Advances in Molecular Diagnosis of Tuberculosis

Dr VM Katoch

MJAFI 2003; 59 : 182-186

Accurate and early diagnosis of tuberculosis is important for its effective management. Several methods for the diagnosis of tuberculosis are available which include tuberculin test, radiological examination and other imaging methods and sputum smear microscopy. Characteristic histopathology is a very important approach but there could be problems to get representative specimen and non specific features could be a problem. The immunology is often not conclusive as antibodies and delayed type hypersensitivity response persist long after the subsidence of sub-clinical or clinical disease. Sputum smear microscopy has both the problems of sensitivity and specificity. Culture is more sensitive and is presently the yardstick for diagnosis, but the time required and frequent negative results in paucibacillary specimens are important limitations. During the last decade, major advances in understanding the genetic structure of mycobacteria have been made. Based on this newer knowledge about the specific gene sequences, several gene probes/gene amplification systems for tuberculosis have been developed [1,2]. These molecular tools and methods can be used for the confirmation of identity of isolates, direct detection of gene sequences from the clinical specimens and also molecular detection of drug resistance.

DNA probes : Identification of mycobacteria is a lengthy and tedious exercise. For rapid and specific identification of *Mycobacterium tuberculosis* and other mycobacteria several DNA probes have been developed [1-5]. Commercially marketed probes for *M tuberculosis* and *M avium* are also available [1,2]. 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 growth early (such as BACTEC, Septi-Chek, MGIT) these are of great help in rapidly confirming the diagnosis as identity can be established within 1 or 2 days with gene probes as compared to much longer time required with classical biochemical tests. For direct confirmation of diagnosis from the clinical specimens, these methods are not very sensitive and need more than 10000 organisms in the specimen for positivity.

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 types of mycobacterial species including *M tuberculosis*, *M leprae*, *M avium*, *M gardoniae* etc [1-5]. These probes target rRNA, ribosomal DNA, spacer and flanking sequences. Commercially available rRNA targeting probes have been reported to be useful for quick identification of mycobacterial isolates [2]. These probes were earlier radio-labelled but have now been developed into chemiluminescent techniques [4]. rRNA targeting probes are 10-100 fold more sensitive than DNA targeting [5] and may be used to confirm the diagnosis directly in the clinical specimens in a good proportion of cases. However, the lowest detection limit is around 100 organisms. At present, these are mainly useful for rapid identification of isolates in tuberculosis.

Gene amplification methods : For the diagnosis of tuberculosis gene amplification, several techniques based on polymerase chain reaction (PCR) and isothermal amplification assay [1] have been developed.

(i) 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. Such techniques involve amplification of specific gene regions followed by hybridization with species specific probes [6,7], sequencing and RFLP analysis [8]. At Central JALMA Institute for Leprosy (CJIL) Agra, two PCR-RFLP assays based on in-house designed mycobacterial specific primers and targeting 16S rRNA and spacer plus flanking sequences have been developed (Singh et al, under publication). While PCR-sequencing approach can be applied by reference laboratories, the hybridization and RFLP approaches are easily workable in clinical mycobacteriology laboratories.

(ii) PCR methods for detection of *M tuberculosis* from clinical specimens : PCR techniques represent the ultimate in sensitivity and under optimum conditions are expected to detect 1-10 organisms. After adequate

evaluation and precautions for avoiding contamination are taken, these assays can play a very useful role in early confirmation of diagnosis in paucibacillary and very early stages of mycobacterial diseases. A variety of PCR methods have been developed for *M tuberculosis* and other mycobacteria [1]. These PCR assays target either DNA or rRNA. Further, these include assays based on conventional DNA based PCR, nested PCR, RT-PCR etc. targeting insertion and repetitive elements, various protein encoding genes and ribosomal RNA. 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 [8-20]. It would be difficult to comment on their relative merits as limited comparative application data is available. Despite initial apprehensions, these assays are now extensively being used all over the world. PCR assay system for tuberculosis is also commercially available from ROCHE [20] and has been reported to be reproducible, sensitive as well as specific. These methods can also be adapted for in-situ application for confirmation of histological diagnosis.

