

This method is a simple mycobacteriophage based system used for both detection and drug susceptibility testing for M. tuberculosis. This test is based on the ability of viable M. tuberculosis to support the replication of an infecting mycobacteriophage. Non infecting exogenous phages are inactivated by the addition of virucidal solution. The phage continues to replicate and these are then amplified by the introduction of a non pathogenic rapid growing helper cell host (M. smegmatis). This progeny phage undergoes cycles of infection, replication and lysis, which is seen as clear plaques in a lawn of the helper cells. The number of plaques generated is directly proportional to the number of viable M. tuberculosis cells containing the phage. To determine the susceptibility, the number of plaques in a drug free control is compared with the number of plaques in the presence of drug. The absence of plaques in the drug-containing sample indicate that the strain is susceptible to the drug and the presence of plaques indicates that viable tubercle bacilli have survived and that the strain is resistant to the anti mycobacterial drug. (Wilson et al., 1996)

2.6.5.f. E-test

This method is performed for the determination of drug susceptibility using strips that contain exponential gradients of impregnated drugs. Strips containing
the drug of choice are applied on the surface of an agar medium inoculated with the test strains, after a period of incubation for 72 hours. The MIC is taken as the point at which the ellipse intersects the strip. This is a simple method which can be adopted in smaller laboratories that do not have the necessary facilities to prepare complex susceptibility testing media. However, biosafety cabinets are essential for this method because of the possibility of aerosol spread (Kakkar et al., 2001)

2.6.5.g. MB/BacT mycobacterium detection system

It is a new fully automated and rapid method. Due to the non radiometric colorimetric detection adopted in this method, the need for radio isotopes and risk of cross contamination during readings as in the BACTEC 460 system, are eliminated. Therefore, this method may be preferred to BACTEC 460. *M.tuberculosis* suspension was added to the culture bottles as drug free and drug containing bottles. Drug susceptibility testing was continuously monitored in the MB/BacT instrument until a positive and a negative result was obtained and the results were interpreted. Bemer et al demonstrated the absence of false susceptibility and false resistance by this method (Bemer et al., 2004).

2.6.5.h. ESP culture system II

The ESP Culture System II instrument (AccuMed International, Westlake, Ohio [formerly Difco) is a fully automated, nonradioactive system providing noninvasive, continuous monitoring of mycobacterial cultures by measuring
changes in headspace pressure due to gas production or consumption during 
microbial growth. This property is used for drug susceptibility testing of 
mycobacteria for the first-line drugs. The agreement of this system with BACTEC 
ranges from 93-100% (Bergmann et al., 1998).

2.6.5.i. Mycolic acid index susceptibility method

In this method a linear relationship has been established between the total 
area under the mycolic acid chromatographic peaks of a culture of *M. 
tuberculosis* and log cfu/ml. There was an agreement of 99.5% between this test 
and Tetrazolium microplate assay. (Viader-Salvado et al., 2001)

2.6.6. Rapid metabolic methods

2.6.6.a Alamar Blue assay

This method is a colorimetric method based on a resazurin based 
oxidation–reduction dye for the quantitative measurement of drug susceptibility 
in *M. tuberculosis*. The dye in the oxidized state is blue and pink in the reduced 
state. Microplate Alamar Blue assay (MABA) is easy to perform and has been 
successfully employed for determining susceptibility of *M. tuberculosis*. The MIC 
is defined as the lowest drug concentration, which prevents a colour change from 
blue to pink. The microtitre result is available within 8 days (Franzblau et al., 
1998).
2.6.6.b. Tetrazolium Bromide assay (MTT)

Another similar colorimetric method, using 3-tetrazolium bromide (MTT) as the viability indicator drug susceptibility testing, is also used for rapid detection of drug resistance. In this method, the MIC is taken as the concentration, which prevents the colour change from yellow to violet. MTT results correlate well with Microplate Alamar Blue assay. It is cheaper than MABA and an alternative to other tetrazolium based methods (Lemus et al., 2004).

2.6.6.c. Colorimetric Nitrate Reductase Based Antibiotic susceptibility assay (CONRAS)

This assay depends on the ability of M. tuberculosis to reduce nitrate to nitrite. Nitrate and the drug to be tested are incorporated into the culture medium (L-J). The culture suspension is inoculated into both drug free and drug containing slopes. The reduction of nitrate to nitrite can be tested by using specific reagents, which produce a dark pink colour. The results are available within 7-10 days with a sensitivity and specificity of 100% (Angeby et al., 2002).

