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Microbiology

A Study to Compare Different Methods for Determination of Generation Time of *Mycobacterium tuberculosis* Isolates

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Abstract		Original Research Article

Apart from genes responsible for drug resistance of *Mycobacterium tuberculosis*, dramatic reduction of metabolic activity of tubercle bacilli in chronically infected animals reveals long generation time. This could be an additional explanation of drug resistance. Amongst different methods available to determine generation time, colony count method, which is cheap, easily available and affordable one, is compared with a novel method i.e. DNA spectrophotometry in an attempt to cut short turnaround time. Thus, this study was conducted to compare two different methods (Colony count method and DNA spectrophotometry) for determination of generation time of *Mycobacterium tuberculosis* in vitro. For this, 20 new sputum smear positive patients were placed in the study. *Mycobacterium tuberculosis* strains were isolated in pure culture by decontaminating, liquefying & concentrating sputum sample & anti-tubercular susceptibility tests were performed. From these, 10 all-drug (Rifampicin and Isoniazid) sensitive isolates were chosen and reference strains were procured. Single mycobacterial cell suspension was prepared from each. After proper standardization, they were incubated in CO_2 incubator and 10μl of each culture suspension was inoculated in Middlebrook 7H11 agar plate to measure CFU/ml and from 500 μl of each culture suspension, DNA extraction is done by Phenol-Chloroform method which is followed by Spectrophotometric quantification of OD/ml (at 260nm) at different time-points (Hour 0,18,36,54,72). Generation time was evaluated from graph of the above colony counts using formula. It was observed that serial culturing by counting of CFU is the best method for assessment of generation time while DNA spectrophotometry could not be used for this purpose. In short, colony count method still stands as the gold standard for determination of generation time for mycobacteria whereas DNA spectrophotometry failed poorly to evaluate the same.
Key words: Tuberculosis, *M. tuberculosis,* Colony count, DNA spectrophotometry, Generation time.

INTRODUCTION

Tuberculosis has been scourge of mankind for thousands of years since time immemorial and being an ancient disease it continues to remain a major public health problem worldwide even today. The problem is further complicated particularly by relentless spread of HIV and emergence of drug resistant tuberculosis. Globally, in 2017, there were estimated 10 million incident cases of TB with 1.3 million deaths among Human Immunodeficiency Virus (HIV) negative cases and in India 2.74 million estimated incident cases and 4.1 lac (excluding HIV) deaths occurred in 2017. Moreover, there were 1.23 lac incident cases of (Multi-Drug Resistant / Rifampicin Resistant) MDR/RR-TB estimated in the year 2017[1].

There are so many reasons for treatment failure out of which mutant strains with resistant genes is most significant. But, scarcity of mutant strains of *Mycobacterium tuberculosis* with multi-drug resistant genes in cavitary pulmonary tuberculosis[2], requirement of remarkably long course of therapy to eradicate the pathogen [3], dramatic reduction of metabolic activity of tubercle bacilli in chronically infected animals [4,5], existence of some drug resistant isolates of *Mycobacterium tuberculosis* having very slow rate of growth [6] and even presence of few strains with INH resistance (having no resistance mutation) with significantly higher (upto 67.9hrs) generation time[7], reveal apart from drug resistant

genes, long generation time could be an additional explanation of drug resistance.

This study was planned to evaluate mycobacterial generation time from the colony forming unit (CFU) count and DNA spectrophotometric values. Although, various modern technique have emerged to detect the growth of mycobacteria like, BACTEC radiometric system and the mycobacteria growth indicator tube (MGIT), still they are not only costly but also use of radioactive material is hazardous and the MGIT system hardly gives quantitative result. Groll *et al.* rightly pointed out that although these methods are faster [8, 9] and sensitive [6] (also supported by Chien *et al.* Morgan *et al.* Roberts *et al.*) [8-10] they require expensive equipment and they are inflexible since they depend on a single pre-packaged growth media [10] Chien *et al.* also observed that the rate of contamination is much higher with BACTEC MGIT 960 than conventional Löwenstein-Jensen medium [8]. Thus, keeping all this in mind, methods which are cheap, easily available in any laboratory like CFU count on solid culture media and a new one i.e. DNA spectrophotometry were employed here to evaluate generation time.

