9. SUMMARY AND CONCLUSIONS

*In vitro* activity based on cumulative percentage inhibition of fluoroquinolones

The *in vitro* activity of quinolones, including lomefloxacin, ofloxacin, ciprofloxacin, sparfloxacin, moxifloxacin and gatifloxacin, was evaluated against 55 clinical isolates of *Mycobacterium tuberculosis* by absolute concentration method on Lowenstein-Jensen (L-J) and Middlebrook’s 7H11 media. The activities of these quinolones were of the order lomefloxacin < ciprofloxacin ≤ ofloxacin < sparfl oxacin < GAT ≤ MXF. GAT and MXF showed low minimal inhibitory concentration (MIC) for both ofloxacin resistant and ofloxacin susceptible strains even though some cross resistances were observed. Also, these quinolones showed a high level of activity on MDR strains, thereby indicating their possible role as therapeutic alternatives in the treatment of multi-drug resistant tuberculosis

*In vitro* definition of resistance to gatifloxacin and moxifloxacin

Fifty *Mycobacterium tuberculosis* isolates, consisting of 30 ofloxacin susceptible and 20 ofloxacin resistant strains, were tested for their susceptibility to GAT and MXF using different susceptibility testing methods namely, absolute concentration method on Lowenstein–Jensen medium (LJ), proportion susceptibility testing method (PST) on LJ and 7H11 agar media, and BACTEC radiometric method. The MIC of GAT and MXF was 1µg/ml by the absolute concentration method on LJ. In the PST method on LJ and
7H11, using a criterion of 1% or more growth as resistant, there was 100% agreement with the absolute concentration method at a concentration of 0.5 μg/ml for GAT, and 96% agreement with BACTEC method at a concentration of 0.25μg/ml. For MXF, results by the PST method showed 96% agreement with the absolute concentration method on LJ at a concentration of 1μg/ml and 92% agreement at a concentration of 0.5μg/ml for both absolute concentration method in 7H11 and BACTEC method. Hence, this study has provided a comprehensive definition on resistance for the newer quinolones against *M. tuberculosis*.

**Bactericidal activity of gatifloxacin**

The bactericidal activity of GAT, alone and in combination with isoniazid and rifampin was studied on both exponential and stationary phase cultures of *Mycobacterium tuberculosis*, strain H37Rv revealed the following: On log phase cultures, the bactericidal activity of GAT at 4 μg/ml was rapid and was very similar to that of isoniazid. At concentrations of 0.25 and 4 μg/ml, GAT enhanced the activity of isoniazid. Killing of the stationary phase culture was observed as biphasic. During the first 2 days, GAT at 4 μg/ml slightly increased the limited bactericidal activities of INH and RMP. However, no further additional bactericidal activity was found during further incubation with isoniazid alone or when GAT was added to either INH or RMP. This suggested that the stationary phase culture contained a mixture of bacilli occasionally dividing that are killed during the first 2 days, and true static
persisters in the residual population, mimicking those in human lesions. In view of the failure of GAT to add to the sterilizing activity of INH or RMP during days 2-6 of exposure in the stationary phase culture, its potentials as a sterilizing drug can be understood better by carrying out studies in long term cultures under different growth conditions. These studies may delineate its precise role in shortening the duration of treatment when it is added to current treatment regimen.

**Bactericidal activity of moxifloxacin**

Similar in vitro simulation study with MXF had shown only a moderate bactericidal activity as a single drug on log phase culture, which is comparable but not equal to that of INH. However, the synergistic activity of MXF against actively growing *M. tuberculosis* culture on exponential phase, in combination with INH, resulting 0 cfu in 6 hours which is similar to that of INH and RMP combination was established. In addition MXF also demonstrated an additive activity with INH and RMP. 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. MXF, when combined with INH, it 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. When INH was replaced by MXF in the combination of INH and RMP on the stationary phase culture, it showed equal and similar results as that of INH.
and RMP combination. MXF had greater activity with RMP at 4 μg/ml on all days and resulted in a reduction of 5.85 log₁₀ cfu on the 6th day, whereas INH and RMP combination gave a reduction of 5.5 log₁₀ cfu, thereby exhibiting an excellent activity. The potent activity of MXF with other anti tuberculosis drugs including first line drugs, established MXF as 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.