2.7. GENOTYPIC METHODS

2.7.1. Direct sequencing

Since drug resistance in M. tuberculosis is due to certain specific changes that occur in the chromosomes, molecular methods for mutation detection are vital for drug susceptibility testing. Among various molecular methods DNA
sequencing is considered as the “gold standard”. There are two different methods in sequencing, 1. the Maxam and Gilbert method and 2. the Sanger method. Maxam and Gilbert method uses end-labeled probe and partial chemical cleavage is achieved at all the bases with particular chemicals (Maxam et al., 1980). Sanger method uses dideoxy nucleotides to allow chain termination at each base and the sequence is defined by the length of the fragment and the base. This is the most frequently used method (Sanger et al., 1977). The main advantage of DNA sequencing is that it not only detects all the known and unknown mutations but also localizes them. The disadvantages are radiolabelling and the number of manipulations that are required, but these are being overcome in the automated sequencers where each ddNTP is fluorescently labeled with a different colour.

2.7.2. PCR-Single Strand Confirmation Polymorphism (PCR-SSCP)

This method is based on the property of single – stranded DNA to fold into a tertiary structure whose shape depends on its sequence. Single strands of DNA differing by only one or a few bases will fold into different conformations with different mobilities on a gel, producing what is called single strand conformation polymorphisms (SSCP). After PCR amplification of the region of interest of the gene, the amplified DNA strands are separated by heat denaturation followed by cooling to allow single strand separation and folding into a characteristic conformation, whose different electrophoretic mobilities are
detected on a gel (Orita et al., 1989). This method is used for the detection of resistance to rifampicin, isoniazid, streptomycin and ciprofloxacin.

### 2.7.3. PCR-hetroduplex formation (PCR-HDF)

This method is performed by mixing amplified DNA from the test organism and susceptible control strain to obtain hybrid complementary DNA. If a resistant strain is present, the mutation will produce a hetroduplex which has a different electrophoretic mobility compared with the homodupiex hybrid. Williams et al evaluated a PCR-based universal heteroduplex generator assay for direct detection of riampicin susceptibility of *M.tuberculosis* from sputum specimens. This method has a sensitivity of 83% and a specificity of 98.2% (Williams et al., 1998).

### 2.7.4. Dideoxy fingerprinting method

This method adopts both SSCP and dideoxy DNA sequencing. The first step involves the PCR amplification of ds DNA template, followed by a cycle sequencing reaction in which one dideoxy nucleotide triphosphate and a radio labeled primer are used. Then the products obtained are heat denatured and run on a non-denaturing gel followed by autoradiography. Any change in the mobility pattern or any formation of an additional band could be viewed. Though this method requires less turn around time, it cannot detect 100% of mutations and also is more expensive than most other techniques (Felmlee et al., 1995)
2.7.5. Solid phase hybridization assay (LiPA)

The Line Probe assay (LiPA) is a commercial test kit for the detection of rifampicin resistance. It is based on the hybridization of amplified DNA from culture strains or clinical samples to ten probes encompassing the core region of \textit{rpo B} gene of \textit{M.tuberculosis}, which are immobilized on a nitro cellulose strip. If hybridization of the amplified DNA occurs to the mutation specific probes which is accompanied with the absence of hybridization to the wild type specific probes, then the mutation is present. The hybrids formed can be read by an immuno enzymatic procedure, which facilitates coloured precipitate formation thus enabling the visual reading of the results. The advantages of this method are its simplicity, speed and the non-labeling procedure involved (De Beenhouwer \textit{et al.}, 1995).

2.7.6. Dot –Blot hybridization strategy

This method is a reliable PCR-based dot-blot hybridization strategy to detect mutations conferring drug resistance. Different loci in six genes associated with drug resistance to INH, Rifampicin, Streptomycin and Ethambutol were included in the strategy. This is a rapid, reproducible method (Victor \textit{et al.}, 1999).

