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Shashikant Vaidya
Clinical Pathology Department, Haffkine Institute, Mumbai, India

Shreyasi Muley
Haffkine Bio-Pharmaceutical Corporation Ltd. Mumbai, India

M.G. Kulkarni
T.N. Medical College and B.Y.L. Nair Charitable Hospital, Mumbai, India

Geeta Koppikar
Breach Candy Hospital Trust, Mumbai, India

AbhayChowdhary
Grant Medical College and Sir J.J. Group of Hospitals, Mumbai, India

Correspondence
D Shashikant Vaidya
Clinical Pathology Department, Haffkine Institute, Mumbai, India

To study bactericidal activity of Isoxyl along with Isoniazid and Rifampicin, alone and in combination against standard strain of *Mycobacterium tuberculosis* using time kill kinetics against log phase and stationary phase cultures

Shashikant Vaidya, Shreyasi Muley, MG Kulkarni, Geeta Koppikar and Abhay Chowdhary

Abstract
Minimum Bactericidal Concentration (MBC) test is the one most frequently used to quantitate bactericidal potency. The MBC values can then be compared with concentrations attainable in blood and tissues to achieve a basis for considerations as to whether the bactericidal effect can be anticipated in the clinical situation. Also the rate of killing can be assessed thus giving a dynamic picture of drug action. It also helps to confirm the results of synergism obtained by checkerboard studies and helps to determine the potential for the antibiotic combination to demonstrate enhanced bactericidal activity as compared to each of the individual drugs**.** With the increasing prevalence of Mycobacterial infections, the development of new antimycobacterial agents and strategies for treating Mycobacterial infections is of paramount importance, As drug development is a long and expensive process, it becomes predominant to reexamine drugs that were formerly deemed effective against TB and increase the permeability of the Mycobacterial cell wall. One such drug is Isoxyl (ISO). ISO is an old drug, used for the clinical treatment of TB in 1960's. The log phase, sometimes called the logarithmic phase, is a period characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the present population. If growth is not limited, doubling will continue at a constant rate so both the number of cells and the rate of population increase doubles with each consecutive time period. The stationary phase is often due to a growth-limiting factor such as the depletion of an essential nutrient, and/or the formation of an inhibitory product such as an organic acid. In this study we have evaluated bactericidal activity of INH and RF alone and in combination with ISO by time kill kinetic study *in vitro* against log phase and stationary phase culture against standard strain of *Mycobacterium tuberculosis*. Synergistic bactericidal effects between ISO & INH and RF& ISO were evident in exponential phase cultures but were absent in the stationery phase cultures. The ability of ISO particularly at their higher concentrations to the increase the bactericidal activity of INH or RF was lost at the culture changed from exponential phase to stationery phase. The result of the present study suggests that ISO could be particularly useful in the first few days of treatment of pulmonary TB, when the majority of the bacterial population is actively multiplying in cavity walls. ISO is expected to have fairly high bactericidal activity at the start of treatment. The low level of bactericidal activity on stationery phase cultures suggest that, though they would still continue to inhibit growth, ISO would not be effective in shortening the duration of treatment. The results shown here with the combined medicament, ISO was in our opinion not unsatisfactory in bactericidal activity.

Keywords: *Mycobacterium tuberculosis*, Isoxyl, minimum bactericidal concentration

1. Introduction
The bactericidal (killing) effect of a drug is usually based on determination of the number of surviving colony forming units per milliliter of liquid medium after a specified period of incubation. For the aerobic bacteria the minimum bactericidal concentration (MBC) is most often defined as the lowest concentration of an agent killing 99.9% of the inoculum within 18 to 24 hours of incubation. It was stressed that this 99.9% criterion was suggested arbitrarily [1] and that there is no evidence that such endpoints as 98 or 99% are inferior to 99.9% for prediction of clinical outcome of chemotherapy.

Generally chose the criterion of 99% as being more reproducible than 99.9% for MBC determination, due to the fact that with some drugs the period of time required to reach the 1000-fold decrease in the inoculum can go beyond limits established for the length of incubation.