MATERIAL & METHODS

In this study, consecutive 20 new sputum smear positive pulmonary tuberculosis patients of all age groups and sexes, giving informed consent and having no co-morbid illness were recruited. Moreover, reference strains of *Mycobacterium tuberculosis*, H37Rv and H37Ra were included in this study. Patients with diabetes mellitus, pregnancy, malignancy, HIV infection, & patients on immuno-suppressive agents and transplant recipients were excluded from the study group.

Mycobacterium tuberculosis isolates were obtained in pure culture by decontaminating, liquefying & concentrating sputum sample & later on identified using standard staining and colony morphology with biochemical characteristics. Then, anti-tubercular susceptibility tests were performed by standard economic variant of 1% Proportion method [11]. From these isolates, 10 all-drug (Rifampicin and Isoniazid) sensitive isolates were chosen and from each isolate, single mycobacterial cell suspension was prepared in Middlebrook 7H9 broth. After proper standardization, they were incubated in CO_2 incubator and 10μl of each culture suspension was inoculated in Middlebrook 7H11 agar plate to measure CFU/ml and from 500 μl of each culture suspension, DNA extraction was done by Phenol-Chloroform method with ethanol precipitation [12, 13] which was followed by Spectrophotometric quantification of OD/ml (at 260nm) (after purity check of extracted DNA) [14] at different time-points (Hour 0,18,36,54,72).
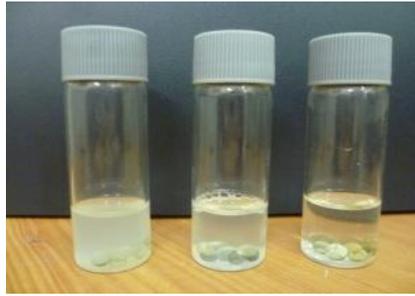
WORK PLAN FOR EACH SAMPLE:
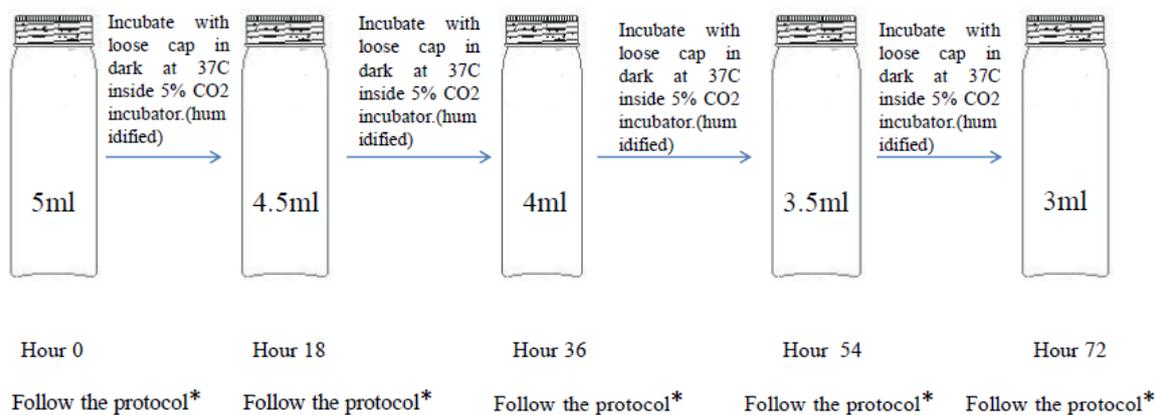
PROTOCOL (FOR EACH STEP)