2.7.7. DNA micro arrays method

This is a new approach for genotypic detection of resistance which is based on hybridization of amplified DNA to high density oligonucleotide arrays on a glass miniaturized support which enables to examine large amounts of
sequence in a single hybridization step. The use of additional probes for other drugs in the same solid support would be an advantage of this technique (Troesch et al., 1999).

2.7.8. RNA/RNA mismatch analysis

This method involves the amplification of the desired region of the test strains by PCR followed by *in vitro* transcription leading to single stranded RNA formation. The RNAs obtained from test and reference wild strains are hybridized and the duplex is then treated with RNase enzyme. The RNase cleaves any mismatched duplex, which will be seen as multiple bands on agarose gel (Kusunoki et al., 1995).

2.7.9. Reverse transcriptase PCR

This method is based on the detection of the internal sequence derived from 71 kDa heat shock protein mRNA in tubercle bacilli, heat-treated in the presence of rifampicin. The target sequence is amplified by RT-PCR followed by agarose gel electrophoresis (Nash et al., 1997).

2.7.10. Cleavage fragment length polymorphism

This is a new mutation scanning method. It was evaluated and compared with PCR-SSCP and PCR-Restriction Fragment Length polymorphism (RFLP) to detect antimicrobial resistance in *M. tuberculosis*. It appears to be more efficient
han SSCP and RFLP for the detection of mutations in large amplicons (Sreevatsan et al., 1998).

Although these methods are highly specific, most of them rely on technically demanding procedures and in some cases, need specialized and costly equipment.

The first phase in the development of the chemotherapy for tuberculosis, started in the late 1940s with the introduction of several potent anti-TB drugs like streptomycin followed by para amino salicylic acid, isoniazid, and pyrazinamide. A landmark study carried out by Tuberculosis Research Centre in the late 50’s proved beyond doubt that tuberculosis patients can be effectively treated at home without added risk of relapse or infection of family members. This concept has revolutionized tuberculosis treatment globally. However, prolonged treatment resulted in noncompliance to treatment by many patients. This problem was overcome by the introduction of intermittent regimens that facilitated supervision of drug administration as well as reducing chronic toxicity and drug cost. The 70’s saw the introduction of short course chemotherapy, which reduced the treatment period to six – eight months. In most of these regimens, four potent drugs viz., rifampicin, isoniazid, pyrazinamide and either streptomycin or ethambutol are given together with an initial intensive phase of 2 or 3 months daily or intermittently. This resulted in reducing the toxicity, although the bulk of dosage increased affecting to some extent patient compliance.
The emergence of drug resistance in *M. tuberculosis* has been associated with a variety of management, health provider and patient-related factors. These include (i) deficient or deteriorating TB control programmes resulting in inadequate administration of effective treatment; (ii) poor case holding, administration of sub-standard drugs, inadequate or irregular drug supply and lack of supervision; (iii) ignorance of health care workers in epidemiology, treatment and control; (iv) improper prescription of regimens; (v) interruption of chemotherapy due to side effects; (vi) non adherence of patients to the prescribed drug therapy; (vii) availability of anti-TB drugs across the counter, without prescription; (viii) massive bacillary load; (ix) illiteracy and low socio-economic status of the patients; (x) the epidemic of HIV infection; (xi) laboratory delays in identification and susceptibility testing of *M. tuberculosis* isolates; (xii) use of non-standardized laboratory techniques, poor quality drug powders and lack of quality control measures; and (xiii) use of anti-TB drugs for indications other than tuberculosis.

In this context the fluoroquinolones were introduced in the chemotherapy of tuberculosis in the early eighties. Among quinolones available at that time, ofloxacin demonstrated bactericidal activity against *M. tuberculosis* showing good penetration in macrophages. Although the addition of fluoroquinolones in the place of ethambutol to the existing short course chemotherapy regimen, it facilitated to reduce the treatment period to 3-4 months when the drugs are administrated daily. However, addition of ofloxacin as one of the constituent drugs in the intermittent chemotherapy along with Isoniazid, rifampicin and...
pyrazinamide, resulted with a high relapse rate. Hence, there was a need to use other modified derivatives of fluoroquinolones with higher bactericidal activity to shorten the duration of chemotherapy and also to treat patients with intermittent chemotherapy. As of now, the value of the fluoroquinolones really lies in the treatment of MDR-TB.

