Discussion
8. DISCUSSION

Fluoroquinolones are one of the few classes of antimicrobial agents that are being increasingly used in the treatment of human diseases. They are being preferred because of their wide spectrum and their stable physicochemical properties. Although Nalidixic acid and its other derivatives with enhanced antibacterial activities were being used in the treatment of urinary tract infections, and respiratory diseases among others, the usage of fluoroquinolones in the treatment of tuberculosis began with the observation of Tsukamura et al (Tsukamura et al., 1985). His first report with ofloxacin in the treatment of 19 chronic drug resistant tuberculosis patients gave an impetus to its usage based on their bacteriological response and excellent tolerability even when the drug was given for 6-9 months. (Tsukamura et al., 1985). Parallely, several in vitro studies were conducted to determine their MIC; in vitro simulation studies were carried out to determine their EBA and SA; EBA studies were conducted in patients to determine their killing effect on actively growing population of M. tuberculosis present in the sputum, which represent the bacterial population present in the inner side of the cavitary wall. These observations have enabled several investigators to use ciprofloxacin and ofloxacin in the treatment of drug resistant tuberculosis although no report has been made available so far from randomized controlled trials on this group of patients. Centers for Disease Control and Prevention (CDC) has stated its potentials for the treatment of drug resistant tuberculosis in 1993 (AmericanThoracicSociety 1994). In the updated

A recently conducted controlled clinical study at the Tuberculosis Research Centre, suggested that ofloxacin-containing regimens might permit substantial shortening of the treatment duration for 4 months from the current treatment period of 6 months. This is a significant step forward in the treatment protocol considering the excellent cure rate with low relapse rate observed in the study (Tuberculosis Research Centre 2002). In the succeeding years several fluoroquinolones with an enhanced \textit{in vitro} activity became available. Of these, moxifloxacin and gatifloxacin had shown much highest activity than other quinolone derivatives (Hu \textit{et al.}, 2003). Their long half life, high area under the concentration-time curve, their tolerability and their ability in enhancing the potential of other anti tuberculosis drugs have made these drugs more attractive.

Many animal studies carried out with MXF and GAT showed substantially a greater activity with other anti tuberculosis drugs. In a murine model of tuberculosis Cynamon \textit{et al}., 2000, had found similar activity with GAT and MXF. GAT had sufficient bactericidal activity, both alone and in combination with ethinomide, with or without pyrazinamide. This treatment concept provided an alternative treatment regimen for the treatment of MDR-TB patients (Alvirez-Freites \textit{et al.}, 2002). In another study they showed that a short course (6 month) regimen containing GAT, ethinomide and pyrazinamide has the potential to achieve a durable cure of tuberculosis. (Cynamon \textit{et al.}, 2003). Another mouse
model study had shown the enhanced activity of MXF along with ethinomide in BALB/c mice, infected with *M.tuberculosis*. This is also an important finding for the treatment of MDR-TB patients (Fattorini *et al.*, 2003). Nurembuerger *et al* showed in a murine model that the combination of MXF, rifampin, and pyrazinamide reduced the time needed to eradicate *M.tuberculosis* from the lungs of infected mice by up to 2 months when compared with the standard regimen of isoniazid, rifampin and pyrazinamide, suggesting that MXF-containing regimen has the potential to substantially shorten the duration of therapy to cure human TB to 4 months or less. (Nuernberger *et al.*, 2004)

There are many reports available with no previous history of previous treatment except for a well conducted study by Kohno *et al.* (Kohno *et al.*, 1992) with ofloxacin-containing treatment regimen of 9 months duration and a limited study carried out with ciprofloxacin-containing regimen by Mohanty *et al.* (Mohanty *et al.*, 1993).

Many clinical trials are being conducted with GAT by investigators in Berlin and in other places by the Tuberculosis Research unit with support from National Institute of Health. The CDC's Tuberculosis Trials Consortium has begun their study with MXF under a Clinical Trials Cooperative Research and Development Agreement.

Although all these studies have enlarged the scope of using MXF and GAT in the treatment of tuberculosis, there still remained considerable lacunae about their *in vitro* definition of MIC and their exact mechanism of killing of actively growing tubercle bacilli and also their action on bacilli present in semi dormant or
persisting conditions. There are also not many molecular studies conducted on the resistant and susceptible strains from the disease endemic regions in delineating the mechanism of resistance. Hence, an attempt has been made at present study to address the following.

1. *In vitro* determination of the inhibitory and bactericidal activity of MXF and GAT

2. *In vitro* simulation studies, attempting to mimic *in vivo* conditions

3. Determination of resistance by molecular methods by comparing the mutation pattern of resistant strains and their susceptibility pattern.
8.1. In vitro activity of different fluoroquinolones against *Mycobacterium tuberculosis*

The *in vitro* activity of different fluoroquinolones like lomefloxacin, ofloxacin, ciprofloxacin, sparfloxacin, MXF and GAT were studied for their cross resistance and compared their activities against both susceptible and resistant strains of *Mycobacterium tuberculosis*. It was found that the MIC of these fluoroquinolones ranged from 4 -16, 2 - 4, 2 - 4, > 1-1, 0.25 – 1 and 0.25 – 1 µg/ml for lomefloxacin, ofloxacin, ciprofloxacin, sparfloxacin, MXF and GAT respectively.