MBC test is the one most frequently used to quantitate bactericidal potency, although it is not commonly used in the clinical laboratory. The NCCLS proposed guidelines to describe techniques for testing aerobic bacteria that grow well overnight in Muller-Hinton broth. These techniques are aimed at determining the killing effect of the antimicrobial agent in a liquid medium within a specified period of cultivation [2]. The MBC values can then be compared with concentrations attainable in blood and tissues to achieve a basis for considerations as to whether the bactericidal effect can be anticipated in the clinical situation [3].

Time kill kinetics technique determines killing of bacteria and therefore the results are likely to be more relevant for situations in which bactericidal therapy is desirable. Also the rate of killing can be assessed thus giving a dynamic picture of drug action. It also helps to confirm the results of synergism obtained by checkerboard studies and helps to determine the potential for the antibiotic combination to demonstrate enhanced bactericidal activity as compared to each of the individual drugs [4]. This is a different approach to assess that the anti-infective efficacy of antibiotics is to use the pharmacokinetic-pharmacodynamic models based on time-kill curves. Time –kill curves can follow the microbial killing and the growth as a function of both time and antibiotic concentration. Antibiotic concentration can either be held constant or changed to mimic an *in vivo* concentration profile, be it either in plasma or at the infection site. The resulting kill curves can be subsequently analyzed with appropriate pharmacokinetic-pharmacodynamic models [5].

Finally these pharmacokinetic-pharmacodynamic models then aid to optimize dosage regimens based on a rational, scientific approach. The advantage of these *in vitro* models is that they allow direct comparison of the effects of various concentration profiles and provide means for a much more detailed assessment of the pharmacokinetic-pharmacodynamic relationship than the simple use of Minimum Inhibitory Concentration (MIC)**.** Kill curves can be and have been used to study anti-infective effects both in animal and *in vitro* models, with the advantage of providing more detailed information about the time course of antibacterial effect.

With the increasing prevalence of Mycobacterial infections, the development of new antimycobacterial agents and strategies for treating Mycobacterial infections is of paramount importance, while *in vitro* methods such as radiometric and calorimetric assays are important to determine the MICs of antimicrobials and effective doses of antimycobacterial drugs and should also be evaluated in a macrophage model to ensure intracellular drug effectiveness [6]. As drug development is a long and expensive process, it becomes predominant to reexamine drugs that were formerly deemed effective against TB and increase the permeability of the Mycobacterial cell wall. One such drug is Isoxyl (ISO). ISO is an old drug, used for the clinical treatment of TB in 1960's. Studies demonstrated modest therapeutic efficacy of ISO monotherapy in cases of untreated pulmonary TB of various degree of difficulty. The NCDDG group led by DR Patrick Brennan recently evaluated this drug and found it to be effective against MDR strains of *Mycobacterium tuberculosis* (MTB). ISO, a thiourea (thiocarlide, 4, 4 –diisoamyloxythiocarbanilide) demonstrated potent activity against standard strain of *MTB.* (Phetsuksiri *et al*., 1999). It had been noted that it strongly inhibited mycolic acid synthesis in *Mycobacterium bovis*.

The log phase, sometimes called the logarithmic phase or the *exponential phase,* is a period characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the present population. If growth is not limited, doubling will continue at a constant rate so both the number of cells and the rate of population increase doubles with each consecutive time period. The stationary phase is often due to a growth-limiting factor such as the depletion of an essential nutrient, and/or the formation of an inhibitory product such as an organic acid. Stationary phase results from a situation in which growth rate and death rate are equal. The number of new cells created is limited by the growth factor and as a result the rate of cell growth matches the rate of cell death.

In this study we have evaluated bactericidal activity of INH and RF alone and in combination with ISO by time kill kinetic study *in vitro* against log phase and stationary phase culture and in murine cell line against standard strain and MDR strain of *Mycobacterium tuberculosis*

2. Material and Methods

Bactericidal activity of INH and RF alone and in combination with ISO was evaluated by time kill kinetic study *in vitro*. [8, 9] Standard strain of MTB H37Rvwas tested in the experimentation. These strain was supplied by P.D. Hinduja Hospital and Medical Research centre, Mumbai. Medium used for growth was Sterile Dubos broth with glucose and albumin supplements with 0.05% Tween 80. (Himedia laboratories) and sterile Lowenstein Jensen Medium (LJM) slants. (Himedia laboratories Pvt). Drugs used were ISO (Cayman Chemicals), INH (Lupin Laboratories), RF (Lupin Laboratories).

