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Research Article

# ASSOCIATION OF GENERATION TIME WITH ANTI-TUBERCULAR DRUG(S) RESISTANCE PATTERN OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES AMONG TREATMENT FAILURE PULMONARY TUBERCULOSIS PATIENTS

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**ABSTRACT**

**Objective:** The emergence of drug resistance has complicated tuberculosis (TB) scenario and is associated to treatment failure. The causative agent, *Mycobacterium tuberculosis* is usually slow growing and has been implicated as a contributing factor for drug tolerance and development of resistant strains. On the other hand, if rapidly growing bacilli, with shorter generation time emerge, mutations may lead to the development of drug resistance. From the hypothesis, this study was aimed to explore the whether there is any association between the generation time of Mycobacteria with their distinct drug resistant pattern.

**Methods:** *In-vitro* generation time was determined from 77 mycobacterial isolates with varied drug resistance pattern, i.e. rifampicin resistant (RIFR), isoniazid resistant, multi-drug resistant (MDR), the sensitive clinical strains along with reference strains. The minimal inhibitory concentration was also determined for the respective resistant groups.

**Results:** Among the individual group of clinical isolates, there was a significant negative association between generation time and drug resistance pattern of RIFR isolates.

**Conclusion:** Keeping the current upsurge of the MDR-TB epidemic in India and the influence of generation time on dosing schedule and treatment strategy, necessary customization of dosing and therapeutic planning seemed urgent to minimize the operational and clinical potential for development of drug resistance among treatment failure pulmonary TB patients in this country.

**Keywords:** *Mycobacterium tuberculosis,* Generation time, Multi-drug resistant, Treatment failure.

## INTRODUCTION

Tuberculosis (TB) continues to be a major public health problem and is currently the second largest infectious cause of death worldwide [1]. About 58% of these new infections were reported from Asian countries, with India being the largest contributor of incident TB infections in the world (125-299 per 100,000 populations), accounting for 26% of the total global cases [1].

Since the anti-tubercular drugs (ATD) have become available, improper regimen, indiscriminate usage, and less than optimal adherence have undermined the potential benefits - largely by facilitating the emergence of drug-resistant strains, particularly the multi-drug resistant (MDR) variety [2,3]. Expectedly, patients with prior exposure to anti-TB therapy turned out to be the ones more vulnerable to developing drug resistance [4]. However, it was perturbing to note that even the newly diagnosed cases had a considerable propensity of having MDR, with possible contributing factors being spontaneous mutation and transmission of resistant strains from others patients harboring MDR-TB [5,6].

Among the various categories of TB, treatment failure groups are associated with high mortality with worst treatment outcomes [7]. Treatment failure patients associated with MDR have higher propensity of spreading the infection to the new individuals.

It is important to note that *M. tuberculosis* radically reduces both its growth and metabolism in unceasingly infected animals, doubling only once every 100 hrs or more [8,9]. Since close to all antibiotics preferentially kill rapidly replicating bacteria [10,11], it has been hypothesized that the reduction of growth and metabolic activity of these dormant populations is responsible for the "drug-tolerance" observed during infection [12,13]. A large proportion of bacilli in cavitating lung lesions are either slow growing or dormant, and these are insensitive to isoniazid (INH) therapy as deteriorated transcriptional retort leads to drug tolerance [14].

Accordingly, it is apparent that promulgation of dormant or non-replicating mutants with longer generation time could contribute to treatment failure. In addition, it is known that generation time of *M. tuberculosis* is normally 15-18 hrs [15] and as evident from *in-vitro* experiments, if a culture of *M. tuberculosis* is exposed to certain drugs for some time, it takes several days (the "lag period") before new growths to occur [16]. Thus, it is also speculated that, if strains with shorter generation time appear and proliferate, drug regimen (thrice a week) might result in treatment failure giving false impression of drug-resistant TB particularly MDR-TB.

Subsequently, considering the above facts and keeping the current upsurge of MDR along with treatment failure epidemic in India, the present study is directed to evaluate and correlate the generation time with the varied resistance pattern of the mycobacterial isolates from treatment failure pulmonary TB (PTB) cases.

## METHODS

### Study isolates

A total of 77 strains (72 clinical and 5 reference strains) of *M. tuberculosis* isolates; 18 samples each (based on equal proportional distribution) from treatment failure drug resistant categories (INH-resistant

[INHR], rifampicin resistant [RIFR], and MDR), 18 samples from dual sensitive (both INH&RIF) group were recruited from Indian Reference Laboratory, Beliaghata, Kolkata. Five reference strains (H37Rv, H37Ra, RIFR TRC, INHR TRC and HRR TRC) were also recruited.

