

A Brief Introduction to Major Diagnostic Methods Used for Tb

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TAXONOMY AND CLASSIFICATION OF MYCOBACTERIA

The genus *Mycobacterium*, is the single genus within the family of *Mycobacteriaceae*, in the order *Actinomycetales* [1]. On the basis of growth rate of mycobacteria, the genus *Mycobacterium* can be divided into two major groups, the slow-growing species e.g. *M. tuberculosis*, *M. bovis* and *Mycobacterium leprae*, which in general are pathogenic, and the fast-growing species e.g. *M. smegmatis*, which in general are opportunistic or non-pathogenic bacteria.

Mycobacterium Tuberculosis Complex (**MTC**) refers to the group of species capable of causing tuberculosis. Members of MTC have 99.9% similarity at the nucleotide level and identical 16S rRNA sequences but differ widely in terms of their host tropisms, phenotypes, and pathogenicity. The classical species of MTC included the well characterized *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*. Newly recognized additions to the MTC include *M. caprae* [2] and *M. pinnipedii* [3,4] and *M. canettii* [5] often commonly referred as *M. tuberculosis* subsp. *Canetti* [6]. Other than these 7 well established members, *M. mungi* was also recently described as MTC member [7].

Also, there are two rare MTC variants, the so-called dassie bacillus [8,9] and *M. orygis*[10] whose standing within MTC remains to be defined [11].

Some MTC members are exclusively human pathogens e.g. *M. tuberculosis*, *M. africanum* and *M. canettii* whereas others have a wide host spectrum e.g. *M. bovis*.

CHARACTERISTICS OF *M. TUBERCULOSIS*

Notable characteristics of *M. tuberculosis* include its slow growth, with a generation time of 18-24 hours, dormancy or non-replicating state characterized by low metabolic activity [12], and intracellular pathogenesis. *M. tuberculosis* is aerobic to facultative anaerobe an aerobe with highly impermeable cell wall composed of peptidoglycan, arabinogalactan and mycolic acids, surrounded by a non-covalently linked outer capsule of proteins and polysaccharides. Often described as Gram-positive, however the high density of lipids in the cell wall prevents accurate Gram staining [13]. *M. tuberculosis* is typically visualized by acid-fast staining e.g. Ziehl-Neelsen stain and appears as a rod shaped red bacillus.

TUBERCULOSIS DISEASE

TB is described as an infection caused by the members of MTC [14]. TB is a contagious, usually chronic, and highly variable disease, mostly transmitted by inhalation of the airborne tubercle bacilli, and primarily affects the lungs but may spread to other organs of the body. It is often characterized by fever, cough and difficulty in breathing, inflammatory cell infiltrations, formation of tubercles, caseation, pleural effusion, and fibrosis. Major risk factors for developing TB include malnutrition, diabetes [15], and use of tobacco [16] and alcohol.

On the basis of anatomical sites involved, TB can be classified as pulmonary and extra-pulmonary.

Pulmonary Tuberculosis

Based on the revised standard case definitions for TB by the World Health Organization (WHO) in 2013, pulmonary TB refers to any bacteriologically confirmed or clinically diagnosed case of TB that involves the lung parenchyma or the tracheobronchial tree [17].

Pulmonary TB manifests in various forms, e.g. latent, incipient, subclinical and clinical or active TB. Approximately 5 to 10% of individuals who get infected with *M. tuberculosis* progress to clinical TB, whereas the remaining individuals usually develop a latent infection. Latent Tuberculosis Infection (**LTBI**), defined as a state of persistent immune response to prior-acquired *Mycobacterium tuberculosis* antigens without evidence of clinically manifested active TB, affects about one-third of the world's population. Approximately 10% of people with LTBI will develop active TB disease in their lifetime, with the majority developing it within the first five years after initial infection [18,19].

Active infection with *M. tuberculosis*, whether primary or reactivated, is identified only when progression to bacteriologically detectable disease occurs. Active TB, represents the late stage of the chronic disease process, and may manifest as noncavitary paucibacillary or cavitary multibacillary disease [20].

During the transition from latent to active TB, patients often undergo an asymptomatic state with some detectable manifestations indicative of disease. This asymptomatic state is referred to as incipient TB in immunocompetent individuals, where it is relatively contained; as compared to immunocompromised individuals where it is referred to as subclinical TB and is largely associated with loss of containment [21].