1. Vortex.
2. 10μl was inoculated on Middlebrook 7H11, incubated as above and CFU were counted after 21days.
3. 500μl was taken for DNA extraction and corresponding OD values were evaluated by spectrophotometer.
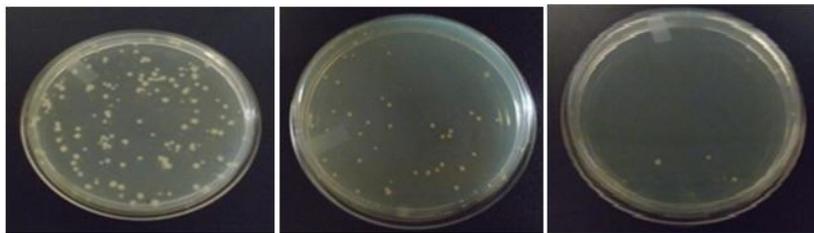
Fig-1: Work plan for each sample

Fig-2: Middlebrook 7H9 broth showing serial dilution

Generation time (g) was evaluated from graph (steep portion of growth curve) of the above colony counts using standard formula where N_1 and N_2 are the population number (CFU/ml or OD/ml) at time t_1 and t_2 respectively[15, 16].

$$g = \frac{0.301\ (t_2 - t_1)}{Log_{10}N_2 - Log_{10}N_1}$$

Fig-3: Middlebrook 7H11 agar showing CFU from serial dilutions

The experiment was performed in triplicate and average value is taken and appropriate statistical tests were applied as and when required.

Fig-4: UV-VIS Spectrophotometer (HALO DB-20, Dynamica GmbH, Austria)
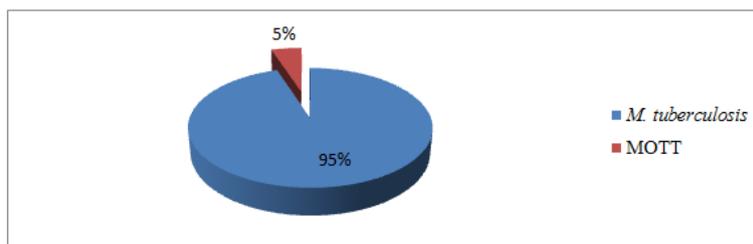
RESULTS

In the present study, a total of twenty (20) isolates from new sputum smear positive (for AFB) patients were included and out of that, 19 (95%) were *Mycobacterium tuberculosis* isolates and 1 (5%) was MOTT.(Figure 5).

Fig-5: Pie diagram showing distribution of Mycobacterium spp. among clinical isolates from new sputum smear positive patients

Among 19 (95%) *Mycobacterium tuberculosis* isolates obtained from new sputum smear positive patients, 18 (94.74%) isolates were sensitive to both Rifampicin (RIF) and Isoniazid (INH) and 1(5.26%) was resistant to INH only. But no isolate could be observed which was resistant either to RIF or both of the drugs (Table 1).

Table-1: Distribution of drug resistance pattern of *Mycobacterium tuberculosis* isolated from new sputum smear positive (N) (n=19)
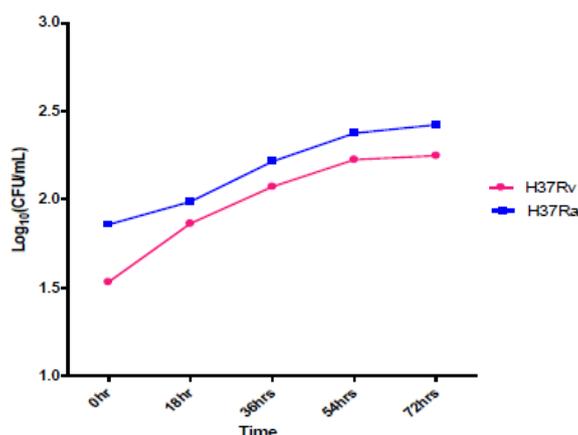
Drug resistance pattern	New sputum smear positive (19) No. (%)
Sensitive to both	18 (94.74%)
Monoresistance to Rifampicin	0
Monoresistance to Isoniazid	1(5.26%)
Resistance to both	0

The mean generation time of reference strains were found to be 16.33 hrs for H37Rv and 23.76 hrs for H37Ra by serial CFU count and using the standard formula. The experiment was performed in triplicate and mean value is taken (Figure 6).