**Determination of mycobacterial generation time**

*Preparation of single cell suspension*

Single cell suspension was prepared following the method by Chakraborty *et al*. [17]. Briefly, Loopful colonies (log phase) of *M. tuberculosis* in solid L-J medium were transferred in a vial consisting of normal saline with 4-5 glass beads. It was then vortexed for repeated times. The suspension was kept undisturbed all night allowing larger bacterial clumps to settle down. The upper part of the bacterial cell suspension was carefully transferred in a sterile vial. The suspension was centrifuged, and the pellet was finally dissolved in 1 mL sterile distilled water. 100 µL from the above suspension was used for Z-N staining. The stained sample was diluted before take the count in hemocytometer.

*Enumeration of mycobacterial suspension*

Based on hemocytometer counting, the mycobacterial cell suspension was adjusted to desired number of cells [17]. The mycobacterial suspension was incubated in the dark at 37°C inside $CO_2$ incubator (Heal Force, Shanghai, China) with 5-10% $CO_2$ and agitated daily. At different time points, i.e. at 0, 15, 30, 45, 60 hrs - mycobacterial suspension was inoculated on Middlebrook 7H11 agar for colony forming units (CFU) count. The bottles were further incubated accordingly.

**Culture and CFU count**

After incubation the developed CFU were counted and expressed as a number of CFU/ml and mean of the three sets were taken into consideration for that particular time point and for a given strain.

**Calculation of generation time**

The generation time was calculated [18] by taking 5 different time points, i.e., at 0, 15, 30, 45 and 60 hrs; three sets of experiment were carried out for each parameter (CFU/mL) and then the mean of the three values were taken for consideration for growth curve preparation and generation time estimation. Distinct growth curves were prepared taking time on X-axis and mean CFU/mL on Y-axis.

**Minimal inhibitory concentration (MIC) value determination of *M. tuberculosis* strains**

MIC values were used as the quantitative estimation of drug resistance in the corresponding isolated organisms. MIC of all the clinical isolates and reference strains was determined by following the CDC guidelines [19] where absolute critical concentration of INH was 0.2µg/mL and RIF was 1 µg/mL, above which the mycobacterial clinical isolates are resistant to both the drugs. The MIC test was performed by MGIT 960 (BD) in 7 mL MGIT-BBL tubes (by following manufacturer instructions).

**Statistical analyses**

Values for MIC (for the drugs INH and RIF) and generation time were determined as the mean of three simultaneously repeated observations. The association between MIC and generation time was determined using Spearman's rank correlation coefficient (using Graph Pad Prism Software Version 5.00) and mixed linear regression (using SAS Version 9.3.2).

**RESULTS**

**Determination of mycobacterial generation time**

The mean generation/doubling time determination by CFU counting was found to be functional as the gradual increase of the appearing colonies on 7H11 plates were observed (5 references strains and 5 clinical isolates) with respect to 0, 15, 30, 45 and 60 hrs time points. The appeared colonies over different time points were used to prepare the growth curve and determination of generation time from the curve.

**Correlation of mycobacterial generation time with drug resistance pattern**

The result showed that among INHR group, the value of MIC's ranged between 0.4 and 5 µg/mL with preponderance of 0.4 to 1 µg/mL. However, two samples were found to be highly resistant to INH (5 µg/mL). In the case of RIFR group, the MICs ranged between 2 and 32 µg/mL with the majority of 2 to 16 µg/mL and four samples were found to be exceedingly resistant to RIF (32 µg/mL). While, among MDR group, the MICs of INH ranged between 0.4 and 3 µg/mL with high resistance to INH was 3 µg/mL, whereas the MIC's of RIF ranged between 2 and 32 µg/mL, with highly resistance to RIF was 32 µg/mL.

Interestingly, a significant correlation was observed only among the RIFR organisms of treatment failure group between their generation time and MICs of RIF *in-vitro* (Spearman's rank correlation coefficient $r^2$=0.3300, p=0.0092) (Fig. 2b and Table 1). However, the correlation between generation time and MIC among INHR and MDR groups by both the above said methods lacks sufficient statistical power (Fig. 2a, c, d and Table 1).

**DISCUSSION**

The determination of mycobacterial growth rate is a complex process due to its slow growing nature as well as the clump formation in the media. The generation/doubling time of *M. tuberculosis* has been studied by some investigators previously [20-23] and are mainly based on the reference strains of *M. tuberculosis* [24]. Moreover, no studies have been conducted till date on how the generation time of *M. tuberculosis* varies according to their drug resistance pattern.

In this work, we compared the generation time of H37Ra and H37Rv along with the various pulmonary clinical isolates of mycobacteria with their distinct drug resistance pattern (Fig. 1).

