RECENT ADVANCES IN TUBERCULOSIS DIAGNOSTIC TECHNIQUES

Wg Cdr PK MENON ^{*}, Lt Col K KAPILA ⁺,
Brig VC OHRI [#]

ABSTRACT

Tuberculosis is re-emerging as an important cause of morbidity and mortality in man. This article outlines current strategies available for the diagnosis of tuberculosis, and its applicability. Fluorescent staining, modified culture methods, antigen detection, ELISA based assays against various antigen preparation and recent advances in molecular techniques have been outlined. Present strategies being developed at Armed Forces Medical College for the early diagnosis, speciation, antibiotic sensitivity testing and epidemiologic testing have also been alluded to.

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Introduction

With the increasing incidence of HIV infection, tuberculosis has reemerged as an important cause of morbidity and mortality in developed countries. Ineffectively executed therapeutic schedules have resulted in the emergence of multi drug resistant strains of *M. tuberculosis*. The classical techniques for the culture, identification and antimicrobial susceptibility testing of mycobacteria suffer a drawback in that the organism is fastidious and grows slowly. *M. tuberculosis* is the prototype of the slow growing acid fast mycobacteria. The TB complex includes *M. tuberculosis* and its variants (Asian, African I & II), *M. bovis* and its variant BCG. The expanding population of immuno compromised individuals has transformed the group known as MAIS complex (*M. avium*, *M. intracellulare* and *M. scrofulaceum*) from environmental opportunists to a rather frequent clinical problem. The need to identify this group rapidly and distinguish it from TB complex is another area of importance so that timely and appropriate chemotherapy can be given [1].

Scope of the problem

M. tuberculosis today infects 1.7 billion people worldwide and is the most common infectious disease of mankind. Current estimates indicate 8 million new cases and 3 million deaths every year worldwide, of which an increasing number are attributable to coinfection with HIV (Estimated in 1995 to be 8.9% globally). The complacency that had arisen from the initial decline that was seen for some time has now been

replaced by a sense of utmost urgency [2].

The increase in the incidence of tuberculosis and emergence of drug resistant strains has enhanced the need to look at faster methods for rapid diagnosis. The time between submission of a sample for examination and of getting the laboratory report with identification and drug sensitivity takes a minimum of 8-12 weeks by traditional techniques. Understanding the molecular and cellular mechanisms of mycobacterial genetics, host-pathogen relationships and drug resistance would help design effective molecular techniques for rapid diagnosis and early initiation of appropriate therapy.

An outline of tests used for identification of mycobacteria

Mycobacteria are classified as category three pathogens and should be processed in biological safety cabinets (class I or II) with a class III containment facility. The techniques currently utilised for the detection of mycobacterial diseases are [3]:

Direct methods

Microscopy or culture,

Mycobacterial speciation by biochemical assays

Mycobacterial antigen detection by monoclonal sera

Analysis of lipid composition by chromatography

Detection of DNA or RNA of mycobacterial origin

Indirect methods

Detection of IgG or IgM antibodies against mycobacteria

^{*} Reader, Department of Microbiology, Armed Forces Medical College, Pune 411 040, ⁺Classified Specialist (Pathology and Microbiology), Command Hospital (NC), C/o 56 APO [#] Commandant, 167 Military Hospital, C/o 56 APO.

Cellular immunity via skin tests.

Microscopy

The hallmark of detection is still direct microscopy of Ziehl Neelsen stained slides and identification of cultured mycobacteria by biochemical tests. Microscopy is the easiest and quickest diagnostic test but has limited sensitivity ($>10^5$ bacteria/ml) and cannot identify bacterial species. Microscopy using a Ziehl-Neelsen or Kinyoun staining procedure is rapid, cheap and easy. The sensitivity varies depending on the source of the sample and the mycobacterium involved. Best results are obtained in respiratory samples. Sensitivity ranges from 46-78% and the specificity is virtually 100%. Centrifugation and fluorochrome staining with ultraviolet microscopy markedly increases the sensitivity of microscopy [4].

Sample concentration methods

The choice and preparation of specimens is an important necessity for culture [4]. In pulmonary tuberculosis sputum is the ideal specimen. Induced sputum and bronchial washings are better than gastric washings and laryngeal swabs which may have many contaminating environmental mycobacteria. Isolation of mycobacteria from sputum is based on the relative resistance of these organisms to chemicals (e.g. sodium hydroxide, oxalic acid, sulphuric acid, various detergents). When treated with these chemicals viscous sputum is converted to a homogeneous sample and the number of unwanted fungi and bacteria is reduced while most of the mycobacteria survive. Decontamination is unnecessary for CSF and other material collected aseptically.

