Newer diagnostic techniques for tuberculosis

V.M. Katoch

Central JALMA Institute for Leprosy & Other Mycobacterial Diseases (ICMR), Agra, India

Received July 16, 2003

Diagnosis of tuberculosis is mainly based on clinical features, histopathology, demonstration of acid fast bacilli (AFB) and isolation of Mycobacterium tuberculosis from the clinical specimens. These techniques have limitations of speed, sensitivity and specificity. During the last two decades several rapid techniques for detection of early growth (5-14 days as compared to 2-8 wk with conventional methods) have been described which can help in obtaining the culture and sensitivity reports relatively early. Prominent among such methods are BACTEC, mycobacterial growth indicator tuber (MGIT), Septi-check, MB/ BacT systems. This growth can be established by rapid methods based on lipid analysis and specific gene probes, PCR-RFLP methods and ribosomal RNA sequencing. Advances in knowledge about genetic structure of tubercle bacillus helped develop gene probes and gene amplification methods for identification and detection of tubercle bacillus, from culture or directly in clinical specimens and molecular detection of drug resistance. While the gene probes can help in rapid identification of isolates, gene amplification methods (PCR as well as isothermal) developed for diagnosis of tuberculosis are demonstrably highly sensitive specially in culture negative specimens from different paucibacillary forms of disease. With these molecular methods drug resistant mutants for drugs like rifampicin can be detected with reasonable certainty within hours. These gene probes, gene amplification methods and in situ approaches offer unparalleled capability to enhance the diagnosis of tuberculosis in near future.

Key words Gene probes - Mycobacterium tuberculosis - PCR - rapid culture - tuberculosis

Early confirmation of the diagnosis of tuberculosis is a challenging problem specially in case of paucibacillary and extra-pulmonary forms. Conventional methods available for diagnosis namely, tuberculin test, radiological examination and other imaging methods and sputum smear microscopy have their own limitations. Histopathology is characteristic but there could be problems to get representative specimen, and non specific features. Immunology is often not conclusive as antibodies and delayed type hypersensitivity response persist for a long time after the subsidence of sub-clinical or clinical disease. Sputum smear microscopy requires 10,000 to 1,00,000 organisms/ml and acid fast bacilli (AFB) could be any pathogenic or saprophytic mycobacteria. Smear microscopy can be made much more convenient by using various fluorochrome dyes such as auramine and rhodamine1.

Accurate identification of Mycobacterium tuberculosis through culture is presently the yardstick for diagnosis, but the time required and frequent negative results in paucibacillary specimens are important limitations. During the last two decades, several methods for achieving early growth of M. tuberculosis have been developed2. Some of the important methods are:

(i) BACTEC system: This assay system developed by Becton Dickinson, is based on generation of
radioactive carbon dioxide from substrate palmitic acid. This method has been extensively used all over the world and growth can be detected in 5-10 days in this system. Inclusion of NAP (beta nitro alpha acetylamine beta hydroxy propiophenone) helps in distinguishing *M. tuberculosis* (inhibited) from other mycobacteria. This system has been widely used for drug susceptibility testing and is currently used as a comparative standard.

**(ii) MGIT (Mycobacteria growth indicator tube):** This method has also been developed by Becton Dickinson and growth is detected by a non-radioactive detection system using fluorochromes for detection and drug screening. This system helps in early detection (7-12 days) of mycobacterial growth and has been reported to be useful for drug susceptibility testing but the experience is limited.

**(iii) MB/BacT:** This system (Organon Technika) is adapted from strategy of colorimetric detection earlier tried for detection of bacterial growth in blood cultures. This has been reported to be useful for drug susceptibility testing of *M. tuberculosis*.

**(iv) Septi-Chek:** This is a biphasic medium system (Roche) consisting of an enriched selective broth and a slide with nonselective Middlebrook agar on one side and with two sections on other side- one with NAP and egg containing agar, second with chocolate agar for detection of contamination. This system has also been found to be quite useful for rapid detection of growth of mycobacteria.