In first experiment, 50 ml of sterile Dubos broth with glucose and albumin supplements with 0.05% tween 80 was distributed into 5 sterile flasks. In four flasks, drug solutions namely INH (1 mcg/ml), RF (1 mcg/ml) and ISO (1 mcg/ml and 5 mcg/ml) at various concentrations were added, individually. 5th flask was treated as positive control in which no drug solution was added. It was used in parallel with the other flasks. Log phase test culture was inoculated into the flask containing sterile medium with or without various concentrations of the individual drug. The culture density was adjusted to 10^5-10^6 CFU/ml. The flasks were incubated at 37^0c for 6 days under stationary condition. Samples were drawn from each flask for viable count at 0, 2, 4 & 6th day. Viable counts were obtained by diluting 1ml of sample drawn to 10^{-1} to 10^{-3} dilution to reduce the drug carry over effects and inoculated 0.1 ml of dilution on LJM slants in duplicate. LJM slants were incubated at 37 ^{0}c for 21 days and number of colonies was counted on each LJM slant. Counts were expressed as CFU / ml.

In 2nd experiment 50 ml of sterile Dubos broth with glucose and albumin supplements with 0.05% tween 80 was distributed into 5 sterile flasks. In first flask, drug solutions namely INH (1 mcg/ml) were added, individually, while in 2nd, 3rd and 4th flask drugs at various concentrations were added in combinations namely INH +RF (1 mcg/ml and 1 mcg/ml), INH+ ISO (1 mcg/ml and 1 mcg/ml) and INH +

ISO (1 mcg/ml and 5 mcg/ml) respectively. Two dilutions of ISO were tested in combination studies, one high (5 mcg/ml) and another low (1 mcg/ml). 5th flask was treated as positive control in which no drug solution was added. It was used in parallel with the other flasks.

In 3rd experiment 50 ml of sterile Dubos broth with glucose and albumin supplements with 0.05% Tween 80 was distributed into 5 sterile flasks. In first flask, drug solutions of RF (1 mcg/ml) were added, individually, while in 2nd, 3rd and 4th flask drugs at various concentrations were added in combinations namely INH +RF (1 mcg/ml and 1 mcg/ml), RF+ISO (1 mcg/ml and 1 mcg/ml) and RF + ISO (1 mcg/ml and 5 mcg/ml) respectively. Two dilutions of ISO were tested in combination studies one high (5 mcg/ml) and another low (1 mcg/ml).

Same experiments were repeated using inoculums as stationary phase culture.

Concentrations of ISO are selected as per the MIC of test strain. Killing curves of the organism were plotted after calculating the $\log_{10}$ CFU V/S time for each of the incubations.

3. Results

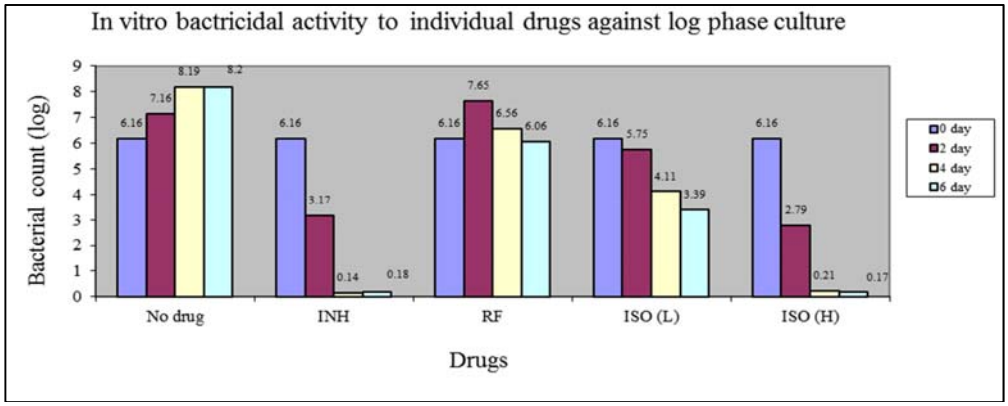

Fig 1: *In vitro* bactericidal activity to individual drugs against log phase culture