It was interesting to note that, among all the strains, the lowest *in-vitro* generation time was observed by H37Rv, i.e. 12.47 hrs. However, the study by Manca *et al.,* in 1999, [25] showed 1.6 times higher generation time by this same strain *in-vitro.*

In our study, the overall *in-vitro* mean generation/doubling time of the clinical isolates was found to be 15.07 hrs. Among the individual group of clinical isolates, RIFR showed the least generation time, i.e., 16.015 hrs. The reasons might be the experimental procedures adopted by us in our laboratory setting, i.e. factors include culture and CFU counting of the appeared colonies, maintaining temperature of 37°C to the individual 7H11 agar plates at 5% $CO_2$ in $CO_2$ incubator, responsible for the better growth and hence better determination of the generation of individual organisms.

Interestingly, it has been found that the generation time of INHR from treatment failure cases was significantly higher compared to other groups. Some studies have found INHR strains having mutation in *KatG* gene which signifies loss of catalase-peroxidase activity. Moreover, INH

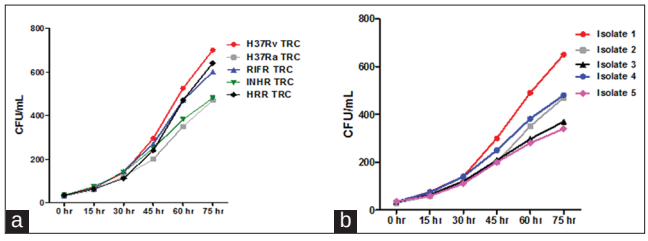


**Fig. 1: Growth curve of the mycobacterial reference strains (TRC). (a) The generation time was calculated from the linear portion of the growth curve for each bacteria. Growth curve of the treatment failure mycobacterial isolates, (b) The generation time was calculated from the linear portion of the growth curve for each bacteria**