**(v) Reporter phages:** Mycobacterial specific phages and reporter genes like luciferase have been successfully used for detection of growth and for assessing the drug susceptibility to anti-TB drugs. Indication of viability could be either emission of light from organism due to activation of luciferase gene or production of a plaque on an indicator strain of mycobacteria. Results can be obtained in 48 h and such systems are commercially also available (Biotec/Medispan).

**(vi) Other methods:** E-test has been reported to be useful for drug susceptibility testing in *M. tuberculosis*. Flow cytometry has also been observed to be a rapid method for drug susceptibility testing but it is technology-intensive.

**Rapid identification of mycobacterial isolates**

Another important step towards early diagnosis is rapid identification of mycobacterial isolates from conventional cultures or rapid methods like BACTEC and others. Traditionally biochemical tests are used for identification of mycobacteria. These are, however, time consuming and sometimes ambiguous results are obtained. To overcome these limitations chemical methods based on lipid profiles, hybridization with specific gene probes, Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) methods such as gene for hsp 65 kDa protein, katG, and rRNA genes and sequencing of 16S rRNA have been described.

**(i) Analysis of lipid profiles:** Mycobacteria have characteristic lipid profiles. These lipid profiles can be analysed by HPLC or HP-TLC and quick identification of mycobacterial isolates can be done. This strategy has been successfully used in several laboratories.

**(ii) DNA probes:** Based on information about specific gene sequences well defined oligonucleotide probes for identification of various clinically relevant mycobacteria have been developed and are readily available. These include probes for identification of *M. tuberculosis*, *M. avium* and several other mycobacteria. These probes are being used in several countries for rapid confirmation of the identity of mycobacterial isolates. When used along with newer methods of detection of the early growth (such as BACTEC, Septi-Chek, MGIT) these are of great help in rapidly confirming the diagnosis as identity of isolate can be established within 1 to 2 days with gene probes as compared to much longer time required with classical biochemical tests.

**(iii) Ribosomal rRNA based probes:** In recent years, ribosomal RNA gene region has been extensively explored for designing systems for ribosomal DNA fingerprinting and for development of probes as well as gene amplification assays for various of
mycobacterial species including *M. tuberculosis*, *M. leprae* and *M. avium* etc. These probes target rRNA, ribosomal DNA, spacer and flanking sequences. Commercially available rRNA based probes have been reported to be useful for quick identification of mycobacterial isolates. These probes were earlier radiolabeled but have now been developed into chemiluminescent techniques. rRNA targeting probes are 10-100 fold more sensitive than DNA targeting and may be used to confirm the diagnosis directly in the clinical specimens in a good proportion of cases; the lowest detection limit is around 100 organisms (Table I). At present these are useful mainly for rapid identification of mycobacterial isolates.

(iv) Gene amplification methods for identification: Techniques may also be used for confirmation of the identity of isolates but the problem of carry over from the original inoculum needs to be kept in mind. Different strategies to identify the isolates from cultures and also directly from the clinical specimens have been described. These include amplification of specific gene regions followed by hybridization with species specific probes, sequencing and RFLP analysis such as hsp 65 kDa gene, *katG* and rRNA genes (a CJIL system under publication) have been described. These PCR-RFLP assays help in quick identification of pathogenic mycobacteria including *M. tuberculosis* from the culture isolates as well as directly from the clinical specimens. While PCR-sequencing approach can be applied by reference laboratories the hybridization and RFLP approaches are easily practicable in clinical mycobacteriology laboratories.

(v) Gene amplification methods for direct detection of *M. tuberculosis* sequences from clinical specimens: Gene amplification techniques have made a major impact on the diagnosis of mycobacterial diseases. These methods may be classified as those based on polymerase chain reaction (PCR) and others based on isothermal amplification reactions. Gene amplification techniques are highly sensitive and under optimum conditions may detect 1-10 organisms (Table I). If systems are adequately standardized, evaluated and precautions for avoiding the contamination are taken, these assays can play a very useful role in early confirmation of diagnosis in paucibacillary extra-pulmonary forms of tuberculosis.