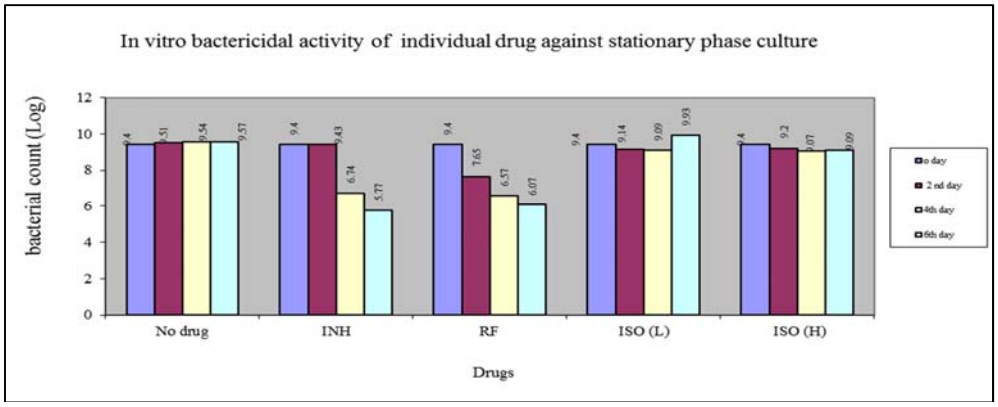

Fig 2: *In vitro* bactericidal activity of individual drug against stationary phase culture

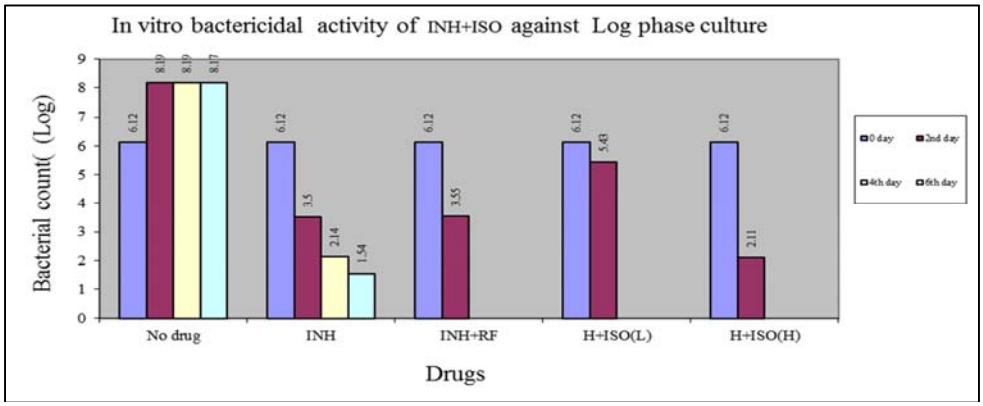

Fig 3: *In vitro* bactericidal activity of INH+ISO against Log phase culture

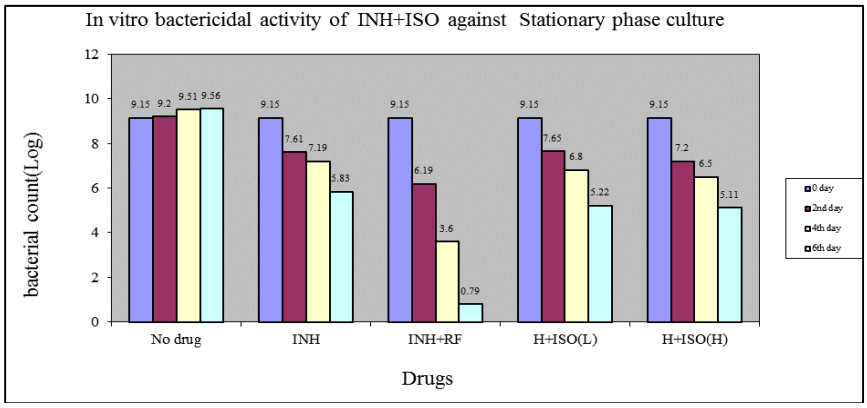

Fig 4: *In vitro* bactericidal activity of INH+ISO against Stationary phase culture

