SIGNIFICANCE OF H37Rv MTB

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Introduction

One of the most important, yet often neglected, tasks in any routine microbiology laboratory is to maintain cultures of *Mycobacterium tuberculosis* (MTB) H37Rv & H37Ra strains. These strains are vital for their role, as the control strains, in identification and drug-resistance testing for tuberculosis disease besides their critical significance, as the challenge strain for any vaccine/drugs evaluation and related Bio-medical research. *M. tuberculosis* H37Rv is the most commonly used laboratory strain and was first isolated in 1905. Since then it is widely used in Microbiology laboratories, as a control strain, worldwide since 1940s [1]. However, over a period of time, it has been observed that the high virulent character of H37Rv is lost, perhaps due to repeated subculture on artificial media, mutation or lack of animal-passaging for restoring its virulence in an appropriate biological host**.** *In-vitro* serial passage of micro-organisms may produce important changes in their phenotypic characteristics [2]. There is a need to reassess the virulent nature of H37Rv or else prepare for its appropriate replacement with a well defined, genetically sequenced, high virulent local field strain for its future usage, as a laboratory control strain besides its vital application as a challenge strain in related research studies involving testing/evaluation of new drugs and vaccines against tuberculosis. While aiming at possible elimination of TB through prophylactic means, a step forward from just control/containment of TB menace that has latently infected one-third of the world's population and is estimated to cause 9 million new cases, 2 million deaths each year worldwide [1], the importance of quality control in every laboratory aspect has become essential.

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What is H37RV?

The term H37RV, first used to describe variants "R" and "S," but these letters did not refer to the words rough and smooth, to describe colonial variants. Instead the terms were used to describe those variants resistant (R) or susceptible (S) to the environment of the culture medium, even though both colonial variants were rough. If the original designations had persisted, the virulent variant of the neo-type strain would be H37S.The inapt choice of the letters R and S was realized, and changed the designation of the virulent variant from S to Rv (for rough virulent). Thus the term H37Rv was used for the first time, and the virulence of this variant was compared with that of the avirulent colony form H37Ra .Historically, *M. tuberculosis* H37Ra is the avirulent counterpart of virulent strain H37Rv and both strains are derived from their virulent parent strain H37, which was originally isolated from a 19 year-old male patient with chronic pulmonary tuberculosis by Edward R. Baldwin in 1905 [3].In order to obtain stable avirulent derivatives of H37, in 1935, William Steenken carried out a dissociation study based on aging of H37 bacilli on solid egg media [4]. The parental virulent H37 was inoculated onto solid egg media at pH 6.2. The resulting culture was allowed to age for 3–4 months at 37°C. By the end of the extended incubation, the original dry, discrete colonies lysed and transformed into a confluent viscous mass. In the midst of the viscous mass, secondary growth with different colony morphology emerged. The new growth, when picked and cultured on fresh media, produced no disease in guinea pigs [5] [6] and was designated H37Ra ("a" for avirulent).

The virulent counterpart (*i.e.*, H37 but with rough colony morphology) was named H37Rv ("v" for virulent) and the original H37 was discontinued. H37Ra and its virulent counterpart H37Rv have been widely used as reference strains for studying virulence and pathogenesis of *M. tuberculosis* worldwide since 1940s and H37Ra is also used as an adjuvant to boost immunogenicity during immunization [7].

Relevance of control/reference MTB strain

There are a number of reasons why a microbiology laboratory needs to maintain MTB reference strains in good condition besides their periodical testing for any unwanted changes that are known to happen. These control cultures may fall into one or more of the following categories:

1. Reference strains for quality control of culture media and methods.
2. In the preparation of inoculated specimens for quality control and training purposes.
3. Reference strains for the development and validation of new methods.
4. Pathogens and spoilage organisms in the investigation of contamination problems.
5. Cultures used in microbiological assays.
6. Well defined high virulent MTB isolates required for research purposes.

Reference MTB cultures are required for establishing acceptable performance of media (including test kits), for validating methods, for verifying the suitability of test methods and for assessing or evaluating ongoing performance. The laboratories must use reference strains of micro-organisms obtained directly from a recognized national or international collection, where these exist. Reference strains may be subculture once to provide reference stocks. Purity and biochemical checks should be made in parallel as appropriate. It is recommended to store reference stocks in aliquots either deep-frozen or lyophilized. Working cultures for routine use should be primary subcultures from the reference stock. If reference stocks have been thawed, they must not be refrozen and reused. Working stocks should not normally be subculture. Usually not more than five generations (or passages) from the original reference strain can be subcultured, if defined by a standard method or laboratories can provide documentary evidence that there has been no change in any relevant property especially its high/low virulent character. Commercial derivatives of reference strains may only be used as working cultures. [8]

How to maintain/restore Virulence of H37Rv?