Culture methods

The recommended practice is to culture on both solid and liquid media. Solid media include egg based media such as Lowenstein-Jensen, Coletso or agar based media such as Middlebrook 7H10. Liquid media include Kirchner or Middlebrook 7H9 broth. There are three rapid methods: Automated systems, the radiometric Bactec 460 and 9000 (Becton Dickinson Instrument systems, Sparks, MD, USA) are used mainly for anti-mycobacterial drug susceptibility testing; the non-automated systems include the biphasic detection on solid media using Middlebrook 7H11 agar and a recent commercial product the Mycobacteria Growth Indicator Tube (MGIT) system. It uses 7H9Broth, and an oxygen sensitive fluorescence sensor to indicate microbial growth. Fluorescence occurs earlier in MGIT system than the appearance of turbidity or micro-colonies. The currently recommended culture pro-

cedure involves the use of two solid media and one liquid medium [5]. Using the Bactec radiometric system, the time of culture can be reduced to 2 weeks. The speed of this system makes it suitable for drug sensitivity testing.

Antigen detection

Gas chromatography

An alternate approach available is the identification of tuberculostearic acid (TBSA) in clinical samples. TBSA is a cell wall fatty acid of mycobacteria and other actinomycetales such as *Nocardia* and actinomycetes and can be detected by gas chromatography-mass spectrometry. The detection of TBSA in samples of CSF is one of the good rapid approaches in the diagnosis of TB meningitis. However, it requires special equipment and is labour intensive [6].

Enzyme Linked Immunosorbent Assay (ELISA) for antigen detection ELISA using double antibody sandwich procedures have been used for the detection of mycobacterial antigens in sputum, CSF and pleural and ascitic fluids. These tests lack specificity because polyclonal antibodies are used. Use of monoclonal antibodies have increased the specificity. Wade et al (1990) have used rabbit polyclonal antibodies against sonicated extract of *M.tuberculosis* to capture antigen in clinical samples. The captured antigen was revealed by purified *anti-M.tuberculosis* antibody conjugated with horse radish peroxidase. The test was able to detect antigen with a sensitivity of 100% and specificity of 97% in CSF and body fluids [7].

Agglutination

Latex particle agglutination assays and RPHA have also been used. Agglutination with sheep red blood cells sensitised with a monoclonal antibody directed against lipoarabinomannan (LAM) was used to detect antigens in patients with TB meningitis. However, some false positives were detected in the control group with pyogenic meningitis. However, advent and enhanced sensitivity of the PCR technique has overshadowed the use of antigen detection methods for the diagnosis of tuberculosis [8].

Antibody detection

ELISA based methods

ELISA based upon covering a 96 well microtiter plate with antigen, reacting this with test antisera, developing colour with an enzyme linked antihuman Ig conjugate is the simplest and most commonly used assay. Another method is to measure antibody response to individual epitopes by competitive inhibition

of the binding of labelled monoclonal antibodies (solid phase antibody competition test-SACT) by epitope specific antibodies present in the patient serum. A more sensitive modification is the SACT-SE wherein the mouse monoclonal is unlabelled and antimouse antibodies are used to detect epitope specific competitive inhibition. Dot and strip immunoassays using antigens bound to nitrocellulose membranes, incubated with test sera and binding detected by antihuman globulin enzyme linked antibodies. Various crude, semi-purified, purified and monoclonal antibody epitope directed antigens have been described. The various antigens have a sensitivity of about 70% and specificity of about 95% in detecting mycobacterial infections [9] as compared to ZN staining which has a sensitivity of 55% and specificity of 99%.

Serology is required where specimen collection is difficult such as in children or when infection is at inaccessible sites (cerebral, pericardial, GIT and bone involvement), prominent fibrosis, paucibacillary disease (lung fibrosis and constrictive pericarditis) and HIV with low bacillary counts in sputum. In smear positive pulmonary tuberculosis the 38 kDa antigen ('Ag S', 38kDa antigen, TB 72 Mab epitope binding) is immuno dominant and the first to appear. High titers are seen in recurrent and extensive disease with poor prognosis. Successful continued therapy results in a decrease in titer. Reappearance or marked rise in titer suggest inadequate therapy or noncompliance [10]. In smear negative pulmonary tuberculosis the ELISA for antibodies against LAM is 82% sensitive and 92% specific. In extra pulmonary tuberculosis the SACT-SE is sensitive (77%) and highly specific (97%) [11]. Disease progression can be monitored by decreasing antibody titers following commencement of therapy. The antibody to the 38 kDa antigen appears at 2-4 weeks and to 65 kDa/TB 72 appears at 1-4 months [12].