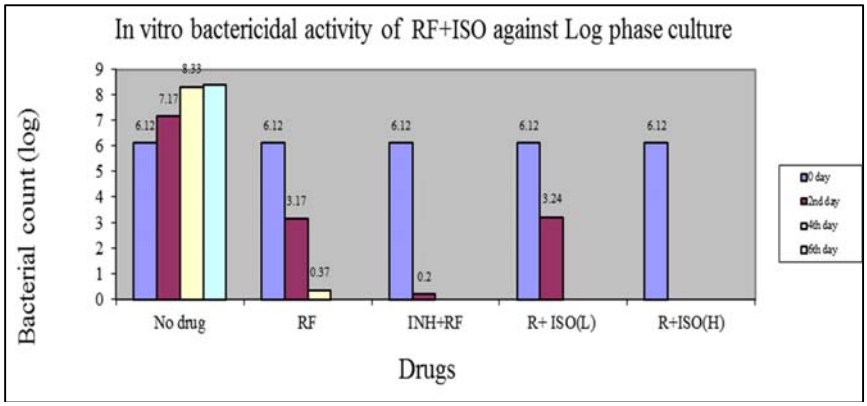

Fig 5: *In vitro* bactericidal activity of RF+ISO against Log phase culture

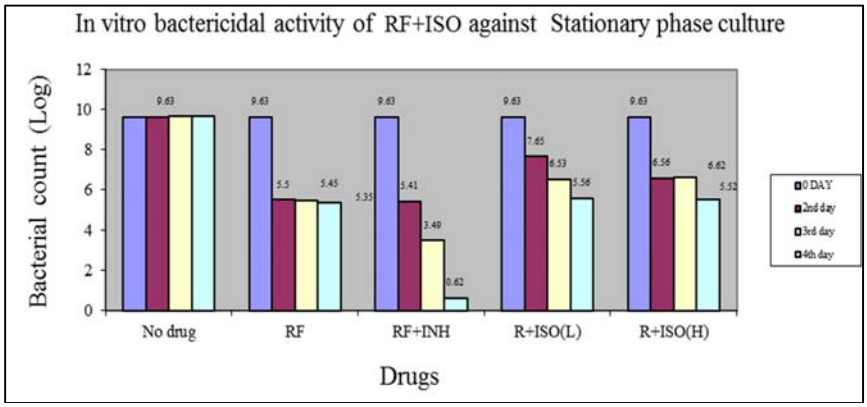

Fig 6: *In vitro* bactericidal activity of RF+ISO against Stationary phase culture

4. Discussion

4.1 *In vitro* bactericidal activity ISO, RF and INH individually and in combination.

Various types of *in vitro* models of bactericidal activity have been devised, the main classes are those with constant antibiotic concentration, which study the effects of a constant concentration of drug against bacteria as a function of time and those with variable antibiotic concentrations fluctuated by dilution or diffusion**.**

In present study we have used both the models to study time-kill kinetic**.** The standard strain of MTB was selected for the time kill studies. The results of the two experiments, one on the exponential phase culture and the other on the stationery phase culture were presented separately. The two antibiotics, INH and RF along with ISO were studied individually and in combination. In study with individual drugs using Standard strain of MTB following results were observed. There was continued growth of the drug-free exponential phase culture but no growth of the stationery phase culture.

In the exponential phase culture RF, INH & ISO at the higher concentrations, all had similar bactericidal activities. There was significant association between INH (1 mcg/ml), RF (1 mcg/ml) and ISO (5 mcg/ml), whereas ISO at the lower concentration showed lesser bactericidal activities.

In stationery phase cultures however RF and INH retained moderate levels of bactericidal activity while ISO, even at their higher concentrations had much less activity with standard strain of *M. tuberculosis*. There was significant difference between the activities of INH (1 mcg/ml), RF (1 mcg/ml) and ISO (5 mcg/ml). While there was significant association between the activities of ISO at 1 mcg/ml and 5

mcg/ml. When ISO was added to INH, in the exponential phase culture, the combination was more bactericidal than INH alone. There was no significant difference between the activities of INH and INH +ISO treated cultures. Each of the combinations namely ISO + INH with the higher ISO concentrations (5 mcg/ml) was nearly as bactericidal as INH + RF combination and while the combinations with lower ISO concentration (1 mcg/ml) were somewhat less bactericidal. When ISO was added to RF, in the exponential phase culture, the combination was more bactericidal than RF alone. There was no significant difference between the activities of RF and RF +ISO treated cultures.