(iii) Isothermal amplification techniques : In these techniques, different enzymes other than taq polymerase are used and various steps of amplification are completed at one temperature only. Strand displacement amplification (SDA) system using isothermal amplification for *M tuberculosis* (Becton - Dickinson) has been described and found promising [21]. Another important assay based on this approach is gene probe amplified *M tuberculosis* direct test which employs the isothermal amplification of *M tuberculosis* complex rRNA followed by detection of amplicon with acridinium ester-labelled DNA probe [22]. Third important approach for isothermal gene amplification is QB replicase based gene amplification which involves production of RNA in the amplification reaction using QB replicase as the enzyme and reaction at fixed temperature (example 37°C) and sensitivity upto one colony forming unit has been reported for *M tuberculosis* [23].

(iv) Experience of application of gene amplification assay in clinical situations:

Various gene amplification assays have been tried for detection of *M tuberculosis* specific sequences directly from the clinical specimens. Some of the important experiences are summarized below :

a. Pulmonary cases : Pulmonary tuberculosis constitutes the major form of tuberculosis in clinical practice. Various PCR methods have been tried for confirmation of diagnosis from sputum, bronchoalveolar lavage etc. In general, different amplification methods (PCR as well as isothermal) have been found to be

positive in 95-100% of smear/culture positive specimens whereas the positivity ranges from 40-60% in smear negative paucibacillary pulmonary disease [9,13,16,20-24].

b. Neurotuberculosis : Tuberculous meningitis and tuberculoma of brain are two important manifestations of neurotuberculosis and are very important because of high morbidity and mortality associated with these forms. These forms of extra pulmonary tuberculosis are difficult to diagnose because of low bacillary numbers present in the CSF which is the usual clinical specimen available for diagnosis. Biopsy is often difficult. PCR has been extensively tried for the detection of *M tuberculosis* specific sequences in the CSF as well as biopsy specimens. As compared to 5-20% positivity with demonstration of AFB and mycobacterial cultures, PCR has been found to be positive in 50-70% of specimens from cases having cardinal features as well as biochemical/cytological evidence of neurotuberculosis [10,18,25].

c. Pleural effusion : The involvement of pleura can be diagnosed by appropriate pleural biopsy and examination of pleural fluid but it is time consuming and direct detection of *M tuberculosis* is rare. PCR can help in confirming the diagnosis in more than 60% of specimens which are negative for mycobacterial culture [14].

d. Ocular tuberculosis : Like various other extra pulmonary forms, the demonstration of mycobacteria is difficult in cases of ocular tuberculosis. In chronic iridocyclitis the establishment of etiology is a challenging task. PCR has been successfully used to confirm the diagnosis in the aqueous/vitreous fluid [25,26].

e. Cutaneous tuberculosis : Tuberculosis manifests in more than a dozen clinical forms in skin. While the characteristic histology and demonstration of organism is used to confirm the diagnosis, in some case, there could be problems. PCR has been used extensively to confirm the diagnosis of cutaneous tuberculosis [27,28]. In case of skin tuberculosis, positivity in the range of 50-60% has been observed as compared to very low positivity by culture.

f. Lymph glands : 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. Positivity rates varying from 40-90% have been reported by PCR in case of tubercular lymphadenitis [29-32].

g. Bone, kidney, genital tuberculosis etc. : Tuberculosis involves different bones, joints, genito-urinary 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 and kidneys [33,34]. PCR has been reported to be useful in confirming the clinical diagnosis of bone tuberculosis [35] and also in the demonstration of tuberculosis in the bones of mummies [36].

Sensitivity and specificity : There has been a genuine concern about 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 such as appropriate sample collection, extraction and assay, use of immunomagnetic beads [37] and capture resins [38], have been described to improve the sensitivity of PCR assays.