### PCR methods

A variety of PCR methods have been developed for detection of specific sequences of *M. tuberculosis* and other mycobacteria. These PCR assays may target either DNA or rRNA/and these could be based on conventional DNA based PCR, nested PCR and RT-PCR. Targets include insertion and repetitive elements, various protein encoding genes, ribosomal rRNA etc. Developments in this area have been very rapid and a large number of PCR assays targeting different gene stretches of *M. tuberculosis* have been described. Indian laboratories have been active in development of PCR methods for detection of

<table>
<thead>
<tr>
<th>Technique</th>
<th>Sensitivity</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA targeting probe</td>
<td>10,000-00,000 copies</td>
<td>Identification of isolates</td>
</tr>
<tr>
<td>rRNA targeting probe</td>
<td>100-1000 copies</td>
<td>Identification of probes; also limited application on clinical specimens</td>
</tr>
<tr>
<td>Gene amplification assays</td>
<td>1-10 copies</td>
<td>Direct application on clinical specimens; also used to identify isolates by PCR-RFLP/sequencing</td>
</tr>
</tbody>
</table>

Superscript numerals denote reference numbers
*M. tuberculosis*. Different Indian investigators have used separate gene targets like MPB 64\(^{33}\), repetitive sequences\(^{36,50}\), GC repeats\(^{37}\), devR\(^{38}\), 38kD\(^{39}\), TRC 4\(^{40}\), IS 1081\(^{41}\) and a system patented by Central Drug Research Institute (CDRI), Lucknow. Some of these assays\(^{30,33}\) have been repeatedly found to be reproducible, highly sensitive and specific in double blind evaluations. IS-1081 based system\(^{41}\) has been further modified and a new nested PCR target of this gene has been developed at Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad. These assays have been reported to be quite promising in confirming the diagnosis of different forms of tuberculosis. A PCR assay system for tuberculosis which is commercially available\(^{42}\) has been found to be reproducible, sensitive as well as specific\(^{44}\). These methods can also be adapted for in situ application for confirmation of histological diagnosis.

Real time PCR has been investigated for rapid and specific detection of *M. tuberculosis* in the clinical specimens\(^{44}\). This strategy can be used for confirming the diagnosis and also monitoring the progress.

**Isothermal amplification techniques**: These techniques represent a major step forward in the application of gene amplification technology without thermal cycler. The important methods are:

(i) *Strand displacement amplification (SDA)*: A SDA system for *M. tuberculosis* has been described and found promising\(^{46}\). It is an isothermal *in vitro* nucleic acid amplification technique which is based upon the ability of HincII to nick the unmodified strand of hemiphosphorothioate form of its recognition site and the ability of exonuclease deficient klenow to extend 3’ end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions.

(ii) *Gene probe amplified Mycobacterium tuberculosis direct test*: This assay employs the isothermal amplification of *M. tuberculosis* complex rRNA followed by detection of amplicon with acridinium ester-labelled DNA probe\(^{29,47,48}\). Reports about the application of this system are encouraging\(^{29,48}\).

(iii) *Q-beta (QB) replicase based gene amplification*: This approach involves production of RNA in the amplification reaction using QB replicase as the enzyme and reaction at fixed temperature (for example 37°C). Using a suitable combination of capture and detector probes, sensitivity up to one colony forming unit has been reported for *M. tuberculosis*\(^{49}\). Further, the inhibitors of PCR were observed to have no effect on this assay.

**Experience of application of gene amplification assay in clinical situations**

There is a growing experience of use of gene amplification assays for detection of *M. tuberculosis* specific sequences directly from the clinical specimens. Some of the important experiences are summarized below:

(i) *Pulmonary cases*: Pulmonary tuberculosis constitutes the major form of tuberculosis in clinical practice. The organisms are demonstrable in multibacillary forms by acid fast staining and also by culture. While these methods are simple, these have the problem of sensitivity as well as specificity and are also time consuming. Various PCR methods have been tried for confirmation of diagnosis from sputum\(^{50,51}\), bronchoalveolar lavage etc. Gastric lavage specimens are considered to be particularly useful for isolation of *M. tuberculosis*\(^{52}\) and also PCR in childhood cases of pulmonary tuberculosis in which it was difficult to obtain sputum specimens\(^{53}\). In general different amplification methods (PCR as well as isothermal) have been found to be positive in 95-100 per cent of smear/culture positive specimens whereas the positivity ranges from 40-60 per cent in smear negative paucibacillary pulmonary disease\(^{29,36,32,35,36,42,47,51}\).