For this study, ofloxacin susceptible and resistant *M. tuberculosis* strains were tested to evaluate the activity of other fluoroquinolones including lomefloxacin, ciprofloxacin, sparfloxacin, MXF and GAT. The results of this study indicate that the activity of these fluoroquinolones were in the order of lomefloxacin < ciprofloxacin ≤ ofloxacin < sparfloxacin < GAT = MXF. These findings are in agreement with another recent *in vitro* study on the sterilizing activities of fluoroquinolones against rifampin-tolerant populations of *M. tuberculosis* in which ciprofloxacin showed the least bactericidal activity while ofloxacin and levofloxacin had greater activities, and MXF and GAT had the greatest activities (Hu *et al.*, 2003).

Also in the present study, the MICs of the fluoroquinolones (lomefloxacin, ofloxacin, ciprofloxacin, sparfloxacin, MXF and gatifloxacin) were 2-32 times higher for ofloxacin resistant strains compared to ofloxacin susceptible strains. Although cross resistance at different levels has been observed among these
fluoroquinolones, they are still active against MDR strains indicating that there was no cross resistance between the fluoroquinolones and the other first line anti TB drugs (Ruiz-Serrano et al., 2000). The susceptibility pattern of resistant strains exhibited a low level of disagreement between L-J and 7H11, probably due to lower binding of the drugs in 7H11 medium, which has lower protein content.

GAT and MXF showed a greater activity than the other fluoroquinolones against ofloxacin resistant strains and also against MDR-TB isolates suggesting that these new fluoroquinolones might be useful in the treatment of MDR-TB. The absence of cross resistance of GAT and MXF with other anti-tuberculosis drugs observed in this study might help in planning experiments to rule out any antagonism between these two drugs and also to look for augmentation of the activity of fluoroquinolones.

Among these newer fluoroquinolones, a good pharmacokinetic profile, absence of photo toxicity and greater activity make both GAT and MXF the drugs of choice. From this it is possible to propose that even ciprofloxacin and ofloxacin resistant patients may be benefited by treatment with GAT or MXF containing regimens because of their enhanced activity even on such resistant strains due to their low MIC. Also, the presence of C8 methoxy group in GAT and MXF reduces the selection of resistant mutants and gives them a greater activity. Their enhanced activity could also be due to an increase in the release of double stranded DNA breaks from drug–gyrase-DNA complex (Lu et al., 2001).
8.2. Definition of resistance to newer fluoroquinolones, GAT and MXF

As WHO Expert Committee on Antibiotics in their second report, stated “Bacterial resistance to antibiotics is the principal obstacle to their successful therapeutic use”. When resistance develops during a course of treatment, it may deprive an antibiotic of its proper therapeutic effect in the patient being treated. For this reason, the estimation of bacterial sensitivity or resistance to antibiotics has assumed great importance. Such estimations are an essential prerequisite for the rational use of antibiotics and for preserving the efficacy of this important group of therapeutic substances”. Hence, there is a need to determine the susceptibility pattern of a strain to a drug. The therapeutic index for a given drug is the difference between the \textit{in vitro} MIC and the drug levels obtained in blood. This index is high for isoniazid and rifampicin giving a good result in treatment. Though the MIC determination gives the maximum information about bacterial sensitivity, in order to recommend a suitable antibiotic, it is highly necessary to consider a large array of different factors such as medium composition, pH, inoculum size, and incubation time. Hence, it is essential to determine the critical concentration to know whether the causative organism is sensitive to this concentration of antibiotic achieved at the site of infection. The breakpoint concentration or critical concentration of a drug is the concentration that will distinguish a strain to be susceptible or resistant, it should be somewhere between highest MIC found among the wild strains and the lowest MIC found among the isolates considered as resistant.
The data on critical concentration for classical second line and other newer drugs are largely fragmentary or lacking altogether. Our aim was to develop a definition of resistance to newer fluoroquinolones in order to help for better treatment. So far there are no definitions of resistance to quinolone derivatives such as GAT and MXF. This is the first report on the critical concentration of GAT and MXF. It has been observed that the MICs of new fluoroquinolones, GAT and MXF are 1 μg/ml and the critical concentration of these fluoroquinolones varies according to the medium used and the techniques followed. These strains resistant to other fluoroquinolones have increased susceptibility to GAT and MXF (low MIC) on L-J. The discrimination approach (Canetti et al., 1969) was used to compare methods of sensitivity testing. For this purpose, the criterion of 1% is chosen to discriminate between predominantly sensitive and predominantly resistant groups of culture as percentage, which determined the efficiency of each method in the detection of resistance. By this, 0.5 μg/ml was determined as the critical concentration for GAT on LJ and 7H11 and 0.25 μg/ml by BACTEC method in 12B medium, and it was 1 μg/ml on LJ, and 0.5 μg/ml on 7H11 and by BACTEC method for MXF.