Each of the combinations namely ISO + RF with the higher ISO concentrations (5 mcg/ml) was nearly as bactericidal as INH + RF combination and while the combinations with lower ISO concentration (1 mcg/ml) were somewhat less bactericidal. While ISO + INH combination, with ISO at higher concentration, showed significant difference in bacterial count in comparison to INH treated culture. There seems to be synergistic activity between INH and ISO at higher concentration of ISO. With higher ISO concentration with RF and INH there was significant decrease in the viable count at the end of 6 days that is indicative of synergism and demonstrating an enhanced bactericidal activity over of the individual drug. In contrast, in stationery phase cultures, the additions of ISO had hardly any effect on the activity of INH or RF and the combinations were much less active than INH+RF. Thus the ability of ISO particularly at their higher concentrations to the increase the bactericidal activity of INH or RF was lost as the culture changed from exponential phase to stationery phase.

When ISO was added to RF or INH, there was probably an increase in the level of bactericidal activity against exponential phase organisms and not in the stationery phase cultures. In summary therefore, synergistic bactericidal effects between ISO & INH and RF& ISO were evident in exponential phase cultures but were absent in the stationery phase cultures. The result of the present study suggests that ISO could be particularly useful in the first few days of treatment of pulmonary TB, when the majority of the bacterial population is actively multiplying in cavity walls. Here they were supplied with air and when the risk of selection of resistant mutants to accompanying drugs is greatest, they would also be valuable in killing bacilli that grow actively after a bacteriological relapse that happens during and after the end of treatment. In view of their considerable bactericidal activity against the exponential-phase cultures, they would be expected to have fairly high bactericidal activity at the start of treatment. No estimates have been published of the Early Bactericidal Activity of ISO**.**

On the other hand, the low level of bactericidal activity on stationery phase cultures suggest that, though they would still continue to inhibit growth, ISO would not be effective in shortening the duration of treatment. Thus, if the drug combination does not contain another sterilizing drug, treatment could be continued for at least 12 months, as was the custom before the advent of the effective sterilizing drugs like RF & PZ [9]. There is no direct evidence of the sterilizing activity of ISO in human disease. In conclusion, routine estimation of the bactericidal activities of drugs against stationery or new stationery phase cultures of MTB is desirable in the preclinical phase of drug development as an indication of sterilizing activity. The work is however, necessary to establish the method of assessment by comparing its results with estimates of sterilizing activities of drugs against experimentally induced murine TB and in patients with pulmonary TB by comparison with relapse rates after chemotherapy and the proportion of patients with easy sputum conversion [9]

Clinical experiences with ISO in combination therapy of freshly evaluated TB showed negotiation reached at the end of third months of therapy was 84.6%. After fourth months of therapy in all cases a microscopic and cultured sputum conversion was reached. Upto the negativation reached the antibiograms showed in no instance resistance to INH, Streptomycin, Ethionamide and ISO. The freshly evaluated and untreated TB needs the combination treatment. The results shown here with the combined medicament, ISO was in author's opinion not unsatisfactory in bactericidal activity [10].

5. Conclusion

Synergistic bactericidal effects between ISO & INH and RF& ISO were evident in exponential phase cultures but were absent in the stationery phase cultures. The ability of ISO particularly at their higher concentrations to the increase the bactericidal activity of INH or RF was lost at the culture changed from exponential phase to stationery phase. The result of the present study suggests that ISO could be particularly useful in the first few days of treatment of pulmonary TB, when the majority of the bacterial population is actively multiplying in cavity walls. ISO is expected to have fairly high bactericidal activity at the start of treatment. The low level of bactericidal activity on stationery phase cultures suggest that, though they would still continue to inhibit growth, ISO would not be effective in shortening the duration of treatment. The results shown here with the combined medicament, ISO was in our opinion not unsatisfactory in bactericidal activity.

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