

LABORATORY METHODS FOR DIAGNOSIS OF TUBERCULOSIS- THE APPROACH AND CHALLENGES

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Abstract

Tuberculosis (TB) remains a major global health problem, particularly in many of the developing countries including India. Some of the factors that have substantially contributed to the number of multi-drug resistant tuberculosis (MDR- TB) and extensively drug resistant tuberculosis (XDR- TB) cases both in general and among HIV infected persons are- the delay in the diagnosis as well as delayed determination in the drug susceptibility of the isolated organism. In the present article, an attempt has been made to review various techniques/methods available for the diagnosis of tuberculosis and their applications along with the advantages and disadvantages/ limitations.

Key words: Tuberculosis, laboratory diagnosis, direct and indirect methods

Introduction

Tuberculosis (TB) remains a major global health problem, particularly in many of the developing countries including India^[1]. The disease is a slow progressive, chronic granulomatous infection caused by *Mycobacterium tuberculosis*, an intracellular pathogen that is capable of establishing and causing life-long infection in humans. About 9 million new cases of TB are reported all over the world killing around 1.5 million people every year^[2]. In India, approximately 12-15 million people are currently suffering from the disease of which about half a million of the affected persons die each year. An increasing trend of the disease associated with the HIV infection has been recorded all over the world and in India alone, out of around 3.5 million HIV infected persons about 1.8 million individuals are co- infected with TB^[3].

The situation however, has aggravated further with the emergence of multi- drug resistant strains which have been detected to all the major anti- TB drugs (MDR- TB) and extensively drug resistant

tuberculosis (XDR- TB) that was detected in 2006^[4]. As the XDR- TB is resistant to several first and second line anti- TB drugs, the treatment options are therefore, limited and thus the mortality rates are extremely high. Therefore the prevention of MDR- TB and XDR- TB must receive great emphasis which might be achieved by the early diagnosis and immediate institution of the treatment. One sputum positive case of TB may be infective to as many as 10- 15 persons over a period of around one year. From the viewpoint of public health perspectives, poorly supervised or even incomplete treatment of TB cases is worse than no treatment at all as such cases remain infectious and may infect others with the drug- resistant strains.

Diagnostic methods

Two approaches that are followed in the diagnosis of TB are- (1). The detection of mycobacteria or its antigenic products (**Direct method**), and (2). Host specific immune responses to the organism (**Indirect method**).

Direct method:

Microscopy- The microscopic examination of Ziehl- Neelsen (ZN) stained smears is of both clinical

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and epidemiological importance in assessing the patient infectiousness. The method is cost- effective and convenient which is likely to remain as one of the important tools in diagnosing TB and to monitor the treatment progress of the patients in our country. Under RNTCP, Govt. of India has taken measures of good quality control of sputum examination by imparting sufficient training in bringing out skilled/ technical manpower along with improved quality- controlled reagents and standardized microscopy. However, the method has limitations as it requires at least 5×10^3 to 10^4 bacilli/ ml of sputum/ sample for the detection by the microscopic examination.

Culture methods

All the clinical samples of suspected TB cases should be cultured that detects as few as 10 organism/ ml. of the specimen. Also the growth of organism is necessarily required for the species identification as well as drug susceptibility testing. The major constraint of culturing mycobacterium employing conventional methods i. e., Lowenstein- Jensen medium is the slow growth of the organism which necessitates an incubation period of minimum four weeks, additionally requiring another four weeks for testing of the anti- TB drugs. Moreover, the choice and preparation of specimens prior to culturing by various pre- treatment procedures affects the sensitivity which mainly depends on the de-contamination techniques so employed.

Various culture methods that are available for primary isolation of mycobacterium include micro-colony detection on solid media, broth cultures for microscopic observation of micro- colonies development, Septi-check AFB method (Roche), ESP II culture system (Difco), MB/BacT system (Organon Teknika), BACTEC 460 radiometric system and MGIT 960 system (Becton Dickinson).

Both the automated BACTEC 460 and MGIT 960 systems allow rapid growth of mycobacterium detecting either $^{14}\text{CO}_2$ or O_2 utilization, respectively

and are extremely useful in reporting early within 7- 14 days along with the sensitivity pattern of the organism to the anti- TB drugs.

Upcoming methods

BacT/ Alert is another rapid colorimetric, fully automated method having online data access system, however its high running cost and the costly instrument are the impediments for its use^[5]. In comparison, E test is a simple method based on the use of E test strips containing gradients of impregnated antibiotics applied on to the surface of solidified media inoculated with the test strain. While the results are easily obtainable with rifampicin, modifications are essentially required for INH^[6]. Also flow cytometry has been utilized for rapid testing of drug susceptibility which is a sophisticated technique requiring a costly instrument.

A number of techniques utilizing oxidation-reduction properties of various indicator dyes/ systems viz. alamar blue, resazurin, nitroblue tetrazolium have been advocated for the early detection of growing organism or its inhibition by different drugs^[7]. Some systems utilizing mycobacterial phages and reporter genes like luciferase for the detection of the organism growth as well as assessing the drug susceptibility to anti-TB drugs that are also available commercially (Biotec/ Medispan)^[8].

Identification of mycobacterial isolates

Mycobacterial identification at species level is carried out employing various methods ranging from conventional biochemical tests to modern molecular biological techniques. The traditionally-used phenotypic and biochemical tests are however, time-consuming and sometimes inconclusive results are obtained. To overcome this, various rapid methods such as chemical method based on lipid profiles, hybridization with specific gene probes, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method such as gene for hsp 65 kDa protein, kat G and rRNA genes, and sequencing of 16 rRNA have been evaluated^[3,5].

Analyses of lipid profiles

Mycobacteria have characteristic lipid profiles that can be analyzed employing gas chromatography, HPLC or HP-TLC. This approach has been useful in several laboratories in quick identification of the mycobacterial isolates.