Virulence of clinical isolates of *M.tuberculosis* is determined by injecting laboratory animals with the bacterial suspension prepared from the growth scrapped from Lowenstein Jensen (L-J) medium standardised on the basis of its optical density [9][10] or actual viable count. Virulence is assessed in terms of ability of bacilli to disseminate from the site of infection to the target organs in form of tubercle lesions in an appropriate animal model [11]. In the guinea pig animal model, six weeks post infection extent of disease is measured by scoring the tubercle lesions Virulence of tubercle bacilli was particularly related to the extent of the disease in the spleen of infected guinea pigs [12] A refinement in the above

mentioned method was achieved by means of microbial enumeration tubercle bacilli recovered from the lungs, liver, spleen and lymph nodes six weeks after infection. For the colony counts on spleen homogenates, separate sterilized Teflon-glass grinding tubes containing 5/4.5 ml gel saline are used for each spleen for mechanical homogenization. The number of colony forming units (CFU) is determined by inoculation serial tenfold dilution of each homogenate on L-J Media. Colonies of *M.tuberculosis* are counted on 28th day of incubation at 37^{o}C [13]. For subsequent animal passage, the cells harvested from the spleen tissue homogenate is prepared, in a dose 0.5ml of suspension containing about a million colony forming units (cfu) and injected in a pair of albino guinea pigs. After necropsy, scoring of lesion and processing of spleen tissue homogenate for viable counts to assess the virulence status is repeated. [14].

Three week old culture of H37Rv strain is subjected to virulence assay in NTI-bred albino guinea pigs. Briefly, 1.0 mg of cells harvested from a 3 week culture on L-J medium /0.5ml of suspension containing about a million colony forming units (cfu) is injected in a pair of albino guinea pigs through subcutaneous route in the medial aspect of the left thigh. The subcutaneous route of infection is used as it is the closest to the aerosol route in terms of deposition of bacilli in a defined anatomical site, followed by local growth, progressive dissemination through lymphatics into the circulation and eventually the hematogenous seeding of other organs to multiply and produce gross disease in the spleen, liver & lungs. Guinea pigs are then sacrificed 6 weeks post infection. Post-mortem examination is carried out immediately after death. The body weight of each animal is recorded before infection, weekly during post-infection and just before sacrifice. The animals are subjected to dissection. Gross lesions of the target organs namely spleen, liver, lungs and lymph glands are to be scored. For the colony counts on spleen homogenates, separate sterilized teflon-glass tissue grinding tubes containing 5/4.5 ml of gel-saline are used for each spleen for mechanical homogenization. The number of colony forming units (cfu) is determined by inoculating serial ten fold dilutions of each homogenate on L-J media, as per the standard procedures. Colonies of *M.tuberculosis* H37Rv are counted on 28th day of incubation at 37 C by an independent reader. [15]

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After necropsy, scoring of lesions and processing of spleen tissue homogenate for viable counts to assess the virulence status is repeated as described above. As such, periodical testing and retaining/restoring the high virulent character of *M.tuberculosis* H37Rv is maintained through animal passage in IAEC approved limited number of NTI-bred albino guinea pigs, as and when required.

Alternatives to H37Rv strain

Genetic differences have arisen in some stocks of $H_{37}Rv$ that are maintained in different laboratories or have diverged from the original strain sequenced or mutation caused by independent outbreaks of drug resistance [16] or may be due to loss of deletion regions [17**]** Or due to relocation of transposons, changes in copy number of tandem repeats, and other genomic characteristics often exploited for genotyping [18]. Ongoing evolution has also been observed in vitro via accumulation of genetic differences during serial passaging of cultures [19]**.** Some evolution is driven by selection largely for mutations that accelerate growth in vitro. For example, loss of PDIM (phthiocerol dimycocerosate) synthesis, which is often associated with reduced virulence, results in more rapid growth in laboratory media, [20] Moreover, changes in *Mycobacterium bovis,* passaged over multiple generations resulted in its avirulent character, that is no longer able to cause disease and thus used as BCG vaccine [21]. However, while some characteristics are selected, many genetic changes likely occur due to simple genetic drift as some changes in BCG resulted in decreased virulence, the majority of alterations may not have been affected [22]