Table-2: Generation time calculation of reference strains

Reference Strains	Hour 0 $X10^2$/ml	Hours 18 $X10^2$/ml	Hours 36 $X10^2$/ml	Hours 54 $X10^2$/ml	Hours 72 $X10^2$/ml	Generation Time (Hrs)
H37Rv	34	73	118	168	177	16.33
H37Ra	72	97	164	237	265	23.76

Fig-6: Growth curve of reference strains

Likewise, the mean generation times of ten (10) all drug sensitive isolates from new sputum smear positive patients are shown below in Figure 6. The experiment in each case was performed in triplicate and mean value is taken.

Table -3: Generation time calculation from CFU count of drug sensitive isolates (n=10)

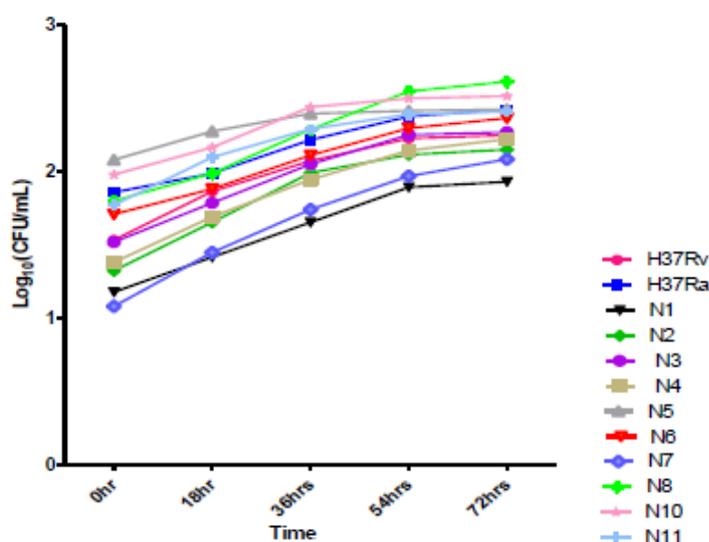| Drug sensitive Isolates	Hour 0 $X10^2$/ml	Hours 18 $X10^2$/ml	Hours 36 $X10^2$/ml	Hours 54 $X10^2$/ml	Hours 72 $X10^2$/ml	Mean Generation Time (Hrs)
N1	15	26	45	78	85	22.68
N2	21	45	98	130	141	16.02
N3	33	61	112	178	185	20.31
N4	24	49	88	139	166	17.48
N5	120	188	248	259	265	45.04
N6	51	76	129	197	231	23.58
N7	12	28	55	93	121	14.72
N8	63	97	193	352	410	18.13
N10	95	146	275	315	327	19.70
N11	59	125	195	245	256	16.62

Fig-7: Growth curve of Drug sensitive isolates (n=10)

The calculation of mean generation times of ten (10) all drug sensitive isolates from new sputum smear positive patients were attempted from serial OD values of DNA spectrophotometric measurements, using the standard formula are shown below in Table 4. The experiment in each case was performed in triplicate and mean value is taken.

Table-4: Generation time calculation from OD values of drug sensitive isolates (n=10)

Drug sensitive Isolates	Hour 0 X2/ml	Hours 18 X2/ml	Hours 36 X2/ml	Hours 54 X2ml	Hours 72 X2/ml	Mean Generation Time (Hrs)
N1	0.056	0.050	0.044	0.051	0.055	*
N2	0.057	0.048	0.051	0.045	0.041	*
N3	0.044	0.047	0.056	0.048	0.047	71.20
N4	0.045	0.040	0.034	0.041	0.025	*
N5	0.054	0.048	0.067	0.042	0.035	*
N6	0.051	0.040	0.054	0.031	0.015	*
N7	0.054	0.057	0.046	0.051	0.047	*
N8	0.046	0.060	0.034	0.041	0.025	*
N10	0.054	0.057	0.051	0.048	0.047	*
N11	0.047	0.038	0.051	0.025	0.041	*

* Generation time could not be calculated.