Antigen detection assays

Various techniques such as sandwich- and inhibition enzyme-linked immunosorbent assay (ELISA), latex agglutination and reverse passive haemagglutination have been utilized for the detection of different mycobacterial antigens in cerebrospinal fluid and in pleural fluid. These assays were found to be successful in detecting the whole antigens (*viz.* mycobacterial sonicates and extracted glycolipids, PPD) as well as various antigenic fractions (Ag5 or 38 kDa, AgA60, 45/47kDa Ag, Ag Kp90, 30kDaAg, P32 Ag, cord factor or trehalose dimycolate and lipoarabinomannan-LAM) detecting the antigens as low as 3-20 ng/ml. of the samples^[3]. Also antigen capture ELISA employing monoclonal antibody and a dipstick test have been found quite sensitive and specific for detection of LAM in sputum and to some extent, in urine samples too. Immuno-chromatographic techniques (ICT) are now commercially available for rapid diagnosis of TB by the antigen detection.

Molecular methods

Molecular methods have an advantage of speed and specificity which have extensively been utilized not only for the diagnosis but also in understanding of drug resistance in TB^[9,10]. A variety of PCR methods have been developed for the detection of specific sequences of *M. tuberculosis* and other mycobacterium species employing conventional DNA based PCR, nested PCR and RT-PCR. Separate gene targets like MPB 64, repetitive sequences, GC repeats, dev R, 38 Kd, TRC 4 and IS 1081, and even some of their modifications have been utilized and were found highly sensitive and specific.

With the advent of isothermal amplification techniques which represents a major progress that do not require a thermal cycle. Some of the important methods available are- standard displacement technique, gene probe amplified *M. tuberculosis* and Q-beta (QB) replicase based gene amplification. In a proper controlled setup, some of these techniques are highly sensitive detecting even one colony-forming unit of the organism and reported to have not effected by the inhibitors of PCR.

Mutations at the target sites are considered as the most important mechanism in TB responsible for the drug resistance of an isolate. As DNA sequencing is the gold standard for detection of mutations in the genes responsible for the drug resistance, simple approaches such as line probe hybridization assay, PCR-SSCP, PCR hetero duplex formation, PCR-RFLP etc. have been utilized for their detection. A line probe assay for rifampicin is one of the commercially-available tests (Inno-LIPA) that is simple to perform in a small laboratory having limited facilities. Further the drug resistance may vary with different drugs even though it is quite stable and reliable with rifampicin. Also such mutations may differ geographically as variable results have been reported by different workers from place to place. Therefore, alternate mechanisms such as permeability barriers, inactivation of drug by the bacterial enzymes and exclusion of drugs by efflux pumps employing micro- array and proteomic approaches have been explored further that hold a great promise in near future^[9]. Recently, a new method named 'FAST-Rif' (fluorometric assay for susceptibility testing of rifampicin) and Gene Type MTB plus test as a marker for MDR-TB and XDR-TB have also been devised for rapid and easy detection of genotypic rifampicin resistance^[11,12]. Also a combination of two FDA approved drugs Clavulanate and Meropenem which are effective against bacteria like *Esch. coli* have recently reported to block completely the growth of 13 strains of XDR-TB in a study conducted at Albert Einstein college of

Medicine, N. York and National Institute of Allergy and Infectious Diseases, US^[2].

Indirect method

Serological tests

Most of the serological tests used in the past have low sensitivity particularly, in cases of smear- negative HIV- positive patients. Also these assays were expensive and very often have difficulty in differentiating between *M. tuberculosis* (Mtb) and other mycobacterium species infections in the past. Some of the tests that have been developed recently that overcame these limitations are- ELISA and the ICT employing antigens such as LAM, 38 kDa or AgA60 in detection of specific antibodies. Also ELISA has been evaluated in detection of antibodies to super-oxide dimutase, an important secretory protein of Mtb^[3]. ICT are now commercially available for rapid diagnosis of TB by the antibody detection.

Miscellaneous diagnostic methods

Newer tests with defined mycobacterial antigens such as MPB64 patch test has been devised which in contrast to tuberculin testing (Mantoux), showed the ability to distinguish the sensitized individuals from those suffering from active disease. Further, ESAT-6 (6 kDa) is a specific antigen that induces IFN gamma production by T lymphocytes of the TB patients. The antigen is recognized by T lymphocytes of TB patients but not by the T lymphocytes of the BCG vaccinated, thus making the test quite specific and reliable^[3] Also estimation of nitric oxide and protein carbonyl in serum of TB patients have been reported recently and the levels correlated well with each other^[13]. These changes are, however a reflection of increased host's defense mechanism at the cellular level against the organism that requires to be examined further in relation to the specificity.

Conclusions

The diagnostic procedures essentially require certain

features such as sensitivity, specificity and speed along with cost- effectiveness for its reliability and wider applications. Smear examination/ microscopy has its inherent limitations, therefore culture methods are essentially required to be utilized along with the former for better results. As such, culture methods still remain the gold standard for the diagnosis as well as in validating other diagnostic tests for TB. In contrast, molecular methods are highly useful particularly, in the extra- pulmonary cases, if special facilities are available. With advent of various ICT based tests specifically detecting different mycobacterium antigenic fractions as well as antibodies against some of the antigenic fractions which has greatly revolutionary the facilities now available in the diagnosis of TB. Also substantial progress has been made in bringing out simpler techniques that may be utilized in early diagnosis as well as detection of drug susceptibility of the isolated organism in near future. In absence of the facilities for carrying out the molecular assays, inclusion of the ICT based tests in detection of both the antigen and antibody along with smear examination and culture facilities may serve the purpose well in a particular setup.

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