GAT and MXF are new third generation quinolones. Although cross resistance has been observed between these and other fluoroquinolones, pathogens resistant to ofloxacin, ciprofloxacin and lomefloxacin, have shown increased susceptibility to GAT and MXF. Unlike the other fluoroquinolones, the absence of halide in GAT and MXF at C-8 position minimizes the potential for photosensitivity reaction. Compared with other fluoroquinolones, these drugs
have potent antitubercular activity, which is similar or somewhat superior to that of sparfloxacin.

Animal model studies have also demonstrated the superior activity of GAT and MXF against tubercle bacilli. GAT has shown sufficient *in vivo* activity in the murine model of tuberculosis (Alvirez-Freites et al., 2002). Grosset *et al.* have suggested a new regimen containing MXF, rifampicin and pyrazinamide as having the potential to shorten the duration of anti-tuberculosis treatment in man because MXF had activity superior to isoniazid in animals (Yoshimatsu *et al.*, 2002; Nuermerberger *et al.*, 2004), and its activity was enhanced in combination with isoniazid. Other workers have also suggested the use of MXF in multiple-drug regimens for human tuberculosis (Miyazaki *et al.*, 1999), and it has been reported that the combination of MXF and ethionamide had potential activity in the treatment of infection by MDR-TB strains in animal models (Fattorini *et al.*, 2003).

GAT and MXF have good pharmacokinetic profiles in tissue and cellular components in humans (Stass *et al.*, 2002; U.S.Pharmacist. 2002). Like ofloxacin, both GAT and MXF have good metabolic stability and long half-life of 7.1–13.9 hrs and 11–14 hrs, respectively, with an enhanced activity even at lower concentrations. GAT is rapidly absorbed, with the maximum serum concentration (*C*\textsubscript{max}) increasing in linear progression with increased dosage. For example, administration of GAT in a single dose of 200, 400, 600 and 800 mg resulted in serum concentrations of 2.0, 3.8, 5.3, and 7.0μg/ml, respectively.
GAT also shows good intracellular penetration and targeting of lung tissue. (U.S. Pharmacist. 2002)

MXF also has a high bioavailability of 90% (U.S. Pharmacist. 2002) and the maximum serum concentration is reached within 1-4 hrs, with a $C_{\text{max}}$ of 4.0 to 5.6 μg/ml for an 800 mg dose. (Stass et al., 2002) Since the serum concentrations achieved are higher than the MICs obtained with susceptible and resistant strains for GAT and MXF, it is possible that they may have activity on strains resistant to other fluoroquinolones, and even if the MIC of GAT and MXF increases with ofloxacin resistance, these two fluoroquinolones may still be used in the treatment of (MDR-TB) multi drug resistant tuberculosis.

The serum concentration for both MXF and GAT achievable in tissues is higher than the MIC of the same, which covers the MIC of highly resistant strains to other fluoroquinolones.

In this study, we assumed that there is complete cross-resistance between ofloxacin and newer fluoroquinolones such as GAT and MXF and then determined the susceptibility of *M. tuberculosis* isolates to these drugs by absolute concentration method on LJ. We found that all the ofloxacin–susceptible strains were susceptible and all the ofloxacin–resistant strains were resistant to GAT and MXF at a concentration of 1 μg/ml with an agreement of 100%. For MXF, 28 of the 30 ofloxacin susceptible strains were susceptible and 19 of the 20 resistant strains were resistant with an overall agreement of 94%.

The same cultures were tested by proportion method on LJ and 7H11, and also by the BACTEC radiometric method to compare the drug susceptibility.
patterns and to establish the critical test concentrations of GAT and MXF for these methods.

By the discrimination approach, (Canetti et al., 1969) 100% agreement was seen with absolute concentration method for GAT and 94% for MXF, at a concentration of 1µg/ml, and 0.5µg/ml and 1µg/ml for GAT(100%) and MXF(96%), respectively by the PST method. Although results were available faster by the absolute concentration method, the influence of inoculum size and viability of the organisms always remain as a limiting factor with this procedure. While the proportion method is not affected by either of the above methods, it takes a longer time for the results to become available when LJ medium is used.

In the proportion method on 7H11 agar medium, for both GAT (100%) and MXF (92%), at 0.5 µg/ml gave good agreement with the absolute concentration method on LJ at 1 µg/ml. The major advantage of using 7H11 is that results become available by 3 weeks because this medium, by being transparent, permits early detection of colonies.

In the BACTEC radiometric method, 0.25 µg/ml and 0.5 µg/ml concentrations gave 96% and 92% concordance for GAT and MXF, respectively, with the definition arrived at using absolute concentration method on LJ medium at 1µg/ml. These results are not surprising since BACTEC 12B is a liquid medium with less protein content, and with a lesser binding of the drugs to this lower amount of proteins, the drugs may be more active in this milieu and the results obtained may also be more precise. The major advantage of this method is the early availability of results, usually within 8-10 days, which is highly
beneficial to patients harboring drug-resistant organisms. Further, the BACTEC method, being a semi-automated technique with standardized media and reagents, provides a means of introducing standardization in the laboratory. For the resource poor countries, the constraint with this method is the high cost, both initial and recurring. However, this constraint could be partially offset by the ease of use and early availability of results, which enables controlling the transmission of disease in these regions with high prevalence of tuberculosis.
8.3. Bactericidal activity of GAT against *Mycobacterium tuberculosis* on logarithmic and stationary phase culture - an *in vitro* simulation study

Any new drug considered for therapeutic use should have high early bactericidal activity (EBA) to prevent the emergence of drug resistance, and high sterilizing activity (SA) to shorten the duration of treatment and prevent relapse. The *in vitro* experiment described here was the first attempt to study the activity of GAT, alone and in combination with other anti-tuberculosis drugs, on log phase and stationary phase growth of *M.tuberculosis*. This study demonstrated an excellent early bactericidal activity of GAT.