DISCUSSION

The present study was undertaken to compare experiments like CFU count and DNA spectrophotometry in measuring generation time. However, in this study, most of the isolates of new sputum smear positive patients were *Mycobacterium tuberculosis* and only 5% were the *M. fortuitum*. It has been seen that in Indian studies, *Mycobacterium tuberculosis* has always been found as the major cause of mycobacterial infections and the proportion of NTM has varied from less than 1% to 28% which is at par with our observation [17].

In this study, most of the isolates are sensitive to both of the drugs i.e. RIF and INH. This observation is supported by the Indian studies where the prevalence of MDR-TB, defined as resistance to INH and RIF with or without resistance to other drugs, is 2% to 3.5% in new cases [1] and overall emergence of resistance to RIF is only 2% of patients despite a high level (18%) of initial resistance to INH, either alone or in combination with other anti-tubercular drugs [18]. Moreover, the incidence of monoresistance to INH among new sputum smear positive patients as recorded by researchers like, Ramachandran *et al.* (TRC, Chennai) [19], Santha *et al.* [20] were 5.4%, 5.3%, that simulates our observation i.e. 5.26% although it is varied widely from the figure of Paramasivan *et al.* [21] *i.e.*12.8%.

Various workers observed that culture method is appropriate for assessment of generation time. Youmans in 1946 determined the culture cycle of the virulent human type tubercle bacillus, strain H37Rv by the use of micro-Kjeldahl nitrogen determinations and from the logarithmic portion of the growth curve, the growth rates and generation times were calculated [22]. Under the conditions of the experiment the generation times were found to vary between approximately 1.5

days and 3.5 days [22]. To overcome the shortcomings of this procedure, (*i.e.* particularly using very large inoculum size) later in 1949,Youmans *et al.* used decimal dilutions of virulent human type tubercle bacilli (varying from 10^{-1} to 10^{-8} mg moist weight) as inocula in the tubes of three types of liquid media containing basal medium with or without crystalline bovine albumin and beef serum and determined the time at which visible subsurface growth of each inoculum first appeared and ultimately, by plotting the logarithms of the inocula employed against the time at which visible growth of each inoculum first appeared, a linear relationship was found and slope of the straight line so obtained yielded the growth rate and the generation time [23] Pope *et al.* used the BACTEC 9240 continuous blood culture system with standard aerobic medium (Plus Aerobic/F) [24] to determine time to positivity as an indicator of the growth rate by modifying the method of Youmans and Youmans[23] O'Sullivan *et al.* [7] also employed a modified Youmans and Youmans method to calculate the generation time [23, 24] of *Mycobacterium tuberculosis* isolates by using an automated liquid culture system, the MB/BacT ALERT 3D. Others like Zimhony *et al.* Chien *et al.* Morgan *et al.* Roberts *et al.* Compared the conventional culture media like Löwenstein-Jensen medium and Middlebrook liquid and solid media with the other methods regarding mycobacterial growth and recovery [8-10, 25]. In our study, the generation time of reference strains like H37Rv was 16.33 hrs, which closely matches the data obtained by O'Sullivan *et al.* (15.9hrs)[7], although different methodology was followed here.

In this study, scope of a novel method like DNA spectrophotometry was also assessed regarding the calculation of generation time. Many researchers like Lambrecht *et al.* Bettencourt *et al.* Zimhony *et al.* Klann *et al.* have attempted to measure mycobacterial generation time from spectrophotometry directly from bacterial suspension [25-28] and Groll *et al.* by measuring quantitatively the reduction of resazurin by spectrophotometry[6] But only, Hiriyanna *et al.* [29] in their study of 'Deoxyribonucleic acid replication time in *Mycobacterium tuberculosis* H37Rv' employed DNA increment method (designed for measuring the increment in the amount of DNA after inhibition of initiation of fresh rounds of replication initiation) to measure the rate of deoxyribonucleic acid (DNA) chain growth in *Mycobacterium tuberculosis* H37Rv growing in Youman and Karlson's medium at 37°C with a generation time of 24 h. In this study to evaluate generation time of mycobacteria by DNA spectrophotometry, it was found that complete extraction of mycobacterial genomic DNA is really a hard nut to crack because robust and waxy cell wall renders mycobacteria difficult to lyse [13] and obviously, mycobacterial species as such are very slow growing organisms, accounting for small amounts of starting material. Jain *et al.* concluded that to extract