Molecular Methods

With the recent publication of the entire genome of *M.tuberculosis*. DNA and RNA amplification techniques will be increasingly used for the diagnosis of tuberculosis [13]. They have a much higher sensitivity than conventional methods and results are available within 24-48 hours. An additional advantage of such systems is the direct identification of the species and detection of drug resistance without having to undertake biochemical tests. Nucleic acid amplification techniques are unnecessary when reliance can be placed on the interpretation of a positive microscopy result, when this is in accordance with the clinical

findings. Amplification techniques should be performed when microscopy is negative, clinical suspicion is high, or on a growth for speciation. Amplification based procedures can also be useful in early detection of drug resistance.

PCR based techniques

The initial studies on PCR detection of *M.tuberculosis* focused on the gene coding for the 65 kDa antigen. A 383 base pair (bp) fragment was amplified and then hybridised to species specific oligonucleotide probes. However, the technique was less sensitive since it contained only one copy of the gene per bacterium [14].

Later studies for detection of *M.tuberculosis* detected the presence of IS 6110 which may be present upto 16 times in the *M.tuberculosis* genome. It is 1361 base pairs (bp) long. The central section shows no heterogeneity. A 123 bp fragment of this sequence was shown to be present in *M.tuberculosis*, *M bovis* and *M.simiae* [15]. Other genes targeted for amplification to detect *M.tuberculosis* were MPB 64 (specific for the *M.tuberculosis* complex, a 336 bp sequence (De Wit et al), a 396 bp sequence (Del Portillo) and a 969 bp sequence (Altarmirano). However, one should follow a strict protocol for guard against false positives and false negative results [16].

Various other molecular strategies for detection of mycobacteria have been developed of which the important ones available are amplification of rRNA sequences which detected highly conserved regions of the ribosomal RNA present in high copy numbers within the bacterium [17]. Probes are commercially available to detect variable regions of rRNA specific to different mycobacteria and have been used for epidemiological typing [18]. PCR can be combined with Bactec or regular culture techniques to rapidly identify organisms after growth has been detected.

Molecular susceptibility testing for first line drugs INH and rifampicin is based on the fact that there is a mutation in the *Cat* gene and the *rpoB* gene. This can be detected by a PCR amplification of the gene fragment followed by a simple electrophoresis in denaturing acrylamide gels for single stranded conformational polymorphism (SSCP) analysis [19,20]. The above procedures enhance early diagnosis of drug resistant infections by *M. tuberculosis* and initiation of appropriate therapy.

Multiplex PCR:

Multiplex PCR is a recent modification wherein the target DNA is exposed to multiple primer sets and

amplified by conventional PCR. The primers are so designed that each set gives a band of different molecular weight and can be readily visualised and distinguished by UV transillumination following electrophoresis on ethidium bromide gel. A multiplex polymerase chain reaction (PCR) assay, based on one step amplification and detection of three different mycobacterial genomic fragments, was designed for differentiation between *Mycobacterium bovis* and *Mycobacterium tuberculosis*. This may be a very useful tool for the rapid and specific differentiation of related mycobacteria and easy to use in medical microbiology laboratories [21-24].

Although PCR is a powerful tool for diagnosis of many infectious diseases including *M. tuberculosis*, false positive results can accrue as a result of poor laboratory techniques by which amplicons find their way into various samples that are otherwise negative. This method of diagnosis, once standardised in a laboratory, may be used to diagnose *M. tuberculosis* from all clinical samples including tissue.

Probe based techniques

DNA probe technology centers on the ability to melt the target organism's DNA into two strands, introduce a labelled probe that is complementary to a portion of the chromosome, lower the temperature to allow the labelled probe to anneal to the specific segment of target chromosome and finally detect the signal of the bound label. The first generation of probes for the detection and identification of *Mycobacterium* species from cultures of clinical specimens used ^{125}I as the label. However, it entailed the handling of radioactive material and had a very short shelf life. To combat this two non-radioactive DNA hybridisation assays were marketed: The SNAP system (Syngene, Inc) uses an alkaline phosphatase label covalently linked to the oligonucleotide (Short single strand chain of DNA) probe. The AccuProbe system (Gen-Probe) is a chemiluminescent probe involving the hydrolysis of an acridium ester. Molecular genetics has resulted in an impressive array of DNA hybridisation probe systems that are non-radioactive, have a longer shelf life and can be incorporated readily into a clinical microbiology laboratory. However, they are suitable for cultures and not for clinical samples which have relatively smaller number of organisms [18].