GAT was remarkably bactericidal against log phase organisms, second only to INH. When combined with INH, it greatly increased bactericidal activity at both its high and low concentrations. However, RMP substantially inhibited the activities of both GAT concentrations. When GAT was added to INH+RMP there was a small increase in activity observed, which was greater with the higher than the lower GAT concentration (p<0.001). With the stationary phase culture, the effect during the first 2 days was distinguished from those during the next 4 days. During the first 2 days, INH was slightly bactericidal, but it had no further bactericidal activity during days 2-6. Since INH is a cell wall antagonist, these findings suggest that the majority of the bacterial population in the stationary culture was occasionally dividing (A), and was killed during the first 2 days, leaving a remainder population (B) not susceptible to INH because it was no longer dividing. In contrast, RMP continued to kill during the entire 6-day period, though more slowly during days 4-6 (Fig 11B). This suggests that the bacilli in
population A had higher metabolic rates than those in population B, since the rate of kill by RMP is slowed down by factors reducing bacillary metabolism. Considering the effects during the first 2 days on population A, GAT increased the bactericidal activities of INH (Fig 12B) and RMP (Fig 13B). However, on population B during the succeeding 4 days, GAT was not bactericidal alone (Fig 11B) and failed to demonstrate any such synergistic action with RMP. It did not alter the activities of INH (p<0.001) (Fig 12B) or RMP (p<0.001) (Fig 13B) and no assessment during days 2-6 could be made when it was added to INH+RMP (Fig 14) since negative cultures were obtained after day-2. It showed that the addition of GAT does not appreciably increase the sterilizing activity of INH or RMP, and we have not been able to assess its activity against both drugs together nor against regimens with pyrazinamide.

To what extent do these findings reflect on the probable response of pulmonary tuberculosis to regimens incorporating GAT. Are the bacilli in the log phase and the stationary phase cultures similar to those in human lesions in their response to drugs? The first study of early bactericidal activity (EBA) measured the changes in cfu counts of M. tuberculosis in sputum during the treatment of patients with pulmonary tuberculosis with 22 different combinations of INH, RMP, pyrazinamide, ethambutol and streptomycin (Jindani et al., 1980). A recent re-analysis of the data showed an initial 2-day period, when INH was the predominant drug killing at the fastest rate of about log_{10} 0.6 cfu / day, and unaffected by other drugs in the regimen (Jindani et al., 2003). In contrast, regimens with RMP, but without INH, killed at a lower rate of about log_{10} 0.3 cfu /
day (Jindani et al., 2003). Actively growing organisms are thought to be killed during this initial phase. These findings are in substantial agreement with our findings on the log phase culture, which showed INH to be the most bactericidal drug and RMP less so. After the first 2 days, the rate of kill in sputum slowed down substantially to about \( \log_{10} 0.12 \text{ cfu/d} \). The organisms are now thought to be persisters. The predominant bactericidal drug changed to RMP, while INH no longer appeared to contribute to bactericidal activity. This again mirrors the relative activities of RMP and INH in the stationary phase culture. Thus, the behavior of the log phase culture to different drugs reflects the first 2 days of therapy while the stationary phase culture reflects the behavior of drugs during subsequent treatment.

We now have to see whether population A or population B in the stationary culture most resembles the persisters found in human lesions. There is substantial evidence that INH has no bactericidal activity in regimens that start with 4 drugs and continue with RMP+INH. The absence of any effect of INH has already been observed in the first EBA study. Clinical trials also provided evidence. Thus, in such regimens (abbreviated conventionally as 2SHRZ/RH) in 8 trials, relapses occurred in 5.2% of 1225 patients with initially sensitive strains and in 8.2% of 61 with initially resistant strains, a non-significant difference (Mitchison 2000). In a recent trial (in a 2EHRZ/RH regimen) the corresponding proportions were 3.7% of 190 and 4.0% of 23 patients, respectively (Jindani et al., 2004). In contrast to these findings with INH, bactericidal action throughout chemotherapy is due entirely to RMP, together with pyrazinamide in the initial
phase (Mitchison 2004). This continuing action of RMP is also shown in its bactericidal action on the stationary culture throughout the 6-day period (Fig 11B). While no assessment has yet been made of the gene expression status of *M. tuberculosis* in sputum, the slow bactericidal activity of INH on population A, and the complete absence of such activity on population B, strongly suggests that it is population B that resembles the lesional persisters. If this so, our finding show that addition of GAT does not appreciably increase the sterilizing activity of INH or RMP, though we have not been able to assess its activity against both drugs together nor against regimens with pyrazinamide.