PCR technology for diagnosis of tuberculosis - Indian scenario :

Indian scientists have been very active in the development of PCR methods for detection of *M tuberculosis*. Different investigators have used separate gene targets like MPB 64 [10], repetitive sequences [13], GC repeats [14], dev R [15], 38kD [17], TRC 4 [18], IS 1081 [19] and a patented system from CDRI Lucknow. IS-1081 based system has been further modified and a new nested PCR target of this gene has been developed at CDFD, Hyderabad. These assays have been reported to be quite promising in confirming the diagnosis of different forms of tuberculosis. Department of Biotechnology (DBT) has made intense efforts to evaluate indigenously developed PCR assays by conducting wet-workshops and by supplying coded samples. The assays developed at CDRI - Lucknow, AIIMS - New Delhi [10, 15] have been consistently found to be of acceptable sensitivity as well as specificity for detection of *M tuberculosis* specific sequences from sputum. These are being further evaluated in extra pulmonary specimens. The evaluation of other systems is continuing. Further, a PCR assay for differentiation of *M tuberculosis* and *M bovis* has been proposed from Dr HK Prasad's laboratory at AIIMS and is being evaluated.

Molecular methods for the detection of drug resistance : Over the last one decade different target loci associated with susceptibility/resistance to rifampicin, INH, ethambutol, pyrazinamide and other

anti-tubercular drugs have been structurally and functionally analysed [39]. Novel mutations in the *rpoB* gene of Indian strains of *M tuberculosis* have been described [40]. While in case of rifampicin, mutations (known as well as novel) have been found to be associated with resistance in more than 95% of the isolates, the mechanism in case of INH, quinolones streptomycin etc., are not well understood and no mutations in the known target loci in 30-50% of isolates resistant to these drugs have been found [40]. Other mechanisms like efflux pumps are being investigated. The detection of mutations in the target genes can be identified by PCR amplification of target loci and subsequent analysis by PCR-SSCP, probe hybridization, sequencing etc. Some approaches like PCR amplification followed by hybridization are easy to apply. One such assay is commercially marketed by Inno-Genetics [41]. While this assay has been found to be reproducible and promising, it is not applicable to a number of Indian strains which have novel mutations. Based on new information about the type of mutations prevalent in Indian strains, probes for direct detection of rifampicin resistance have been developed and are being evaluated (CDFD/JALMA/AIIMS/NDTBC etc.). 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.

To conclude, several probes and gene amplification for the diagnosis of tuberculosis are now available. In addition, several rapid methods for early detection of growth and also viability determination including reporter phages are available and improve our capability to manage tuberculosis in a more effective way. More experience of their clinical application will be required for inspiring the necessary confidence in the molecular methods which certainly offer the scope to achieve highest sensitivity as well as specificity.

References

1. Katoch VM, Sharma VD. Advances in the diagnosis of mycobacterial diseases. *Indian J Med Microbiol* 1997;15:49-55.
2. McFadden JJ, Kunze Z, Seechum P. DNA probes for detection and identification. In : McFadden J editor, *Molecular Biology of the Mycobacteria*, Surrey University Press, UK. 1990;139-72.
3. Katoch VM, Kanaujia GV, Shivannavar CT et al. Progress in developing ribosomal RNA and rRNA gene(s) based probes for diagnosis and epidemiology of infectious diseases especially leprosy. In : Sushil Kumar, Sen AK, Dutta GP, Sharma RN, editors. *Tropical Diseases - Molecular Biology and Control Strategies*. A CSIR Publication, New Delhi. 1994;581-87.
4. Kaminski DA, Hardy DS. Selective utilization of DNA probes for identification of *Mycobacterium* species on the basis of