Extrapulmonary Tuberculosis

Extrapulmonary Tuberculosis (**EPTB**) refers to any bacteriologically confirmed or clinically diagnosed case of TB involving organs other than the lungs, e.g. pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, meninges. According to WHO, miliary TB is classified as pulmonary TB because there are lesions in the lungs and tuberculous intra-thoracic lymphadenopathy or tuberculous pleural effusion, without radiographic abnormalities in the lungs, are considered as extrapulmonary TB. A patient with both pulmonary and extrapulmonary TB is classified as a case of pulmonary TB [17]. The anatomical sites of EPTB may vary according to geographic location, population groups and a wide variety of host factors [22,23]. Lymph node TB accounts for nearly half of all the non respiratory sites [24] and is usually found to be most prevalent extrapulmonary TB [25,26] however some studies report pleura as the most common site of extra-pulmonary involvement followed by the lymph nodes [22,27,28]. The epidemiology and risk factors of EPTB differ from those of pulmonary TB, and the proportion of EPTB has increased over the past two decades [29].

DIAGNOSIS OF TB

The timely diagnosis of TB is very important to start the anti-tuberculosis treatment. Primary methods to diagnose the infection in TB endemic and low-income countries include mainly microscopy, chest X-rays and microbial culturing. Diagnosing EPTB is often challenging because the procedures to obtain tissue from extrapulmonary sites may be technically difficult or unavailable. Also, number of bacilli present in extrapulmonary fluids and tissues are often smaller than those found in the lungs, resulting in the low smear or culture positivity rates. Serodiagnosis could offer solutions to some of these problems. Diagnostic tests of extrapulmonary TB and other bacteriologically negative cases, and tests for vaccine-induced immunity need critical consideration [30]. WHO guidelines for LTBI recommend that either interferon-gamma release assays or Mantoux tuberculin skin test should be used [19].

The brief details of some important TB diagnostic methods are given below:

AFB Staining

Conventionally, Acid Fast Bacilli (**AFB**) testing, microbial culturing and Tuberculin Skin Test (**TST**) are used to diagnose TB. AFB testing is the simplest way to detect acid fast bacilli in the clinical samples of TB patients. This rapid method is specific in areas with high TB prevalence but has modest sensitivity ranging from 20% to 80% in different settings. The sensitivity is compromised as >10,000 bacilli/ml of sputum are required for the positive test [31]. In addition, its sensitivity is low in extra-pulmonary tuberculosis, Childhood TB and HIV co-infection [32].

Culturing

Culturing of *M. tuberculosis* from clinical samples is thought to be “gold standard test” for the diagnosis of TB. This method can detect 100 bacilli/mlin clinical samples like sputum, bronchial washings and extra-pulmonary samples [31]. This method is much more sensitive but it requires approximate 6- 8 weeks to grow. Moreover, culture failure of *M. tuberculosis* is 10- 20% [32,33]. An automated BACTEC radiometric system was introduced in 1980's which detects the amount of $^{14}\text{CO}_2$ by the growth of bacilli. Although it is rapid but has several limitations: colony morphology can't be observed, mixed cultures recognition is difficult, over-growth by contaminants, expensive, radioisotopes disposal and extensive use of needles [34]. A fluorescence based Mycobacteria Growth Indicator Tube (**MGIT**) system has also been used in clinical settings to detect and recover mycobacterial but the rate of recovery with the MGIT system was slightly inferior to the BACTEC system [35, 36].

Tuberculin Skin Test

The Tuberculin Skin Test (**TST**) is based upon the fact that *M. tuberculosis*, being an intracellular pathogen, sensitizes the T- cells of patients and delayed hypersensitive response is measured using the Purified Protein Derivatives (**PPD**) to give rise a skin reaction. This method was widely used over the past 50 years; however, the major limitation is that the proteins in PPD are shared by many other mycobacterial species as well, which reduces the test specificity. Therefore, people previously exposed to non-TB mycobacteria or BCG vaccinated can give false positive results. Also, there is chance of false negative results in cases of disseminated TB, HIV co-infected TB patients, immunosuppressed patients due to transplantation or haemodialysis [33,37].

Nucleic Acid Amplification Tests

Nucleic acid amplification tests (**NAATs**) are used to amplify DNA segmentsspecific to *M. tuberculosis* complex. NAATs are can detect specifically both pulmonary and extra-pulmonary TB but sensitivity is low and variable. Sensitivity is highest in case of smear positive cases while lower in extra-pulmonary and smear negative cases. Generally, the accuracy of these NAATs is heterogenous [35,38]. The IS6110 is widely used as an amplification target and the sensitivity range of 4-80% and specificity of 80-100% [30].A novel, simplified and rapid NAAT is Loop Mediated Isothermal Amplification (**LAMP**). This test has low specificity as it can detect non-

tuberculous mycobacteria thus limiting its application in high TB burdened countries. Xpert® MTB/RIF is the PCR based test that utilizes the instrument called GeneXpert. GeneXpert is the molecular platform used to diagnose *M. tuberculosis* and rifampicin resistance. It is used to detect the mutation in *rpoB* gene. This test can be applied directly on sputum sample and is quite rapid. However, this test cannot be used to monitor the treatment as it can't differentiate between live and dead bacterial cells [39].