In summary, our results with GAT show that it has many of the same features as INH, particularly great bactericidal activity against multiplying organisms but very limited sterilizing activity against persisters. This would suggest that, like INH, it would be effective in retreatment regimens that do not contain RMP, since in regimens without RMP, INH is a slowly sterilizing drug (Mitchison 2000). The concept is in agreement with assessments of retreatment regimens in murine tuberculosis (Cynamon et al., 2003).
8.4. Bactericidal activity of moxifloxacin against *Mycobacterium tuberculosis* on logarithmic and stationary phase culture – an *in vitro* simulation study

To shorten the duration of treatment, new drugs are needed to augment the activity of the standard regimen against persisting *Mycobacterium tuberculosis* that, by virtue of slow or intermittent multiplication, are less susceptible to killing by most of the presently available antituberculosis drugs. Although there are studies reported on animals models (Nuermberger *et al.*, 2004) and in humans (Veziris *et al.*, 2003) to evaluate the activity of MXF alone and in combination with other antituberculosis drugs, only few studies were conducted with standard regimen containing INH and RMP (Jindani *et al.*, 2003). The standard early bactericidal activity (EBA) technique has its limitations in measuring the sterilizing activity of a drug against bacilli that persist despite effective drug treatments and these persisters are responsible for prolonging the treatment period, whereas *in vitro* bactericidal activity on stationary phase culture predicts the sterilizing activity of a drug. A recent report on the activity of MXF against rifampicin tolerant persisters was observed as a rational model to measure the activity of the drug against persisters (Hu *et al.*, 2003). Following this, the present experiment has been carried out to evaluate the activity of MXF on log phase as well as on 30 days old cultures, alone and in combination with other standard first line drugs INH and RMP. This study revealed that as a single drug, MXF had shown only a moderate bactericidal activity on log phase culture. This observation is in agreement with a recent observation made in an editorial,
which stated that MXF activity is comparable but not equal to that of INH (Gosling et al., 2004). On stationary phase culture, MXF had shown remarkable activity, both at low and high concentrations which is greater than RMP alone on both slowly multiplying organisms and also against true static populations as observed in the earlier study (Experiment III) with GAT (Miyazaki et al., 1999). MXF when combined with INH had shown a greater activity like INH and RMP combination and even at lower concentration, MXF activity was greatly augmented by the addition of INH. These findings support the report of the mouse model, where combination of MXF and INH, was highly effective (Dong et al., 2000). The present study revealed that when INH was replaced with MXF, the combination of MX2 and RMP on the stationary phase culture, showed similar results equivalent to that of INH and RMP combination, which is also in agreement with another murine model study reported by Neurnberger et al (2004). They also reported that combination of MXF with standard regimen replacing INH, 2RMZ/4RM resulted in 0 cfu both in spleen and lung on the 4\textsuperscript{th} month itself, whereas 2HRZ/4RH resulted 0.39 log cfu in lung and 0 cfu at the 2\textsuperscript{nd} month in spleen, suggesting that in the standard regimen, MXF might be used to replace INH and have the potential to substantially shorten the duration of therapy for the treatment of human tuberculosis.

It was also seen that, MXF (4 μg/ml) had greater activity with RMP on stationary phase culture on all days and gave a reduction of 5.85 log\textsubscript{10}cfu on the 6\textsuperscript{th} day, and with INH and RMP combination gave a reduction of 5.5 log\textsubscript{10}cfu, thereby exhibiting an enhanced activity. Further, synergistic activity of MXF in
combination with INH, against actively growing *M. tuberculosis* culture on exponential phase, resulting 0 cfu in 6 hours was observed which was similar to the activity of INH and RMP combination that resulted in 0 cfu within 6 hours. On exponential culture in three drug combination of INH, RMP and MX2, resulted a 0 cfu at 2 hrs whereas INH and RMP combination showed 0 cfu at 6 hrs and thereby demonstrating additive activity of MXF with INH and RMP.

As reported by others, the potent activity of MXF with other anti tuberculous drugs including first line, second line and third line drugs seemed to be a powerful alternative for the treatment of tuberculosis where isoniazid and rifampicin cannot be used, which is the main feature of multi drug resistant TB (Veziris *et al.*, 2003). The reported findings of an *in vitro* model on rifampicin tolerant persisters are again in agreement with our findings where RMP had shown a reduction of 1.6, 3.1, and 3.3 log₁₀ cfu/ml but RMX2 showed a reduction of 2.7, 4.9 and 5.9 log₁₀cfu/ml on 2nd, 4th and 6th days respectively on stationary phase cultures.

MXF and GFX are the recently introduced C-8 methoxy fluoroquinolones with greater *in vitro* and *in vivo* activities in murine models than other fluoroquinolones. Superior activities of MXF and GAT have been attributed to the special structure activity relationship of the C-8-methoxy substitution in the chemical structure of the prototype of fluoroquinolones, to have potent activity on microbials. In addition, the development of acquired resistance to the new drugs would be less because of their lower mutant prevention concentrations (Dong *et al.*, 2000). MXF has a favorable pharmacokinetic profile in humans with 86 to
92% oral bioavailability with a maximum concentration in serum of approximately 4 μg/ml, a half life in serum of 9 to 12 hrs and a good penetration into the intracellular space (Stass et al., 2002). Hence a good pharmacokinetic profile and in vitro bactericidal and sterilizing activity makes the drug as a potent one against *M. tuberculosis*.