mycobacterial DNA from sputum samples successfully, a strong physical treatment to weaken the mycobacterial cell wall, use of a detergent and enzyme (chemical method) for lysis of the mycobacterial cell wall, DNA purification steps including treatment with proteinase K to remove proteins, DNA purification step with or without phenol and DNA precipitation in ethanol or isopropanol are essential steps for extraction of mycobacterial DNA from clinical samples[12] Kaser *et al.* used various matrix materials (zirconia beads or ceramic or glass beads) and homogenized the samples with a mechanical bead beater device [13], Bates *et al.* and Banavaliker *et al.* suggested further improvement of lysis could be achieved by use of suitable chemical (particularly with the use of a detergent) and enzymatic digestion of bacterial cell wall [30, 31] and Jain et al recommended use of either sodium dodecyl sulphate (SDS) or Triton X-100 in their study[12]. In the present work various methods *i.e.* physical, chemical and combination of both were tried to obtain maximum yield of mycobacterial DNA [12, 13] Although these methods described by many researchers are useful for PCR related studies, complete quantification of DNA is not seen particularly as the number of bacilli in the inocula in the present work were very less and the scope of application of amplification method was beyond question. So, out of so many methods followed [12, 13], combined method was seen to be most rewarding. One of the major hurdles in mycobacterial DNA extraction was at the lysis step. So to overcome that, processing of the sample with Lysis buffer, Lysozyme, SDS and Proteinase K, sequentially was largely followed from the study of Jain *et al.* [12] with necessary modifications like increment in concentration and or incubation time so as to obtain higher and purer DNA yield. Extraction of DNA using phenol: chloroform: iso-amyl alcohol improved the quantity as well as the quality of target DNA. As per study of Jain *et al.* protocols not using phenol and/or chloroform for extracting DNA were found to be unsatisfactory[12] Noordhoek *et al.* [53] observed that phenol extraction of DNA removes inhibiting substances from those samples in which inhibitors were present even after DNA extraction with guanidinium thiocyanate (GuSCN) and silica particles. Still, Brisson-Noel *et al.* [33] estimated that 5% of clinical specimens contain some inhibiting component(s), which are not removed by phenol extraction of DNA. But, Querol *et al.* achieved 97% PCR positivity by using phenol: chloroform: iso-amyl alcohol extraction followed by isopropanol precipitation of DNA [34] Similarly, some workers reported that the use of phenol and chloroform for extraction and ethanol and/or isopropanol for precipitation of DNA surely improves the yield of the purified target DNA, which finally results in increased sensitivity of PCR [35, 36]. That is why, in this study, DNA was extracted by using phenol: chloroform: iso-amyl alcohol method and the Phenol-chloroform extraction and Ethanol precipitation method was followed as described by Kaser *et al.* [13] After proper

purity checking of DNA it was seen that increment of DNA amount does not match with growth of bacteria and their gradual yield was so erratic that generation time could not be calculated.

CONCLUSION

There are ample reasons to believe that apart from drug resistant genes, long generation time could be an additional explanation of drug resistance. To evaluate generation time of mycobacteria, methods which are cheap, easily available in any laboratory like CFU count on solid culture media and a novel one i.e. DNA spectrophotometry were employed here. But, the observations of the present study showed that colony count method still stands as the gold standard for determination of generation time for mycobacteria while DNA spectrophotometry failed poorly to evaluate the same. Because, it was so found that, in spite of all attempts 100% yield of mycobacterial genomic DNA is really far from reality as human error during pipetting and separating aqueous phase leads to loss of bacterial DNA. Moreover, even apart from genomic DNA, DNA from other organelles could come into play and complicate the picture. Thus, DNA spectrophotometry as a tool to assess generation time of mycobacteria should not be opted.

ACKNOWLEDGEMENT

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