T- cell Based Assays

T- cell based assays are used to diagnose latent TB by measuring the level of interferon γ from T- cells sensitized with antigens specific to *M. tuberculosis*. The antigens which are used in this assay can elicit an interferon- γ response. On the basis of antigens used, two main types of the tests are available: assays based on PPD (Quantiferon®) and assays based on the antigens belonging to RD1 region i.e. ESAT-6 and CFP-10 and RD11 region i.e. TB7.7 [40-42]. These Interferon Gamma Release Assays (**IGRAs**) generally show more specificity than TST. Since the proteins used in these assays are encoded by RD1 that is absent from *M. bovis* BCG so, it is helpful to distinguish actual latent infection from purely BCG vaccination-induced responses[33].

Serological Assays

A number of serological assays or have been developed over the years to detect antibodies circulating in blood like complementation fixation tests, radioimmunoassay, haemagglutination tests and Enzyme Linked Immunosorbant Assays (**ELISAs**).

ELISA assay is quite common and user friendly format used in diagnosis of diseases. Typically, ELISA assay is performed in 96- well plate by passively immobilization of antibodies, antigens or proteins in the wells of ELISA pate. There are different ELISA formats which are used depending upon the type of analysis. All the ELISA formats have some common elements: plate coating, blocking, probing and signal detection (Figuer1.2). To choose the one assay type depends upon the type of detecting analyte and reagents availability.

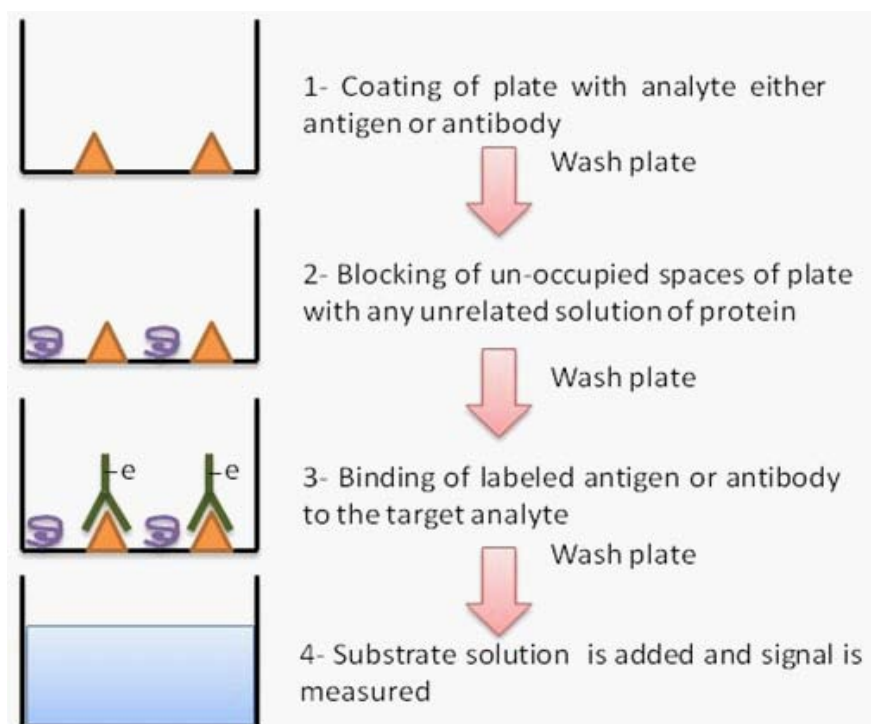


Figure1.2: Basic steps of ELISA assay. The assay involves coating with analyte, blocking of plate, probing and signal detection.

These antigen-antibody based assays could replace microscopy to diagnose TB and easy to perform in high burden TB endemic countries because of their speed and simplicity. Moreover, for extra-pulmonary cases, invasive procedures are used to get sample for analysis. However, a serodiagnostic test based upon antibodies detection can replace such invasive procedures for getting samples from disease site. However, none of the commercially available ELISA kits showed promising results and thus are not implemented [43-46]. WHO conducted the meta-analysis and systematic review of accuracy of in-house ELISA kits for diagnosis of TB and developed the policy regarding the use of ELISA in 2011. Due to the inconsistent and variable ELISA results, WHO recommended that these tests should not be used for diagnosis of TB. However, they stated clearly in their 2011 policy that further research to identify new/alternative point-of-care tests for TB diagnosis and/or serological tests with improved accuracy is strongly encouraged. Appropriate study design including study population, eventual follow-up is important factors while performing such research [47].

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