(ii) *Neurotuberculosis*: PCR has been extensively tried for the detection of *M. tuberculosis* specific sequences in the CSF as well as biopsy specimens in cases of tubercular meningitis and tuberculoma of brain. As compared to 5-20 per cent positivity with demonstration of AFB and mycobacterial cultures, PCR has been found to be positive in 50-70 per cent of specimens from cases having cardinal features as well as biochemical/cytological evidence of neurotuberculosis\(^{33,40,54}\).
(iii) Pleural effusion: The diagnosis of pleural involvement in tuberculosis is made by appropriate pleural biopsy and examination of pleural fluid, it is time consuming and direct detection of M. tuberculosis is rare. PCR can help in confirming the diagnosis in more than 60 per cent of specimens which are negative for mycobacterial culture

(iv) Ocular tuberculosis: Like various other extra pulmonary forms, the demonstration of mycobacteria is difficult in cases with ocular tuberculosis and in cases of chronic iridocyclitis the establishment of aetiology is a challenging task. PCR has been shown to confirm the diagnosis in 50-70 per cent cases of ocular tuberculosis using aqueous/vitreous fluid as specimens.

(v) Cutaneous tuberculosis: The tuberculosis manifests as more than a dozen of clinical forms in skin. While the characteristic histology and demonstration of organism is used to confirm the diagnosis in some cases, there could be problems. PCR has been used extensively to confirm the diagnosis of cutaneous tuberculosis from unfixed biopsies as well as paraffin embedded section. In cases of skin tuberculosis, the positivity in the range of 50-60 per cent has been observed as compared to very low positivity by culture.

(vi) Lymph glands: The tuberculosis of cervical as well as other lymph glands is one of the important forms of childhood tuberculosis. Different lymph glands may be involved even in adults. PCR has been used to confirm the diagnosis in case of lymphadenopathy. The positivity rates varying from 40-90 per cent have been reported by PCR in case of tubercular lymphadenitis.

(vii) Bone, kidney, genital tuberculosis: Tuberculosis involves different bones, joints, genitourinary tract and almost all organs of human body. The bacillary load in these specimens is usually low. PCR has been found to be useful in confirming the diagnosis in a substantial number of cases of tuberculosis of female genital tract. PCR has been found to be useful in confirming the clinical diagnosis of bone tuberculosis.

Sensitivity and specificity of diagnostic methods

In general, gene amplification methods have been found to be highly sensitive and specific for diagnosis of tuberculosis directly from clinical specimens. Depending upon the bacteriological status and copy number of target sequence, sensitivity has ranged from 70-100 per cent whereas specificity between 80-100 per cent has been reported by different methods.
investigators (Table II). While figures may vary from series to series, overall trends are similar and promising.

**False positive and false negative results**

There has been a genuine concern of false positivity due to contamination occurring in clinics and laboratories. The problem of false positivity can be substantially reduced by proper laboratory design, strict discipline about collection and processing of the specimens, handling of reagents and use of certain blocking reagents. Further the application of *in situ* PCR approach removes the doubts about contamination and will be very useful to the pathologists for arriving at a confirmed diagnosis. In case of false negative results several strategies can be used to improve the sensitivity. While there are individual problems of appropriate sample collection, extraction and assay, very small number of organisms and inhibitors in paucibacillary specimens are specially important. It has been observed that by using immunomagnetic beads and capture resins, the sensitivity of PCR assays can be significantly improved.