The drug efficiency is considered to be good during the continuation phase therapy, since it has good sterilizing activity in addition to early bactericidal activity. The present finding reiterates the potential activity of MXF on both actively growing and also against persisting bacteria.
8.5. Bactericidal activities of moxifloxacin and gatifloxacin in various combinations with standard drugs in a new acidic model of persistent *Mycobacterium tuberculosis*

The experimental model used, for the first time, an acid pH modeling the presumed acid environment of extra cellular bacilli in liquefying cavities where the great majority of bacilli in sputum originate (Canetti 1955). In the Zhang model for the activity of PZA (Zhang *et al.*, 2003), the pro-drug PZA is converted to pyrazinoic acid in the bacterial cell. It is then excreted and enters the cell again in its protonated form which penetrates the cell by diffusion according to the Henderson-Hasselbach equation. The extent of penetration depends on the concentration and pH. To get penetration with concentrations below the peak concentration of 50 µg/ml PZA, it is necessary to have a pH at 6.0 or slightly less. At this pH, growth may be slowed but it still continues actively. A more acid pH would cause irregular inhibition of growth.

This experiment mimicked fairly the behavior of the bacterial populations in the cavities of pulmonary tuberculosis, as sampled in sputum. During the experiment, there was an initial phase lasting 2-4 days in which there was a highly variable rapid kill with a mean of $-0.36 \log \text{ cfu/ml/day}$, followed by a slower consistent, exponential kill with a mean of $-0.106 \log \text{ cfu/ml/day}$. These are similar to the bi-exponential kills, measured in sputum, occurring during treatment of pulmonary tuberculosis with streptomycin/INH/RMP/PZA (SHRZ) when the regression coefficients were $-0.48 \log \text{ cfu/ml/day}$ during the first 2 days and $-0.109 \log \text{ cfu/ml/day}$ during days 2-28 (Brindle *R et al.*, 2001). Furthermore, the
sputum data showed much higher variability in the measures of daily kill during the first 2 days (SD = 0.55) as compared to those during days 2-28 (SD = 0.048), similar to the present *in vitro* findings. Presumably these two phases (Canetti 1955, Jindani *et al.* 2003) are due to the presence of two distinct bacterial populations in the 30-day culture and in patients. The majority population was killed rapidly during the first few days, probably because it was growing rapidly, perhaps as a result of rapid adaptation from micro-aerophilic conditions in the Erlenmeyer flasks to air during aliquoting in the *in vitro* system and also to the presence of air in tuberculous cavities. The minority population, consisting of persisters in cavities, and perhaps of bacilli micro-aerophilically adapted *in vitro* was killed later and much more slowly. Since the persisters are responsible for prolonging treatment, the best *in vitro* model of drug action that might shorten treatment is therefore the kill during days 4-21.

Acidification of the medium to pH 5.9 creates a further important resemblance to the environment of cavitary bacilli since it is necessary to postulate an acid environment of the bacilli, created by inflammation, for PZA to be bactericidal (Mitchison 1985). Acidification caused a slowing of the growth rate leading to a reduction of the bactericidal action of RMP, as has been shown previously (Dickinson *et al.*, 1981). Thus, the counts on a 30-day stationary phase 7H9 medium culture at normal pH, had reached 1.0 log cfu/ml at day 6 when exposed to RMP (Paramasivan *et al.*, 2005) whereas the count in acid medium was still 4.45 log cfu/ml/day at day 21 (Table 14). The acid medium also allowed estimates to be made on all of the various drug combinations over the
entire 21-day period. The second effect of the acid medium was to demonstrate
the bactericidal action of PZA (McDermott et al., 1954), which was found to be as
active as RMP. The regression coefficient was −0.108 which agrees well with an
estimate of −0.114 for PZA during days 2-14 when given in monotherapy to
patients (Jindani et al., 2003). PZA appeared to inhibit slightly the action of RMP
resembling its inhibition of the bactericidal activity of INH in vitro (Dickinson et al.,
1977). In the acid medium, INH had little bactericidal activity whereas greater
activity was shown by RMP and PZA, mimicking their relative activities in
pulmonary tuberculosis. It seems that the use of acid medium greatly improves
the model as a mimic of the bactericidal action of drugs during the treatment of
pulmonary tuberculosis.

The substitution of either of the fluoroquinolones for INH in the control
regimen of RMP/PZA/INH failed to increase its bactericidal effects, as the
estimates of the regression coefficients over days 4-21 were slightly lower (−
0.096 and −0.095 log cfu/ml/day, for MXF and GAT, respectively) when the
fluoroquinolones were substituted than for the control regimen itself (−0.106 log
cfu/ml/day). Similar conclusions were drawn from the regression coefficients over
days 0-21 and from the final cfu counts at 21 days. The reason for the major
discrepancy between this negative finding and the large effect of the similar
substitution in the recent mouse experiment is unclear. A potential limitation of
mouse experiments with drug combinations could be due to interference in
absorption of one drug by others in the combination (Dickinson et al., 1992).
Such an alteration in absorption of RMP and PZA was ruled out in the
experiment by separating the timing of the dose of RMP from the doses of the other drugs and by measuring their concentrations in plasma of the mice (Nuememberger et al., 2004).