DNA Fingerprinting:

Is useful in detecting cluster of infections especially in HIV cases. Chromosomal DNA is digested with restriction enzyme Pvu II, separated on agarose gel electrophoresis, transferred onto a membrane, probed

with a fragment of IS 6110 previously labelled with horse radish peroxidase, which catalyses the oxidation of luminol, and light generated detected by exposure to an X-ray cassette. IS 1081 shows a single pattern in BCG and not shared by *M. tuberculosis* or virulent *M. bovis* [25].

Random amplification of polymorphic DNA (RAPD) Typing

Random amplification of polymorphic DNA (RAPD) typing uses short primers, without any specific sequence homology to the bacterial genome, to randomly hybridise to initiate DNA polymerisation. On amplification by a routine PCR the primers serve to amplify the region of DNA between them. Thus amplification produces DNA finger prints that differ when electrophoresed and the ladder visualised in ethidium bromide gels. The method has the advantage that no prior knowledge of the template DNA sequence is required, can be applied to any organism, and the DNA finger prints yielded differ according to the degree of relatedness of the strain under investigation [26]. There has been no report on RAPD typing of mycobacteria in world literature. At AFMC we have started to examine the usage of RAPD typing of mycobacteria as an alternative to DNA fingerprinting methodology.

Ligase chain reaction

A heat stable DNA ligase has been identified and is finding use in the mycobacteriology laboratory. DNA ligase functions to link two strands of DNA together to continue a double-strand segment. The seal can reliably take place only if the ends are complementary and are an exact match. With the requirement for an exact match in mind one can visualise the ability to detect a mismatch of one nucleotide such as can occur in a mutation (possibly dictating drug resistance or perhaps virulence) [27].

Luciferase reporter gene assay

Jacobs and colleagues have evaluated a novel reporter gene assay system for the rapid determination of drug resistance based on the gene coding for luciferase. Luciferase is an enzyme that, in the presence of ATP, interacts with luciferin and emits light (identified as the light producing system in fireflies). This gene has been inserted into the mycobacterial bacteriophage. Once the mycobacteriophage attaches to the mycobacterium, the phage DNA is injected into the bacterium where the viral genes are expressed. Because the luciferase gene is within the viral genome, luciferase is produced within the organism. In the

presence of ATP, the reporter gene results in the emission of light which can be measured. Because the amount of light is related to the amount of ATP available, the reporter assay can distinguish between those producing very little from those that produce regular amounts. This fact may be made use of in utilising the luciferase reporter assay in the study of drug resistance in *M. tuberculosis* [28].

Transcription mediated assay

16s RNA exists in multiple copies as compared to genomic DNA. Hence 16s RNA detection techniques are more sensitive. TMA is an isothermal reaction wherein T7 RNA promoter sequences are carried at one end of a primer. This results in many copies of RNA, which are converted into cDNA by reverse transcriptase. The method is extremely sensitive and has been used in the "Gene Probe Amplified Mycobacterium assays (Gen Probe) which combines transcription-mediated amplification of target rRNA with amplicon detection by a chemiluminescent DNA probe for the rapid detection of *Mycobacterium tuberculosis*.

Strand displacement amplification assay

This is a fascinating new isothermal technique which uses two sets of primers, one external to the other, two enzymes (a strand displacing DNA polymerase and a restriction enzyme), and a modified deoxynucleoside triphosphate. It is able to detect less than 10 target copies. Amplified fragments are less than 200 base pairs.

Conclusions and Scenario at AFMC

Given the current cost factors involved, it is unlikely that every lab will be able to carry out both nucleic acid amplification and culture routinely on all samples. Each diagnostic center must evaluate for its own population and economic situation, the cost-effectiveness and cost-benefits of the available diagnostic techniques. At AFMC we have started PCR based diagnosis of *M. tuberculosis* and are presently standardising multiplex PCR, mycobacterial drug sensitivity testing by PCR SSCP and mycobacterial finger printing by RAPD typing.

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