**PCR for detection of drug resistance**

It is well known that conventional methods for drug susceptibility testing for tuberculosis are time consuming. Except for phage based methods other rapid methods also need about 7-10 days to obtain a result. Because of these limitations molecular methods have become attractive alternatives for detection of drug resistance in tuberculosis. Over the last one decade different target loci associated with susceptibility/resistance to rifampicin, isoniazid (INH), ethambutol, pyrazinamide and other antitubercular drugs have been structurally and functionally analysed. These mutations can be identified by PCR amplification of target loci and subsequent analysis by PCR-SSCP (Single strand conformation polymorphism), probe hybridization, sequencing etc. Some of approaches like PCR amplification followed by hybridization are easy to apply. One such assay is commercially available. While this assay has been found to be reproducible and promising, it is not applicable to a number of Indian strains and correlation up to 60 per cent has been observed in some of these studies due to unknown mutations; some mutations also being present in strains with low degree of rifampicin resistance. In India, these mutations have been scanned in *M. tuberculosis* isolates from different types of tuberculosis and from different geographical locations. In case of rifampicin, mutations (known as well as novel) have been found to be associated with resistance in more than 95 per cent of the isolates the multidrug resistance. However, the mechanism in case of INH, quinolones, streptomycin *etc.*, are not well understood and no mutations in the known target loci in 30-50 per cent of isolates resistant to these drugs have been found. Other mechanism like efflux pumps may be responsible. Based on new information probes for direct detection of rifampicin resistance are being developed. These methods can thus reduce the time period for detection of drug resistance and can be helpful to clinicians as well as epidemiologists interested in the surveillance of drug resistance in tuberculosis.

**Application of gene amplification methods - difficulty in comparing with gold standard**

Cultivation of *M. tuberculosis*, though not foolproof, is considered the Gold standard for the diagnosis of tuberculosis. However, this Gold standard lacks sensitivity and is negative in specimens from majority of paucibacillary cases. This poses great dilemma for comparing gene amplification methods which are vastly more sensitive but have danger of false positivity due to contamination. While *in situ* PCR is specific for other gene amplification assays, other evidences should be carefully interpreted and should be correlated with other clinical, histological, cytological, biochemical, imaging and therapeutic parameters.

Due to various limitations of false negativity and danger of false positivity, no clear guidelines for application of gene amplification methods are available. American Thoracic Society recommended to use PCR as additional evidence in specimens smear positive for AFB. For smear negative cases cautious approach of using gene amplification as one
of the evidences would be preferable, which should be repeated in cases of doubt. As the advantages are many, the use of these methods with appropriate quality assurance should be encouraged.

Molecular methods: Indian perspective

Indian institutions cover a big range of technical expertise and financial affordability. Use of probes for rapid identification of growth is an attractive option. For a laboratory with good load and which is using rapid methods for early growth detection, this should not add to cost significantly. If 10-25 isolates/growths are checked for identity simultaneously, additional cost for each isolate, using a non-radioactive detection system like digoxigenin (DIG), should not be more than 150-200 rupees. Scientific and technical personnel from Mycobacteriology laboratories handling extra-pulmonary specimens, specimens from non-responders and research specimens can easily be trained to use these methods. Similarly for a PCR system using primers, which are not patented, cost should be in similar range as the prices of primers and reagents have considerably been reduced during the last 4-5 yr. These estimates will vary from laboratory to laboratory and will need to be worked out. Considering the advantages of rapidity, sensitivity and specificity, the cost is not much and should not be a deterrent in the adoption of molecular methods for diagnosis of tuberculosis.

Serological methods continue to be attractive choice because of ease of their application in the field and smaller laboratory settings. While the problem of false positive results of antibody detection with ill defined preparations is a problem in endemic countries, the antigen detection methods will be more specific. Combination of defined antigenic preparations like antigen 5, novel PPE antigens, excretory-secretory proteins etc., may lead to methods with desired sensitivity and specificity.

To conclude, the newer methods for detection of growth of M.tuberculosis along with gene probe and gene amplification technologies have provided excellent tools for rapid identification of isolates, direct detection of mycobacterial pathogens from clinical specimens and finally detection of drug resistance. Wider experience of the clinical application would be required for optimizing and if necessary, improving these methods to achieve the highest sensitivity and specificity.

Acknowledgment

The author thanks GPS Jadaun, Anuj Gupta and Rahul Sharma for help in preparation of the manuscript and financial support of agencies like LEPRA, WHO and DBT in carrying out various studies reported is gratefully acknowledged.

References


\textit{Reprint requests}: Dr V.M. Katoch, Director, Central JALMA Institute for Leprosy & Other Micobacterial Diseases (ICMR), Tajganj, Agra 280001, India
e-mail: jalma@sancharnet.in
rohinik@sancharnet.in