A drug-drug interaction at the bacillary level was assumed to occur. The present experiments fail to demonstrate such an interaction. In contrast, the addition of either quinolone to the control regimen increased the speed of kill during days 4-21 from \(-0.106\) log cfu/ml/day for the control regimen to \(-0.145\) and \(-0.139\) log cfu/ml/day, for MXF and GATI, respectively. The difference between these rates of kill of \(0.039\) and \(0.033\) log cfu/ml/day, respectively, is about 3 times the SD within the experiment (table 14) and therefore highly significant. These increases of \(0.039\) and \(0.033\) log cfu/ml/day can be compared with an increase of similar regression coefficients of \(0.050\) log cfu/ml/day (-0.109 minus -0.059) over the 2-28 day period in a comparison of the effect of adding both RMP and PZA to regimens for treating pulmonary tuberculosis (Brindle R et al., 2001). This comparison suggests that addition of either quinolone to a standard regimen should result in a substantial shortening of treatment. Finally, MXF and GAT appeared to have closely similar bactericidal activities. Although, GAT alone seemed more bactericidal than MXF, this was mainly due to a greater kill during the highly variable initial 4 days of exposure. In both comparisons of 3-drug and 4-drug regimens, the regression coefficients over days 0-21 and days 4-21 were almost identical. In conclusion, the findings suggest that there would be an appreciable reduction of the duration of treatment if either MXF or GAT were added to a standard regimen in the treatment of pulmonary tuberculosis.
This view differs from the more guarded conclusion of experiments limited to only 6 days with normal culture medium (Paramasivan et al., 2005), and demonstrates the value of the longer exposure period obtained with acid medium. However, no such shortening of treatment would be expected in substituting either quinolone for INH in a standard regimen. The bactericidal effects of MXF and GAT in the drug combinations were found to be closely similar.
8.6. Amplification of quinolone resistance determining region (QRDR) of \textit{gyr A}, sequencing and to determine the level of resistance by comparing with the susceptibility pattern of the same

In this study mutations in the QRDR segment of \textit{gyr A} was evaluated in 118 clinical isolates. Mutations were seen at codon, 90, 91, 94, 95, 68, and 109. Two new mutations were identified in drug susceptible strains. The R68G, (non synonymous mutation) was associated with a highly sensitive phenotype (MIC <2 \(\mu\)g/ml of ofloxacin) while the L109V (synonymous mutations) was associated with moderate sensitive phenotype of ofloxacin (Table 17). Interestingly, these two mutations co-inherited along with S95T polymorphism. Five sensitive strains did not have any mutations. Since 97.9\% of susceptible strains showed no mutations, the genotypic analysis is highly specific and could help in early diagnosis of quinolone resistance.

As described by us earlier (Sulochana \textit{et al.}, 1999) any \textit{M. tuberculosis} strains which had shown a resistance of \(\geq 8 \mu\)g/ml on LJ by absolute concentration method is considered as a resistant strain. We have had 71 such strains fulfilling the above criteria included in the study as ofloxacin resistant isolates. Among the OF \(^{r}\) isolates 32/71 had mutations at codon 90, 91 and 94, which were the most frequently mutated codons as reported by others (Siddiqi \textit{et al.}, 2002). Of these 32 OF \(^{r}\) strains, A90V mutation in 7 strains was associated with low level resistance (MIC <16 \(\mu\)g/ml) (Table 18). These 7 strains also had S95T mutations. The S91P mutation was seen in a single strain along with S95T mutations and its level of resistance is not known. Twenty four strains had 4
Table 18: Distribution of mutations in both susceptible and resistant strains

<table>
<thead>
<tr>
<th>Mutations</th>
<th>No of strains</th>
<th>Percentage of mutations</th>
<th>Susceptibility pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>R</td>
<td>S%</td>
</tr>
<tr>
<td>S95T</td>
<td>40</td>
<td>67</td>
<td>85</td>
</tr>
<tr>
<td>R68G</td>
<td>1</td>
<td>0</td>
<td>2.12</td>
</tr>
<tr>
<td>L109V</td>
<td>1</td>
<td>0</td>
<td>2.12</td>
</tr>
<tr>
<td>S91P</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D94G</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>D94N</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D94A</td>
<td>0</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>D94V</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>A90V</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