2.8. Reasons for the selection of quinolones as drugs of choice

The availability of ofloxacin in the early nineties brightened the scope of tuberculosis treatment on two specific areas. The first one aimed at shortening of treatment and second one pertaining to treat MDR-TB cases. These ideas became crystallized due to several studies carried out in diverse settings. These include *in vitro* simulation experiments done at TRC (Herbert *et al.*, 1996), the multi-centric EBA studies carried out by Prof. Mitchison (Sirgel *et al.*, 2000) the randomized clinical trial carried out by Khono *et al* (1992) and a controlled clinical trial of a shorter duration carried out at TRC (TuberculosisResearchCentre 2002). These studies suggested that ofloxacin may have certain amount of sterilizing activity.

At a later time-point it was found that the newer quinolones, moxifloxacin and gatifloxacin (C-8 methoxy quinolones) demonstrated greater *in vitro* activity than ofloxacin and ciprofloxacin (C-8 Hydrogen quinolones) (Hu *et al.*, 2003). Further, closely related C-8 methoxy compounds were more active against mutants than its C-8 hydrogen compounds like ofloxacin and ciprofloxacin (Lu *et al.*, 2001). In experimental murine tuberculosis, moxifloxacin and gatifloxacin
were found to have more sterilizing activity than ofloxacin (Alvirez-Freites et al., 2002; Nuermberger et al., 2004). Although the above observations were promising, considering what has been achieved with ofloxacin, there remained still substantial gaps towards our understanding about the full potentials of newer quinolones in the treatment of tuberculosis. Hence an attempt has been made to conduct the following studies to evaluate the antimycobacterial activity of these newer quinolones.
3. AIMS AND OBJECTIVES

3.1. AIM

To determine the *in vitro* activities of quinolones against *M.tuberculosis*

3.2. Objectives

1. To study the *in vitro* activity of different quinolones viz: gatifloxacin (GAT), moxifloxacin (MXF), sparfloxacin, ofloxacin (OFX), ciprofloxacin, and lomefloxacin by absolute concentration method on LJ and 7H11 medium and to determine the presence of any cross resistance.

2. To determine *in vitro* definition of resistance to gatifloxacin and moxifloxacin using different susceptibility test methods against clinical isolates of *M.tuberculosis* which were resistant and susceptible to ofloxacin.

3. To determine the bactericidal activity of gatifloxacin, alone and in combination with isoniazid (INH) & rifampicin (RMP) on logarithmic and stationary phase cultures of *Mycobacterium tuberculosis*.

4. To determine the bactericidal activity of moxifloxacin alone and in combination with INH and RMP on logarithmic and stationary phase cultures of *Mycobacterium tuberculosis*.

5. To determine the bactericidal activities of moxifloxacin and gatifloxacin in various combinations with standard drugs in a new acidic model of persistent *Mycobacterium tuberculosis*.
6. a. To amplify quinolone resistance determining region (QRDR) of *gyr A*
b. Sequencing of PCR product of *gyr A* region for the detection of mutations.
c. To compare the susceptibility pattern of these strains with mutations, to determine the level of resistance
### 3.3 Table 2: Experimental Design

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>In vitro activity of different quinolones to study the presence of cross resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Experiment 2</td>
<td>An <em>in vitro</em> study to determine the definition of resistance to gatifloxacin and moxifloxacin against <em>M. tuberculosis</em> using different established susceptibility test methods</td>
</tr>
<tr>
<td>3</td>
<td>Experiment 3 &amp; Experiment 4</td>
<td>Bactericidal activity of gatifloxacin and moxifloxacin alone and in combination with INH &amp; RMP on logarithmic and stationary phase cultures of <em>M. tuberculosis</em></td>
</tr>
<tr>
<td>4</td>
<td>Experiment 5</td>
<td>Bactericidal activities of moxifloxacin and gatifloxacin in various combinations with standard drugs in acidic condition against stationary phase <em>M. tuberculosis</em></td>
</tr>
</tbody>
</table>
| 5 | Experiment 6 | a. To amplify quinolone resistance determining region (QRDR) of *gyr A*  
b. Sequencing of PCR product of *gyr A* region for the detection of mutations  
c. To compare the susceptibility pattern of these strains with mutations, to determine the level of resistance |