- cord formation in primary BACTEC cultures. J Clin Microbiol 1995;33:1548-50.
5. Sharma RK, Katoch K, Shivannavar CT et al. Comparisons of sensitivity of probes targeting RNA vs DNA in leprosy cases. Indian J Med Microbiol 1996;14:99-104.
 6. De Beenhouwer H, Liang Z, De Rijk P et al. Detection and identification of mycobacteria by DNA amplification and oligonucleotide specific capture plate hybridization. J Clin Microbiol 1995;33:2994-8.
 7. Suffys PN, Silva Rocha AD, Oliviera MD et al. Rapid identification of mycobacteria to the species level using INNO-LIPA mycobacteria, a reserve hybridization assay. J Clin Microbiol 2001;39:4472-82.
 8. Telenti A, Marchesi F, Balz M, Bally F, Bottget E, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 1993;31:175-8.
 9. Hermans PW, Schultema RJ, Soolingen DV et al. Specific detection of *Mycobacterium tuberculosis* complex strains by polymerase chain reaction. J Clin Microbiol 1990;28:1204-13.
 10. Shankar P, Manjunath N, Mihan KK et al. Rapid diagnosis of tuberculosis meningitis by polymerase chain reaction. Lancet 1991;337:3-7.
 11. Sritharan V, Barker Jr EH. A simple method for diagnosis of *M tuberculosis* in clinical samples using PCR. Mol Cell Probes 1991;5:385-95.
 12. Eisenach KD, Gifford MD, Bates JH, Crawford JT. Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction. Amer Rev Resp Dis 1991;144:1160-3.
 13. Reddi PP, Talwar GP, Khandekar PS. Molecular cloning and characterization of contiguously located repetitive and single copy sequences of *Mycobacterium tuberculosis* : development of PCR based diagnosis assay. Int J Lepr 1993;61:227-35.
 14. Verma A, Dasgupta N, Aggrawal AN et al. Utility of a *Mycobacterium tuberculosis* GC rich repetitive sequence in the diagnosis of tuberculosis pleural effusion by PCR. Indian J Biochem Biophys 1995;32:429-36.
 15. Singh KK, Nair MD, Radhakrishnan K et al. Utility of PCR assay in diagnosis of En-Plaques tuberculoma of the brain. J Clin Microbiol 1999;37:467-70.
 16. Kox LFF, Rhienthong D, Miranda AM, et al. A more reliable PCR for detection of *Mycobacterium tuberculosis* in clinical specimens. J Clin Microbiol 1994;32:672-8.
 17. Kadival GV, D'Souza CD, Kulkarni SP et al. A highly specific polymerase chain reaction for detection of *M tuberculosis*. Indian J Tuberc 1996;43:151-4.
 18. Narayanan S, Parandaman V, Narayanan PR et al. Evaluation of PCR using TRC-4 and IS6110 primers in detection of tuberculous meningitis. J Clin Microbiol 2001;39:2006-8.
 19. Ahmed N, Mohanty AK, Mukhopadhyay et al. PCR-based rapid detection of *Mycobacterium tuberculosis* in blood from immunocompetent patients with pulmonary tuberculosis. J Clin Microbiol 1998;36:3094-5.
 20. Baevig KG, Litchy MB, Jungkind DL et al. Evaluation of AMPLICOR PCR direct detection of *Mycobacterium tuberculosis* from sputum specimens. J Clin Microbiol 1995;33:2582-6.
 21. Spargo CA, Haaland PD, Jurgensen SR et al. Chemiluminescent detection of strand displacement amplified DNA from species comprising *Mycobacterium* complex. Mol Cell Probes 1993;7:395-404.
 22. Jonas V, Alden MJ, Curry JJ et al. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by amplification of rRNA. J Clin Microbiol 1993;33:2410-16.
 23. Shah J, Liu J, Buxton D, Hendrix A. Q-beta replicase amplified assay for detection of *Mycobacterium tuberculosis* directly from clinical specimens. J Clin Microbiol 1995;33:1435-41.
 24. Beerbal, Sachan AS, Singh D et al. Application of polymerase chain reaction for detection of *M tuberculosis* in sputum specimens. Indian J Tuberc 1999;46:235-8.