S-sensitive, R-resistant, HS highly sensitive, MS moderately sensitive, HR-highly resistant, MR-moderately resistant, LR-Low resistant
different mutations at codon 94 (D94G-10; D94N-1; D94A-11 and D94V-2) making it as a mutational ‘hot spot’ as reported by others (Siddiqi et al., 1998). Two of these mutations (D94G and D94N) were associated with high level resistance (MIC >64 μg/ml) while the remaining two (D94A and D94V) with moderate level of resistance (MIC ≥32 μg/ml). The D94N and D94V mutations were always seen along with S95T polymorphism. Strains with D94A (9/11) and D94G (9/10) mutations, also had the S95T polymorphism. The specific polymorphism at different codons and the corresponding amino acid changes could be correlated with higher or lower level of resistance. Two strains, which exhibited a high level of resistance with an MIC of 128 μg/ml, did not have any mutations. The PCR amplification and sequencing of gyr B for those samples, revealed no mutation in the gyr B region which is again a confirmation of the previous reports (Kocagoz et al., 1996). It might be related to the mutations outside the QRDR of gyr A and it requires further elucidation. No studies reported gyr B mutations for resistance, except a study on laboratory selected fluoroquinolone resistant strain of Mycobacterium tuberculosis H37Ra (Yew et al., 2000). The polymorphism at codon S95T, showed no significant impact on fluoroquinolone susceptibility. No double mutations except with S95T, a natural polymorphism which occurred even in highly resistant strains, were found in laboratory strains.

This gyr A region is highly conserved in Escherichia coli, Staphylococcus and Campylobacter jejuni and M. tuberculosis. This study confirms the universal pattern of gyr A mutations and there is no geographical clustering of isolates in
terms of mutations which is in compliance to the earlier reports for the clinical isolates in different parts of the world. Hence, the presence of mutations could be a diagnostic tool for detecting the resistance of fluoroquinolone drugs and the type of mutation would also help in determining the treatment regimen. It will be interesting to conduct a study in future to find the variations in the structural gene of gyr A in clinical isolates from MDR patients after treatment with fluoroquinolones. Till date there was no novel mutations reported, which is again confirmed by these findings. This is in contrast to the evidence of new mutations found in rpo B region in Indian clinical isolates. This might be due to drug pressure (Mani et al., 2001). Since fluoroquinolones are getting increasingly used for other diseases, a constant surveillance on mutation becomes mandatory. All these fluoroquinolones exhibited cross resistance among themselves, at different levels on the clinical isolates of Mycobacterium tuberculosis. Among these fluoroquinolones, GAT, MXF and sparfloxacin were found to have greater activity than ofloxacin and ciprofloxacin. These drugs showed no cross resistance between other anti tuberculosis drugs and had greater activity even on MDR strains.

Efflux mediated resistance has been described in the mycobacteria (Takiff et al., 1996) and an efflux pump of the MFS group, Lfr A has been identified in Mycobacterium smegmatis (Liu et al., 1996; Takiff et al., 1996). Lfr A mediated resistance is apparently limited to the more hydrophilic fluoroquinolones. Its exact role in the resistance at present is unclear and its contribution to fluoroquinolones efflux has only demonstrated in a fluoroquinolone sensitive strain of
M. smegmatis harbouring a plasmid-borne copy of the Ifr A gene (Takiff et al., 1996).

8.6.1 PCR-RFLP Assay

Gyr A being a gene with housekeeping functions is highly conserved in various mycobacterial species. In the gene, as shown by PCR, QRDR region is found to be even more conserved in all mycobacterial species. This prompted us to evaluate the QRDR region for species identification by PRA method. Species identification is not only of academic interest but it is also of clinically important, since identification provides useful information on the epidemiology and pathogenesis of the organism which would help in the successful treatment of the patient.

Currently available methods are based on phenotypic and biochemical tests and are time consuming; they are also labour-intensive procedures requiring expensive equipment such as HPLC. PCR-RFLP Assay (PRA) technique certainly fits these requirements better than other molecular methods.

This method is rapid, simple and precise because it employs PCR. It can differentiate numerous species of mycobacteria within a single experiment.

In this study we adapted PCR-RFLP assay using gyr A gene which has been undertaken to see the difference in the profile of 23 standard species of mycobacteria. Though most of the species produced the same pattern, the clinically important species M. tuberculosis, M. avium, and M. intracellulare produced a different profile with two different restriction enzymes. Since these
species are the most important mycobacterial pathogens, \textit{Bgl I} and \textit{Hga I} could be used to differentiate these species. Since the profile is seen with less number of bands i.e. presence of one or two bands in the different restriction pattern, the results were easier to read. This assay has to be validated with other clinical isolates of mycobacteria.

\textit{Mycobacterium avium} complex (MAC) is a group of environmental mycobacteria found widely in soil, water and aerosols and causes diseases in animals and human (Inderlied \textit{et al.}, 1993). \textit{M.avium} and \textit{M.intracellulare} belong to the MAC group with overlapping phenotypic characteristics. Hence their precise identification is usually difficult. They appear to be significantly different both clinically as well as genetically. More than 90\% of MAC isolates from AIDS patients are \textit{M.avium}. Even biochemical tests for the identification of MAC do not accurately resolve differences between these species. Hence, the isolates are simply reported as members of MAC. Several PRA methods have been so far developed based on different genes of mycobacteria like \textit{16S rRNA} gene, \textit{hsp 65} and \textit{rpoB}. In this context \textit{gyr A} gene could serve as one more candidate for PRA based identification of mycobacteria. However, \textit{gyr A} intein , a protein of mycobacteria is a privileged host for protein intron because though the intein sequences are overall well conserved with in the same allelic family, the degree of homology between intein varies. Hence apart from the QRDR region, the \textit{gyr A} intein may also serve as a valuable candidate for mycobacterial species identification.