Due to above mentioned observations certain laboratories and research centers have opted for use of other alternatives to H37RV such as Erdmann strain, CDC 1551 etc., which are the local field strains isolated from the human sputum isolates. Strain Erdmann was isolated from human sputum by W.H Fieldman in 1945, at the mayo clinic, Rochester, MN and deposited with the Trudeau mycobacterium culture collection in 1946. Due to its consistently high virulence, it has been widely used as a standard consistently high virulence; it has been widely used as a standard virulence laboratory strain for virulence and immunization studies [23] CDC 1551 is a recent clinical isolate which was responsible for an outbreak of TB in a rural area of the USA in 1995. This strain (often referred to as the "Oshkosh" strain, but more properly CDC-1551) was isolated from a male children's clothing factory worker and was shown to be

highly contagious, infecting approximately 80% of his co-workers and social contacts. The CDC-1551 strain was also demonstrated to be highly virulent in mice, producing several orders of magnitude more organisms than the H37Rv strain when inoculated into the lungs of mice. The strain has not caused wide-spread disease in man and is pan-drug sensitive. The *Mycobacterium tuberculosis CDC1551* genome was sequenced and found to have a total of 4189 genes. The genome is a circular chromosome of 4,403,765 base pairs with an average G + C content of 65.6% [24]. In India, research laboratories like Indian Institute of Science (IISc) and NTI at Bangalore have worked on the high virulent character of a local high virulent field strain (NTI-83949), isolated from a TB patient. Genome of this well defined strain NTI-83949 has been sequenced and found to different from other reported strains [25] and its high virulent character also tested in albino guinea pig model at NTI besides using it successfully as a challenge strain for new vaccine research (under publication).

Future Challenges

The whole genome sequence of the H_{37}Rv strain of *Mycobacterium tuberculosis* by Stewart Cole and colleagues in 1998 provided a breakthrough in tuberculosis (TB) research. [26] The mechanisms by which *M. tuberculosis* causes disease have remained largely unknown until the improvement made recently *via* the application of modern molecular genetic tools, including genomic sequencing of the common lab virulent reference strain H37Rv the clinical isolate CDC1551, *M. bovis* and *M. bovis* BCG strains. In an attempt to determine the virulence determinants the genetic and phenotypic differences between these strains have been studied as a result of which some genes were found for example, the *eis* (enhanced intracellular survival) gene and *erp* (exported repetitive protein) genes enhance *M. tuberculosis* survival in macrophages *ivg* (in vivo growth) of *M. tuberculosis* H_{37}Rv confers a more rapid in vivo growth rate to *M. tuberculosis* H_{37}Ra. [27] Aside from the identified virulence factors, genomic differences such as insertions, deletions and single nucleotide polymorphisms have been found in both virulent and attenuated *Mycobacteria.* [28]

Since long time the strain H_{37}RV has been continuously passaged and, as its virulence in humans is unknown, there have been concerns as to whether this may have led to attenuation of virulence (Jacobs *et al.*, 1996). However, it has maintained virulence in animal models Through lack of knowledge or experience in culture maintenance many isolates of H37Rv are no longer virulent for their

common laboratory animal hosts. Therefore the available highly resistant strain $H_{37}Rv$ is not recommended as a QC strain. The notion of H37Rv as a standard reference strain should be used with some caution. The reason behind the loss of virulence may be due to the following factors.

Due to failure of the laboratory-maintained H37Rv strain of MTB obtained from several laboratories in India to cause disease owing to loss of virulence from repeated subculture, the use of a well characterized human sputum isolate of MTB characterized at NTI (NTI83949) as challenge strain in guinea pig experiments is highly recommended as a parallel comparative control strain.

Most of the lab in India using H37RV as a standard high virulent strain, it appears to have lost its original high virulent character due to repeated in-vitro passaging. Therefore the need of the hour is to identify local high virulent strain after ensuring its high virulence by genotypic methods. Already attempts by Indian scientists have been able to identify and characterise MTB as a replacement / parallel strains that can be tested or explored for its usage as a control / reference strain. This also opens up an opportunity to have a stock of such strains that can be maintained at national institutes as a repository and can be supplied to the laboratories upon request.

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