 25. Lin JJ, Harn HJ, Hsu YD et al. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction assay of cerebrospinal fluid. J Neurol 1995;242:147-52.
 26. Gunisha P, Madhavan HN, Jayanthi U et al. Polymerase chain reaction using IS-6110 Primer to detect *M tuberculosis* in clinical samples. Indian J Pathol Microbiol 2001;44:97-102.
 27. Arora SK, Kumar B and Sehgal S. Development of a polymerase chain reaction dot blotting system for detecting cutaneous tuberculosis. Brit J Dermatol 1999;142:1-6.
 28. Margall N, Baselga E, Coll P et al. Detection of *Mycobacterium tuberculosis* DNA by the polymerase chain reaction for rapid diagnosis of cutaneous tuberculosis. Br J Dermatol 1996;135:231-6.
 29. Narayanan S, Parandaman V, Rehman F et al. Comparative evaluation of PCR using IS 6110 and a new target in the detection of tuberculous lymphadenitis Curr Sci 2000;78:1367-70.
 30. Kwon KS, Dh CK, Jang HS et al. Detection of mycobacterial DNA in cervical granulomatous lymphadenopathy from formalin fixed paraffin embedded tissue by PCR. J Dermatol 2000;27:355-60.
 31. Singh KK, Muralidhar M, Kumar A et al. Comparison of in house polymerase chain reaction with conventional techniques for the detection of *Mycobacterium tuberculosis* DNA in granulomatous lymphadenopathy. J Clin Pathol 2000;53:355-61.
 32. Rimek D, Tyagi S, Kappe R. Performance of an IS 6110 - based assay and the COBAS AMPLICOR MTB PCR system for the detection of *Mycobacterium tuberculosis* complex DNA in human lymph node samples. J Clin Microbiol 2002;40:3089-92.
 33. Ferrara G, Cannone M, Guadagnino A et al. Nested polymerase chain reaction on vaginal smears of tuberculous cervicitis. Acta Cytol 1999;43:308-12.
 34. Mirlina ED, Lantsov VA, Semenovskii AV et al. Diagnostic values of polymerase chain reaction test in females with genital tuberculosis. Problemy Tuberkuleza. 1988;1:46-8.
 35. Sun Y, Zhang Y, Lu Z. Clinical study of polymerase chain reaction technique in the diagnosis of bone tuberculosis. Zhonghua Jie He He Hu Xi Za Zhi 1997;20:145-8.
 36. Zink A, Haas CJ, Reischl U et al. Molecular analysis of skeletal tuberculosis in an ancient Egyptian population. J Med Microbiol 2001;50:355-66.
 37. Mazurek GH, Reddy V, Murphy D, Ansari T. Detection of *M tuberculosis* in cerebrospinal fluid following immuno-

- magnetic enrichment. J Clin Microbiol 1996;34:450-3.
38. Amicosante M, Re neldi L, Trenti G et al. Inactivation of inhibitors by *Mycobacterium tuberculosis* DNA amplification in sputum by using capture resin. J Clin Microbiol 1995;33:629-30.
39. Musser JM. Antimicrobial agent resistance in Mycobacteria : Molecular genetic insights. Clinical Microbiol Rev 1995;8:496-514.
40. Siddiqi N, Shamim M, Hussain S et al. Molecular characterization of multidrug resistant isolates of *M tuberculosis* from Indian patients. Antimicrob Agents Chemother 2002;46:443-50.
41. De Beenhouwer H, Lhiang Z, Jannes G et al. Rapid detection of rifampicin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assay. Tubercle Lung Dis 1995;76:425-30.

JOURNAL INFORMATION

The journal is indexed/abstracted by ExtraMED, Index Medicus of Southeast Asia, International Abstracts of Biological Sciences, Abstracts of World Medicine, IndMED, Hygiene and Tropical Disease Abstracts and EMBASE.

The IndMED database is accessible on internet at the website <http://indmed.nic.in>

Guidelines for authors appear in January issue every year.

Authors' Index, Subject Index and Contents of the Volume appear in October issue every year