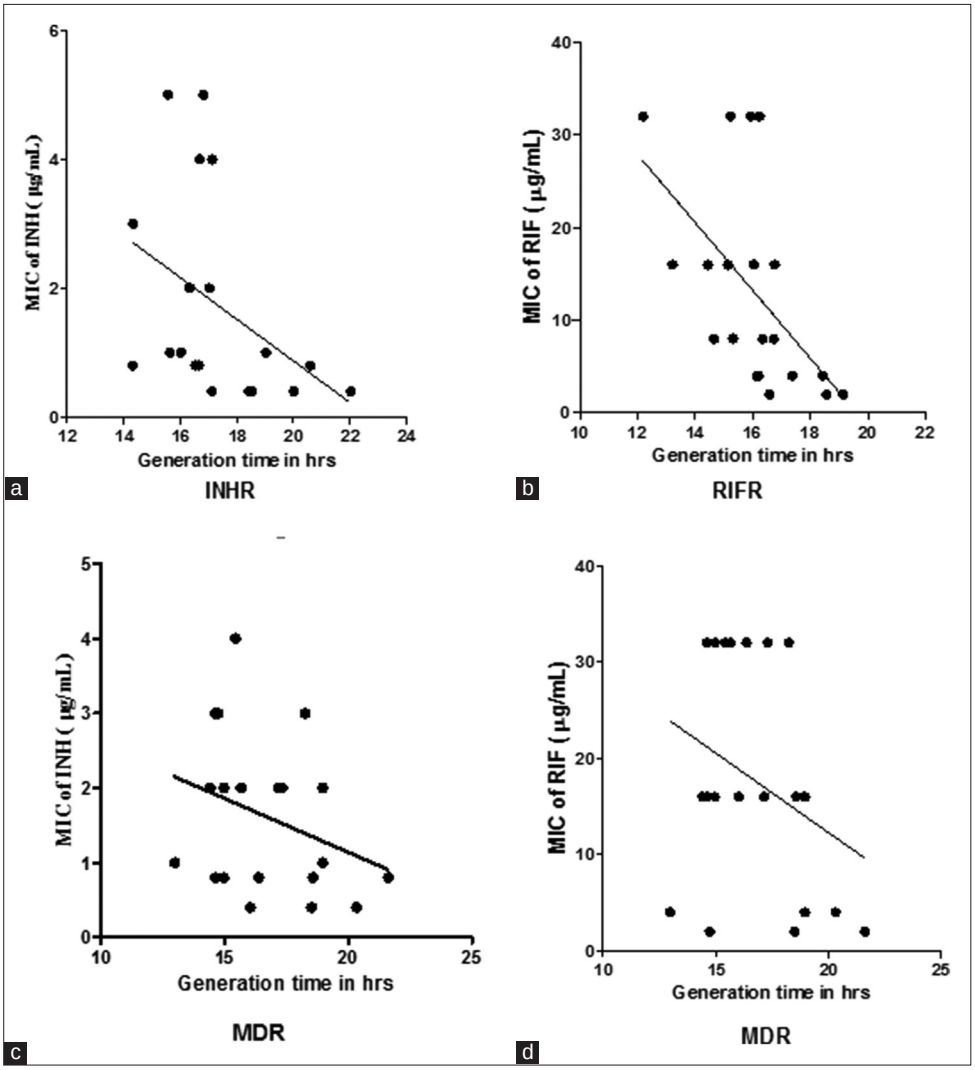


**Fig. 2: Correlation between *in-vitro* mycobacterial generation time and minimal inhibitory concentration (MIC) of isoniazid (INH) and rifampicin (RIF) drugs. The linear regression showing the relation between *in-vitro* mycobacterial generation time and minimal inhibitory concentration of INH and RIF drugs. (a) Non-significant correlation was observed among the generation time and MIC of INH R group with MIC'S of INH (n=18, Spearman's rank correlation coefficient), (b) Significant correlation was observed among the generation time of RIFR group with MIC'S of RIF (n=18, Spearman's rank correlation coefficient; $r^2$=0.3300, p=0.0092). The solid line represents the linear regression curve of best fit, (c) Non-significant correlation was observed among the generation time of multi-drug resistant (MDR) group with MIC'S of INH (n=18, Spearman's rank correlation coefficient), (d) non-significant correlation was observed among the generation time of MDR group with MIC'S of RIF (n=18, Spearman's rank correlation coefficient)**

acts on cell wall mycolic acids and prevents the formation of envelope of the organism. Hence, INHR organisms might takes a longer time to synthesize envelope and cell wall structure which give rise to higher generation time [26].

The MIC, in our study, was found to be rationale with prior findings where highest resistance to INH and RIF, in our study, was found to be 5 and 32 µg/mL, respectively [27,28]. Correlations of the concentration of individual drugs (INH and RIF) with the generation time of the organisms were found interesting (Fig. 2).

The correlation between mycobacterial generation time and drug resistance pattern revealed that there existed a statistically significant negative association between generation time and MIC for RIF while there seemed to be a similar negative association for INH also but result lacked power (Table 1). Data are unavailable in this context and none has demonstrated any correlation between generation time with the MIC of INH and RIF among the treatment failure group. We believe, as RIF has got late sterilizing activity on the bacterium [26] and this might help the bacteria to undergo in a stage of dormancy. Some metabolically active *M. tuberculosis* might escape the bactericidal effect of RIF. Furthermore, some bacteria might leave the dormant stage and starts multiplying between the time periods for next administration of the drug.

Moreover, increased size of inoculums accelerates growth [23] and this may shorten the doubling/generation time which corroborates our result. RIF might get no/less effect on increased bacterial population and this helps in developing resistance. Although only one partially comparable study has been done [29]. Our study revealed association between the *in-vitro* generation time and drug resistance pattern in the case of RIFR bacteria which showed significantly shorter generation time *in-vitro*.

## CONCLUSIONS

It can be concluded that higher the resistance to RIF, lesser the generation time. There seemed to be a fairly strong negative association between mean generation time and MIC for commonly prescribed first line ATD among the treatment failure group. Keeping the current upsurge

**Table 1: The correlation between *in-vitro* MIC and the generation time of drug resistant *M. tuberculosis***

| Mycobacterial isolate | Mean generation time (h) | Correlation between MIC and mean generation time | |
|---|---|---|---|
| | | $r^2$ | p value |
| INH-mono-R | 17.247 | 0.1643 | 0.0743 |
| RIF-mono-R | 16.015 | 0.3300 | 0.0092 |
| Resistant to both INH and RIF | 16.711 | 0.0973<br>0.0969 | 0.1791<br>0.1811 |
| **Drugs** | **Regression between MIC and mean generation time** | | |
| | **Coefficient (95% CI)** | **SE** | **p value** |
| INH | −0.197 (−0.409-0.017) | 0.107 | 0.0711 |
| RIF | −1.611 (−3.149-−0.067) | 0.769 | 0.0409 |

The regression analyses revealed that there existed a statistically significant (at α=0.05) negative association between generation time and MIC for RIF (β=−1.611, p=0.0409) while there seemed to be a similar negative association for INH also (β=−0.197, p=0.0711). *M. tuberculosis*: *Mycobacterium tuberculosis*, MIC: Minimal inhibitory concentration, CI: Confidence interval, SE: Standard error, INH: Isoniazid resistant, RIF: Rifampicin resistant

of the MDR-TB epidemic in India and the influence of generation time on dosing schedule and treatment strategy, necessary customization of dosing and therapeutic planning seemed urgent to minimize the operational and clinical potential for development of drug resistance among treatment failure PTB patients in this country.

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