**Bactericidal activity of gatifloxacin and moxifloxacin in acid model**

The bactericidal activities of moxifloxacin and gatifloxacin were measured alone and in different combinations with isoniazid, rifampin and pyrazinamide against a 30-day, undisturbed, stationary phase culture in a micro-aerophilic environment, at a pH of 5.9 to model cavitary pH and to allow pyrazinamide activity. Measurements of colony forming units (cfu) over a 21-day period, indicated a rapid, irregular fall in counts during the first 4 days followed by a slower consistent kill during days 4 – 21 with mean kills of −0.36 (SD = 1.37) and −0.106 (SD = 0.0057) log₁₀ cfu/ml/day, respectively. The 4-21 days kill seemed the best assessment of bactericidal activity against persisting bacilli that prolong treatment because it reproduced speeds of kill in human cavities due to isoniazid, rifampin and pyrazinamide. These were similar to the biexponential kills measured in sputum, occurring during the treatment of pulmonary tuberculosis with standard short course chemotherapy consisting of streptomycin, INH, RMP and pyrazinamide. Presumably 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. With this measure, the substitution of either of the quinolones for isoniazid in the control regimen of rifampin, pyrazinamide and isoniazid did not increase bactericidal activity. However, adding moxifloxacin or gatifloxacin to the control regimen resulted in a significant increase in bactericidal action, considered sufficient to shorten the treatment of pulmonary tuberculosis appreciably. Moxifloxacin and gatifloxacin had closely similar activities in all drug combinations. Thus, support is given to reducing the treatment period in pulmonary tuberculosis by substitution of moxifloxacin for ethambutol, but not by its substitution for isoniazid. This view differs from the more guarded conclusion of experiments limited to only 6 days with normal culture medium 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.

**Amplification of QRDR of gyr A, sequencing and determining the level of resistance**

The molecular mechanism for quinolone resistance in Indian clinical strains has been studied in large numbers, collected at different time periods from different parts of this country. Mutations in the QRDR segment of gyr A was evaluated in 118 clinical isolates consisting of 71 ofloxacin resistant
(OF<sup>r</sup>) and 47 ofloxacin sensitive (OF<sup>s</sup>) strains. Mutations were seen at codons, 90, 91, and 94 in resistant strains. The S95T polymorphism was seen as the most common mutation, which was seen both in drug resistant 91.6% and susceptible strains 89% and accounted for 90% of all the mutations seen. Of these 32 OF<sup>r</sup> strains, A90V mutation in 7 strains was associated with low level resistance (MIC < 16 μg/ml). These 7 strains also had S95T mutations. The S91P mutation was seen in single strain along with S95T mutations and its level of resistance is not known. Twenty four strains had 4 different mutations at codon 94 (D94G-10; D94N-1; D94A-11 and D94V-2) making it as a mutational ‘hot spot’. Two of these mutations (D94G and D94N) were associated with high level resistance (MIC > 64 μg/ml) and the remaining two (D94A and D94V) with moderate level of resistance (MIC ≥ 32 μg/ml).

The comparison of conventional method with the sequencing method showed a sensitivity of 95.7% and a specificity of 45%. The resistance observed might be due to the mutations at different region in gyr A or due to the efflux mechanism. It also revealed its conservative nature and the uniqueness in the development of mutation and their level of resistance for a particular type of mutation.

 gyr A being a highly conserved region in mycobacterial species was evaluated for its utility in species identification. Totally 23 mycobacterial species have been tested for this PCR-RFLP assay. The enzyme Bgl I gave double band for both <i>M. tuberculosis</i> and <i>M. intracellulare</i> but single band for <i>M. avium</i>, while Hga I gave double band for <i>M. tuberculosis</i> and <i>M. avium</i> but
single band for *M. intracellulare*. Hence it is feasible by using both *Bgl* I and *Hga* I to differentiate clinically important species such as *M.tuberculosis*, *M.avium* and *M.intracellulare*.

To sum up, the definition of MIC for gatifloxacin and moxifloxacin was determined by various test procedures for the first time. The enhanced activity of gatifloxacin and moxifloxacin when compared to other quinolones was demonstrated by cumulative percentage inhibition study. The *in vitro* simulation experiments at neutral pH have proved the potentials of gatifloxacin and moxifloxacin in augmenting the activity of INH and RMP on log phase cultures and also demonstrated convincingly their early bactericidal and synergistic activities with other key anti TB drugs. The activity of gatifloxacin on stationary phase growth of *M.tuberculosis* revealed the presence of organisms in the fluid culture system in a bi-phasic state. This necessitates further elucidation in a suitable test system to understand different metabolic state of the organisms. However, the enhanced activity of moxifloxacin on stationary phase cultures and moxifloxacin augmenting the activity of INH and RMP present in this phase further demonstrated its potential use as a sterilizing drug. The activities of these two quinolones on long term stationary phase growth of *M.tuberculosis* under microaerophilic condition at acidic pH had shown that addition of gatifloxacin and moxifloxacin to the control regimen containing RMP, PZA and INH resulted in a significant increase in bactericidal action. And also these two quinolones exhibited
similar activity in all drug combinations. These findings indicate that addition of either quinolone to a standard regimen should result in substantial reduction in treatment. This novel acidic model mimicking extracellular acidic environment similar to what is seen in lung lesions also requires further elucidation. Finally, the molecular study to determine quinolone resistance revealed the presence of mutations at codons 90, 91 and 94 in the QRDR segment of the gyr A gene in resistant strains. Detailed analyses had shown a correlation between various mutation pattern and different levels of quinolone resistance. The usefulness of QRDR segment of the gyr A gene as a target for mycobacterial species identification was evaluated and need further validation in clinical isolates.